Effects of Temporally Controlled Huntingtin Expression and Autophagy Upregulation in *Drosophila melanogaster*

A thesis

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By

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Dedicated to

Maa, Babai and Tim

Contents

Declaration

I declare that the matter presented in my thesis entitled "**Effects of temporally controlled Huntingtin expression and autophagy upregulation in *Drosophila melanogaster***" is the result of studies carried out by me at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Dr. Sheeba Vasu and that this work has not been submitted elsewhere for any other degree. In keeping with the general practice of reporting of scientific observations, due acknowledgement has been made wherever the work described has been based on the findings of the other investigators. Any omission, which might have occurred by oversight or error of misjudgement, is regretted.

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<u>Certificate</u>

This is to certify that the work described in the thesis entitled "**Studying the effects of temporally controlled Huntingtin expression and autophagy upregulation in *Drosophila melanogaster*"** is the result of investigations carried out by **Payel Ganguly** in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

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Acknowledgement

Synopsis

Previous studies in the laboratory have shown that expression of pathogenic human Huntingtin (HTT-Q128) in circadian pacemaker neurons (ventro lateral neurons, LN_v) of *Drosophila*, leads to immediate behavioural arrhythmicity in constant darkness and loss of neuropeptide Pigment Dispersing Factor (PDF) specifically from a subset of LN_v - the small LN_v (sLN_v), while the large LN_v (lLN_v) are intact. In the present study, we have attempted to examine effects of temporally restricting HTT expression in LN_v on neuronal function and behaviour using an inducible Gene Switch system that enables HTT induction only in the presence of a progesterone analogue RU. We used this system to ask whether larval versus adult specific HTT induction alters the selective vulnerability of sLN_v and circadian behavioural dysfunction (arrhythmic locomotor activity) under constant darkness. It was seen that either adult-restricted or larval-restricted HTT induction were each sufficient to render flies arrhythmic, albeit to different magnitudes. Moreover, the onset of arrhythmicity was delayed compared to flies expressing HTT throughout. Both these treatments were also sufficient to cause loss of PDF in sLN_v; but at a slower rate compared to flies expressing HTT throughout. Therefore, the selective susceptibility of sLN_v does not seem to depend on the life-stage at which HTT is expressed, but the rate of loss seems to be affected by the duration of HTT expression.

We also attempted to study if the process of autophagy (which has previously been shown to be affected in several neurodegenerative disorders including Huntington's disease) can help attenuate the severity of the disease in our *Drosophila* circadian model. We screened three autophagy genes *atg1*, *atg5* and

atg8a. These genes are involved at different stages in the autophagy induction, recruitment of other Atg proteins and fusion of autophagosomes to the lysosome. We find that upregulation of *atg8a* along with expression of pathogenic HTT in the LN_v improves rhythmicity and delays disappearance of PDF from sLN_v. Our studies help understand the contributions of timing of HTT expression and autophagy in the progression of Huntington's disease in *Drosophila melanogaster*.

Chapter 1: Introduction

Neurodegenerative diseases

Neurodegenerative diseases (NDs) are typically characterized by adult onset, progressive loss of neurons from specific regions in the brain, cellular dysfunction and cell death. Human NDs could arise from a variety of genetic as well as environmental causes (Lessing and Bonini, 2009). In 2005, the World Health Organization reported that NDs such as Alzheimer's, Parkinson's and other dementia make up around 14% of the global burden and the numbers are going to rise as life expectancy increases (World Health Organization. Neurological disorders: Public Health Challenges (World Health Organization, Geneva.2006)). NDs have visible symptoms but little is known about their underlying pathogenicity, hence there are few cures and effective treatments. However, several studies have suggested that the general mechanisms of aggregate accumulation and inclusion formation may be a common feature of these conditions and therefore an intricate understanding of the underlying cellular processes could prove crucial in developing effective therapies. The most prevalent NDs include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Polyglutamine (PolyQ) diseases.

PolyQ diseases

PolyQ diseases comprise of a group of nine neurodegenerative disorders, caused by expanding nucleotide repeats CAG coding for glutamine (Q) (Margolis and Ross, 2001). These diseases include Huntington's disease (HD), spinal and bulbar muscular dystrophy (SBMA), dentatorubral and pallidoluysian Atrophy (DRPLA) and other forms of spino-cerebellar ataxia (SCA). Specific subsets of neurons are vulnerable to these diseases but there is considerable overlap in the brain regions

including the basal ganglia, brainstem nuclei, cerebellum and spinal motor nuclei (Ross, 1995). It has also been reported that there is no homology among the genes causing these disorders except for the polyglutamine stretch itself (Ross, 2002). Of all the PolyQ diseases HD has been most extensively studied for disease pathogenesis partly, because it is caused by a single gene mutation (Ross, 2002).

Huntington's disease

The genetic basis of HD was indentified in 1993 (HDCRG, 1993). HD is an inherited fatal autosomal dominant neurodegenerative disorder caused by expansion of CAG repeats in exon 1 of the *Huntingtin (htt)* gene, which encodes the HUNTINGTIN protein (HTT). Asymptomatic individuals have fewer than or equal to 35 repeats (Tobin and Signer, 2000). However, CAG repeats greater than 35 results in the manifestation of the disease (Rubinsztein et al., 1996) suggesting a sharp threshold for alteration in protein structure or function. An inverse relationship exists between the age of HD onset and the number of CAG repeats i.e. greater the number of repeats, earlier the age of onset (Rubinsztein, 2002). Furthermore, expansions greater than 70 lead to juvenile onset (Duyao et al., 1993). Expanded CAG repeats are unstable and they show a propensity for expansion when transmitted through the male germline (Ranen et al., 1995). This results in anticipation where the age of onset decreases in successive generations (Ross et al., 1993). HD pathogenesis has evidence for gain-of-function mutation mechanism with homozygotes having almost identical phenotype to that of heterozygotes. It has also been shown in humans that loss of one of the two *htt*

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gene (hemizygous condition) does not result in the onset of the disease (Rubinsztein, 2002).

The primary symptoms of HD are characterized by altered personality, involuntary choreiform movement, loss of motor co-ordination, cognitive impairment and psychiatric disturbances (Vonsattel et al., 1985). The most susceptible region in the brain is the striatum where the medium spiny neurons show altered morphology with respect to density, shape and size of spines (Rubinsztein, 2002). With the progression of the disease there is also neuronal loss in the cortex, thalamus, substantia nigra pars reticulate and in the subthalamic nucleus (Reiner et al., 1988).

An important pathological feature of HD is misfolding and aggregation of mutant HTT protein resulting in the formation of inclusion bodies and neuronal degeneration in specific regions of the brain (Ross and Poirier, 2004). Even though the inclusions are seen in the affected regions of the brain, degeneration is not limited to those neurons (Ross and Poirier, 2004). It has been seen that inclusions are denser in populations of large interneurons, which are intact, than in the medium spiny projection neurons, which are selectively lost (Kuemmerle et al., 1999). Whether these aggregates are harmful or neuroprotective has been the subject of much debate. Some studies have indicated a correlation between PolyQ containing inclusions and disease progression (Davies et al., 1997). Aggregates may cause pathology by sequestering various proteins away from their normal sites of function or by disrupting cellular processes (Yamamoto et al., 2000). Covalent modifications of the proteins may facilitate aggregation by cleavage or phosphorylation, thus initiating the conversion of a protein into an abnormal conformation (Ross and Poirier, 2004). Other studies have dissociated inclusions

from pathology and cytotoxicity. For instance, Saudou and his co-workers have shown that decreasing the proportion of inclusions in cell aggregates by inhibiting ubiquitination increased cell death (Saudou et al., 1998). In case of SCA1 and SCA13 proteins, neuronal cells in the brain with visible aggregates appear healthier than those without it (Nagaoka et al., 2003), thus lending support for the latter hypothesis. This issue remains to be resolved and so far no consensus has been reached. In their review, Ross and Poirier, have hypothesized that the toxicity may be associated with the intermediate steps of the molecular cascade than the inclusion themselves which are the final visible products (Ross and Poirier, 2004). Therefore, inhibition early in the pathway of polymerization could be beneficial to the cell by preventing the formation of potentially toxic intermediates.

Processes that get impaired in HD

The detailed mechanism of HD pathogenesis is yet to be deciphered. The pathogenicity observed is probably a summation of multiple factors rather than a single initiating event. Current theories of HD pathogenesis suggest that there is transcriptional dysregulation, ubiquitin-proteasome dysfunction, synaptic pathology, excitotoxicity, mitochondrial dysfunction, trafficking of autophagosomes and impaired axonal transport. Some of these processes are discussed below:

1. Transcriptional dysregulation

One of the early events contributing towards pathogenicity could be, the abnormal binding of mutant HTT (mHTT) to proteins regulating transcription, thereby altering levels of gene transcription (Sugars and Rubinsztein, 2003). PolyQ

repeats occur in transcription factor proteins, suggesting that wild type HTT may play a role in transcription by interacting with these proteins. The function of HTT in the nucleus is still unresolved; while some studies report localization in the nucleus (DiFiglia et al., 1995) other studies show that it represses transcription in the nucleus (Kegel et al., 2002). Gene expression profile of the HD brain reveals that gene expression levels are prominently altered in the motor cortex and the caudate nucleus (Hodges et al., 2006). The affected genes were those essential for glucose and lipid metabolism and also involved in oxidative stress response. Studies have also suggested that cleavage of HTT may be an important step towards dysregulation of transcription (Chan et al., 2002). mHTT undergoes abnormal association with proteins like cAMP response element binding protein (CBP), which functions in a neuroprotective way via a secondary signalling cascade (Nucifora et al., 2001). Thus, mHTT with its altered binding ability inhibits and disrupts the transcription of genes required for maintaining neuronal homeostasis.

2. Ubiquitin- proteasome dysfunction

The cell ensures that newly synthesized polypeptides fold into optimal conformations. However, if mutations or stress impair protein folding, the cell has developed mechanisms to defend against those misfolded and aggregated proteins. The primary line of defence is through molecular chaperones which facilitate refolding of those damaged proteins (McClellan and Frydman, 2001). If native conformation of protein is not achieved, the protein is targeted for degradation by the ubiquitination-proteasome pathway (Goldberg et al., 1996) which is responsible for many quality control functions in the cell. The presence of traces of ubiquitin, chaperones and proteasome in the inclusions indicate the failure of

these defences in clearing aggregates. Aggregate protein toxicity can be rescued by overexpression of chaperone proteins in neuronal cultures.

3. Synaptic pathology

Decreased synaptic vesicle density and neurotransmitter release is seen in transgenic mouse model (R6/2), which could be result of high levels of mHTT in the presynaptic terminals (DiFiglia et al., 1995). Smith and co-workers (2005) showed that decreased neurotransmitter release is probably because of abnormal associations between mHTT and presynaptic protein (Smith et al., 2005). Post synaptic defects in HD may also arise from impaired interaction between mHTT and PSD-95, a protein that plays a key role in regulation of synaptic plasticity and synaptogenesis (Che et al., 2000). Wild-type HTT associates with the SH3 domains of PSD-95, a scaffold protein that links NMDA and kainite receptors to PSD (F and Kim, 2002). mHTT decreases its interaction with PSD-95, leading to increased NMDA receptor sensitivity and excitotoxic cell death (Sun et al., 2001).

4. Mitochondrial dysfunction and oxidative damage

Disruption of normal mitochondrial function results in metabolic insufficiency, oxidative damage, excitotoxicity and ND. Many studies have indicated that mitochondrial dysfunction may contribute towards many NDs (Beal, 2000). Ultrastructure studies indicate the presence of abnormal mitochondria in the brain and in mHTT expressing lymphoblasts. HD patients also show greater signs of oxidative damage in the striatum and cortex, leading to DNA breaks. Loss of mitochondrial membrane potential impairs ATP synthesis and reduces calcium uptake leading to neuronal cell death (Ruan et al., 2004). In mutant HTT knock-

in mice, both mitochondrial respiration and ATP production are inhibited (Milakovic and Johnson, 2005).

5. Disruption of autophagy

Autophagy refers to a catabolic process by which dysfunctional organelles and misfolded proteins are delivered to the lysososme for degradation (Cuervo, 2004; Levine and Klionsky, 2004). The process of autophagy is tightly regulated by a set of genes called autophagy related genes (*atg*). The process involves three steps starting with induction of autophagy, formation of autophagosomes and finally fusing with lysosomes facilitating degradation by lysosomal enzymes. Mice models with incompetent autophagy processes have neurodegeneration in certain regions of the brain and protein inclusions, making it evident that autophagy is required for neuronal homeostasis and quality control mechanisms in the cell (Hara et al., 2006; Komatsu et al., 2006). Failure of autophagy in neurons can lead to the accumulation of aggregate- prone proteins and neurodegeneration. It has also been reported that in HD the processes of cargo loading and the fusion of the autophagosomes to lysosomes are mostly impaired (Martin et al., 2015).

6. Impaired axonal transport

The process of axon transport is the key to neural dynamics and is responsible for transporting membranous organelle and signalling molecules towards the nerve terminal (anterograde direction) and back towards the cell body (retrograde direction). The organelles include vesicles, mitochondria and elements of smooth endoplasmic reticulum. These particles are actively transported through tracks aligned along the main axis of the axon and these tracks are the microtubules, they provide the framework on which specific organelles move by means of molecular

motors. Motor molecules for anterograde transport are kinesin and kinesin related proteins, a large family of ATPases, each of which transports different membrane cargo. Rapid transport also occurs in retrograde direction, from nerve endings towards the cell body. The force necessary to move organelles in this direction is thought to be generated by dynein. Impairment of axonal trafficking systems can have a devastating effect on both neuronal function as well as survival. Studies have shown that HTT aggregates block transport in narrow axons. Truncated version of huntingtin inhibits both anterograde and retrograde transport in giant squid axons, mammalian tissue culture and flies models (Gunawardena et al., 2003; Lee et al., 2004). Recent studies also point towards the fact that impaired axonal transport of mitochondria could be contributing significantly to HD pathology (Cattaneo et al., 2005).

Putative functions of HTT

Fly and mice models have provided useful insights into normal huntingtin protein functions. Wild-type HTT is expressed in many different tissues and is particularly enriched in the brain (Cattaneo et al., 2005). The protein is predominantly cytoplasmic and is localized to various subcellular compartments, including the endoplasmic reticulum, golgi body, neuritis and synapses (DiFiglia et al., 1995; Kegel et al., 2002; Velier et al., 1998). HTT may also have a role to play in the nucleus (Xia et al., 2003). Studies in mice have shown that HTT is necessary for development, as homozygous knockout mice exhibit embryonic lethality (Duyao et al., 1995; Nasir et al., 1995). Conditional inactivation of HTT in mice also leads to early onset of symptoms and development-related abnormalities suggesting the importance of HD in adulthood as well as

postembryonic period (Cattaneo et al., 2005). Additionally it has also been seen that expression of mutated HTT (mHTT) in knock-out mice rescues lethality (Leavitt et al., 2001). In vitro and in vivo data show that wild-type HTT, but not the mutant protein, facilitates the transcription and production of a brain-derived neurotrophic factor (BDNF), in the cortical neurons that project to the striatum (Gauthier et al., 2004). Wild-type huntingtin interacts with huntingtin-associated protein (HAP1) to bring about vesicular BDNF transport from the cortex to the striatum (Gauthier et al., 2004). HTT is present at synaptic terminals as well where it binds to PSD-95, a key molecule in synaptic transmission (Sun et al., 2001). HTT also associates with clathrin-coated vesicles, endosomal compartments and microtubules thereby mediating clathrin-coated endocytosis (Li et al., 2003). Recent studies have shown that HTT acts a scaffold for selective autophagy (Ochaba et al., 2014; Rui et al., 2015). The wild-type function of huntingtin suggests that it has beneficial functions. Therefore its loss in HD patients could enhance the toxic effects of mutant protein contributing to pathogenicity.

Lessons from animal models

A wide range of studies have been done in invertebrates as well as vertebrates to identify the normal functions of HTT. Understanding the normal function of HTT is important to design effective therapeutic interventions.

1. Parker and colleagues (2007), found impaired neuronal function in *Caenorhabditis elegans* (*C.elegans*), which was due to the overexpression of HTT exon 1 with either 88 or 128 repeats (Parker et al., 2007). Even though this neuronal dysfunction was PolyQ length dependent, it was not

accompanied by cell death. Studies using HTT exon-1Q150 *C.elegans* model showed sensory neuronal impairment but no cell death (Faber et al., 2002).

2. In mice, loss of HTT leads to lethality at the embryonic stage (Duyao et al., 1995, Nasir et al., 1995, Zeitlin et al., 1995). Early mortality, motor phenotype, sterility together with progressive neurodegeneration is seen in mice models with conditional inactivation of HTT in the mouse brain at either postnatal or embryonic stages (Dragatsis et al., 2000). Studies have also suggested that HTT has a role to play in ciliogenesis since loss of HTT from mouse cells leads to reduced primary cilia formation (Keryer et al., 2011). HTT knockdown and mutant HTT also impairs the processes of axonal transport of vesicles, mitochondria and autophagosomes (Gauthier et al., 2004, Martinez-Vicente et al., 2010, Trushina et al., 2004; Wong and Holzbaur, 2014).

3. In contrast to the above studies, the role of HTT in fruit flies *Drosophila melanogaster* (dHTT) is likely to be slightly different. HTT loss of function flies develop normally but the same flies expressing human mutant exon 1 HTT shows progressive degeneration (Zhang et al., 2009). It is yet unclear whether the role of dHTT was similar to that of mammalian HTT. Studies have shown that there is conservation of function in mammalian HTT and dHTT with respect to axonal transport as dHTT-knockout flies had impaired axonal transport (Zala et al., 2013). Not unlike mammalian HTT, dHTT interacts with molecular motor dynein, synaptic vesicles and micrtoubules (Zala et al., 2013). Studies have also demonstrated that expression of dHTT in mice treated with *htt* si

RNA can compensate for the loss of functional mouse HTT thereby lending evidence towards functional conservation of HTT in these widely divergent species (Godin et al., 2010). Recently, it has also been shown that loss of HTT in *Drosophila* results in loss of starvation induced autophagy as well as selective autophagy pointing towards the same (Ochaba et al., 2014; Rui et al., 2015).

4. Studies from Zebrafish have shown that HTT is required for cellular iron utilization since depletion of HTT leads to symptoms of cellular iron deficiency and some development related disorders (Lumsden et al., 2007).

5. *Dictyostelium discoideum* cells without endogenous HTT were viable but were unable to show the typical polarized morphology and they also had difficulties in surviving in nutrient limiting condition. Their development was delayed and they produced defective fruiting bodies (Myre et al., 2011).

Fly as a model organism

Drosophila melanogaster is one of the best invertebrates for modelling several aspects of biology of higher organisms. They have been used for over a century and hence been well characterized. Comparative genome analysis reveals that nearly 50 % of fly genes have similar genes in mammals (Rubin et al., 2000). Additionally, there is conservation of basic molecular mechanisms which include regulation of gene expression, neuronal connectivity, cell signalling and cell death (Adams et al., 2000). Fruit flies have a relatively smaller genome with four pairs of chromosomes and about 15,500 genes as opposed to humans with 23 pairs of

chromosomes and 22,000 genes. This indicates that there is less redundancy in the fly system. The fly has a well structured nervous system of around 300,000 neurons with an architecture that segregates behaviours such as aggression, olfaction, vision, circadian rhythms, sleep, learning and memory (Hartenstein et al., 2008). By studying these behaviours in flies we can gain an understanding of the basic pathways underlying them. The wide array of impressive genetic tools available to manipulate the fly system allows us to perform a variety of experiments. The most common being the classical GAL4/UAS system (Brand and Perrimon, 1993) which provides a simple way of targeted gene expression and also allows us to have spatial control. There are inducible systems like gene switch (Osterwalder et al., 2001; Roman et al., 2001) and temperature sensitive GAL80 transgene with the UAS construct which facilitate both spatial and temporal control. Fly models have been used for large scale genetic screens (Bier, 2005). The fly is also an excellent in vivo system for performing high-throughput screening of chemical libraries for compounds with therapeutic potential (Agarwal and Prasad, 2005). The short generation time, high fecundity and relatively low cost of maintenance makes it especially useful.

Flies in neurodegenerative diseases, especially Huntington's disease

Drosophila melanogaster is being used as a model for studying human disease genes, including those responsible for developmental disorders, cancer, cardiovascular disease, metabolic and neurodegenerative diseases, and for genes required for the function of the visual, auditory and immune systems. 75 % of known human disease genes have a homolog in *Drosophila* (Chien et al., 2002;

Reiter et al., 2001). In the fly, the functions of both normal and aberrant gene can be analyzed by generating mutants, or by introducing the human form of diseased gene into the fly genome. With these advantages, the fly system has been widely used for studying neurodegenerative diseases. *Drosophila* model of neurodegenerative diseases faithfully mimic several features of human disease such as decline in longevity, progressive neurodegeneration, visible protein aggregates, deficits in motor and cognitive function and the dependence of age-of-onset and severity on nucleotide repeat length (Marsh and Thompson, 2006). The organization of *Drosophila* central nervous system allows monitoring of selective neurodegeneration by using tools to follow the fate of specific subsets of neurons. Dominant NDs like HD can be modelled particularly well in flies since they are caused by gain of function mutation in single genes which can be engineered to be expressed in flies and recapitulate phenotypes seen in human diseases (Marsh et al., 2003). Apart from mimicking the pathogenicity, the fly model also offers simple tests to assay for motor defects and neurodegeneration, the most common one being climbing ability and integrity of photoreceptor cells of the eye. Motor defects are assayed by exploiting the negative geotropic behaviour of flies and counting the number of flies that can climb to the top of a tube in a definite window of time (Marsh et al., 2003). *Gmr* and *elav* are the two most widely used drivers for neurodegeneration studies. *Elav* is a broad driver which targets all the neurons of the nervous system and *gmr* targets neurons and supporting cells in the eye. Expression of PolyQ protein by elav does not give rise to any aberrant morphology but with gmr driver extensive degeneration is seen in the eye giving a "rough" eye phenotype (Jackson et al., 1998; Lee et al., 2004). Several groups studied the aberration in eyes either by characterizing the rhabdomeres or by

estimating the retinal depth, they observed that there was no difference between experimental and control flies when they emerge from pupae but the degeneration progresses with age, both progression and degree of degeneration also depends on the length of PolyQ that is being expressed (Gunawardena et al., 2003; Jackson et al., 1998; Lee et al., 2004; Romero et al., 2008; Warrick et al., 1998). Owing to their small size and fully sequenced genome, large scale screening approaches are easy to perform. A plethora of genetic and pharmacological screens have been used in the HD model of fly to find out the molecular components of the pathways associated with neurodegeneration. The genetic screens aim to identify suppressors as well as enhancers of disease phenotype and how they function in PolyQ diseases. The study by Branco and co-workers, in 2008 showed that some modifier genes behave similarly in SCA1 and HD models while others seem to have disease specific effects and some act in opposite ways as well (Branco et al., 2008). Genome wide RNAi screening has also facilitated the identification of modifiers of aggregate formation in the *Drosophila* (Doumanis et al., 2009, Zhang et al., 2010). Studies also demonstrate that high throughput yeast two-hybrid screening for protein interactions combined with genetic validation in *Drosophila* was highly useful in identifying HTT interacting modifiers (Kaltenbach et al., 2007). These screens allowed us to gain information about other processes associated with HD and how anomalies in these processes could contribute towards neurodegeneration. Flies are good candidates for large scale drug screening as well. They are mostly used as secondary screening assays for drugs indentified via other methods (Agarwal and Prasad, 2005; Apostol et al., 2003).

Circadian model to study Huntington's disease

Even though HD is being studied and analyzed over the past two decades, scientists lack a clear understanding of the underlying pathogenicity. The understanding lacks at the level of individual neurons, sequence of events, inability of associating it to a particular behaviour and also in establishing the link between the definitive timing of aggregate formation and actual degeneration. This could be because of the widespread expression of HTT in human patients and current HD animal models. The *Drosophila* HD models have so far been characterized using a broad subset of neurons or the eye tissue. A broad driver will target many neurons (pan-neuronal), rendering it difficult to identify the origin and the sequence of cellular events that follow and a driver targeting the eye will primarily give morphological phenotype and very little cellular information. So we reasoned that targeting a smaller subset of neurons and tweaking expression levels of mutant HTT (mHTT) may give us a better control of the system in answering questions with respect to the progress of pathogenicity. Therefore, the *Drosophila* circadian model is an ideal system because of its genetic tractability, well characterized neural circuitry, robust and quantifiable behavioural readouts like daily rhythms in activity rest, eclosion, mating and egg-laying (Sheeba, 2008).

Endogenous biological oscillator with a near 24 hour periodicity (circa = almost, dian = day) is referred to as a circadian clock. They are temperature compensated, self sustainable, can entrain i.e. synchronize to external time cues such as light and temperature. They function within organisms to provide a daily temporal framework and allow organisms to modulate biological events both at the

physiological and the behavioural level allowing them to anticipate the changing environment conditions of the day. Many elegant genetic tools available in *Drosophila* have enabled identification of the molecular mechanisms (Allada, 2003) and location of neuronal groups (Kaneko and Hall, 2000) that are essential components of the circadian machinery. At the molecular level, the clock involves a principal feedback loop with PERIOD and TIMELESS negatively feeding back to CLOCK and CYCLE along with two additional loops. It is believed that the principal loop establishes the rhythm of the clock, the additional loops are responsible for phase and amplitude of the core and output rhythms. The discovery of clock genes and their successful cloning paved the path for understanding the neural circuit in the brain of the fly. The circadian pacemaker neurons are believed to be essential for the regulation of behavioural rhythms (Helfrich-Forster, 1998). The classification into different neuronal groups is based on the expression of clock proteins (PERIOD) and neuropeptide Pigment Dispersing Factor (PDF). There are approximately 150 circadian neurons in the *Drosophila* brain and they appear to be connected via neurites with wide arborization patterns. It can be broadly classified into lateral neurons (LN) and dorsal neurons (DN) cells. The LN cells can be further divided into three clusters: dorsal lateral (LN_d), large ventral lateral (lLN_v) and small ventral lateral (sLN_v) neurons. The dorsal neurons (DN) cells consist of DN1, DN2 and DN3 cells (Fig 1).
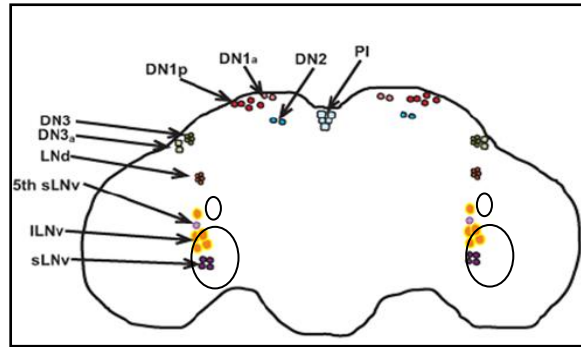
Fig 1. Schematic representation of the circadian pacemaker cells in adult *Drosophila nmelanogaster* with the known members of the various neuronal subgroups are represented as 8 bilaterally placed coloured dots. The PDF expressing LN_v are indicated in the black circle; the small in dark purple and the large in yellow. (Sheeba, 2008) , Fig.1A.)

PDF acts as a circadian clock output factor and it is believed to be the principal neurotransmitter within the circadian circuit. The cloning of *Pdf* from *Drosophila melanogaster* (Park and Hall, 1998) made it possible to address the role this peptide plays in control of the circadian locomotor rhythms. It was shown that PDF is expressed in lLN_v and sLN_v and is secreted at these axonal termini. In adults, PDF is released in a rhythmic manner and PDF levels vary cyclically in the terminus of the sLN_v cells peeking between ZT 2-4 and with a trough between ZT 14-16. Flies bearing the loss of function pdf^{01} show loss of anticipatory morning activity and advanced evening activity under light dark (LD) conditions (Renn et al., 1999). These mutants become arrhythmic under constant darkness (DD) (Renn et al., 1999). RNA-interference mediated knockdown of PDF in the lLN_v resulted in disappearance of the PDF signal with no effect on the free running period but knockdown of PDF from the sLN_v made the flies completely arrhythmic in constant conditions (Shafer and Taghert, 2009). The study of

disconnected (disco) mutants showed that the LN clusters are most important for persistence of circadian rhythm in locomotor activity. These mutants have no functional LN cluster but occasional presence of a single sLN$_v$ is capable of eliciting robust rhythms (Helfrich-Forster, 1998). *Drosophila melanogaster* shows bimodal activity with prominent morning and evening peak. Studies have shown that PDF expressing LN$_v$ are dispensable for evening activity but required for morning activity and PDF negative LN$_d$ can induce evening behaviour in the presence of PDF positive LN$_v$ (Grima et al., 2004; Stoleru et al., 2004). The circadian signals from the neural clusters in the brain are transferred downstream to primary targets of the master clock cells and from these, cells signals may be transferred electrically towards behaviourally relevant centres (Helfrich-Forster, 2005). The discovery of the PDF receptor contributed more to our understanding of the signalling pathways involved in the circadian clock. The arrhythmic behaviour of PDF receptor null mutants in constant conditions established its role in regulating circadian rhythms (Hyun et al., 2005, Lear et al., 2005, Mertens et al., 2005). The PDF receptor Han is expressed in 13 pairs of neurons in adult fly brains: four lLN$_v$, one LN$_d$, seven DN1s, and one DN3. So it has been hypothesized that the PDF signals produced by lLN$_v$ and sLN$_v$ are transmitted to four lLN$_v$s, one LN$_d$, seven DN1s, and one DN3 through Han receptors. All these studies point towards the fact that neuropeptide PDF along with its receptor has multiple roles in the organization of the circadian clock.

In addition to the previously discussed issues, major problems seen in HD patients are circadian and sleep related defects. HD patients show atrophy in key brain structures like brain stem and lateral hypothalamus that are involved in the regulation of sleep. Transgenic R6/2 mice that carry the HTT mutation have

provided useful insights into the molecular basis of circadian rhythm in HD. In this particular mouse model, pharmacological treatments restore circadian rhythm via regulating circadian gene expression and also by hindering cognitive decline (Pallier et al., 2007). Studies using this same mouse model have helped scientists arrive at the conclusion that management of sleep wake cycle could also help in ameliorating cognitive function (Pallier et al., 2007). Recent studies have also suggested that it is time to integrate circadian clocks and neurodegenerative diseases because clock-relevant processes might intervene in protein aggregation by regulating their physiological mechanisms thereby contributing towards toxicity (Hastings and Goedert, 2013). These studies provided the incentive towards using this model system to understand defects due to mHTT expression both at the cellular and behavioural level.

Studies have shown that pathogenic HTT expression in the pacemaker neurons causes selective loss of PDF signal from the sLN_v, but not from the lLN_v and renders the flies arrhythmic in constant darkness (DD) (Sheeba et al., 2010). We aimed to determine whether the expression of HTT during the pre-adult stage alone versus adult stage alone can alter selective vulnerability of the sLN_v. Our studies reveal that selective loss of PDF from sLN_v occurs irrespective of when pathogenic HTT was expressed. However, the rate of this loss was faster when pathogenic HTT was expressed as both pre-adults as well as adults. In terms of behaviour, arrhythmicity onset was earliest in flies expressing pathogenic HTT throughout, followed by those with adult induced expression and then by flies with larval induced expression. Further, recent studies have indicated that fly is good model to understand the role of autophagy in neurodegeneration (Neufeld and Baehrecke, 2008). Therefore we screened for three autophagy genes *atg1*,

atg5 and *atg8a*. These genes are involved at three different stages in the autophagy pathway. We show that upregulation of *atg8a* in flies expressing pathogenic HTT in the PDF neurons improves rhythmicity and slows down loss of PDF from sLN$_v$. These results are described in further detail in the next two chapters.

Chapter 2: Temporal restriction of pathogenic Huntingtin expression reduces its toxicity in circadian pacemaker neurons

Introduction

Previous studies

Previous results from the laboratory suggest that expression of pathogenic human HTT (HTT-Q128) in PDF expressing LN_v pacemaker neurons of *Drosophila melanogaster* render the fly behaviourally arrhythmic in constant darkness (DD) at 25°C. Flies expressing HTT-Q128 appear arrhythmic as early as the first day in DD. Even though these flies express pathogenic HTT-Q128, they are rhythmic under LD 12:12 at 25°C and have bimodal activity profiles with a clear anticipation of morning and evening transitions. The overall levels of activity and sleep are also not different from controls. Additionally, it was observed that life span of flies expressing HTT-Q128 in the LN_v was not significantly lower than the flies expressing non-pathogenic human HTT (HTT-Q0).

At the cellular level, expression of HTT-Q128 in the pacemaker neurons causes selective loss of the circadian neurotransmitter PDF from the sLN_v cell bodies, while the lLN_v are spared. This loss appears to occur either at the pupal stage or very early in the adult stage, since PDF is detectable in sLN_v cell bodies and dorsal projection in the larval stage. However, in adults the sLN_v cell bodies do not stain with anti-PDF and appear to be absent, while their dorsal projections are intact. In many NDs including HD, subpopulations of neurons are selectively affected, leading to the progressive failure of few neurons and circuits, but the basis of such selective neuronal vulnerability is not clear. Studies suggest that the toxic effects might not be due to cell-type specific features rendering specific neuronal subsets increasingly vulnerable (Han et al., 2010). This selective vulnerability could be explained by the difference in their biochemical content, morphology and connectivity among the neuronal cell types (Han et al., 2010).

Affected neurons show marked reduction in the number of axon fibres and synaptic proteins early in the course of HD (Levine and Klionsky, 2004; Li et al., 2003; Reiner et al., 1988) and is accompanied by electrophysiological disturbances (Laforet et al., 2001). Selective susceptibility could also be due to cell specific factors such as neuronal projections, differential protein expression, calcium homeostasis, metal storage, and neurotransmitter transport (Double et al., 2010).

Rationale for my study

In the model system used in our studies, the ventral lateral neuronal subset of the circadian pacemaker circuit in *D.melanogaster,* the sLN$_v$ appear early in the first instar larval stage and the lLN$_v$ are detectable only during middle to late pupal stage (Helfrich-Forster et al., 2007). There is a 7 day lag between the appearances of the PDF positive sLN$_v$ versus lLN$_v$ during development. This difference in the timing of development of the two LN$_v$ subsets may be a factor in the selective vulnerability of sLN$_v$ to pathogenic HTT. The difference in the rate of disappearance of PDF signal could be attributed to two factors- the total duration of HTT expression in the cells or the stage specificity of HTT expression. The increased susceptibility of the sLN$_v$ to HTT as compared to the lLN$_v$ could be because of the fact that the sLN$_v$ begin to express HTT approximately 7-9 days prior to the lLN$_v$. If so, it would be expected that given enough time (7-9 days more), lLN$_v$ should also show loss of PDF similar to sLN$_v$. However, PDF was present in lLN$_v$ of 40 day old *pdf>Q128* flies. Also, it has been shown that the HTT-Q128 expressing lLN$_v$ continue to show normal electrical activity, even after 20 days from emergence (Sheeba et al., 2008). These results suggest that differences in the duration of HTT expression alone cannot explain the selective

susceptibility of the sLN$_v$. We went on to test the second possibility that HTT might exert a developmental stage-specific effect that contributes to loss of PDF in the early appearing sLN$_v$ but not the later appearing lLN$_v$.

We aimed to determine whether the expression of HTT during the pre-adult versus adult stage alone can alter selective vulnerability of the sLN$_v$. In the process we also asked how the circadian rhythms are disrupted due to temporally altered expression of pathogenic protein.

To achieve temporal control of tissue-specific expression, it was necessary to use an inducible system. It was not possible to use temperature sensitive tubulin Gal80 system since studies in our lab suggest that warm temperatures of 29°C can have direct effects on the toxicity of HTT-Q128. Therefore, we adopted the inducible Gene Switch system to target expression of HTT in the circadian pacemaker neurons- the lLN$_v$ and the sLN$_v$ (~16 cells in the brain) to either the pre-adult or adult stages of the fly (Depetris-Chauvin et al., 2011). This system allows one to have spatial as well as temporal control.

The *Gene Switch* system is based on a chimeric gene that encodes a GAL4 binding domain, the human progesterone receptor-ligand-binding domain, and the activation domain from the human protein, p65. In presence of antiprogestin, RU486, the chimeric molecule binds to upstream activation sequences (UAS) leading to ligand-inducible activation of downstream gene of interest, while no expression is detected in the absence of the ligand (Osterwalder et al., 2001; Roman et al., 2001).

My studies reveal that expression of HTT-Q128 in pre-adult plus adult stages led to early age arrhythmicity onset, whereas adult restricted HTT-Q128 expression

was sufficient to delay onset of arrhythmicity. Furthermore, larval restricted HTT-Q128 induction resulted in onset of arrhythmicity of adults, albeit at a later stage. The selective vulnerability of sLN$_v$ in terms of PDF loss was not due to a life-stage specific effect of HTT-Q128, but the rate of loss was synergistically faster with pre-adult and adult expression.

Materials and Methods-

Fly lines

Transgenic UAS lines for human *htt* gene carrying the coding region for first 548 aa of *htt* gene with non-pathogenic form (0 Q repeats i.e *w;UAS-HTT-Q0A;+*) and a line containing a pathogenic tract of 128 glutamine repeats (Q128 i.e *w;UAS-HTT-Q128:+*) were used, provided by Troy Littleton, MIT, USA (Lee et al., 2004). Transgenic PDF driven *Gene Switch* (*w^{1118};+;pdf>GS*) lines were obtained from Maria Fernanda Ceriani , Laboratorio de Gene´tica del Comportamiento, Buenos Aires, Argentina (Depetris-Chauvin et al., 2011).

Fly maintenance

Flies were grown and maintained at 25°C in vials containing standard cornmeal medium under LD 12:12 (~250 lux), except those with RU486 (Mifepristone, Sigma). RU food was dissolved in 80% ethanol (Stock of 4mg/ml) and added to standard cornmeal medium to a final concentration 750 µg/ml (henceforth, RU). The same amount of 80% alcohol was also added to food for control flies (henceforth, vehicle) (Depetris-Chauvin et al., 2011). Virgin females of *pdf>GS* were crossed to males of UAS line to obtain flies of desired genotype. Henceforth, *pdf-GS>Q0* and *pdf-GS>Q128* will be used to denote flies carrying

the *Gene Switch* GAL4 driver along with UAS-HTTQ0 and UAS-HTTQ128 constructs respectively. The crosses were set up in standard medium for about 2 days and then transferred to vials with RU or vehicle containing medium. Flies developing in RU food were collected in food vials with vehicle only immediately after eclosion (RV) ensuring that pdf-GS driven UAS-HTT protein induction was restricted to the pre-adult stage. For flies receiving RU only as adults, flies developing in vehicle were collected in RU food vials post eclosion (VR) ensuring that pdf-GS driven UAS-HTT protein induction was restricted to adult stages only.

Locomotor activity-rest rhythm assay

For all experiments virgin male flies of age 2-3 days were used. The day of eclosion was considered as age 0 day. Flies were placed into glass tubes (3 mm inner diameter and 65 mm long) and recorded using *Drosophila* activity monitors (DAM V) that record movement of flies across the length of the glass tube. An infra-red (IR) emitter-sensor pair is situated such that movement of the fly breaks the IR beam (TriKinetics, Waltham, USA; www.trikinetics.com) and is recorded as activity. The activity of flies was recorded in DD 25°C in an incubator (Sanyo MIR-154).

Flies were assayed for 21 days and fresh food was provided after every four days to ensure that RU levels had not been depleted. Care was taken to minimise disturbance while transferring flies into fresh tubes by giving food change on that time of the day when flies were maximally active. A dim red light was used to aid in visibility in otherwise constant darkness.

Analysis of activity-rest rhythm from DAM system

Activity was recorded in 1min bins. Raw time series data from individual flies were then binned into 15min. Data obtained during DD conditions was analyzed using the CLOCKLAB software from Actimetrics using the Chi Square periodogram with a significance of 0.01 to determine the whether the flies were rhythmic with a circadian period, to estimate period and robustness of the rhythm. Data was analysed using three different age windows- age 3-9 days, 10-16 days, and 17-23 days. The percentage rhythmicity across age windows was also calculated.

Statistical analysis

Statistical analysis of data was performed using the software Statistica 7.0. For activity/ rest behaviour, a 3-way ANOVA was performed to compare the period and amplitude of the two genotypes across three age windows with a p-value of <0.05. Age, treatment and genotype were considered as fixed factors. The ANOVA was followed by Post hoc comparisons of means using Tukey's honest significance test (HSD).

Immunocytochemistry

Adult fly brains were dissected at age day 1, 6 and 8. The dissections were carried out in 1X PBS. Brains were fixed with 4% paraformaldehyde (PFA) at room temperature for 30 minutes, followed by three washes with 1X PBS-0.5% Triton X-100 (0.5% PBT) at intervals of ten minutes . 10% horse serum in 0.5% PBT was used for blocking. The brains were blocked for 1 hour at room temperature followed by 6 hours at 4 °C. Primary antibodies rabbit anti-PDF

(1:30,000) and mouse anti-HTT (1:500) (Millipore) was added to the samples and were incubated at 4 °C for 48 hours. After removal of primary antibody, six washes were given with 0.5% PBT. Then secondary antibody, anti-rabbit Alexa 488 (1:3000) and anti-mouse Alexa 546 (1:3000) was added and tissues incubated for 24 hours at 4 °C. This was followed by six washes with 0.5% PBT. The whole brains were cleaned and mounted on slides using 70% glycerol in 1XPBS media with the ventral side facing upward. Imaging was done using Zeiss microscope (Axio Z1).

Results

1. Gene Switch inducible system facilitates targeting of circadian pacemaker neurons:

To validate the *Gene Switch* inducible system with the *pdf* promoter (*pdf-GS*) in combination with HTT-PolyQ flies carrying pathogenic UAS-HTT-Q128 or non-pathogenic UAS-HTT-Q0 transgenes driven by *pdf-GS* were fed <u>R</u>U or <u>v</u>ehicle during both larval as well as adult stages. Henceforth these treatments will be denoted as **RR** and **VV** respectively, where the first letter refers to the larval stage and the second to the adult stage. Flies undergoing RR treatment would be expected to either express HTT-Q128 or HTT-Q0 throughout their life (both pre-adult as well as adult stages) as opposed to their VV controls where neither HTT-Q0 nor HTT-Q128 would be expressed at any stage. To enable quantification of age-specific effects of the treatments, data was analysed in three age windows consisting of seven consecutive days: days 3-9 = age window 1; days 10-16 = age window 2 and days 17-23 = age window 3. In case of both the genotypes, VV treated flies were rhythmic across all the three age windows as expected (Fig 1A,

left panels, 1B). *pdf-GS>Q128* flies under RR regime were arrhythmic in all the age windows as opposed to the *pdf-GS>Q0* flies, which were rhythmic throughout (Fig 1A, right panels, 1B). *pdf-GS>Q0* and *pdf-GS>Q128* flies subjected to VV treatment had a period ≈24 hour across all the age windows with no significant difference between the two genotypes (Tukey's HSD, *p=0.99*) (Table 1, 3 and 5). RR treated *pdf-GS>Q0* flies, exhibited a period of ≈25 hours across all the three age windows (Table 1) which was significantly higher than the same genotype under VV treatment (Tukey's HSD, *p=0.000948*) (Table 3). The RR treated *pdf-GS>Q128* flies also exhibited a period >24 hours in the first two age windows while the same genotype under VV had a period ≈24 hours across age windows (Table 1). The period values were not computed for *pdf-GS>Q128* flies in RR treatment in the third age window since all the flies were arrhythmic (Table 1). There was no difference in the robustness of rhythm of *pdf-GS>Q0* flies across age windows in both RR and VV treatments (Tukey's HSD, *p=0.08*). The *pdf-GS>Q128* flies in VV treatment had a significantly higher robustness of rhythm in the last two age windows as compared to the first age window (Fig 3B, right panel) (For age window 1 and 2, Tukey's HSD, *p=0.000021*) (For age window 1 and 3, *p=0.0005*). Robustness of rhythm under VV treatment was significantly higher than all other treatments in the second age window. With VV treatment there was no difference between the *pdf-GS>Q0* and *pdf-GS>Q128* flies with respect to robustness of rhythm in any of the age windows (Tukey's HSD, *p=0.89*). Robustness could not be computed for *pdf-GS>Q128* flies in RR treatment in the last age window since all the flies were arrhythmic (Fig 3B, left panel and Table 2). Thus, we confirmed that the *pdf>GS* system can reliably cause arrhythmia in the LN$_v$, possibly via induction of HTT-Q128.

2. Adult-specific expression of HTT-Q128 disrupts the circadian activity/rest rhythms

To examine the effect of HTT expression when temporally restricted to either pre-adult stage or only-adult stages, both *pdf-GS>Q0* and *pdf-GS>Q128* were subjected to two kinds of treatments- RU containing food only as adults, while larvae were fed vehicle (henceforth **VR**) or RU food only as larvae, while adults were fed vehicle (henceforth **RV**). Flies developing in vehicle were collected in RU food vials post eclosion (VR) and flies developing in RU were collected in food with vehicle immediately after eclosion (RV) ensuring that *pdf-GS* driven HTT-PolyQ protein induction was restricted to the adult and pre-adult stages respectively. *pdf-GS>Q0* flies were rhythmic in both the treatments as expected (Fig 2A, lower panels). The *pdf-GS>Q128* flies, with VR treatment, were rhythmic to begin with i.e. in the first age window (Fig 2A, top left panel) with percentage rhythmicity of around 70% (Fig 2B). However, in the last age window, the percentage rhythmicity decreased to zero (Fig 2B). The results of the VR treatment suggest that pathogenic HTT expression has adult-specific effects that are evident almost immediately in about 30% of the flies (Fig 2B and 3A). However, within the next two age windows most flies become strongly affected showing that adult specific expression can cause dysfunction of sLN$_v$ within 7 -14 days. Upon RV treatment, the *pdf-GS>Q128* flies showed percentage rhythmicity as high as 86% in the first age window which decreased to 60% and then to 55 % in the last two age windows (Fig 2A, top left and 2B). It shows that pathogenic HTTQ128 induction restricted to larval stages may offer an initial reprieve to rhythmicity of adults, but at least half the flies develop symptoms of dysfunctional sLN$_v$ after two weeks as adults even though active HTT-Q128 production has

stopped possibly as early as pre-pupal stage when larvae had stopped feeding on RU food.

The change in level of rhythmicity was accompanied by period changes as well. *pdf-GS>Q0* flies had a period >24 hours when they were subjected to the VR treatment (Table 1) and they did not differ from *pdf-GS>Q128* flies in the first two windows (For age window 1, Tukey's HSD, *p=0.70*) (For age window 2, Tukey's HSD, *p=0.73*). In VR treatment the period of *pdf-GS>Q0* in the second age window was significantly higher than the other two age windows (For age window 1 and 2, Tukey's HSD, *p=0.000018*) (For age window 2 and 3, Tukey's HSD, *p=0.028301*) (Table 1 and Table 3). The *pdf-GS>Q128* flies showed periods >24 hours for both VR and RV treatments across age windows (Table 1).

For RV treatment, the robustness of rhythm of *pdf-GS>Q0* flies in the second and third age window was also significantly higher than the first window (For age window 1 and 2, Tukey's HSD, *p=0.000161*) (For age window 1 and 3, Tukey's HSD, *p=0.014836*) (Table 5). However, there was no difference in robustness of rhythm between *pdf-GS>Q0* and *pdf-GS>Q128* in two age windows for both VR and RV. Period and amplitude could not be computed for *pdf-GS>Q128* flies in VR treatment in the last age window since all the flies were arrhythmic (Fig 2 and Table 3 and 4). On comparing *pdf-GS>Q128* across RU treatments, the % arrhythmicity in the first age window changed in the following manner: flies expressing HTT-Q128 throughout (RR) > flies with HTT-Q128 induced as adults (VR) > flies with HTT-Q128 induced as pre-adults (RV) (Fig 3A). In the second age window also this trend continued. In the third age window both RR and VR had no rhythmic flies, whereas in RV treatment 50% of the flies were still

rhythmic. Thus, with increase in duration of expression of HTT-Q128 (in VR treatment), greater fraction of flies become arrhythmic.

3. Selective loss of PDF signal from sLN$_v$ when HTT expression is restricted either to pre-adult or adult stages

pdf-GS>Q0 flies when subjected to RR and VV treatment showed PDF expression in sLN$_v$ and lLN$_v$ cell bodies as well as both dorsal projections (DP) and contralateral projections (CP) (data not shown). Under VV treatment, *pdf-GS>Q128* flies showed the presence of PDF positive sLN$_v$ and lLN$_v$ at both day 1 and 8 (Fig 4, 5 and 6) with intact projections. No HTT signal was detected confirming that in the absence of RU, there was no leaky expression of HTT (Fig 4). In *pdf-GS>Q128* flies under RR, PDF in the sLN$_v$ could not be detected at either day 1 or 8, whereas PDF was present in lLN$_v$ (Fig 4, 5 and 6). At day 1, HTT in lLN$_v$ was diffuse with few puncta (Fig 5), whereas on day 8 it was completely punctate (Fig 6). At both stages PDF was present in CP and DP with mostly punctate HTT in DP.

Among 1 day old *pdf>Q128* flies in VR, nearly half of the hemispheres examined showed the presence of PDF positive sLN$_v$ and the other half lacked PDF signal in the sLN$_v$ (Fig 4 and 5). lLN$_v$ were stained with PDF in all samples and HTT was mostly punctate (Fig 5). At day 8, these flies mostly lacked PDF positive sLN$_v$ and retained PDF in lLN$_v$ (Fig 4 and 6). HTT was highly punctate and was found outside the cells also (Fig 6). CP and DP showed the presence of PDF and punctate HTT on days 1 and 8. Thus, with adult-restricted HTT-Q128 expression the loss of PDF from sLN$_v$ appears to be slower compared to flies expressing HTT-Q128 throughout.

Similar to 1 day old *pdf-GS>Q128* in RV, 1 day old *pdf-GS>Q128* in VR showed anti-PDF stained sLN$_v$ in half of the hemispheres and the others lacked them and lLN$_v$ showed presence of PDF (Fig 4 and 5). HTT in sLN$_v$ and lLN$_v$ was mostly punctate (Fig 5). PDF was present in CP and DP. 8 day old flies mostly lack PDF positive sLN$_v$ but had PDF signal in the lLN$_v$ (Fig 4 and 6). HTT was undetectable at day 8 indicating that post removal of RU, the production of HTT having ceased, residual HTT has been cleared (Fig 6). PDF was present in CP and DP. Thus, with larval restricted HTT-Q128 induction, the loss of PDF from sLN$_v$ appears to be slower compared to flies expressing HTT throughout. Thus, regardless of whether HTT-Q128 is expressed as pre-adults or adults, there was loss of PDF signal in the sLN$_v$ although the rate was lowered. This indicates that sLN$_v$ vulnerability to HTT-Q128 does not seem to be due a stage specific effect of HTT-Q128, but the rate of loss is a accumulative effect of pre-adult plus adult expression.

Discussion

The inducible *Gene Switch* system enables spatial as well as temporal control of HTT expression in the LN$_v$, such that HTT is expressed only in the presence of progesterone – analogue RU. This system was used to ask whether pre-adult versus adult HTT expression alters the selective vulnerability of sLN$_v$ and circadian function under constant darkness. In our studies, flies of both genotypes that received VV were rhythmic, expressed PDF in both the sLN$_v$ and lLN$_v$ as expected, and there was no HTT staining indicating absence of leaky expression of HTT. The *pdf-GS>Q128* flies subjected to RR treatment were completely arrhythmic and this is in consonance with the finding that PDF expression was

lacking in the sLN$_v$ but not the lLN$_v$ and was characterized by presence of HTT puncta in the cell bodies as well as in the arbors. This helped us to validate the inducible gene expression system in our model.

To determine effects of stage specific expression of HTT, we induced HTT-Q128 expression only in the adult stage (VR) or only in the larval stage (RV). Flies expressing HTT-Q128 only as adults were mostly rhythmic initially and rhythmicity decreased across age windows with the fly being completely arrhythmic in the last age window. The early age rhythmicity of these flies can be explained by the presence of PDF expressing sLN$_v$ in 1-day-old flies and middle age arrhythmicity due to complete loss of PDF expressing sLN$_v$. The slower rate of sLN$_v$ loss in the VR, despite HTT-Q128 levels being high enough to form puncta could be a consequence of HTT being induced only post eclosion. As a result, HTT-Q128 was present for a shorter duration of time which might be insufficient to cause sLN$_v$ dysfunction in terms of PDF loss. Given sufficient time, HTT-Q128 does lead to complete PDF loss from sLN$_v$ as evident from 8-day-old flies.

Flies with HTT-Q128 expression induced only as larva were about 86% rhythmic initially and this decreases with age; but 50% of the older flies were still rhythmic. Here again, early age rhythmicity seems to be a consequence of presence of PDF in sLN$_v$ at day 1. Despite complete loss of PDF signal in the sLN$_v$ at day 8, half of the flies remained rhythmic. This suggests that absence of PDF from sLN$_v$ cell bodies does not necessarily always result in arrhythmicity. This is very surprising, since downregulation of PDF from sLN$_v$ was sufficient to render flies arrhythmic (Shafer et al., 2009). It is possible that despite loss of PDF from sLN$_v$

cell bodies in this treatment, the PDF in the projections could be cycling rendering flies rhythmic. It is also possible that, even though the end result is PDF loss, the HTT-Q128 induced PDF loss and PDF loss via RNAi mediated downregulation have very different functional consequence. Another explanation is that in 8 day old flies no HTT was detected. In the absence of HTT-Q128, even though PDF in sLN$_v$ once lost was not restored, the overall health of sLN$_v$ may have improved. As a functional consequence of improvement of health of sLN$_v$, flies might be rhythmic even in the absence of detectable PDF in cell bodies of sLN$_v$. These results suggest that loss of PDF from sLN$_v$ cell bodies does not always translate into arrhythmicity in activity/rest behaviour. Also, continuous HTT induction as adults seems to be required for arrhythmicity to be sustained. The slower rate of sLN$_v$ loss in RV treatment, despite HTT-Q128 being induced right at the larval stage could be due to lack of active induction of HTT-Q128 immediately post-eclosion. In the absence of continued HTT induction post-eclosion, the HTT punctae already formed may get cleared over time as evident on day 8 when no HTT was detected. The larval induced HTT levels might not be sufficient to cause complete loss of PDF from sLN$_v$ at early age day 1. But, it could have set in motion events leading to complete PDF loss from sLN$_v$ over time, as observed on day 8, even though at this age HTT was not detected. In order to get a better understanding of the dynamics of HTT induced over time, it would be important to determine the time taken for complete HTT induction post RU administration, the duration for which expression can be sustained for a given dose of RU and time taken for complete clearance of HTT after RU removal.

We found that both adult induced HTT expression and larval induced HTT expression were sufficient to render flies arrhythmic at middle and late age

windows to different magnitudes. Both these treatment are also sufficient for loss of PDF stained sLN_v; but at a slower rate compared to flies expressing HTT throughout. In conclusion, the selective susceptibility of sLN_v does not seem to be due to the time at which HTT expression begins. However, rate of PDF loss from sLN_v does seem to depend on both the duration of HTT expression in sLN_v and active HTT induction as adults.

Flies expressing HTT-Q128 only as pre-adults (RV), despite having no HTT at age 8 have no PDF expressing sLN_v and yet a half of them are rhythmic. This indicates that behavioural rhythms might be a reflection of both the presence of PDF in sLNv and a consequence of HTT presence on cellular health and function. This result also suggests that loss of PDF from sLN_v due to HTT-Q128 induction as larvae cannot be overcome by stopping induction of HTT as adults and hence, PDF loss is an irreversible change. To examine whether irreversibility of PDF loss is due to sensitivity of the larval stage alone and if behavioural arrhythmicity in adults is reversible, further studies using *Gene Switch* to cause HTT expression only for short durations during adulthood, either early or late adult life would be useful.

In the early age window the percentage of rhythmic flies decreases in the order: flies with HTT induced as pre-adults only > HTT induced as adults only > HTT expressed throughout. Thus, in terms of behaviour, flies expressing HTT as pre-adults and adults show the most severe phenotype. In terms of loss of PDF in sLN_v also, HTT expression throughout is most severe with the other two treatments (VR and RV) showing a delayed loss. More time-resolved dissections along with detailed quantification of PDF and HTT staining intensity and HTT

aggregate size and number would provide clarity regarding progression of cellular degeneration. In summary, temporally regulating HTT expression in PDF positive LN$_v$ has helped us gain a broad idea of the time at which HTT expression might be important and given us many interesting leads to follow upon.
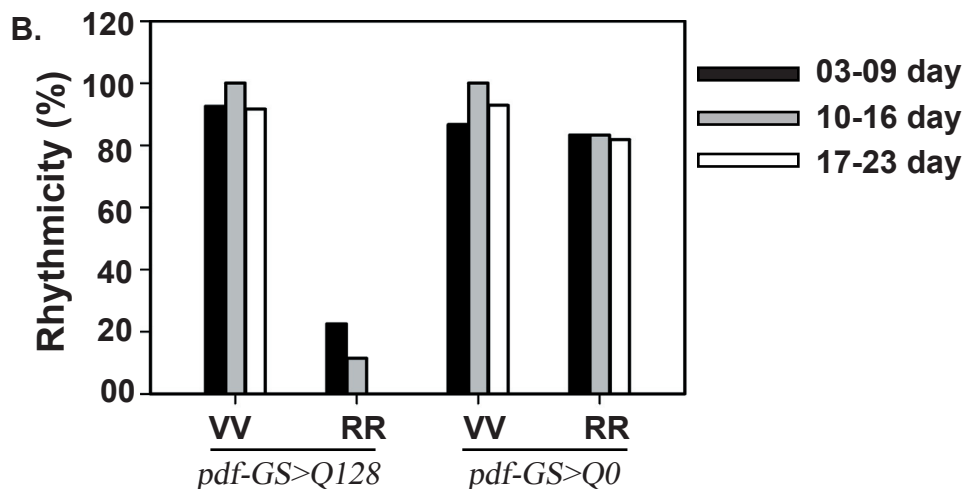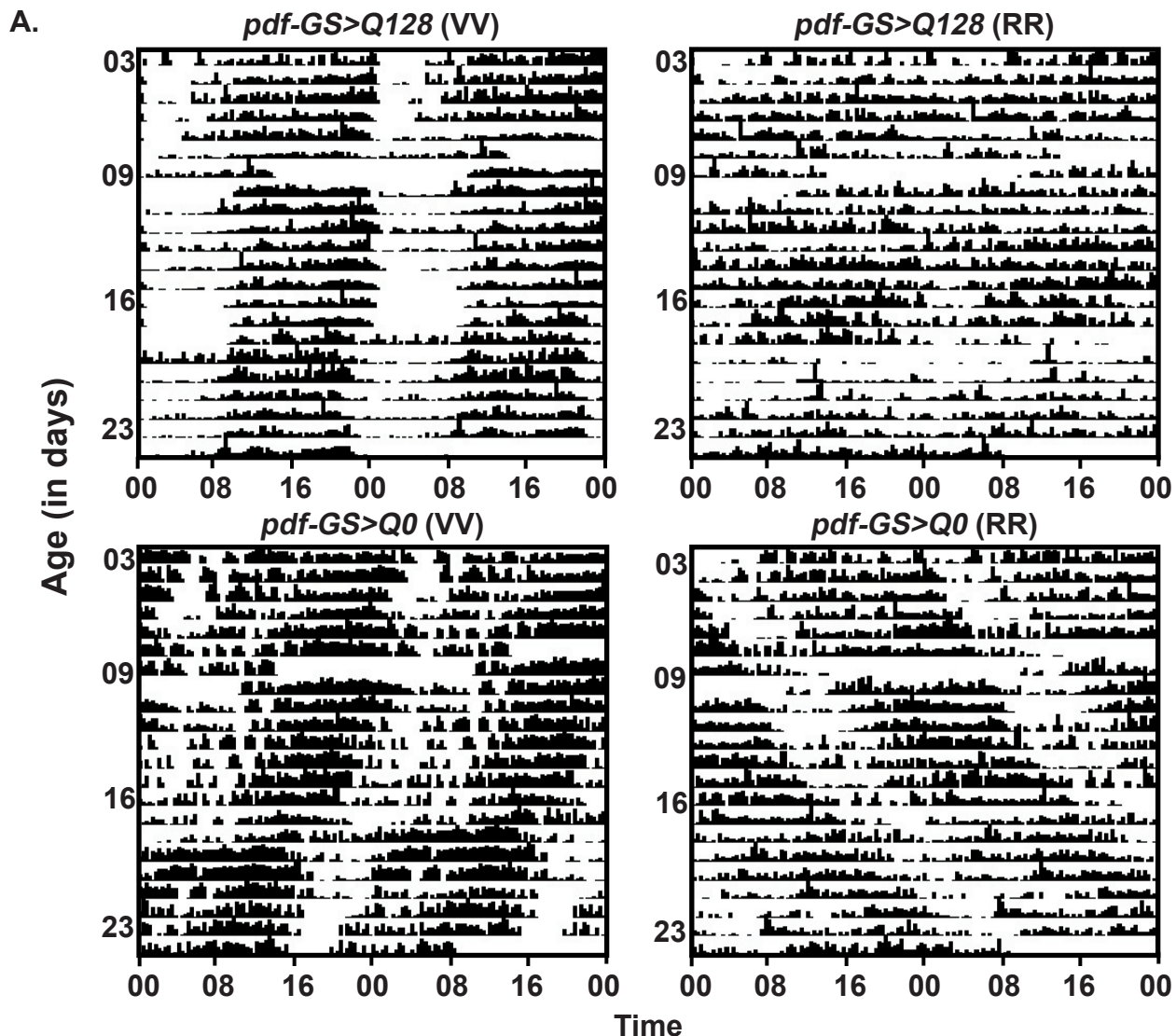
**Figure 1: *pdf>GS* inducible system allows targetting of circadian pacemaker neuons.** (A) Representative normalised double-plotted actograms of activity/rest behaviour of virgin male flies in DD at 25°C for 21 days (day 3-23). The x-axis depicts time of the day (0-48h) and the y-axis represents consecutive days. The respective genotypes and treatments are indicated above each actogram. *pdf-GS>Q128* flies fed with RU were arrhythmic (top right panel) and with vehicle were rhythmic (top left panel). (B) The percentage of rhythmic flies in DD 25°C is plotted against three age windows. *pdf-GS>Q128* flies fed with RU during development and adult stages (RR), are mostly arrhythmic across all the three age windows and while those fed on vehicle (VV) are rhythmic throughout.
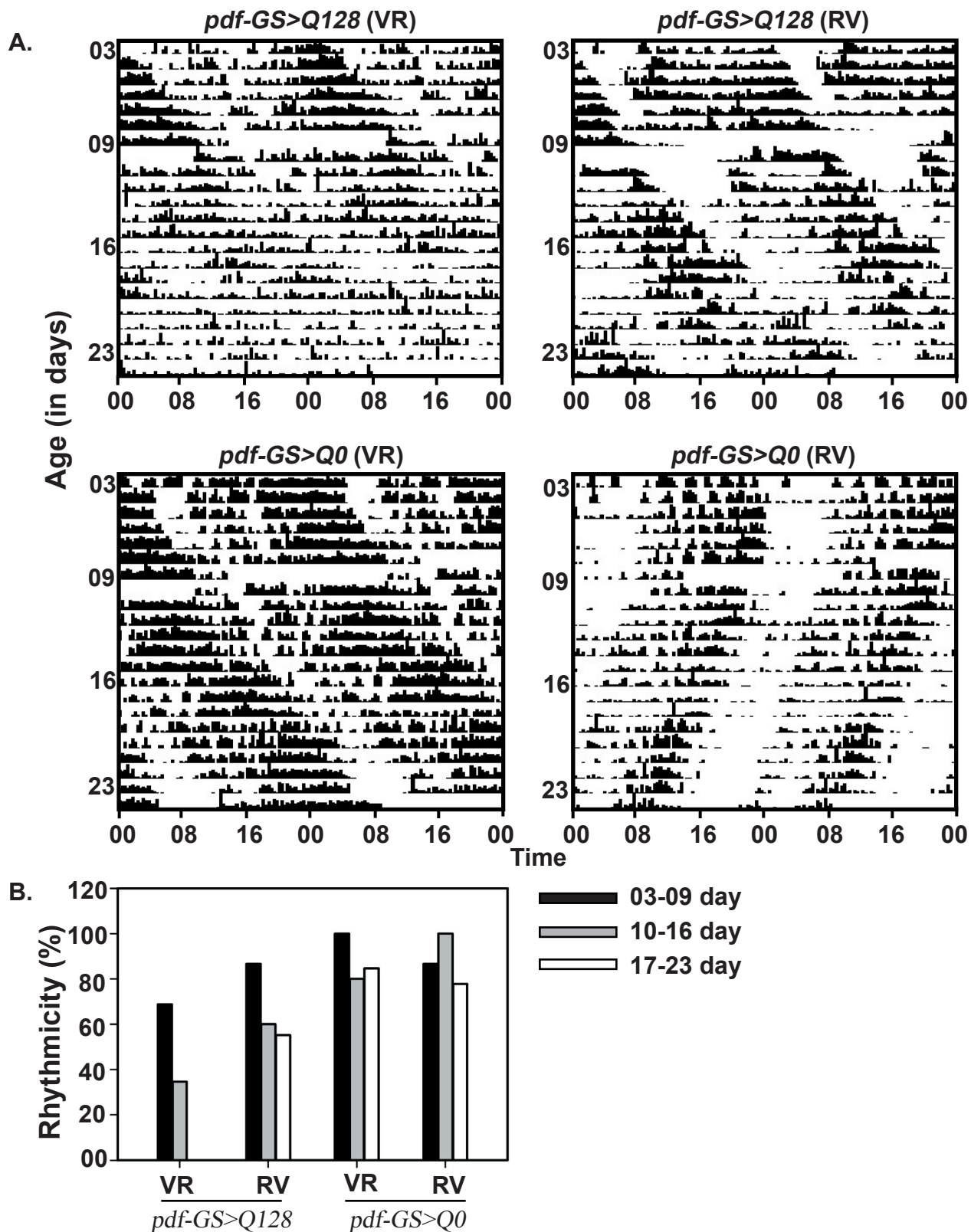
Figure 2 : Adult specific expression of HTT-Q128 in the pacemaker neurons disrupts the circadian locomotor activity. (A) Representative normalised double-plotted actograms of activity/rest behaviour of virgin male flies in DD at 25°C for 21 days (age 3-23). The x-axis depicts time of the day (0-48h) and the y-axis represents consecutive days. The respective genotypes and treatments are indicated above each actogram. (B) The percentage of rhythmic flies in DD 25°C subjected to VR and RV treatment for all the age windows. VR treatment renders the flies arrhythmic in the last age window while RV treatment decreased the percentage of rhythmic flies across age, from being 86% rhythmic in the first age window to 50 % in the last age window.
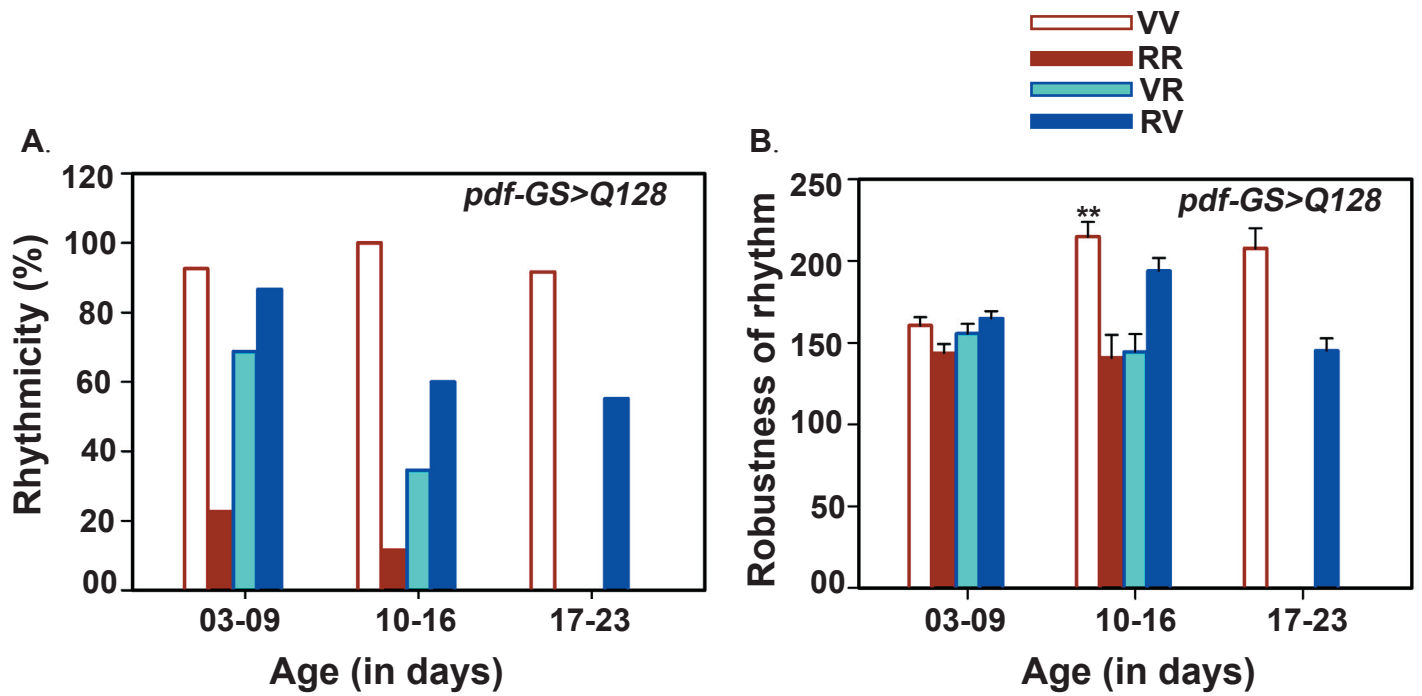
Figure 3 : Rhythmicity and robustness of rhythm of *pdf-GS>Q128* across treatments and age windows. (A) The percentage of rhythmic flies in DD 25°C are plotted for *pdf-GS>Q128* in all the four treatments. Flies in VV treatment are rhythmic as expected and those in RR treatment are completely arrhythmic. Flies in VR and RV treatment were rhythmic to begin with and gradually became arrhythmic. (B) Robustness of rhythm in the three age windows. Robustness of rhythm under VV treatment was significantly higher than all other treatments in the second age window. Asterisks indicate *p*<0.05. Error bars are S.E.M.
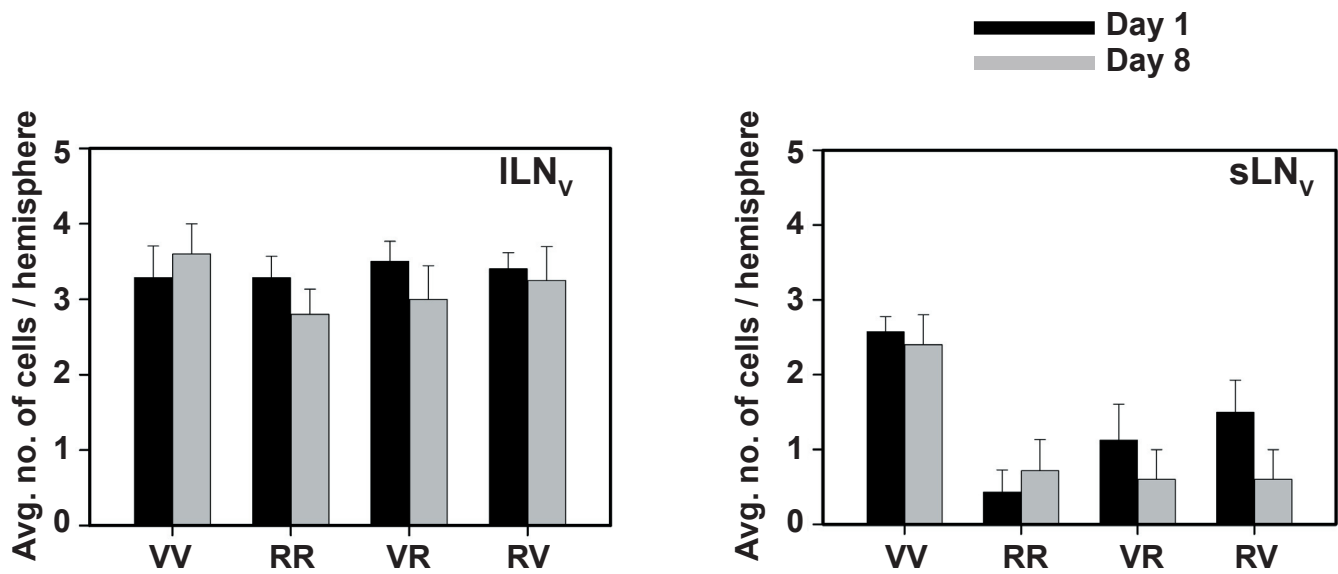
Figure 4: Selective loss of PDF-immunoreactivity in the small LN$_v$ irrespective of when HTT-Q128 is expressed. The average number of LN$_v$ (± SEM) detected using anti-PDF in day 1 and 8 are plotted for *pdf-GS>Q128* in four different treatments. The *pdf-GS>Q128* flies in VV have both sLN$_v$ and lLN$_v$ at both day 1 and 8. The *pdf-GS>Q128* flies in other treatments have PDF signal in the lLN$_v$ but the number of sLN$_v$ with PDF signal are less at both day 1 and 8.

Fig 5: An image of 1 day old adult *Drosophila* whole brain of *pdf>Q128* immunostained with antibodies against PDF (green), and HTT (red). Arrow: sLN$_v$, Arrow head: lLN$_v$, Block arrow: Diffuse HTT, Chevron: Punctate HTT. *pdf-GS>Q128* flies in VV treatment (first panel) shows PDF staining in both sLN$_v$ and lLN$_v$ with no HTT staining indicating no induction. Those in RR treatment (second panel) show PDF staining in only lLN$_v$ with diffuse HTT staining in cell bodies. In VR treatment (third panel) PDF staining is seen in both lLN$_v$ and sLN$_v$ with diffuse HTT staining. In RV treatment (last panel) PDF staining is seen in lLN$_v$ and faint outline of the sLN$_v$ and with punctate HTT staining. Scale bar = 50 μm.
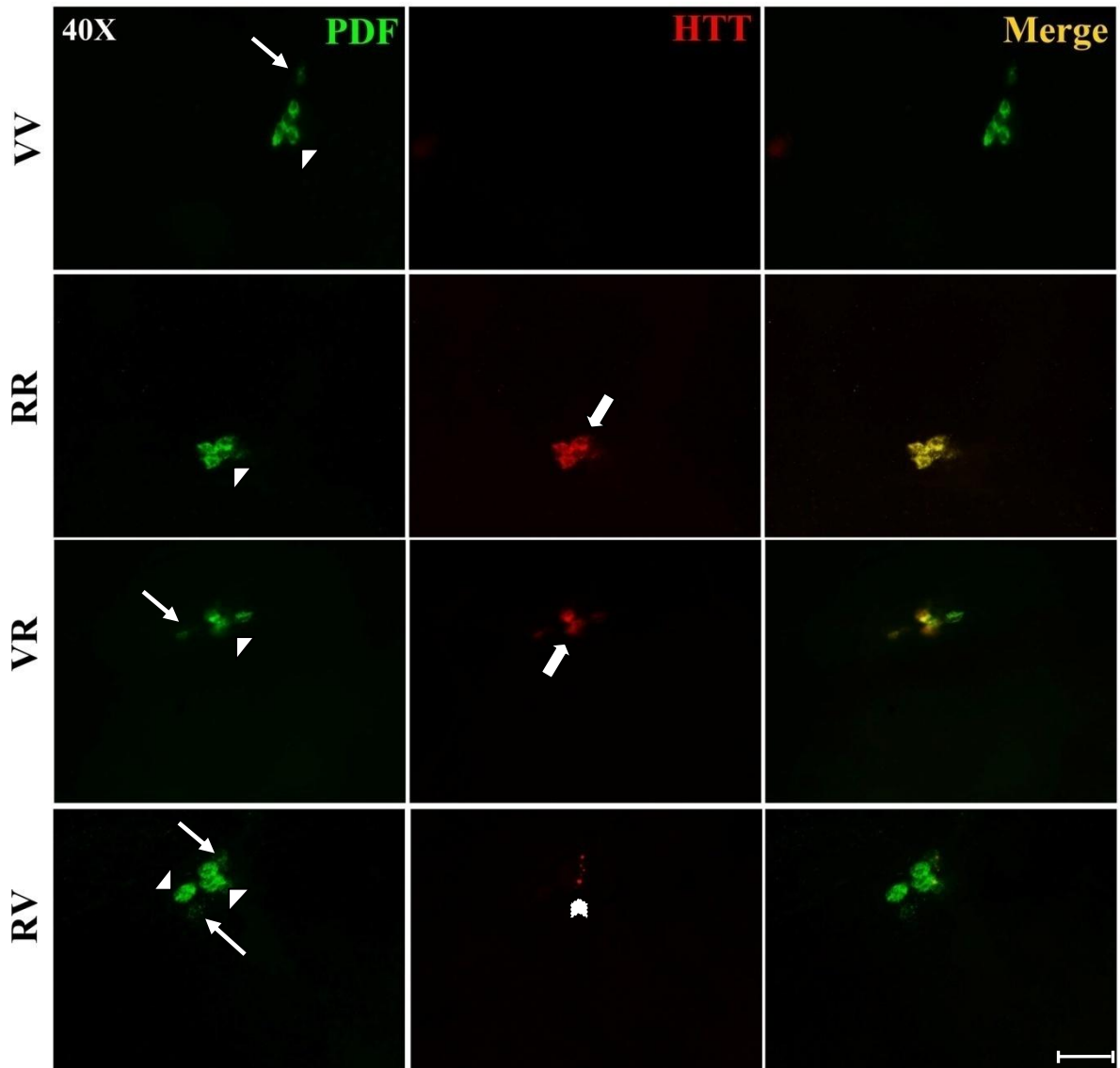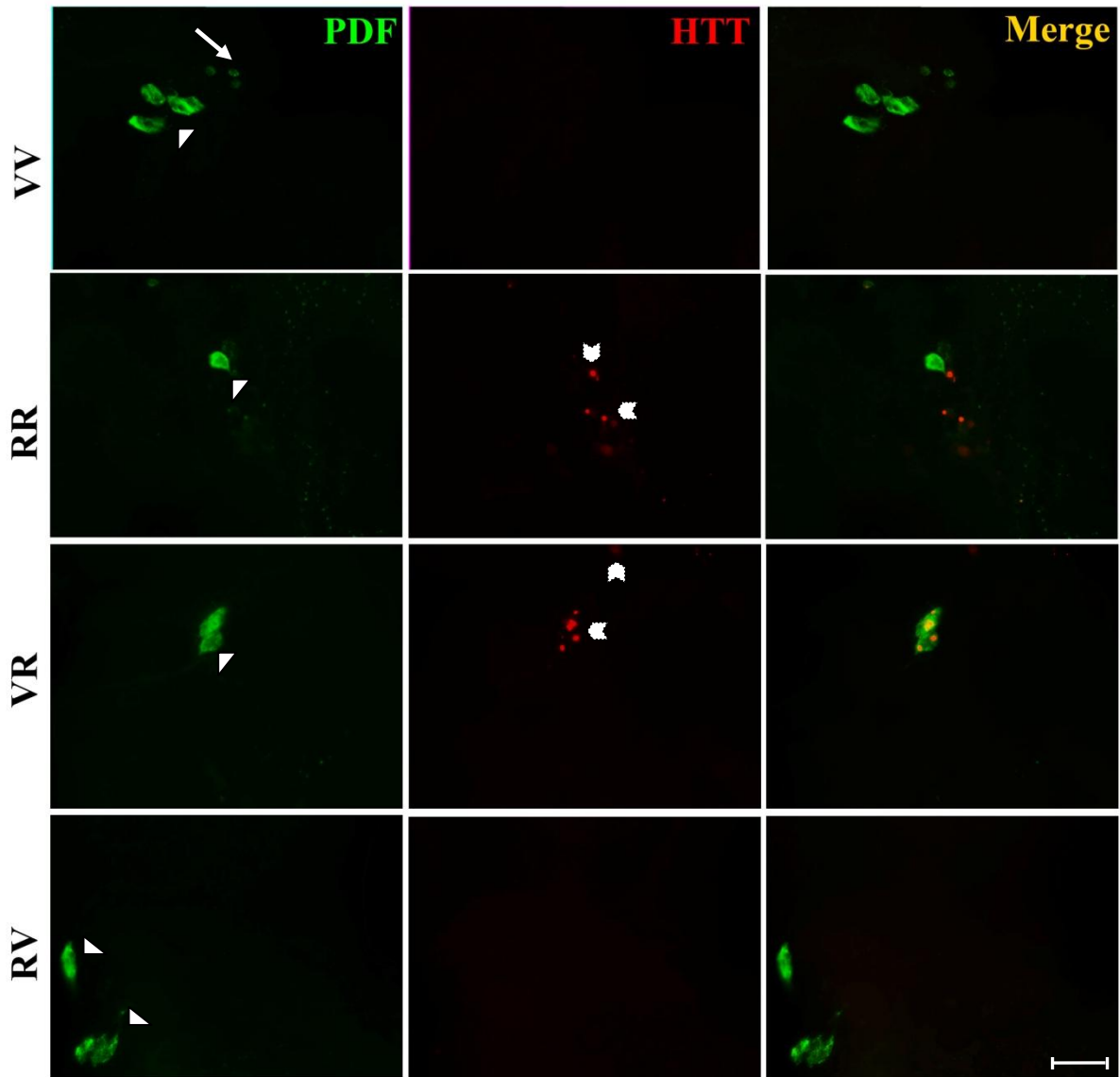
Fig 6: An image of 7 day old adult *Drosophila* whole brain of *pdf>Q128* immunostained with antibodies against PDF (green), and HTT (red). Arrow: sLN$_v$, Arrow head: lLN$_v$, Block arrow: Diffuse HTT, Chevron: Punctate HTT. *pdf-GS>Q128* flies in VV treatment (first panel) shows PDF staining in both sLN$_v$ and lLN$_v$ with no HTT staining indicating no induction. Those in RR treatment (second panel) show PDF staining in only lLN$_v$ with punctate HTT staining in cell bodies. In VR treatment (third panel), PDF staining is seen only in lLN$_v$ with punctate HTT staining. In RV treatment (last panel) PDF staining is seen in lLN$_v$ with no HTT staining indicating that there is no longer HTT induction. Scale bar = 50 µm.

Mean Period (h ± sem)				
Genotype	Treatment	Age window 1	Age window 2	Age window 3
pdf-GS>HTT-Q128	VV	23.85 ± 0.05	23.59 ± 0.02	23.55 ± 0.01
pdf-GS>HTT-Q128	RR	25.00 ± 0.29	24.53 ± 0.29	NA
pdf-GS>HTT-Q128	VR	25.32 ± 0.16	25.82 ± 0.43	NA
pdf-GS>HTT-Q128	RV	25.04 ± 0.09	26.07 ± 0.15	25.02 ± 0.30
pdf-GS>HTT-Q0	VV	23.86 ± 0.12	23.46 ± 0.04	23.33 ± 0.16
pdf-GS>HTT-Q0	RR	25.13 ± 0.11	25.13 ± 0.50	25.00 ± 0.31
pdf-GS>HTT-Q0	VR	24.83 ± 0.15	26.47 ± 0.35	25.39 ± 0.31
pdf-GS>HTT-Q0	RV	23.73 ± 0.11	23.54 ± 0.03	23.46 ± 0.06

Table 1: Mean period in control and treated groups calculated for each free running phase. NA indicates not applicable.

Robustness of Rhythm (a.u ± sem)				
Genotype	Treatment	Age window 1	Age window 2	Age window 3
pdf-GS>HTT-Q128	VV	160.59 ± 5.01	214.81 ± 9.04	207.72 ± 12.24
pdf-GS>HTT-Q128	RR	143.75 ± 5.47	140.72 ± 14.11	NA
pdf-GS>HTT-Q128	VR	155.56 ± 6.02	144.39 ± 10.84	NA
pdf-GS>HTT-Q128	RV	164.89 ± 4.37	165.97 ± 4.84	145.16 ± 7.50
pdf-GS>HTT-Q0	VV	166.63 ± 8.86	182.73 ± 11.40	182.09 ± 9.43
pdf-GS>HTT-Q0	RR	165.22 ± 4.75	141.18 ± 9.44	135.13 ± 8.66
pdf-GS>HTT-Q0	VR	154.56 ± 5.46	147.32 ± 10.31	153.50 ± 8.42
pdf-GS>HTT-Q0	RV	137.57 ± 5.95	193.87 ± 7.94	181.69 ± 12.91

Table 2: Robustness of rhythm (arbitrary units) estimated from the amplitude of the periodogram for each age window of flies that were rhythmic in control and treated groups calculated for each free running phase.

Effects	df	MS	F	p-value
Age	1	2.6	5.6	0.0193
Genotype	1	6.8	14.8	<0.001
Treatment	3	38	82.3	<0.001
Age*Genotype	1	0	0	0.846
Age*Treatment	3	5.6	12.1	<0.001
Genotype*Treatment	3	16.2	35	<0.001
Age*Genotype*Treatment	3	3.9	8.4	<0.001

Table 3: Results of ANOVA on period for *pdf-GS>Q0* and *pdf-GS>Q128* for the first two age windows for all the four treatments (VV, RR, VR, RV).

Effects	df	MS	F	p-value
Age	1	3610	3.917	0.048996
Genotype	1	51	0.055	0.815111
Treatment	3	12173	13.209	<0.0001
Age*Genotype	1	72	0.078	0.780555
Age*Treatment	3	6966	7.559	0.000077
Genotype*Treatment	3	1366	1.483	0.220006
Age*Genotype*Treatment	3	8263	8.967	0.000012

Table 4: Results of ANOVA on robustness of rhythm for *pdf-GS>Q0* and *pdf-GS>Q128* for the first two age windows for all the four treatments (VV, RR, VR, RV).

Effects	df	MS	F	p-value
Age	2	1.8	6.7	0.001548
Genotype	1	42.6	157.7	<0.00001
Treatment	1	35.3	130.8	<0.00001
Age*Genotype	2	2.3	8.5	0.000279
Age*Treatment	2	2.7	10.0	0.000074
Genotype*Treatment	1	33.4	123.7	<0.00001
Age*Genotype*Treatment	2	1.8	6.6	0.001726

Table 5: Results of ANOVA on period for *pdf-GS>Q0* and *pdf-GS>Q128* for all the three age windows for VV and RV treatment.

Effects	df	MS	F	p-value
Age	2	15858	12.071	0.000011
Genotype	1	25	0.019	0.891309
Treatment	1	23286	17.725	0.000039
Age*Genotype	2	1189	0.905	0.406030
Age*Treatment	2	1373	1.045	0.353488
Genotype*Treatment	1	12398	9.437	0.002423
Age*Genotype*Treatment	2	13733	10.453	0.000048

Table 6: Results of ANOVA on robustness of rhythm for *pdf-GS>Q0* and *pdf-GS>Q128* for all the three age windows for VV and RV treatment.

Chapter 3: Upregulation of autophagy gene *atg8a* mitigates the toxicity of pathogenic Huntingtin in circadian pacemaker neurons

Introduction

Autophagy can be broadly defined as an intracellular process of degradation of cytoplasmic components within the lysososome. Both autophagy and ubiquitin mediated proteolysis are essential for quality control mechanisms of the cell and represents the bulk of degradation processes (Klionsky and Emr, 2000).

Autophagy can typically be divided into three types based on how cargo is delivered to the lysosome:

1. **Macroautophagy**: This is a conserved bulk clearance mechanism in eukaryotic cells, mainly activated during starvation consisting of three main stages: autophagosome formation, maturation and fusion with lysosome (Mizushima, 2005).

2. **Microautophagy**: This particular process involves the engulfment of the cytoplasm directly by the lysosome without involvement of intermediate transport vesicles (Kaushik et al., 2010).

3. **Chaperone mediated autophagy**:.

   This requires the substrate protein's pentapeptide sequence (Lys Phe Glu Arg Gln) to be recognised and selected by chaperone (Hsp 70) protein. This substrate–chaperone complex is recognized by LAMP2A, the lysosomal chaperone-mediate autophagy receptor. The protein substrate is then unfolded and translocated across the lysosomal membrane and is degraded in the lysosome (Kon and Cuervo, 2010).

Both chaperone mediated autophagy and microautophagy are poorly understood. Macroautophagy is fairly well characterized and will be referred to as autophagy from now on. Autophagy dysfunction has been one of the contributing factors in

several neurodegenerative disorders. The process of autophagy is tightly controlled by a set of genes collectively referred to as autophagy genes (*atg*). Genetic screening has identified around 33 different *atg* genes in yeast and many of which have mammalian homologues (Radad et al., 2015). The high degree of conservation suggests that autophagy has critical function across species. Autophagy involves a series of steps starting with initiation of autophagy, elongation of autophagosomes, cargo recognition, maturation of autophagosomes and fusion with lysosome leading to degradation.

Initiation and Elongation. The autophagy process can be regulated through the serine/threonine kinase mTOR (mammalian target of rapamycin pathway), which suppresses autophagosomes formation under nutrient rich conditions by phosphorylating Atg1 and Atg13. Initiation of autophagy when triggered by starvation leads to the dephosphorylation and activation of *atg1* complex. This in turn leads to the activation of the class III PI3 kinase Vsp34. The activated Vsp34 generates phosphatidylinositol 3-phosphate, which is an important lipid component of autophagosomes (Fimia et al., 2007; Liang et al., 2008; Ravikumar et al., 2010).

During the initiation stage, the autophagic membrane forms a phagophore and then elongates. Previously it has been demonstrated that endoplasmic reticulum, golgi body and plasma membranes could be the sources of this elongating membrane (Hailey et al., 2010; Yen et al., 2010; Yla-Anttila et al., 2009). It has been shown recently that endoplasmic reticulum and golgi body contact site is the source of autophagosomes formation (Hamasaki et al., 2013).

Initiation and elongation involves two major protein conjugations- conjugation of Atg12 to Atg5 and the microtubule–associated protein light chain (LC3-I), which is an ortholog of yeast Atg8, also present in *Drosophila* (Ravikumar et al., 2010). Atg12 and Atg5 are responsible for elongation of the autophagic membranes. The LC3-I protein is conjugated to phosphatidylethanolamine to form LC3-II, which is found on the inner and outer surfaces of autophagosomes and upon completion of autophagosomes formation Atg5-Atg12 conjugation is lost (Mizushima et al., 1998; Mizushima et al., 2003). LC3-II continues to remain attached to the autophagosomes after fusion with lysosome, thus serving as a marker for autophagy.

Cargo **recognition.** Presence of cargo adaptors i.e. molecules which enable autophagosomes to identify specific substrates are required for cargo recognition (Pankiv et al., 2007). Precise molecular mechanisms of cargo recognition are yet to be established. Autophagic receptors, like Atg19 and Atg34, have been identified that are responsible for selective transport of vacuolar enzymes by autophagic pathways (Leber et al., 2001; Scott et al., 2001). Other receptors like p62 and BRCA1 have also been identified, that aid in autophagic degradation of ubiquinated protein aggregates (Bjorkoy et al., 2005; Kirkin et al., 2009).

Maturation and Fusion. Autophagosome formation occurs in the cytoplasm and it is then transported along microtubules towards the microtubule organizing center, where lysosomes are abundant (Luzio et al., 2007). Here, the autophagosomes fuse to lysosomes and are then often referred to as autolysosomes. The cargo is then degraded by lysosomal hydrolases.

General functions of autophagy

Basal level of autophagy is critical for maintaining cellular homeostasis by selectively removing misfolded and aggregate-prone proteins and damaged organelles. It is also required during development and differentiation. It has been reported that autophagy is responsible for removal of non-vital cells/tissues and also remodelling functions during erythropoiesis, adipogenesis and lymphopoiesis (Aburto et al., 2012; Mizushima and Levine, 2010).

Among different types of cells in the body, autophagy is of particular importance for neuronal cells. Deletion of *atg7* in Purkinje cells resulted in progressive dystrophy and degeneration of axon terminals of these cells (Komatsu et al., 2007). Autophagy is also involved in synaptic plasticity (Lee, 2012). Therefore, autophagy is active in healthy neurons and maintained such that it does not deplete small cytoplasmic nutrient reserves (Hara et al., 2006; Komatsu et al., 2006).

Autophagy in neurodegenerative diseases

Recently, several studies have linked autophagy to neurodegenerative diseases (NDs). Many NDs like Alzheimer's, Parkinson's and PolyQ diseases have been characterized by low autophagic flux which leads to accumulation of autophagy substrates and toxicity. The pathological role of aggregates is debatable, but the presence of aggregates is directly linked to neuronal toxicity and the removal of the aggregates is often correlated with improvement in phenotype and health of cells (Yamamoto and Simonsen, 2011).

The fact that mHTT accumulates in the neurons suggest that a defect in macroautophagy could inhibit the clearance of the mutant protein (Sarkar and Rubinsztein, 2008). In Huntington's Disease (HD), the process of autophagy induction is probably not impaired because earlier reports have suggested that there are increased numbers of autophagosomes in mouse striatal neurons expressing truncated or full length human HTT, in human brains with HD and in HD mouse models (Davies et al., 1997; Sapp et al., 1997). Recent evidences have also pointed towards the fact that HTT functions as a scaffold in selective autophagy (Ochaba et al., 2014; Rui et al., 2015). Studies in *C. elegans* have shown that inactivation of *atg* increased the accumulation of HTT in the sensory neurons (Jia et al., 2007). Autophagy defects in HD have been attributed to the failure of cargo sequestration and presence of empty autophagosomes (Martinez-Vicente et al., 2010) which means autophagic vacuoles are unable to recognize cytosolic cargo in HD cells.

Autophagy is regulated by both mTOR dependent and independent pathways, which have multiple components that could be targeted for therapeutic manipulation. Studies have shown that modulation of autophagy could be a therapeutic strategy in NDs. It has been reported that rapamycin, an mTOR inhibitor, can attenuate neurodegeneration in both flies and mice models of HD by improving behaviour as well as motor performance (Ravikumar et al., 2002). Trehalose which is a sugar has also been implicated as a drug to regulate protein processing abnormalities in HD patients (Fernandez-Estevez et al., 2014). Studies in several model systems (mammalian cell lines, fly and rodent models) have shown improvement in HD phenotypes following treatment with various autophagy inducers (Sarkar, 2013).

Further, recent studies have indicated that the fly with its virtue of simple circuits and elegant genetic studies has emerged as a good model to understand the role of autophagy in neurodegeneration (Neufeld and Baehrecke, 2008). Therefore, we asked whether in the circadian HD model where a small number of cells are targeted and a robust behavioural phenotype can be scored, upregulation of autophagy could ameliorate HTT-Q128 induced phenotype both at the cellular and behavioural levels. Here, we show that upregulation of *atg8a* in flies expressing pathogenic HTT in the PDF neurons resulted in sustained improvement in rhythmicity and a delayed loss of PDF from sLN$_v$.

Materials and Methods

Fly lines

Transgenic UAS lines for human *htt* gene carrying the coding region for first 548 amino acids of *Htt* gene with non-pathogenic form (0 glutamine repeats, Q0 i.e *w;UAS-HTTQ0:+*) and a line containing a pathogenic tract of 128 glutamine repeats (Q128 i.e *w;UAS-HTTQ128:+*) were used, provided by Troy Littleton, MIT (Lee et al., 2004). Transgenic UAS lines carrying *atg genes* were used in this study (procured from Bloomington Stock Center, Bloomington, Indiana). The UAS-lines used were *atg1* (BL51655), *atg5* (BL8731) and *atg8a* (BL51656). Driver line used was *w^{1118};pdfGAL4;+* and *w^{1118};+;+* served as the wild type control in all the experiments.

Fly maintenance

The flies wear reared in LD 12:12 at 25°C and recorded at DD 25°C. Each of these UAS lines were balanced using a quadruple balancer w^{1118};*IF/Cyo;TM6,Tb/MKRS,Sb*. Balanced lines were crossed to each other such that each fly had both the UAS *htt* transgene and UAS *atg* gene. Females of *pdfGAL4* line were crossed to males of UAS line containing *htt* and *atg*, to obtain flies of desired genotype (*pdfGAL4/UAS HTT; UAS atg*). Activity rest rhythm of individual flies was assayed using DAM recording system.

Locomotor activity-rest rhythm assay

The method used is same as the chapter 2 except that here fresh food change was given on the 8th day and 15th day to prevent the food from drying up. The food change was given in DD using a red lamp. The assay was repeated four times with similar results.

Analysis of activity-rest rhythm from DAM system

Refer to Chapter 2 methods.

Statistical analysis

Software Statistica 7.0 was used. For activity/ rest behaviour, a 2-way ANOVA was performed to compare the period and amplitude of various genotypes across three age windows with a p-value of <0.05. Age and genotype were considered as fixed factors. To determine r, a measure of consolidation of activity across days, raw data was binned in one hour bins and r was then calculated in a lab generated program in MATLAB. r values were subjected to a, 2-way ANOVA with age and

genotype as fixed factors with at $p<0.05$ followed by Post hoc comparisons of means using Tukey's honest significance test (HSD).

Immunocytochemistry

Refer to Chapter 2 methods.

Results

1. Screening of autophagy lines

Three autophagy lines with genes of *atg1*, *atg5* and *atg8a* fused with UAS were used for the preliminary screen. These genes are involved at three different stages in the autophagy pathway. Atg1 induces autophagy, Atg5 is a part of the conjugation system and recruits other Atg proteins for the fusion of autophagosomes to lysosomes and Atg8a is further downstream, it facilitates the fusion of autophagosome to the lysosome. Our results show that co-expression of Atg8a with HTT-Q128 in the LN_v reduces the extent of arrhythmicity when compared to flies expressing HTT-Q128 alone under constant conditions of DD 25°C. The overexpression of Atg8a results in a rhythmicty of around 70% across all the three age windows (Table 1). However, the upregulation of *atg1* and *atg5* did not consistently increase rhythmicity (Table 1).

2. Upregulation of *atg8a* attenuates the pathogenic effects of HTT-Q128 on activity/rest behaviour

Upregulation of the autophagy gene, *atg8a*, in the *Drosophila* pacemaker neurons makes the flies behaviourally rhythmic. Three independent experiments were

performed and the results were consistent across experiments. To enable quantification of age-specific effects, data was analysed in three age windows consisting of seven consecutive days: days 3-9 = age window 1; days 10-16 = age window 2 and days 17-23 = age window 3. Percentage rhythmicity was computed across all the three experiments for the three age windows. Flies expressing HTT-Q128 were 10 % rhythmic in the first age window and 0% arrhythmic in the next to age windows (Fig 1, 2 and Table 2). It was seen that across three experiments the fraction of rhythmic HTT-Q128 flies over-expressing Atg8a were 78.05% in age window 1, 66.62% in the second age window and 70.4% in the last age window (Fig 2 and Table 2). There was a main effect of age window and genotype on rhythmicity (Table 3). Fraction of rhythmic *pdf/Q128;atg8a* flies were significantly higher compared to the *pdf/Q128* flies (Tukey's HSD, *p=0.000150*). However, the rhythmicity of *pdf/Q128;atg8a* flies were significantly lower than the GAL4 control and its Q0 controls which were nearly 100% rhythmic across all the age windows (Fig 2 and Table 4). .

Since the results of the three independent experiments were consistent, I will discuss the results of one with respect to their clock properties (period and robustness of rhythm) and day-wise consolidation of activity for 21 days (age 3d to age 23d). There was a main effect of age and effect of interaction between age and genotype on period (Table 5). *pdf/Q0;atg8a* flies had a slightly longer period in age windows 2 and 3 and differed significantly from *pdf/Q128;atg8a* (For age window 1, Tukey's HSD, *p=0.000018*) (For age window 2, Tukey's HSD, *p=0.000190*) (Fig 1 and 3A). The periodicities of UAS controls, experimental groups and other GAL4 controls are tabulated in Table 6 and Table 7.

Although a higher fraction of flies were rhythmic when Atg8a was co-expressed with HTT-Q128, the robustness of rhythm of *pdf/Q128;atg8a* flies was poor, thereby indicating only a partial rescue (Fig 3B). There was main effect of both age and genotype on the robustness of rhythm (Table 8). The robustness of rhythm of *pdf/Q128;atg8a* flies was significantly lower than *pdf/Q0;atg8a* in all the age windows and from *pdf/Q0;atg8a* and *pdf/+* flies in the last two age windows (Fig 3B and Table 11). The robustness of UAS controls was similar and not different from GAL4 controls (Table 9 and 10).

Estimates of rhythmicity, period and robustness were all calculated across 7-day windows thus giving us some indicators to compare across three stages in the life of the adult fly. In order to obtain a higher temporal resolution of changes in rhythm parameters we also estimated 'r' which is a measure of the extent of consolidation of activity across a 24 hr window, thus providing a marker of the locomotor behaviour on a daily basis. r was estimated for individual flies for each day from age 3-23 days. It was seen that *pdf/Q128;atg8a* flies have significantly higher r values compared to *pdf/Q128* flies on days 6, 7, 9, 10, 18 and 23. *pdf/Q128;atg8a* had a significantly lower r compared to *pdf/Q0;atg8a* on days of 9, 10, 11, 12, 13, 14 and 18 (Fig 4). This suggests that upregulation of Atg8a in the LNv of HTT-Q128 flies gives a partial rescue.

3. HTT-Q128 co-expression with Atg8a slows down the selective loss of PDF from sLN$_v$

To determine how Atg8a over expression may have caused reduction in pathogenicity of HTT-Q128, we examined the health of LN$_v$ in terms of PDF and HTT expression. *pdf/Q0* controls as expected show the presence of PDF in both

sLN$_v$ and lLN$_v$ cell bodies and projections at both day 1 and 7 (Fig 5, 6, 7, 8, 9 and 10). The HTT is diffuse in both sLN$_v$ and lLN$_v$ at both day 1 and day 7 (Fig 6, 7 and 9). One day old *pdf/Q128* flies did not show PDF signal in the sLN$_v$, but showed presence of intact lLN$_v$ with PDF signal (Fig 5 and 6). HTT in lLN$_v$ was both punctate and diffuse. PDF was mostly seen only in the dorsal projections (DP) with punctate HTT (Fig 8). Co-expression of Atg8a with HTT-Q128 resulted in presence of PDF signal in the sLN$_v$ and lLN$_v$ on day 1 (Fig 5, 6 and 7). HTT was punctate in sLN$_v$ and diffuse in lLN$_v$ (Fig 6 and 7). PDF was present in both dorsal and contralateral projections (CP) with diffuse HTT in CP and punctate HTT in most DP (Fig 8). Seven day old *pdf/Q128* flies did not show PDF in sLN$_v$ with PDF present in lLNv (Fig 5 and 9). HTT in lLN$_v$ and DP were punctate (Fig 9 and 10) and PDF was present in CP and DP (Fig 10). Flies co-expressing Atg8a with HTT-Q128 at day 7 also showed PDF only in lLN$_v$ (Fig 5 and 9) with punctate HTT in lLN$_v$ (Fig 9). PDF was present in both CP and DP and HTT was punctate in DP (Fig 10). The number and size of puncta were not quantified. Visual observations showed that in day 1 *pdf/Q128* flies, HTT in lLN$_v$ was both diffuse and punctate, whereas those in *pdf/Q128;atg8a* was diffuse. Also with age, both *pdf/Q128* and *pdf/Q128;atg8a* flies showed HTT aggregates more often. In conclusion, co-expressing Atg8a with HTT-Q128 delayed the loss of PDF from sLN$_v$ and aggregation of HTT in lLN$_v$.

Discussion

The results of our studies revealed that co-expression of Atg8a with HTT reduced the pathogenic effect of HTT-Q128 in the circadian pacemaker neurons of *Drosophila melanogaster*. Previous results in our laboratory and my own results confirm that HTT-Q128 expression in the PDF neurons disrupts the circadian locomotor behaviour and the flies become arrhythmic in constant darkness (DD). Arrhythmicity was evident on day 1 of DD and these flies continued to be arrhythmic for the next 21 days. At the cellular level, it was found that there was selective loss of PDF from the sLN$_v$ cell bodies but not the the lLN$_v$. The loss of PDF signal from the sLN$_v$ can explain their arrhythmic activity since PDF in the sLN$_v$ is required for persistence of rhythm in DD (Renn et al., 1999). In these flies the LN$_v$ were characterized by the presence of HTT puncta. Previous studies in the laboratory suggest that such aggregates are detectable even at the larval stage (Pavitra Prakash and Vasu Sheeba, unpublished work). The dorsal and contralateral projections even though intact based on PDF signal also harboured HTT puncta. The frequency of occurrence of puncta in the projections was more at day 7 than at day 1, especially near the dorsal projections. At a later stage (day 7), the aggregates were no longer restricted to the cell bodies. However, these results cannot distinguish between the mere lack of production of the neurotransmitter, PDF and the degeneration of sLN$_v$ cell bodies. Flies co-expressing Atg8a with HTT-Q128 were mostly rhythmic in constant darkness over age. However, the rhythmicity was poor in terms of robustness but they showed significantly higher daily activity consolidation than *pdf/Q128* flies. Hence, expression of Atg8a in *pdf/Q128* flies improves many of the rhythm properties at the behavioural level. This behavioural improvement seems to be a

functional consequence of improvements at the neuronal level. Our studies reveal that at the cellular level, for 1 day old *pdf/Q128;atg8a* flies, PDF was present in both sLN$_v$ and lLN$_v$ and HTT was diffused in the lLN$_v$ and punctate in sLN$_v$, as opposed to the flies expressing HTT-Q128 alone. At day 7, in *pdf/Q128;atg8a* flies, PDF signal from the sLN$_v$ was no longer present similar to the *pdf/Q128* but the lLN$_v$ were intact. Even though the lLN$_v$ were intact, they had HTT puncta. The dorsal projection and the contralateral projections were intact with occasional presence of puncta. The presence of PDF signal in the sLN$_v$ at an earlier age can explain their rhythmic behaviour albeit weak in the first age window. So overexpressing Atg8a is probably improving the general health of the LN$_v$ and delaying the pathogenic effects of HTT-Q128. Since Atg8a has been shown to be an important protein that enables fusion of the autophagosomes to lysosome, one would expect that overexpression of Atg8a might hike up the basal autophagy levels and help in clearance of aggregates. But our results reveal that aggregates are present in 1 day old flies of both arrhythmic *pdf/Q128* flies lacking PDF in sLN$_v$ and rhythmic *pdf/Q128;atg8a* flies with intact sLN$_v$. Based on our current results, co-expression of Atg8a in *pdf/Q128* flies does not seem to reduce aggregation. But HTT in lLN$_v$ of *pdf/Q128;atg8a* flies were all diffuse, whereas only about 50% of lLN$_v$ of *pdf/Q128* were diffuse. Better resolved dissections with aggregate quantification might reveal finer differences. Thus, expression of Atg8a in *pdf/Q128* flies improves their circadian phenotype both at the behavioural and cellular level.

To monitor the role of autophagy modifiers and to understand the age dependent progression of HD, it would be practical to work with an intermediately toxic form of HTT-Q50, which causes delayed onset of arrhythmicity (post 14 days of

age) and delayed PDF loss from sLN$_v$ (Vasu Sheeba, Keri J. Fogle, Saima Rashid, Shannon Farris, Todd C Holmes, unpublished work). Downregulation of autophagy in the pacemaker neurons could accelerate the loss of PDF from the sLN$_v$ leading to earlier onset of arrhythmicity.

Studies have shown that in HD, autophagy is affected at several steps including a defect in cargo loading, trafficking of autophagosomes, and decreased fusion between autophagosomes and lysosomes leading to a build-up of toxic materials in the cytoplasm and empty autophagosomes (Martin et al., 2014). Therefore, one could speculate that upregulation of *atg8a*, which is membrane marker for both autophagosomes and autolysosomes, brings about rescue by facilitating fusion of the autophagosomes to the lysosomes, thereby decreasing the cellular load and improving overall cellular function. To further investigate the health of these cells and corroborate our present findings, one could check if the PDF release in the projections oscillate as expected in wild type sLN$_v$ and examine whether oscillation of PERIOD protein are also intact. At a mechanistic level, it would be important to know whether autophagy is down regulated in HTT-Q128 expressing PDF neurons and if yes, then it would be crucial to determine the age at which this happens. It would be relevant to know whether upregulation of a*tg8a* actually results in a subsequent increase in autophagy. It would be interesting to look at the interaction between HTT-Q128 and Atg8a to elucidate the mechanism by which the rescue is being brought about.

To understand the role of Atg8a in a comprehensive way, one could look at other interacting partners of Atg8a, like Rab 7 (Hyttinen et al., 2012). Rab 7, a member of small GTPases, is known to be responsible for maturation of autophagosomes and endosomes (Hyttinen et al., 2012). Therefore, modulation of Rab 7 activity in

diseases caused by toxic effects of protein aggregates may be a useful therapeutic target. In our model system, study of such interacting partners would be helpful such that upregulation of autophagic processes improves neuronal health and could potentially delay arrhythmicity to a later stage. It would also be interesting to discern the stage in the lifecycle of our HD fly model when upregulation of autophagy is crucial. We could use the spatio-temporal Gene Switch system to get a stage-specific or duration-specific autophagy induction. To clarify the role of autophagy in HD, one could also look at chemical modifiers of autophagy to check if ingestion of certain chemical compounds can facilitate aggregate clearance and in turn improve the health of the cell. In conclusion, our data suggests that upregulation of autophagy via Atg8a enhances both neuronal function and its associated behaviour in HD flies. This study paves the way for understanding possible mechanisms through which autophagy might restore cellular health and associated function at the behavioural level in HD flies.
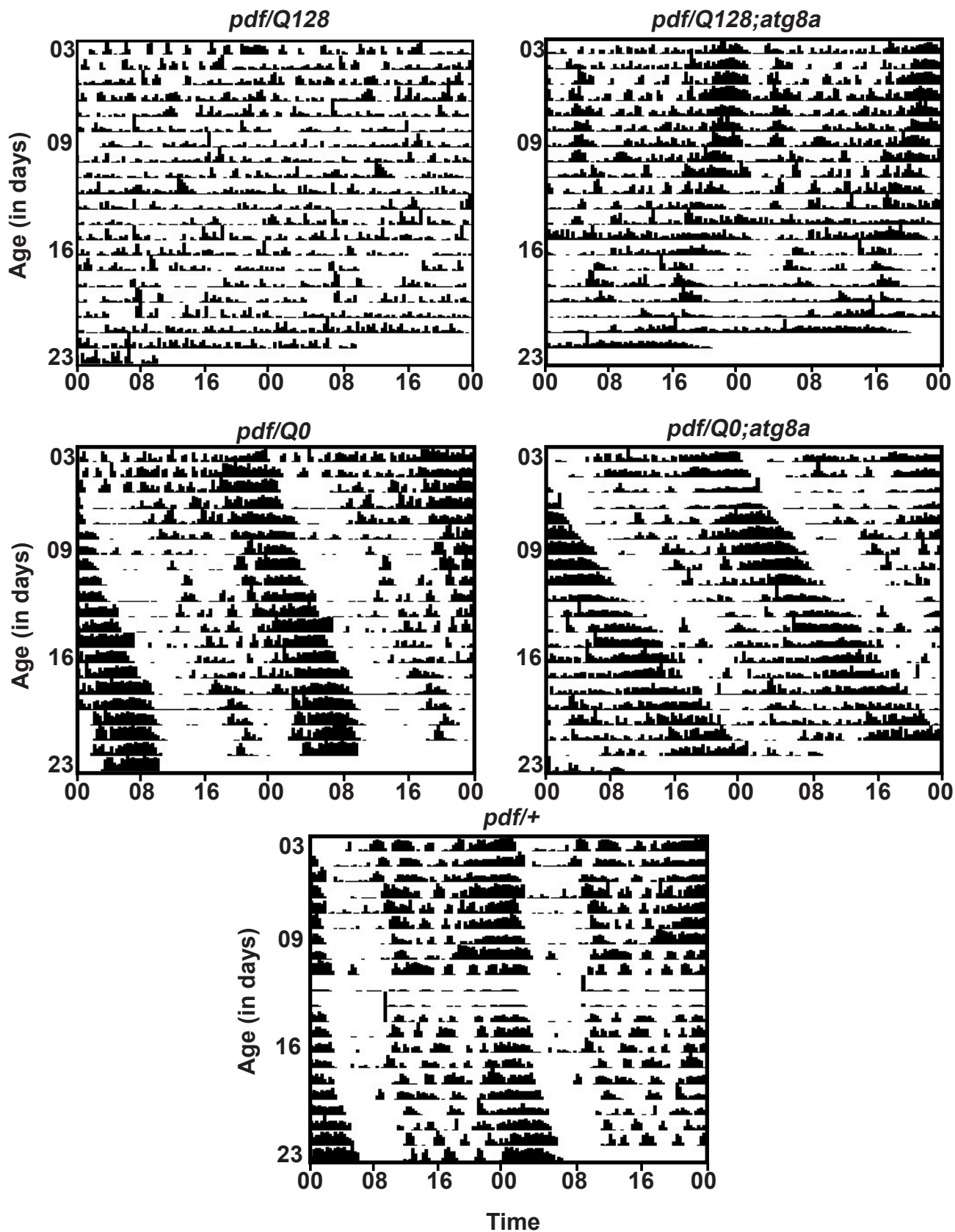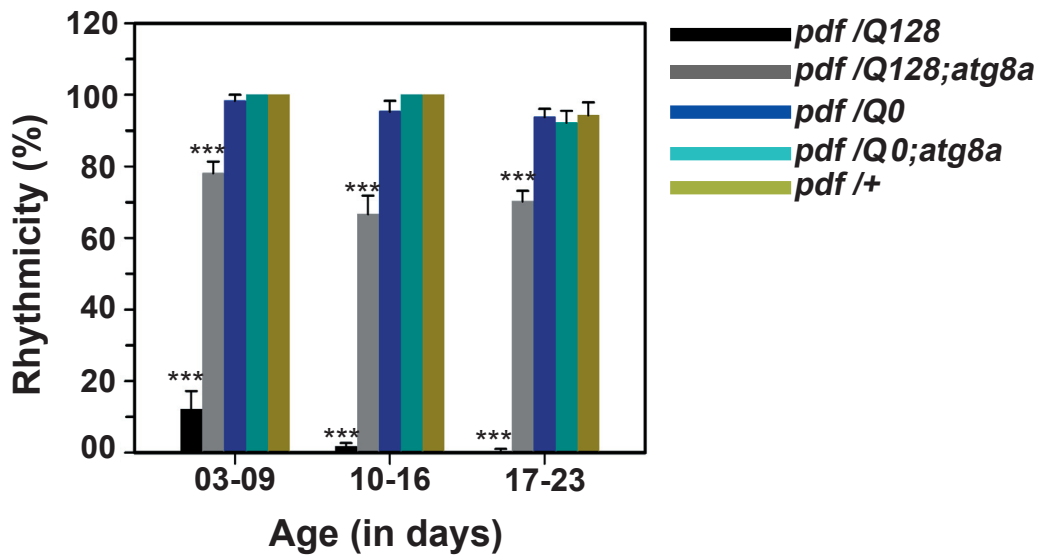
Figure 1: Representative normalised double-plotted actograms of activity/rest behaviour of virgin male flies in DD at 25⁰C for 21days (day 3-23). The x-axis depicts time of the day (0-48h) and the y-axis represents consecutive days. The respective genotypes are indicated above each actogram.

Figure 2: Co-expression of Atg8a with HTT in the LN$_v$ enhances rhythmicity across age. The percentage rhythmicity of adult flies averaged over four independent experiments is plotted against three age windows. The percentage of rhythmic *pdf/Q128* flies are significantly lower than all the other genotypes across age windows. *pdf/Q128;atg8a* flies have significantly lower rhythmicity from all the other genotypes except *pdf/Q128* in the three age windows. Asterisks indicate significant difference within each age window between *pdf/Q128* and *pdf/Q128;atg8a* genotypes and all other genotypes at *p<0.05*. Error bars are S.E.M.
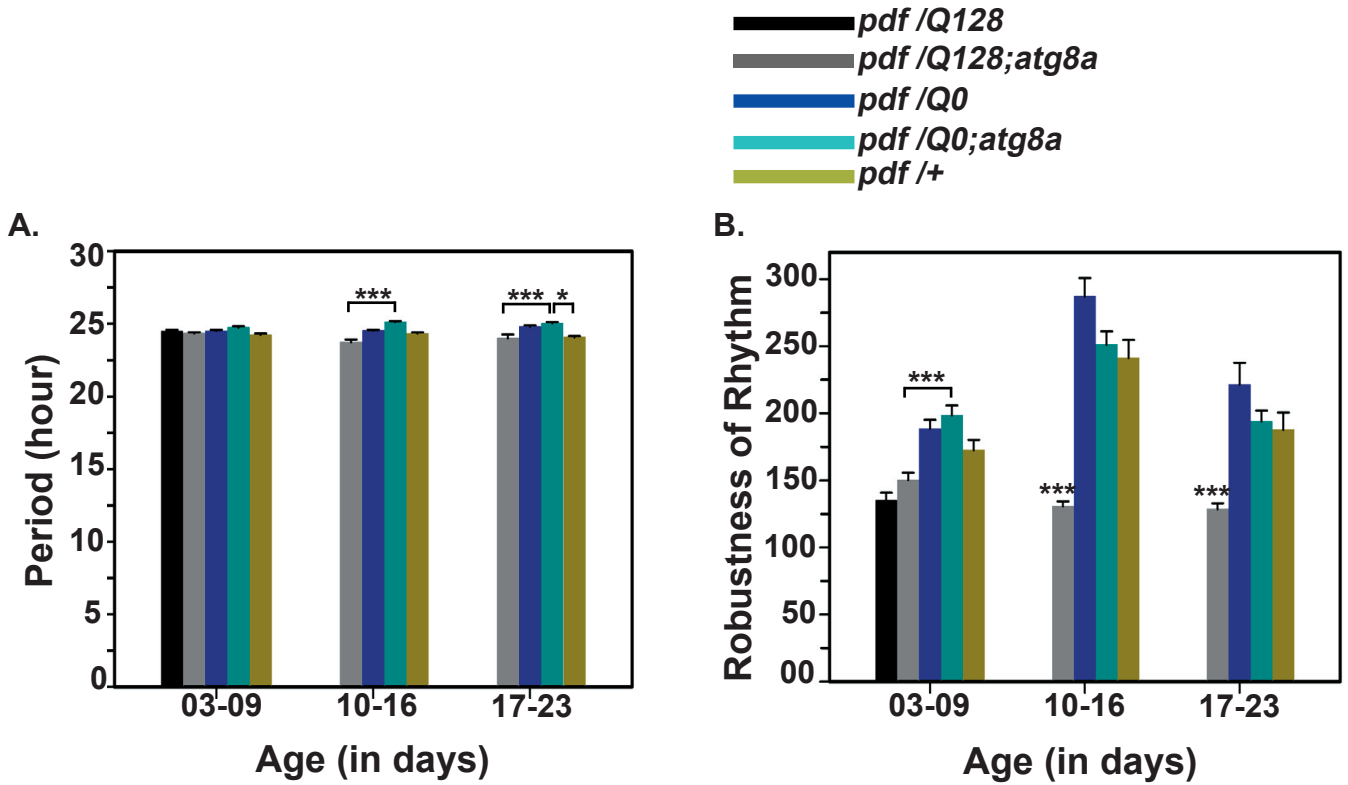
Figure 3: Co-expression of Atg8a with HTT in the LN$_v$ renders flies rhythmic with a poor robustness. *pdf/Q128* flies were arrhythmic in the last two age windows. A. Mean Period of the flies are plotted against three age windows. Period of *pdf/Q128; atg8a* is significantly lower than *pdf/Q0;atg8a* in the last two age windows. The period of *pdf/Q0;atg8a* is significantly higher than *pdf/+* in the last age window. B. Robustness of rhythm of flies plotted against three age windows. The robustness of rhythm of *pdf/Q128;atg8a* is significantly lower than *pdf/Q0;atg8a* in the first age window and all the other genotypes in the last two age windows. Asterisks indicate significant difference between *pdf/Q128;atg8a* and *pdf/Q0;atg8a* in the first age window and between *pdf/Q128;atg8a* and all other genotypes in the other age windows at *p<0.05*. Error bars are S.E.M.
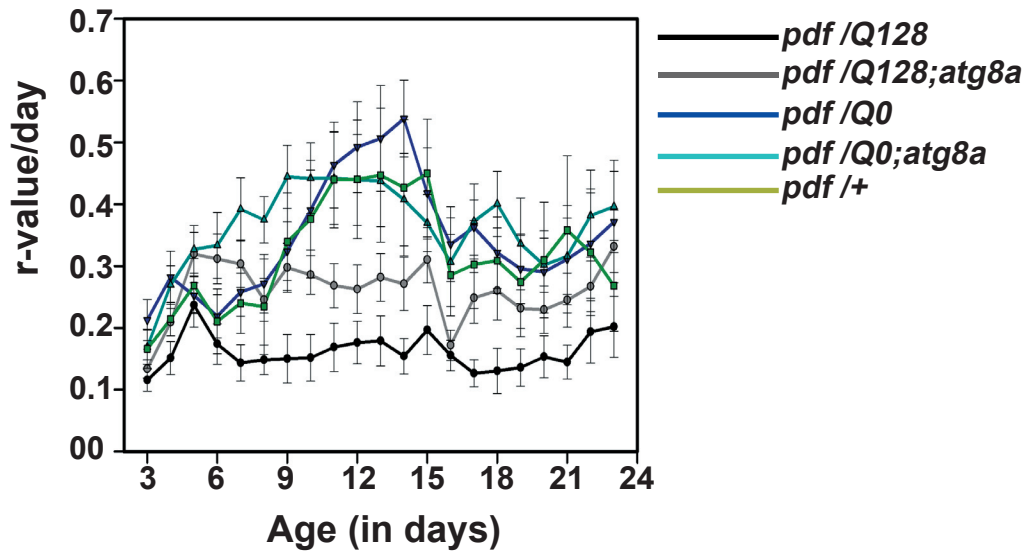
Figure 4: Co-expression of HTT with Atg8a in the LN$_v$s brings about enhanced consolidation of activity in DD 25°C across ages. The r-value for each day is plotted against age. The r-value of *pdf/Q128* flies flies are significantly lower than all the other genotypes except *pdf/Q128;atg8a* from days 9 to15. The *pdf/q128* flies are significantly different from *pdf/Q128;atg8a* flies at days 7,9,10,11,18 and 23. Error bars are 95% CI.
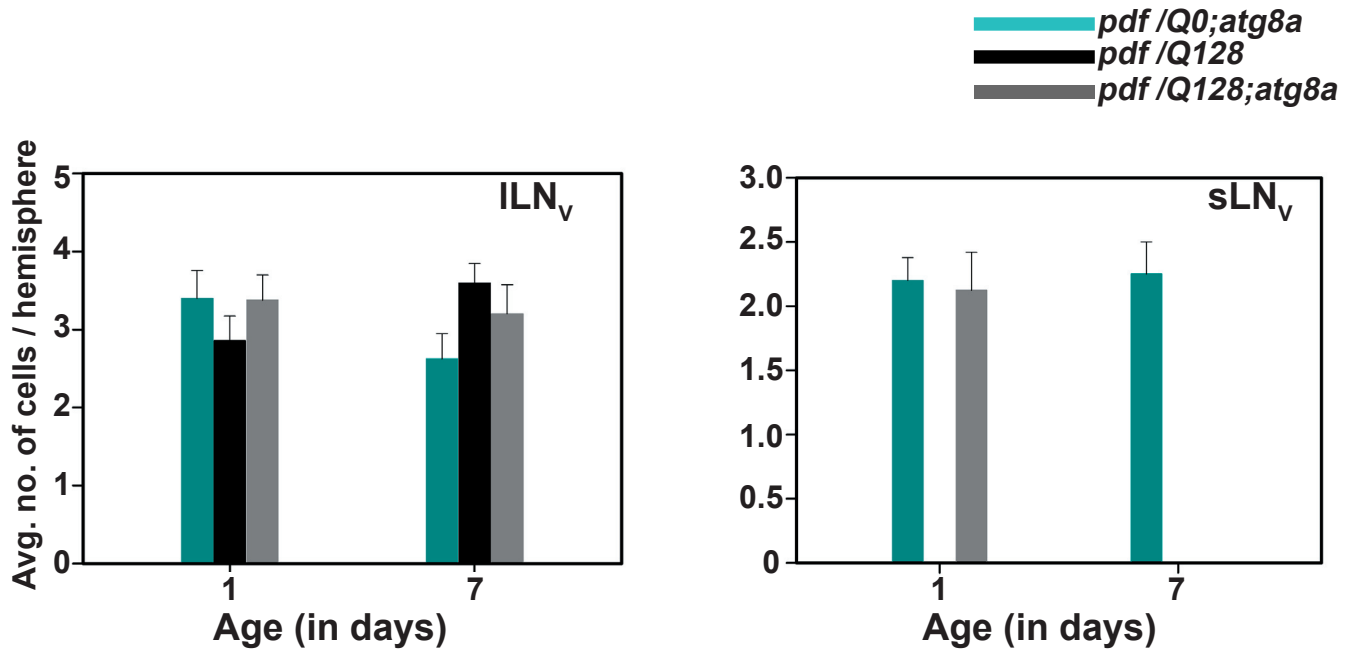
Figure 5: Co-expression of HTT with Atg8a in the LN$_v$ delays the selective loss of PDF immunoreactivity from the sLN$_v$. The average number of LN$_v$ (± SEM) detected using PDF staining at day 1 and 7 are plotted for the experimental and control flies. The *pdf/Q0;atg8a* flies have both sLN$_v$ and lLN$_v$ at both day 1 and 7. The *pdf/Q128* flies lack PDF signal in the sLN$_v$ but the lLN$_v$ are intact in both day1 and day 7. The *pdf/Q128;atg8a* flies have PDF signal in both the sLN$_v$ and lLN$_v$ at day 1 but the signal from the sLN$_v$ disappears at day 7 so they are not visible. Error bars are S.E.M.

Day 1

Fig 6: Representative images of 1 day old adult *Drosophila* whole brain immunostained with antibodies against PDF (green), and HTT (red) with lLN$_v$ in focus. Arrow: sLN$_v$, Arrow head: lLN$_v$, Block arrow: Diffuse HTT, Chevron: Punctate HTT. *pdf/Q0;atg8a* (top panel) shows PDF staining in both sLN$_v$ (arrow) and lLN$_v$ (arrow head) with diffuse HTT staining (block arrow). *pdf/Q128* (middle panel) shows PDF only in the lLN$_v$ with punctuate (chevron) and diffuse HTT(block arrow) staining in cell bodies and PDF signal in the sLNv are missing. *pdf/Q128;atg8a* (bottom panel) shows PDF in both sLNv and lLNv, with puncta in sLNv and diffuse in lLNv. Scale bar = 50 µm.

Day 1

Fig 7: Representative images of 1 day old adult *Drosophila* whole brain immunostained with antibodies against PDF (green), and HTT (red) with sLN$_v$ in focus. Arrow: sLN$_v$, Arrow head: lLNv, Block arrow: Diffuse HTT, Chevron: Punctate HTT. *pdf/Q0;atg8a* (top panel) shows PDF staining in both sLN$_v$ and lLN$_v$ with diffuse HTT staining. *pdf/Q128;atg8a* (bottom panel) shows PDF in both sLN$_v$ and in the lLN$_v$, with puncta in sLN$_v$ and diffuse HTT in lLNv and also outside the cell body. Scale bar = 50 µm.
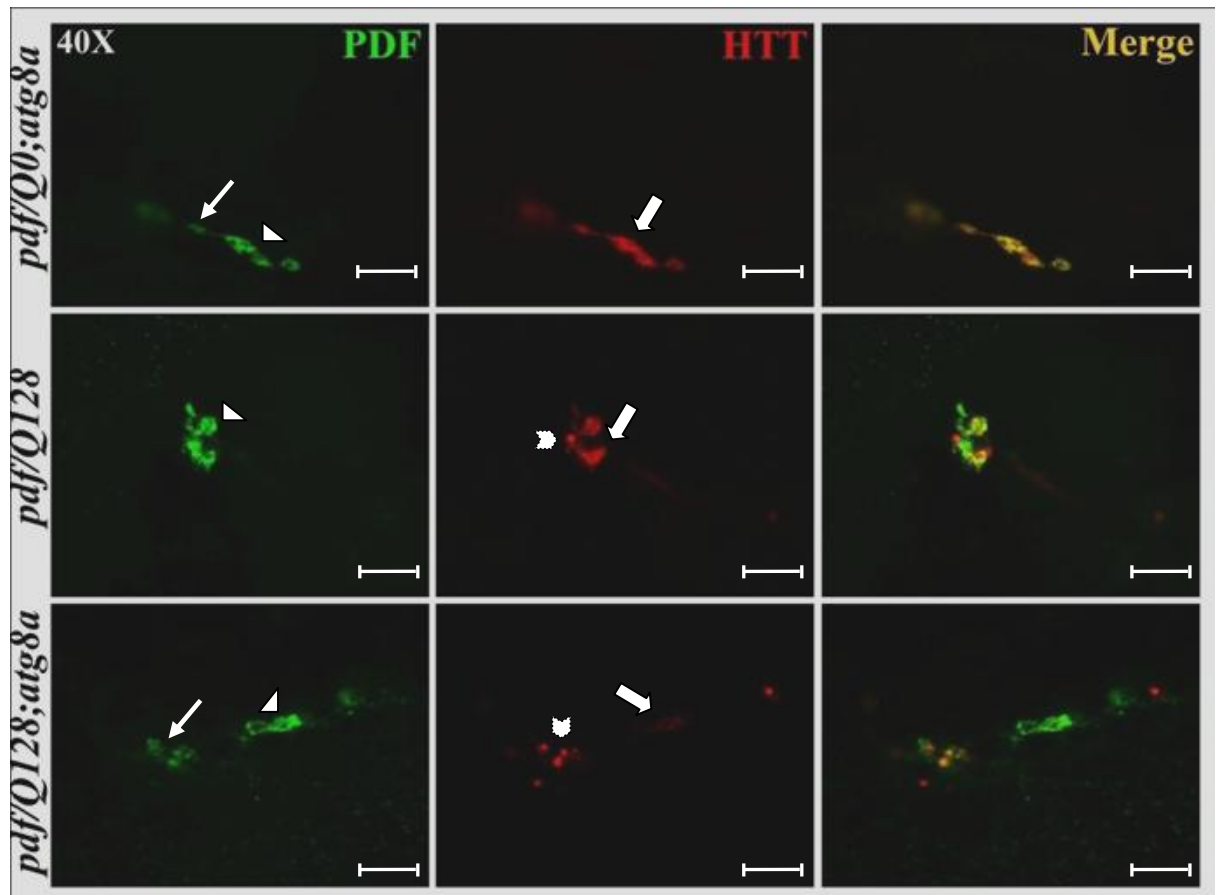
Day 1

Fig 8: Representative images of 1 day old adult *Drosophila* whole brain immunostained with antibodies against PDF (green), and HTT (red). Arrow: Dorsal projection, Arrow head: contralateral projection, Block arrow: Diffuse HTT, Chevron: Punctate HTT. *pdf/Q0;atg8a* (top panel) shows PDF staining in both dorsal and contralateral projections with diffuse HTT staining. *pdf/Q128* (middle panel) shows PDF only in the dorsal projections of the sLN$_v$ with punctate HTT staining in the dorsal projections. *pdf/Q128;atg8a* (bottom panel) shows PDF in both dorsal and contralateral projections, with occasional presence of puncta in the dorsal projections. Scale bar = 50 μm.
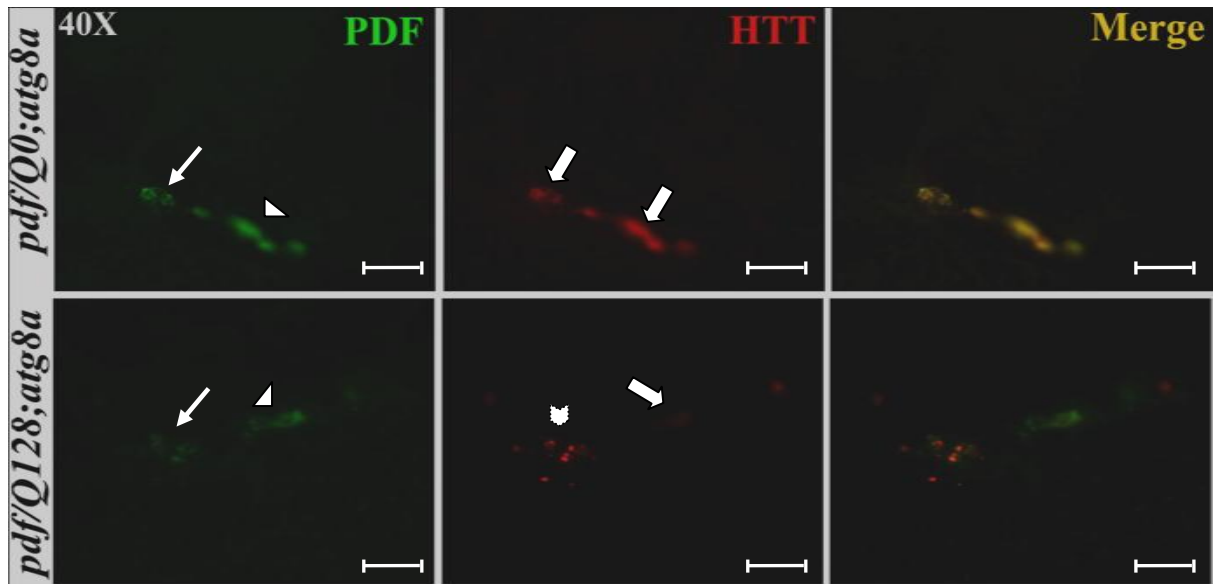
Day 7
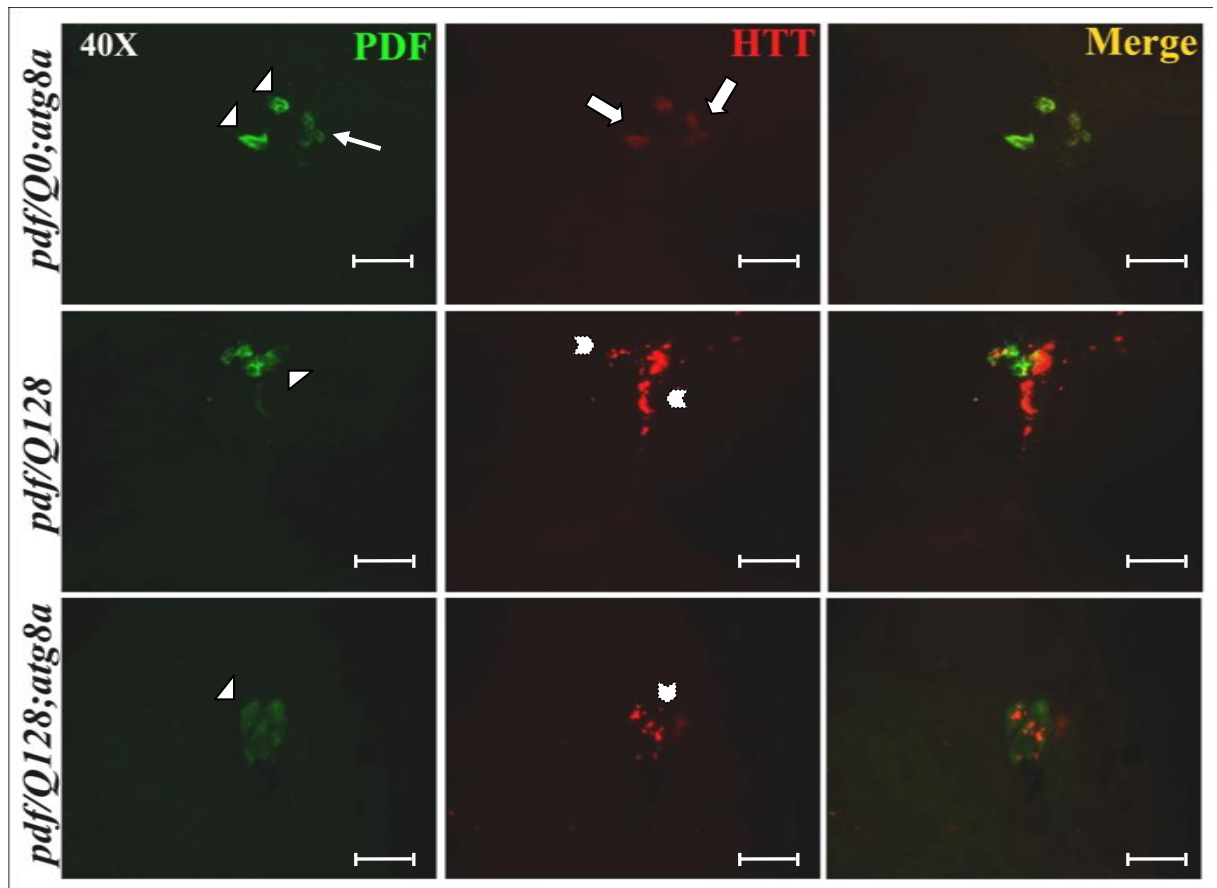
Fig 9: Representative images of 7 day old adult *Drosophila* whole brain immunostained with antibodies against PDF (green), and HTT (red). Arrow: sLN$_v$, Arrow head: lLN$_v$, Block arrow: Diffuse HTT, Chevron: Punctate HTT. *pdf/Q0;atg8a* (top panel) shows PDF staining in both sLN$_v$ and lLN$_v$ with diffuse HTT staining. *pdf/Q128* (middle panel) shows PDF only in the lLN$_v$ with punctuate HTT staining in cell bodies and also outside it. *pdf/Q128;atg8a* (bottom panel) shows PDF only in the lLN$_v$, with puncta in the lLN$_v$. Scale bar = 50 μm.
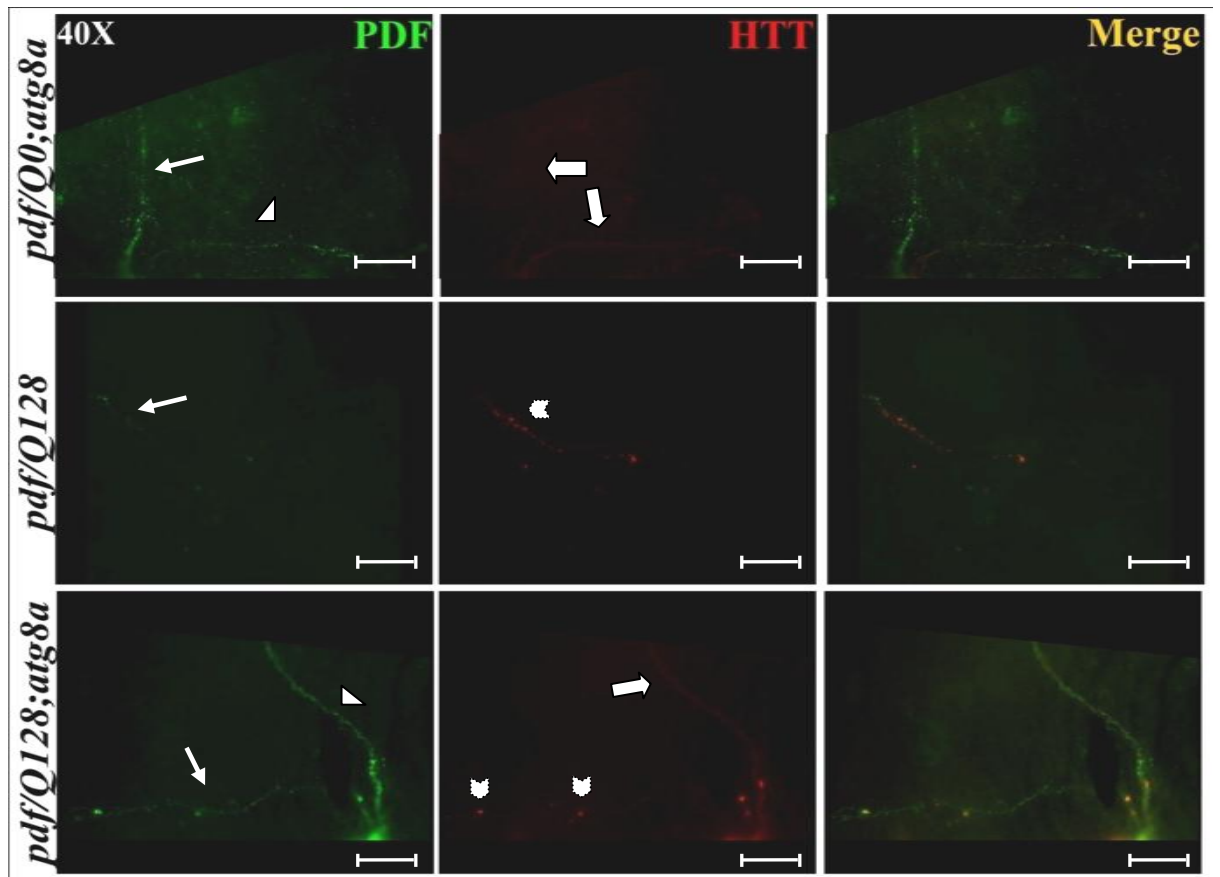
Day 7
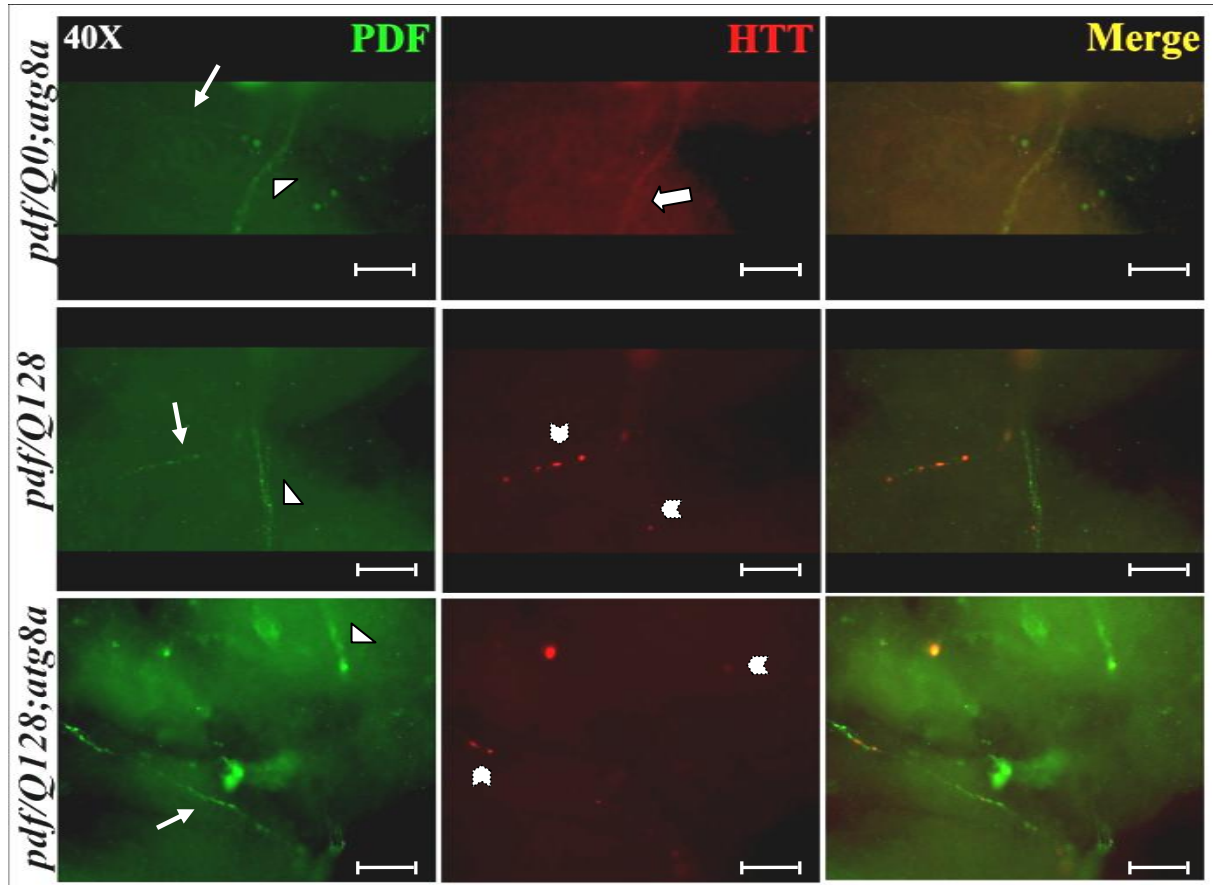
Fig 10: Representative images of 7 day old adult *Drosophila* whole brain immunostained with antibodies against PDF (green), and HTT (red). Arrow: Dorsal projection, Arrow head: contralateral projection, Block arrow: Diffuse HTT, Chevron: Punctate HTT. *pdf/Q0;atg8a* (top panel) shows PDF staining in both dorsal and contralateral projections with diffuse HTT staining. *pdf/Q128* (middle panel) shows PDF in the contralateral projections and dorsal projections with punctuate HTT staining. *pdf/Q128;atg8a* (bottom panel) shows PDF in both dorsal and contralateral projections, with occasional presence of puncta in the dorsal projections. Scale bar = 50 μm.

Rhythmicity (%)			
Genotype / Age	**Day 3-9**	**Day 10-16**	**Day 17-23**
pdf/Q0	93.33	100	100
pdf/Q128	10	0	0
pdf/Q128;atg8a	75	65.2	73.68
pdf/Q128;atg1	28	33.33	33.33
pdf/Q128;atg5	26	20	13.33

Table 1: Percentage of rhythmicity (%) for the autophagy lines screened. The co-expression of *atg8a* with HTT-Q128 results in increased rhythmicity in all the three age windows.

Rhythmicity (%)			
Genotype / Age	**Day 3-9**	**Day 10-16**	**Day 17-23**
pdf/Q128	12.07	1.70	0
pdf/Q128;atg8a	78.06	66.63	70.20
pdf/Q0	98.33	95.31	93.77
pdf/Q0;atg8a	100	100	92.25
pdf/+	100	100	94.23

Table 2: Average percentage rhythmicity shown. Data represents four independent experiments.

Effect	df	MS	F	p-value
Genotype	4	4.11560	272.338	0.000000
Age	2	0.18565	12.285	0.000055
Genotype*Age	8	0.01744	1.154	0.347304

Table 3: Results of ANOVA on rhythmicity for *pdf/Q128*, *pdf/Q128;atg8a*, *pdf/Q0*, *pdf/Q128;atg8a* and *pdf/+*tabulated for four independent experiments. There is a main effect of age and genotype.

Genotype / Age	Age window 1	Age window 2	Age window 3
pdf/Q0	0.000251	0.000251	0.047742
pdf/Q0;atg8a	0.001491	0.028119	0.169005
pdf/+	0.000251	0.000251	0.286408

Table 4: Shows the significant difference between *pdf/Q128;atg8a* and other control for average rhythmicity across age windows (p values from Tukey's HSD).

Effect	df	MS	F	p-value
Genotype	2	0.0	0.0	0.956577
Age	3	13.9	22.4	0.000000
Genotype*Age	6	1.6	2.6	0.016730

Table 5: Results of ANOVA on mean period for *pdf/Q128*, *pdf/Q128;atg8a*, *pdf/Q0*, *pdf/Q128;atg8a* and *pdf/+*.

Mean Period (h ± sem)			
Genotype / Age	**Age window 1**	**Age window 2**	**Age window 3**
pdf/Q128	24.5 ± 0.1	NA	NA
pdf/Q128;atg8a	24.34 ± 0.09	23.73 ± 0.19	23.4 ± 0.28
pdf/Q0	24.5 ± 0.09	24.54 ± 0.06	24.8 ± 0.09
pdf/Q0;atg8a	24.73 ± 0.1	25.12 ± 0.07	25.03 ± 0.09
pdf/+	24.24 ± 0.1	24.33 ± 0.08	24.06 ± 0.11

Table 6: Mean period calculated for each free running phase for *pdf/Q128*, *pdf/Q128;atg8a*, *pdf/Q0*, *pdf/Q128;atg8a* and *pdf/+*. NA indicates not applicable.

Mean Period (h ± sem)			
Genotype / Age	**Age window 1**	**Age window 2**	**Age window 3**
pdf/atg8a	23.93 ± 0.07	24.57 ± 0.08	24.1 ± 0.1
w;+;+	23.69 ± 0.05	23.7 ± 0.16	23.29 ± 0.24
Q0;atg8a/+	23.82 ± 0.11	23.9 ± 0.08	23.56 ± 0.14
Q128;atg8a/+	23.62 ± 0.09	23.93 ± 0.15	23.88 ± 0.21
pdf/+	24.02 ± 0.08	24.47 ± 0.08	23.88 ± 0.15
Q0/+	23.79 ± 0.05	23.9 ± 0.09	23.62 ± 0.12
Q128/+	23.94 ± 0.07	24.18 ± 0.08	23.64 ± 0.06
atg8a/+	23.7 ± 0.07	23.74 ± 0.09	23.37 ± 0.04

Table 7: Mean period for UAS and Gal4 controls calculated for each free running phase.

Effect	**df**	**MS**	**F**	*p-value*
Genotype	2	55194	37.972	<0.0001
Age	3	137273	94.441	<0.0001
Genotype*Age	6	16126	11.094	<0.0001

Table 8: Results of ANOVA on mean robustness of rhythm for *pdf/Q128*, *pdf/Q128;atg8a*, *pdf/Q0*, *pdf/Q128;atg8a* and *pdf/+*.

Robustness of Rhythm (a.u. ± sem)			
Genotype / Age	Age window 1	Age window 2	Age window 3
pdf/Q128	134..98 ± 5.9	NA	NA
pdf/Q128;atg8	150.26 ± 5.51	130.77 ± 3.61	128.50 ± 4.52
pdf/Q0	188.53 ± 6.62	287.08 ± 13.76	221.32 ± 16.39
pdf/Q0;atg8	198.38 ± 7.52	251.03 ± 10.15	193.95 ± 8.19
pdf/+	172.43 ± 7.79	241.1 ± 13.55	187.66 ± 12.92

Table 9: Robustness of Rhythm (arbitrary units) estimated from the amplitude of the periodogram for each age window of flies that were rhythmic for *pdf/Q128*, *pdf/Q128;atg8a*, *pdf/Q0*, *pdf/Q128;atg8a* and *pdf*. NA indicates not applicable.

Robustness of Rhythm (a.u. ± sem)			
Genotype / Age	Age window 1	Age window 2	Age window 3
pdf/atg8a	268.76 ± 11.65	273.24 ± 13.75	280.73 ± 13.47
w;+;+	200.79 ± 9.81	154.75 ± 9.42	127.37 ± 11.27
Q0;atg8a/+	213.54 ± 11.43	198.26 ± 9.92	160.41 ± 10.11
Q128;atg8a/+	206.99 ± 15.57	211.63 ± 10.88	173.44 ± 14.16
pdf/+	209.56 ± 10.00	229.03 ± 15.97	184.57 ± 13.45
Q0/+	236.67 ± 12.97	224.02 ± 16.14	190.58 ± 11.74
Q128/+	221.03 ± 10.14	201.74 ± 13.71	152.48 ± 10.86
atg8a/+	219.75 ± 17.66	264.25 ± 14.92	232.83 ± 13.85

Table 10: Robustness of Rhythm (arbitrary units) estimated from the amplitude of the periodogram for each age window of flies that were rhythmic for UAS and GAL4 controls for each age window.

Genotype / Age	Age window 1	Age window 2	Age window 3
pdf/Q0	0.055795	0.000018	0.000018
pdf/Q0;atg8a	0.000027	0.000018	0.000018
pdf/+	0.776692	0.000018	0.000295

Table 11: Shows the significant difference between *pdf/Q128;atg8a* and other control for robustness of rhythms across age windows (p values from Tukey's HSD).

pdf/Q0			
pdf/Q0;atg8a	0.000027	0.000018	0.000018
pdf/+			

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