Mechanisms underlying circadian behavioral alterations in mouse models of Alzheimer's disease

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June 2017

A dissertation presented to the Graduate Faculty of the
University of Virginia in Candidacy for the
Degree of Doctor of Philosophy

Neuroscience Graduate Program

University of Virginia
September 2023

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Acknowledgements

I owe much to many people for this dissertation, for my time in graduate school, and for getting here to begin with.

First, thank you to Dr. Heather Ferris and all the members of the Ferris Lab. I have appreciated Heather's support and tried to learn from the clarity of her thinking and planning. It was a privilege to watch the lab get established and grow. I was also lucky to work with my fellow graduate students Dr. Lisa Post and Josh Milstein, who were always helpful and encouraging no matter how busy they were. A special thanks to Dr. Josh Kulas, an extraordinarily generous scientist and mentor and friend. Thanks also to Cherry Guo for her work on this project and all the work she has done with me in the lab.

Thank you to the many members of the UVA community who have helped me on this work. Thanks to the generosity of the members of the Güler and Liu labs in teaching me the techniques I used here. Thanks also to the faculty members who contributed to this project. The members of my committee, Dr. Sarah Kucenas, Dr. George Bloom, Dr. Ali Güler, Dr. Alex Kuan, and Dr. Norbert Leitinger, were not just consistently supportive but also brought out aspects of my project and my data that I couldn't see on my own. Thanks also to Dr. Iggy Provencio whose questions and enthusiasm helped bring together important aspects of this work. And thanks to the NGP administrators, departmental staff, and vivarium staff for all of their work and support.

Thanks also to my friends in Charlottesville who have given me company through this long process. Thank you to Matt and Keric for many good dinners and jokes and nights in. Thanks to Courtney for many good desserts and for all the kindness and commiseration. And thanks again to Josh for lots of sad talks and happy talks and science talks, whatever the occasion called for.

Finally, thank you to my family. Thanks to my mom and dad for all your curiosity and care, commitment and hard work. Dessie, you're an extraordinary person and you make me laugh more than anyone I know. Lastly thank you to Taylor for your love and companionship. Your thoughtfulness and caring are an inspiration for me to try to be a better person.

I'm very grateful to you all.

Preface

The four chapters of this dissertation present my work on circadian rhythms in models of Alzheimer's disease. At the end I have appended two additional sections, literature reviews focusing on oxidative stress and lipid oxidation in Alzheimer's disease. These are the products of another project which has not been finished and does not contribute to the narrative of this circadian research. Nevertheless the project has been a significant portion of the research that I have carried out in my training here, so these two appendices are included to represent that still-unfinished work.

Chapter 1 Introduction

Here I will present my research on alterations to circadian behavior and light sensitivity in mouse models of Alzheimer's disease (AD). In this chapter I will provide a review of the relevant literature that informs my research project. First I will introduce Alzheimer's disease, discussing the relevant cellular and molecular mechanisms of the disease and how it is studied. Next I will introduce circadian rhythms and how the brain uses environmental timing cues to synchronize the body's clock. Finally I will discuss the bidirectional relationship between AD and disruptions to circadian rhythms.

1.1 Alzheimer's disease

Alzheimer's disease is an aging-related neurodegenerative disorder and the most common cause of dementia. More than 6 million Americans are estimated to be living with the disease, with that number expected to rise dramatically in the coming decades (Alzheimer's Association, 2023). In addition to the high prevalence and severe effects of AD on patients, loved ones, and caregivers, it imposes a heavy burden on the healthcare industry, with an estimated more than $300 billion spent yearly on AD care (Alzheimer's Association, 2023). AD results in progressive cognitive impairment and memory loss, accompanied by characteristic pathological conditions in the brain. Here I will review the relevant background on AD: the pathological hallmarks of the disease, the associated glial and immune activation, how the disease progresses through the brain, and how mouse models are used to study the disease.

1.1.1 Pathological hallmarks

Amyloid beta (Aβ) is one of the pathological hallmarks of AD. It is a toxic peptide which makes up amyloid plaques in the AD brain. Amyloid precursor protein (APP), a protein whose normal

function is poorly understood but which is expressed by neurons and some other cell types, is enzymatically cleaved to produce the shorter peptide fragment Aβ. Aβ is produced in healthy people but is effectively cleared from the brain through several mechanisms, including microglia phagocytosis, glymphatic flow, and meningeal lymphatic drainage (Tarasoff-Conway et al., 2015). Aβ begins to accumulate when the rate of production is too high for the rate of clearance from the brain to compensate.

As soluble Aβ is no longer effectively cleared from the brain, its concentration increases and it can begin to agglomerate, producing different sized structures which interact with the brain environment differently. The most classically identifiable form in AD is amyloid plaques. These are large, insoluble, extracellular deposits of Aβ. While plaques are the largest Aβ species, they are not the most toxic, and they may even protect against more toxic forms of Aβ by sequestering them into insoluble aggregates (Y. Huang et al., 2021; Yuan et al., 2016). Smaller and still soluble Aβ aggregates such as oligomers and protofibrils are thought to be more toxic than the larger fibrils and plaques (Hampel et al., 2021).

Aβ accumulation is the best studied mechanism in the pathogenesis of Alzheimer's disease. Familial Alzheimer's Disease (FAD) is a rare and aggressive form of the disease, and unlike most cases of AD it is monogenic. FAD is caused by mutations in APP or in one of two genes involved in the cleavage of APP into Aβ, presenilin 1 and 2 (PS1 and PS2) (Janssen et al., 2003). That these mutations are sufficient to drive AD is proof that Aβ plays a core role in AD pathogenesis. Additionally, targeting Aβ as a therapeutic technique has recently shown positive results in delaying progression in AD patients (Mintun et al., 2021; van Dyck et al., 2023). But intriguingly, some people have very high levels of Aβ in their brains while remaining cognitively normal and not showing any of the other signs of AD (Aizenstein et al., 2008; Katzman et al.,

1988). Amyloid load in the brain is also not the best predictor of AD clinical stage (Hanseeuw et al., 2019). So while Aβ certainly plays a core role in AD pathogenesis and progression, it is clearly not the only mechanism at play in the disease.

The other classic pathophysiological hallmark of AD is neurofibrillary tangles (NFTs), composed of misfolded and hyperphosphorylated tau. Tau is a protein encoded by the gene MAPT. It is best known for its role in binding and stabilizing microtubules in neuronal axons, but it has a variety of other less well characterized functions. In AD, tau undergoes a series of changes, including hyperphosphorylation, misfolding, and mislocalization (reviewed in Naseri et al., 2019). Pathological tau forms the core of neurofibrillary tangles, toxic aggregates of protein that form inside the neurons in AD. Tau pathology correlates more directly with cognitive decline in AD than Aβ does (Hanseeuw et al., 2019). In addition to its role in AD, pathological tau plays a role in a variety of other neurodegenerative diseases, including frontotemporal dementia, chronic traumatic encephalopathy, corticobasal degeneration, and others.

1.1.2 Gliosis and neuroinflammation

The accumulation of amyloid and tau pathology in AD is accompanied by a variety of other changes in the brain microenvironment, including gliosis and neuroinflammation. These two related processes can be detected early in disease progression. They are of great interest because of research showing that glia and immune activation can play both protective and destructive roles in the disease depending on genetics, disease stage, and other factors (Fakhoury, 2018; Hansen et al., 2017).

Microglia have extensive links to AD pathogenesis and undergo significant changes over the course of the disease. We know that microglia play a role in AD risk because some AD risk genes, most notably TREM2, are expressed primarily or exclusively by microglia (McQuade &

Blurton-Jones, 2019). As the resident phagocytes of the brain, microglia are important for the clearance of extracellular waste such as Aβ. When this process fails, Aβ accumulates in the brain and can form toxic oligomers and protofibrils. Microglia can also phagocytose synapses, a process which is normal during neurodevelopment but which may contribute to neurodegeneration under pathological conditions (Hong et al., 2016). A drug which blocks microglia activation was found to protect AD model mice against memory deficits and neurodegeneration (J.-S. Park et al., 2021), further demonstrating the detrimental role that microglia activation can play.

One consequence of microglia activation is that immune signaling molecules are altered in AD. This phenomenon has been studied at multiple levels, from *in vitro* to mouse models to humans. Microglia treated *in vitro* with Aβ release a variety of cytokines, including tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), and IL-6 (Akama & Eldik, 2000; Lue et al., 2001). Mouse models of AD have been studied more extensively, and different models have been reported to show increased levels of pro-inflammatory cytokines including TNFα, IL-1β, IL-1α, IL-17, IL-6, and GM-CSF (Janelsins et al., 2005; Jin et al., 2008; Manji et al., 2019; Patel et al., 2005). Data from humans is more sparse and sometimes conflicting due to patient heterogeneity and the difficulty of collecting samples from human subjects. TNFα expression is reportedly increased in the brain (L. B. Wood et al., 2015), while another study found no increase in TNFα expression but significant increases in IL-1β, IL-10, and IL-33 (Morimoto et al., 2011). IL-1β is found in microglia surrounding plaques in AD patients (Heneka et al., 2015), while elevated TNFα in the cerebrospinal fluid (CSF) predicts progression from mild cognitive impairment (MCI) to AD (Tarkowski et al., 2003). While data on immune signaling in the human brain during AD is limited and more research is needed to understand the roles of specific cytokines, it appears that

immune activation in the central nervous system (CNS) is a common marker in AD. This inflammation is thought to contribute to disease progression and cognitive decline in AD (reviewed in Kinney et al., 2018).

Microglia activation also contributes to oxidative stress in the AD brain (reviewed in Simpson & Oliver, 2020). Oxidative stress is a well-studied mechanism in AD which arises early in the disease course and directly contributes to neuronal dysfunction and death. NADPH oxidase (NOX), a class of enzymes with several different isoforms which produce the reactive oxygen species superoxide, are primarily expressed in the brain in microglia (though they are also found in astrocytes and endothelial cells). NOX1 and NOX3 are elevated in the brains of AD patients, including at early stages of disease progression (de la Monte & Wands, 2006), and NOX2 is activated in microglia in AD brains (Shimohama et al., 2000). Treatment of cultured microglia with Aβ results in increased NOX2 expression (Geng et al., 2020). Disease model research suggests that this upregulated NOX may play a role in disease progression: knocking out NOX2 in a mouse model of AD decreased oxidative stress in those brains and protected those mice against behavioral deficits in a memory task (L. Park et al., 2008). Interestingly, in spite of the improved memory function, Aβ levels were not decreased in NOX2 knockout (KO) AD model mice. This supports the hypothesis that ROS generation is increased in response to Aβ, at least in part through microglia-expressed NOX, and that this oxidative stress contributes to neurodegeneration.

Oxidative stress, both from glial activation and other mechanisms, is an important topic of research in AD. There is substantial evidence of oxidative stress in AD brains, which have evidence of heightened lipid oxidation, protein oxidation, and oxidative DNA damage (Aksenov et al., 2001; Lovell & Markesbery, 2007; see Appendix II). While it was not examined in this

research and will not be addressed at length here, the role of oxidative stress and astrocytic antioxidant functions in neurodegenerative diseases is the topic of Appendix I.

Astrocytes are also activated in AD, at least partially in response to the microglia activation discussed above. Cytokines released by activated microglia, specifically IL-1α, TNFα, and C1q, act on astrocytes *in vitro* to induce a neurotoxic state termed "A1" activation (Liddelow et al., 2017b). Importantly, that study found astrocytes resembling these neurotoxic astrocytes *in vivo* in AD and other neurodegenerative diseases. Aβ administered to astrocytes *in vitro* can complement these cytokines and contribute to a similar neurotoxic activation state (LaRocca et al., 2021). Aβ treated astrocytes can promote tau phosphorylation and cell death in co-cultured neurons (Garwood et al., 2011), and inhibiting astrocyte activation in a tauopathy model decreased tau pathology and neurodegeneration (Litvinchuk et al., 2018). A recent study provided important evidence that this astrocyte activation plays a role in pathology in human disease as well. As mentioned above, Aβ is present in the brains of many people who do not develop significant tau pathology or cognitive impairment. A population of cognitively normal but Aβ-burdened subjects were tested for biomarkers of tau pathology and astrocyte activation (Bellaver et al., 2023). In subjects with detectable astrocyte activation, Aβ and tau biomarkers were positively correlated, but in subjects without astrocyte activation tau was not correlated with Aβ. Further, in subjects whose tau levels were measured longitudinally, astrocyte activation predicted more dramatic increases of in tau pathology in the future. These results provide strong evidence supporting the hypothesis that astrocyte activation plays an important role in mediating disease progression after Aβ accumulation begins.

1.1.3 Neurodegeneration and staging

Amyloid and tau pathology, neuroinflammation, gliosis, and other mechanisms all converge on one process in the AD brain: neurodegeneration. The progressive loss of synapses and neurons is the last pathophysiological hallmark of the disease, and it is directly responsible for the memory deficits and dementia that characterize AD. Because of this neurodegeneration the volume of the brain in AD patients declines dramatically over the course of the disease. Before neurons begin to die, they experience synaptic dysfunction and progressive synapse loss. Neuronal pathology eventually progresses and results in the death of neurons. AD affects different regions of the brain differently and at different times in the disease course, and the spread of AD pathology through the brain follows a predictable pattern. This disease progression is commonly categorized using the Braak staging system: tau pathology first appears in the entorhinal cortex, then limbic regions including the hippocampus, and later spreads widely through the neocortex (Braak & Braak, 1995).

The suprachiasmatic nucleus (SCN), discussed below for its role in circadian rhythms, appears to be in some ways protected from this pathological progression. Plaques and NFTs are rare in the SCN even late in the disease course, with plaques appearing in the SCN much less commonly than in other nearby hippocampal regions (Baloyannis et al., 2015; Stopa et al., 1999). Despite this, the SCN does undergo degeneration, with SCN volume and cell count decreased in the brains of late stage AD patients (Swaab et al., 1985; Y.-H. Wu et al., 2007), as well as decreased synapse number and morphological complexity in the SCN in earlier stage AD brains (Baloyannis et al., 2015). The ratio of glia to neurons, which is increased in conditions of gliosis or neuronal death, is sometimes used as a proxy for neurodegeneration. Glia:neuron ratio in the SCN is elevated in AD (Harper et al., 2008; Stopa et al., 1999). Studies of specific cell

populations in the SCN have found decreased number of arginine vasopressin (AVP) expressing neurons in early- and late-stage disease (Y.-H. Wu et al., 2006). In summary, the SCN is relatively protected from amyloid and tau pathology, and while it does undergo degeneration in AD this research has mostly been performed in the late stages of the disease.

The retina, as part of the CNS, is susceptible to AD pathology as well (reviewed in Mirzaei et al., 2020). In fact, because the neurons of the retina can be observed in living subjects much more easily than those inside the skull, there has been significant interest in imaging AD pathology in the retina as a readout of disease risk or progression. The retina accumulates Aβ plaques in AD which can be detected by retinal imaging in live patients (Koronyo et al., 2017; Tadokoro et al., 2021). These plaques can appear in the retina early in the disease course, before the onset of cognitive symptoms (Koronyo-Hamaoui et al., 2011). Neurodegeneration can also be observed *in vivo* in the eye, where AD patients have thinner retinas and retinal nerve fiber layers as measured by the non-invasive technique optical coherence tomography (OCT) (Coppola et al., 2015; Iseri et al., 2006). Other AD pathological features cannot yet be detected *in vivo*, but have been identified in post mortem tissues. Tau phosphorylation is found in the retina in AD (den Haan et al., 2018; Grimaldi et al., 2019; Schön et al., 2012), though these studies conflict on whether NFTs are present. Microglia and astrocyte activation, along with pro-inflammatory signaling, are also increased in the retina in AD patients (Grimaldi et al., 2019). In summary, the retina experiences the core pathophysiological features of AD found in other parts of the brain, including pathological Aβ and tau accumulation, gliosis, neuroinflammation, and neurodegeneration.

1.1.4 Mouse models of AD

Many mouse models of AD are based on mutations identified in human patients with familial Alzheimer's disease (FAD), an aggressive and early-onset form of the disease. These pathogenic mutations are introduced into mice, which do not normally develop Aβ plaques or neurofibrillary tangles, to drive one or both of these pathological features of AD. Different mouse models of AD vary in the severity of their symptoms, speed of disease progression, regional differences in pathological burden, and many other factors depending on which mutations are used and how they are expressed. The three models used in my studies reflect some of this diversity.

The 3xTg mouse carries mutations in APP, PS1, and MAPT, resulting in a progressive accumulation of Aβ plaques and neurofibrillary tangles (Oddo et al., 2003). By 6 months of age 3xTg mice have developed plaques, but NFTs are not observed until 12 months of age (Oddo et al., 2003). In this model some memory impairment is detectable at 4 months, before the presence of plaques (Billings et al., 2005). This model is useful for studying concurrent Aβ and tau pathology and may capture phenotypes resulting from the interactions between the two pathological processes.

The 5xFAD model carries mutations in APP and PS1, driving aggressive amyloid pathology but no neurofibrillary tangles (Oakley et al., 2006). Plaques can be detected in these mice by as early as 2 months old (Richard et al., 2015). Memory deficits are observed later, by 6 months (Jawhar et al., 2012). We use this model to study Aβ pathology in the absence of mutant tau and neurofibrillary tangles.

The PS19 mouse, also called Tau P301S, is a model of tauopathy (Yoshiyama et al., 2007). It carries a mutation in MAPT identified in cases of frontotemporal dementia which drives the accumulation of neurofibrillary tangles. These mice develop neurofibrillary tangles (Yoshiyama

et al., 2007) and memory deficits (Takeuchi et al., 2011) by 6 months. It models tau pathology similar to that observed in AD but in the absence of mutations driving Aβ production and plaque development.

1.2 Circadian rhythms

Circadian rhythms are any rhythmic biological changes which follow a roughly 24 hour cycle. A wide variety of functions, from the behavioral level to the molecular, are controlled by circadian rhythms. In this section I will introduce the machinery of the molecular circadian clock, how the brain maintains and synchronizes whole-body circadian rhythms, and how light information is detected in the retina for circadian purposes.

1.2.1 Molecular circadian rhythms

Cells throughout the body maintain their own molecular circadian clocks through what is called the transcriptional-translational feedback loop (TTFL), a set of transcription factors and their inhibitors whose interactions result in a self-sustaining cycle of gene expression and protein activity. These circadian transcription factors have extremely broad effects, with more than 40% of protein-coding genes found to be rhythmically expressed in at least some tissues in mice (R. Zhang et al., 2014).

The molecular circadian clock is composed of two halves, termed the positive and negative arms. The positive arm is driven by the BMAL:CLOCK complex, which acts as a transcription factor for a broad set of genes. Among those is are its inhibitors PER and CRY, which form a complex that inhibits the activity of the positive arm of the clock. Thus the activity of the positive arm induces the negative arm. PER and CRY are then gradually degraded, allowing for another increase in BMAL:CLOCK activity. This cycle lasts approximately 24 hours and forms the core

of the molecular circadian clock. This core clock is modified and strengthened through inputs from other systems, including the circadian transcription factors RORα and REV-ERBα/β.

1.2.2 Circadian processing in the SCN

While individual cells can maintain their own molecular circadian rhythms, other signals are required to synchronize these individual rhythms to create a coherent rhythm for the body. The brain structure most responsible for this task is the suprachiasmatic nucleus (SCN). The SCN is a region in the ventromedial hypothalamus, directly above the optic chiasm. It is responsible for entrainment, the matching of the biological clock to the timing of the external world. The SCN controls glucocorticoid secretion from the adrenal gland, and glucocorticoid signaling sets the circadian clock in cells throughout the body.

Unlike other tissues, the SCN is able to maintain a robust, synchronized circadian rhythm without external entrainment cues. When an animal lacks environmental entrainment cues, or zeitgebers, they are said to be “free running”. In these conditions, healthy animals continue following a ~24h clock dictated by the pacemaker in the SCN. This is due to complex intra-SCN signaling relying on, among other mechanisms, the neurotransmitter γ-aminobutyric acid GABA), the neuropeptides arginine vasopressin (AVP) (Yamaguchi et al., 2013) and vasoactive intestinal peptide (VIP) (Mazuski et al., 2018), and astrocytes as well as neurons (Brancaccio et al., 2017, 2019). These signals synchronize the clocks of the neurons of the SCN to maintain a coherent rhythm in the absence of external time cues, as well as allowing the SCN to adjust that rhythm in response to circadian cues, principally light.

The circuitry of photoentrainment has been studied extensively (Fig. 1.1) (reviewed in Golombek & Rosenstein, 2010). In brief, specialized retinal ganglion cells in the retina (discussed below) project to the SCN via the retinohypothalamic tract (RHT), signaling primarily via glutamate and

the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP). The RHT synapses on the ventrolateral SCN, also called the SCN core, which contains VIP+ cells. VIP signaling from the SCN core is essential for synchronizing the dorsolateral SCN, also called the shell,

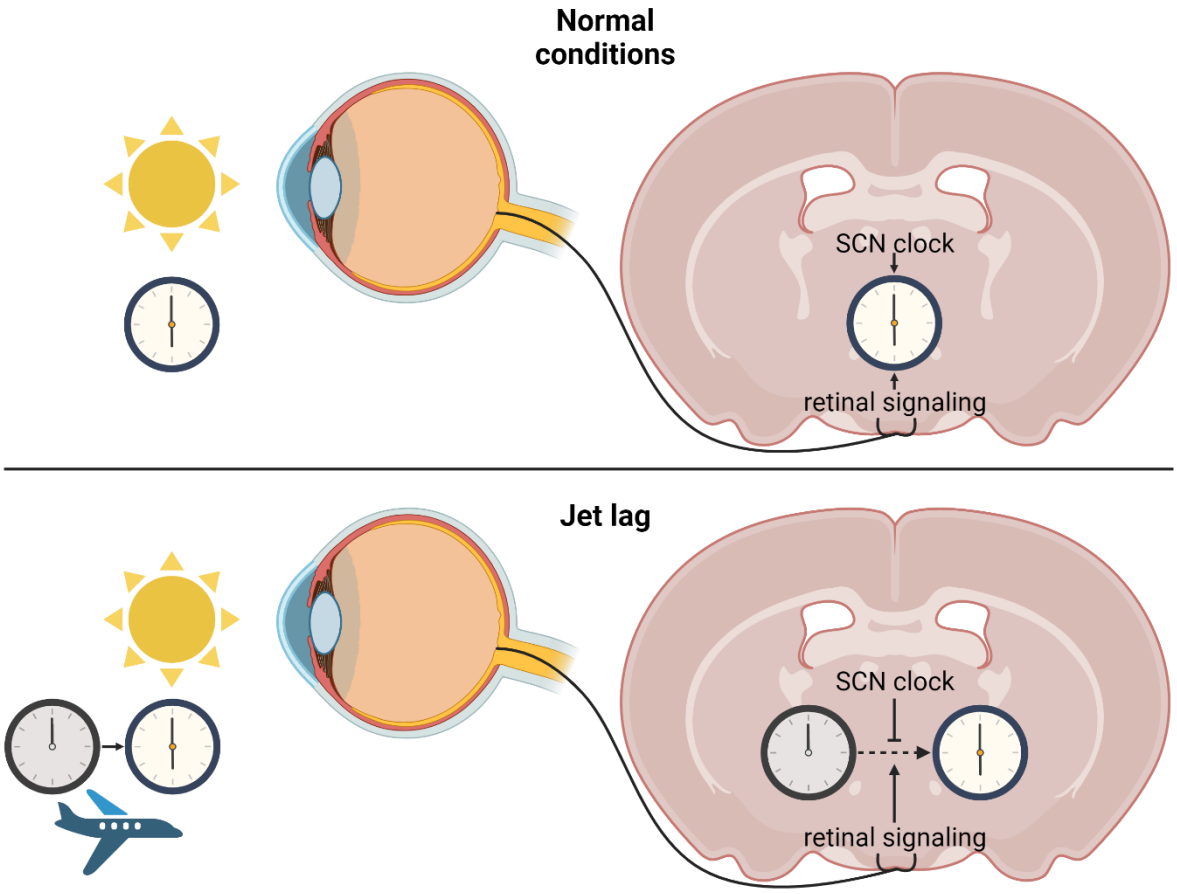

Figure 1.1: Jet lag and re-entrainment in the brain

Under normal conditions, the circadian clock of the suprachiasmatic nucleus (SCN) is synchronized with the light cycle of the environment, so retinal signals about light conditions (in this case morning sunlight) and the SCN clock are in agreement. When the external light cycle changes rapidly, as in the case of someone flying six time zones east, light signals from the retina indicate that it is morning while the SCN clock indicates that it is midnight. The SCN is out of phase with the environmental light cycle, resulting in jet lag. Retinal signaling to the SCN promotes shifting the clock to match the new light cycle, but this is resisted by the SCN. Over time the SCN clock is shifted, or re-entrained, to match the light cycle. Figure created with BioRender.com.

which contains AVP+ neurons. The SCN shell is predominantly responsible for synchronizing the clocks of the rest of the body (Evans et al., 2015).

1.2.3 Circadian light sensing in the retina

The most important cue used by the SCN to set the body's clock is light. In mammals, information about light conditions are received by the SCN from the retina. Interestingly, mice which lack the photoreceptor cells of the retina, which are responsible for detecting light for image-forming visual processing, are still capable of using light cues to synchronize the body's circadian rhythms (Freedman et al., 1999). This is because the cells in the retina which send light information to the SCN, the intrinsically photosensitive retinal ganglion cells (ipRGCs), function differently than the RGCs responsible for image-forming vision, which receive all their light information from photoreceptors. ipRGCs receive inputs from photoreceptor cells, but they also express melanopsin, a photosensitive protein which allows for direct sensing of light by the ipRGCs.

Melanopsin and the melanopsin-containing ipRGCs play essential roles in circadian entrainment. Mice knocked out for OPN4, the gene encoding melanopsin, have decreased phase shifting in response to light pulses (Panda et al., 2002). If ipRGCs are genetically ablated, photoentrainment is lost and animals begin to free run – their behavioral circadian rhythms persist but become completely untethered from the light:dark cycle (Güler et al., 2008; Hatori et al., 2008). This contrasts with the results of SCN ablation: in these animals, behavioral rhythmicity is completely lost (Stephan & Zucker, 1972). Therefore, the SCN is necessary for generating a coherent circadian rhythm, but ipRGCs are necessary for matching that rhythm to light cues from the environment and shifting the rhythm in response to changes in the light cycle.

ipRGCs come in different varieties, with different melanopsin expression levels, morphologies, electrophysiological properties, and projection patterns (reviewed in Do, 2019). The SCN is innervated by two ipRGC subtypes: M1 cells, which make up 80% of retinal SCN inputs; and M2 cells, which make up the remaining 20% (Baver et al., 2008). M1 cells express melanopsin at high levels and have strong intrinsic electrophysiological responses to light (Hattar et al., 2006). M2 ipRGCs express lower levels of melanopsin and are less intrinsically photosensitive than M1 cells (Schmidt & Kofuji, 2009). M3 cells are similar to M2 but with slightly different dendritic morphology, while other ipRGC types express melanopsin at levels too low to readily detect by immunohistochemistry and do not project to the SCN, performing other functions in non-image forming and image-forming visual circuits (Do, 2019).

The experiments here focus on circadian rhythms, but ipRGCs have roles in other visual pathways as well. The pupillary light reflex (PLR), the automatic constriction of the pupils in response to light, relies on ipRGCs (S.-K. Chen et al., 2011; Güler et al., 2008). Mice have heightened alertness or anxiety-like behaviors in light compared to darkness, and this behavior is mediated by ipRGCs (Milosavljevic et al., 2016; G. Wang et al., 2023). ipRGC activity also modulates sleep and wakefulness (Altimus et al., 2008; Lupi et al., 2008) and body temperature (Rupp et al., 2019) independent of the effects of the ipRGCs on circadian rhythms. ipRGCs are central to circadian rhythms but have a wide variety of other roles in light-sensitive behaviors.

1.3 Circadian rhythms in Alzheimer's disease

Circadian rhythms intersect in multiple important ways with AD, and the effects of AD on circadian rhythms are the focus of this dissertation project. Here I will introduce the circadian disruptions observed in AD, how circadian disruptions might contribute to AD progression, and what is known about altered circadian rhythms in mouse models of AD.

1.3.1 Circadian symptoms in AD

While AD is characterized by memory and cognitive symptoms, these are not the only nor the earliest symptoms. Sleep and circadian disruptions are common in AD and appear early in the disease course, frequently before the onset of cognitive deficits and clinical diagnosis (Musiek et al., 2015).

The best characterized of these symptoms are sleep disruptions. A majority of AD patients report multiple disordered sleep traits, including getting up during the night, wandering during the night, and excessive sleeping during the day (Tractenberg et al., 2003). A recent meta-analysis of studies of Aβ and sleep found a significant positive correlation between Aβ42/40 ratio and total sleep time (Harenbrock et al., 2023), indicative of short sleep correlating with severity of AD pathology. And while they are also reported in late stages of the disease, sleep disruptions are often a very early symptom of AD, preceding the onset of cognitive decline by years (Musiek et al., 2015). Sleep disruptions do not only affect the wellbeing of AD patients; they dramatically increase the difficulty and stress of caring for AD patients (Gallagher-Thompson et al., 1992; Kang et al., 2009; Musiek et al., 2015; Tractenberg et al., 2003).

In addition to sleep disruptions, changes to daily activity rhythms are also reported (reviewed in Leng et al., 2019). Studies have found more fragmented rest-activity rhythms in those with preclinical as well as symptomatic AD (Hooghiemstra et al., 2015; La Morgia et al., 2016; Musiek et al., 2018). Data on the phase of activity rhythms is conflicting, with one study reporting that AD patients have a delayed phase of activity (J. L. Wang et al., 2015), while studies of patients with MCI have reported advanced activity phases (Naismith et al., 2014; Ortiz-Tudela et al., 2014). One study also found a significantly decreased amplitude of daily activity rhythms (La Morgia et al., 2016), though the others cited here do not replicate this

finding. In short, the literature supports an increased fragmentation in daily rest-activity rhythms in AD while suggesting that other parameters of these rhythms may also be affected.

Another circadian symptom of AD is sundowning, which describes the increased severity of emotional and behavioral symptoms in AD patients later in the day compared to earlier. Studies differ on the prevalence of sundowning among AD patients, with one study reporting rates as high as 66% (Gallagher-Thompson et al., 1992) and more recent studies reporting 19-28% (Angulo Sevilla et al., 2018; Pyun et al., 2019). Insufficient or disordered sleep is associated with dramatically higher risk for sundowning behaviors (Angulo Sevilla et al., 2018; Pyun et al., 2019). This suggests that the sleep disruptions discussed above contribute to sundowning, though non-sleep-dependent pathways may also contribute (Todd, 2020).

Though they are significantly more difficult to study than behavioral changes, there is also evidence of disrupted molecular circadian rhythms in AD patients. One study measuring transcription of circadian clock genes in postmortem tissue found a delayed phase of BMAL1 expression in AD patients compared to healthy controls in multiple brain areas (Cermakian et al., 2011). Another found decreased rhythmicity of clock gene expression in early- and late-stage AD in the pineal gland, the major target of the SCN and the source of the circadian entraining molecule melatonin (Y.-H. Wu et al., 2006). This likely contributes to the observed changes in melatonin expression in AD. Even in pre-clinical subjects with normal cognition, melatonin levels in the CSF are decreased (Zhou et al., 2003) and day-night rhythmicity of melatonin levels in the pineal gland are lost (Y.-H. Wu et al., 2003).

1.3.2 Potential circadian contributions to AD progression

Circadian alterations in AD are also of great interest because they may play a role in the development of AD. As discussed above, circadian and sleep disruptions are observed early in

the disease course, before the onset of cognitive symptoms. Recent research has examined whether these early circadian disruptions may not simply be results of early AD pathology but might contribute to disease progression.

Clues that sleep disturbances may influence AD, rather than simply being symptoms of AD, can be found in the many longitudinal studies examining sleep and later AD risk. Meta-analyses of this literature have concluded that there are strong correlations between sleep disturbances (including unusually short or long sleep, fragmented sleep, or subjectively low quality sleep) and later AD risk (Bubu et al., 2017; L. Shi et al., 2018; W. Xu et al., 2020). Many of these studies have follow-up times of less than 10 years between sleep measurement and cognitive testing. Given the long preclinical phase of AD, this means that some of these correlated sleep disruptions are likely prodromal symptoms rather than causal risk factors. Stronger evidence for a causal role for sleep disruptions comes from the studies with long follow-up times. In one large study of sleep length, people who slept for less than 6 hours a night in their 50's and 60's had a 30% higher risk of AD at age 70 than those who regularly slept for around 7 hours (Sabia et al., 2021). Another study which measured sleep length and quality and then followed up to administer cognitive tests 16-26 years later found that low sleep quality, short sleep length, or abnormally long sleep length all correlated with lower cognitive score later in life (Virta et al., 2013). Another study measuring sleep length and testing for dementia an average of 15 years later found that dementia risk was doubled in subjects who had slept less than 7 hours each night (Lutsey et al., 2018). While more research is needed looking at sleep and AD risk over longer time scales, the evidence thus far showing sleep disturbances many years before AD diagnosis suggests that sleep may play a causal role in AD development.

Experiments studying the effects of sleep deprivation in mouse models supports this hypothesis. Chronic sleep deprivation in young adult mice causes later-in-life memory deficits and increases their susceptibility to Aβ and tau pathology, even in those not genetically predisposed to AD (Owen et al., 2021). Sleep deprivation in an AD model before the onset of AD pathology resulted in more severe memory deficits and Aβ and tau pathology once those pathological markers arose (Niu et al., 2022). Interestingly, the effect of sleep deprivation on AD risk may be modulated by APOE genotype: in a mouse model expressing different human APOE isoforms, sleep deprivation significantly exacerbated AD pathology in APOE4 mice but not in APOE3 (C. Wang et al., 2023). Unlike the observational human studies of sleep, these experiments using direct interventions demonstrate that sleep disruptions can increase susceptibility to and exacerbate progression of AD pathology.

The role of sleep in Aβ clearance from the CNS may explain the connection between impaired sleep and AD progression. In AD mouse models, Aβ levels in the interstitial fluid of the brain change rhythmically over the course of the day, peaking during the active period and decreasing during the inactive period, and sleep deprivation increases Aβ levels (Kang et al., 2009). This is due to an increase in the rate of clearance of Aβ and other molecules from the brain during sleep (Xie et al., 2013). These findings have been recapitulated in healthy humans by measurements of Aβ in the CSF (Ooms et al., 2014) and by PET scanning (Shokri-Kojori et al., 2018). Thus, sleep disruptions before the onset of AD may increase Aβ accumulation in the brain, accelerating progression of the disease or increasing the likelihood of it arising. The role of sleep in Aβ clearance therefore provides one possible mechanistic explanation for the correlation between disrupted sleep and later life AD risk.

Circadian disruptions may also contribute to AD risk through other mechanisms regulated by the circadian clock. Single nucleotide polymorphisms in the core circadian genes CLOCK and ARNTL (BMAL1) have been associated with increased AD risk (H. Chen et al., 2013; Q. Chen et al., 2015). Circadian transcription factors regulate many AD-associated processes in the cells of the brain. Knockout of the clock gene BMAL1 is associated with astrocyte activation in models of amyloid pathology (McKee et al., 2022) and tau pathology (Sheehan et al., 2021), and is associated with increased astrocytic protein degradation pathways (McKee et al., 2023; Sheehan et al., 2023). AD-related processes in microglia are also circadian regulated. The rate of microglia phagocytosis of Aβ in 5xFAD mice fluctuates in a circadian manner, and inhibiting the circadian repressor proteins REV-ERBα and β increases this process (J. Lee et al., 2020). The interactions between different transcription factors in the molecular circadian clock are complex and bluntly inhibiting or knocking out components of the clock does not replicate the circadian alterations observed in AD. However, these results demonstrate that multiple pathways associated with AD progression are circadian regulated, and alterations to circadian rhythms which might impair these processes is another possible mechanism by which circadian disruptions could increase AD risk.

1.3.3 Circadian rhythms in mouse models of AD

The circadian alterations described above result in challenging symptoms and may contribute to disease progression, but the mechanisms underlying these circadian disruptions are still poorly understood. Mouse models of AD are an important tool for studying these mechanisms, and research in these models has found varied evidence of alterations to molecular and behavioral circadian rhythms.

Circadian transcription and protein expression are affected in multiple AD mouse models (reviewed in Sheehan & Musiek, 2020). Changes to the amplitude and phase of rhythmic clock gene expression are observed in some AD models, including 3xTg (Bellanti et al., 2017), 5xFAD (H. Song et al., 2015a), and PS19 (Han et al., 2022) mice.

Sleep disruptions are observed in AD models, recapitulating some of the altered sleep patterns in AD patients. These sleep phenotypes include sleep fragmentation, decreases in rapid eye movement (REM) sleep, and decreases in total sleep, phenotypes which are found in some, but not all, AD mouse models (reviewed in Drew et al., 2023). 5xFAD mice have more fragmented sleep than wild types at 4-6 months, with the effect being stronger in females than males, and total sleep duration is decreased in females but not males (Sethi et al., 2015). PS19 mice have decreased REM sleep by 9 months and decreased total sleep by 11 months (Holth et al., 2017). 3xTg mice, on the other hand, are not found to exhibit altered sleep duration or electrophysiological properties (Kent et al., 2018).

Non-sleep circadian behavior in constant light-dark (LD) and dark-dark (DD) conditions is also affected in some AD models, including behavioral phenotypes reminiscent of the circadian symptoms found in AD patients (reviewed in Sheehan & Musiek, 2020). When anxiety tests are administered at different times of day, AD model mice exhibit more severe anxiety-like behavior later in their active period compared to earlier (Bedrosian et al., 2011), a phenotype similar to sundowning in AD patients. Behavioral data suggest some altered rhythms in 3xTg mice: a shortened free running period has been reported in one study (Adler et al., 2019), and several studies find decreased amplitude of daily behavioral rhythms (Adler et al., 2019; Sterniczuk et al., 2010; M. Wu et al., 2018), but another study found *increased* amplitude (Knight et al., 2013). Research is more limited in 5xFAD mice, but decreased amplitude of rhythmic locomotor

activity has been observed (Dong et al., 2022; H. Song et al., 2015b). One study reported no circadian locomotor behavioral differences in PS19 mice (Chalermpalanupap et al., 2018), but the examination of circadian behavior here was limited and to my knowledge no other studies have examined further in this model.

Entrainment, the matching of the biological clock to the daily rhythm of the world, has been less well studied than other behaviors in AD mouse models. One technique for studying behavioral entrainment is through a jet lag paradigm, where a mouse's LD cycle is advanced and their re-entrainment to this new light cycle is observed. A normal SCN resists being immediately re-entrained to a new light cycle, and the mismatch between the SCN clock and the environmental clock is experienced as jet lag (Fig. 1.1). Rapid re-entrainment, i.e. faster recovery from the jet lag, can indicate a weakness of the intrinsic SCN clock and an inability to resist out-of-phase light cues (Yamaguchi et al., 2013). The studies which have examined jet lag in AD model mice have not found significant differences in male 3xTg mice (González-Luna et al., 2021) or APP/PS1 AD model mice (Kent et al., 2019; Otalora et al., 2012). However, a different test of entrainment in female 3xTg mice showed a non-significant trend towards greater entrainment to a light pulse in female 3xTg mice (Sterniczuk et al., 2010). The literature on altered entrainment in AD models is thus limited and somewhat mixed. In my research I examined re-entrainment in multiple AD mouse models to explore how this circadian behavior could be affected in AD.

Chapter 2 Altered circadian entrainment in mouse models of Alzheimer's disease

Adapted from (Weigel et al., 2023).

2.1 Abstract

Circadian symptoms have long been observed in Alzheimer's disease (AD) and often appear before cognitive symptoms, but the mechanisms underlying circadian alterations in AD are poorly understood. We studied circadian re-entrainment in AD model mice using a "jet lag" paradigm, observing their behavior on a running wheel after a six hour advance in the light:dark cycle. Female 3xTg mice, which carry mutations producing progressive amyloid-β and tau pathology, re-entrained following jet lag more rapidly than age-matched wild type controls at both 8 and 13 months of age. This re-entrainment phenotype has not been previously reported in a murine AD model. Because microglia are activated in AD and in AD models, and inflammation can affect circadian rhythms, we hypothesized that microglia contribute to this re-entrainment phenotype. To test this, we used the colony stimulating factor 1 receptor (CSF1R) inhibitor PLX3397, which rapidly depletes microglia from the brain. Microglia depletion did not alter re-entrainment in either wild type or 3xTg mice, demonstrating that microglia activation is not acutely responsible for the re-entrainment phenotype. To test whether mutant tau pathology is necessary for this behavioral phenotype, we repeated the jet lag behavioral test with the 5xFAD mouse model, which develops amyloid plaques, but not neurofibrillary tangles. As with 3xTg mice, 7-month-old female 5xFAD mice re-entrained more rapidly than controls, demonstrating that mutant tau is not necessary for the re-entrainment phenotype. Together, these experiments

demonstrate novel circadian behavioral phenotypes with accelerated re-entrainment to shifted photic cues in AD model mice which are not dependent on tauopathy or microglia.

2.2 Introduction

Altered circadian rhythms are a common symptom of Alzheimer's disease (AD). These alterations appear early in the disease, before hallmarks such as memory impairment, amyloid-β (Aβ) plaques, and neurofibrillary tangles (Musiek et al., 2015). AD circadian symptoms include sleep disruptions and a greater severity of behavioral symptoms later in the day, known as sundowning. Circadian disruptions are also observed at the molecular level, with alterations in circadian clock gene expression in the brains of AD patients (Cermakian et al., 2011). These circadian alterations are particularly interesting because they may play a role in disease progression: sleep can facilitate Aβ clearance from the brain (Shokri-Kojori et al., 2018; Xie et al., 2013) and poor sleep quality in adulthood is a risk factor for AD later in life (Sabia et al., 2021). Additionally, sleep disruptions caused by altered circadian rhythms significantly increase the difficulty of caring for AD patients (Kang et al., 2009; Musiek et al., 2015). Thus understanding the mechanisms of circadian disruption in AD could have both important preventative and therapeutic potential.

Many circadian phenotypes seen in humans with AD are recapitulated in mouse models of AD. Certain AD models demonstrate changes to the free running period (the intrinsic period of an animal's circadian behavior when kept in constant darkness) and activity in light or dark phases (Sheehan & Musiek, 2020). AD model mice also score better in anxiety tests earlier in their active period compared to later (Bedrosian et al., 2011), a phenotype reminiscent of sundowning in AD patients. Circadian alterations are recapitulated at the molecular level as well, with

changes to the amplitude and phase of rhythmic clock gene expression in some AD models, including 3xTg (Bellanti et al., 2017) and 5xFAD (H. Song et al., 2015a) mice.

Other facets of circadian rhythms have been less well studied in AD models. Entrainment is the process of synchronizing the biological circadian clock with the daily rhythm of the environment. In this study we tested circadian behavior in models of AD using a "jet lag" protocol. We found that female 3xTg mice re-entrain more rapidly than wild type (WT) controls. We then examined neuroinflammation, amyloid, and tau pathology as possible contributors to this altered circadian behavior.

2.3 Results

2.3.1 3xTg mice have accelerated circadian re-entrainment

To test the re-entrainment behavior of AD model mice, we first studied female 3xTg mice. The 3xTg mouse model of AD carries pathogenic mutations in amyloid precursor protein (APP), presenilin 1 (PS1), and human tau (MAPT), resulting in progressive accumulation in the brain of Aβ plaques and neurofibrillary tangles. Sex-specific circadian behavioral alterations have previously been observed in 3xTg mice (Sterniczuk et al., 2010), and female 3xTg mice have a more rapid progression of AD pathology than males (Dennison et al., 2021). In a photic phase shift experiment, which measures the shifting of circadian behavior caused by a brief pulse of light during the dark phase, female 3xTg mice showed a trend towards greater phase shifting while males did not (Sterniczuk et al., 2010). We examined re-entrainment in these mice using a shifted light-dark (LD) cycle, simulating travel across 6 time zones and subsequent "jet lag". This behavior is not altered in male 3xTg mice (González-Luna et al., 2021), but female mice, which have more severe AD pathology than males, have not been studied in this paradigm. We allowed female 8-month-old 3xTg and B6129SF2/J wild type (WT) control mice to entrain to a

12:12 L:D light cycle and monitored their behavior on a running wheel. At this age, female 3xTg mice have relatively mild Aβ and tau pathology (Fig. 2.1A). After full entrainment and habituation to the running wheel, the LD cycle was advanced by 6h (Fig. 2.1B). The onset of nightly running was measured in the days following the light cycle shift. 3xTg activity onset was

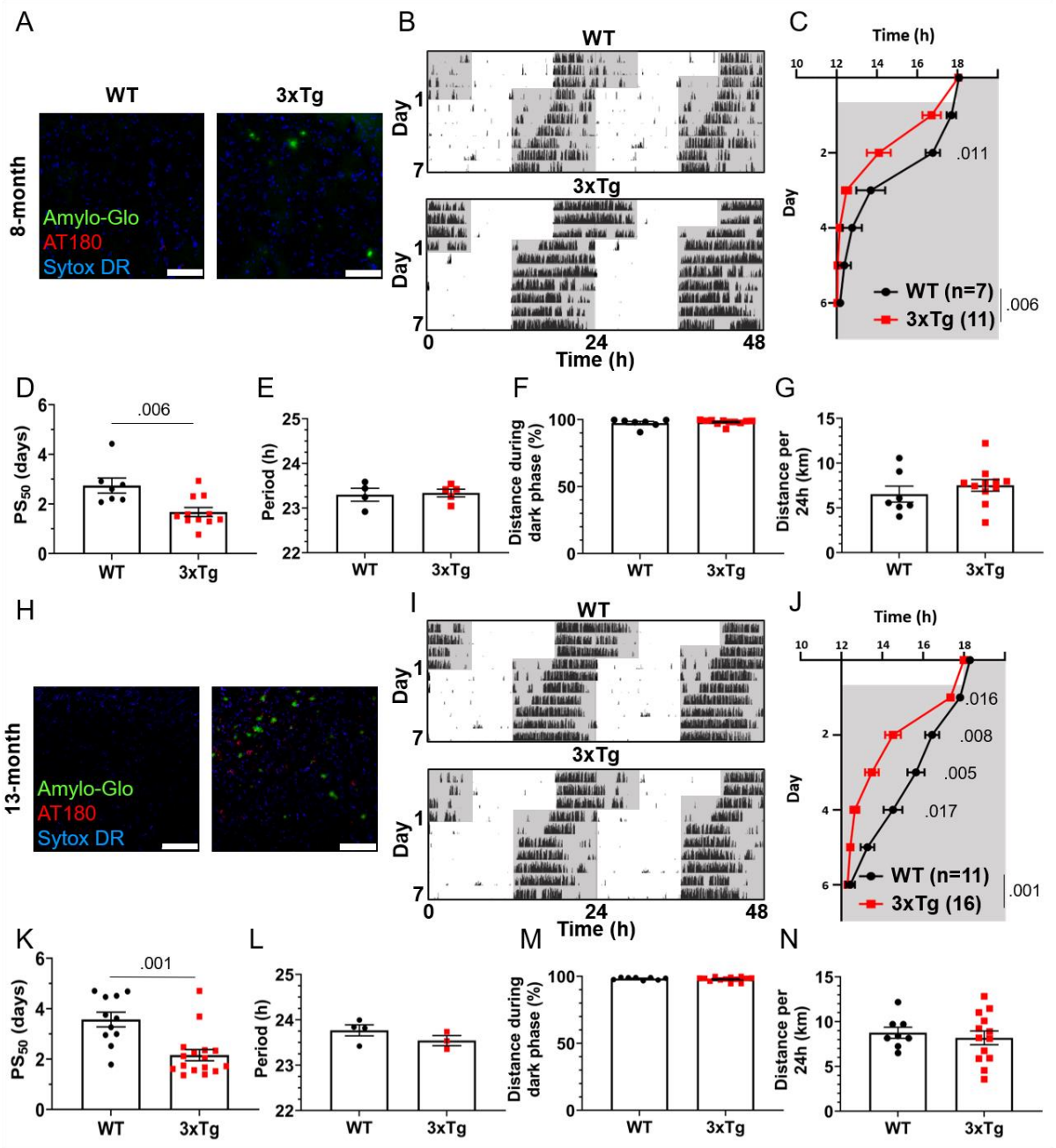

Figure 2.1 *legend on next page*

Figure 2.1: Altered circadian re-entrainment in 3xTg mice

(A) Representative images from the hippocampus of 8-month-old (mo) B6129SF2/J wild type (WT) and 3xTg mice. Aβ plaques are stained with Amylo-Glo (green), phosphorylated tau is stained with AT180 (red), and nuclei are stained with Sytox-DR. Scale bars =100μm.
(B) Representative double-plotted actograms of 8mo WT and 3xTg mice subjected to a 6h phase advance. Light and dark phases of the LD cycle are represented by white and grey background, respectively.
(C) Group analysis of activity onset in 8mo mice, with grey representing darkness as in (B). Mixed model with Sidak post hoc comparison, n=7-11.
(D) Time to 50% of total phase shift (PS_{50}) in 8mo mice from (C), n=7-11.
(E) Free-running period (averaged over 7 days) in 8mo mice maintained in constant darkness.
(F) Percent of running performed during the dark phase and (G) total distance run in 24 hours (averaged over two 24h periods) in 8mo mice, n=7-11.
(H-N) Same as (B-G) but in 13mo mice. n=11-16 in (I-K), 3-4 in (L), 8-13 in (M-N).
All analyses are two tailed Student's t-tests unless otherwise noted. All data plotted as mean ± SEM.

significantly earlier than WT following the light cycle shift on day 2 after the shift (Fig. 2.1C), demonstrating more rapid re-entrainment. We calculated the number of days each mouse took to complete half of the re-entrainment, the 50% phase shift (PS_{50}), and found that mean PS_{50} was 1.07 days earlier in 3xTg than WT mice ($p<.006$) (Fig. 2.1D). We examined free-running period when kept in 24h darkness (Fig. 2.1E) and preference for running during the dark phase in a 12:12 LD cycle (Fig. 2.1F) and found no difference between 3xTg and WT in these other aspects of circadian behavior. Total running was not affected by genotype (Fig. 2.1G), suggesting that the wheel running re-entrainment phenotype is not influenced by the hyperactivity or perseverative behavior sometimes observed in AD mouse models.

To test this phenotype as AD pathology progresses with aging, we repeated this experiment using 13-month-old female 3xTg and WT mice, which have more advanced amyloid and tau pathology (Fig. 2.1H-I). Behavior onset after the light cycle shift was significantly earlier in 13-month 3xTg than WT mice on days 1-4 after the shift (Fig. 2.1J). Mean PS_{50} at 13 months was 1.41 days faster ($p<.001$) in 3xTg than WT (Fig. 2.1K). Free running period and preference for

running in the dark phase were not affected by genotype (Fig. 2.1L-M). Total running was again not affected by genotype (Fig. 2.1N). These results show that 3xTg mice re-entrain more rapidly in a jet lag paradigm at multiple stages of pathological progression.

To explore whether age plays a role in entrainment in these mice, we compared PS_{50} data from 8- and 13-month-old 3xTg and WT mice by 2-way ANOVA. There is a significant effect of age ($p<.016$) as well as genotype ($p<.001$) in re-entrainment speed. Post-hoc comparisons did not find significant differences between 8- and 13-month-old mice within either genotype.

2.3.2 Microglia depletion does not alter circadian re-entrainment in 3xTg mice

As neuronal loss does not appear in AD until long after the development of circadian symptoms, we sought other possible mechanisms underlying this behavioral phenotype. Microglia are heavily implicated in AD pathophysiology and microglia activation can contribute to disease progression. In the 3xTg model, the brain has elevated levels of microglia-produced pro-inflammatory cytokines (J. S. Park et al., 2021) and microglia activation and proliferation can be observed before the development of Aβ plaques (Janelsins et al., 2005). We observe activated microglia in 3xTg mice at 13mo, where they cluster around Aβ plaques and display a more amoeboid morphology (Fig. 2.2A). Microglia depletion in AD models decreases neuroinflammatory signaling without acutely altering amyloid and tau pathology and in some studies can partially restore memory deficits (E. E. Spangenberg et al., 2016). We hypothesized that activated microglia and neuroinflammation could contribute to the circadian re-entrainment phenotype observed in 3xTg mice and microglia depletion would rescue the re-entrainment phenotype. We used the colony stimulating factor 1 receptor (CSF1R) antagonist Plexxikon 3397 (PLX) to rapidly deplete microglia from the brain. Following re-entrainment to the shifted light cycle in Fig. 2.1, 13-month 3xTg or WT mice were switched to control or PLX chow (600mg/kg)

for 7 days to deplete microglia (Fig. 2.2B). PLX treatment effectively depleted microglia from the brain in WT and 3xTg mice, reducing the number of microglia in the ventromedial hypothalamus, the region containing the SCN, by >98% (Fig. 2.2C-D). After 7 days of PLX or control treatment, light cycles were advanced by 6h and running wheel behavior monitored (Fig. 2.2E). PLX treatment did not rescue the more rapid re-entrainment in 3xTg mice, and there was

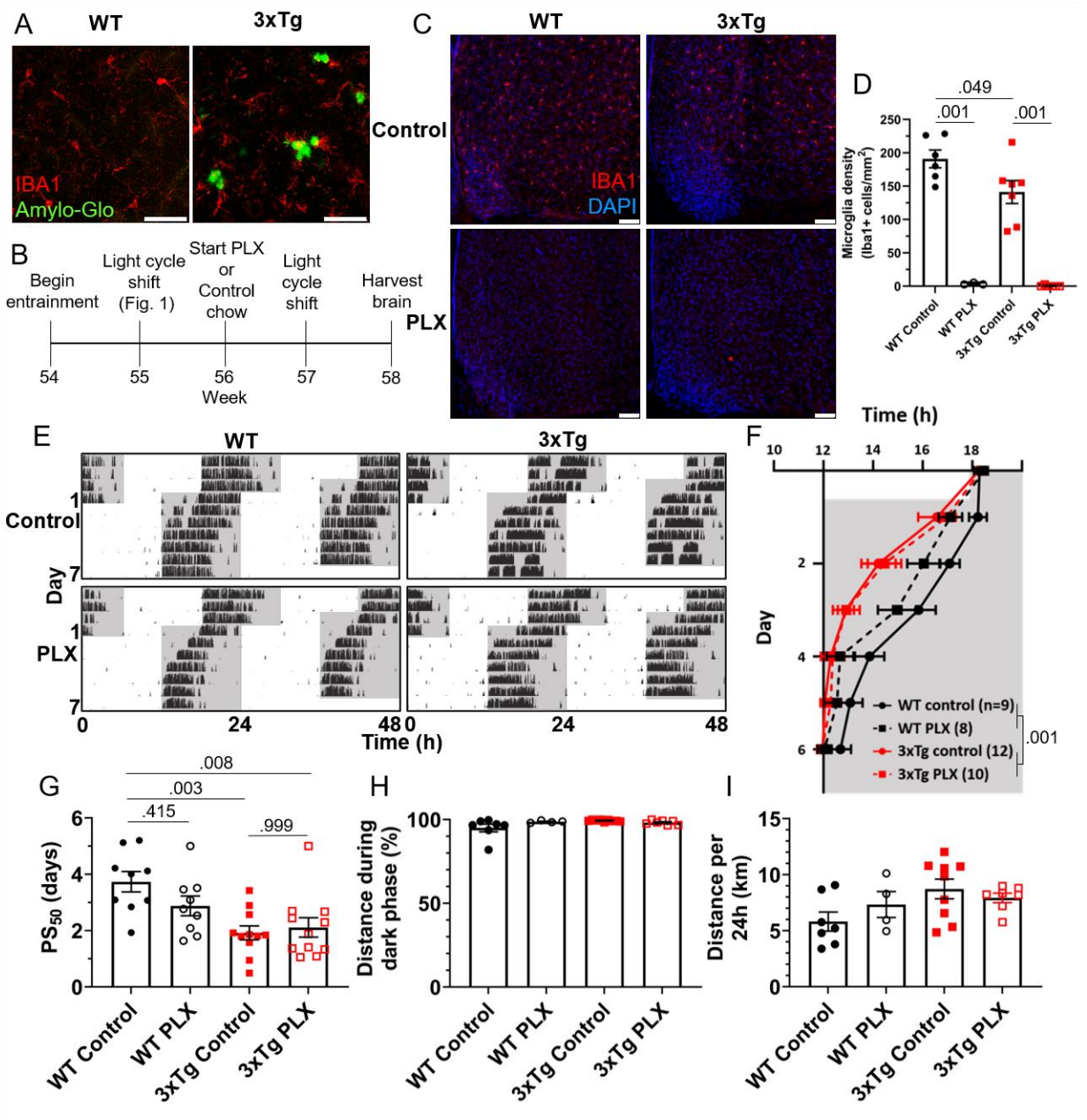

Figure 2.2 *legend on next page*

Figure 2.2: Microglia depletion does not rescue circadian re-entrainment phenotype in 3xTg mice

(A) Representative images of hippocampal microglia morphology in 13mo 3xTg and B6129SF2/J WT mice. Aβ plaques are stained with Amylo-Glo (green) and microglia are marked by staining for IBA1 (red). Scale bars =50μm.
(B) Timeline of experiment. After completing the light cycle shift experiment in Fig. 2.1H-N, 13mo WT and 3xTg mice were fed Plexxikon 3397 (PLX) chow to deplete microglia or control chow for 7 days before beginning light cycle shift.
(C) Representative images of microglia depletion in the SCN and surrounding region in WT and 3xTg mice fed control or PLX diets. Microglia labeled by staining for IBA1 (red). Scale bars =100μm.
(D) Quantification of microglia in the ventromedial hypothalamus, the region containing the SCN, in WT and 3xTg mice fed control or PLX diets.
(E) Representative double-plotted actograms of 13mo wild type (WT) and 3xTg mice, treated with control or PLX chow, subjected to a 6h phase advance. Light and dark phases of the LD cycle are represented by white and grey background, respectively.
(F) Group analysis of activity onset, with grey representing darkness as in (E), n=8-12. Mixed model with Sidak post hoc comparison.
(G) Time to 50% of total phase shift (PS_{50}) in mice from (E), n=8-12.
(H) Percent of running performed during the dark phase and (I) total distance run in 24 hours (averaged over two 24h periods), n=4-9.
All analyses are 2-way ANOVAs with Sidak *post hoc* comparison unless otherwise noted. All data plotted as mean ± SEM.

no significant effect of treatment on time of running onset (Fig. 2.2F). As in Figure 2.1, behavior onsets were earlier in 3xTg than WT mice, with a significant effect of genotype on time of running onset ($p<.001$). PLX treatment did not significantly alter PS_{50} in 3xTg mice (Fig. 2.2G). There was a nonsignificant trend towards higher PS_{50} in WT control vs. PLX-treated mice ($p<.415$). There was no significant effect of treatment on PS_{50} ($p<.316$), but the effect of genotype on PS_{50} was again significant ($p<.001$). Thus, acute microglia depletion did not rescue the re-entrainment phenotype observed in 3xTg mice. PLX treatment also did not alter other running behaviors measured, with no significant effect of genotype or treatment found in percent running during the dark phase or total distance traveled (Fig. 2.2H-I). These data demonstrate

that re-entrainment remains altered in microglia-depleted 3xTg mice, suggesting that microglia and neuroinflammation are not acutely responsible for this circadian phenotype.

2.3.3 5xFAD mice have accelerated circadian re-entrainment

3xTg mice carry mutations driving pathological Aβ and tau expression in the brain. To probe whether both of these pathological proteins are necessary in order to produce the re-entrainment phenotype we observed in 3xTg mice, we studied re-entrainment in 5xFAD mice. The 5xFAD model expresses mutant APP and PS1 transgenes, but no mutant tau transgene, and thus develops aggressive amyloid pathology without neurofibrillary tangles. 5xFAD mice show altered molecular circadian rhythms and circadian behavior (J. Lee et al., 2020; H. Song et al., 2015a). Based on the observed behavioral phenotype in 8-month-old 3xTg mice, which have amyloid pathology but little tauopathy, we hypothesized that Aβ is sufficient to alter re-entrainment. We studied female 5xFAD mice at 7 months of age, at which time they have extensive Aβ plaques (Fig. 2.3A). We repeated the jet lag experiment described above in these aged 5xFAD mice (Fig. 2.3B). Behavior onset was significantly earlier in 5xFAD mice than WT mice on days 2-4 after the shift (Fig. 2.3C), and mean PS_{50} was reached 2.27 days earlier ($p<.006$) (Fig. 2.3D). Free-running period and preference for running during the dark phase were not significantly affected by genotype (Fig. 2.3E-F). Total running distance was not altered in 5xFAD mice, suggesting that hyperactivity or perseverative behavior were not responsible for altered performance on the running wheel in this model (Fig. 2.3G). These results closely recapitulate the findings in aged 3xTg mice. Thus, amyloid pathology in the absence of mutant tau is sufficient to alter circadian re-entrainment in these AD mouse models.

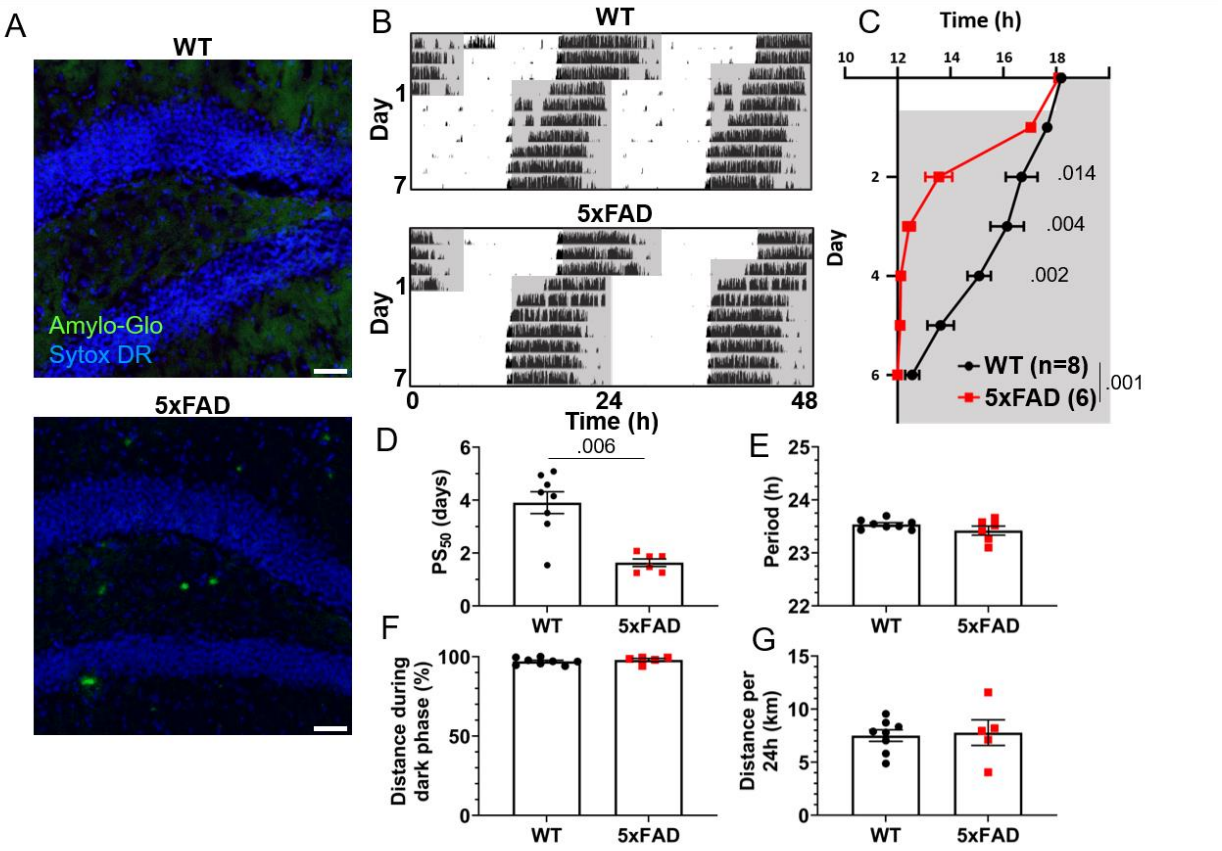

Figure 2.3: Altered circadian re-entrainment in 5xFAD mice

(A) Representative images from the hippocampus of 7mo 5xFAD and littermate control WT mice. Aβ plaques are stained with Amylo-Glo (green) and nuclei are stained with Sytox-DR (blue). Scale bars = 50μm.
(B) Representative double-plotted actograms of 7mo WT and 5xFAD mice subjected to a 6h phase advance. Light and dark phases of the LD cycle are represented by white and grey background, respectively.
(C) Group analysis of activity onset, with grey representing darkness as in (B). Mixed model with Sidak post hoc comparison, n=6-8.
(D) Time to 50% of total phase shift (PS_{50}) in mice from (C), n=6-8.
(E) Free-running period (averaged over 7 days) in mice maintained in constant darkness, n=6-8.
(F) Percent of running performed during the dark phase and (G) total distance run in 24 hours (averaged over two 24h periods), n=6-8.
All analyses are two tailed Student's t-tests unless otherwise noted. All data plotted as mean ± SEM.

2.3.4 PS19 mice do not have accelerated re-entrainment

Re-entrainment is accelerated in a genetic model which drives both Aβ and tau pathology and a model which only carries mutations driving Aβ pathology, showing that the behavioral

differences are downstream of Aβ pathology and that NFTs are not necessary for the phenotype. Next we asked whether tauopathy was sufficient to alter re-entrainment in the absence of Aβ pathology.

To explore this question, we repeated the jet lag experiment with the PS19 mouse model (also called the Tau P301S mouse). PS19 mice carry a mutant form of human MAPT which causes frontotemporal dementia and parkinsonism in humans (Sperfeld et al., 1999). The mice develop aggressive tauopathy and neurodegeneration, but they do not carry amyloidogenic mutations or develop Aβ plaques (Yoshiyama et al., 2007). We repeated the jet lag experiment described above in 7-month-old female PS19 mice, which have detectable pTau staining in the hippocampus (Fig. 2.4A-B). There were no significant differences between PS19 and WT mice in behavior onset, PS_{50}, free-running period, preference for running during the dark phase, or total running distance (Fig. 2.4C-G).

PS19 mice, unlike 3xTg and 5xFAD, do not demonstrate accelerated re-entrainment in the jet lag test. The rapid re-entrainment of WT controls for this model may obscure fine differences between groups. However, the absence of a trend towards more rapid re-entrainment in this tauopathy model further supports the hypothesis that the re-entrainment phenotype is driven by amyloid, rather than tau, pathology.

To explore whether background strain plays a role in entrainment in these mice, we compared PS_{50} data from 7-month-old WT controls in the 5xFAD experiment (which are on a C57BL/6J background) to 7-month-old WT controls in the PS19 experiment (which are on a B6C3

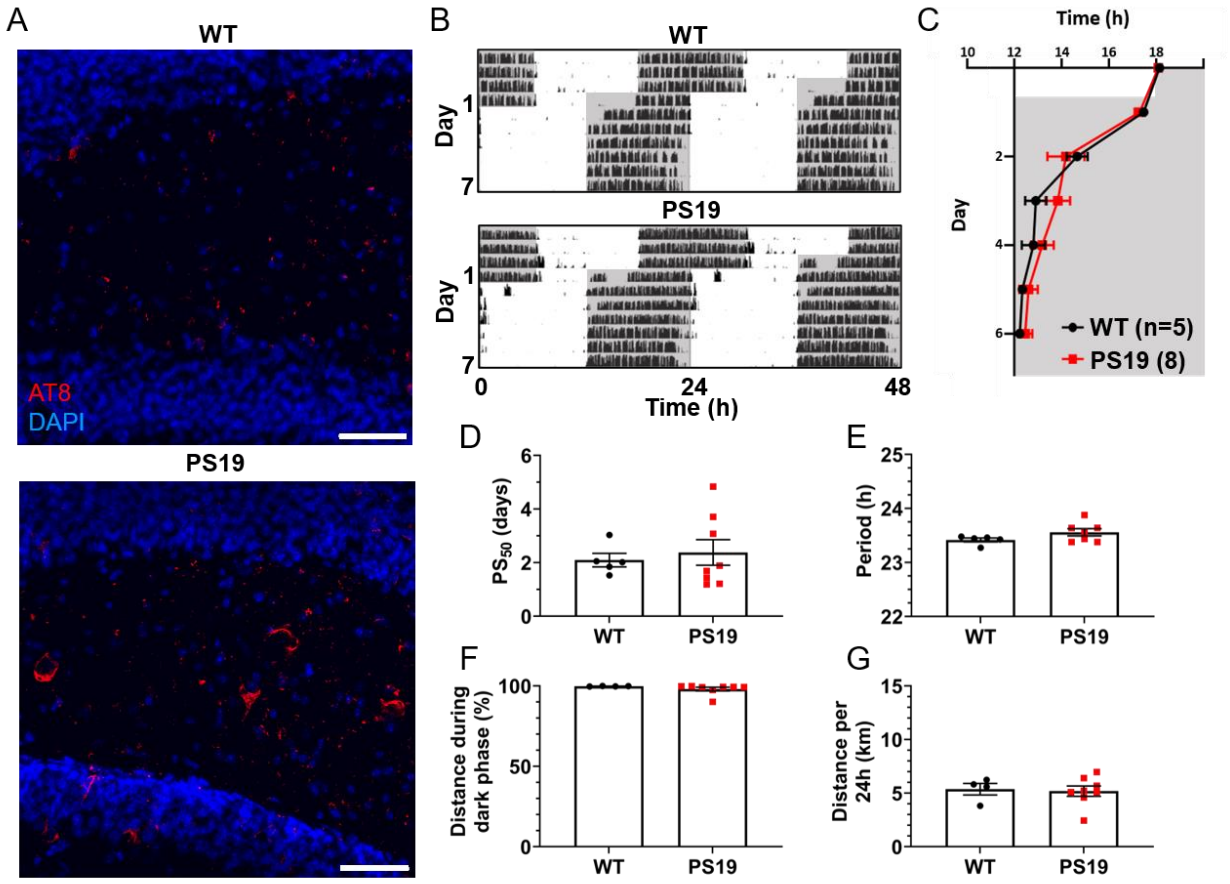

Figure 2.4: No altered circadian re-entrainment in PS19 mice

(A) Representative images from the hippocampus of 7-month-old 5xFAD and littermate control WT mice. Phospho-tau is labeled with AT8 (red) and nuclei are stained with DAPI (blue). Scale bars = 50µm.
(B) Representative double-plotted actograms of 7mo PS19 and littermate control WT mice subjected to a 6h phase advance. Light and dark phases of the LD cycle are represented by white and grey backgrounds, respectively.
(C) Group analysis of activity onset, with grey representing darkness as in (A). Mixed model with Sidak post hoc comparison, n=5-8.
(D) Time to 50% of total phase shift (PS50) in mice from (B), n=5-8.
(E) Free-running period (averaged over 7 days) in mice maintained in constant darkness, n=5-7.
(F) Percent of running performed during the dark phase and (G) total distance run in 24 hours (averaged over two 24h periods), n=4-8.
All analyses are two tailed Student's t-tests unless otherwise noted. All data plotted as mean ± SEM.

background) by t-test. B6C3 controls re-entrained significantly faster than C57BL/6J controls

($p<.008$). Re-entrainment therefore is partially dependent on background strain.

2.3.5 Re-entrainment does not correlate with body weight changes

The SCN is the central timekeeper for the body, but peripheral clocks can receive entrainment cues through other mechanisms as well. Feeding is a potent entrainment cue for the molecular clock of the liver (Stokkan et al., 2001), and insulin/insulin-like growth factor 1 signaling can affect entrainment of circadian locomotor rhythms (Crosby et al., 2019). AD is associated with altered insulin signaling (Kulas et al., 2020), and 3xTg mice exhibit a metabolic phenotype resulting in greater body weight than B6129SF2/J controls (Robison et al., 2020). We found significantly higher body weight in our 3xTg mice compared to WT at 8 months ($p<.001$) and at 13 months ($p<.001$). Further, in some mice we observe significant weight loss over the course of their time housed in running wheel cages for circadian behavioral experiments, which could result in metabolic changes that would influence insulin signaling and other metabolic processes. 3xTg mice in our experiments showed significantly greater weight loss than WT at 8 months ($p<.001$) and 13 months ($p<.001$). Thus it is possible that metabolic differences between AD model mice and controls could contribute to the observed re-entrainment phenotype.

To examine this, we measured body weight before being placed in running wheel cages, body weight at the end of the experiment, and % weight change over the course of the experiment. All of these were charted against PS_{50} to determine whether body weight or weight change correlated with speed of re-entrainment. If the accelerated re-entrainment phenotype were driven by weight loss, we would expect a significant positive correlation between % weight change and PS_{50}. In 8-month-old 3xTg and WT mice, we see no significant correlation (Fig. 2.5A). If the accelerated re-entrainment phenotype were driven by high body weight in 3xTg mice, we would expect to find a significant negative correlation between body weight and PS_{50}. 8-month-old 3xTg and WT mice did not show any significant correlation between pre- or post-experiment body weight and

re-entrainment speed (Fig. 2.5B-C). Similar results were found in 13-month-old 3xTg and WT mice (Fig. 2.5D-F). These metabolic differences are thus not likely to contribute to the observed re-entrainment phenotype.

5xFAD mice are not reported to demonstrate the metabolic phenotype observed in 3xTg mice, and our 7-month-old 5xFAD mice weighed significantly less than controls at the beginning of their wheel running experiments ($p<.014$). These mice showed an insignificant trend towards a

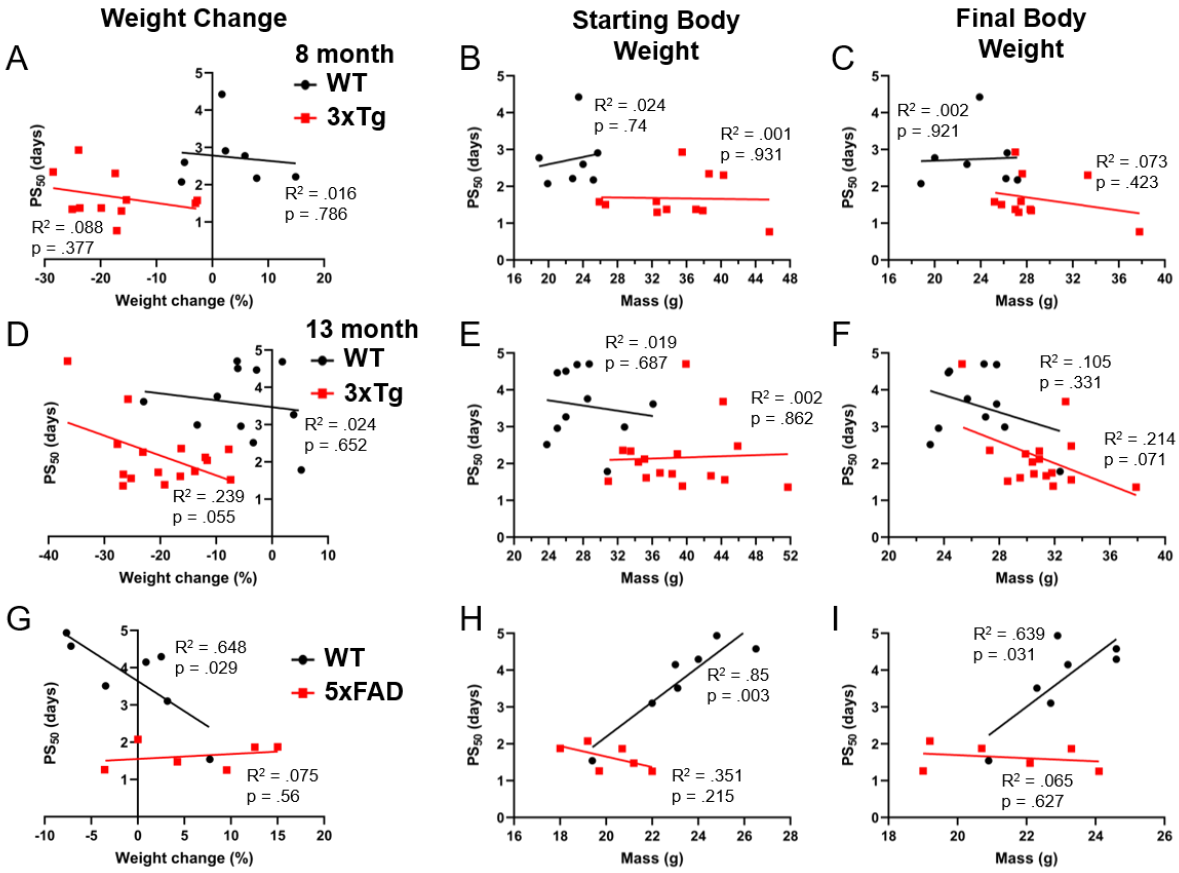

Figure 2.5: Associations of weight loss and body weight with jet lag re-entrainment

(A) Percent weight change over the course of wheel running in 8-month-old WT and 3xTg mice plotted with time to 50% re-entrainment (PS_{50}).
(B) Body weight at the beginning of wheel running plotted with PS_{50}.
(C) Body weight at the end of wheel running plotted with PS_{50}.
(D-F) Same as (A-C) with 13-month-old WT and 3xTg mice.
(G-I) Same as (A-C) with 7-month-old WT and 5xFAD mice.
All analyses are linear regressions.

greater increase in body weight over the course of the experiment ($p>.083$). In 5xFAD mice there are no significant correlations between % weight change or body weight and PS_{50} (Fig. 2.5G-I). Interestingly, in WT mice in this experiment there is a significant negative correlation between weight change and PS_{50} ($p=.029$), and positive correlations between PS_{50} and initial body weight ($p=.003$) and final body weight ($p=.031$). These correlations suggest that metabolism may play a role in re-entrainment, but it appears to be strain-specific and is not observed in AD model mice.

In summary, there is no significant correlation between body weight or weight loss and re-entrainment speed in 3xTg mice, and 5xFAD show the same re-entrainment phenotype but do not have increased body weights or weight loss. Thus, we conclude that these metabolic differences are not responsible for accelerated re-entrainment in AD model mice.

2.3.6 SCN structure and pathology are not grossly altered in AD models

The SCN is the master entrainer of the body's circadian rhythms, and AD pathology-induced deficits in the region could contribute to the re-entrainment phenotype. The SCN is reported to undergo degeneration late in AD progression (Swaab et al., 1985; Y.-H. Wu et al., 2007), but it carries a relatively light load of plaques and NFTs compared to other nearby brain regions (Baloyannis et al., 2015; Stopa et al., 1999). We sought to assess whether we could detect AD pathology and gross alterations to SCN cell populations in AD model mice.

We first stained for Aβ plaques and tau phosphorylation in the SCN of 3xTg mice at 8 months (Fig. 2.6A) and 13 months (Fig. 2.6B). We found no plaques or pTau staining at either age. Plaques were also not present in the SCN in 5xFAD mice (Fig. 2.6C). Aβ and tau pathologies could still be present in the region, but they would have to be less advanced forms of pathology that could not be detected by these methods, such as Aβ oligomers or other tau with

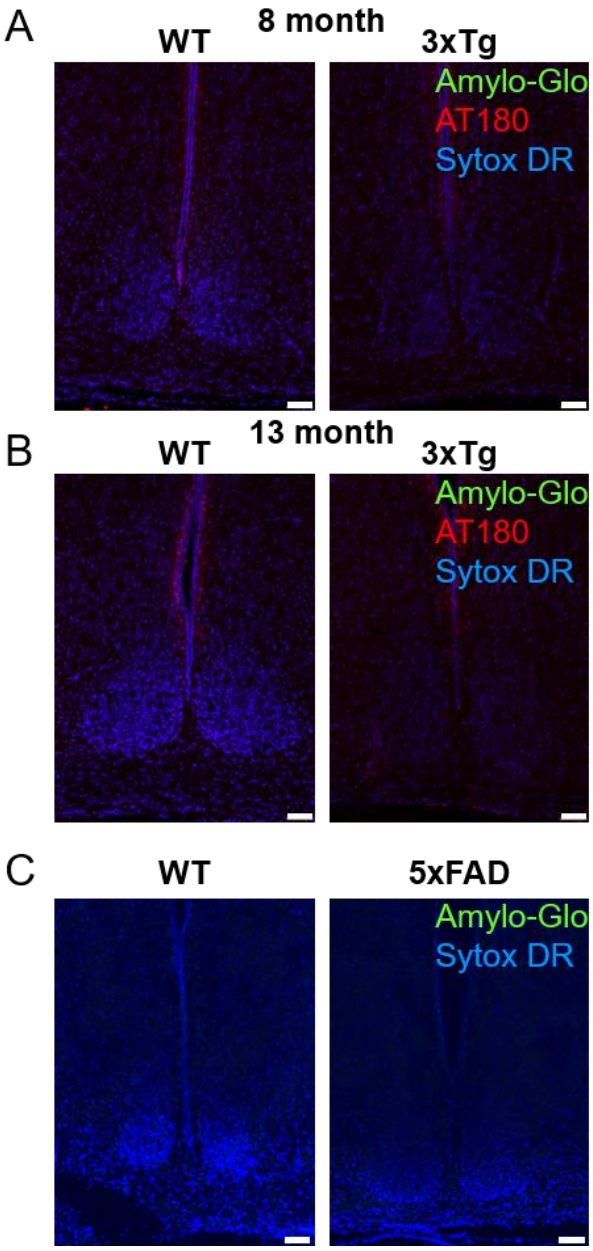

Figure 2.6: Amyloid and tau pathology are not detected in the SCN of AD models

Stains of the SCN and surrounding region with Sytox-DR for nuclei (blue), AmyloGlo for Aβ plaques (green), and AT180 for phosphorylated tau (red) in WT and 3xTg mice at (A) 8 months, and (B) 13 months. (C) Stains with Sytox-DR for nuclei and AmyloGlo for Aβ plaques in WT and 5xFAD mice at 7 months.
Scale bars =100μm.

modifications not detected by the AT180 antibody. We conclude, however, that the re-entrainment phenotype is not dependent on the direct effects of Aβ plaques or NFTs in the SCN.

We also briefly examined some cell type markers in the SCN. AVP is a neuropeptide expressed in cells in the SCN shell which plays an important role in entrainment (Yamaguchi et al., 2013), and AVP expression may be decreased in AD (Harper et al., 2008; R. Y. Liu et al., 2000). We stained 13-month-old WT and 3xTg mice for AVP and did not observe a qualitative difference (Fig.

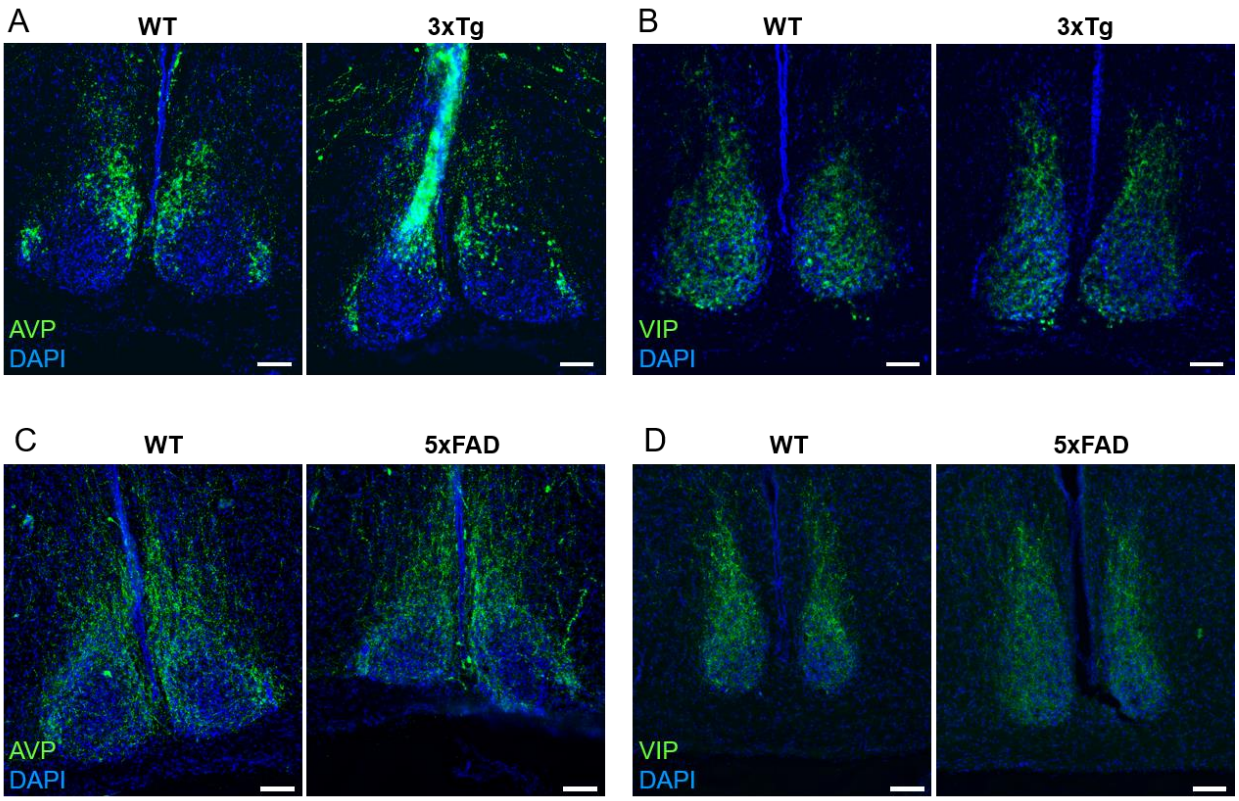

Figure 2.7: Gross changes to AVP and VIP expression are not observed in the SCN of AD models

Representative images from 13-month-old WT and 3xTg brains of (A) arginine vasopressin (AVP) and (B) vasoactive intestinal peptide (VIP).
(C-D) Same as (A-B) in 8-month-old WT and 5xFAD brains.
Scale bars = 100μm.

2.7A). Given the small size and regional heterogeneity of the SCN, small variations in tissue slicing and staining can make large differences in cell counting or fluorescence measurements, so we did not assess these stains quantitatively. Next we stained for VIP, another neuropeptide expressed in the SCN which is involved in entrainment (Shen et al., 2000). There was no qualitative difference in 3xTg mice (Fig. 2.7B). 7-month-old 5xFAD mice also did not show apparent changes in AVP or VIP expression (Fig. 2.7C-D).

2.4 Discussion

We found that two amyloid-based AD mouse models have significantly accelerated re-entrainment in a jet lag paradigm. This behavioral phenotype was not affected by the depletion of microglia and did not depend on the presence of mutant tau. Together these results uncover a novel circadian phenotype in AD model mice driven by Aβ.

This is the first report, to our knowledge, of altered jet lag behavior in AD model mice. Previous studies in APP/PS1 mice (Kent et al., 2019; Otalora et al., 2012) and male 3xTg mice (González-Luna et al., 2021) did not show altered re-entrainment in the jet lag paradigm. However, the APP/PS1 model has been criticized as not replicating some circadian phenotypes observed in AD patients (Sheehan & Musiek, 2020) and most studies find less severe pathophysiology and behavioral phenotypes in male than female 3xTg mice (Dennison et al., 2021). Given the variability between different AD models, our finding of the same robust re-entrainment phenotype in two different models is important for validating that this phenotype is not the result of peculiarities of a specific genetic model. We do not observe alterations to free running period or daily activity patterns, two measures of circadian behavior in which differences have been reported in some studies with 3xTg mice (Adler et al., 2019; M. Wu et al., 2018). Previous research has not found altered free running period in 5xFAD mice (Nagare et al., 2020), consistent with our findings here. We did observe notable differences between WT mice in our studies depending on age (Fig. 2.1D, K) and strain (Fig. 2.1D, Fig. 2.3D, Fig. 2.4C). Indeed, given the very rapid re-entrainment of littermate controls of PS19 mice, the B6C3 background strain in this model may not be well suited to detecting fine differences in re-entrainment behavior, and other tauopathy models may exhibit behavioral differences. These heterogeneous

results highlight the importance of sex, age, and background strain in circadian behavioral experiments.

Modulating neuroinflammation in AD is a promising field of research, and microglia can both contribute to and protect against disease progression. As neuroimmune activation and circadian disruptions both appear early in the progression of AD, and inflammation can modulate circadian rhythms, microglia seemed a promising possible mechanism underlying circadian symptoms. However, we found that microglia depletion did not rescue jet lag behavior in 3xTg mice. The microglia depletion in our experiment was acute and longer treatment may have other effects on circadian behavior, but our results suggest that acutely targeting inflammation in AD may not directly ameliorate circadian disruptions. Retinal microglia are also likely not acutely involved in the jet lag phenotype as CSF1R inhibitors effectively deplete microglia in the retina as well as in other regions of the brain (Dharmarajan et al., 2017; Y. Huang et al., 2018). We did observe a trend towards accelerated re-entrainment in WT mice after microglia depletion, which may suggest a role for microglia in circadian regulation in the healthy brain. A study reported altered circadian behavior in rats after using a targeted diphtheria toxin approach to deplete microglia (Sominsky et al., 2021), although these results may have been influenced by sickness behavior induced by the diphtheria toxin approach. Another recent study found that microglia depletion alters sleep in mice (H. Liu et al., 2021), but did not study circadian-regulated behaviors. More recent research, however, has found no effect of PLX treatment on re-entrainment in a jet lag paradigm in WT mice (Matsui et al., 2023).

Accelerated recovery from jet lag is not one of the circadian symptoms of AD, but this phenotype may reflect underlying circadian disruptions. Strategies for managing sundowning and improving sleep-related symptoms in AD patients include maintaining strict light schedules,

increasing exposure to intense light during daylight hours, and decreasing exposure to light during the night (Mitolo et al., 2018). AD patients also have decreased amplitude of rhythmic circadian gene expression (Cermakian et al., 2011). This may indicate a weak biological circadian clock, making them heavily dependent on external cues for its maintenance. Rapid re-entrainment may indicate a decrease in the coupling and synchrony of neurons in the SCN, resulting in an intrinsic circadian timekeeper less resistant to being shifted by misaligned photic entrainment cues. A very similar jet lag phenotype is observed in mice genetically knocked out for AVP signaling (Yamaguchi et al., 2013), which impairs interneuronal communication in the SCN. Some reports find decreased AVP expression in the SCN of AD patients (Harper et al., 2008; R. Y. Liu et al., 2000), though this has been disputed (J. L. Wang et al., 2015), and previous reports have found decreased expression of AVP in the SCN of 3xTg mice (Sterniczuk et al., 2010). We observed no qualitative differences in AVP and VIP expression in AD model mice, but quantitative analysis of gene or protein expression should be explored in the future. Further studies will be needed to determine if changes in SCN signaling contribute to this phenotype and how amyloid pathology drives those changes.

Several other questions are also raised by these results that should be explored further. First, we observe accelerated re-entrainment in 3xTg mice at 8 months, relatively early in the pathological progression in the model, but still after the reported onset of some cognitive symptoms (Billings et al., 2005). Circadian disruptions in humans can appear very early in the disease course, many years before clinical diagnosis. A jet lag test in younger 3xTg and 5xFAD mice, before the onset of cognitive deficits, could explore whether re-entrainment in mouse models reflects this aspect of human disease. Second, male mice should be studied to explore whether there are sex differences in the re-entrainment phenotype. Third, in these experiments we only studied

locomotor behavior as a readout of circadian rhythms. Future experiments could examine the molecular clocks of both the SCN and peripheral tissues by collecting tissue at different circadian times in the days after jet lag to determine whether the accelerated behavioral re-entrainment is also reflected in re-entrainment of the circadian transcriptional clock.

In summary, we demonstrate that AD model mice exhibit strikingly altered circadian re-entrainment. This appears to be driven by pathogenic Aβ and does not require the presence of microglia or mutant tau.

2.5 Methods

2.5.1 Mice

All animal experiments were conducted in accordance with the University of Virginia Institutional Animal Care and Use Committee. Animals were housed in a temperature and humidity controlled vivarium (22-24°C, ~40% humidity) and were provided with food and water ad libitum. 3xTg experiments were conducted with 8- to 13-month-old female 3xTg mice on a B6129 background (Oddo et al., 2003) (Jackson Laboratory #034830), with age-matched B6129SF2/J (Jackson Laboratory #101045) females as wild type controls. 5xFAD experiments were conducted with 7-month-old female heterozygous 5xFAD mice on a C57BL/6J background (Oakley et al., 2006) (Jackson Laboratory #034848), with littermates genotyped as not expressing the mutant transgene serving as wild type controls. PS19 experiments were conducted with 7-month-old female heterozygous PS19 mice on a B6C3 background (Yoshiyama et al., 2007) (Jackson Laboratory #008169), with littermates genotyped as not expressing the mutant transgene serving as wild type controls. For microglia depletion experiments, mice were given chow formulated with PLX3397 (660mg/kg) or control chow for 7 days before light cycle shift and were maintained on PLX or control chow for the remainder of the experiment.

2.5.2 Behavioral analysis

Behavioral testing protocol was adapted from (Grippo et al., 2017). Mice were individually housed in cages (Nalgene) containing running wheels in light-tight boxes which were illuminated with timed fluorescent lights ($590x10^{12}$ photons/cm2/s). Wheel running data were collected and analyzed with ClockLab software (Actimetrics). Activity onset was automatically detected by ClockLab software and when necessary, corrected by eye by an experimenter blinded to genotype and treatment group. Mice were allowed to habituate to running wheel cages and entrained to a 12hr:12hr LD cycle for at least 7 days before experiments began.

In jet lag re-entrainment trials, the onset of the dark phase was abruptly advanced by 6 hours and running wheel activity was recorded for at least 7 days after light cycle shift. PS_{50} values were calculated using Prism software (GraphPad) by fitting a sigmoid dose-response curve to onset times in days 0-6 after light cycle shift (Kiessling et al., 2010). Total running distance and preference for running in the dark were measured after all mice had completely re-entrained after a phase shift and were averaged across 2 days. To determine free running period, after all mice had completely re-entrained they were switched to DD and period was calculated from the onset of activity across 7 days.

2.5.3 Histological analysis and imaging

All mice used for histological analysis were sacrificed from ZT5-9. Mice were anesthetized with a ketamine:xylazine solution and transcardially perfused with chilled phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were dissected and post-fixed in 4% PFA for 24h at 4°C. Brains were cryoprotected by incubating >24h in 15% sucrose in PBS followed by 30% sucrose before being frozen and sectioned at 30μm with a cryostat (Leica CM 1950). For stains using primary antibodies raised in mice, sections were blocked using Mouse on

Mouse blocking solution (Vector Laboratories) for 2h at room temperature. Otherwise, brain sections were incubated with blocking solution (5% bovine serum albumin, 2% horse serum, 1% Triton X-100 in PBS) for 2h at room temperature. Primary antibodies were diluted in blocking solution and placed on sections overnight at 4°C. Sections were washed and incubated with secondary antibodies, diluted in blocking solution, for 2h at room temperature. Sections were washed and if applicable were treated with DAPI (Sigma-Aldrich) (1:2000 in PBS for 15min), Sytox Deep Red (Thermo Scientific) (1:2000 in PBS for 30min), and/or AmyloGlo (Biosensis) per manufacturer protocol. Sections were washed and then mounted with ProLong Gold mounting media (Thermo Fisher).

Primary antibodies used were anti-phospho-tau AT180 (mouse, Invitrogen MN1040, 1:250), anti-phospho-tau pThr231 (rabbit, Invitrogen 701056, 1:500), anti-Iba1 (rabbit, Wako 019-19741, 1:300), anti-AVP (rabbit, Cell Signaling D8T3K, 1:100), and anti-VIP (rabbit, Cell Signaling D8J1V, 1:200). Secondary antibodies used were donkey anti-rabbit Alexa Fluor 594 (Invitrogen A21207), donkey anti-rabbit Alexa Fluor 647 (Invitrogen A32795), donkey anti-mouse Alexa Fluor 594 (Invitrogen A21203), and donkey anti-mouse Alexa Fluor 647 (Invitrogen A11012), all diluted 1:1000.

Images were acquired with a Keyence BZ-X800 fluorescence microscope and images were stitched using BZ-X800 Analyzer software. Cell quantification was performed with ImageJ. For quantification of microglia, a 0.36mm^2 square was drawn in the ventromedial hypothalamus and all Iba1+ cells were counted.

2.5.4 Statistical analysis

All data are presented as mean ± SEM. Statistical analyses were performed with Prism software (GraphPad). Time of onset of running after jet lag was analyzed by mixed model with Sidak

post-hoc comparisons. In PLX experiment, other data were analyzed by 2-way ANOVA with Sidak post-hoc comparisons. All other data were analyzed by two tailed Student's t-test. Differences between groups were determined to be statistically significant when $p<.05$.

2.6 Acknowledgements

We thank R. Grippo and Q. Tang for help with behavioral experiments, U. Eyo for providing PLX3397 chow, and Virginia Alzheimer's Disease Center for providing antibodies. This research was funded by grants from the Owens Family Foundation, Commonwealth of Virginia's Alzheimer's and Related Diseases Research Fund and K08DK097293 to HAF, T32GM139787 to TKW and R35GM140854 to ADG.

Chapter 3 Altered light sensing as a contributor to circadian phenotypes in Alzheimer's disease models

Adapted from (Weigel et al., 2023).

3.1 Abstract

We previously found that AD model mice have altered circadian behavior in a jet lag re-entrainment test. This phenotype was not dependent on microglia or on mutant tau expression. The specific mechanisms underlying this altered circadian behavior are unknown. Entrainment to photic cues relies on light sensing by specialized cells in the retina, the intrinsically photosensitive retinal ganglion cells (ipRGCs), which signal to the suprachiasmatic nucleus (SCN). We hypothesized that, independent of alterations to circadian processing in the SCN, altered ipRGC light sensing contributed to the re-entrainment phenotype in AD mice. To test this, we began by examining negative masking, a behavior which relies on ipRGC signaling but is not dependent on the SCN. We found that 3xTg mice exhibit heightened negative masking compared to controls, demonstrating greater sensitivity to light as a behavioral cue. We performed jet lag again under dim light conditions and found that 3xTg mice re-entrain much more rapidly than WT mice. Finally, we examined the retinas of these mice and found that they had more ipRGCs than controls. Together these findings indicate that 3xTg mice have heightened sensitivity to light at the level of the retina, which may contribute to their accelerated re-entrainment.

3.2 Introduction

Visual information is carried from the retina to the rest of the brain by retinal ganglion cells (RGCs), which receive information from the specialized photoreceptor cells of the retina, the rod

and cone cells. A specialized class of RGCs, however, are able to sense light directly. These intrinsically photosensitive retinal ganglion cells (ipRGCs) receive inputs that carry light information detected by rods and cones and are directly photosensitive through the protein melanopsin. ipRGCs are responsible for non-image-forming visual functions, including light sensing for circadian timekeeping.

One of the most important roles for ipRGCs is in entraining the circadian clock to photic cues. Lighting cues sensed in the retina are carried by the ipRGCs to the SCN, the central circadian timekeeper of the brain. When photic zeitgebers are misaligned with the SCN clock, the SCN clock can be gradually shifted or re-entrained to match the light cycle (Fig. 1.1). ipRGCs are essential for this re-entrainment to photic cues. Melanopsin KO mice exhibit impaired photoentrainment (Panda et al., 2002), and when ipRGCs are genetically ablated mice completely lose their ability to entrain to photic signals (Güler et al., 2008).

ipRGCs are involved in other non-image-forming visual functions as well, including masking. Masking is a phenomenon wherein changes in light conditions can alter normally circadian-controlled behaviors without first altering the circadian pacemaker. For example, a mouse running in the dark may stop running if the lights are suddenly turned on, in spite of it still being the animal's active phase. As in the case of entrainment, melanopsin KO mice have impaired masking (Mrosovsky & Hattar, 2003) while mice which have had ipRGCs genetically ablated exhibit no masking at all (Güler et al., 2008). In contrast with photoentrainment, masking is not reliant on the SCN. Though direct activation of VIP+ neurons in the SCN can produce masking-like outcomes in experimental settings (B. Collins et al., 2020; Mazuski et al., 2018; Paul et al., 2020), there is no evidence that the SCN contributes significantly to masking under normal circumstances, and masking is preserved in SCN-ablated animals (Redlin & Mrosovsky, 1999).

The circuitry which controls masking is less well understood, but studies in several model organisms implicate regions of the thalamus (Gall et al., 2013; Q. Lin & Jesuthasan, 2017; Redlin et al., 1999).

Given their important roles in non-image-forming visual functions, ipRGCs have been the subject of some interest in disease research. ipRGC loss in a model of glaucoma results in impaired visual function (Gao et al., 2022). ipRGC loss has been reported in the late stages of AD (La Morgia et al., 2016). This was also observed in one AD mouse model (Carrero et al., 2023). However, ipRGCs have not been examined in 3xTg mice or at earlier stages of AD progression. The retina, like other regions of the brain, is subject to the pathological processes of AD. Aβ aggregates and phosphorylated tau are detectable in the retina in AD (Hart et al., 2016; Koronyo et al., 2017), and evidence of RGC degeneration can be measured *in vivo* (Coppola et al., 2015; Iseri et al., 2006). The effects that these changes have on non-image-forming vision are not well understood.

We hypothesized that the changes that AD pathology induces in the retina would result in altered sensing and processing of photic cues for circadian purposes, potentially contributing to the re-entrainment phenotype that we observed in AD model mice (discussed in Chapter 2). Therefore we tested light sensitivity in these mice. We examined masking and jet lag under dim lighting conditions to test SCN-dependent and independent behaviors that depend on ipRGC signaling. Then we stained the retinas of these mice for signs of AD pathology and for ipRGCs.

3.3 Results

3.3.1 3xTg mice exhibit heightened sensitivity to photic cues

The jet lag re-entrainment paradigm relies on the retina to detect photic entrainment cues and the SCN to shift the biological clock in response to those cues. We next tested negative masking, a test of the behavioral response to light that depends on the retina but is preserved in SCN-ablated animals (Redlin & Mrosovsky, 1999). A previous study found increased negative masking in male 3xTg mice (González-Luna et al., 2021), but did not examine behavior at low light levels or

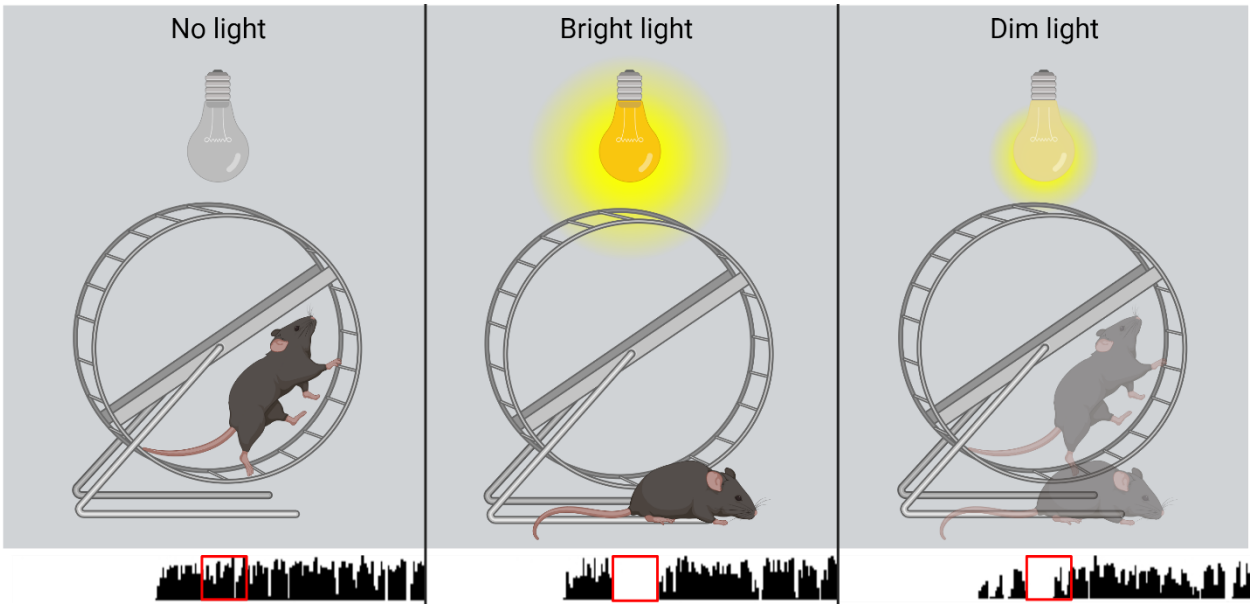

Figure 3.1: Diagram of masking experiment

Masking is a phenomenon wherein light can alter normally circadian controlled behavior, such as wheel running in mice. In this experiment, we monitor mice in wheel-running cages on a 12 hour:12 hour light:dark schedule. Example actograms on the bottom of the diagram depict running at different times of day, with black ticks representing wheel rotations. Mouse locomotor activity is high during the dark phase and low during the light phase. To test masking, lights of different intensities are turned on for a 1-hour block during the dark phase (depicted with red boxes in actograms). Wheel running decreases dramatically when bright lights are turned on (middle panel), demonstrating negative masking. When dimmer lights are turned on, mice decrease their wheel running by varying degrees (right panel). A greater decrease in wheel running is interpreted as a more dramatic negative masking response, or a greater sensitivity to light in the masking task. Figure created with BioRender.com.

in females. To measure negative masking, we gave a one-hour pulse of light of different intensities beginning one hour after the onset of the dark phase of a 12:12 LD cycle and monitored running wheel activity of 8-month-old 3xTg and WT mice during that time (Fig. 3.1). By comparing to running during uninterrupted darkness, we could calculate the degree to which

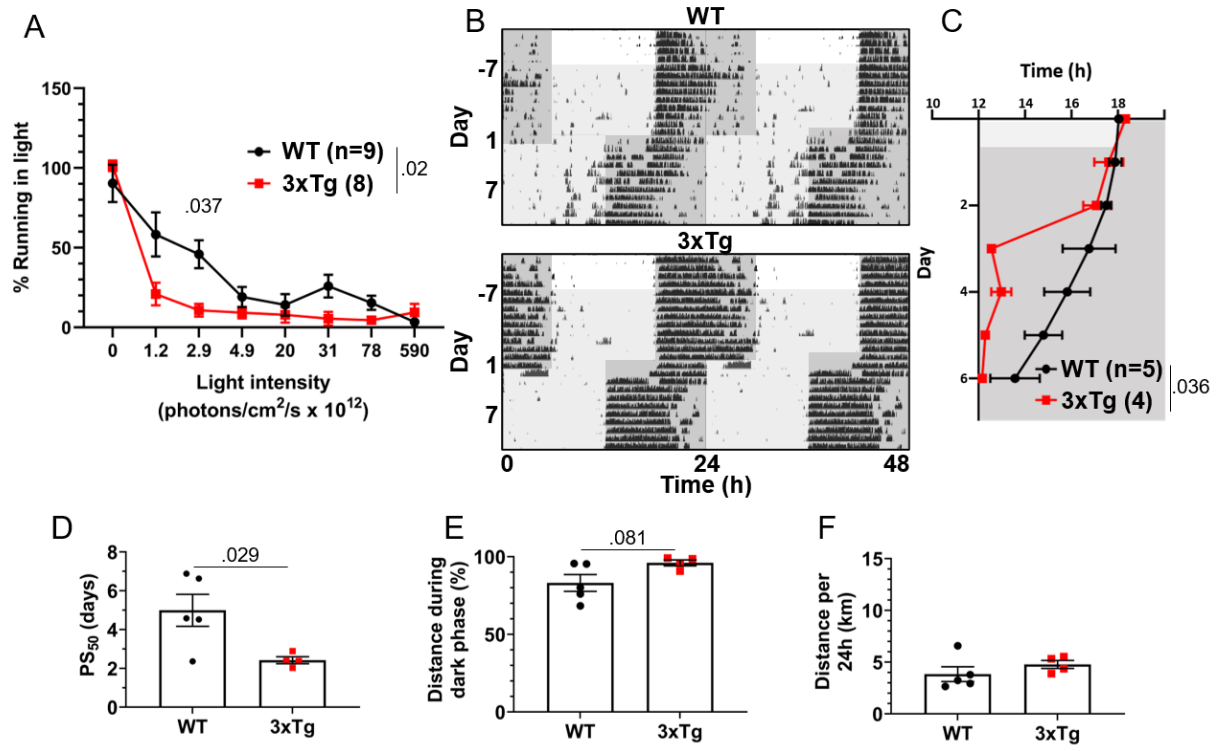

Figure 3.2: Visual circadian function is altered in 3xTg mice

(A) Reduction in running caused by light pulses of increasing intensity during the dark phase in 8-month-old 3xTg and B6129SF2/J WT mice, n=8-9. Lights of different intensities were turned on from ZT13-14 and distance run in that period was compared to running during the same period of constant darkness the previous night.
(B) Dim light jet lag trial representative double-plotted actograms. Lighting was switched from $590x10^{12}$ to $2.9x10^{12}$ photons/cm^2/s. 7 days later, LD phase was advanced by 6h. Dark phase represented by dark grey background, dim light represented by light grey.
(C) Group analysis of activity onset, with grey representing darkness as in (F). Mixed model with Sidak post hoc comparison, n=4-5.
(D) Time to 50% of total phase shift (PS_{50}) in mice from (G), n=4-5.
(E) Percent of running performed during the dark phase and (F) total distance run in 24 hours (averaged over two 24h periods), n=4-5.
All analyses are two tailed Student's t-tests unless otherwise noted. All data plotted as mean ± SEM.

different intensities of light masked running behavior, and therefore measure the sensitivity of the mouse to circadian light cues in a non-SCN-dependent system. We found a significant difference in negative masking behavior between 3xTg and WT mice ($p<.02$), and 3xTg running was significantly more suppressed by low-intensity $2.9x10^{12}$ photons/cm^2/s lighting than WT ($p<.037$) (Fig. 3.2A). Unexpectedly, this demonstrates an elevated responsiveness to photic circadian signals in AD model mice, raising the possibility that increased sensitivity to the photic re-entrainment cue in the jet lag paradigm may contribute to more rapid re-entrainment.

To address this question, we repeated the jet lag experiment with these mice under dim lights. Mice were switched from the $590x10^{12}$ photons/cm^2/s lighting conditions used for previous experiments to only $2.9x10^{12}$ photons/cm^2/s, the intensity in the masking experiment where we found the most significant difference between 3xTg and WT. Mice were kept on a 12:12 LD cycle with these dim lights for 7 days, during which time all mice maintained their entrainment (Fig. 3.2B). After 7 days the jet lag phase advance was performed, still under dim lights, and re-entrainment was observed. 3xTg mice re-entrained significantly more rapidly than WT ($p<.036$) (Fig. 3.2C), with a PS_{50} a dramatic 2.54 days earlier ($p<.029$) (Fig. 3.2D). The magnitude of this difference appears greater than in either of the 3xTg jet lag experiments performed under bright lights.

Also notably, after re-entrainment under dim light, some WT animals displayed unusual running behavior, including considerable bouts of running before the onset of the dark phase (see Fig. 3.2B, upper panel). This was not observed in 3xTg mice. This resulted in a trend ($p=.081$) towards decreased preference for running in the dark phase in WT mice (Fig. 3.2E). Total running was not significantly different between the groups (Fig. 3.2F). Overall, the masking and dim light experiments demonstrated that 3xTg mice respond more intensely than WT mice to

dim light, both as a masking stimulus and an entrainment cue. This supports the hypothesis that altered retinal function contributes to differing circadian behavior.

3.3.2 3xTg mice have a greater density of ipRGCs

We next analyzed the retinas of aged 3xTg mice by immunohistochemistry. Consistent with findings from AD patient retinas, we observed Aβ and tau phosphorylation in the retinas of 13-month-old 3xTg mice (Fig. 3.3A-B). We then stained for RBPMS, a pan-RGC marker. We did not observe gross changes to retinal morphology or the RGC layer (Fig. 3.3C) and quantification of RBPMS+ cells revealed no significant change in RGC density in 3xTg retinas ($p>.298$) (Fig. 3.3D). A slight trend towards decreased RGC density in the 3xTg retinas may suggest that some retinal degeneration is beginning in the model at this age, but this would need to be explored further with larger groups of mice or at a later age. Together, these results demonstrated that AD pathology is present in the retina of 3xTg mice, but by 13 months it has not yet caused dramatic degeneration.

Next we examined ipRGCs, the cells in the retina responsible for transmitting lighting for non-image-forming vision, including to circadian circuits. In order to be able to quantify ipRGCs, which are relatively scarce, we stained whole-mounted retinas for melanopsin, a marker for ipRGCs (Fig. 3.3E). Interestingly, we found a 64% increase in the density of ipRGCs in the retinas of these 3xTg mice compared with WT ($p<.019$) (Fig. 3.3F). All retinal tissue samples were collected between ZT5-8 and ipRGC density was averaged across images from the four quadrants of the retina to minimize the effects of rhythmicity (Hannibal et al., 2005) and regional

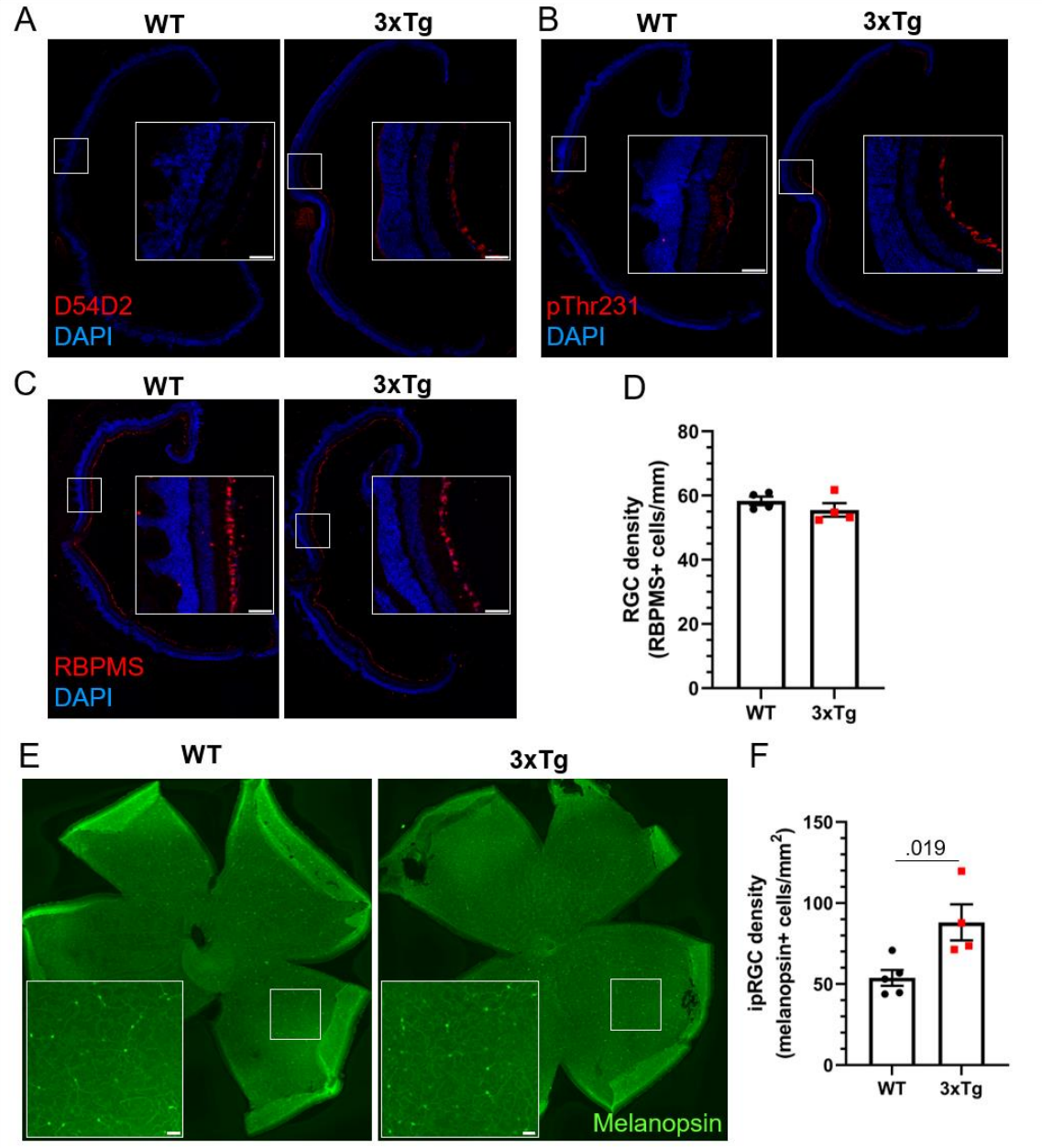

Figure 3.3: Increased ipRGC density in 3xTg retinas

13-month-old WT and 3xTg sagitally sectioned retinas were stained with DAPI and (A) D54D2 for Aβ, (B) pThr231 for phospho-tau, and (C) RBPMS for retinal ganglion cells.
(D) Quantification of (C).
(E) Retina whole mounts from WT and 3xTg mice stained for melanopsin to identify ipRGCs.
(F) Quantification of (E).
All analyses are two tailed Student's t-tests. All data plotted as mean ± SEM. Scale bars = 50μm.

disparities (Dacey et al., 2005) in melanopsin expression. This increase in the ipRGC population

may explain the observed heightened sensitivity to light in non-image-forming visual tasks. When the lights turn on at the beginning of the new light cycle in the jet lag task, a mouse with more ipRGCs might receive that photic stimulus as a more intense entrainment cue than a mouse with fewer ipRGCs. This is one possible contributor to their accelerated re-entrainment in jet lag in 3xTg mice.

3.4 Discussion

In this study we examined female 3xTg mice for their light sensitivity for purposes of circadian behavior. We found an increase in negative masking, suggesting that an increased response to circadian photic cues is not entirely SCN dependent. We also found dramatically faster re-entrainment in a jet lag test performed under dim lighting conditions, indicating that light intensity plays a role in the difference between 3xTg and WT mice in this task. Finally we examined the retinas of these mice, finding AD pathology accompanied by an increased density of ipRGCs in 3xTg mice. Together, these results demonstrate that 3xTg mice have increased light sensitivity in circadian behavioral tasks, possibly because of an increase in ipRGCs in the retina. This light sensitivity may contribute to the accelerated re-entrainment we observe in AD model mice.

The finding of increased ipRGC density in 3xTg retinas is surprising. The reported ipRGC loss in AD (La Morgia et al., 2016) and an AD mouse model (Carrero et al., 2023) suggests changes in the opposite direction of what we observed. Retinal inflammation and some indications of retinal degeneration have been found in 3xTg mice, though that study did not examine RGC number (Grimaldi et al., 2018). The limited research on visual ability in 3xTg mice has not reported deficits (King et al., 2018), while some decrease in RGC activity can be observed by electroretinography (Frame et al., 2022). Our finding in 3xTg mice of heightened responses to

photic cues in two circadian behavioral paradigms, as well as increased ipRGC density, runs contrary to these findings and suggests that in some models, or at some stages of disease progression, ipRGC signaling may be increased rather than decreased. Indeed, while human data show a decrease in ipRGCs in postmortem AD retinas (La Morgia et al., 2016), another study did not find deficits in the pupillary light reflex, an ipRGC-dependent task, in pre-symptomatic AD subjects (Oh et al., 2019). Thus, the reported decline in ipRGC number in AD may be specific to disease stage. Some evidence suggests that ipRGC number declines with age in mice (Semo et al., 2003) and humans (La Morgia et al., 2016), which may slow re-entrainment in aged WT mice. ipRGC density in 3xTg retinas over aging should be studied to determine whether this decline is modified and whether that may contribute to the observed increased ipRGC count at 13 months. More research will be necessary to determine the causes of differences between disease stages.

The heterogeneity of the ipRGC population should also be further studied in these mice. Some ipRGC subtypes express melanopsin at very low levels and would be undetectable by the IHC technique used here. One possible explanation for the increase in melanopsin+ cells in the 3xTg retinas could be an increase in melanopsin expression in non-M1 ipRGCs rather than an increase in the number of the ipRGC population. As these non-M1 ipRGCs have different functions and projection patterns than M1 ipRGCs (reviewed in Do, 2019), such a change in the ipRGC population could have consequences for diverse visual circuits and behaviors. This possibility is explored further in Chapter 4.3.

The difference in ipRGCs between WT and 3xTg mice may run deeper than their relative numbers. Previous research has reported changes in ipRGC morphology in AD (La Morgia et al., 2016). ipRGCs express melanopsin throughout their dendrites and also use them to receive

inputs from bipolar cell carrying light information from rod and cone cells, so changes in dendritic arborization could dramatically influence ipRGC responsiveness to light. Staining retinas for melanopsin and choline acetyltransferase (ChAT) and imaging by confocal microscopy would allow for detailed analysis of ipRGC morphology. ChAT labels cells in the inner plexiform layer (IPL) of the retina, providing a landmark by which to categorize the layers of the retina in which ipRGC dendrites are found (Schmidt et al., 2008). Differing cell morphologies and depths of dendritic projections characterize the different ipRGC subtypes (Do, 2019), so these imaging analysis would also allow us to detect subtype-specific differences between WT and AD model retinas. This could offer insight into what other ipRGC-mediated behaviors might be altered in AD models, as different ipRGC subtypes project to different brain regions and are involved in different types of visual processing.

Electrophysiology could also be used to study functional differences in light sensing between AD and WT mice. Electroretinography can be used to study whole-retina electrical responses to lights, and has been used in AD model research (Carrero et al., 2023), but it does not specifically measure ipRGC activity. For that, patch-clamp electrophysiology can be used to measure individual ipRGC responses to light pulses of different intensities and under different conditions (Do, 2022). This technique allows for isolating ipRGC-intrinsic photosensitivity by blocking signaling from rod and cone cells during ipRGC recording (Do, 2022), permitting further study into whether differences in ipRGC activity are cell-intrinsic or not. These experiments would allow us to assess whether ipRGCs are functionally different between AD and WT mice, providing further evidence to explain differences in light sensing between mice, and might point towards the cell types most responsible for these differences.

We have previously found that 3xTg and 5xFAD mice both re-entrain more rapidly than controls in a jet lag protocol. Currently we have only examined the light sensitivity of 3xTg mice and do not know if 5xFAD also share these masking and ipRGC phenotypes. These experiments should be repeated in 5xFAD mice. We predict that they would have similar results, and given the dramatic difference in re-entrainment between 5xFAD and WT mice, the visual and retinal phenotypes may be even more stark in 5xFAD than 3xTg mice. If this is the case, it would be consistent with the hypothesis that altered retinal function and heightened light sensitivity contribute to accelerated re-entrainment in AD model mice. If 5xFAD mice do not show these phenotypes, though, it suggests that either (1) different mechanisms are converging to cause the same re-entrainment phenotype in the two AD models, or (2) the retinal phenotype in 3xTg mice is not related to the re-entrainment phenotype, which is actually caused by some other mechanism which is shared between the two models. Such a divergence between the two groups could be the result of differences in background strain, age, or the genetic manipulations made in each model.

In summary, we found significantly heightened negative masking and accelerated re-entrainment under dim light in 3xTg mice. 3xTg mice also had a higher density of ipRGCs in their retinas. This indicates heightened sensitivity to light for circadian purposes in these mice and points to an increase in ipRGCs as a possible mechanism behind this phenotype.

3.5 Methods

3.5.1 Mice

All animal experiments were conducted in accordance with the University of Virginia Institutional Animal Care and Use Committee. Animals were housed in a temperature and humidity controlled vivarium (22-24°C, ~40% humidity) and were provided with food and water

ad libitum. 3xTg experiments were conducted with 8- to 12-month-old female 3xTg mice on a B6129 background (Oddo et al., 2003) (Jackson Laboratory #034830), with age-matched B6129SF2/J (Jackson Laboratory #101045) females as wild type controls.

3.5.2 Behavioral analysis

Behavioral testing protocol was adapted from (Grippo et al., 2017). Mice were individually housed in cages (Nalgene) containing running wheels in light-tight boxes which were illuminated with timed fluorescent lights ($590x10^{12}$ photons/cm^2/s). Wheel running data were collected and analyzed with ClockLab software (Actimetrics). Activity onset was automatically detected by ClockLab software and when necessary, corrected by eye by an experimenter blinded to genotype and treatment group. Mice were allowed to habituate to running wheel cages and entrained to a 12hr:12hr LD cycle for at least 7 days before experiments began.

In the jet lag re-entrainment trial, the onset of the dark phase was abruptly advanced by 6 hours and running wheel activity was recorded for at least 7 days after light cycle shift. PS_{50} values were calculated using Prism software (GraphPad) by fitting a sigmoid dose-response curve to onset times in days 0-6 after light cycle shift (Kiessling et al., 2010). Total running distance and preference for running in the dark were measured after all mice had completely re-entrained after a phase shift and were averaged across 2 days. To determine free running period, after all mice had completely re-entrained they were switched to DD and period was calculated from the onset of activity across 7 days.

In masking trials, light intensity was decreased by wrapping fluorescent lights in neutral density filter films and measured with a spectrometer (Sekonic Spectrometer C-800). Illuminance was measured in lux, and photon flux, summed from 380 to 780nm, was calculated using formulas in the supplementary materials in (R. J. Lucas et al., 2014). A 1hr light pulse was delivered from

ZT13-14. Percent running in light was calculated by comparing to the activity measured during the same period of constant darkness on the preceding day for each mouse. Individual trials were separated from each other by 3-4 days.

3.5.3 Histological analysis and imaging

All mice used for histological analysis were sacrificed from ZT5-9. Mice were anesthetized with a ketamine:xylazine solution and transcardially perfused with chilled phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA).

Retinas were dissected and stained as in (Gao et al., 2022) with minor modifications. For retinal sectioning, eye cups were dissected and post-fixed in 4% PFA for 30min at room temperature. Eye cups were cryoprotected in 30% sucrose in PBS overnight at 4°C, frozen, and sectioned on the cryostat at 14µm. Sections were again post-fixed with 2% PFA for 30min at room temperature. Sections were then blocked and stained. Tissues were incubated with blocking solution (5% bovine serum albumin, 2% horse serum, 1% Triton X-100 in PBS) for 2h at room temperature. Primary antibodies were diluted in blocking solution and placed on slides overnight at 4°C. Sections were washed and incubated with secondary antibodies, diluted in blocking solution, for 2h at room temperature. Sections were washed and treated with DAPI (Sigma-Aldrich) (1:2000 in PBS for 15min). Sections were washed and then mounted with ProLong Gold mounting media (Thermo Fisher).

For retina whole-mounts, eye cups were dissected and post-fixed with 2% PFA for 1h on ice. They were then washed and blocked with blocking solution described above for 2h at room temperature. They were incubated with primary antibody diluted in blocking solution overnight on a shaker at 4°C. They were then washed and incubated with secondary antibody diluted in

blocking solution overnight on a shaker at 4°C. Finally the retinas were removed from the eye cup, cut to create four quadrants, and flat mounted with ProLong Gold mounting media.

Primary antibodies used were anti-phospho-tau pThr231 (rabbit, Invitrogen 701056, 1:500), anti-RBPMS (rabbit, Abcam ab152101, 1:500), anti-amyloid D54d2 (rabbit, Cell Signaling 8243, 1:200), and anti-melanopsin (Panda et al., 2002) (rabbit, 1:2500). Secondary antibodies used were donkey anti-rabbit Alexa Fluor 594 (Invitrogen A21207) and donkey anti-rabbit Alexa Fluor 647 (Invitrogen A32795).

Images were acquired with a Keyence BZ-X800 fluorescence microscope and images were stitched using BZ-X800 Analyzer software. Cell quantification was performed with ImageJ. For quantification of RGCs in sectioned retinal tissue, 500-700μm lines were drawn along the RGC layer beginning 300μm to either side of the optic nerve exit point and all RBPMS+ cells were counted. For quantification of ipRGCs in retina whole mounts, four 0.25mm^2 squares were drawn in each retina at equal distances from the optic nerve exit point and melanopsin+ cells were counted in each, with cell density being calculated as an average of these counts.

3.5.4 Statistical analysis

All data are presented as mean ± SEM. Statistical analyses were performed with Prism software (GraphPad). Masking and time of onset of running after jet lag was analyzed by mixed model with Sidak post-hoc comparisons. All other data were analyzed by two tailed Student's t-test. Differences between groups were determined to be statistically significant when $p<.05$.

3.6 Acknowledgements

We thank R. Grippo and Q. Tang for help with behavioral experiments, J. Cole for assistance with retinal staining, Virginia Alzheimer's Disease Center for providing antibodies, and I.

Provencio for advice and antibodies. This research was funded by grants from the Owens Family Foundation, Commonwealth of Virginia's Alzheimer's and Related Diseases Research Fund and K08DK097293 to HAF, T32GM139787 to TKW and R35GM140854 to ADG.

Chapter 4 Discussion and future directions

4.1 Summary

This dissertation work studied the circadian behavior of AD mouse models, focusing on re-entrainment after jet lag. I found that AD model mice demonstrate accelerated re-entrainment. Further exploration of this phenotype pointed towards Aβ pathology, and away from tau and neuroinflammation, as a causal factor in this behavioral difference. I next examined vision in these mice, finding that AD model mice were more sensitive to light in another behavioral task, and observing an increased population of cells in the retina that may contribute to these phenotypes.

In this chapter I will expand on these conclusions, offer hypotheses that could explain some of these results, propose future directions to test those hypotheses, and discuss the implications of this work for AD research. First I will discuss the role(s) that the SCN may play in altered re-entrainment in AD, attempting to interpret the results from Chapter 2 through avenues other than the one explored in Chapter 3. Then I will address possible causes and consequences of the altered retinal function observed in Chapter 3. Next I will discuss other aspects of entrainment and circadian rhythms that were not explored here and how they may also be affected in these models. I will conclude with a discussion of the implications this research has for our understanding and treatment of Alzheimer's disease.

4.2 Re-entrainment and altered SCN function in AD

I found accelerated re-entrainment in a jet lag paradigm in multiple AD mouse models. Immediate future directions following from these results, including performing jet lag tests in younger 3xTg and 5xFAD mice, in older PS19 mice, and studying molecular rhythms in the SCN

in these AD models, were discussed in Chapter 2.4. Here I will discuss the SCN (introduced in Chapter 1.2.2) in greater depth. It is the brain area which controls entrainment and the most obvious candidate region for explaining a re-entrainment phenotype. Indeed, genetic perturbations to the SCN can produce behavior in a jet lag paradigm very similar to that of the AD model mice in these experiments (Yamaguchi et al., 2013). Therefore, while we found altered visual function that could explain the re-entrainment phenotype in our mice, it is worth considering possible mechanisms in the SCN which may also contribute to accelerated re-entrainment. Different neuronal subpopulations and astrocyte functions in the SCN will be discussed as possible causes of SCN clock alterations that may affect re-entrainment.

4.2.1 Altered neuronal populations in the SCN

The SCN is a heterogeneous region in which neurons have different characteristics and functions. Several neuronal populations in the SCN have been directly implicated in entrainment and may contribute to the re-entrainment phenotype observed in AD model mice (Fig. 4.1).

AVP is a neuropeptide heavily expressed in the shell region of the SCN. AVP+ neurons receive inputs from the VIP+ neurons of the SCN core, and project onto other AVP+ neurons as well as out of the SCN. When V1a and V2b receptors, the receptors for AVP, are knocked out or pharmaceutically inhibited, mice in a jet lag experiment re-entrain much more rapidly than controls (Yamaguchi et al., 2013). Thus, loss of AVP signaling can produce accelerated re-entrainment and may play a role in the phenotype we observe in AD model mice.

The neuropeptide VIP is another regulator of re-entrainment. VIP is expressed in neurons in the SCN core which receive inputs from the RHT and then project to the AVP-expressing neurons of the SCN shell. VIP or VIP receptor agonist treatment of SCN cultures can induce phase shifts of electrical activity and AVP release *in vitro* (Reed et al., 2001; Watanabe et al., 2000), and

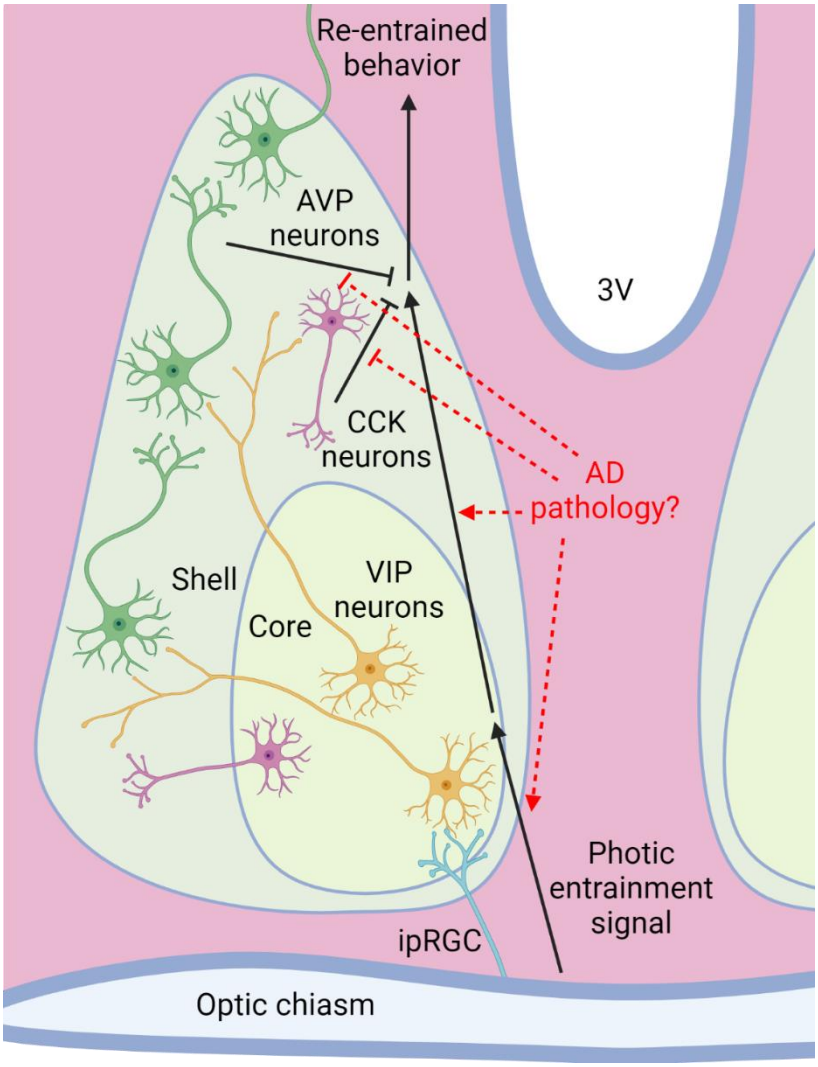

Figure 4.1 Re-entrainment signaling in the SCN

A diagram of the interactions between different cell types in the suprachiasmatic nucleus (SCN) during re-entrainment. Light signals detected in the retina are carried by intrinsically photosensitive retinal ganglion cells (ipRGCs) to the SCN. There they synapse on vasoactive intestinal peptide (VIP) + neurons in the SCN core. VIP+ cells carry this entrainment signal to cells in the SCN shell, including arginine vasopressin (AVP) and cholecystokinin (CCK) + cells. These cells, which maintain and reinforce the SCN clock, will resist phase shifting in response to photic entrainment signals that are out of phase with the SCN clock, thus slowing re-entrainment. SCN outputs integrate these competing messages and signal to other structures to synchronize the circadian clocks of other brain and peripheral tissues. AD pathology could lead to accelerated re-entrainment by strengthening photic entrainment signaling (either from ipRGCs or VIP+ neurons), or by impairing the mechanisms which resist out-of-phase entrainment signals (AVP+ or CCK+ neurons). Figure created with BioRender.com.

knocking out VIP signaling impairs the ability of cells in the SCN to synchronize with each other, resulting in profoundly abnormal circadian behavior (Aton et al., 2005; Harmar et al., 2002). Interestingly, mice re-entrained more rapidly in a jet lag paradigm when they *overexpress* the human version of the VIP receptor $VPAC_2R$ (Shen et al., 2000). This contrasts with AVP signaling, where AVP receptor KO resulted in more rapid re-entrainment (Yamaguchi et al., 2013).

This suggests a circuit in which VIP signaling drives re-entrainment in jet lag while AVP signaling reinforces the SCN internal clock and resists mismatched photic cues, acting as a brake on re-entrainment (Fig. 4.1). This is supported by data on re-entrainment of the molecular clock in different regions of the SCN after jet lag. In rats, the phase of PER1 transcription shifts rapidly in the VIP-expressing core, synchronizing with the new light cycle within a day, while it takes more than a week for the AVP-expressing shell to fully shift (Nagano et al., 2003). According to this model of re-entrainment, if these signaling pathways are involved in the accelerated re-entrainment phenotype observed here, we would expect (1) decreased AVP signaling in AD, i.e. a weaker brake on re-entrainment; and/or (2) increased VIP signaling in AD, i.e. a stronger force for re-entrainment.

Some research has suggested that AVP signaling in the SCN is decreased in AD. Decreased AVP expression has been reported in the SCN of AD patients (Harper et al., 2008; R. Y. Liu et al., 2000), though this has been disputed (J. L. Wang et al., 2015). One study of 3xTg mice supported this, reporting decreased numbers of AVP+ cells in the SCN of 10- to 11-month-old male 3xTg mice (data for females was not reported) (Sterniczuk et al., 2010). Given the small size and regional variations in the SCN, staining we performed in these experiments for AVP and other SCN markers did not lend itself to quantification. We did not observe qualitative differences

between AD model mice and controls in AVP expression in the SCN. In future experiments, more comprehensive sectioning and staining to capture the entire SCN could be performed to allow for reliable quantification of SCN neuronal populations in our animals. Alternatively, Western blotting and qPCR on SCN tissue would give quantitative data on neuropeptide protein and RNA expression. Given the evidence in the literature suggesting decreased AVP signaling in the AD brain, this hypothesized mechanism merits further examination.

Data on VIP expression in AD is less supportive of the hypothesis that it contributes to the re-entrainment phenotype observed here. One study examining postmortem human SCN tissue found significantly decreased VIP+ cell count in females ages 41-65 with AD, as well as trends in the same direction among male and older female patients (Zhou et al., 1995). VIP+ cell count is also reportedly decreased in the SCN of male 3xTg mice (Sterniczuk et al., 2010). We did not observe qualitative differences in VIP staining. To my knowledge there are no reports of increased VIP or $VPAC_2R$ expression in the SCN in AD patients, so evidence to support a role for enhanced VIP signaling in accelerated re-entrainment is lacking.

AVP- and VIP-expressing neurons are the best studied cell populations in the SCN, but another population expressing the neuropeptide cholecystokinin (CCK) also plays a role in entrainment. CCK is expressed in some neurons in both the core and shell of the SCN and labels a distinct population from AVP+ or VIP+ cells. A mouse knocked out for CCK did not show dramatically altered circadian behavior (Hannibal et al., 2010), but a more recent study which ablated CCK+ neurons in the SCN entirely, rather than just knocking out the gene, demonstrated important roles for this cell population (Xie et al., 2023). Mice lacking CCK+ neurons, or those which had SCN CCK+ neurons chemogenetically inhibited, re-entrained more rapidly than controls in a jet lag paradigm. Calcium imaging to measure the daily activity rhythms of cells in the SCN in a jet lag

paradigm found that CCK+ neurons shifted more slowly than VIP+ neurons (Xie et al., 2023). Together, these results suggest a role for CCK+ neurons as a brake on re-entrainment, similar to AVP+ neurons. Loss of these cells or decreases in their activity are therefore possible SCN-intrinsic mechanisms by which AD pathology could drive accelerated re-entrainment. Decreased CCK levels have been observed in the CSF of AD patients (Plagman et al., 2019), though this may reflect changes in cortical CCK+ cells (Mazurek & Beal, 1991) rather than ones in the SCN. Research is still needed to determine how CCK+ cells in the SCN are affected in AD. Curiously, CCK signaling may also be involved in light sensing for circadian purposes in the retina (Shimazoe et al., 2008), and thus could be acting at multiple levels of the photoentrainment pathway.

4.2.2 Altered astrocyte functions in the SCN

While these neuronal populations are all important for entrainment and the maintenance of circadian rhythms, another cell type may also contribute to SCN disruptions in AD: astrocytes. Astrocytes play important roles in regulating extracellular neurotransmitter levels throughout the brain, and recent research has implicated this process in SCN function (Fig. 4.2). Extracellular glutamate in the SCN, which is derived from astrocytes, fluctuates rhythmically (Brancaccio et al., 2017, 2019). Extracellular glutamate levels can be raised in SCN cultures by inhibiting glutamine synthetase (GS), an enzyme which breaks down glutamate in astroctyes, or by blocking astrocytic glutamate uptake by inhibiting the glutamate transporters excitatory amino acid transporter 1 (EAAT1, also known as GLAST) and EAAT2 (aka GLT-1) (Brancaccio et al., 2017). Either of these glutamate-increasing treatments causes neurons to become desynchronized and blunts the amplitude of clock gene expression. Decreases in astrocytic glutamate clearance capacity might therefore result in a weakened SCN clock. Notably, single cell RNA sequencing

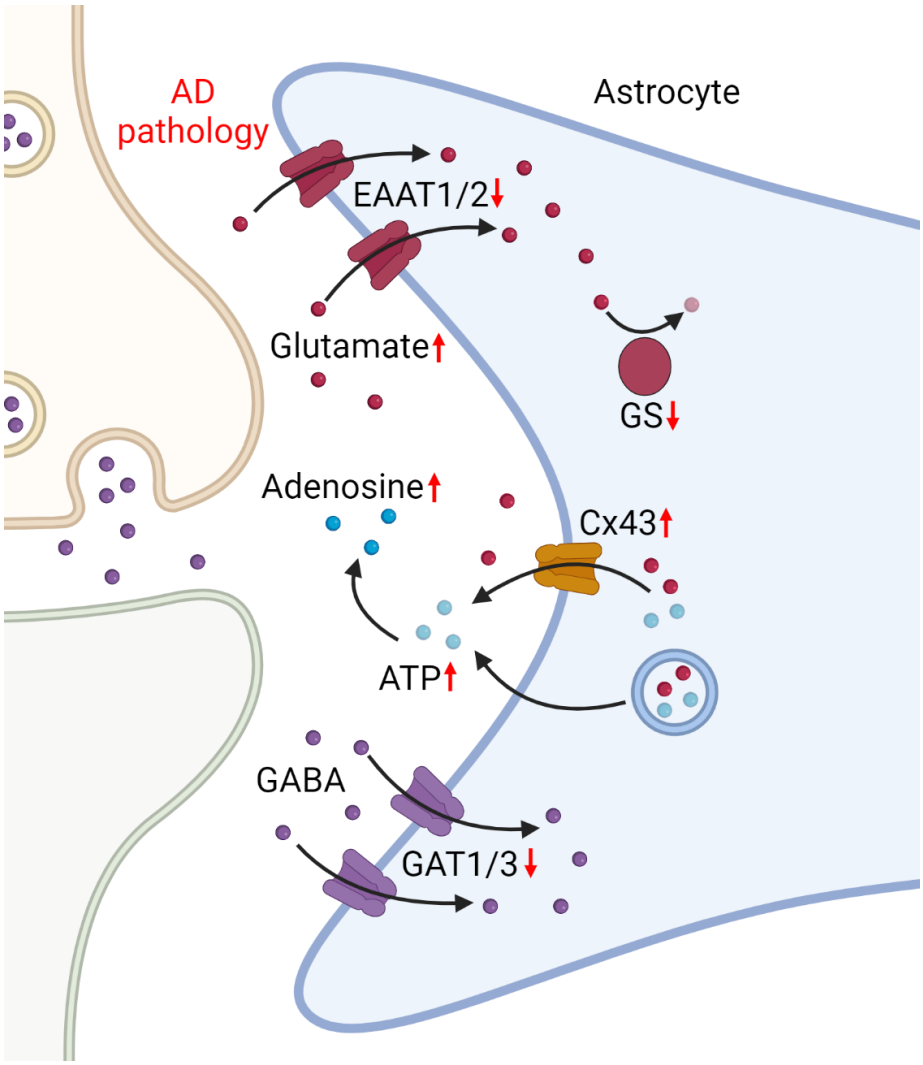

Figure 4.2 Mechanisms of astrocyte neurotransmitter regulation in the SCN

A diagram of neurotransmitter regulatory mechanisms in astrocytes that may contribute to altered re-entrainment in the SCN. Astrocytes clear the excitatory neurotransmitter glutamate from the extracellular space through the transporters excitatory amino acid transporter (EAAT) 1 and 2, as well as converting glutamate to glutamine through the action of the enzyme glutamine synthase (GS). EAAT and GS expression are decreased in AD, while extracellular glutamate is increased. Astrocytes release ATP (as well as glutamate) via connexin 43 (Cx43) hemichannels and via vesicular release. In the extracellular space this ATP is converted to adenosine. Cx43 expression is increased in AD, as are extracellular ATP and adenosine. Astrocytes also regulate extracellular levels of GABA, the primary neurotransmitter of SCN neurons, through expression of GABA transporter (GAT) 1 and 3. GAT1 and 3 expression are decreased in AD. In AD these altered neurotransmitter regulatory mechanisms may result in increased extracellular glutamate and adenosine and decreased rhythmicity of GABA levels, possibly contributing to a weakened SCN circadian clock and more rapid re-entrainment. Figure created with BioRender.com.

(scRNAseq) of astrocytes from AD and control brains showed downregulation of SLC1A3 and SLC1A2 (encoding EAAT1 and 2, respectively), as well as GLUL (encoding GS) (Sadick et al., 2022). Protein levels of EAAT1 and 2 are also decreased in the brain in AD (Jacob et al., 2007), as are protein levels and enzymatic activity of GS (Robinson, 2000; Smith et al., 1991). These studies of expression of astrocytic glutamate clearance mechanisms were performed on cortical tissue. It is unclear if the same is true for the SCN.

In addition to decreased glutamate clearance, astrocytes could contribute to elevated glutamate levels through increased glutamate release. Astrocytic glutamate release, like uptake, is rhythmic in the SCN (Brancaccio et al., 2017). Astrocytes treated with Aβ *in vitro* released glutamate by a mechanism dependent on connexin 43 (Cx43) hemichannels (Orellana et al., 2011). Cx43 is upregulated in the brain in AD and in 5xFAD mice (Angeli et al., 2020; Nagy et al., 1996). Astrocytes can also release glutamate via secretory vesicles (Ni & Parpura, 2009), another process which may become dysregulated under pathological conditions (Bal-Price et al., 2002). If astrocytes lose their capacity for regulating glutamate levels in the SCN during AD, this may contribute to desynchronization of the SCN clock and downstream circadian behavioral deficits. This could be a result of impaired glutamate clearance and breakdown, an increase in glutamate release, or a combination of both. Given the prominent role that glutamate excitotoxicity plays in neurodegeneration in AD, astrocytic glutamate regulation in the SCN is a promising target of future research.

Astrocytes play important roles in maintenance of the SCN clock through other signaling pathways as well. The circadian rhythm of extracellular GABA levels is important for the SCN clock and is dependent on astrocytes (Barca-Mayo et al., 2017; Patton et al., 2023). GABA is the primary neurotransmitter utilized in intra-SCN communication by neurons, but as with

glutamate, astrocytes are important for regulating extracellular levels of the molecule. Astrocytes express the GABA transporters GAT1 and GAT3 rhythmically, and impaired astrocytic GABA uptake is associated with altered circadian behavior (Barca-Mayo et al., 2017). Extracellular GABA levels and GAT expression are both rhythmic in the SCN and cycle in antiphase to each other, with astrocytic GATs upregulated during the day, removing GABA from the extracellular space, and GATs decreasing during the night, allowing extracellular GABA to rise (Patton et al., 2023). As with the glutamate transporters discussed above, expression of both SLC6A1 (encoding GAT1) and SLC6A11 (encoding GAT3) is decreased in astrocytes in AD brains (Sadick et al., 2022). Altered rhythmic GABA clearance is thus another plausible mechanism by which astrocytic dysfunction may alter SCN function, though given that GABAergic signaling is generally thought to be less severely affected than glutamatergic signaling in AD, glutamate probably remains a likelier explanation. A side note on one of the studies discussed above: global BMAL1 KO had been associated with premature aging (Kondratov et al., 2006) and cognitive impairments (Wardlaw et al., 2014), and here an astrocyte-specific BMAL1 deletion was found to impair performance in memory tasks, not just alter circadian behavior (Barca-Mayo et al., 2017). Astrocytic circadian rhythms are clearly important to many of the processes and phenotypes related to AD and deserve more extensive treatment than can be provided here.

A last astrocytic mechanism known to affect entrainment is adenosine signaling. In response to endocannabinoid signaling, astrocytes release ATP, which is quickly converted to adenosine in the extracellular space. Adenosine can affect SCN neurons and influence entrainment (Hablitz et al., 2020). Astrocytes release ATP when treated with Aβ *in vitro* (E. S. Jung et al., 2012; Orellana et al., 2011), and as with glutamate this ATP release was dependent on Cx43 (Orellana et al., 2011). While changes in adenosine signaling are observed in AD (reviewed in Launay et al.,

2023), changes in astrocyte adenosine release that may affect SCN function need further exploration.

4.3 Altered ipRGC population and function in AD

While the SCN is essential for entrainment, more rapid re-entrainment may be driven by changes in the retina rather than the SCN. Heightened light sensitivity could cause the shifted light schedule to be perceived as a more potent entrainment stimulus in AD model mice. I found increased sensitivity to light in 3xTg mice in a negative masking experiment, a task dependent on ipRGCs and not on the SCN. I examined the retinas of these mice and found more melanopsin+ cells, which we interpret as an increase in the number of ipRGCs. Some immediate future directions following from these results, including studying ipRGC morphology and electrophysiology in 3xTg retinas and repeating masking and retina experiments in 5xFAD mice, were discussed in Chapter 3.4. Here I will discuss at greater length possible explanations for the observed change in the ipRGC population, other interpretations of the melanopsin data, and implications of these findings for non-circadian functions in AD.

4.3.1 Increased ipRGC signaling

Our finding of increased ipRGC density in 3xTg mouse retinas fits well with the re-entrainment and masking data, but it came as a surprise nonetheless. Previous research has found decreased ipRGC density in human AD and in a mouse model (Carrero et al., 2023; La Morgia et al., 2016). However, those studies have important differences from the ones presented here which may explain these divergent findings. First, the data finding decreased ipRGCs in AD patients only examined the latest stages of the disease (La Morgia et al., 2016). More research on the dynamics of the ipRGC population over disease progression will be needed before we know whether ipRGCs play a role in the circadian symptoms in early AD. Regarding the recently

published finding of decreased ipRGC density in a mouse model of AD, this study used the APP/PS1 model (Carrero et al., 2023) rather than the 3xTg in our experiment. APP/PS1 mice reportedly lack the accelerated re-entrainment phenotype that we observe in 3xTg and 5xFAD mice (Kent et al., 2019; Otalora et al., 2012). Given that their behaviors differ, it is not surprising that we find that the pathophysiology in these models also differs. In fact, this difference circumstantially hints that the increased ipRGCs we observe are responsible for the altered re-entrainment behavior in 3xTg mice, as an AD model lacking this pathophysiology in the retina also lacks the behavioral phenotype.

Nevertheless, the finding of increased ipRGC density in an AD model is strange, and without further experiments we can only speculate as to the cause for this phenotype. Because ipRGC maturation occurs quite early in development (J. A. Lucas & Schmidt, 2019) and RGCs are not thought to replenish in adult mammals, it is unlikely that AD pathology is inducing proliferation and generation of new ipRGCs. One possibility is that ipRGC development is altered in the 3xTg model, resulting in a lifelong increase in ipRGC number. Because of the early age at which ipRGCs differentiate and mature, this would more likely be a quirk of the genetic manipulation in the mice rather than a result of AD pathology. This hypothesis could be tested by examining 3xTg mice at just a few weeks old, at which point ipRGCs will have likely finished their maturation (J. A. Lucas & Schmidt, 2019) but long before 3xTg mice begin to show AD pathology.

Another possibility is that ipRGC density is increased in AD model mice because AD pathology modifies normal aging-related changes in the retina which result in decreased ipRGC density. The previously discussed study examining ipRGCs in late-stage AD patients also found a significant negative correlation between age and ipRGC density in healthy control subjects (La

Morgia et al., 2016). Likewise, the study examining ipRGCs in APP/PS1 mice found a significant decline in ipRGC count in WT mice between 6 and 12 months of age (Carrero et al., 2023). Neither of these studies, though, found that ipRGC density significantly decreased with aging in AD groups. The reasons for this decline in ipRGCs with aging are not known, and further research will be needed to identify possible mechanisms that might be altered by AD pathology. Though the mechanisms underlying this ipRGC loss with aging have not been researched, the dynamics of the RGC population more generally may offer some suggestions. Mitochondrial dysfunction, oxidative stress, and autophagy, three interrelated mechanisms which are altered in both healthy aging and AD, have all been implicated in RGC degeneration in aging-related retinal diseases (Abu-Amero et al., 2006; Bell et al., 2020; Moreno et al., 2004). Regardless of the mechanism behind it, the hypothesis that increased ipRGC counts in AD mice are a result of an altered rate of aging-related RGC death could be tested by examining ipRGC numbers across aging in AD model and control mice.

4.3.2 Differential expression of melanopsin within ipRGCs

In Chapter 3, an increase in melanopsin+ cells detected by IHC was interpreted as an increase in ipRGCs. However, melanopsin staining is not capable of detecting all ipRGCs. The ipRGC subtypes M4, M5, and M6 all express melanopsin at low enough levels not to be detected by IHC. Therefore, an alternate explanation for an increase in melanopsin+ cells could be an increase in melanopsin expression among ipRGCs that otherwise would not have been detected (Fig. 4.3). It is unlikely that ipRGCs can switch subtypes in adulthood, as they are differentiated from each other very early in development (J. A. Lucas & Schmidt, 2019) and to my knowledge there have been no reports of such switching after development. More plausible is an increase in melanopsin expression in non-M1-3 cells leading to more of them becoming detectable. In this

manner melanopsin+ cells measured by IHC could be increased without there being an increase in the total number of ipRGCs.

While some mechanisms are known to regulate melanopsin expression, discussed below, the effects of AD pathology on melanopsin levels have not been studied. Further experiments would be necessary to generate hypotheses as to how melanopsin levels might be increased in ipRGCs that would otherwise go undetected by IHC. This could be explored by performing scRNAseq in the retinas of AD genetic model mice or mice which have received intraocular Aβ injections. This would show the transcriptional changes that are undergone in ipRGCs when exposed to AD pathology and could point to cell-intrinsic and cell-extrinsic mechanisms which might influence melanopsin expression levels. Follow-up experiments could attempt to modify these mechanisms in 3xTg mice and test whether this affects jet lag and masking behavior.

Alternatively, melanopsin expression could be increased as an artifact of other mechanisms less directly relevant to AD pathology. ipRGC melanopsin expression adapts to light, with melanopsin levels inversely correlated with light exposure (Hannibal et al., 2005). Therefore behavioral or physiological differences which decreased irradiance (light exposure) on the retina, such as squinting or more constricted pupils, would be predicted to increase melanopsin expression. Baseline pupil diameter is decreased in APP/PS1 AD model mice (Liebscher et al., 2016). The relevance of this data is questionable, though, as these mice do not share several aspects of retinal and circadian physiology we found in the experiments presented here (discussed in Chapter 4.3.1). To my knowledge these phenotypes have not been reported on in 3xTg or 5xFAD mice. Melanopsin mRNA levels also fluctuate rhythmically under DD conditions in rats (Sakamoto et al., 2004), showing that melanopsin is circadian regulated independently of any light regulation. Thus, apparent differences in melanopsin expression levels

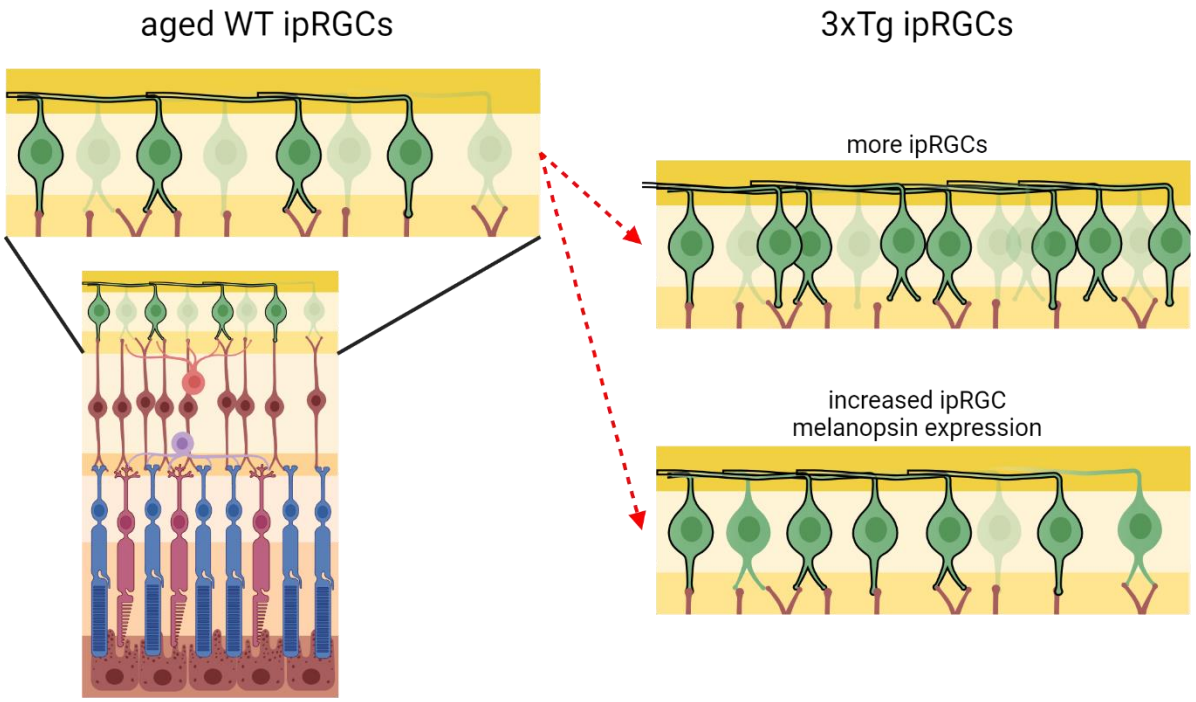

Figure 4.3 Possible mechanisms of increased melanopsin+ cells in the 3xTg retina

Intrinsically photosensitive retinal ganglion cells (ipRGCs) detect and transmit light information for non-image-forming vision. ipRGCs are scarce relative to other RGCs, but in this figure their density is exaggerated and other RGCs omitted. ipRGCs can be identified by their expression of melanopsin, but only some subtypes of ipRGCs express the protein at high enough levels to detect by immunohistochemistry (IHC) (depicted here as either dark or pale green). When we stained aged WT and 3xTg mice, we found more melanopsin+ cells in the 3xTg retina. Two interpretations of this result are depicted here. In one, 3xTg retinas contain more ipRGCs. In the other, ipRGCs which were previously undetectable by IHC have upregulated melanopsin expression and become detectable, resulting in an apparent increase in melanopsin+ cells without a change in ipRGC number. Figure created with BioRender.com.

could be a result of, rather than a cause of, circadian alterations. We did not assess squinting, pupillary diameter, or retinal molecular rhythms in these experiments and cannot rule these mechanisms out as possible explanations for increased detection of melanopsin+ cells.

The interpretation that increases in detected melanopsin+ cells represent an increase in melanopsin expression rather than an increase in ipRGC count could be examined in several ways. The hypotheses that increased observed melanopsin+ cells are an artifact of lighting or

retinal circadian rhythms could be tested relatively easily. By observing the eyelids and pupils of live mice under normal lighting conditions we could determine whether irradiance on the retina may be decreased in AD model mice. Because the light-induced suppression of melanopsin expression appears to be relatively short-lived, largely washing out after 5 days in DD (Hannibal et al., 2005), mice could be maintained in DD for a week before harvesting retinas and quantifying melanopsin+ cells, which would be expected to eliminate any differences based on irradiance in the retina. To address the question of circadian regulation of melanopsin expression, by collecting retinal tissue at different ZTs and performing qPCR to measure rhythmic circadian gene transcription, we could determine whether the clock in the retina is different between AD and control mice and whether this is a possible contributor to differences in melanopsin detection.

A more demanding experiment could address the question of melanopsin expression levels conclusively by using genetic tools rather than immunohistochemistry to detect ipRGCs. By crossing 3xTg and control mice to a melanopsin reporter mouse (Ecker et al., 2010), which allows highly sensitive detection of melanopsin-expressing cells, we could quantify all melanopsin-expressing cells in the retina. This would allow for quantification of all subtypes of ipRGC, regardless of melanopsin expression levels, allowing us to determine conclusively whether ipRGC counts are elevated in our AD model mice. This would also be useful in quantifying and analyzing the morphology of other ipRGC cell types in the AD model retina to determine whether there are other changes which went undetected by IHC.

4.3.3 Altered retinohypothalamic signaling

For understanding the contributions of ipRGCs to altered circadian behavior, ipRGC signaling to the SCN is more important than simple ipRGC density. Given the increased ipRGC count and the

heightened masking response, we would predict that ipRGC inputs to the SCN are increased in 3xTg mice. This could be explored through staining and tracing techniques. A simple method is to inject a tracer molecule, such as a fluorescently labeled cholera toxin B, into the eye (Carrero et al., 2023). This tracer is taken up by RGCs, including ipRGCs, and spreads down their axons to label their projections. Imaging of the SCN with this tracer molecule would then allow for quantification of the ipRGC inputs to the SCN, which could be a more sensitive and useful measure than simply the number of ipRGC cell bodies in the retina. Increased ipRGC inputs to the SCN in AD model mice would strengthen the hypothesis that heightened light sensitivity in the retina contributes to the circadian phenotypes we observe.

To delve deeper into retinohypothalamic signaling, electrophysiology could be performed to test the responsiveness of the SCN to light received in the retina. By dissecting out the SCN while still connected to the optic nerves and retinas, the electrophysiology of the SCN response to retinal stimulation can be studied (K. Y. Wong et al., 2007). 3xTg and WT SCNs and retinas could be dissected out, their retinas stimulated with increasing intensities of light, and activity in the SCN measured to determine whether the SCN is indeed more responsive to light in 3xTg mice than WT. Electrophysiological assessment of ipRGCs would also be helpful. Heightened ipRGC excitability (either through differences in melanopsin activity, rod and cone signaling to ipRGCs, or other mechanisms) could also result in accelerated re-entrainment by increasing light sensitivity. Recording from ipRGCs and SCN neurons could help disentangle the contributions of these two structures to the re-entrainment phenotype. If exposing the retina to light of an equal intensity resulted in more excitation in the SCN in 3xTg than WT tissue, that would strongly support the hypothesis that 3xTg mice are more sensitive to light and that this affects their circadian rhythms. If 3xTg SCN cultures are not acutely more responsive to retinal light

exposure, that would suggest that mechanisms downstream of the RHT are responsible for the differences in circadian behavior.

4.3.4 Non-circadian functions of ipRGCs

The finding of heightened negative masking raises the question of whether other ipRGC-dependent behaviors may be altered in AD model mice. As was briefly discussed in Chapter 1.2.3, some ipRGC subtypes don't project to the SCN at all and instead mediate the effects of light on various other behaviors and states, including the pupillary light reflex (PLR) (S.-K. Chen et al., 2011; Güler et al., 2008), alertness and anxiety (Milosavljevic et al., 2016; G. Wang et al., 2023), sleep and wakefulness (Altimus et al., 2008; Lupi et al., 2008), and body temperature (Rupp et al., 2019). We found altered negative masking, a well characterized ipRGC-dependent task, but these other functions of ipRGCs are exciting possibilities for future research.

Probably the best-studied non-circadian ipRGC function is the pupillary light response, by which the pupil constricts in response to light. PLR is profoundly disrupted in mice which have had their ipRGCs ablated (Güler et al., 2008). PLR has been studied in AD with somewhat conflicting results (reviewed in Chougule et al., 2019), but some studies have found increased latency to constriction or decreased constriction velocity in AD patients (Fotiou et al., 2007, 2015), indicative of an impaired PLR. A study of cognitively normal subjects with early AD pathology did not find these PLR deficits (Oh et al., 2019). Confounding interpretations of the role of ipRGCs in differences in PLR in AD patients is the fact that the PLR is heavily dependent on cholinergic signaling from the Edinger Westphal nucleus (EWN). Cholinergic deficits are prevalent in AD and the EWN is susceptible to AD pathology early in the disease course (Scinto et al., 2001). If ipRGC sensitivity is increased in AD, then, this may be obscured by deficits downstream from the retina in PLR circuitry. Given the simplicity of the test and its use in

human subjects, however, the PLR may still be a valuable test for non-circadian ipRGC dependent behavior in future experiments.

Heightened anxiety-like behavior has been reported in many AD models, including 3xTg (Y.-L. Zhang et al., 2016) and 5xFAD mice (H. Li et al., 2022; Locci et al., 2021), but the role of light sensing and ipRGCs in this phenotype has not been well explored. Mice are nocturnal prey animals, and thus typically avoid open or brightly lit areas. This trait forms the basis of most tests of anxiety-like behavior in mice. When given a choice between a sheltered/dark area or an open/bright one, some mice will heavily prefer to spend time in the sheltered/dark area, while some will explore both areas; these are interpreted as more or less anxiety-like behaviors, respectively. Light sensing plays an obvious role in this phenotype, and research has found that ipRGCs in particular are important. Bright light induces more anxiety-like behavior than dim light in an open field test in WT mice, but melanopsin KO mice do not show this increased anxiety-like behavior in response to bright light (Milosavljevic et al., 2016). Conversely, the same study found that chemogenetically activating ipRGCs increases anxiety-like behavior in an open field test and elevated plus maze. More recent research has uncovered the circuitry underlying this phenomenon: ipRGC projections to the central amygdala (G. Wang et al., 2023). Heightened ipRGC sensitivity to light, as we found in AD model mice, might result in heightened anxiety-like behavior through this mechanism in tests which are performed under lighting conditions that activate ipRGCs.

Indeed, this explanation might help explain conflicting reports of both increased and decreased anxiety-like behavior in some mouse models of AD (reviewed in Kosel et al., 2020). While some of these disparities are likely due to differences in age, sex, and background strain between experiments, the role of ipRGC-dependent light-induced anxiety-like behavior merits further

examination. Many of these studies do not adequately report the lighting conditions under which these experiments were performed, leaving open the question of whether light sensitivity could contribute to elevated anxiety-like behavior in AD model mice. To explore this, open field and elevated plus maze tests could be performed under bright light and then in darkness or under dim red light so as to avoid activating ipRGCs. If heightened light sensitivity does contribute to anxiety-like phenotypes in AD mice, one would predict significantly higher anxiety-like behavior in AD mice compared to wild type in tests performed under bright light, but less or no difference when performed in darkness. These results would suggest that anxiety-like behavior in AD model mice is driven by heightened retinal light sensitivity rather than higher baseline levels of arousal or fear, a significant change in the interpretation of many behavioral results in the field.

4.4 Other factors in re-entrainment

The experiments here focused on how re-entrainment is affected by AD pathology, but a variety of other factors also contribute to re-entrainment speed in these experiments and in the literature.

4.4.1 Effects of sex, aging, and strain differences on re-entrainment

One notable gap in our research is the lack of male mice in the experiments and subsequently our inability to assess sex differences in these phenomena. We elected to study only female mice because of their more dramatic phenotypes in the 3xTg model (Dennison et al., 2021), and for the sake of consistency continued with only females in other models as well. There is reason to believe that there may be sex differences in this phenotype, as male 3xTg mice reportedly do not have accelerated re-entrainment in a jet lag paradigm (González-Luna et al., 2021). In that study, however, neither the 3xTg nor the WT mice appear fully entrained before the beginning of the phase shift in some of the jet lag experiments, so these results may not be entirely reliable. More research should be performed to assess sex differences in re-entrainment.

Aging also appears to affect re-entrainment. In our 3xTg jet lag experiments in Chapter 2, mice at 13 months re-entrained more slowly than at 8 months. This is in accordance with two previous studies performed in C57BL/6 mice which found significantly slower re-entrainment of locomotor activity in old (>19 months) versus young (<9 months) mice (Sellix et al., 2012; Valentinuzzi et al., 1997). It is unlikely that this is due to a weak and easily perturbed SCN rhythm in young mice compared to old. More likely is the explanation that signals from the retina are less potent re-entrainment cues in older mice, as has been previously suggested (Hood & Amir, 2017). 3xTg mice in our experiments did not appear to have as dramatic a slowing in their re-entrainment with age as their WT controls. This would fit with an age-dependent decline in ipRGC number or signaling that is somehow slowed in AD model mice, as discussed in Chapter 4.3.1. This interpretation, however, is complicated by one account of re-entrainment in aged mice (Sellix et al., 2012). Although behavioral re-entrainment was slower in old mice than young, re-entrainment of the SCN (as measured by Per2 transcription) was actually *faster* than in young mice, while the clock in several peripheral tissues re-entrained *more slowly* (Sellix et al., 2012). Similar results were found in rats, where the arcuate nucleus and pineal gland (both downstream of the SCN in entrainment signaling) re-entrained faster in old rats while the aged liver struggled to re-entrain at all (Davidson et al., 2008). This suggests a role for peripheral clocks in determining the speed of behavioral re-entrainment. Peripheral clocks may also be important for the re-entrainment phenotype observed here in AD model mice and merit further study.

Finally, strain differences also appear to have effects on re-entrainment speed. All three AD models used in these experiments are on different background strains: B6129SF2/J (for 3xTg), C57BL/6J (for 5xFAD), and B6C3 (for PS19). These WT controls showed dramatic differences

between their re-entrainment speeds, with B6C3 mice re-entraining significantly faster than C57BL/6J at 7 months. This could represent differences between strains in light sensing, SCN function, or some other system.

4.4.2 Microglia depletion and re-entrainment

As discussed in Chapter 2, we hypothesized that the neuroinflammation and gliosis associated with AD and present in AD models contributed to the re-entrainment phenotype we observed. To test this, we performed a jet lag experiment in 13-month-old female WT or 3xTg mice which had received either control chow or chow containing the CSF1R inhibitor PLX3397 for 7 days. PLX3397 was highly effective in depleting microglia from the hypothalamus, including the SCN. But microglia depletion did not rescue the accelerated re-entrainment observed in our AD model mice, with 3xTg control and 3xTg PLX groups performing the same in the jet lag test. From this we concluded that microglia activation is not acutely responsible for the altered re-entrainment behavior in AD model mice. This experiment raised other questions, however, that I will address here.

First, this experiment was only able to test for very acute effects of microglia activation on circadian behavior, and the chronic gliosis and inflammation present in the AD brain may still contribute to the phenotype. Activated microglia have many effects that could persist long after microglia themselves are removed: for example, microglia phagocytose synapses in AD model mice (Hong et al., 2016), and microglial synaptic pruning can have persistent, perhaps permanent, effects on circuits (Schafer et al., 2012). Microglia therefore could contribute to this behavioral phenotype through some such long-lasting effect of activation. To test this, we could perform this microglia depletion for longer periods of time before behavioral testing.

Depleting microglia over a very long period of time before behavior testing could help answer other questions related to this project as well. Previous research using CSF1R inhibitors to deplete microglia in 5xFAD mice has found that chronically depleting microglia in mice that already have extensive Aβ plaques can partially protect against neurodegeneration but does not alter Aβ load (E. E. Spangenberg et al., 2016). Unexpectedly, chronically depleting microglia in younger 5xFAD mice, starting before the onset of Aβ plaques, largely prevents those plaques from forming as long as microglia remain depleted (E. Spangenberg et al., 2019a). These mice still produce Aβ, but instead of aggregating into plaques in the brain parenchyma, it appears to largely accumulate in the blood vessels of the brain. This phenotype offers an interesting possibility to examine another question in this project: are Aβ plaques necessary for the re-entrainment phenotype we observed? We do not observe plaques in the SCN or retinas of our AD model mice, so we hypothesize that plaques are not necessary for the behavioral phenotype. But perhaps plaques exert an effect on this phenotype from other regions of the brain. 5xFAD mice chronically treated with CSF1R inhibitor before plaque formation offer an intriguing model which has abundant soluble Aβ but very few Aβ plaques. We have already established that neurofibrillary tangles are not necessary for the phenotype, but by performing a jet lag experiment in these mice, we could test whether the dramatic re-entrainment phenotype we observed in 5xFAD mice persists in the near absence of plaques.

4.5 Implications for AD research and treatment

As mentioned in Chapter 2, accelerated re-entrainment has not been noted as a circadian symptom of AD. However, it may share some important similarities with the circadian disruptions that are found in the disease. The mechanism explored in Chapter 3, heightened light sensitivity due to changes in the ipRGC population, suggest a possible explanation for disrupted

sleep and rhythmic behavior: AD patients may experience mismatched light cues (such as a night light) as more intense re-entrainment cues than others, leading to frequent disruptions to their clocks. Other possible mechanisms leading to weakened SCN rhythms, as discussed in Chapter 4.2, may contribute to circadian symptoms more directly by impairing the ability of the SCN to synchronize its internal clock and the body's peripheral clocks. Our findings of accelerated re-entrainment in AD model mice have important implications for explaining some aspects of the circadian disruptions in AD.

4.5.1 AD therapeutics and circadian rhythms

The field of AD treatment has had major accomplishments recently, with clinical trials for multiple anti-amyloid monoclonal antibody treatments demonstrating decreases in AD pathology and slowed cognitive decline (Mintun et al., 2021; van Dyck et al., 2023). These are the first treatments to successfully alter the course of the disease rather than treating individual symptoms. These antibody treatments have distinct downsides, though. They are administered as frequent injections and treatment will have to be continued over long periods. They will be extremely expensive, making them heavy burdens on health insurance systems and keeping them out of reach of many who could benefit from the treatments. They can have severe side effects, including cerebral edema and hemorrhage. Their efficacy is also limited, not curing the disease, only partially slowing progression. Despite all this, the new therapies are important. First, they offer the possibility of a slower cognitive decline for people with AD, meaning more time spent with few or no cognitive impairments before the disease progresses. Second, they validate the amyloid hypothesis of AD in a treatment paradigm: decreasing amyloid pathology early in the disease can decrease other pathological markers and delay cognitive decline. This confirms that amyloid can be an effective target for AD treatment, offering hope for future therapies.

Our finding that accelerated re-entrainment in AD models does not require neurofibrillary tangles and appears to be driven by amyloid pathology is interesting in this context. I would predict that similar antibody treatment in our AD model mice, if administered early enough, would be effective in preventing circadian behavioral alterations. Lecanemab is formulated to preferentially target Aβ protofibrils, a soluble form of the molecule, and effectively lowers soluble Aβ levels in humans and in mice (Tucker et al., 2015). Our mice do not accumulate plaques in the SCN or retina, but we did not test for the presence of protofibrils, which might be present in one or both of these sites in our mice. For that reason lecanemab may be more effective in this case than the monoclonal antibody therapy donanemab, for example, which preferentially targets plaques (DeMattos et al., 2012). An interesting question would be to test whether the accelerated re-entrainment can be rescued by treatment beginning *after* the onset of the circadian phenotype. If the cause of this behavior is an increase in the number of ipRGCs, I would not expect amyloid-clearing therapy to modify it after the circadian phenotype has developed. If the phenotype is caused by some acute effect of soluble Aβ in the SCN, however, then perhaps an anti-amyloid antibody could rescue the phenotype after it has already begun to present.

Related questions remain for these treatments in humans. Thus far clinical trials have not reported any data on sleep or circadian rhythms in anti-amyloid antibody trials. Likewise they have not reported on amyloid buildup in the retina. Future studies should examine circadian rhythms and sleep in treated AD patients to determine whether these treatments are able to rescue or slow the progression of these symptoms in the same manner that the treatments slow cognitive decline. If they do not, as seems very possible for symptoms such as sleep disruptions which

begin very early in the disease course, other treatments will be needed to address the circadian symptoms of AD.

4.5.2 Light therapy in AD

Managing light exposure in AD patients by increasing exposure to bright light during daylight hours and/or decreasing exposure to light during the night have been used to treat circadian and sleep symptoms in AD, with some successes (reviewed in Mitolo et al., 2018). Several studies, both of patients in care facilities and living at home, have found that bright light treatment during daytime hours improved sleep quality and duration (Ancoli-Israel et al., 2002, 2003; Cremascoli et al., 2022; Figueiro et al., 2014, 2015). These studies also found a strengthening and/or phase shift of daily activity rhythms, demonstrating the ability of these light stimuli to entrain patients. One of these studies also examined the circadian rhythms of caregivers living with AD patients, who were also exposed to the ambient bright light therapy, and found that their daily activity rhythms showed strengthened circadian rhythms (Figueiro et al., 2015). This effect may reflect a combination of the alleviated challenge of managing circadian disruptions in their partners (Musiek et al., 2015) and their own entrainment response to the light stimulus.

Importantly, these light interventions appear to affect other AD symptoms as well. Mood symptoms such as agitation and depression are improved in some light therapy studies (Figueiro et al., 2015; Onega et al., 2016). This is consistent with the agitation observed in sundowning being attributed in part to circadian disruptions. Memory and cognitive symptoms also may be modulated by light interventions. In a study in which patients received either bright or dim light treatment found that the bright light group had significantly improved cognitive scores compared to the dim light group at 1.5 and 3.5 years after treatment onset (Riemersma-van der Lek et al., 2008). Though there is not a statistically significant change in the rate of cognitive decline

between the groups, the data also suggest that the light treated group may be partially protected against disease progression. In a shorter study of just 4 weeks of light therapy, one study found an improvement in cognitive performance over baseline (Cremascoli et al., 2022). This shorter study supports the claim that a portion of the cognitive impairments in AD are not due to irreversible neurodegeneration but may be reversible, the result of acute dysfunctions in sleep or circadian rhythms. Thus sleep- and circadian-modifying treatments may be useful both for acutely treating AD symptoms and for slowing disease progression.

4.5.3 Jet lag paradigm as a research tool

The re-entrainment phenotype we observed in a jet lag behavioral test is a novel and useful tool for future AD research. The effect is strong, with observed changes in PS_{50} ranging from 39% in 8-month-old 3xTg mice to 58% in 5xFAD mice. This, combined with the consistent phenotype in two distinct genetic models, makes it an unusually robust phenotype among AD behavioral tests. Circadian behavior in AD mouse models is highly variable between models (Sheehan & Musiek, 2020) and often difficult to analogize to the circadian symptoms in AD (for example, what effect does a shortened free running period have on behavior in humans living in environments with daily light-dark rhythms?). Accelerated re-entrainment in the jet lag paradigm, on the other hand, may suggest a weakened SCN clock and/or heightened light sensitivity, both of which have obvious implications for human behavior. For these reasons the jet lag paradigm offers an opportunity for testing future potential AD therapeutics, or therapeutics for other diseases, for their effects on a phenotype related to circadian alterations in disease.

Though this dissertation has focused narrowly on AD, many other diseases are associated with circadian disruptions, and the jet lag paradigm has served as a valuable research tool in some of these other fields. Parkinson's disease (PD) often presents with sleep disruptions (reviewed in

Gros & Videnovic, 2020). Much like AD, circadian disruptions can appear many years before clinical diagnosis in PD (Leng et al., 2020), leading to speculation that circadian rhythms may contribute to PD risk or progression. Light therapy in PD patients may decrease these circadian symptoms and even some motor symptoms (Willis & Turner, 2007). Interestingly, studies in two different mouse models of PD provided different results in jet lag studies: one found more rapid re-entrainment (Fifel & Cooper, 2014), while another found that re-entrainment was significantly slowed in their mutant mice (Pfeffer et al., 2018). The latter paper speculated that dopamine signaling in the retina may be involved in this phenotype, but the actual mechanisms underlying re-entrainment phenotypes in these models are not known. Circadian and sleep symptoms are also observed in Huntington's disease (HD) and may contribute to disease progression (reviewed in Voysey et al., 2021), and again light therapy has been found to alleviate some motor symptoms in a mouse model of HD (H.-B. Wang et al., 2017). One study performing jet lag in an HD model, however, found no difference in re-entrainment speed to a jet lag phase advance (though the HD mice took longer to re-entrain in a phase delay jet lag experiment) (N. I. Wood et al., 2013). Similar results were found in the same mouse model for phase shifting in response to a short light pulse (Ouk et al., 2019). Interestingly, ipRGCs degenerate early in the disease progression in this HD model (M.-S. Lin et al., 2019).

4.6 Conclusion

The causes of circadian disruptions in AD are still poorly understood. These experiments demonstrate a circadian behavioral phenotype in AD model mice and identify potential mechanisms underlying that phenotype. Changes in the retina in these AD model mice point to a novel possible explanation for this altered behavior. Further exploration of these phenomena

could help explain the mechanisms behind circadian disruptions in AD and improve our management and treatment of those symptoms.

Appendix I Astrocyte oxidative stress and antioxidant mechanisms in neurodegenerative disease

I.1 Introduction

Neurodegenerative diseases are a family of CNS disorders characterized by progressive death of neurons. The incidences of many neurodegenerative diseases are rising, as a result of aging populations and possibly other factors. This will place significant strain on healthcare systems in the future. Treatment options for these diseases are limited and none of them can currently be cured. While the causes of most cases of neurodegenerative disorders are still not known, research has uncovered common mechanisms involved in many neurodegenerative disorders. These include neuroinflammation, protein misfolding and aggregation, excitotoxicity, and oxidative stress. Neurodegenerative diseases are ultimately diseases of neuron death and neurons are by far the most commonly studied cells in these diseases, but many of these shared mechanisms can be modified by other cell types, particularly glial cells. Astrocytes are a subset of glia that play an important role controlling oxidative stress in the brain. On the other hand, they can also contribute to neuronal oxidative stress and are susceptible to oxidative damage themselves. Astrocyte defenses against and contributions to oxidative stress are important topics of research in understanding neurodegenerative disease.

This review focuses on Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). These neurodegenerative disorders have different etiologies and pathophysiological hallmarks, but all three have strong associations with astrocyte pathology and oxidative stress. There are also currently few effective disease modifying therapies for these diseases, emphasizing the importance of further research into druggable mechanisms. This

review will assess the evidence linking astrocytes and oxidative stress to neurodegenerative disease before examining astrocyte oxidant and antioxidant mechanisms and how they are affected by or contribute to neurodegeneration.

I.2 Astrocyte dysfunction in neurodegenerative disorders

Astrocytes are the most plentiful glial cell type in the CNS and perform a wide variety of roles in maintaining homeostasis in the adult brain. These include blood-brain barrier maintenance, neurotrophic factor expression, immune defense, waste clearance, and metabolic support. In disease states, though, astrocytes can rapidly damage neurons by decreasing these neuroprotective mechanisms and upregulating potentially neurotoxic ones. The maintenance of neuroprotective astrocyte functions and repression of neurotoxic ones is necessary for maintaining neuronal health and brain homeostasis. In neurodegenerative disorders, astrocytes undergo changes that can be alternately protective or harmful. This review will focus on impaired or deleterious functions of astrocytes in neurodegenerative diseases, but it should not be forgotten that research has also demonstrated protective roles for astrocytes and astrocyte activation (A. W. Kraft et al., 2013; Nielsen et al., 2010).

Astrocyte activation is a phenomenon that is widely studied in neurodegeneration research, but it is a poorly defined phrase. Traditionally, astrocyte activation is measured by hypertrophy and increased glial fibrillary acidic protein (GFAP) expression. More recently, different categories of activated astrocytes have been proposed: A1, or neurotoxic, induced by LPS; and A2, or neuroprotective, induced by ischemia (Zamanian et al., 2012). “A1 astrocytes” have been identified in Alzheimer’s, Parkinson’s, ALS, and other neurodegenerative diseases based on their increased expression of complement component 3 (C3) (Liddelow et al., 2017a). The A1/A2 astrocyte activation classification system has come under criticism, however, as overly simplistic

and not representative of the heterogeneity of astrocyte functions (Cunningham et al., 2018). This review will avoid using the A1/A2 nomenclature in favor of directly presenting expression of astrocyte genes and proteins in order to more precisely describe the effects of astrocyte activation in disease.

Various astrocyte activation profiles and effects on neurons have been studied in the context of neurodegeneration. Two of the best characterized mechanisms are excitotoxic injury and inflammatory signaling. The evidence for astrocyte involvement in neurodegenerative disease and the importance of these mechanisms is reviewed here. Another promising common mechanism for astrocyte pathological effects in neurodegeneration is oxidative stress, which will be the focus of the rest of this review.

I.2.1 Astrocytes in Alzheimer's disease

Alzheimer's is primarily a disease of neurons, but astrocyte involvement in AD is well established. Astrocytes have long been observed to associate with amyloid plaques (Beach & McGeer, 1988; Itagaki et al., 1989) and extracellular neurofibrillary tangles (NFTs) (Irwin et al., 2012; Probst et al., 1982). Astrocyte activation increases over the course of AD and correlates more strongly than amyloid plaque load with dementia severity (Vehmas et al., 2003). Astrocytes are also the highest expressers in the brain of ApoE, the most significant genetic risk factor for late onset AD, and Clu, another common risk gene (Y. Zhang et al., 2014).

Activated astrocytes in AD induce immune activation that can promote neuronal damage. Astrocytes from APP/PS1 mice have upregulated expression of genes involved in the complement pathway and other immune responses, while they downregulate genes involved in pathways such as cholesterol synthesis, synaptogenesis, and neuronal support (Orre et al., 2014). Primary human astrocytes treated with amyloid β oligomers secrete elevated levels of the

proinflammatory cytokine IL-6 and chemokine CCL2 (Montoliu-Gaya et al., 2018) Astrocytic JAK2-STAT3 signaling may play a role in this immune activation in AD. Selectively inhibiting JAK2-STAT3 in astrocytes of APP mice results in decreased astrocyte activation as measured by GFAP, as well as decreased proinflammatory gene expression (Ceyzériat et al., 2018). This STAT3 inhibition also decreased amyloid plaque formation and improved spatial learning (but not memory) in the Morris Water Maze task.

Astrocytes can also contribute to excitotoxic damage. Astrocytes are important in the healthy brain to uptake glutamate at synapses with glutamate transporters. Amyloid β treatment of cultured mouse astrocytes decreases cell surface levels of the glutamate transporter GLT-1, resulting in decreased capacity for clearing extracellular glutamate (Scimemi et al., 2013). This is in accordance with human AD studies finding decreased EAAT2, the human homolog of GLT-1 (S. Li et al., 1997; J. E. Simpson et al., 2010). This decreased capacity for astrocyte glutamate uptake promotes excitotoxic stress in neurons.

I.2.2 Astrocytes in Parkinson's disease

As with amyloid β and NFTs in Alzheimer's disease, astrocytes can interact with pathologically aggregated α-synuclein in Parkinson's disease. α-synuclein aggregates can be found within astrocytes in PD brain tissue (Braak et al., 2007), and increased number of α-synuclein containing astrocytes correlates with greater neuronal loss in the substantia nigra (Wakabayashi et al., 2000). Astrocyte GFAP staining, a marker of activation, is increased in the substantia nigra of PD patients (Y. J. C. Song et al., 2009). Perhaps the most compelling evidence that astrocytes play a causal role in PD development, though, is that mice that express a mutant α-synuclein protein specifically in astrocytes develop PD-like motor symptoms and neurodegeneration in the substantia nigra (Gu et al., 2010).

Rodent *in vivo* and *in vitro* experiments have demonstrated an important role for astrocyte immune signaling in PD. Microarray analysis of rat astrocytes treated with extracellular α-synuclein shows increased expression of pro-inflammatory cytokines including IL-1α, IL-1β, and IL-6, as well as genes in a variety of other pro-inflammatory processes (H.-J. Lee et al., 2010). In another study, an agonist for glucagon-like peptide-1 receptor (GLP1R) was protective in two mouse PD models through a mechanism involving interactions between microglia, astrocytes, and neurons (Yun et al., 2018). Astrocytes were found to be responsible for killing neurons in an *in vitro* fibrillar α-synuclein treatment system, but this neurotoxic astrocyte activation could be prevented by inhibiting microglial proinflammatory signals to astrocytes, including IL-1α, TNFα, and C1q. The specific activated astrocyte factors responsible for neurotoxicity in this model were not identified, but these experiments compellingly illustrated a system of immune signaling between microglia and astrocytes that promotes astrocyte-mediated neurodegeneration.

I.2.3 Astrocytes in ALS

There is direct evidence of astrocytes playing a central role in neurodegeneration in mouse familial ALS models. In chimeric mice where different cells in a single organism express either wild type or mutant SOD1, WT astrocytes can delay degeneration of nearby mutant motor neurons, and conversely WT motor neurons can develop ALS-like pathology when in proximity to SOD1 mutant astrocytes (Clement et al., 2003). This finding was clarified in subsequent *in vitro* studies which found that SOD1 mutation was not sufficient to induce degeneration in motor neurons, but that conditioned media from SOD1 mutant astrocytes was sufficient to induce degeneration in WT motor neurons (Di Giorgio et al., 2007; Nagai et al., 2007).

As in other neurodegenerative diseases, there is compelling evidence of alterations in astrocytic glutamate regulation playing a mechanistic role in ALS. GLT-1 levels in ALS patient spinal cord

and motor cortex are decreased by 60% and 70%, respectively (Rothstein et al., 1995). This dramatic decrease in astrocytic glutamate transporter is also observed in rodent ALS models (Bendotti et al., 2008; Bruijn et al., 1997; Howland et al., 2002). Though the signature pathophysiological sign of ALS is motor neuron degeneration, astrocyte death is also observed in the spinal cord of a SOD1 mutant mouse model (Rossi et al., 2008). These astrocytes degenerate through an excitotoxic pathway, and inhibiting this glutamate-dependent astrocyte damage extends survival in mice.

Inflammatory molecules also contribute to motor neuron death in ALS models. SOD1 mutant astrocytes secrete the proinflammatory cytokine interferon-γ, which promotes the degeneration of motor neurons *in vitro* through LIGHT signaling in neurons (Aebischer et al., 2011). Knocking out LIGHT delays disease progression *in vivo*, demonstrating the functional importance of this immune signaling pathway in motor neuron degeneration. In another study, wild type human embryonic stem cell-derived motor neurons were susceptible to degeneration when cocultured with SOD1 mutant astrocytes (Di Giorgio et al., 2008). A major molecular mediator of this degeneration was astrocyte-secreted prostaglandin D2, and prostaglandin D2 receptor inhibition in neurons partially rescued SOD1 mutant astrocyte-induced degeneration. Prostaglandin D2 is a signaling molecule important in a variety of immune functions.

I.3 Oxidative stress in neurodegenerative disorders

The brain is highly metabolically active, consuming 20% of the oxygen in the body. This oxygen consumption is necessary to produce the large amount of energy required to sustain brain activity, but it comes with costs. One of those is the generation of reactive oxygen species (ROS). ROS in eukaryotes are primarily generated as a byproduct of mitochondrial respiration or as the product of certain enzymatic reactions, often involved in immune defense. ROS can

damage many biological molecules, including lipids, proteins, and DNA, and high ROS levels are cytotoxic. Cellular ROS originates in two main forms, superoxide and hydrogen peroxide, both of which can be produced by mitochondrial respiration or enzymatic activity. These molecules can be harmful in several ways (Imlay, 2008; Winterbourn, 2013). Hydrogen peroxide can react directly with cysteine residues to damage proteins, but most cytotoxic effects of these two molecules are less direct. Hydrogen peroxide and superoxide can participate in a Fenton reaction in which they oxidize metal ions such as iron or copper. This reaction then produces a hydroxyl radical and, depending on the redox state of the metal ion, it may generate a powerfully oxidative metal ion. Hydroxyl radicals produced in this reaction are highly reactive and can induce lipid peroxidation, protein oxidation, and oxidative DNA damage.

Because oxidative damage is so destructive ROS levels must be kept under tight homeostatic control. Cellular antioxidant systems have evolved to reduce ROS and repair or clear oxidatively damaged lipids, proteins, and DNA. Some of these antioxidant mechanisms will be discussed in section 4 below in the context of astrocytes and neurodegeneration. Under pathological conditions the balance between ROS generation and antioxidant defenses can become tilted towards dangerous oxidant levels, a state called oxidative stress.

Oxidative stress in the brain can be measured in a variety of ways. ROS themselves are often difficult to detect, with hydroxyl radicals having half-lives as short as one nanosecond (Sies, 1993). Thus, oxidative stress is often measured through proxies. Lipid peroxidation resulting from free radical reactions with lipids can be measured by assaying for thiobarbituric acid reactive substances (TBARS) or 4-hydroxynonenal (4-HNE), byproducts of lipid peroxidation (Benzie, 1996). Protein oxidation can be measured with assays that detect protein carbonyl groups, a product of oxidation of some amino acid side chains (Dalle-Donne et al., 2003). DNA

oxidative damage can be measured by detecting molecules such as 8-OHdG, an oxidation product of the deoxyribonucleotide guanosine (Valavanidis et al., 2009). Other techniques can more directly measure ROS levels by using oxidation-sensitive fluorescent dyes. Some of these dyes are specifically sensitive to superoxide or are targeted to the mitochondria rather than the cytoplasm, allowing for greater specificity in identifying the type and subcellular origin of ROS. Using this variety of different techniques, oxidative stress has been observed in many neurodegenerative disorders and there is strong evidence implicating ROS as an important mechanism in both the initiation of disease and in actual neuronal death.

I.3.1 Oxidative stress in Alzheimer's disease

Of all neurodegenerative diseases, Alzheimer's disease is the one with the most well-established links with oxidative stress. Analysis of human tissue shows increases in a variety of markers of oxidative stress in AD. Lipid peroxidation, the result of oxidative damage to membranes, is elevated in AD brains (Markesbery & Lovell, 1998; Subbarao et al., 1990). Oxidative DNA damage is elevated in AD brains (Lovell & Markesbery, 2007; Mecocci et al., 1994; J. Wang et al., 2005), with mitochondrial DNA seemingly more severely affected than nuclear DNA (Mecocci et al., 1994). Protein oxidation is elevated in the hippocampus and other brain regions in AD (Aksenov et al., 2001; Hensley et al., 2002). Many of these markers of oxidative stress have also been observed in mouse models of AD (Belkacemi & Ramassamy, 2012; Butterfield & Boyd-Kimball, 2018).

Evidence suggests that oxidative stress is not just correlated with AD but rather is an important mechanism in pathogenesis. Oxidative DNA damage is elevated in blood in AD (Mecocci et al., 2002; Migliore et al., 2005), and surprisingly this is also observed in patients with mild cognitive impairment (MCI) (Migliore et al., 2005), which often progresses to AD but does not have

observable amyloid plaque or neurofibrillary tangle pathology. Increased lipid and protein oxidation are also found in both CNS and peripheral samples from MCI patients (Butterfield et al., 2006; Petersen et al., 2001; Praticò et al., 2002). These studies are interesting because they show that elevated oxidative stress in AD is also present outside of the CNS, but also because they demonstrate that increased oxidative stress precedes the appearance of classic AD pathology. *In vitro* experiments have provided mechanistic support for the hypothesis that oxidative stress can drive AD pathology. Pharmacological inhibition of the electron transport chain, resulting in disrupted mitochondrial function and increased ROS levels, leads to increased amyloid β production that can be partially rescued by antioxidant treatment (Leuner et al., 2012). Cells treated with hydrogen peroxide (Misonou et al., 2000) or 4-HNE (Tamagno et al., 2005), a toxic product of lipid peroxidation, also increase amyloid β secretion. Tau pathology is also promoted by oxidative stress. Inducing oxidative stress in cultured cells by depleting the antioxidant glutathione results in tau hyperphosphorylation (Su et al., 2010), and reactions between hyperphosphorylated tau and 4-HNE promote the assembly of tau into filaments (Pérez et al., 2000).

While oxidative stress can promote Alzheimer's pathology, the reverse is also true, with amyloid β and tau pathology driving increased oxidative stress. Mice that overexpress mutant APP or tau and APP have disrupted mitochondrial function and increased ROS levels (Hauptmann et al., 2009; Rhein et al., 2009). Amyloid oligomers and fibrils can have direct ROS-producing effects, generating hydrogen peroxide and hydroxyl radicals through interactions with metal ions (X. Huang, Atwood, et al., 1999; X. Huang, Cuajungco, et al., 1999; Mayes et al., 2014). Treatment of cultured neurons, astrocytes, and microglia with amyloid β induces increased ROS generation (Schilling & Eder, 2011; Urrutia et al., 2017; Yan et al., 1996; Yatin et al., 2000). This positive

feedback loop, where ROS can promote amyloid β and amyloid β pathology can drive increased ROS, points to oxidative stress as a powerful player in the initiation and progression of AD.

Given the evidence that oxidative stress is an important mechanism of neurodegeneration in AD, antioxidants have been examined as possible treatments. Although vitamin E (α-tocopherol, an antioxidant) showed promise in some mouse model studies (Nakashima et al., 2004; Sung et al., 2004), it has failed to show benefits in human trials for MCI or AD (Lloret et al., 2009; Petersen et al., 2005). Using a different approach, mitochondrial-targeted antioxidants decrease oxidative stress and neuronal death *in vitro* and in a mouse model of AD (Kwon et al., 2016; Manczak et al., 2010). As with other attempted therapeutic strategies for AD, no antioxidant treatment has been approved in spite of often promising preclinical evidence. These antioxidant trials have likely failed for many of the same reasons as other AD trials: differences between mouse models and human disease, inadequate delivery of therapeutics to the correct brain regions or cell types, difficulty stratifying AD patients into different populations that might respond to different drug classes, and failure to treat patients early enough in the disease progression. Combatting oxidative stress remains a compelling therapeutic approach to AD, but these problems must be addressed if new antioxidant therapies, possibly with more targeted action, can be successful.

I.3.2 Oxidative stress in Parkinson's disease

As in Alzheimer's disease, Parkinson's disease is associated with increased oxidative stress. 4-HNE is increased in PD, indicating increased lipid peroxidation (Yoritaka et al., 1996). Markers of oxidative DNA damage are elevated in PD (Alam, Jenner, et al., 1997; Nakabeppu et al., 2007). Protein carbonylation is also increased (Alam, Daniel, et al., 1997; Floor & Wetzel, 1998).

Oxidative stress can also be detected in the periphery in PD patients, with increased ROS and oxidative DNA damage found in blood cells (Migliore et al., 2002; Prigione et al., 2006).

The mechanisms that connect Parkinson's disease to this oxidative stress are still being studied. As in the case of Alzheimer's disease, PD pathology and ROS generation appear to constitute a positive feedback loop with each driving more of the other. MPTP, a drug used to model PD because it selectively kills dopaminergic neurons, increases α-synuclein expression in a mitochondrial ROS signaling dependent mechanism (Je & Kim, 2017). On the other hand, there is significant evidence that PD pathology promotes ROS generation. iPSC-derived neurons from patients with mutant *SNCA*, a cause of familial Parkinson's disease, produce more superoxide and hydrogen peroxide than controls (Deas et al., 2016). They are also more sensitive to challenge with oligomeric α-synuclein, which results in dramatically increased ROS production. α-synuclein fragments can also promote microglial expression of the ROS-producing enzyme NADPH oxidase, resulting in increased microglia-produced ROS (S. Wang et al., 2016). 6-hydroxydopamine (6-OHDA), a molecule used to induce dopaminergic neurodegeneration in rodents, also promotes ROS production. 6-OHDA induces expression of NADPH oxidase in dopaminergic neurons, and NADPH oxidase knockdown protects against 6-OHDA (Choi et al., 2012). The MPTP model also induces the upregulation of neuronal NADPH oxidase and subsequent ROS generation (Mandir et al., 1999). NADPH oxidase is also upregulated in dopaminergic neurons in PD patients (Choi et al., 2012; Mandir et al., 1999).

Parkinson's disease is characterized by a preferential degeneration of dopaminergic neurons in the substantia nigra, and part of this increased vulnerability of dopaminergic neurons may be due to the susceptibility of dopamine to oxidation. Dopamine is subject to autoxidation, producing cytotoxic oxidized dopamine as well as ROS (Fornstedt et al., 1990). ROS and oxidized

dopamine are elevated in dopaminergic neurons derived from PD patient iPSCs, and decreasing dopamine production or oxidation in those neurons results in decreased α-synuclein production (Burbulla et al., 2017). Oxidized dopamine, however, is not essential to pathogenesis in a mouse PD model. Tyrosine hydroxylase KO mice, which are incapable of synthesizing dopamine, are still susceptible to the MPTP model (Hasbani et al., 2005). While dopamine oxidation appears to contribute to disease, there are likely other mechanisms contributing to dopaminergic neuron susceptibility to neurodegeneration in PD.

Given the extensive evidence of oxidative stress as an important mechanism in Parkinson's disease, antioxidants have been considered as potential therapies. Vitamin E has been tested in several trials with mixed results, but the largest and highest quality trial did not find a benefit of Vitamin E supplementation (Parkinson Study Group, 1993, 1998). Coenzyme Q_{10} (CoQ_{10}), a component of the electron transport chain as well as a mitochondrial free radical scavenger, is protective in the MPTP model in mice and nonhuman primates (Beal et al., 1998; Horvath et al., 2003). However, the largest human trial to date found no effect of CoQ_{10} (Beal et al., 2014). Another mitochondrial antioxidant, MitoQ, has also failed in a human trial (Snow et al., 2010). Thus far antioxidant therapies have not shown benefits in humans in large, placebo-controlled trials. However, for many of the same reasons discussed above regarding antioxidant trials in AD, this does not prove that the oxidative stress hypothesis in PD is not a druggable target. By the time that motor symptoms appear and PD can be diagnosed, as many as half of the dopaminergic neurons in the substantia nigra may be dead and the others are already severely stressed (Snow et al., 2010). L-DOPA, the best-known PD treatment, can supplement dopamine levels in the absence of healthy dopaminergic neurons. Antioxidant therapies, on the other hand, would be dependent on supporting the health of living dopaminergic neurons, and thus may only

be effective before a large fraction of neurons have already degenerated. This would suggest that antioxidants may be more effective as prevention or during the pre-clinical phase of PD, when antioxidant support could prevent degeneration of stressed neurons.

I.3.3 Oxidative stress in ALS

ALS is another neurodegenerative disorder associated with oxidative stress at multiple levels. 4-HNE levels are increased in the spinal cord in ALS (Perluigi et al., 2005). Protein oxidative damage is elevated (Ilieva et al., 2007). Mitochondrial and nuclear DNA oxidative damage are also increased (Bogdanov et al., 2000; Dhaliwal & Grewal, 2000). ALS is also linked to oxidative stress through the genetic risk conferred by mutations to SOD1, an important antioxidant protein. The possible role of mutant SOD1 in promoting ROS generation in ALS is discussed in section 4.2 below.

In vivo and *in vitro* models recapitulate the increased oxidative stress observed in ALS patients. Mutant TAR DNA-binding protein 43 (TDP-43), a familial ALS risk gene, induces oxidative stress and mitochondrial dysfunction in a motor neuron-like cell line (Duan et al., 2010). The best characterized ALS model, the SOD1 mutant, promotes extensive ROS production in microglia, astrocytes, and neurons (Kruman et al., 1999; Y. Liu et al., 2009; Marchetto et al., 2008). Hexanucleotide repeat expansion in C9ORF72, the most common form of familial ALS, also results in increased mitochondrial ROS and oxidative damage *in vitro* (Lopez-Gonzalez et al., 2016). Antioxidant treatment in these mutant cells protects against DNA damage, demonstrating that toxicity in C9ORF72 is at least partially mediated by ROS induction. *In vivo* evidence also demonstrates the importance of oxidative stress in these models. For example, the lifespan of SOD1 mutant mice is extended when the ROS generating enzyme NADPH oxidase is inhibited or knocked out (Harraz et al., 2008; Marden et al., 2007; D.-C. Wu et al., 2006).

Mitochondrial dysfunction also plays an important role in ALS, at least in part through increasing oxidative stress. SOD1 mutant mouse spinal cords have impaired activity of electron transport change complexes (C. Jung et al., 2002). Mitochondrial structural abnormalities and impaired mitochondrial transport are observed *in vitro* in the TDP-43, C9ORF72, and SOD1 models (Dafinca et al., 2016; De Vos et al., 2007; Magrané et al., 2014). Similar mitochondrial structural defects have also been observed in the spinal cord of sporadic ALS patients (Sasaki & Iwata, 2007). Mutant SOD1 could be part of a feedback loop promoting this mitochondrial dysfunction, as misfolded SOD1 is known to associate with mitochondria and induce mitochondrial damage and ROS production (Pickles et al., 2013, 2016). ROS can oxidize SOD1 and promote misfolding and aggregation (Rakhit et al., 2002). SOD1 misfolding can damage mitochondria, increasing ROS, which increases SOD1 oxidation and misfolding, further perpetuating mitochondrial dysfunction and oxidative stress. Though TDP-43 is less well studied than SOD1, it may also participate in a similar feedback loop, as it is a target of oxidation (Cohen et al., 2012) and TDP-43 aggregates cause mitochondrial dysfunction (Y.-F. Xu et al., 2010).

Antioxidant therapy has been more successful in ALS than in other neurodegenerative diseases. A variety of antioxidant treatments with different antioxidant properties, including some that are specifically targeted to the mitochondria, improve survival in mouse ALS models (Bond et al., 2018; Miquel et al., 2014; Petri et al., 2006). Most compellingly, though, one of the only FDA approved drugs for ALS is the antioxidant edaravone. Edaravone appears to act primarily as a scavenger for peroxynitrite (Banno et al., 2005; Fujisawa & Yamamoto, 2016), a potent oxidative and nitrosative species, and it effectively decreases plasma markers of oxidative stress in ALS and stroke (Nagase et al., 2016; Uno et al., 2005). In mouse models of ALS edaravone delays

symptom progression and decreases motor neuron degeneration (Ikeda & Iwasaki, 2015; Ito et al., 2008). The most recent placebo-controlled trial of edaravone showed significant protection against decline in a subset of ALS patients (Abe et al., 2017), and in 2017 edaravone was approved by the FDA for the treatment of ALS.

I.4 Astrocyte antioxidant mechanisms in neurodegenerative disorders

One of the many important roles astrocytes play in maintaining CNS homeostasis is regulating redox balance. Astrocytes use a variety of antioxidant mechanisms not just to control oxidative stress associated with the metabolic functions of astrocytes, but also to support neurons and other CNS cell types in reducing the oxidative damage that they produce (Fig. I.1). These mechanisms are necessary for normal CNS function and neurodevelopment. In various neurodegenerative disorders, however, these astrocyte antioxidant mechanisms are impaired. This can result in increased astrocyte oxidative stress, driving astrocyte activation and pro-inflammatory or neurotoxic astrocyte phenotypes. Altered astrocyte antioxidant responses can also impair antioxidant support for neurons, promoting neuropathology and neurodegeneration. Here we will examine the most significant antioxidant mechanisms in the brain, the roles astrocytes play in these mechanisms, and how these antioxidant mechanisms are disrupted in neurodegenerative diseases.

I.4.1 Glutathione

Glutathione (GSH) is a short polypeptide important for a wide variety of antioxidant responses. GSH can donate an electron to reduce ROS nonenzymatically or serve as a cofactor in enzymatic reactions. It also plays important roles in detoxifying xenobiotics and modifying proteins, among other functions.

GSH acts as an electron donor to scavenge free radicals or reduce the products of oxidative damage. This can occur nonenzymatically or enzymatically. Free GSH can react with ROS in the cytosol to form water or hydrogen peroxide. Enzymatic GSH-dependent reduction is primarily mediated by glutathione peroxidases, a family of enzymes that catalyze the reduction of peroxides, particularly hydrogen peroxide and lipid peroxides (Liddell, Hoepken, et al., 2006).

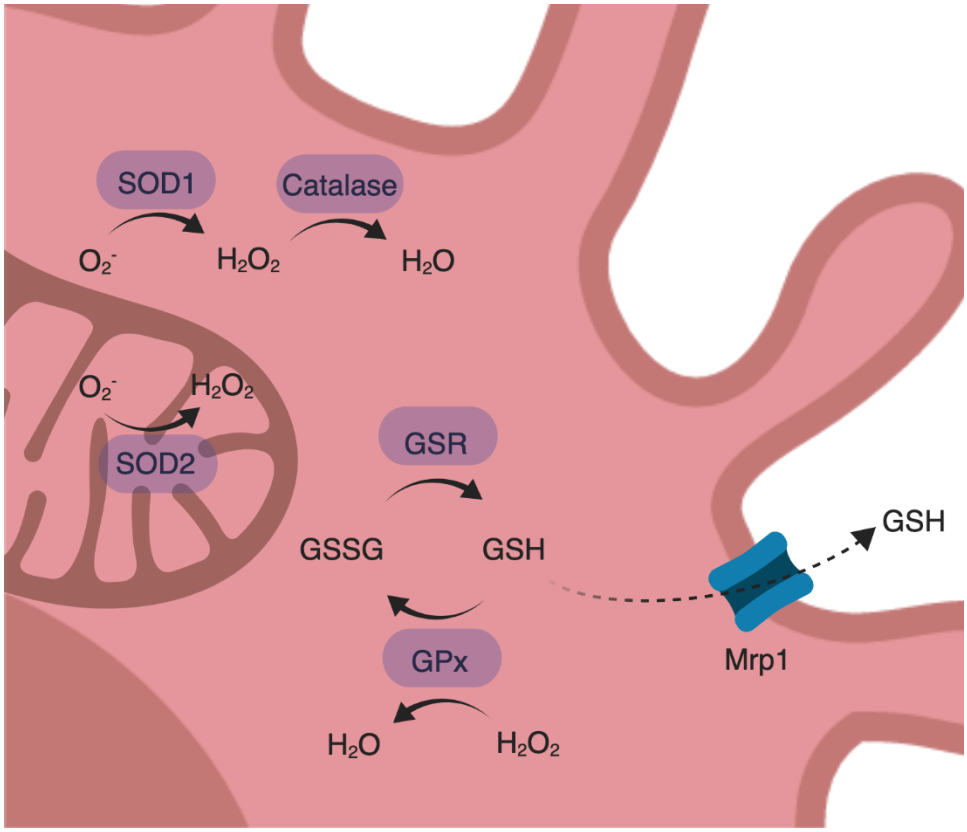

Figure I.1 Antioxidant systems in astrocytes

Superoxide ($O2^-$) can be converted to hydrogen peroxide (H2O2) by superoxide dismutase 1 (SOD1) in the cytoplasm and SOD2 in the mitochondria. Hydrogen peroxide can be converted to water by catalase or glutathione peroxidase (Gpx). Glutathione (GSH) is necessary for glutathione peroxidase activity as well as other redox systems. It can be oxidized to glutathione disulfide (GSSG), which can be regenerated to glutathione through the NADPH-dependent activity of glutathione reductase (GSR). Astrocytes also use multidrug resistance protein 1 (MRP1), an ATP binding cassette transporter, to secrete glutathione to support antioxidant function in other cells in the brain. Figure created with BioRender.com.

Glutathione peroxidase uses GSH to reduce these peroxides, producing water and oxidized GSH as a byproduct.

When GSH is oxidized it can react with another molecule of oxidized GSH to form glutathione disulfide (GSSG). The enzyme glutathione reductase can then regenerate two molecules of GSH from one molecule of GSSG in an NADPH-dependent reaction. Glutathione reductase is expressed broadly in the brain, as all cell types utilize the GSH/GSSG redox pair and must regenerate GSH from the GSSG produced by antioxidant activities (Y. Zhang et al., 2014). Intracellular GSH is far more abundant than GSSG at baseline, high ROS conditions can drive the oxidation of more GSH to GSSG by glutathione peroxidase and nonenzymatic reactions, so the [GSH]:[GSSG] ratio can be used as a measure of cellular oxidative stress.

Astrocytes have robust GSH-dependent antioxidant mechanisms. Intracellular GSH is higher in astrocytes than in neurons, giving them a greater capacity for reducing exogenous ROS (Dringen et al., 2002; Makar et al., 1994; Raps et al., 1989). Glutathione peroxidase, the enzyme that mediates GSH-dependent reduction of hydrogen peroxide and organic peroxides, is expressed in astrocytes and promotes survival when astrocytes are treated with peroxides (Liddell, Dringen, et al., 2006; Liddell, Hoepken, et al., 2006). Astrocytes are also high expressers of glutathione reductase to reduce GSST after the actions of glutathione peroxidase, and the [GSH]:[GSSG] ratio rapidly declines upon acute ROS treatment but then returns to normal through the action of glutathione reductase (Liddell, Dringen, et al., 2006; Liddell, Hoepken, et al., 2006).

Astrocytes also contribute to neuronal GSH stores. While GSH is recycled from GSSG in neurons and other cells in the CNS, GSH is also synthesized *de novo* to replace loss from the GSH/GSSG cycle because of breakdown or export. GSH is a tripeptide consisting of glycine, glutamate, and cysteine, of which cysteine is the limiting factor. γ-glutamylcysteine synthetase,

the rate limiting enzyme in GSH synthesis, is expressed at dramatically higher levels in astrocytes than in neurons (Makar et al., 1994). Astrocytes secrete GSH with the transporter multidrug resistance protein 1 (Mrp1, also known as ABCC1) (Hirrlinger et al., 2002; Minich et al., 2006). This secreted GSH is cleaved into its component amino acids in the extracellular space (Johnson et al., 2012), and neurons are then able to uptake the cysteine through excitatory amino acid transporters (EAATs) and synthesize GSH (Y. Chen & Swanson, 2003). This exogenous cysteine is necessary for neurons to maintain GSH stores *in vitro* (Sagara et al., 1993).

Given the importance of GSH mechanisms for controlling oxidative stress in the brain, it is unsurprising that alterations to GSH levels, [GSH]:[GSSG] ratio, and glutathione peroxidase activity have been associated with many CNS disorders. In Parkinson's disease, GSH is decreased by nearly 50% and the [GSH]:[GSSG] ratio is increased by nearly 30% in the substantia nigra (Sofic et al., 1992). One study reported increased GSH linked to astrocyte proliferation in other brain regions in PD (Mythri et al., 2011), possibly as a compensatory mechanism for elevated oxidative stress. Astrocytes from parkin KO mice have increased intracellular GSH but decreased secreted GSH, as well as decreased glutathione peroxidase activity (Solano et al., 2008), pointing to a possible role for parkin in antioxidant support of neurons. As GSH is apparently severely disrupted in PD, GSH supplementation has been considered as a possible treatment. N-acetylcysteine (NAC), a precursor in GSH synthesis, is protective in a rat model of PD (Rahimmi et al., 2015) and is currently in a trial in humans. If it proves successful, it will likely be through increased cysteine in the brain leading to increased astrocyte GSH synthesis and secretion.

In AD GSH is decreased in the brain (Saharan & Mandal, 2014), and serum glutathione peroxidase is decreased in patients with AD or MCI compared to controls (Padurariu et al., 2010). Severity of cognitive decline in AD is correlated with decreased GSH levels in the hippocampus and frontal cortex (Mandal et al., 2015a). Interestingly, amyloid β treatment decreases GSH in astrocytes and neurons cocultured with astrocytes (Abramov et al., 2003). Another *in vitro* study found a short-term increase in astrocyte GSH release in response to amyloid β, but after longer treatments astrocyte GSH release declined below the levels of controls (Ye et al., 2015). In addition, in the late stages of a mouse model of AD levels of Mrp1, the astrocyte GSH exporter, are decreased (Ye et al., 2015). This points strongly to a specific effect of AD pathology on astrocyte GSH antioxidant support for neurons. Given that oxidative stress is an important driver of neuronal death in AD, this failure of astrocyte antioxidant support is likely one mechanism by which amyloid β promotes neurodegeneration. In studies of rat AD models, NAC administration protected against amyloid β-induced memory deficits (More et al., 2018; Shahidi et al., 2017), but there are currently no NAC or GSH supplementation trials in humans for AD. The data relating AD to GSH antioxidant activity is strong, and points particularly to amyloid β induced impairment of astrocyte GSH synthesis and export, reaffirming the importance of astrocyte antioxidant support in neurodegeneration.

GSH antioxidant systems are also disrupted in ALS. Decreasing GSH in *in vitro* and *in vivo* mouse models exacerbates neurodegeneration and accelerates disease progression (Chi et al., 2007; Vargas et al., 2011). Cortical GSH was decreased by 31% in a study of a small number of ALS patients compared to controls (Weiduschat et al., 2014), and glutathione peroxidase activity in human serum and brain tissue were also decreased (Moumen et al., 1997; Przedborski et al., 1996). However, ALS model mice with genetically increased or decreased glutathione

peroxidase expression do not show altered disease progression (Cudkowicz et al., 2002), and GSH and the GSH precursor N-acetylcysteine (NAC) have failed in human trials as therapeutic approaches for ALS (Chili et al., 1998; Louwerse et al., 1995). These conflicting data suggest that while GSH antioxidant systems are impaired in ALS, this impairment is likely not a major contributor to disease pathology.

I.4.2 Superoxide dismutase

Superoxide is a highly reactive oxygen species that can be produced as a byproduct of oxidative phosphorylation or through the enzymatic activity of NADPH oxidase. Superoxide dismutases (SODs) are the primary cellular defense against superoxide. SODs use metal ions to convert two molecules of superoxide into oxygen and hydrogen peroxide. The hydrogen peroxide produced in this reaction is still a reactive oxygen species, but it is less damaging than superoxide and can be cleared by other antioxidant mechanisms such as glutathione peroxidase or catalase. Mammals express three different SODs. SOD1, also known as CuZn-SOD, is present throughout the cytoplasm of the cell and in the mitochondrial intermembrane space. SOD2, also known as Mn-SOD, is present in the mitochondrial matrix. It is particularly important in the control of superoxide produced through oxidative phosphorylation in the mitochondria. SOD3 is a member of the same family as SOD1, but is secreted and interacts with the extracellular matrix or plasma membrane, converting superoxide in the extracellular space. While SOD3 has not been extensively studied in the brain, SOD1 and SOD2 have long been studied in astrocytes and in neurodegenerative disease.

As discussed in section 3 above, oxidative stress in astrocytes can impair their neuroprotective effects and activate their neurotoxic processes. SODs play an important role in controlling this astrocytic oxidative stress, thereby sparing neurons these adverse effects. SOD2 and catalase

(discussed below in 4.3) are upregulated in astrocytes exposed to hydrogen peroxide (Nahirnyj et al., 2013). In one study of *in vitro* astrocytes, glucose deprivation induces the expression of sirtuin 1 (SIRT1) (Y. Cheng et al., 2014), a protein associated with longevity that is important in a variety of stress responses. Glucose deprivation also upregulates astrocytic SOD2 and catalase, and upregulation of both is impaired by SIRT1 inhibition. Interestingly, ROS levels were not elevated in glucose deprived astrocytes, but addition of a SIRT1 inhibitor to glucose deprived astrocytes resulted in elevated ROS, suggesting that SIRT1-dependant SOD2 and catalase may be important in controlling astrocytic oxidative stress during nutrient deprivation.

SOD activity in tissue from AD brains is decreased compared to controls (Omar et al., 1999). SOD1 overexpression protects against MPTP toxicity to dopaminergic neurons in the substantia nigra in a mouse model of PD (Przedborski et al., 1992), and mice with decreased SOD2 expression are more susceptible to MPTP and other pharmacological models of neurodegeneration in the substantia nigra (Andreassen et al., 2001).

SOD1 is commonly linked with neurodegeneration because of its association with ALS. SOD1 mutations were the first identified genetic cause of familial ALS (Bruijn et al., 1997; Rosen et al., 1993). ALS-causing SOD1 mutations account for an estimated 20-25% of cases of familial ALS (Pasinelli & Brown, 2006). These mutations are autosomal dominant and include nonsense and missense mutations that can be found in all exons of the gene. Evidence of the importance of astrocytes in ALS is particularly strong in SOD1 mutant models. SOD1 mutant motor neurons cocultured with wild type astrocytes have improved survival over those cocultured with mutant astrocytes (Clement et al., 2003), and mutant astrocytes can induce degeneration in wild type motor neurons (Aebischer et al., 2011; Di Giorgio et al., 2008; Nagai et al., 2007). Astrocytic mutant SOD1 may induce motor neuron disease through a variety of mechanisms which are still

under dispute. Impaired SOD1 activity may result in increased superoxide levels and oxidative stress. Strangely, some early studies suggested that mutant SOD1 may be capable of aberrant enzymatic activity that could produce ROS rather than clear it (Bruijn et al., 1998; Gurney et al., 1994). More recent studies suggest that altered SOD1 catalytic activity is likely not the primary mechanism by which mutant SOD1 promotes neurodegeneration. The most compelling evidence against this hypothesis come from studies in which wild type SOD1 is overexpressed alongside mutant SOD1 in mice. Contrary to what would be predicted if mutant SOD1 is pathogenic because of decreased or altered enzymatic activity, these mutant/overexpressed WT SOD1 mice had more severe neurodegenerative phenotypes than mice that only expressed the mutant SOD1 (Deng et al., 2006; Jaarsma et al., 2000). This points towards other pathogenic mechanisms for mutant SOD1, likely including the formation of SOD1 aggregates. The effects of these SOD1 aggregates on astrocyte oxidative stress are discussed in section 5.

I.4.3 Catalase

Catalase is a protein that plays an overlapping role with glutathione peroxidase by breaking down hydrogen peroxide. It uses an iron ion to catalyze the conversion of two molecules of hydrogen peroxide to two molecules of water and one of oxygen. Hydrogen peroxide is a common product of oxidative phosphorylation as well as of immune defense, and the homeostatic control of hydrogen peroxide levels are a necessary process throughout the body. Overexpression of catalase can protect against some adverse effects of aging (Selvaratnam & Robaire, 2016), demonstrating the importance of catalase in controlling oxidative damage during aging.

Catalase levels and activity are affected in neurodegeneration, and some catalase-based interventions have shown benefits in rodent models of neurodegenerative diseases. Catalase

activity is dramatically decreased in Alzheimer's disease brains (Omar et al., 1999) and Parkinson's disease brains (Desagher et al., 1996) compared to controls. In a fly model of a common genetic cause of ALS as well as frontotemporal dementia (FTD), *C9ORF72* poly(GR) repeat expansion, overexpression of catalase decreases toxicity resulting from the mutation (Lopez-Gonzalez et al., 2016). Catalase has been explored as a potential novel treatment for Parkinson's disease. Exosomes or macrophages loaded with catalase can cross the BBB and deliver catalase to the brain, resulting in decreased ROS, decreased microglial and astrocytic activation, and improved neuronal survival in a rodent model of PD (Haney et al., 2015; Klyachko et al., 2017; Y. Zhao et al., 2011). However, the exosome technique was found to primarily deliver catalase to neurons, suggesting that benefits from this technique are more likely due to catalase activity in neurons than in astrocytes (Haney et al., 2015).

All cell types in the brain express CAT, the gene encoding catalase, but astrocytes and endothelial cells are the highest expressers (Y. Zhang et al., 2014). Neuronal cultures are protected against hydrogen peroxide toxicity by coculture with astrocytes, and this protective effect is dependent upon astrocytic catalase activity (Desagher et al., 1996). This neuroprotective response to ROS by astrocytes is mediated in part by Nrf2, a transcription factor that drives genes involved in antioxidant defense. Astrocytic Nrf2-mediated transcription protects both astrocytes and neurons against hydrogen peroxide treatment (A. D. Kraft et al., 2004; Shih et al., 2003). Astrocyte-specific Nrf2 overexpression in a mouse model of ALS results in delayed disease onset and progression (Vargas et al., 2008) and Nrf2 in astrocytes, but not in other brain cell types, is protective in a mouse PD model (P.-C. Chen et al., 2009). A subsequent study using proteomics to identify the protein-level changes induced by Nrf2 signaling in astrocytes identified catalase and a glutathione synthetic enzyme among the targets of Nrf2, but it identified

Nrf2-induced catalase as particularly important in protection against hydrogen peroxide toxicity (Dowell & Johnson, 2013). This points to catalase, and possibly GSH, as likely mediators of astrocyte Nrf2-dependent protection against oxidative stress and neurodegeneration in *in vitro* and *in vivo* models. Direct research on the particular contributions of astrocytic catalase compared to catalase in other cell types is limited. However, given the high expression of catalase in astrocytes and the well documented neuroprotective roles of astrocytic Nrf2, the evidence points to a role of astrocytic catalase in protection against neurodegeneration that should be studied further.

Neuroinflammatory signaling in the brain during neurodegeneration may affect catalase activity in the brain. Neurodegenerative diseases are accompanied by increased inflammation and are frequently associated with elevated IL-1β (Leal et al., 2013; Mendiola & Cardona, 2018; W.-Y. Wang et al., 2015). Interestingly, astrocytes increase ROS production and downregulate catalase in response to IL-1β (Sheng et al., 2013). Through this downregulation of astrocytic catalase, cytokine signaling may be one mechanism by which astrocytic ROS production and oxidative stress are increased in neurodegenerative disorders.

I.5 Astrocyte ROS production in neurodegenerative disorders

Astrocytes play many neuroprotective roles in healthy brains as well as in neurodegeneration, including the antioxidant mechanisms reviewed in section 4 above. On the other hand, astrocytes also generate ROS. Under nonpathological conditions astrocytic ROS can perform signaling functions in the cell and excess ROS is neutralized by astrocytic antioxidant mechanisms. In neurodegeneration, however, astrocytes can dramatically increase ROS production. If this increased ROS production is not balanced by increased astrocytic antioxidant capacity, redox balance in astrocytes can be upset, resulting in oxidative stress in astrocytes and surrounding

neurons. Increased astrocytic ROS production in neurodegeneration occurs through two primary mechanisms, reviewed below: NADPH oxidase enzymatic activity and oxidative phosphorylation (Fig. I.2).

I.5.1 NADPH oxidase

NADPH oxidase is primarily involved in immune responses to pathogens, but it can be highly expressed in sterile injuries and in neurodegeneration. NADPH oxidase is membrane-bound protein complex which oxidizes NADPH to $NADP^+$ in order to produce superoxide in the extracellular space or within a phagosome. The highly reactive superoxide is a potent antimicrobial, explaining the utility of NADPH oxidase in controlling infection, but its activity in neurodegeneration is more poorly understood. NADPH oxidase has several isoforms. In nonpathological conditions NOX2 is the most common in the brain, where it is primarily expressed by microglia (Y. Zhang et al., 2014). However, other cell types and other NADPH oxidase isoforms can be expressed in the brain: human astrocytes express NOX4 (G. Cheng et al., 2001), mouse microglia express NOX1 (Cheret et al., 2008), and astrocytes and neurons upregulate NOX2 and NOX4 in subarachnoid hemorrhage (L. Zhang et al., 2017).

As discussed briefly in section 4.3, inflammatory signals in the brain are commonly elevated in neurodegenerative disease and may contribute to oxidative stress. Fetal human astrocyte cultures treated with IL-1β have elevated ROS, but this elevation in ROS can be rescued by NADPH oxidase inhibition with the drug diphenyleneiodonium (DPI) (Sheng et al., 2013). This

demonstrates that the inflammatory environment in neurodegeneration could contribute to increased oxidative stress through increased activity of NADPH oxidase.

Astrocytic NADPH oxidase may contribute significantly to the oxidative stress observed in AD. Amyloid β treatment of rat astrocyte cultures and astrocyte-neuron cocultures depletes astrocytic GSH, depolarizes astrocytic mitochondrial membranes, and induces death in both astrocytes and neurons (Abeti et al., 2011; Abramov et al., 2004). However, all of these effects can be blocked

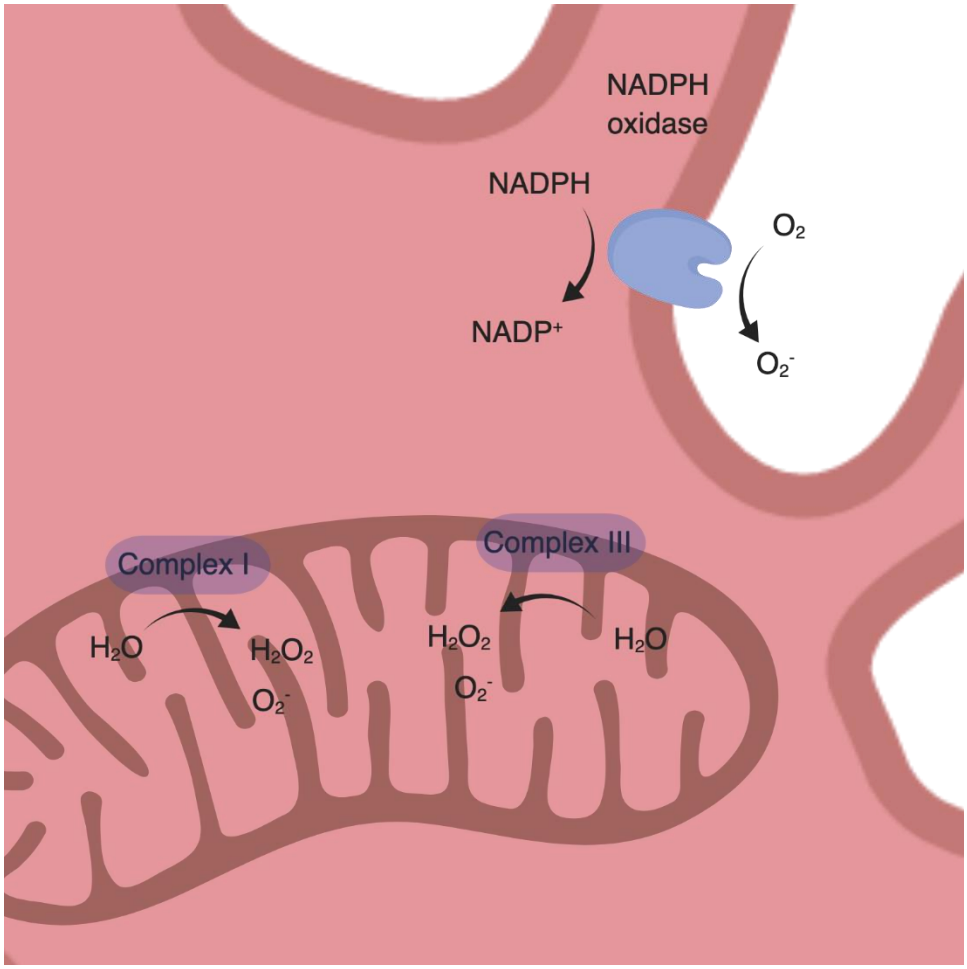

Figure I.2 ROS-generating systems in astrocytes

Mitochondrial ROS are generated by electron trainsport chain complex proteins, particularly complexes I and III, which can generate hydrogen peroxide (H2O2) and superoxide ($O2^-$) from water. The other major source in astrocytes is NADPH oxidase, which oxidizes NADPH to NADP+ in order to convert oxygen to superoxide outside of the cell. Figure created with BioRender.com.

by inhibiting NADPH oxidase (Abramov et al., 2004). Amyloid β increases NOX2 expression in cultured rat astrocytes, and NOX2 is elevated in human AD brains and colocalizes with astrocytes (Wyssenbach et al., 2016).

In ALS the role of NADPH oxidase has been disputed. NADPH oxidase is increased in SOD1 mutant mouse spinal cord and deletion of NOX1 or NOX2 was reported to increase survival in a mouse model of ALS (Marden et al., 2007; D.-C. Wu et al., 2006), but more recently another group failed to replicate this finding with either NOX1 or NOX2 deletion or NADPH oxidase inhibitor treatment (Seredenina et al., 2016).

Interestingly, microglial NADPH oxidase has been identified as a driver of astrocyte activation in mouse models of PD (Hou et al., 2017; Q. Wang et al., 2015). In these studies, mice treated with LPS or MPTP to induce inflammation and neurodegeneration in the substantia nigra had decreased astrocyte activation and neurodegeneration when NADPH oxidase was pharmacologically inhibited or when NOX2 was knocked out. Cultured astrocytes treated with activated microglia conditioned media increased expression of iNOS, the cytokine TNFα, and neurotrophic factors, but these astrocyte transcriptional changes were blocked by treating the microglia with an NADPH oxidase inhibitor (Hou et al., 2017). This supports the hypothesis that microglia drive astrogliosis through NADPH oxidase activity. However, because other cell types can also express NOX2, repeating this experiment with microglia-specific NOX2 knockout mice would more definitively show that microglia are primarily responsible for driving this NADPH oxidase-dependent astrocyte response *in vivo*.

I.5.2 Mitochondrial ROS production

Though mitochondrial respiration is typically an efficient process for producing energy while transferring electrons from NADH and $FADH_2$ to oxygen, electrons can leak from the electron transport chain and produce ROS in the mitochondria. The primary reactive byproducts of oxidative phosphorylation are superoxide and hydrogen peroxide, both of which can be produced by electron leaks at many of the redox centers in the electron transport chain. Superoxide is thought to be produced primarily when electrons leak from mitochondrial complexes I and III, reacting with oxygen and generating superoxide (Dröse & Brandt, 2012; H.-S. Wong et al., 2017). Hydrogen peroxide is also produced at these sites and on other electron transport chain proteins, but it can also be generated by the breakdown of superoxide into hydrogen peroxide by mitochondrial SOD2 (H.-S. Wong et al., 2017). Mitochondrial ROS generation can increase or decrease depending on the substrates utilized for respiration, the glycolytic/oxidative balance of ATP generation, the energetic needs of the cell, and other factors. Elevated mitochondrial ROS is observed in several neurodegenerative disorders, and astrocytes play an important role in this mitochondrial oxidative stress.

Neurons and astrocytes have dramatically different metabolic profiles, and this results in differences in mitochondrial ROS generation between the two cell types. Though both cell types are capable of glycolysis and oxidative phosphorylation, neurons tend to utilize oxidative phosphorylation for most of their energy needs while astrocytes are more reliant on glycolysis (Bélanger et al., 2011). In fact, astrocytes are able to survive and appear to function normally while relying entirely on glycolysis for ATP generation, as shown in astrocyte-specific mitochondrial complex IV knockout mice which are incapable of normal mitochondrial respiration (Supplie et al., 2017). Mitochondrial activity is therefore significantly different

between astrocytes and neurons. Perhaps counterintuitively, astrocytes produce more mitochondrial ROS than neurons *in vitro*, reportedly due to the decreased mitochondrial complex I supercomplex assembly in astrocytes (Lopez-Fabuel et al., 2016).

Mitochondrial dysfunction in astrocytes has been associated with Parkinson's disease in several studies. Mitochondrial complex I activity is decreased in the substantia nigra of PD patients (Mizuno et al., 1989; Schapira et al., 1990). Several toxins used to induce PD-like degeneration of the dopaminergic neurons, including MPTP, inhibit mitochondrial complex I and result in increased ROS production (Niranjan, 2014; Przedborski et al., 2004). Cultured mouse astrocytes treated with α-synuclein oligomers display altered mitochondrial morphology and increased mitofusin 1, a mitochondrial fusion protein associated with mitochondrial stress (Lindström et al., 2017). These astrocytes have decreased ATP levels, indicating functional impairment to mitochondria in response to α-synuclein. Human iPSC-derived astrocytes also have decreased ATP levels and altered mitochondrial morphology when treated with α-synuclein (Rostami et al., 2017). Interestingly, healthy astrocytes in this system preferentially transfer mitochondria to α-synuclein loaded astrocytes, showing that, at least *in vitro*, astrocytes are capable of supplying mitochondria to surrounding cells as a protective mechanism against α-synuclein toxicity.

Although in the context of ALS mitochondrial oxidative stress has been primarily studied in neurons, there is compelling evidence that mitochondrial ROS production in astrocytes plays an important role in the disease. Cultured rat astrocytes from a SOD1 mutant ALS model produce elevated superoxide levels in their mitochondria (Cassina et al., 2008). These mutant astrocytes have impaired mitochondrial coupling and induce motor neuron death in coculture with wild type motor neurons, but astrocytic mitochondrial coupling and motor neuron survival were rescued when the astrocytes were treated with mitochondrial-targeted antioxidant compounds.

These experiments compellingly show that in the SOD1 mutation model of ALS, astrocytic mitochondrial oxidative stress is an important mechanism for SOD1-driven motor neuron degeneration and a possible therapeutic target for future research. One possible mechanism for this phenomenon is lysyl-tRNA synthetase (KARS), a protein involved in translation of mitochondrial proteins. Mutant SOD1 binds KARS and induces misfolding and aggregation, thereby impairing mitochondrial protein synthesis (Kawamata et al., 2008). This protein synthesis impairment could contribute to the mitochondrial oxidative stress that leads to motor neuron degeneration in ALS.

Alzheimer's disease is strongly linked to mitochondrial dysfunction, and elevated astrocyte mitochondrial ROS production has been studied in multiple experimental systems. iPSC-derived astrocytes from AD patients with a *PSEN1* mutation that causes early onset AD have been studied for metabolic changes compared to controls. These AD astrocytes have significantly higher basal respiration and lower basal glycolysis than controls as measured by Seahorse assay, as well as elevated ROS levels (Oksanen et al., 2017). Somewhat contradicting these results, another study found that treating neonatal rat astrocytes with amyloid β resulted in decreased basal and maximal oxygen consumption and eventually resulted in mitochondrial depolarization (Abeti et al., 2011). The contradictory findings in these studies could be due to the differences in time scale between the acute amyloid β treatment versus the chronic effects of *PSEN1* mutation, or because of differences in the model systems of rat neonatal astrocytes versus human iPSC-derived astrocytes. In both cases, however, mitochondrial dysfunction is observed, resulting in increased astrocytic ROS generation.

One possible mechanism in AD-related disrupted astrocyte mitochondrial respiration is insulin and insulin-like growth factor 1 (IGF-1) signaling. Insulin resistance and decreased IGF-1 levels

have long been observed in the AD brain (Ferreira et al., 2018), and intranasal insulin administration has been explored as a possible AD treatment with mixed results (Avgerinos et al., 2018; Chapman et al., 2018). The IGF-1 receptor (IGFR) is decreased in the brains of aged mice (Logan et al., 2018; Sonntag et al., 2005). IGFR KO mouse astrocytes *in vitro* have decreased oxidative phosphorylation efficiency and increased mitochondrial ROS production compared to control astrocytes, as well as decreased amyloid β uptake (Logan et al., 2018).

I.6 Conclusion

Alzheimer's disease, Parkinson's disease, and ALS are all devastating diseases with often insufficient or nonexistent options for treatment. Research into these diseases is made more difficult by the fact that the etiologies of these diseases are poorly understood and only a small fraction of cases of each can be traced to a known genetic cause. However, some of their shared pathological mechanisms offer hope for treatments that could alter disease course for both sporadic and familial forms of neurodegenerative diseases. The evidence for astrocyte-modulated oxidative stress as a causal factor in these diseases is strong, from *in vitro* experiments through rodent models and into human disease. Still, with the notable exception of edaravone in ALS, antioxidant treatments in neurodegenerative diseases have had at best mixed results in human trials. The evidence reviewed here points to possible future directions that may be more successful in treating neurodegenerative diseases. Future antioxidant therapies may be more successful by targeting specific contributors to oxidative stress in neurodegeneration, such as NADPH oxidase or astrocytic mitochondrial function. Neurodegenerative disease therapeutics may also fail because they are started too late in disease progression. In this case, oxidative stress is a promising target for future trials as it appears early in the progression of many of these

diseases. These are all promising research topics, and further research on the role of astrocytes in controlling or contributing to oxidative stress in neurodegenerative diseases is needed.

Appendix II Oxidized cholesterol species as signaling molecules in the brain: diabetes and Alzheimer's disease

Previously published as (Weigel et al., 2019).

II.1 Abstract

Type 2 diabetes is associated with adverse central nervous system effects, including a doubled risk for Alzheimer's disease (AD) (Gudala et al., 2013) and increased risk of cognitive impairment (Geijselaers et al., 2015; Roberts et al., 2014), but the mechanisms connecting diabetes to cognitive decline and dementia are unknown. One possible link between these diseases may be the associated alterations to cholesterol oxidation and metabolism in the brain. We will survey evidence demonstrating alterations to oxysterols in the brain in AD and diabetes and how these oxysterols could contribute to pathology, as well as identifying research questions that have not yet been addressed to allow for a fuller understanding of the role of oxysterols in AD and diabetes.

II.2 Introduction

Type 2 diabetes is a driver of Alzheimer's disease (AD) symptomatology, likely through multiple mechanisms that result in a doubled risk of AD (Gudala et al., 2013). Diabetes and AD share several common brain phenotypes which have been proposed as possible links between the disease. AD and diabetes both increase insulin resistance, inflammation, and oxidative stress in the brain (Arnold et al., 2018; Pugazhenthi et al., 2017). Vascular disease associated with diabetes increases risk of dementia (Exalto et al., 2013; Haroon et al., 2015), and both hypoglycemia (Whitmer et al., 2009) and hyperglycemia (Bangen et al., 2016; Macauley et al.,

2015) may also contribute to AD risk. Diabetes and AD have also both been linked to disruptions in cholesterol metabolism in the CNS (T.-Y. Chang et al., 2017; C.-C. Liu et al., 2013; Suzuki et al., 2010). The brain is highly cholesterol dense, and cholesterol is essential for neuronal functions such as synapse formation, vesicle fusion, and membrane receptor clustering (Allsopp et al., 2010; Fukui et al., 2015; C.-C. Liu et al., 2013). Genetic evidence strongly implicates cholesterol in AD. The largest known genetic risk factor for late onset AD, the most common form of the disease, is the ϵ4 allele of apolipoprotein E (ApoE) (Musiek et al., 2015). ApoE is a lipid-binding protein that forms high density lipoprotein (HDL)-like particles to transport cholesterol and other lipids throughout the brain. Other risk genes for AD are also associated with cholesterol, including ApoJ/CLU (which interacts with ApoE to form cholesterol-carrying lipoproteins) (J. A. Chen et al., 2015; Lambert et al., 2009, 2013), ABCA7 (a membrane cholesterol transporter) (J. A. Chen et al., 2015; Steinberg et al., 2015), and SORL1 (an ApoE receptor involved in internalizing cholesterol-carrying lipoproteins) (J. A. Chen et al., 2015; Lambert et al., 2013). Studies of rodent models of diabetes have shown decreased cholesterol synthesis in the brain (Suzuki et al., 2010). Direct modulation of cholesterol synthesis in the brain has been proposed as a possible therapeutic strategy in AD, and cholesterol synthesis inhibitors can decrease amyloid β and tau phosphorylation *in vitro* (Parsons et al., 2007; van der Kant et al., 2019). Observational studies in humans have reported that use of the cholesterol synthesis-impairing statins is correlated with decreased AD risk (Jick et al., 2000; Wolozin et al., 2000), but randomized placebo-controlled trials have not found statins to decrease AD incidence (R. Collins et al., 2002; Houx et al., 2002; Mcguinness et al., 2016). Other than through alterations in biosynthesis, cholesterol metabolism could affect brain health through changes in oxidized cholesterol species, known as oxysterols. Levels of these molecules are altered in AD

and diabetes, and may have broad effects on important mechanisms in neurodegeneration including neuroinflammation, CNS cholesterol regulation, and neuronal health and survival.

II.3 Oxysterols

Cholesterol can be oxidized at various positions and by enzymatic or non-enzymatic mechanisms, resulting in a variety of structurally and functionally distinct oxysterols. The 7-carbon of cholesterol is particularly vulnerable to autoxidation, yielding oxysterols including 7-ketocholesterol (7KC) and 7β-hydroxycholesterol (7βOHC). Thus 7KC and 7βOHC production is a function of ROS levels in cells (Fig. II.1). Other oxysterols are produced enzymatically, primarily by members of the cytochrome P450 family: 7α-hydroxycholesterol (7αOHC) is produced by CYP7A1, 25-hydroxycholesterol (25OHC) by cholesterol 25-hydroxylase (CH25H), 24(S)-hydroxycholesterol (24(S)OHC) by CYP46A1, and 27-hydroxycholesterol (27OHC) by CYP27A1. In addition to their enzymatic production, 7αOHC and 25OHC can also be generated non-enzymatically.

II.4 Oxysterol production and regulation in the brain

The oxysterol content of the brain differs from that of the blood and other peripheral tissues because of CNS-specific patterns of lipid composition and oxysterol generation and export. The blood-brain barrier (BBB) is impermeable to cholesterol, meaning that cholesterol in the brain must be synthesized *in situ*. In the adult brain this is performed primarily by astrocytes, which utilize some of that cholesterol and export the rest for use by neurons and other glia. This cholesterol can subsequently be oxidized, either to serve as a signaling molecule, to facilitate cholesterol clearance from the CNS, or as a consequence of oxidative stress. Unlike cholesterol, these less hydrophobic oxysterols can be cleared from the brain by crossing the BBB, with subsequent transport via the blood to the liver. Permeability of the BBB to oxysterols means that

they can also cross from the blood into the brain. In humans the flux of the oxysterols 24(S)OHC, 7βOHC, and 7KC is out of the brain (Crick et al., 2015), while flux of 27OHC is into the brain (Heverin et al., 2005).

Enzymatic cholesterol oxidation differs in the brain compared to other tissues. 24(S)OHC is a CNS-specific oxysterol that is essential for regulating brain cholesterol content. Because cholesterol cannot cross the BBB and is not broken down locally, excess in the brain is converted to the BBB-permeable oxysterol 24(S)OHC by CYP46A1 expressed in neurons. 24(S)OHC is then exported to the blood where it is carried by LDL to the liver for degradation and excretion.

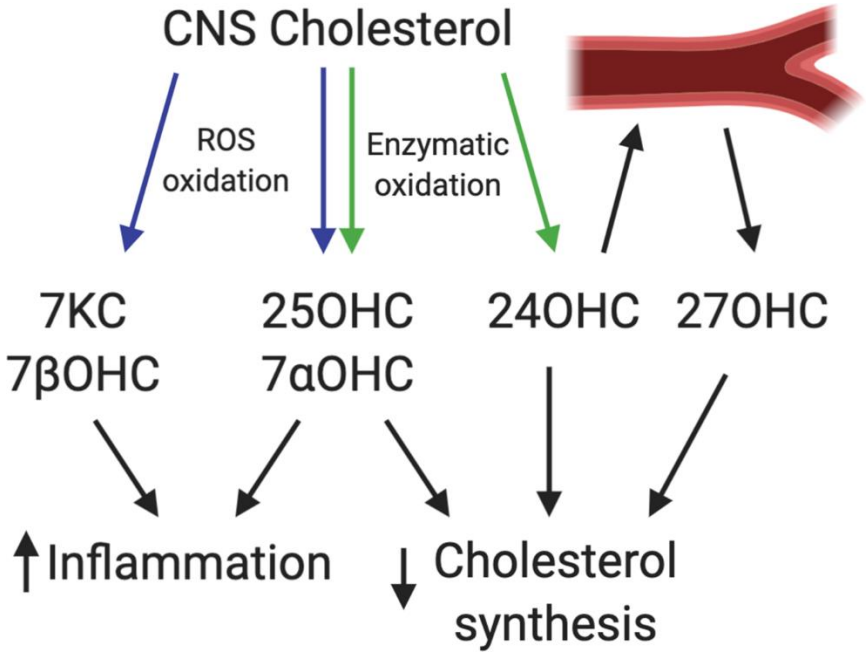

Figure II.1 Oxysterols in the brain

Oxysterols are produced from cholesterol by autoxidation or enzymatic oxidation. Non-enzymatically produced oxysterols include 7-ketocholesterol (7KC) and 7β-hydroxycholesterol (7βOHC). Other oxysterols are produced predominantly enzymatically, including 24(S)-hydroxycholesterol (24OHC), which exits the brain through the blood. 7α-hydroxycholesterol (7αOHC) and 25-hydroxycholesterol (25OHC) are produced both enzymatically and non-enzymatically. 27-hydroxycholesterol (27OHC) is produced in the periphery and enters the brain through the blood. These oxysterols can contribute to neuroinflammation and decreased cholesterol synthesis, two mechanisms thought to contribute to neurodegeneration. Figure created with BioRender.com.

Because it has such an important role in cholesterol clearance and not just as a signaling molecule, 24(S)OHC is the most prevalent oxysterol in the brain. Regulation of CYP46A1 is not well understood, and at the transcriptional level it is not altered by cholesterol, oxysterols, or statin treatment (Ohyama et al., 2006). Its activity can be increased *in vitro* by direct interactions with neurotransmitters, particularly glutamate (Mast et al., 2017), and several drugs have been found to modulate CYP46A1 activity (Mast et al., 2012, 2014; Shafaati et al., 2010). The antiretroviral efavirenz increases CYP46A1 activity *in vitro* and increases 24(S)OHC levels *in vivo* (Mast et al., 2014), while the antifungal voriconazole inhibits CYP46A1 and decreases 24(S)OHC production *in vivo* (Shafaati et al., 2010). These treatments induce broader alterations to brain cholesterol metabolism, with the CYP46A1 antagonist causing decreased cholesterol synthesis in the brain and CYP46A1 agonist inducing increased brain cholesterol synthesis. In both cases this results in unchanged total brain cholesterol levels. That a CYP46A1 agonist would increase cholesterol synthesis may seem paradoxical, as 24(S)OHC is a negative regulator of the transcription factor promoting cholesterol synthesis (Radhakrishnan et al., 2007). However, cholesterol is also an inhibitor of cholesterol synthesis through this same system (Radhakrishnan et al., 2004), and increased activity of CYP46A1 will decrease cholesterol levels and relieve this inhibition of cholesterol synthesis. This demonstrates a complex mechanism in the brain that senses and responds to changes in cholesterol levels and oxidation to maintain cholesterol homeostasis in the CNS. CYP46A1 inhibition induces astrocyte activation in the retina (Fourgeux et al., 2014), suggesting that in addition to synthesizing the bulk of the cholesterol in the brain, astrocytes may also be responsible for detecting changes in brain cholesterol metabolism and compensating for them. The role that CYP46A1 and 24(S)OHC plays in the brain, facilitating the clearance of excess cholesterol by the blood to the liver, is

performed in other tissues by CYP27A1 and its oxysterol product 27OHC. 27OHC levels are higher in the blood than in the brain, resulting in a flux of 27OHC into the brain from the periphery (Heverin et al., 2005). Though this oxysterol is not primarily derived from the CNS, it may play important roles in health and pathology in the brain, discussed below.

25OHC, another important oxysterol in the brain, is produced by CH25H. It is not necessary for cholesterol export from the brain and instead serves primarily as a signaling molecule. 25OHC is best studied in immune function, and CH25H activity is induced in macrophages by treatment with LPS or the cytokines IFN-α, IFN-β, and IFN-γ (Diczfalusy et al., 2009; K. Park & Scott, 2010). 25OHC acts at multiple levels of the immune response: it acts as a signaling molecule to amplify inflammatory activation in macrophages (Gold et al., 2014), but it also has direct antimicrobial effects, impairing virus entry and replication (Blanc et al., 2013; S.-Y. Liu et al., 2013). The role of 25OHC in the brain is poorly understood. 25OHC is elevated in the brain in X-linked adrenoleukodystrophy, where it promotes microglial activation and oligodendrocyte death through NLRP3 inflammasome activation (Jang et al., 2016). The roles that 25OHC may play in the healthy brain need to be further studied.

In addition to tissue-specific patterns of enzymatic cholesterol oxidation, non-enzymatic oxysterol production plays important roles in the brain. The brain is particularly susceptible to non-enzymatic cholesterol oxidation because of the high concentration of cholesterol combined with high oxygen consumption and ROS generation in the CNS. Unlike enzymatically generated oxysterols, production of these species cannot be regulated at the transcriptional or enzymatic level. The rate of production of non-enzymatically generated oxysterols in the brain varies as a function of the balance between ROS generation and antioxidant function, with more oxysterols produced when ROS generation outstrips the cell's ability to reduce reactive species before they

cause oxidative damage to lipids. While non-enzymatic oxysterol production cannot be directly regulated, levels of these oxysterols can be regulated through mechanisms of clearance. ATP binding cassette (ABC) efflux pumps play important roles in exporting oxysterols from cells in the brain, and the expression of these transporters is one mechanism by which intracellular oxysterol levels can be regulated. In fact, different oxysterols can regulate each other by modulating efflux pump expression. As an example, 24(S)OHC treatment induces expression of ABCG1 through activation of the liver X receptor (LXR). Cultured neurons pre-treated with 24(S)OHC have increased resistance to 7KC-induced apoptosis through an increase in ABCG1-mediated 7KC efflux (Okabe et al., 2014).

Because cholesterol and oxysterols are not broken down in the brain, clearance of these molecules requires their export across the BBB and into the blood so that they can be transported to the liver. This export process is still not fully understood, but it is thought to be driven by passive diffusion and active transporters in the neurons and glia and at the endothelial cells of the BBB. Sidechain-oxidized oxysterols (such as 24(S)OHC, 27OHC, and 25OHC) are particularly capable of being expelled from lipid membranes, allowing them to diffuse across plasma membranes and into the blood (Meaney et al., 2002). Active transport may also play a role in export of oxysterols across the BBB, as an inhibitor of organic anion transporter proteins (OATPs) decreases efflux of 24(S)OHC from the brain in rats (Ohtsuki et al., 2007).

II.5 Oxysterols in AD and diabetes

Oxidative stress is an important mechanism in AD. AD is characterized by both increased ROS production and decreased antioxidant function in the CNS. Treatment of neurons, astrocytes, and microglia with amyloid β increases ROS generation *in vitro* (Schilling & Eder, 2011; Urrutia et al., 2017; Yan et al., 1996; Yatin et al., 2000), and fatty acid peroxidation, a result of oxidative

damage to membranes, is increased in AD brains (Markesbery & Lovell, 1998; Subbarao et al., 1990). Meanwhile the important antioxidant polypeptide glutathione (GSH) is decreased in the brain in AD (Saharan & Mandal, 2014), and the severity of cognitive decline in AD is correlated with decreased GSH levels in the hippocampus and frontal cortex (Mandal et al., 2015b). As cholesterol autoxidation is a function of ROS levels, this increased ROS generation and decreased antioxidant function in AD results in increased generation of non-enzymatically produced oxysterols. Levels of the ROS-generated oxysterols 7KC and 7βOHC are elevated in cerebral cortical tissue from AD patients (Testa et al., 2016). 7KC is also elevated in the blood in AD (Liang et al., 2016), likely as a result of the ability of oxysterols to cross the BBB into the periphery.

Oxidative stress is also observed in diabetes, though it is far less well studied in the brain than in AD. Fatty acid peroxidation resulting from oxidative damage to lipid membranes is increased in the brain in Zucker diabetic rats (Raza et al., 2015), a genetic model of type 2 diabetes, and in rats treated with streptozotocin (STZ) (Sözbir & Nazıroğlu, 2016), a model of type 1 diabetes. Studies of high fat diet (HFD), which can induce symptoms of metabolic syndrome in rodents, have examined oxidative stress in the brain more extensively. HFD increases ROS and oxidative damage while decreasing GSH levels and the activity of superoxide dismutase (SOD), an antioxidant protein, in the hypothalamus in rats (Cavaliere et al., 2018). HFD also elevates oxidative stress and decreases antioxidant function in the hippocampus in mice, and these effects can be rescued by melatonin, which can act as an antioxidant (Alzoubi et al., 2018). The increased oxidative stress observed in these models suggest that non-enzymatically produced oxysterols would be elevated in the brain in diabetes similar to what is seen in AD. The only study directly measuring oxysterols in the brain in diabetes models found that rats treated with

STZ have elevated 7KC and 7βOHC in the cortex (Romano et al., 2017, 2018). While oxysterols in the brain are poorly studied in diabetes, oxysterols have been found to be elevated in the blood of diabetes patients (Endo et al., 2008) and is correlated with HbA1c levels, a measure of glucose control (Samadi et al., 2019). 7βOHC is also elevated in the blood in diabetes patients (Ferderbar et al., 2007). Oxysterols in the brains of diabetes patients still have not been measured, but these findings from studies of rodent brains and human blood suggest that, like AD, diabetes likely results in increased levels of non-enzymatically produced oxysterols in the brain.

Enzymatically-produced oxysterols are also affected by AD and diabetes, though some of these results are still disputed. 24(S)OHC has been reported to be both increased (H.-L. Wang et al., 2016) and decreased (Benussi et al., 2017; Kölsch et al., 2004; Testa et al., 2016) in the brain and CSF of AD patients. Some of these conflicting results may come from differences in disease stage being studied, where 24(S)OHC may be elevated in early AD but decrease when neurodegeneration progresses and the neurons that express CYP46A1 begin to die, but more research on this topic is necessary. Though it has not been studied in the brain in human diabetes, 24(S)OHC is decreased in the brain of a rodent diabetes model (Romano et al., 2018). 27OHC, which is derived from the periphery but can cross the BBB, is elevated in AD brains (Heverin et al., 2004; Testa et al., 2016). Again, data on oxysterols in the brain in diabetes is limited, but enzymatically-produced oxysterols are likely altered in this disorder as well. STZ-treated insulin-deficient rats have decreased 24(S)OHC and 27OHC in the cerebral cortex (Romano et al., 2018). In humans, 27OHC and 25OHC are elevated in the blood in patients with type 2 diabetes compared to controls (Murakami et al., 2000). These differing results regarding 27OHC may be attributable to the importantly distinct effects on peripheral metabolism from the obesity and insulin resistance of type 2 diabetes versus the rodent model of lean, insulin-deficient type 1

diabetes. The peripheral metabolic effects of type 2 diabetes are likely responsible for alterations in both blood and brain 27OHC levels, as it is produced in the periphery and then diffuses into the brain, but these changes may have substantial signaling effects in the brain.

II.6 Oxysterol toxicity and signaling functions

Both enzymatically- and non-enzymatically produced oxysterols have broad signaling functions involving a number of pathways that are altered in diabetes and AD. Here we will discuss CNS cholesterol regulation, oxidative stress, and inflammation.

Perhaps the most important role for oxysterols in the brain is in regulating cholesterol synthesis and transport. Numerous genes identified as risk factors for AD are related to cholesterol transport and metabolism in the brain, including ApoE (Musiek et al., 2015), ApoJ/CLU (J. A. Chen et al., 2015; Lambert et al., 2009, 2013), ABCA7 (J. A. Chen et al., 2015; Steinberg et al., 2015), and SORL1 (J. A. Chen et al., 2015; Lambert et al., 2013). Cholesterol synthesis in the brain is also decreased in multiple mouse models of diabetes (Suzuki et al., 2010). Brain cholesterol metabolism is altered in aging: cholesterol synthesis is decreased (Boisvert et al., 2018; Thelen et al., 2006) and cortical cholesterol content is decreased (Svennerholm et al., 1994), though this decreased cholesterol level has not been observed in the hippocampus (Thelen et al., 2006). Cholesterol synthesis is driven by the transcription factor sterol regulatory element-binding protein-2 (SREBP2), which controls transcription of the genes involved in the cholesterol synthetic pathway. SREBP2 resides in the ER, bound to the proteins SCAP and Insig, which retain SREBP2 in the ER. Cholesterol and oxysterols facilitate the interaction between SCAP and Insig that retain SREBP2 in the ER. In sterol-poor conditions 25OHC levels decline, destabilizing the SCAP-Insig interaction and allowing SREBP2 to be transported to the Golgi. There it undergoes proteolytic cleavage to release its transcriptionally active N terminus, which

travels to the nucleus and drives transcription of cholesterol synthetic genes. Oxysterols, particularly 24(S)OHC and 25OHC, are repressors of the transport of SREBP2 to the Golgi and subsequent induction of cholesterol synthesis (Radhakrishnan et al., 2007).

Oxysterols can contribute to both oxidative stress and antioxidant defense. Several oxysterols, most notably 7KC, are known to induce ROS generation (Nury et al., 2014; Palozza et al., 2010). Treatment with high concentrations of 7KC induces apoptosis in cultured macrophages in an oxidative stress-dependent mechanism (Leonarduzzi et al., 2006). Because 7KC is both a product and an inducer of ROS generation, it may contribute to a positive feedback loop resulting in increased oxidative stress in the brain. On the other hand, other oxysterols can counteract increased oxidative stress. 24(S)OHC can activate sirtuin 1 (SIRT1), a histone deacetylase protein involved in regulating transcription of important antioxidant genes (Testa et al., 2018).

Oxysterols can additionally act as potent immune signaling molecules. 7KC in particular has been studied in the context of inflammation. Neuroinflammation is an important component of AD pathology and is thought to play a mechanistic role in neurodegeneration. Peripheral immune activation has long been identified in diabetes, but more recent research has demonstrated neuroinflammation in response to high-fat diet (Fu et al., 2012; Guillemot-Legris & Muccioli, 2017; Noronha et al., 2019; Spencer et al., 2017). 7KC activates the immune sensor toll like receptor 4 (TLR4) (J.-D. Huang et al., 2014) and can induce the secretion of pro-inflammatory cytokines from macrophages and microglia. In the brain this may contribute to neuroinflammation, gliosis, and increased risk for neurodegeneration.

II.7 Microglia and oxysterols in disease

Microglia are the brain's resident immune cells and play an active role in surveying the brain and responding to a variety of insults. Unlike other tissue-specific macrophages, which originate from bone marrow progenitor cells, microglia derive from the yolk sack and migrate to the brain during development (Ginhoux et al., 2013). Microglia are motile cells which extend numerous branched processes from their cell bodies and are well-known to undergo morphological changes in response to pro-inflammatory stimuli. Like peripheral macrophages, microglia are capable of phagocytosing pathogens and cellular debris to protect the brain, and are thought to play an important role in regulating synaptic connections (Colonna & Butovsky, 2017).

Abundant evidence has linked microglia to neurodegenerative disease. Microglial activation is a widely observed phenomenon in mouse models of AD and in human AD (Hansen et al., 2017). Mutations in Trem2, a cell surface protein found on microglia are a risk factor for AD (Jonsson et al., 2013; Pottier et al., 2013). Pro-inflammatory shifts in microglial phenotype have also been observed in response to HFD and in mouse models of diabetes (Baufeld et al., 2016). Recent research has shown that pharmacologic depletion of microglia from the brains of mice maintained on a HFD reduces food intake and weight gain (Valdearcos et al., 2017). However, depleting microglia in mouse models of AD can surprisingly result in a loss of parenchymal amyloid plaques (Sosna et al., 2018; E. Spangenberg et al., 2019b). Thus, microglia appear to play a complex role in the brain response to disease and are capable of influencing the progression of brain diseases.

It is well characterized that peripheral immune cells phagocytose oxidized cholesterol laden lipoproteins. When cholesterol levels are elevated in the periphery, macrophages consume excess oxidized cholesterol molecules and shift to an inflammatory phenotype (Chistiakov et al., 2016).

While macrophage sequestration of oxysterols is likely protective, eventually, buildup of these cholesterol-laden immune cells in blood vessel walls is associated with vascular damage and ultimately atherosclerosis. Compared to macrophage biology, much less is known about how oxysterols, including 7KC, impact microglia (Fig. II.2). Early work by Chang et al. demonstrated that some oxysterols, including 25OHC, may act directly on the N9 microglial cell line, potently stimulating induction of iNOS, increasing the inflammatory state of the cells and inducing toxicity (J. Y. Chang et al., 1998). They subsequently demonstrated that treatment of N9 cells with micromolar concentrations of 25OHC potently induces c-Jun signaling and that this effect could be inhibited by treatment with PPAR receptor agonists (J. Y. Chang & Liu, 2001). This research helped lay the groundwork establishing that oxysterols act as pro-inflammatory inducers in microglial cells *in vitro*.

Several groups have contributed to the understanding of how oxysterols drive microglial activation in disease models and *in vivo*. A large fraction of the brain's cholesterol content is found in myelin. Demyelinating diseases, such as multiple sclerosis, significantly increase 7KC levels in CSF, likely due to myelin breakdown (Diestel et al., 2003). 7KC itself, in turn, is a robust inflammatory activator of the BV2 microglial cell line, suggesting that 7KC may be a signal to microglia for debris engulfment. 7KC induces iNOS expression and promotes nuclear accumulation of the pro-inflammatory transcription factor NFκB. Poly ADP-ribose polymerase 1 (PARP-1) modifies proteins post-translationally via conjugation of ADP-ribose moieties, a process which has been shown to play an important role in immune cell differentiation and adaptation to extracellular danger signals (Rosado et al., 2013). Interestingly, siRNA targeting of PARP-1 is sufficient to block the pro-inflammatory and toxic effects of 7KC in cultured BV2 cells and in brain tissue/microglia co-cultures. This work suggests that, in addition to 7KC being

elevated in demyelinating diseases and AD, it is sufficient to promote neurotoxicity. Moreover, the neurotoxic effects may be dependent on microglia. This concept of PARP-1 playing an essential role in oxysterol induced inflammation was further demonstrated in studies examining 15α-hydroxicholestene (15HC), another oxidized cholesterol metabolite that is significantly increased in the serum of MS patients. 15HC stimulated a robust increase in iNOS and TNFα in primary microglial cultures and robustly activated PARP-1 enzyme activity (Farez et al., 2009). The oxysterol induced PARP-1 is dependent on the pattern recognition receptor TLR2. This suggests that some oxysterols promote their inflammatory effects by activating cell microglial pattern recognition receptors in the brain to induce a neuroinflammatory cascade. In addition to TLR2, TLR4 can also act as a receptor for 7KC (J.-D. Huang et al., 2014). Interestingly, TLR driven activation of PARP-1 has also been implicated as a mechanism for Aβ microglia activation, suggesting that oxysterols and Aβ may synergistically stimulate microglia through a common signaling pathway (Kauppinen et al., 2011; S. Liu et al., 2012).

The retina can often provide significant insights into the biology of the brain. Oxysterols may play a particularly important role in the retina. 7KC content increases in the mouse retina with age (Indaram et al., 2015). It has also been implicated in diabetic retinopathy. Either as part of an oxidized LDL complex or on its own, 7KC can induce apoptosis of pericytes (Fu et al., 2012). 7KC stimulates retinal microglia, changing their morphology and causing increased expression of pro-inflammatory cytokines. Moreover, LPS primed retinal microglia were found to have increased inflammasome activation by increased NRLP3 induction in response to 7KC. This resulted in significantly increased secretion of cytokines including TNFα, IL6 and IL1β. 7KC can also lead to the downregulation of neurotrophic factor expression in microglia, including brain derived neurotrophic factor (BDNF), suggesting that elevated levels of 7KC may promote

inflammation and disrupt important factors in maintaining neuronal survival and function. These findings were consistent with work which demonstrated that 7KC inflammasome induction also occurs in human microglia (G. Shi et al., 2015).

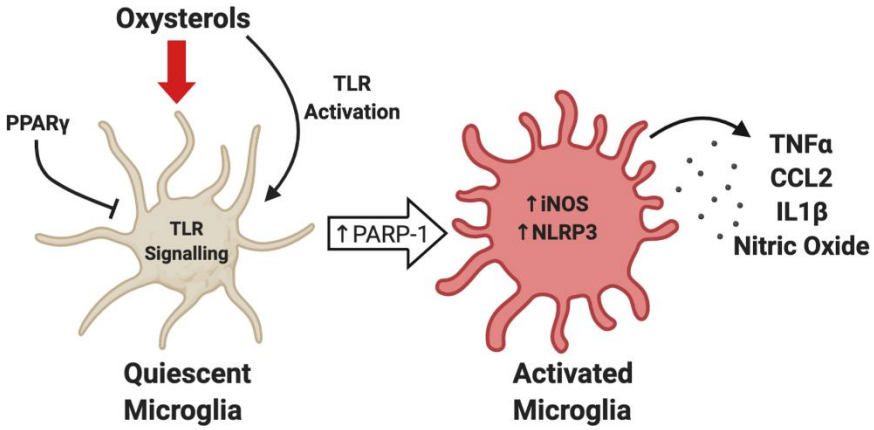

Figure II.2 Overview of Microglia Activation by Oxysterols

Oxysterols including 7-ketocholesterol stimulate microglia by activating toll like receptors (TLRs). TLR activation drives an intracellular signaling cascade dependent on kinases including Akt, P38, PKC, PI3K and ERK1/2. TLR signaling activates PARP-1 enzyme activity and ultimately shifts microglia to a pro-inflammatory activated state. Oxysterol activation of microglia can be prevented by treatment with PPARγ receptor agonists. Oxysterol activated microglia have activated inflammasome markers including increased NLRP3 and caspase 1. iNOS expression is also robustly upregulated. Activated microglia then secrete a variety of immune signaling molecules including TNFα, CCL2, IL1β and nitric oxide. Figure created with BioRender.com.

While the pro-inflammatory effects of oxysterols on microglia have been consistently observed, how inflammatory signals in the brain affect the production of oxysterols in cells is poorly understood. In a recent study BV2 microglial cells were treated with LPS and several oxysterols were measured over 24 hours (Mutemberezi et al., 2018). Non-enzymatically produced 7KC was significantly increased, while several enzymatically produced oxysterols, including 25OHC and 27OHC, were downregulated. This is consistent with the notion that LPS stimulation will cause increased ROS production, causing increased non-enzymatic cholesterol oxidation in turn. LPS also drives changes in the expression of cholesterol oxidizing cytochrome P450 enzymes,

increasing the cholesterol synthetic pathway CYP7B1 expression, while reducing CYP27A1 and CYP46A1 expression. In addition to LPS driving oxysterol profile changes, Interleukin-4 is also capable of changing oxysterol levels. Thus it appears that oxysterol production is dynamically regulated in microglia in response to environmental signals.

Taken together these studies demonstrate a clear role for oxysterols, and particularly 7KC, as potent pro-inflammatory molecules which are sufficient to activate microglia. The effect of oxysterols on microglia is modeled in Fig. II.2. Oxysterol levels have been shown to increase in metabolic disease and in AD (reviewed above), thus increased oxysterol levels may play an important role in these diseases by promoting microglial mediated inflammation to exacerbate the neurodegenerative process.

II.8 Conclusion

Oxysterol levels are altered in AD and diabetes, reflecting changes in both oxidative stress and enzymatic regulation of cholesterol oxidation. These oxysterols are potent signaling molecules and may contribute to mechanisms of neurodegeneration. 7KC in particular is elevated in AD and diabetes, is an inflammatory signaling molecule, and can induce immune activation in microglia. Enzymatically produced oxysterols such as 25OHC and 24(S)OHC, known modulators of cholesterol metabolism, are also altered in AD and diabetes. Increased neuroinflammation and decreased cholesterol synthesis are possible mechanisms in the progression of neurodegeneration and changes in oxysterols in the brain may contribute to them both. Still, many questions remain to be answered. The effects of diabetes on brain oxysterol levels are understudied. The cell-type specific effects of oxysterols in the brain are also mostly unexamined. The pro-inflammatory effects of 7KC and other oxysterols on microglia are discussed here, but oxysterols likely have variable and important effects on different cells.

Astrocytes are the main cholesterol producers of the brain, oligodendrocytes contain dense cholesterol deposits in their myelin, and neurons depend on cholesterol in the dynamic membranes at their synapses; oxysterols could have distinct effects on all these cells in neurodegeneration. Further research will give a clearer picture of the mechanistic roles that oxysterols may play linking diabetes and AD.

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