RESEARCH PAPER

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Effects of *PINK1* mutation on synapses and behavior in the brain of *Drosophila melanogaster*

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Mutations in the *PINK1* gene are responsible for typical symptoms of Parkinson's disease. Using *Drosophila melanogaster* mutant *PINK1*^{B9} and after *PINK1* silencing with RNAi using transgenic lines, we observed defects in synapses and behavior. The lack or reduced expression of *PINK1* prolonged sleep during the day (nap) and decreased the total locomotor activity during 24 h, in addition to a decrease in climbing ability and a reduced lifespan. In the brain, *PINK1* mutants had a lower level of Bruchpilot (BRP), a presynaptic scaffolding protein that is crucial for neurotransmission in all type of synapses in *Drosophila*. In addition, other proteins that are involved in synaptic transmission; Rab5, Syntaxin and Wishful Thinking were also decreased in abundance in mutants, except Synaptotagmin. Transmission electron microscopy (TEM) also confirmed less and abnormal synaptic vesicles at tetrad synapses in the visual system of *PINK1* mutants. The lower level of BRP and longer day sleep observed was also detected in *white* mutants, which were examined to test the effect of the *white* background on the *PINK1*^{B9} strain. The reduced locomotor activity and longer day sleep in *PINK1* mutants and after decreasing the PINK1 level in neurons seem to be correlated with a decrease in mitochondria number during the day, when they normally peak, and with impaired synaptic transmission.

Key words: Parkinson's disease, Bruchpilot, Rab5, Syntaxin, Synaptotagmin, Wishful Thinking, *white* gene, motor activity, circadian rhythm, sleep, synaptic plasticity, mitochondria

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative disorders characterized by degeneration of dopaminergic neurons in the substantia nigra of the brain. PD, the familial form, is caused by mutations in ~150 genes while the sporadic form can be induced by exposure to toxins. One of the common mutations observed in the familiar form of PD is mutation in the *PINK1 (PARK6)* gene, which encodes PTEN-induced putative kinase 1 (PINK1). In normal cells PINK1 accumulates on the surface of damaged mitochondria and together with the E3 ubiquitin ligase PARKIN, initiates mitophagy, a process of autophagy of damaged mitochondria (Matsuda et al. 2010). PINK1 contains an N-terminal mitochondrial targeting sequence (MTS), a transmembrane sequence (TMS), and a Ser/Thr kinase domain located at the C-terminus (Eiyama and Okamoto 2015). Under normal conditions PINK1 is translocated to the mitochondrial membrane, where MTS is cleaved by the mitochondrial processing peptidase (MPP) (Greene et al. 2012), and the TMS by Presenilin-associated rhomboid-like protein (PARL) (Deas et al. 2011). PINK1 without the MTS and TMS domains is transferred to the cytoplasm, where it is degraded mainly by the ubiquitin-proteasome system (Yamano and Youle 2013). When mitochondria are damaged, they do not maintain the inner membrane potential, and in effect the MTS and TMS cannot be cleaved and PINK1 is stabilized on the outer mitochondrial membrane (OMM) (Lazarou et al. 2012). After PINK1 attachment to the translocase complex of the mitochondrial outer membrane (TOM), it phosphorylates PARKIN, which causes its activation and recruitment into the OMM (Chen and Dorn 2013). In the next step, PINK1, to-

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gether with PARKIN, targets mitofusins located on the mitochondrial surface for proteasomal degradation, leading to whole organellum degradation (Thomas et al. 2014). During PD, in the result of the *PINK1* mutation, damaged and old mitochondria, which are the source of high amounts of free radicals, cannot be degraded and thus they cause dysfunction or death of neurons. The most sensitive structures to malfunctioning mitochondria are synapses, because of the high energy requirement for synaptic transmission. Mitochondria provide ATP for the formation and transport of synaptic vesicles and for neurotransmitter exocytosis. Moreover, mitochondria are involved in uptake and release of calcium ions (Ly and Verstreken 2006), which regulate release of neurotransmitters from synaptic vesicles to the synaptic cleft. Disorders in calcium release from mitochondria and in ATP production affect motor and cognitive functions, similar to those observed in PD.

Various aspects of PD and other neurodegenerative diseases are often studied using the fruit fly, *Drosophila melanogaster*, as a model organism. Molecular and behavioral disorders in the *Drosophila* model of PD are similar to those observed in mammalian PD models (Feany and Bender 2000, Lu and Vogel 2009). In *Drosophila* PINK1 is involved in the mitochondrial fission/fusion process and *PINK1* mutants have already been described (Yang et al. 2006). Mutations in *PINK1* cause inhibition of mitochondrial fission and in result the appearance of large and swollen mitochondria (Poole et al. 2008). One of the substrates of PINK1 is mitofusin (Mfn), a protein responsible for mitochondrial fusion. PINK1 with PARKIN directs Mfn to the degradation pathway and thus it contributes to the process of mitochondrial fission (Ziviani et al. 2010). Mutation of *PINK1* results in reduced production of ATP (Park et al. 2006), indirect flight muscle degradation (Yang et al. 2006) and disorders in the locomotor activity of flies. In addition, *PINK1* mutation causes degeneration of dopaminergic neurons (Wang et al. 2006), a marker of Parkinson's disease, abnormal synaptic transmission and accumulation of synaptic vesicles (Morais et al. 2009).

One of the most important proteins involved in synaptic transmission in *Drosophila* is the presynaptic protein Bruchpilot (BRP) (Kittel et al. 2006). BRP is the human homolog of ELKS/CAST/ERC [CAST - cytoskeletal matrix associated with the active zone (CAZ)-associated structural protein, also called ERC (ELKS, Rab6-interacting protein 2, and CAST)] proteins and it is responsible for the accumulation of calcium channels in the active zone and release of neurotransmitter. BRP is expressed in all synapses as two subunits BRP190 and BRP170 (Wagh et al. 2006). It has been shown that a reduced level of BRP results in motor disorders (Wagh et al. 2006) similar to that present in flies with mutations causing PD symptoms. Besides BRP other proteins such as Rab5, Syntaxin, Synaptotagmin and Wishful Thinking (WIT) are crucial for synaptic transmission. Rab5 is a major protein that mediates membrane trafficking with the specialized early endosome domain. Rab5 takes part in synaptic vesicle maturation during synaptic transmission (Hoop et al. 1994, Stenmark 2009, Wucherpfennig et al. 2003). Wucherpfennig et al. (2003) reported that lack of Rab5 causes locomotor defects, abnormal morphology of synaptic terminals and a reduced size of synaptic vesicles. Syntaxin protein is involved in synaptic vesicle fusion in the presynaptic active zones and it mediates exocytosis (Sieber et al. 2006, Ullrich et al. 2015). In turn Synaptotagmin is an essential protein for the release of neurotransmitter into the synaptic cleft because it binds Ca^{2+} that triggers vesicle fusion (Geppert et al. 1994, Shields et al. 2017). WIT regulates synaptic growth, the number of active zones in presynaptic elements and maintains the amplitude of excitatory junction potentials (Aberle et al. 2002).

In our present study, we found a correlation between the level of BRP protein and motor disorders caused by *PINK1* mutation. We showed that *PINK1* mutants have less BRP and all other proteins studied, except Synaptotagmin. In addition, the *PINK1* mutation affects sleep, increasing sleep during the day (nap), which leads to a decrease of total activity during 24 h.

METHODS

Animals

The following strains were used for the experiments: Canton S, w^{1118} (null mutation of the gene *white* encoding the ABC transporter) (Krstic et al. 2013), $PINK1^{B9}$ (point deletion of the gene encoding PINK1 kinase) (Park et al. 2006), *elav*-GAL4 (expressing the yeast transcription factor GAL4 under control of the *elav* promoter) (DiAntonio et al. 2001), *21D*-GAL4 (expressing the yeast transcription factor GAL4 in L2 neurons of the lamina, the first optic neuropil) (Weber et al. 2009), UAS-*Valium10* (expressing *GFP* and *Valium* under UAS control) (Ni et al. 2009), UAS-*PINK1*RNAi (expressing interfering RNA for *PINK1*) (Yang et al. 2006) and UAS-*mitoGFP* (expressing GFP with a mitochondrial import signal) (Pilling et al. 2006).

Since the strain $PINK1^{B9}$ used in our experiments has the *white* background, which may affect results, we used *white* mutants as a control in addition to wild type flies Canton S, and a strain with *PINK1* RNAi expressed in neurons (*elav*-GAL4>UAS-*PINK1*RNAi) to decrease the level of PINK1 in neurons. The *White* gene encodes

the ABC transporter that is one of the most important membrane transporters (Ewart et al. 1994) and is involved in many physiological processes. As a control for the RNAi strain (*elav*-GAL4>UAS-*PINK1*RNAi) we used *elav*-GAL4>UAS-*Valium10.* The level of gene expression silencing in the *elav*-GAL4>UAS-*PINK1*RNAi strain was equal to 74%.

Transgenic strains were obtained from the Bloomington Drosophila Stock Center. Flies were maintained on a standard yeast-cornmeal-agar medium at 25 ± 1°C, under a day/night cycle (12 h of light and 12 h of darkness; LD 12:12). To downregulate *PINK1* expression in neurons, *elav*-GAL4 females were crossed to UAS-*PINK1RNAi* males and *elav*-GAL4 females were also crossed to UAS-*Valium10* males to express the VALIUM vector in neurons as the control in the RNAi experiments (Ni et al. 2009). To visualize mitochondria in the L2 cells of the first optic neuropil (lamina) of the optic lobe *21D*-GAL4 females were crossed to UAS-*mitoGFP* males.

Locomotor activity and sleep analysis

Males, 1–2 days old (N=32), were transferred to small glass tubes containing the sugar-agar food medium. Vials were located in DAMS monitors (Drosophila Activity Monitoring System, TriKinetics) and placed in an incubator (25°C). Monitors were equipped with infrared sensors, which recorded the activity of the flies inside the vials every 5 min. For the first 5 days, monitors were held in LD 12:12 (12 h of light and 12 h of darkness) conditions and then for 6 days in constant darkness (DD). Results from the second day of recording were analyzed to estimate the total activity and duration of sleep during the day and during the night [(Microsoft Excel plugin – BeFly kindly donated by E. Green from Genetics, University of Leicester) (Rosato and Kyriacou 2006) and Python 22 (http://www.python.org/)]. Sleep in flies is defined as time in which they do not change their position for at least 5 min. The experiment was repeated three times. In LD 12:12 and DD the rhythm of locomotor activity was also examined, and the period of the circadian locomotor activity rhythm was measured in DD.

Immunohistochemistry

Males, 7 and 35 days old, were decapitated at four times points: 1 h after lights-on (ZT1), 4 h after lights-on (ZT4), 1 h after lights-off (ZT13) and 4 h after lights-off (ZT16). Heads were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; pH 7.4) for 3 h at 4°C. Next, they were washed in PBS two times for 10 min and then cryoprotected by incubation in 12.5% sucrose for 10 min and 25% sucrose overnight at 4°C. Heads were then embedded in Tissue-Tek (Thermo Scientific, frozen medium), frozen in liquid nitrogen and 20 nm cryostat sections were cut. Sections were washed in PBS for 30 min, then washed two times in phosphate buffer with added 0.2% Triton X 100 (PBT) for 10 min, once in 2% PBT for 5 min and three times in 0.5% PBT for 5 min. Next, they were incubated in 5% Normal Goat Serum (NGS) in 0.5% Bovine Serum Albumin (BSA) for 30 min at room temperature. Subsequently sections were incubated with primary antibodies mouse nc82 against Bruchpilot protein, diluted 1:20 (Developmental Studies Hybridoma Bank) in 2% NGS in 0.5% PBT for 3 days at 4°C, or with rabbit anti-GFP antibodies (Novus Biologicals) diluted 1:1000 in 2% NGS in 0.5% PBT for 1 day at 4°C. Afterwards sections were washed six times in 0.2% BSA in 0.2% PBT for 5 min, blocked in 5% NGS in 0.2% BSA for 30 min and incubated overnight at 4°C with secondary antibodies [Cy3 conjugated goat anti-mouse antibodies (Jackson Immuno Research) diluted 1:500 or Alexa488 conjugated goat anti-rabbit antibodies (MolecularProbes) diluted 1:1000, respectively]. After the incubation, sections were washed twice in 0.2% BSA in 0.2% PBT for 10 min, six times in 0.2% PBT for 5 min and twice in PBS for 10 min. Finally, they were mounted in Vectashield medium (Vector) and examined with a Zeiss Meta 510 Laser Scanning Microscope or Zeiss Axio Imager M2 fluorescence microscope.

Quantification of Immunolabeling

To measure the fluorescence intensity of BRP in the first optic neuropil (lamina) of the *Drosophila* optic lobe, we used confocal images of the lamina cross sections. We used the lamina because in our earlier studies we found that in this optic neuropil, tetrad synapses formed between the eye photoreceptor terminals and lamina cells, oscillate during the day and night (Pyza and Meinertzhagen 1993, Woznicka et al. 2015) and this rhythm is correlated with the circadian changes of BRP in tetrad synapses (Górska-Andrzejak et al. 2013). For the present study, we randomly selected 5–10 distal cartridges (the second and third row of cartridges from the lamina cortex) where BRP can be measured in tetrad synapses and measured the fluorescence intensity with ImageJ software (NIH, Bethesda). In the distal lamina tetrad, synapses outnumber other synapse types in the lamina (Meinertzhagn and O'Neil 1999). The fluorescence intensity of images was converted to gray values and the mean gray value (the sum of the gray values of all pixels in the area divided by the number of pixels within the selection) was calculated. Results from one

head were averaged and a background signal was subtracted. For each strain 15–25 measurements were collected, and the experiment was repeated three times.

The fluorescence intensity of the GFP-labeled mitochondria was measured from images of longitudinal sections of the lamina neuropil. The intensity of 2 random areas of the lamina neuropil was analyzed by ImageJ software as described above and results from one head were averaged. The experiment was repeated three times.

Western Blot

Males, 7 days old (N=30), were frozen in liquid nitrogen 1 h after lights-on and decapitated. Heads were homogenized by sonication in 30 µl of Laemmli buffer with protease inhibitor (Boehringer, Mannheim). Homogenates of heads were incubated for 30 min at 4°C and frozen at -20°C until centrifugation. The homogenates were centrifuged at 13,200 rpm for 1 h at 4°C. Supernatants were collected and denatured at 85°C for 5 min. Total protein level was measured by Quant-iT Protein Assay Kit and Qubit fluorometer (Invitrogen). Afterwards, 20 µg of protein from each supernatant was subjected to electrophoresis (NuPAGE 4–12% bis-Tris gels, Invitrogen) at 165 V for 40 min and then blotted by electrotransfer onto a PVDF membrane (Invitrogen) at 30 V for 60 min. The membrane was blocked in 5% non-fat dry milk in PBS with 0.1% Tween 20 (TBS) for 1 h at 4°C and incubated with primary antibodies; anti-BRP (nc82, dilution 1:1000) and anti-α tubulin (dilution 1:20000), anti-WIT (23C7, dilution 1:1000), anti-Synaptotagmin (3H2 2D7, dilution 1:2), anti-Syntaxin (8C3, dilution 1:1000) from the Developmental Studies Hybridoma Bank, and anti-Rab5 (diluted 1:1000, Abcam) in 1% BSA in 0.1% TBS overnight at 4°C. Next, the membrane was washed 5 times in 0.1% TBS for 10 min and incubated with the secondary antibody conjugated with HRP (dilution 1:10000, Abcam) in 1% BSA in 0.1% TBS for 1 h at room temperature. After this the incubation membrane was washed 5 times in 0.1% TBS and immunodetected with the ECL detection system (Perkin Elmer). Densitometric analysis of Western Blots was performed by ImageJ. The experiment was repeated three times.

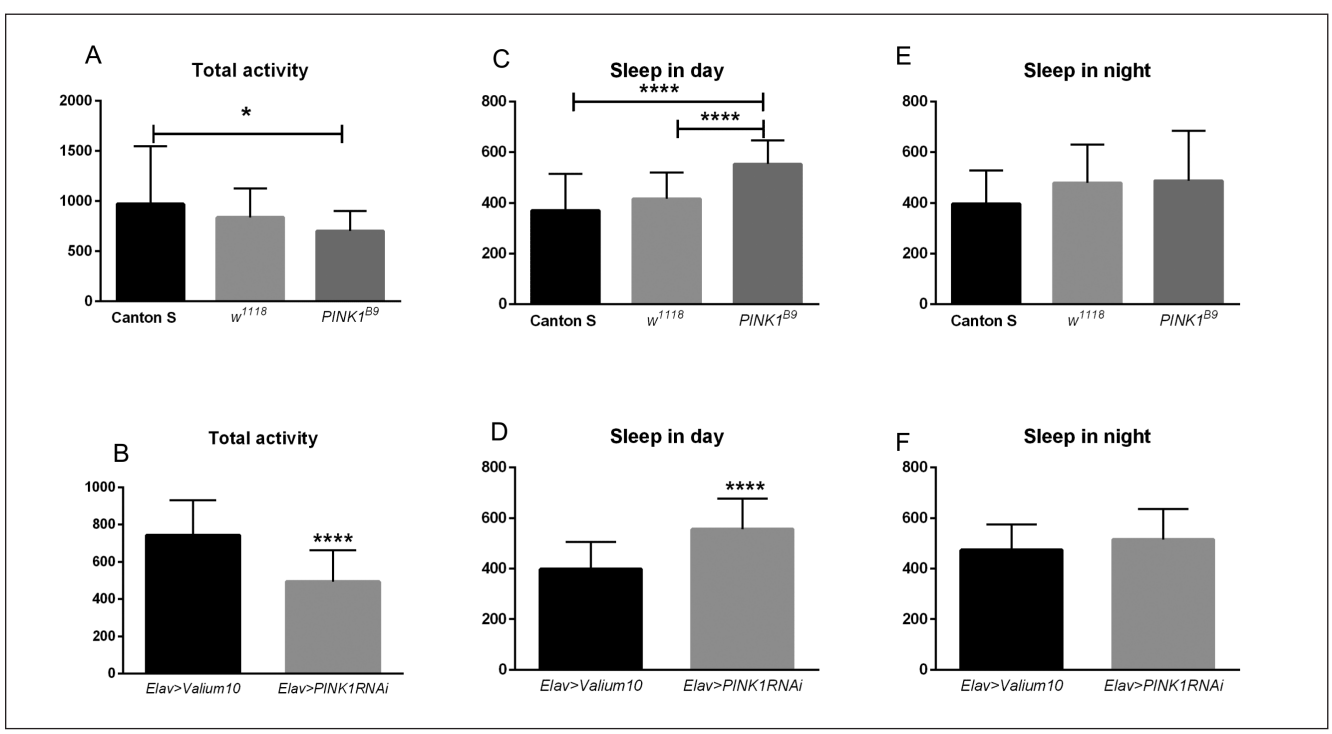

Fig. 1. *PINK1* mutation and *PINK1 RNAi* in neurons cause locomotor activity impairment. (A) and (B) Total activity from the second day of locomotor activity recording in LD 12: 12 conditions (12 h of light and 12 h of darkness). Charts show time of total activity in minutes for each genotype. (A): the total activity time was the lowest in *PINK1* mutants in comparing with *white* mutants and wild type Canton S. (B): flies with silenced *PINK1* in neurons had also reduced activity when compared with the control (four stars represent $p<0.01$, one star represents $p<0.05$) (B). (C) and (D) Sleep duration in the day/light phase of LD 12: 12 conditions. *PINK1*^{B9} and *w*^{1118} flies had prolonged day sleep (nap) (one star represents $p<0.05$) in comparing with wild type strain (C). Flies with silenced *PINK1* in neurons also exhibited longer sleep during the day than control flies (four stars represent $p<0.01$) (D). (E) and (F) Sleep duration in the night/dark phase of the second day of LD 12: 12 conditions. *PINK1*^{B9}, *w*^{1118} and Canton S had the same sleep duration during the night (E). The duration of sleep at night did not change also in *elav*-GAL4>UAS-*PINK1*RNAi flies (F).

Transmission Electron Microscopy (TEM)

Heads of 1-week old males were dissected one hour after lights-on and fixed in cacodyl-buffered PFA (2.5%) and glutaraldehyde (2%) primary fixative for 2 h. They were post-fixed in OsO_4 (2%) in veronal acetate buffer for 1 h. Subsequently, the heads were dehydrated in a series of alcohols and propylene oxide and embedded in Poly/Bed 812 resin (Polysciences). Ultrathin sections (65 nm thick) of the lamina were cut and contrasted with uranyl acetate and lead citrate. Images of tetrad synapses in the lamina were taken using a Jeol JEM 2100 HT TEM. The experiment was repeated 3 times. 10 images were taken per 1 repetition.

Statistics

The statistical analyses were performed using GraphPad Prism 6. Data were examined for distribution normality, and statistical tests were chosen accordingly. For lifespan results the Kaplan-Meier test was used. The Wilcoxon–Mann–Whitney and Kruskal–Wallis tests were performed to assess differences in the fluorescence intensity correlated with BRP protein levels from confocal images, GFP fluorescence intensity of mitochondria, sleep, total activity, period of the circadian rhythm of locomotor activity and for climbing assays. For Western Blot data the one-way ANOVA and Tukey tests were used.

RESULTS

The effect of PINK1 on locomotor activity

Recordings of flies' locomotor activity showed that the activity level during 24 h of *PINK1*^{B9} was lower when compared with w^{1118} and Canton S (Fig. 1A). The activity of *PINK1* RNAi flies was also lower than the control *Valium10* (Fig. 1B). Sleep in both *PINK1*^{B9} and w^{1118} flies was increased but only during the day (Fig. 1C). Similar results were also obtained in flies with *PINK1 RNAi*, which exhibited longer sleep during the day (Fig. 1D), whereas sleep during the night was unchanged (Fig. 1E, F).

The effect of *PINK* mutation on synapses

BRP level, measured as the fluorescence intensity after immunolabeling in the lamina at ZT1 (Fig. 3A-C)

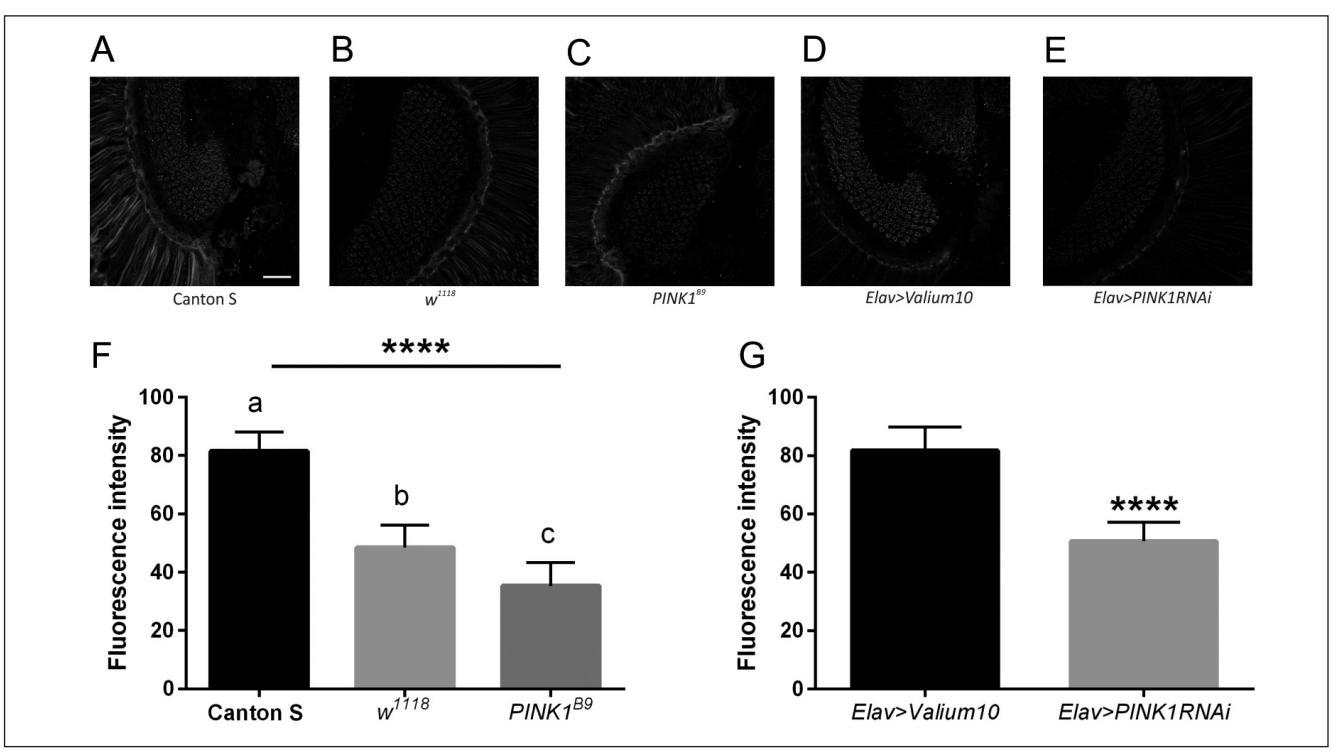

Fig. 2. *PINK1* and *white* mutations are responsible for the reduced Bruchpilot (BRP) level in tetrad synapses in the lamina. (A-E) Immunolabeling of BRP in tetrad synapses of the examined strains with nc82 antibodies. Reaction was carried out in the lamina sections of flies collected at ZT1 (one hour after lights-on). Scale bar – 20 μm. (F) and (G) The fluorescence index of BRP. Charts show the fluorescent intensity correlated with BRP level. Statistically significant differences (four stars and a,b,c represent $p<0.05$) are between all genotypes in both (F) and (G) charts. *PINK1*^{B9} and *elav*-GAL4>UAS- *PINK1*RNAi had lower level of BRP in tetrad synapses in comparing with other strain studied.

was the lowest in *PINK1*^{B9} flies (Fig. 2B), however, it was also lower in the *white* mutation in comparison with Canton S Flies and with silenced *PINK1* in neurons there was also a reduced BRP level in the lamina (Fig. 2G). In contrast, the daily rhythm of the BRP level in tetrad synapses, with two peaks at ZT1 and ZT13, was not changed in both mutants; *PINK1*^{B9} and w^{1118}, in comparison with Canton S flies.

The reduced level of BRP was also detected in the whole brains of the studied flies. Western Blot analysis showed a lower level of both BRP isoforms BRP170 and BRP190 in *PINK1*^{B9} and *elav*-GAL4>UAS-*PINK1RNAi* (Fig. 3A-C) when compared with the controls Canton S and *elav*-GAL4>UAS-*Valium10*, respectively. BRP level in w^{1118} was also lower than in Canton S but this reduction was not statistically significant. The daily rhythm in changes of the BRP level was not affected by aging. Moreover, the BRP level at ZT1 was similar in young (7 days old) and older (35 days old) flies of the Canton S and other strains studied: w^{1118}, *PINK1*^{B9} and *elav*-GAL4>UAS-*PINK1RNAi.*

PINK1 mutants also exhibited reduced levels of other proteins involved in synaptic transmission. In these mutants, the levels of Syntaxin and Rab5 were lower when compared with the controls Canton S and w^{1118} (Fig. 4A-B). The abundance of Wishful Thinking (WIT) was also lower compared with the control w^{1118} (Fig. 4C), in contrast to Synaptotagmin, where the level was similar in all genotypes studied (Fig. 4D). Moreover, morphology of the synaptic vesicles studied in tetrad synapses in the visual system in *PINK1* mutants was changed when compared with *white* and Canton S controls (Fig. 5). The synaptic vesicles of *PINK1* mutants had broken membranes and most of them were darker (higher electronic density) compared with the control strains and their number was reduced (Fig. 5).

Daily oscillations of mitochondria number in the first optic neuropil

Since sleep in the mutant studied was affected only during the day we also measured daily changes in mitochondria number in neurons. We selected L2 interneurons of the lamina, one of the four postsynaptic cells in tetrad synapses and analyzed the fluorescence intensity of GFP-labeled mitochondria in L2 (Fig. 6A) at different time points. The obtained results showed a significantly higher signal during the day (ZT1 and ZT4) than during the night (ZT13 and ZT16) (Fig. 6B). This means that the number of mitochondria increases during the day, when insects are more active in locomotor activity, rather than during the night. Their number is not correlated with the two peaks, at ZT1 and ZT13, in the

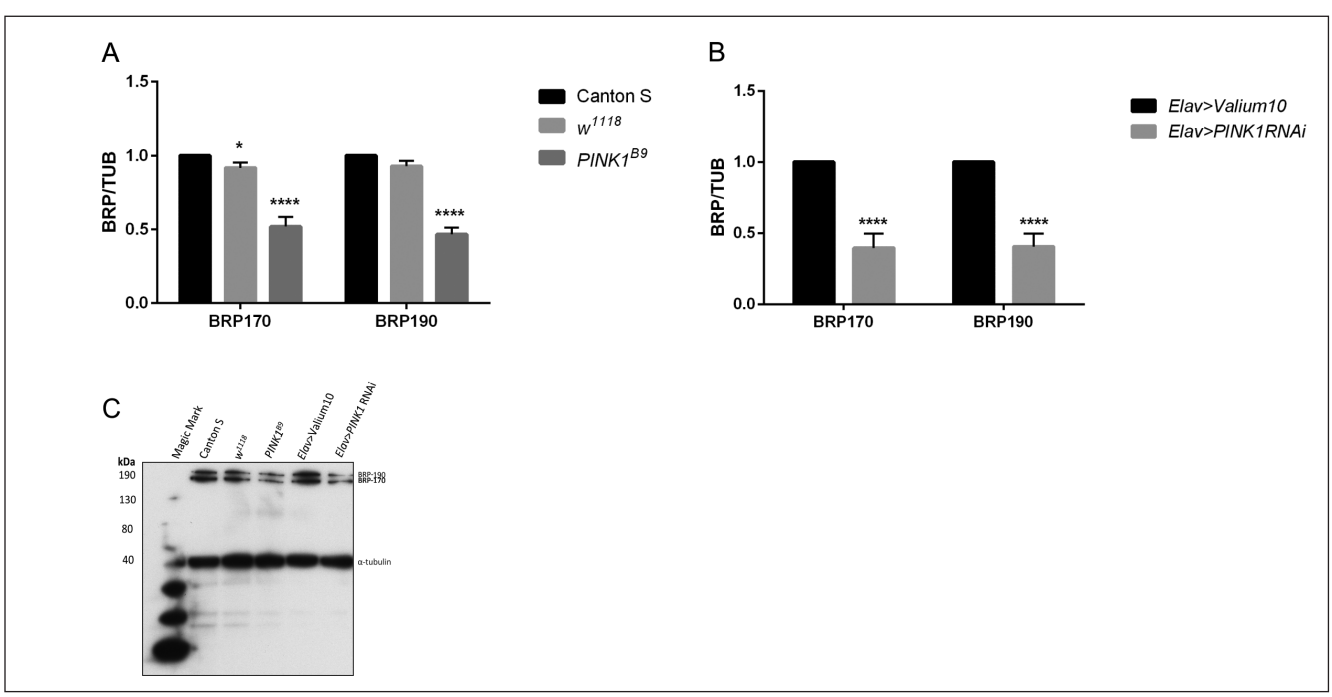

Fig. 3. *PINK1* and *white* mutations are responsible for the reduced Bruchpilot (BRP) level in the brain. (A) and (B) Densitometric analysis of BRP190 and BRP170 isoforms in Canton S, *PINK1*^{B9}, w^{1118} (A), *elav*-GAL4>UAS-*Valium* and *elav*-GAL4>UAS-*PINK1*RNAi (B). BRP level was standardized to Canton S and *elav*-GAL4>UAS-*Valium* in (A) and (B), respectively. Statistically significant differences (four stars represent p<0.01, and one star represents p=0.05) are between *PINK1*^{B9} and Canton S and between *elav*-GAL4>UAS-*PINK1*RNAi and *Valium* controls. (C) Result of Western Blot of BRP in whole brain homogenates of all genotype studied.

number of tetrad synapses with postsynaptic elements that are also in L2 interneurons.

DISCUSSION

The malfunction of Mitochondrial Complex I, oxidative stress and aggregation of abnormal/misfolded proteins are typical molecular symptoms of Parkinson's disease (PD) (Dawson and Dawson 2003). They lead to a decrease in mitochondria number, and the lack of energy may be responsible for subsequent neurodegeneration (Li et al. 2017), thus this may be correlated with the observed motor and non-motor disorders in PD. It was previously shown that in *PINK1* mutants the level of ATP is low, indicating dysfunction of mitochondria (Liu et al. 2011). In the present study, we found that *PINK1* mutation causes not only motor disorders and reduced activity but also affect synapses and synaptic transmission in the brain and neuromuscular junctions. Motor disorders have already been reported in PD animal models (Feany and Bender 2000). Moreover, in *PINK1* mutants of *Drosophila* morphological abnormalities of indirect flight muscles (Park et al. 2006) and apoptosis of muscle cells have been observed (Clark et al. 2006).

As reported by other authors both mutations also affect lifespan and the climbing ability of flies, which were both decreased (data not shown). In the present study we showed that in addition both *PINK1* and *white* mutants have abnormal sleep and their total activity is decreased. The duration of sleep was lengthened during the day but not during the night in comparison with wild type Canton S flies, and in addition, total activity was decreased in *PINK1* mutants. Longer sleep during the day shortens time for living functions and behavior in *Drosophila*, which are concentrated during the day and at the beginning of the night. The effect of *PINK1* mutation on sleep and daily activity was also confirmed using a *Drosophila* strain in which *PINK1* gene expression was reduced in neurons. These flies also showed longer

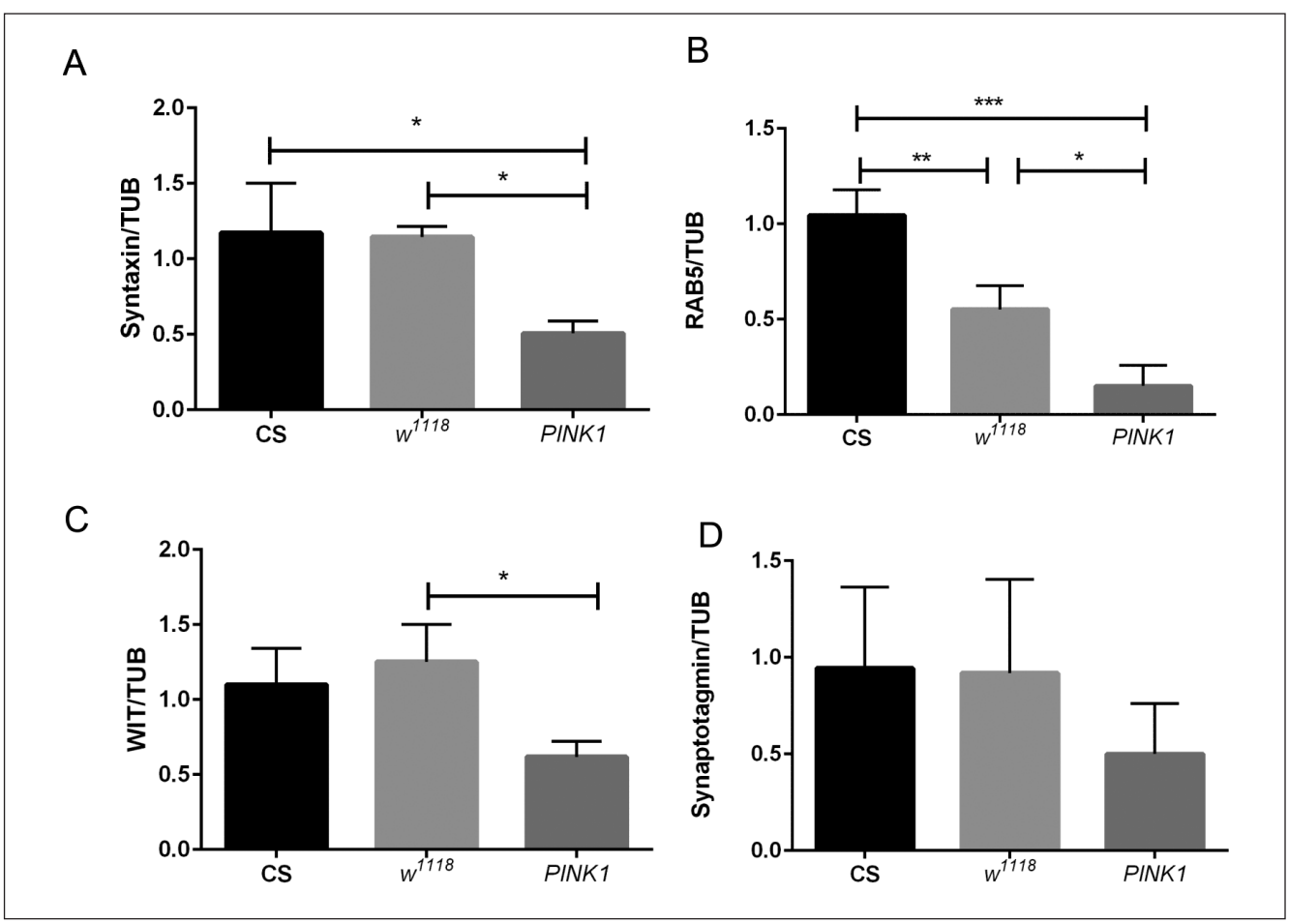

Fig. 4. PINK1 and white mutations reduce levels of other proteins involved in synaptic transmission in the Drosophila brain. (A-D) Densitometric analysis of Syntaxin (A), Rab5 (B), Wishful Thinking (C) and Synaptotagmin (D) in Canton S, w^{1118} and $PINK1^{9}$ whole heads. Protein levels were standardized to Tubulin. Statistically significant differences are represented by three stars (p=0.01), two star (p<0.05) and one star (p=0.05).

day sleep and a reduced total activity time in 24 h when compared with controls. This result suggests a role for PINK1 in maintaining sleep and activity phases. Interestingly, behavioral changes were observed only during the day when the number of mitochondria is normally higher than during the night. Since *PINK1* mutation disrupts the functions of mitochondria and their fission and fusion processes, we hypothesize that changes in the sleep/activity pattern of flies during the day might be correlated with the impairment of mitochondria in the brain during the day.

PINK1 mutation directly affects synapses by decreasing the level of the presynaptic protein BRP and other proteins (Rab5, Syntaxin and WIT) involved in synaptic

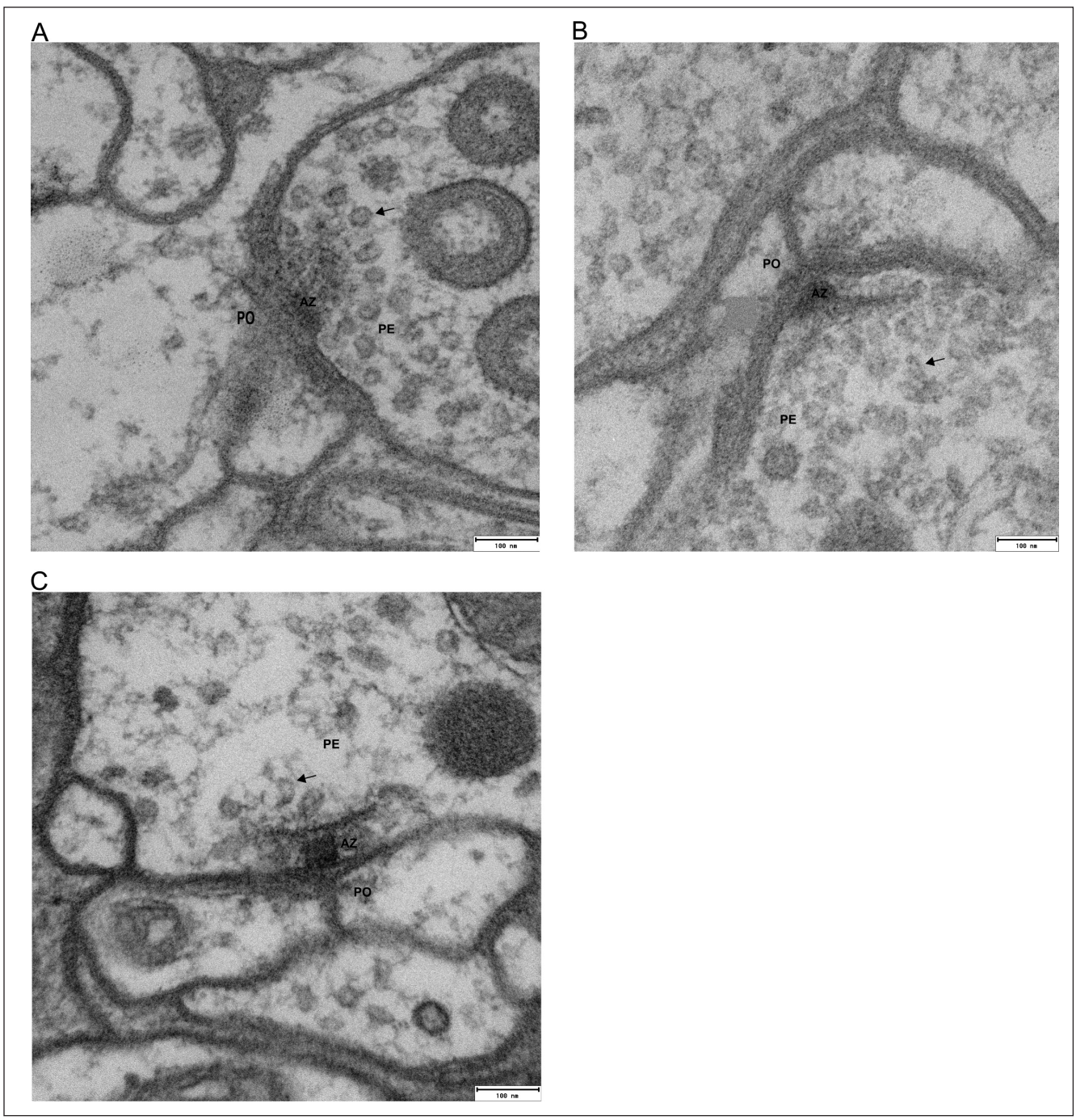

Fig. 5 *PINK1* mutation affects morphology of synaptic vesicles. (A-B) TEM micrographs of tetrad synapses of Canton S (A), w^{1118} (B) and $PINK1^{9}$ (C). Arrow – synaptic vesicles, PE – presynaptic element, PO – postsynaptic element, AZ – active zone.

transmission in the brain of *Drosophila melanogaster*. In addition to the brain, we examined BRP and synaptic vesicles in tetrad synapses in the visual system. In these types of synapses in the distal lamina BRP can predominantly be measured in tetrad synapses and it is known that the number of tetrad synapses and the BRP level in these synapses oscillate during the day and night with the same pattern (Górska-Andrzejak et al. 2013, Meinertzhagen and Pyza 1999, Woźnicka et al. 2015). The number of tetrad synapses peaks twice, in the morning and in the evening and is also higher after light exposure (Pyza and Meinertzhagen 1993). Both peaks during the day are correlated with a high level of BRP, swelling of postsynaptic interneurons of tetrad synapses (Pyza and Meinertzhagen 1999) and peaks of locomotor activity rhythm in *Drosophila* (Górska-Andrzejak et al. 2013). We found that the *PINK1* mutation reduces BRP level in tetrad synapses, however, the circadian rhythm in the BRP level is maintained. The fact that daily expression of BRP in the lamina of *PINK1* mutants was not affected is surprising since an involvement of mitochondria in neuroplasticity and synaptic plasticity in the brain is well documented. However, this observation and behavioral data suggests that circadian plasticity in the visual system is less dependent on the activity of mitochondria than in other parts of the brain.

Since a similar result was found in the case of BRP in whole brain homogenates, the impairment of synapses occurs in all synapses in the brain and probably in neuromuscular junctions where BRP is also a presynaptic protein (Wagh et al. 2006).

Because of low transmission levels in mutants, postsynaptic cells and muscles can degenerate. *PINK1* mutants and flies with *PINK1 RNAi* had a lower level of BRP in the brain when compared with Canton S, w^{1118} and *Valium10* controls. This indicates that *PINK1* mutation affects synaptic contacts in the nervous system because of increasing the production of reactive oxygen species (ROS) (Chien et al. 2013). It has already been shown that the reduced level of BRP in *brp* mutants causes similar motor disorders to that observed in our study (Wagh et al. 2006). However, *PINK1* mutation also causes degeneration of dopaminergic neurons (Yang et al. 2006), and perhaps may also affect other types of neurons that regulate motor activity.

Furthermore, we showed that genetic background, *white* mutation, may also cause a reduction of BRP but this effect was seen only in tetrad synapses in the photoreceptor terminals and not in whole head homogenates. This result is correlated with an important function of WHITE in the eye. In *Drosophila white* mutants atypical pigment granules were found in the eye (Schraermeyer and Dohms 1993) and degeneration of the retina (Ambegaokar and Jackson 2010). The ABC transporter encoded by the *white* gene transports, in addition to pigment in photoreceptors, ions, amino acids, peptides and sugars across membranes (Savary et al. 1996) and *white* mutants show various abnormalities including a low level of neurotransmitters (Borycz et al. 2008). The level of the other proteins studied, except Rab5, was not significantly changed in *white* mutants.

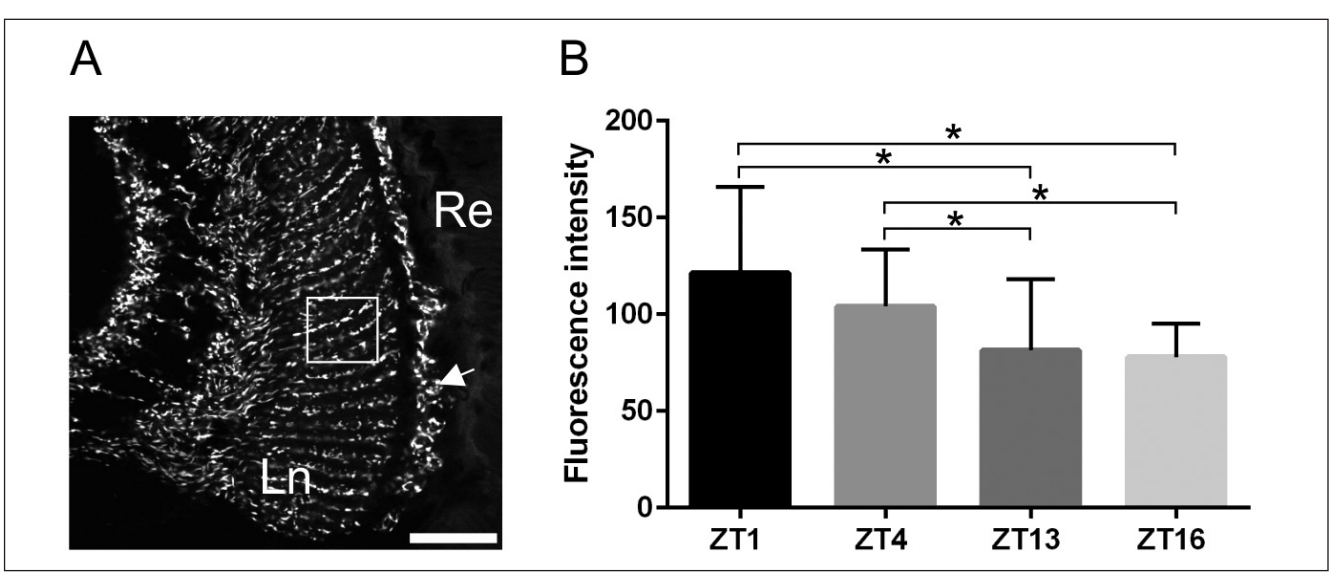

Fig. 6. The number of mitochondria in the lamina L2 interneurons oscillates during the day. (A) Localization of mitochondria labeled with GFP in L2 interneurons of the lamina of *21D*-GAL4>UAS-*mitoGFP*. Re – retina, Ln – lamina neuropil, Arrow – cell bodies of L2 neurons, Frame – measurement area, Scale bar – 20μm. (B) The fluorescence index of GFP-labeled mitochondria in the lamina neuropil measured in different time points of the day of *21D*-GAL4>UAS-*mtoGFP*. Statistically significant differences (one star represents p=0.05) are between ZT1 and ZT13, ZT16; ZT4 and ZT13, ZT16.

CONCLUSIONS

In the present study we proved that PINK1 is required for maintaining sleep during the day and synaptic transmission by the regulation of synaptic protein levels. The lack of PINK1 results in the reduction of synaptic proteins responsible for exocytosis of neurotransmitters, which in turn may cause previously described motor and sleep disorders. We also showed that using *white* background, to create transgenic strains, leads to motor and non-motor disorders in those flies. We suggest using w^{1118} as an additional control for strains with *white* background, because the comparison between Canton S and w^{1118} may give information on the effects of *white* mutation itself on the examined processes.

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