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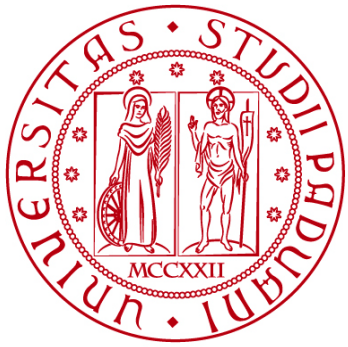

TESI DI LAUREA

EFFECT OF USP14 INHIBITION ON SLEEP DISTURBANCE IN A DROSOPHILA MODEL OF PARKINSON'S DISEASE

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ABSTRACT

Sleep disturbances and circadian disruptions are associated with aging and neurodegenerative disorders. However, while alterations in 24-h circadian rhythms and sleep are common in older adults, they seem to be more severe in people that suffer age-related neurodegenerative disease. The mechanistic link between circadian rhythms and neurodegeneration is not fully understood, although proposed underlying pathways include alterations in protein homeostasis (proteostasis). Loss of proteostasis is well documented in neurodegenerative conditions, and seems to depend on the progressive pathological decline in the proteolytic activity of two major degradative systems: the ubiquitin-proteasome and the lysosome-autophagy system. Indeed, promoting proteasome or autophagy activity increases lifespan, and rescues the pathological phenotype of animal models of neurodegeneration, presumably by enhancing the degradation of misfolded proteins and dysfunctional organelles, which are known to accumulate in these models and to induce intracellular damage. While many studies investigate the effect of potentiating proteostasis to scavenge intracytoplasmic neurotoxic aggregates, very little attention has yet been paid to explore the potential link between alteration in protein homeostasis and (in)stability of core components of the circadian clock in neurodegenerative conditions.

In this work we want to exploit drosophila models of Parkinson's Disease (PD) to investigate the potential beneficial effect of enhancing proteostasis in the context of circadian and sleep disturbances. Our data show that inhibition of deubiquitinating enzyme USP14, which is known to enhance proteasome and autophagy activity, ameliorates sleep disturbances and circadian defects that are associated to two drosophila models of PD: the PINK1 and Parkin KO flies.

1. INTRODUCTION

1.1. Parkinson's disease: clinical symptoms and potential causes

Parkinson's disease (PD) is the second most common degenerative disease of the central nervous system, which affects around 0.3% of the population (Raza et al., 2019). The pathological hallmarks that characterize PD are the accumulation of filamentous, cytoplasmic inclusions consisting mainly of α-synuclein aggregations in the form of Lewy bodies (LB) in the substantia nigra of the brain, and the selective loss of dopaminergic neurons with consequently depleted dopamine (DA) levels. These deficiencies lead to motor dysfunction, with symptoms such as bradykinesia, resting tremor, muscular rigidity, and postural instability (Kalia & Lang, 2015).

PD is mainly a sporadic disorder, of largely unknown etiology. The disease can result from genetic and environmental factors and approximately 10% of all cases have a genetic origin and show early manifestation, with currently at least 23 disease-segregating loci identified. In the 1997, α-synuclein (*SNCA*) was described as the first gene associated with an autosomal dominant form of PD. Later, four other genes linked with autosomal (*LRRK2*) or recessive (*Parkin*, *PINK1, DJ-1*) onset forms of PD were described. So far, mutations in six genes are identified in autosomal dominant (AD) forms of PD: *SNCA*, *LRRK2*, *VPS35*, *EIF4G1*, *DNAJC13*, and *CHCHD2*. Loss of function mutations of *Parkin*, *PINK1*, and *DJ-1* are responsible for autosomal recessive (AR) forms of PD, which are more frequently associated with early onset (Hernandez et al., 2016).

Most of the proteins encoded by these genes (PINK1, Parkin and DJ1 in particular) are important for mitochondrial homeostasis and function. Accumulation of dysfunctional mitochondria can indeed contribute to cell demise in different ways. Defective mitochondria can enhance oxidative stress, which further exacerbates protein misfolding. Moreover, they can also lead to inflammation derived from mitochondrial release of damage associated molecular patterns (mtDAMPs), and impair calcium homeostasis. PINK1, Parkin and DJ1 play a fundamental role in preventing damage originating from dysfunctional mitochondria. In particular, PINK1 and Parkin cooperate to induce the degradation of damaged mitochondria via autophagy, while DJ1 exerts neuroprotective effects by enhancing the antioxidant defence of the cell (Figure 1) (Kalia & Lang, 2015).

Genetic and sporadic forms manifest similar clinical symptoms and indistinguishable PD hallmarks, including dopaminergic neuron loss, intracellular neurotoxic inclusions, mitochondria abnormalities, oxidative stress and inflammation. Therefore, sporadic PD will likely benefit from studies of the molecular pathways which are impaired in familiar cases. Indeed, the identification and the study of these rare genetic forms identified oxidative stress, alteration of the ubiquitin proteosome system (UPS) and autophagy, and mitochondrial dysfunction as causal factors in the pathophysiology of PD (Kalia & Lang, 2015).

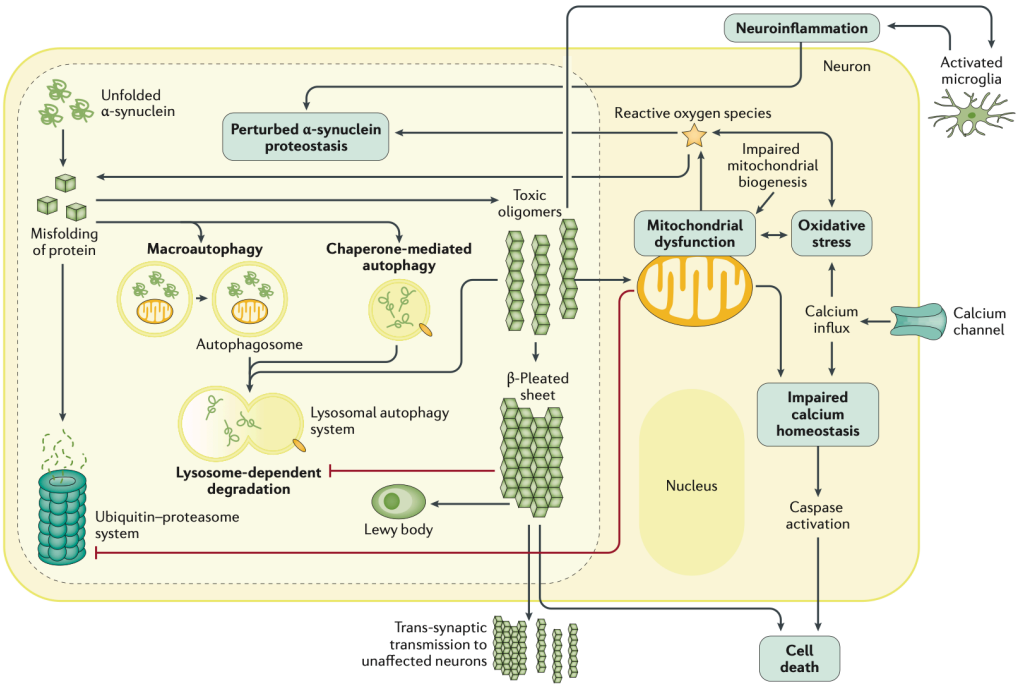

Figure 1. Molecular pathways implicated in Parkinson's disease. *α-synuclein can be found in unfolded conformation in the cytoplasm. The unfolded forms are initially ubiquitinated and eliminated by the ubiquitin-protein system (UPS) or autophagy. Mutation of α-syn or the alterations in the UPS and autophagy lead to fibrillar α-syn aggregates and formation of neurotoxic Lewy bodies. Protofibrils can also induce directly toxicity causing oxidative stress, which leads to depletion in ATP levels, as well as impairment in the UPS and additional accumulation of aggregates. Toxic oligomers can also affect mitochondrial function, and exacerbates oxidative stress. Mitochondrial dysfunction can lead to mitochondrial release of damage associated molecular patterns (DAMPs; ROS, mtDNA, mtRNA) that promote neuroinflammation, and impair calcium homeostasis, leading to cell demise. In familiar forms of PD (such as PINK1 and Parkin), reduction in the complex I activity, caused by PINK1 mutations, contributes to reactive oxygen species formation (ROS), oxidative stress and lose of membrane potential, which causes the opening of the mitochondrial permeability pore (mPTP), and the subsequent release of cytochrome c release to trigger apoptosis. Several works demonstrated the neuroprotective effects of Parkin, DJ-1 and PINK1 against mitochondrial dysfunction. In particular, DJ-1 executes its neuronal defence mechanism against oxidative insults. Parkin and DJ1 collaborate to maintain the normal UPS function. Moreover, Parkin and PINK1 are involved in the mitochondrial quality control, inducing the degradation of damage mitochondria via mitochondrial autophagy (from Poewe et., 2017).*

1.2. Non-motor symptoms of PD

In addition to above mentioned primary motor function impairments, non-motor features are also frequently present in PD, and they include neuropsychiatric symptoms (depression, cognitive dysfunction and dementia), sleep disorders, and autonomic symptoms (bladder disorders, orthostatic hypotension, erectile impotence). These non-motor symptoms (NMSs) have gained increasing relevance for their impact on quality of life of PD patients and their contribution to institutionalization at advanced disease.

Interestingly, these non-motor symptoms are not only common in early PD, but they can also manifest before the onset of the classical motor symptoms. In this premotor phase, the pathogenic process of PD is presumed to be underway, involving regions of the peripheral and central nervous system in addition to the

dopaminergic neurons of the *substantia nigra pars compacta* (SNpc) (Raza et al., 2019).

In particular, sleep disorders are found in two-thirds of PD patients and they include symptoms like insomnia, rapid eye movement, vivid dreaming, increased nocturnal activity, more fragmented sleep, REM sleep behaviour disorder (RBD) and excessive daytime sleepiness. Because sleep behaviour is controlled by circadian rhythmicity, the above-mentioned sleep disorders can be a consequence of dysfunctional circadian clock in PD patients. Indeed, although circadian disorders are not frequently mentioned among the common PD symptoms, several studies in rodent PD models have shown a correlation between PD-associated neurodegenerative phenotype and circadian dysfunction. For example, the overexpression of α-synuclein in mice was reported to correlate to deficits in circadian locomotor activity (Kudo et al., 2011). Moreover, neurotoxin-based models that are known to selectively destroy dopaminergic neurons in the brain and induce Parkinsonism, such as treatment with 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), recapitulate an alteration in the levels of some clock genes, and an aggravation of motor symptoms. A study on monkeys showed that MPTP treatment leads to a direct alteration of rest-activity cycles and cognitive deficits very early after exposure (Vezoli et al., 2011). In MPTP mouse model, the disruption of the circadian rhythm exacerbates motor deficit by triggering neuroinflammatory reaction and degeneration of the nigral-dopaminergic neuronal system (Lauretti et al., 2017). Furthermore, rats and SH-SY5Y cells treated with 6-OHDA showed a reduced level of SIRT1, an enzyme that affects circadian rhythms and the expression of clock-controlled genes (Wang et al., 2018).

The mechanistic link between circadian rhythms and PD neurodegeneration is not fully understood, although proposed underlying pathways include alterations in protein homeostasis (i.e.proteostasis) (Leng et al., 2019).

Importantly, disturbances in sleep-wake cycle precede the onset of the cognitive decline and motor symptoms by many years, supporting the hypothesis that circadian rhythm disruption might not simply be a consequence of PD but a pathogenic component of the disease that can contribute to neurodegeneration. Clinical diagnosis of PD is mainly based on the presence of motor symptoms, which usually appear when more than 50% of dopaminergic neurons have already been lost. Since the diagnosis occurs too late for neuroprotective treatments to work, it would be fundamental to find a way to diagnose the disease before the appearance of motor symptoms and implement a therapy at the premotor phase to prevent or delay the development and progression of disease (Kalia & Lang, 2015). Thus, sleep disturbances, as well as non-motor symptoms in general, may offer a pre-symptomatic window for the treatment of this disease, allowing earlier intervention with therapeutic drugs (Julienne et al., 2017).

1.3. Animal models of PD

Animal models of PD are quite useful to understand the pathogenesis of this disease. Even though they don't mimic completely the progression and pathology of the disease, they are still helpful in the development of therapies to treat PD. Animal models of PD can be obtained either by neurotoxins treatment or genetic manipulations. Whether the causative factor is a toxic molecule or a mutated gene, the perfect animal models of PD does not exist. Nevertheless, the neurotoxin-induced vertebrate models of PD are suitable for investigating disease-modifying therapies, since they have already proved predictive, while genetic animal models of PD are useful to investigate the early processes of degeneration in the nigrostriatal DA system (Shimohama & Hisahara, 2011).
While the neurotoxins models will be described briefly (see below), we will extensively illustrate the genetic models with a specific focus on *Drosophila melanogaster*.

1.3.1. Synthetic neurotoxins models

MPTP and 6-OHDA are two neurotoxins able to reproduce Parkinsonism (akinesia, rigidity, and tremor) inducing severe neuronal degeneration in the substantia nigra with specific loss of dopaminergic neurons, and causing Lewy bodies (LBs) and Lewy neurites (LNs) formation.
Environmental exposures, for examples to pesticides, are also thought to be involved in the pathogenesis of sporadic PD. The pesticide Rotenone is a strong inhibitor of complex I, which is located at the inner mitochondrial membrane. Exposure to rotenone causes many features of PD, including nigrostriatal dopaminergic degeneration. Models treated with this neurotoxin developed Parkinsonism with symptoms like bradykinesia, fixed posture, and rigidity. The herbicide Paraquat (PQ) has been suggested as a risk factor for PD because of its similarity with 1-methyl-4-phenylpyridinium (MPP^+, the active metabolite form of MPTP). These models develop symptoms similar to those generated by MPTP treatment (Shimohama & Hisahara, 2011).

1.3.2. Genetic models of PD: eye on fly

The discovery of familiar forms of PD caused by mutation in specific genes led to the identification of common pathways in the pathogenesis of PD, and the development of several genetic animal models. Murine or non-human primate models often prove to be costly and time-consuming. Moreover, mammalian models are often unable to recapitulate all the pathological symptoms of PD. For examples, α-synuclein, Parkin or DJ-1 KO mice models fail to show loss of nigrostriatal DA neurons and DA-agonist-responsive behavioural abnormalities. For these reasons, new models such as zebrafish, fruitflies, nematodes and anurans have gained relevance in the last years.
In particular*, Drosophila melanogaster* is being increasingly used as animal model to study PD. Flies are very small and easy to maintain, and they have a short life cycle, which allow for a large quantity of flies to be produced within a short

period. From a genetic point of view, *Drosophila* is an ideal model organism, as it has four pairs of chromosomes compared to 23 pairs in humans. They are easier to manipulate: compared to that of the human, the genome of the fly is much smaller at 5% of its size. However, 15500 genes are present in the flies as compared to 2200 genes in humans, and more than 60% of the Drosophila genes are the same, as they originate from a common ancestor.

Importantly, pathology observed in human PD can be accurately reproduced in *Drosophila*, with an area-specific and age-dependent loss of DA neurons, as well as LBs and LNs formation. Moreover, homologues for several PD causing genes such as PINK1, Parkin, DJ-1 and LRRK2 are encoded by the fly genome. As opposed to mammalian models of PD, downregulation or transgenic expression of these PD-related genes in flies often reproduces a very obvious phenotype.

In particular, transgenic expression of human α-synuclein or human LRRK2 in *Drosophila* results in the loss of dopaminergic neurons, impaired locomotor activity and early mortality, while double KO of the two *Drosophila* DJ-1 alleles (α and β) does not translate into an obvious phenotype, (i.e. flies are viable and fertile, with normal lifespan and no DA neurons loss) (Shimohama & Hisahara, 2011). Perhaps the best-characterized *Drosophila* models of PD are the *Pink1* and *park* mutant flies. The serine/threonine kinase domain of *Drosophila* Pink1 shares 43% amino acid sequence homology with human PINK1. Silencing of *Pink1* in the fly has been observed to induce PD-like phenotypes such as abnormal wing posture, degeneration of flight muscles, abnormal mitochondrial morphology (swollen mitochondria with disorganized cristae) and depletion of dopamine levels. *Drosophila Pink1* knockout results in similar phenotype changes and climbing defects, but with milder age-dependent dopaminergic cell death (Park et al., 2006).

The E3 domains of *Drosophila* parkin (encoded by *park*) share 42% homology of the amino acid sequence with those of human Parkin. *park* knockdown causes age-dependent motor damage and severe loss of dopaminergic neurons. In knockout flies, age-dependent motor impairment and dopaminergic death are mild, while male infertility levels and climbing defects associated with muscle degeneration are elevated (Ganesan & Parvathi, 2021). Pink1 and parkin are certainly involved in the genetics and pathogenesis of PD. But are the loci associated with PD located in the same signalling pathway or is the disease the result of multiple independent damages that cause the loss of DA neurons? *Drosophila* models provided evidence for a genetic interaction between *park* and *Pink1* genes. Indeed, overexpression of parkin rescued the male infertility and mitochondrial defects of the *Pink1* mutants, while the double mutants lacking both *Pink1* and *park* show identical muscle damage as observed in single mutants. So, Pink1 and parkin appear to share the same functional path, with Pink1 located upstream of parkin (Clark et al., 2006).

Additionally, non-motor features such as impaired memory and disturbed circadian rhythms were noted in *Pink1*/*park* mutant flies. $park^{1/\Delta 21}$ and $Pink1^{B9}$ mutants show fragmented sleep, with frequent awakenings and a decrease in the length of the sleep sessions and an increase in their frequency, and they fail to anticipate dawn and dusk (even if less evidently). Anticipation is another

feature that characterizes circadian rhythms, by which flies start to increase their activity around 3h before the light is turned on or off. Morning anticipation (MA) is regulated by ventral lateral neurons (LNvs) that secrete pigment-dispersing factor (PDF). Interestingly, in *park*$^{1/\Delta 21}$ and in *Pink1*^{B9} mutants it was observed an excess of endoplasmic reticulum (ER)-mitochondria contacts, that cause abnormal lipid trafficking, and disrupts the production of PDF-containing vesicles, leading to defective release of PDF and thus explaining the anticipation defect in these PD models (Valadas et al., 2018).
Pink1^{B9} and *park*^{25} mutants display relatively normal activity in light-dark conditions (LD) and maintain some discernible rhythmicity in their activity in total darkness conditions (DD). However, there appears to be less of a distinction between the periods of activity and inactivity, and relative night-time activity seems to be elevated. Furthermore, most *Pink1*^{B9} flies were totally arrhythmic, while more of the *park*^{25} flies tended to be weakly rhythmic (Julienne et al., 2017). Overall, these studies suggest that *Pink1*/*park* mutant flies can represent an ideal model to study circadian defects in PD, and serve as powerful tool to investigate the molecular mechanism underlying the potential link between circadian cycle disorders and PD.

1.4. Proteostasis and PD

Proteostasis, or protein homeostasis, is the dynamic regulation and maintenance of a functional proteome through a coordinated network of events that rapidly correct unwanted proteomic changes. This mechanism is necessary for cell metabolism, organelles biogenesis, and stress adaptation.
Cellular proteostasis is often compromised during aging and it is also compromised in many neurodegenerative diseases, like PD. Considering its involvement in human age-related pathologies, cellular proteostasis has gained increasing relevance as an unexplored potential therapeutic target.
Proteostasis is regulated by chaperones and two degradation systems, the ubiquitin-proteasome system (UPS) and autophagy. Chaperones are proteins that help *de novo* folding of proteins as well as their assembly and disassembly processes. When folding is not possible, chaperones target the unfolded protein for degradation (Kaushik & Cuervo, 2015).
The proteasome is a multicatalytic protease made of two subunits, the core particle (20S), and the regulatory particle (19S). Ubiquitination is essential to tag and induce the degradation of the target protein by the proteasome. The regulatory particle of the proteosome recognizes ubiquitinated substrates and delivers them for degradation to the core particle. Energy from ATP hydrolysis is required to unfold the proteins and translocate them to the core particle where they will be degraded. Proteasome is the most abundant protease in the cytosol, thus UPS is the primary way for rapid degradation of short-lived, misfolded, and damaged proteins.
Autophagy is responsible for degradation of large, heterogeneous cytoplasmic materials such as protein aggregates and dysfunctional organelles that are too large for proteasome. The substrates are therefore delivered to the lysosome for

degradation. Different types of autophagy have been identified (macroautophagy, microautophagy, and chaperone-mediated autophagy) and they differ in the way the cargo is delivered to the lytic compartment. In chaperone-mediated autophagy, proteins expose a pentapeptide motif (KFERQ), which is recognized by heat shock protein HSC70 which in turn binds to lysosomal-associated membrane protein 2A (LAMP2A). The target proteins are therefore unfolded and translocated into the lumen where they are degraded. In microautophagy, cytoplasmic material is trapped in the lysosome by a small membrane invagination. Macroautophagy implies the engulfment of the substrate by the phagophore, a double-membrane structure which expands around the substrate and subsequently closes to form the autophagosome. The autophagosome fuses with the lysosome, and the material is degraded (Dikic, n.d.) (Figure 2).

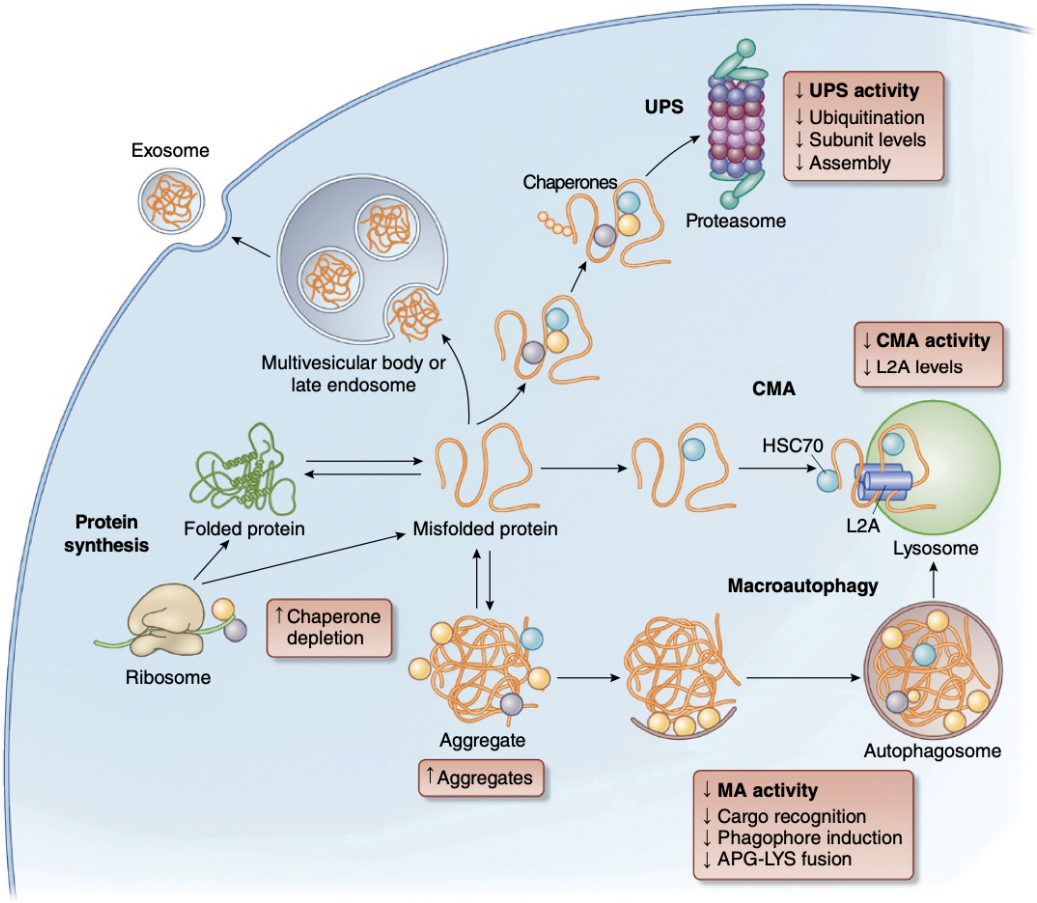

Figure 2. Chaperones, UPS and autophagy regulate proteostasis. *Chaperones and two degradation systems, the ubiquitin-proteasome system (UPS) and autophagy, regulate proteostasis. Chaperones help de novo proteins folding and their assembly and disassembly processes: if folding is not possible, chaperones target proteins for degradation by the UPS. Degradation by the UPS is induced by ubiquitination that is essential for the protein to be recognized and delivered to the proteasome. If cytoplasmic materials are too large to be delivered to the proteosome, the substrates are engulfed by a double membrane structure (the phagophore), which expands around the substrates and closes to form the autophagosome. Autophagosome delivers its content to the lysosome where degradation occurs. This event is known as macroautophagy or autophagy for short. Changes with age in intracellular proteostasis systems are highlighted in the boxes (from Kaushik and Cuervo, 2015).*

Organelle-specific proteostasis mechanisms also exist. For example, the endoplasmatic reticulum (ER) is involved in the folding and transport of many proteins, thus it needs a robust system of quality control. So, the ER can undergo degradation through the process of ER-phagy, which is a specialized form of autophagy assisted by FAM134B protein. In this case, degradation includes not only unfolded proteins but also whole regions of the ER membrane. The proteasome is also involved in the proteostasis of this organelle through the unfolded proteins response (UPR). Activation of the UPR decreases protein translation levels and increases the production of molecular chaperones. If this response is not sufficient, the protein is retrotranslocated for degradation in the cytoplasm by cytosolic proteasome through a process called endoplasmic reticulum-associated degradation (ERAD) (Kaushik & Cuervo, 2015).

In addition to ER-phagy, mitochondria proteostasis is also essential, given the abundance of ROS that can originate from this organelle. ROS are involved in multiple cellular pathways, but excessive ROS can damage the cell. Thus, cells need to be protected from cytotoxic ROS production by quality control mechanisms. Mitophagy is one of these quality control mechanisms that control the selective degradation of ROS-producing mitochondria by autophagy. This is a complex process of multiple events that in the canonical form involves the activation of the PINK1/Parkin proteins, both mutated in familiar PD. Under normal conditions, PINK1 levels are maintained low, as the protein is continuously targeted to the mitochondria through its mitochondrial targeting sequence, imported into mitochondria by the TIM/TOM complex and cleaved by presenilin-associated rhomboid-like (PARL) protease. Cleaved PINK1 retrotranslocates to the cytosol and it is degraded by the proteasome. When the mitochondrial membrane depolarizes due to damage, PINK1 fails to be imported into mitochondria and the protein accumulates on the outer mitochondrial membrane (OMM). Accumulated PINK1 is autophosphorylated and activated, and it phosphorylates ubiquitin on serine 65 (Ser65), which recruits Parkin. Parkin phosphorylation and activation by PINK1 leads to the polyubiquitination of several substrates on the OMM, including VDAC and mitofusins Mfn1 and Mfn2. Ubiquitination and phosphorylation of such membrane proteins recruit autophagic receptors, and promote the formation of the autophagosome (Yoo & Jung, 2018). At this point, the double-membrane autophagosome fuses with lysosomes, forming an autolysosome, in which the mitochondrion will be degraded, and its components recycled (Rodger et al., 2018). Importantly, mitochondria undergo fission to decrease their size in order to be efficiently engulfed by the autophagosome.

Mutations in PD proteins PINK1 or Parkin can lead to an impairment of this process of selective mitochondrial autophagy (Raza et al., 2019).

The failure of mitochondrial homeostasis plays an important role in PD pathogenesis, as dopaminergic neurons require high bioenergetic demand and are therefore sensitive to the disruption of mitophagy (Mouton-Liger et al., 2017). Moreover, accumulation of dysfunctional mitochondria contributes to oxidative stress and may lead to inflammation derived from mitochondrial release of DAMPs from the deranged organelle. As for ER, additional

mitochondrial quality control mechanisms are in place, which involve the activation of chaperones and proteases. The mitochondrial UPR (mtUPR) is similarly activated to increase chaperone content in mitochondria. Unfolded proteins are cleaved by the mitochondrial caseinolytic peptidase (ClpXP) into small peptides that are translocated into the cytosol (Kaushik & Cuervo, 2015) (Figure 3).

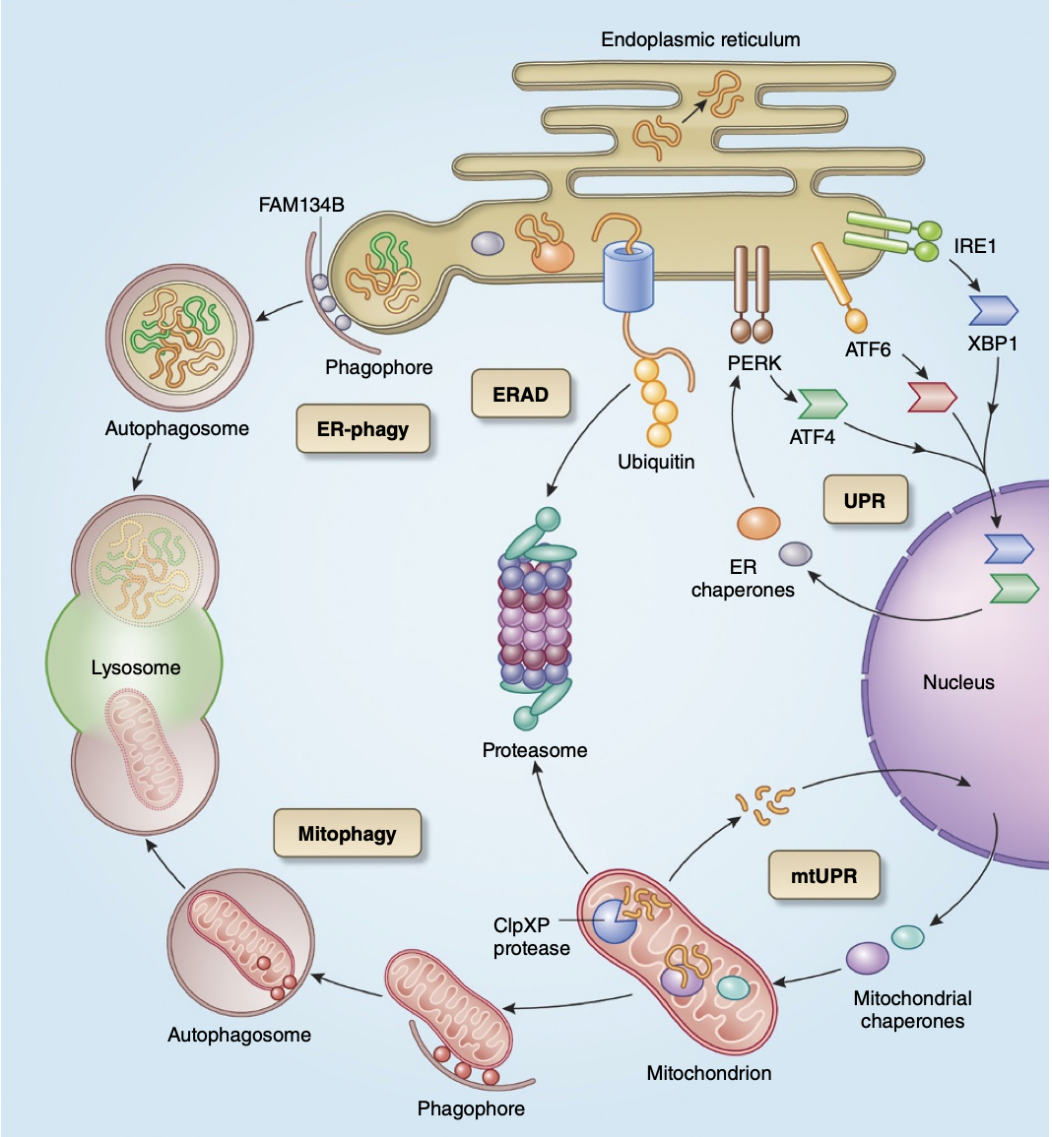

Figure 3. Organelle specific proteostasis. *The endoplasmatic reticulum (ER) and mitochondria can be degraded through ER-phagy and mitophagy, respectively. These two processes are specialized forms of autophagy in which the whole organelles undergo degradation. The proteasome is also involved in the proteostasis of ER and mitochondria through the unfolded proteins response (UPR) and mitochondrial UPR (mtUPR). In the ER, activation of the UPR decreases protein translation and increase chaperones content. If this response is not sufficient, unfolded proteins are retrotranslocated for degradation in the cytoplasm by the proteasome (ERAD). The mitochondrial UPR (mtUPR) is similarly activated to enhance expression of chaperones and to attenuate translation. Unfolded mitochondrial proteins are cleaved by the ClpXP protease and translocated into the cytosol (from Kaushik and Cuervo, 2015).*

Proteins and organelles proteostasis networks are fundamental for the cell: both ER stress conditions and mitophagy malfunctioning are involved in age-related disorders, PD in particular, with two PD related proteins (PINK1 and Parkin) that are directly controlling mitochondrial proteostasis. Moreover, accumulation of unfolded proteins and intracytoplasmic aggregates, as a result of defective proteostasis mechanisms, is a fundamental hallmark of PD (Kaushik & Cuervo, 2015).

1.4.1. Deubiquitinating enzymes (DUBs) and their role in proteostasis

The aberrant accumulation of neurotoxin proteins that characterizes neurodegenerative disorders is linked not only to their overproduction, but also to their reduced degradation. Among the processes that lead to protein degradation, the one mediated by the UPS accounts for 80-90% of cellular proteolysis. As already seen, proteins are ubiquitinated and targeted to proteasome for degradation. However, ubiquitination is a reversible process that can be counteracted by deubiquitinating enzymes (DUBs). DUBs are a large group of proteases that cleave ubiquitin from proteins. In the human genome there are more than one hundred DUBs classified into 7 subfamilies. Over the last decade, DUBs have started to be considered as an attractive therapeutic candidate to enhance protein and organelle homeostasis, mitochondrial proteostasis in particular. Different studies indicate that several DUBs such as ataxin-3 (ATXN3), USP14, USP15, USP30 and USP35 can regulate ubiquitin-dependent mitophagy. Indeed, Parkin-mediated mitochondrial ubiquitination can be specifically counteracted by DUBs. USP15, USP30, and USP35 are able to inhibit mitophagy, counteracting Parkin activity on its targets. Likewise, specific inhibition of these DUBs can enhance mitophagy, presumably by stabilizing ubiquitination on mitophagy targets, thus enhancing degradation. Interestingly, Parkin itself can be target of deubiquitination, which is required to facilitate the translocation of Parkin to damaged mitochondria, and thus mitophagy. Two DUBs, USP8 and ATXN3, have been found to directly target and deubiquitinate Parkin. In this respect, activation of these specific DUBs can enhance Parkin translocation and increase mitophagy (Liu et al., 2021).

1.4.2. USP14

USP14 is a DUB intrinsically associated with the proteasome that belong to the ubiquitin-specific protease (USP) family. The UPS plays a major role in the regulation of proteins degradation via the proteasome and lysosome and maintains cellular homeostasis by removing misfolded and possibly toxic proteins. The catalytic domains of USP14 consist of a typical USP architecture, with additional USP14 specific domains, namely Fingers, Palm, and Thumb. USP14 binds ubiquitin using its Fingers domain and a binding groove between the Palm and Thumb domains. However, proteasomal DUBs must be highly regulated and even when assembled into the proteasome, USP14 is not constitutively in an active state. Indeed, USP14 activity is regulated by the

presence of loop segments, BL1 and BL2, that limit the access of ubiquitin to the catalytic site by spreading into the ubiquitin binding groove. Only when these loops are displaced, allowing the access of the ubiquitin C-terminus to the binding groove of USP14, the enzyme activates (Figure 4).
Interestingly, USP14 has been proposed to suppress the degradation of its targets, by promoting the dissociation of the substrate before the commitment step. In particular, by rapidly deubiquitinating its targets, USP14 decreases the dwelling time of its targets on the proteasome, thus suppressing their degradation. Consistent with this mechanism of action, inhibition of USP14 activity in mammalian cells stimulates the degradation of specific proteasome substrates, while USP14 activation by AKT seems to suppress the degradation of multiple proteins (de Poot et al., 2017).

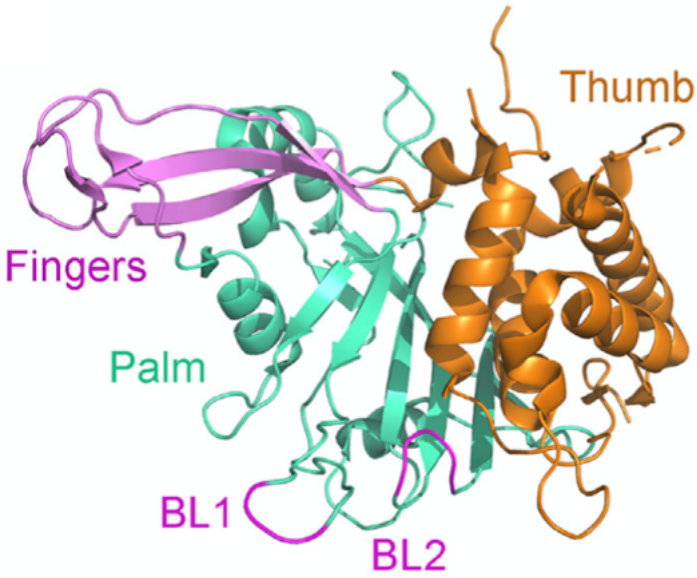

Figure 4. Structure of USP14. *The structure of USP14 consists in specific domains namely Fingers, Palm and Thumb. USP14 binds ubiquitin using its Fingers domain and a binding groove between the Palm and Thumb domains. The activity of USP14 is regulated by the presence of BL1 and BL2 loop segments that limit the access of ubiquitin to the catalytic site (from Wang et al., 2022).*

As already seen, USP14 promotes ubiquitin (Ub) chains disassembly during substrates degradation, so that Ub molecules can be reutilized. In addition to this catalytic role, USP14 is also responsible of allosteric regulation of the proteasome, since it is able to both activate and inhibit proteins degradations. Indeed, when there is no Ub conjugate bound, the activity of the proteasome is low and USP14 inhibits the hydrolysis of ATP and the entrance of the substrate in the core particle. In this way, no ATP is wasted and there is no degradation of non-ubiquitinated proteins. However, when USP14 binds Ub chains, it promotes the entrance of the substrate into the core particle and ATP hydrolysis, in order to ensure efficient degradation of ubiquitinated substrates. This activated state is maintained as long as USP14 binds a ubiquitin chain. Then, when the substrate is degraded, USP14 returns to the quiescent basal state (Kim & Goldberg, 2017).

In 2010, Finley and colleagues identified IU1 as the first highly selective inhibitor of proteasome-bound USP14. In the absence of proteasomes, USP14 was insensitive to IU1, suggesting that IU1 binds specifically to the activated form of USP14 bound to the proteasome (B. H. Lee et al., 2010).
Importantly, the suppression of USP14 activity, either by knocking down USP14 or by IU1, leads to Beclin1-dependent autophagy (Xu et al., 2016), highlighting for the first time the fundamental importance of USP14 activity not only in the regulation of the UPS, but also autophagy. In more details, USP14 seems to regulate autophagy by negatively controlling K63 ubiquitination of Beclin-1. K63 is a lysine residue, target of USP14, present in Beclin-1, a protein with a critical role in the regulation of autophagy. Inhibition of USP14 (either by siRNA knockdown or IU1 inhibitor) leads to high levels of K63-linked ubiquitination of Beclin-1. This is correlated with an increased interaction of Beclin-1 with Atg14L and UVRAG, two important regulators of Vps34 complex activity. Vps34 activity is critical for the production of PtdIns3P, which is a key signal in autophagosome formation. Thus, in the proposed model, increased K63 ubiquitination of Beclin-1 upon USP14 inhibition determines an increased association of Atg14L and UVRAG with Beclin-1, which promotes autophagy (Xu et al., 2016).
A recent study published in our lab confirmed the “autophagic” effect of USP14 pharmacological and genetic inhibition. Furthermore, in the same study we demonstrated that USP14 inhibition promotes mitophagy in several cell lines, including primary fibroblasts from PD patients. Importantly, genetic or pharmacological inhibition of USP14 ameliorates the defective phenotypes of two *Drosophila* models of PD, which are known to accumulate defective mitochondria (the PINK1 and Parkin KO flies). Genetic or pharmacological inhibition of USP14 in PINK1 or Parkin KO flies led to an increase in life expectancy, improved climbing ability, and it completely recovered mitochondrial defects and function, presumably by enhancing protein and organelle homeostasis (Chakraborty et al., 2018).

Defects in the activity of the UPS and/or autophagy have been reported during aging and in many neurodegenerative diseases, PD in particular. Indeed, accumulation of misfolded, aggregated proteins and dysfunctional organelles, is a specific hallmark of PD, which can undoubtedly contribute to neurodegeneration. Downregulation of specific DUBs has the potential to enhance the degradation of protein aggregates and damaged organelles, thus promoting the survival of neuronal cells. Because of the unique capability of USP14 to enhance both proteasome activity and autophagy, USP14 inhibition may offer additional advantages over other DUBs in promoting cellular proteostasis. Moreover, the existence of highly potent and selective inhibitors (IU1 and derivatives) makes USP14 a very attractive target for therapeutic intervention.

1.5. The circadian clock

The circadian clock is an internal timing system that allows adapting and, most importantly, anticipating the daily changes that occur on our planet. It controls important physiological events such as sleep-wake cycle, locomotor activity, preparation for food intake during the active phase, and many other fundamental biological events.
At the systemic level, the circadian clock is composed by three major elements: (i) a core oscillator, which generates the rhythmicity; (ii) inputs pathways that allow the organism to perceive the external stimuli; and (iii) output pathways (represented by the circadian phenotypes), which are generated by the cyclic transcription of clock-controlled output genes (Patke et al., 2020).
More specifically, at the cellular level, the circadian clock is composed of highly specialized neurons in which the circadian rhythms are generated (*core oscillator*). These neurons receive inputs from daylight (*inputs pathways*), which are used to synchronize the approx. 24hrs rhythm of the internal clock to the exact 24hrs daily rhythm of planet Earth. Light is the most important synchronizing stimulus, but this is not the only one, and additional stimuli (collectively called *Zeitgebers,* from the German words *Zeit*, meaning "time", and *geben*, "to give"), such as food intake and exercise, help synchronizing the internal clock to a 24hrs period. At the systemic level, the circadian rhythms that assemble the clock allow the alignment of biological functions with regular and predictable environmental daily changes, to optimize the performance of the entire organism (*output pathways*) (Patke et al., 2020).
Importantly, a self-sustained, cell autonomous internal clock generates the circadian rhythm, which in fact persists even in the absence of external signals. Circadian oscillations are generated by evolutionary conserved transcriptional-translational feedback loop (TTFL), which takes approximately 24 hours to complete. In this loop, positive elements promote the rhythmic transcription of the negative elements that inhibit in a feedback loop the activity of the positive elements (please see more details in the next paragraph).
Disruption of these rhythms can lead to the development of diseases, such as metabolic syndromes, cancer and cardiovascular diseases. Thus, the maintenance of proper network coordination within the clock is essential for health and well-being.

1.5.1. The circadian clock in Drosophila

Circadian mechanisms were firstly characterized in the fruit fly *Drosophila melanogaster*. The first phenotypic mutants in *Drosophila* were described by Konopka and Benzer in 1971, the *period* mutants per^S, per^L, per^0. The mutants were identified following a genetic screen for the timing of eclosion, which in wild type flies normally occurs in the morning. Per^0 was found to be substantially arrhythmic, while per^S, per^L exhibited a shorter and a longer period of eclosion, respectively. Both the eclosion rhythm and the locomotor activity of flies were affected: per^0 locomotor activity was abolished, showing no evident periodicity,

while *per*^{S} and *per*^{L} locomotor activity was characterized by a shorter and longer period respectively (Konopka & Benzer, 1971).
In the decade between 1990 and 2000 several other genes have been identified in Drosophila, which mutations correlate to aberrant circadian phenotype, and have been therefore classified as core components of the circadian clock.
In flies, the circadian oscillation is composed of two main TTFLs, the Per/Tim Feedback loop and the Clock Feedback loop. These two loops are interlocked by two common transcription factors, Clock and Cycle that regulate the expression of the circadian genes (please see below).

The Per/Tim Feedback Loop

Clock proteins PERIOD (PER) and TIMELESS (TIM) are two core components of the TTFL, a system by which circadian oscillations are generated. These two proteins physically associate and translocate to the nucleus, where they repress the transcription of their own genes *period* (*per*) and *timeless* (*tim*), by suppressing the activity of two transcription factors, CLOCK (CLK) and CYCLE (CYC). Transcription factors CLK and CYC bind as heterodimers to the E-Box sequences (CACGTG) in the promoter region of *per* and *tim*, promoting their transcriptions. *per* and *tim* mRNA start to accumulate with a peak at the end of the day, while the corresponding proteins PER and TIM accumulate only in the middle/late night, four to six hours after *per* and *tim* mRNAs peak. This delay is due to the rapid degradation of PER via a proteasome-dependent mechanism that is controlled by the kinase DOUBLE-TIME (DBT), and is mediated by the ubiquitin ligase SLIMB. The DBT/PER complex becomes stabilized (i.e. fails to be degraded) only when TIM reaches high enough levels to bind to the DBT/PER complex. The PER/TIM/DBT complex enters into the nucleus, where PER binds to CLK/CYC, and removes this complex from the E-Box sequence, ultimately inhibiting *per* and *tim* transcription (Patke et al., 2020). Additional kinases, such as Casein Kinase 2 (CKs), which phosphorylates PER, and SHAGGY (SGG), which phosphorylates TIM, contribute to the phosphorylation status of the PER/TIM/DBT complex, which in its hyperphosphorylated form enters the nucleus, and represses CLK/CYC activity by promoting the phosphorylation and degradation of CLK (Yu & Hardin, 2006) (Figure 5).
Thus, changes in the levels of *per* and *tim* mRNAs are controlled by their functional gene products PER and TIM, which regulate their own gene expression through an autoregulatory negative feedback loop (So & Rosbash, 1997). Ultimately, this mechanism allows daily fluctuation of the proteins PER and TIM, which accumulate during the night, inhibit the activity of the CLK/CYC transcriptional complex, and are degraded in the morning. The control of PER and TIM oscillations largely depends on post-translational modifications (phosphorylation and ubiquitination to drive their degradation).

The Clock Feedback Loop

The same heterodimer CLK-CYC is also controlling the initial transcription of two additional genes, which are also important components of the biological clock: *pdp1ε* (*PAR-domain protein 1 epsilon*) and *vrille*. These two genes are transcribed

into mRNA, and translated into the corresponding proteins, PDP1 and VRILLE (VRI), during the late day/early night. VRI quickly accumulates into the cytosol, and enters the nucleus where it binds to specific sequences within the *clk* and *Pdp1ε* genes (called the V/P Box sequences), to negatively regulate the transcription of *clk* and *Pdp1ε* genes. When VRI levels are low as a consequence of inactivation of CLK by the *per*/*tim* feedback loop, PDP1ε levels can increase and the protein replaces VRI on the V-P Box sequences, reactivating the transcription of *clk*. Therefore, the initial inhibition of clk and *Pdp1ε* genes by VRI, followed by the activation of the transcription of clock by PDP1ε, generates a second feedback loop which allows the oscillatory expression of clk (Cyran et al., 2003).
As a result, the *clk* gene is rhythmically expressed, reinforcing the circadian oscillation (Figure 5).

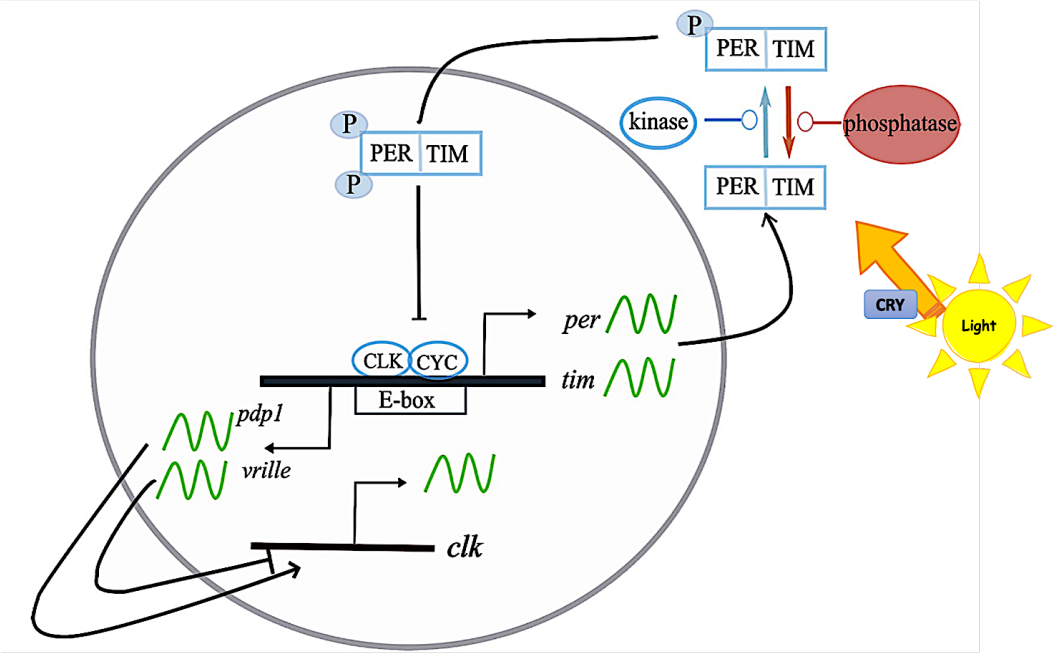

Figure 5. A two loop model for the Drosophila clock. *Two interconnected transcription feedback loops lie at the core of the Drosophila molecular clock. CLK and CYC bind to the promoters of per and tim, directly activating their transcription. Once the TIM-PER complex enters into the nucleus, it inhibits CLK/CYC activity. In addition, CLK/CYC complex directly activates the transcription of two other clock genese, vri and Pdp1ε. Consequently, Clk transcription is first repressed by VRI, and then activated by PDP1ε. Repression and activation of Clk are separated by the different phases of VRI and Pdp1ε proteins (from Xue et Zhang, 2018, modified).*

<u>The role of light</u>

As we all know very well, the sun does not rise and set at the same time every day in the different periods of the year. During the summer the light appears much earlier than in the winter, when the days are shorter and the sun rises later. Therefore, there must be a process that resets and synchronizes the clock every day to the external stimulus of light.
How does the *Drosophila* clock receive and integrate inputs from daylight? The light reaches the clock neurons through the compound eyes, the ocelli and the

Hofbauer Buchner eyelet, which are three different photoreceptive organs, containing different rhodopsins (Helfrich-Förster, 2020). Light can also directly reach the clock neurons through the blue light photoreceptor cryptochrome (CRY), which is present only in a specific subsets of pacemaker neurons and in the compound eyes (Yoshii et al., 2008). CRY accumulates in fly neurons during the night. During the day, CRY is activated following a conformational change that depends on light exposure, and binds to TIM to trigger its degradation. In particular, the TIM/CRY complex is recognized by JETLAG (JET), which targets TIM for proteasome-dependent degradation(Koh et al., 2006). During this event, CRY levels also decrease, because when TIM is almost completely degraded, CRY becomes a target of JET, it is therefore ubiquitinated and degraded (Peschel et al., 2009).
The lack of TIM causes PER to be vulnerable to DBT-dependent phosphorylation and degradation. Thus, the PER/DBT complex is degraded, and the clock resets.

The anatomy of the fly circadian clock

The Drosophila central circadian clock network is made up of approximately 150 neurons symmetrically distributed in the two hemispheres of the brain. This clock network is organized into multiple oscillatory units that are differentially coupled to one another (Hermann-Luibl & Helfrich-Förster, 2015). The dorsal groups of neurons, dorsal neurons 1-3 (DN_{1-3}), are in the dorsal area of the fly brain. The other neurons are located laterally, and are called lateral neurons (LPN, LN_ds, l-LN_vs, and s-LN_vs). These neurons are distributed in different areas of the fly brain, and they express different neuropeptides. In particular, the two groups of LN_vs (except one of the five small LN_vs (s-LNvs)) express the neuropeptide Pigment-dispersing factor (Pdf) and are required for the fly's morning peaks of activity, which anticipate the light's turning-on. They are therefore considered to be the "Morning Oscillator" (Grima et al., 2004) (Figure 6).
Pdf encodes a secreted biologically active neuropeptide that acts via a specific G-protein-coupled receptor to trigger intracellular signalling. It has a prominent role in the physiology of circadian rhythms, as well as contributing to other processes such as control of flight and digestion. *pdf*^{0} mutants do not display lights-on anticipatory activity (Grima et al., 2004). The evening peaks of activity rely on the Pdf-negative LN_ds and 5th s-LN_v, which are responsible for the anticipation of lights-off and are considered the "Evening Oscillator" (Grima et al., 2004).

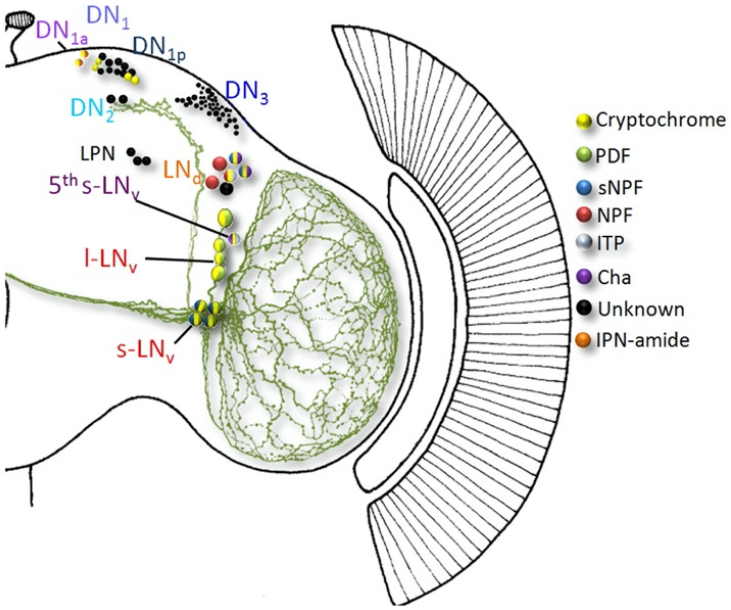

Figure 6. The location of the circadian clock. *The Drosophila circadian clock is made up of 150 neurons symmetrically distributed in the two hemispheres of the brain. Three neuronal groups are called dorsal neurons 1-3 (DN_{1-3}) and they are located dorsally. The other four groups are located more laterally and are therefore called lateral neurons (LN_d, l-LN_v, LPN, and s-LN_v). The l-LNvs and the s-LN_vs are expressing the neuropeptide pigment-dispersing factor (PDF) (from Peschel et al., 2009).*

1.5.2. The role of proteostasis in the regulation of the molecular clock

The degree of feedback repression of the transcription of *per* and *tim* is a key regulator of the circadian clock. In fact, overexpression of *per* shortens the circadian period, whereas a reduction of *per* levels lengthens the period. Predictably, events that affect the synthesis or degradation of clock proteins can contribute to the precise timing of the circadian clock by introducing delays or accelerating the TTFL. In this scenario, alterations in proteostasis can affect protein stability of core components of the clock, and alter rhythmicity. Among all the clock components, PER was shown to be an essential rate-limiting component, and high-amplitude oscillations in PER levels were investigated as fundamental determinants of the duration and phase of the circadian 24h cycle. Importantly, studies in mammals as well as *Drosophila* demonstrated that post-translational modifications (PTMs) of PER are crucially important in affecting PER stability, and they can therefore impinge on rhythmicity (C. Lee et al., 2001). Indeed, in both mammals and flies, the stability of clock proteins is regulated by coordinated phosphorylating and de-phosphorylating events that tag clock proteins (TIM, PER and CLK in particular) for proteasome-dependent degradation. In addition to phosphorylation, ubiquitination is also an important PTM that can control protein stability by targeting the protein to degradation. Thus, the interplay between ubiquitination and deubiquitination can regulate the physiological protein levels, and therefore it can contribute to create cyclical patterns of protein accumulation in the clock mechanism (Srikanta & Cermakian, 2021).

2. AIMS OF THE STUDY

Parkinson's Disease is a complex, multifactorial neurodegenerative disease, which often occurs sporadically in aged population. Patients develop a very obvious locomotor phenotype, with symptoms such as bradykinesia, resting tremor, muscular rigidity, and postural instability. At the cellular level, PD pathology is characterized by the presence of intracytoplasmic neurotoxic aggregates of misfolded proteins and dysfunctional organelles, resulting from proteostasis failure. By the time motor symptoms manifest, ninety percent of dopaminergic producing neurons are lost, which makes therapeutic intervention ineffective. Non-motor symptoms, such as constipation and olfactory deficits, are also very common in PD, and include alteration in the sleep-wake cycle, which is controlled by the circadian clock. These non-motor symptoms seem to precede the onset of the motor symptoms by many years, offering a window of therapeutic intervention that could delay - or even prevent - the progression of the disease.

Loss of proteostasis is well documented in PD, and seems to depend on the progressive pathological decline in the proteolytic activity of the ubiquitin-proteasome and the lysosome-autophagy system. However, while many studies investigate the effect of potentiating proteostasis to scavenge intracytoplasmic neurotoxic aggregates in PD models, very little attention has yet been paid to explore the possibility that alteration in proteostasis may affect the stability of core components of the circadian clock, and influence sleep behaviour observed in the prodromal phase.

Thus, in this study we want to exploit two well-established *Drosophila* models of PD, the PINK1 and Parkin KO flies, to investigate the effect of enhancing proteostasis in the context of circadian and sleep disturbances. We will enhance proteostasis by inhibiting the activity of deubiquitinating enzyme (DUB) USP14. USP14 is a DUB, belonging to ubiquitin-specific processing (USP) family, which negatively regulate autophagy and the ubiquitin proteasome system (UPS). Hence, its inhibition is predicted to both enhance the activity of the UPS, and autophagy. Furthermore, highly specific and potent inhibitors of USP14, such as IU1, are available. Previous studies in my lab have shown that genetic downregulation of USP14 or its pharmacological inhibition by specific inhibitor IU1, activates the proteasome and induces autophagy and mitophagy in different cell models. Remarkably, in our hands USP14 inhibition recovered locomotor behaviour of PINK1 and Parkin KO flies, and it extended flies' lifespan.

Published works indicate that PINK1 and Parkin KO flies also manifest abnormal circadian rhythmicity and disturbed sleep-wake cycle (with some discrepancies between studies), raising the question whether USP14 inhibition would also ameliorate these non-motor phenotypes.

With that in mind, the central aims of this internship thesis are:

(i) to characterize the PINK1 and Parkin KO flies in the context of circadian rhythmicity and sleep-wake cycle. In particular, we want to explore in depth sleep and circadian rhythmicity of these flies by recording locomotor activity

using the TriKinetics *Drosophila* Activity Monitor (*DAM*), to strengthen what is currently published in literature.
(ii) to test the effect on circadian rhythmicity and sleep-wake cycle of genetically downregulating USP14 in PINK1 and Parkin KO flies.

3. MATERIALS AND METHODS

3.1. *Drosophila* husbandry and lines generation

Flies and crosses were maintained under standard conditions at 23°C on agar, cornmeal and yeast food. Control (w^{1118}) and driver lines (Gal4) were obtained from Bloomington Drosophila Stock Center. $PINK1^{B9}$ and $park^{25}$ mutants have been described before (Greene et al., 2003; Park et al., 2006. See Table X for details.) and were provided by Dr. A. Whitworth. The UAS USP14 RNAi line was obtained from VDRC Stock Center. Parental lines were already present in the lab stock, so we had to collect virgins and males of the right genotypes and set the crosses, as illustrated in Table X.

Table 1. Description of mutant lines utilized.

Genotype	Nature of the lesion	Reference
$Pink1^{B9}$	Deletion of 570bp of Pink1 sequences	Park et al., 2006
$park^{25}$	The first three exons of the park gene have been deleted as well as half of exon 4	Greene et al., 2003

Table 2. Mating schemes.

Background wild type			
Driver	**Cross**	**Flies analyzed (♂)**	
	w1118 x UAS USP14 RNAi	UAS USP14 RNAi/+	Control of USP14i
Act5C-GAL4 (II)	w1118 x FM7-GFP/Y; Act5C-GAL4	Act5C-GAL4/+	Control of driver Act5C
Act5C-GAL4 (II)	UAS USP14 RNAi x FM7-GFP/Y; Act5C-GAL4	UAS USP14 RNAi/Act5C-GAL4	USP14i with driver Act5C
tim-GAL4 (II)	yw; tim-GAL4 x w1118	tim-GAL4/+	Control of driver tim
tim-GAL4 (II)	yw; tim-GAL4 x UAS USP14 RNAi	tim-GAL4/UAS USP14 RNAi	USP14i with driver tim
Pdf-GAL4 (I)	Pdf-GAL4 x w1118	Pdf-GAL4/Y	Control of driver Pdf
Pdf-GAL4 (I)	Pdf-GAL4 x UAS USP14 RNAi	Pdf-GAL4/Y; UAS USP14 RNAi/+	USP14i with driver Pdf
elav-GAL4 (I)	elav-GAL4 x w1118	elav-GAL4/+	Control of driver elav
elav-GAL4 (I)	elav-GAL4 x UAS USP14 RNAi	elav-GAL4/Y; UAS USP14 RNAi/+	USP14i with driver elav

Background $Pink1^{B9}$			
Driver	**Cross**	**Flies analyzed (♂)**	
	lethal/FM7-GFP; Gla/CyO x UAS USP14 RNAi	FM7-GFP/Y; UAS USP14 RNAi/CyO	
	$Pink1^{B9}$/FM7-GFP x FM7-GFP/Y; UAS USP14 RNAi/CyO	$Pink1^{B9}$/Y; UAS USP14 RNAi/+	Control of USP14i in $Pink1^{B9}$ background

Act5C-GAL4 (II)	Pink1^{B9}/FM7-GFP x FM7-GFP/Y; Act5C-GAL4	Pink1^{B9}/Y; Act5C-GAL4/+	Control of driver Act5C
Act5C-GAL4 (II)	Pink1^{B9}/FM7-GFP; Act5C-GAL4/(CyO) x FM7-GFP/Y; UAS USP14 RNAi/CyO	Pink1^{B9}/Y; Act5C-GAL4/UAS USP14 RNAi	USP14i with driver Act5C
tim-GAL4 (II)	lethal/FM7-GFP; Gla/CyO x yw/Y; tim-GAL4	FM7-GFP/Y; tim-GAL4/CyO	
tim-GAL4 (II)	Pink1^{B9}/FM7-GFP x FM7-GFP/Y; tim-GAL4/CyO	Pink1^{B9}/Y; tim-GAL4/+	Control of driver tim
tim-GAL4 (II)	Pink1^{B9}/FM7-GFP; Gla/CyO x FM7-GFP/Y; tim-GAL4/CyO	Pink1^{B9}/FM7-GFP; tim-GAL4/CyO	
tim-GAL4 (II)	Pink1^{B9}/FM7-GFP; tim-GAL4/CyO x FM7-GFP/Y; UAS USP14 RNAi/CyO	Pink1^{B9}/Y; tim-GAL4/UAS USP14 RNAi	USP14i with driver tim

Background park^{25}			
Driver	**Cross**	**♂**	
	yw; Sp/CyO; Sb/TM6B x UAS USP14 RNAi	yw/Y; UAS USP14 RNAi/Sp; TM6B/+	
	CyO/+; park^{25}/Sb x yw/Y; UAS USP14 RNAi/Sp; TM6B/+	UAS USP14 RNAi/CyO; park^{25}/TM6B	
	park^{25}/TM6B x UAS USP14 RNAi/CyO; park^{25}/TM6B	UAS USP14 RNAi/+; park^{25}/park^{25}	Control of USP14i in park^{25} background
Act5C-GAL4 (II)	yw; Sp/CyO; Sb/TM6B x FM7-GFP/Y; Act5C-GAL4	yw/Y; Act5C-GAL4/Sp; TM6B/+	
	park^{25}/TM6B x yw/Y; Sp/CyO; Sb/TM6B	CyO/+; park^{25}/Sb	
Act5C-GAL4 (II)	CyO/+; park^{25}/Sb x yw/Y; Act5C-GAL4/Sp; TM6B/+	Act5C-GAL4/CyO; park^{25}/TM6B	
Act5C-GAL4 (II)	park^{25}/TM6B x Act5C-GAL4/CyO; park^{25}/TM6B	Act5C-GAL4/+; park^{25}/park^{25}	Control of driver Act5C
Act5C-GAL4 (II)	Act5C-GAL4/CyO; park^{25}/TM6B x UAS USP14 RNAi/CyO; park^{25}/TM6B	UAS USP14 RNAi/Act5C-GAL4; park^{25}/park^{25}	USP14i with driver Act5C
tim-GAL4 (II)	yw; Sp/CyO; Sb/TM6B x yw/Y; tim-GAL4	yw/Y; tim-GAL4/Sp; TM6B/+	
tim-GAL4 (II)	CyO/+; park^{25}/Sb x yw/Y; tim-GAL4/Sp; TM6B/+	tim-GAL4/CyO; park^{25}/TM6B	
tim-GAL4 (II)	park^{25}/TM6B x tim-GAL4/CyO; park^{25}/TM6B	tim-GAL4/+; park^{25}/park^{25}	Control of driver tim
tim-GAL4 (II)	UAS USP14 RNAi/CyO; park^{25}/TM6B x tim-GAL4/CyO; park^{25}/TM6B	UAS USP14 RNAi/tim-GAl4; park^{25}/park^{25}	USP14i with driver tim

3.2. Genotyping

After generating the lines, genotypes were checked by PCR.
For DNA extraction, one adult fly was put in a 1.5 ml Eppendorf and on ice to anesthetize the animal. The fly was mashed with a pipette tip for 10 seconds before adding 50 µl Squishing buffer (Tris-HCl pH 8.2 10 mM, EDTA 1 mM, NaCl 25 mM, Proteinase K 200 µg/ml) and pipetting. Samples were then incubated at 37°C for 20 minutes and subsequently heated to 95°C for 2 minutes to inactivate

the Proteinase K. Samples were next centrifuged for 2 minutes at top speed and supernatant was collected.
1 µl of extracted DNA was added to the PCR mix (5x FIREPol® Master Mix Ready to Load, Solis BioDyne) and the samples were briefly spun down. Based on the targets we wanted to amplify, different primers and thermocycling conditions were used.

Table 3. Primers and thermocycling conditions used.

TARGET	PRIMERS	THERMOCYCLING CONDITIONS
Pink1B9	dPINK1 FW: AACGTTGAAGTAGGCGCATT dPINK1 RV GGCTGGCTGTAGTGGAAGAC	Initial denaturation: 95°C, 2 min Denaturation: 95°C, 30s Annealing: 55°C, 30s Extension: 72°C, 2 min 45s (x30) Final extension: 72°C, 5 min Hold: 4°C
Park25	dParkin FW: CTTTACCATCCCCCAATCAA dParkin RV CCTGGCTGAACATTTTGTC	Initial denaturation: 95°C, 2 min Denaturation: 95°C, 30s Annealing: 60°C, 40s Extension: 72°C, 3 min 30s (x30) Final extension: 72°C, 5 min Hold: 4°C

1% Agarose gel was prepared by mixing and boiling 0.5 g UltraPure Agarose (Invitrogen) with 50 ml TAE buffer 1X. After cooling down, 5 µl of SYBR Safe DNA Gel Stain (Invitrogen) were added. 20 µl of each sample were loaded along with 5 µl of Gene Ruler 1kb DNA ladder (ThermoFisher), to determine the size of DNA fragments. Gel ran for 30 minutes at 100 V. The PCR products were analyzed under UV light.

3.3. RNA extraction, reverse transcription and qRT-PCR

To check the correct knockdown of USP14 by the USP14 RNAi construct expressed through the UAS/Gal4 system, we assessed USP14 mRNA levels in control flies (Act5cGal4/+) and in flies with UAS USP14 RNAi under the control of the ubiquitous Act5cGal4 driver (Act5cGal4/UAS USP14 RNAi).
For RNA extraction, whole flies were homogenized in TRI Reagent (Zymo Research) and incubated for 5 min atRT. Chloroform was added and the samples were incubated for 15 min in ice. Samples were then centrifuged at 14000 g for 15 min at 4°C, so that lipids, denatured proteins and DNA precipitate, while the RNA remains in the aqueous phase. Once the separation of the phases was obtained, the supernatant phase containing the RNA was taken from each tube and collected in a new tube. At this point, samples were treated with isopropanol (the volume used is equal to the volume of aqueous solution taken containing the RNA). After the first centrifugation step at 4°C with isopropanol for RNA precipitation, the supernatant obtained was discarded and a 75% ethanol solution was added to the pellet to wash it. This was followed by another centrifugation step at 4°C for 15 min and then the supernatant was discarded.

Once the ethanol evaporated, the pellet was resuspended in RNase free water and samples were then further purified by precipitation with LiCl 8M.
Finally, the RNA was quantified with the Eppendorf BioPhotometer D30 (Eppendorf) by measuring absorbance at 260 nm (to verify the presence of RNA), at 280 nm (to verify the presence of protein contamination) and at 230 nm (to verify the presence of phenol or carbohydrates contamination). The ratios A260/A280 and A260/230 should be higher than 1.9 for a good purification.

For each sample, 1 µg of RNA was used for first-strand cDNA synthesis with the SensiFAST cDNA Synthesis Kit (Bioline) following the kit protocol. Three technical replicates were loaded for each sample and the expression level of Rp49 was set as an internal amplification control for normalization. qRT-PCRs were performed using the HOT FIREPol SolisGreen qPCR Mix (Solis Biodyne) on a QuantStudio 5 Real-Time PCR System (Thermofisher). The sequences of qPCR primers used are:

USP14_FW: AGCTCAGAAGAGGATCCCGA
USP14_RV: CGGCTCACCAAGTAAGTTCG
Rp49_FW: ATCGGTTACGGATCGAACAA
Rp49_RV: GACAATCTCCTTGCGCTTCT

3.4. Assessment of locomotor activity: *Drosophila* activity monitoring system DAM

Locomotor rhythms of 3-10 days old individual male flies were recorded with Drosophila activity monitors DAMSystem˝ (Drosophila Activity Monitor System ˝) (Trikinetics Inc, Waltham, MA, USA). Briefly, the system records activity from individual flies maintained in sealed tubes placed in activity monitors, in which an infrared beam is directed through the midpoint of each tube. Glass tubes (about 5 mm in diameter) that contain food substance at one end and plugged with sponge plug at the other end were used in this experiment. Flies were anesthetized with CO_2 and the males of the right genotype were selected and transferred each on a single activity tube with the help of a paintbrush. The tube was closed with a sponge plug to avoid the fly from escaping during the experiment. Tubes were then inserted in the activity monitors and fixed with a rubber band to prevent them from moving and to ensure that the infrared beam passes the tube at the center position. Each time a fly crosses the beam an “activity event” is measured and events detected over the course of each consecutive sampling interval are summed and recorded over the course of the experiment for each fly. Flies were kept for 3 days in 12 h_12 h LD cycles before being transferred to constant-dark conditions (DD) in which they remained for at least 7 days. The experiments were performed at constant temperatures of 23°C.
After the run of the experiment, data are pre-processed using the “DAMFileScan111X” software from TriKinetics, which allows to specify experiment start and end dates/times, consolidate recorded bins into longer interval (bin) lengths, and create individual .txt files for each fly. Data are saved

in 30min bin length for circadian analyses and 1min bin length for sleep analyses (Pfeiffenberger et al., 2010) (Figure 7).

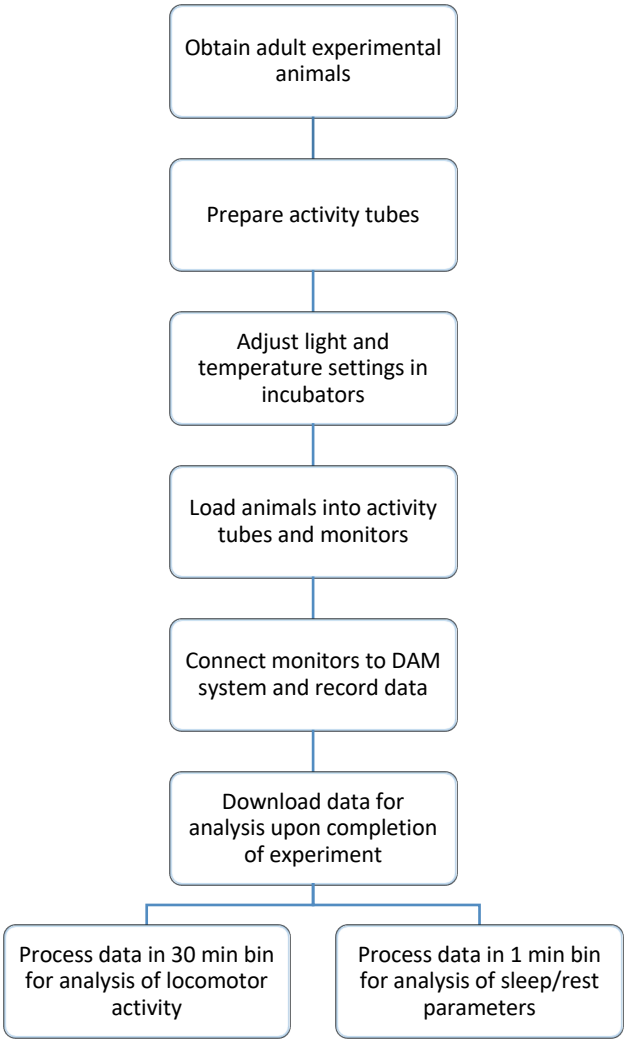

Figure 7. Flowchart outlining the major steps for assaying locomotor activity rhythms in Drosophila (from Chiu et al., 2010, modified).

Circadian analysis

Activity data have been analysed using the FaasX (Fly Activity Analysis Suite for MacOS) software, developed by M. Boudinot and François Rouyer (Institut de Neurobiologie Alfred Fessard, CNRS, France). The FaasX generates tabulated text and graphical rendering on circadian activity collected by DAMSystem such as:

- double plot of daily activity (Actogram);
- average activity at each time interval throughout an entrained circadian cycle (Eduction) and
- period analysis (periodogram).

Morning Index was calculated for each fly using the following mathematical approach (Seluzicki et al., 2014):

$$M.Index = \frac{\left(\sum activity.3h.before.light.on\right)}{\left(\sum activity.6h.before.light.on\right)} - 0.5$$

When the locomotor activity in the 3 hours before lights-on is higher than the one in 6 hours, the M. Index value is >0, indicating a morning onset anticipation, while when the activity 3 and 6 hours before lights-on is equivalent, the value is 0, indicating the absence of the morning anticipation. The same was done for evening anticipation, with Evening Index calculated before lights-off.
Rhythmicity was calculated using two types of analysis, autocorrelation, which allows to identify a periodic pattern in a series of data, and MESA (maximum entropy spectral analysis) which estimates the periodicity.

Sleep analysis

The activity data collected by the DAMS (Trikinetics, USA) activity monitors were analyzed using custom R scripts, designed by prof. Mauro Zordan (Department of Biology, University of Padova). The scripts were designed to access the data files generated by the DAMS activity software and prepare the data for inspection, for the generation of graphs and for statistical analysis. In particular, a first script was designed to batch process the raw activity data files in order to pre-process the raw data (i.e. perform 30min. binning and calculate sleep-specific parameters) and to save the resulting data-frames as files to be used by the second script. Based on the files saved by the first script, the second script was designed to perform comparisons between sleep data from different experimental conditions/genotypes. This results in the generation of graphical representations (based on the ggplot2 package by Hadley Wickham) of the sleep parameters being compared (i.e. total length of time spent sleeping, number and length of sleep bouts, both during the day and during the night). For average daily sleep profile sleep is calculated by measuring the minutes that the fly does not move every 30 minutes. 5 minutes is the minimum time for the fly to be considered asleep, while if it does not move for more than 30 minutes it is considered dead. In addition, this script also performs the statistical analysis of such comparisons based on ANOVA with adequately corrected (Tukey HSD) multiple comparisons.

3.5. Statistical analysis

Statistical analysis was performed using GraphPad PRISM or R software as described in detail below.

Analysis of activity
Statistical analysis of circadian activity was performed using GraphPad PRISM. Statistical significance was determined by Kruskal-Wallis nonparametric ANOVA (to account for non-normality of samples). Each bar of the bar charts (total

activity and period) represents mean ± SEM of the indicated parameter for the indicated genotype.
Stacked bars (rhythmicity, EA and MA) represent the percentage of flies showing the indicated phenotype for each genotype. Significance was determined by Contingency Fisher's exact test. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$. “n” indicates the number of flies analyzed per genotype.

Analysis of sleep
Statistical analysis of sleep behaviour was performed using R software . Significance was determined by Tukey’s HSD multiple comparison test. For each box plot, the median is shown with the upper and lower quartile and the 0^{th} and 100^{th} percentile; *: $p<0.05$ **; $p<0.01$; ***: $p<0.001$

4. RESULTS

4.1. Genetic inhibition of USP14 lengthens the circadian period of *Drosophila melanogaster*

USP14 is a deubiquitinating enzyme (DUB) intrinsically associated with the proteasome that belongs to the ubiquitin-specific protease (USP) family. USP14 suppresses the degradation of its targets by promoting the dissociation of the substrate from the proteasome before the commitment step. Hence, inhibition of USP14 stimulates proteasome-dependent degradation of its substrates, and it can overall enhance proteostasis.

In mammals, USP14 appears to be also a clock-relevant DUB that contributes to the maintenance of the circadian clock. In particular, USP14 activity has been shown to affect the circadian period by slowing down the degradation of clock protein PER. Accumulation of PER generates a time delay in the removal of the inhibition of Clock and Cycle, which is required to the maintenance of a robust circadian clock (D'Alessandro et al., 2017).

With that in mind, we wanted to address whether USP14 could similarly affect the circadian clock in flies.

To this aim, we developed and validated a GAL4/UAS-based RNAi approach (Hales et al., 2015) for cell-specific downregulation of USP14 in specific tissues of the fly. This requires a "driver" which is made of a tissue-specific promoter placed at 5' of the gene encoding the yeast GAL4 transcription factor, and the RNAi against the gene of interest placed at 3' of the upstream activating sequence (UAS). Transgenic flies carrying either of the two constructs alone do not express the RNAi of interest, however when crossed into the same fly, the tissue-specific promoter will drive the expression of GAL4, which downregulates the gene of interest in the specific tissue. We used four different drivers (*elav*, *tim*, *Pdf* and *Actin*) to express USP14 RNAi in different target cells. In particular, *elav* (embryonic lethal abnormal visual), whose gene product encodes an RNA-binding protein expressed in all neurons, allows downregulating USP14 in the whole nervous system of adult flies. *timeless* (*tim*) encodes a key component of the TIM-PER complex, required for the generation of circadian rhythms. This protein is mainly expressed in the brain of the fly, in particular in LN_ds and LN_vs neuronal clusters. Thus, *tim*-GAL4 driver is used as a marker for these specific neurons. Neuropeptide Pdf (pigment dispersing factor), which is secreted by a subset of circadian neurons (the so called "morning oscillators"), can be used as a selective marker for those neurons. Finally, Actin is an ubiquitous-expressed protein, which allows expression of *USP14* RNAi in the whole fly. We used these four different drivers to dissect the effect of *USP14* downregulation in the whole fly versus specific subsets of clock neurons that are known to be involved in circadian oscillation (i.e. TIM and Pdf expressing neurons) (Figure 1A).

Locomotor activity and circadian behavior of the abovementioned transgenic flies was recorded using the TriKinetics *Drosophila* Activity Monitor (*DAM*). In this system, flies are held individually in small horizontal glass tubes intersected by an infrared beam. When a fly is active it breaks this beam and activity is

recorded. Flies were entrained in light/dark (LD) conditions for three days and then shifted to constant darkness (DD) for seven days to monitor endogenous circadian locomotor activity. We found that downregulation of *USP14* in the tested tissues did not affect total activity of the flies (Figure 1B), nor rhythmic behavior in LD conditions (Figure 1C). A similar lack of effect was observed for evening anticipation (EA; i.e. increased activity just before the transition to lights-off) (Figure 1D), whereas morning anticipation (MA; i.e. increased activity just before the transition to lights-on) was decreased in flies in which USP14 was selectively downregulated in Tim expressing neurons (Figure 1E). Importantly, specific knockdown of USP14 in Tim+ and Pdf+ cells significantly lengthened the period of rhythmic flies from 23.8hrs to 24.5hrs and from 23.7hrs to 24.2hrs, respectively (Figure 1F). Control flies (*tim-GAL4/+* and *pdf-GAL4/+*) maintained circadian locomotor activity, indicating that nonspecific effects from UAS/GAL4 system in Tim+ or Pdf+ cells did not cause loss of circadian activity *per se*.

In summary, genetic inhibition of USP14 in Tim+ and Pdf+ cells lengthened the circadian period of flies, and partially affected their ability to anticipate transition to lights-on. Importantly, this effect was specific for Tim and Pdf expressing neurons, as it was not observed in other neuronal cells. Rhythmicity and total activity did not seem to be affected by USP14 downregulation.

4.2. *Drosophila* PINK1 and Parkin mutant (KO) flies display reduced total activity and weakened circadian rhythms

Published works indicate that PINK1 and Parkin KO flies manifest abnormal circadian rhythmicity and disrupted sleep-wake cycle, with some discrepancies between studies. Thus, we wanted to explore in depth circadian rhythmicity and total activity of these flies, to strengthen *Drosophila* as animal model for investigating non-motor symptoms of PD.
To this aim, we used $park^{25}$ and $Pink1^{B9}$ null strains, which were extensively used before to study PD motor phenotype. In particular, $park^{25}$ and $Pink1^{B9}$ null mutant flies display phenotypes such as abnormal wing posture, degeneration of flight muscles, mitochondrial dysfunction and loss of dopaminergic neurons, which are highly reproducible and translate into climbing and flight defects (Greene et al., 2003; Park et al., 2006). We monitored locomotor activity of wild type and $Pink1^{B9}$ and $park^{25}$ mutant flies by using the *Drosophila* Activity Monitor. Flies were kept in light/dark (LD) conditions for three days and then shifted to constant darkness (DD) for seven days to monitor endogenous circadian locomotor activity. Wild type flies displayed morning and evening peaks in activity, with characteristic anticipation before lights-on (morning anticipation, MA) or lights-off (evening anticipation, EA). They were active during the day and relatively inactive at night (Figure 2A). They displayed a total activity of about 500 beam-recorded counts per day (Figure 2B) and maintained circadian rhythmicity (Figure 2C), with a period of 23.6hrs (Figure 2D). Moreover, most of them displayed morning and evening anticipation (Figure 2E-F).
Both mutants displayed reduced total activity (Figure 2B). Moreover, in constant

conditions, a higher percentage of *Pink1^{B9}* flies were arrhythmic (Figure 2C). Among those flies that remained rhythmic, we recorded a significant lengthening of the circadian period compared to wild type flies (from 23.6hrs to 26.2hrs) (Figure 2D). Also, *Pink1^{B9}* flies appeared to have almost completely lost their ability to anticipate light transitions, and a significantly smaller percentage of them displayed morning (Figure 2E) and evening anticipation (Figure 2F).
The endogenous rhythmicity was affected also in *park^{25}* flies, although to a minor extend: a higher percentage of flies remained rhythmic compared to *Pink1^{B9}* flies (Figure 2C), and the period was only slightly, but significantly lengthened (Figure 2D). Like the PINK1 KO flies, a significantly larger percentage of *park^{25}* mutant flies failed to anticipate morning and evening clues (Figure 2E-F).

Thus, *park*^{25} and *Pink1^{B9}* mutant flies display an impairment in their circadian behaviours. In LD conditions, they seem to be less active during the day, with disrupted morning and evening anticipation. In constant conditions, a smaller percentage of flies were rhythmic, and displayed a longer circadian period.

4.3. USP14 downregulation in PINK1 mutant flies rescues the circadian defects of these flies

We recently reported that genetic and pharmacological inhibition of USP14 in PINK1 and Parkin KO flies rescued the locomotor phenotype associated with these flies. In particular, PINK1 and Parkin mutant (KO) flies displayed reduced climbing and flight ability, shorter lifespan, and degeneration of the muscle of the thorax. The mutant genotypes also develop mitochondrial dysfunction (reduced mitochondrial respiration, deranged mitochondrial shape and ultrastructure), and loss of DA neurons in the drosophila brain PPL1 cluster. Inhibition of USP14 activity almost completely normalizes the abovementioned phenotypes, and lengthens PINK1 and Parkin KO lifespan to control conditions (Chakraborty et al., 2018).
With that in mind, we wanted to assess whether USP14 downregulation can also effectively recover the circadian phenotype associated to PINK1 and Parkin KO flies.
To this aim, we downregulated USP14 either in the whole fly, with the actinGal4 driver, and in the Tim expressing neuron, as our previous data indicate that USP14 inhibition had the most readable effect in this specific subset of circadian neurons (see Figure 1). Flies of the desired genotypes were tested for locomotor and circadian behavior as previously described.
We monitored total activity (Figure 3B), percentage of rhythmicity (Figure 3C), periodicity of the rhythmic flies, EA and MA (Figure 3D-F) of PINK1 KO flies in which USP14 was downregulated in the Tim+ clock neurons. We observed a rescue in the total activity of the PINK1 KO flies (Figure 3B), and in all the circadian parameters that we tested, except for the period (Figure 3C-F). As observed for wild type flies, USP14 downregulation in Tim+ cells lengthened the circadian period of PINK1 KO flies.

Interestingly, downregulation of USP14 in the whole animal with Actin promoter did not seem to be as effective in preventing the development of the circadian phenotype of the PINK1 flies, in that it only rescued circadian rhythmicity (Figure 4A-F). This result suggests that the effect of USP14 inhibition is specific for clock neurons.

These results indicate that USP14 downregulation in PINK1 mutant flies rescues the circadian defects of these flies. The effect seems to be particularly evident when we used a clock specific driver like Tim.

4.4. USP14 downregulation in Parkin mutant flies does not rescue the circadian phenotype of these flies

We next investigated the effect of USP14 downregulation in Parkin KO background. As done before, we used the UAS/GAL4 system and cell-specific drivers tim and actin to downregulate USP14 in Parkin KO flies. Downregulation of USP14 in Tim+ clock neurons did not recover the circadian phenotype of these flies (Figure 5). The same lack of effect was observed when USP14 was downregulated in the whole fly with Actin promoter (Figure 6).

Thus, as opposed to PINK1 flies, downregulation of USP14 in Parkin KO background does not rescue the circadian phenotype of these flies.

4.5. *Drosophila* PINK1 and Parkin KO flies display altered sleep behavior

In our previous analysis we demonstrated that PINK1 and Parkin KO flies display defects in circadian rhythmicity with comparable phenotype between the two genotypes. The aberrant circadian phenotype includes decrease in total activity of the flies, disrupted rhythmicity, reduced ability to respond to changes to external clues (measured as their ability to anticipate lights on and off), and longer circadian period. Interestingly, USP14 downregulation in Tim+ clock neurons almost completely recovered PINK1 KO flies, whereas it did not prevent the development of circadian defects in Parkin KO background.
Because the circadian clock controls sleep behaviour, we wanted to assess sleep-wake cycle in PINK1 and Parkin KO flies, and investigate whether USP14 inhibition can rescue potential sleep defects in these mutant backgrounds.
To this aim, we monitored sleep behaviour of flies of the indicated genotypes using the DAM monitoring system previously described. Sleep episodes in flies are defined as time in which flies do not change their position for at least 5 min. Recordings from the second day were analysed to estimate sleep performance during the 24hrs period (12hrs of light and 12hrs of darkness) (Figure 7A). Wild type flies (w1118) exhibited characteristic sleep behaviour: they seemed to be relatively active during the day and inactive at night, with a pick of inactivity during the day (“siesta”), four to five hours after light went on (Figure 7A, grey line). Parkin KO flies exhibited a similar trend, although they seemed to be less

active during the day (Figure 7A, green line). On the contrary, PINK1 mutants seemed to sleep a lot more, especially during the day (Figure 7A, blue line).
Consistent with this analysis, we indeed observed a significant increase in total sleep in PINK1 and Parkin mutant flies (Figure 7B), either during the light phase and in the dark phase (Figure 7C and 7D, respectively). Interestingly, the number of sleep episodes during the light phase was significantly higher in Parkin mutants (Figure 7E), while *Pink1^{B9}* flies displayed fewer episodes of sleep during the dark phase (Figure 7F). PINK1 KO flies also exhibited longer episodes of sleep in both light and dark phases (Figure 7G-H). The number and the length of the sleep episodes is important to estimate sleep fragmentation.

In summary, *Pink1^{B9}* and *park^{25}* flies seem to sleep more compared to wild type. Moreover, the quality of sleep appeared to be significantly different between wild type and the mutant genotypes. In particular in PINK1 mutants, sleep is less fragmented with flies displaying fewer but longer episodes of sleep.

4.6. USP14 downregulation in PINK1 mutant flies rescues the sleep defects of these flies

Next, we wanted to investigate whether USP14 downregulation affects the sleep behavior of PINK1 and Parkin flies. Once again, we used the UAS/GAL4 system and cell-specific drivers Tim and Actin to downregulate USP14 in Tim expressing neurons, and in the whole fly. Recordings from the second day were analysed, and we estimated sleep performance during 12hrs of light and 12hrs of darkness of PINK1 KO flies in which USP14 was downregulated in Tim+ neurons. Like PINK1 KO flies (*Pink1^{B9}*, dark blue line), parental lines (*Pink1^{B9}*;USP14 RNAi/+ and *Pink1^{B9}*;tim-GAL4/+, red and orange lines respectively) displayed increased sleep during the day compared to wild type flies (w1118; grey line). Importantly, downregulation of USP14 in Tim+ neurons of PINK1 flies (*Pink1^{B9}*;tim-GAL4>USP14 RNAi, light blue line) completely rescued sleep performance of PINK1 flies. In particular, flies of the indicated genotype almost completely phenocopied wild type flies in that they were active during the day and they rested during the night (Figure 8B).
In perfect agreement with the sleep plot described in Figure 8B, the parental lines (*Pink1^{B9}*;USP14 RNAi/+ and *Pink1^{B9}*;tim-GAL4/+, red and orange graph bars respectively) exhibit a sleep behavior comparable to *Pink1^{B9}* (dark blue graph bar). Notably, USP14 downregulation in Tim+ neurons completely normalized total sleep of PINK1 KO flies (Figure 8C-E).
Similar results were obtained when USP14 was downregulated in the whole animal with the Actin promoter fly (Figure 9).

Thus, genetic inhibition of USP14 in *Pink1^{B9}* flies normalizes the sleep behavior of PINK1 KO flies.

4.7. USP14 downregulation in Parkin mutant flies does not normalize the sleep phenotype of these flies

We next investigated the effect of USP14 downregulation in Parkin KO background. Downregulation of USP14 in Tim+ clock neurons did not recover the sleep defects of these flies (Figure 10). The same lack of effect was observed when USP14 was downregulated in the whole fly with Actin promoter (Figure 11).

In summary, downregulation of USP14 in Parkin mutants does not normalize the sleep defects of these flies.

4.8. Results Figures

Figure 1.

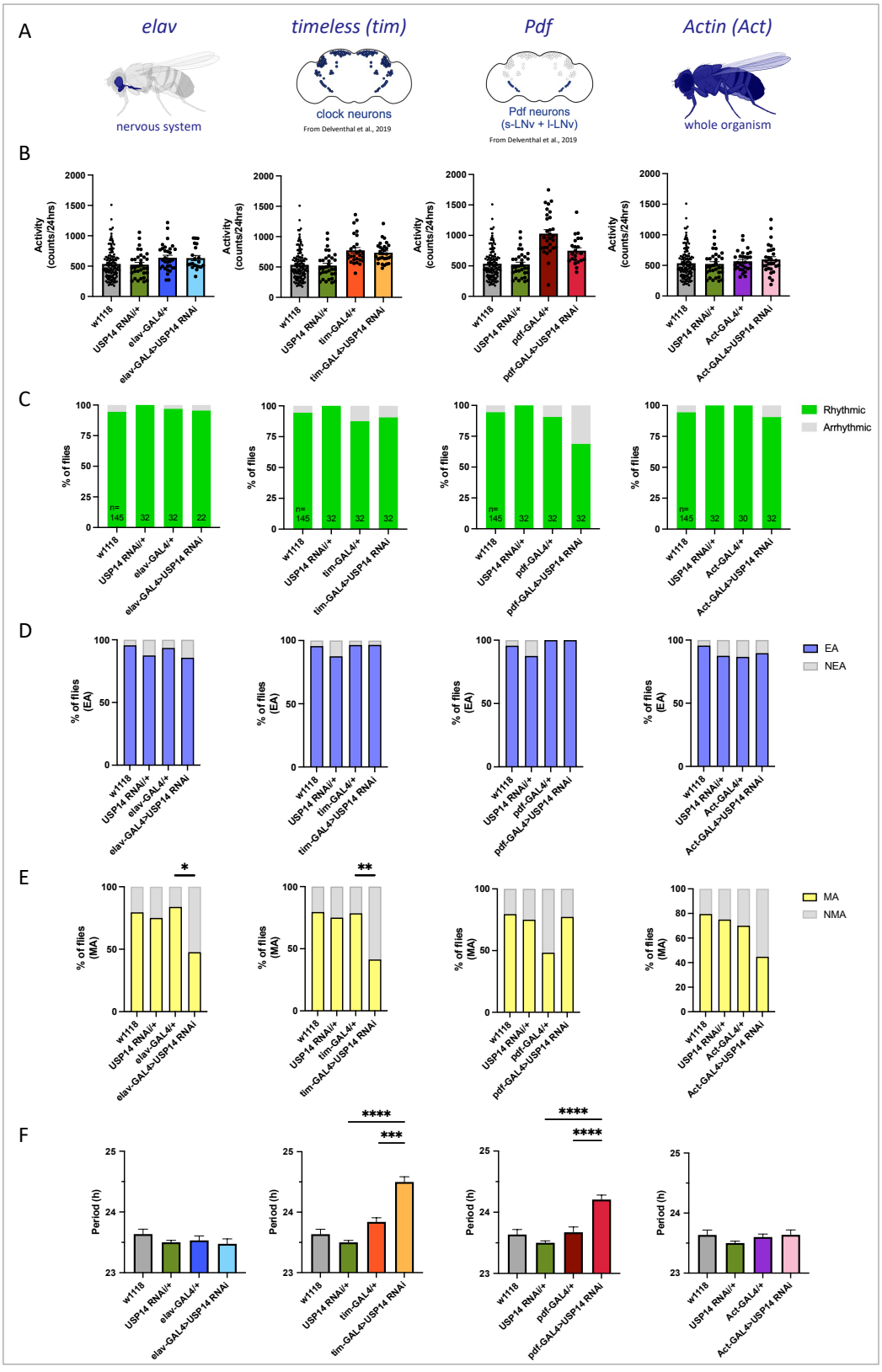

Figure 1. Cell-specific genetic inhibition of USP14 lengthens the circadian period of Drosophila melanogaster, and partially affects their Morning Anticipation. *(A) Diagram showing neurons and tissues targeted by USP14 RNAi using elav-GAL4, tim-GAL4, pdf-GAL4 and Act-GAL4, respectively. (B) Bar charts showing total activity during the second day of locomotor activity recording in LD 12:12 conditions (12h of light and 12h of darkness). Bar graph expresses mean ± SEM of total activity of the indicated genotypes measured as the number of time that the fly intersected the infrared beam of the recording apparatus in 24hrs. (C) Stacked bars showing percentage of rhythmic and arhythmic flies. (D) Stacked bars indicating the percentage of flies that show evening anticipation (EA) or not (NEA). EA was calculated as described in materials and methods. (E) Stacked bars indicating the percentage of flies that show morning anticipation (MA) or not (NMA). MA was calculated as described in materials and methods. (F) Bar charts showing the length of the period of rhythmic flies for the indicated genotype.*

Figure 2.

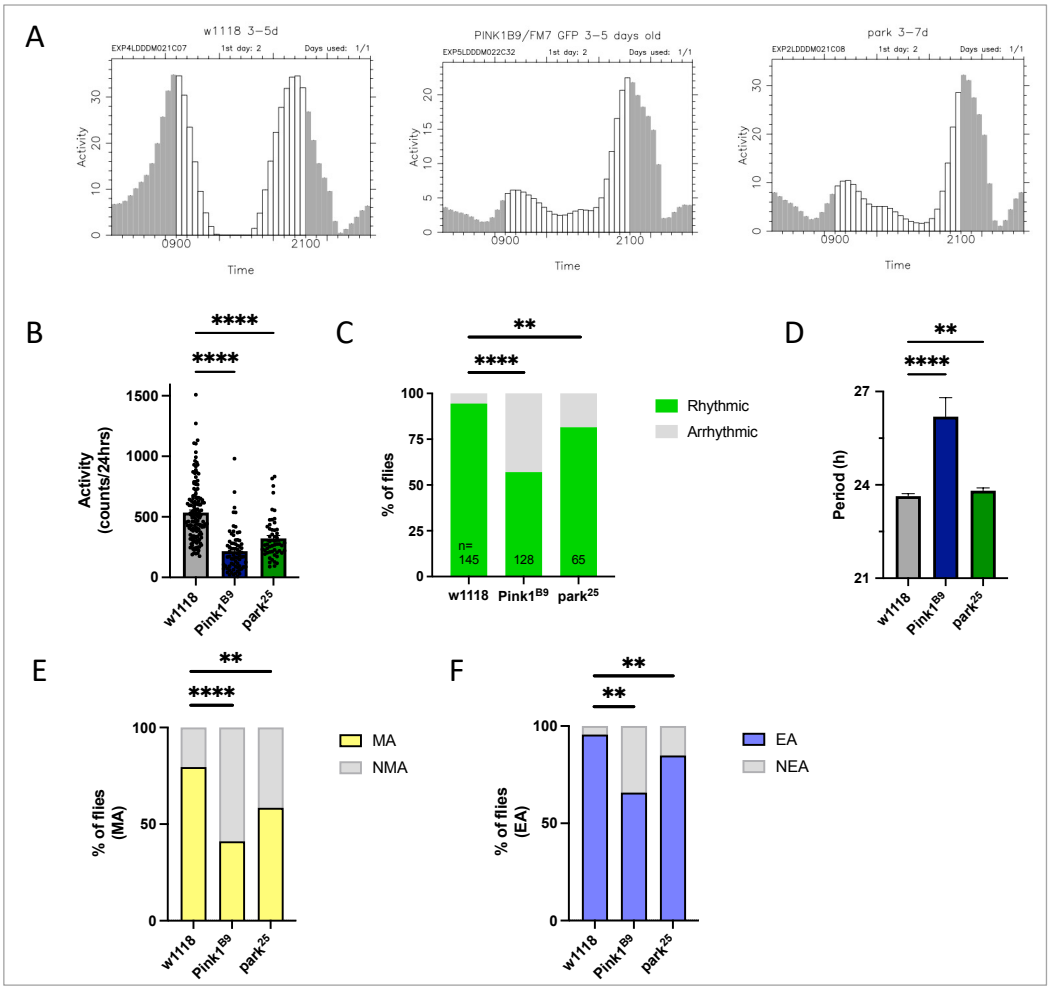

Figure 2. Drosophila PINK1 and Parkin mutant (KO) flies display aberrant circadian rhythmicity, with lower levels of activity during the day and disrupted morning and evening anticipation. *(A) Representative eduction charts showing the activity of a single fly in the second day of LD 12:12 conditions (12 h of light and 12 h of darkness). Each bar represents the activity counts every 30 minutes. (B) Bar charts showing total activity during the second day of locomotor activity recording in LD. Charts show total activity for each genotype as beam crosses per 24hrs. (C) Stacked bars showing percentage of rhythmic and arhythmic flies. (D) Bar charts showing the period of rhythmic mutant flies. (E) Stacked bars indicating the percentage of flies that show morning anticipation (MA) or not (NMA). (F) Stacked bars indicating the percentage of flies that show evening anticipation (EA) or not (NEA).*

Figure 3.

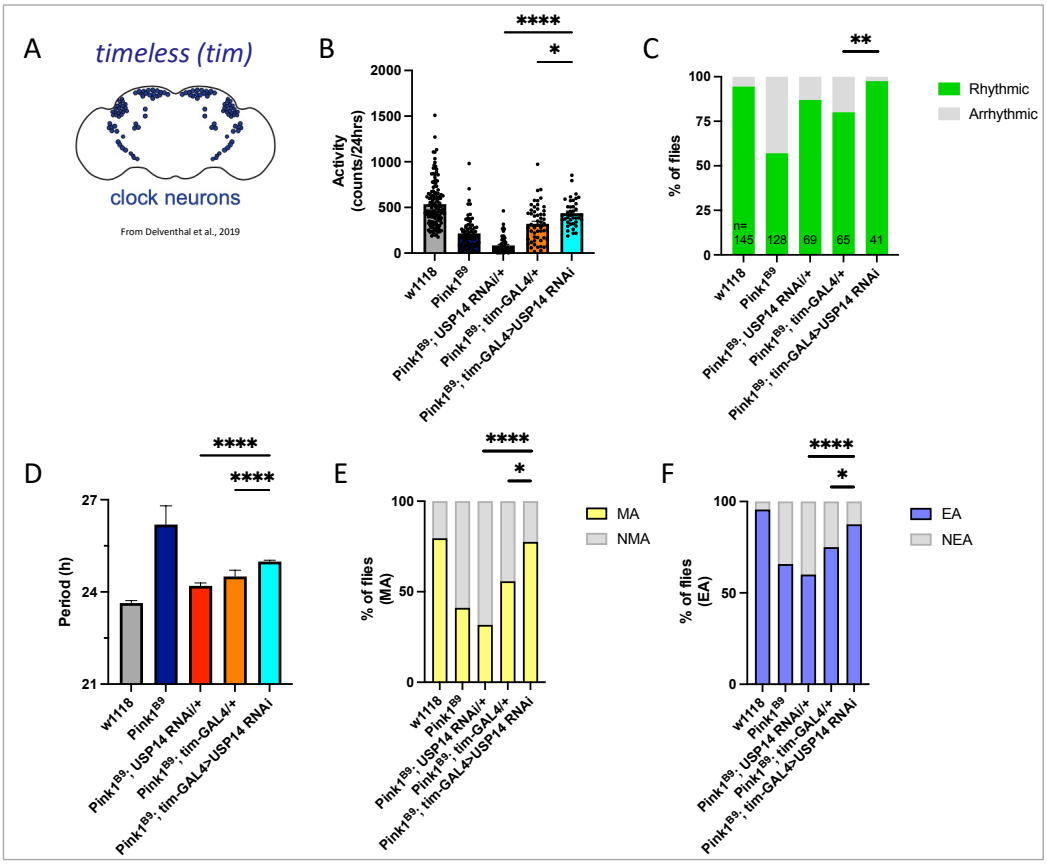

Figure 3. Tim-specific genetic inhibition of USP14 in PINK1 mutant flies rescues the circadian defects*. (A) Diagram of the clock neuron subset targeted by USP14 RNAi using tim-GAL4. (B) Bar chart showing total activity during the second day of locomotor activity recording in LD. Charts show total activity for each genotype as beam crosses per 24hrs. (C) Stacked bars showing percentage of rhythmic and arhythmic flies. (D) Bar chart showing the period of rhythmic flies with clock neurons-specific inhibition of USP14. (E) Stacked bars indicating the percentage of flies that show morning anticipation (MA) or not (NMA). (F) Stacked bars indicating the percentage of flies that show evening anticipation (EA) or not (NEA).*

Figure 4.

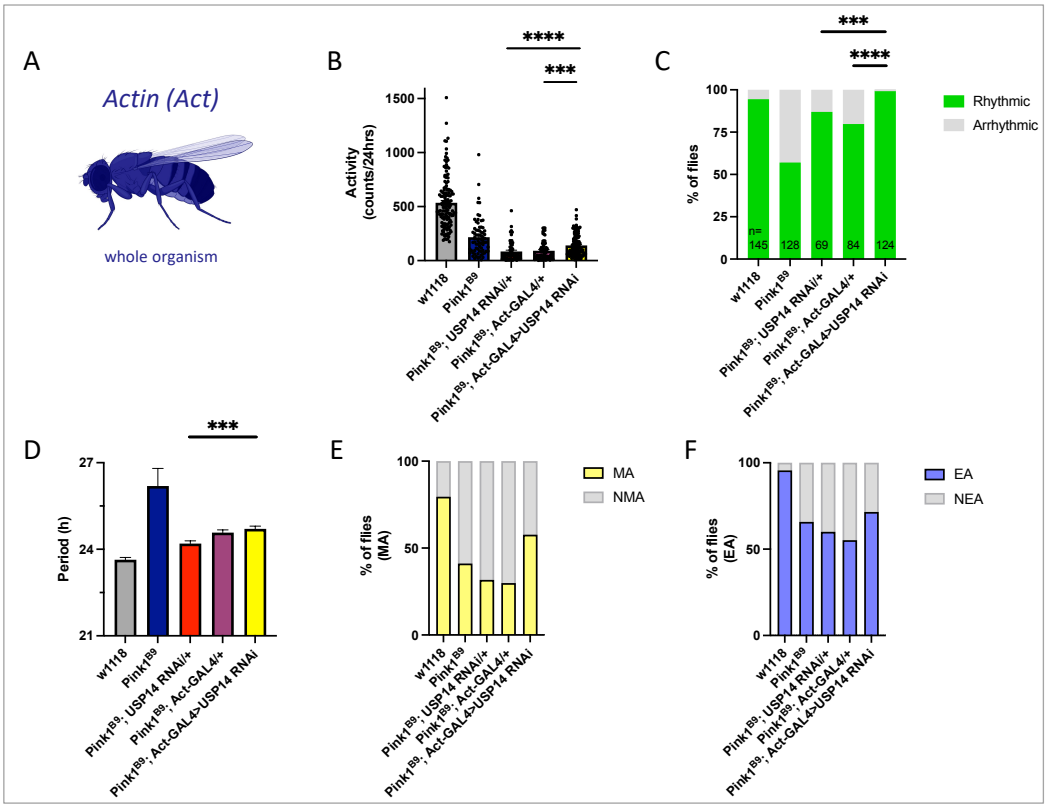

Figure 4. Act-driven genetic inhibition of USP14 in PINK1 mutant flies rescues total activity and rhythmicity. *(A) Diagram of tissues targeted for inhibition of USP14 using Act-GAL4. (B) Bar chart showing total activity during the second day of locomotor activity recording in LD. Charts show total activity for each genotype as beam crosses per 24hrs. (C) Stacked bars showing percentage of rhythmic and arhythmic flies. (D) Bar chart showing the period of rhythmic flies with ubiquitous inhibition of USP14. (E) Stacked bars indicating the percentage of flies that show morning anticipation (MA) or not (NMA). (F) Stacked bars indicating the percentage of flies that show evening anticipation (EA) or not (NEA).*

Figure 5.

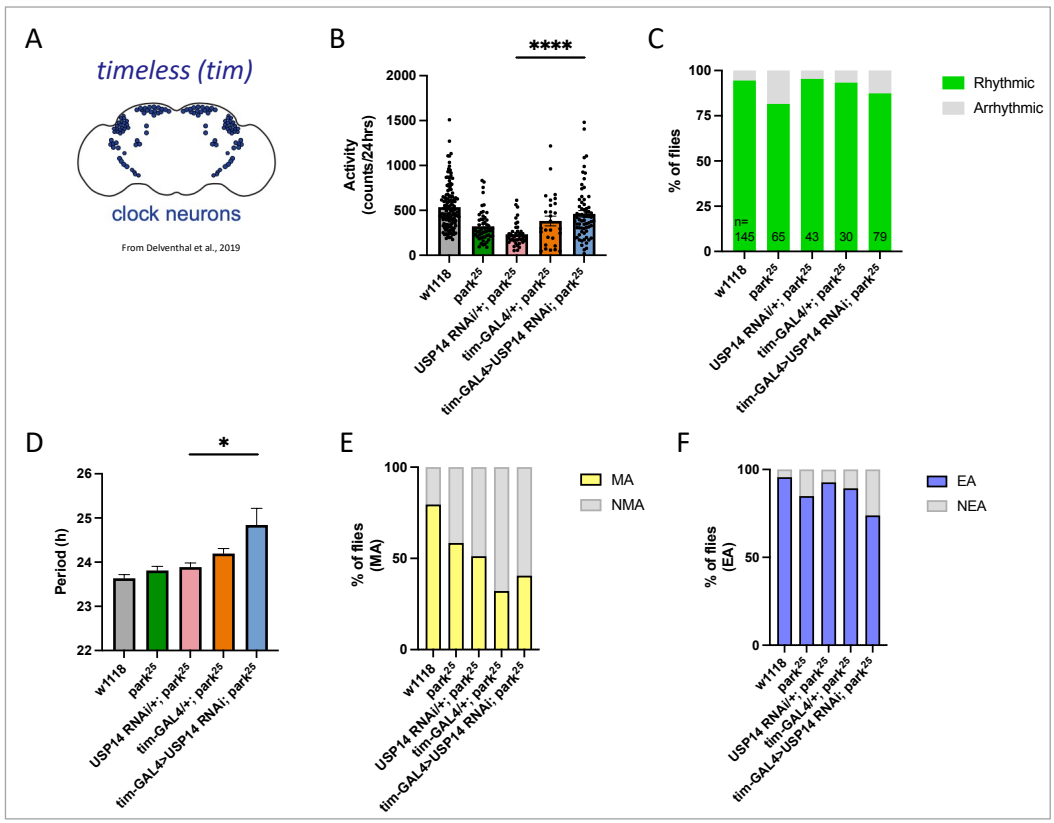

Figure 5. Tim-specific genetic inhibition of USP14 in Parkin mutant flies does not rescue the circadian defects of these flies. *(A) Diagram of clock neurons targeted for inhibition of USP14 using tim-GAL4. (B) Bar chart showing total activity during the second day of locomotor activity recording in LD. Charts show total activity for each genotype as beam crosses per 24hrs. (C) Stacked bars showing percentage of rhythmic and arhythmic flies. (D) Bar chart showing the period of rhythmic flies with clock neurons-specific inhibition of USP14. (E) Stacked bars indicating the percentage of flies that show morning anticipation (MA) or not (NMA). (F) Stacked bars indicating the percentage of flies that show evening anticipation (EA) or not (NEA).*

Figure 6.

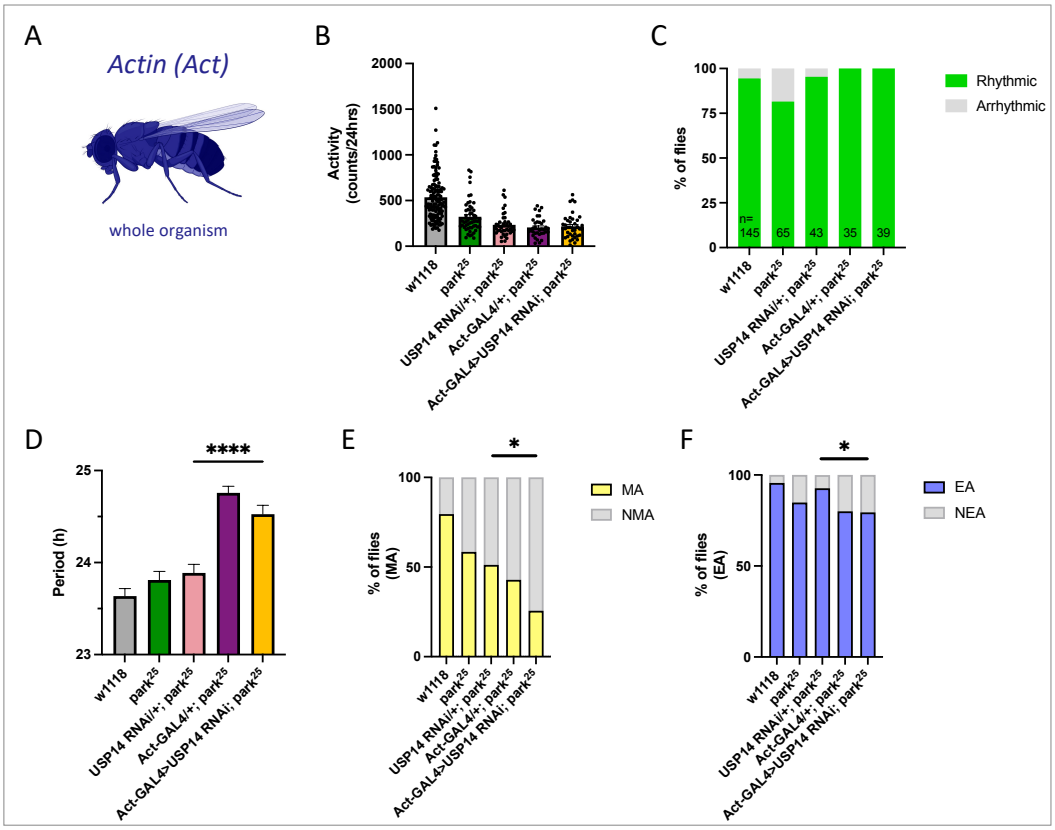

Figure 6. Act-driven genetic inhibition of USP14 in Parkin mutant flies does not rescue the circadian defects of these flies. *(A) Diagram of tissues targeted for inhibition of USP14 using Act-GAL4. (B) Bar chart showing total activity during the second day of locomotor activity recording in LD. Charts show total activity for each genotype as beam crosses per 24hrs. (C) Stacked bars showing percentage of rhythmic and arhythmic flies. (D) Bar chart showing the period of rhythmic flies with ubiquitous inhibition of USP14. (E) Stacked bars indicating the percentage of flies that show morning anticipation (MA) or not (NMA). (F) Stacked bars indicating the percentage of flies that show evening anticipation (EA) or not (NEA).*

Figure 7.

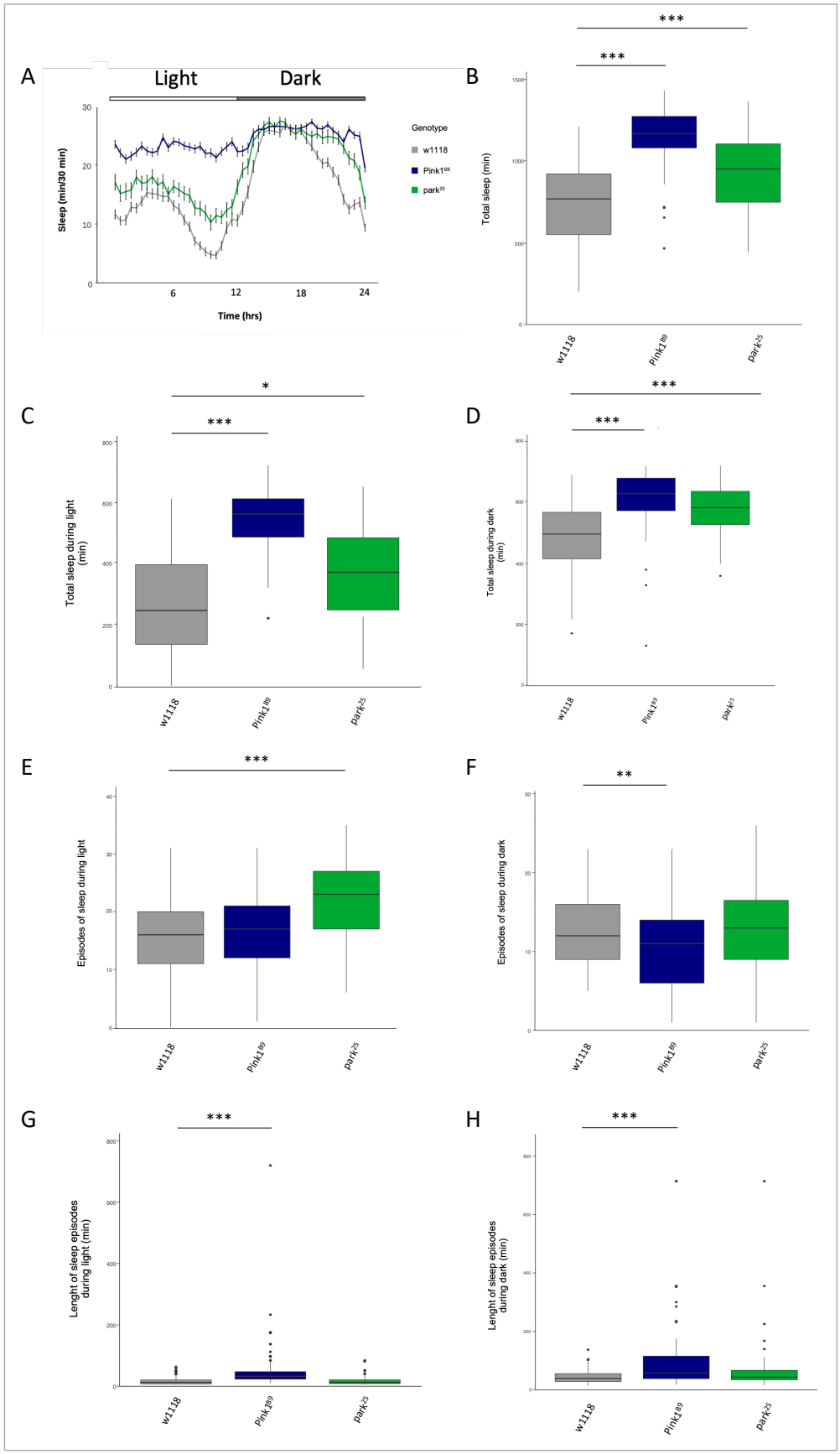
A
Light
Dark
Sleep (min/30 min)
Time (hrs)
Genotype
w1118
Pink1^B9
park^25
B
Total sleep (min)
C
Total sleep during light (min)
D
Total sleep during dark (min)
E
Episodes of sleep during light
F
Episodes of sleep during dark
G
Lenght of sleep episodes during light (min)
H
Lenght of sleep episodes during dark (min)
w1118
Pink1^B9
park^25

Figure 7. Drosophila PINK1 and Parkin mutant (KO) flies display aberrant sleep behavior. *(A) Average daily sleep profile of the indicated genotypes from the second day of LD 12:12. (B) Total sleep amount (in minutes) during the second day of LD 12:12 conditions. (C) Sleep amount (in minutes) in the day/light phase of LD 12:12 conditions. (D) Sleep amount (in minutes) in the night/dark phase of LD 12:12 conditions. (E) Number of sleep episodes in the light/day phase of LD 12:12 conditions. (F) Number of sleep episodes in the dark/night phase of LD 12:12 conditions. (G) Length of the episodes of sleep (in minutes) in the light/day phase of LD 12:12 conditions. (H) Length of the episodes of sleep (in minutes) in the dark/night phase of LD 12:12 conditions.*

Figure 8.

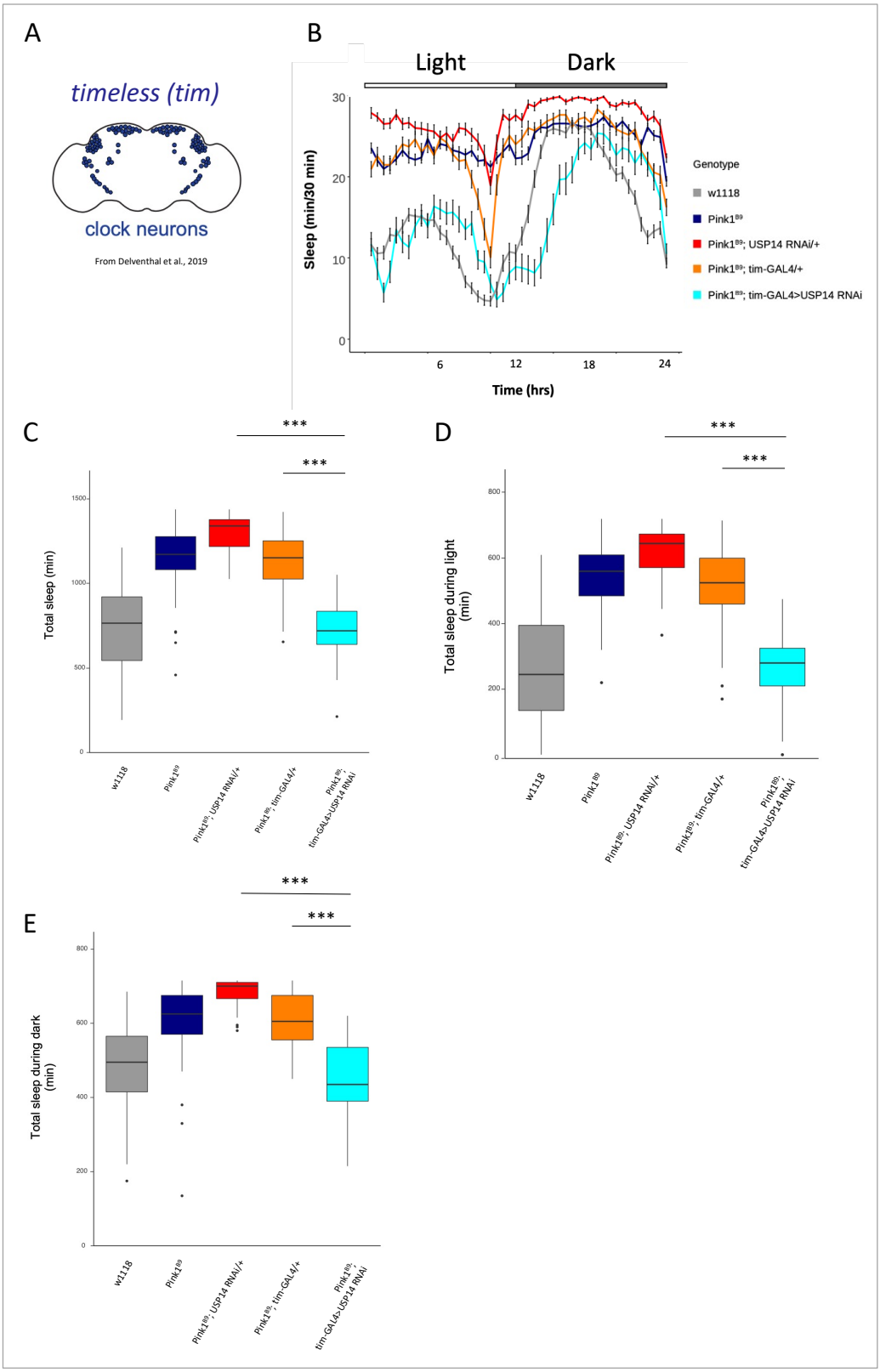
A
timeless (tim)
clock neurons
From Delventhal et al., 2019
B
Light
Dark
Sleep (min/30 min)
Time (hrs)
Genotype
w1118
Pink1B9
Pink1B9; USP14 RNAi/+
Pink1B9; tim-GAL4/+
Pink1B9; tim-GAL4>USP14 RNAi
C
Total sleep (min)
D
Total sleep during light (min)
E
Total sleep during dark (min)
***

Figure 8. Tim-specific genetic inhibition of USP14 in PINK1 mutant flies rescues the sleep defects. *(A) Diagram of clock neurons targeted for inhibition of USP14 using tim-GAL4. (B) Average daily sleep profile of the indicated genotypes from the second day of LD 12:12. (C) Total sleep amount (in minutes) during the day of LD 12:12 conditions. (D) Sleep amount (in minutes) in the light/day phase of LD 12:12 conditions. (E) Sleep amount (in minutes) in the dark/night phase of LD 12:12 conditions.*

Figure 9.

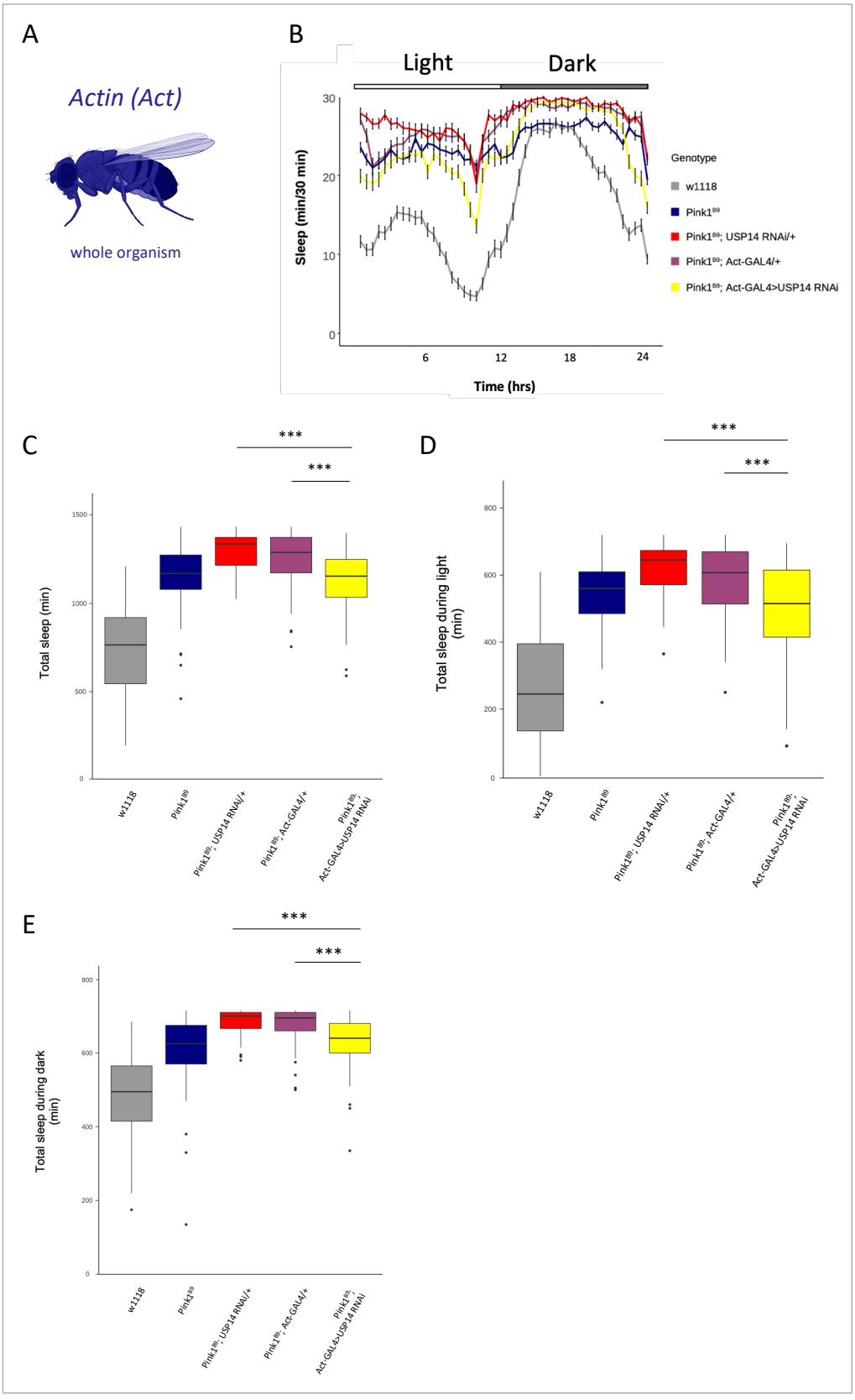
A
Actin (Act)
whole organism
B
Light
Dark
Sleep (min/30 min)
Time (hrs)
Genotype
w1118
Pink1B9
Pink1B9; USP14 RNAi/+
Pink1B9; Act-GAL4/+
Pink1B9; Act-GAL4>USP14 RNAi
C
Total sleep (min)
***
***
w1118
Pink1B9
Pink1B9; USP14 RNAi/+
Pink1B9; Act-GAL4/+
Pink1B9; Act-GAL4>USP14 RNAi
D
Total sleep during light (min)
***
***
E
Total sleep during dark (min)
***
***

Figure 9. Act-driven genetic inhibition of USP14 in PINK1 mutant flies rescues total sleep. *(A) Diagram of tissues targeted for inhibition of USP14 using Act-GAL4. (B) Average daily sleep profile of the indicated genotypes from the second day of LD 12:12. (C) Total sleep amount (in minutes) during the day of LD 12:12 conditions. (D) Sleep amount (in minutes) in the light/day phase of LD 12:12 conditions. (E) Sleep amount (in minutes) in the dark/night phase of LD 12:12 conditions.*

Figure 10.

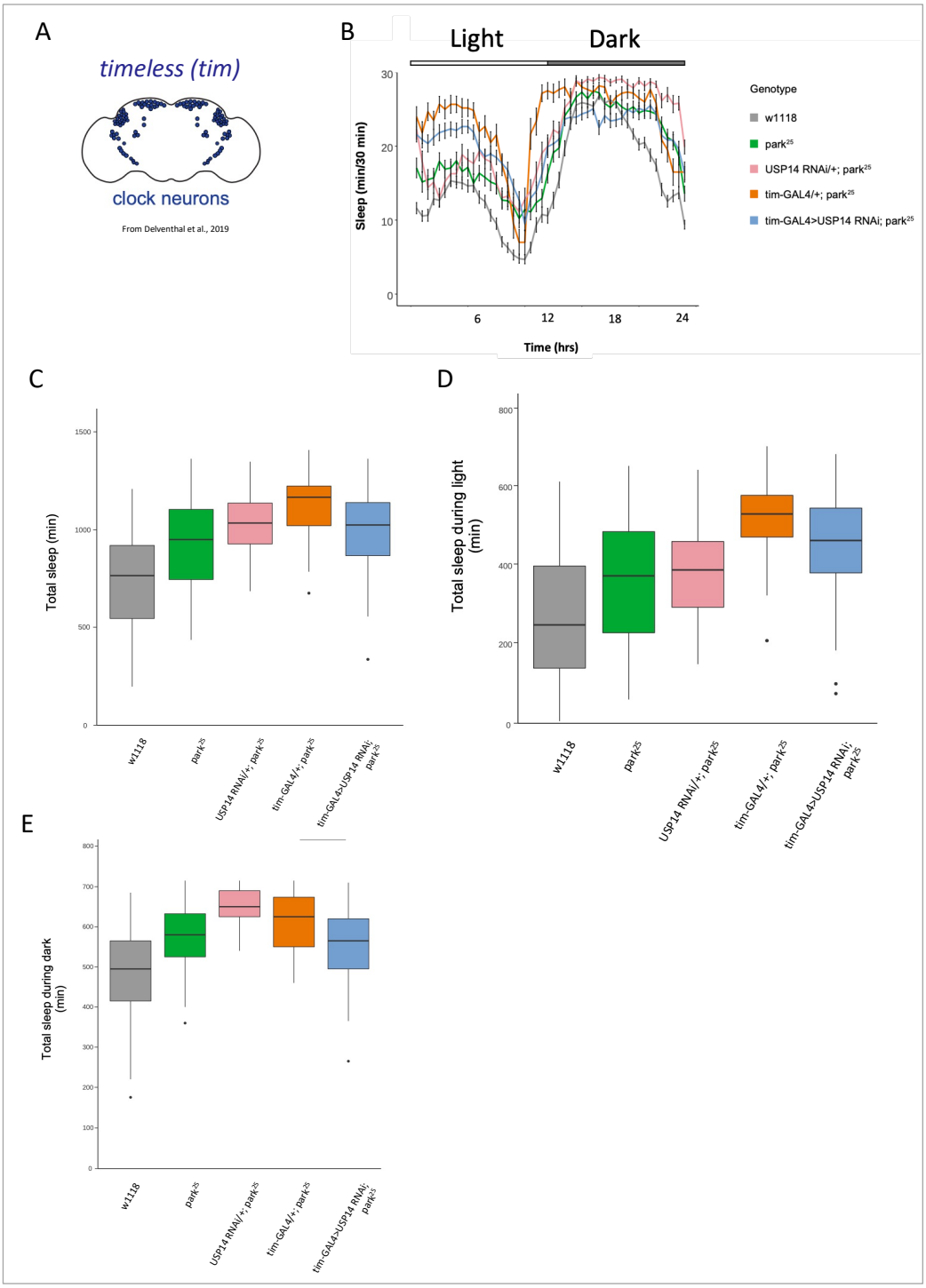
A
timeless (tim)
clock neurons
From Delventhal et al., 2019
B
Light
Dark
Sleep (min/30 min)
Time (hrs)
Genotype
w1118
park25
USP14 RNAi/+; park25
tim-GAL4/+; park25
tim-GAL4>USP14 RNAi; park25
C
Total sleep (min)
D
Total sleep during light (min)
E
Total sleep during dark (min)

Figure 10. Tim-specific genetic inhibition of USP14 in Parkin mutant flies does not rescue any of the sleep defects. *(A) Diagram of clock neurons targeted for inhibition of USP14 using tim-GAL4. (B) Average daily sleep profile of the indicated genotypes from the second day of LD 12:12. (C) Total sleep amount (in minutes) during the day of LD 12:12 conditions. (D) Sleep duration (in minutes) in the light/day phase of LD 12:12 conditions. (E) Sleep amount (in minutes) in the dark/night phase of LD 12:12 conditions.*

Figure 11.

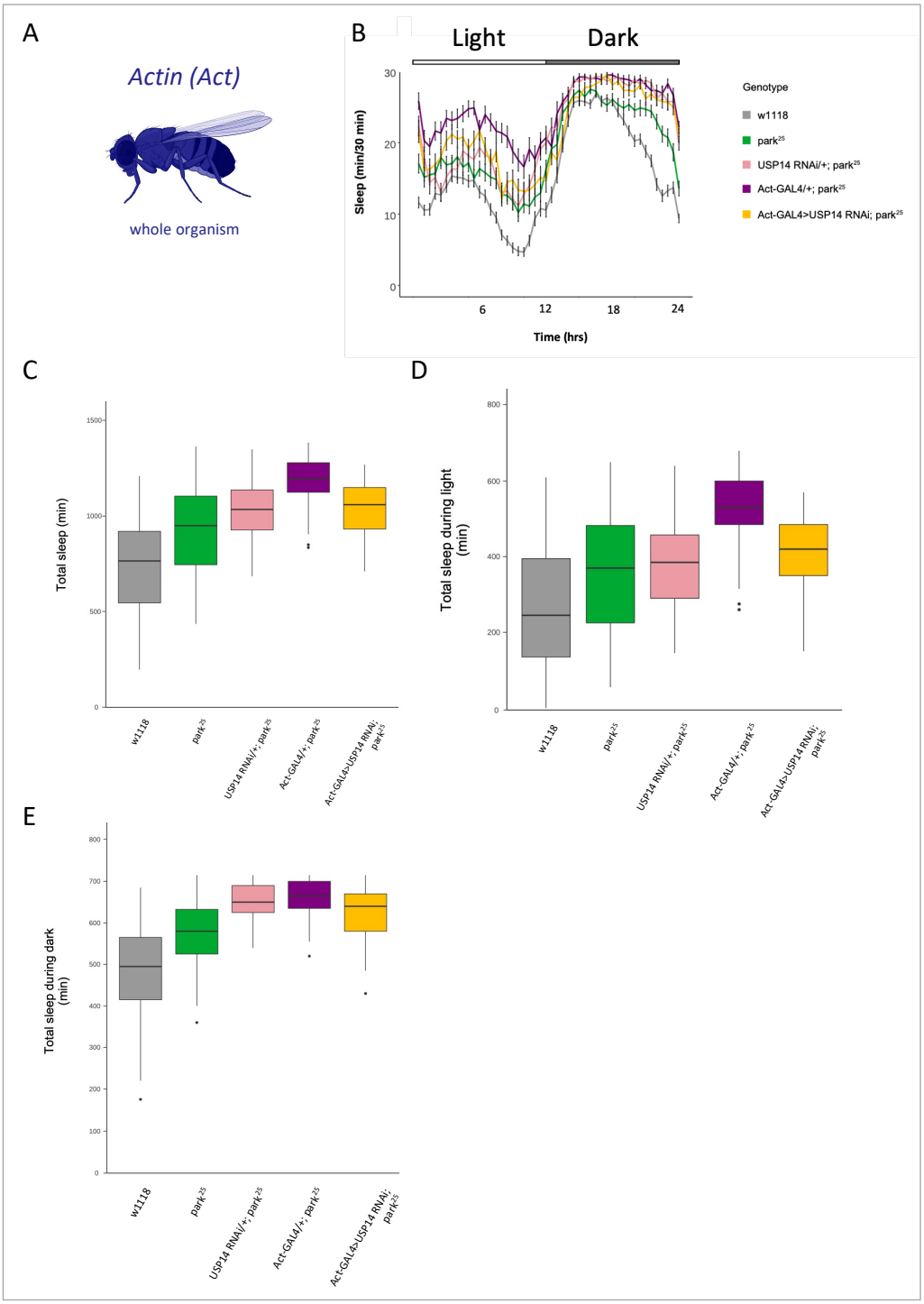
A
Actin (Act)
whole organism
B
Light
Dark
Sleep (min/30 min)
Time (hrs)
Genotype
w1118
park25
USP14 RNAi/+; park25
Act-GAL4/+; park25
Act-GAL4>USP14 RNAi; park25
C
Total sleep (min)
D
Total sleep during light (min)
E
Total sleep during dark (min)

Figure 11. Act-driven genetic inhibition of USP14 in Parkin mutant flies does not rescue any of the sleep defects. *(A) Diagram of tissues targeted for inhibition of USP14 using Act-GAL4. (B) Average daily sleep profile of the indicated genotypes from the second day of LD 12:12. (C) Total sleep amount (in minutes) during the day of LD 12:12 conditions. (D) Sleep amount (in minutes) in the light/day phase of LD 12:12 conditions. (E) Sleep amount (in minutes) in the dark/night phase of LD 12:12 conditions.*

5. DISCUSSION

Parkinson's disease (PD) is a multifactorial devastating degenerative disease of the central nervous system, which affects around 0.3% of the population (Raza et al., 2019). PD is characterized by intracellular accumulation of α-synuclein aggregates that form the Lewy bodies (LB) in the substantia nigra of the brain, and cause the selective loss of dopaminergic neurons with consequently depleted dopamine (DA) levels. Motor dysfunctions such as bradykinesia, resting tremor, muscular rigidity, and postural instability are the main symptoms that characterize PD.

Besides motor impairments, non-motor dysfunctions are commonly present in PD, and they include neuropsychiatric symptoms (depression, cognitive dysfunctions and dementia), sleep disorders, and autonomic symptoms (bladder disorders, orthostatic hypotension, erectile impotence).

Sleep disorders such as insomnia, rapid eye movement, vivid dreaming, increased nocturnal activity, more fragmented sleep, REM sleep behaviour disorder (RBD), and excessive daytime sleepiness, are found in two-thirds of PD patients. These symptoms have gained considerable relevance since they largely affect the quality of life of patients.

Sleep behaviour is controlled by circadian rhythmicity. Thus, in the case of a dysfunctional circadian clock, sleep behaviour can be affected, resulting in sleep disturbances.

Importantly, defects in sleep-wake cycle precede the onset of the cognitive decline and motor symptoms by many years. Thus, circadian rhythm disruption might not simply be a consequence of PD but a pathogenic component of the disease that can contribute to neurodegeneration. In PD patients, motor symptoms tend to appear when more than 50% of dopaminergic neurons have already been lost and itis therefore too late for neuroprotective treatments to be effective. For this reason, it would be fundamental to diagnose the disease before the appearance of motor symptoms, and find a therapy that can counteract the loss of DA neurons in the premotor phase to prevent or delay the development and progression of the disease (Kalia & Lang, 2015).

Thus, sleep disturbances may represent a pre-symptomatic window for the treatment of PD, allowing earlier intervention with therapeutic drugs (Julienne et al., 2017).

The mechanistic link between circadian rhythms and PD is poorly understood, but proposed underlying mechanisms suggest alterations in protein homeostasis (i.e., proteostasis) (Leng et al., 2019).

Proteostasis, defined as the maintenance of a functional proteome, is gradually lost during aging. In neurodegenerative diseases, like PD, loss of proteostasis seems to be exacerbated, and depends on the pathological decay in the proteolytic activity of the ubiquitin-proteasome and the lysosome-autophagy system, the two major degradative system of the cell. Indeed, promoting the activity of the ubiquitin proteasome system (UPS) or autophagy increases lifespan, and rescues the pathological phenotype of animal models of neurodegeneration, presumably by enhancing the degradation of misfolded

proteins and dysfunctional organelles, which are known to accumulate in these models of neurodegeneration. While many studies investigate the protective effect of enhancing proteostasis to eliminate intracytoplasmic neurotoxic aggregates, very little attention has yet been paid to explore the protective potential of altering proteostasis in the context of circadian dysregulation in neurodegenerative conditions.

With that in mind, we wanted to investigate whether enhancement of proteostasis, which we can induce by inhibiting the activity of deubiquitinating enzyme (DUB) USP14, ameliorates the circadian phenotype and sleep disturbances of two well-established *Drosophila* models of *PD*, the PINK1 and Parkin KO flies. USP14 is a proteasome-associated DUB that negatively regulates autophagy and the UPS. Thus, inhibition of USP14 enhances the activity of the UPS and autophagy. Importantly, genetic and pharmacological inhibition of USP14 in PINK1 and Parkin KO flies rescues the locomotor and cellular phenotype associated with these flies, presumably by enhancing proteostasis (Chakraborty et al., 2018).

Does USP14 inhibition exert a similar protective effect in the case of circadian defects?

5.1. Genetic inhibition of USP14 lengthens the circadian period of *Drosophila melanogaster*

To address this question, we set off by characterizing the effect of USP14 downregulation in different cells and tissues. We used four different drivers (*elav*, *tim*, *Pdf* and *Actin*) to express USP14 RNAi in different target cells. We found that in Tim+ and Pdf+ cells, the downregulation of USP14 significantly affected the circadian behavior in that it prolonged the circadian period of flies, and partially affected their ability to anticipate transition to lights-on. Rhythmicity and total activity did not seem to be affected by USP14 downregulation. Importantly, the effect of USP14 inhibition was readable only in Tim and Pdf expressing neurons, and not in other neuronal cells, highlighting the potential physiological importance of USP14 activity in clock-relevant neurons

5.2. *Drosophila* PINK1 and Parkin mutant (KO) flies display reduced total activity and weakened circadian rhythms

Next, we wanted to investigate the effect of USP14 inhibition in PINK1 and Parkin KO flies. Previous works identified circadian defects in PINK1 and Parkin mutants, although with some discrepancies between studies. In particular, Julienne H. and co-authors showed a decrease in total activity in PINK1 and Parkin flies, and a significant reduction in rhythmicity. Among those flies that remained rhythmic, authors did not observe a significant difference in the circadian period (Julienne et al., 2017). In contrast with these results, another study showed that Parkin KO flies displayed a significant increase in total activity and reduced nighttime sleep, and a significant lengthen of the circadian period (Doktór et al., 2019). The same study reported a difference in the circadian expression of clock genes *per*, *tim* and *clk* in Parkin mutant, which translated at the protein level in aberrant

circadian expression of protein PER. A parallel study reported a trend towards a decrease in total activity of PINK1 KO flies, while a significant decrease in total activity was shown when PINK1 was specifically downregulated in *elav* expressing cells. In this study, PINK1 mutant flies displayed increased daytime sleep, whereas night-time sleep was not affected (Doktór et al., 2018).
Thus, before assessing the effect of USP14 inhibition in PINK1 and Parkin KO background, we wanted to further characterize these mutants to strengthen what was currently available in literature.
As described in the abovementioned studies, we also observed defective circadian behaviour in $park^{25}$ and $Pink1^{B9}$ mutant flies, although with some discrepancies compared to previous studies. In particular, in LD conditions, PINK1 and Parkin flies seem to be less active during the day, with reduced ability to anticipate morning and evening clues. Moreover, the percentage of rhythmic flies was significantly reduced in both mutant genotypes. Among those flies that remained rhythmic, the circadian period was significantly lengthened in both mutants.
We also wanted to assess the sleep-wake cycle of these mutants to monitor sleep behaviour and compare our results with what was available in literature. We found that both $Pink1^{B9}$ and $park^{25}$ flies sleep more during the night and also during the day compared to wild type. Moreover, the quality of sleep appeared to be different between wild type and the mutant genotypes. In particular, PINK1 mutants displayed fewer but longer episodes of sleep during the night, and equal number but longer episodes of sleep during the day (i.e. longer "siesta"). Parkin mutants on the other hands did not show a significant difference in terms of number of sleep episodes and their duration during the night, while the number of sleep episodes during the day was significantly bigger compared to wild type.

Thus, Drosophila PINK1 and Parkin KO flies display altered sleep behaviour, with an overall increase in total sleep.

5.3. Downregulation of USP14 in PINK1 KO flies rescues the circadian defects and sleep behaviour of these flies

We next moved to address the effect of USP14 downregulation in PINK1 and Parkin mutants. We downregulated USP14 either in the whole fly, with the actinGal4 driver, and in the Tim expressing neuron, as our data indicate that USP14 inhibition had the most readable effect in this specific subset of circadian neurons. Importantly, downregulation of USP14 almost completely rescued the circadian defects of PINK1 KO flies. The rescuing effect of USP14 was particularly evident when we specifically downregulated USP14 in Tim expressing neurons, further highlighting the potential physiological importance of USP14 inhibition for clock neurons lacking PINK1 expression. Furthermore, downregulation of USP14 almost completely rescued the sleep defects of PINK1 KO flies. Once again, the effect was most readable when USP14 was specifically downregulated in Tim+ cells.

Of note, USP14 inhibition did not ameliorate the circadian phenotype and the sleep defects of Parkin KO flies, highlighting an important difference between the two genotypes. One possible interpretation for this lack of effect is that Parkin expression is required for the protective effect of USP14 inhibition. This seems plausible if we consider that Parkin is an E3-ubiquitin ligase that might be important for the “proteostastatic” effect of USP14 inhibition.

6. CONCLUSIVE REMARKS AND FUTURE PERSPECTIVES

PD is characterized by accumulation of misfolded proteins and dysfunctional mitochondria, which can be at least in part ascribed to defective mechanisms of proteostasis. Thus, approaches that can reduce the levels of toxic aggregates and/or dysfunctional organelles by enhancing proteostasis are of potential therapeutic interest. In particular, activation of autophagy and the ubiquitin proteasome system (UPS) can enhance proteostasis and promote the selective elimination of defective mitochondria and misfolded proteins that are known to accumulate in pathological conditions. In this scenario, the “proteostatic” effect of USP14 inhibition could be a valuable approach, due to its unique ability to both enhance the UPS, autophagy and mitophagy. The protective effect of USP14 inhibition has been described in several disease models, however this is the first time that the protective effect of USP14 inhibition has been explored in the context of circadian dysfunction in PINK1 and Parkin KO flies modelling PD. It remains to be clarified how USP14 inhibition promotes the normalization of the circadian phenotype of PINK1 flies. One tempting hypothesis is that clock-relevant proteins, like Timeless or Period, are direct targets of USP14, so that in inhibiting conditions, their levels are affected.
Thus, further studies are required to consolidate the results presented in this work, and to clarify the underlying mechanism of suppression.

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