Characterisation of circadian rhythms in adolescent BLOC-1 deficient and wild-type mice

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ABSTRACT:

A gap in our knowledge on circadian rhythms is how they build up during early postnatal life and importantly during adolescence, a critical and vulnerable window for brain development, highly sensitive to environmental stressors, including circadian disruptions. Hence, the aim of my project was to characterize activity rhythms in adolescent wild-type (C57b/6j) mice as well as in a strain lacking the Biogenesis of lysosome-related organelles complex-1 (BLOC-1). In particular, we wanted to answer some basic questions such as how these cycles develop, change and strengthen during this life-period, how different they are from those observed in adult mice, and whether sex plays a role in the build-up of the rhythms. The activity rhythms were recorded using both running wheels and infrared (IR) sensors. In general, the adolescent mice displayed highly variable sleep/wake cycles, with apparent sex-divergent effects but no genotypical differences. The wheel recordings did provide more robust recordings, while IR recordings seemed more sensitive and better at capturing activity during the inactive phase, along with behavioral disturbances, such as a phase delay that is commonly observed in human adolescents. With both recording modes providing valuable outputs, we will be able to construct a longitudinal (age-related) actogram spanning from the beginning of adolescence (postnatal (P) day 25) to full adulthood (P90). These findings provide novel information on the shaping of sleep/wake cycles during a vulnerable window of brain development.

INTRODUCTION:

Circadian misalignment has been linked to maladies such as diabetes and cancer, as well as psychiatric disorders (depression and schizophrenia) (Baron and Reid, 2014), along with negative impact on cognitive functions. Adolescence provides a time when the developing brain shows extreme plasticity, rendering it highly sensitive to environmental influence, including circadian disruptions, which can affect its circuitries and proper wiring (Konrad et al., 2013). Little is known on sleep/wake cycles during this critical window for brain development, and or how they are shaped, a quite important issue in light of the presence of several circadian disruptors and mal-habits in the present world, including the use of electronic devices late at night, the presence of continuous light, even dim, throughout the night, the early start of schools that does not keep in account the peak of sleep in adolescents and busy schedules (scholastic, sportive and extra-curricular activities) (Touitou et al., 2017). Hence, the aim of this project was to characterize the activity rhythms in adolescent wild-type mice and in mice lacking the Biogenesis of lysosome-related organelles complex-1 (BLOC-1).

The function(s) of BLOC-1 in brain and during its development has only begun to be dissected out. This complex, contains 8 subunits forming a linear complex of about 300 angstrom (Lee et al., 2012), has been mostly studied in the context of the biogenesis of lysosome-related organelles such as melanosomes and platelet dense granules (Falcón- Pérez et al., 2002; Moriyama & Bonifacino, 2002). In humans, its absence has been associated with Hermansky-Pudlak syndrome (HPS), a rare genetic disorder characterized by defective biogenesis of melanosomes, platelet dense granules and other lysosome-related organelles (reviewed by Bowman, Bi-Karchin, Le, & Marks, 2019). Few patients carrying mutations in three BLOC-1 subunits have been identified, including a 6-year-old male who displays HPS as

well as motor and language development delays (Bryan et al., 2017). Furthermore, albeit not without controversy, genetic variations in BLOC-1 subunits have also been linked to deficits in cognitive abilities and increased susceptibility for neurodevelopmental psychiatric disorders in humans (Kircher et al., 2009; Luciano et al., 2009; Narr et al., 2009; Varela-Gomez et al., 2015; reviewed by Hartwig et al., 2018).

Several mouse models carrying mutations in one of the BLOC-1 subunits are available and present with different as well as common phenotype (Swank et al., 1998). Sandy mice, for instance, carry a mutation in the gene encoding dysbindin (*Dntbp1*; Li et al., 2003), and are present not only with reduced mRNA and protein levels in the prefrontal cortex and the hippocampus (Talbot, 2009; Ghiani & Dell'Angelica, 2011; Hartwig et al., 2018), but also for all the other subunits, hence, the entire complex is downregulated (Talbot, 2009; Ghiani et al, 2010). Early studies have associated allelic variants in Dntbp1 with an increased risk for schizophrenia (Kendler, 2004; Straub et al., 2002). Such association did not survive (Farrell et al., 2015), but prompted a series of studies to elucidate the role of BLOC-1 in brain functions. It is now evident that mice lacking stable BLOC-1, because of a mutation in *Dntbp1* or *Bloc1s6*, the gene encoding for the pallidin subunit are present with cognitive and behavioral deficits (reviewed by Ghiani and Dell'Angelica, 2011, Bhardwaj et al., 2009; Cox et al., 2009; Ryder and Faundez, 2009; Talbot, 2009; Papaleo et al., 2012; Carr et al., 2013; Glen et al., 2014; Larimore et al., 2014, 2017; Bhardwaj et al., 2015a; Spiegel et al. 2015; Yuan et al., 2016; Petit et al., 2017; Chang et al., 2018; Lee et al., 2018). Similar results were obtained in Drosophila melanogaster lacking functional BLOC-1 (Cheli et al., 2010; Shao et al., 2011; Dickman et al., 2012; Mullin et al., 2015; Chen et al., 2017).

Altered sleep/wake cycles have been reported in both sandy, although only under constant light (Bhardwaj et al., 2015b), and pallid mice (Lee et al, 2018). Male pallid mice exhibited less sleep at the beginning of the resting phase (insomnia?) than wild-type mice, along with a highly fragmented sleeping pattern in normal light-dark conditions. The strength of the activity rhythms in the mutants was significantly reduced with more fragmentation and lower precision than in age-matched wild-types. These effects were accompanied by subtle morphological changes in the central circadian clock (the suprachiasmatic nucleus, SCN), including a larger SCN and increased expression of the relative levels of the clock gene Per2 product during the day, and unaltered neuronal activity rhythms. Altered PER2 protein levels were also observed in the hippocampus along with reduced pCREB/tCREB ratio during the day (Lee et al., 2018). In addition, the pallid mice present with sex-divergent and brain region specific maldevelopment, with the males and the hippocampus being the most sensitive to the lack of BLOC-1 (Lee et al., 2020).

Altogether the findings described above suggest that lack of BLOC-1 in mice has a negative impact on sleep/wake cycles, cognitive functions and elicits poor performance in behavioral tasks. Mice lacking BLOC-1 display cognitive and behavioral impairments reminiscent of those presented by individuals with intellectual and developmental disabilities. The circadian clock drives rhythms in the signaling pathway(s) in the brain areas involved in cognitive functions, such as the hippocampus (Stephan and Kovacevic, 1978; Wang et al., 2009; Phan et al., 2011; Fernandez et al., 2014; Shimizu et al., 2016). The present study aimed at investigating and characterizing the buildup and shaping of the activity rhythms during a vulnerable window of brain development, adolescence, when wiring and consolidation of brain circuits occurs, in particular considering that the modern lifestyle has plenty of circadian

disruptors and stressors targeting the juvenile population, which can negatively impact cognitive functions and could lead to lifelong disabilities.

MATERIALS AND METHODS:

2.1 Animals

All experimental protocols used in this study were approved by the University of California, Los Angeles (UCLA) Animal Research Committee (ARC 2009-022). UCLA Division of Laboratory animal recommendations for animal use and welfare, as well as National Institutes of Health guidelines, were followed. BLOC-1-deficient male pallid (B6.Cg-Bloc1s6pa/J) and "wild-type" (WT) control strain (C57BL/6J) were from our breeding colony maintained at UCLA. The pallid strain carries a non-sense mutation in the Blos1s6 gene (also known as Pldn) encoding pallidin (Huang et al., 1999), which is an essential component of BLOC-1 (Falcón-Pérez et al., 2002; Moriyama and Bonifacino, 2002), while containing no mutations in the Dtnbp1 gene encoding dysbindin. A total of 31wild-type and 32 BLOC-1-deficient pallid mice, both males and females, were used in this study. More specifically, there were 15 Male wild-type adolescent, 12 Female wild-type adolescent, 2 Male wild-type adult, 2 Female wild-type adult, 10 Male Pallid adolescent, 9 Female Pallid adolescent, 2 Male Pallid adult, and 6 Female Pallid adult (Table 1). Mice were weaned and separated by sex and genotype in housed groups of two to four. They remained in group housing until individually housed during activity recording in a 12:12 h LD light-controlled room with

controlled temperature and humidity. They had access to standard chow diet and water ad libitum for the entirety of the experiment.

2.2 Cage Activity

Methods used were similar to those described previously (Li et al., 2015; Loh et al., 2015). Mice were singly housed in cages with up to 8 mice enclosed in custom light-tight cabinets. This setup allowed for continuous sleep behavior recording over an extended period of time. Mice were housed in cages with wheels (11.5 cm diameter, Mini Mitter, Bend, OR, United States) or/and Infrared sensors to record their locomotor activity. Under the LD 12:12 conditions, the time of lights-on defines ZT0, whereas the time of lights-off is defined as ZT 12. In this experiment, the ZT 0 was 06:00 Pacific Standard Time (PST), while ZT 12 was 18:00 PST. The 12:12 h LD cycle mice were needed as a control in order to get necessary data for the adolescent mice who do not have extensive previous activity recordings. The adolescent mice were placed into the custom light-tight cabins and activity recording started at age P25. The adult mice were placed in at P60. The adolescent mice were removed from the coffin and Wheel and/or IR recording stopped at P90 before being moved into immobility sleep-defined behavior recording (Methods 2.3), while the adult mice were stopped and moved at P105.

Wheel revolutions were recorded in 3 min bins, and 14 days of data under each condition were averaged and analysed to determine the period and rhythmic strength (Li et al., 2015; Loh et al., 2015). The locomotor activity rhythms were analysed using the periodogram analysis combined with the $\chi 2$ test with $P = 0.05$ significance level (Actimetrics, Wilmette, IL) on the raw data. Activity amount over 24 h was determined by averaging 14 days of wheel revolutions (rev/h). Nocturnality was defined as the % of total activity within a 24-h cycle that occurred in the night. The number of activity bouts and the average length of bouts were determined using

Clocklab (Actimetrics, Wilmette, IL), in which each bout was counted when activity bouts were separated by a gap of 21 min (maximum gap: 21 min; threshold: 3 counts/min). The onset variability was determined using Clocklab by drawing the best-fit line over the 14 days and averaging the differences between activity onset and best-fit regression of each day. Precision was determined by calculating the daily variation in onset from a best-fit regression line drawn through 14 days of activity in LD using the ClockLab programme (Actimetrics, Wilmette, IL, United States).

Activity analysis from the 3 min bin Wheel recording was also represented as a waveform using Excel. The 3 minute bins for each individual mouse were smoothed over in each cell by averaging the previous 29 3-minute bins with next 20 3-minute bins after. 27 days of data were analyzed for each mouse starting at the age P27 representing day 0 and ending on P53. The results from all these mice were then averaged and separated based on genotype and sex.

2.3 Recording Immobility Sleep-Defined Behavior

Behavior was measured with video recording in combination with an automated mouse tracking analysis software system as previously described (Li et al., 2015; Loh et al., 2015). WT and pallid mice were singly housed in transparent cages under a 12:12 h light-dark (LD) cycle. For the LD cycle ZT0 corresponds to 06:00 PST, and ZT12 corresponds to 18:00 PST. Adolescent mice were placed into sleep recording at P54, while the adult mice were placed into sleep recording at P105. Mice were transferred into sleep at the onset of their active phase following their final phase advance in order to ensure minimal disruption. Mice were housed in see-through plastic cages containing bedding, but without the addition of nesting material. Video capture of a side-on view of each cage was obtained, and was not obstructed by the top mounted food bin or water bottle. Cages were under constant infrared LED lighting. Video was captured

using infrared surveillance cameras (700TVL SONY Effio-E with 2.8–12 mm zoom; Gadspot Inc., City of Industry, CA, United States) equipped with IR850 infrared philtre (Neewer Technology Ltd., Guangdong, China). ANY-maze software (Stoelting Co., Wood Dale, IL) was used to track the animals as described by Fisher and colleagues (Pack et al., 2007; Fisher et al., 2012), who found 99% correlation between immobility-defined and EEG-defined sleep using an immobility detection threshold set to 95% of the area of the animal immobile for 40 s. Continuous recording was performed over 5 days and data collected from days 2 and 3 were used for further analysis. There were randomized visits (1/day) by the experimenter to confirm mouse health and video recording. Collected sleep data were exported in 1 min bins, and the total amount of sleep was determined by summing the duration of sleep in the day (Zeitgeber Time, ZT0-12) or night (ZT12-24). The number of sleep bouts in the day or night was counted by using the ClockLab programme (Actimetrics, Wilmette, IL, United States). The sleep onset was automatically detected by the ClockLab software analysis function. Briefly, ClockLab first bins the activity or sleep record into activity/sleep bouts. Once activity/sleep bout was counted when activity was separated by a gap of 21 min or more. The time of the first activity/sleep bout after at least a 6-h period of inactivity is considered the onset. An average waveform of hourly sleep from both days was produced per genotype, light scheme, and age group.

RESULTS:

Wheel running is used routinely to analyze rhythms in activity in rodents. They are a reliable source to capture and characterize sleep/wake cycles and associated abnormalities. First, we have analyzed the activity of adolescent wild-type (males n=17; females n=9) as well as adolescent BLOC-1 deficient (males n=11; females n=11; Table 1) mice by using both wheel running and infrared (IR) camera recordings in the same cage setup. While, we were able to successfully determine that adolescent mice, although small in size, were able to move the wheels and, thus, could record their circadian rhythms of activity, the IR cameras were unable to capture the mice movements while they are running on the wheels (see example in Figure 1). IR recordings were, for instance, able to pick up a phase delay, a phase shift in the circadian rhythm to later in the day, in adolescent mice that are common in humans. This analysis showed that the rhythms in activity appear to be affected by both sex and genotype, therefore, for the subsequent activity recordings, the mice were housed in cages equipped with wheels or IR cameras, so that one recording mode does not confound the other, as both provide valuable outputs.

To begin we analyzed the WT rhythms, in order to build-up a baseline of the sleep/wake cycles during adolescence. The adolescent wild-type males, albeit with some variability, possess robust activity rhythms even at this young age (Figure 2A). Similarly, to our previous findings in adult mice (Lee at al., 2018), adolescent BLOC-1 deficient male mice display a high mouse to mouse variability in their rhythmic output (Figure 2B&C). Figure 2B shows a well entrained mouse with minimal activity during the inactive phase, while the representative actogram in Figure 2C shows a mutant with weak activity rhythms. The wild-type females (Figure 2D) showed to be well entrained, along with some of the mutants (panel E). However, we observed again some variability with some mutant females displaying high level of activity outside the

active phase (Figure 2F).

Next, we computed the wheel activity by sex and genotype across the age range, from the beginning of the juvenile period (P25) to adulthood (P60) and constructed the waveforms shown in figure 3. From this analysis, it can be appreciated that for both sexes and genotypes the animals' wheel running activity strengthens over time to become stable and acquire a strong precision. The adolescent wild-type males appeared to take longer (P27-P53) to build up robust rhythms. They displayed the lower amplitude throughout the age-period, and it took about 13-14 days to reach the maximum amplitude, after that these became very stable, with strong precision (Figure 3A). The BLOC-1 deficient males (Figure 3B) took about 8 days to increase the amplitude of the rhythms to wild-type levels, and the amplitude was bigger than in wild-type, and with some variability. On the other hand, females (Figure 3C&D) seemed to achieve robust and stable rhythms earlier than the males (about 4.5-5 days.) They also exhibited rhythms with higher amplitude than the males throughout the entire period. Interestingly, the females (both genotypes) displayed a small burst of activity near the nadir of the inactive phase, which was more pronounced in the mutant females, especially towards the end of this developmental window (figure 3D). Conversely, the wildtype males showed these burst only early in adolescence until about P35, and the BLOC-1 deficient males only between P25 and P30.

These findings showed that both WT and the mutants seem to exhibit a greater variability in the robustness of their rhythms compared to adult mice, in particular the rhythms of activity show to have a divergent shaping and buildup in males and females from both genotypes.
We have previously reported that adult (P90) males, but not females, BLOC-1 deficient mice show disrupted sleep/wake cycles (Lee et al., 2018; and Sharma et al., unpublished results). The present findings appear to be in line with our previous work showing sex-divergent effects in

BLOC-1 deficient mice, with the males being more susceptible.

DISCUSSION:

Originally the IR recordings were completed in conjunction with Wheel recordings to see if one mode of recording provided an advantage to the other. The actogram recordings from the wheels are dramatically cleaner than the IR recordings. The IR recording may not be able to properly distinguish mouse movement when they are spatially locked on the running wheel during their active phase. On the other hand, the IR recording provides the advantage of being able to clearly record mouse offsets and disturbances when they may be active during their inactive phase, but do not run on the wheel. The difference in Wheel and IR can be seen in Figure 1 (IR recording) and Figure 2 (Wheel recording). The Wheel recordigns show a clear rhythm with great power, while the IR recordings show lower power but a much more variable rhythmic output. In the IR recordings the adolescent mice show phase delays which are common to humans (Figure 1D), arguing for the necessity of them in recording the whole picture of activity. We have shown that Wheel recordings provide a more robust rhythm, while IR recordings are able to capture a wider range of offsets in the sleep phase. After taking into account these differences and importance of both wheel and IR recording we will be planning on separating the mice in IR and Wheel groups and recording more longitudinally from P25 to P90. More specifically the mice will be separated into four LD adolescent groups: PA Wheel, PA IR, WT Wheel, WT IR. The reason for separating the recordings is due to the fear that the IR is picking up weak or no recordings during the active phase when the mouse is fixed into position on the running wheel.

When looking at the wheel recording data the adolescent mice have a highly variable rhythmic output and are more vulnerable to a phase changing environment. To counter the view that schizophrenia is a synaptic transmission disorder, another view suggests that during development genetic variants and environmental factors disrupt normal neural connectivity such as through neuronal migration or dendritic arborization (Ghiani et al., 2010). In support of this developmental hypothesis BLOC-1 is shown to interact with a subset of SNARE (soluble Nethylmaleimide-sensitive factor attachment protein receptor) proteins previously implicated in neurite outgrowth and may help explain the seen neurite outgrowth effects. Changes in the DG may lead to learning and memory defects as it is the critical hub for memory formation and subsequent storage in the CA3 region (Lee et al., 2018). Future studies need to be conducted in the future correlating how phase disturbances during development may be linked to cerebral changes that affect learning and memory in later years of life.

Our primary objective was to gain a deeper understanding of adolescent rhythms in LD which presents a knowledge gap in the literature. The preliminary finding of the adolescent mice phase delay that resembles the same phase delay in humans is exciting as it points towards adolescent mice providing a complementary circadian model to human development, and deserves further investigation. However, the robust variability between mice indicates more mice data needed before clear correlations can be made. We also plan to analyze the onset, offset, power, recovery variability, and week-to-week variability in all of the mouse groups to better find differences between sex/age/genotype. All mice groups will be contained in the same type cage with the same bedding regardless of recording. By separating these we should have more true recordings for each recording mode as well as a better representation of the full developmental cycle into adulthood.

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Male Pa Adolescent - Wheel	4
Male WT Adolescent - Wheel	17
Male Pa Adult - Wheel	0
Male WT Adult - Wheel	0
Female Pa Adolescent - Wheel	4
Female WT Adolescent - Wheel	9
Female Pa Adult - Wheel	0
Female WT Adult - Wheel	0
Male Pa Adolescent - IR only	9
Male WT Adolescent - IR only	0
Male Pa Adult - IR only	0
Male WT Adult - IR only	0
Female Pa Adolescent - IR only	7
Female WT Adolescent - IR only	0
Female Pa Adult - IR only	0
Female WT Adult - IR only	0

Table 1: Summary of the 63 mice that have completed recording.

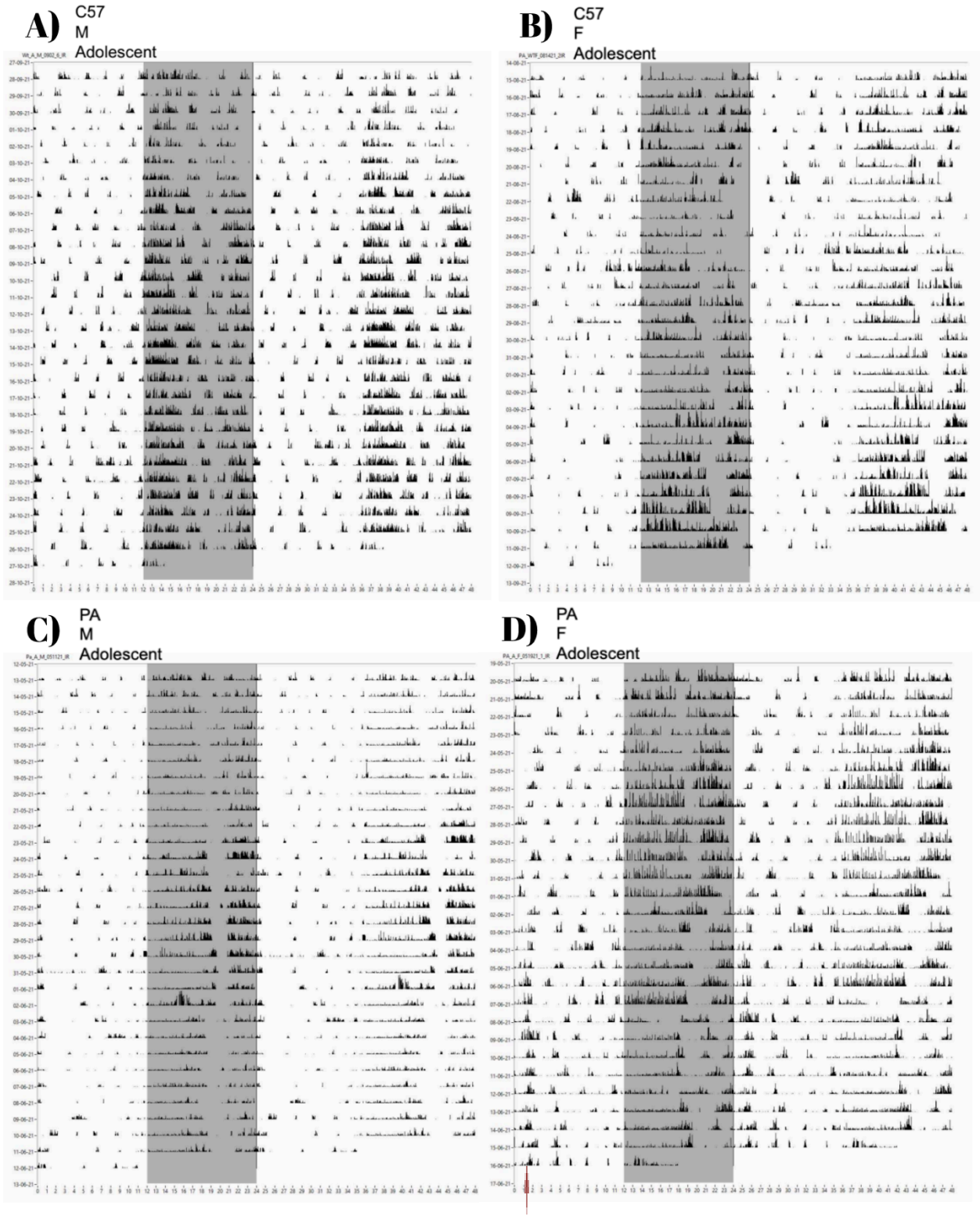

Figure 1: Examples of infrared (IR) sensor recording actograms from 4 adolescent mice kept on an 12-12 LD light-scheme. The gray shading represents lights off, i.e. the active phase of the

mice. Figure1A&B depict wild-type mice, while Figure 1C&D are BLOC-1 deficient mice. The IR recordings shown provide a weaker/less robust signal, however this mode of recordings allows more ‘awakenings’ to be picked up during the sleep inactive phase. The red arrow in Figure 1D displays how IR recordings can pick up a phase delay in the mice that are less likely to be registered in wheel recording when the mouse may wake up during its inactive phase but not run on the wheel.

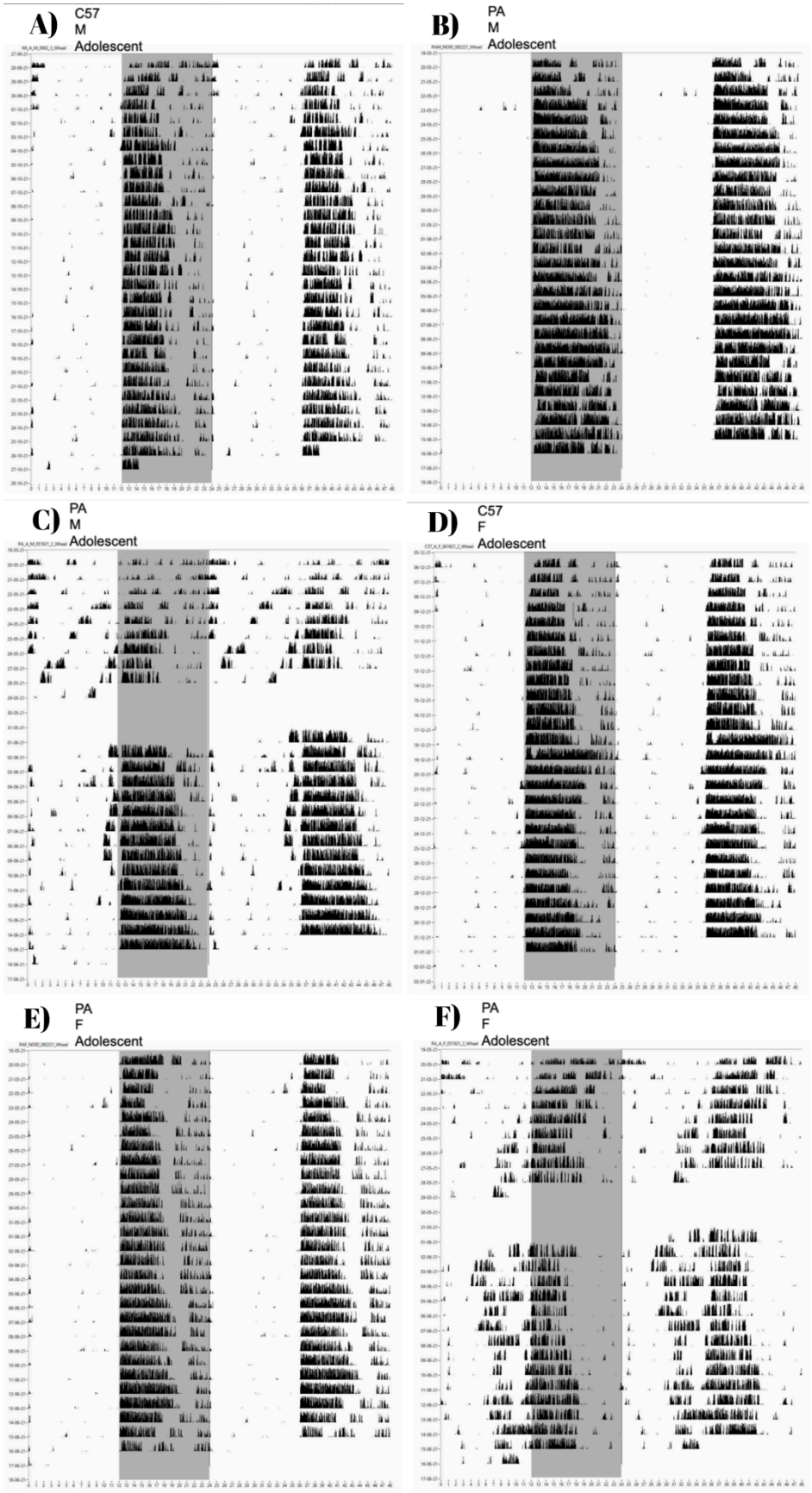
A)
C57
M
Adolescent
B)
PA
M
Adolescent
C)
PA
M
Adolescent
D)
C57
F
Adolescent
E)
PA
F
Adolescent
F)
PA
F
Adolescent

Figure 2: Examples of wheel recording Actograms from 6 adolescent mice kept on an 12-12 LD light-scheme. The gray shading represents lights off, i.e. the active phase of the mice. Figure 2A shows a wild-type Male mouse to act as a baseline control. Figure 2B and 2C show the strong variability in rhythmic output between two male BLOC-1 deficient mice, resembling previous findings in adult mutant males from our group. Similarly, Figure 2D shows a wild-type female mouse and Figure 2E and 2F show the strong variability in rhythmic output between two female BLOC-1 deficient mice. The actograms in Figure 2C and 2F suggest that there may be sex-divergent effects in this mutant strain, with females showing weaker and more variable activity rhythms, however this needs further confirmation. These wheel recordings help provide evidence of strong and clear onset of sleep/wake rhythms.

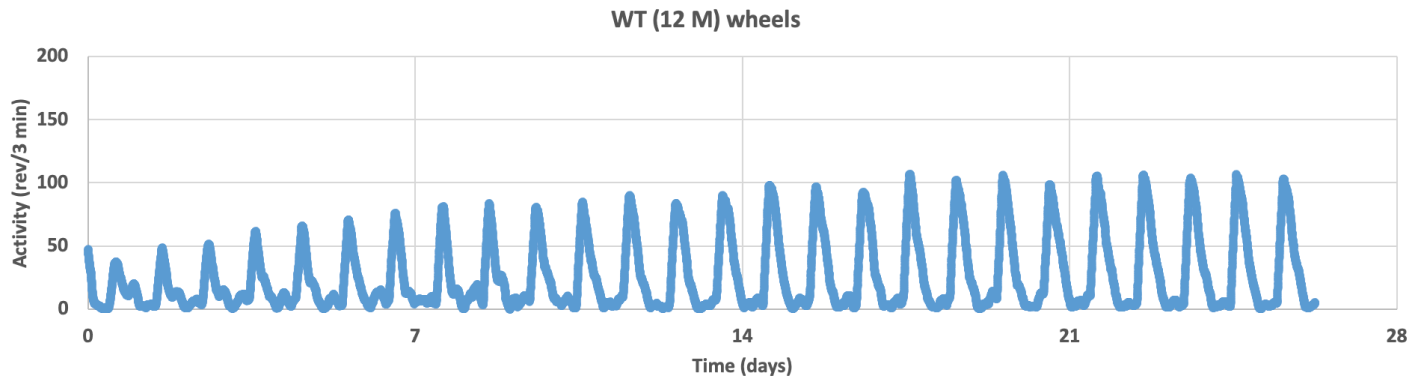

Figure 3A: Wheel recording Waveform from 12 wild-type male mice.

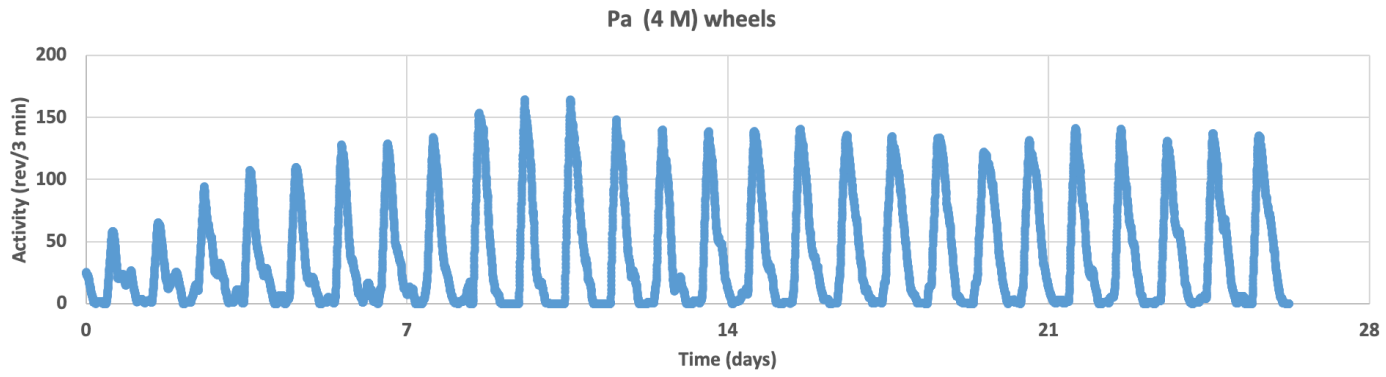

Figure 3B: Wheel recording Waveform from 4 BLOC-1 deficient male mice.

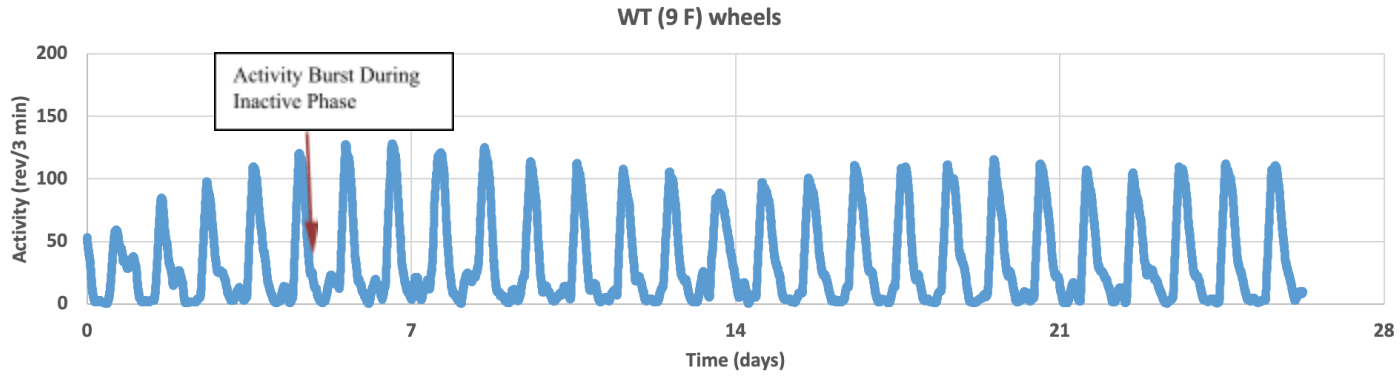

Figure 3C: Wheel recording Waveform from 9 wild-type female mice.

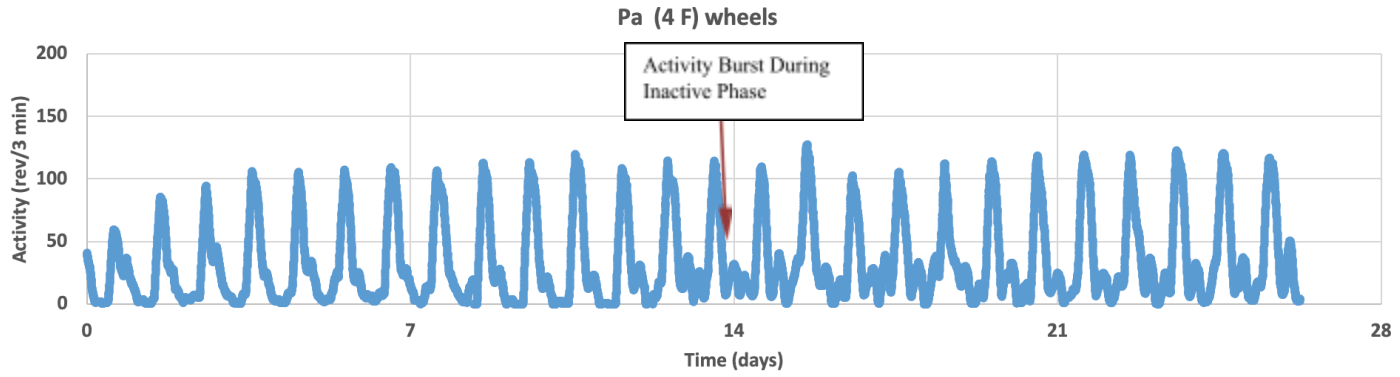

Figure 3D: Wheel recording Waveform from 4 BLOC-1 deficient female mice.

Figure 3: Waveform Analysis of Wheel Recording Data from 12 Male Wild-Type Mice (Figure 3A), 4 Male Pallid Mice (Figure 3B), 9 Female Wild-Type Mice (Figure 3C), and 4 Female Pallid Mice (Figure 3D). The presented data is over a 27 day period starting at the age P27 as day 0 and ending on P53. Comparing the Males (Figure 3A&B) to the Females (Figure 3C&D) it appears that the Females exhibit a more consistent rhythm while it takes the Males more days during this developmental period as their wheel running activity increases over time. In all groups there is also a slight burst of activity near the nadir of the inactive phase, however it is more prevalent in the female mice and indicated with a red arrow.