COGNITIVE CHANGES AND CIRCADIAN TIMEKEEPING DISTURBANCES IN AGING ACROSS SEXES

An Undergraduate Research Scholars Thesis

by

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RESEARCH COMPLIANCE CERTIFICATION

Research activities involving the use of human subjects, vertebrate animals, and/or biohazards must be reviewed and approved by the appropriate Texas A&M University regulatory research committee (i.e., IRB, IACUC, IBC) before the activity can commence. This requirement applies to activities conducted at Texas A&M and to activities conducted at non-Texas A&M facilities or institutions. In both cases, students are responsible for working with the relevant Texas A&M research compliance program to ensure and document that all Texas A&M compliance obligations are met before the study begins.

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This project required approval from the Texas A&M University Research Compliance & Biosafety office.

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ABSTRACT

Cognitive Changes and Circadian Timekeeping Disturbances in Aging Across Sexes

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Cognitive changes in aging and Alzheimer's disease (AD) are often accompanied by pronounced disturbances of circadian timekeeping, especially the sleep-wake cycle. Normal circadian timekeeping has an important impact on human health and performance by providing the temporal coordination of internal processes to ensure their occurrence at the "right time" relative to each other and the external environment. The aging of the rodent circadian system is characterized by changes comparable to those in human aging and AD. Common disturbances in the sleep-wake rhythms of aged rodents include alterations in circadian activity. However, not all aged rodents show these changes, demonstrating the variability characteristic of human aging in pre-dementia or mild cognitive impairment (MCI). Because the aging population also shows variability in onset and magnitude of cognitive impairment, we explored the relationship between these cognitive deficits and sleep disturbances during aging in mice.

The circadian rhythm of locomotor activity was continuously analyzed for 30-40 days in young (3-5 mo), middle-aged (12-14 mo), and aged (18-24 mo) mice. We then evaluated the mice in the Barnes maze for learning and memory performance. Aged mice exhibited significant

cognitive impairment in conjunction with striking changes in their circadian patterns of activity. Data from middle-aged animals (12-14 mo) showed that changes in circadian activity occur before deficits in learning and memory. Interestingly, we observed a gender-specific relationship between cognitive impairment in the Barnes maze and increased variability in daily onset times of circadian activity in aged female mice (20-24 mo). This data is the foundation of our model to further understand the relationship between circadian synchronization and cognitive impairment, and to probe possible mechanisms of underlying age-related changes.

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I want to thank all the student researchers at my lab who had helped with small tasks along the way: William Jackson, Jocelin Reyes, Judy Muayad, Wajihah Chaudhary and Emma Tran. Thanks also go to my friends, colleagues, and the department faculty and staff for making my time at Texas A&M University a great experience. Finally, thanks to my girlfriend, Emma Bender, for her encouragement and my parents for their patience, love, and support.

The data for Cognitive Changes and Circadian Timekeeping Disturbances in Aging Across Sexes were analyzed by Dr. Karienn Montgomery, Dr. David Ernest, and Andrew Powell. The analyses depicted in Cognitive Changes and Circadian Timekeeping Disturbances in Aging Across Sexes were conducted by the department of Neuroscience & Experimental Therapeutics and are currently unpublished.

All other work conducted for the thesis was completed by the student independently.

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authors and do not necessarily represent the official views of the Department of Neuroscience & Experimental Therapeutics.

1. INTRODUCTION

Cognitive decline is the clinical hallmark of dementia and Alzheimer's disease (AD), and although significant effort has been applied towards research, there is still no cure or prevention for the disease, and the relationship between behavioral and molecular markers is not entirely known. The hippocampus, the region of the brain responsible for spatial memory formation, is severely affected in AD. Atrophy of this region is correlated to the progressive decline seen in dementia, which results in failure of episodic memory recollection. Chronic sleep disturbances are also observed with more frequency in aging, and it is reported that at least 2/3 of nursing home residents suffer from irregular sleep patterns, such as difficulties falling asleep, waking up, and staying asleep (Cipriani et al. 2015).

Cognitive impairment has been linked to disruptions in circadian patterns (Devan et al. 2001; Craig and McDonald 2008). This is not surprising, as it is well-documented that hippocampal memory consolidation occurs during sleep (Ji and Wilson 2007). Sundowning syndrome, which is the emergence or worsening of neuropsychiatric symptoms near sunset, is linked to poor outcomes in patients with dementia (Canevelli et al. 2016). This phenomenon is related to the light-dark cycle and circadian rhythmicity (Bliwise 1994). Sleep patterns rely on circadian timekeeping, and disruptions of circadian physiological processes can disrupt sleep patterns. (Weissova et al. 2016).

Mice models have been long been utilized as effective model organisms for human aging and circadian influenced behaviors (Bedrosian and Nelson 2013; Ackert-Bicknell et al. 2015). It is also very helpful that mice and humans share remarkable similarities between brain structures and functions, and that aged mice exhibit similar hippocampal deficits and performance

variability to those observed in humans (Gallagher and Rapp 1997). This, in combination with the low economic cost and short life span of mice, makes them ideal candidates for studying hippocampal memory and learning.

Entrainment in mice is the physiological process that occurs when mice adapt to the light-dark cycle of their local environment, after a brief acclimation period of a couple of days. After acclimation, mice become active shortly after ambient light has decreased (Kim et al. 2018). In mice, disturbances of the sleep-wake cycle include alterations in this entrainment process, and such disruptions occur naturally as mice age (Tahara et al. 2015). However, there is no current model that allows for the study of these disruptions in aging, especially within the framework of age-related cognitive decline. Furthermore, there is potential for using circadian changes as an early predictor of cognitive decline (Targa et al. 2021). This knowledge is important for the study of potential preventative methods for cognitive decline or AD because, by the time a patient is diagnosed with AD, substantial hippocampal deficits can already be detected, and patients are left with few options for treatment. Anything that contributes to earlier diagnoses can be invaluable to slowing the regression of cognition and inspiring novel therapeutic treatments.

In this study, we explore the relationship between cognition, circadian activity, and sex across the lifespan of the C57Bl6 and VGAT-ChR2(H134R)-EYFP mouse models [which has previously been used by our lab in aging studies and shows no difference to control C57BL6; (Bang et al. 2021)]. Furthermore, our study offer insight into whether circadian disturbances appear alongside the development of cognitive impairment, or prior to future cognitive dysfunction, providing an effective model for future aging studies.

2. METHODS

2.1 Animals and Treatment

Breeding pairs of VGAT-ChR2(H134R)-EYFP [Stock 014548;(Zhao et al. 2011)] and matched NTg controls (C57Bl6) were obtained from Jackson Labs, and we established a long-term colony of mice across the lifespan of both sexes and all genotypes. The number of mice per experiment is detailed in Table 2.1. Mice were maintained in the AAALAC-accredited vivarium at the Texas A&M University Health Science Center under controlled conditions (22–25°C; lights 0700–1900 h; mouse chow and water ad lib) in accordance with policies of the Texas A&M University Laboratory Animal Care Committee and NIH guidelines. Mice were euthanized using isoflurane and the was brain quickly removed for subsequent in vitro studies (not included in this manuscript).

Table 2.1: *Animal numbers used in circadian and cognitive experiments.*

	Circadian Experiment					
Age	Male C57Bl6	Female C57Bl6	Male VGAT	Female VGAT	Total Males	Total Females
Young	8	8	3	3	11	11
Middle-aged	10	10	0	0	10	10
Aged	14	15	5	5	19	20
	Barnes Maze				Total Males	Total Females
Young	8	8	3	3	11	11
Middle-aged	10	11	0	0	10	11
Aged	14	15	5	5	19	20

2.2 Wheel Activity

Mice were housed individually in cages and maintained under a 12:12 light-dark (LD) cycle and their circadian rhythm of free wheel-running activity was continuously recorded for 30-40 days. Wheel-running activity was continuously recorded, summed, and stored in 10-min bins using a computer running Dataquest IV data acquisition software (Data Sciences, Inc., St. Paul, MN). Graphical records of circadian activity rhythms were generated and analyzed using ClockLab data analysis software (ActiMetrics, Evanston, IL). During entrainment to LD 12:12, the onset of activity for a given cycle was identified as the first bin during which an animal attained 10% of peak running-wheel revolutions (i.e., intensity). To measure the angle of entrainment, least-squares analyses were used to establish a regression line through the daily onsets of activity during the period of entrainment (30 days), and then the number of minutes before (positive) or after (negative) the time of lights-off in the LD cycle (1800 hr) was determined for each animal. Total daily activity was calculated by averaging the number of wheel revolutions per 24 hours over the 30-day interval of analysis. Activity duration was then determined by measuring the time interval between the daily activity onsets and offsets.

2.2.1 Data Analyses

Statistical analyses were performed on the raw data using a one-way ANOVA to determine the significance of treatment effects on circadian properties and quantitative parameters of the activity rhythm, and Fischer's LSD post hoc analyses were applied if necessary. Two-way ANOVAs were used in some cases to investigate the influence of sex and age on the parameters.

2.3 Behavioral Experiments

2.3.1 Barnes Maze

The Barnes circular platform maze is a 36-inch diameter circular platform on a 4 ft stand with twenty 2-inch holes evenly spaced around the circumference, where a black box (escape tunnel) was placed underneath one of the holes (San Diego Instruments, San Diego, Ca). Four bright lights were positioned above the maze as an aversive stimulus to cause the mice to seek out the escape tunnel using spatial cues. Between each trial, the platform and escape tunnel were cleaned with 70% ethanol and water, and video was acquired using a color GigE camera. Data were quantified using Ethovision 16 video tracking software (Noldus, Leesburg, VA, USA).

2.3.1.1 Habituation

During habituation, a mouse was placed on the table for 5 minutes and allowed to explore the maze without an escape box, under dim lights. Next, mice were placed in a 2 liter transparent glass beaker, under aversive lighting. After 1 minute, the mouse was gently guided to the escape hole, and the lights are turned off.

2.3.1.2 Learning Training Trials

In all subsequent trials, the animals were placed in the center of the table under a 2-liter glass beaker. The aversive lights were then turned on and the mouse was allowed to navigate the maze using spatial cues. Each mouse was allowed 180 seconds to locate and enter the escape tunnel per trial. If the mouse was unable to locate the escape hole after 180 seconds, it was gently guided to the correct hole location and allowed to enter the escape tunnel. Once the mouse entered the escape tunnel (either guided or on its own), it remained in the tunnel for one minute before returning to its home cage. Each day, the animal would attempt four trials spaced 15 minutes apart for a total of sixteen learning training trials, for 4 days.

2.3.1.3 Probe Testing

48 hours after learning trials, a single probe trial is performed. In this trial, the escape is removed, and mice are allowed to search for 3 minutes, after which they are removed from the maze.

2.3.1.4 Search Strategies and the Spatial Cognitive Score

A detailed description of search strategies can be found in (Illouz et al. 2016). Each learning trial is analyzed (blinded experimenter) and given a score as follows:

Hippocampal dependent strategies: direct (no error; score= 1), corrected (searched + or – 1 immediate hole, score= 0.75), focused (searched + or – 3 immediate holes, score= 0.5) and long correction (mouse searches across the target and immediately corrects toward correct hole, score =0.5).

Non-hippocampal strategies: the serial search (animal methodically searches holes one by one, score =0.25), random (search without a clear strategy and target hole identified by chance, score=0), and failure (animal searches but does not find the target, score=0).

Examples are shown in Figure 2.1. Scores from each trial were summed and graphically presented as a Cognitive Score.

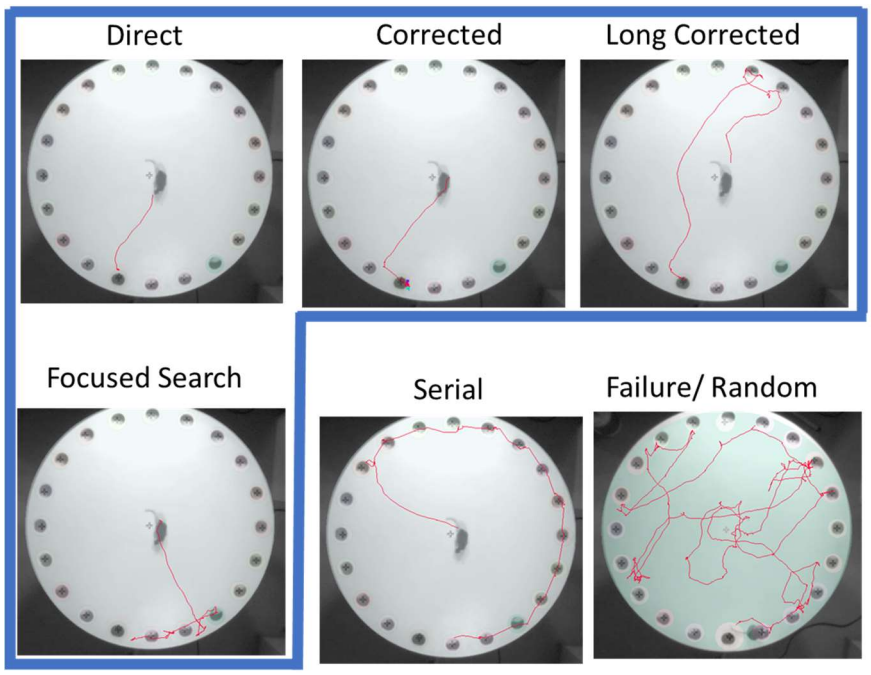

Figure 2.1: Examples of search strategies. *Images were acquired using Ethovision Software by Noldus.*

2.3.1.5 Data Analysis

The video detection software can acquire and analyze multiple variables, but the distance traveled (cm) until the animal finds the target hole is used to show whether the animals have learned the location of the escape during learning trials. Speed (cm/s) influences the latency (s) variable and can be used to demonstrate whether a group is struggling with motivation or motor performance. Data were analyzed with repeated measures analysis of variance (ANOVA) across days for sex and age.

For probe trials, the percent of the time in the target quadrant is used to quantify the animal's insistence about the escape hole's location. Quadrants each contain five possible locations for escape, and the escape hole is in the middle of the target quadrant. Only data from the first 30 seconds are analyzed because mice typically give up searching after approximately

30 seconds. Group means from the probe trial were analyzed with one-way ANOVAs for each sex.

3. RESULTS

3.1 Wheel-running Activity

3.1.1 Actograms and Wheel-running Activity

The circadian activity was monitored constantly while mice were in the 12:12 LD environment. This activity can be plotted into an actogram such as the one shown in Figure 3.1, which shows wheel-running activity for a single young mouse across periods of light and dark. The white regions are periods when the lights in the room are on, with the gray regions representing times when the lights are off. Mice are nocturnal animals and wheel-running activity is detected almost immediately after the lights are switched off. This timeframe of activity initiation is referred to as the activity onset period.

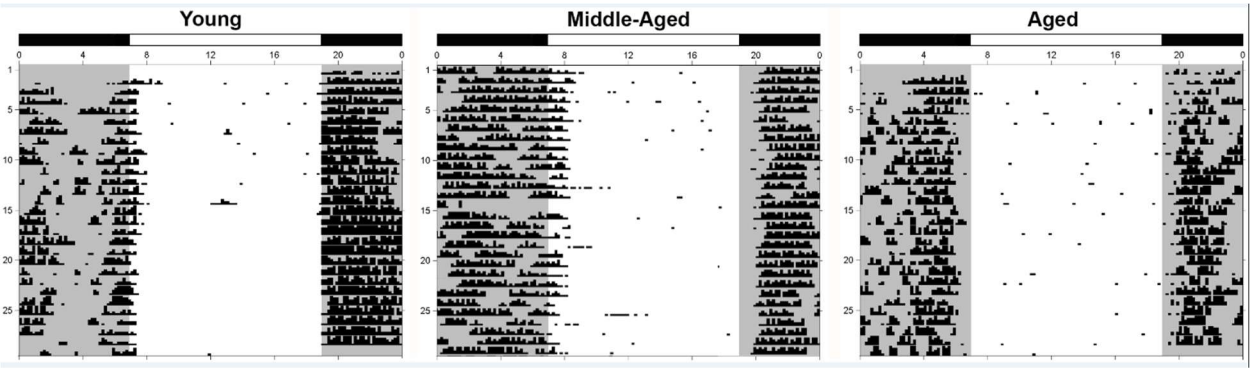

Figure 3.1: Effects of aging on circadian patterns of mouse wheel-running activity during light-dark entrainment*. Representative records of circadian wheel-running behavior comparing the activity rhythms of young (left), middle-aged (middle), and aged (right) WT mice during entrainment to LD 12:12. Actograms are plotted over a 24-hr period. The closed bars at the top and shading on the records signify the timing of the dark phase in the LD 12:12 cycle.*

The young mouse represented in Figure 3.1 exhibited moderate activity during the 12-hour light period during their first few days in the room. Over time, however, the mouse adapts to the light-dark cycle of the room and showed almost no activity during the light periods. As

mice age, their ability to entrain to an unfamiliar environment decreases, and more light period activity is observed. Aged mice actograms exhibit a much higher level of activity onset variability since they are more likely to run during periods of light.

During exposure to LD 12:12, entrainment of the activity rhythm was observed in all young, middle-aged, and aged mice. The activity rhythms of middle-aged and aged mice were distinguished by an altered phase angle of entrainment to LD 12:12 such that their daily onsets of activity were delayed and occurred at later times relative to young animals, commencing up to 30-70 min after lights-off for some animals.

3.1.2 Male Mice

The average values for Ψ between activity onsets and lights-off were shown in Figure 3.2 -25.9 + 3.98 min in middle-aged mice, -27.888 + 3.75 min in aged animals and were significantly different [$p<0.05$] from that observed in young mice (-9.391 + 1.664 min; one way ANOVA, $F(2,33)= 9.55$, $p<0.0005$).

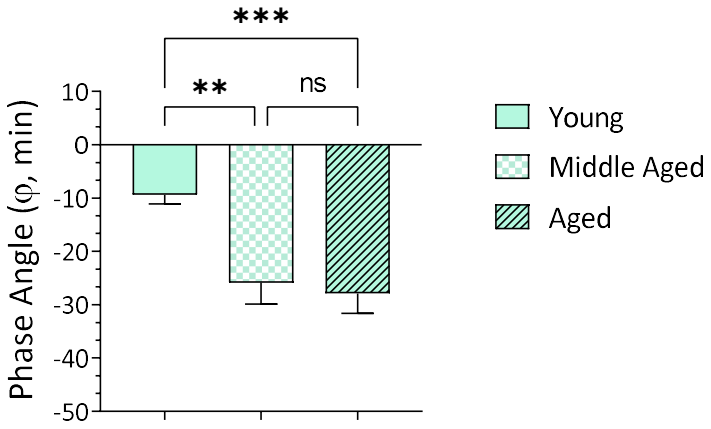

Figure 3.2: Effects of aging on circadian entrainment wheel-running activity in males. *The phase angle between daily activity onsets and lights-off during LD 12:12 entrainment in young, middle-aged, and aged mice. Bars represent the mean (± SEM) phase angle of entrainment (Ψ) to LD 12:12 in minutes. Negative values indicate that daily onsets of activity occur after lights-off whereas positive values denote that activity onsets precede the end of the light phase.*

In addition to the delayed onsets of daily activity, middle-aged and aged mice showed unstable patterns of entrainment to LD 12:12 as indicated by high variability in the timing of their activity onsets between successive days (Figure 3.3). During exposure to LD 12:12, the activity onsets in individual middle-aged and aged mice occurred at (earlier or later) times that differed on average by 10.067 min and 21.847 min, respectively from the preceding day, whereas the average day-to-day variability in activity onset times of young animals was only 6.449 min. The absolute day-to-day variation in the onsets of activity in aged animals was significantly greater [$p<0.05$] than that observed in the other 2 cohorts ($F(2,41)= 26.543$, $p<0.0001$). The aged mice show significantly higher levels of variability when compared to middle-aged ($p<0.0001$) and young mice ($p<0.0001$). Young and middle-aged mice showed insignificant differences in activity onset variability ($p=0.1741$).

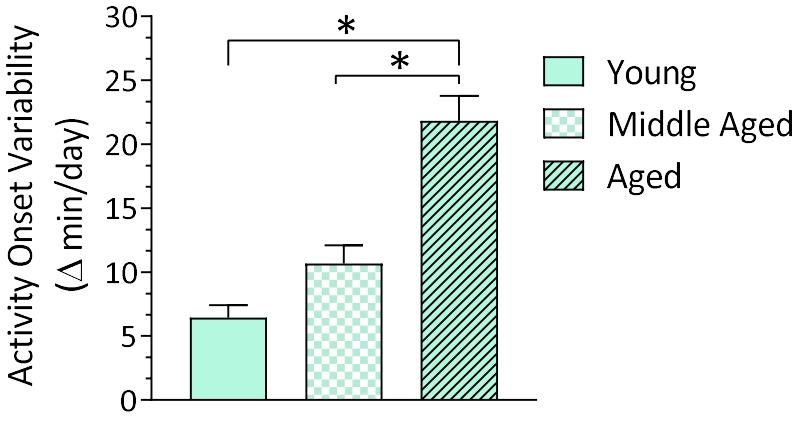

Figure 3.3: Effects of aging on circadian entrainment wheel-running activity in males. *Absolute day-to-day variability in daily onsets of activity. Absolute differences in the timing of activity onsets on successive days were analyzed in individual animals and bars represent the mean (± SEM).*

Daily wheel-running activity, while mice were in the wheeled housing was recorded in revolutions per 24 hours. Male mice age groups were compared in Figure 3.4 ($F(2,41)=19.292$, $p<0.0001$). Young and middle-aged males exhibited similar levels of activity ($p=0.3570$).

However, aged male mice exhibited significantly lower activity than young ($p<0.0001$) and middle-aged ($p<0.0001$) males.

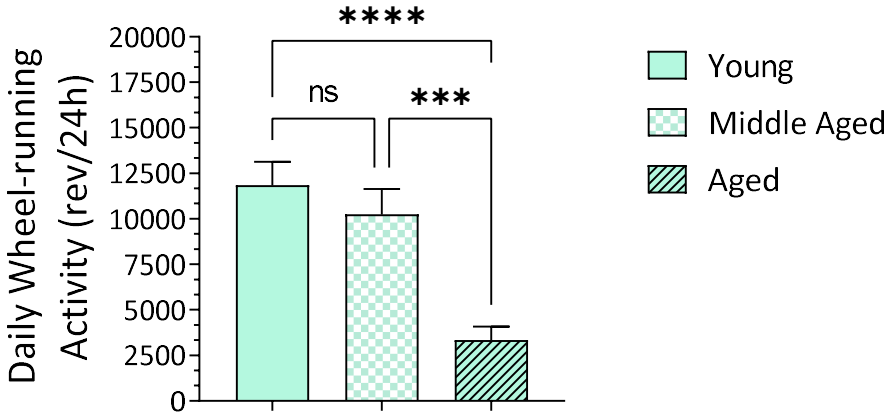

Figure 3.4: Effects of aging on wheel-running activity in males. *The Total daily wheel-running activity of young, middle-aged, and aged mice. Bars depict average wheel revolutions per 24 h (± SEM).*

3.1.3 Female Mice

Comparable results were observed in the female cohort. The average values for Ψ between activity onsets and lights-off were –37.9179 + 4.224 min in middle-aged mice, which was significantly higher than -27.116 + 2.675 min in aged animals and that observed in young mice (- 11.748 + 2.115 min (one way ANOVA, $F(2,34)= 14.949$, $p<0.0001$). The difference can be observed in Figure 3.5.

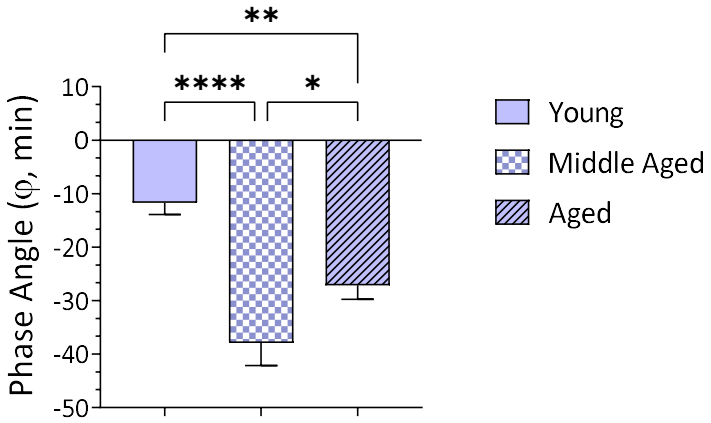

Figure 3.5: Effects of aging on circadian entrainment wheel-running activity in females. *The phase angle between daily activity onsets and lights-off during LD 12:12 entrainment in young, middle-aged, and aged mice. Bars represent the mean (± SEM) phase angle of entrainment (Ψ) to LD 12:12 in minutes. Negative values indicate that daily onsets of activity occur after lights-off whereas positive values denote that activity onsets precede the end of the light phase.*

Just like in males, female middle-aged and aged mice showed unstable patterns of entrainment to LD 12:12 (Figure 3.6). During exposure to LD 12:12, the activity onsets in individual middle-aged and aged mice occurred at (earlier or later) times that differed on average by 15.540 min and 19.611 min, respectively from the preceding day, whereas the average day-to-day variability in activity onset times of young animals was 5.136 min ($F(2,43)= 22.790$, $p<0.0001$). In contrast to the males, middle-aged female mice showed significantly more absolute day-to-day variation in the onsets of activity in young animals only ($p<0.0001$). This may mean that changes in circadian activity can be detected earlier in females versus males. The aged mice show significantly higher levels of variability when compared to young mice ($p<0.0001$) but not middle-aged ($p=0.0663$).

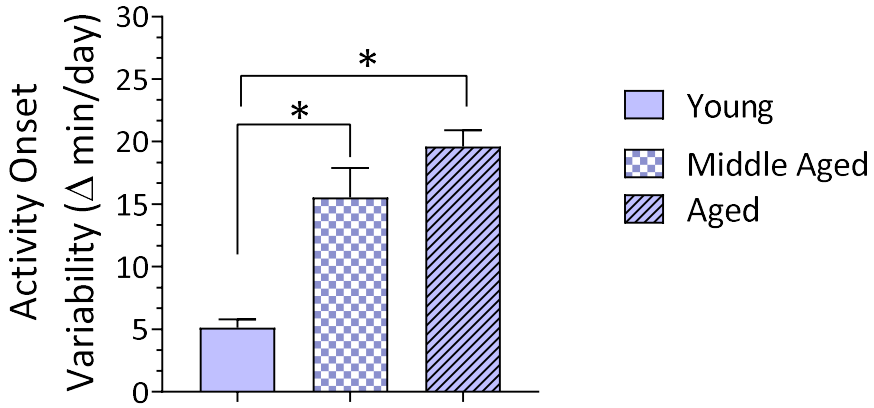

Figure 3.6: Effects of aging on circadian entrainment wheel-running activity in females. *Absolute day-to-day variability in daily onsets of activity. Absolute differences in the timing of activity onsets on successive days were analyzed in individual animals and bars represent the mean (± SEM).*

Daily wheel-running activity (Figure 3.7) while females show that middle-aged and young mice were comparable (p=0.224), even though there was a main effect of age (F(2,43)= 37.247, p<0.0001). This was driven by the lower activity seen in aged mice, which differed from the 2 other groups (p<0.0001).

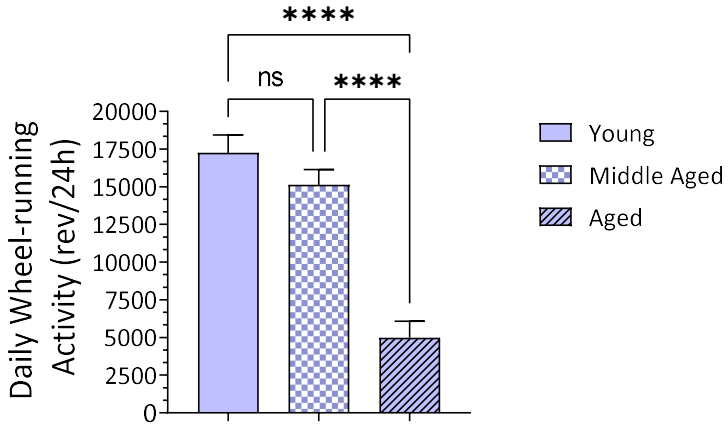

Figure 3.7: Effects of aging on wheel-running activity in females. *The Total daily wheel-running activity of young, middle-aged, and aged mice. Bars depict average wheel revolutions per 24 h (± SEM).*

3.2 Behavioral Tests

Circular Barnes mazes tests were conducted on all mice to evaluate spatial learning and memory. The following results are grouped by sex.

3.2.1 Barnes Maze Performance in Males

As mice age, motor performance declines. In the Barnes maze, this activity difference translates into longer speeds while searching. Figure 3.8 shows differing speeds of male mice age groups ($F(2,41)=14.080, p<0.0001$) across training days.

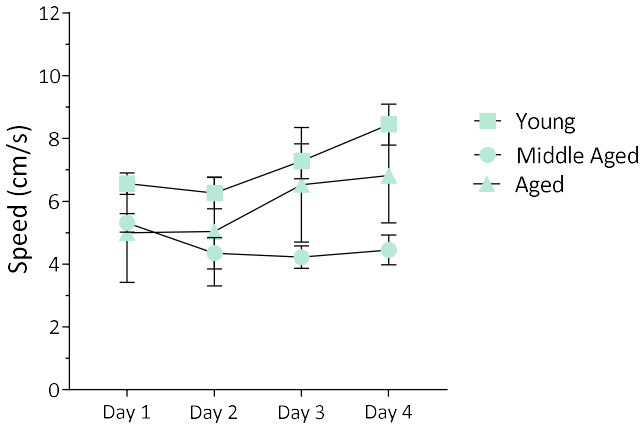

Figure 3.8: Effects of aging on motor performance in the Barnes Maze Performance. *The speed during trials across 4 days of learning trials (mean ± SEM).*

Middle-aged male mice are showing significant differences in speed from young males(<0.0001), while young and aged males are also different ($p=0.0004$). Middle-aged males and aged males displayed similar rates of speed ($p=0.0643$).

Due to these highly variable rates of speed, latency (s) is not an accurate measurement for learning performance in the Barnes maze, therefore, distance traveled (cm) until escape is used for assessment of search efficiency. Figure 3.9 shows male distance traveled until escape across all male mice groups ($F(2,41)= 3.684$, $p=0.0338$). This difference is driven by an age, with aged males showing the longest path to the escape hole as compared to young ($p=0.0128$) males.

Middle-aged and young males performed almost indistinguishably from each other (p=0.5834). Middle-aged and aged males also showed no significant differences in the Barnes learning curve (p=0.0923).

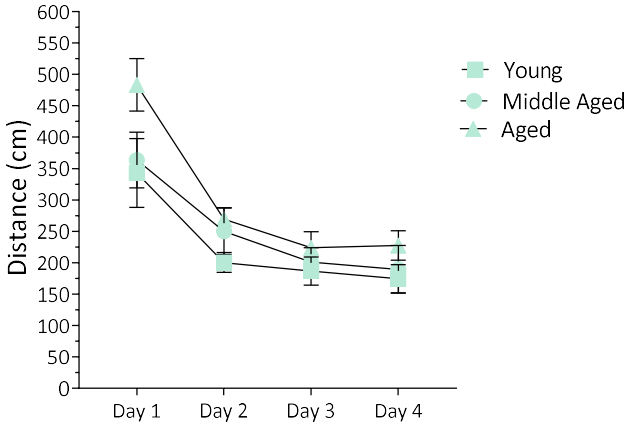

Figure 3.9: Effects of aging on Learning in the Barnes Maze Male Performance. *The distance to the target-hole average per day across 4 days of learning trials (mean ± SEM).*

To further analyze the behavioral performance in our cohorts, we calculated cognitive scores (Index) for each age group based on the procedures outlined in the methods. Higher cognitive scores convey higher use of hippocampal and spatial memory techniques in the Barnes maze. Cognitive scores for male age groups are shown in Figure 3.10 and aged just as it is observed with distance, aged mice show significantly lower scores than the other 2 cohorts ($F(2,40)= 14.610$, $p<0.0001$). Middle-aged and young male mice showed similar levels of hippocampal engagement (p=0.9219). Young and aged male mice (p=0.0013) were slightly less significant than the middle-aged and aged comparison (p=0.0008).

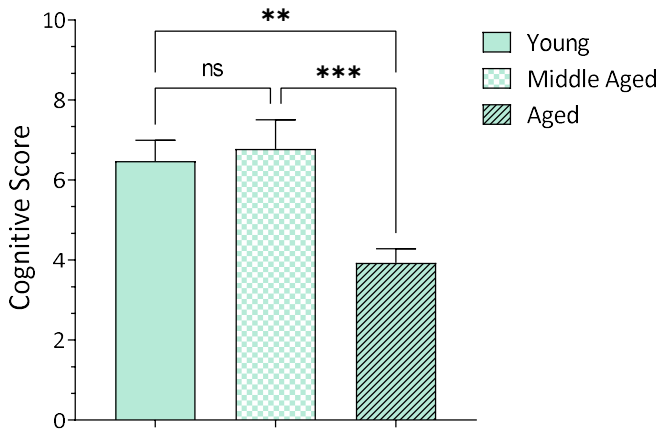

Figure 3.10: Effects of aging on Learning in the Barnes Maze Male Performance. *The cognitive score is based on strategies during learning trials (mean ± SEM).*

A probe test can be used to access the degree of memory performance in mice. Aged male mice did trend toward a lower value (38%) in comparison to middle-aged (51%) and young (51%) mice (Figure 3.11), but all groups were statically insignificant from each other ($F(2,41)=1.493, p=0.2366$).

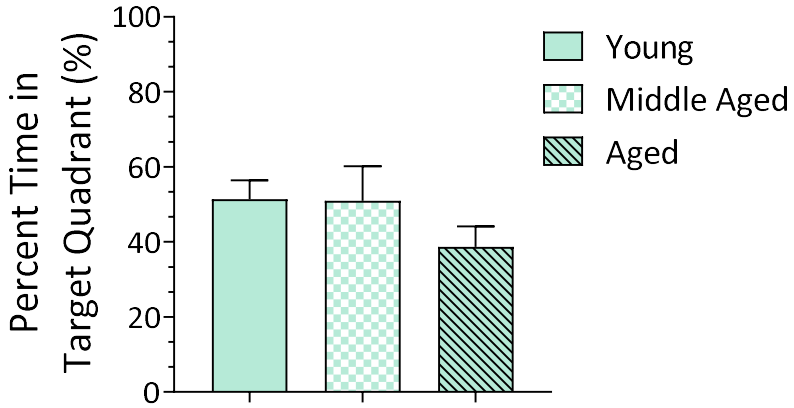

Figure 3.11: Effects of aging on Memory in the Barn es Maze Male Performance. *The percent time spent in the target quadrant is shown for all ages (mean ± SEM).*

3.2.2 Barnes Maze Performance in Females

As seen with the male mice, young female mice also showed significantly higher speeds (cm/s) than their middle-aged and aged cohorts (Figure 3.12; $F(2,40)=14.136$, $p<0.0001$). However, the young females were significantly faster than middle-aged ($p<0.0001$) and aged females ($p<0.0001$). Aged and middle-aged females showed similar speeds during Barnes maze trials ($p=0.6803$).

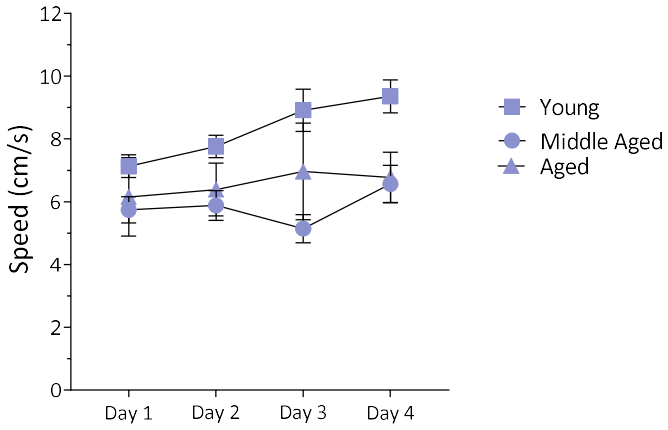

Figure 3.12: Effects of aging on motor performance in the Barnes Maze Female Performance. *The speed of female mice during trials across 4 days of learning trials (mean ± SEM).*

The total distance traveled until escape for female mice is shown in Figure 3.13, and we observed an age difference between the groups ($F(2,40)4.267$, $p=0.0209$). Upon post hoc analysis, we noted that young females learned like middle-aged females ($p=0.6593$) but were significantly different from aged females ($p=0.0112$). Middle-aged and aged females also showed different path distances until escape in the Barnes maze learning trials ($p=0.0426$).

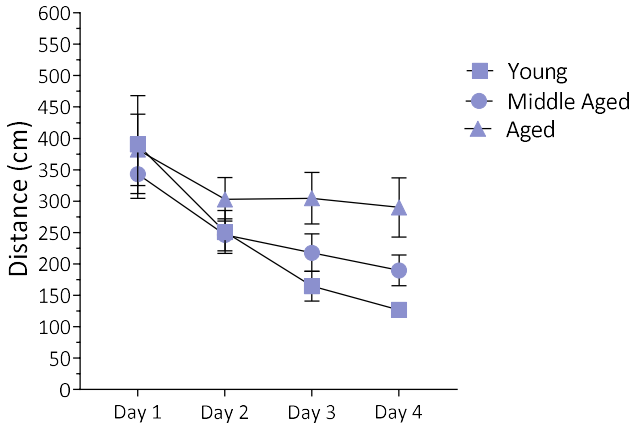

Figure 3.13: Effects of aging on Learning in female mice Barnes Maze Female Performance. *The distance to the target-hole average per day across 4 days of learning trials (mean ± SEM).*

Cognitive scores for female search strategies in the Barnes maze are shown in Figure 3.14. Young females did not differ from middle-aged (p=0.6878) but were significantly better than the aged females (p=0.0248). Aged females had the lowest cognitive score on average but were not statically different from middle-aged females (p=0.1849).

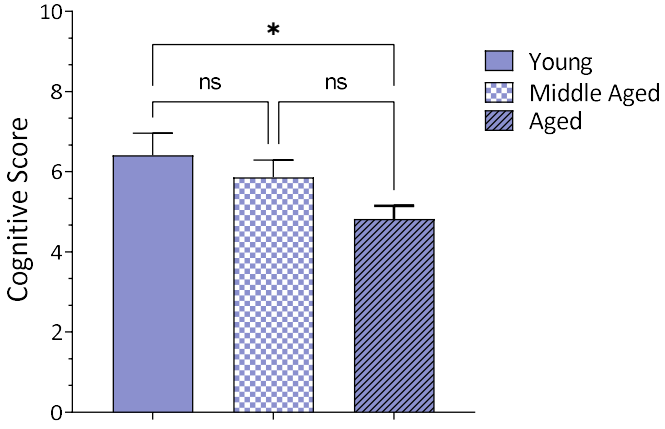

Figure 3.14: Effects of aging on Learning in the Barnes Maze Female Performance. *The cognitive score was based on strategies during learning trials with female mice (mean ± SEM).*

Interestingly, we saw an age effect in the probe test for females ($F(2,40)=3.232$, $p=0.0500$), shown in Figure 3.15. Aged females showed significantly less time in the target quadrant than young (27% versus 49% respectively; $p=0.0280$). Middle-aged (46%) mice were not significantly different from young ($p=0.7616$) or age females ($p=0.0676$).

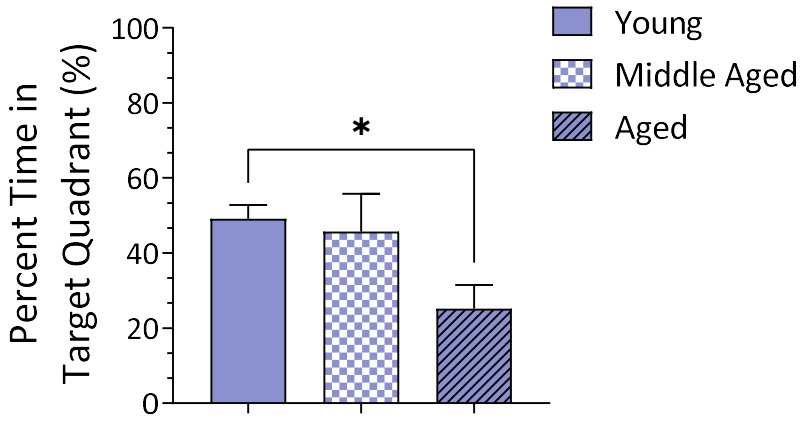

Figure 3.15: Effects of aging on Learning in Barnes Maze Female Performance*. The percent time spent in the target quadrant is shown for all ages (mean ± SEM).*

4. CONCLUSION

Predictive biomarkers are essential for the development of novel therapeutics and earlier diagnoses of age-related cognitive decline. This study sheds some light on the behavioral changes that occur before the onset of significant age-related cognitive decline, and how these results differ across sexes.

We did not distinguish between C57Bl6 and VGAT-ChR2(H134R)-EYFP mice because the circadian activity had already been analyzed for genotype differences in Bang et al. (2021) and no differences were detected. In this previous study, our lab also found that the young and aged cohorts showed significantly different phase angles and activity onset variability. We expanded these results by adding a middle-aged group and separating the data by sex. Furthermore, we believe we are one of the first to look at circadian activity metrics of comparison between young, middle-aged, and aged mice's cognitive behavior.

In the current study, we found that middle aged mice showed increased levels of variability in entrainment to light in comparison to young, particularly in the female cohort, which showed differences in phase angle and activity onset. The significance of photopic exposure on entrainment of circadian rhythms in rodents has long been established, but the molecular pathways for this entrainment are still unknown (Shigeyoshi et al. 1997). The extent to which sex influences this entrainment is also not known (Krizo and Mintz 2014). Our study highlights the need for careful analysis of sex-dependent circadian behaviors. Mice exhibit baseline sex differences in many circadian variables that could affect their performance, such as different levels of food anticipatory activity (Aguayo et al. 2018). In our study, however, mice always had access to food, and food anticipatory activity is therefore controlled. Identification of

other sex-linked influences is important to understand the degree to which circadian behaviors differ between sexes.

Another variable in which sex differences are commonly observed is motor activity. However, differences in motor activity are related to differences in motor function and physical ability and do not factor in measures of circadian rhythmicity or cognition. Sex hormones have some perceivable effect on nearly every cell in the body, so it is not surprising to see circadian differences across sexes. Sex hormone agonists or antagonists may be potentials avenues for further detangling circadian timekeeping differences across sexes (Joye and Evans 2021).

In human studies, the relationship between aging, sex, and cognitive decline is often obscured by numerous co-morbidities present in elderly populations (Campos Costa, Nogueira Carvalho, and Fernandes 2013). Humans and mice demonstrate remarkably similar patterns of sleep disorder onset, near the middle of their respective lifespans (Carrier et al. 2017). However, women tend to spend more time asleep and sleep more mindfully than males do (Carrier et al. 2017). Women need longer periods of rest than men to recover from similar levels of sleep deprivation (Corsi-Cabrera et al. 2003). Literature on sex-linked circadian differences in humans is surprisingly lacking, and more studies focusing on sex hormones' effects on human sleep-wake cycles are needed before sound conclusions can be made.

Because we know that there are sex differences in circadian activity, we analyzed male and female Barnes data separately. The learning curves, Figures 3.9 and 3.13, decreased with aging, with aged mice performing poorer than their younger counterparts across both sexes. Figures 3.10 and 3.14 show the cognitive score for both sexes, which displayed a significant difference between aged and young mice. Cognitive score is a quantification of the strategies employed by mice during Barnes maze navigation and may be a more sensitive measure of

cognition than Barnes maze learning curves. Interestingly, the middle-aged females' cognitive scores were not different from those of aged females. In contrast, middle-aged males had a significantly higher cognitive score than aged males. This difference was not observed in learning curves collected during the Barnes maze trials. Female aged mice did exhibit less insistence in the probe test (27%, Figure 3.15), a deficit that was not seen in the male aged cohorts (38%, Figure 3.11). These data suggest that middle-aged females may have begun age-related decline. The middle-aged females displayed significantly higher levels of circadian impairment and trivial differences in cognition. This implies that the areas of the brain responsible for circadian regulation are impaired sooner than areas of the brain responsible for cognition.

The combination of short-term circadian monitoring followed by Barnes maze testing is a novel technique. A future study could monitor only middle-aged mice, wait for those mice to age, and then evaluate Barnes maze performance of said mice to see the extent to which circadian impairment predicts later cognitive decline. Barnes maze deficits in C57B16 mice typically appear at 14 months of age, so monitoring the circadian activity of mice prior to 14 months of age, will aid in developing a preclinical model that can be used in future mechanistic studies. Future studies should include monitoring of estrous cycle activities in females as well.

In human populations, sex is a significant factor in developing Alzheimer's or dementia (Lindsay et al. 2002). Women are also significantly more likely to enter cognitive decline than men (Beam et al. 2018). However, women's longer lifespan might confound this claim (Barford et al. 2006).

Sleep disruptions correlate directly with dementia, Alzheimer's, and mortality (Shi et al. 2018). The data provided in this study suggest how these disruptions might fit into the overall

progression of the disease and suggest that impairment of circadian timekeeping presents before cognitive impairment. Analysis of the precise molecular mechanisms that regulate circadian timekeeping and its relationship with cognition may provide potential therapeutic targets for the prevention of Alzheimer's and dementia.

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