Investigating the contribution of autophagy modulation and feeding regimes in mitigating the toxicity of mutant Huntingtin protein in Drosophila melanogaster

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by

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Dedicated to my family and the red eyed tiny insect with which I worked

DECLARATION

I hereby declare that the work enveloped in this thesis entitled, "*Investigating the contribution of autophagy modulation and feeding regimes in mitigating the toxicity of mutant Huntingtin protein in Drosophila melanogaster*" has been the result of constant investigations carried out by me under the supervision of **Prof. Sheeba Vasu** at the Neuroscience Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India and the work has not been submitted elsewhere for the award of any degree or diploma. In keeping with the general practice in reporting the scientific observations, due acknowledgment has been made whenever the work described is based on the findings of other investigators. Any omission that might have occurred due to oversight or error in judgment is regretted.

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CERTIFICATE

I hereby certify that the work described in this thesis entitled, "*Investigating the contribution of autophagy modulation and feeding regimes in mitigating the toxicity of mutant Huntingtin protein in Drosophila melanogaster*" has been carried out by Mr. Ankit Sharma under my supervision at the Chronobiology and Behavioral Neurogenetics Laboratory, Neuroscience Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India and that it has not been submitted elsewhere for the award of any degree or diploma.

Prof. Sheeba Vasu
(Research Supervisor)

Synopsis

Neurodegenerative diseases (ND) are characterized by age-dependent degeneration of neurons which impacts various aspects of physiology and behaviour of the sufferers. Some of the well-characterized neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Amyotrophic lateral sclerosis (ALS). These disorders affect millions of people worldwide and are characterized by the presence of protein aggregates and selective loss of cells of specific brain regions eventually causing cellular and behavioural defects. Motor and psychiatric changes are common to all NDs. Apart from motor and psychiatric changes, perturbation in sleep and circadian behaviours are also commonly observed in patients and are proposed to be a risk factor for NDs. With their increasing prevalence and limited therapeutic success, most of which are symptomatic NDs impose a serious health challenge in the near future. It is proposed that by 2040, NDs will be the second leading cause of death after heart disease. Hence, there is an urgent need for therapeutic strategies that can at least delay the progression of such age-related disorders.

Huntington's disease is a monogenic, autosomal dominant disorder which is an outcome of the expansion of CAG codon repeats in exon 1 of the *Htt* gene. It is among one of the nine known CAG/poly-Q repeat disorders. The length of CAG repeats varies between 15-30 in wild-type alleles of the gene. However, an increase beyond 40 leads to the manifestation of disease phenotypes (McColgan & Tabrizi, 2018; Roos, 2010; Ross & Tabrizi, 2011). The translated polyglutamine/poly-Q tract in the HTT protein causes structural and functional changes. Perturbation in mitochondrial functioning, vesicular transport, protein homeostatic pathways, and transcriptional alterations are reported in HD. Like other neurodegenerative disorders, HD is characterized by the presence of mutant HTT protein, selective loss of neurons (striatal region of

the brain), and behavioural defects (including motor, psychiatric symptoms, sleep, circadian perturbations, etc.). Despite the accumulation of a large body of knowledge regarding systemic and cellular phenotypes, no therapeutic intervention exists that can delay its progression, and all available ones are symptomatic treatments. Even those strategies which show amelioration in preclinical studies have not been successful in clinical trials.

In addition to motor and psychiatric changes, perturbation in sleep, circadian and metabolic changes are reported in patients and pre-clinical models of HD. Changes in activity, melatonin, blood pressure rhythms, heart rate variability, increased sleep latency, high nighttime awakening, and excessive daytime sleep are reported in HD patients and model organisms (Mohammadi et al., 2023; Morton et al., 2005; Musiek & Holtzman, 2016). Recent observations also suggest that sleep, circadian, and metabolic defects are prodromal symptoms in HD. Proper functioning of sleep and the circadian circuit is crucial for an organism and any perturbation in them can have ill effects on the physiology. Given this, it is hypothesized that perturbation in sleep and circadian circuits can potentially aggravate neurodegenerative phenotypes and therapeutic interventions that can improve the functioning of sleep and might delay the progression of HD and associated phenotypes. **Using *Drosophila melanogaster* as a model organism, I explored therapeutic interventions that can improve the functioning of the circadian and sleep circuits with the hypothesis that it can also slow down the progression of HD-associated phenotypes.**

Drosophila is a well-established model organism for studying neurodegenerative disorders. 60% genome homology with humans along with functional homology in a large fraction of genes make it a suitable model organism to understand diverse biological processes including various known disorders. Its short lifespan, low-cost maintenance, available genetic toolkit, and ability to record diverse behaviours are its advantages. In context with neurodegenerative disease, studies

have shown that pan-neuronal expression of neurodegenerative proteins (e.g. mutant human *Htt*, Aβ, α-synuclein, etc.) in *Drosophila* recapitulate various neurodegenerative phenotypes including the formation of protein aggregates, selective loss on neurons, and behavioural defects (Bolus et al., 2020; Chan & Bonini, 2000; Lu & Vogel, 2009). Furthermore, sleep and circadian behaviours and their underlying genetic and neuronal circuits are well-studied in *Drosophila*. Flies exhibit rhythmicity in many behaviours and physiological processes and are controlled by a set of circadian pacemaker neurons located in the fly brain. The proper function of the clock neurons is important for several rhythmic behaviour, particularly locomotor activity. The clock neurons express certain genes which comprise the core molecular components of the circadian clock such as period (*Per*), timeless (*Tim*), clock (*Clk*), and cycle (*Cyc*) (Sheeba, 2008). Additionally, it is possible to specifically target different subsets of neurons that are important for specific behaviours such as sleep, thermos-sensitivity, and carbohydrate & lipid metabolism. Overall, our detailed understanding at both behavioural and cellular levels and the ability to manipulate a diverse set of neurons make fly an excellent model organism to explore diverse questions.

I expressed a region of the human mutant *Htt* gene containing an abnormal expansion of 128 CAG repeats (HTT-Q128) in flies to explore how genetic and behavioural interventions can impact the progression of HD-associated phenotypes. The genetic approach was to modify a known proteostasis pathway, namely the autophagy pathway whereas the behvaioural approach was to introduce a period of fasting on a daily basis in a regime termed as Time Restricted Feeding (TRF). The details of both are given below.

The autophagy pathway maintains cellular homeostasis in healthy cells by degrading unwanted proteins and organelles (Kenney & Benarroch, 2015). This process is known to be compromised in NDs including Huntington's disease (Cao et al., 2021; Guo et al., 2018; Klionsky, 2007; Martin

et al., 2015). Given accumulation of mutant proteins is observed in many ND conditions, it was thought that harnessing the autophagy pathway can mitigate the toxicity caused by mutant proteins. In HD models, pharmacological induction of the autophagy pathway is shown to mitigate the toxic effect of mutant HTT protein (Ravikumar et al., 2004, 2008; Sarkar et al., 2005, 2007; Vodicka et al., 2016; Williams et al., 2008). Here **I report the results of a systematic study where genetic modulation of autophagy is upregulated specifically in a set of neurons which have a clear behavioural phenotype namely rhythmic locomotor activity**. This behaviour is disrupted upon ectopic expression of mutant human *Htt* gene.

In **chapter 2** of my thesis, I report the results of my studies which show that the specific expression of mutant HTT-Q128 protein in a subset of circadian neurons called lateral ventral neurons/Pigment dispersing factor (PDF^{+ve} neurons) perturbs activity rhythm under constant conditions (i.e., constant darkness at 25°C). Changes in activity rhythm were an outcome of the presence of mutant HTT protein aggregates, loss of the targeted neurons, and defective molecular clock in the targeted neurons. Genetic modulation of the autophagy pathway through *Atg8a* (one of the crucial proteins in the autophagy pathway) overexpression leads to a sustained partial behavioral improvement. Detailed cellular characterization revealed that behavioral improvement in activity rhythm is not an outcome of the clearance of protein aggregates despite the involvement of the autophagy pathway. In contrast, I observed a significant increase in the number of mutant HTT-Q128 protein aggregates, which possibly is an outcome of the sequestration of mutant HTT-Q128 protein for degradation. Further, my data shows that partial behavioral improvement is an outcome of improved synaptic communication between the targeted neurons and the downstream neurons. Overall, this study points out that autophagy modulation can improve circadian circuit functioning in presence of mutant HTT-Q128 protein.

Functionally diverse circuits and tissues are known to be affected by the presence of mutant HTT protein and it is important that a therapeutic intervention effectively mitigates the toxicity of the mutant protein from most of them. **Therefore, I asked whether distinct neuronal circuits affected by mutant HTT protein respond effectively to autophagy modulation – a hitherto neglected aspect of previous studies.**

I chose different circuits to express mutant HTT protein and examined whether they respond effectively to autophagy modulation. Towards this end, I expressed the mutant HTT-Q128 protein separately in circuits modulating sleep, circadian clocks, motor, and temperature sensory neurons. I also targeted a set of neurons involved in lipid and carbohydrate metabolism called Dilp2^{+ve} neurons. These studies are reported in **Chapter 3** of this thesis. My studies show that genetic modulation of the autophagy pathway does not improve the functioning of all the circuits affected by mutant HTT-Q128 protein. I find that sleep, circadian circuits, and Dilp2^{+ve} neurons respond robustly to autophagy modulation, while motor and temperature sensory neurons do not show any behavioral improvement on autophagy modulation. Similar results were obtained from pan-neuronal studies wherein improvement in sleep and activity rhythm was observed on *Atg8a* overexpression, while no major improvement was observed in motor functioning. From this data, I concluded that not all circuits affected by mutant HTT protein respond effectively to autophagy modulation. I propose that a possible reason for the low success of autophagy modulators in clinical trials is due to lack of rescue in some neuronal circuits.

I also explored the impact of behavioural intervention approach is slowing the progression of HD in the fly model.

Recent work using various model organisms suggest that poor gut health and altered metabolism can negatively impact brain health and functioning through the gut-brain axis, suggesting that interventions that can manage such symptoms of NDs can have a positive impact on brain-related disorders. Since metabolic defects are predominantly manifested in HD patients: **I explored the impact of TRF regimes (a non-invasive behavioral intervention) in a fly model of HD.**

I used pan-neuronal expression of mutant HTT-Q128 protein in flies and observed diverse behavioral and metabolic phenotypes (**Chapter 4**). Age-dependent decline in motor function, perturbed activity-rest rhythms, and reduced lifespan were observed. Gross metabolic changes including hyperglycemia and altered lipid levels were also observed in flies expressing mutant HTT-Q128 protein. My studies also reveal that in comparison to males, female flies are relatively more resistant to mutant protein toxicity and show delayed behavioral perturbations. Implementation of 12:12 TRF cycles (presence of food during 12hrs of day followed and its absence during 12hrs of the night) on flies expressing mutant HTT-Q128 protein delays certain behavioral phenotypes. In comparison with the flies kept on Ad-libitum food, flies experiencing TRF regimes show a delayed decline in their motor functions. A small increase in lifespan was also observed post-TRF treatment. However, no changes were observed in activity rhythm and sleep phenotypes. Apart from behavioral changes, the implementation of TRF regime also delay the decline in feeding behaviour and body weight mainly in female flies (described in **Chapter 4**). Overall, our results from this section point out that the implementation of TRF regimes can delay the decline in HD associated phenotypes.

In summary, I explored whether autophagy modulation or TRF regimes can impact the progression of Huntington's disease using a fly model. I find that targeting the autophagy pathway can effectively mitigate the toxic effect of mutant HTT protein from sleep and circadian neurons,

pointing out that autophagy modulators can potentially be used to improve sleep and circadian circuit functioning in NDs and potentially in sleep disorders. Further, I show that not all circuits positively respond to autophagy modulation in the presence of the mutant HTT protein. This can be one of the potential reasons behind the low success of autophagy modulators in clinical trials and this study again emphasizes the need for detailed characterization of any therapeutic strategy. My studies show that the implementation of non-invasive behavioral interventions like TRF can delay the decline in phenotypes associated with HD. These results are in line with some recent studies done in the mammalian model of HD. However, in contrast to the available data I did not observe any major changes in the behaviors controlled by sleep and circadian circuits, pointing towards the lack of involvement of core clock neurons in TRF-mediated improvements. Marginal improvement in the lifespan of the flies post-TRF treatment can be an outcome of high toxicity caused by HTT-Q128 protein and hence detailed investigation is required on how TRF regimes impact the progression of the disease-associated phenotypes in a less severe HD model system (e.g., HTT-Q50).

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List of Publications

1) *Ankit Sharma, Kavyashree N, Ravi Manjithaya, Sheeba Vasu. Restoration of sleep and circadian defects by autophagy modulation in Huntington's Disease. ***Journal of Neuroscience*** (https://doi.org/10.1523/JNEUROSCI.1894-22.2023)

2) Anushka Chakravorty, **Ankit Sharma**, Vasu Sheeba, Ravi Manjithaya. Glutamatergic synapse dysfunction in *Drosophila* neuromuscular junctions can be rescued by proteostasis modulation. ***Front. Mol. Neurosci.*** July 2022, 15:842772. (10.3389/fnmol.2022.842772)

3) Link between sleep and neurodegenerative disorders (Article) - **InSC Newsletter 2021** (https://chronobiologyindia.org/insc-newsletter-samay/).

Table of Content

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List of abbreviations

Abbreviations	Full form
ND	Neurodegenerative disease
AD	Alzheimer's disease
PD	Parkinson's disease
HD	Huntington's disease
ALS	Amyotrophic Lateral Sclerosis
Poly-Q	Polyglutamine
HTT	Huntingtin protein
SCA	Spinocerebellar ataxia
SBMA	Spinal and Bulbar Muscular Atrophy
DRPLA	Dentatorubral-pallidoluysian
UHDRS	Unified Huntington's Disease Rating Scale
REM	Rapid eye movement
BACHD	Bacterial artificial chromosome HD
SCN	Suprachiasmatic nucleus
KO	Knockout
ER	Endoplasmic reticulum
NES	Nuclear export signal
PRD	Poly-proline repeat domain
HEAT	HTT, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and lipid kinase Tor
HAP	HTT-associated protein
HIP	HTT-interacting protein
NLS	Nuclear localizing signal
HAP40	Huntingtin-associated protein 40
CBP	CREB-binding protein
SP1	Specificity protein 1
NF-kB	Nuclear factor-kB
GOF	Gain of function
LOF	Loss of function
CREB	cAMP-response element binding protein
SOD	Superoxide dismutase
GPX	Glutathione peroxidase
ROS	Reactive oxygen species
UPS	Ubiquitin proteasomal system
HSP	Heat shock protein
BDNF	Brain-derived neurotrophic factor
TALEN	Transcription activator-like effector nucleases
ASO	Antisense oligonucleotides
RISC	RNA-induced silencing complex
UPS	Ubiquitin proteasomal system
TRF	Time restricted feeding
CMA	Chaperone-mediated autophagy
UPS	Ubiquitin Proteasomal System
HSP	Heat shock proteins
GS1	Glutamine Synthetase 1

LNvs	Lateral ventral neurons
DAM	Drosophila Activity Monitoring
MIP	Maximum intensity projection
Cath-D	Cathepsin-D
s-LNv	Small ventral lateral neurons
PDF	Pigment dispersing factor
DN	Dorsal Neurons
dFB	dorsal Fan-shaped body
dTRPA1	Drosophila Transient Receptor Potential-A1

IPCs	Insulin-producing cells
MIP	Maximum intensity projection

CHAPTER 1: INTRODUCTION

1.1 Neurodegenerative diseases

Neurodegenerative diseases (ND) are sporadic and hereditary diseases characterized by selective and progressive loss of neurons from specific regions in the nervous system. Some of the most prevalent NDs include Alzheimer's disease (AD, Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic Lateral Sclerosis (ALS), etc. Worldwide, millions of people are suffering from either one of these diseases and the numbers are increasing day by day posing a serious health crisis in the upcoming time (World Health Organisation, 2007, Geneva). NDs are characterized by different morphological and pathophysiological features. The most common symptoms of neurodegenerative diseases are cognitive decline and motor impairment. In addition to these, sleep and circadian defects are also commonly observed in all NDs. Given changes in sleep and circadian circuits can impact the physiology of an organism, perturbation in them is proposed to be a risk factor and can potentially aggravate the neurodegenerative phenotypes (Voysey et al., 2020).

The involved disease proteins in NDs (amyloid-β + tau in AD, α-synuclein in PD, expanded polyglutamine (poly-Q) containing Huntingtin protein (HTT) in HD, etc.) form protein aggregates with both nuclear and cytoplasmic localization. The presence of mutant protein aggregates causes cellular changes and leads to disturbed cellular homeostasis which ultimately causes degeneration of cells. Major efforts have been made to identify the molecular causes of these diseases (Dugger & Dickson, 2017). Several intracellular processes, including oxidative stress, metabolism, axonal transport, synaptic, and mitochondrial functioning have been reported to be altered in neurodegenerative diseases (Bossy-Wetzel et al., 2004). Even then, the exact molecular mechanisms involved in the origin and pathogenesis of neurodegenerative diseases remain poorly understood ultimately resulting in poor outcomes based on current therapeutic strategies in their attempts to slow down the progress of any of these disorders.

1.2 Huntington's disease

Huntington's disease (HD) is a monogenic inherited autosomal dominant neurodegenerative condition and was first described by George Huntington in 1872 through the publication of a paper titled "On Choreas" (Huntington, 2003). Apart from its inheritance pattern, George Huntington also pointed out two other predominant characteristics of HD. First is the association of cognitive impairment (termed as 'insanity') and second being that the symptoms generally manifest at mid-life (around 40-45 years of age) followed by the death of the patient within 15-20 years post onset of clinical symptoms (Huntington, 2003). The disease is associated with dance-like propensities/jerky movements (chorea) of those suffering from it, hence also called Huntington's chorea. Early postmortem studies on families suffering from HD also revealed lesions in the basal ganglia with the striatum showing the greatest degree of atrophy (Davenport, 1915).

Even though behavioural characterization for HD was well established, the genetic basis for the disorder was not known till the 1980s. In 1983, the locus for HD was assigned to the 4th chromosome in one of the first successful linkage analysis done using polymorphic DNA markers in humans (Gusella et al., 1983). Later in 1993 the gene responsible for HD was identified and named as *IT15* (later named as *Htt* gene*)* (Macdonald, 1993) Comparative analysis between the wild type and the mutated gene revealed the presence of extended CAG repeats (> 40) in the *Htt* gene as the cause for the disease and is one of many known poly-Q disorders (Aronin et al., 1995; Trottier et al., 1995). Other known CAG/poly-Q repeat diseases are Spinal and Bulbar Muscular Atrophy (SBMA or Kennedy's disease), Dentatorubral-pallidoluysian atrophy (DRPLA or Haw River syndrome), and six types of spinocerebellar ataxias: SCA1, SCA2, SCA3 (Machado Joseph disease), SCA6, SCA7 and SCA17 (La Spada & Taylor, 2003; Orr & Zoghib, 2007). Most of the poly-Q disorders share similar features for poly-Q toxicity (including presence of mutant protein

aggregates, motor defects) and the length of poly-Q repeat shows an inverse correlation with the age of the onset of the disease phenotypes (longer the CAG repeats earlier the disease onset) (Wen et al., 2014).

1.3 Epidemiology

A recent study analyzing published data available from 1983 till 2022 states that the overall prevalence rate for HD is around 3.92 cases per 100,000 individuals which is also reported in other studies (Kay et al., 2017; Medina et al., 2022). However, the global population prevalence of HD appears to show variation across regions (Rawlins et al., 2016). Sub-group analysis revealed that the North American population is associated with the highest frequency (7.43 cases per 100,000 individuals), followed by the European population with a frequency of around 5.65 cases. Asian and African populations are among the ones with the least frequency of around 0.99 and 0.23 cases per 100,000 individuals. The observed variation in the prevalence rate can be an outcome of allelic variations and their frequency of occurrence (Warby et al., 2009). Further, longer baseline CAG repeats in European and American populations (≈19) as compared to the Asian population (≈16) might also contribute to the observed variations.

1.4 Clinical features of HD

Clinical features for HD are diverse and can occur at any stage of the disease progression. The nature and severity of each feature varies across individuals even with similar CAG repeat length. HD progression can be demarcated into three different phases. The first two phases are referred to as pre-symptomatic phases (comprising of the healthy phase and pre-diagnostic phase), and patients show no or very mild behavioural and motor changes in this phase. Following the pre-symptomatic phase is the symptomatic phase, wherein majority of the clinical symptoms are

manifested, and patients die within 15-20 years post onset of the symptomatic phase (**Figure 1.1**). The observed behavioural changes and their severity in the patients is gauged based on standard clinical assessment tool including Shoulson and Fahn capability scale (Shoulson & Fahn, 1979) and Unified Huntington's Disease Rating Scale (UHDRS) - encompassing motor, cognitive, behavioural, and functional assessment scale. The scale has five levels (I – V), wherein I level is associated with mild behavioural changes (Onset of disorder) and V level is associated with severe behavioural defects (advanced stage) in the patients. UHDRS-For Advanced Patient scale is also available for detailed and accurate assessment of behavioural phenotypes for patients in advanced stages of the disease (Stoker et al., 2022; Winder et al., 2018).

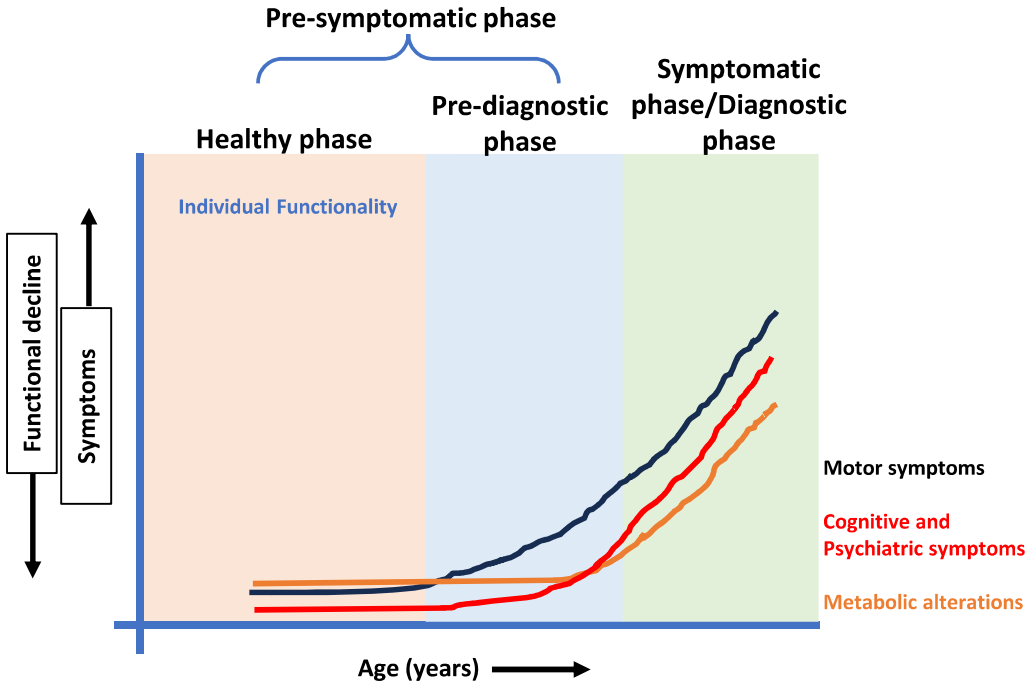


Figure 1.1: Graph depicting different phases of progression of HD. With age, the functionality of a patient drastically decreases and requires caregiver support, while increased manifestation of clinical symptoms are observed (adapted from (Ghosh & Tabrizi, 2018)).

Some of the behavioural defects observed in patients suffering from HD are summarized below and are reviewed in detail in (Bates et al., 2015; Ghosh & Tabrizi, 2018; Novak & Tabrizi, 2011; Sturrock & Leavitt, 2010; Walker, 2007) (Figure 1.2)

Motor symptoms: Both voluntary and involuntary moments are compromised in HD. One of the classical motor symptoms associated with HD is 'chorea' wherein the patient shows uncontrolled rapid and jerky movements. These changes manifest at an early age and deteriorate as the disease progresses. In the early stages of the disorder, changes in fine motor control can be observed (for example – writing) which then deteriorate to a point where the individual has minimal/no control. Dystonia (which includes sustained muscular contractions leading to changes in body posture), myoclonic jerks, rigidness, akinesis (i.e., loss of control of voluntary movements), and difficulties in swallowing are also observed in patients.

Cognitive and Psychiatric symptoms: Minor cognitive impairments can be observed at pre-symptomatic stages. Some of the early cognitive changes that can be observed in patients include personality changes, bradyphrenia (slowness of thoughts), changes in visual attention, and decreased psychomotor speed. Short-term memory impairments are also observed at the later stages of disease progression. In addition to cognitive changes, psychiatric changes are also very common in HD patients. Anxiety, low self-esteem, and irritability issues are also reported in HD patients. Apathy (i.e., lack of interest) and difficulty in initiating active conversations are common. Suicide is one of the major causes of death in HD patients. A study conducted on 4171 patients revealed further pointed that approximately 10% of the patients have attempted suicide and approximately 18% had suicidal thoughts.

Sleep disturbances: Not all patients display sleep defects in HD. Reports suggest around 45-50 % of patients show one or the other sleep defects (Happe & Trenkwalder, 2002). Data available from both patients and model organisms points toward increased sleep latency (i.e. time taken to initiate sleep is increased), decreased and perturbed nighttime sleep with frequent awakening, and increased daytime sleep (Arnulf et al., 2008; Aziz et al., 2010; Goodman et al., 2011; Wiegand et al., 1991). These changes are also accompanied by electroencephalographic abnormalities pointing towards changes in sleep centres in presence of mutant HTT protein. Detailed characterization revealed that changes in sleep are mainly an outcome of disturbances in both the Rapid eye movement (REM) and non-REM phases of sleep (Y. Zhang et al., 2019). Reduced N3 stage and increased sleep spindle density (associated with non-REM stage) is observed in HD patients (reviewed in Herzog–Krzywoszanska & Krzywoszanski, 2019; Ogilvie et al., 2021; Voysey et al., 2021).

Circadian disturbances: Circadian defects are also recorded in HD and are considered to be a clinical symptom for all NDs. Both patients and model organisms show a wide variety of defects. The first description of defects in circadian behaviour came from activity recordings in HD patients, which showed an overall loss of activity rhythm with increased nighttime awakening (Morton et al., 2005). Reduced amplitude of melatonin and associated rhythms which can directly impact activity and sleep rhythms is also well reported (Aziz et al., 2009b). Perturbed activity rhythm, high nighttime activity, reduced sleep levels, and changes in heart rate rhythm have been reported in different pre-clinical model system (Bellosta Diago et al., 2017; Kudo et al., 2011; Leng et al., 2019; Musiek & Holtzman, 2016; Voysey et al., 2020). Changes in rhythmic behaviours were associated with perturbed functioning of the molecular clock in the Suprachiasmatic nucleus (SCN) and other parts of the brain (Morton 2005, Kudo et al., 2011, Piller

et al., 2007). Postmortem studies in patients suffering from HD also point out degeneration in SCN, suggesting that mutant HTT protein impacts the functioning of circadian clocks. Importantly, studies point out that these disruptions in the sleep/wake cycle occur early in the disease progression and so could serve as a biomarker for HD.

Peripheral symptoms: HD was thought to be a brain/neuron associated disorder (Roos, 2010; Stoker et al., 2022b). But as mutant HTT protein is also expressed in various other non-neuronal tissues (for example – liver, heart, muscles, etc.) a wide range of systemic changes are observed in patients (Sathasivam et al., 1999). Severe weight loss (that can be attributed to skeletal muscle wasting and feeding changes), and decreased bone density are observed in patients suffering from HD (Busse et al., 2008; Costa de Miranda et al., 2019; Goodman & Barker, 2011). Gross metabolic changes have been reported in HD patients and multiple model organisms. This is associated with lowered endocrine function as suggested by reduced fat mass (Fain, 2001; Ogilvie et al., 2021a; Süssmuth et al., 2015), insulin resistance, hyperglycemia (possibly an outcome of insulin resistance/low glucose uptake by the tissues) (Andreassen, 2002; Blázquez et al., 2022; Lalić et al., 2008) and altered lipid metabolism (Block et al., 2010; Fain, 2001; Nambron et al., 2016a). Heart failure compromised liver and pancreas functioning are commonly observed in patients suffering from HD and are major cause of death in patients (Bär et al., 2008; Chuang & Demontis, 2021a; Kiriazis et al., 2012; Schroeder et al., 2016). While the observed changes are likely an outcome of expression of mutant HTT protein in the non-neuronal tissues directly affecting their functions, contribution of neuronal dysfunction cannot be ruled out.

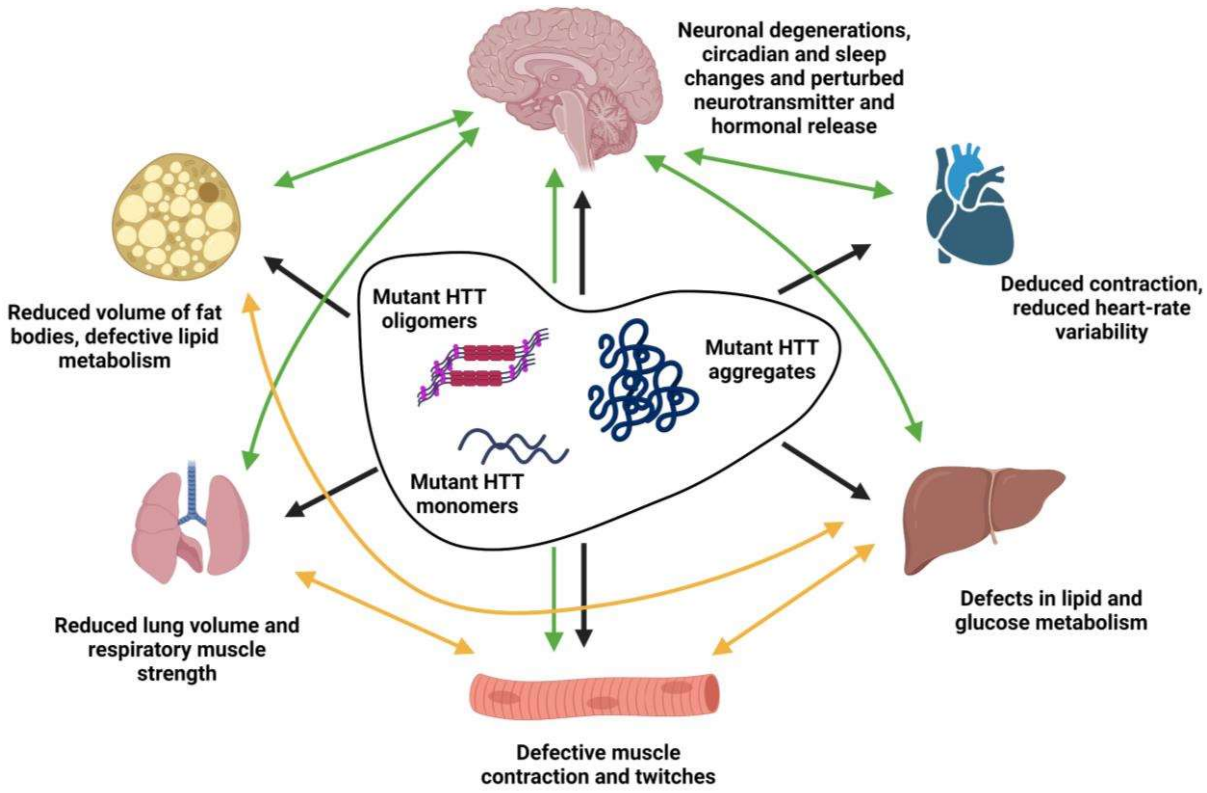


Figure 1.2: Flowchart depicting diverse tissues that are known to be affected by mutant HTT protein in patients and model organisms. Primarily neuronal degeneration is known to take place in the brain, however, tissues like liver, heart, muscles, lungs, etc. are also affected directly or indirectly. The functioning of different tissues is codependent on others, wherein perturbation in brain functioning can impact other tissues (green line) and vice versa. Such interactions coexist between other tissues as well (yellow line).

Most of the observed clinical features in HD are an outcome of changes in the central nervous system **(Figure 1.3)**. Defects in motor defects can be attributed to degeneration and changes in the release of neurotransmitters in the striatal and primary motor cortex (Q. Li et al., 2017). Degeneration in the cerebellar cortex is also reported in later stages of disease progression which further contributes to the severity of motor functions. Detailed characterization of postmortem samples and data from animal models suggest degeneration in the hypothalamus, brain stem, medulla, and locus coeruleus regions of the brain that are involved in the maintenance of sleep in an organism (Mohammadi et al., 2023; Tabrizi et al., 2012). Changes in metabolism can

be attributed to degeneration in the hypothalamic axis (leading to hormonal imbalance) and perturbation in the function of non-neuronal tissues like the liver, pancreas, etc. because of presence of mutant HTT protein aggregates. (reviewed in (Rüb et al., 2016)).

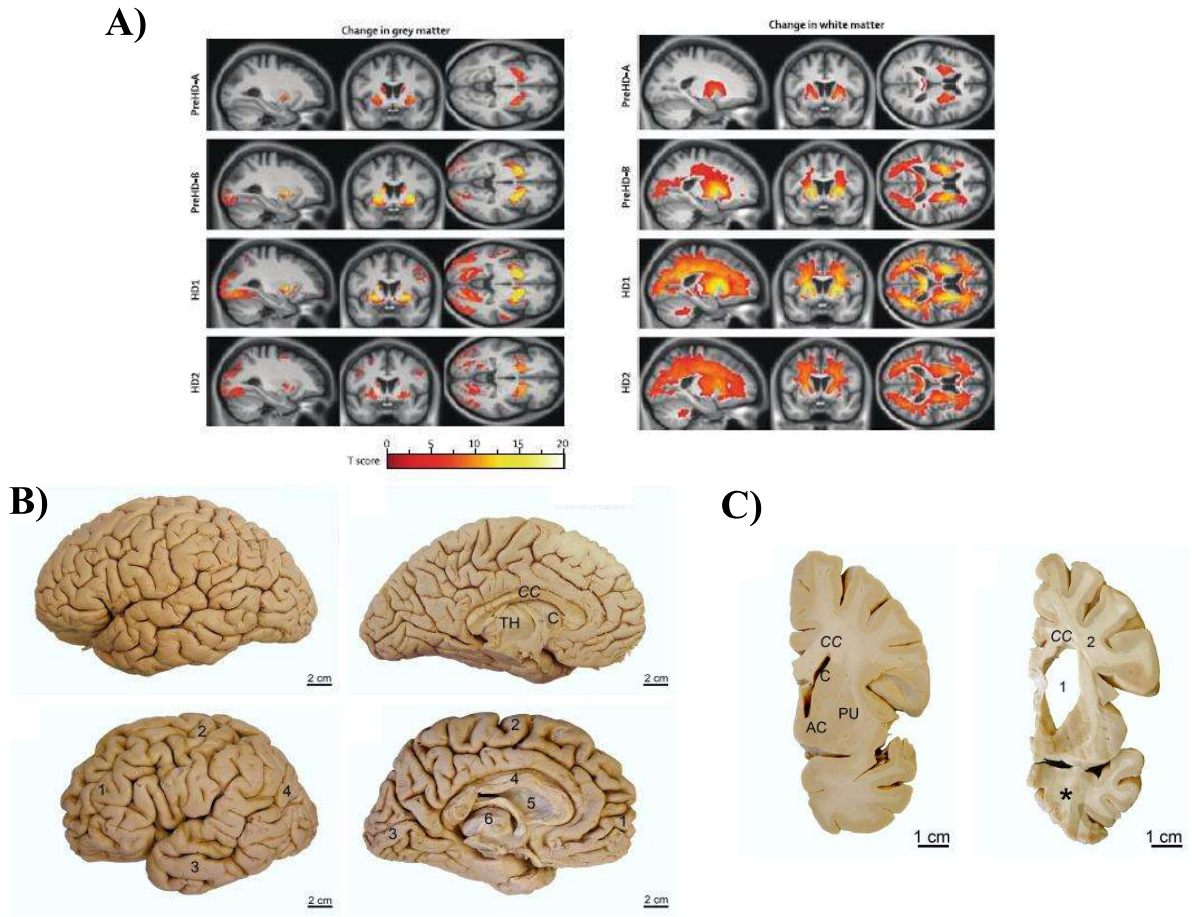


Figure 1.3: Brain atrophy is observed in patients in presence of mutant HTT protein. **A)** Parametric map of patients (at different stages of HD) showing atrophy in different brain regions at baseline, 12 and 24 months. Images showing brain atrophy of the cerebral lobes and subcortical regions in HD (bottom left). **C)** Atrophy of striatum and white matter loss can be observed in patients suffering from HD (bottom right). CC – corpus callosum; TH – thalamus; C – caudate nucleus; PU – putamen; AC – accumbens nucleus) (Figures courtesy (B, C -Rüb et al., 2016; A - Tabrizi et al., 2012)).

1.4.1 Why should we care about perturbed clocks and sleep?

Circadian and sleep disturbances are commonly observed in both aging and neurodegenerative disorders. Proper function of clocks and timely sleep is very important for an organism and their chronic perturbations can impact the physiology of an organism. Chronic disruption of both clocks and sleep reduces neuronal plasticity and causes cognitive defects (Karatsoreos et al., 2011). Studies from model organisms also demonstrate cognitive impairment and decreased hippocampal neurogenesis (Gibson et al., 2010; Kott et al., 2012). Mice lacking core clock protein-*Bmal1* show marked astrogliosis which was evident by 2 months of age and progressed to involve the entirety of the cortex, striatum, and hippocampus (Musiek et al., 2013). These mice also had increased levels of oxidative damage in the cortex and exhibited spontaneous degeneration of presynaptic terminals and diminished cortical functional connectivity. Changes in mitochondrial function that can directly regulate neurodegeneration are well reported after clock and sleep disruption. In *Drosophila*, studies have shown that levels of oxidative stress markers and antioxidants show time-dependent oscillation and is controlled by circadian clocks (Beaver et al., 2012; Krishnan et al., 2008a). Deletion of core clock gene (*Period*) exacerbates oxidative injury, shortens lifespan, and accelerates neurodegeneration (Krishnan et al., 2008a, 2009, 2012). In fly models of Alzheimer's disease, *Period gene* deletion did not hasten neurodegeneration, yet their lifespan was decreased, suggesting that perturbation of clocks and sleep can potentially aggravate neurodegenerative phenotypes.

Data from healthy humans demonstrating the contribution of clocks to neurodegeneration is minimal. Recent observation on young flight staff who routinely flew across time zones and had shorter recovery time between cross-time zone flights had higher cortisol levels and smaller

temporal volume and performed very poorly in cognitive tasks (K. Cho, 2001; K. Cho et al., 2000). In case of patients with AD, genetic polymorphism in one of the clock genes (*Clock*) is linked to an increased risk of AD and an epidemiologic study also demonstrates that diminished clock functioning is associated with increased risk of developing dementia (H. Chen et al., 2013; Q. Chen et al., 2013; Tranah et al., 2011; Y.-K. Yang et al., 2013). On a more mechanistic level, circadian oscillations in the level of the Aβ in cerebrospinal fluid of older adults have been described and suggest possible regulation of Aβ metabolism by the circadian clock, though it does not demonstrate a clear role for these oscillations in the disease process (Huang, 2012; Kang et al., 2009).

Since evidence from patients and model organisms suggest that sleep and circadian perturbations can be observed in the early stages of disease progression, they are now proposed to be a risk factor in NDs and can modulate the progression of the disorder (Leng et al., 2019; Musiek & Holtzman, 2016)**. Hence it is also proposed that interventions that can improve sleep and the functioning of circadian clocks can potentially slow down the progression of neurodegenerative phenotypes** (Colwell, 2021; Musiek & Holtzman, 2016; Voysey et al., 2020)**.**

1.5 Etiology of HD

HD is typically a mid-age disorder, wherein the onset of clinical phenotypes happens at around 35-40 years with a disease duration of around 15-20 years. It is caused by mutation in Huntingtin gene (*Htt*) which is located on the short(p) arm of the 4th chromosome in humans (Macdonald, 1993). The first exon of the *Htt* gene contains CAG trinucleotide repeats which code for polyglutamine/poly-Q track at the protein level (Aronin et al., 1995). The wild-type allele of *Htt* gene contains CAG repeats ranging between 14-32, whereas patients suffering from HD have a repeat length of >40 (DiFiglia et al., 1997; Gusella & MacDonald, 2006; MacDonald et al., 1993). Although a complete penetrance is observed for poly-Q length >40, those with repeat length between 35-40 can show some mild symptoms associated with HD (but not always) (Bessi et al., 2021). An increase in polyglutamine length leads to structural and functional changes in the HTT protein. Changes in HTT protein eventually perturb cellular functioning. There exists an inverse correlation between CAG repeat length and the age of onset of symptoms, wherein longer CAG repeats are correlated with the early onset of symptoms. Studies have found that the CAG repeat partially explains the variance in the age of onset and is proposed that the changes are also influenced by additional environmental and genetic factors (modifier genes) (Andresen et al., 2007; Rosenblatt et al., 2001; Ross et al., 2014). In support of this argument, studies have shown that monozygotic twins suffering from HD do not show similar clinical symptoms suggesting that epigenetic and environmental factors indeed influence disease symptoms and possibly progression of the disease. HD is an autosomal dominant disorder, i.e. mutation in only one copy is sufficient for disease phenotypes (heterozygous condition). Few cases have been reported where both copies of the gene show increased CAG repeats (homozygous condition), however not much differences in the toxicity and the disease phenotypes have been reported (Alexandra Durr, 1999).

1.6 Huntingtin protein

The gene that encodes HTT protein is highly conserved among vertebrates, including mice, rats, pigs, and fish (Z. Li et al., 1999; Matsuyama et al., 2000; Savov et al., 1994). While a homologous gene is identified in invertebrates like fruit flies many significant differences can also be observed in the amino acid sequence of the HTT protein and in structure. One of the prominent differences is that fruit flies and other invertebrates lack poly-Q region in HTT (Candiani et al., 2007).

In humans, the gene that codes for HTT protein has a length of about 180kb and has 67 exons. Two mRNA transcripts of size (~13.7kb & ~10.3kb) are transcribed from *Htt* gene and the longer transcript is predominantly found in adult brain, while the smaller transcript is more widely distributed (Lin et al., 1993; Mort et al., 2015) The gene codes for a large 350-kDa protein. Expression analysis revealed that HTT protein is ubiquitously expressed throughout the body with its highest expression in different brain regions and testis. Other tissues like liver, pancreas, heart, etc. are also positive for HTT protein but with low expression (uniport.org; ID – P42858; DiFiglia et al., 1995; S.-H. Li et al., 1993; Sharp et al., 1995). Subcellular localization of HTT protein is dynamic and complex. HTT protein can attain various conformations and is shown to have cytoplasmic localization (uniport.org; ID – P42858; DiFiglia et al., 1995). Cellular studies further suggest that HTT protein localizes to multiple organs including the nucleus, plasma membrane mitochondria, Endoplasmic reticulum (ER), etc. pointing towards diverse roles of the HTT protein. In neurons, it specifically localizes to microtubules, synaptosomes, etc. (Gutekunst et al., 1995; Hoffner et al., 2002; Kegel et al., 2005; Saudou & Humbert, 2016; Sharp et al., 1995)

Structural characterization revealed that HTT protein contains multiple domains (**Figure 1.4)**. The N-terminal of the protein is characterized in great detail, contains a polyglutamine (poly-

Q) stretch (responsible for disease phenotype), and has a putative nuclear export signal (NES) possibly involved in the translocation of protein out of the nucleus. The following poly-Q repeats are Poly-proline repeat domain (poly-P/PRD) tract found only in mammals, suggesting its recent evolution. Both stretches are polymorphic in human population. Poly-Q stretch is important for the protein function as its deletion impacts neuronal functioning. The poly-P domain allows the protein to interact with several proteins with SH3 (Src homology region 3) and has also been shown to modulate the toxicity of the HTT protein. The N-terminal of HTT protein is predicted to be highly flexible and can attain different structures (alpha-helix, random coil, and extended loop). HTT protein also contains multiple HEAT (HTT, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and lipid kinase Tor) repeats that are distributed along the length of the protein. HEAT domains consist of about 50 aa which have two anti-parallel helices which form a hairpin and serves as a scaffold for multiple cellular activities, provide flexibility to achieve multiple conformation for proper interaction with proteins like HTT-interacting protein (HIP) 1, HIP14, and HTT-associated protein (HAP) 1. The C-terminal of the protein harbours a nuclear localizing signal (NLS), which promotes nuclear entry of HTT protein. Multiple cleavage sites (Caspases, calpain, endopeptidases, etc.) are known to be present in the protein which cleaves the protein in short N-terminal and long C-terminal. Given, N-terminal of the mutant HTT has been shown to impact cellular functioning, these cleavage sites present near the N-terminal are thought to play an important role in HTT-mediated toxicity. HTT protein has been shown to undergo several of posttranslational modification that modulate its functioning (Harjes & Wanker, 2003; Saudou & Humbert, 2016; Schulte & Littleton, 2011; Takano & Gusella, 2002a).

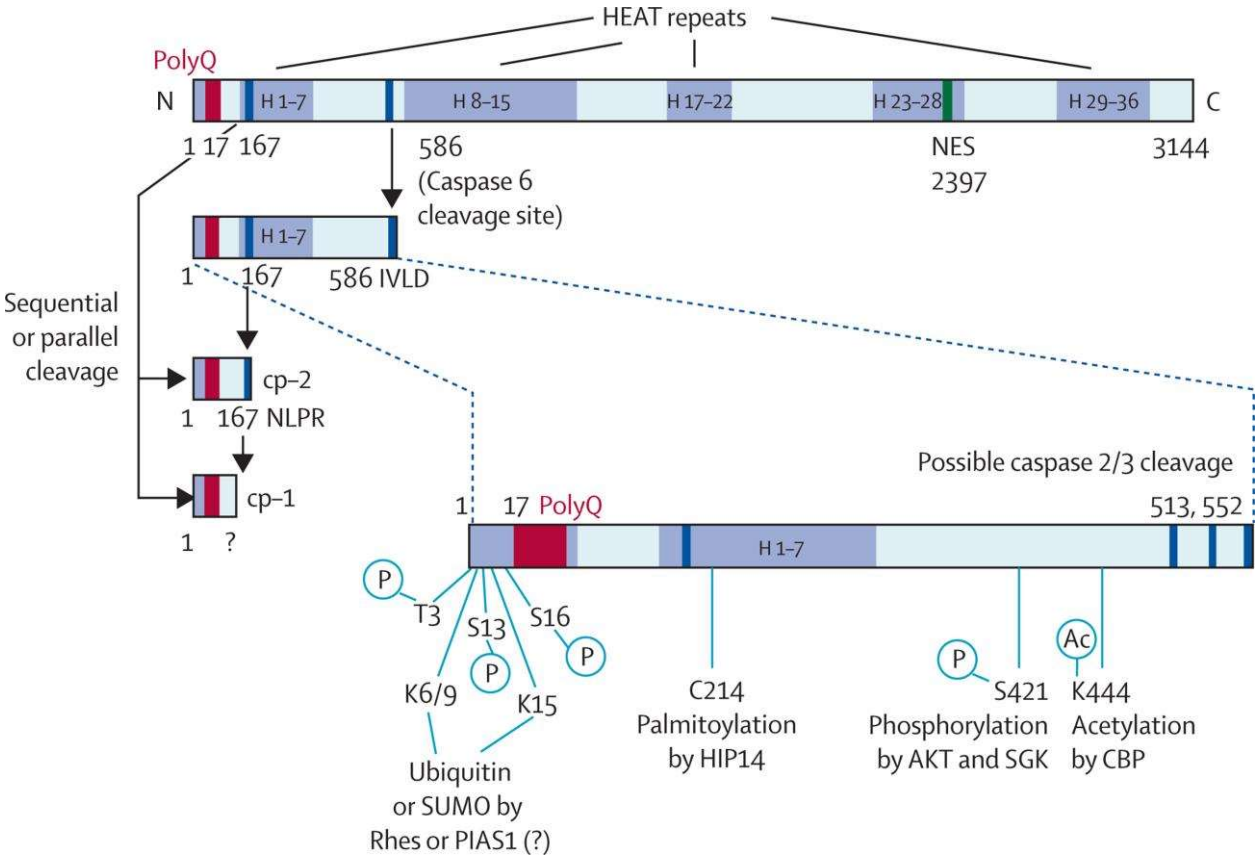


Figure 1.4: HTT protein structure depicting identified domains, protein cleavage and post-translational modification sites. The N-terminal of the protein harbours Poly-Q domain (red-shaded region) responsible for the disease phenotypes. Following the Poly-Q region, Poly-P domain is attached. A putative nuclear import sequence and nuclear export signal (green region) are also present. The protein contains multiple HEAT repeat domains (light blue regions) that are important for the functioning of the protein. HTT protein is known to be cleaved by different endopeptidases and plays an important role in protein aggregation and toxicity. HTT protein functioning is modulated by numerous post-translation modifications on sites spanning the entire protein (Ross & Tabrizi, 2011).

1.7 Functional characterization of HTT protein:

Since early 1990s the involvement of HTT protein in HD was established, yet the exact cellular function of HTT protein is not clear (Ochaba et al., 2014; Zhao et al., 2022). Studies have shown that loss of *Htt* gene expression results in embryonic lethality, pointing toward its role in development possibly because of lack of CNS development (Duyao et al., 1995; S.H. Li et al., 1993; Zeitlin et al., 1995). HTT being a large-size protein is known to interact with a lot of proteins

and is thought to have diverse functions (Schulte & Littleton, 2011). Because of the presence of multiple HEAT repeats, HTT is predicted to play a role as a scaffold protein and is thought to be majorly involved in vesicular transport. HTT protein interactors function in microtubule-based axon trafficking (both anterograde and retrograde). HIP1, HAP40 (Huntingtin-associated protein 40) and HIP-14 help HTT protein to interact with microtubule and associated proteins like kinesin, dynactin, dynein, etc. for both short-range and long-range transport axonal transport (Caviston et al., 2007; Engelender, 1997; Gauthier et al., 2004). Its role in axonal transport is further confirmed by studies in *Drosophila* and cell culture, wherein loss of dHTT or hHTT protein is reported to impact axonal transport, synaptic morphology, and function (Gunawardena et al., 2003; Weiss & Littleton, 2016). Even though HTT protein is reported to be associated with components of molecular motors, its exact role at molecular level is unclear. Loss of HTT protein in Zebrafish causes dysregulation of iron and hemoglobin production (Lumsden et al., 2007). HTT protein is further speculated to be involved in regulating the autophagy pathway. It regulates autophagy by modulating autophagosome trafficking. Multiple domains in the HTT protein are predicted to be similar to the proteins involved in regulating the autophagy pathway, overall pointing out that HTT might impact autophagy.

Beyond development and transport, HTT is shown to have anti-apoptotic effects by means of regulating caspase activity. Its overexpression is beneficial and reduced levels leads to enhanced apoptosis (Leavitt et al., 2006; Rigamonti et al., 2000, 2001). Given the presence of nuclear import and export signals, its role in regulating transcription is also speculated. HTT contains a poly-Q tract and such motifs are known to be present in transcription factors and act as transcriptional regulators. Wild-type HTT binds to numerous transcription factors, including CREB-binding protein (CBP), NeuroD, the specificity protein-1 (SP1), nuclear factor-kB (NF-kB), and the tumor

suppressor protein 53 (p53) (Dunah et al., 2002; Marcora et al., 2003; Steffan et al., 2000; Takano & Gusella, 2002b)). Apart from this HTT has been shown to modulate ciliogenesis, DNA damage repair wherein it localizes to the nucleus and helps in basic excision repair (Saudou & Humbert, 2016).

1.8 HTT: From function to dysfunction

Since decades, the field is trying to explore and understand how the expansion of poly-Q repeats in mutant HTT protein impacts cellular functioning and leads to behavioural defects. Molecular changes reported in HD are broad and still not well understood. Multiple model systems have been generated to better understand the outcome of expression of mutant HTT proteins in neurons (summarized in **Table 1**). Ways that have been proposed by which mutant HTT leads to toxicity are 1) **Gain of function** (GOF) of the mutant protein; 2) **Loss of function** (LOF) of the WT proteins (Basu et al., 2013; Kaemmerer & Grondin, 2019; Paine, 2015). In addition to both GOF and LOF, studies have also argued that the absolute level of wildtype HTT transcripts can also have an impact on the disease phenotypes (named **haploinsufficiency**). Given HTT protein is involved in different cellular functions, changes in the protein levels can modulate disease phenotype. Studies in this context have shown that overexpression of wildtype HTT protein in presence of mutant HTT protein delays behavioural defects. However, these changes can be an outcome of improvement in cellular function in presence of a functional HTT protein, rather than levels. This idea was further supported by another study suggesting that HTT-mediated toxicity is not affected by the level of wild-type copy of HTT protein (Ambrose et al., 1994). Overall, these observations strengthen the idea that the Gain/Loss of function of the mutant HTT protein majorly contributes to the disease phenotype and is discussed below.

1.8.1 Gain of function of HTT

The main feature of HD is presence of mutant protein aggregates. Studies from late 1990s have shown that mutant HTT protein with expanded poly-Q repeats forms protein aggregates in the targeted cells (Aronin et al., 1995; Davies et al., 1997a; DiFiglia et al., 1997). Presence of aggregates that are otherwise not observed in cells is thought to be one of the major causes of cellular toxicity and is achieved via aberrant inter/intra-protein interactions. Different forms of mutant HTT proteins have been reported in both in-vivo and in-vitro studies. Monomers, oligomers, and fibral forms are proposed to be an outcome of the generation of small N-terminal fragments of the mutant HTT protein by enhanced activity of endo-proteases (Fodale et al., 2020a; Wanker et al., 2019). Aggregation process of the mutant protein is a self-initiated process and is mainly dependent on the concentration of mutant HTT protein and time (Boatz et al., 2020; Jarosińska & Rüdiger, 2021). Presence of poly-Q containing N-terminal forms β-sheet (held together by hydrogen bonds) resulting in the outcome of amyloid structures. Accumulation of multiple amyloid structures or fibral form further give rise to large protein aggregates (**inclusions**) (Bäuerlein et al., 2017; Y. E. Kim et al., 2016; Wagner et al., 2018; H. Yang et al., 2020). All forms are present in cytoplasm as well as nucleus of the affected cell. Aggregates are primarily composed of mutant HTT proteins, however many proteins including ubiquitin (DiFiglia et al., 1997), proteasome proteins, chaperones, and transcription factors have shown to be sequestered in the aggregates (Bates et al., 2015a; Cummings et al., 1998; Ravikumar et al., 2004; Warrick et al., 1998).

Even though presence of diverse forms of the mutant protein is well accepted, the question remains which form of the truncated protein is more toxic? Is it the soluble form or the aggregated form? Lack of information on the actual diversity and size criteria make this demarcation even

more complicated. Currently, studies have provided evidences for both arguments. Initial studies in the field proposed that the large insoluble aggregated form of the mutant HTT protein is responsible for cellular toxicity and is achieved by sequestration of other functional proteins (discussed above) (Davies et al., 1997b; DiFiglia et al., 1997; Ordway et al., 1997). The large size of protein aggregates/inclusions can also directly impact the cell functioning by hampering processes like vesicular transport. Aggregate formation and its presence are also linked to defects in transcription, protein folding, perturbation of mitochondrial functioning, and many others. Despite there being the availability of literature that favors aggregate-mediated toxicity, recent literature argues that the insoluble form (monomers, oligomers, protofibrils) of the mutant HTT protein is toxic, and sequestration of soluble form into the aggregated form reduces HTT toxicity (Arrasate et al., 2004; Miller et al., 2010). It is proposed because of the small size and their easy transport, soluble form causes more toxicity by affecting nuclear function, axonal transport, synaptic release and perturb cellular activities by interacting with other proteins involved in RNA processing, cAMP-response element binding protein (CREB) signaling etc.

1.8.2 Loss of function of HTT

While HD is caused by a toxic gain of function due to poly-Q expansion, multiple models suggest that a loss-of-function of the wildtype HTT protein may significantly contribute to several components of disease pathology (Fodale et al., 2020b; Gerasimavicius et al., 2022; Jimenez-Sanchez et al., 2017a). HTT interacts with a large number of effector proteins and also functions in transcription and trafficking processes that can alter the processing and localization of many others. Thus, loss of normal HTT function may have an even wider range of impacts on cell physiology than currently appreciated (explained in **section 1.7**).

1.8.3 Cellular perturbation in presence of mutant HTT

Wild-type HTT protein is proposed to be involved in diverse functions. Expansion of poly-Q repeats have been shown to impact wide range of pathways in the affected cells. Transcriptional changes, disturbed functioning of glial cells, metabolic and proteome changes are reported. Some of the cellular changes that occur in presence of mutant HTT protein are discussed in detail below (**Figure 1.5**).

Synaptic dysregulation and excitotoxicity

Neurotransmitter release is one of the key functions of neurons. There is evidence for synaptic dysfunctions in HD and so much so that HD is considered as a synaptopathy (Joshi et al., 2009; J.-Y. Li et al., 2003; Morton et al., 2001). HTT protein is expressed in axons and synapses. Mutant HTT protein hampers the expression of various synaptic proteins which can impact vesicular density and synaptic release. Further being a scaffold protein, mutant HTT affects the transport of vesicles and possibly their recycling. Changes in synaptic function can be an outcome of either direct effect of mutant HTT protein on transcription or sequestration of synaptic protein in mutant HTT aggregates or can possibly lead to post-translational modification (W. C. M. Lee et al., 2004; Schulte & Littleton, 2011; Smith et al., 2005).

In addition to synaptic dysfunction, excitotoxicity is also considered to be a common cause for neuronal death. Overstimulation of receptors via high release of neurotransmitters or by increased levels of receptors can have ill effects on the functioning of neurons. First evidence to suggest that excitotoxicity can play an important role in HTT-mediated toxicity from studies in rats wherein stimulation of glutamate receptors via kainic acid led to striatal neuron degeneration. More recent evidence also suggests that agonist for NMDAR-type glutamate receptor induces loss

of $GABA^{+ve}$ neurons in striatum. Intrastriatal injection of the NMDAR-specific agonist quinolinate in rats and non-human primates also reproduced some of the behavioral features of HD pointing that excitotoxicity can contribute to HD pathology (Dong et al., 2009; Henningsen et al., 2021; Raymond, 2003).

Transcription Dysregulation

Transcriptional changes are also reported in HD. Gene expression profile of HD brain reveals changes in both directions (Becanovic et al., 2010; Cha, 2007;Hodges A et al., 2006). They are proposed to be one of the early events contributing toward pathogenicity. Mutant HTT protein shows aberrant interaction with multiple transcription factors (CREB, STAT, p53, TBPs, CBP, etc.) and in turn changes their transcriptional activity leading to major cellular changes. Perturbation in transcription can potentially be an outcome of sequestration of proteins and transcription factors in large mutant protein aggregates (Nucifora FC et al., 2001). Mutant HTT has been shown to interact with many important components of core transcriptional machinery including RNA polymerase II, TATA binding protein etc. and can perturb basal level of transcription (Dunah et al., 2002). Mutant HTT can also interfere with chromatin structure. HATs and HDACs are some of the factors to which HTT binds and blocks their activity (Becanovic et al., 2010; Cha, 2007; Pogoda et al., 2021; Tabrizi et al., 2020a).

Mitochondrial Dysregulation

Proper functioning of mitochondria is important for cells (especially for neurons) in order to maintain their day-to-day energy requirements and any defects in their functioning can impact overall cellular health. Evidence for mitochondrial abnormalities in HD was first observed in the 1990s using ultrastructural slides of brain biopsies (M.Gu et al., 1996). Consistent with these

findings, low activity of mitochondrial complex is also observed (Sawant et al., 2021; Sharma et al., 2021; Siddiqui et al., 2012). Changes in mitochondrial functioning are an outcome of downregulation of mitochondrial proteins and loss of membrane potential. Mitochondrial recycling is also impaired in HD wherein fission and fusion is affected which is an outcome of changes in the levels of mitochondrial proteins like DRP1, Mfn1 & 2 (Pantiya et al., 2020; Sawant et al., 2021; Shirendeb et al., 2012; Song et al., 2011). HD patients also show signs of oxidative damage in the striatum and cortex leading to DNA breaks (Ruan Q et al., 2004).

Perturbed glial cell functioning

While a variety of neuronal subpopulation are affected in HD, recent literature points toward changes in non-neuronal cellular population including microglia, astroglia and oligodendroglia. MRI observation on patients also suggests that changes in glial cells might occur early on in the progression of HD pointing out that glial pathology in HD may not simply be secondary to neuronal loss but accompany neuronal degeneration. Postmortem studies have shown changes in white matter (that comprise glial cells) and are associated with disease symptoms including motor dysfunction, cognitive and psychiatric issues. Transcriptional changes in glial cells are also well characterized and point towards major changes in their functioning. Perturbed functioning of oligodendrocytes is observed in HD and might precede neuronal changes. Further, studies have also shown that selective expression of mutant HTT protein in glial cells recapitulates motor phenotypes again pointing towards the contribution of glial cells in HD. Decreased expression of mature oligodendrocyte markers and perturbed myelination process (with thinner myelination) is seen in different model organisms (Cvetanovic & Gray, 2023; Shin et al., 2005; Wilton & Stevens, 2020).

Neuroinflammation and oxidative stress

Presence of mutant HTT protein and its extracellular release results in the expression of pro-inflammatory cytokines and other molecules and exacerbates the response of microglia, triggering the downstream cytokine response. Degeneration of neurons further releases molecular patterns, that further increases the inflammatory response, resulting in a vicious loop that compromises the neuronal functioning (de Araújo Boleti et al., 2020; Jia et al., 2022; Möller, 2010).

Increased reactive oxygen species have been detected in HD. One of the major contributing factors for this is mitochondrial dysfunction, wherein release of free electrons increase ROS levels in the neurons. In addition to this, decreased levels of antioxidants like superoxide dismutase (SOD), glutathione peroxidase (GPX) also contributes to reactive oxygen species (ROS) mediated damage. Increase in ROS damages cell membrane (via increasing cell permeability) and can initiate apoptosis. In addition to this, increased ROS levels also modify lipids, proteins and lead to DNA damage, a common phenomenon associated with (Browne & Beal, 2006; Liu et al., 2017; Stack et al., 2008a; Trushina & McMurray, 2007).

Protein Homeostatic dysregulation

Protein homeostasis refers to maintenance of optimal levels of functional proteins that help organisms respond to changes in their environment. Proper functioning of protein homeostatic pathways is critical for maintenance for cellular homeostasis by degrading unwanted misfolded protein and damaged organelles. Cells utilize different pathways to take care of cellular waste. Ubiquitin proteasomal system (UPS), Heat shock protein (HSPs) and autophagy pathway are known proteostasis pathways. UPS pathway plays an important role in degrading low molecular weight proteins while autophagy has the ability to clear both small and large aggregates and

damaged organelles. Accumulation of protein aggregates is one of the hallmarks of neurodegenerative disorders, suggesting that the functioning of the above-mentioned pathways might be perturbed in NDs.

UPS functioning in HD: The function of UPS is reported to be perturbed in a variety of HD models. Perturbed UPS functioning is an outcome of sequestration of proteosome subunits in mutant HTT aggregates. Disassembly of the proteasome into its substituent components which are then sequestered by HTT aggregates is also postulated as a mechanism to affect UPS activity. However, contrasting reports suggest that no significant change occurs in UPS activity and show that mutant HTT can be degraded by UPS. The later studies do not detect any change in the level of proteins involved in UPS pathway (Harding & Tong, 2018; X.-J. Li & Li, 2011; Ortega & Lucas, 2014).

Autophagy pathway in HD: Autophagy pathway is a multistep pathway and studies have pointed out that mutant HTT proteins impact multiple steps of the pathway. Cargo recognition, maturation and degradation step is shown to be hampered. Sequestration of mTOR (a negative regulator of autophagy pathway) in mutant HTT protein. However, in contrast some other studies have also found out no or very little change in the levels of proteins involved in the autophagy pathway, suggesting that autophagy pathway might not be impaired in HD and can be targeted to mitigate mutant HTT protein toxicity (Croce & Yamamoto, 2019a; Guo et al., 2018; Martin et al., 2015; Menzies et al., 2017; Pircs et al., 2018).

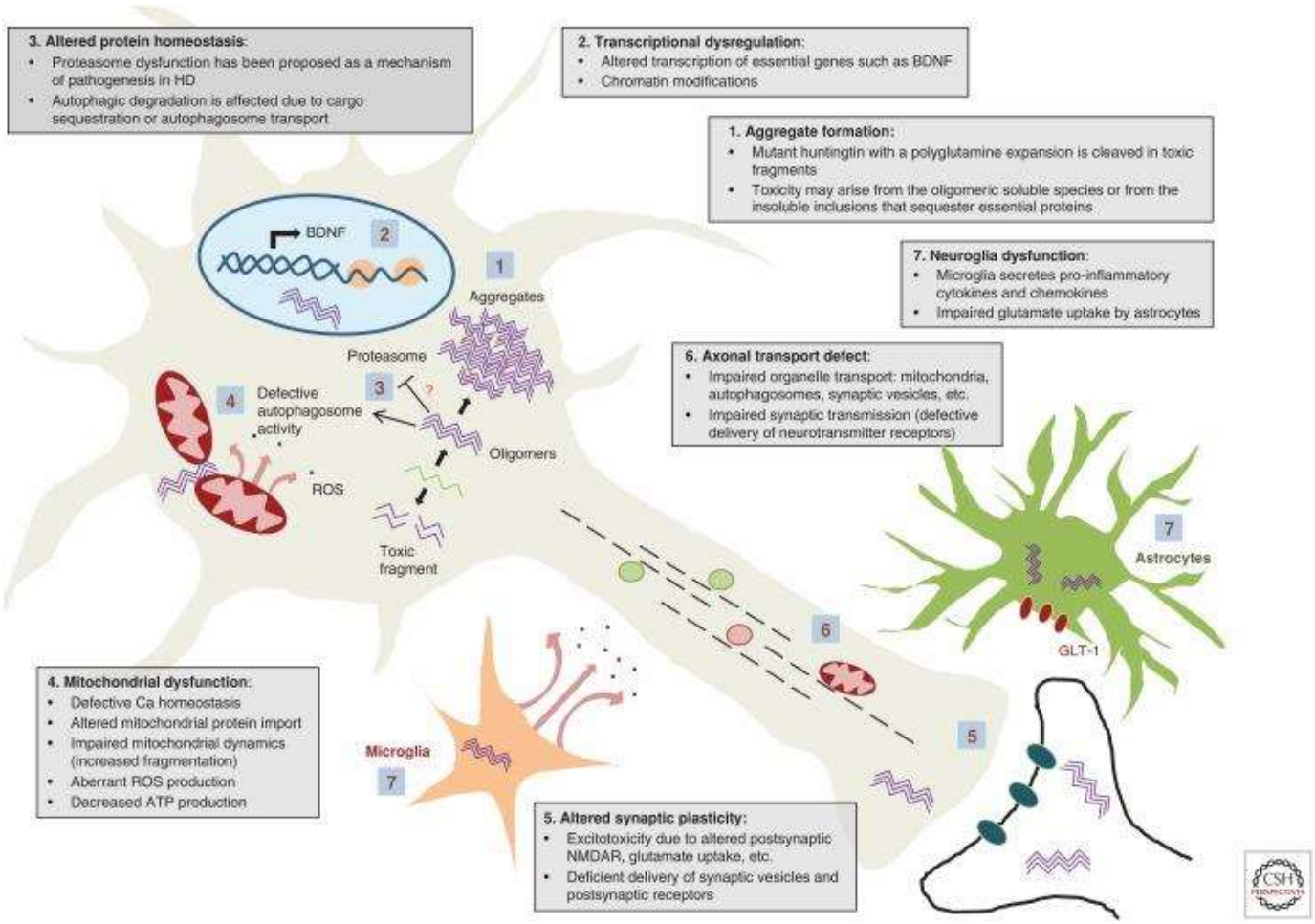


Figure 1.5: Picture depicting cellular processes affected by mutant HTT protein. HTT protein can be present in different forms (monomers, oligomers. fibrils and aggregates) and all of them can impact neuronal functioning. Transcription dysregulation, mitochondrial dysfunction, defects in axonal transport (both vesicular and mitochondrial), and altered synaptic activity is commonly observed in HD. Perturbation in the functioning of glial cells have also surfaced in recent years, suggesting HD is not neuropathy (taken from (Jimenez-Sanchez et al., 2017b).

Table 1: Summarizing different model organisms used to understand the pathology of HD with their associated behavioral defects.

Model	Species	Construct used	Behavioural Symptoms	References
Toxin models				
Quinolinic acid (QA)	Rat and nonhuman primates	Nil	Hyperkinesia, dystonia, dyskinesia, memory defects, Visuospatial deficits	(Foster et al., 1984; Furtado & Mazurek, 1996; Vazey et al., 2006)
3-Nitropropionic acid (3-NP)	Rat, mouse, and nonhuman primates	Nil	Hyperkinesia, dystonia, dyskinesia, hypokinesia, memory defects	(M. Beal et al., 1993; M. F. Beal et al., 1993; Brouillet et al., 1993)
Genetic models				
Htt79Q95, Htt171Q150, Htt57Q128	*C. elegans*	N-terminal fragment with varied lengths of Q repeats	Age-dependent neurodegeneration, mechanosensory defects, morphological abnormalities	(Faber et al., 1999; Parker et al., 2001; Van Pelt & Truttmann, 2020)
Htt132Q75, Htt132Q120, Htt548Q128, N-termHtt208Q128, ***HttQ128FL*** **(full length human HTT protein)**	*Drosophila melanogaster*	N-terminal fragment with varied lengths of N terminal and expanded Poly-Q repeat length/Full-length human mutant *Htt* gene with Q128 poly-Q repeats	Photoreceptor neurons degeneration, reduced larval and adult lethality, motor and climbing defects, electrophysiological abnormalities	(Agrawal et al., 2005; Brand & Perrimon, 1993; Jackson et al., 1998; Kaltenbach et al., 2007; W.-C. M. Lee et al., 2004; Romero et al., 2008)
Rodent models				
R6/1, R6/2	Mice	N-terminal fragment with 67 amino acids containing 116 and 144 Poly-Q repeat length	Hyperactivity, learning and memory impairment, motor defects, short lifespan, seizures, weight loss, sleep and circadian defects	(Dragatsis et al., 2009; Li et al., 2005; Mangiarini et al., 19, 96)
N171-Q82, N118-Q82, N586-Q82	Mice	N-terminal fragment with varied Q repeat length	Weight loss, motor and clasping defects, memory defects, short lifespan	(Schilling, 1999; Tebbenkamp, Green, et al., 2011; Tebbenkamp, Swing, et al., 2011)
HD51	Rat	N terminal fragment of human *Htt* gene	Emotional, cognitive decline, motor	(Bode et al., 2008; Kántor et al., 2006; von

		containing 51 poly-Q repeats	dysfunction, neurodegeneration	Horsten et al., 2003)
Q175	Mice	Exon 1 of human *Htt* gene with 175 poly-Q repeats	Clasping, motor defects, memory defects, reduced lifespan, sleep and circadian defects	(Menalled et al., 2012)
YAC128, YAC48, YAC72	Mice	Full-length human *Htt* gene with 128, 48, and 72 poly-Q repeats	Motor and cognitive defects, Neurodegeneration, memory defects, sleep and circadian defects	(Hodgson et al., 1999; Slow, 2003; Wilcox et al., 2021)
BACHD	Mice, rat	Full-length human *Htt* gene with 97 poly-Q repeats	Motor defects, neurodegeneration, sleep and circadian defects, clasping defects,	(Gray et al., 2008; Gu et al., 2022; Yu-Taeger et al., 2012)
HdHQ92, HdHQ111, HdHQ150, 200	Mice	Modified mice *Htt* gene with variable poly-Q repeats	Motor defects, learning and memory defects, cognitive decline,	(Heng et al., 2007a, 2007b; Tallaksen-Greene et al., 2005; Wheeler, 2000, 2002)
Large mammalian models				
OVT73 transgenic m*Htt* model	Sheep	Full-length *Htt* gene fused with human *Htt* gene promoter containing 73 poly-Q repeats	Motor, sleep, and circadian abnormalities, mild neurological abnormalities. No dystonia or chorea or overt brain difference.	(Bouvery et al., 2003; Clark & Soares Magalhães, 2018; Renee. R. Handley et al., 2016; Jacobsen et al., 2010; Morton et al., 2014; Morton & Howland, 2013; Reid et al., 2013; Sartoretto et al., 2016; Tebbenkamp, Green, et al., 2011)
TgHD-N548, KI-HD-Q85/150	Minipig	N-terminal fragment of human *Htt* gene with 124 poly-Q repeats/full-length KI model with 85 or 150 poly-Q repeats	Uncoordinated limb movement, atypical inactive behaviour, anxiety, dyskinesia, increased perseverative behaviour	(Baxa et al., 2013; Evers et al., 2018; Macakova et al., 2016; Vidinská et al., 2018; D. Yang et al., 2010)
AAV-*mHtt* somatic model, transgenic *mHtt*, KI -*mHtt*	Rhesus macaque, Marmoset	N-terminal (varied ranges from 1-10 exons) human *Htt* gene with 82 or 84 or 67-73 poly-Q repeats,	Cognitive and motor function decline, defects in fine motor skills, anxiety-like behaviours, seizures	(Lallani et al., 2019; Lavisse et al., 2019a, 2019b; Naidoo et al., 2018; S.-H. Yang et al., 2008)

1.9 Therapeutic strategies in HD

Even though the cause of HD was identified decades ago, till date, no treatments are able to delay the progression of the disorder. All treatments are symptomatic and aim to control observed behavioural abnormalities with very limited success. Additionally, many pre-clinical studies have used different strategies (genetic, pharmacological, behavioural interventions) to mitigate the toxic effect of mutant HTT protein, however not many have shown promising results in clinical trials till date (Estevez-Fraga et al., 2020a; O. J. Handley et al., 2006; Jurcau & Jurcau, 2022a; Kanazawa, 2006). In this section, I briefly discuss the current treatments and strategies used to improve symptoms or delay the progression of the disorder.

Symptomatic treatments

HD is characterized by diverse motor and psychiatric symptoms. Excitotoxicity is one of the major causes of cell death. Studies have tried using antagonists for NMDA receptors to rescue striatal degeneration but again had very limited success. Tetrabenazine and deutetrabenazine are two FDA-approved drugs that have been shown to reduce chorea in HD. However, the side effects are not negligible, with depression, anxiety, and increased suicidal risk among the prominent ones. Other psychiatric symptoms of HD, such as depression and anxiety are treated with benzodiazepines, while dementia related to HD with acetylcholinesterase inhibitors (Caron et al., 2018; Estevez-Fraga et al., 2020a; Frank et al., 2016; O. J. Handley et al., 2006; Huntington Study G, 2006). Beneficial effects of rTMS (a brain stimulation technique) have been reported in patients, however detailed investigation with a large sample size is required (Adam & Jankovic, 2008; Priori et al., 1994)

Restorative strategies

BDNF is one of the critical factors for neuronal functioning and its levels were observed to be perturbed in presence of mutant HTT protein (Baydyuk & Xu, 2014; Ou et al., 2021). Reducing the BDNF levels was shown to improve the motor functioning in pre-clinical models (Canals et al., 2004) Genetic manipulations using lentiviruses, dietary and pharmacological interventions for increasing Brain-derived neurotrophic factor (BDNF) mRNA levels have shown improvements in motor function in model organisms and even to some degree in patients (Arregui et al., 2011; Cabezas-Llobet et al., 2018; S.-R. Cho et al., 2007). Serotonin reuptake inhibitors (paroxetine, fluoxetine) have also shown promising results in elevating BDNF level-mediated behaviour improvements.

Apart from elevating BDNF levels, grafting of primordial striatal cells into the striatum of HD mouse model showed improvement. A follow-up study in patients with implantation of embryonic cells into the head of caudate nucleus and putamen showed slow deterioration of symptoms. In addition to this intravenous administration of human neural stem cells in HD mice model showed reduced motor defects, slow loss of striatal neurons. Even though, beneficial effects of stem cell transplant have been observed, further detailed clinical trials are required to validate this approach (Conti & Cattaneo, 2010; Kerkis et al., 2021; Ryu et al., 2004).

Strategies targeting mutant HTT levels

Given that toxicity is an outcome of the expression of the mutant HTT gene and its translation into proteins, strategies that can reduce the level of mutant HTT protein can modulate toxicity. This can be achieved by either modulating the expression of *Htt* gene at DNA or RNA or protein level and are discussed in detail in the reviews (Estevez-Fraga et al., 2020b; Jurcau & Jurcau, 2022b; Pan &

Feigin, 2021; Wild & Tabrizi, 2017). This idea was further strengthened by observation wherein suppression of mutant HTT expression reduces the aggregate load which in turn reduces the toxicity of mutant HTT protein.

Targeting at DNA level: Modulating the expression of mutant *Htt* gene at DNA level can in principle improve many aspects of HD, but this approach is far from being achievable in human patients due to numerous practical and ethical considerations. Nevertheless, the following strategies have been tested in pre-clinical model organisms to modulate the expression of *Htt* gene.

1) Zinc finger proteins bind to specific DNA sequences and can modulate the expression of multiple genes. Two subgroups are known, one with nuclease activity, and another group can suppress the transcription of genes. Currently two zinc finger transcriptional repressors have been tested in pre-clinical models and have shown promising results in terms of behavioural improvements (ZF-KOX1 and TAK-686).

2) CRISPR/Cas9 based approach adopted form an ancient bacterial defence system to degrade foreign DNA particle and modified to introduce changes in the DNA of different organisms. In HD, CRISPR can be used to correct *Htt* alleles by removing CAG repeats or to remove the entire gene. The approach has been tested in cell lines and model organisms, wherein excision of CAG repeats from exon 1 of the *Htt* gene led to improvement in motor symptoms, reduced mutant HTT expression, and astrocyte reactivity. However, concerns about off-target mutations caused by CRISP/Cas9 need to be resolved before using it in clinical trials. Similarly, transcription activator-like effector nucleases (TALEN) which can also be used to remove mutated genes are being developed and can be systematically employed in pre-clinical models to test their efficiency.

Targeting mutant HTT RNA: Antisense oligonucleotides (ASO) and RNAi approaches are well explored in mitigating the toxic effect of mutant HTT protein. Both strategies can selectively reduce the mRNA of the mutant *Htt* gene. ASO lowers HTT transcripts by binding to specific sequence of *Htt* mRNA and RNase-H activity degrades the targeted mRNA. ASOs targeting human *Htt* gene (IONIS-HTT_{RX}) are under clinical trials. In pre-clinical models the molecule reduced brain atrophy and was shown to outlast the effects of drugs. In clinical trials there was dose-dependent decrease in mutant HTT levels in CSF, however detailed results are not published. Given multiple alleles of *Htt* gene exists, two allele specific ASOs (s362037 and rs362331) are also under clinical trials and have been shown to reduce the mutant HTT load in CSF (clinicaltrials.gov, NCT03225833, NCT03225846; NCT03761849).

MicroRNAs (miRNAs) or shRNAs can also lower *Htt* RNA transcripts by recruiting mRNA to RNA-induced silencing complex (RISC). miRNA delivered via direct intrastriatal administration have resulted in a therapeutic benefit in multiple HD mouse models and a sheep model. An outcome of these studies is that AMT-30, a gene therapy is under clinical trials for HD.

It contains a gene that codes for miRNA which is administered as an intrastriatal injection after it was found to be safe and effective in preclinical mouse and primate models (NCT04120493). Another RNAi therapy tested in preclinical trials is VY-HTT01 and has shown promising results with improvement in motor functions. In addition, small, orally administered molecules which modify pre-mRNA of *Htt* gene are under clinical trials (PTC518 - NCT 05358717; LMI070 - NCT05111249). Even though positive effects of strategies that can target mutant mRNA have shown promising results, multiple challenges need to be resolved (specificity, effective delivery to the targeted cells, immune responses if any).

Strategies targeting HTT protein: The main cause of toxicity is accumulation of mutant HTT protein in the cells. Strategies that can clear the accumulated proteins can have beneficial effects on the functioning of the cells and can lead to behavioural improvements. Protein homeostatic pathways are inherently present in the cells and can clear misfolded and damaged proteins. Studies have shown that targeting the ubiquitin proteasomal system (UPS) via genetic means can clear monomeric and oligomeric forms of mutant HTT protein leading to behavioural rescue (DeMarch et al., 2008; B.-H. Lee et al., 2010; Seo et al., 2007; Wong et al., 2008). The UPS can be exploited to target specific substrates for degradation via proteolysis targeting chimaeras (PROTACs) and has been demonstrated successfully to degrade mutant HTT protein in cell lines. However, UPS cannot clear large inclusions. In this context studies have shown that pharmacological or genetic modulation of the autophagy (which has the capacity to clear both monomeric and inclusion of mutant HTT protein) can mitigate the toxicity of mutant HTT protein in different model organisms (discussed in **Chapter 2**). Even though positive effects of modulation of protein homeostatic pathways have been observed in pre-clinical models, none of them have shown promising results in clinical trials.

In addition to this, modulation of HSPs has also shown beneficial effects in preclinical models. Overexpression of HSP40 and HSP70 has been shown to improve behavioural defects by modulating the aggregation process of mutant HTT protein (J. Y. Chen et al., 2018; H. Kim & Gomez-Pastor, 2022; Lang et al., 2021; Prakash et al., 2022a).

Non-invasive interventions: As discussed in section 1.4.1, the perturbation of circadian clocks and sleep can potentially aggravate the neurodegenerative phenotype. Recently a lot of focus has shifted towards lifestyle interventions and their role in modulating the toxic effects of mutant HTT protein. Exercise, blue light therapy, and timed feeding regimes have been shown to boost the

function of circadian clocks and sleep. Studies in this direction have shown that implementation of these lifestyle interventions can delay some of the neurodegenerative phenotypes (like motor function, metabolic alterations, etc.) in patients and model organisms (Frese et al., 2017; Phillips et al., 2022; Playle et al., 2019; H.-B. Wang et al., 2017, 2018a). However, detailed studies are required to assess their effectiveness. Given their low cost in implementation and their limited side effects, these interventions can be easily implemented if proven effective.

Limited number of therapeutic interventions have shown promising results in clinical trials. Lack of detailed understanding about how mutant HTT protein perturbs the functioning of an organism both at cellular and behavioural levels is one of the reasons behind low success rates in clinical trials. Hence, systematic characterization is required, wherein understanding the efficacy of an intervention at multiple levels is important. Understanding the molecular mechanisms and whether different tissues affected by mutant proteins respond to the given intervention is essential. Further, HD being an age-associated disorder, it is also important to explore the effectiveness as the disorder progresses with age.

1.10 Aim of my study

Using *Drosophila* as my model organism I explored the effectiveness of a genetic and a lifestyle intervention in mitigating the toxic effect of mutant HTT protein. Specifically, I genetically modulated the autophagy pathway and implemented a lifestyle intervention - Time-restricted feeding (TRF) regimes and explored if these interventions can slow down the behavioural defects controlled by diverse circuits (including circadian and sleep neurons) in presence of a human mutant HTT protein containing Q128 poly-Q repeats using *Drosophila* as a model organism.

1.11 *Drosophila* as a model system

Studies using model organisms have provided invaluable insights in elucidating the cellular and molecular basis of cellular processes and how these processes are impacted in disease. Among others, *Drosophila melanogaster* (henceforth referred to as *Drosophila*) is a well-established model organism to explore several biological processes and their alteration in diseases. With a homology of approximately 60% many genes and pathways are conserved between flies and humans. Structural and functional similarities exist between human and *Drosophila* brain (like the presence of blood brain barrier and diverse cell types) (Tello et al., 2022). A simple nervous system along with reasonably complex behaviours including memory & learning, sleep, etc., make it an attractive system for a study of neurodevelopment and neurodegeneration. Their low-cost maintenance, short lifespan, and available genetic toolkit offers several advantages that other vertebrate model organisms lack. Using the available tools, one can carry out large scale screens, can record complex behaviours and understand their cellular and molecular regulation with high precision. Given its high homology, one can explore mechanisms underlying disease progression and identify therapeutic targets that can delay the progression of the disorder (Rezaval, 2015; Şentürk and Bellen, 2018).

HD and other neurodegenerative disorders are extensively studied in *Drosophila*. Studies have shown that it re-capitulates polyglutamine induced neurodegeneration. Multiple fly lines expressing varied length of human mutant *Htt* gene have been generated to explore HD. *Drosophila* models have replicated many of the pathogenic processes of HD, such as a formation of mutant protein aggregates, varied age of onset (depending on the construct used), decreased lifespan, impaired motor and cognitive functions, perturbed sleep and circadian behaviors and metabolic alterations (Chan et al., 2002; Marsh and Thompson, 2004; Xu et al., 2015; Rosas-

Arellano et al., 2018b; Bolus et al., 2020). Further, *Drosophila* has been a good pre-clinical model system, wherein multiple pharmacological and lifestyle interventions have shown positive in delaying the progression of the disorder. Thus, *Drosophila* is a powerful model system to test for disease modifiers and develop therapeutic strategies and drugs to delay or even reverse the progression of neurodegenerative disorders.

1.11.1 *Drosophila* circadian circuit as a model system

Drosophila circadian comprises of approximately 150 neurons that are bilaterally distributed in the brain and are named based on their location. Proper function of these neurons is important for bring out overt rhythms including activity-rest, feeding, eclosion, etc. These neurons are the site of the working of the core molecular clock composed of transcription and translation feedback loop, involving multiple proteins. The *Clock* and *Cycle* genes encode proteins that forms the positive limb (inducer) of the feedback circuit. Both CLOCK and CYCLE form heterodimer (CLOCK: CYCLE) and translocate to the nucleus as a heterodimer and initiates the transcription by binding to specific DNA elements in the promoter region of other genes. Specifically, they transcribe a set of genes named *Period* and *Timeless*. Transcription of *Per* and *Tim* results in production of both PER and TIM protein. Both the protein form heterodimers (CLOCK: TIMELESS) that translocate to the nucleus and suppresses their own expression by inhibiting transcriptional activity, allowing the cycle to repeat from a low level of transcription (Sheeba, 2008).

Drosophila circadian circuit has been used as a model system to explore the HD. Studies have shown that expression of human *Htt* gene containing poly-Q repeats in clock neurons recapitulates various cellular and behavioural phenotypes observed in HD (Prakash et al., 2017, 2022b; Xu et al., 2019a). Formation of protein aggregates, selective loss of specific neurons and

perturbation of activity and sleep rhythms is observed. *Drosophila* circadian circuit offers various advantages. One to one correlation exist, wherein proper functioning of clock neurons is important for maintenance of activity and sleep rhythms under different conditions. Further, given the small number of neurons one can also explored in detail cellular and molecular changes on expression of mutant HTT protein. In this study we have also targeted *Drosophila* circadian circuit to explore our questions.

1.12 Objectives of my study

Using the available genetic took kits in *Drosophila* (GAL4-UAS system that is widely used in *Drosophila*) to target specific tissues, I first asked whether genetic modulation of the autophagy pathway can mitigate the toxic effect of mutant HTT protein in a subset of *Drosophila* circadian neurons and if so, what molecular mechanisms are involved (**Chapter 2**). Given, that the functioning of diverse circuits is compromised in NDs including HD, I further explored whether different circuits affected by mutant HTT protein in brain effectively respond to autophagy modulation (**Chapter 3**).

Metabolic changes are commonly observed in HD and Time-restricted feeding (TRF, a non-invasive lifestyle intervention) has been shown to improve metabolic phenotypes across multiple metabolisms associated pre-clinical models. In the final part of my work, I explored whether implementations of TRF regimes can also slow down the progression of HD-associated behavioural phenotypes (**Chapter 4**). Finally, I summarize and discuss the findings from the available and my data with discussion on future avenues that require further attention (**Chapter 5**).

Autophagy and Huntington's disease (HD)

Chapter 2: Does genetic modulation of the autophagy pathway mitigate the toxic effect of mutant Huntingtin (HTT) protein?

A part of the results described in this chapter is a part of the manuscript published in the *Journal of Neuroscience* entitled "Restoration of sleep and circadian behavior by autophagy modulation in Huntington's disease" by Ankit Sharma, Kavyashree Narasimha, Ravi Manjithaya, and Vasu Sheeba (2023). DOI: https://doi.org/10.1523/JNEUROSCI.1894-22.2023

2.1 Introduction:

Autophagy is a well-conserved protein homeostatic pathway employed by cells to clear unwanted toxic and damaged entities (ranging from damaged organelles to protein aggregates to bacteria and viruses) present in the cells (Glick et al., 2010; Parzych & Klionsky, 2014; Yu et al., 2018). It was Christian de Duve who first coined the term 'Autophagy' in the 1960s based on his observation of the double-membraned structures present inside the cells that delivered intracellular materials to lysosomes for degradation (Deter & de Duve, 1967). Even though it was long known that cells utilize the autophagy pathway to degrade damaged organelles and to survive under stressful conditions (like starvation), molecular players involved in the autophagy pathway were identified much later in genetic screens by Yoshinori Ohsumi and other groups across the world from the 1980s to the early 2000s (Klionsky, 2007; Nakatogawa et al., 2009). During the same time, specialized types of autophagy (including microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy) and molecular players regulating these were also discovered. Out of the three, macroautophagy (henceforth referred to as autophagy) is studied in most detail (Farré & Subramani, 2016; Kenney & Benarroch, 2015). At the molecular level, the pathway is subdivided into three main steps. The first step (the initiation step) involves the formation of a double membrane structure called a phagophore. Followed by the initiation step, elongation of the phagophore leads to the formation of an incomplete autophagosome. It is at the elongation step where all the degradative materials are loaded in the incomplete autophagosomes with the help of specific proteins like ATG8a/LC3 and adapter proteins like p62/Ref(2)P, Optineurin, etc. Following the cargo loading step, the double membrane structure is sealed to form a mature autophagosome. The final step of the pathway is the transportation of a mature autophagosome to lysosomes and once the autophagosome and lysosomes are in proximity, fusion of both the vesicles

takes place. Hydrolytic enzymes present in the lysosomes degrade the cargo into basic building blocks which are then reutilized by the cells (Cao et al., 2021; Yamamoto et al., 2023a).

For cellular homeostasis, proper functioning of the autophagy and other protein homeostatic pathways (Ubiquitin Proteasomal System (UPS) and Heat shock proteins (HSPs)) is crucial for all cells including mitotically silenced neurons. In line with this argument, studies have shown that knocking out crucial autophagy genes (like *Atg5*, *Atg7,* etc.) leads to the accumulation of p62-tagged damaged organelles and proteins in the neurons which eventually leads to cell death (Cann et al., 2008; O'Sullivan et al., 2005; Xi et al., 2016). Similar phenotypes are observed not only in neurons but also in other tissues (Kuma et al., 2017; F. Wang et al., 2021). Furthermore, many mutations in autophagy genes are also associated with different disorders like Crohn's disease (linked to *Atg16L1*), childhood ataxia (linked to *Atg5*), breast and ovarian cancers (linked to *BECN1*), and Danon's cardiomyopathy (linked to *Lamp2*), pointing that proper functioning of autophagy pathway is crucial for neurons and tissue homeostasis (Stamatakou et al., 2020; Yamamoto et al., 2023b).

In neurodegenerative disorders (like AD, PD, HD, etc.) autophagy pathway is reported to be compromised and is potentially thought to be one of the reasons for enhanced toxicity of mutant neurodegenerative proteins (Karabiyik et al., 2021a; Menzies et al., 2017). Specifically in the context of Huntington's disease, reports showed that the mammalian target of rapamycin (mTOR- a negative regulator of autophagy pathway) is sequestered in mutant HTT protein aggregates resulting in the induction of the autophagy pathway (Ravikumar et al., 2004). However, data from cell lines and mouse models show that the autophagy pathway is affected both at cargo loading and fusion step in the presence of mutant HTT proteins (Martinez-Vicente et al., 2010). Similar

observations are put forward by a recent study, showing evidence of defective autophagy in patient-derived neuronal culture (Pircs et al., 2022)..

Given the importance of the autophagy pathway in neurodegenerative conditions and its ability to degrade large organelles and aggregated proteins, it was thought that manipulation of the autophagy pathway can mitigate the toxicity by clearing the mutant HTT through lysosomal degradation (Croce & Yamamoto, 2019b). In this context, studies showed that pharmacological or genetic manipulation of the autophagy pathway can mitigate the toxicity of the mutant proteins in HD and other neurodegenerative disorders. Pharmacological induction of the autophagy pathway using Rapamycin (an inhibitor of mTOR protein) has been shown to improve photoreceptor degeneration in *Drosophila* and improve motor function in Ross/Borehett mouse models of HD. This improvement in motor functions is correlated with the reduction of mutant HTT protein aggregates in the striatum region of the brain (Ravikumar et al., 2004). In line with this observation, other studies employing diverse model organisms (*C. elegans*, *Drosophila*, mouse/rodents, and cell lines) have demonstrated that modulation of the autophagy pathway through compounds like Rilmenidine, Trehalose, Resveratrol, Minoxidil, Caplins, etc. also improves photoreceptor degeneration (*Drosophila*), improves motor function in different mouse models of HD and reduces aggregate stress (Menzies et al., 2015; Rose et al., 2010; Sarkar et al., 2007, 2008; Tanaka et al., 2004; Williams et al., 2008). Not only pharmacological agents, but genetic modulation of autophagy pathway through TFEB (major transcription regulator of autophagy pathway), AMPK, Rab5 & Glutamine Synthetase 1 (GS1) overexpression, or calpain inhibition reduces aggregate stress, neurodegeneration and improves mitochondrial function (resulting in reduced oxidative stress) (Fox et al., 2020; Martinez-Vicente, 2020; Ravikumar et al., 2008; Vidal et al., 2012;

Vodicka et al., 2016). These results further strengthen the idea that autophagy modulation can mitigate the toxicity of mutant HTT protein.

Even though experimental data from diverse model organisms strongly point out that the autophagy pathway can be targeted to mitigate the toxicity of mutant HTT protein. However, none of the autophagy modulators have shown promising results in clinical trials (NCT03764215 – Nilotinib; NCT04826692 – Metformin; ACTRN12621001755820 – Trehalose; NCT03515213 – Fenofibrate) pointing that comprehensive understanding is essential before considering the use of the autophagy modulators as a therapeutic strategy.

1) Even though studies have correlated behavioral improvement with the decrease in mutant HTT protein, molecular and cellular changes leading to the clearance of the mutant protein and hence behavioral rescue are not well explored.
2) Most of the studies done till now have explored the short-term effects of autophagy modulation. Given neurodegenerative disorders are age-related disorders i.e., as time progresses their severity increases, studies are needed to explore the long-term effect of autophagy modulation in mitigating the toxic effect of mutant HTT protein.
3) Finally, given with age the mutant HTT protein impacts the functioning of different circuits and neurons, it is important to explore whether most of the circuits affected by mutant HTT protein respond to autophagy modulation (**explored in chapter 3**) (Croce & Yamamoto, 2019b).

Genetic tools available in *Drosophila melanogaster* (fruit flies) offer various advantages. One can target specific neurons and circuits, proper function of which can be directly correlated with specific behaviors (Duffy, 2002). Given this direct correlation, one can associate cellular changes with behavioral changes which is difficult to do in pan-neuronal studies. Hence, with the available

tools one can not only explore cellular changes that lead to behavioral rescues but can also understand whether different circuits respond to autophagy modulation in the presence of mutant protein.

With this, I asked whether genetic modulation of the autophagy pathway can mitigate the toxic effect of mutant HTT protein. If so, what are the molecular mechanisms involved? I used a genetic approach to modulate the autophagy pathway because pharmacological compounds can have off-target effects. I specifically targeted a subset of *Drosophila* circadian neurons named lateral ventral neurons (LNvs). Proper functioning of these neurons is important for the maintenance of activity rhythm under constant conditions (25°C, constant darkness) (Grima et al., 2004; Stoleru et al., 2004), and given the small number of targeted neurons, one can investigate cellular changes leading to behavioral improvements. In this context, earlier studies from our lab and others have shown that expression of human mutant HTT protein containing Q128 polyQ repeats in the lateral ventral neurons (LNvs) recapitulate various features of HD, including the formation of protein aggregates, defects in activity rhythm (behavioral defects), and selective susceptibility of a subset of targeted neurons (Prakash et al., 2017, 2022b; Xu et al., 2019b). Circadian circuit functioning is known to be perturbed in HD and recent literature points out that defects in circadian circuits can potentially exacerbate the neurodegenerative phenotypes. Hence through this study, I also investigated if autophagy modulation can improve the functioning of these neurons in the presence of mutant HTT protein using this model.

In this context, a targeted screen consisting of three crucial autophagy genes named *Atg1*, *Atg5*, and *Atg8a* revealed that *Atg8a* is a potential modifier of behavioral phenotypes observed on the expression of mutant HTT protein (Pavitra Prakash et al., 2022; Payel Ganguly, 2015). Given that the role of *Atg8a* in neurons is not well understood (Ratliff et al., 2015), I carried out detailed

studies to examine the cellular processes that enabled behavioural rescue. In summary, I demonstrate that overexpression of *Atg8a* in presence of mutant HTT protein rescues locomotor activity rhythms and sleep phenotypes. Even though *Atg8a*-mediated rescue was dependent on the autophagy pathway, I observed enhanced aggregation of the mutant protein in the targeted circuits. I further showed that the presence of mutant HTT protein in the circadian neurons hampers the expression of Cathepsin-D (an important lysosomal enzyme), a phenotype rescued in a subset of the targeted neurons upon *Atg8a* overexpression. Finally, I show that *Atg8a* mediated behavioural rescue is possibly an outcome of improved communication of the targeted neurons to the downstream neurons. Overall, this study shows that genetic modulation of the autophagy pathway can largely mitigate the toxicity caused by mutant HTT protein in a subset of *Drosophila* circadian neurons.

2.2 Material and Methods

2.2.1 Fly Lines:

Transgenic fly line with 548 aa of the human Huntingtin (*Htt*) gene either with pathogenic 128 poly-glutamine repeats (w^{1118}*; UAS-Htt128; +*) or controls containing no poly-glutamine repeats (w^{1118}*; UAS-HttQ0; +*) were used for this study and were a generous gift from Troy Littleton, MIT (Lee et al., 2004). *PdfGAL4* driver line was obtained from Todd Holmes, UC Irvine. Other UAS lines: yw^{1118}*; +; UAS-GFP-Atg8a* (BL 51656), UAS RNAi: *yv; +; UAS Atg1RNAi* (BL 26731), *yv; +; UASAtg5RNAi*, and w^{1118}*; +; +* (BL 5905), lines were procured from Bloomington Stock Centre, Indiana. All fly lines were maintained on a standard cornmeal medium under LD12:12 at 25°C.

The *UAS-HttQ128* and *UAS-HttQ128; UAS-Atg8a* lines were used to generate the *w; PdfGal4/UAS-Q128* (mutant) and *w; PdfGal4/UAS-Q128; UAS-Atg8a/+* (rescue) lines which would express mutant HTT-Q128 protein or co-express *Atg8a* and HTT-Q128 in the PDF neurons. The UAS control lines are referred to as *Q128; Atg8a* and their driver control lines as *Pdf/+*. Their corresponding control lines with no poly-Q expansion is denoted *Pdf>Q0, Atg8a*.

2.2.2 Behavioural assays:

Activity-rest rhythm for male flies was recorded by using ***D****rosophila* **A**ctivity **M**onitoring (DAM, Trikinetics™, Waltham, MA) system. 2-3-day old flies of the desired genotypes were loaded in glass tubes containing food at one end and a cotton plug at the other end. Experiments related to *PdfGal4* drivers were done in 7mm glass tubes, and the locomotion data was recorded in 1min bins continuously for 21 days (starting from Day 3, post eclosion) under constant darkness (DD, 25°C). Experiments were performed in incubators manufactured by Sanyo (Japan) or Percival (USA) or in light-tight boxes placed in temperature-controlled cubicles.

2.2.3 Activity-rest data analysis:

For activity rhythm analysis, raw activity count data was scanned and binned in 15min. Data was analyzed with CLOCKLAB software (Actimetrics, Wilmette, IL) using Chi-square periodogram with a cut-off of $p < 0.05$ to determine whether the flies are rhythmic and the amplitude of the periodogram. A fly was considered rhythmic if the periodogram amplitude was above the cut-off and was also validated by visual inspection of its actogram. For circadian circuit data, activity rhythm features such as percentage rhythmicity and robustness of rhythm were calculated over three 7-day windows to track progressive changes. The three temporal windows (age windows or AWs) spanning 21d are designated as age window 1 (AW1: age 3day-9day), age window 2 (AW2:

age 10 day-16day), and age window 3 (AW3: age 17day -23day). To obtain a measure of changes in activity consolidation across days, we calculated '*r*' for each day of individual flies using a custom-made MATLAB code as described in (Prakash et al., 2022).

2.2.4 Immunocytochemistry:

Adult male fly brains were dissected at specified ages in ice-cold PBS and fixed with 4% paraformaldehyde at room temperature (RT) for 45 minutes. 10% horse serum in 0.5% PBT was used as blocking solution. Overnight blocking was done at 4°C. Post-blocking, samples were incubated in primary antibodies for 48h at 4°C. Incubation with secondary antibodies was done for 24h at 4°C. Whole brains were mounted on slides using 70% glycerol in PBS. Primary antibodies used were anti-Huntingtin - Mouse (1:500) (Millipore MAB2166), anti-PDF - Rabbit (1:30,000) (a gift from Michael Nitabach, Yale University), anti-PDF - Mouse (1:5000) (DSHB PDF C7), anti-HSP70 – Mouse (Thermo Scientific), anti-PDF - Rat (1:3000) (a gift from Jae Park), anti-PER - Rabbit (1:20,000) (a gift from Jeffrey C Hall, Brandeis University), anti-GFP - Chicken (1:2000) (Invitrogen), anti-Cathepsin-D (1:250) (CST, 2284) and anti-Ref(2)P - Rabbit (1:500) (Abcam, ab56416). Secondary antibodies Alexa fluors (Invitrogen) (1:3000) anti-rabbit-488, -546 and -647, anti-mouse-546 and -647, and anti-chicken-488 and anti-rat-647 were used.

2.2.5 Image acquisition and quantification:

Brain samples were imaged using Zeiss microscope (LSM 880) either at 20X, 40X (oil-immersion) or 63X (oil immersion) objective with the same - zoom, laser power, gain, and other settings for a given experiment. While the specific settings varied for different experiments, they were kept constant for all the control and experimental genotypes within an experiment. All image analysis was carried out on raw image files. PDF^{+ve} small and large neurons were distinguished based on their position and size. Quantification (cell number) of both the cell types were done manually

based on anti-PDF and -HTT staining by going through each stack of the captured images for all the genotypes.

Aggregate quantification was done using ImageJ. Maximum intensity projection (MIP) images were used for quantification of both aggregated and non-aggregated HTT-Q128 staining. Quantification was done for each brain hemisphere separately by marking the area of the PDF neurons and some initial part of the projection. Thresholding of the MIP images was done keeping consistent parameters for both genotypes. The *analyse particles* tool with size specification of 0.5 to ∞ (for large neurons) and 0.6 to ∞ (for small neurons) was used to obtain measures of inclusion number and size of inclusion. The quantification method did not distinguish between HTT inclusions and spots, resulting in spots being included in the inclusion number and size quantification. Colocalized Ref(2)P-HTT or Cath-D-HTT levels were quantified from the area marking the PDF neurons using the colocalization tool in ImageJ. In small neurons, the number of colocalization events was quantified. However, in large neurons intensity of colocalization was quantified and the same is plotted in the results section.

The signal intensity of mutant HTT, PERIOD protein, and PDF neuropeptide was quantified using maximum-intensity projection images. The raw intensity of the respective proteins/ neuropeptide was quantified by marking the area of the projections or the cell bodies. Further, using the same area, background staining intensity was also quantified. Before plotting and analysis, background intensity was subtracted from raw values.

Wherever representative images are provided, to enable better visualization, adjustments for brightness and contrast have been applied for individual channels separately.

2.2.6 Experimental Design and Statistical analysis

Each assay or experiment included all possible experimental genotypes with age-matched individuals. All statistical analysis was done using Statistica 7. Data for fraction of rhythmic individuals and Cathepsin-D positive cells were compared using the Chi-Square test. m x n and 2x2 Fisher Exact test were done using iCalcu (https://www.icalcu.com/stat/chisqtest.html) and vassarstats (http://vassarstats.net/newcs.html). For pairwise comparisons, *p*-value was adjusted through Bonferroni corrections. Amplitude of periodogram, period, and *r*-values were compared using repeated measures ANOVA, keeping genotype as a fixed factor. Sleep parameters, mutant HTT protein aggregates, and size analysis, large neurons Ref(2)P-HTT-Q128 colocalization, PDF neuropeptide levels were compared using One-Way ANOVA, keeping genotype as a fixed factor. Post-hoc multiple comparisons in all the cases were carried out using Tukey's Honest Significant Difference test with α=0.05. For PDF^{+ve} cell number, PERIOD protein levels, Cath-D-HTT-Q128 colocalization, and PER^{+ve} DN cells quantification were compared using Kruskal – Walli's test was done keeping genotype as a fixed factor. $HSP70^{+ve}$ small and large neuron numbers, non-aggregated HTT protein intensity, small neurons Ref(2)P-HTT-Q128 colocalization were compared using Mann-Whitney U test, keeping genotype as a fixed factor. Statistics and *p*-value till four decimal places are mentioned in the results section. When the values were so small as to have more than 4 zeroes post-decimal, they are represented as $p << 0.05$.

Data related to this chapter is provided in Appendix Chapter 2.

2.3 Results

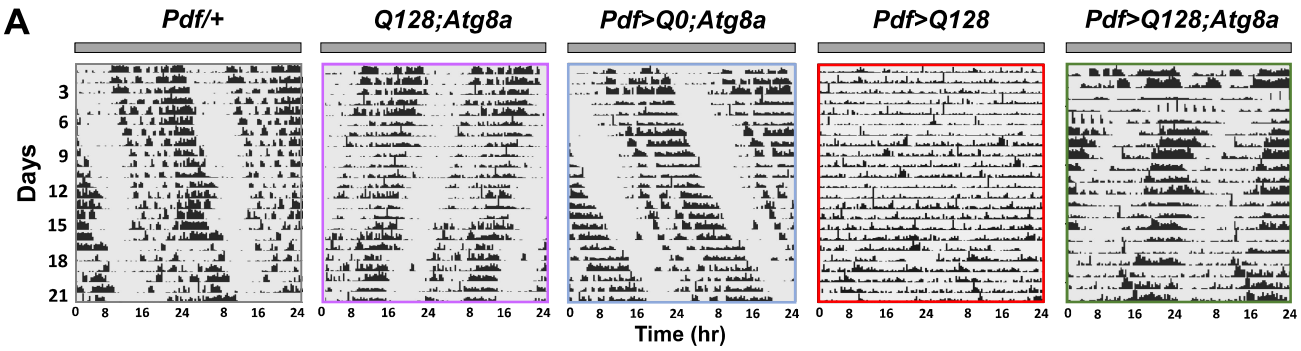
2.3.1 *Atg8a* over-expression in PDF neurons rescues activity rest rhythm under constant darkness (DD)

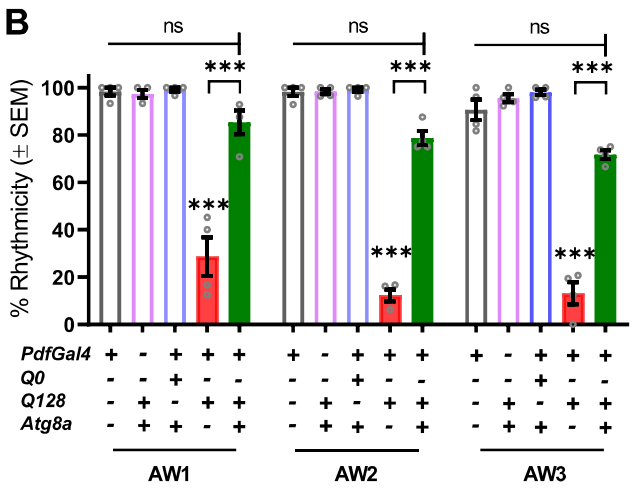
An initial screen from the lab revealed that *Atg8a* is a potential modifier of behavioral defects caused by the expression of human mutant HTT protein in the PDF neurons (Payel Ganguly, 2015; Prakash et al., 2022). Before exploring the cellular changes, I first checked whether *Atg8a* overexpression rescues behavioral defects in PDF neurons. Using a similar approach, I first quantified the fraction of individuals that show rhythmic locomotor activity when *Atg8a* is co-expressed with HTT-Q128 protein in PDF neurons. As expected, almost all control individuals were rhythmic for at least 3 weeks (**Figure 2.1A**). To examine age-dependent changes in rhythmicity, I analyzed features of the rhythm in three consecutive Age Windows (AW – each comprising 7 days; **Figure 2.1B, C, D**). HTT-Q128 expressing flies showed a significant reduction in rhythmicity even as early as AW1 (**Figure 2.1B, red bar**). *Atg8a* over-expression in presence of the mutant HTT-Q128 protein led to a significant improvement in the fraction of rhythmic individuals (**Figure 2.1B, green bar**). However, when I examined the robustness of the rhythm using the amplitude of the periodogram (Chi-square periodogram analysis) I observed that *Atg8a* overexpressing flies exhibited poorer quality of rhythms (**Figure 2.1C, green bar**). I also tabulated the circadian period values of the control and experimental genotypes. Overexpression of *Atg8a* or co-expression of non-pathogenic HTT-Q0 with *Atg8a* in the PDF neurons leads to an increase in the circadian period in the later age windows (**Figure 2.1D**). The few flies that remained rhythmic upon expression of mutant HTT-Q128 protein in the PDF neurons exhibited periodicity close to 24h and did not appear to be different from parental controls (visual observation; no statistical analysis due to low sample size of *Pdf>Q128* flies). Further, in AW1, flies co-expressing

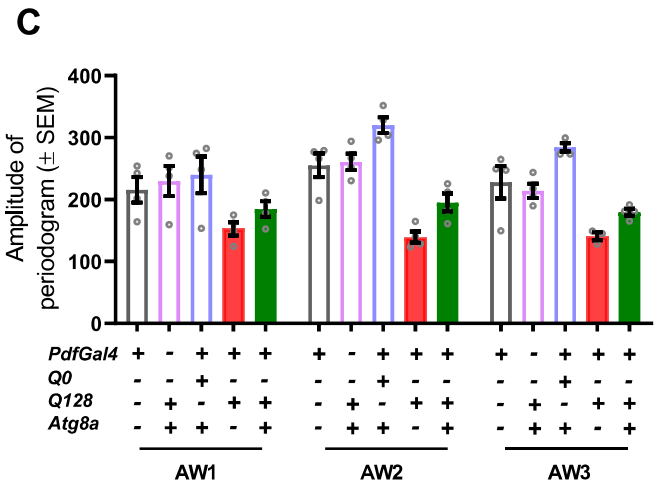
the mutant HTT-Q128 protein with *Atg8a* showed period values comparable to the control genotypes. However, at later age windows, period values decrease (**Figure 2.1D**), possibly because of the presence of mutant HTT-Q128 protein. To obtain greater temporal resolution for the quality of activity rhythm I calculated '*r*', an estimator of consolidation of activity (Prakash et al., 2017, 2022). Control flies (*Pdf>Q0; Atg8a*) showed a higher *r*-value (of around 0.5) and it was maintained consistently across days. HTT-Q128 expression led to a significant reduction in *r*-value (of around 0.2). Co-expression of *Atg8a* with HTT-Q128 led to a significant improvement in the *r*-value and was sustained across days (**Figure 2.1E, green line**).

LNv neurons are also important modulators of sleep (Potdar & Sheeba, 2018; Sheeba et al., 2008) and expression of HTT-Q128 protein leads to sleep defects (Faragó et al., 2019). I asked if *Atg8a* overexpression can also improve sleep defects. I quantified total sleep, length, and the number of sleep episodes for the first 4 days under DD (age 5-8 days). Total sleep of HTT-Q128 expressing flies and those co-expressing *Atg8a* were not different from control genotypes (**Figure 2.1F**). More detailed characterization showed that sleep quality in terms of bout length and number was affected by HTT-Q128 expression (**Figure 2.1G and H, red bar**). I observed that *Atg8a* overexpression led to changes in both, however, significant improvement was only observed in the number of sleep bouts (**Figure 2.1G and H, green bar**). Thus, these results show that *Atg8a* over-expression can rescue the behavioral phenotypes of arrhythmic locomotion and sleep disruptions caused by HTT-Q128 expression in circadian pacemaker neurons.

Figure 2.1

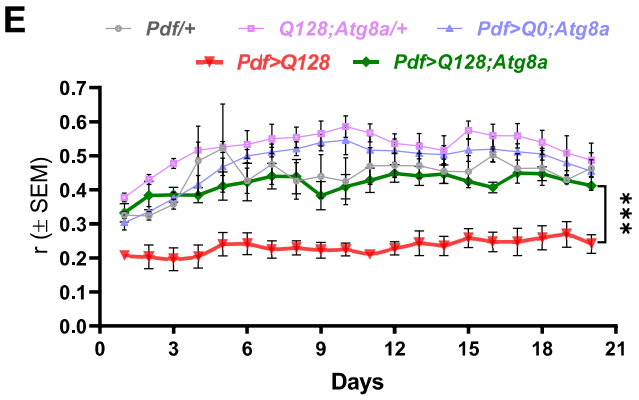


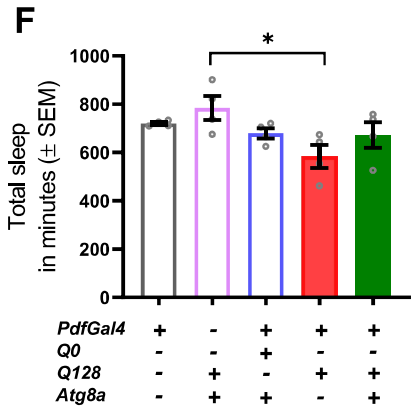


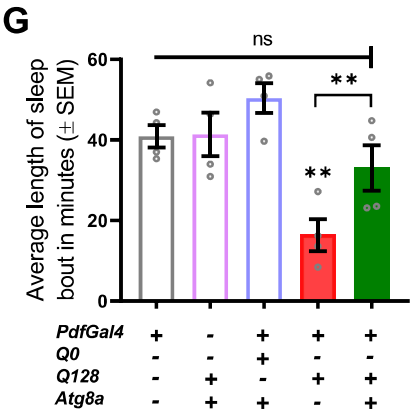


D

Periodicity values for control and experimental genotype (hr)			
Genotypes	AW 1	AW 2	AW 3
Pdf/+	24.01	24.12	24.16
Q128; Atg8a	23.69	23.70	23.76
Pdf>Q0	24.24	24.46	24.39
Pdf>Atg8a	24.18	24.56	24.81
Pdf>Q0; Atg8a	24.66	24.96	25.08
Pdf>Q128	24.11	23.81	23.95
Pdf>Q128; Atg8a	24.40	23.54	23.62







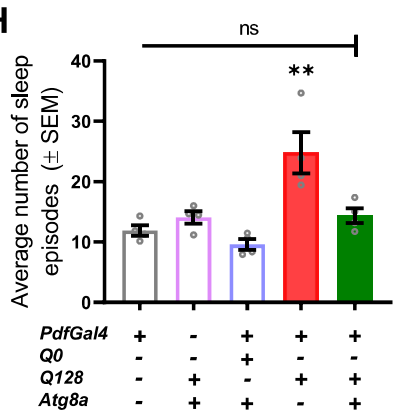


Figure 2.1: ***Atg8a* overexpression in PDF^{+ve} lateral ventral neurons improves activity-rest rhythm in presence of mutant HTT-Q128 protein.**

A) Representative double-plotted actograms for control (*Pdf/+*, *Q128; Atg8a*, *Pdf> Q0; Atg8a*) and experimental (*Pdf>Q128*, *Pdf>Q128; Atg8a*) genotypes, showing activity data for 21 days in constant darkness (DD) at 25°C. **B)** Plot representing quantification of rhythmicity values from four independent replicate experiments for control and experimental genotypes (red bar, green bar) plotted across three age windows (AW1-3; 7 Days each). In comparison with the control genotypes, flies expressing mutant HTT-Q128 protein showed a significant decrease in percentage rhythmicity. Further *Atg8a* over-expression in presence of HTT-Q128 improves the number of rhythmic individuals. **C)** Plots representing mean amplitude values from four independent experiments plotted across three age windows (AW1-3; 7 Days each) for all the genotypes. Atg8a overexpressing flies in presence of mutant HTT-Q128 proteins does not lead to any significant improvement in the amplitude of the periodogram. **D)** Table representing mean period values from four independent replicate experiments for control and experimental genotypes (red bar, green bar) for three age windows (AW1-3; 7 Days each). **E)** Plot representing mean 'r' values from four independent experiments plotted across 20 days for all the genotypes. In comparison with control genotypes, flies expressing mutant HTT-Q128 protein show significantly reduced r value. Significant improvement in the r value was observed on Atg8a overexpression. **F)** Plot representing mean values of total sleep from four independent experiments obtained by averaging total sleep across the first 4 days of the run for all the genotypes. Mutant HTT-Q128 expression and *Atg8a* co-expression in the PDF neurons does not affect the total sleep of the flies and the values were comparable to all the control genotypes (except *Q128; Atg8a*). **G)** Plot representing mean values of length of sleep bout from four independent experiments. Expressing mutant HTT-Q128 protein in PDF neurons led to a significant reduction in the length of sleep bouts. *Atg8a* over-expression led to improvement in the length of sleep bouts however, the values were not comparable to the control genotypes (except *Pdf>Q0; Atg8a*). **H)** Plot representing mean values of the number of sleep episodes from four independent experiments. Expression mutant HTT-Q128 protein in PDF neurons led to a significant increase in the number of sleep episodes. *Atg8a* over-expression significantly reduces the number of sleep episodes.

n> 16 flies for all the genotypes/replicate experiment. (+) and (-) in the bar graphs represent the presence or absence of the gene in the fly. Asterisk on individual genotypes means that the genotype is significantly different from all other plotted genotypes. * $p < 0.05$.

2.3.2 Autophagy pathway is involved in *Atg8a*-mediated behavioral rescue

Even though *Atg8a* is crucial for the autophagy pathway (Nguyen et al., 2016), to the best of our knowledge, it is not known whether its overexpression induces autophagy in neuronal cells. To confirm that indeed autophagy pathway modulation is the basis of the *Atg8a*-mediated behavioral rescue, I first focused on Ref(2)P, a key autophagy adaptor protein (Lippai & Low, 2014), and hypothesized that changes in Ref(2)P levels would reflect the status of the autophagy. Colocalized Ref(2)P was detected in both the experimental genotypes (**Figure 2.2**). In small neurons (3rd instar larva), I observed that co-expression of *Atg8a* with mutant HTT-Q128 protein led to a significant increase in colocalized Ref(2)P-HTT-Q128 levels (**Figure 2.2A, green bar**). No significant change was observed in the large neurons upon *Atg8a* over-expression (Day 1). However, at a later stage (Day 10), a small but significant increase in the Ref(2)P-HTT-Q128 colocalization was observed (**Figure 2.2B, green bar**). Further, a comparison between Day 1 and Day 10 also revealed a significant increase in Ref(2)P-HTT-Q128 colocalization upon *Atg8a* overexpression, however, such an increase was not observed in flies only expressing mutant HTT-Q128 protein. Overall, increased Ref(2)P colocalization to the HTT-Q128 aggregates suggests the involvement of the autophagy pathway in *Atg8a*-mediated rescue.

I further reasoned that if *Atg8a* overexpression was indeed bringing about the rescue via the autophagy pathway, then the downregulation of key autophagy genes should prevent the observed rescue. I first downregulated an upstream autophagy gene, *Atg1* in flies co-expressing HTT-Q128 and *Atg8a* and recorded activity rhythms for two AW. Control flies showed ~ 90% rhythmicity in both age windows (**Figure 2.2C**). Downregulation of *Atg1* alone in the LNvs did not lead to any decrease in the fraction of rhythmic individuals (**Figure 2.2C, black bar**). As expected, flies co-expressing HTT-Q128 and *Atg8a* were rhythmic (**Figure 2.2C, green bar**).

Downregulation of *Atg1* in the rescue background (*Pdf>Q128; Atg8a, Atg1RNAi*) did not lead to any significant reduction in the rhythmicity in the first age window (AW1). However, by the second age window, these flies showed significantly reduced rhythmicity (**Figure 2.2C, filled green bar**). Further to verify these observations, I downregulated another key autophagy gene (*Atg5*) in the rescue background and asked whether it also recapitulates phenotypes same as the *Atg1* downregulation experiment. As expected, all control flies showed near ~ 100% rhythmicity (**Figure 2.2C**), and downregulation of *Atg5* alone in the targeted neurons did not lead to any decrease in the fraction of rhythmic individuals (**Figure 2.2C, black bar**). Expression of mutant HTT-Q128 protein in the targeted neurons leads to a significant reduction in the number of rhythmic individuals. Co-expression of *Atg8a* with mutant HTT-Q128 protein leads to a significant increase in the number of rhythmic individuals (**Figure 2.2C, green bar**). Downregulation of *Atg5* in the rescue background (*Pdf>Q128; Atg8a, Atg5RNAi*) did not lead to any significant reduction in the rhythmicity in the first age window. However, consistent with the *Atg1* downregulation experiment, *Atg5* downregulation in the rescue genotype showed a significant reduction in the number of rhythmic individuals in the second age window (**Figure 2.2C, filled cyan bar**). Overall, compromised rescue at later stages indicates that the autophagy pathway is being harnessed in mitigating the toxicity of HTT-Q128.

Figure 2.2

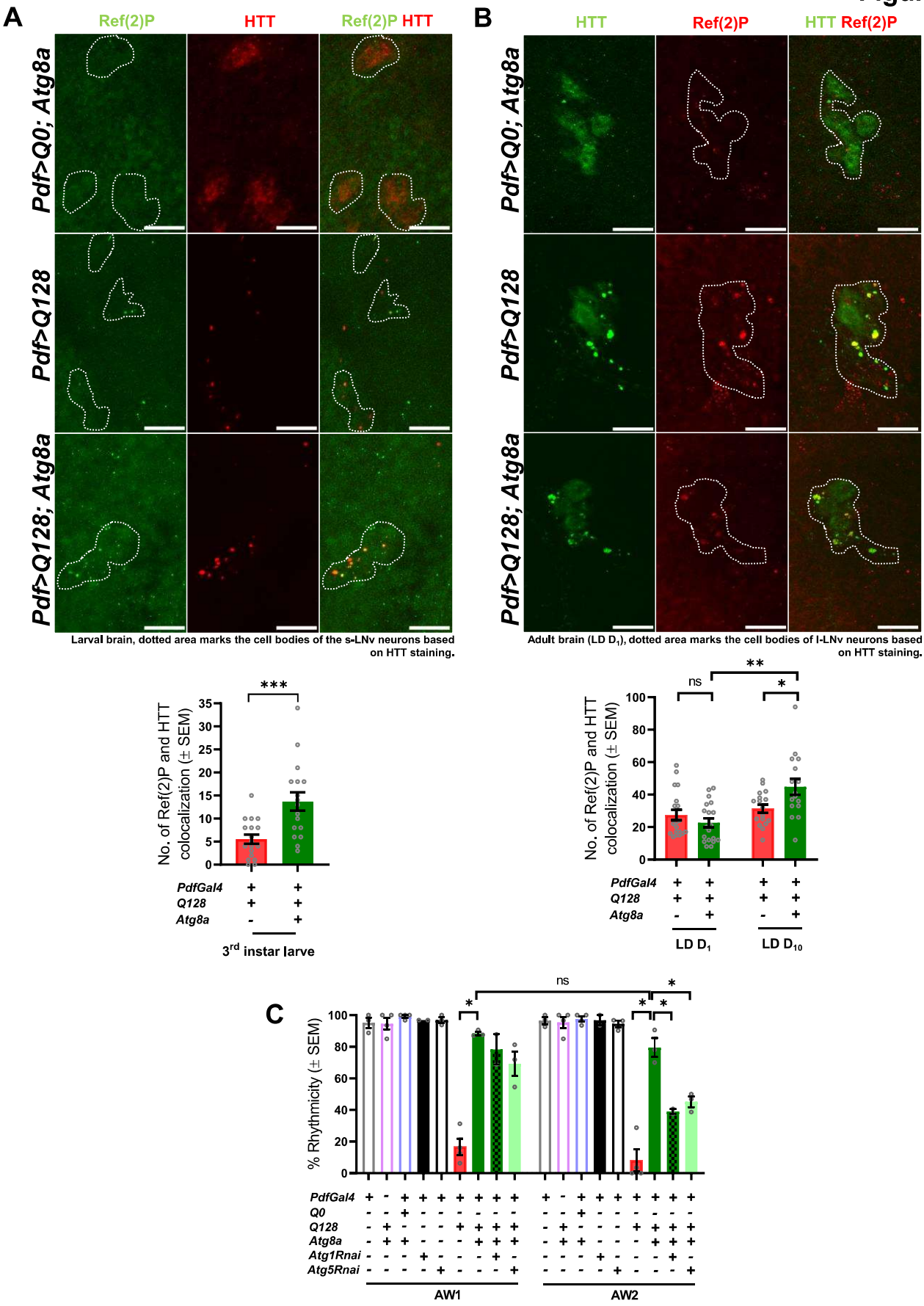

A
Ref(2)P
HTT
Ref(2)P HTT
Pdf>Q0; Atg8a
Pdf>Q128
Pdf>Q128; Atg8a
Larval brain, dotted area marks the cell bodies of the s-LNv neurons based on HTT staining.
B
HTT
Ref(2)P
HTT Ref(2)P
Pdf>Q0; Atg8a
Pdf>Q128
Pdf>Q128; Atg8a
Adult brain (LD D1), dotted area marks the cell bodies of l-LNv neurons based on HTT staining.
No. of Ref(2)P and HTT colocalization (± SEM)
***
PdfGal4
Q128
Atg8a
3rd instar larve
ns
**
*
LD D1
LD D10
C
% Rhythmicity (± SEM)
Q0
Atg1Rnai
Atg5Rnai
AW1
AW2

Figure 2.2: **Increased Ref(2)P levels in the rescue genotype suggests that *Atg8a-mediated* rescue in activity-rhythm is autophagy-dependent.**

A) Representative maximum intensity projection images of small PDF neurons (from L3 stage, larval brain) depicting staining for Ref(2)P protein (marked in green), control (HTT-Q0), and mutant HTT-Q128 (marked in red). Scale bar - 20µm. Plot representing quantification of the Ref(2)P-HTT colocalization events in small neurons for both the experimental genotypes. A significant increase in the Ref(2)-HTT colocalization was observed on *Atg8a* overexpression. n> 16 brain hemispheres (for both genotypes). **B)** Representative maximum intensity projection images (LD Day1, adult brain) of PDF neurons depicting staining for Ref(2)P protein (marked in red), control (HTT-Q0), and mutant HTT-Q128 (marked in green). Scale bar - 20µm. Plot representing quantification of the number of Ref(2)P-HTT colocalization events quantified across two different ages (Day1 and 10) for both the experimental genotypes from large neurons. A significant increase in the Ref(2)P-HTT colocalization was observed with age in flies co-expressing mutant HTT protein and *Atg8a*. n> 16 brain hemispheres (for both genotypes). **p* <0.05. **C)** Plot representing quantification of rhythmicity values quantified across two age windows (8 days each) for all the genotypes. *Atg8a* overexpression results in a significant increase in the number of rhythmic individuals. However, downregulation of *Atg1* or *Atg5* gene in rescue genotype results in a significant reduction in the number of rhythmic individuals with age, suggesting the involvement of the autophagy pathway in *Atg8a* mediated behavioural rescue n> 16 flies/genotype. **p* <0.05.

The dotted area marks the cell bodies of the small neurons. (+) and (-) in the bar graphs represent the presence or absence of the gene in the fly. Asterisk on individual genotypes means that the genotype is significantly different from all other plotted genotypes. * *p* < 0.05.

2.3.3 HTT-Q128 hampers Cathepsin-D expression

Lysosomes are key components of the autophagy pathway. Studies have shown that the activity of lysosomal enzymes involved in the degradation of cargo increases upon autophagy induction (Settembre et al., 2011, 2013; Stransky & Forgac, 2015). I examined the status of lysosomes starting at the 3rd instar larval stages when small neurons already show aggregates of HTT-Q128. I stained for Cathepsin-D (Cath-D) a key enzyme involved in lysosome function (Benes et al., 2008; Sevlever et al., 2008). Cath-D was not observed in the small neurons of control or flies expressing mutant HTT-Q128 protein. However, flies co-expressing HTT-Q128 and *Atg8a* showed punctate staining for the Cath-D in the soma of small neurons (**Figure 2.3A, right top graph**). I further checked whether mutant HTT-Q128 protein aggregates co-localize with Cath-D staining and indeed I observed a significant increase in the number of Cath-D-HTT-Q128 co-localization events in flies co-expressing both mutant HTT-Q128 protein and *Atg8a* (**Figure 2.3A, right bottom graph**).

To examine the large neurons which appear only during pupal stages, I imaged adult brains (on Day 2, post-eclosion). The control genotype showed diffuse and punctate Cath-D staining in the soma. However, very faint or no staining was observed in flies either expressing only HTT-Q128 or co-expressing *Atg8a* with the mutant HTT-Q128 protein (**Figure 2.3B, right top graph**). Colocalization quantification further supports these results, wherein a significantly high colocalization intensity of Cath-D-HTT-Q128 was observed in the control genotype in comparison with the experimental genotypes (**Figure 2.3B, right bottom graph**). Overall, improved Cath-D staining in the larval small neurons further strengthens the idea of autophagy-mediated rescue by *Atg8a* overexpression.

Figure 2.3

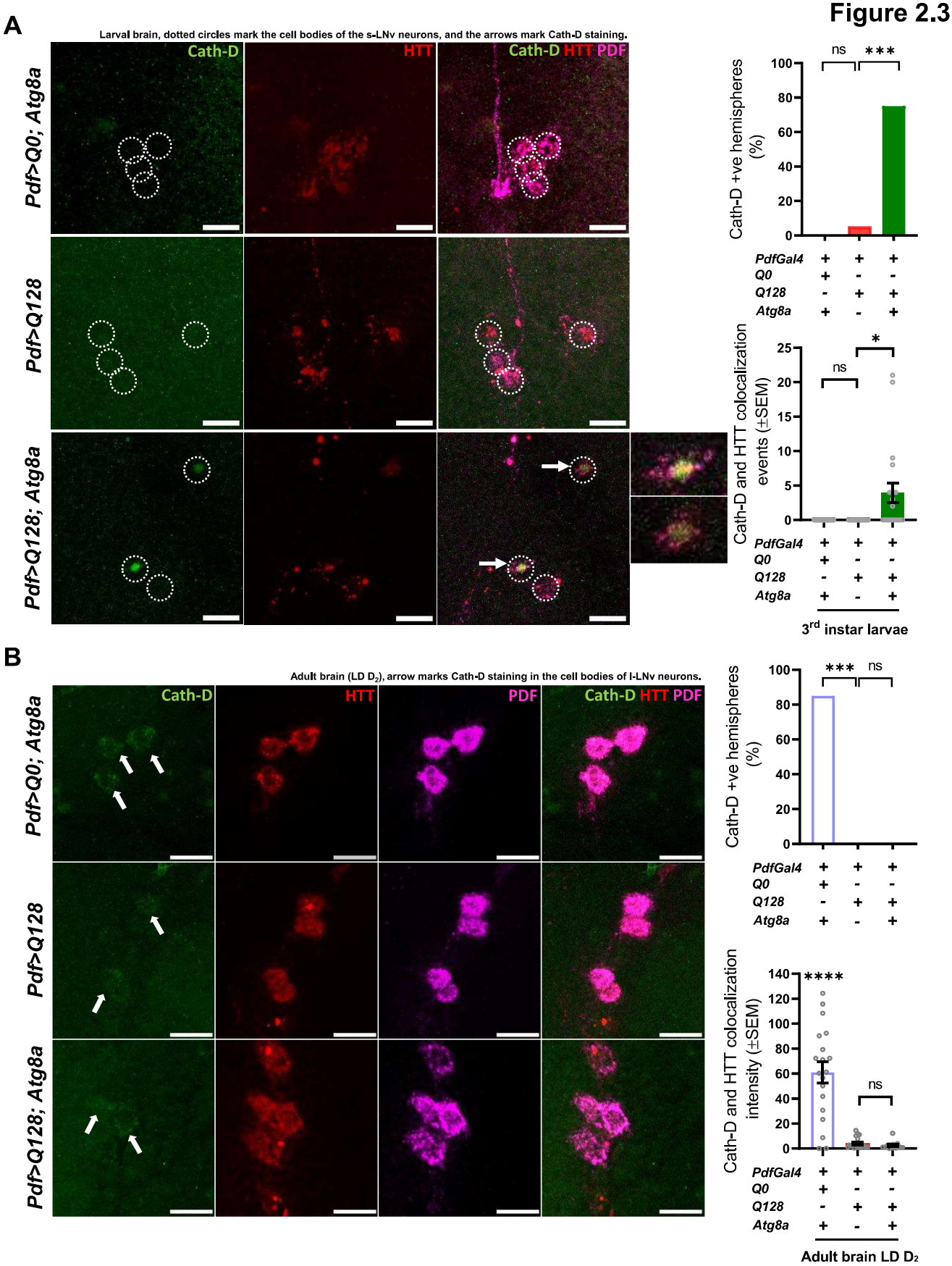

A
Larval brain, dotted circles mark the cell bodies of the s-LNv neurons, and the arrows mark Cath-D staining.
Cath-D
HTT
Cath-D HTT PDF
Pdf>Q0; Atg8a
Pdf>Q128
Pdf>Q128; Atg8a
Cath-D +ve hemispheres (%)
ns
***
PdfGal4
Q0
Q128
Atg8a
Cath-D and HTT colocalization events (±SEM)
*
3rd instar larvae
B
Adult brain (LD D2), arrow marks Cath-D staining in the cell bodies of l-LNv neurons.
PDF
Cath-D and HTT colocalization intensity (±SEM)
****
Adult brain LD D2

Figure 2.3: ***Atg8a* overexpression improves Cathepsin-D staining (possibly lysosome functioning) in the small neurons.**

A) Representative maximum intensity projection images (at L3 stage, larval brain) of small PDF^{+ve} neurons, depicting staining of Cath-D (marked in green), control (HTT-Q0) and HTT-Q128 (marked in red), and PDF neuropeptide (marked in magenta). Scale bar - 20µm. No punctate staining of Cath-D was observed in both the control genotype and flies expressing mutant HTT-Q128 protein. However, Cath-D staining was observed in flies co-expressing both the mutant HTT-Q128 protein and *Atg8a* (top right graph). Further, significantly high Cath-D-HTT-Q128 colocalization events were observed in flies co-expressing both the mutant HTT-Q128 protein and *Atg8a*. n> 14 brain hemispheres/genotype. **B)** Representative maximum intensity projection images (LD Day1, adult brain) of large PDF^{+ve} neurons, depicting staining of Cath-D (marked in green), control (HTT-Q0) and HTT-Q128 (marked in red), and PDF neuropeptide (marked in far-red). Scale bar - 20µm. Control genotype shows diffuses and punctate staining of Cath-D in the large neurons. However, no or very faint Cath-D staining was observed in flies either expressing mutant HTT-Q128 protein or co-expressing mutant HTT-Q128 protein with *Atg8a* (bottom top right graph). Further, colocalization quantification further supports the presence or absence of Cath-D staining in the large PDF neurons (bottom right graph). n> 16 brain hemispheres/ genotype. **p* <0.05.

(+) and (-) in the bar graphs represent the presence or absence of the gene in the fly. Asterisk on individual genotypes means that the genotype is significantly different from all other plotted genotypes. * $p < 0.05$.

2.3.4 Behavioral rescue is not accompanied by improvement in PDF neuropeptide levels (in soma)

PDF is an important signaling molecule only released by the lateral ventral neurons. Expression of mutant HTT-Q128 protein has been shown to affect the levels of PDF neuropeptide (mainly in the soma) in a subset of lateral ventral neurons named small lateral ventral neurons – s-LNv and another subset named large lateral ventral neurons (l-LNv) remain unperturbed (Prakash et al., 2017, Xu et al., 2019). To relate behavioural improvements to cellular changes, I first asked whether these behavioural changes are because of dilution effects caused by expression of two UAS constructs (*UAS-HTT-Q128* & *UAS-GFP-Atg8a*) under a single Gal4 driver. To assess this, I quantified the levels of HTT-Q0 protein from both subsets of neurons in flies only expressing HTT-Q0 protein or co-expressing HTT-Q0 with *Atg8a* in the PDF neurons. Co-expression of HTT-Q0 protein and *Atg8a* does not lead to any decrease in the levels of HTT-Q0 protein in both the subsets of neurons (**Figure 2.4B**). I also recorded activity-rest rhythm for flies only expressing HTT-Q128 protein or co-expressing HTT-Q128 and UAS-GFP (another UAS transgene in place of UAS-*Atg8a* which is expected to be benign but could potentially result in dilution effects) in the PDF neurons. It was observed that co-expression of both UAS constructs in PDF neurons does not lead to any significant improvement in the activity-rest rhythm in comparison to flies only expressing HTT-Q128 protein (**Figure 2.4C**). Overall, both immunocytochemistry and behavioral experiments point out that the expression of two UAS constructs does not decrease the expression of the HTT protein or lead to any improvement in the activity rhythm suggesting that the behavioral rescue on *Atg8a* overexpression is possibly not an outcome of dilution effects.

Now to relate behavioral improvements to cellular changes, I asked whether *Atg8a*-overexpression rescues PDF neuropeptide levels in the soma of the targeted neurons, especially in

the small lateral ventral neurons as they are important for the maintenance of activity-rest rhythm under constant darkness. To access, I quantified the number of detectable LNvs in both the experimental genotypes (*Pdf> HttQ128* and *Pdf> HttQ128; Atg8a*) based on PDF and HTT staining. Flies expressing non-toxic huntingtin protein HTT-Q0 with *Atg8a* showed ≈ 4 small and large LNvs (**Figure 2.4D and E, blue unfilled bar**). I observed that HTT-Q128 expression in the LNv neurons causes a sharp decline in the number of PDF^{+ve} small neurons (**Figure 2.4D, red bar**), and with age, the number further declines. Co-expression of *Atg8a* with mutant HTT-Q128 protein showed a consistent increase in the small neurons number, but the improvement was not significantly different from the mutant protein expressing flies (**Figure 2.4D, green bar**). Furthermore, as expected no change in the number of large neurons was observed in any of the experimental genotypes and the numbers were comparable to the control genotype (**Figure 2.4E, red and green bars**).

Since no changes were observed in the number and PDF levels of large neurons, I asked if their ability to tolerate stressful conditions is better than the small neurons. To examine this, adult fly brains were dissected at two different ages (Day 1, 18, and 36) and were co-immunostained against PDF and Heat Shock Protein 70 (HSP70, a stress marker (Kim et al., 2020)). No HSP70 staining was observed in the small and large neurons of the control genotypes (**Figure 2.5A, top panel**). On Day 1, most of the small neurons of HTT-Q128 expressing flies showed HSP70 staining, while only a few large neurons were $HSP70^{+ve}$ (**Figure 2.5B and C, red bar**). Co-expression of *Atg8a* with the mutant HTT-Q128 protein did not lead to any significant decrease in the number of $HSP70^{+ve}$ small or large neurons (**Figure 2.5B and C, green bar**). When compared between Day 1 and Day 18 or Day 36, no significant difference was observed in the number of $HSP70^{+ve}$ large neurons between experimental genotypes, however a significant reduction in the

number of HSP70^{+ve} small neurons was observed in both the genotypes (**Figure 2.5B**). Taken together, PDF-based cell quantification and HSP70 staining data suggest that *Atg8a* overexpression does not lead to any significant improvement in the number of small neurons. Moreover, HSP70 staining suggests that the large neurons are more resistant to stress, and the gradual decline in the number of HSP70^{+ve} small neurons possibly points toward the loss of these neurons with age.

Figure 2.4: ***Atg8a* overexpression in PDF^{+ve} lateral ventral neurons does not improve the levels of PDF neuropeptide in the small ventral lateral neurons (s-LNv).**

A) Representative maximum intensity projection images (Day1, adult brain) of PDF neurons (large neurons represented by an arrow and small neurons represented by dotted circles) depicting staining of GFP-*Atg8a* (marked in green), mutant HTT-Q128 (marked in red), and PDF (marked in magenta). The right-top cartoon represents the right half of the *Drosophila* brain depicting the position of PDF neurons in the brain. Scale bar - 20µm. **B)** Plot representing quantification of HTT protein intensity from small and large neurons when only HTT-Q0 protein or HTT-Q0 and *Atg8a* protein were co-expressed. No significant decrease in the intensity of HTT protein was observed on the expression of two UAS constructs in the PDF neurons. n> 16 brain hemispheres. **C**) Plot representing % rhythmicity values for control and experimental genotypes. No significant improvement in % rhythmicity was observed when mutant HTT-Q128 protein was co-expressed with UAS-*GFP*. n> 17 flies/genotype. **D)** Plot representing quantification of the number of PDF^{+ve} small neurons based on PDF neuropeptide and HTT-Q128 staining. Mutant HTT-Q128 protein expression led to a significant reduction in the number of small neurons. A small improvement in the number of small neurons was observed on *Atg8a* overexpression. n>12 brain hemispheres (for control genotype), n> 16 brain hemispheres (for experimental genotype). **E)** Plot representing quantification of the number of PDF^{+ve} large neurons based on PDF neuropeptide and HTT-Q128 staining. Control genotype showed approx. 4 PDF^{+ve} large neurons per hemisphere. Mutant HTT-Q128 protein expression in the large neurons does not lead to any significant reduction in the number of neurons. Neither any change was observed on *Atg8a* overexpression in presence of mutant HTT-Q128 protein. n>12 brain hemispheres (for control genotype), n> 16 brain hemispheres (for both experimental genotypes).

(+) and (-) in the bar graphs represent the presence or absence of the gene in the fly. Asterisk on individual genotypes means that the genotype is significantly different from all other plotted genotypes. * $p < 0.05$.

Figure 2.4

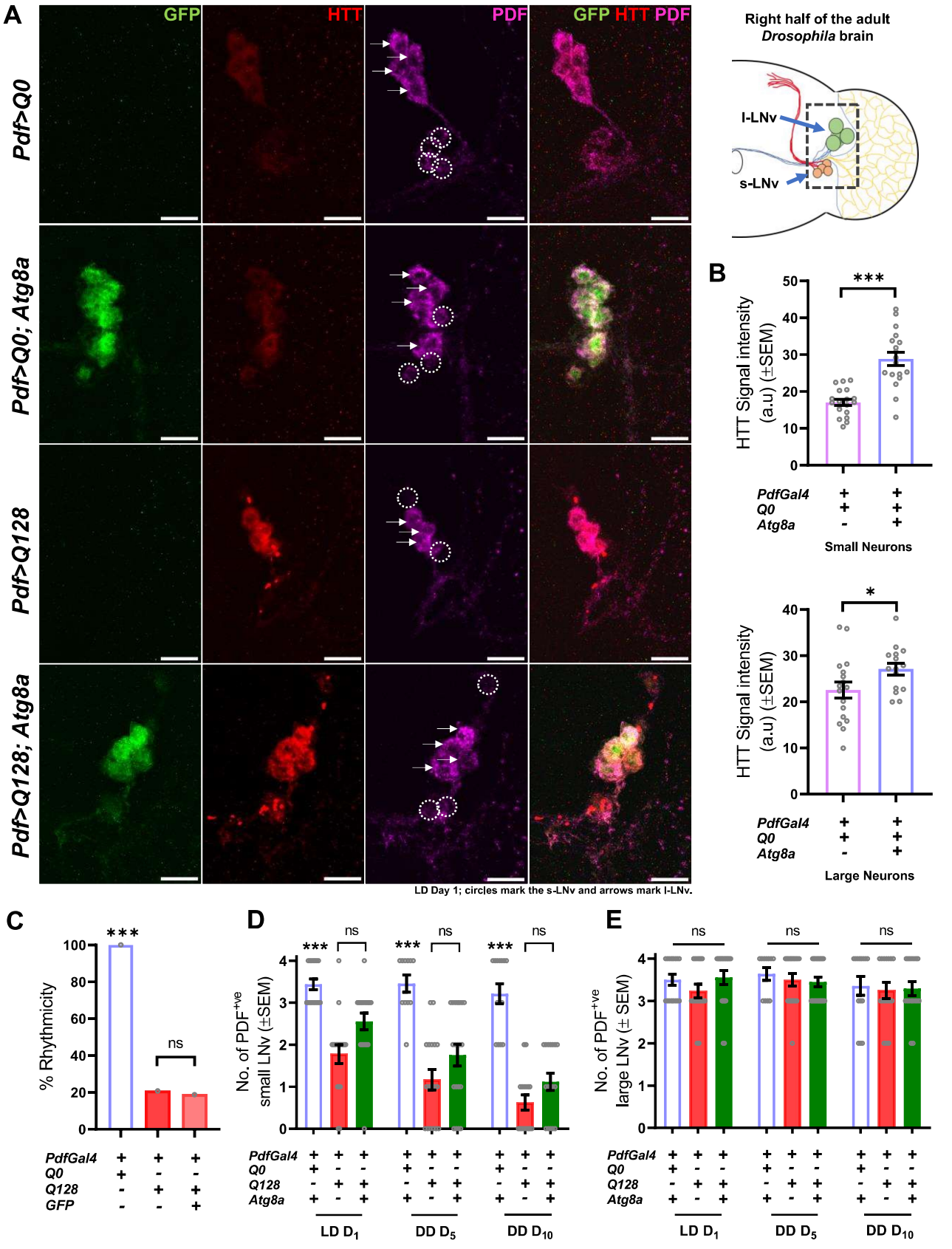

A
GFP
HTT
PDF
GFP HTT PDF
Pdf>Q0
Pdf>Q0; Atg8a
Pdf>Q128
Pdf>Q128; Atg8a
LD Day 1; circles mark the s-LNv and arrows mark l-LNv.
Right half of the adult Drosophila brain
l-LNv
s-LNv
B
HTT Signal intensity (a.u) (±SEM)
***
PdfGal4
Q0
Atg8a
Small Neurons
*
Large Neurons
C
% Rhythmicity
***
ns
PdfGal4
Q0
Q128
GFP
D
No. of PDF+ve small LNv (±SEM)
***
ns
PdfGal4
Q0
Q128
Atg8a
LD D1
DD D5
DD D10
E
No. of PDF+ve large LNv (± SEM)
ns
LD D1
DD D5
DD D10

Figure 2.5

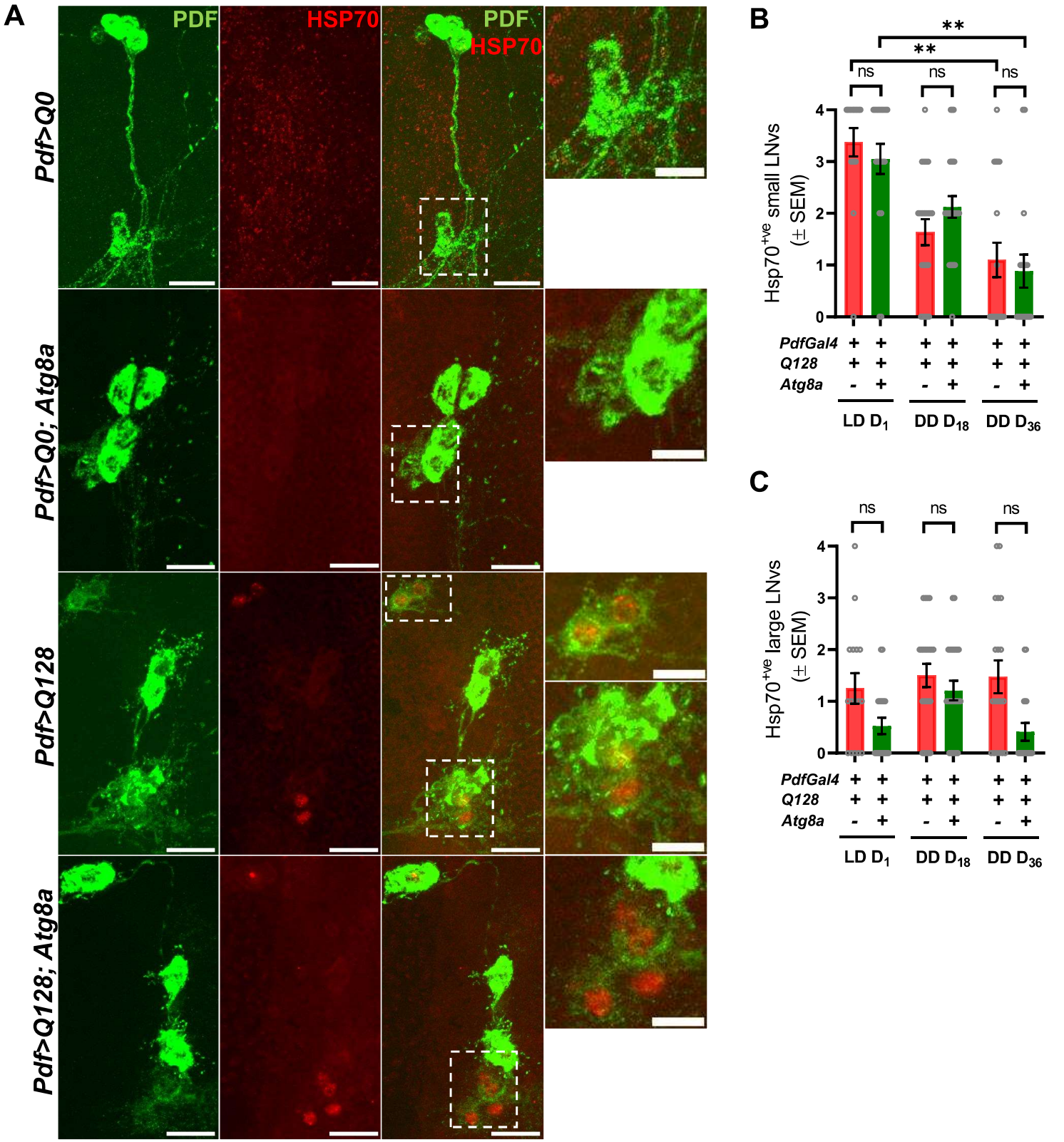

A
PDF
HSP70
PDF
HSP70
Pdf>Q0
Pdf>Q0; Atg8a
Pdf>Q128
Pdf>Q128; Atg8a
B
Hsp70+ve small LNvs
(± SEM)
ns
ns
ns
**
**
PdfGal4
Q128
Atg8a
LD D1
DD D18
DD D36
C
Hsp70+ve large LNvs
(± SEM)
ns
ns
ns
PdfGal4
Q128
Atg8a
LD D1
DD D18
DD D36

Figure 2.5: **Mutant HTT-Q128 induces HSP70 expression in the PDF^{+ve} lateral ventral neurons.**

A) Representative maximum intensity projection images (Day1, adult brain) of PDF neurons (large neurons and small neurons) depicting staining of PDF neuropeptide (marked in green), and HSP70 (marked in red). Scale bar - 20µm. Zoomed in panel shows small neurons. Scale bar - 10µm. **B)** Plot representing quantification of the number of HSP70^{+ve} small neurons. Mutant HTT-Q128 protein expression induces HSP70 protein expression in mainly small neurons at an early age. No significant change was observed in the number HSP70^{+ve} small neurons on *Atg8a* overexpression at both ages. n> 16 brain hemispheres (for both genotypes). **C)** Plot representing quantification of the number of HSP70^{+ve} large lateral ventral neurons. Mutant HTT-Q128 expression led to the induction of HSP70 protein in large neurons but the number of HSP^{+ve} cells is low as compared to small neurons. Further, no significant change was observed in the number HSP70^{+ve} large neurons on *Atg8a* overexpression. n> 16 brain hemispheres (for both experimental genotypes).

(+) and (-) in the bar graphs represent the presence or absence of the gene in the fly. Asterisk on individual genotypes means that the genotype is significantly different from all other plotted genotypes. * $p < 0.05$.

2.3.5 Mutant HTT protein aggregates persist in LNv despite behavioural rescue

The presence of mutant protein aggregates is thought to be toxic to neurons (Davies et al., 1997; DiFiglia et al., 1997). However, other studies have suggested that the soluble form is more toxic (Lajoie & Snapp, 2010; Takahashi et al., 2008). I asked whether the rescue in locomotor activity rhythm upon *Atg8a* over-expression is an outcome of clearance of mutant protein aggregates from the LNvs. To assess this, I quantified the number of mutant protein aggregates (present both in the cell bodies and projections of both the subsets of neurons) and aggregate size from the targeted neurons. Quantification from small neurons was done at the third instar larval stage where mutant HTT-Q128 protein aggregates are present but no loss in the small neurons was observed. For large neurons, quantification was done at Day 1 and Day 10 (post-eclosion). Expression of mutant HTT-Q128 protein led to the formation of protein aggregates in both subsets of LNvs, while no aggregates were observed in flies expressing non-toxic HTT-Q0 construct (**Figure 2.6A and 2.6A**). At the larval stage (quantified at L3), co-expressing HTT-Q128 and *Atg8a* resulted in a significant increase in the number of mutant HTT-Q128 protein aggregates in small neurons (**Figure 2.6B**). Further, when looked at the size of mutant HTT protein aggregates an increase was observed, however, combined all experiments the differences were not significant (**Figure 2.6C)**.

At the adult stage, where the large neurons can be visualized, mutant HTT-Q128 protein (which does not appear to be aggregated) is detected both in the neurons on Day 1 (**Figure 2.6A, right**). I quantified both the aggregated and non-aggregated forms of mutant HTT-Q128 protein from the large neurons. No change was observed in the intensity of the non-aggregated form of mutant HTT-Q128 protein upon *Atg8a* overexpression (**Figure 2.6D, green bar**). The number of large neurons with either the diffuse or aggregated forms of mutant HTT-Q128 protein was also not altered (**Figure 2.6E**). Additionally, in the large neurons *Atg8a* over-expression does not lead

to any significant change in the number of mutant protein aggregates or size (quantified at D1 and D10) (**Figure 2.6F and G, green bar**). Overall, these results show that *Atg8a* over-expression in presence of mutant HTT-Q128 protein led to a significant increase in the number and size of the mutant protein aggregates only in the small neurons.

Figure 2.6

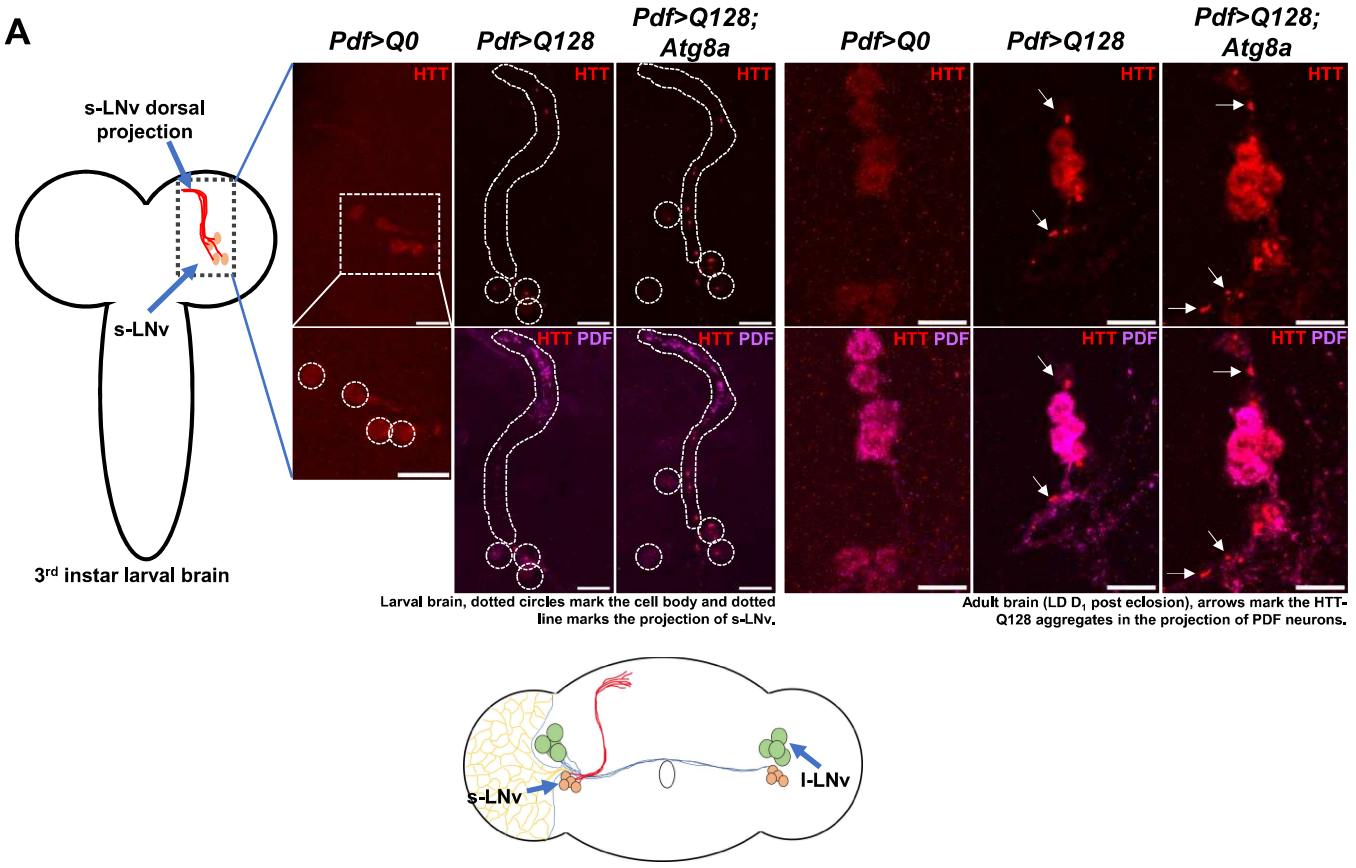

A
Pdf>Q0
Pdf>Q128
Pdf>Q128; Atg8a
Pdf>Q0
Pdf>Q128
Pdf>Q128; Atg8a
s-LNv dorsal projection
s-LNv
3rd instar larval brain
HTT
HTT PDF
Larval brain, dotted circles mark the cell body and dotted line marks the projection of s-LNv.
Adult brain (LD D1 post eclosion), arrows mark the HTT-Q128 aggregates in the projection of PDF neurons.
s-LNv
l-LNv
Aggregate parameters (s-LNv)
Aggregate parameters (l-LNv)
B
No.of HTT aggregates
PdfGal4
Q128
Atg8a
C
Size of HTT aggregates (µm2)
3rd instar larval stage
D
HTT Signal intensity (a.u.) (±SEM)
LD D1
E
Cells with HTT (±SEM)
Diffused+ Aggregates HTT
Diffused HTT
F
No.of HTT aggregates (±SEM)
LD D1
DD D10
G
Size of HTT aggregates (µm2) (±SEM)
ns
*

Figure 2.6: *Atg8a* overexpression increases mutant HTT-Q128 protein aggregates in the PDF^{+ve} small lateral ventral neurons.

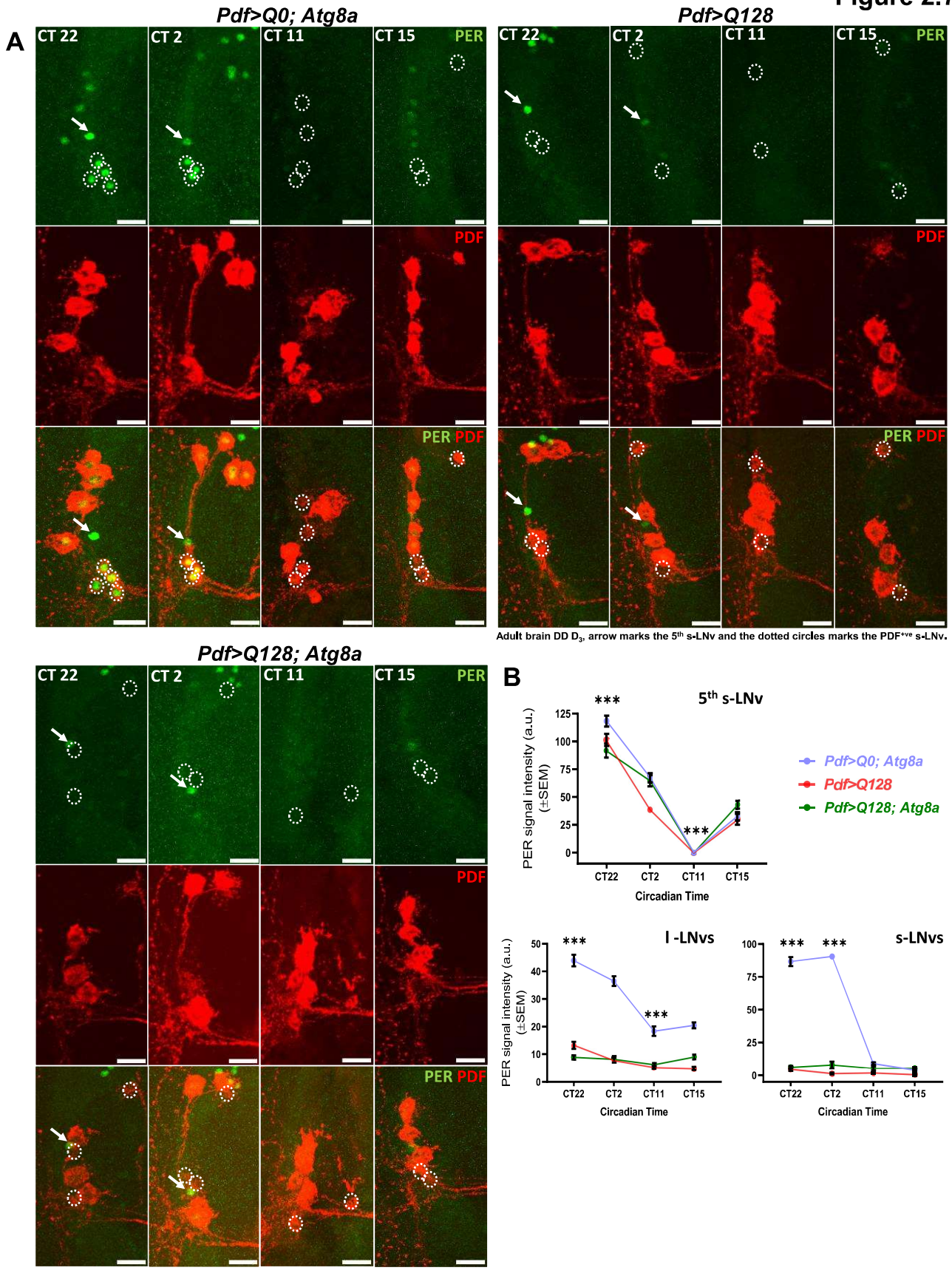
A) Representative maximum intensity projection images of PDF neurons (small - (larva brain; left) and large neurons – (adult brain; right)) depicting staining of mutant HTT-Q128 protein (red) & PDF neuropeptide (marked in magenta). The left cartoon represents the larval *Drosophila* brain depicting the position of small PDF neurons. Dotted circles represent small neuron cell bodies and dotted areas represent small neuron projections. Scale bar - 20μm. **B)** Plot representing quantification of the number of mutant protein aggregates. A significant increase in mutant HTTT-Q128 protein aggregates was observed on *Atg8a* overexpression. Each dot represents an independent experiment. **C)** Plot representing quantification of the size of mutant protein aggregates. An increase in the size of mutant HTT-Q128 protein aggregates was observed on *Atg8a* overexpression. Each dot represents an independent experiment. **D)** Plot representing quantification of signal intensity of non-aggregated mutant HTT-Q128 protein, quantified from the large neurons (on Day1, post eclosion). No change was observed in the intensity of mutant HTT-Q128 protein in flies either expressing only mutant HTT-Q128 protein or co-expressing both *Atg8a* and mutant HTT-Q128 protein. n> 30 neurons (for both experimental genotypes). **E)** Plot representing quantification of the cell numbers positive for either only mutant HTT-Q128 protein aggregates or have both aggregated and non-aggregated mutant HTT-Q128 protein. No significant change was observed in the cell numbers for any of the quantification. n> 30 neurons (for both experimental genotypes). **F)** Plot representing quantification of the number of mutant protein aggregates, quantified from the large neuron cell bodies and initial part of the projections at two different ages (Day1 and 10). *Atg8a* overexpression does not lead to any change in the number of mutant HTT-Q128 protein aggregates. n> 18 brain hemispheres (Day1, for both experimental genotype) and n> 16 brain hemispheres (Day 10, for both experimental genotypes. **G)** Plot representing quantification of the size of mutant protein aggregates. No significant change was observed in the mutant protein aggregates size in either of the genotypes. n> 18 brain hemispheres (Day1, for both the experimental genotypes) and n> 16 brain hemispheres (Day10, for both experimental genotypes).

(+) and (-) in the bar graphs represent the presence or absence of the gene in the fly. Asterisk on individual genotypes means that the genotype is significantly different from all other plotted genotypes. * $p < 0.05$.

2.3.6 Behavioral rescue is not dependent on PERIOD protein oscillation

Daily oscillation of core circadian clock component-PERIOD (PER) in the small LNv is crucial for the maintenance of locomotor rhythm under constant darkness (DD) (Peng et al., 2003; Yang & Sehgal, 2001). Previous studies showed that expression of mutant HTT-Q128 in LNvs disrupts the level and dampens the oscillation of PER protein in both LNv subtypes (Prakash et al., 2017, 2022). To assess whether *Atg8a*-mediated behavioral rescue in the activity-rest rhythm is an outcome of improvement in PER protein levels, I examined four different circadian time points on DD Day 3 (CT22, 2, 11, and 15). The signal intensity of PER protein was quantified from small, large, and PDF^{-ve} 5^{th} s-LNv (as non-targeted control). Control flies showed circadian oscillation of PER protein in all three sets of neurons (**Figure 2.7A and B**). In both the experimental genotypes, a clear time-dependent oscillation of PER protein was observed in 5^{th} s-LNv (**Figure 2.7B, red and green curve, top graph**). Expression of mutant HTT-Q128 protein led to a significant reduction in both the levels and oscillation of PER protein in both small and large neurons (**Figure 2.7B, red curve, bottom graph)**. Interestingly, co-expression of *Atg8a* with mutant HTT-Q128 protein failed to improve levels or oscillation of PER protein in both neuronal subsets (**Figure 2.7B, green curve, bottom graph**). Overall, this result suggests that the observed behavioral rescue is not dependent on PERIOD protein oscillation.

Figure 2.7



Adult brain DD D_3, arrow marks the 5th s-LNv and the dotted circles marks the PDF+ve s-LNv.

Figure 2.7: Atg8a overexpression does not improve PERIOD (PER) protein oscillations in the PDF^{+ve} lateral ventral neurons.

A) Representative maximum intensity projection images (DD Day 3, adult brain) of PDF neurons and 5th small lateral ventral neurons (do not express PDF neuropeptide and served as our control for the PER staining) depicting staining for PER protein (marked in green) and PDF neuropeptide (marked in red) for four circadian time points (CT22, 2, 11 and 15). Arrow depicts 5th s-LNv and dotted circles depict small PDF neurons. Scale bar - 20µm. **B)** Plots representing quantification of the mean signal intensity of PER protein, quantified on DD Day 3 from 5th small lateral ventral neuron (left graph), PDF^{+ve} small neurons (center graph), and large neurons (right graph) at four different circadian time points. 5th small ventral neuron shows time-dependent oscillation of PER protein in all the genotypes. In comparison to the control genotype, expression of mutant HTT-Q128 protein in both small and large neurons significantly hampers the PER protein levels and oscillation in both small and large neurons (red curve). No significant improvement was observed in PER protein levels or oscillation on *Atg8a* overexpression in presence of mutant HTT-Q128 protein in the PDF neurons (green curve). n> 16 brain hemispheres/time point (for control genotype) and n> brain 18 hemispheres/time point (for experimental genotypes).

Asterisk on individual genotypes means that the genotype is significantly different from all other plotted genotypes. * $p < 0.05$.

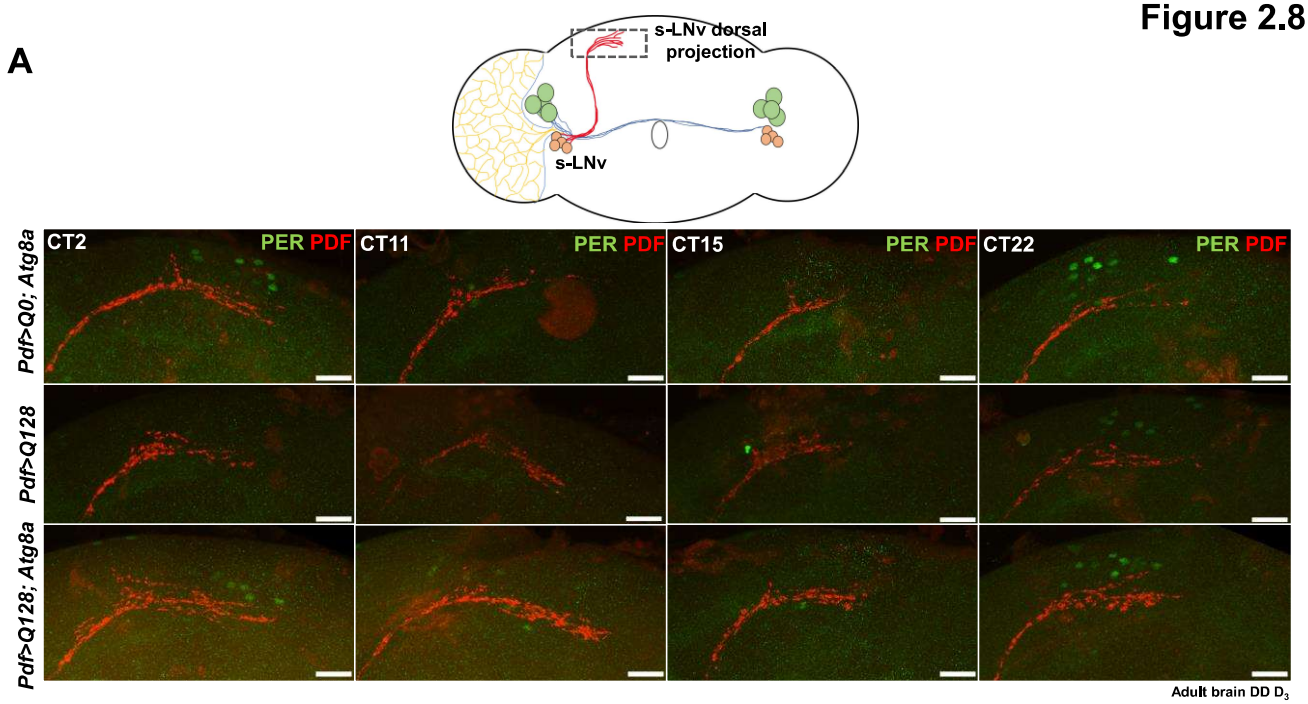
2.3.7 *Atg8a* overexpression improves output from the PDF neurons

The small neurons exhibit a circadian rhythm in the level of Pigment dispersing factor (PDF) neuropeptide in the dorsally located terminal projections and its release at the dorsal projections is critical for synchronizing core clock protein oscillation in the downstream circadian neuronal groups (Peng et al., 2003; Renn et al., 1999). Previously our lab has reported that the expression of mutant HTT-Q128 protein in PDF neurons does not lead to a breakdown in PDF oscillations in the dorsal projections (Prakash et al., 2017). Here also I report that oscillation of PDF neuropeptide occurs in the dorsal projections for all the tested genotypes including flies expressing mutant HTT-Q128 protein (**Figure 2.8A, B**). However, based on our sampling of four-time points across a day, I find that the oscillation of PDF neuropeptide in mutant HTT-Q128 protein expressing flies did not show the gradual rise and fall as seen in the controls (**Figure 2.8B, red bars**). Interestingly, upon co-expression of *Atg8a*, along with HTT-Q128 a gradual rise and fall in the PDF levels were now restored in the dorsal projections (**Figure 2.8B, green bars**).

Since PDF oscillations appear to have been modified by *Atg8a* overexpression, I asked whether the downstream circuits have also been affected. I quantified PER protein oscillation in a subset of circadian clock neurons called Dorsal Neurons (DNs- DN1, DN2, and DN3), which express PDF-receptor (PDFR) and are known to receive input from the s-LNv (Lear et al., 2009; Mertens et al., 2005). DN1s receive input from the PDF^{+ve} s-LNv and communicate with downstream motor centers (Cavanaugh et al., 2014). Since I was not always able to reliably distinguish the DN1 and DN2 subtypes I consider them as one entity in our analysis. In control flies, the number of PER^{+ve} DNs showed the expected oscillation with a significant peak at CT22 and low values at CT 11 and 15 (**Figure 2.8C, blue bars**). Expression of mutant HTT-Q128 protein in PDF neurons led to an overall reduction in PER^{+ve} DNs and a few neurons showed PER

staining at CT22 (**Figure 2.8C, red bars**). *Atg8a* over-expression in the PDF neurons improved PER expression in the DNs, although the distribution of cell numbers was different from controls with ~3-4 cells being detected even at CT15 and there being relatively larger numbers even at CT 11 (**Figure 2.8C, green bars**). Further, I quantified the PER protein intensity and found the expected oscillation in control flies (**Figure 2.8D, blue bars; 2.8E, top graph**). The presence of mutant HTT-Q128 protein in PDF neurons caused an overall reduction in the PER protein in DNs (**Figure 2.8D, red bar; 2.8E, center graph**). Co-expression of *Atg8a* with HTT-Q128 protein in PDF neurons led to a significant improvement in PER protein level in the DNs although only a low amplitude PER protein oscillation was detected (**Figure 2.8D, green bar; 2.8E, bottom graph**). Overall, in presence of mutant HTT-Q128 protein overexpression of *Atg8a* in the PDF neurons improves output from the small neurons.

Figure 2.8


A
s-LNv dorsal projection
s-LNv
CT2
PER PDF
CT11
PER PDF
CT15
PER PDF
CT22
PER PDF
Pdf>Q0; Atg8a
Pdf>Q128
Pdf>Q128; Atg8a
Adult brain DD D3

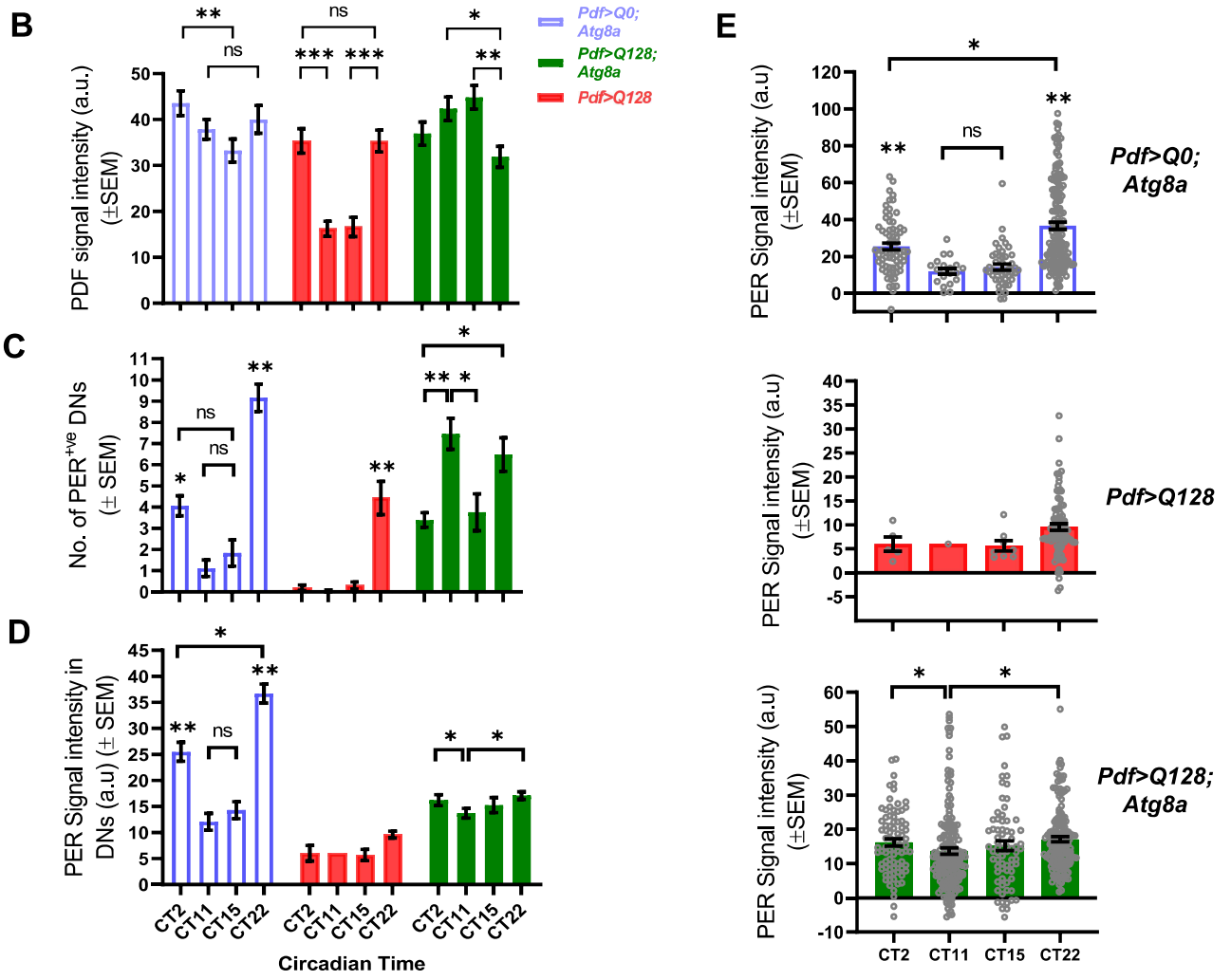

B
PDF signal intensity (a.u.) (±SEM)
Pdf>Q0; Atg8a
Pdf>Q128; Atg8a
Pdf>Q128
C
No. of PER+ve DNs (± SEM)
D
PER Signal intensity in DNs (a.u) (± SEM)
CT2 CT11 CT15 CT22
Circadian Time
E
PER Signal intensity (a.u) (±SEM)
Pdf>Q0; Atg8a
Pdf>Q128
Pdf>Q128; Atg8a
CT2 CT11 CT15 CT22

Figure 2.8: ***Atg8a*** **overexpression improves the output from the PDF^{+ve} small neurons to the downstream neurons.**

A) Representative maximum intensity projection images (DD Day 3, adult brain) of dorsal projections (part of small lateral ventral neurons) and DNs, depicting staining for PER protein (marked in green) and PDF neuropeptide (marked in red) for four circadian time points (CT22, 2, 11 and 15). Scale bar - 20µm. **B)** Plots representing quantification of the mean signal intensity of PDF neuropeptide, quantified from the dorsal projections of small neurons at four different circadian time points on DD Day 3. Time-dependent oscillation of PDF neuropeptide was observed in the control genotype (blue bars). Expression of mutant HTT-Q128 in the PDF neurons does not hamper the oscillation of PDF neuropeptide in the dorsal projections, but like the control genotype, no gradual decrease or increase was observed in the intensity (red bars). *Atg8a* overexpressing flies in presence of mutant HTT-Q128 protein also show PDF oscillation (green bar), but the change in the intensity is distinctly different from what was observed in flies only expressing mutant HTT-Q128 protein. n> 16 brain hemispheres/time point (for control genotype) and n>18 brain hemispheres/time point (for experimental genotype). **C)** Plot representing quantification of the number of PER^{+ve} DN1 and DN2 (together mentioned as DNs) neurons quantified across four different circadian time points. Time-dependent oscillation was observed in the number of PER^{+ve} DNs for the control genotype. Expression of mutant HTT-Q128 protein in the PDF neurons hampers PER expression in the DNs and hardly any cells were visualized at CT2, 11, and 15 time points. Over-expression of *Atg8a* in the PDF neurons improves PER expression in the DNs, however, the oscillation in the cells number was not the same as observed in the control genotype. n> 16 brain hemisphere/time point (for control genotype) and n> 18 brain hemisphere/time point (for experimental genotype). **D)** Plots representing quantification of the mean signal intensity of PER protein, quantified from DNs at four different circadian time points. The control genotype showed a nice time-dependent oscillation of PER protein in the DNs (blue bars). Expression of mutant HTT-Q128 protein in the PDF neurons hampers PER oscillation in the DNs (red bars). *Atg8a* overexpression in the PDF neurons results in a low amplitude PER oscillation in downstream DNs (green bars). n> 16 brain hemispheres/time point (for control genotype) and n> 18 brain hemispheres/time point (for experimental genotype). **E)** Plots (same as Figure 2.8D) representing individual values of PER protein intensity quantified from the DNs.

Asterisk on individual genotypes means that the genotype is significantly different from all other plotted genotypes. * $p < 0.05$.

2.4 Summary and Discussion

Autophagy modulation has been shown to mitigate the toxic effect of mutant HTT protein in different model organisms, however detailed molecular mechanism leading to behavioral and cellular rescues are not well understood. Here, using a genetic approach, I targeted a subset of *Drosophila* circadian neurons whose functioning is directly correlated with self-sustained locomotor rhythms and asked whether genetic modulation of the autophagy pathway within these cells can mitigate the toxicity caused by mutant HTT protein. Additionally, circadian defects are also well documented in both patient and animal models of Huntington's disease. While there is no direct evidence, recent studies have highlighted that strategies that can improve the functioning of circadian circuits can potentially delay the progression of neurodegenerative phenotypes (Pallier et al., 2007; Wang et al., 2017; Whittaker et al., 2018). Hence, I explored whether autophagy modulation can mitigate the toxicity of mutant HTT-Q128 protein in a subset of *Drosophila* clock neurons.

This study shows that overexpression of a key autophagy gene - *Atg8a* in presence of mutant HTT-Q128 protein led to sustained (up to 3 weeks as adults), although partial (low amplitude) rescue of rhythmic locomotion. In addition to the improvement in activity rhythm, *Atg8a* overexpression also ameliorated sleep defects in the flies. Interestingly, despite the clear behavioral rescue, some cellular phenotypes revealed novel and unexpected patterns. Staining for a molecular clock protein - PERIOD revealed that *Atg8a*-mediated behavioral rescue is not dependent on improvement in the oscillation of the PERIOD protein. The maintenance of activity rhythms in absence of PERIOD protein in the small neurons indicates that the protein within these neurons might be dispensable for rhythm sustenance. Two very recent studies support this reasoning and show that PERIOD protein in LNvs is not necessary for the persistence of activity

rhythms under DD but is vital for rhythm strength (Delventhal et al., 2019; Schlichting et al., 2019). Overall, these results point out that autophagy modulations can mitigate the toxicity caused by the mutant HTT protein in circadian neurons.

I show that *Atg8a* overexpression results in increased Ref(2)P-HTT-Q128 colocalization and improves Cathepsin-D expression in the targeted neurons. Further, *Atg1* and *Atg5* downregulation attenuated the *Atg8a*-mediated rescue. These results strongly point towards the involvement of *Atg8a* in regulating the autophagy pathway in neurons. Two recent studies lend support to this observation, wherein they show that *Atg8a* positively regulates the autophagy pathway (Hwang et al., 2022; Jacomin et al., 2020).

Basal level of autophagy may vary among neuronal subtypes, and they may clear cargo at different rates (Tsvetkov et al., 2013). My finding of low level of Ref(2)P colocalization in small neurons also points toward possible differences in the level of autophagy between the two subsets of neurons. This can be an outcome of differences in the size of the two targeted neurons. However, cathepsin-D staining also supports this notion, as small neurons failed to show any Cathepsin-D staining in control flies while large neurons showed both diffused and punctate staining. These inherent differences between large and small neurons can possibly explain why small neurons are more susceptible to aggregate stress. The lack of Cathepsin-D staining in large lateral ventral neurons on *Atg8a* overexpression along with lower HSP70 suggest that large neurons have greater ability to tolerate stress. This is also supported by the RNAseq studies which indicate that large neurons are well equipped with pathways required for tackling stressful conditions (Kula-Eversole et al., 2010; Ma et al., 2021). A recent study from our lab also shows that overexpression of HSP70 protein can decrease toxicity of the mutant HTT-Q128 protein in the small neurons leading to improvement in activity-rest rhythm (Prakash et al., 2022). The lack of change in aggregate

number in the large neurons also points toward lack of autophagy induction upon *Atg8a* overexpression. Given these differences, it will be interesting to examine stage-specificity of *Atg8a*-mediated autophagy induction in the large neurons and whether inherent differences in protein homeostatic pathways are one of the reasons for the selective susceptibility of small neurons.

Despite the involvement of the autophagy pathway in *Atg8a*-mediated rescue of behavior, I did not observe any decrease in mutant HTT protein aggregates in the circadian and sleep neurons. Interestingly, I observed an increase in the levels of HTT aggregates on *Atg8a* overexpression in both circuits. Increased aggregation in the targeted neurons may be an outcome of defects at fusion steps of the autophagy pathway which result in the accumulation of aggregates in these neurons (Martinez-Vicente et al., 2010). Further studies are required to investigate whether boosting the fusion step of the autophagy pathway can reduce the aggregate stress and enhances the behavioural rescue. Studies have also shown that soluble forms of mutant HTT are more toxic to neurons and increased aggregation helps neurons to better deal with toxicity (Arrasate et al., 2004; Miller et al., 2010). It could be possible that increased aggregation and Ref(2)P-HTT-Q128 colocalization is a major reason for the partial rescue in behaviour. The prolonged survival of large neurons despite the presence of a high aggregate load is not surprising. Similar observations have been reported for mammalian cortical neurons, where high aggregate load is not correlated with cell death (Gutekunst et al., 1999; Kuemmerle et al., 1999). It further suggests that the large neurons are more competent in tackling stress, as no major defects were observed in the large neurons despite the accumulation of mutant HTT aggregates.

Synaptic transmission of neuropeptides and neurotransmitters is critical for proper behavioral and physiological outputs. In neurodegenerative disorders including HD, defects in

axonal transport, vesicular fusion, and release of neurotransmitters or neuropeptides are seen (Li et al., 2001; Q. Xu et al., 2013). Here I show that the presence of mutant HTT protein in LNvs hampers the expression of PDF neuropeptide in the soma of small neurons. However, the axonal projections of these neurons show strong PDF staining and oscillation. The presence of PDF staining in axons suggests that the vesicular transport of PDF mRNA or peptides in the axons is not significantly affected by HTT. Loss of PDF, PERIOD, and Cathepsin-D staining in the cell bodies of the small neurons further suggests that HTT-mediated defects are more prominent in cell bodies. Further, the oscillation of PDF neuropeptide in the neuronal processes hints toward contribution from other cells including glia in maintaining the PDF oscillations. This observation is further supported by a recent study, which shows the involvement of glial cells in regulating PDF oscillations in the projections of the small neurons (Damulewicz et al., 2022). Lack of PERIOD protein expression and oscillation in the downstream dorsal neurons on mutant HTT expression in the small neurons point toward defective synaptic communication between the small and dorsal neurons which might be the case with the sleep neurons as well *Atg8a* overexpression in the small neurons led to an improvement in PERIOD protein levels in dorsal neurons, suggesting that the behavioral rescue is possibly an outcome of improvement in synaptic communication between the small neurons and downstream dorsal neurons. I speculate that this improved synaptic communication is an outcome of increased aggregations leading to decreased toxicity of mutant HTT in the neurons, or that *Atg8a* being a vesicular protein improves PDF release from the small neurons.

In summary, I present evidence for compromised autophagy in circadian pacemaker circuit when mutant HTT protein is expressed and demonstrate that genetic upregulation of *Atg8a* enables the circadian pacemaker circuit to drive behavioral rhythmicity in locomotion. Further, this study

suggests that this occurs possibly through improvement in synaptic output of the circuits. I propose that *Atg8a* in the targeted neurons enhances autophagy, which in turn increases the aggregation of mutant HTT protein and rescues the strength of connections with downstream neurons in the circadian circuit, thus enabling overall rescue of circadian and sleep phenotypes.