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Dedication

It feels impossible to distill in the space of a page the contribution of friends, family, and loved ones to this dissertation and my time at The University of Chicago, as I truly would not be here without their support. These paragraphs are poor compensation for the time and care you spent helping me grow into the person and scientist I am today. To my parents, Judy Cocchiarella Beach and Dan Beach, even though you might have no idea what I've been doing for the last 6 years or why I'm having mental breakdowns over mouse brains, you've always met me with endless support, curiosity, and love. Your parenting guided me towards a love of learning that brought me to grad school, and your models of compassion, work ethic, and humor are what allowed me to succeed. And if all else failed, there was always whiskey.

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Quote

"We walk the corridors, searching the shelves and rearranging them, looking for lines of meaning amid leagues of cacophony and incoherence, reading the history of the past and our future, collecting our thoughts and collecting the thoughts of others, and every so often glimpsing mirrors, in which we may recognize the creatures of the information."

-Jorge Luis Borges

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Abstract

Life has evolved within a dynamic environment. In order to anticipate and prepare for recurrent environmental changes, organisms have internalized biological solutions to challenges imposed through sensory stimuli. Circadian rhythms have evolved to provide synchrony with the predictable daily oscillators of environmental cues, and interactions among circadian oscillators throughout the brain and the body influence the adaptive coordination of the organism as a whole. To abstract relevant information, organisms must constantly sample not only rhythmic features from the environment— such as light— but also more transient stimuli— such as olfactory cues. Olfaction allows for active sampling of crucial survival information including predator identification, social recognition, and sourcing of food. Importantly, odor stimuli exert powerful control over learning and memory networks through the unique anatomical structure of olfactory pathways, which in turn predispose the olfactory system to be more vulnerable to neurodegenerative diseases. This research examines how desynchrony between internal biology and external cues can impact overall organismal health, and in turn how neurodegenerative disease alters perception of environmental stimuli. First, I examined how internal desynchrony within the molecular machinery of the circadian clock affects behavior in the face of environmental challenge by testing the hypothesis that a core circadian clock gene, per2, is responsible for circadian clock responses to light. Lack of functional per2 prevented the normal circadian period lengthening and compression of active period in response to light by increasing the magnitude of phase advance in response to late evening/early morning light and decreasing the magnitude of phase delay in response to early evening light (Chapter 3). In this way, destabilization of the internal biological clock machinery through the functional mutation of the per2 gene can affect the behavioral response to environmental stimuli. Experiments in Chapter 4

disrupted circadian rhythms by manipulating exogenous stimuli: the timing of light and food, to examine whether circadian disruption exacerbated symptoms of neurodegenerative disease. In the APP/PS1-21 mouse model of Alzheimer's Disease (AD), mice were fed either only at night or only during the day, the latter of which induced internal circadian desynchrony. Circadian desynchrony itself idiosyncratically altered performance in some behavioral cognitive and emotional tests, but it did not clearly exacerbate AD behavioral pathology. Finally, in Chapter 5, I aimed to characterize how neurodegenerative disease alters perception of environmental cues, by characterizing olfactory dysfunction in this same model of AD during the initial stage of disease. An objective of this study was to identify behavioral markers for diagnosis of AD early in development (Chapter 5). APP/PS1-21 mice at the initial stages of pathology showed reduced discrimination between odorants in a non-rewarded paradigm. However, discrimination was recovered under conditions of reward, indicating that mice may be able to recruit other neural systems to compensate for impaired olfaction, when sufficiently motivated by appetitive stimuli. This presents olfactory discrimination as a potential site for behavioral diagnosis of early-stage AD. Together, these experiments identify novel interactions among sensory features of the environment and their influence on brain function, behavior, and symptoms of disease.

Chapter 1: Background and General Information

Life has evolved within a dynamic environment. To anticipate and prepare for recurrent environmental changes, organisms have internalized biological solutions to challenges imposed through sensory stimuli. For example, the Earth's 24-hour rhythm of alternation between light and darkness has resulted in the development of biological rhythms in nearly every organism. Biological rhythms in physiology and behavior are essential for adaptive interactions within this rhythmic environment. Circadian rhythms (CRs) have evolved to provide synchrony with the predictable daily oscillations of environmental cues, such as the light cycle, food availability, presence of predators, and temperature changes, through a process known as entrainment. In organisms from bacteria to humans, CRs drive a myriad of daily rhythms in behavior and physiology to promote both (1) internal synchrony among innumerable biological systems within the individual, and (2) synchrony of the individual with the rhythmically-changing environment. Well-described CRs including sleep and wakefulness, arousal, ingestive behavior, hormone secretion, and immune response, positioning circadian rhythms at a pivotal point for impact on health and disease (Benca et al., 2009; Coogan & Wyse, 2008; Sahar & Sassone-Corsi, 2009; M. E. Young & Bray, 2007). It has been well established that the interactions between circadian oscillators across the body influence adaptive coordination within the organism as a whole. This dissertation aims to better understand how CRs support organismal homeostasis through experiments that examine how environmental perturbations alter the coupling dynamics between circadian clocks, and the impact of this altered coordination on physiology and behavior.

In ideal conditions, internal physiology and behavior maintain a stable phase relationship with the environment. This requires both a functional internal circadian network that can internalize environmental time cues to coordinate organismal physiology, as well as stable

environmental conditions with which an organism is able to align its behavior and physiology (Pittendrigh, 1960). In addition to rhythmic features of the environment, to abstract relevant information, organisms must constantly sample features from the environment, including both regularly-recurring rhythmic events (e.g., sunrise sunset, seasonal changes in day length) and more transient stimuli (e.g., sudden food appearance, olfactory stimuli). Indeed, olfaction allows for active sampling of crucial survival information including predator identification, social recognition, and sourcing of food. Importantly, odor stimuli exert powerful control over learning and memory networks through the unique anatomical structure of olfactory pathways. Unlike other sensory systems, olfactory information is transmitted directly from the sensory organ to the cortex, without passing through the thalamic sensory relay system (Haberly & Price, 1977; Price & Powell, 1971), although the olfactory cortex may perform the function of an olfactory thalamus (Kay & Sherman, 2007). As a result, olfactory sensory information garners privileged access to cortico-limbic structures, making it an interesting domain for investigating how the sensory stimuli from the environment are integrated into cortical representations to impact behavior (Merrick et al., 2014). Similarly, photic circadian time information is registered by the brain and is subjected to its most essential calculations without being processed by the thalamic input system, instead gaining access to the central circadian pacemaker via a direct retinal-hypothalamic projection (Golombek & Rosenstein, 2010; Hannibal, 2002, 2021; R. F. Johnson et al., 1988). Despite these broad similarities, investigations into how circadian and olfactory information interact to modulate behavior are uncommon.

The following research program examines how desynchrony between internal biology and external cues can impact organismal health. First, I examined how internal desynchrony within the molecular machinery of the circadian clock affects behavior in the face of

environmental challenge by testing the hypothesis that a core circadian clock gene is responsible for circadian clock responses to light (Chapter 3). Second, in a mouse model of Alzheimer's Disease (AD), I induced a state of profound circadian misalignment and evaluated the impact of internal circadian desynchrony on disease progression (Chapter 4). Finally, I aimed to characterize the progressive olfactory dysfunction in this same model of AD, with an objective of identifying behavioral markers for diagnosis of the disease early in development (Chapter 5). To provide a context for this work, background in circadian rhythms, misalignment, Alzheimer's Disease, and olfactory dysfunction is first considered.

Central coordination of bodily timekeeping

To adapt and respond appropriately to daily environmental changes, organisms have evolved biological clocks that entrain to the 24-hour day. So crucial to functioning are circadian clocks, that these molecular timekeepers exist in every cell of the body in both the brain and the periphery. These clocks are organized hierarchically to create a circadian network, with a superordinate (master) circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus (Rusak & Zucker, 1979). Bilateral ablation of the SCN results in total elimination of circadian rhythms in locomotor activity, which can then be reinstated through implantation of SCN donor tissue (Ralph et al., 1990; Rusak & Zucker, 1979; Stephan & Zucker, 1972), demonstrating the necessity of the SCN in rhythm generation (H. Li & Satinoff, 1998; Silver et al., 1996). The SCN relies primarily on photic cues to synchronize with the environment, where light information detected by the retina is transmitted directly to the central pacemaker to confer information about the time and phase of solar day. This induces a change in the onset of the clock within the SCN, corresponding to behavioral changes in subsequent cycles (Meijer &

Schwartz, 2003). In order to keep the vast network of cellular and tissue-level clocks in phase, the central clock must be able to not only perceive environmental time cues and integrate this time information into the clock machinery, but also transmit time information across the circadian network through humoral and electrical signals (Guo et al., 2006; Silver et al., 1996) and integrate feedback from peripheral clocks back into its molecular machinery (Pittendrigh, 1960). Central coordination of circadian rhythms thus serves two primary adaptive functions: (1) it allows for internal physiological functioning to maintain specific phase relationships with recurrent daily changes in the environment, and (2) it allows for internal alignment of physiological processes between and among tissues. These two features of the circadian network allow organisms to anticipate and prepare for recurrent daily changes in the environment and adjust their physiology and behavior accordingly (Daan & Pittendrigh, 1976; Pittendrigh & Daan, 1976).

Because individual cells each contain their own circadian clocks, these cells must be synchronized within their respective tissues, and tissues and organs coordinated into stable phase relationships with one another. To keep this web of circadian pacemakers in sync, cellular clocks adjust their phase and amplitude in response to stimuli that contain reliable time information, integrate this information into their molecular machinery, and communicate this information to other cells (Schibler & Sassone-Corsi, 2002). While the central circadian pacemaker receives light input through a direct mono-synaptic connection with the retina and therefore is strongly entrained to the ambient light/dark cycle, peripheral clocks often rely on non-photic cues (Pezuk et al., 2010). Under conditions free of external time cues (*i.e.,* continuous darkness or illumination), rhythms exhibit 'free running' features (Daan & Pittendrigh, 1976). They drift out of phase with the external 24h world at a period dictated by the endogenous speed of the

organisms' circadian oscillator. Remarkably, even in the absence of an external time cue, but with a few exceptions (Damiola et al., 2000; Stokkan et al., 2001), the countless circadian rhythms in the body free run indefinitely, locked in phase with one another (Dibner et al., 2010). However, this phase coordination can be challenged by the sensitivity of different clocks to various time cues (Yamazaki et al., 2000).

In complex multicellular organisms, the circadian network accommodates the conflicting needs for both rigid homeostatic mechanisms and flexible allostatic mechanisms to remain aligned with the environment. The vast array of circadian clocks throughout the body must be autonomous and self-sustaining to predict and adaptively respond to recurrent daily environmental features. However, these same clocks must also continuously adapt to changes within the environment and the body's own internal signals to organize individual cellular clocks and tissue level oscillators into a coherent network, thus coordinating an organism's behavior and physiology precisely with its environment. Understanding the mechanisms by which biological clocks individually keep time and integrate diverse time cues to communicate with one another is paramount to understanding how circadian biology affects psychological and physiological health.

Entrainment: the central circadian pacemaker

Because temporal adaptation is so vital to survival, organisms must adapt their behavior and physiology to align with predictable, recurrent environmental cues, a process known as entrainment. The environmental cues that confer time information to drive entrainment are known as *Zeitgebers*, from the German for 'time giver,' the most salient *zeitgeber* being light. During the process of entrainment, the endogenous period of a biological rhythm (also called tau;

τ: the speed at which an endogenous clock runs in the absence of environmental time cues) takes in the period of the entraining stimulus (T). In order for an organism to entrain to environmental cues, it must reset the period and phase of the free-running pacemaker each day, correcting for the difference between the period of the environmental time cue and the endogenous clock, adjusting τ to T (Pittendrigh & Daan, 1976). There are two contrasting formal models describing the mechanisms of entrainment: the parametric model and the non-parametric model. The non-parametric model describes how entrainment occurs through instantaneous phase shifts in response to discrete light pulses, since the clock is differentially sensitive to light cues at different phases of the circadian cycle. Phase response curves (PRCs) provide information about the sensitivity of the clock across the circadian day and the magnitude of the shift imposed by a *zeitgeber* at each phase. PRCs for light are generated by first transferring an organism to constant darkness, measuring the endogenous circadian rhythm, and then administering light pulses of a specific intensity and duration at each phase of the circadian cycle and measuring the magnitude of the resultant phase shift. Light pulses in early subjective night (e.g., CT15) will produce high amplitude phase delays, while pulses later in the subjective night (e.g., CT20) generate high amplitude phase advances. Under natural conditions, these time periods correspond to twilight hours, and indeed stable entrainment can be achieved in the lab with only one or two light pulses (or a 'skeleton' photoperiod). On the other hand, the parametric model describes the sensitivity of the circadian pacemaker to long-duration light stimuli (*e.g.*, constant illumination). The intensity of the light stimuli can also modify aspects of the circadian oscillator through continuous action. Jurgen Aschoff first described the response of nocturnal and diurnal animals to light of different intensities (Aschoff, 1960; Beaulé, 2009). For nocturnal species, such as mice, Aschoff's rule states that as light intensity increases, tau increases, and the active period

duration (alpha) decreases. Parametric and non-parametric effects on the circadian pacemaker likely act in concert to generate entrainment (An et al., 2022; Daan, 2000).

The formal actions of light on the circadian pacemaker are also reflected in physiology (see Fig.1 below). Photic cues received by rods and cones, as well as intrinsically photosensitive retinal ganglion cells, form direct monosynaptic projections with the SCN via the retinohypothalamic tract (RHT) (R. F. Johnson et al., 1988). Signaling by the RHT onto to SCN is primarily glutamatergic (Hannibal, 2002). The SCN also receives direct serotonergic input from the median Raphe Nucleus (Meyer-Bernstein & Morin, 1996) and neuropeptide Y (NPY) signaling from the intergeniculate leaftlet (IGL) (Harrington, 1997), important for transducing non-photic time cues (Mrosovsky, 1995; Webb et al., 2014). The retinorecipient ventral core of the SCN contains mostly non-oscillatory cells and is responsible for performing initial processing of information from input pathways (Albers et al., 2017). These calbindin (CalB)-expressing cells gate the photic entrainment of vasopressin (VP)-containing cellular circadian oscillators in the dorsal SCN shell (Hamada et al., 2003; LeSauter et al., 2009). The synchronization within the SCN is achieved via intracellular neurotransmitter and neuropeptide interactions including gamma aminobutyric acid (GABA), gastrin-releasing peptide (GRP), and vasoactive intestinal peptide (VIP) in the ventral core. GRP induces the phosphorylation of ERK and subsequent signaling cascade, resulting in the expression of *Per1* and *Per2* mRNA in cells in the SCN shell. VIP in particular is thought to drive network synchrony, as loss of VIP signaling resulted in desynchronization of SCN cells (Maywood et al., 2006). GRP, VIP, and GABA work synergistically to sustain the network-level oscillation of the SCN as well as mediate photic entrainment (Aton et al., 2005; Hamnett et al., 2019; Jones et al., 2018; Maywood et al., 2006; Ono et al., 2021). VIP neurons also provide timed GABAergic stimuli to output pathways,

suppressing activity during particular times of day and thus regulating the timing of physiological functions, such as heart rate and corticosterone secretion (Paul et al., 2020). SCN shell neurons contain both the receptor for VIP and also express arginine vasopressin (AVP), which plays a role in driving autonomous network synchrony and stabilization of circadian rhythms (Herzog et al., 2017; Mieda, 2019; Shan et al., 2020).

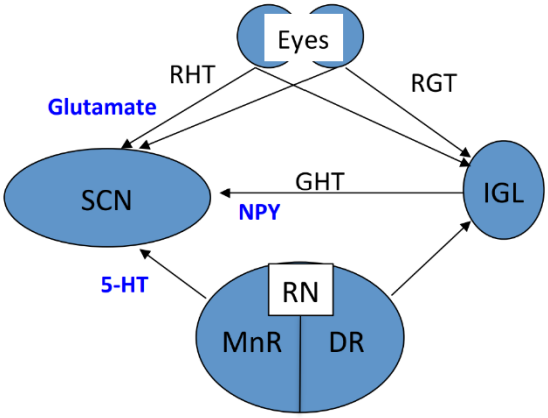

Figure 1: Direct and indirect pathways of entrainment into the SCN

RHT = retinohypothalamic tract; IGL = intergeniculate leaflet of the thalamus;
GHT = geniculohypothalamic tract; RN = Raphe nucleus; MnR = median Raphe nucleus;
DR = dorsal Raphe nucleus

Glutamatergic input from the retinohypothalamic tract signals light information gathered from the eyes. Serotonergic input from the median Raphe nucleus and neuropeptide Y from the intergeniculate leaflet communicate time information from non-photic cues to the SCN. These three pathways mediate entrainment of the SCN.

Entrainment: peripheral clocks

Peripheral tissues, in which every cell contains tissue-specific clock controlled genes, can create rhythmic responses to environmental input specific to that tissue (Balsalobre et al., 1998). For example, clocks within liver cells are primarily entrained by food, whereas the SCN

clock is preferentially entrained by light (Damiola et al., 2000; Stokkan et al., 2001). This sets up reciprocal coupling dynamics between the central and peripheral oscillator systems. Light acts through the SCN clock to control feeding time, while feeding entrains the liver clock. Because of this, altering the relationship between light and food cues can alter this coupling relationship, potentially shifting the phase of these oscillators to establish new temporal relations with one another. A concrete example of such dynamics is evident in the phenomenology of food entrainment. "Food anticipatory activity" (FAA) paradigms are capable of disentangling food-entrainable and light-entrainable circadian oscillators. In such paradigms, experiments are performed in which food is available only during a small window of time each day (usually 3-4h). This offering commonly occurs during the normal rest phase of the animals. When this feeding regimen continues for many days, animals become active in anticipation of the interval of food availability (*i.e.*, in the daytime, when they are normally inactive), while still maintaining a robust nocturnal pattern of activity. Crucially, the rhythm of gene expression within the SCN remains phase-locked to the light-dark cycle, while restricted feeding rapidly entrains the liver to the time of food availability (Stokkan et al., 2001). Thus, the expression of the light-entrainable and food-entrainable oscillators are revealed to be separate from one another in time (*i.e.*, in 'phase'). Experiments in which animals are food deprived for several days in a row after such daytime restricted feeding (RF) regimens have indicated that FAA is the result of an endogenous oscillator rather than a masking response, as animals continue to become active at the previous time of food delivery, even when no food is offered (Mistlberger, 1994).

Multiple clocks and misalignment

Circadian clocks are responsive to multimodal environmental stimuli including light, food, and social cues. This offers flexibility but also presents a system in which conflicting time signals from these cues can lead to misalignment between central and peripheral oscillators and/or between endogenous clocks and the environment. For example, circadian clocks in the SCN and clocks in the liver are typically in phase with one another. However, these clocks can be rapidly decoupled by phase misaligned (Hara et al., 2001), high-frequency (ultradian) feeding (de Goede et al., 2018), or even transiently by phase shifts in the light cycle, which suggests independence of these oscillators. Using a per1-luciferase rat model to identify the rhythmic expression of the clock gene per1, Yamazaki and colleagues found that phase advances or delays in the light dark cycle shifted circadian responses in the SCN, peripheral tissues, and behavior at different speeds (Yamazaki et al., 2000). Additionally, researchers investigated the phase and period of peripheral tissue clocks in SCN-lesioned mice with a per2-luciferase reporter (Yoo et al., 2004). Although the phase relationships between peripheral oscillators were disrupted following the SCN lesion, each tissue maintained a characteristic period, suggesting independence of these oscillators from the SCN. The SCN shifted almost immediately to the new light cycle, while the liver clock had not fully shifted even after 6 cycles. Additionally, restricted feeding (RF) paradigms where mice are allowed a 4h window that was 7h advanced from normal feeding time, followed by a 7h shift in the light dark cycle, show that while the peak of clock gene expression in the SCN followed this advance, no change in phase occurs in the liver clock (Damiola et al., 2000). Other high-frequency feeding paradigms provide food at 6 even intervals over the course of a day, eliminating the daily rhythm in feeding behavior while still maintaining the day/night difference in body temperature and locomotion. This results in no differences in temporal dynamics in the SCN but notable differences in rhythmic expression of genes involved

in glucose and lipid metabolism (de Goede et al., 2018). Taken together, these pieces of evidence suggest independence of the light-entrained oscillator in the SCN and the food-entrained oscillator in the periphery, despite their strong coupling under standard environmental conditions.

A major theoretical concept in the field of circadian biology lies in better understanding how central and peripheral circadian clocks interact with one another. There are many ways to alter the peripheral-central phase relationship. These include jet lag paradigms, presentation of light at night, repeated/constant phase shifting, and shift work models. These paradigms allow insight into how the coupling relationship between central and peripheral clocks impacts health.

Misalignment paradigms:

Jet-lag

A potent example of circadian misalignment both between internal oscillators themselves and between the internal milieu and the environment is evident in individuals who engage in long trans-meridian travel in short intervals of time (so called jet lag). In these individuals the internal clock ends up out of synchrony with the external conditions of the new time zone, and jet lag symptoms such as poor sleep, daytime tiredness, and appetite loss occur while the clock adapts to the new time (Forbes-Robertson et al., 2012; Haimov & Arendt, 1999). In animal models of jet lag, it has been demonstrated that oscillators that generate CRs in different traits adjust to the new time zone at different speeds (Abraham et al., 2010; Yamazaki et al., 2000), altering communication between oscillators and coordination of behavior and physiology with the new circadian phase. Jetlag paradigms have established how age, sex, and molecular clock

components interact. However, understanding how central and peripheral clocks communicate also has practical/clinical importance. Indeed, a number of studies suggest that misalignment of CRs plays a central role in numerous health disorders including cardiovascular disease (Merikanto, Lahti, Puolijoki, et al., 2013), diabetes (Chellappa et al., 2021; Mason et al., 2020), obesity (Arble et al., 2009; Baron et al., 2011), cancer (Kogevinas et al., 2018; Marinac et al., 2015; Salamanca-Fernández et al., 2018; Straif et al., 2007), and mood disorders (Baron & Reid, 2014; Chellappa et al., 2020; Lewy, 2009; Zou et al., 2022). Additionally, simulated chronic jet-lag paradigms impair memory task performance (Krishnan & Lyons, 2015; Smarr et al., 2014). This is readily modeled in mice as well: misalignment between central and liver oscillatory networks has, for example, been shown to lead to metabolic dysbiosis and obesity in WT mice (Mukherji et al., 2015).

Shift work

People who engage in 'shift work' live for several days of the week 8-16 hours out of phase with their non-shift working social environment; on 'off days,' they commonly adjust their activity/rest cycles back to a phase that matches that of their non-shift working peers, only to shift back to the lagged schedule when work resumes – amounting to a major jet lag event every week (Czeisler & Gooley, 2007; Wittmann et al., 2006). Shift work causes circadian misalignment among central and peripheral oscillators, and has been linked to adverse metabolic consequences in humans (Scheer et al., 2009; F. Wang et al., 2014). For example, a higher risk of metabolic syndrome was found in night shift workers (Cheng et al., 2021; X. Yang et al., 2021), despite evidence that shift workers are more physically active than non-shift workers (Loef et al., 2017, 2018). Forced desynchrony protocols in which individuals are forced to adopt days outside the range of circadian entrainment (*e.g.*, 28h days) allow for additional empirical study of this

phenomenon. Notably, when subjects' food intake and sleep rhythms were 12h out of phase with their habitual rhythms, subjects exhibited physiological markers indicative of a prediabetic state (Scheer et al., 2009). In shift work, partitioning of activity to times other than daytime represents a circadian misalignment between peripheral signals (including food intake) and the central clock, which is entrained to (adapted to align with) environmental cues such as the light/dark cycle.

Sex Differences in Circadian Rhythms

Biomedical research has long omitted the use of females in pre-clinical research, largely due to a belief that, because females have hormonal cycles, they exhibit more variability than males. For this reason, single-sex studies of males have outnumbered females 5.5 to 1 (Beery & Zucker, 2011). However, randomly cycling females are no more variable than males on any behavioral, morphological, physiological, or molecular trait (Prendergast et al., 2014). This is not to say females and males are biologically identical, as there exist sex differences in many biological processes from pain signaling to drug metabolism (Zucker et al., 2022). Circadian rhythms research is no exception to sex disparities in samples, despite sex differences in circadian organization and activation being long evident.

Sex hormones exert influence on circadian free-running period, where removal of gonads and thus endogenous circulating hormones lengthens period, and replacement with exogenous hormones shortens period (Albers, 1981; Morin et al., 1977). The first systematic report of sex differences in chronobiology and their biological underpinnings showed that circadian period changes in responses of hamsters to estradiol are sexually dimorphic and depend on organizational effects of androgens early in life (Zucker et al., 1980). Specifically, testosterone

treatment early in life masculinizes the SCN and prevents the normal female-typical estradiol-induced shortening of tau in adulthood. Estrogen impacts both the phase and amplitude of activity, with elevated levels causing phase advances, consolidation of activity to the dark phase, and increases in total amount of activity (Morin et al., 1977). Estradiol also impacts the function of the SCN clock in response to environmental cues. For example, exogenous estradiol administration in rodents enhances precursor transcription factors to the expression of clock genes in the SCN (Abizaid et al., 2004), altering responsiveness to light pulses administered in the late subjective night (Blattner & Mahoney, 2015). Additionally, a greater number of estrogen receptors of alpha and beta subtypes are found in the SCN of female compared to male mice (Vida et al., 2008). These subtypes are responsible for different circadian consequences of estrogen. ERα increases total activity, activity amplitude, and proportion of activity consolidated to the dark phase, while ERβ delays acrophase (time of peak activity) but advances activity onset (Royston et al., 2014).

Sex differences in responses to circadian misalignment have also been identified in many areas of physiology and behavior, but careful comparison of these differences in a controlled misalignment paradigm is still far from completion. In humans, females have been shown to have a greater impairment than males in cognitive performance during shift work (Santhi et al., 2016). Some studies have also shown sex differences in prevalence of diabetes mellitus and metabolic syndrome in shift workers, though results have not always been consistent (Gan et al., 2015; Guo et al., 2006; Karlsson et al., 2001; Khosravipour et al., 2021; X. Yang et al., 2021). Additionally, female shift workers exhibit higher risk for developing breast cancer (Hansen, 2017; Szkiela et al., 2020), with the International Agency for Research on Cancer (IARC) classifying shift work involving circadian disruption as a possible human carcinogen

(Stevens et al., 2011). These differences might be results of baseline sex differences in circadian organization. For example, females have shorter endogenous circadian periods compared to males (Duffy et al., 2011), which could make adjustment to a later work schedule more taxing. Males have also been shown to have more stable entrainment (Davis et al., 1983), which may represent a sex difference in the reliance on and sensitivity to external time cues. In this way, sexually dimorphic circadian clock organization could impact misalignment both in terms of external and internal synchrony, and this clock organization could be mediated through differences in sex hormones.

Circadian misalignment and health

CRs in mammals are strongly tied to many facets of cardiovascular, metabolic, and cognitive health (Benca et al., 2009; Coogan & Wyse, 2008; Sahar & Sassone-Corsi, 2009; M. E. Young & Bray, 2007). For example, participants were subjected to a 28h day to dissociate endogenous circadian rhythmicity from behavior; during periods in which subjects slept and ate 12h out of phase from their endogenous rhythm (maximum circadian misalignment) they exhibited increased mean arterial pressure, insulin, and glucose secretion (Scheer et al., 2009). This mirrors a study done by Baron and colleagues who reported a correlation between calories consumed after 8:00pm and BMI (Baron et al., 2011). Similarly, mice fed during the light (inactive) phase consumed the same number of calories but gained twice as much weight as mice fed only during the active phase (Arble et al., 2009). Additionally, mice fed a high-fat diet only during the active phase did not develop metabolic syndrome, unlike those that were fed ad libitum or during the light phase (Hatori et al., 2012).

Chronotype is an individual's preferred timing of sleep and activity, and human chronotypes can be classified on a continuous scale (*MCTQ*, n.d.). Operationally, chronotype is often measured through the Munich Chronotype Questionnaire (MCTQ), which assesses sleep behavior via the midpoint of sleep on free days corrected for sleep debt accumulated on work days (Juda et al., 2013; Wittmann et al., 2006). Environmental influences such as sex, age, and genetic variations contribute to chronotype. Though humans show large variations in chronotype, we are subjected to the same social obligations, such as school and work. These social schedules can interfere considerably with preferred times of sleep and activity. Social jet lag refers to a form of circadian misalignment arising from the discrepancy between sleep/wake on work days versus on free days (Wittmann et al., 2006). Individuals with evening chronotypes, who initiate sleep onset later, show the greatest misalignment between free and working days, leading to considerable sleep debt on work days. Similar to other forms of circadian misalignment, social jet lag is associated with adverse health outcomes (Caliandro et al., 2021), such as development of metabolic disease (Roenneberg et al., 2012). Additionally, there is an association between evening chronotype and risk for development of mood disorders such as depression (Baron & Reid, 2014; Chelminski et al., 1999; Gaspar-Barba et al., 2009; Hirata et al., 2007; K. M. Kim et al., 2020; Kitamura et al., 2010; Merikanto, Lahti, Kronholm, et al., 2013) and bipolar disorder (Ahn et al., 2008; Chung et al., 2012; Giglio et al., 2010; Mansour et al., 2005; Wood et al., 2009). Interestingly, there is an association between the alteration in some circadian clock proteins and mania-like behaviors (hyperactivity, decreased sleep, lowered depression-like behavior, and increased reward sensitivity) in mice (Roybal et al., 2007). Additionally, decreased circadian amplitude and higher intra-daily variability is associated with higher risk of developing mild cognitive impairment and/or Alzheimer's dementia (P. Li et al., 2020; Tranah et al.,

2011). There are strong links between circadian misalignment and decreased health outcomes in many facets of physiology and behavior, and the direct manipulation of this misalignment remains to be explored.

Alzheimer's Disease

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by deterioration of cognitive functions and other neuropsychiatric symptoms (Ballard et al., 2011; Scheltens et al., 2016). Though, the disease is most commonly associated with cognitive issues such as memory loss, patients can also exhibit language impairment, motor skill issues, and perception issues that contribute to impairments in performing daily life activities (Neugroschl & Wang, 2011). AD is currently the most prevalent form of dementia (a decline in both intellectual functioning and the capacity for independence in daily activities), representing 2/3 of dementia cases and 50 million patients worldwide, and this number is projected to double every 5 years (Breijyeh & Karaman, 2020). Despite the debilitating nature and prevalence of this disease, there currently exist only two disease-modifying treatments, one of which has recently been the source of some controversy due to poor efficacy in modifying disease-related cognitive and functional measures (Tampi et al., 2021; Tan, 2022). Also complicating treatment, AD exhibits a long pre-clinical phase of ~20 years, and an average clinical duration of only 8-10 years (Masters et al., 2015). This late diagnosis in terms of disease etiology means treatment is administered well into disease progression. For this reason, recent efforts have investigated the potential of early lifestyle interventions to delay AD progression or prevent development entirely (Dhana et al., 2020).

On a physiological level, AD is characterized by an accumulation of aggregated amyloid and tau proteins throughout the brain, leading to the development of beta-amyloid plaques and tau tangles respectively (Armstrong, 2009; Serrano-Pozo et al., 2011; Spires-Jones & Hyman, 2014). Amyloid proteins are produced by the breakdown of amyloid precursor protein (APP) by secretases (Crouch et al., 2008; Selkoe & Hardy, 2016). The location of these secretases is altered by presenilin (PS1 and PS2), with PS1 increasing the likelihood of producing protein lengths that are more likely to form aggregated 'clumps' (Jankowsky et al., 2004; Kikuchi et al., 2017). These aggregated amyloid proteins accumulate between neurons, disrupt cell function and prevent nutrient distribution, eventually killing the cells (Abramov et al., 2009; Cras et al., 1991; Selkoe & Hardy, 2016). Additionally, these protein deposits activate an immune response, triggering inflammation and autophagy of cells (Hur et al., 2020; M.-M. Wang et al., 2018; S. D. Yan et al., 1996). Importantly, amyloid plaques begin forming at least two decades before tau pathology and the onset of clinical behavioral impairment (Bateman et al., 2012; Sasaguri et al., 2017). Neurofibrillary tangles form via the detachment and aggregation of tau proteins from the microtubules that structurally support neurons (Grundke-Iqbal et al., 1986). These tangles block neuronal transport and synaptic communication (Gómez-Isla et al., 1997; Spillantini & Goedert, 2013). Interestingly, plaque and tangle processes appear to be connected, where once amyloid plaque aggregation reaches a critical threshold, there is a rapid spread of tau throughout the brain (Ismail et al., 2020; Tanzi & Bertram, 2005). This impaired synaptic connectivity and eventual cell death leads to reduced cognitive function over the course of the disease (Hyman et al., 2012; Neugroschl & Wang, 2011).

AD mouse models

A common tool for investigating AD treatments are transgenic mouse models expressing AD genetic variants. While there are many AD transgenic mouse strains, mice expressing cDNA encoding familial AD–linked human Swedish mutation of amyloid precursor protein mutation (APPSWE) and presenilin 1 deleted in exon 9 (PS1ΔE9) variants - known as APP/PS1 mice - are among the most commonly studied models (Trinchese et al., 2004). Overexpression of these transgenes results in overproduction of APP and PS1 splice variants that increase amyloid-beta plaque formation (Jankowsky et al., 2004). APP/PS1 mice also display AD-related behavioral phenotypes including cognitive impairment (Sadowski et al., 2004), increased behavioral anxiety (Meng et al., 2020) and other related symptoms (Malm et al., 2011). This mouse model, however, also produces amyloid aggregates in peripheral tissues (*e.g.,* blood vessles, liver, and gastrointestinal tract) in addition to the brain, complicating interpretation of whether behavioral impairment is due to an impact of these aggregates on other functions (L. Zhang et al., 2021). To solve this problem, the APP/PS1-21 mouse uses a Thy1 promoter to express these AD transgenes, making Aβ plaques brain specific (Radde, 2006). Because plaques in this model are only present in the brain, rigorous testing of how peripheral-specific interventions to manipulate peripheral-central alignment, such as food timing, can impact disease etiology in the central nervous system is merited. The APP/PS1-21 mouse also develops plaques as early as 6 weeks of age and has a highly stereotyped pattern of plaque deposition, making it an ideal model in which to test how interventions early in plaque development can impact the etiology of AD.

Alzheimer's Disease and Circadian Rhythms

AD patients show clear circadian disturbances in addition to the more commonly recognized behavioral symptoms (Coogan et al., 2013; Hatfield et al., 2004; Hu et al., 2009; Skene & Swaab, 2003; Witting et al., 1990; Y.-H. Wu et al., 2006). AD patients exhibit nighttime restlessness and daytime drowsiness (Coogan et al., 2013; Hu et al., 2009), fragmentation and reduced amplitude of CRs (Hatfield et al., 2004; van Someren et al., 1996), altered rhythmic expression of melatonin (Y.-H. Wu et al., 2006), and a shift in diurnal feeding pattern with more calories consumed earlier in the day (K. W. H. Young et al., 2001; K. W. H. Young & Greenwood, 2001). Neural dysfunction and neurodegeneration has been observed within the SCN, but it is unknown whether this is a consequence or a contributor to AD development (Leng et al., 2019; Singer & Alia, 2022; J. L. Wang et al., 2015). Transgenic mouse models for Alzheimer's disease have also demonstrated circadian changes. Both mouse and drosophila AD models demonstrate a bidirectional relationship between the development of AD and circadian misalignment (Chauhan et al., 2017), where circadian dysfunction increases likelihood of Alzheimer's development and/or Alzheimer's development increases circadian dysfunction. For example, AD transgenic mice with deletion of a prominent circadian gene, BMAL1, demonstrated increased plaque formation relative to AD transgenic mice with a functioning circadian clock (Kress et al., 2018). Additionally, reduced circadian rhythm amplitude and power and delayed phase predicted later development of dementia in women (Tranah et al., 2011). These reciprocal interactions between disease states and circadian functioning complicate understanding of how circadian rhythms contribute to AD etiology. This represents a major unmet need in our understanding of how the alteration of circadian rhythms may inform AD treatment before the onset of clinical impairment. **A causal relationship has yet to be established between misalignment of circadian clocks in the brain and body and**

increased AD pathology. Should circadian misalignment be shown to impact plaque deposition in a mouse model of AD, this could translate to a site for preventive or even a treatment mechanism for human patients.

Circadian-Olfactory Axis

In addition to the circadian clock within the SCN, evidence indicates that the olfactory system also contains a circadian oscillator (Granados-Fuentes et al., 2004; Guilding & Piggins, 2007). It stands to reason that this might translate to an impact of time of day on olfactory sensitivity (Amir et al., 1999), which raises interesting questions about autonomy/interactions of olfactory network and SCN oscillators. Using a forced desynchrony protocol in which contributions from intrinsic circadian physiology can be divorced from the influence of time of day (number of hours awake), odor detection was found to vary across circadian phase for human adolescents (Herz et al., 2018). Specifically, peak olfactory sensitivity occurred during early biological night, reaching a minimum during biological day. This circadian variation in olfactory sensitivity points to a bidirectionality between these two systems. Lesioning the SCN in mice does not abolish rhythms in the olfactory system (Granados-Fuentes et al., 2011), but removing the olfactory bulb (OB) alters the period of locomotor activity rhythms (Granados-Fuentes et al., 2006; Perret et al., 2003; Pieper & Lobocki, 1991). Given the aforementioned influences of the circadian clock on AD, attention should be paid to the reciprocal influences of olfaction and circadian function, and how these might be impacted by degeneration in response to disease, such as in AD.

Early onset olfactory dysfunction in AD

Furthering the connection between olfaction and AD, APP/PS1 transgenic mice and human patients with AD both demonstrate amyloid beta mediated degeneration in the brain, beginning with early degeneration in the olfactory bulb (Ubeda-Bañon et al., 2020). This pattern mirrors the etiology of neurodegenerative disorders like Alzheimer's with olfactory deficits preceding clinical onset of cognitive decline (Vasavada et al., 2015). Increasingly severe olfactory dysfunction in both magnitude and progression over time correlates with greater clinical impairment in AD patients (Velayudhan et al., 2013). Patients with more severe clinical impairments exhibited lower smell identification scores, and during a 3-month follow up, more rapid decline in olfactory identification scores correlated with greater symptomatic severity. Different types of olfactory dysfunction are differentially impacted by various neurodegenerative diseases. Types of olfactory dysfunction include detection (ability to perceive the olfactory stimulus), identification (correctly naming olfactory stimuli), discrimination (identifying the odd olfactory stimulus out of a set), and recognition (identifying an originally presented olfactory stimulus among several choices following a delay). While the definitions of these types of olfaction vary throughout the literature, it is commonly accepted that detection relies on peripheral perceptual ability of the subject while the other olfactory parameters depend somewhat on integration from higher cognitive areas. Compared to other human neurodegenerative diseases, olfactory identification and recognition were found to be more severe in AD, while the olfactory detection threshold was not similarly impaired (Rahayel et al., 2012). This indicates that olfactory impairment in AD might involve more advanced olfactory cognitive processing than in other neurodegenerative diseases, positioning olfactory dysfunction as an important early tool for differentiating and recognizing Alzheimer's Disease in order to implement early treatment efforts.

Olfactory discrimination in AD transgenic mice

Discrimination between olfactory parameters that are more reliant on perception (*i.e.,* detection) or on higher cognitive computations can be evaluated both in humans and animal models, since the olfactory system is highly conserved across species (Ache & Young, 2005). Additionally, similar to human AD patients, olfactory dysfunction correlates with amyloid burden in AD mouse models, with plaque deposition beginning in the olfactory bulb. Odor cross-habituation assesses multiple olfactory parameters including novel odor investigation, odor learning and memory (habituation) and discrimination (cross-habituation). APP/PS1 mice demonstrate deficiencies in odor habituation and increased latency to habituate across odor presentations starting at only 3 months of age (Wesson, Levy, et al., 2010). This is likely related to the early onset of amyloid deposition in the olfactory bulb beginning at only 3 months of age in that model, prior to plaque deposition in any other brain area. APP/PS1 mice perform worse than wild-type (WT) mice on olfactory discrimination between attractive and aversive odors and have a longer latency to uncover food pellets (Yao et al., 2016). Taken together these data suggest that the ability of AD transgenic mice to identify and differentiate scents may be impaired due to plaque deposition. However, this has yet to be empirically examined in the APP/PS1-21 mouse model, which develops plaques only within the brain. The stereotyped plaque development of APP/PS1-21 mice, beginning in the olfactory bulb, allows for testing of olfaction at the very beginning of plaque deposition and long before onset of cognitive behavioral impairment. **Should differences in olfaction be present at this early stage of plaque deposition, this could inform development of an early detection paradigm for human patients.**

Aims

Here I describe experiments that examine the role of coordination between the endogenously rhythmic individual and its rhythmic environment on metabolic and neurological health. In Chapter 3, I use a genomic manipulation of the circadian pacemaker to examine how disruption of the internal circadian clock affects coordination/entrainment with environmental lighting cycles in a sex-specific manner. In Chapter 4, I examine how circadian misalignment impacts metabolic and behavioral endpoints in an Alzheimer's transgenic mouse model (APP/PS1-21). Finally, in Chapter 5, I examine olfactory function in APP/PS1-21 mice at the onset of plaque deposition with experiments that examine the potential diagnostic utility of the early-onset olfactory dysfunction evident in AD and other neurodegenerative diseases. Our bodies are both responsive to and dependent on environmental cues. The following work seeks to understand how environmental time cues and olfactory information interact with behavior and physiological health.

Chapter 2: Materials and Methods Common to Many Experiments

Below, I summarize and provide an overview of the experimental methods and concepts used in the following work. Experimental methods, reagents, calculations, and procedures are provided at a more extensive level of detail within the respective Chapters 3-5.

Circadian Manipulations

Mouse locomotor activity (LMA) was recorded during multiple circadian manipulations in Chapters 3 and 4 to measure entrainment to environmental cues such as light and food, as well as endogenous, free-running rhythms under constant conditions. Standard chronobiology metrics are outlined below.

Activity monitoring and telemetry

Mice were housed in polypropylene cages equipped with passive infrared motion detectors (PIR) to monitor locomotor activity, as previously described in detail (Prendergast et al., 2012). All cages were equipped with overhead passive infrared (PIR) sensors to monitor locomotor activity. Breaks in the infrared beam were recorded in 1-minute bins using the ClockLab Acquisition software (Actimetrics, Wilmette, IL, USA).

Circadian activity measures

Activity data was visualized and analyzed using Clocklab Analysis 6 software (Actimetrics). Characterization of circadian chronotypes in mice were performed via methodology described previously (Zheng, 1999; Zheng et al., 2001): double-plotted actograms were scored by an experimenter blind to sex, photoperiod, genotype, and/or food manipulation. Total daily activity counts in LD were derived from Clocklab over a 10-day interval. Parametric

statistical tools such as an analysis of variance (ANOVA) are not suitable for the detection of rhythmicity (Refinetti et al., 2007), so Fourier-based techniques, in which time series are decomposed into summations of different sin waves, are often used. Lomb-Scargle periodigram (LSP) allows for generation of a Fourier-like power spectrum even when using a sample of unevenly sampled data, accomodating data drop out better than other methods (Ruf, 1999). Clocklab Analysis 6 software (Actimetrics) computes LSP and displays periodigram peaks, with an adjustable significance level. LSP can underestimate the strength of the circadian component, so onsets and offsets of activity are often called by hand with phase shifts quantified using projected onsets (Refinetti et al., 2007). In Chapter 4, locomotor activity during the time of food availability relative to overall activity was compared between feeding groups using ClockLab Analysis 6 software (Actimetrics, Wilmette, IL, USA) to analyze circadian distribution. Activity profiles and actograms showing the average of activity across selected days for each group were also generated using Clocklab.

Actogram

Mouse activity over successive days is visualized using actograms. The x-axis indicates time of day, and the y-axis denotes successive days of the experiment. Annotations across the top abscissae indicate the timing of the light/dark cycle, white lights on being represented by a white bar and lights off being represented by a black bar. See Fig. 2 below for example.

Onset, offset, alpha

Activity onset is the time at which the active phase begins, and offset denotes the time the inactive/rest phase begins. In a nocturnal animal such as a mouse, activity onset begins around lights off, and offset begins around lights on each day. The time elapsed between activity onset and offset is referred to as alpha, the duration of the active phase.

Onset tau

When a mouse is placed in constant conditions it begins to free-run at the speed of its endogenous clock, referred to as tau. Tau can be calculated by using activity data collected from mice housed in constant conditions using ClockLab's least-means-squared line drawn through consecutive days of activity onsets, with the slope of that line being the speed of the endogenous circadian clock. Circadian period was also calculated using a Lomb-Scargle Periodogram analyses (LSP) on 10 days of activity data (Ruf, 1999; Tackenberg & Hughey, 2021).

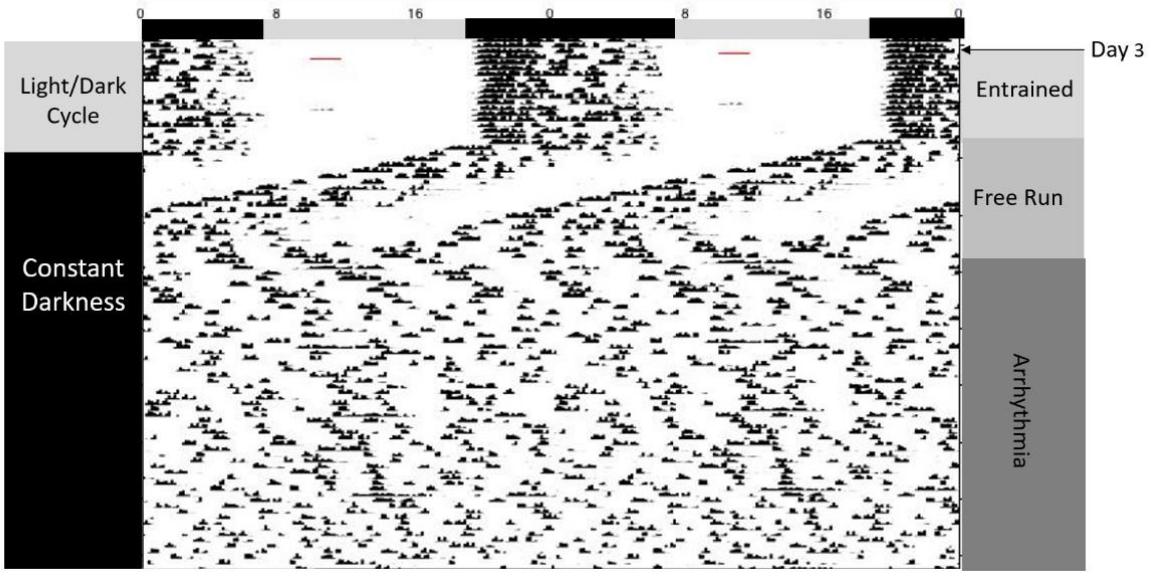

Figure 2: Representative Double-Plotted Actogram

The x-axis depicts time, with two days of activity plotted side by side. The y-axis depicts successive days. Light and dark bars across the top of the plot denote lights on and lights off times of a photocycle. Tick marks denote activity counts in 1-minute bins. This actogram demonstrates three states of rhythmicity including entrainment to a light-dark cycle, free-running activity in constant conditions (in this case constant darkness; DD), and arrhythmic activity.

Behavioral Tests

Four types of behavioral testing were used throughout these experiments to assess cognitive behaviors related to neurodegenerative disease and olfaction. (A) marble burying as a measure of behavioral anxiety, (B) T-maze spontaneous alternation as a measure of spatial working memory, (C) olfactory cross-habituation to test the ability of mice to habituate and generalize to odors, and (D) a digging task to assess the ability of mice to discriminate between a rewarded odor and related odors.

Marble Burying Test

The marble burying test has been purported to measure compulsive anxiety and repetitive behavior, common clinical features of Alzheimer's Disease and dementia. However, some experiments have claimed an increase in marble burying equates to increased compulsive anxiety (Cipriani et al., 2013; Gitlin et al., 2012), while other studies have equated reduced marble burying with increased compulsive anxiety (Broekkamp et al., 1986; de Brouwer et al., 2019; Deacon, 2006; Londei et al., 1998; Njung'e & Handley, 1991; Takeuchi et al., 2002; Thomas et al., 2009). Reduced marble burying has also been described as denoting decreased exploratory behavior (Moreno et al., 2017), and neophobia (Kemppainen et al., 2014; T.-K. Kim et al., 2012; Vepsäläinen et al., 2013). Alzheimer's transgenic mice with mutations in APP and PSEN1 (APP/PS1) have been shown to bury fewer marbles than Ntg mice (Day et al., 2023; Keszycki et al., 2023; T.-K. Kim et al., 2012), though there exist reports demonstrating the opposite effect (Peng et al., 2021; Torres-Lista et al., 2015; Q. Zhang et al., 2018), complicating interpretation of this task. We aimed to assess how this behavior was altered in a specific strain of AD transgenic mice before and after experimental interventions. Trials consisted of placing each mouse in a clean cage filled with 3cm of corncob bedding for 10 minutes to acclimate to the new

environment and then returning the mouse to their original cage. Then, 15 marbles were spaced evenly within the cage, and the mouse was placed back in this cage for 10 minutes. Marble burying was performed at ZT 2-4 and ZT 14-16 for all mice. The number of marbles buried (covered >50% with bedding) and the number of marbles moved after the 10-minute period was recorded and analyzed by an experimenter blind to treatment group.

T-Maze Spontaneous Alternation

Spontaneous alternation exploits the natural exploratory behavior of rodents to assess spatial working-memory performance, with a higher percentage of alternation indicating better cognitive behavioral performance. Spontaneous alternation (SA) tests were carried out using a T-maze with 15cm high walls that consisted of a 10cm x 10cm starting box off of a 40cm long start arm with two 20cm long choice arms extending on either side. The start box and the 2 choice arms were blocked off from the start arm with guillotine doors. All trials were recorded with a camcorder equipped with infrared night vision positioned above the T-maze. The mouse was placed inside the start box for 5 seconds before the start door was opened. For the first trial, only one choice arm door was opened (forced trial). The mouse exited the start box to the start arm, then to the open choice arm, and eventually wandered back into the start box, at which point the start box door was closed for another 5 seconds. For all subsequent trials, both choice arm doors were raised upon opening of the start box door. Once the mouse exited the start box and selected a choice arm, the other choice arm was closed off. If the time of a trial exceeded 2 minutes, the mouse was carefully placed back into the start box and a new trial was started. This free choice procedure was repeated 13 times for each mouse. The entire SA procedure was performed at ZT 2-4 and ZT 14-16 for all mice. Experimenters blind to condition recorded the number of spontaneous alternations made (entering the right goal arm after the left goal arm was entered

and vice versa). The number of alternating entries made was divided by the total number of free choice entries made and times by 100 to get a percent of alternation.

Olfactory Cross-Habituation Testing

Testing took place in a conventional cage devoid of bedding material. Mice were tested in one session, either during the light phase between ZT6-8 or during the dark phase between ZT18-20. Odors were diluted to a standard vapor pressure of 1Pa in mineral oil and applied to filter paper in 60μL aliquots, which were enclosed inside a metal tea ball to prevent contact of the liquid odor with the testing chamber or animal, yet still allow for volatile odor delivery. Odors were delivered in 11 successive trials of 50s each where the tea ball was placed in the center of the testing chamber, with a 5-minute inter-trial interval. The test consisted of 1 presentation of a blank (plain mineral oil), followed by 3 presentations of propanol (Habituation Odor; OHab). The test odors were then presented, interleaved with presentations of OHab to reinforce habituation. The order of test odors was pseudorandomized across mice, with an attempt to balance the order of presentation across subjects within a condition (sex, genotype, and time of testing) as much as possible. The duration of time spent investigating (snout-oriented sniffing within 1cm of the odor) was recorded across trials by a single observer blind to genotype.

Odor-Cued Digging Task

Mice were trained to dig in a small glass petri dish (60mm diameter) filled with corncob bedding for a food reward (1/4 honey nut cheerio, stale to decrease olfactory cues from the cereal) until they reached criterion (initiated digging within 10sec). Mice were then presented with 2 dishes, one scented with odorant diluted in mineral oil, and one unscented with just mineral oil. The mice were then trained to dig in the scented dish for a reward until they reached

criterion (digging in correct dish 90% of the time). Each test trial was 20 seconds long, after which the animal was removed from the arena and placed back in the start chamber. Odor testing sessions began with 10 training trials in which the mouse learned to dig in response to the training odor and avoid digging in the unscented control dish. The mouse was then tested on a set of odors (including the trained odor) that were presented in counterbalanced order across mice. In these test trials, no reward was present in the dish. The amount of time spent digging in the scented dish was recorded. To avoid extinction, one to three reinforcement trials with the trained odor were interspersed within the testing trials. Generalization was measured as significant digging in an odor other than the training odor. Identification was measured as significant digging in the training odor.

Genotyping

Genotyping of all mice bred in our vivarium was done using primers from the Jackson Lab Website and using specification for the Platinum Taq Polymerase (Life Technologies, Invitrogen catalog number: 10966-018). For each individual PCR reaction: 16.65 uL of DNAase free H2O, 2.5 uL of 10X PCR Buffer with no $MgCl_2$, 0.75 uL 50mN $MgCl_2$, 0.5 uL 10mM dNTP mix, 0.5 uL of each primer, and 0.1 uL of Taq were added to a master mix and thoroughly mixed by pipetting up and down. 22 uL of master mix were aliquoted and added to 3 uL of DNA from HotShot (Truett et al., 2000). Representative APP/PS1-21 gels with genotype interpretation are in Fig. 3 below.

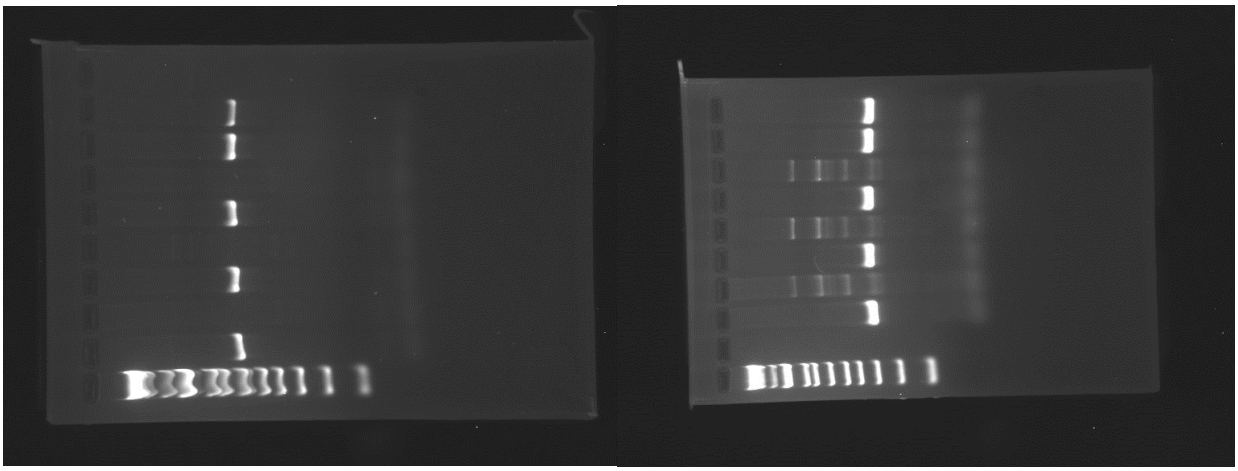

Figure 3: Representative APP and PS1 gels and interpretation of genotype

Representative gels denoting APP (left) and PS1 (right). Resultant bands (amplicon sizes) per Jackson Lab Website were as follows: PS1 = ~300 bp, APP = ~500 bp. Due to cointegration of the transgenes, for routine analysis only genotyping for APP was done, with PS1 genotyping done only sporadically as a control and for genotype confirmation.

Statistics

Differences between groups in each experiment were evaluated in most instances with parametric statistics in the form of an analysis of variance (ANOVA); the F statistic is robust to violations of sample size inequality or normality (Lindman, 1974). To control for alpha inflation and Type I error, pairwise comparisons were performed using two-tailed t-tests, where justified by a significant omnibus F statistic, except in instances of *a priori* planned comparisons. In the case of *a priori* planned comparisons despite a non-significant F statistic, a Tukey/Kramer test was used to evaluate pairwise comparisons while controlling for familywise alpha. When there were unequal groups, an equality of variance F-test was performed to assess any violations of the assumption of equal variance before performing the ANOVA. If an F-test indicated a violation in the assumption of variance, nonparametric methods were used. To evaluate differences in factors with 3 levels, a Kruskal-Wallis Test for overall differences with a Dunn correction to evaluate pairwise comparisons was used. Comparisons between factors with two levels were evaluated

with a Mann-Whitney U Test. All statistical comparisons were performed using StatView software (SAS). Differences were considered significant if $p<0.05$, unless otherwise specified by a specific non-parametric method (where noted).

In olfactory behavioral tests, sniff time and digging time data were normalized to z scores (zero mean and unit standard deviation) for each mouse for each test day, as reported in earlier studies (Kay, 2003; Kay et al., 2005; Nusser et al., 2001). We normalized in this fashion because sniffing and digging times varied widely across mice, and this made for easy application of post hoc comparisons. Normalized data were analyzed with analysis of variance (ANOVA), followed by pairwise comparisons using two-tailed t-tests, where justified by a significant omnibus F statistic.

Chapter 3: Aberrant parametric and non-parametric responses to light in mice with a mutation in the core clock gene Period2 (Per2): effects of sex and gene dosage

Abstract

Circadian rhythms are driven by a molecular clock which generates self-sustaining light-synchronized oscillations with a period of ~24h. The effect of light is two-fold: to shift the period and phase of endogenous rhythms to align them with the environment, an effect referred to as entrainment, and to directly inhibit or stimulate behavior, an effect referred to as masking. In the laboratory, endogenous circadian rhythms can be observed following the removal of external time cues, such as the alternation of light and dark, as well as through responses to acute light pulses. The relative contributions of several individual circadian clock genes to entrainment and masking effects of light have been examined, but these investigations have systematically excluded reporting data from females. Numerous sex differences exist in the circadian system. Thus, we do not know the extent to which sex affects the role of specific clock genes. Here we examined circadian parametric responses to constant light of different intensities (DD→dimLL→brightLL→DD) and non-parametric responses to discreet light pulses in mice with zero (*per2*$^{m/m}$; MUT) or one (*per2*$^{-/m}$; HET) copy of a functional *per2* gene, and in wild-type (WT) controls. Lack of functional *per2* dampened activity and prevented the circadian period lengthening and active phase length (alpha) compression in LL predicted by Aschoff's Rule. Additionally, functional *per2* gene copy number affected the ratio of phase delay and phase advance in a gene dosage dependent fashion, with MUTs and HETs showing increased phase advances relative to WTs. Together, these results demonstrate that *per2* mediates parametric and non-parametric entrainment to light and does so differently in males and females.

Background

In mammals, the circadian pacemaker is located in the suprachiasmatic nucleus of the hypothalamus (SCN) and facilitates entrainment to the environmental light:dark cycle through a direct photic input that is distinct from the image forming visual system. Direct projections from retinal ganglion cells to the hypothalamus convey photic information to the SCN, forming the retinohypothalamic tract (RHT) (Morin & Allen, 2006). Photic entrainment is lost after bilateral transection of the optic nerve or RHT, but not after bilateral transection of the optic tract, the canonical visual imaging pathway (R. F. Johnson et al., 1988). So important is the process of circadian entrainment to the environment, it operates on a distinct photic input pathway, allowing those who are blind but still have intact RHTs to be spared from a number of circadian entrainment deficiencies that are present in individuals whose blindness is due to retinal pathology. In this way, the SCN has direct access to photic information from the environment to facilitate alignment of endogenous circadian rhythms to environmental conditions (Czeisler et al., 1995).

The SCN is a collection of ~20,000 neurons, which contain molecular and biochemical autoregulatory oscillators (Silver et al., 1996). This transcriptional-translational feedback loop (TTFL) is generated by 10-12 core clock genes, which reciprocally inhibit and stimulate each other's expression, cycling with a period of approximately 24 hours as its protein products degrade (Reppert & Weaver, 2002). The core loop consists of the transcription factor CLOCK and brain and muscle Arnt-like protein-1 (BMAL1), which dimerize and bind to an E-box promoter to initiate transcription of the Period (*per1*, *per2*, *per3*) and Cryptochrome (*cry1*, *cry2*) genes. This promotes expression of per and cry genes and thus of PER and CRY proteins, which oligomerize, translocate back into the nucleus, and inhibit the action of CLOCK:BMAL1, thereby inhibiting their own transcription (Dunlap, 1999; Shearman, Sriram, et al.,

2000). Secondary loops primarily composed of REV-ERBα and RORα regulate this core loop by acting on bmal1 transcription, with REV-ERBα inhibiting and RORα promoting this transcription (Guillaumond et al., 2005). In particular, REV-ERBα is highly expressed in the liver (Cho et al., 2012), where one of the most important peripheral circadian clocks exists, especially in regards to metabolic function.

As described in Chapter 1, there exist multiple animal models of circadian misalignment with the external environment. Environmental cues interact with the genome to direct changes in physiology and behavior, and our relatively comprehensive knowledge of the TTFL provides abundant opportunity to examine gene-environment interactions in the mediation of circadian entrainment and misalignment. Mutations in TTFL genes result in diverse chronotypes, depending on the particular gene affected and the environmental conditions under which the organism is maintained. For example, full clock knockout (KO) mice like those resulting from deletion of BMAL1 or combinations of PER (per1/*per2*) (Zheng, 1999) and CRY (cry1/cry2) (Van der Horst, 1999) show immediate circadian arrhythmia when placed into constant conditions like constant darkness (DD). In genomically intact mice, a free run (FR) at the animal's endogenous period (tau) indicates the presence of a circadian clock in constant conditions. We can use this comparison to conclude that BMAL1, per1/2 double, and cry1/2 double knockout mice do not have a functional master circadian pacemaker. These mice do, however, exhibit synchrony within a light dark cycle, though this relative coordination is thought to be a masking response. Interestingly, in most reports these mutant mice all exhibit food entrainment, indicating that peripheral clocks are still functional (Feillet et al., 2006; Iijima et al., 2005; Pendergast et al., 2009; Storch & Weitz, 2009; Van der Zee et al., 2008).

Other TTFL mutants exhibit profoundly disrupted, but not deleted, circadian clocks (Baggs et al., 2009). $Per1^{-/-}$ and $Per3^{-/-}$ mutants show a short-period endogenous clock. REV-ERBα$^{-/-}$ and $Clock^{-/-}$ mice also display a short endogenous tau, while $Clock^{\Delta 19/\Delta 19}$ mice (with a mutation producing an aberrant clock protein that cannot promote transcription of CCGs) show a long tau chronotype. Again, these mutants all continue to exhibit food anticipatory activity (Pendergast & Yamazaki, 2018). Interestingly, food anticipatory activity in the *Per2*$^{-/-}$ mutant mouse has been heterogeneous across studies (Chavan et al., 2016; Feillet et al., 2006; Pendergast et al., 2017; Storch & Weitz, 2009). This is likely due to differences in experimental protocols, but could potentially point to a more integrated role of *per2* in peripheral entrainment (Carneiro & Araujo, 2012).

The *Per2*$^{m/m}$ mouse, where the PAS region of the *Per2* gene is deleted, has a disrupted clock. It has been argued to function as a damped oscillator, capable of variable numbers of oscillations in the absence of zeitgeber input, but not indefinite persistence. Thus it has a phenotype in which circadian functions are still evident under certain conditions. *Per2* mice, for instance, entrain to a light dark cycle and show a transient FR in constant darkness (DD) with a short tau for many cycles before going arrhythmic (Zheng, 1999, 2001). Rhythmicity can then be restored with a 6h light pulse, indicating the circadian network is labile. Important features of this mouse model include their shortened tau of approximately 22 hours and their ability to synchronize/mask with ultradian light dark (LD) cycles. Data from our laboratory indicate that a higher proportion of mutant females exhibit arrhythmia in DD (Riggle, Onishi, et al., 2022), and that mutant females are superior in their masking response to a high frequency (ultradian) LD cycle (unpublished observation). This points to *Per2*$^{m/m}$ mice as a potential model for studying

circadian instability, and for examining sex as a biological variable in the genomic regulation of circadian behavior.

The genome of a species is constantly in fluctuation, shaped by both neutral events and natural selection. One common cause of genomic variation is due to changes in gene dosage, or a variation in the number of copies of a gene within the genome due to insertions, deletions, or amplifications of genome segments (Basilicata & Valsecchi, 2021; Rice & McLysaght, 2017). Genes can also exist as allele pairs, where alternate forms of a gene that arise by mutation can be found at the same location on a chromosome. To be heterozygous for a trait is to have two different alleles for it, whereas homozygous individuals express identical alleles. Differences between two alleles of a gene can give rise to differences in functioning. Individual alleles often have genetic dominance relationships in which the dominant allele overrides the expression of the recessive allele to drive the phenotype (Lewis & Simpson, 2023; Mendel, n.d.). In such a situation, a heterozygote would display a homozygous dominant phenotype. However, many genes also exhibit incomplete dominance or codominance (*Genetic Dominance*, n.d.). Most human physical characteristics, such as hair, eye color, height, and skin color, are determined by incomplete dominance, where the expression of alleles are combined to express an intermediate phenotype. Blood type is an example of codominance (ex. AB blood), which occurs when alleles do not exhibit a dominant or recessive allele relationship and instead each allele is able to add phenotypic expression. Foundational studies of circadian clock genes and behavior in mice rarely subject heterozygotes to the same phenotyping examinations that are applied to homozygous and WT mice. For example, Zheng and colleagues evaluated circadian period differences in heterozygous *per2* mice with 10 days of entrainment to a 12:12 photoperiod followed by 22 days in DD and found no significant differences in circadian period between heterozygous (n=13) and

WT mice (*n*=13), concluding there exists no Per2 HET phenotype (Zheng, 1999). However, it was also noted in this report that two heterozygotes transiently lost rhythmicity in DD, while all WT mice retained rhythmicity throughout the DD interval. Despite this potential difference in oscillator stability, the subsequent study of *per2* heterozygotes has been neglected due to their proposed similarity to WT mice in terms of endogenous tau.

The neglect of heterozygotes is not exclusive to *per2*, however, but endemic to the study of circadian gene mutant mice. The foundational papers establishing *per1* and *per3* mutant mice did not evaluate heterozygotes at all (Bae et al., 2001; Shearman, Jin, et al., 2000). A mutation manipulating another core clock gene, bmal1 (through loss of PAS protein MOP3), evaluated some circadian parameters of the heterozygote including entrainment to an LD cycle, endogenous circadian period, amplitude, and total activity counts and found no significant differences from WT mice (Bunger et al., 2000). The authors subsequently concluded that the *Mop3* mutant allele is recessive, as the phenotype did not differ from WTs. While this investigation was more thorough than reports on *per* heterozygotes, the authors still failed to test phase shifting in response to light pulses in the *Mop3* heterozygote, or circadian behavior in conditions of constant light. It therefore seems a hasty assumption to presume the *Mop3* gene recessive based entirely on limited behavioral observations. Mice with mutations in the core circadian gene *Clock* are an exception to the rule of subtle heterozygote differences in behavior, as mice heterozygous for *Clock* mutation exhibit pronounced differences in circadian period (tau>24h), large phase shifts in response to light pulses, and reduced amplitude of oscillations in the SCN (Vitaterna et al., 1994, 2006). As the *Clock* mutant allele is a dominant negative mutation, effects on behavioral output of the circadian clock may be more identifiable than in situations of codominance or incomplete dominance. Thus the extent to which gene dosage and

genetic inheritance impact the role of clock genes on circadian behavior remain to be sufficiently examined.

Examination of clock gene phenotypes are also augmented by challenging mice with more than just DD. Indeed, these paradigms that further challenge how the clock copes with exogenous photic perturbations, such as misalignment paradigms, exposure to constant conditions of various light intensities, and discreet light pulses during certain phases of the circadian cycle, stand to reveal phenotypes that are not evident in DD. For example, *Per2*$^{m/m}$ mice are able to synchronize their activity with ultradian LD cycles while WT mice cannot (Riggle, Kay, et al., 2022). I previously examined circadian responses to a high-frequency light dark cycle (3L:3D; hfLD) in mice with zero (*per2*$^{m/m}$; MUT) or one (*per2*$^{-/m}$; HET) copy of a functional *per2* gene, and in wild-type (WT) controls (Beach, 2019; MA thesis). Sex and *per2* copy number dramatically altered behavioral responses to these challenging LD cycles. WT mice of both sexes exhibited free running activity with a period (tau) greater than 24h. As predicted by other reports of enhanced masking in *Per2*$^{m/m}$ mice, MUT mice synchronized with the hfLD cycle, exhibiting clear positive masking responses to darkness (a response that was more robust in females). HET mice, however, not only exhibited different behaviors in hfLD as compared to WT and MUT mice, but biological sex categorically predicted these responses: females free ran with a tau<24h whereas males exhibited circadian patterns of activity (clear daily onsets and offsets) that remained phase locked to the time of dark onset in the prior LD photocycle (12L:12D). These data suggest that *per2* gene copy number and sex interact to affect pacemaker function.

Parametric entrainment paradigms offer yet another protocol that may reveal subtle effects of circadian manipulation. Aschoff first described the response of nocturnal and diurnal

animals to light of different intensities (Beaulé, 2009). For nocturnal species such as mice, Aschoff's rule states that as light intensity increases, tau increases, and the active period duration (alpha) decreases. *Per2* seems to be a key player in mediating this parametric response. *Per2*$^{m/m}$ mice display short circadian periods with tau<24h with increased light intensity in constant light conditions (LL) (Steinlechner et al., 2002), the opposite of what is predicted by Aschoff's rule. Alpha response to light shows the same effect. In WT mice, the clock gene m*per2* is acutely upregulated by discrete light pulses and *Per2* protein levels stay constantly elevated under LL conditions (Muñoz et al., 2005). Constant light in this way appears to prevent the degradation of *per2* protein without constantly inducing m*per2* expression, enhancing the phase delaying of the circadian clock and thereby lengthening tau. Interestingly, this has only been studied in male mice with small sample sizes. Given the clear sex differences evident in circadian organization of *Per2*$^{m/m}$ mice (Riggle, Onishi, et al., 2022), this raises the question of how sex and *per2* interact to mediate Aschoff's rule. To directly examine this, experiments in this chapter aimed to document Aschoff-like behavior of mice with different dosages of the *per2* gene in constant conditions of various light intensities, including constant darkness, dim constant light, and bright constant light.

Light can act on the mechanism of the circadian clock in a continuous way, as in the case of the parametric entrainment studied by Aschoff, but also in a discreet way, as in the case of non-parametric entrainment (Daan & Pittendrigh, 1976; De Coursey, 1960). Pittendrigh acted as Aschoff's foil, investigating how the circadian clock undergoes resetting in response to discrete light pulses. In stable entrainment, the free running period of the circadian clock is corrected each day by light falling at a particular phase in each cycle, corresponding to a phase shift of the correct magnitude on the following cycle (Pittendrigh, 1981). Phase response curves (PRCs)

provide information about the magnitude and direction of a phase shift that will be delivered when a zeitgeber hits a particular phase in the circadian cycle (Daan & Pittendrigh, 1976; *PRC Atlas*, n.d.). PRCs for lights are generated by transferring an organism into DD, measuring the period of the endogenous oscillator, then providing a discreet light pulse of specified intensity and duration at different phases of the circadian cycle. In Type 1 PRCs, light pulses early in subjective night produce phase delays, while pulses in late subjective night produce phase advances. *Per2*'s role in non-parametric responses to light is equivocal, though these mice consistently exhibit a larger phase advance region on their PRC compared to WT mice (Albrecht et al., 2001; Pendergast et al., 2010; Steinlechner et al., 2002). This larger advance region was almost equal in size to the delay region of the PRC, corresponding to no period lengthening in LL (Pendergast et al., 2010; Steinlechner et al., 2002). Thus, there is a link between parametric and non-parametric responses to light. Additionally, these parametric responses have only been evaluated in homozygous *Per2* mutants, leaving the gene dosage effect of *per2* on non-parametric responses to light yet to be investigated. To address these questions, experiments in this chapter also investigated acute, non-parametric effects of discrete light pulses on the circadian locomotor response of mice with zero ($per2^{m/m}$; MUT) or one ($per2^{-/m}$; HET) copy of a functional *per2* gene, and in wild-type (WT) controls. The results of these experiments illuminate the role of the clock gene *per2* and biological sex in generating circadian rhythms in response to light, both discrete and continuous.

Materials and Methods

Experiments

Experiment 1 tested the hypothesis that the clock gene *per2* and biological sex interact to mediate Aschoff's Rule effects on circadian period (tau) and length of active phase (alpha). Experiment 2 evaluated the impact of *per2* and sex on non-parametric effects of light.

Animals

Adult male and female *Per2*$^{-/-}$ mice (B6.Cg-*Per2*^{tm1Brd} *Tyr*$^{c-Brd}$/J; Stock #003819) and their wild-type (WT) littermates (*Per2*$^{+/+}$*)* were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were backcrossed to C57BL/6-Tyr^{c-Brd} mice to produce mice homozygous for both the *Per2*^{tmBrd} target mutation and the recessive *Tyr*$^{c-Brd}$ mutation (MUT: *Per2*$^{m/m}$), as well as mice heterozygous for these mutations (HET: *Per2*$^{+/m}$*)*. *Per2*$^{+/+}$ littermates were used as WT controls. Mice of both sexes (*Per2*$^{m/m}$: n=7 females, n=9 males; *Per2*$^{+/m}$*:* n=9 females, n=9 males; WT: n=9 females, n=9 males) between 7 and 11 months of age were single housed in conventional cages with wirebar lids and without microisolator filters in a 12L:12D photocycle of approximately 150-200 lux. Experiments were performed within a single room with cage location in the room chosen randomly. Mice had *ad libitum* access to standard rodent diet (Irradiated Teklad Global 18% Rodent Diet 2918, Envigo RMS) and filtered drinking water. Cage changing was performed at two-week intervals. All mice were acclimated to PIR cages for at least one week prior to data collection. The integrity of experimental LD cycles and constant condition treatments was continuously monitored and verified by dataloggers (HOBO,UX90, Onset Comp). Estrous cycles of females were not monitored. At the conclusion of the experiments homozygous WT and *Per2*$^{m/m}$ and heterozygous *Per2*$^{+/m}$ genotypes were confirmed in all mice by PCR using the protocol described for this genotype by JAX (see below). All

procedures related to animal use were approved by the University of Chicago Institutional Animal Care and Use Committee.

Genotyping

Homozygous WT, heterozygous *Per2*$^{+/m}$, and homozygous *Per2*$^{m/m}$ mice were included in these analyses. Genotyping of all mice bred in our vivarium was done using primers from the Jackson Lab Website [5' 3': Common Forward (TTC CAC TCT GTG GGT TTT GG), Wild Type Reverse (AAA GGG CCT CTG TGT GAT TG), and Mutant Reverse (GCC AGA GGC CAC TTG TGT AG)] and using specification for the Platinum Taq Polymerase (Life Technologies, Invitrogen catalog number: 10966-018). For each individual PCR reaction: 16.65 uL of DNAase free H2O, 2.5 uL of 10X PCR Buffer with no $MgCl_2$, 0.75 uL 50mN $MgCl_2$, 0.5 uL 10mM dNTP mix, 0.5 uL of each primer, and 0.1 uL of Taq were added to a master mix and thoroughly mixed by pipetting up and down. 22 uL of master mix were aliquoted and added to 3 uL of DNA derived via HotShot (Truett, Heeger et al. 2000), from ear clips collected prior to onset of the studies. We used the following PCR Protocol on a thermocycler: (1) 94 C for 2 minutes, (2) 94 C for 20 seconds, (3) 65 C for 15 seconds, with a -0.5 C decrease with each cycle, (4) 68 C for 10 seconds, (5) repeat steps (2-4) 10 times, (6) 94 C for 15 seconds, (7) 60 C for 15 seconds, (8) 72 C for 10 seconds, (9) Repeat (6-8) 38 times, (10) 72 C for 2 minutes, (11) 10 C for 2 minutes, (12) End. 8 uL of the resultant PCR products, and a 100 Bp to 2000 Bp Ladder (Thermofisher catalog # 15628050) for reference were mixed with 1.4-1.5 uL of loading dye (Thermo Scientific catalog number: R0611), loaded on a 2% agrarose gel with 2.5uL of Ethidium Bromide (stock solution: 10mg/mL), and visualized. Resultant bands (amplicon sizes) per Jackson Lab Website were as follows: mutant (m/m) = ~200 bp, heterozygote (m/+) = ~200 bp and 297 bp, and wild type (+/+) = 297 bp.

Photoperiod manipulations

For experiment 1, mice were maintained in 12L:12D for 21 days, at which time the housing room was switched to continuous darkness (DD). After 28 days in DD (2, 2-week epochs), the housing room was switched to 28 days (2, 2-week epochs) of constant dim light (dimLL; <10lux), followed by 28 days (2, 2-week epochs) of constant bright light (LL; >300lux). Dim LL (<10 lux) was achieved by projecting one portable 1000 lumen LED work light against an adjacent wall to the animal rack, controlling for even lux distribution across the animal position grid. Bright LL (>300 lux) was achieved by projecting multiple portable 5000 lumen LED work lights onto an adjacent wall, again controlling for even lux distribution across the animal rack. Mice were then released back into DD for 21 days to evaluate circadian aftereffects. For experiment 2, non-parametric effects of light were tested using a modification on Aschoff's Type II protocol (Evans et al., 2004), whereby mice were transferred to a 12L:12D cycle for 3 weeks, then released into DD for 24 hours and subsequently administered a 15 minute light-pulse (1000 lux) during either the delay (projected ZT15, pZT15) or advance (pZT22) region of their Phase Response Curve (PRC), for a detailed schematic see Fig. 4. Specifically, WT mice received the pZT15 (Phase Delay) light pulse 2h:56min after ZT12, where ZT12=24h after the LD-DD transition, this is based on endogenous period of 23.95. HETs received the ZT15 light pulse 2h:36min after ZT12 (based on endogenous period of 23.71), and MUTs received the ZT15 light pulse 1h:28min after ZT12 (based on endogenous period of 22.65). For phase advances, WT mice received the pZT22 light pulse 2h:4min before ZT0, where ZT0=36h after the LD-DD transition. HETs received the ZT22 pulse 2h:25min before ZT0, and MUTs received the ZT22 pulse 3h:55min before ZT0. In sum, pZT15 mice received light pulses after 25.5-27h in DD, and pZT22 mice received light pulses after 37.5-39h in DD. Delay (ZT15) pulses were administered

to all animals first, followed by 10 days in DD and 1 month in LD before administration of the Type II Aschoff procedure for phase advance pulses.

Circadian Activity Measures

Activity data was visualized and analyzed during each light intensity epoch using ClockLab Analysis 6 software (Actimetrics, Wilmette, IL, USA). Actograms were generated and quantitative analyses were performed in Clocklab 6. Actoograms were first visually assessed for rhythmicity in photic manipulation by an observer blind to genotype and sex. To evaluate active period length (alpha), activity onsets and offsets were identified across the whole period of each photic manipulation where rhythmic locomotor activity was determined (*i.e.*, all 21 days). For evaluation of all other circadian parameters, epochs were 10days in length beginning 2 days after cage change and ending 2 days before the next cage change. Characterization and quantitative evaluation of circadian chronotypes in mice were performed via methodology previously described for this mutant (Zheng, 1999): double-plotted activity records were evaluated by an experimenter blind to sex and genotype for entrainment in LD, free running circadian period in constant conditions (DD, dimLL, bLL), and total activity counts, including active and rest phase activity. Circadian period was calculated using a Lomb-Scargle Periodogram analysis (LSP) on each 10-day epoch of activity data (Ruf, 1999; Tackenberg & Hughey, 2021). In some cases where the LSP on 10-days of activity data did not meet the significance threshold, but activity onsets and offsets were identifiable throughout the whole photic manipulation to generate an alpha value, we report only the alpha value in the absence of a tau value. This effect was only present in conditions of constant light, with 1 male MUT and 1 female MUT in the first epoch of dimLL, 9 mice in bLL1 (3 female HET, 2 male HET, 1 female MUT, 3 male MUT), and.5 mice in bLL2 (2 F WT, 1 female HET, 1 male MUT, 1 female MUT) displaying this phenotype. Phase

shifting in response to light pulses was determined by fitting a regression line through 6 consecutive activity onsets before (LD) and after (DD) and determining the displacement between the two regression lines on the first day after the light pulse. The first 3 days post-pulse were excluded from the analysis to allow for transients.

Statistical Analyses

Analyses of variance (ANOVAs) were performed to decrease chances of Type I error. If a statistically significant F-statistic was achieved, unpaired T-tests were performed using Statview 5.0 (SAS Institute, Cary, NC). To determine differences in sequential exposures to constant conditions of different light intensities, paired T-tests were performed. When there were unequal data dropouts from groups, an equality of variance F-test was performed to assess any violations of the assumption of equal variance before performing the ANOVA. If an F-test indicated a violation in the assumption of variance, nonparametric methods were used. To evaluate differences in factors with 3 levels, a Kruskal-Wallis Test for overall differences with a Dunn correction to evaluate pairwise comparisons was used. Comparisons between factors with two levels were evaluated with a Mann-Whitney U Test. Spearman Rank Correlations were performed to evaluate paired comparisons between sequential exposures to constant conditions of different light intensities. When investigation into interactions between sex and genotype was merited by our *a priori* hypotheses despite a non-significant ANOVA, a Tukey/Kramer test was used to evaluate pairwise comparisons. Differences were considered significant if $p<0.05$, unless otherwise specified by a specific non-parametric method (where noted).

Results

Parametric Entrainment

Circadian Period Length. All mice entrained their locomotor activity rhythm to an LD photocycle (Fig.4-5), as observed previously (Steinlechner et al., 2002; Zheng, 1999, 2001). When initially placed under DD, there were significant differences among genotypes ($p<0.0001$) and between sexes ($p<0.002$), and no interaction between genotype and sex ($p=0.2771$; Fig. 4-5). Functional *per2* gene copy number significantly impacted period in epoch 1 of DD, contrary to previous findings (Zheng, 1999). WT mice exhibited the longest average tau (23.95h; $p<0.002$), MUTs the shortest average tau (22.54h; $p<0.0001$), and HETs displayed an intermediate circadian period (23.62h; $p<0.002$; Fig. 5). Additionally, males displayed a slightly longer circadian period than females (tau=23.464 and 23.243, respectively). A Tukey/Kramer post hoc test indicated this sex difference was driven by MUTs and WTs, as females in both of these groups displayed a shorter tau than the males ($p<0.05$ for both: Mean diff= -0.397, Crit diff=0.261; Mean diff= -0.244, Crit diff=0.18, respectively). This sex difference was not evident in the HETs ($p>0.05$; Mean diff= -0.106, Crit diff=0.344). This overall sex difference in the first epoch of DD was eliminated in the second epoch ($p=0.1372$). Again, there was a significant impact of genotype ($p<0.0001$) and no interactions (p-0.6232). The difference in period length between WT and HET mice disappeared in the second epoch of DD ($p=0.2465$), though MUTs still had a significantly shorter tau ($p<0.0001$; Fig5). The initial difference between HETs and WTs in circadian period that later disappeared could indicate some latency on the part of HETs in establishing a stable circadian period in DD. Though no sex differences or interactions were significant in the ANOVA, we investigated our a priori hypothesis with a Tukey/Kramer post hoc test, which showed a significant sex difference in HETs, with female HETs displaying a shorter tau compared to males ($p<0.05$, Mean dif= -0.272, Crit diff=0.245).

Upon exposure to constant illuminance of <10lux (dimLL), there was again an impact of genotype ($p<0.0001$), but not of sex ($p=0.5104$), with no interactions ($p=0.9769$). Again, functional *per2* gene copy number significantly impacted circadian period (Fig. 4-5), with WTs displaying the longest (24.26h; $p<0.005$), MUTs the shortest (22.70h; $p<0.0001$), and HETs an intermediate tau (23.99h; $p<0.005$). WT and HET mice lengthened their circadian period from DD to dimLL ($p<0.002$ and $p<0.05$; respectively), while MUT mice did not significantly change their circadian period ($p=0.44$; Fig. 5). A Tukey/Kramer post-hoc test showed no significant sex differences in dimLL1 ($p>0.05$; HET Mean diff= -0.15, Crit diff=0.275; MUT Mean diff= -0.088, Crit diff=0.906; WT Mean diff= -0.072, Crit diff=0.252). No genotype significantly changed their circadian period between epochs of dimLL ($p=0.2174$,$p=0.1649$,$p=0.2368$), so the second epoch of dimLL reflected similar differences as the first epoch, with a significant main effect of genotype ($p<0.0001$), and no effect of sex ($p=0.7514$) and no interaction effect ($p=0.6291$). MUTs remained with a significantly shorter circadian period of 22.41h ($p<0.0001$), HETs an intermediate circadian period of 23.87h ($p<0.0001$), and WTs the longest period of 24.34h ($p<0.0001$; Fig. 5). Similar to the first epoch, a Tukey/Kramer post-hoc test showed no significant sex differences in dimLL2 ($p>0.05$; HET Mean diff= -0.061, Crit diff=0.432; MUT Mean diff= -0.125, Crit diff=0.458; WT Mean diff= -0.1, Crit diff=0.162).

When mice were placed into bright constant illumination (bLL; >300lux), approximately half of the HETs (5 females, 4 males), all of the female MUTs, and a third of the male MUTs were behaviorally arrhythmic (LSP value below threshold). After two weeks in bLL, this distribution somewhat changed, with a greater number of male HETs (8/9) and about half of the WTs (2 females, 6 males) displaying arrhythmia, while half the female and most of the male mutants (2/3) recovered rhythmicity above LSP threshold. These group dropouts in circadian

rhythmicity resulted in a violation of the assumption of variance in both epochs of bLL for tau (F test: $p<0.001$). Therefore, epochs of bLL were evaluated using nonparametric methods. A Kruskal-Wallis Test for overall differences with a Dunn correction to evaluate pairwise comparisons was used to evaluate differences between genotypes, and comparisons between sexes were evaluated with a Mann-Whitney U Test. Spearman Rank Correlations were performed to evaluate paired comparisons between epochs.

WTs exacerbated the period lengthening seen in dimLL in bLL, displaying a significantly longer circadian period than MUTs ($p<0.0005$) in the first epoch of bLL (bLL1; Fig. 4-5). This continued in the second epoch of bLL (bLL2; $p<0.0001$) as WTs further lengthened their circadian period in response to constant bright light from an average of 25.269h to 25.795h ($p<0.05$), while MUTs actually reduced their circadian period in prolonged bLL from an average of 23.808h to 23.242h ($p<0.05$). Thus, MUTs did not significantly lengthen their circadian period between DD and bLL2 despite exposure to constant light ($p=0.5447$), while HETs ($p<0.01$) and WTs did ($p<0.0001$). HETs did not display a difference in circadian period length from WT or MUT groups in bLL1 ($p=0.0784$ and $p=0.0349$; where a significant p-value must be below 0.0167), but a difference from MUTs in bLL2 did appear ($p=0.0001$), due to the further shortening of circadian period displayed by MUTs (Fig. 5). Sex differences were also evident in bLL, where females exhibited a longer circadian period than males in both epochs ($p<0.001$ and $p<0.0005$, respectively). In the first two weeks of constant bright light exposure, females had an average circadian period of 25.45h, compared to the 24.458h average tau displayed by males. Females subsequently lengthened their circadian period in prolonged bLL (to an average of 25.462h; $p<0.05$), while males shortened their circadian period to an average of 23.467h in bLL2 ($p<0.05$).

Though group dropouts prevented statistical analysis in bLL with parametric methods that would allow for looking at genotype and sex interaction effects, our *a priori* hypotheses permitted a more in-depth investigation, which we analyzed with Tukey/Kramer. Male and female WTs (Fig. 4; left plots) lengthened period in bright constant light ($p<0.0005$; Fig. 5), and this was especially evident for females ($p<0.05$, Mean Diff.=0.506, Crit. Diff.=0.259). Upon exposure to bLL, HETs struggled to maintain rhythmicity (Fig. 4, middle plots), with half of females (5 of 9) and males (4 of 9) going arrhythmic (ARR). By the end of bLL, half (4 of 9) the female and 8 out of 9 male HETs were ARR. However, animals remaining rhythmic lengthened their circadian period like WTs ($p<0.05$; Fig. 5) and did not show sex differences in period length ($p>0.05$, Mean Diff.=0.922, Crit. Diff.=1.908). MUTs (Fig. 4, right plots) also struggled to retain rhythmicity in bLL. When first exposed to bLL, every MUT female went ARR (Fig. 5). But, in the second epoch of bLL, the half of the females that recovered rhythmicity (4 of 9) lengthened tau to an average of 24.39 hours (Fig. 5), though this was not statistically different from tau in dimLL, likely due to increased variability ($p=0.1570$). Conversely, 1/3 of the male MUTs went ARR in the first epoch of bLL, but the ones remaining rhythmic (6 of 9) showed Aschoff-like period lengthening with an average circadian period length of 23.81 hours ($p<0.02$; Fig. 5). But, when all but 1 male had recovered rhythmicity in bLL2, MUT males showed a shorter circadian period again, which was not significantly different from tau in dimLL ($p=0.2682$).

Finally, when mice were released back into DD, there was a significant effect of genotype ($p<0.05$), but no significant main effect of sex ($p=0.7254$) or interaction ($p=0.3266$; Fig. 4-5). The genotype effect was driven by MUTs displaying a significantly shorter circadian period (23.07h) compared to WTs (23.86h, $p<0.05$) and HETs (23.77h, $p<0.005$; Fig. 5). HETs

and WTs did not show significant differences in tau ($p=0.7647$). Tukey/Kramer again showed no sex differences in this final epoch of DD ($p>0.05$: HET Mean diff= -0.314, Crit diff=0.508; MUT Mean diff=0.157, Crit diff=0.854; WT Mean diff=0.902, Crit diff=1.241). Additionally, a paired T test was performed to assess differences in period length from the first 2 epochs of DD, prior to constant light exposure. Compared to the first epoch of DD, WTs and HETs did not display any differences in circadian period ($p=0.8205$ and $p=0.1216$, respectively) during the final DD epoch, while MUTs did display an increased circadian period in the final epoch of DD compared to the first ($p<0.05$; Fig. 5). Compared to the second epoch of DD, WTs, HETs, and MUTs did not display any differences in circadian period during the final DD epoch ($p=0.4832$, $p=0.5795$, $p=0.7394$, respectively).

Alpha length. In a 12:12 photocycle, there was a significant main effect of genotype on alpha duration ($p=0.0145$), but no main effect of sex ($p=0.262$), and no interaction effects ($p=0.3999$). MUT mice have a longer alpha duration (12.50h) than WT (11.86h) and HET (11.73h) mice due to their positive phase angle of entrainment (Fig. 6), whereby they begin their activity in advance of lights off because of their short endogenous clock period ($p<0.05$ and $p<0.01$ respectively). WT and HET mice did not significantly differ from each other in length of active period in 12:12 ($p=0.5519$). In constant darkness (DD), there were no genotype ($p=0.4801$) or sex (0.8661) differences in the length of alpha, and no interaction effects ($p=0.8169$). While mice did not exhibit different active period durations in DD, WTs and HETs showed an increase in alpha from LD to DD ($p<0.0001$ and $p<0.0001$, respectively), while MUTs did not significantly change alpha duration from LD ($p=0.0815$; Fig. 6). When mice were transferred to dimLL, there was again a significant main effect of genotype ($p<0.02$) but not of sex ($p=0.3354$), and no interaction effects ($p=0.2096$). Mutants had a significantly longer alpha

duration than WTs ($p<0.02$) and HETs ($p<0.05$), who did not significantly differ from each other ($p=0.7955$). Thus, WT and HET mice compressed their alpha from DD to dimLL ($p<0.05$ and $p<0.05$, respectively), while MUTs did not change the duration of their alpha between DD and dimLL ($p=0.986$; Fig. 6). While WT and HET mice showed the alpha responses predicted by Aschoff's Rule in DD and dimLL, MUT mice failed to show this response: they did not expand alpha in response to constant darkness (DD) or compress alpha in response to dim constant light (dimLL).

When mice were exposed to bLL, there was a significant main effect of genotype ($p<0.005$) but not sex ($p=0.2777$), and no interaction effects ($p=0.7133$). This was driven by significant alpha compression among WTs, who displayed shorter alpha duration than HETs ($p<0.01$) and MUTs ($p=0.0005$). While HETs did not change alpha length from dimLL, MUTs and WTs compressed their alpha in bLL ($p=0.0002$ and $p<0.0001$, respectively; Fig. 6). Though we did not observe a main effect of sex in the ANOVA, our *a priori* hypothesis allowed us to probe deeper into this interaction between genotype and sex by performing a Tukey/Kamer post-hoc comparison between these factors. The Tukey/Kramer still did not show significant differences between sexes within each genotype (Fig. 6), but indicated a trend for female MUTs to have a longer alpha duration than male MUTs (HET: Mean diff=0.503, Crit diff=3.72; MUT: Mean diff=1.309, Crit diff=1.444, WT: Mean diff=0.165, Crit diff=1.586). Because mice altered rhythmicity status over the course of the two epochs in this photoperiod (13 mice missing alpha values in bLL1, 20 mice missing alpha values in bLL2), bLL was also split into two epochs for alpha analysis. An F test showed that individual epochs of bLL violated the assumption of equal variance for an ANOVA ($p<0.05$). Therefore, individual epochs of bLL were evaluated using nonparametric methods. A Kruskal-Wallis Test for overall differences with a Dunn correction to

evaluate pairwise comparisons was used to evaluate differences between genotypes, and comparisons between sexes were evaluated with a Mann-Whitney U Test. There was a significant effect of genotype in bLL1, with WTs exhibiting a shorter alpha duration than HETs ($p<0.002$) and MUTs ($p<0.01$). There was no significant effect of sex ($p=0.8826$) in bLL1. There was no impact of genotype ($p=0.3156$) or sex ($p=0.4903$) in bLL2.

Finally, when mice were released into DD after bLL exposure, a significant main effect of genotype ($p<0.02$) was observed. There was no main effect of sex ($p=0.2702$), though there was a significant interaction between sex and genotype ($p<0.05$). MUTs displayed a significantly longer alpha duration than WTs ($p<0.05$) and HETs ($p<0.05$), and this was specifically driven by MUT females, who displayed longer alpha duration than female HETs ($p<0.02$) and female WTs ($p<0.005$). MUT males did not significantly differ from WT males ($p=0.757$) or HET males ($p=0.8417$). Additionally, A paired t-test showed significant differences in alpha duration between the first and last bouts of DD for HETs ($p<0.02$) and WTs ($p<0.01$), but not for MUTs ($p=0.4933$). Based on our *a priori* hypotheses, we evaluated pairwise comparisons for sex and genotype differences between the first and last bouts of DD. While no significant differences were present, there was a trend for female WTs to show compressed alpha in the last epoch of DD ($p=0.0564$), and this change was not evident in male WTs ($p=0.1107$). Female and male HETs also showed a trend for compressed alpha in the final DD epoch compared to the first ($p=0.0753$ and $p=0.0777$, respectively). Female and male mutant mice did not change alpha duration from the first epoch of DD to the last ($p=0.2507$ and $p=0.1889$, respectively).

Activity counts. Across all photoperiod manipulation, MUTs in general were less active than HETs and WTs, and females demonstrated higher activity than males. In a 12:12 photocycle, there were no significant genotype ($p=0.2964$) or interaction (0.4247) effects of sex

and genotype on the magnitude of activity in the active phase (alpha), but there was a significant main effect of sex ($p<0.02$), whereby female mice had higher activity during the active phase (611.22 ±113.66 counts for females and 361.412±77.86 counts for males). There were no main or interaction effects for inactive phase activity (rho). Again, for total activity during the LD cycle, there were no significant genotype ($p=0.3698$) or interaction ($p=0.3908$) effects of sex and genotype on the magnitude of activity, but there was a significant main effect of sex ($p<0.02$) whereby female mice had higher overall activity (771.86±123.60 counts for females and 480.875±98.38 counts for males). In DD during the active phase, there were significant genotype ($p<0.05$) and sex ($p<0.05$) differences, and no interaction effects ($p=0.2258$). Genotype differences were driven by mutant mice, who displayed lower activity compared to WT mice ($p<0.02$). There were no significant differences between WT and HET mice ($p=0.3221$) and between HET and MUT mice ($p=0.1206$). Again, females had higher activity during the active phase than males ($p<0.05$). There were no significant differences in inactive phase activity counts (genotype: $p=0.3368$, sex: $p=0.7585$, interaction: $p=0.0806$) or total activity counts (genotype: $p=0.0529$, sex: $p=0.0637$, interaction: $p=0.2066$). In dimLL, there were significant differences in alpha counts, rho counts, and total counts. In active phase activity, there were significant genotype ($p<0.05$) and sex differences ($p<0.001$), but no interaction effects. MUTs had lower activity during the active phase than HETs ($p<0.05$) and WTs ($p<0.01$), and females had higher activity than males ($p<0.0005$). In the inactive phase, there were no genotype ($p=0.0548$) or interaction effects ($p=0.1818$), but there were significant sex differences ($p<0.05$), where females had higher activity ($p<0.02$). The total activity in dimLL showed significant genotype ($p<0.05$) and sex ($p<0.001$) differences, and no significant interactions ($p=0.2118$) that followed the same pattern as alpha activity. MUTs had lower activity than HETs ($p<0.05$) and

WTs ($p<0.01$), and females had higher activity than males ($p<0.0005$). In bLL during the active phase, there was a significant effect of sex ($p<0.02$) where females had higher activity ($p<0.002$), but not a significant effect of genotype ($p=0.8877$) and no interaction ($p=0.8674$). There were no significant differences in rho (genotype: $p=0.3764$, sex: $p=0.0613$, interation: $p=0.9997$). Once again, total activity counts in bLL showed a sex difference ($p=0.002$) but no genotype ($p=0.7686$) or interaction ($p=0.932$) effects. In the second epoch of DD after constant light exposure, there was a significant main effect of sex ($p<0.01$) and genotype ($p<0.05$) on activity counts during the active phase, with MUTs showing reduced activity compared to HETs and WTs ($p<0.02$), and females showing greater activity than males ($p<0.01$). While the ANOVA showed a sex difference in inactive phase activity ($p<0.05$), the unpaired T test was not significant. Total activity showed a sex difference ($p<0.01$) with females displaying higher activity ($p<0.005$) but no significant main effect of genotype ($p=0.1006$) or interaction ($p=0.7705$).

Nonparametric Entrainment

Phase Delay. In response to light pulses administered during the phase delay region (Fig. 7) of the circadian cycle (ZT15, corresponding to early subjective night), all but two female MUTs went arrhythmic, increasing variability (Fig. 8). Altogether, 8 MUTs, 5 HETs, and 4 WTs lost measurable behavioral rhythmicity following a delay pulse. An F test showed the equality of variance assumption was violated between groups ($p<0.01$), so an overall ANOVA would not be statistically valid. Therefore, we again performed nonparametric tests to analyze differences. A Kruskal-Wallis Test showed no differences between genotype in phase delay ($p=0.1647$; Fig. 8).

Additionally, a Mann-Whitney U Test showed no significant sex differences in phase delay ($p=0.4967$).

Phase Advance. Group sizes fluctuated somewhat for the phase advance pulse (Fig. 7; administered during late subjective night; ZT22) as well, with all groups except female WTs missing subjects due to arrhythmia or lack of sufficient activity to select onsets. Group sizes were as follows: 9 WT females, 6 WT males, 6 HET females, 7 HET males, 5 MUT females, 8 MUT males. An F test showed a violation in the equality of variance assumption ($p<0.05$), preventing an ANOVA from being statistically valid. Therefore, we again used nonparametric methods to assess group differences. A Kruskal-Wallis Test indicated significant differences between genotypes ($p=0.0001$), with MUTs exhibiting larger phase advances than WTs ($p<0.0001$) and HETs ($p=0.0002$; Fig. 8). HETs and WTs did not display differences in phase advances from each other ($p=0.3626$). There was also an impact of sex on phase advance, with males displaying larger phase advances (mean=1.249±0.20h) than females (mean=0.593±0.10h; $p<0.02$).

Advance-Delay Ratio. To get an idea of the Advance-Delay ratio (Fig. 9), we again applied nonparametric testing and found a difference between genotypes ($p<0.002$), driven by MUTs displaying a more equal Advance-Delay Ratio compared to WTs ($p<0.01$). There was no significant effect of sex on Advance Delay Ratio ($p=0.0738$). MUTs showed an average phase delay of 3.09 hours and average phase advance of 1.81 hours, corresponding to the most even Advance-Delay Ratio of 0.585 (advance was 58.5% of delay). On the other hand, WTs delayed 1.96 hours on average and advanced 0.41 hours on average, corresponding to a ratio of 0.204 (advance was 20.4% of delay). Though HETs did not significantly differ from MUTs ($p=0.5165$)

or WTs (p=0.0438), they delayed 1.8 hours and advanced 0.66 hours on average, corresponding to a ratio of 0.366 (advance was 36.6% of delay).

Discussion

The present report identifies novel roles of sex and the clock gene *per2* in driving behavioral circadian rhythms. These data show that as light intensity increases, complete lack of functional *per2* prevents the lengthening of circadian period predicted by Aschoff's Rule (Fig. 4-5). MUT mice did not significantly lengthen their circadian period in response to increased constant light, maintaining a consistent tau throughout constant darkness, dim constant light, and bright constant light. This suggests that either functional *per2* is essential for period lengthening in response to increased light intensity (at the level of the molecular clock itself), or that it increases the threshold at which light induces Aschoff effects (at the level of the clock inputs), confirming previous research (Steinlechner et al., 2002) and extending it to females. The latter possibility is supported by our observation that HET mice do not lengthen alpha in dim constant light to the same extent as WT mice, but show no differences from WT mice in length of circadian period in bright constant light (Fig. 6). Thus, the magnitude of photic input sufficient to induce Aschoff-like effects might be increased for mice missing functional copies of the *per2* gene. Additional support for this is seen in the alpha compression demonstrated by mutant mice only in response to bright constant light (Fig. 6). Interestingly, HET mice significantly reduced alpha in dim constant light but not in bright constant light, indicating that the effects of light on tau and alpha are somewhat separable. It could be the case that alpha can only be compressed by constant light to a certain extent, but the magnitude of light necessary to induce alpha compression differs as a function of functional *per2* gene copy number, as both HETs and MUTs displayed a similar average alpha duration by the end of bLL (11.458h and 11.1h, respectively;

compared to 9.133h alpha duration of WTs). Though not significant, the alpha compression exhibited by MUTs appeared particularly prominent for male *per2* mice (Fig. 6), supporting a potential interaction between sex and genotype in mediating Aschoff's Rule.

It was essential to test whether the mediation of Aschoff's Rule was dependent on sex, as previous research has been done on males or animals of unspecified sex (Spoelstra, 2004; Steinlechner et al., 2002). Additionally, previous research has shown sex differences in circadian stability of *per2* mutant mice (Riggle, Onishi, et al., 2022), and we found some evidence for a role of sex in the prevention of tau lengthening during exposure to constant light (Fig. 5). While we initially observed a sex difference in the first epoch of constant darkness for all genotypes, where males displayed a longer circadian period than females, only HETs maintained this sex difference in the second epoch of DD (Fig. 5). No sex differences were evident in dim constant light, but bright constant light reversed the pattern seen in DD, with females across genotypes displaying longer circadian period than males. Interestingly, females progressively lengthened tau during exposure to bright constant light, while males shortened their tau over this same period. However, the significant group dropouts in bLL make it difficult to conclusively determine the interaction between sex and *per2* in the period lengthening ascribed by Aschoff's Rule. For example, all female mutants were arrhythmic in the first epoch of bLL (Fig. 5), while 8 out of 9 male HETs were arrhythmic in the second epoch of bLL, necessitating the use of nonparametric methods in both epochs to analyze circadian parameters and preventing the investigation of interactions between genotype and sex. A more specific investigation into the interaction between sex and *per2* in mediating the response to constant light of high intensity is merited.

Additionally, the present study extended previous research by investigating *per2* gene dosage effects through the inclusion of heterozygotes. Previous research suggested no circadian differences between *per2* HETs and WTs, as they did not show group differences in LD and DD (Zheng, 1999), except for occasional transient loss of rhythmicity by HETs. We find a similar effect here, with HETs exhibiting similar circadian periods to WTs in a light-dark cycle and constant darkness (Fig 4-5). Additionally, we unmasked differences between HETs and WTs in response to constant light (Fig. 5-6). In dim constant light, HET mice did not show the same tau lengthening as WTs (Fig. 5), though they did exhibit alpha compression (Fig. 6). However, in bright constant light, HETs lengthened their period to a similar tau as WTs (Fig. 5) despite continuing to exhibit the same active phase duration as in dimLL (Fig. 6). Despite this recovery to WT period length in bLL, the genotype level differences in dimLL could indicate some dosage effect of *per2* in period lengthening response to low intensity light. Muñoz et al., 2005 showed that m*Per2* is constitutively expressed during LL and acts as a molecular basis of phase delaying in response to light. Therefore, dimLL may not be intense enough to activate the latent *mper2* in HETs, preventing them from showing the same degree of tau lengthening as WTs. Additionally, our lab has previously tested HETs in a 3:3 high-frequency light-dark (hfLD) cycle, and found that they exhibit categorically different responses from WTs and mutants. In particular, male HETs look as if they are still entrained to the previous LD cycle. Given that hfLD is interpreted as dimLL by the circadian system, if HETs do not show tau lengthening or alpha compression in dimLL, this would cause them to appear to be locked on to the previous LD cycle in terms of period and active phase length in hfLD.

Investigations into the differences between the magnitude of phase delay compared to magnitude of phase advance in response to light pulses have been used to predict how animals

will alter circadian period and alpha in response to constant light (Pendergast et al., 2010), aligning parametric (*i.e.,* circadian period alteration in response to the constant action of light on the clock) and non-parametric (*i.e.,* circadian period alteration in response to discrete light pulses) actions of light on the clock. Thus, we also sought to understand if alterations in non-parametric response to light pulses were altered by lack of functional *per2*, and if this fit within the context of our findings of parametric response to constant conditions. Here we found that *per2* MUTs exhibited phase delays and phase advances to light at pZT14 and pZT22, respectively. In the present study, the magnitude of phase delay was larger than the magnitude of phase advances by approximately 2-fold. Previously, Pendergast et al. (2010) found that $Per2^{-/-}$ mice (a slightly different functional mutation of *per2*) exhibited comparable phase advances and phase delays; in contrast, WT mice exhibited smaller advances than delays. Spoelstra et al. (2004) using $per2^{Brdm1}$ mice (the same mutant used in our experiments), found advances and delays in both MUT and WT mice, but smaller advances and larger delays. Finally, Albrecht et al. (2001) did not observe phase delays at ZT14 in *per2* mutants, while normal phase advances remained intact. However, effects of ZT14 light pulses on tau appeared evident in representative actograms depicted in this report. Our understanding of these results can be enhanced by taking a deeper look at the Phase Response Curves (PRCs) generated by Pendergast et al. for male and female mice of a knockout *Per2* strain ($mPer2^{ldc}$; $Per2^{-/-}$) and the PRC generated by Spoelstra et al. for $Per2^{tmBrd}$ ($Per2^{m/m}$) male mice. Examination of the PRC allows us to investigate how *per2* mice may differ in their responses to delay pulses administered at ZT14, as in Spoelstra and Albrecht, and ZT15, as in our experiments. $Per2^{-/-}$ mice show lower amplitude phase delays compared to WT mice, but the difference doesn't become evident until after ZT14. Thus, our light pulse at ZT15 may capture a greater magnitude delay than the ZT14 pulse of Spoelstra and

Albrecht, contributing to a more equal Delay/Advance ratio (Fig. 9). Where differences exist between the present report and those of prior studies, the methods of generating the PRC may contribute. Prendergast used DD, as did Spoelstra in a subset of studies. Albrecht and Spoelstra also used Aschoff Type II protocols with light pulses delivered in the 12h immediately preceding the LD-DD transition, whereas in the present report we used the modified Aschoff Type II protocol described by Evans and Gorman (2004): allowing 1 full cycle in DD to elapse before light pulses were delivered.

Due to their short endogenous period, Spoelstra et al. brought to light that MUTs may never fully entrain to a light-dark cycle and instead merely mask to it with a positive phase angle of entrainment. Though we timed the light pulses to account for the shorter endogenous period, if MUTs were never fully entrained to the LD cycle, pulses would occur 30 minutes earlier in the circadian cycle than intended. Interestingly this would actually bring our ZT15 delay pulse closer in time to ZT14, making our results more comparable to that of Spoelstra and Albrecht. At ZT14-15, the PRC for *Per2*$^{-/-}$ mice demonstrated comparable phase delays with WT mice, with differences in the magnitude of phase delaying not evident until ZT16. This is in agreement with our results (Fig. 8-9) and that of Spoelstra et al., who also found no differences in the phase delaying of *per2* mutants and WT mice. The discrepancy of the results of our experiment, Spoelstra et al.'s, and Pendergast et al.'s with Albrecht et al. is unclear, though various methodological differences (sexes and ages of mice, intensity of light pulse, number of crosses to C57Bl6 background strain, etc.) are likely at play. Our reduced Delay/Advance ratio relative to Pendergast et al. is easily explained by both our failure to administer delay or advance pulses at the peak of amplitude for *per2* mice, and by Pendergast's methodology for computing the ratio via the total area under the curve for advance and delay regions, as we calculated our ratio from

the average amplitude delay/advance in response to two discrete pulses only. In our experiment, MUTs exhibited a delay/advance ratio of .59, while WTs had a ratio of .20. Interestingly, HETs displayed an intermediate ratio of .37, demonstrating that *per2* gene dosage impacts non-parametric entrainment. *Per2* gene dosage impacted phase delay and phase advance differently, with more influence over phase advances and no significant impact of genotype on phase delay (Fig. 8-9). While mice displayed an average phase delay of 2.21 hours, MUTs had the largest average phase advance of 1.81 hours, and HETs had a larger average phase advance than WTs (0.66 hours and 0.41 hours, respectively), contributing to their intermediate delay/advance ratio. Future work should expand upon our findings here by evaluating the full PRC of $Per2^{m/m}$ MUT and HET mice in comparison to WTs, in particular with the inclusion of both males and females, to compute a comprehensive Delay/Advance ratio from area under the curve. This ratio can predict the response of the circadian system to light as well as inform our understanding of the flexibility of the circadian oscillator as determined by interactions between sex and the expression of these clock genes.

In this study, we also aimed to investigate circadian aftereffects, whereby exposure to a previous environment alters the speed of the clock. In particular, we examined the speed of the circadian clock and length of the active period in DD epochs before and after exposure to constant light. Aftereffects typically refer to entrainment aftereffects, but here we sought to determine if exposure to constant conditions exerted similar, persistent effects on circadian organization, and in a sex-specific manner. In terms of circadian period length, only MUTs showed changes in tau from the first epoch of DD to the last, with a longer tau in the last epoch of DD compared to the first (Fig. 5). No genotypes showed changes in circadian period length between the second epoch of DD (just before exposure to constant light) and the final epoch of

DD (after exposure to constant light). Therefore, aftereffects of constant conditions on circadian period length were not evident. In terms of active phase length changes, there was a trend for female WTs to show decreased alpha duration in the second bout of DD ($p=0.0564$), while other groups did not demonstrate this trend. As Aschoff's rule states that increased light intensity will increase circadian period and decrease active period length, female WTs in particular seem to exhibit these aftereffects of constant light into DD. This could be due to female WTs exhibiting the most pronounced extension of tau and compression of alpha in bLL compared to other groups (Fig. 5-6). These results could also imply that functional *per2* and sex interact to generate circadian aftereffects, though more specific research is needed to confirm this. For example, investigating how gonadectomized mice (with or without exogenous hormone administration) change circadian period length and alpha after entrainment to days of different lengths would give insight into how sex hormones contribute to circadian aftereffects of light exposure. Additionally, when mice were first transferred to constant conditions of a different light intensity, several groups would exhibit exaggerated responses (*i.e.,* MUTs drastically shortening tau in the first epoch of DD, and then lengthening over time; Fig. 5). Given that some of these responses were in the opposite direction predicted by aftereffects (prior exposure to a LD cycle should not induce shortening of circadian period), it's possible these are caused by some form of behavioral arousal that then settles by the second epoch of each constant condition. For this reason, we have chosen to visualize and focus on the second epoch in each constant condition.

These experiments probed the stability of the circadian network in the presence or absence of *per2* with several strategies aimed at examining processes that contribute to entrainment to the light dark cycle. These data reveal that the clock gene *per2* alters two core features of the circadian network. Firstly, *per2* impacts the speed and amplitude of the

endogenous clock itself. Additionally, *per2* impacts how the circadian system entrains to both parametric and non-parametric environmental cues, in this case the intensity and timing of light. Lack of functional *per2* prevented the lengthening of circadian period in response to constant light predicted by Aschoff's Rule (Fig. 4-5), and impacted alpha compression in a sexually diphenic way (Fig. 6), with a trend for MUT males but not females to compress active phase length in response to increase in light intensity. Partial lack of functional *per2* also impacted response to light, with HETs increasing circadian period in dim constant light to a lesser degree than WT mice, but increasing circadian period in higher intensity constant light to match WTs (Fig. 5). Additionally, HETs exhibited alpha compression in dimLL but did not further reduce alpha duration in response to greater light intensity (Fig. 6). *Per2* and sex also impacted the amplitude of the circadian clock, with MUTs showing decreased activity and females showing increased activity across conditions. Finally, lack of functional *per2* impacted the response to non-parametric light pulses during the delay and advance portions of the circadian cycle in a dose-dependent way (Fig 8-9). WTs showed a delay to advance phase shift ratio of around 0.2, MUTs exhibited a ratio of about 0.6, and HETs fell almost exactly in the middle with a ratio of around 0.4.

Increased responsiveness to light pulses may reflect a lower amplitude oscillator. Consider a small (low-amplitude) versus a large (high-amplitude) swinging pendulum: it takes more energy to disturb the large pendulum. A low amplitude circadian oscillator, like a low-amplitude pendulum, is easier to disrupt. Mice with low amplitude circadian oscillators would show increased responsiveness to light stimuli, and would be more likely to lose rhythmicity, both of which we see in the *per2* mouse. Lack of functional *per2* overall may reduce the amplitude of the molecular pacemaker and increase responsiveness to light during both the

advance and delay portions of the circadian cycle, but especially the advance portion. However, we cannot exclude an effect of *per2* on light perception (pacemaker input). As has been previously asserted, this balance between advance and delay portions could prevent normal lengthening of circadian period in response to constant light and be the driver behind circadian period differences (Pendergast et al., 2010). Together, this report is the first to indicate a role of gene dosage of *per2* and sex in driving parametric and nonparametric entrainment to light, supporting the concept that stability of the circadian system can be impacted both internally and by external environmental factors.

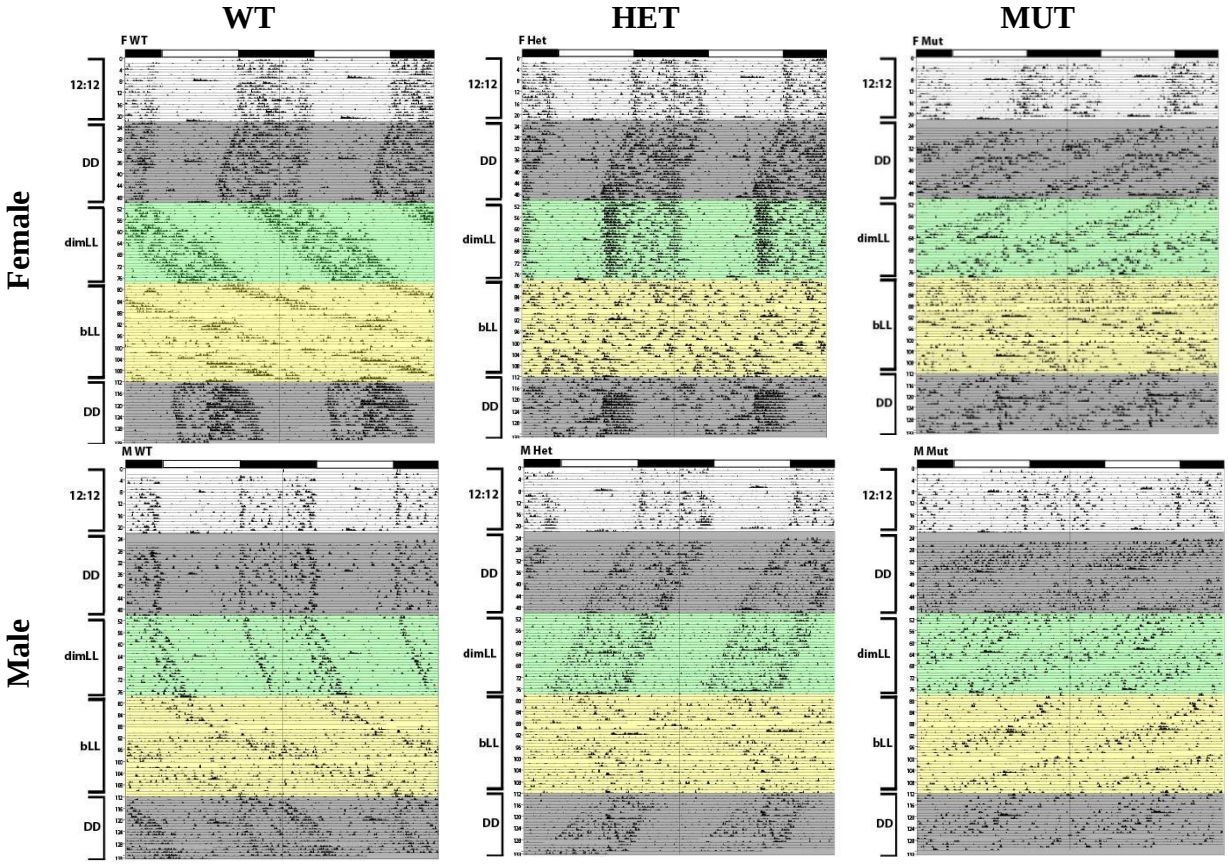

Figure 4: Representative actograms for each group in constant conditions of different light intensity

Double-plotted home cage locomotor activity (LMA) record for mice housed in various photoperiods and constant conditions: 12:12 (LD, white), with lights on and lights off represented with the white and blacks bars at the top of the actogram, respectively; constant darkness (DD; grey); dim constant light (dimLL; green); bright constant light (bLL; yellow). Male and female WTs (left plots) showed Aschoff-like effects on circadian period, with females (top left) showing slightly shorter period than males (bottom left) in DD and longer period than males in LL. HETs (middle plots) show much the same response as WTs in 12:12, DD, and dimLL. Upon exposure to bLL, HETs struggled to maintain rhythmicity, with half of females and most of the males going arrhythmic (ARR). HETs remaining rhythmic lengthened their circadian period similar to WTs. MUTs (right plots) showed a categorically different response to these photic manipulations. Males and females entrain to the light-dark cycle with a positive phase angle of entrainment due to their very short tau of ~22h, as seen in DD. Similar to WTs and HETs, females show a shorter tau than males in DD. In dimLL, MUTs do not show period lengthening and there were no differences between sexes. Additionally, MUTs struggled to retain rhythmicity in bLL. When first exposed to bLL, every MUT female went ARR. But, in the second epoch of bLL, the half of the females that recovered rhythmicity exhibited some period lengthening, though not significantly from dimLL tau. Conversely, 1/3 of the male MUTs went ARR in the first epoch of bLL, but the ones remaining rhythmic showed Aschoff-like period lengthening. But, when all but 1 male had recovered rhythmicity in bLL2, MUT males showed a shorter circadian period again, matching the period from dimLL.

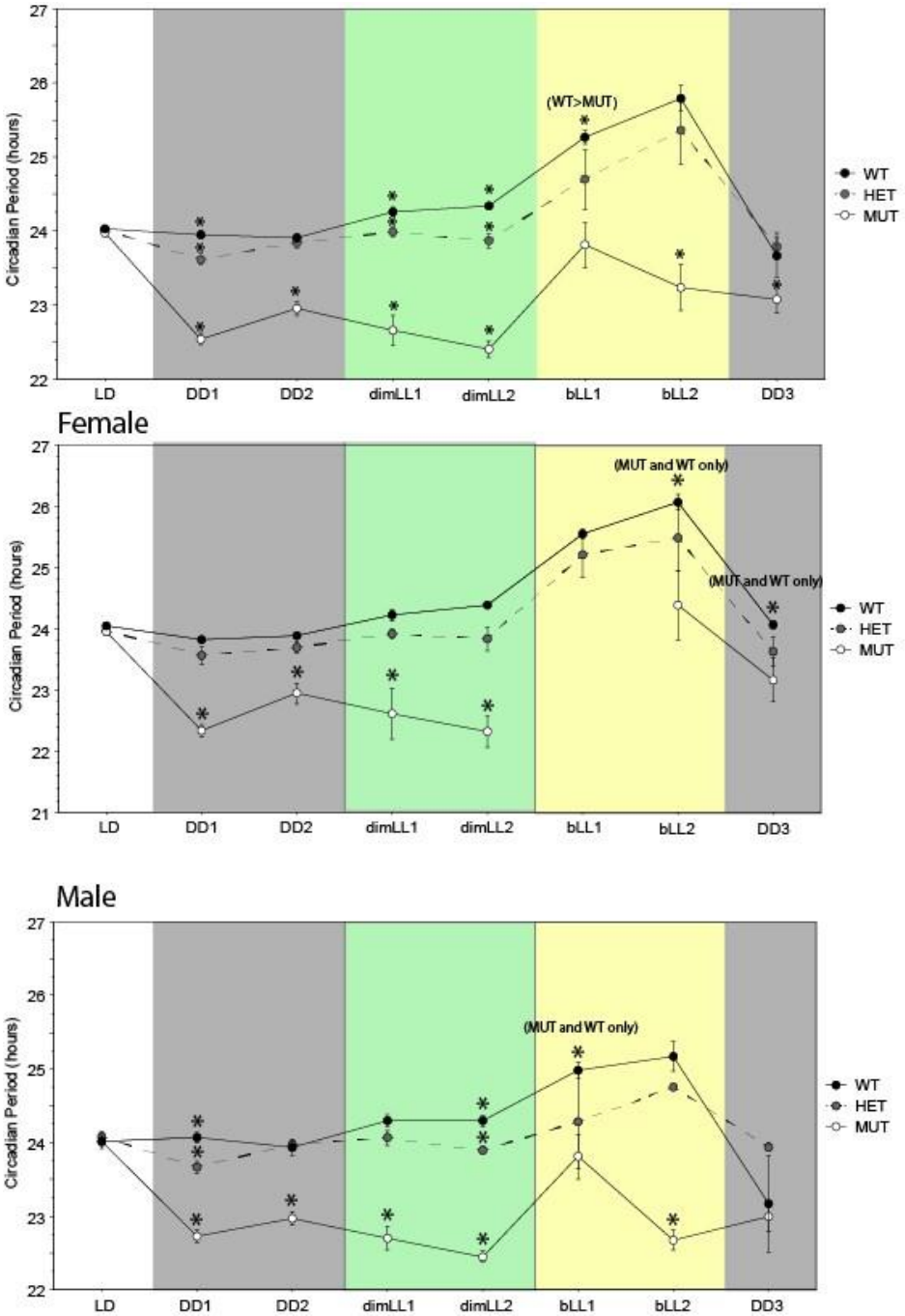

Figure 5: Circadian period changes in response to light of different intensity

Circadian period in hours is shown for each photoperiod: a 12:12 light-dark cycle (LD), the first two epochs of constant darkness (DD; grey), two epochs of dim constant light (<10lux, dimLL; green), two epochs of bright constant light (>300lux, bLL; yellow), and the third epoch of DD (DD3; grey). Due to lack of significant main effect of sex due to uneven group sizes across epochs, and to allow for easier visualization, genotype differences collapsed across sex are shown in the top plot. Circadian period differences broken apart by sex are also depicted in the bottom two plots. Asterisks indicate significant differences for that genotype within that photic manipulation ($p<0.05$). Error bars represent ±1SEM.

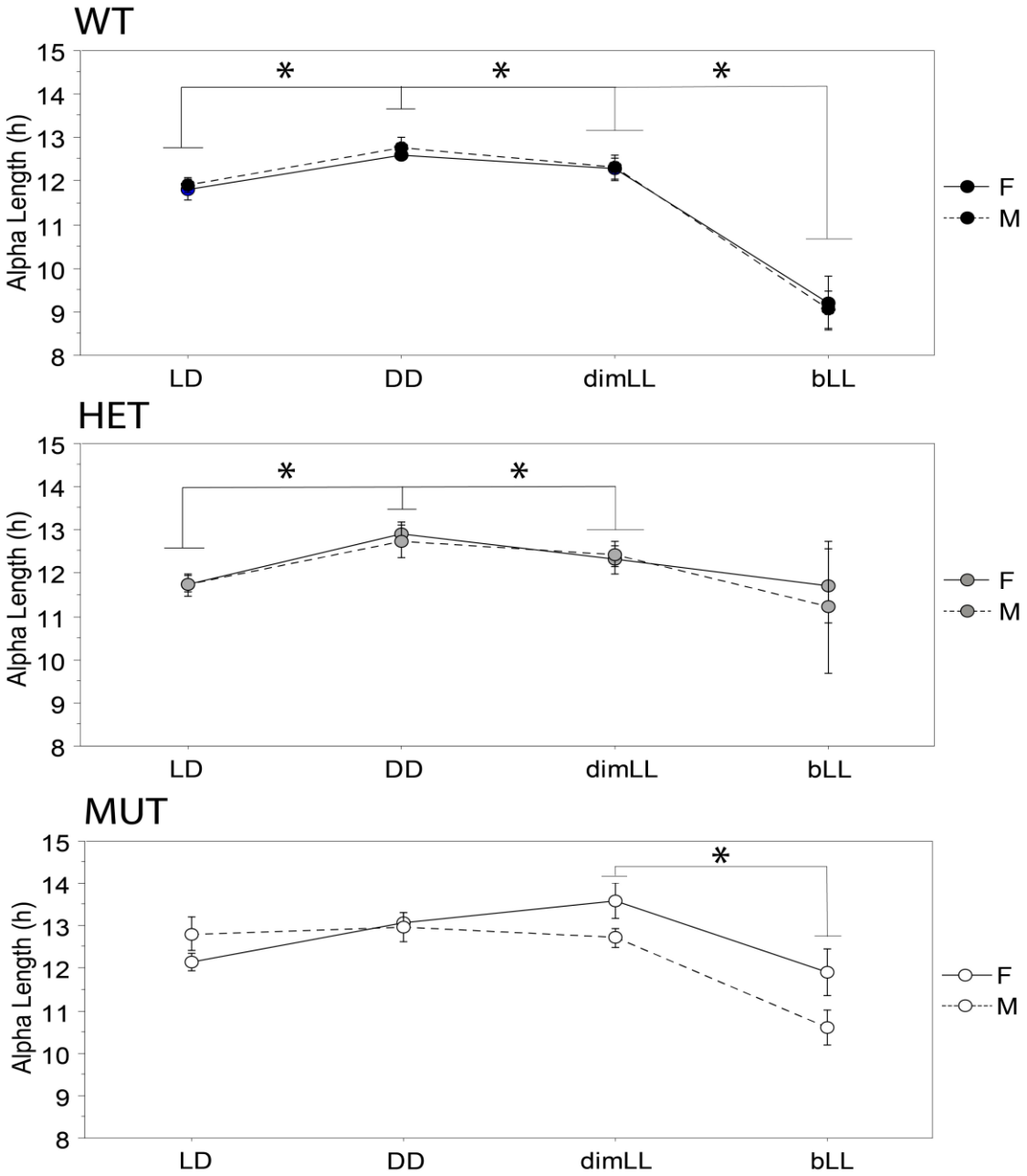

Figure 6: Alpha changes in response to light of different intensity

Length of active period (alpha) is shown for a 12:12LD cycle and constant conditions of increasing light intensity. WTs (top panel) and HETs (middle panel) showed alpha expansion in response to DD and alpha compression in response to dimLL, as predicted by Aschoff's Rule. WTs showed increased alpha compression in bLL while HETs did not. MUTs (bottom panel) did not show change in alpha length in any condition except bLL, where they showed alpha compression, but not to the same extent as WTs. Error bars represent ±1SEM. Asterisks indicate significant differences for that genotype ($p<0.05$).

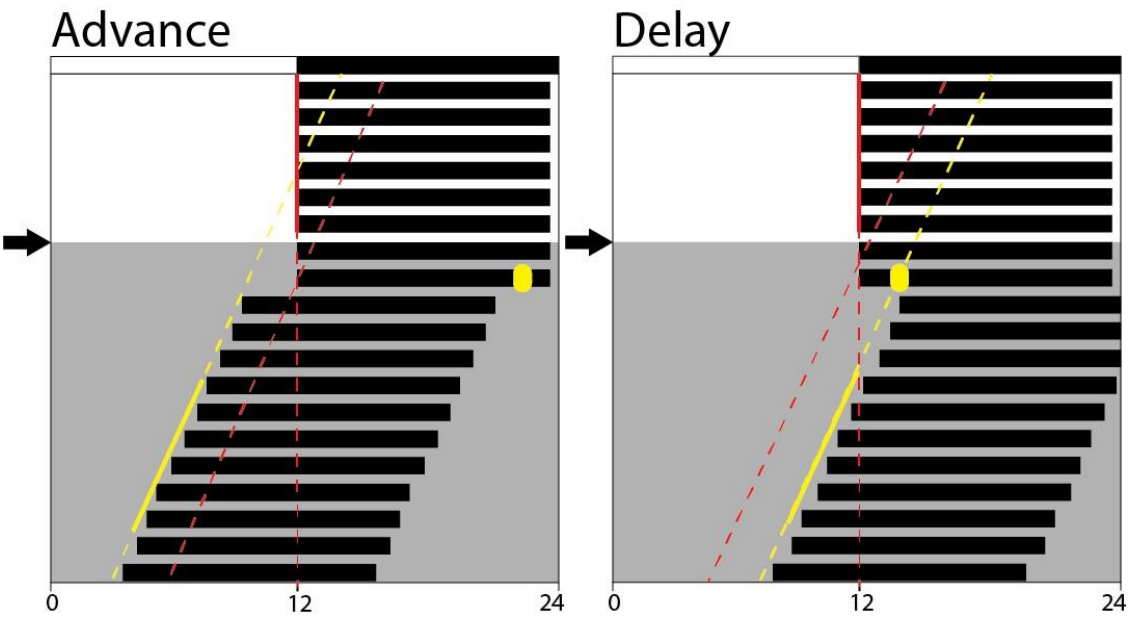

Figure 7: Schematic illustrating non-parametric Aschoff Type II phase shift protocol

After 3 weeks in a 12:12LD cycle, mice were transferred to constant darkness for 24h (arrow), after which a 1000 lux, 15-minute light pulse during either the advance (ZT22) or delay (ZT15) portion of the circadian cycle (yellow dot). One regression line was fit to the onset of activity for 6 days before the light pulse (solid red line). A second regression line was fit to the onset of activity for 6 days following the light pulse, excluding the first 3 post-pulse onsets (yellow line). Phase shift was calculated to account for the phase change between projected pre-pulse onsets and post pulse onsets (*i.e.*, difference between dotted red and yellow lines).

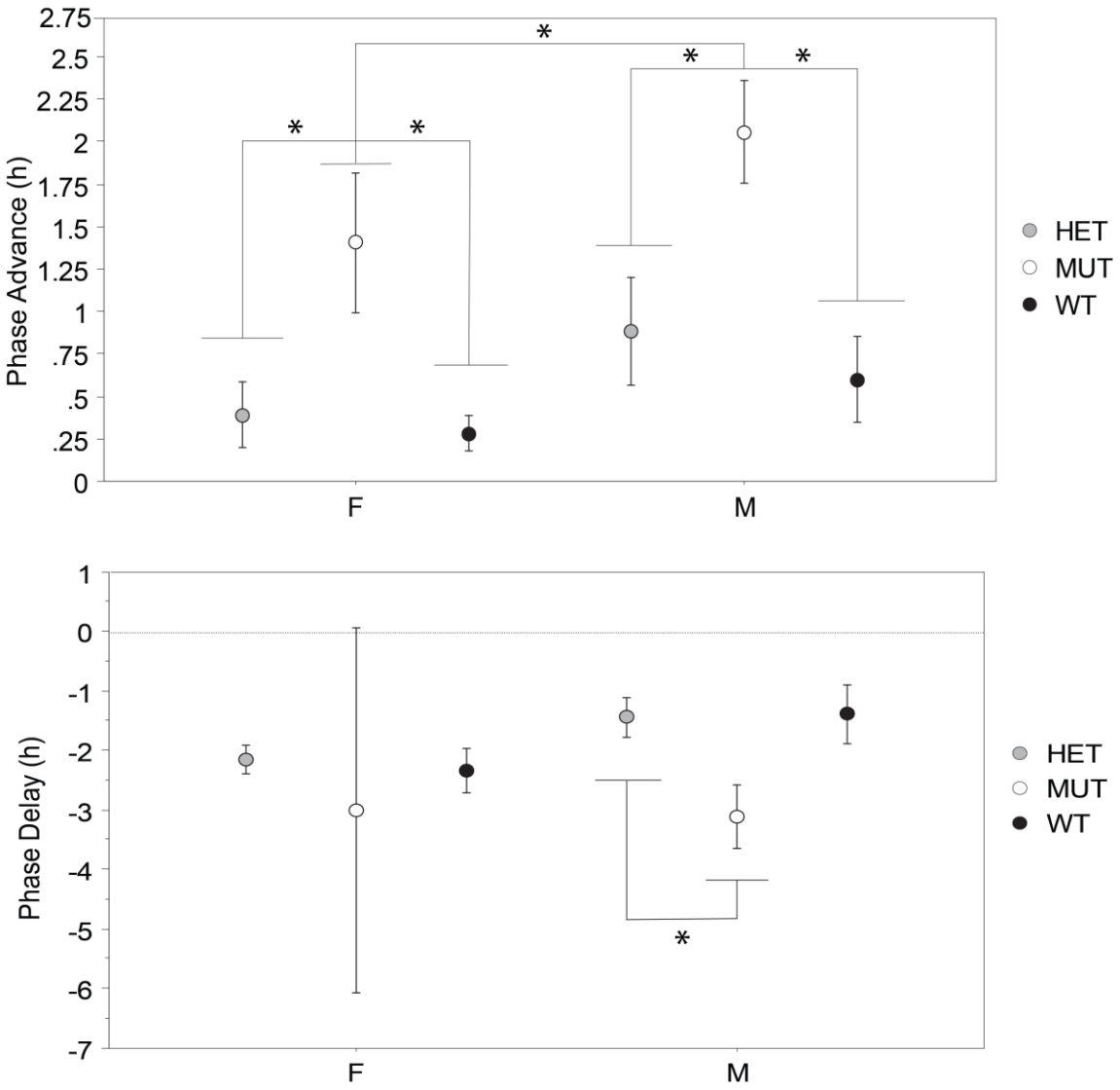

Figure 8: Phase advances and phase delays by sex and genotype

MUTs showed larger phase advances than HETs and WTs, which did not show significantly different phase advances from each other (top plot). Males also showed overall higher phase advances than females. Dropouts due to arrhythmia post-delay-pulse increased variability for female MUTs, and no significant differences among genotype were observed in females (bottom plot). MUT males showed a significantly larger phase delay compared to HET males, and showed a trending difference from WT males. WTs and HETs did not show significant differences in delay magnitude. Error bars represent ±1SEM. Asterisks indicate significance ($p<0.05$).

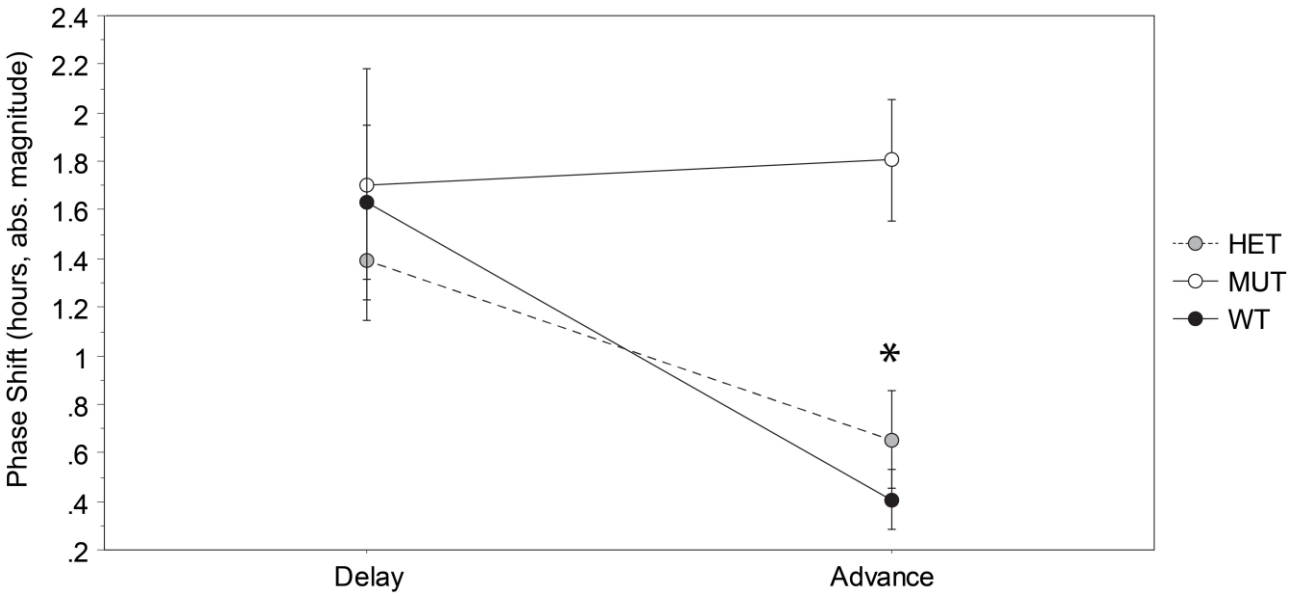

Figure 9: Phase Advance-Delay Magnitude

Absolute value for phase shifts during the delay and advance pulse plotted by genotype. Sex was collapsed for ease of visualization due to similar trend of genotype across sex and lack of significant sex differences in phase delays. MUT mice did not show significantly different magnitude phase delays and advances, giving them a more equal Delay/Advance Ratio. HETs and WTs showed a significantly smaller phase advance compared to a phase delay. Error bars represent ±1SEM. Asterisks indicate significant difference between delay and advance magnitude ($p<0.05$).

Chapter 4: Effect of circadian timed feeding on disease progression, circadian coordination, and behavior in the APP/PS1-21 Alzheimer's transgenic mouse

Abstract

Circadian rhythms have evolved to provide synchrony with the predictable daily oscillations of environmental cues, and interactions among circadian oscillators throughout the brain and body influence the adaptive coordination of the organism as a whole. By manipulating the timing of endogenous stimuli that represent salient time cues to distinct circadian clocks throughout the body, internal circadian desynchrony can be induced. Recent research has suggested that time-restricted feeding (TRF), whereby food intake is restricted to a particular window each day, may be useful in reducing or delaying neurodegeneration. We sought to apply TRF to the APP/PS1-21 mouse model of Alzheimer's Disease (AD), by manipulating the time of food availability to drive both circadian synchrony and circadian misalignment. APP/PS1-21 mice did not exhibit overt differences from non-transgenic (Ntg) in circadian parameters, but did exhibit baseline differences in some cognitive behavioral tests, with female APP/PS1-21 mice in particular exhibiting cognitive deficits. Circadian misalignment through timed feeding idiosyncratically altered performance in some cognitive behavioral tests but did not clearly exacerbate AD behavioral pathology.

Background

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by deterioration of cognitive functions, such as memory loss, language impairment, and even motor and perceptual skills (Ballard et al., 2011; Scheltens et al., 2016). These symptoms drastically impair quality of life, and progressive neuron loss eventually results in death (Neugroschl &

Wang, 2011). However, the pathology of AD begins well before these behavioral changes. The aggregation of beta-amyloid into extracellular plaques, in particular, begins at least two decades before the accumulation of tau tangles and behavioral impairment (Bateman et al., 2012; Sasaguri et al., 2017). In this way, understanding how the development of amyloid plaques impacts behavior and how plaque aggregation can be reduced is especially important for advancing research towards early interventions for AD.

A common tool for investigating AD treatments are transgenic mouse models expressing AD genetic variants. While there are many AD transgenic mouse strains, mice expressing cDNA encoding familial AD–linked human APPSWE and PS1ΔE9 variants - known as APP/PS1 mice - are among the most commonly studied for their production of amyloid plaques (Sasaguri et al., 2017). APP/PS1 mice display AD phenotypes including amyloid-beta plaque formation, cognitive impairment, increased behavioral anxiety and other related symptoms (Trinchese et al., 2004). However, the traditional APP/PS1 mouse model produces amyloid aggregates in peripheral tissues (*e.g.*, blood vessels, liver, and gastrointestinal tract) in addition to the brain, complicating interpretation of whether behavioral impairment is due to an impact of these aggregates on other functions (L. Zhang et al., 2021). The APP/PS1-21 mouse, on the other hand, uses a Thy1 promoter to express these AD transgenes, making Aβ plaques brain specific (Radde, 2006). This allows for more specific testing of central versus peripheral interactions within AD. Additionally, this strain develops plaques as early as 6 weeks of age, making it an ideal model in which to test interventions that could ameliorate already-present AD.

Several studies have found marked sex differences in plaque deposition and response to interventions in APP/PS1-21 and APP/PS1 transgenic mice (Dodiya et al., 2019; X. Li et al., 2016; J. Wang et al., 2004). This is comparable to the sex differences we see in human AD

patients, with women at greater risk of developing AD (Zhu et al., 2021). APP/PS1 female mice exhibit increased plaque deposition and greater spatial working memory impairment compared to APP/PS1 male mice (Mifflin et al., 2021; J. Wang et al., 2004). These differences may be dependent on differences in the gut microbiome. APP/PS1-21 mice given an antibiotic cocktail (ABX) that depletes gut microbiota had sexually diphenic responses (Dodiya et al., 2019, 2020). Namely, males given ABX had reduced plaque development while females given ABX did not. Other interventions related to metabolic factors seem to also have sexually diphenic results; APP/PS1 female mice showed greater improvement in spatial working memory than male APP/PS1 mice after undergoing a running exercise intervention (Zhou et al., 2018). Additionally, female APP/PS1 mice exhibit increased oxidative stress and impaired cellular energy metabolism in peripheral organs compared to males (J. Wu et al., 2016). This marks an important avenue for delving into sex differences within AD development and treatment, especially with consideration for metabolic factors.

Because of the late diagnosis in terms of disease etiology, recent efforts investigating how multidimensional approaches that center on altering lifestyle factors may be used to delay or prevent AD development (Dhana et al., 2020). Accessible lifestyle interventions such as the manipulation of the timing of light and food, are well positioned for investigation. AD patients exhibit disruption in coordination with food and light cues within the environment, including sleep fragmentation, decreased daytime activity, and altered food intake, to name a few, suggesting circadian rhythm disruption (Coogan et al., 2013; Musiek et al., 2015; van Someren et al., 1996; Vitiello & Prinz, 1989). In fact, timed bright light exposure has been used to treat some of these symptoms of AD in human patients, bolstering sleep efficiency, increasing daytime wakefulness, and reducing evening agitation (also referred to as sundowning, a common AD

behavioral symptom) (Figueiro, 2017; Sharma et al., 2021). Might the timing of environmental cues such as light and food be used early in disease progression to alter disease progression itself?

The timing of food is a salient cue for organizing physiology and behavior ecologically, and mistimed feeding is a potent circadian disruption manipulation that can be administered in the laboratory. In order to understand how circadian disruption due to mistimed feeding impacts the circadian system, it is first necessary to establish how circadian clocks in the body respond to the timing of food intake. Light acts to entrain the SCN clock in the brain, which serves as the master regulator of the phase relationship among peripheral circadian clocks, including the liver clock (Izumo et al., 2014). However, these peripheral clocks also use tissue-specific time cues to entrain their clocks (S. Zhang et al., 2020). For example, the liver entrains to time of food availability and related metabolic cues (Frazier et al., 2022; Tahara et al., 2011; Yamajuku et al., 2012). When food is available *ad libitum*, rodents consume the majority of their food during the dark phase during a window controlled by the circadian network (Challet, 2019). If food availability occurs outside of the time determined by the SCN (*i.e.*, during an abnormal time relative to the light cycle), the liver preferentially follows the food cue (Damiola et al., 2000; Stokkan et al., 2001). Thus, altering the relationship between light and food cues can alter the coupling relations between central and peripheral clocks, potentially shifting the phase of these oscillators to establish new temporal relations with one another. Outside of the lab, factors such as shift work or the constant availability of highly palatable foods prevalent in contemporary society can drive peripheral clocks out of alignment with the SCN light-entrainable clock (Pickel & Sung, 2020), and this misalignment is associated with diseases ranging from metabolic syndrome and diabetes (F. Wang et al., 2014) to cancer (Salamanca-Fernández et al., 2018).

Therefore, manipulating internal circadian alignment through the timing of food and light presents a strategy for probing how circadian misalignment may contribute to disease progression.

In fact, recent research has investigated how manipulating the alignment of central and peripheral circadian oscillators through timed feeding impacts neurodegenerative disease. Researchers investigated the impact of circadian misalignment on Huntington's Disease (HD), a disease of the extrapyramidal motor system due to loss of dopaminergic neurons in the basal ganglia. In the Q175 mouse model of HD, restricting food access to a 6-hour window during the midpoint of the active phase for 3 months improved locomotor activity rhythms, motor performance, and reduced HD-markers in the striatum to WT levels (H.-B. Wang et al., 2018). Together these data suggest that timed feeding, presumably via its effects on alignment of central and peripheral circadian oscillators (in non-specific tissues), reduced HD pathogenesis in this mouse model. Therefore, driving circadian alignment through the timing of food and light cues reduces etiology of the neurodegenerative disease, HD. Thus, a promising avenue for further exploring the pathogenesis of other neurodegenerative diseases such as AD lies in examination of the links between circadian disruption and AD progression.

Thus, in order to further explore the connection between circadian dysfunction, sex, and AD development, we altered the phase relations of light-driven and food-driven circadian rhythms by manipulating the timing of food availability as a non-photic zeitgeber. In this study, APP/PS1-21 mice of both sexes were subjected to a feeding regimen designed to entrain peripheral metabolic oscillators (Damiola et al., 2000; Stokkan et al., 2001). Depending on the timing of the food window, specifically depending on whether food was only available coincident with the active phase (when mice would normally eat most of their food) or

coincident with the rest phase (when mice eat only a small fraction of their food), we predicted that this will either drive alignment or misalignment, respectively, between the central circadian clock and peripheral metabolic clocks.

Several animal models of AD have investigated the relationship between intermittent fasting (IF) and AD etiology, with some studies showing reduction in amyloid plaques and others showing non-significant results (Cremonini et al., 2019; Gudden et al., 2021; Martin et al., 2006; Nasaruddin et al., 2020; Park et al., 2020; Shin et al., 2018; J. Zhang et al., 2017). These differences in findings are likely due to great disparity in IF procedure and animal model chosen. Specifically, some studies employed alternate-day fasting rather than a daily time-restricted feeding (TRF) paradigm in which food is restricted to a smaller window of availability, neglecting the circadian component inherent within IF (Halagappa et al., 2007; J. Zhang et al., 2017). The present study aims to rigorously test the contribution of circadian dynamics within AD using TRF, both at the "incorrect" (anorexic) time of the circadian cycle in order to drive misalignment (TRF-light) and at the "correct" (food ingestive) time to enhance circadian alignment (TRF-dark).

In order to confirm that a timed feeding paradigm is producing behavioral circadian disruption, it is necessary to measure whether the differences in circadian entrainment occur between mice that are fed at different times of day. Because activity is observed even when the food availability is shifted to the inactive phase and has been shown to be driven by its own peripheral circadian clock (Mistlberger, 1994), activity during the window of food availability will be used to determine how the mice align their activity to the time of day that food is presented relative to their overall central circadian rhythm, as measured by overall locomotor activity. This will be one metric by which we can assess the relative coordination between

peripheral clocks driven by food cues, and the clock in the brain that drives overall locomotor activity. One aim of this experiment, then, will be to use the extent to which mouse behavior is altered as an indirect measure of circadian disruption, and then to examine the extent to which this disruption is related to the severity of AD disease progression and behavioral symptomatology.

Numerous endpoints can be used to evaluate disease progression in AD mouse models. Physiological endpoints in APP/PS1 mouse models are generally targeted at assessing brain amyloidosis (*i.e.*, plaque deposition). While there are many techniques aimed at accomplishing this, immunoassays in which proteins are tagged by specific antibodies, such as immunohistochemistry (IHC) and enzyme-linked immunosorbent assays (ELISAs), are among the most common (Jiang et al., 2018). Though ELISAs are common, this technique was developed for monomeric proteins and thus is less likely to accurately detect aggregates, such as amyloid plaques (Janssen et al., 2015). Immunohistochemical (IHC) staining, on the other hand, can effectively detect aggregates, and affords the additional benefit of retaining information about spatial distribution of the protein of interest within the brain (Deng et al., 2011). The gold standard monoclonal antibody for assessing brain amyloidosis with IHC is 3D6 (3D6 Alzforum, n.d.), as it detects all amyloid beta species with amino-terminal aspartic acid, essential for the fibrillation of amyloid proteins and subsequent plaque formation and neurotoxicity (Sargaeva et al., 2009; Shi et al., 2022).

Behavioral assays of AD progression have also been developed and validated in AD mouse models, including the APP/PS1-21 mouse. APP/PS1-21 mice exhibit impaired cognitive flexibility, as measured by reversal learning (Radde, 2006; Van den Broeck et al., 2019), impaired learning and memory as assessed by the novel object recognition task (NOR) (Becerril-

Ortega et al., 2014; Scholtzova et al., 2008), impaired spatial learning and memory as assessed by performance on the Morris Water Maze (MWM) (Pihlaja et al., 2018), and impaired nesting ability and social interaction (C. Li et al., 2015; Owona et al., 2019; Z.-Y. Zhang et al., 2014; Z.-Y. Zhang & Schluesener, 2013). APP/PS1 mice also exhibit reduced performance on the MWM and other spatial working memory tasks, including spontaneous alternation (Kemppainen et al., 2014; T.-K. Kim et al., 2012; McClean et al., 2011; Vepsäläinen et al., 2013), impaired reversal learning (Dempsey et al., 2017; McClean et al., 2011; Pietropaolo et al., 2012), reduced NOR and novel odor recognition (Dempsey et al., 2017; McClean et al., 2011; Q. Zhang et al., 2018), and reduced spontaneous exploratory activity (Kemppainen et al., 2014). Changes in marble burying have also been reported in APP/PS1 mice, though there is some discrepancy in interpretation. Some studies have regarded this behavior as a measure of object neophobia, as APP/PS1 showed reduced marble burying (Kemppainen et al., 2014, 2015; T.-K. Kim et al., 2012; Vepsäläinen et al., 2013). However, some studies showed an increase in marble burying for APP/PS1 mice, and cast this to be an assay of compulsive-like behavior (Peng et al., 2021; Q. Zhang et al., 2018). This discrepancy really illustrates the difficulty with cognitive testing in animal models, and the necessity to consistently validate behaviors across models in order to determine face validity. However, each of these behavioral phenotypes has construct validity with AD in humans (*i.e.*, impaired spatial working memory, cognitive flexibility, etc.) and therefore afford critical insights into neurophysiological progression of AD and how it may respond to environmental manipulation. We first want to assess how marble burying is impacted in the APP/PS1-21 model and determine the efficacy of this paradigm as a neophobia or compulsive anxiety test, and how this is impacted by progressive plaque deposition. We will also evaluate AD progression and how it is impacted by circadian disruption and timed feeding using

a spontaneous alternation paradigm to assess spatial working memory (Hughes, 2004; Prieur & Jadavji, 2019), as this cognitive behavior is shown to be particularly impacted by Alzheimer's Disease (Guariglia, 2007; Kirova et al., 2015).

Methods

Table 1: Circadian Misalignment and Alzheimer's Abbreviations

Abbreviations	
AD	Alzheimer's Disease
HD	Huntington's Disease
APP/PS1-21; Tg	Transgenic AD (experimental) mouse
Ntg	Nontransgenic (control) mouse
LD	12:12 light dark cycle
DD	Constant darkness
LL	Constant light
ZT	*Zeitgeber* time, time relative to photic stimulus with ZT12 being lights off
AL	*Ad libitum, Ad lib* food available
TRF-D	Dark-phase fed, food only available during 6h midpoint of dark phase
TRF-L	Light-phase fed, food only available during 6h midpoint of light phase
SA	Spontaneous Alternation task of spatial working memory
MSFI	Mass-specific food intake; grams of food intake per gram of body weight per day

Animals

APPSWE/PS1L166P (APP/PS1-21) mice were obtained from a colony maintained by the Sisodia lab at University of Chicago. The mouse line was originally obtained from M Jucker; University of Tubingen, Tubingen, Germany (*APPPS1 | ALZFORUM*, n.d.; Radde, 2006). Mice

were maintained on a C57BL6Cj background and bred within a colony managed by our lab. Homozygous nontransgenic and heterozygous transgenic APP/PS1-21 mice were paired to produce heterozygous APP/PS1-21 (transgenic; to be used as experimental mice) and homozygous nontransgenic (Ntg; to be used as control) offspring. Mice of both sexes (transgenic: n=39 females, n=32 males; Ntg: n=32 females, n=35 males) were single housed in conventional cages with wirebar lids and without microisolator filters in a 12L:12D photocycle of approximately 150-200 lux. Experiments were performed within two neighboring vivarium rooms with inverse light-dark cycles. Mice were randomly assigned to either *ad libitum* or time-restricted access to standard rodent diet (Irradiated Teklad Global 18% Rodent Diet 2918, Envigo RMS). All mice had *ad libitum* access to filtered drinking water. Cage changing was performed at two-week intervals, during which time food weight and body weight were measured. All mice were acclimated to cages for at least one week prior to data collection. The integrity of experimental LD cycles was continuously monitored and verified by dataloggers (HOBO,UX90, Onset Comp). Experiment 2 was conducted in two identical rounds. Estrous cycles of females were not monitored. All procedures related to animal use were approved by the University of Chicago Institutional Animal Care and Use Committee. At the conclusion of the experiments homozygous nontransgenic (Ntg) and heterozygous transgenic (Tg) APP/PS1-21 genotypes were confirmed in all mice by PCR using the protocol described for this genotype by Radde (2006).

Genotyping

Only homozygous Ntg and heterozygous Tg mice were included in these experiments (a priori criterion). Genotyping of all mice bred in our vivarium was done using primers ordered from the Jackson Lab Website [5’ → 3’: PS1 Forward (CAG GTG CTA TAA GGT CAT CC), PS1

Reverse (ATC ACA GCC AAG ATG AGC CA), APP1 (CGA CAG TGA TCG TCA TCA CCT), APP4 (CTT AGG CAA GAG AAG CAG CTG)] and using specification for the Platimun Taq Polymerase (Life Technologies, Invitrogen catalog number: 10966-018). For each individual PRC reaction: 17.15 uL of DNAase free H2O, 2.5 uL of 10X PCR Buffer with no MgCl2, 0.75 uL 50mN MgCl2, 0.5 uL 10mM dNTP mix, 0.5 uL of each primer (either APP1 and APP4 OR PS1F and PS1R), and 0.1 uL of Taq were added to a master mix and thoroughly mixed by pipetting up and down. 22 uL of master mix were aliquoted and added to 3 uL of DNA derived via HotShot (Truett, Heeger et al. 2000), from ear clips and tail clips collected prior to or at the conclusion of the studies, respectively. We used the following PCR Protocol on a thermocycler (S1000; Bio-Rad): (1) 95 C for 3 minutes, (2) 95 C for 30 seconds, (3) 58 C for 1 minute, (4) 72 C for 1 minute, (5) repeat steps (2-4) 30 times, (6) 72 C for 5 minutes, (7) End (hold at 4 C). 8 uL of the resultant PCR products, and a 100 Bp to 2000 Bp Ladder (Thermofisher catalog # 15628050) for reference were mixed with 1.4uL of loading dye (Thermo Scientific catalog number: R0611), loaded on a 2% agrarose gel with 2.5uL of Ethidium Bromide (stock solution: 10mg/mL), and visualized. Resultant bands (amplicon sizes) per Jackson Lab Website were as follows: PS1 = ~300 bp, APP = ~500 bp. Due to cointegration of the transgenes, for routine analysis only genotyping for APP was done, with PS1 genotyping done only sporadically as a control and for genotype confirmation.

Study Design

Experiment 1: A 2 (sex; male and female) x 2 (genotype; Ntg and Tg) design (n=3-4/group) was used to assess cognitive behavior in the marble burying test and spontaneous alternation (SA) test at the onset of plaque development (6 weeks), as well as circadian parameters including

entrainment to a 12:12LD cycle, endogenous circadian period in constant darkness (DD), and response to constant light (LL).

Experiment 2: A 2 (sex; male and female) x 2 (genotype; Ntg and Tg) x 3 (feeding manipulation; *ad libitum*, TRF-L, TRF-D) design (n=8-18/group) was used to assess how time-restricted feeding impacts AD progression. A subset of these mice (n=2-7/group) were also monitored for locomotor activity to verify behavioral circadian disruption. Food manipulation began at 6-weeks of age, with mice either receiving food *ad libitum*, during the 6h midpoint of the light (inactive) phase (TRF-L), or during the 6h midpoint of the dark (active) phase (TRF-D). Food manipulation continued for 12 weeks. During week 11, behavioral tests (marble burying and spontaneous alternation) were administered. Mice were sacrificed and tissues collected at the end of the 12 weeks of food manipulation.

Lighting Cycles

Experiment 1 was a pilot study which examined circadian dynamics and cognitive behavior at the onset of plaque deposition. Mice remained in a 12:12 LD cycle until 6 weeks ±3 days of age, during which time they underwent behavioral tests. After at least 2 weeks in 12:12, these mice were put into constant darkness (DD) for 2 weeks, followed by constant light (LL) for 2 weeks. This first cohort of mice (n=3-4 per group) was not subjected to any food manipulation and food and water were available *ad libitum*. Mice (n=8-11 per group) in Experiment 2 stayed in a 12:12 photocycle throughout the TRF experiment.

Time Restricted Feeding Manipulation

Mice in the Experiment 2 (n=8-16/group) were entrained to a 12:12 photoperiod for a minimum of 2 weeks prior to any treatment. Mice began food manipulation at 40-50 days of age and were

randomly assigned to a feeding condition: *ad lib*, food available for 6h during the midpoint of the light phase (ZT 3-9), or food available for 6h during the midpoint of the dark phase (ZT 15-21). By definition, ZT 12 occurs at the onset of the dark (active) phase. Food was available via food hopper, which was taken out of the cage to prevent food consumption outside of the 6h time block. The mice were held in this condition for 12 weeks.

Fecal Pellet Collections

Fresh fecal pellets were collected in sterile 1.5ml centrifuge tubes at ZT9 and ZT21 on either week 2 or week 4 of TRF treatment, and during week 11 of treatment. These pellets were collected with the goal of characterizing the intestinal microbial community structure via 16S at a future date. Fecal samples were immediately frozen on dry ice and subsequently stored in -80℃.

Necropsy and Tissue Harvesting

Tissues were collected during the ZT3-5 window at the end of week 12 of food manipulation. Necropsy was performed according to procedures approved by Animal Care and Use Protocols. Briefly, mice were deeply anesthetized under 3-4% isoflurane/O_2 gas. After confirmation of deep anesthesia, the heart was accessed through abdominal incision. Blood was collected from the right ventricle with a 25-guage needle and stored in buffered sodium citrate on ice. Following blood collection, the descending aorta was clamped and mice were perfused with 0.9% saline (pH 7.4) for 3 minutes. Brains were then excised and the two hemispheres were bisected; the right hemisphere was postfixed in 4% paraformaldehyde and the left hemisphere immediately frozen on dry ice and stored in -80℃. Liver, small and large intestine, and cecum were collected and immediately frozen on dry ice and stored in -80℃. Immediately following necropsy, plasma was separated from blood cells by centrifugation at 2,000 rpm for 10 minutes at -4℃ and stored at

-80℃. These brains were collected with the goal of characterizing soluble and insoluble Aβ levels via MSD MesoScale extraction at a later date (Dodiya et al., 2021).

Locomotor Activity Analysis

All cages in Experiment 1 (n=3-4) and a subset of select cages in Experiment 2 (n=2-7 per group) were equipped with overhead passive infrared (PIR) sensors to monitor locomotor activity (Prendergast et al., 2015). Sensors were mounted 22cm above the floor of the home cage and recorded a movement event when 3 of the 27 zones were crossed within the cage (beam breaks). Breaks in the infrared beam were recorded in 1-minute bins using the ClockLab Data Collection system and analyzed using ClockLab Analysis 6 software (Actimetrics, Wilmette, IL, USA). For Experiment 1, locomotor activity in a 12:12LD cycle, constant darkness (DD), and constant light (LL) was analyzed to determine circadian period and onset variability by drawing a best-fit line over 2-week bouts. For Experiment 2, locomotor activity during the time of food availability relative to overall activity was compared between TRF-light, TRF-dark, and ad lib groups to analyze circadian distribution. Activity profiles were averaged by group across the full length of the 12 weeks of TRF manipulation.

Marble Burying Test

Marble burying tests were administered to assess how this behavior is altered in the APP/PS1-21 mouse. This test has been purported to measure compulsive anxiety and repetitive behavior, common clinical features of Alzheimer's Disease and dementia, though some experiments have claimed an increase in marble burying equates to increased compulsive anxiety (Cipriani et al., 2013; Gitlin et al., 2012), while other studies have equated reduced marble burying with increased compulsive anxiety (Broekkamp et al., 1986; de Brouwer et al., 2019; Deacon, 2006; Londei et al., 1998; Njung'e & Handley, 1991; Takeuchi et al., 2002; Thomas et al., 2009).

Reduced marble burying has also been described as denoting decreased exploratory behavior (Moreno et al., 2017), and neophobia (Kemppainen et al., 2014; T.-K. Kim et al., 2012; Vepsäläinen et al., 2013). The majority of these studies demonstrate reduced marble burying behavior in APP/PS1 mice, so these latter explanations seem to show the most face validity. However, we aimed to evaluate if the APP/PS1-21 model recapitulates this response to marble burying. Marble burying was conducted with a procedure modified from (Amodeo et al., 2012), with 10 minute rather than 30 minute trials. Trials consisted of placing each mouse in a new cage filled with 3cm of corncob bedding for 10 minutes to acclimate to the new environment and then returning the mouse to their original cage. Then, 15 marbles were spaced evenly within the cage, and the mouse was placed back in this cage for 10 minutes. Marble burying was performed at ZT 2-4 and ZT 14-16 for all mice in Experiment 1 and was performed at ZT 2-4 for all mice in Experiment 2. The number of marbles buried (covered >50% with bedding) and the number of marbles moved after the 10-minute period was recorded and analyzed by an experimenter blind to treatment group.

T-Maze Spontaneous Alternation

Spontaneous alternation exploits the natural exploratory behavior of rodents to assess spatial working-memory performance, with more alternation between arms of the maze indicating better cognitive behavioral performance. Percentage of alternation (number of alternating entries divided by the total number of free choice entries into maze arms) has been shown to decrease in APP/PS1 mice relative to WT mice (Chaney et al., 2018). Spontaneous alternation was conducted as previously described by Gerlai in order to minimize handling and thus stress on the mice (Gerlai, 1998). Spontaneous alternation (SA) tests were carried out using a T-maze with 15cm high walls that consisted of a 10cm x 10cm starting box off of a 40cm long start arm with

two 20cm long choice arms extending on either side. The start box and the 2 choice arms were blocked off from the start arm with guillotine doors. All trials were recorded with a camcorder equipped with infrared night vision positioned above the T-maze. The mouse was placed inside the start box for 5 seconds before the start door was opened. For the first trial, only one choice arm door was opened (forced trial). The mouse exited the start box to the start arm, then to the open choice arm, and eventually wandered back into the start box, at which point the start box door was closed for another 5 seconds. For all subsequent trials, both choice arm doors were raised upon opening of the start box door. Once the mouse exited the start box and selected a choice arm, the other choice arm was closed off. If the time of a trial exceeded 2 minutes, the mouse was carefully placed back into the start box and a new trial was started. This free choice procedure was repeated 13 times for each mouse. The entire SA procedure was performed at ZT 2-4 for all mice in Experiments 1 and 2, and at ZT 14-16 for all mice in Experiment 1. Experimenters blind to condition recorded the number of spontaneous alternations made (entering the right goal arm after the left goal arm was entered and vice versa). The number of alternating entries made was divided by the total number of free choice entries made and multiplied by 100 to get a percent of alternation.

Locomotor Activity Analysis

For Experiment 1, locomotor activity in a 12:12LD cycle, constant darkness (DD), and constant light (LL) were analyzed to determine circadian period and onset variability by drawing a best-fit line over 2-week bouts. For Experiment 2, circadian period and locomotor activity during the time of food availability relative to overall activity was compared between TRF-light, TRF-dark, and ad lib groups to analyze circadian distribution. Average activity profiles for each group were generated in ClockLab. Circadian period was calculated using a Lomb-Scargle Periodogram

(LSP) analysis (ClockLab; threshold: P=0.001) on 10 days of activity data (Ruf, 1999; Tackenberg & Hughey, 2021). Periods between 22 and 26 hours were considered in the circadian range. Total daily activity counts, active phase activity, and rest phase activity in each photoperiod were derived from Clocklab over 10-day epochs and aggregated in Excel.

Statistical Analyses

Analyses of variance (ANOVAs) were performed to decrease chances of Type I error. If a statistically significant F-statistic was achieved, unpaired T-tests were performed using Statview 5.0 (SAS Institute, Cary, NC). Differences were considered significant if $p<0.05$. If an F test showed a violation of the assumption of variance for an ANOVA, nonparametric analyses were performed. Kruskal-Wallis Tests were used to assess differences between feeding conditions, and if a significant H statistic was found, pairwise comparisons would be analyzed with a Bonferroni/Dunn. Sex and Genotype differences were each analyzed with a Mann-Whitney U Test. Experiment 2 was conducted in two rounds. Datasets from both rounds were combined as no statistical differences were observed between rounds.

Results

Experiment 1 – circadian, emotional, and cognitive behavior in APP/PS1-21 mice

Circadian Phenotyping. There was no main effect of genotype ($p=0.9439$) or sex ($p=0.4455$) and no interaction effects ($p=0.2001$) on circadian period in 12:12 (Fig. 10), indicating all mice entrained to the 12:12 LD cycle. Again, there was no main effect of genotype ($p=0.7742$) or sex ($p=0.5887$) and no interactions ($p=0.5887$) for circadian period (tau) in DD, demonstrating no differences in endogenous circadian period length. There was a significant main effect of sex on tau in LL ($p<0.002$), with females displaying a longer circadian period than

males ($p<0.001$), with a mean difference of 0.77 hours. However, there were still no genotype ($p=0.1165$) or interaction ($p=0.1090$) effects on tau in LL. There were significant main effects of sex ($p<0.01$) and genotype ($p<0.02$) on onset variability in 12:12 (Fig. 10), and a significant interaction between sex and genotype ($p<0.005$), with female APP/PS1-21 mice displaying more onset variability than all other groups ($p<0.01$). There were no main effects of genotype ($p=0.1298$ and $p=0.3513$, respectively) or sex ($p=0.5384$ and $p=0.2817$, respectively) on onset variability in DD and LL, and no interaction effects ($p=0.6937$ and $p=0.3150$, respectively), demonstrating that transgenic females may have some difficulty in entrainment, but this effect disappears in constant conditions.

Cognitive Behavioral Tests. All mice (n=3-4/group) were administered marble burying and spontaneous alternation tasks during the light and dark phase (Fig. 11). There was a significant main effect of sex ($p<0.05$) and genotype ($p<0.001$), but not of time of test ($p=0.7305$) on marbles buried, with no significant interactions ($p=0.3596$; $p=0.5041$; $p=0.4597$; $p=0.9855$). The effect of sex on marbles buried was not significant, but trending ($p=0.0658$), with females burying fewer marbles than males. Additionally, APP/PS1-21 mice buried significantly fewer marbles than Ntg mice ($p<0.0005$). There was also a significant main effect of sex ($p=0.0001$) and genotype ($p<0.0005$) on marbles moved, and no significant effect of time of test was observed ($p=0.6959$). There was also a significant interaction between sex and genotype ($p<0.05$), but no other significant interactions ($p=0.8033$; $p=0.5705$; $p=0.4792$). No sex difference was evident in Ntg mice for marbles moved ($p=0.1277$), with both sexes moving on average 10.5 marbles. However, APP/PS1-21 females moved significantly fewer marbles than APP/PS1-21 males ($p=0.0001$), moving only 1.6 marbles on average compared to the 9.8 marble average of males. In fact, APP/PS1-21 males did not differ from Ntg males in number of marbles

moved (p=0.1713). These results point to APP/PS1-21 female mice in particular exhibiting deficits in marble burying and moving behavior. No significant main effects or interactions were observed in spontaneous alternation (sex: p=0.0816; genotype: p=0.2219; time of test: p=0.2429), though there was a trend for an impact of sex, where females showed reduced alternation (p=0.082).

Experiment 2 – The impact of time restricted feeding on AD behavioral progression

***Circadian activity profiling*:** 61 mice (n=2-10/group) from the first round of Experiment 2 were monitored for locomotor activity throughout the 12-week feeding paradigm (Fig. 12-13). Activity over the course of the 12 weeks of timed feeding treatments was separated into 6 10-day epochs, with each epoch starting 2 days after cage change and ending 2 days before the subsequent cage change. An F test showed a violation of the assumption of equality of variance for an ANOVA in individual epochs as well as the average of all epochs, due to the unbalanced group sizes. Therefore, nonparametric analyses were performed. Kruskal-Wallis Tests were used to assess differences between feeding conditions, and if a significant H statistic was found, pairwise comparisons would be analyzed with a Bonferroni/Dunn. Sex and Genotype differences were each analyzed with a Mann-Whitney U Test.

Total activity counts

Genotype: There was no significant impact of genotype on average total daily activity in any epoch (p=0.9646; p=0.9528; p=0.9058; p=0.7387; p=0.5243; p=0.6275).

Sex: There was a significant impact of sex on average total daily activity in all epochs, with females displaying higher activity than males ($p<0.05$; $p<0.05$; $p<0.02$; $p<0.01$; $p<0.01$; $p<0.005$).

Feeding Group: A Kuskal-Wallis Test for epoch 1 showed a significant effect of feeding condition ($p<0.002$): TRF-L fed mice exhibited significantly less average total daily activity than TRF-D ($p<0.005$) and AL ($p<0.005$) groups, who did not differ from each other ($p=0.6099$). In epoch 2 and all other epochs, there was again an impact of feeding condition ($p<0.01$; $p<0.05$; $p<0.02$; $p<0.005$; $p<0.02$), with TRF-L mice exhibiting less activity than TRF-D mice ($p<0.01$; $p<0.02$; $p<0.005$; $p<0.002$; $p<0.01$), though they no longer showed different activity counts from AL ($p=0.0381$; $p=0.1256$; $p=0.0397$; $p=0.0534$; $p=0.1311$, where $p<0.0167$ is significant). TRF-D and AL groups again did not differ ($p=0.2306$; $p=0.1926$; $p=0.1943$; $p=0.0710$; $p=0.1172$).

Analysis of Circadian Activity

Most studies using FAA limit food to a 4-5h window of the day (usually the light phase for nocturnal rodents), and operationally define FAA as activity during a window of time preceding the food availability window. The present study design did not permit such a methodology, as food was available for a longer window of time, covering half of the light phase (TRF-L) or half of the dark phase (TRF-D). In order to draw connections between circadian rhythmic disruption consequent to TRF and changes in behavior, I quantified TRF-induced circadian disruption by evaluating activity during 3 intervals of the circadian cycle for each mouse: (1) the amount of activity expressed during the dark (active) phase, (2) the amount of activity during the 3h window preceding the window of food availability (termed FAA), and (3) the amount of activity during the window of food availability. Activity in each interval was

expressed as a % of total daily activity. Because these data were also in violation of the assumption of equality of variance for an ANOVA due to markedly different sample sizes, nonparametric methods were used to assess group differences.

Food anticipatory activity (FAA) interval

Genotype: There was no significant impact of genotype on % activity during the FAA window for any epoch ($p=0.8592$; $p=0.5543$; $p=0.5250$; $p=0.8708$; $p=0.6048$; $p=0.4077$).

Sex: There was no significant impact of sex on the partitioning of % activity to the FAA window for any epoch ($p=0.7938$; $p=0.9884$; $p=0.5911$; $p=0.4245$; $p=0.4950$; $p=0.8617$).

Feeding Group: There was a significant effect of TRF treatment on percent FAA in epoch 1 ($p<0.0001$), with TRF-D mice showing higher activity than AL ($p=0.0001$) and TRF-L ($p<0.0001$) mice during this period. TRF-L mice showed the lowest activity during the FAA window, significantly lower than AL mice ($p<0.005$). In all other epochs, there was again a significant impact of feeding group on FAA ($p<0.0001$ for epochs 2-6). TRF-L mice still showed the lowest activity during this period, significantly lower than TRF-D ($p<0.0001$ for epochs 2-6) and AL ($p<0.0001$ for epochs 2-6) mice, though in these latter epochs TRF-D and AL mice no longer differed from each other ($p=0.0173$; $p=0.0684$; $p=0.1828$; $p=0.9941$; $p=0.4173$, where $p<0.0167$ is significant). When averaged across all epochs, TRF-L mice partitioned 9.14% of their activity to the FAA window, while AL mice partitioned 18.56% and TRF-D mice partitioned 21.14% of their daily activity to this same window.

Food availability interval

Genotype: There was no significant impact of genotype on the consolidation of activity to the window of food availability for any epoch ($p=0.9764$; $p=0.9058$; $p=0.9882$; $p=0.5059$; $p=0.8825$; $p=0.8360$).

Sex: There was no significant impact of sex on the consolidation of activity to the window of food availability for any epoch ($p=0.3094$; $p=0.5614$; $p=0.8163$; $p=0.6631$; $p=0.8163$; $p=0.2281$).

Feeding group: There was a significant impact of feeding group on activity during the window of food availability in epochs 1 and 2 ($p=0.0005$; $p<0.001$), with TRF-D mice spending a significantly higher percentage of their overall activity in this 6h window compared to AL ($p=0.0001$; $p<0.0001$) and TRF-L ($p=0.001$; $p<0.005$) mice, which did not significantly differ from each other ($p=0.8902$; $p=0.6340$). These relations changed in epochs 3-6, though there was still a significant impact of feeding condition ($p<0.02$; $p<0.02$; $p<0.005$; $p<0.001$). TRF-D mice partitioned more of their activity than AL mice to the window of food availability ($p=0.001$; $p<0.02$; $p<0.0002$; $p<0.0001$), and AL and TRF-L groups still did not differ from each other ($p=0.4924$; $p=0.1815$; $p=0.853$; $p=0.0878$), but the difference between TRF-D and TRF-L disappeared ($p=0.0299$; $p=0.0928$; $p=0.1015$; $p=0.0424$, where $p<0.0167$ is significant). Together these data indicate that TRF-L mice were able to alter their activity to better align with the window of food availability after the first 2 epochs (4 weeks). When averaged across all epochs, AL mice allocated 26.36±3.57% of their daily activity to the window of food availability (they were not restricted to eating only in this window, so they did not need to partition their activity to align with it), TRF-D mice allocated 42.99±1.44% of total activity to the window of food availability, and TRF-L mice allocated 31.08±3.09% of their activity to this window ($p<0.05$; Fig. 14).

Dark phase activity

Genotype: There was no impact of genotype on the consolidation of activity to the dark phase in any epoch ($p=0.1602$; $p=0.3219$; $p=0.4077$; $p=0.3366$; $p=0.3593$; $p=0.3440$).

Sex: There was no impact of sex on the consolidation of activity to the dark phase in any epoch ($p=0.4678$; $p=0.8960$; $p>.9999$; $p=0.6526$; $p=0.3680$; $p=0.2281$).

Feeding condition: Feeding condition had an impact on percentage of activity allocated to the dark phase for all epochs ($p<0.0001$ for all epochs), with TRF-D mice allocating the most activity ($p<0.001$; $p<0.0001$; $p<0.0005$; $p<0.0001$; $p=0.0001$; $p<0.0001$), AL mice an intermediate amount ($p<0.001$; $p<0.0001$; $p<0.0005$; $p<0.0001$; $p=0.0001$; $p<0.0001$), and TRF-L the least ($p<0.0001$ for all epochs). Averaged across all epochs, TRF-D mice allocated 87.04%, AL 76.78%, and TRF-L mice 50.66% of their overall activity to the dark phase (Fig. 14).

Summary of activity data reduction

Taken together, the data indicate that, overall, TRF-D increased dark phase activity and TRF-L augmented light phase activity. The synergy of darkness and food especially augmented food window activity in TRF-D mice, but TRF-L mice were also able to significantly increase food window activity during the light phase during the latter epochs in a way that was statistically indistinguishable from TRF-D mice.

Body weight and food intake

To investigate how metabolic factors were impacted by our TRF manipulations, 139 mice (n=8-16/group) from Experiment 2 had food weight (to calculate average daily food intake) and body weight measured at the time of cage change every 2 weeks. There was a significant main effect of sex ($p<0.0001$) and genotype ($p<0.01$) on average body mass (Fig. 15), with no significant interactions. Feeding condition did not significantly impact body weight ($p=0.8872$). In general, males weighed more than females ($p<0.0001$), and Ntg mice weighed more than APP/PS1-21 mice ($p<0.001$). There was no significant main effect of sex ($p=0.1679$) or genotype ($p=0.7161$) on food intake. However, there was a significant main effect of feeding condition ($p<0.0001$) and a significant interaction between sex and feeding condition (Fig. 15; $p<0.05$). *Ad lib* animals ate more than animals in TRF groups ($p<0.005$). TRF-D and TRF-L males did not differ in food intake ($p=0.7108$), while TRF-D females ate more than TRF-L females ($p=0.0001$). We computed mass-specific food intake (MSFI) to control for the effects of body mass on food intake. MSFI was calculated as food intake per day divided by body weight (grams of food eaten per gram of body mass). There was a significant main effect of sex ($p<0.0001$) and feeding manipulation ($p<0.0001$) on MSFI, and a significant interaction between sex and feeding group ($p<0.0001$) and between genotype and feeding condition ($p<0.02$; Fig. 15). *Ad lib* fed mice ate the most per body mass ($p<0.001$), mice fed only in the dark phase ate an intermediate amount per body mass ($p<0.001$), and mice fed only in the light phase ate the least per body mass ($p<0.0001$). Sex differences in MSFI were evident in *ad lib* ($p<0.0001$) and TRF-D mice ($p<0.0001$), but not for TRF-L mice ($p=0.1432$). As TRF-L showed the lowest MSFI (average=0.155), this likely represents a floor effect. The largest sex difference was evident in *ad lib* fed mice, with females exhibiting an average 0.219 MSFI and males an average 0.172 MSFI.

This corresponds to females eating 27% more than males when fed *ad lib*. TRF-D females, on the other hand, ate 19% more than TRF-D males (female average=0.193; male average=0.162). TRF-L fed mice displayed an average MSFI of 0.155. Genotype differences in MSFI were evident for *ad lib* mice ($p<0.05$), but not for TRF-D ($p=0.5336$) or TRF-L ($p=0.7116$) mice. This suggests that restricted feeding alone is exerting a stronger influence over MSFI than genotype. *Ad lib* fed APP/PS1-21 mice ate 11% more per body mass than *ad lib* fed Ntg mice (APP/PS1-21 average=0.205; Ntg average=0.184). TRF-D fed mice showed an average MSFI of 0.179, while TRF-L fed mice had an average MSFI of 0.155.

Cognitive Behavioral Tasks

77 mice (n=3-11) from Experiment 2 underwent marble burying testing. There was a significant main effect of feeding condition on marbles buried ($p=0.0005$), and no main effect of genotype ($p=0.0978$) or sex ($p=0.6322$) and no significant interactions ($p>0.0823$). *Ad lib* fed and dark-fed mice did not bury a different number of marbles from each other ($p=0.3886$), but both buried more marbles than TRF light-fed mice ($p<0.0001$ and $p<0.02$, respectively; Fig. 16). In addition to number of marbles buried, we also assessed differences in number of marbles moved. There were significant main effects of genotype ($p<0.01$) and feeding condition ($p<0.01$) on marbles moved, but no significant main effect of sex ($p=0.2766$), though there was a significant interaction between genotype and sex ($p<0.02$). Ntg mice moved significantly more marbles than APP/PS1-21 mice ($p<0.02$) and did not exhibit a sex difference in marbles moved ($p=0.7021$; Fig. 16). However, similar to the findings of Experiment 1 (Fig. 11), there was a trend for female APP/PS1-21 mice to move fewer marbles than APP/PS1-21 males ($p=0.0652$). Additionally, *ad*

lib fed mice moved more marbles than either TRF group ($p<0.02$), who were not significantly different from each other ($p=0.7935$).

109 mice (n=7-11) were administered the Spontaneous Alternation task. Spontaneous alternation measures the number of alternations to the arm of the maze that was not visited on the previous trial, divided by the total number of trials, and times 100 (to result in a percentage). 50% alternation is chance level, and healthy WT mice show alternation around 70% (d'Isa et al., 2021). Spontaneous alternation showed no significant impact of any factors, though there was a trend for an interaction between sex and genotype ($p=0.052$). This interaction suggested that transgenic females showed greater alternation than Ntg females, but the opposite was true for males (Fig. 16).

Discussion

Here we evaluated baseline circadian and cognitive behavioral phenotypes of the APP/PS1-21 mouse model at the onset of plaque deposition and investigated the behavioral impact of time-restricted feeding in conjunction with the plaque development exhibited by this transgenic mouse model of AD. We found few baseline circadian differences in this mouse strain, indicating the APP/PS1-21 mouse can entrain to a LD cycle and exhibits an endogenous period similar to Ntg littermates early in plaque development (Fig. 10). However, there were some baseline cognitive behavioral differences, with female APP/PS1-21 mice in particular showing reduced marble burying and moving, and a trend for reduced spontaneous alternation compared to other groups, suggesting APP/PS1-21 mice might be particularly susceptible to cognitive behavioral deficits (Fig. 11). We expanded upon this study of baseline differences in the APP/PS1-21 mouse to investigate how timed feeding and, in particular, circadian

misalignment interacted with sex and plaque development to impact behavioral impairment. Circadian misalignment through timed feeding idiosyncratically altered performance in some cognitive behavioral tests but did not clearly exacerbate AD behavioral pathology (Fig. 16-17). Below, I will put these results into context with a discussion of how our findings fit in the greater framework of AD pathophysiology and behavior.

To establish a baseline circadian phenotype in the APP/PS1-21 mouse at the onset of plaque deposition, we conducted a preliminary experiment to assess entrainment to a normal 12:12 light-dark cycle as well as endogenous circadian period in constant darkness and constant light (Experiment 1; Fig 10). The circadian dynamics of the APP/PS1-21 mouse have not been previously characterized, though we confirmed this genotype exhibits similar behavior to the APP/PS1 mouse, which develops plaques more globally and at a slower pace. Previous research has found few differences in circadian parameters between APP/PS1 mice and Ntg controls (Otalora et al., 2012; Oyegbami et al., 2017). APP/PS1 mice at 6, 9, 12, and 19 months demonstrate no difference in circadian period, response to phase shifts, total activity, number or duration of activity bouts, or food anticipatory behavior compared to Ntg controls, though transgenic mice display slightly delayed activity onset and increased activity in the second half of the night, which is reminiscent of the fractured circadian activity in human AD patients (Kent et al., 2019; Sheehan & Musiek, 2020). We did not find evidence of marked differences in circadian parameters in the APP/PS1-21 mouse, as transgenic mice only exhibited circadian differences from Ntgs in terms of slightly altered activity onset (Fig 10). Additionally, we confirmed that sex differences in circadian period persist in this transgenic model, with female mice exhibiting longer circadian periods in constant light. We similarly did not observe an impact of genotype on circadian coordination to food restriction in Experiment 2 (Fig. 12-13).

Taken together, these findings suggest a lack of dramatic circadian differences between Ntg and APP/PS1-21 mice, though there may be some circadian instability manifesting as alterations in activity onset.

Behavior in Experiment 2 demonstrated clear circadian disruption driven by timed feeding. Feeding during the 6h midpoint of the light or dark phase was sufficient to change locomotor activity (Fig. 12-14), despite mice failing to exhibit normal FAA. Time-fed mice in general consolidated their activity to accommodate the time of food availability (Fig. 14). When the food availability window overlapped with the active phase, activity was more pronounced, as evidenced through the higher proportion of dark phase activity in TRF-D mice compared to AL mice (Fig. 14). We did not observe an impact of genotype or sex on this consolidation of activity, though these factors could be masked by the more salient food availability cues. Previous experiments have reported an impact of sex on food anticipatory activity, with male mice exhibiting a higher-amplitude food anticipatory activity and quicker onset of FAA than female mice (Aguayo et al., 2018; Z. Li et al., 2015; Michalik et al., 2015). Male mice also exhibit FAA for a palatable snack even under *ad lib* chow conditions, while female mice do not show FAA but do track the timing of the snack (Hsu et al., 2010). Additionally, restricted feeding under constant darkness modulates and even synchronizes the endogenous circadian clock in female mice, while male mice exhibited only slight phase shifting, indicating the circadian network of females may be more sensitive to food-related cues (Mei et al., 2021). Though we did not observe significant sex differences here, our limited sample sizes and restricted feeding window differed from traditional FAA paradigms and may have been sufficient to mask these differences in the presence of extremely salient food and light cues.

We also evaluated the impact of restricted feeding on factors linked to metabolic functioning, including food intake and body weight (Fig. 15). TRF impacted mass-specific food intake without affecting body mass, indicating an effect specifically on caloric intake. Other reports have documented the opposite, with mice consuming equivalent caloric intake and physical activity levels under TRF and *ad lib* conditions, but demonstrating changes in body mass according to timing of food intake (Arble et al., 2009; Hatori et al., 2012). Those reports have shown that light-phase feeding with a high-fat diet exacerbates metabolic syndrome, while feeding this same diet only during the dark phase protects against development of metabolic issues. Interestingly, these and other studies investigating the impact of TRF on food intake and weight gain are done using high-fat diet or in metabolically-challenged mouse models, such as the leptin-deficient ob/ob mouse (Arble et al., 2009, 2011; Bray et al., 2010, 2013; Chaix et al., 2019; Hatori et al., 2012; Oishi & Hashimoto, 2018; Reznick et al., 2013; Sherman et al., 2012; Yasumoto et al., 2016). Our experiment investigated TRF under normal chow conditions and included Ntg controls. We observed that *ad lib* fed mice consumed the most food while light-phase-fed mice consumed the least, and dark-phase-fed mice an intermediate amount, despite no differences in bodyweight between groups (Fig 15). A previous study investigating mice under normal chow conditions similarly found that light-fed mice eat more than dark-fed mice, though increased weight gain under light-fed conditions was also observed (Bray et al., 2013). Another experiment investigated mice fed normal chow during a consistent 12h phase amidst a jet lag paradigm in which light cycles were advanced by 6 hours every 2-3 days (Oike et al., 2015). These mice showed isocaloric food intake compared to *ad lib* fed mice under the same jet lag paradigm but did not demonstrate a comparative increase in body mass, body fat, and glucose tolerance. Finally, a study comparing high-fat and low-fat timed feeding during the active phase

found that the high-fat fed group exhibited a phenotype indicative of increased satiation and reduced stress (Sherman et al., 2012). Taken together, these findings point to a potential tradeoff between nutrient density and length of the food availability window in contributing to metabolic dysbiosis. Without the intense metabolic disruption of a high-fat diet or a pre-existing metabolic challenge, mice may engage in some type of compensatory mechanism whereby they balance caloric intake and timing of food consumption to maintain body mass. Indeed, Bray et al., 2010 demonstrated that mice fed a contiguous 12h-low and 12h-high fat diet adjusted food intake and energy expenditure to maintain normal physiological functioning. If mice auto-regulate food intake under normal chow conditions to reduce calories during the "wrong" time of day or exhibit more efficient calorie usage during the "correct" time of day, this could contribute to differences in food intake levels and protect against weight gain or other negative metabolic consequences.

Though *ad lib* conditions showed genotypic differences in MSFI, with APP/PS1-21 mice exhibiting hyperphagia, time-restricted feeding normalized these genotypic differences (Fig. 15). In addition to reduced body weight, Alzheimer's transgenic mice have been previously reported to exhibit increased frequency and duration of feeding bouts (Knight et al., 2012; Pugh et al., 2007; Vloeberghs et al., 2008), mirroring the hyperphagia and weight loss seen at all stages of Alzheimer's Disease progression in human patients (Poehlman & Dvorak, 2000; Shea et al., 2018; P.-N. Wang et al., 2004). In fact, weight loss often occurs prior to the onset of clinical impairment and may predict the development of dementia (Alhurani et al., 2016; Barrett-Connor et al., 1998; Cova et al., 2016; Jimenez et al., 2017; D. K. Johnson et al., 2006). Similar to behaviors observed in human patients, APP/PS1-21 mice exhibit hyperphagia but did not weigh more than Ntg mice (Fig. 15). However, no genotype differences were evident in either of the

TRF conditions, indicating restricted feeding alone is exerting a stronger influence over MSFI than plaque load. Therefore, latent effects of plaque deposition on MSFI in APP/PS1-21 mice are masked by restricted feeding, no matter the circadian phase the food availability window occurs during.

We observed several idiosyncratic effects of treatment variables on marbles buried and marbles moved, but these differences manifested only as main effects, thus at present we cannot determine contributions of other treatment variables on the effects of misaligned feeding or APP/PS1-21 females on these related but different marble-related behaviors. Specifically, mice subjected to misaligned feeding buried fewer marbles, and this was not dependent on genotype. Mice that were subjected to TRF (during the light or the dark phase) also moved fewer marbles than AL mice, and this was particularly evident for APP/PS1-21 females (Fig. 16). However, what this behavior indicates is somewhat open to interpretation (de Brouwer et al., 2019). Marble burying has been used as a proxy for compulsive anxiety because marble burying behavior is attenuated by anxiolytic and antidepressant drugs (Broekkamp et al., 1986; de Brouwer et al., 2019; Deacon, 2006; Londei et al., 1998; Njung'e & Handley, 1991; Takeuchi et al., 2002; Thomas et al., 2009). Compulsive anxiety has also been observed in human AD patients (Gitlin et al., 2012). Anxiety and compulsive behavior should result in increased marble burying behavior (Broekkamp et al., 1986; Cipriani et al., 2013; Gitlin et al., 2012; Pyter et al., 2009), and increased marble burying has been observed in APP/PS1 mice (Schemmert et al., 2019). However, the majority of research has documented reduced marble burying in APP/PS1 mice (Day et al., 2023; Keszycki et al., 2023; T.-K. Kim et al., 2012; Vepsäläinen et al., 2013). These and other researchers have claimed that, rather than compulsive anxiety, marble burying measures something more akin to neophobia, reduced exploratory motivation, disengagement

with the task, and/or apathy-like behavior (Day et al., 2023; Moreno et al., 2017). Our results would support this explanation, but the effect should be carefully interpreted. We observed differences in marble burying even at the very onset of plaque development (6 weeks of age), as APP/PS1-21 mice buried and moved fewer marbles than Ntg mice in Experiment 1 (Fig. 11). Most studies on Alzheimer's transgenic mouse strains do not assess behavioral impairment until at least 6 months of age (C. Li et al., 2015; L. Li et al., 2020; Owona et al., 2019; Scholtzova et al., 2008; Z.-Y. Zhang et al., 2014), as full-blown pathology in terms of global neuronal loss is not present until 8 months of age (Radde, 2006). We observed reduced marble burying behavior even at the onset of plaque development, which indicates that this behavior may be a result of baseline genotypic differences rather than something due to plaque development itself. In Experiment 2, APP/PS1-21 mice again moved fewer marbles, but this effect was only evident in female APP/PS1-21 mice, irrespective of feeding treatment (Fig. 16). Marble burying behavior per se was exacerbated by misaligned feeding, with mice that were only fed during the light phase burying fewer marbles, irrespective of sex and genotype. Our results confirm that AD transgenic mice that develop plaques exhibit reduced marble burying and moving, and extend this finding to the APP/PS1-21 mouse model of accelerated plaque development. Additionally, because marble burying was also reduced by light-phase feeding, misaligned feeding itself also affects this paradigm, independently of plaque load or background genotypic differences.

At plaque development baseline, there were no differences between APP/PS1-21 and Ntg mice in spontaneous alternation performance when tested during either the light phase or the dark phase, though there was a trend for female APP/PS1-21 mice to exhibit reduced alternation (Fig. 11). While we did not see significant differences in spontaneous alternation at 4.5 months of age, we did observe a trend for APP/PS1-21 females to exhibit more alternation than Ntg

females (Fig 17). This trend is not in line with our hypothesis, as we expected transgenic mice to show reduced spontaneous alternation, equating to impairments in spatial working memory. However, our findings fit in with the discrepancy within the literature for spatial working memory deficits in later onset APP/PS1 mice (van Heusden et al., 2021). While some studies have documented working memory deficits in these transgenic mice (Chaney et al., 2018; H. Y. Kim et al., 2015; X. Wang et al., 2017), others have failed to find differences between APP/PS1 and Ntg mice (Chaney et al., 2018; Harrison et al., 2009; Lalonde et al., 2004; Reiserer et al., 2007). Of note, the experiments that found spatial learning deficits were conducted on much older mice (12 months of age and older) that were well into disease progression, while the studies that did not see spatial learning deficits included younger mice closer to onset of plaque development at 6 months of age. In fact, Chaney et al. tested these mice at 3 time points and did not observe deficits in spontaneous alternation until 18 months of age. While we are not aware of another experiment testing spontaneous alternation in the APP/PS1-21 mouse, the data from the later onset APP/PS1 mouse suggests this test may not be sensitive enough to capture early changes in spatial working memory. In future studies, it would be interesting to test spontaneous alternation at a more advanced time point (closer to 8 months in the APP/PS1-21 mouse) after previous intervention, such as time restricted feeding.

In the design of this experiment, we aimed to assess not only the behavioral impact of our TRF manipulations but also the neurological plaque accumulation. While we did not directly assess the plaque load in our mice via immunohistochemical staining, this is a promising future step to confirm the cellular impact of peripheral metabolic manipulation. Prior research has documented that peripheral manipulations in APP/PS1-21 mice can have an impact on plaque deposition, in particular implicating the gut microbiome (Wasén et al., 2022). Metabolic

manipulations, such as calorie restriction, reduced plaque load, likely through the normalization of the gut microbiome (Cox et al., 2019). Additionally, APP/PS1-21 mice exhibit greater plaque deposition in response to increased bacterial burden, such as bacterial sepsis (Basak et al., 2021). Conversely, when the gut microbiome is suppressed through antibiotic treatment or abolished through a germ-free model, plaque load is reduced in APP/PS1-21 mice (Dodiya et al., 2019, 2020; Harach et al., 2017). Importantly, these reductions in plaque load require microglia (Dodiya et al., 2021), presenting a clear link between peripheral and central factors. Thus, it would be an interesting future direction of research to analyze the fecal pellet samples we collected in this study, both at the onset of food manipulation and at the study endpoint, to investigate if microbiome alterations over time could be a potential mechanism for mediating central-peripheral alignment and subsequent plaque load.

Hundreds of failed clinical trials targeting Alzheimer's Disease over the last 20 years have demonstrated that a single-target pharmacological approach to treatment is not effective (Cummings et al., 2022; C. K. Kim et al., 2022). In that time, only two drugs, monoclonal antibodies designed to bind and eliminate plaques, have been approved as a disease modifying treatment, though one of these drug has since failed to show much efficacy (*Biogen Plans Regulatory Filing for Aducanumab in Alzheimer's Disease Based on New Analysis of Larger Dataset from Phase 3 Studies | Biogen*, n.d.). However, strategies employing multimodal approaches, such as nutrition and exercise, have shown promise in ameliorating AD (Chu et al., 2022; Cremonini et al., 2019; Valenzuela et al., 2020). Because circadian rhythms can be bolstered or disrupted by the timing of eating and activity, we investigated the accessible intervention of timed feeding as a preventive approach. Circadian dysfunction has been well-documented in Alzheimer's Disease patients and presents even before onset of clinical

impairment. These experiments aimed not only to establish a baseline of circadian functioning at the onset of plaque deposition and farther into disease etiology in a transgenic mouse model of Alzheimer's Disease, but also to manipulate peripheral-central biological rhythm alignment by presenting aligned or conflicting time cues to the brain and periphery to assess how circadian alignment may contribute to plaque development and disease progression.

Alzheimer's Disease in particular faces issues with translation from animal models to human patients. Currently no animal models recapitulate all aspects of the disease or are yet predictive of the efficacy for disease intervention in the clinic. Animal models of Alzheimer's Disease in general address one of the three neurological phases leading to AD pathology (Sasaguri et al., 2017). The first preclinical phase is described by plaque deposition prior to neurological symptoms, which the APP/PS1 and APP/PS1-21 mouse models attempt to recapitulate. The second phase is marked by tauopathy, progressive neurodegeneration, and mild cognitive impairment (MCI). Finally, the third phase, consists of significant neurodegeneration and subsequent dementia, and represents what is referred to as clinical AD. As these experiments aimed to address preclinical AD, we chose the APP/PS1-21 mouse model as a vector to assess whether early interventions can affect plaque accumulation and carried out critical experiments in validating this model compared to the APP/PS1 mouse.

The present report was not sufficient to conclude that time-restricted feeding had an effect on Alzheimer's-related disease progression. Our experimental endpoint was chosen to assess plaque load in the brain prior to clinical behavioral impairment to avoid the potential of a plaque load ceiling being reached and confounding interpretation between groups. However, we wanted to assess whether significant environmental manipulation could exacerbate behavioral impairment to such a degree that cognitive changes would be evident even at this early

timepoint, as has been occasionally observed (Becerril-Ortega et al., 2014). We did find evidence that driving peripheral food cues out of alignment with central light cues was sufficient to exacerbate behavioral pathology in this mouse model prior to normal onset of behavioral impairment, as in reduced marble burying (Fig 16). However, we did not see an improvement in behavioral pathology at this timepoint by driving central-peripheral alignment through dark-phase feeding (Fig 16-17). Given the increased compulsive anxiety induced through misaligned feeding, it could be possible circadian rhythms and peripheral time cues are interacting to produce alterations in cognition, though this may be independent of plaque deposition. Circadian rhythms may operate independently of peripheral time cues to generate enhanced plaque development, and misalignment between central and peripheral cues does not alter this progression. However, it is also possible that we analyzed behavioral impairment too early in disease pathology to catch any difference, as pathology was not exacerbated enough by misalignment to produce extensive behavioral effects. In this case, testing cognitive behavior at a later time point would allow us to answer whether alignment between central and peripheral cues early in disease pathology can reduce plaque development and delay clinical impairment.

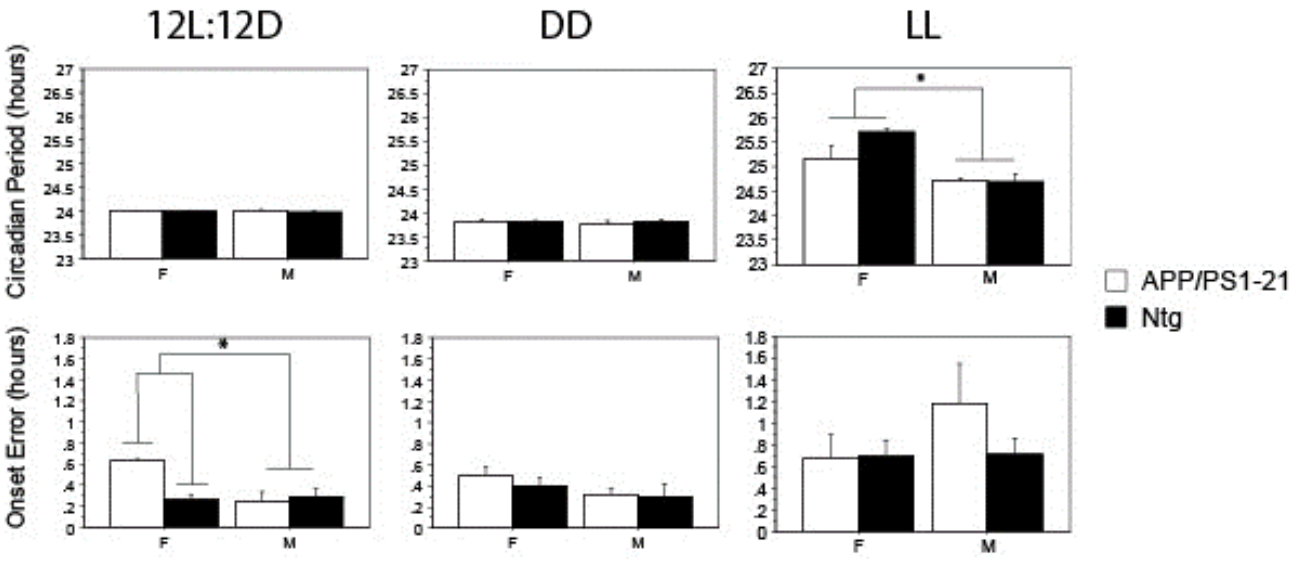

Figure 10: Circadian period and onset variability of APP/PS1-21 mice

Average circadian period (top plots) and activity onset variability (bottom plots) for APP/PS1-21 and Ntg mice during each photoperiod (12:12) or constant condition (DD, LL) is shown. While there were no group differences in circadian period length in 12:12 (top left), female APP/PS1-21 mice exhibited increased onset variability compared to other groups (bottom left). In DD, no group differences in tau (top middle) or onset variability (bottom middle) were exhibited. Females showed longer circadian period in LL compared to males (top right), though there were no differences in onset variability between groups in LL (bottom right). Error bars represent ±1SEM. Asterisks indicate significant differences ($p<0.05$).

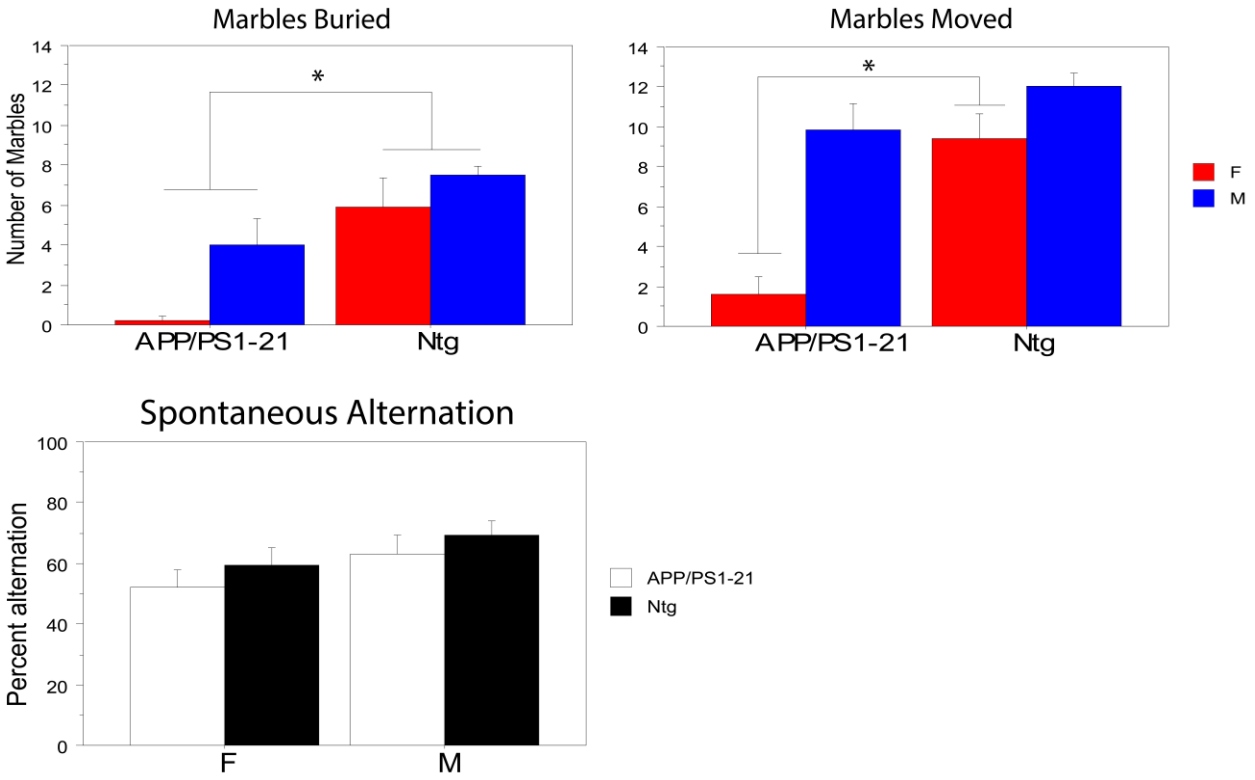

Figure 11: Cognitive behavioral tests at plaque development baseline in APP/PS1-21 mice

Experiment 1 cognitive test results for APP/PS1-21 and Ntg male (blue) and female (red) mice. APP/PS1-21 mice showed reduced marble burying (top left) and moving (top right) compared to Ntg mice, driven by APP/PS1-21 females. There were no significant differences in Spontaneous Alternation (percent of alternation between choice arms over total number of trials) between groups in Experiment 1 (bottom). Error bars represent ±1SEM. Asterisks indicate significant differences ($p<0.05$).

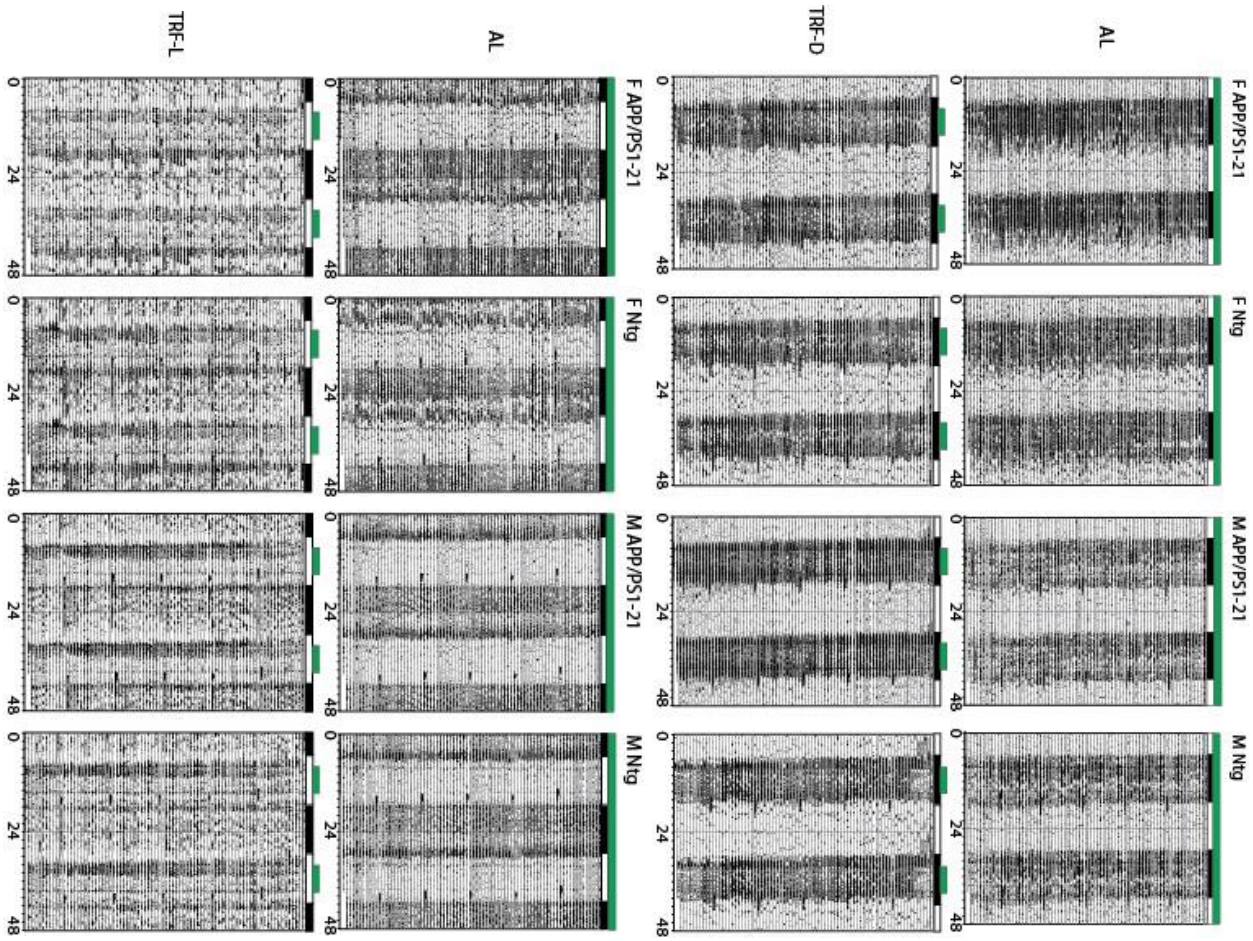

Figure 12: Averaged actograms under TRF and AL conditions

Averaged actograms for APP/PS1-21 and Ntg mice under each feeding condition in Experiment 2. Double-plotted home cage locomotor activity (LMA) record for mice housed in a 12:12 photoperiod, with lights on and lights off represented with the white and black bars at the top of the actogram, respectively. The window of food availability is denoted by the green bar above the lights on/off bar.

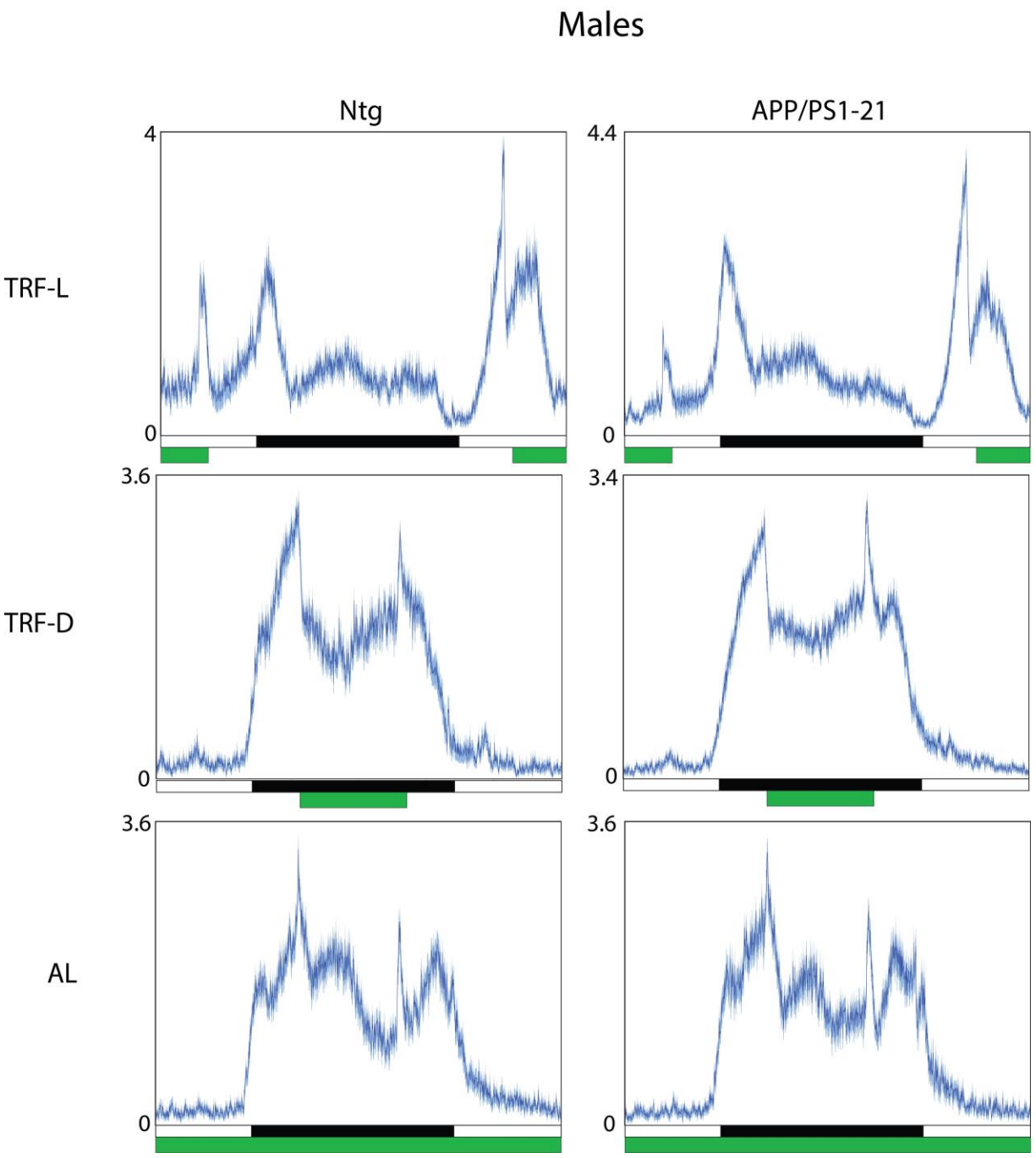

Figure 13: Average activity profile for TRF and AL conditions

Average activity profile for each group in Experiment 2. Males are shown in the top plot and females in the bottom plot. X-axis shows lights on (white) and off (black) times, as well as time of food availability (green), y-axis denotes amplitude of activity profile. ±1SEM is shown around activity profile waveform (light blue). Disruption in the circadian waveform is evident for light-phase-fed (TRF-L) groups, while dark-phase feeding (TRF-D) exacerbates the dip in the active phase for both genotypes.

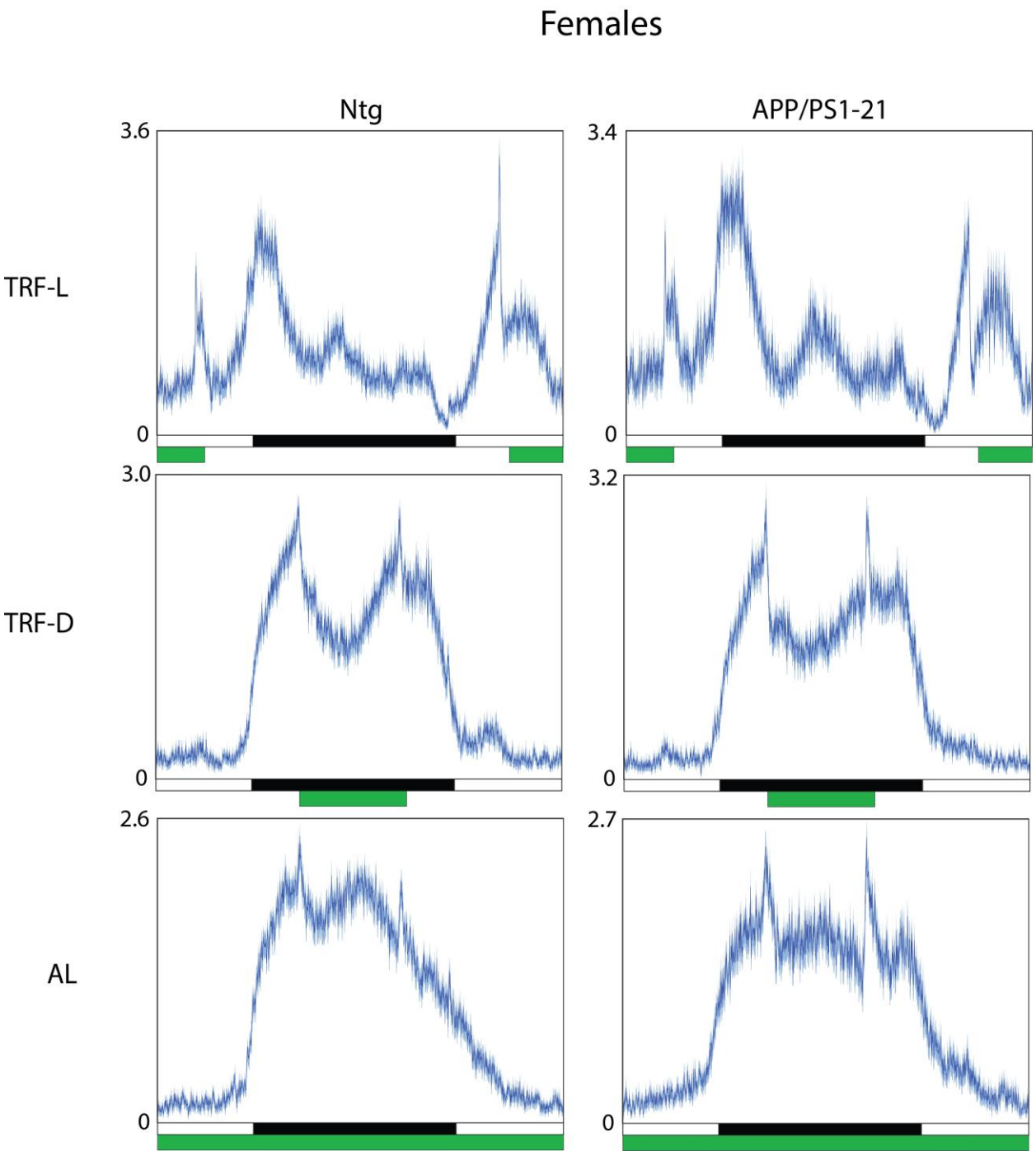

Figure 13 (cont.)
Average activity profile for each group in Experiment 2. Males are shown in the top plot and females in the bottom plot. X-axis shows lights on (white) and off (black) times, as well as time of food availability (green), y-axis denotes amplitude of activity profile. ±1SEM is shown around activity profile waveform (light blue). Disruption in the circadian waveform is evident for light-phase-fed (TRF-L) groups, while dark-phase feeding (TRF-D) exacerbates the dip in the active phase for both genotypes.

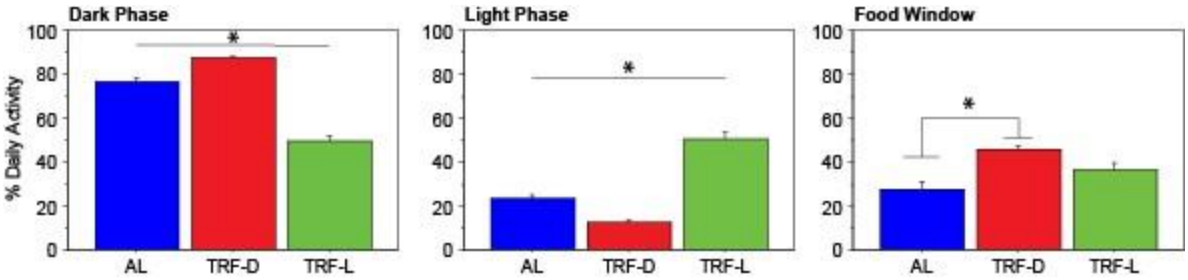

Figure 14: Average diurnal and food-driven activity for TRF and AL conditions

Average counts during the dark phase (left), light phase (middle), and window of food availability (right) for each feeding condition. Y-axis denotes activity expressed as percentage of total daily activity. Dark-fed mice were more activity in the dark phase and less active in the light phase compared to other groups. On the other hand, light-fed mice split their activity between the dark and light phase evenly. There was no difference in percent of activity consolidated to the window of food availability among TRF groups, indicating both dark- and light-fed mice were tracking the timing of food. Error bars represent ±1SEM. Asterisks indicate significant differences ($p<0.05$).

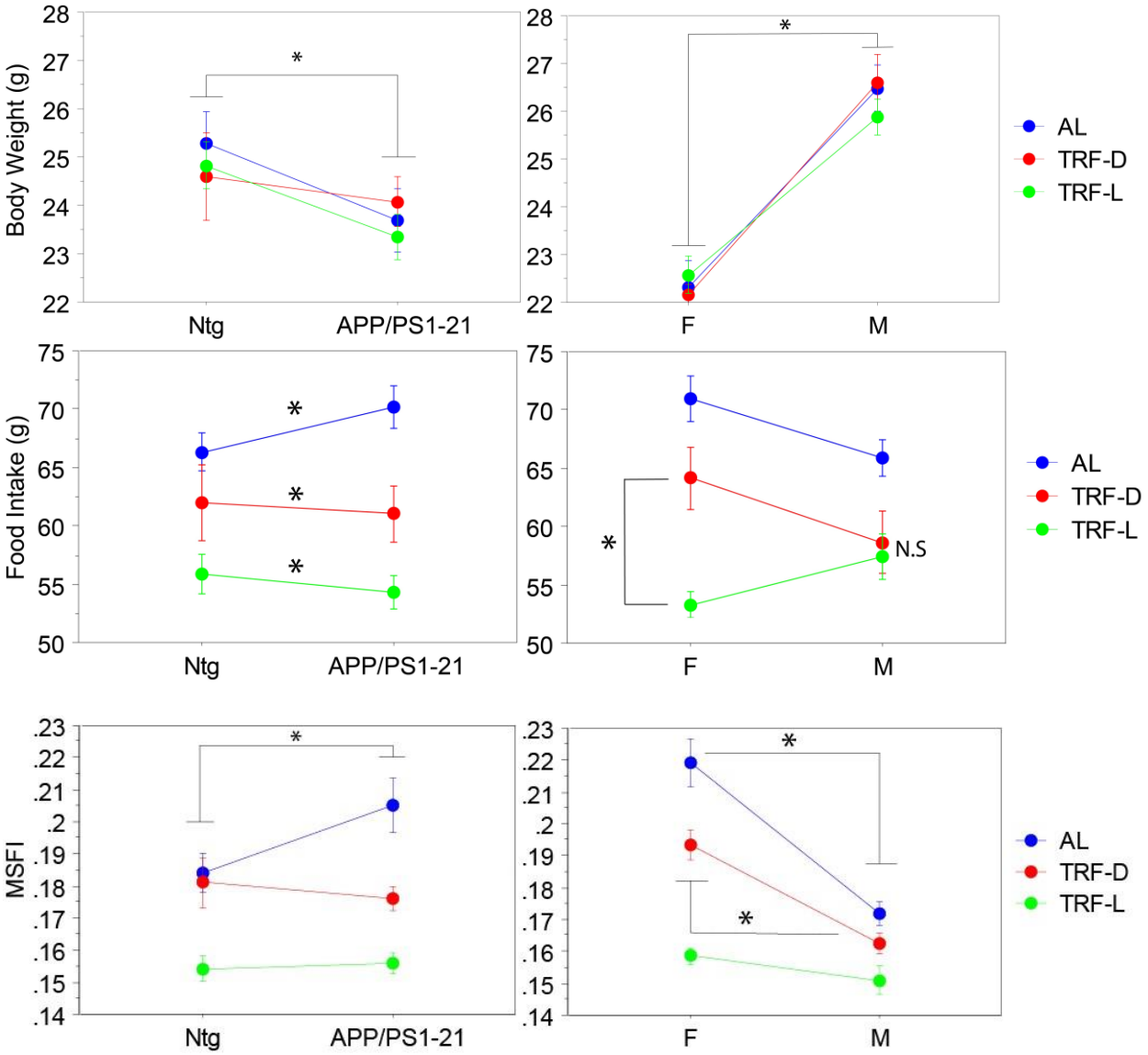

Figure 15: Food intake, but not body weight, is dependent on feeding condition

Body weight was not impacted by feeding condition (top plots), but was impacted by genotype (top left), with APP/PS1-21 mice weighing less than Ntg mice. Body weight was also impacted by sex (top right), with female mice weighing less than males. Food intake was not impacted by genotype but was impacted by feeding condition (middle plots), with AL mice eating the most, TRF-D mice eating an intermediate amount, and TRF-L mice eating the least (middle left). TRF males did not differ in food intake, but TRF-D females ate more than TRF-L females (middle right). Mass Specific Food Intake (MSFI) for each group in Experiment 2 was impacted by feeding condition (bottom plots). Genotype differences in MSFI were evident for *ad lib*, but not for TRF-D or TRF-L mice (bottom left). Sex differences in MSFI were present for *ad lib* and TRF-D mice, but not for TRF-L mice (bottom right). Error bars represent ±1SEM. Asterisks indicate significant differences ($p<0.05$).

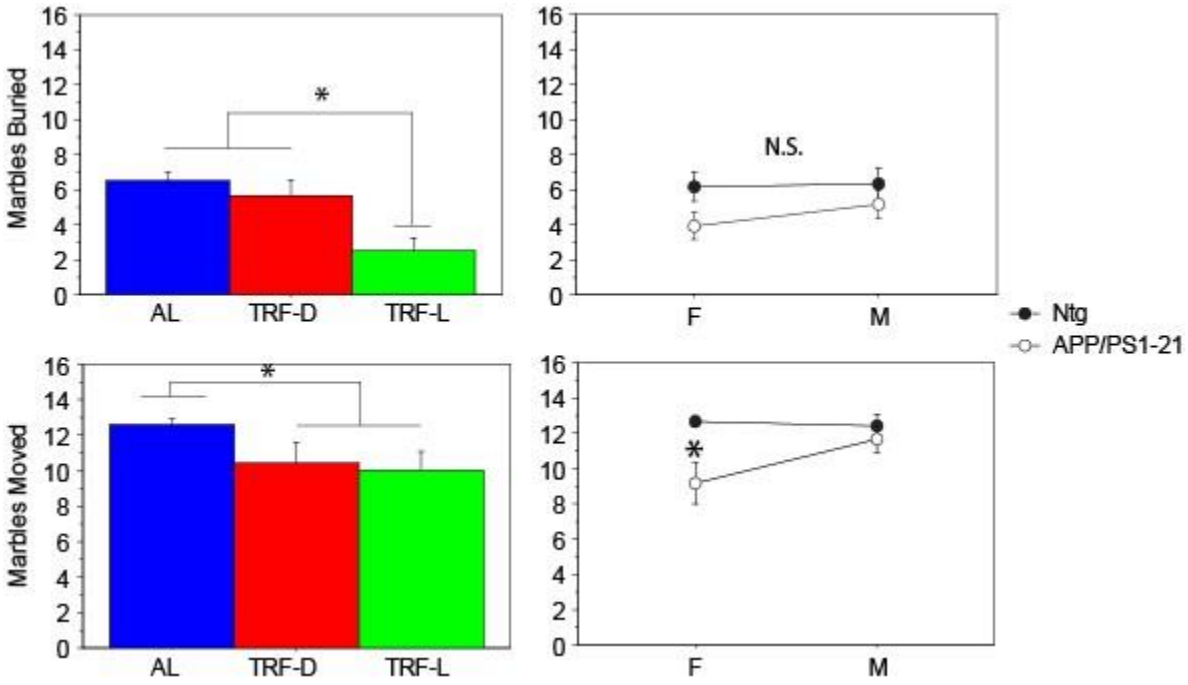

Figure 16: Marble burying behavior in TRF

Marble Burying Task performance for mice in Experiment 2. The Y axis shows number of marbles buried (top plots) or moved (bottom plots). There was no impact of sex or genotype on marbles buried (top right). However, there was an impact of feeding condition on marbles buried, where light-fed mice buried fewer marbles than *ad lib* and dark-fed mice (top left). Males did not show a genotype difference in marbles moved, but there was a significant difference in marbles moved for female mice, with APP/PS1-21 females moving less than Ntg females (bottom right). Again, there was an impact of feeding condition on marbles buried, with *ad lib* fed mice moved more marbles than either TRF group, which did not significantly differ from each other in marbles moved (bottom left). Error bars represent ±1SEM. Asterisks indicate significant differences ($p<0.05$).

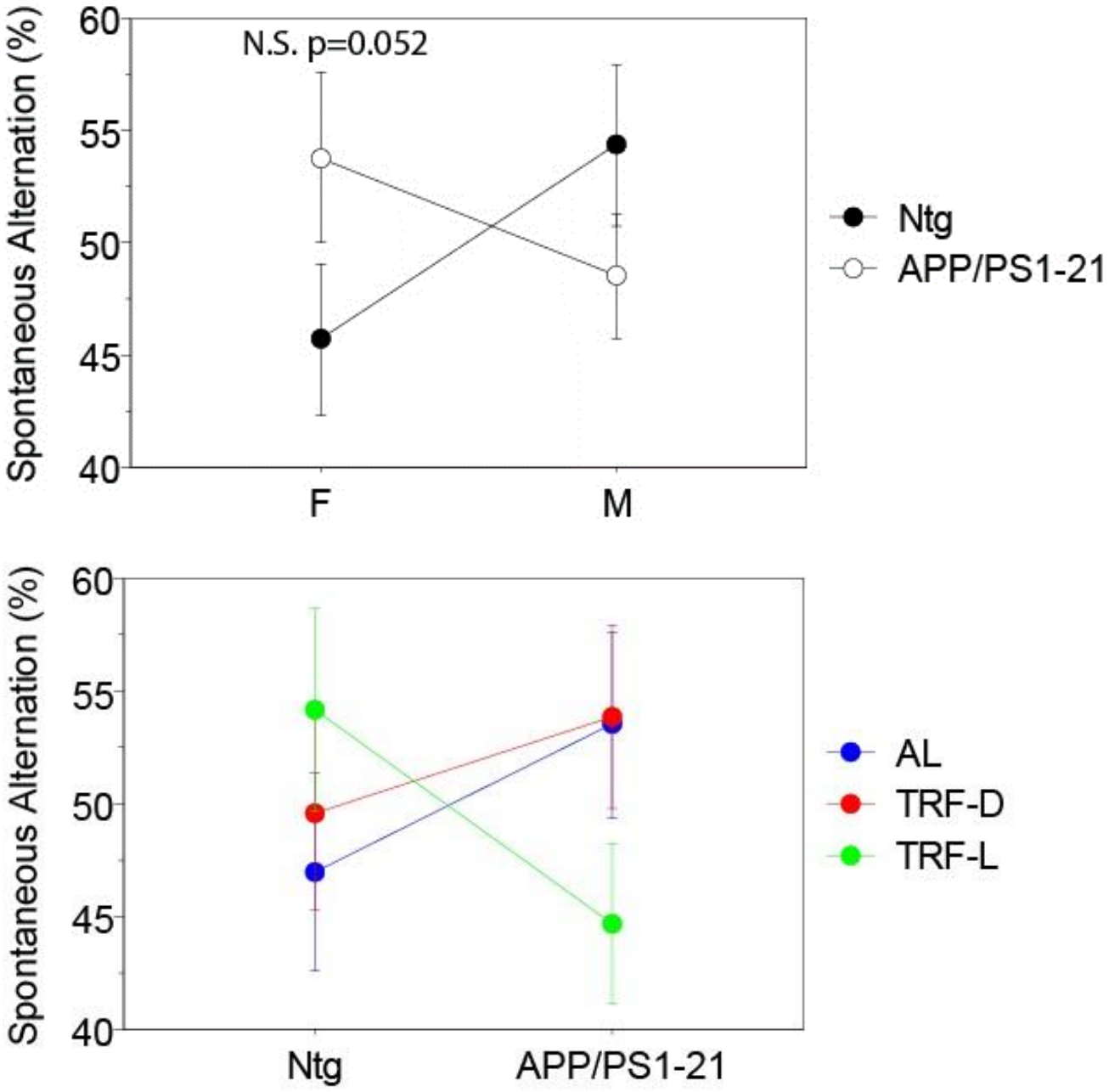

Figure 17: Spontaneous Alternation

Spontaneous alternation in Experiment 2. Y-axis denotes percent of trials mice showed alternation between choice arms, with 100% alternation denoting alternation to the opposite choice arm on every trial. There was no significant main effect of sex, genotype, or feeding condition on SA, but there was a trend for an interaction between sex and genotype (p=0.0520), where APP/PS1-21 female mice were trending to show increased alternation compared to Ntg females (top plot). Though not significant (p=0.1421), we also plotted the interaction between genotype and feeding condition for visualization (bottom plot). Error bars represent ±1SEM.

Chapter 5: Olfactory dysfunction in the APP/PS1-21 transgenic mouse model of Alzheimer's

Background

Olfactory stimuli are a crucial feature of the environment harnessed by organisms to promote adaptive functioning. Olfaction allows for active sampling of crucial survival information including predator identification, social recognition, and sourcing of food. Importantly, odor stimuli exert powerful control over learning and memory networks through the unique anatomical structure of olfactory pathways. Unlike other sensory systems, olfactory information is transmitted directly from the sensory organ to the cortex, without having to pass through the thalamic sensory relay system (Ongür & Price, 2000; Shepherd, 2005). Thus, olfactory stimuli have a direct route into the Olfactory Bulb (OB), which has been hypothesized to fulfill the role of an olfactory thamalus (Kay & Sherman, 2007). The OB is an integral part of the limbic system, a set of structures including the hippocampus and amygdala that are involved in learning and memory (Sullivan et al., 2015). The OB has dense bidirectional connections with both the amygdala and the entorhinal cortex, which is the main route into and out of the hippocampus for sensory information (Carpenter, 1972; Shipley & Ennis, 1996). Additionally, because of the unique anatomical organization and its exposure to pathogens from the nose, it has been argued that the OB is especially vulnerable to pathological protein aggregation, such as that seen in neurodegenerative diseases, which then triggers the spread of pathology throughout the brain (Rey et al., 2018). This makes the OB an interesting site of research for diseases impacting these cognitive processes, such as Alzheimer's Disease.

Relevant to the OB's central position in the limbic system, neurodegenerative disease patients exhibit early degeneration in the OB that precedes that of the rest of the brain (Attems et al., 2014; Ubeda-Bañon et al., 2020), mapping onto early olfactory deficits that precede clinical

onset of cognitive decline (Vasavada et al., 2015). However, the impact of many neurodegenerative diseases on olfactory function complicates the potential of olfactory tests for specific diagnoses. To tease apart the impact of different types of neurodegenerative disorders on olfaction, different facets of olfactory function must be evaluated. Compared to other neurodegenerative diseases, certain types of olfactory dysfunction were found to be more severe in AD (Rahayel et al., 2012), including the ability to correctly identify olfactory stimuli themselves (identification) or among other stimuli after a delay (recognition). Importantly, the actual sensory capacity of AD patients does not appear to be as affected as in other neurodegenerative diseases, like Parkinson's Disease (PD), as measured by the threshold at which patients are able to smell an odorant. This indicates that olfactory impairment in AD might involve more recruitment from limbic areas rather than pure sensory dysfunction. This positions olfactory dysfunction as an important early tool for differentiating and recognizing Alzheimer's Disease in order to implement early treatment efforts.

The several types of olfaction can be behaviorally separated through the administration of different behavioral paradigms. Sensory threshold is evaluated through the initial investigation of an odor at a given concentration as compared to a habituated blank. Odor identification is evaluated through olfactory habituation, or the progressive reduction in behavioral responsiveness to a repeated similar stimulus (Rose & Rankin, 2001; Thompson & Spencer, 1966). Finally, odor discrimination is seen through investigation of novel "test" odors after the subject has been habituated to a single odor, relying on the innate tendency of mice to spend more time investigating a stimulus that is perceived to be novel. The degree to which mice respond (or cross-habituate) to a novel odorant depends upon both the persistence of the memory of the habituated odor as well as the perceptual or structural similarity of the novel odorant to the

previously habituated odorant (Cleland et al., 2002). The degree of cross-habituation declines as odors become less similar (Linster et al., 2002). In previous research, AD transgenic mice which overexpress human APP (APP/PS1 mice) demonstrate deficiencies in odor habituation starting at only 3 months of age, coinciding with the onset of plaque deposition in the OB (Wesson, Levy, et al., 2010). This suggests that the ability of AD transgenic mice to identify and differentiate odors may be impaired even early in disease etiology due to plaque deposition within the OB before other brain areas. However, this research was performed with small sample sizes, and odorants were not controlled for volatility. Additionally, APP/PS1 mice produce amyloid aggregates in peripheral tissues (*e.g.,* blood vessels, liver, and gastrointestinal tract) as well as in the brain, complicating interpretation regarding whether behavioral impairment is due to an impact of these aggregates on other functions (L. Zhang et al., 2021). The APP/PS1-21 mouse model, with transgenes tied to the neuron-specific Thy1 promoter, allows for more specific testing of the impact of plaque deposition in the brain on olfactory dysfunction.

Several features of olfaction that rely on higher-order cognitive processing, including odor recognition and discrimination, can be evaluated in the olfactory cross-habituation test (Wesson, Levy, et al., 2010). This paradigm involves presenting a mouse with an odorant to habituate to over successive trials (OHab), followed by presentation with a novel odorant (Test Odorant). The odor cross-habituation or habituation/dishabituation protocol (also called simply a 'habituation' protocol (Cleland et al., 2002)) allows for measurement of 1) novel odor investigation responses, 2) odor learning and memory (habituation), and 3) odor discrimination (cross-habituation), making it an ideal behavioral paradigm for evaluating the impact of plaque development on several parameters of olfaction at once (Bath et al., 2008; Coronas-Samano et al., 2016; Freedman et al., 2013; Mandairon et al., 2009; McNamara et al., 2008; Tucker et al.,

2012; M. Yang & Crawley, 2009). These three features rely on different neurological and perceptual properties, and deficits in any of these responses can indicate degeneration in various areas of the system.

Olfactory Cross-Habituation

Novel Odor Orienting Response. Novel odor investigation response could be an indicator of arousal/motivation, short-term habituation, or simple perceptual differences. This type of behavior is distinguished from classic habituation by classifying the amount of investigation upon the first presentation of a stimulus, rather than dampening of response during successive presentations of the same stimulus. A previous report showed that APP transgenic mice exhibit elevated odor investigation behavior (*i.e.*, more overall sniffing) despite having similar numbers of sniff bouts relative to WT mice in response to the first presentation of novel odors, suggesting that the observed difference in novel odor investigation was not due to changes in perception or motivation, but instead due to deficient ability to habituate in the short-term (Wesson, Levy, et al., 2010). Importantly, odor structure and volatility were not reliably controlled in this study, presenting an important gap in our understanding of how plaque deposition impacts novel odor investigation. Investigative response to odors on a short time scale like this is supported by group III metabotropic glutamate receptors in the olfactory piriform cortex (Best & Wilson, 2004; Wada et al., 1998). Blocking these receptors prevents cortical adaptation, a key driver in adaptation of odor perception, resulting in a failure to habituate normally to presentation of an odor stimulus (Pellegrino et al., 2017). In fact, the previously-reported differences in novel odor investigation in the APP transgenic mouse do not come online until after 16 months of age, the time when plaque deposition begins in the piriform cortex in the APP/PS1 slow plaque deposition mouse (Wesson, Levy, et al., 2010).

Short Term Habituation. Habituation describes the process of reduced investigation of a stimulus over successive presentations. If mice are unable to habituate properly, this could indicate altered odor perception or impaired odor recognition. APP/PS1 mice display impairments in this classic habituation starting at 3-4 months of age, coinciding with the beginning of plaque deposition in the glomerular layer in that mouse model (Wesson, Levy, et al., 2010). This precedes plaque deposition in any other brain area, indicating that impaired habituation at this early stage is driven by plaque development in the olfactory bulb. Importantly, habituation deficits increase in severity as plaque deposition starts in the piriform cortex after 16 months of age in APP/PS1 mice, at which point the previously discussed deficits in novel odor investigation come online. If Ntg and APP/PS1-21 mice demonstrate similar habituation regardless of age and progression of plaque deposition, this would indicate that plaque load does not impact habituation. However, if habituation in APP/PS1-21 mice is not affected at the onset of plaque deposition but is affected after more advanced plaque development, this could indicate habituation is impacted by advanced plaque deposition throughout the cortex, or that plaque deposition in the OB must reach a certain threshold to impact this behavioral response. Alternatively, Ntg mice could show enhanced habituation relative to APP/PS1-21 mice even at the onset of plaque deposition, indicating that plaque deposition within the OB itself is responsible for this deficit and that habituation does not depend on extensive plaque development branching into other cortical areas.

Odor Discrimination. Cross-habituation describes the ability of the animal to differentiate between a habituated odor and a novel odor and represents a measure of spontaneous odor discrimination. Greater investigation of a novel odor indicates discrimination between the two odors, while reduced investigation reflects generalization. Importantly, cross-

habituation eliminates many of the confounds usually needed to test odor discrimination including food deprivation, reward, training, etc. While few discrimination studies have been done in Alzheimer's transgenic lines, prior research has demonstrated that APP/PS1 mice show increased generalization driven by plaque deposition in the glomerular layer (Wesson, Levy, et al., 2010). Ntg mice tend to generalize only to odors 1 carbon away (Chan et al., 2017; Cleland et al., 2002; McNamara et al., 2008; Wilson, 2000), as perceptual similarity is proportional to structural similarity in these aliphatic alcohols (Cleland & Linster, 2002; Nusser et al., 2001). If APP/PS1-21 mice show reduced ability to differentiate between the habituated odor and novel odors (*i.e.,* through reduced sniffing of test odors), this would indicate that plaque development impairs odor discrimination. If discrimination is impacted at the onset of plaque deposition, this would indicate that plaque development in the OB drives this impairment and could present a potential diagnostic target for differentiating AD behaviorally. If behavioral impairment is only present well into plaque deposition, this would indicate that advanced plaque deposition throughout the cortex and OB is required for impaired odor discrimination.

Discrimination under conditions of reward

Olfactory discrimination can also be tested through appetitive conditioning to test the ability of mice to discriminate between a trained, rewarded odorant and closely related odorants (or odorant mixtures). The digging task uses a reward associated with one of the odorants to drive behavioral discrimination, unlike the cross-habituation paradigm that does not involve reward associations and measures untrained responses to odor stimuli. If APP/PS1-21 transgenic mice have impaired discrimination under reward conditions, they should over-generalize to odors that are perceptually and structurally less similar to the rewarded odor. This would indicate that plaque load reduces the ability to differentiate odors, regardless of reward association. If this

difference is present at the onset of plaque development, then plaque load in the OB could be responsible for driving this difference. However, if this impaired discrimination does not come online until well into plaque deposition, the impairment might be more dependent on other cortical areas. This reward-based discrimination task should engage different neural systems relative to a non-rewarded task such as the cross-habituation test. Prior research has demonstrated that olfactory bulb and piriform cortex neurons respond differently to the same odors presented under different reward contexts (D. Wang et al., 2019), and that mice are better able to discriminate closely related odors under reward conditions (Cleland et al., 2002). Thus, if these areas are impacted in APP/PS1-21 mice, the outcome of a discrimination task reliant on reward might differ from performance on a task that is not dependent on reward associations (cross-habituation). If we observe differences in discrimination between the outcomes of these two tasks, this would demonstrate a reliance on different cognitive processes under conditions of reward, and could enhance our understanding of early detection mechanisms for AD.

Methods

Experiments

Experiment 1 consisted of a pilot study to determine the behavioral responsiveness of APP/PS1-21 mice and Ntg controls to odorants of 3 different concentrations, to control for baseline perceptual differences that might confound interpretation of results for the behavioral tasks of Experiment 2. Experiment 2a evaluated several aspects of olfactory function, including habituation to an odor and generalization to structurally similar odorants (cross-habituation). Experiment 2b expanded upon the results from 2a to determine if odor-discrimination ability in APP/PS1-21 mice is influenced by appetitive conditioning processes.

Animals

Adult male and female APPSWE/PS1L166P (APP/PS1-21) mice and their wildtype littermates on a C57Bl6Cj background were obtained from S. Sisodia's lab at the University of Chicago (original stock from M. Jucker, University of Tubingen, Tubingen, Germany). The aggressive APP/PS1-21 mouse model of amyloidosis expresses familial AD-linked APPSWE and PS1L166P transgenes driven by the neuron-specific Thy1 promoter. This transgenic mouse exhibits Aβ deposition beginning in the olfactory bulb and extending out into the cerebral cortex as early as 6 weeks of age.

Mice were group housed in conventional cages with wirebar lids and without microisolator filters in a 12L:12D photocycle of approximately 150-200 lux. Mice had *ad libitum* access to standard rodent diet (Irradiated Teklad Global 18% Rodent Diet 2918, Envigo RMS) and filtered drinking water. Cage changing was performed at one-week intervals. The integrity of experimental LD cycles was continuously monitored and verified by dataloggers (HOBO,UX90, Onset Comp). Estrous cycles of females were not monitored. All procedures related to animal use were approved by the University of Chicago Institutional Animal Care and Use Committee. At the conclusion of the experiments homozygous Ntg and heterozygous *APP/PS1-21* genotypes were confirmed in all mice by PCR using the protocol described for this genotype by JAX (see below).

Genotyping

Only homozygous nontransgenic and heterozygous APP/PS1-21 mice were included in these experiments (a priori criterion). Genotyping of all mice bred in our vivarium was done using primers ordered from the Jackson Lab Website [5' → 3': PS1 Forward (CAG GTG CTA TAA GGT CAT CC), PS1 Reverse (ATC ACA GCC AAG ATG AGC CA), APP1 (CGA CAG

TGA TCG TCA TCA CCT), APP4 (CTT AGG CAA GAG AAG CAG CTG)] and using specification for the Platimun Taq Polymerase (Life Technologies, Invitrogen catalog number: 10966-018). For each individual PRC reaction: 17.15 uL of DNAase free H2O, 2.5 uL of 10X PCR Buffer with no MgCl2, 0.75 uL 50mN MgCl2, 0.5 uL 10mM dNTP mix, 0.5 uL of each primer (either APP1 and APP4 OR PS1F and PS1R), and 0.1 uL of Taq were added to a master mix and thoroughly mixed by pipetting up and down. 22 uL of master mix were aliquoted and added to 3 uL of DNA derived via HotShot (Truett, Heeger et al. 2000), from ear clips and tail clips collected prior to or at the conclusion of the studies, respectively. We used the following PCR Protocol on a thermocycler: (1) 95 C for 3 minutes, (2) 95 C for 30 seconds, (3) 58 C for 1 minute, (4) 72 C for 1 minute, (5) repeat steps (2-4) 30 times, (6) 72 C for 5 minutes, (7) End (hold at 4 C). 8 uL of the resultant PCR products, and a 100 Bp to 2000 Bp Ladder (Thermofisher catalog # 15628050) for reference were mixed with 1.4uL of loading dye (Thermo Scientific catalog number: R0611), loaded on a 2% agrarose gel with 2.5uL of Ethidium Bromide (stock solution: 10mg/mL), and visualized. Resultant bands (amplicon sizes) per Jackson Lab Website were as follows: PS1 = ~300 bp, APP = ~500 bp. Due to cointegration of the transgenes, for routine analysis only genotyping for APP was done, with PS1 genotyping done only sporadically as a control and for genotype confirmation.

Olfactory Habituation Threshold Testing

In Experiment 1, mice of both sexes (*APP/PS1-21:* n=8 females, n=11 males; *Ntg:* n=4 females, n=2 males) were subjected to behavioral testing between 7 and 9 weeks of age. Ntg littermates were used as positive controls. Mice were screened for olfactory sensory deficits using an odor cross-habituation task against the mineral oil diluent for the odorant. The odorant *n*-amyl acetate was diluted in mineral oil to 3 different concentrations with correspondingly

different theoretical vapor pressures (0.1Pa, 1Pa, 3Pa). 60μL of mineral oil or mineral oil plus odorant was applied to filter paper (0.5in x 0.5in), which was enclosed inside a metal tea ball to prevent contact of the liquid odor with the testing chamber or animal, yet still allow for volatile odor delivery. Odors were delivered in 4 successive trials of 50s each where the tea ball was placed in the center of the testing chamber, with a 5-minute inter-trial interval. Testing took place in a conventional cage devoid of bedding material. All mice were tested at ZT6-8 during the light phase for 4 consecutive daily sessions. Day0 consisted of 4 trials of just mineral oil; Day1 consisted of 3 mineral oil habituation trials followed by 1 test trial with 0.1Pa n-amyl acetate in mineral oil; Day2 consisted of 3 mineral oil habituation trials followed by 1 test trial with 1Pa n-amyl acetate in mineral oil; Day3 consisted of 3 mineral oil habituation trials followed by 1 test trial with 3Pa n-amyl acetate in mineral oil. The duration of time spent investigating (snout-oriented sniffing within 1cm of the odor) was recorded across trials by a single observer blind to genotype.

Olfactory Cross-Habituation Testing

In Experiment 2a, Ntg and APP/PS1-21 mice of both sexes were screened for olfactory deficits using an odor cross-habituation task between 7 and 9 weeks of age (*n*=9-11 per group). Mice were tested again on the same task at 15-17 weeks of age to assess olfactory deficits under conditions of advanced plaque deposition (*n*=5-6 per group). Odors (n=5 aliphatic alcohols; propanol[3C], butanol[4C], pentanol[5C], hexanol[6C], heptanol[7C]) were diluted to a standard vapor pressure of 1Pa in mineral oil and applied to filter paper in 60μL aliquots, which were enclosed inside a metal tea ball to prevent contact of the liquid odor with the testing chamber or animal, yet still allow for volatile odor delivery. Odors were delivered in 11 successive trials of 50s each where the tea ball was placed in the center of the testing chamber, with a 5-minute

inter-trial interval. Testing took place in a conventional cage devoid of bedding material. Mice were either tested in the light phase at ZT6-8 or during the dark phase at ZT18-20 during the course of 1 session. The test consisted of 1 presentation of a blank (plain mineral oil), followed by 3 presentations of propanol (Habituation Odor; OHab). The test odors (4C-7C) were then presented, interleaved with successive presentations of OHab to reinforce habituation (See Fig.18 for diagram of experimental setup). The order of test odors was counterbalanced across mice. The duration of time spent investigating (snout-oriented sniffing within 1cm of the odor) was recorded across trials by a single observer blind to genotype. Odor dilutions were as follows:

Table 2: Aliphatic Alcohol Dilutions for Olfactory Behavioral Tests

	For 1Pa in 50ml of mineral oil (MO)
Propanol [3C]	0.2 μL
Butanol [4C]	0.9 μL
Pentanol [5C]	3.3 μL
Hexanol [6C]	11.1 μL
Heptanol [7C]	35.3 μL

Olfactory-Cued Digging Task

In experiment 2b, mice of both sexes (*APP/PS1-21:* n=10 females, n=10 males; *WT:* n=10 females, n=10 males) were trained using a protocol developed and modified according to Linster and Hasselmo (1999) and presented elsewhere (Linster & Hasselmo, 1999; Nusser et al., 2001). The testing chamber consisted of a polycarbonate cage similar to the home cage but fitted with a dividing door to separate the starting and testing chambers. Mice were trained to dig in a small glass petri dish filled with corncob bedding for a food reward (1/4 stale Honey Nut

Cheerio™- stale to decrease the odor of the reward) until they reached criterion (initiated digging in the odor dish within 10sec). They were then presented with 2 dishes, one scented with odorant (1Pa *n*-amyl acetate, 36.1μL in 50 mL MO), and one unscented (just MO). The mice were then trained to dig in the scented dish for a reward, with the side of the odor dish randomized across trials. Each test trial was 20 seconds long, after which the animal was removed from the arena and placed back in the start chamber. Odor testing sessions began with 10 training trials in which the mouse learned to dig in response to the dish with the training odor (1 Pa propanol [3C]) and avoid digging in the unscented control dish. The mouse was then tested on the same set of aliphatic alcohols used for the habituation test, including propanol, plus an unrelated odor (ethyl 2-methyl butyrate [EMB], 2.4μL in 50 mL MO) presented in randomized order across mice. In these test trials, no reward was present in the dish. The amount of time spent digging in the scented dish was recorded. To avoid extinction, three reinforcement trials with the trained odor were interspersed between every one to two testing trials. Identification was measured as significant digging in the training odor dish. Generalization was measured as significant digging in an odor other than the training odor.

Statistics

Sniff time and digging time data were normalized to z scores (zero mean and unit standard deviation) for each mouse for each test day, as reported in earlier studies (Kay, 2003; Kay et al., 2005; Nusser et al., 2001). We normalized in this fashion because sniff and digging times varied widely across mice, and this made for easy application of post hoc comparisons. Normalized data were analyzed with analysis of variance (ANOVA) for carbon chain length. To control for alpha inflation and Type I error, pairwise comparisons were performed using two-

tailed t-tests, where justified by a significant omnibus F statistic. All statistical comparisons were performed using StatView software (SAS). Differences were considered significant if $p<0.05$.

For Experiment 2a (Olfactory Cross-Habituation), data were split by time of testing (*i.e.*, between tests conducted during the dark phase or during the light phase) for easier visualization. Data for Experiment 2a was analyzed according to the olfactory functional behaviors that were assessed: The first (Blank) trial was analyzed for group differences in novel odor orienting response, the following 3 trials with the 3-carbon aliphatic alcohol (3C; OHab) were analyzed for group differences in habituation, and the remaining trials were analyzed for discrimination (cross-habituation) between OHab and test odorants. The sniff times for 3C (OHab) trials that were interleaved between test odorant trials were averaged for each animal, and this value was used for comparison with sniff times to each test odorant. Data for experiment 2b (Olfactory Digging Task) were assessed for differences in discrimination, operationally defined as digging in the 3C (propanol) odor dish compared to digging in the other test odor dishes. As there were two unrewarded 3C test trials, digging times were averaged for these two trials and this average value was used for comparison with digging times on other test odorants.

Results

Experiment 1

Experiment 1 was a pilot study used to determine the concentration of odorant that would be above threshold at the onset of plaque deposition, which begins in the APP/PS1-21 mouse at 6 weeks of age. This odor concentration would then be used for all other olfactory behavioral tests to control for any differences in odor detection threshold between genotypes and sexes. Sniffing behavior in response to first presentation of three different concentrations of a test odor (*n*-amyl

acetate) after habituation to OHab (mineral oil) was measured in Ntg and APP/PS1-21 mice. We did not observe main effects of genotype or sex in response to test odors of any concentration, though there was a significant interaction between genotype and sex in response to *n*-amyl acetate at 0.1Pa, where there was a trend for Ntg females to sniff more than APP/PS1-21 females (p=0.0508). Therefore, we selected 1Pa as the test odor concentration for future olfactory behavioral tests, as this was the next lowest concentration where significant differences between genotypes in odor detection threshold were not evident.

		0.1Pa		1Pa		3Pa	
	DF	F	*p*	F	*p*	F	*P*
Genotype	1	0.322	0.5880	0.027	0.8751	1.728	0.2301
Sex	1	3.320	0.1112	0.370	0.5622	0.098	0.7636
Geno*Sex	1	9.369	**0.0183**	0.197	0.6703	0.001	0.9756

Experiment 2a: Cross-Habituation at onset of plaque deposition

Dark Phase

Novel Odor Orienting Response: A main effect of genotype ($p<0.05$), but not of sex ($p=0.2847$), was observed in novel odor orienting response in the dark phase (response to the first presentation of plain mineral oil [MO], Fig 19), with no significant interaction between these factors ($p=0.5238$). Specifically, APP/PS1-21 mice showed increased sniffing to the initial (Blank/MO) trial compared to Ntg mice ($p<0.05$), corresponding to an increase in novel odor orienting response for APP/PS1-21 mice.

Habituation: Though APP/PS1-21 mice trended towards sniffing the first presentation of 3C less than Ntg mice ($p=0.0544$) in the dark phase, there was no significant main effect of

genotype for the next two presentations of 3C ($p=0.8367$ and $p=0.1421$, respectively). There were also no significant main effects of sex ($p=0.5703$ and $p=0.2230$, respectively), or interaction effects between genotype and sex ($p=0.7884$ and $p=0.5821$, respectively) for the first two presentations of 3C (OHab). However, there was a significant main effect of sex for the third habituation trial ($p<0.02$), where female mice sniffed more than male mice ($p<0.01$), indicating females show less habituation to 3C than males (Fig 19). There was still no main effect of genotype on this third presentation of OHab ($p=0.1421$) and no interaction ($p=0.7545$), indicating that mice of different genotypes did not exhibit differences in habituation. A paired t-test comparing sniff times between Blank and the first presentation of 3C (OHab) showed a significant reduction in sniff time for 3C ($p<0.0001$). All groups except Ntg females exhibited significant differences between Blank and the first presentation of 3C (Ntg F: $p=0.2040$; Ntg M: $p<0.05$; APP/PS1-21 F: $p=0.0001$; APP/PS1-21 M: $p<0.001$). There were no significant differences in sniff time between the first and second presentations of OHab ($p=0.9622$). Similarly, there was not a significant difference in sniff time between the second and third presentations of OHab ($p=0.6113$), or between the first and third presentations ($p=0.3411$). A paired t-test between the third presentation of 3C and the average sniff times for the 3C trials that were interleaved between test trials showed a significant difference in sniff time ($p<0.001$), driven in particular by a reduction in sniff time for the latter 3Cs for female mice (Ntg: $p<0.005$; APP/PS1-21: $p<0.05$). Male Ntg and APP/PS1-21 did not show differences in sniff times between the third OHab trial and the average of the OHab trials interleaved between test trials ($p=0.3151$ and $p=0.9583$, respectively), indicating that female mice may be more sensitive to habituation than males. This suggests that while mice eventually habituate to OHab, habituation is not evident until more than three presentations of 3C. In an ANOVA assessing average sniff

times to the 3C trials interleaved between test trials, there was no significant main effect of genotype ($p=0.9585$) or sex ($p=0.8810$), and no significant interaction ($p=0.4975$), indicating habituation was maintained across the test for all groups.

Discrimination: When mice were tested on the cross-habituation test odors during the dark phase, we observed a significant main effect of genotype ($p<0.02$) and carbon chain length ($p<0.05$), as well as a significant interaction between these two factors ($p<0.05$). There was not a significant main effect of sex in the dark phase ($p=0.3093$). Ntg mice were able to discriminate most of the odorants from the habituated odorant: sniffing the 4-carbon odorant ($p<0.001$), the 6-carbon odorant ($p<0.02$), and the 7-carbon odorant ($p<0.01$) more than 3C (Fig. 20). However, APP/PS1-21 mice did not discriminate these odorants from the habituated odorant ($p=0.8711$, $p=0.3643$, $p=0.2834$, respectively). Interestingly, neither genotype sniffed the 5-carbon odorant more than OHab (Ntg: $p=0.6158$; APP/PS1-21: $p=0.0757$). There were no significant interactions between genotype and sex ($p=0.4329$), carbon length and sex ($p=0.5956$), and genotype*carbon length*sex ($p=0.2526$).

Light Phase

Novel Odor Orienting Response: When mice were tested in the light phase, there were no significant main effects of genotype ($p=0.3088$) or sex ($p=0.2593$) on sniffing in response to the initial (Blank, mineral oil) trial, and no interaction between these factors ($p=0.0967$). There was therefore no significant difference in novel odor orienting response between groups when testing was administered in the light phase (Fig. 19).

Habituation: There were no significant main effects of genotype ($p=0.6902$, $p=0.1592$, and $p=0.5300$, respectively), sex ($p=0.6263$, $p=0.5873$, and $p=0.7543$, respectively), or

interaction effects between the two ($p=0.2511$, $p=0.6238$, and $p=0.4210$, respectively) for the first three presentations of 3C (OHab), indicating that groups did not exhibit differences in habituation during the light phase (Fig. 19). Paired t-tests were performed to assess for differences between successive presentations of 3C (OHab). A significant reduction in sniff time was observed for the first presentation of OHab compared to sniff times for Blank ($p<0.05$), driven by female Ntg mice ($p<0.05$). Additionally, there was reduced sniffing in response to the second presentation of OHab compared to the first ($p=0.001$), driven by APP/PS1-21 females ($p<0.02$). While there were no significant differences in sniff times between the second and third presentations of 3C ($p=0.5957$), sniff times in response to the third presentation of 3C were still significantly reduced compared to the first presentation of 3C. Finally, there was a reduction in sniff time for the average of all OHab trials presented between test trials (*i.e.*, OHab used to compute the difference in discrimination) compared to the third presentation of OHab (*i.e.*, final OHab in trials used to compute habituation; $p<0.005$), and this was driven by Ntg females ($p<0.02$) as no other groups showed a difference in sniff times between these OHab metrics (M Ntg: $p=0.8159$; F APP/PS1-21: $p=0.0913$; M APP/PS1-21: $p=0.0871$). Overall, this suggests that mice successfully habituated to 3C, and females may have a lower habituation floor. Finally, an ANOVA looking at differences in average sniff times for the 3C trials interleaved between test trials showed no significant main effects of genotype ($p=0.4066$) or sex ($p=0.3993$), and no significant interaction ($p=0.0800$).

Discrimination. For test trials of odorants with different carbon chain lengths, there was a significant main effect of genotype ($p<0.05$) and carbon chain length ($p<0.0001$), and significant interactions between carbon chain length and sex ($p<0.005$) and between genotype*carbon chain length*sex ($p<0.05$). There was not a significant main effect of sex

(p=0.0629), although perhaps a marginal difference. Ntg male mice showed the opposite response expected for the cross-habituation task, as there was more likely to be discrimination shown for odorants with lower carbon chain lengths (*i.e*., more similar to OHab), but mice generalized more often to odors with longer carbon chain lengths (*i.e*., more dissimilar to OHab; Fig. 21). Compared to OHab (3C), there was increased sniffing in response to 4C ($p<0.002$), and this was driven primarily by APP/PS1-21 males ($p<0.05$). All other groups failed to show differences in sniff times between 3C and 4C (Ntg F: p=0.1314; Ntg M: p=0.1528; APP/PS1-21 F: p=0.1596). Similarly, there was increased sniffing for 5C compared to 3C (p=0.0002), driven primarily by Ntg males (p=0.0002), who sniffed 2 times longer than other groups, though APP/PS1-21 males also showed increased sniffing in response to 5C ($p<0.01$). Females failed to show differences in sniff times between 3C and 5C (Ntg: p=0.0634; APP/PS1-21: p=0.7824). No groups showed differences in sniff times between 3C and 6C (Overall: p=0.6549; Ntg F: p=0.3128; Ntg M: p=0.2954; APP/PS1-21 M: p=0.3805; APP/PS1-21 F: p=0.9317). There was an overall trend for increased sniff times for 7C relative to 3C (p=0.0551), driven by APP/PS1-21 males ($p<0.05$), though no other groups showed differences in sniff times between 3C and 7C (Ntg F: p=0.0706; Ntg M: p=0.7654; APP/PS1-21 F: p=0.9800).

Cross-Habituation in more advanced plaque deposition

Dark Phase

Novel odor orienting response: In an ANOVA to assess differences in response to the Blank (mineral oil) trial, we did not observe a significant main effect of genotype (p=0.4681) or sex (p=0.2387), and no significant interaction (p=0.9984), indicating that all groups showed a similar novel odor orienting response (Fig. 21).

Habituation: There were no significant main effects of genotype ($p=0.3173$, $p=0.6647$, and $p=0.9966$, respectively), sex ($p=0.2234$, $p=0.9236$, and $p=0.0514$, respectively), or interaction effects between the two ($p=0.4969$, $p=0.5423$, and $p=0.4064$, respectively) for the first three presentations of 3C (OHab), indicating that groups did not exhibit differences in habituation during the dark phase (Fig. 22). A paired t-test between Blank and the first 3C trial did not show a significant decrease in sniff time ($p=0.0561$), although this value can be considered marginal. Additionally, mice exhibited increased sniff times in the response to the second presentation of 3C compared to the first presentation ($p<0.05$), and this was particularly driven by APP/PS1-21 mice ($p<0.05$). Ntg mice did not show a difference between the first and second presentations of 3C ($p=0.4413$). While there was a significant reduction in sniff time for the third presentation of 3C compared to the second ($p<0.05$), there was not a significant difference in sniff times for the third 3C presentation compared to the first presentation of 3C ($p=0.8276$). Taken together, this indicates that mice did not habituate to 3C within these first 3 trials. However, a paired t-test between sniff times in response to the third 3C presentation compared to the average sniff times for the 3C trials that were interleaved between test trials showed a reduction in sniff times for the latter ($p<0.005$), with both Ntg mice ($p<0.05$) and APP/PS1-21 mice ($p<0.05$) exhibiting reduced average sniff time for these later 3C trials. This indicates that it may take more presentations of a stimulus to induce habituation in older mice. These average sniff times for the 3C trials interleaved between test trials were also assessed for group differences with an ANOVA. There was a significant main effect of sex ($p<0.005$), where female mice sniffed more than males. There was no main effect of genotype ($p=0.541$) and no significant interaction ($p=0.3730$).

Discrimination: There was a significant main effect of carbon length on sniff time in response to test odors in the dark phase ($p<0.05$), but no significant main effect of genotype ($p=0.8593$) or sex ($p=0.5425$), and no significant interactions (Fig. 22). All mice showed increased sniffing relative to 3C for all test odors (4C: $p<0.01$; 5C: $p<0.005$; 6C: $p<0.05$; 7C: $p<0.005$).

Light Phase

Novel odor orienting response: In an ANOVA to assess differences in response to the Blank (mineral oil) trial, we did not observe a significant main effect of genotype ($p=0.6193$) or sex ($p=0.3096$), and no significant interaction ($p=0.9230$), indicating that all groups showed a similar novel odor orienting response (Fig. 22).

Habituation: There were no significant main effects of genotype ($p=0.1547$, $p=0.1002$, and $p=0.5127$, respectively), sex ($p=0.3984$, $p=0.5520$, and $p=0.2413$, respectively), or interaction effects between the two ($p=0.1547$, $p=0.8368$, and $p=0.2408$, respectively) for the first three presentations of 3C (OHab), indicating that groups did not exhibit differences in habituation during the light phase (Fig. 22). A paired t-test between Blank and the first 3C trial did not show a significant decrease in sniff time ($p=0.4353$), though Ntg males significantly reduced sniff times for 3C compared to Blank ($p<0.05$). Additionally, mice did not show significant differences between the first and second presentations of 3C ($p=0.1919$), with the exception of Ntg males showing increased sniff times for the second presentation of OHab compared to the first ($p<0.02$). There was a significant reduction in sniff time for the third presentation of 3C compared to the second ($p<0.002$) and compared to the first presentation ($p<0.05$). Taken together, this indicates that the mice showed some impairment in habituation

over the first 3 OHab trials. A paired t-test between sniff times in response to the third 3C presentation compared to the average sniff times for the 3C trials that were interleaved between test trials indicated showed a reduction in sniff times for the latter ($p<0.05$). This again could indicate that it may take more presentations of a stimulus to induce habituation in older mice and that habituation occurs slightly faster in the light phase compared to the dark phase for some groups. There were also group differences in average sniff times for the 3C trials interleaved between test trials, with a significant main effect of genotype ($p<0.02$) and a significant interaction between genotype and sex ($p<0.0001$), but no main effect of sex ($p=0.8615$). Male Ntg mice showed increased sniffing compared to female Ntg mice, but male APP/PS1-21 mice showing reduced sniffing compared to female APP/PS1-21 mice.

Discrimination: Again, there was a significant main effect of carbon length on sniff time in response to test odors in the light phase ($p<0.01$), but no significant main effect of genotype ($p=0.3562$) or sex ($p=0.7020$), and no significant interactions (Fig. 23). All mice showed increased sniffing relative to 3C for all test odors (4C: $p<0.02$; 5C: $p<0.01$; 6C: $p<0.005$; 7C: $p<0.001$).

Relationship between cross-habituation at different phases of plaque deposition

Dark Phase

In order to assess any differences in average sniff times for the 3C trials interleaved between test odorant trials, we ran an ANOVA on the z-scored average of these 3C trials with genotype, sex, and testing age as factors (early or later in plaque deposition timeline), none of which showed significant main effects ($p=0.0698$, $p=0.01518$, $p=0.1797$, respectively). However, there was a significant interaction between sex and testing age ($p<0.01$), where females did not

show differences between testing at each age but males showed reduced average sniffing time for 3C trials when they were older ($p<0.005$). An ANOVA run on the z-scored cross-habituation test trial data with genotype, sex, carbon chain length, and testing age as factors showed a significant main effect of carbon chain length on sniff times ($p<0.001$), but no main effects of any other factors (Geno: $p=0.0841$; Sex: $p=0.1925$; Test age: $p=0.5160$) and no significant interactions ($p>0.1440$). However, all variables except carbon chain length (power=0.966) were underpowered (genotype=0.391; sex=0.241; testing age=0.097). All mice showed increased sniffing relative to 3C for all test odors (4C: $p<0.001$; 5C: $p<0.002$; 6C: $p<0.002$; 7C: $p<0.0001$).

Light Phase

There were group differences in average sniff times for the 3C trials interleaved between test trials, with a significant main effect of testing age ($p<0.0005$) and a significant interaction between genotype and sex ($p<0.0001$), a significant interaction between genotype and testing age ($p=0.001$), and a significant interaction between genotype, sex, and testing age ($p=0.001$). Female mice in early testing do not show differences by genotype in sniff duration ($p=0.2238$), but in late testing female APP/PS1-21 mice show increased sniff time for 3C relative to Ntg females ($p<0.0001$). Male mice at both ages show a genotype difference, with Ntg males sniffing 3C more than APP/PS1-21 males (Early test: $p<0.0001$; Late test: $p<0.02$). An ANOVA run on the z-scored cross-habituation data with genotype, sex, carbon chain length, and testing age (early or later in plaque deposition timeline) as factors showed a significant main effect of carbon chain length on sniff times ($p<0.01$), and a trend for impact of testing age ($p=0.0542$), but no main effects of genotype ($p=0.0622$) or sex ($p=0.1899$). There was a significant interaction between carbon chain length and testing age ($p<0.005$), where mice at both time points sniffed

5C more than 3C, but only mice tested later sniffed the other test odors more than 3C (see table below for significance). This could indicate mice perform better on this task when they are older, but more likely denotes z-scoring sniff times for each mouse for each session may not accurately capture differences in test performance over time.

	4C	5C	6C	7C
Early Test	$p=0.1101$	$p<0.01$	$p=0.4774$	$p=0.6850$
Later Test	$p<0.02$	$p<0.01$	$p<0.005$	$p<0.001$

Changes over time

We also z-scored within mouse across both test times rather than z-scoring only for each session, in order to get a better idea of how mice altered their behavior over time. This resulted in an ANOVA with significant main effect of test age ($p<0.0007$), but no main effect of any other variable (genotype: $p=0.3300$; sex: $p=0.4770$; test time: $p=0.2714$; carbon length: $p=0.4208$). There was also a significant interaction between genotype and testing age ($p<0.005$) and between sex and testing age ($p<0.01$). Mice at the onset of plaque deposition showed increased sniff times for test odorants compared to older mice ($p=0.001$), and this was driven by APP/PS1-21 mice at the onset of plaque deposition sniffing more than APP/PS1-21 mice further into plaque deposition ($p<0.0001$; Fig. 24). Ntg mice did not show changes in sniff times between testing ages ($p=0.8523$). Additionally, older males showed reduced sniff times compared to younger males ($p<0.0001$), while females did not show differences in sniff times across testing ages ($p=0.5858$; Fig. 26). These results overall indicate the expected overall decrease in sniff times due to anosmia for APP/PS1-21 mice further into plaque deposition and illustrate an interesting

sex difference indicating males might be particularly susceptible to olfactory dysfunction compared to females.

Experiment 2b: Olfactory discrimination under conditions of reward

The digging task allowed us to test the influence of reward-based learning on odor identification and discrimination. We did not observe any differences between groups in digging times for C3 during unrewarded test trials, with no significant main effect of genotype ($p=0.4173$) or sex ($p=0.0645$) and no interactions ($p=0.9012$), indicating all groups were able to learn the reward association for this odorant. A significant main effect of carbon chain length ($p<0.0001$) and a significant interaction between sex and carbon chain length ($p<0.05$) were observed in digging times for dishes with test odors (3C, 4C, 5C, 6C, 7C, EMB), though there was no significant main effect of sex ($p=0.1620$). Under conditions of reward, we did not observe genotype differences in discrimination, with no significant main effect of genotype ($p=0.0730$; Fig. 23-24)). There were no other significant interactions ($p>0.1894$). Overall, female mice were better than male mice at discriminating odorants under conditions of reward, with sex differences most evident for odors most similar to 3C (Fig. 23). Compared to the 3C dish, female mice dug less in the 4C ($p=0.0002$) and 5C ($p<0.0001$) dishes, while males did not differ in digging times between the 3C and 4C ($p=0.4854$) or 5C ($p=0.0837$) dishes. This suggests females were able to discriminate between 3C and more similar odors, while males were not. Interestingly, all mice indicated difficulty in discriminating the 3C and 6C odors, with neither sex differing in digging times between 3C and 6C dishes (F: $p=0.0759$; M: $p=0.3828$). However, both sexes were able to differentiate the most dissimilar aliphatic alcohol, 7C, from 3C, displaying decreased digging time in the 7C dish compared to the 3C dish (F: $p=0.0001$; M:

p=0.0002). Additionally, both sexes were able to discriminate the unrelated odorant, EMB, from 3C: displaying significantly decreased digging times for the EMB dish (F: $p<0.0001$; M: $p<0.02$).

While there were no overall differences in average digging time in test odor dishes (Geno: p=0.1050; Sex=0.2042; Geno*Sex: 0.6794), there were differences in average digging times in blank dishes between groups. This could be an indicator of how well mice were paying attention, or how well mice were able to inhibit the desire to dig after being trained not to dig in the blank dish. There was a significant main effect of genotype on this behavior ($p<0.0001$), with Ntg mice digging more in blank dishes than APP/PS1-21 mice ($p<0.0001$). There was also a significant main effect of sex ($p<0.02$), with female mice digging more in blank dishes than male mice ($p<0.01$). There was not a significant interaction between genotype and sex (p=0.0890). Additionally, we evaluated the number of times a mouse dug in a blank dish before digging in the test dish for each test odorant. For every test odorant except 6C and EMB, we observed between 1 and 3 instances of a mouse digging in the blank dish first (overall, a mouse dug first in a blank dish in 3.3% of all test trials), with only one mouse digging in a blank dish before a test odor dish more than one time (APP/PS1-21 female, in the second 3C unrewarded trial and the 7C unrewarded trial). The highest likelihood of digging in the blank dish prior to the test dish occurred during the second unrewarded 3C trial (7.9%; 1 male Ntg, 1 male APP/PS1-21, 1 female APP/PS1-21), indicating mice might be switching their strategy after not finding a reward in the first unrewarded 3C trial.

Discussion

Cognitive symptoms of AD do not manifest until long after the onset of plaque deposition, posing a major obstacle to early detection and treatment. AD, like many neurodegenerative diseases, often begins with early olfactory perceptual deficits (Alves et al., 2014; Barresi et al., 2012). This could be because of to the olfactory system's unique vulnerability to pathological protein aggregates, such as amyloid plaques (Rey et al., 2018). However, the commonality of olfactory dysfunction to many neurodegenerative diseases presents an issue with specific diagnoses of AD (Wesson, Wilson, et al., 2010). Some research has suggested that types of olfactory dysfunction may be disease-specific, because AD patients showed greater impairment in olfactory recognition than in detection threshold as compared to patients with Parkinson's Disease (PD)-related dementias (Rahayel et al., 2012). However, the extent to which plaque deposition early in disease etiology drives specific changes in olfactory function has not been fully evaluated. Our study overcomes some of the weaknesses of earlier experiments, particularly addressing sex differences and time of day. This allowed us to more specifically evaluate olfactory dysfunction in early plaque development of a mouse model of AD (Wesson, Levy, et al., 2010). With the experiments presented here, we aimed to more specifically evaluate olfactory dysfunction early in plaque development, while controlling for sex differences and circadian parameters.

Our results demonstrate significant differences in novel odor orienting response and discrimination that manifest early in plaque deposition (Figs. 19-21), similar to effects seen in other AD models (Wesson et al., 2011; Wesson, Levy, et al., 2010). APP/PS1-21 mice showed increased sniffing in response to novel odor (Blank trial) relative to Ntg mice, which was especially evident when testing occurred during the dark phase. Though increased sniffing in response to novel odor could indicate increased arousal and motivation or reduced ability to

habituate in the short term, the findings from Wesson et al. (2010) lend support to the latter hypothesis (Wesson, Levy, et al., 2010). The behavioral differences in the marble burying task reported for the APP/PS1-21 mouse in Chapter 4 also suggest that this transgenic model exhibits reduced exploratory motivation, bolstering support for impairment of very short-term habituation in APP/PS1-21 mice. Other tests assessing exploratory behavior in APP/PS1 mice have indicate similar findings of reduced exploratory behavior (Benito et al., 2017; McClean et al., 2011), making it unlikely that arousal or motivation is responsible for the observed increase in novel odor response we see here. Therefore, the increased sniffing of APP/PS1-21 mice in response to the Blank trial (Fig. 19) could represent a deficit in very short-term habituation, with transgenic mice failing to reduce sniffing of an individual odor over the course of a single trial. Previous research pointed to plaque deposition in the piriform cortex (PCX) in driving this response (Wesson, Levy, et al., 2010). Additionally, at the onset of plaque development, APP/PS1 mice have also been shown to display abnormal OB and PCX hyperactivity, which corresponds to impairments in habituation (Wesson et al., 2011). Thus, it is possible the increased sniffing in response to the Blank trial observed in APP/PS1-21 mice may be due to hyperactivity within this olfactory circuit. Future research should investigate if this hyperactivity exists within the APP/PS1-21 mouse at the onset at plaque deposition, and if driving early olfactory hyperactivity could worse disease progression, or vice versa (Wesson et al., 2011).

We did not observe genotype differences in normal habituation to the 3C odor over repeated trials (Fig. 19), though females and particularly APP/PS1-21 females tended to take more trials to habituate. This is somewhat in contrast to the findings of Wesson et al. (2010), in which a longer latency to habituate across odor presentations was observed for APP/PS1 mice even at the age of initial plaque deposition onset, though only an 8% difference in habituation

index from WTs was observed (as compared to a 31% difference when well into plaque deposition). However, that experiment lacked the level of control that ours had in terms of group sizes, time of administration of behavioral testing, and investigation of sex as a factor, which may contribute to differences from our results. Importantly, another experiment from the same group of researchers did not observe differences in habituation for those same AD transgenic mice at the start of plaque development, despite testing mice at the same age and with the same olfactory paradigm (Wesson et al., 2011). Therefore, novel object investigation (possibly short-term habituation), habituation over multiple trials to an odorant, and discrimination of odorants in the cross-habituation task seem to represent discrete, separable underlying processes, which are differentially affected by or sensitive to early plaque deposition. Habituation can be conceptualized as a type of olfactory identification or recognition (namely, the ability to correctly identify olfactory stimuli themselves or to distinguish them from other stimuli after a delay). Interestingly, impairments in sensory habituation and odor identification performance have been noted for PD patients (Cavanagh et al., 2018; Iravani et al., 2021). Taken together, this suggests that odor habituation alone is not an appropriate assay to reliably detect early plaque-related changes in AD and may better represent a marker of general neurodegeneration.

Our experiment demonstrated that APP/PS1-21 mice exhibit odor discrimination deficits early in plaque deposition, and this was especially evident when the cross-habituation paradigm was administered during the dark phase (Fig. 20). APP/PS1-21 mice overgeneralized to all odors during the dark phase, indicating a failure in discrimination, while Ntg mice discriminated most odors from 3C. Previous research on APP/PS1 mice did not demonstrate deficits in discrimination until well into plaque deposition (Wesson, Levy, et al., 2010). However, previous experiments administered the cross-habituation paradigm only in the light phase. In our

experiment, when mice were tested during the light phase, neither genotype discriminated most of the test odors, with the exception of males discriminating 5C from 3C (Fig. 21). This represents a clear circadian difference in habituation generalization, and highlights the importance of the time of administration for cognitive or perceptual testing. Mice are nocturnal, making it more ecologically valid to administer behavioral tests during the dark phase and to test at the same time for all subjects (however, given the impact of light pulses, care must be taken to avoid exposing the mice to light during the dark phase). Therefore, olfactory discrimination testing, when applied to human patients, should be aligned with an ecologically relevant testing time.

We observed some strange behavioral responses to some of these aliphatic alcohols in our experiments, specifically 5C and 6C. During the cross-habituation task done at 7-9 weeks of age, Ntg mice in the dark phase were able to discriminate every odor from 3C except for 5C (Fig. 20), and in the light phase male mice (especially Ntg) showed a large uptick in sniffing in response to 5C (Fig. 21), which was not evident for 4C or 6C. During the digging task, none of the test groups were able to discriminate 6C from 3C, though female mice were able to discriminate all other test odors (Fig. 25). It is unclear why these odors would be driving these aberrant responses, since previous research suggests 5C and 6C would be perceptually dissimilar from 3C (Cleland & Linster, 2002; Yoder et al., 2014). We are confident that there was little influence of baseline perceptual deficits in the cross-habituation paradigm, because in Experiment 1 we determined a concentration for odorants that were above detection threshold for APP/PS1-21 mice as well as their Ntg littermates, and this 1Pa concentration was used for all behavioral tests. It is possible that our odor dilutions did not completely cancel out the chain length effects on volatility, which scales inversely with chain length. However, similarity of the

5C or 6C alcohols to 3C specifically was only observed for one group each in different conditions or tasks (*e.g.*, during the dark phase or for Ntg males), which complicates interpretation. Thus, it would be informative in the future to test another set of aliphatic odorants, such as acids or esters, to see if our results translate more broadly.

A key component of the study was to evaluate how several characteristics of olfactory function that rely on different perceptual and cognitive computations were impacted by early plaque development, and whether a reward paradigm could override any observed deficits. Crucially, despite demonstrating a deficit in discrimination during the cross-habituation paradigm (Fig. 19), APP/PS1-21 mice did not show a deficit in discrimination in our appetitive conditioning task (digging task) using the same odor set (Fig. 23-24). This suggests that while early plaque deposition does induce olfactory dysfunction, the deficit can be overridden by other mechanisms that are only activated under a reward paradigm. This provides not only crucial information about the design of a diagnostic behavioral test for human patients, but additional insight into how the olfactory circuit functions in early AD.

In humans, OB volume and shape is associated with olfactory dysfunction (X. Yan et al., 2022). In particular, OB volume correlates with increased olfactory identification ability in humans, but not with detection or discrimination ability (Buschhüter et al., 2008), which suggests that these tasks may depend more on other cortical areas. The OB forms dense bidirectional connections with many brain areas, including the limbic system and Piriform Cortex (PC) (Carmichael et al., 1994; Gottfried, 2010; Gottfried & Zald, 2005; Haberly, 2001; Schoenbaum & Eichenbaum, 1995; Wilson & Rennaker, 2010). The limbic system includes structures such as the amygdala and hippocampus, crucial for emotion and memory processing; and even anecdotally, the relationship between smell, memory, and affective state is as obvious

as Proust's madeleine or the memory of your grandma's pie. The PC has been implicated in odor discrimination, with bilateral lesions of PC impairing odor discrimination (Chapuis et al., 2013). Additionally, lesions of piriform cortex have been seen to impair discrimination of complex odor mixtures but not simple odorants (Staubli et al., 1987). Early activity in the PC indicates odor identity but does not predict choice during olfactory behavioral tasks (P. Y. Wang et al., 2020). This suggests the PC plays a role in odor identity encoding. The PC also sends projections to the Orbitofrontal Cortex (OFC), which is not only the principal neocortical target of the olfactory network, but itself also projects back to limbic structures (Gottfried & Zald, 2005). Relevant to our findings, cell ensemble firing in the OFC can reliably show odorant discrimination in olfactory behavioral tasks, and also correctly assign the likelihood of reward associated with a given odorant (Schoenbaum & Eichenbaum, 1995). This could indicate that, in the absence of reward, early plaque deposition in the OB is sufficient to result in odor discrimination deficits, but that these deficits can be rescued through recruitment of other cortical structures, such as limbic structures, the PC, and/or the OFC, under conditions of reward. Future research should probe this pathway further. For example, lesioning or inactivating these nuclei in the APP/PS1-21 mouse and evaluating if there is a subsequent failure to discriminate under conditions of reward would give vital insights into how the olfactory circuit is impacted by Alzheimer's progression.

The high incidence of olfactory dysfunction in patients with some types of neurodegenerative diseases, particularly those that may produce dementia, indicates olfactory paradigms may be useful for early detection of disease (Duff et al., 2002; Velayudhan et al., 2013). Additionally, olfactory dysfunction in behavioral paradigms correlates with amyloid burden in AD mouse models, corresponding with plaque deposition in the olfactory bulb

beginning earlier than in any other brain area in these mice (Wesson, Levy, et al., 2010). There is some indication that human degeneration begins in the OB as well (Brozzetti et al., 2020; Rey et al., 2018; Ubeda-Bañon et al., 2020; J. Wang et al., 2010). Importantly, the literature has suggested that different types of olfactory dysfunction might be more affected than other types in different neurodegenerative disorders, with AD patients exhibiting more deficits in olfactory tasks relying on higher-order cognitive function than in simple perception (Rahayel et al., 2012). These facets of olfactory dysfunction can be tested with simple behavioral paradigms in both rodents and humans. If olfactory function is negatively impacted early on in AD progression, and in a distinguishable pattern from other neurodegenerative diseases, then a specific olfactory test may permit early detection of AD, allowing for early treatments or interventions. Our findings suggest that olfactory discrimination deficits are a promising direction for diagnosis, and differing performances under non-rewarded and rewarded conditions could be particularly informative. Future work should be targeted at evaluating these olfactory paradigms in mouse models of similar neurodegenerative diseases, such as Parkinson's Disease, to evaluate whether the presentation of early olfactory dysfunction reliably differs between different types of dementia-like animal models.

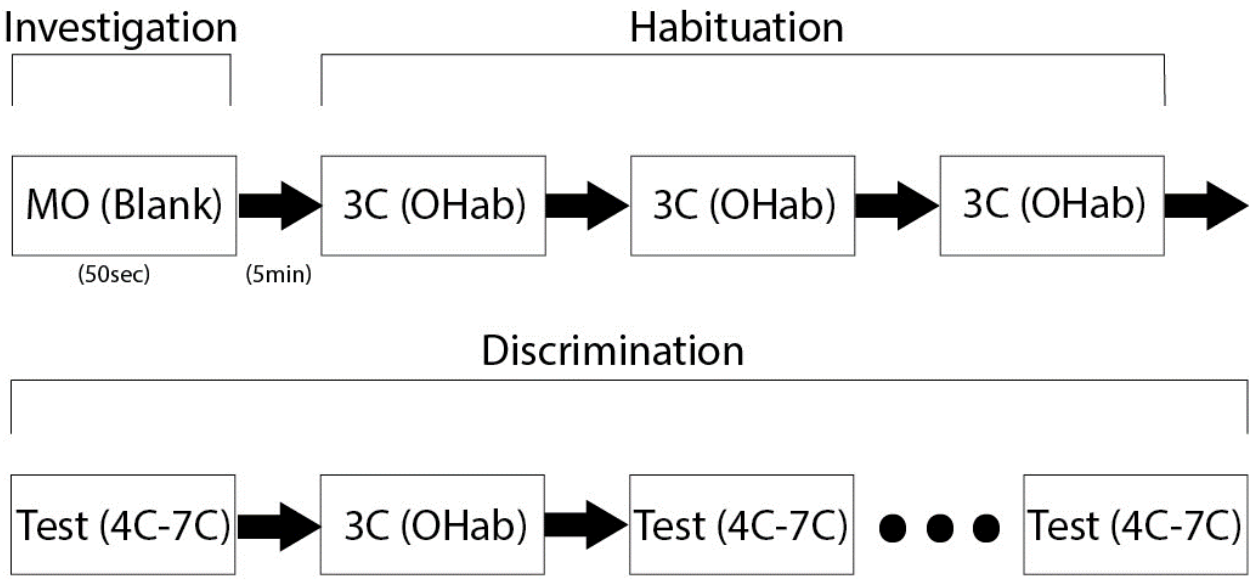

Figure 18: Diagram of Olfactory Cross-Habituation setup

Mice of both sexes were screened for olfactory deficits using an odor cross-habituation task between 7-9 weeks of age (n=9-11). A subset of these mice were tested again on the same task at 15-17 weeks of age to assess olfactory deficits under conditions of advanced plaque deposition (n=5-6). Odors (n=5 aliphatic alcohols; propanol[3C], butanol[4C], pentanol[5C], hexanol[6C], heptanol[7C]) were diluted to a standard vapor pressure of 1Pa in mineral oil and applied to filter paper in 60μL aliquots, which were enclosed inside a metal tea ball to prevent contact of the liquid odor with the testing chamber or animal, yet still allow for volatile odor delivery. Odors were delivered in 11 successive trials of 50s each where the tea ball was placed in the center of the testing chamber, with a 5-minute inter-trial interval. Testing took place in a conventional cage devoid of bedding material. Mice were tested either in the light phase at ZT6-8 or during the dark phase at ZT18-20. The test consisted of 1 presentation of a blank (plain mineral oil), followed by 3 presentations of propanol (Habituation Odor; OHab). The test odors (4C-7C) were then presented in pseudorandom order, interleaved with successive presentations of OHab to reinforce habituation.

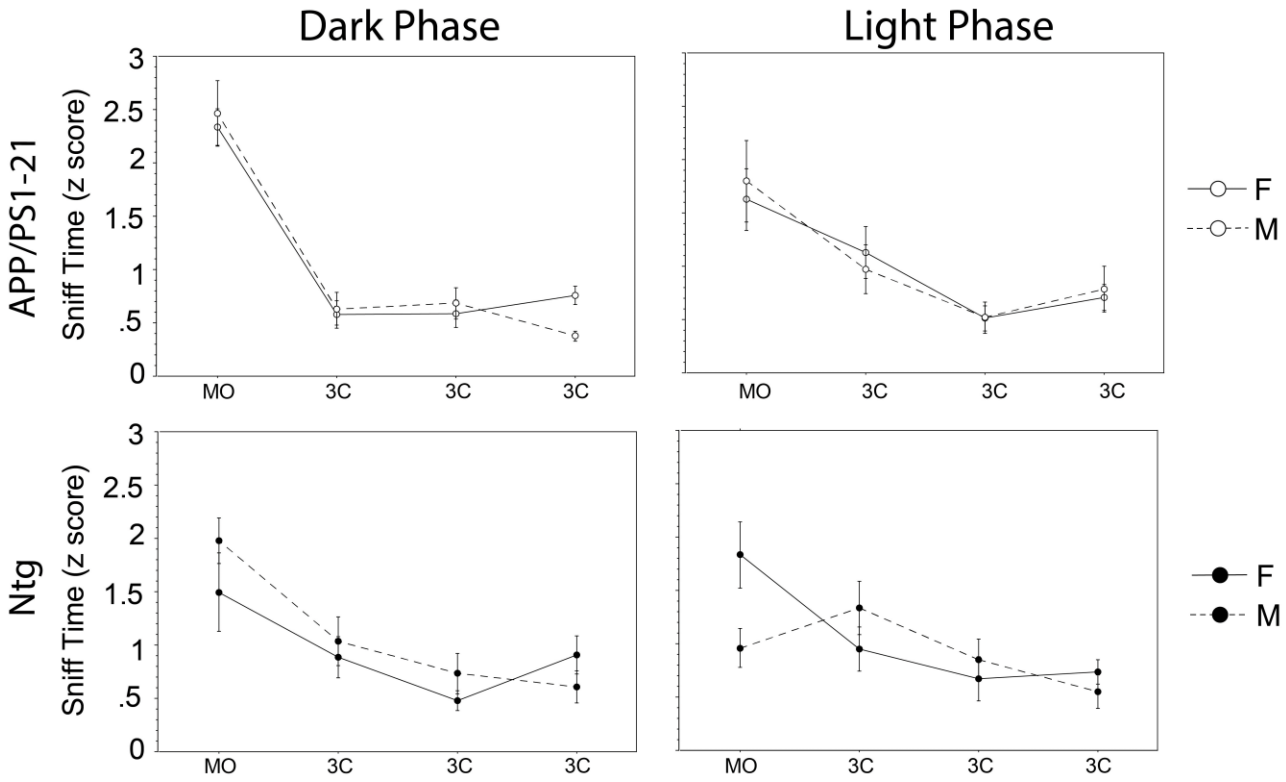

Figure 19: Novel odor orienting response and habituation at the onset of plaque deposition

APP/PS1-21 mice sniffed the Blank trial (MO) longer than Ntg mice when tested in the dark phase, and this difference disappeared during light phase testing. No significant differences between genotypes were observed in habituation to 3C (OHab). Error bars represent ±1SEM.

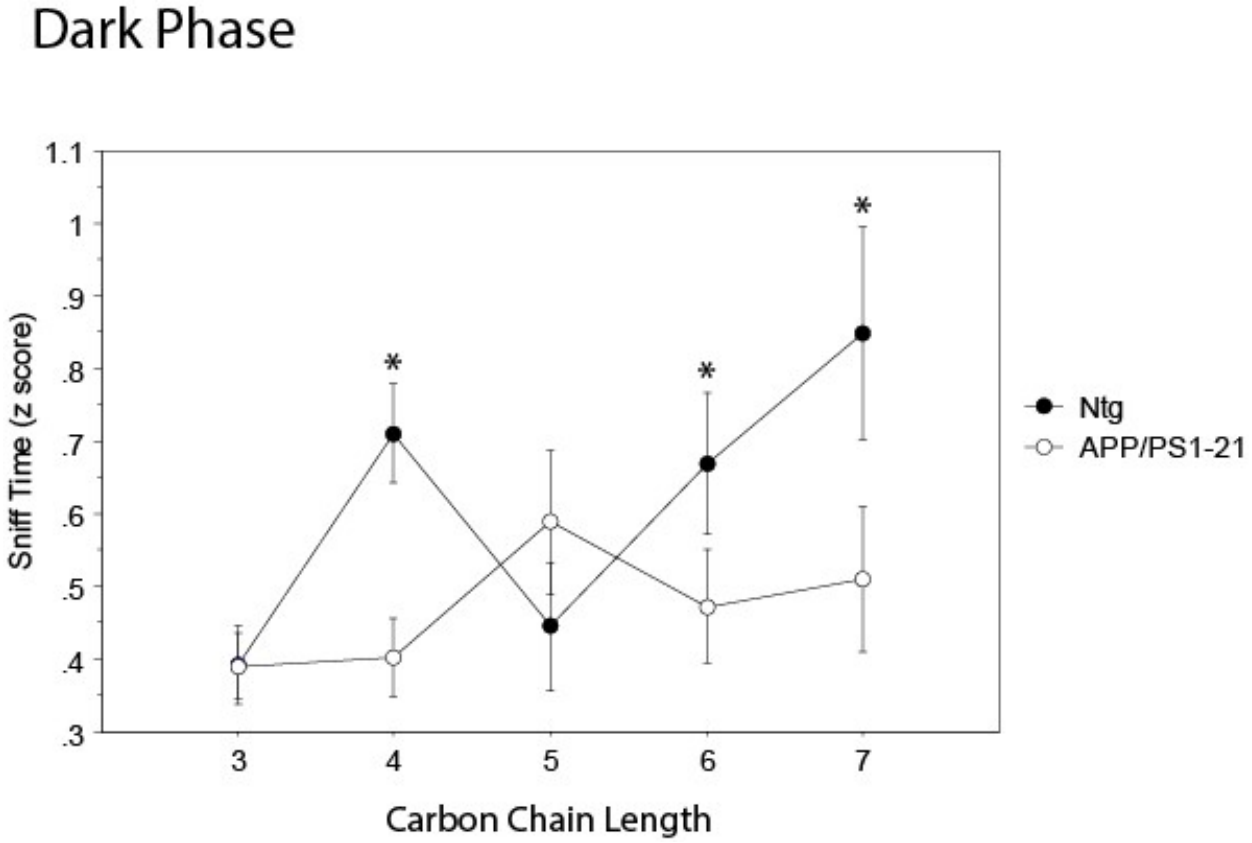

Figure 20: Olfactory cross-habituation in the dark phase

Significant genotype differences were observed in discrimination during the cross-habituation paradigm when tested during the dark phase. Ntg mice discriminated most odors (with the exception of 5C, which all groups generalized to), whereas APP/PS1-21 mice generalized to every odor. Error bars represent ±1SEM. Asterisks indicate significant difference in sniff time from 3C (OHab) ($p<0.05$).

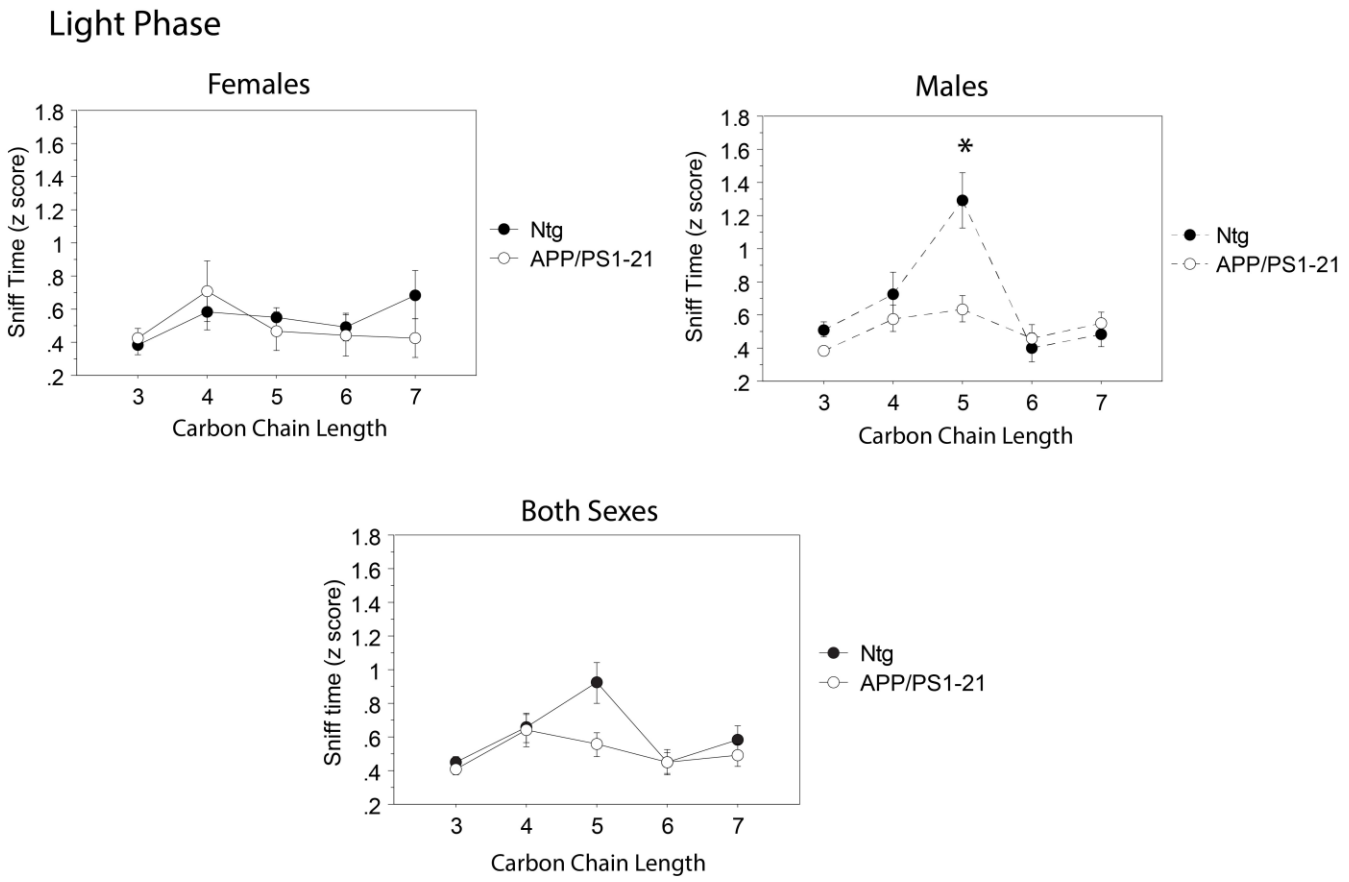

Figure 21: Olfactory cross-habituation in the light phase

When tested during the light phase, there was an interaction between genotype, carbon chain length, and sex driven by the increased sniffing response to 5C displayed by male mice. Sniff times for test trials collapsed across sexes is included in the plot on the bottom for easy visualization, though statistical marking was not permitted on this plot due to a lack of an interaction between carbon chain length and genotype (only the three-way interaction between carbon chain length, genotype, and sex was significant). In general, mice showed the opposite response predicted for cross-habituation, and were more likely to discriminate between more similar odors (4C and 5C) and more likely to generalize to longer carbon odors (6C and 7C). Error bars represent ±1SEM. Asterisks indicate significant difference in sniff time from 3C (OHab) ($p<0.05$).

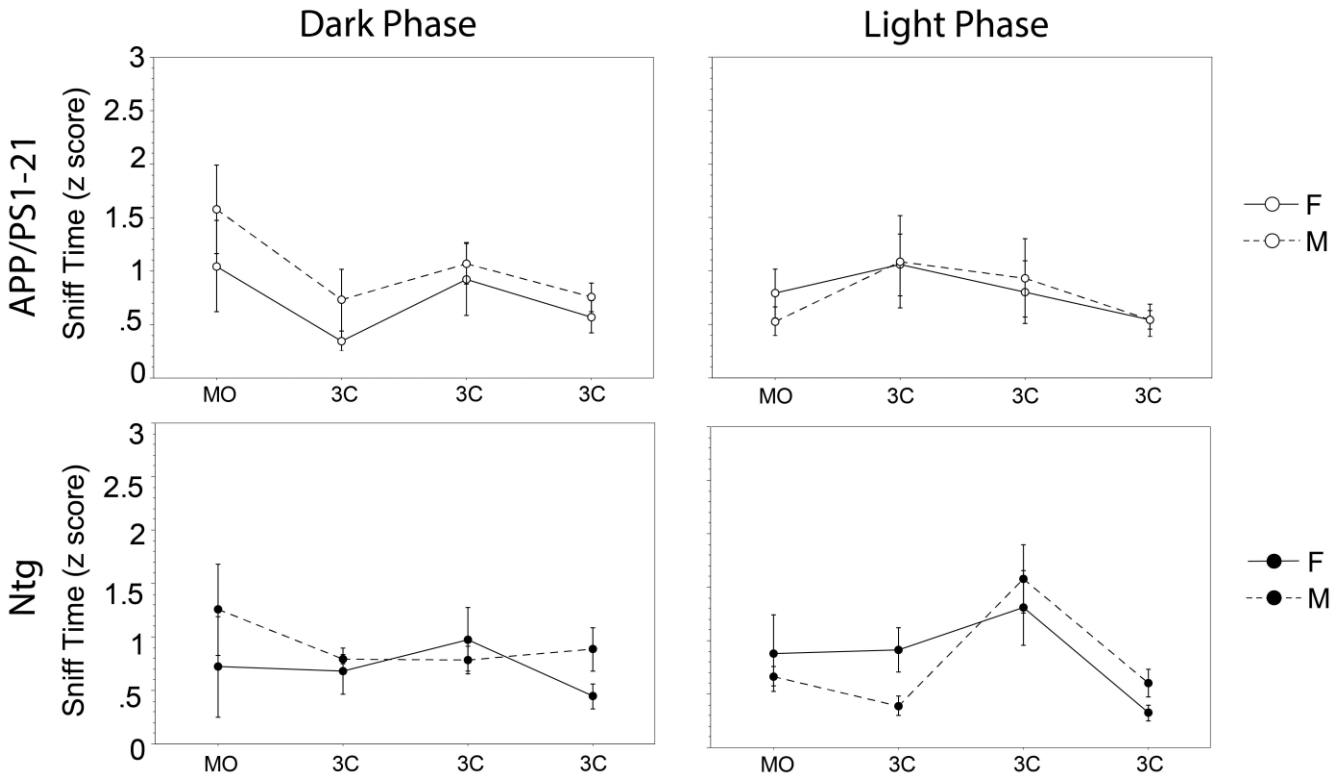

Figure 22: Novel odor orienting response and habituation after more progressed plaque deposition

No significant differences between genotypes were observed for the Blank trial (MO) or habituation to 3C. Mice exhibited a blunted novel odor orienting response that was not significantly different from the first presentation of 3C (OHab), unlike the response of younger mice. Mice in the dark phase did not show a reduction in sniff time over the first 3 presentations of OHab, though reduced sniff time to later presentations of OHab was evident (the trials interleaved between test odorants, not shown), demonstrating a latency to habituate. Mice in the light phase did show a reduction in sniff times over the first three presentations of OHab. Error bars represent ±1SEM.

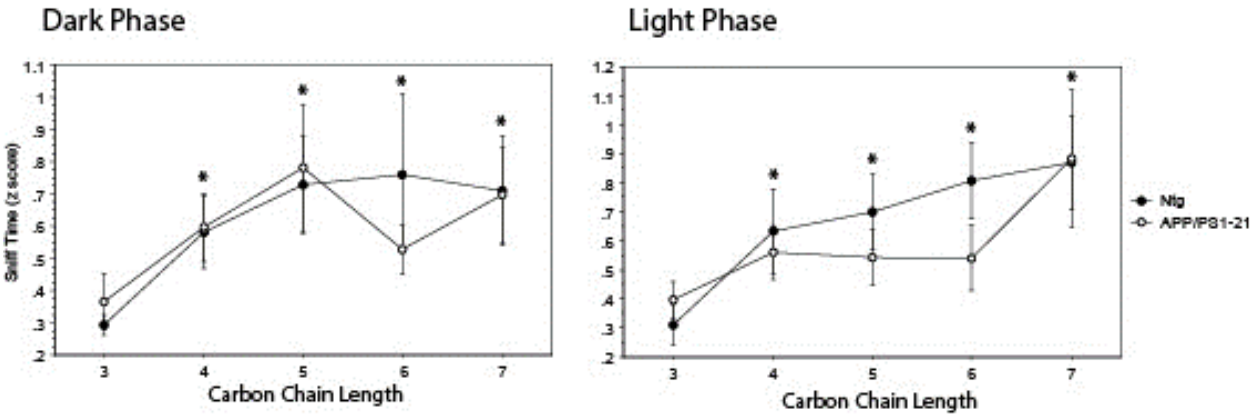

Figure 23: Discrimination/Cross-Habituation after more advanced plaque deposition

No genotype or sex differences in discrimination were observed. All mice during both light phase and dark phase testing successfully discriminated all test odors from 3C (OHab). Error bars represent ±1SEM. Asterisks indicate significant difference in sniff time from 3C (OHab) ($p<0.05$).

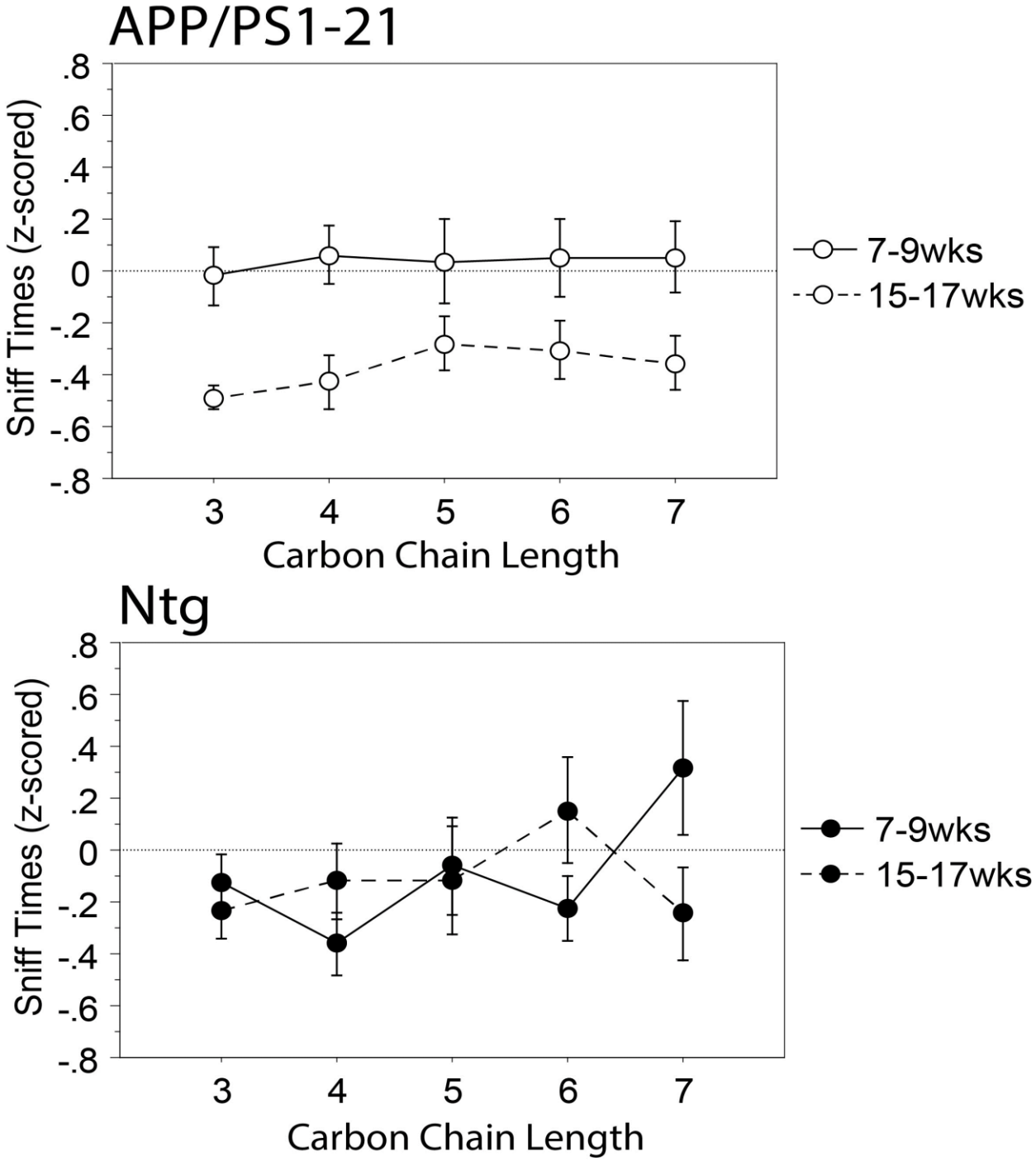

Figure 24:Cross Habituation Across Ages and Genotypes

When mice were z-scored across both test times rather than within one session (*i.e.*, across mouse rather than across day), we saw a significant interaction between test age and genotype, with no effect of carbon chain length. APP/PS1-21 mice at the onset of plaque deposition (7-9 weeks old, solid line) showed overall higher sniff times compared to older mice (15-17 weeks old, dashed line), while Ntg mice did not show changes in sniff times between testing ages. Error bars represent ±1SEM.

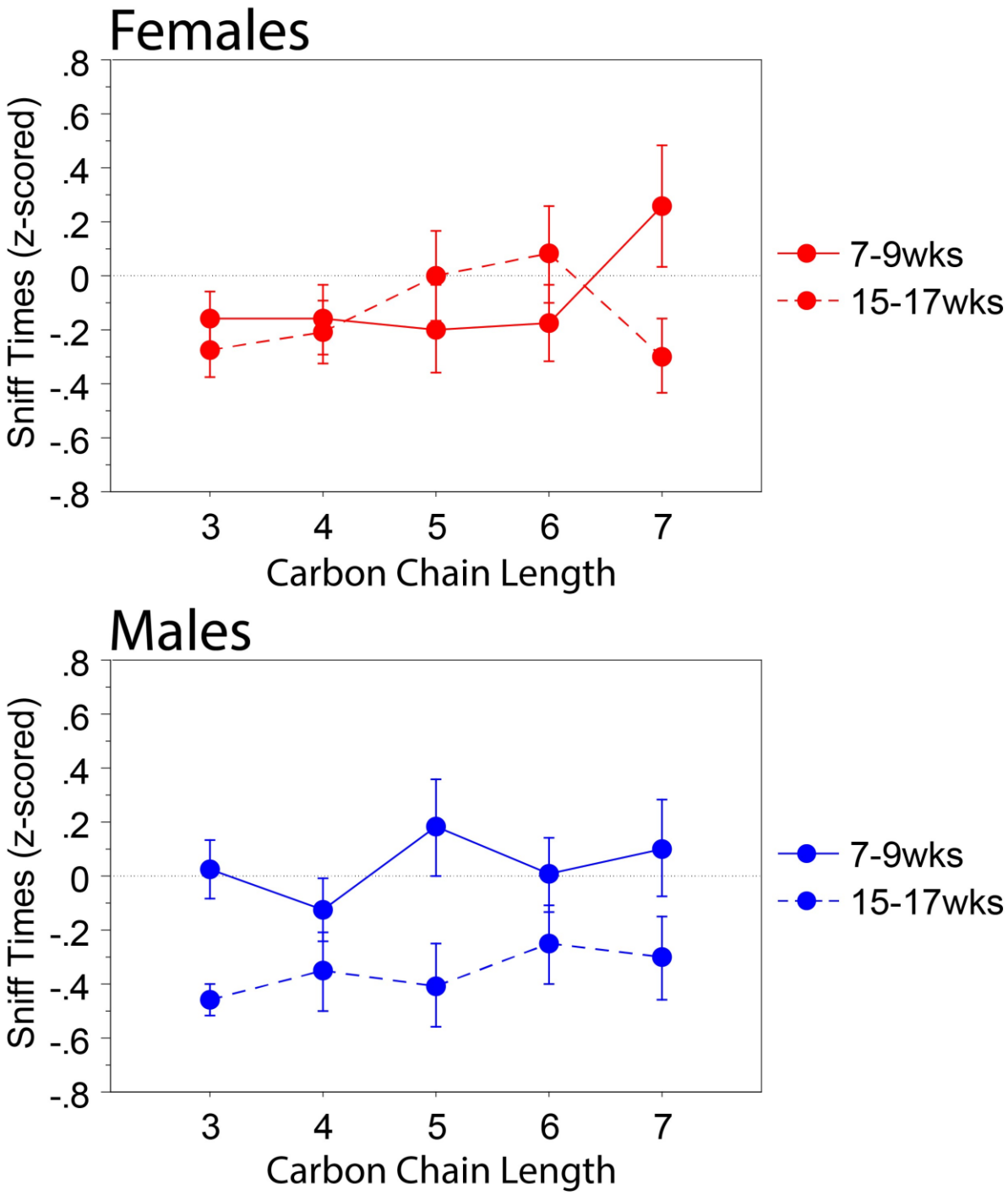

Figure 25: Cross Habituation across ages and sexes

When mice were z-scored across both test times rather than within one session (*i.e.*, across mouse rather than across day), we saw an interaction between testing age and sex, with no impact of carbon chain length. Older male mice (15-17 weeks old, dashed blue line) showed reduced sniff times compared to younger male mice (7-9 weeks old, solid blue line), while females did not show differences in sniff times across testing ages (top plot, red). Error bars represent ±1SEM.

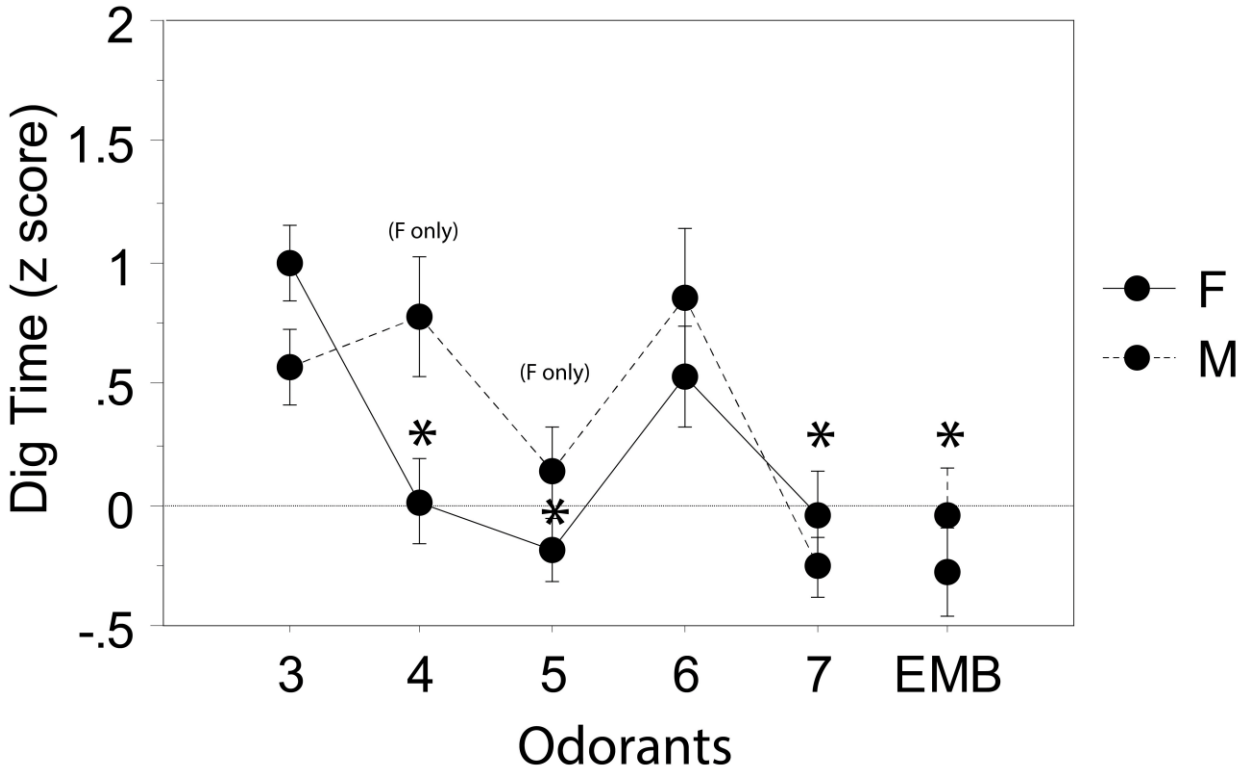

Figure 26: Discrimination in the digging task is impacted by sex

There was a significant main effect of carbon chain length and a significant interaction between sex and carbon chain length, but no main effect of genotype in reward-based discrimination learning. Only female mice could discriminate lower carbon chain odorants (4C and 5C). No mice could discriminate 6C, but both males and females could discriminate very dissimilar odorants (7C and EMB). Error bars represent ±1SEM. Asterisks indicate significant difference in sniff time from 3C (OHab) ($p<0.05$).

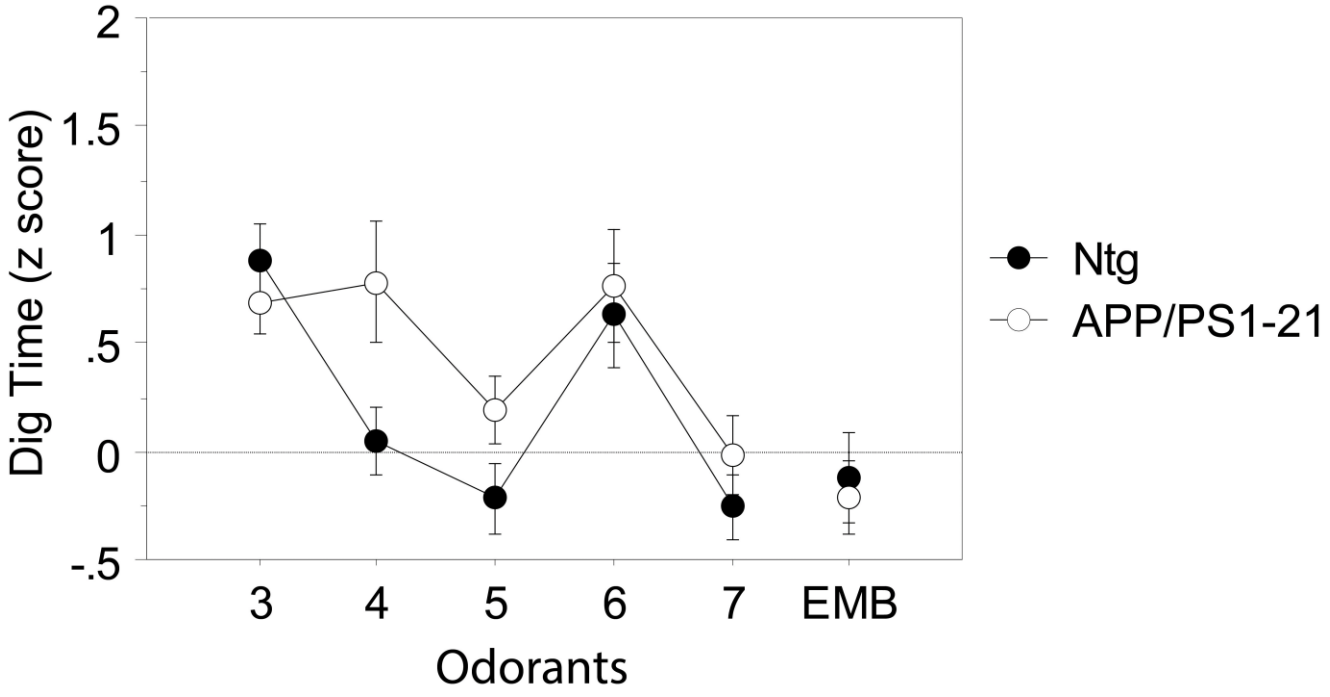

Figure 27: Discrimination in the digging task is not impacted by genotype

There was no main effect of genotype. Error bars represent ±1SEM.

Chapter 6: General Discussion

Here I summarize the main findings of each experiment and integrate the data so as to provide general insights into the role of environmental coordination in circadian network dynamics and amyloidosis. In Chapter 3, I showed that mice lacking functional copies of a core clock gene, *per2*, did not exhibit normal circadian period lengthening and active phase compression in response to light, reflected in a more even ratio between phase advances and phase delays relative to WT mice, and this was particularly evident in mice lacking both functional copies. In Chapter 4, I found that time-restricted feeding altered consolidation of locomotor activity as well as performance on a cognitive behavioral task, independent of the development of amyloid plaques. Therefore, this circadian disruption paradigm did not clearly exacerbate Alzheimer's Disease (AD) behavioral pathology prior to the age at which significant neurodegeneration begins. In Chapter 5, I showed that olfactory discrimination is impaired by early plaque deposition in the Olfactory Bulb, but these deficits in olfactory discrimination disappear under conditions of reward.

Together, these experiments characterize the influence of environmental factors (light, food, and olfactory cues) on behavior. Endogenous circadian disruption by functional mutation of the clock gene *per2* impacted coordination with the environment through differential responsivity to light cues at different phases of the circadian cycle, giving new insight into the role of *per2* in setting the period and phase of the clock. Exogenous circadian disruption through conflicting environmental cues (timing of food and light) affected metabolic and cognitive behavioral responses. This occurred independent of plaque deposition, suggesting that the degree of circadian alignment is not a key driver of pathology in the APP/PS1-21 mouse model of AD. Lastly, olfactory function is differentially affected by early AD-related plaque deposition,

reinforcing olfactory testing as a potential avenue for early AD diagnosis. Additionally, impaired olfactory discrimination can be recovered by appetitive conditioning early in plaque deposition, indicating that other neural systems might be recruited early in disease etiology to compensate for olfactory deficits. Examining environmental coordination in the context of AD development provides crucial insights into how accessible interventions and diagnostic tools may be developed.

In order to assess complex questions in depth, research often aims to analyze parts of a problem in their most reduced state from a specific lens (*i.e.*, biological rhythms OR olfaction OR psychoneuroimmunology, etc.) and/or from a given domain (*i.e.*, behavioral OR systemic interactions OR cellular/molecular biology). While this type of approach certainly offers merits, it can create artificial divides in our understanding of multifactorial complex problems such as the development, diagnosis, and prevention of disease. For example, theories of Alzheimer's Disease development such as the amyloid hypothesis (Beyreuther & Masters, 1991; Glenner & Wong, 1984; Selkoe & Hardy, 2016), which assume accumulation of beta amyloid as the principal cause of disease, resulted in many researchers and clinicians focusing on targeting and destroying this specific biological hallmark of AD. However, likely because plaque deposition starts long before clinical diagnosis of disease and because disease etiology is extremely complex (Ferrari & Sorbi, 2021), drugs targeting only this aspect have not shown great efficacy. Since AD patients show altered circadian rhythmicity, energy metabolism, inflammation, and olfactory function, we aimed to use broad, multidimensional approaches that address all of these systems, rather than targeting just one. Additionally, we focused on accessible interventions and diagnostic approaches, manipulating exposure and response to basic environmental stimuli such as light, food, and smell.

Our findings generated interesting connections between scientific domains. To highlight one example, we observed hyperphagia in female APP/PS1-21 transgenic mice in our circadian misalignment experiments that could be recovered through timed feeding (Chapter 4), suggesting altered metabolic response driven by plaque deposition that could be altered by environmental conditions. Human AD patients also exhibit altered glucose and energy metabolism in the brain and periphery (Batra et al., 2023; Kang et al., 2017; Kumar et al., 2022; Yin et al., 2016). And, in our investigation into the olfactory system in AD (Chapter 5), we found research showing that hyper-metabolism was also observed in both human AD patients (Meadowcroft et al., 2020) and APP/PS1 mice at the onset of plaque development (Wesson et al., 2011). This combined with the olfactory system showing early susceptibility to Alzheimer's disease supports the notion that the olfactory system could be acting as a hub to seed and spread misfolded proteins such as plaques throughout the cortex (Rey et al., 2018; Ubeda-Bañon et al., 2020). Since we saw that changing environmental conditions such as the timing of food intake could alter hyperphagia, might environmental changes also alter the hypermetabolism in the olfactory system in AD? Mechanisms that alter the activity within the olfactory circuit, such as olfactory enrichment (Rusznák et al., 2018; Veyrac et al., 2009), present an interesting future direction in understanding the pathogenesis and treatment of AD through the manipulation of neuronal activity. It could be the case that olfactory enrichment drives increased plaque deposition through greater hyperactivity within the circuit, or that neurogenesis in the olfactory bulb within the olfactory bulb and hippocampus in response to enrichment can counteract disease progression. In any case, our investigation into the peripheral metabolic changes of APP/PS1-21 mice in response to food intake led to a question of the central metabolic changes within the olfactory

bulb that may drive disease progression. In this way, this research program highlights how a multidimensional approach can bolster our overall understanding of not only the underlying biology of AD, but also how to best address this complex disease with accessible environmental interventions.

Epilogue:

“It’s not that you have to achieve anything, it’s that you have to get away from where you are.”

-Marguerite Duras

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