THE ROLE OF LIGHT SIGNALING ON ASTROCYTIC MORPHOLOGICAL PLASTICITY IN THE ADULT MALE RAT SUPRACHIASMATIC NUCLEUS

BY

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DISSERTATION

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ABSTRACT

The body's master clock lies at the base of the hypothalamus immediately above the optic chiasm. Because of the intimate connection to the optic chiasm, this hypothalamic nucleus was named the suprachiasmatic nucleus (SCN). Environmental light signaling conveys time of day and seasonal changes to the SCN via the retinohypothalamic tract (RHT). The SCN then relays this information to the brain and the rest of the body through synaptic signaling and indirectly through circadian regulation of hormonal signaling. The SCN is unique in that in the absence of external signaling, circadian rhythms will persist. This is accomplished through a transcription-translation feedback loop consisting of both positive and negative transcription factors. The interactions of the players within this loop create a near 24 hour rhythm.

Although research within the SCN has focused primarily on neuronal signaling astrocytes comprise nearly a third of the total number of cells within the nucleus based on stereological analysis. Moreover, the astrocyte cytoskeletal marker, glial fibrillary acidic protein (GFAP), is expressed in much higher levels compared to other local hypothalamic regions containing neuronal fibers. GFAP allows for the rough estimation of the overall astrocyte cell shape and despite a lack of in vivo polymerization dynamics, in vitro GFAP filaments have been shown to be dynamically regulated by phosphorylation by known kinases. Additionally, the synaptic signals encoding light information, glutamate and pituitary adenylate cyclase activating peptide (PACAP), have been shown to bind to receptors that activate kinases responsible for GFAP phosphorylation.

Based on this work, we hypothesized that the RHT regulates astrocytic cytoskeletal dynamics within the SCN of the male rat. To show this we established that GFAP immunofluorescence is significantly different between early day and early night within the SCN. We then showed that this observable difference is likely due to a shift in GFAP polymerization state from filaments into soluble

monomers. Second, we clearly show that this polymerization shift is regulated by the optic nerve and not a circadian phenomenon. We further establish that long term enucleation decreases the overall GFAP levels relative to other local hypothalamic regions, suggesting that the higher levels of GFAP within the SCN is regulated by the optic nerve. Lastly, in order to establish a model system to study effects RHT signals have on SCN astrocytes we characterized the polymerization state of GFAP within the brain slice. Moreover, we studied the effects of glutamate and PACAP on the brain slice. In conclusion, we have determined that signals from the RHT drive the observed levels of GFAP as well as the polymerization state of the GFAP cytoskeleton in the adult male rat SCN.

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CHAPTER ONE

INTRODUCTION

1.1 Circadian rhythms synchronize internal physiology to external cues

Life has evolved through the optimization and synchronization between the external environment and internal physiological process. Light has been a consistent environmental cue that serves as a daily oscillation that organisms used to predict availability to food and mates, avoid predation, coordinate arousal or sleep states, and anticipate daily physiological stress. Optimal physiology can be anticipated and prepared for by varying brain activity and hormonal release that play roles in arousal, hunger, body temperature, blood pressure, and others. This integration and synchronization of internal rhythms to environmental cues is conserved from single cells to complex organisms, emphasizing the importance of circadian rhythms.[72]

1.2 The master clock is in the Suprachiasmatic Nucleus

In the absence of light, organisms continue to exhibit behavioral rhythms in a near 24-hour cycle. The anatomical origin of biological rhythms in mammals resides within a small, bilateral nucleus found immediately dorsal to the optic chiasm within the anterior hypothalamus. This master clock is named the suprachiasmatic nucleus (SCN) and was located through electrical ablation and resultant loss of behavioral rhythms in rodents.[67,109] Spontaneous circadian membrane excitability and activity that continues in the absence of a *zeitgeber*, German for "time giver," is a unique property of the SCN.[47] This network circadian rhythm within the SCN arises from the interplay between three cellular oscillators; redox/metabolic state of the system, spontaneous membrane excitability of neurons within the circuit, and a molecular transcriptional-translational feedback loop (TTFL) found in neuronal cells of the SCN.[164] These oscillators allow cells within the SCN to synchronize and create a coherent rhythm capable of

output to local targets within the brain. Moreover, each oscillation has the ability to influence the rhythms of the other two.[6,164]

Redox state, membrane excitability, and gene oscillations are not independent. As one changes, the states of the other two shift to stay in phase. The oscillatory cells within the SCN are reduced during the day and oxidized at night.[42] Moreover, Wang et al. showed that the redox state of the SCN could be shifted by adding either an oxidizing or reducing agent and subsequently, peak membrane excitability is shifted.[164] The availability of redox-sensitive cofactors that membrane channels and transcription factors require for activation allows redox state to affect neuronal activity state and gene expression.

Spontaneous membrane activity in acute SCN brain slices was first observed in 1982 by Green and Gillette, and Groos et al.[47,49] The peak of spontaneous firing occurs around circadian time 7 (CT), which corresponds to 7 hours after lights have been turned on. This predictable and reproducible output signal of SCN activity has been used to characterize input and signaling mechanisms.[25,41,131,154,155]

The core TTFL is a molecular oscillator comprised of positive and negative transcription elements. The positive elements, CLOCK and BMAL, and negative transcription elements, PERIOD and CRYPTOCHROME (PER and CRY), regulate gene expression in a near 24-hour period. CLOCK and BMAL proteins bind to ebox transcription elements within *Per* and *Cry* genes resulting in increased transcription. PER and CRY proteins are post-translationally modified and heterodimerize within the cytoplasm and are subsequently transported back into the nucleus where they inhibit CLOCK and BMAL binding of promoter elements. This results in a decrease in *Per* and *Cry* transcription. This positive-negative transcriptional dance creates the genetic circadian temporal loop (Figure 1.1).[168]

At the tissue level, the SCN cytoarchitecture is commonly defined by the peptides expressed by neurons within a distinct region. Based on this criterion, we differentiate the SCN into a ventrolateral "core" and a dorsomedial "shell".[6] The "core" receives input from the retina directly (via the

retinohypothalamic tract, or RHT) and indirectly (via the geniculohypothalamic tract, or GHT).[125] This region expresses vasoactive intestinal peptide (VIP), gastrin releasing peptide (GRP), and SAAS.[5] It is responsible for light signaling integration and intra-SCN synchronization, which is important for a coherent circadian rhythm.[6] The "shell" expresses arginine vasopressin (AVP or anti-diuretic hormone) and serves to send efferent signals from the SCN, which synchronizes other brain-endocrine axes and subsequently the rest of the organism.[112] The synchronicity of the individual cells within the SCN is an emergent property of the circuit as a whole. This is achieved by intraSCN synaptic and peptidergic signaling. The importance of the inter-cellular signaling is shown when synaptic signaling is blocked by tetrodotoxin (TTX) bath in tissue culture, SCN rhythmicity is dampened and signal output is attenuated.[32] Additionally, when SCN neurons are in a dispersed culture, each cell exhibits its own rhythm and phase.[167] Moreover, the period and phase of individual SCN neuronal rhythms varies widely between cells.[166] Within the SCN, peptidergic signaling is possibly more important than synaptic.[58] The SCN releases peptides in discrete temporal windows.[62] To illustrate the input of peptidergic signals, animal models have been created in which the genes encoding highly expressed peptides, or their cognate receptors, have been genetically deleted. For example, animals lacking vasoactive intestinal peptide (VIP, a highly expressed peptide within the SCN) or VIP's primary receptor within the SCN, VPAC2, have weak or absent behavioral and neuronal activity rhythms.[19] Moreover, diffusible factors from transplanted fetal SCN cells, enclosed within a membrane preventing extension of synapses into the parenchyma, can restore behavioral and physiological rhythms in animals with ablated SCNs. The grafts failed to restore serum hormonal rhythms, establishing a role for synaptic signaling in some physiological rhythms.[84,87,142]

1.3 Information input to the SCN

The SCN serves as the arbiter of external-internal homeostatic balance. To this end, the "core" SCN receives major environmental information from the optic nerve (retino-hypothalamic tract; RHT),

reciprocal signals from the intergeniculate leaflet of the thalamus (geniculo-hypothalamic tract; GHT) and from the median raphe nucleus within the brainstem.[135] Less substantial innervation from the brainstem. Each projection transmits unique information about brain and environmental state.

The RHT transmits information about the presence and intensity of environmental light. This is done by intrinsic photosensitive retinoganglion cells (ipRGCs) that contain a membrane-bound light sensitive protein, melanopsin.[56,57,110,114,144] When light hits an ipRGC, melanopsin depolarizes the cell and a signal is transmitted down the RHT. The signal of light from the RHT to the SCN is transmitted by glutamate and PACAP, via axon terminals that innervate the retinorecipient area, or ventrolateral SCN. Glutamate induces neuronal excitability, seen in immediate early gene transcription, and subsequently induces clock gene transcription. This signaling results in a shift of the subsequent peak membrane firing, pulsatile release of neuropeptides, and synaptic output from the SCN. This is affected by both NMDA and non-NMDA receptors, initiating a variety of intercellular and intracellular signaling mechanisms.[29,30,41,71,155]

Another route for photic input arises from the IGL and projects into the core of the SCN. The IGL receives visual image information from the environment through the optic radiation.[111,113] This information is initially processed and efferent information is sent to higher cortical regions while one projection goes back to the SCN core. These axons release neuropeptide-Y (NPY), which can shift the clock and modulate RHT input, and GABA, which has a variable input role when considering the extent of GABA-ergic neurons within the SCN.[40,100]

The third afferent tract to the SCN comes from the median dorsal raphe nucleus. These projections are serotonergic, regulating arousal state, and encoding internal homeostatic and behavioral information. The median dorsal raphe, via serotonergic signaling, can induce phase shifts during the day and can modulate light-induced phase shifts during the night in the SCN.[13,14] Ablation of the

serotonergic input into the SCN results in larger phase advances and delays, showing the inhibitory and neuromodulatory effect of the median raphe.[138]

Additional input into the SCN comes from the caudal hypothalamus, brainstem, and the pontine tegmentum and basal forebrain. These regions target the SCN "shell" primarily and release histamine, noradrenaline, and acetylcholine, respectively.[105,135] Together, these input signals to the SCN allow for internal physiology to synchronize to important environmental cycles.

1.4 SCN efferent targets and signals

After integration of afferent information from the RHT, GHT, and median raphe nucleus, the SCN sends synchronization signals to the rest of the brain in the form of diffusible signals and efferent projections. Most of the direct synaptic targets of the SCN lie adjacent in the hypothalamus. Some of the best studied targets of the SCN are the subparaventricular zone (sPVZ), paraventricular nucleus (PVN), supraoptic nucleus (SON), ventromedial preoptic area (vmPOA), and the ventromedial and dorsal medial hypothalamus (vMH and dMH, respectively).[26,112,151,174]

Output signals from the SCN can be in the form of diffusible factors, that likely work in a paracrine manner. Evidence for diffusible signals were produced by ablation of an animal's SCN followed by grafts fetal SCN tissue enclosed in a mesh that prevented outgrowth of neuronal projections.[84,142] Animals that had their SCN ablated lost behavior rhythmicity and hormonal rhythmicity. SCN grafts restored the behavior rhythms but failed to return hormonal rhythms. Animals that received grafts also exhibited the circadian period length of the graft animals instead of their own phenotype, demonstrating the importance of diffusible signals from the SCN.[142]

1.5 The body's clock is plastic

The SCN receives signals from both the external and internal environment. In order to maximize the synchronization between the two, the SCN can shift its peak activity rhythm earlier or later, corresponding to an earlier sunrise or later sunset. A shift earlier is referred to as a phase advance and a shift to a later time is a phase delay. Whether the SCN advances or delays depends on the nature and timing of the signal.[41] This selective gating by the SCN allows for proper integration and interpretation of stimuli. Environmental light is a signal to which all rodents respond. During the day, the SCN is insensitive to light signals; the animal is synchronized to the ambient day-night cycle and the signal of light fails to elicit a phase-changing response in the SCN. However, if light signaling arrives at the SCN during the entrained dark/night, it signals desynchronization and phase-adjustment follows. The nocturnal timing of the signal determines the change in phase of the clock. If an animal is exposed to light during the early night, the animal's activity rhythm is delayed. If light is given during the late night, then an advance occurs. Historically, light signaling has been studied through animal behavior onset. Rodents will run on cage wheels, which can be monitored by computer software. Thus, researchers can monitor when animals are awake and when they sleep, allowing for light pulses to be administered at specific clock times, even when the animal is in total darkness. In order to parse the signaling mechanisms of SCN shifts, acute or organotypic slices are used. This can be accomplished due to the fact that the SCN maintains many biological rhythms in constant conditions.

Photic input is not the only route whereby an animal's internal clock can be changed. Projections from the median raphe nuclei release serotonin onto the SCN and can induce phase advances during the subjective day and modulate phase shifts from photic input at night.[99,126,129,145] Input from the IGL through release of the neuropeptide NPY has the ability to modulate RHT input and shift the clock when applied by itself.[37,74,172] Cholinergic input from the forebrain and mesopontine nuclei shift rhythms at night allowing for additional non-photic feedback from internal physiological state.[1,41,66,89] Taken together, input independent of RHT signaling can modulate light induced phase

shifts or shift the SCN independently, ensuring internal physiological cycles are represented in rhythmogenesis over the course of the day.

1.6 Astrocytes, GFAP, and astrocyte rhythms

Neuroscientists of the 19th century originally classified and studied the brain and its cellular components based on morphological features. Sketches were made of dye filled cells and separated into neurons and non-neuronal cellular components.[73,123] These non-neuronal cells filled the spaces between neurons, offer structural rigidity for neuronal scaffolds, and provide nutritional support to the more electrically active components of the brain.[165] These cells were considered a new cell type, the neuroglia. Neuroglia were later subdivided into many different specific cell types such as astrocytes, oligodendrocytes, microglia, and tanycytes. Each cell type has a specific role: astrocytes encircle and modulate synapses and blood vessels, oligodendrocytes wrap axons increasing signaling velocity, microglia are the brains phagocytic immune system, and tanycytes line the large fluid filled ventricles of the brain.

Although astrocytes do not exhibit membrane firing as seen in neurons, they do change membrane potentials in response to synaptic activity.[27] Some types of astrocytes can buffer their electrical activity by forming gap junctions with neighboring astrocytes creating a syncytia allowing for spreading over large domains. Astrocytes exhibit local and network Ca^{2+} fluctuations that correspond to astrocyte excitability. Classification of astrocytes are based on location, where fibrous astrocytes are in white matter while protoplasmic astrocytes are found in grey matter. Within the grey matter of the brain, protoplasmic astrocytes establish and maintain discrete spatial domains (or tiles) that can encompass as many as 100,000 synapses.[21] These domains are not established at birth but are developed over time and are likely influenced by the neuronal activity within their locale.[20] Astrocytes also have distinct shapes which can differ within and between brain regions (Figure 1.3).

Evidence is building that astrocytes contribute to the emergence of intelligence and higher learning in organisms. Astrocytes in humans are much larger and exponentially more complex than rat or mouse astrocytes.[120,121] Amazingly, a study from Han et al. in 2013 successfully grafted human astrocyte precursor cells into neonate mouse brains.[53] Precursors grew into mature human astrocytes that were phenotypically similar to normal human astrocytes. These animals not only had stronger long term potentiation in the hippocampus of brain slices but also performed better than wild type mice in memory and learning tests. This study establishes astrocytes as key players in the establishment of learning and intelligence in mammals.

In regards to circadian biology, astrocyte cultures from whole brain cultures have robust clocks.[91] Astrocytes express many genes, including the TTFL, in a circadian manner.[128] Yet astrocytes seem to lack a crucial quality that SCN neurons seem to have; the ability to maintain cell-to-cell synchronous rhythms. Cultured astrocytes, in the absence of a *zeitgeber* become progressively desynchronized.[91] Co-cultured organotypic SCN tissue can rescue the synchronicity of the cultures.[128] Also, if a known circadian synchronization signal, such as VIP, is given to an astrocytic culture, the cells become synchronized and maintain their synchrony, so long as the signal is repeated in a circadian manner.[91] If either the synchronizing SCN or administered synchronizing agent are removed, the culture again becomes desynchronized in a manner of days. The direct role that astrocytes play in SCN signaling and circadian rhythms hasn't been established. Prosser et al. showed that disruption of astrocyte gap junctions resulted in a shift in peak SCN rhythm.[130]

1.7 Astrocytes play many roles in the brain

When first discovered, astrocytes were considered merely supportive cells. They were lumped into a group of cells that were electrically inactive, non-neuronal and given the name glia, or "glue." Scientific study for nearly 100 years was reserved for neurons since the glial cells weren't thought to

contribute significantly to memory and other functional aspects of the brain. Recently, however, interest has spiked with the appreciation of the tripartite synapse that consists of the pre- and post-synaptic neurons and surrounding astrocytes that insulate, support, and signal within the synapse (Figure 1.2).

Astrocytes serve as the guardians of the blood-brain-barrier, enwrapping the endothelial vasculature with end-feet. Brain volume and fluid regulation is closely controlled by astrocytes through the water channel aquaporin-4 (AQP-4).[11] They also screen metabolites, osmolites, and other humoral factors before passage into the brain parenchyma.

Astrocytes play a pivotal in synapse formation during development through axonal growth by physical and chemotactic cues.[140,173] In a striking study, astrocyte vesicular D-serine release is integral for synaptic development and maturation in adult neurogenesis in the hippocampus.[150] Additionally, astrocytes are key in neuronal protection and cerebral responses to injury and infection. Astrocytes respond to neuronal injury by increasing cell volume and GFAP expression, creating glial scars that sequester the injured brain from healthy tissue and may facilitate proper healing of damaged axons.[35,78]

1.8 GFAP expression, regulation, and degradation

Astrocytes within the CNS can be distinguished from neurons by cell shape, membrane conductance, and by protein markers expressed primarily by astrocytes. Cell shape and membrane conductance are both used to identify astrocytes in electrophysiology experiments. In situ immunohistochemical experiments that do not require live astrocytes utilize markers expressed only in astrocytes to identify these cells for study. Although astrocytes and neurons express many common markers, aldehyde dehydrogenase 1 (Aldh1), S100β (a calcium sequestering protein), and GFAP are generally considered astrocyte specific.[143,157] Although Aldh1 and S100β are considered to be pan astrocytic markers, these proteins give vague representations of cell location and shape. Alternatively, GFAP is a key structural intermediate filament expressed primarily in mature astrocytes within the CNS

that not only marks astrocytes but also gives a good representation of the cell's morphology. Originally considered to be a static protein, research now shows that GFAP expression, polymerization state, and degradation is closely regulated by canonical signaling.[69]

GFAP expressing astrocytes can be found throughout the CNS of mammals. Structural differences within the CNS express higher levels of GFAP, such as the glial limitans (physical borders of the brain) and blood vessels. Regional GFAP differences within the brain also exist. For example, the SCN and intergeniculate leaflet, which both receive retinal input, express much higher levels of GFAP compared to the other regional nuclei.[115] Astrocytes mature from radial glial cells and begin expressing GFAP in response to local pituitary adenylate cyclase activating peptide (PACAP) signaling. PACAP drives GFAP expression through calcium dependent signaling activation of the transcription complex DREAM.[24] What determines the amount of GFAP expressed in quiescent mature astrocytes within the CNS is unknown. Inflammatory signals, hypoxia, and traumatic injury to the brain induces GFAP expression and astrocyte swelling through a process known as gliosis.[171]

As an intermediate filament, GFAP has been thought of as a static cytoskeletal filament that provides structural support. GFAP monomers dimerize in a parallel orientation through interactions within their head domains. GFAP dimers assemble antiparallel (head-tail) into filaments. Assembly and filament formation is regulated by phosphorylation of the head domain. Phosphorylation sites have been identified at four serine residues Ser-8, Ser-13, Ser-17, Ser-34 as well as threonine-7.[70] Protein kinase A (PKA – cAMP dependent protein kinase), protein kinase C (PKC – IP3 dependent kinase), and calmodulin dependent kinase 2 (CaMKII) have been shown to phosphorylate GFAP. Through phosphorylation cells can rapidly create a pool of dynamic monomers that aid in astrocytic process motility.

GFAP stability and degradation in vivo are poorly understood. Gigaxonin, a protein shown to mediate intermediate filament degradation in both neurons and astrocytes, appears to facilitate ubiquitination of GFAP, targeting monomers to the proteasome.[88] It is not understood however, what regulates gigaxonin or how it interacts with GFAP. Paradoxically, phosphorylation is the only known driver of GFAP filament depolymerization and has been shown to protect GFAP from degradation.[152]

1.9 GFAP has physiological roles in astrocytic homeostatic processes

GFAP is an astrocytic intermediate filament and is primarily expressed in mature astrocytes after postnatal day 25 (P25). It is seen in the developing brain while *in utero* and increases as the animal ages.[68] As part of the cell cytoskeleton, GFAP functions as a cellular transportation highway, trafficking proteins from the soma to membrane. GFAP plays a role in movement of glutamate transporters out to the membrane in response to network activity via an unidentified mechanism.[149] However, GFAP filaments do not extend all the way to the cell membrane. The filaments end some distance from the membrane and smaller filaments interact and extend from GFAP, primarily actin and ezrin.[80]

Although GFAP is a key cytoskeletal element in astrocytes, animals that have GFAP genetically deleted develop normally in the laboratory. The astrocytes within these animals have smaller volumes with fewer and less complex cell extensions.[2] Interestingly, the loss of GFAP results in stronger LTP in the hippocampus of brain slices, implying a role for GFAP in synaptic signaling.[98] However, the loss of GFAP is not always beneficial. Mice with GFAP genetically deleted have lower survival rates when there is direct cerebral damage or trauma.[122] Animals that were exposed to cerebral infection and immune inflammation had poorer outcomes.[148] Additionally, GFAP knockout animals that were challenged with head trauma had severe cerebral damage compared to control animals, implying a structural role for GFAP in overall brain stability. Astrocytes near these insults would increase GFAP expression and

partition the damaged brain from healthy tissue through a glial scar, preventing further damage and abnormal regrowth of neural circuits.

Previous studies indicate the total GFAP does not change with the day-night cycle but GFAP immunoreactivity is reduced in the SCN if the animal is kept in constant darkness or enucleated immediately after birth.[68,118] Animals that are placed back into a recurrent lighting schedule (LD; 12:12) have a concurrent increase in GFAP immunoreactivity, implying a role for light signaling in GFAP expression. Circadian changes in SCN astrocyte morphology and GFAP have been studied since the 1990's.[82] The results of these studies, however, have been contradictory and inconclusive. Differences between the methodology, SCN region, and the methods of analysis have resulted in a lack of agreement on the role of GFAP and astrocytes. Some of the earliest circadian GFAP immunohistochemistry-based studies were carried out by Lavaille and Serviere in the Syrian hamster SCN.[82] Their results reported that astrocytes exhibited differences between the light phase and dark phase of the hamster's diurnal cycle. They reported that GFAP staining in astrocytes exhibited a significant increase and networked expression during the light phase with reduced expression at night.[82] These researchers have continued studying the hamster SCN and have produced several interesting papers showing diurnal changes in fluorescent images of GFAP immunoreactivity, with the highest immunoreactivity during the day and lowest at night.[82,83] Lavaille et al also showed that this diurnal variation of GFAP immunoreactivity continued if the animal was kept in constant darkness, albeit reduced in amplitude, and was lost if the animal was enucleated.[81]

Leone et al looked at GFAP in the mouse SCN and found similar patterns as that seen by Servier and Lavialle.[86] However, Moriya et al did an almost identical study and found no circadian differences in the GFAP expression in the mouse SCN.[116] The method used by these groups utilizes two dimensional brightfield microscopy at low magnifications (typically 20X objective lenses) on 3-3'-diaminobenzidine (DAB) stained tissue.[162]. This technique, though rapid and quantifiable, yields information about the

total GFAP expression in the SCN, similar to an optical Western blot, but fails to discriminate the expression levels within individual astrocytes. Moreover, the technique utilizes a thresholding method for optical density that could potentially miss finer GFAP filaments.[162]

Other groups have used electron microscopy to look at the finer, non-GFAP containing processes. Elliot and Nunez concluded from their transmission electron microscopy (TEM) study that the SCN astrocytes did not have diurnal differences in glial-neuronal membrane apposition.[33] However, this study utilized a small sample of rats and did not specify a region of the SCN studied. Likely the most complete series of studies to date have been completed by the French group headed by Becquet, Bosler, and Girardet. These studies show astrocytic diurnal changes in both GFAP and the finer, non-GFAP containing processes of the adult rat SCN.[16,45,97] The GFAP change was found to be in out of phase to the hamster rhythm seen by Servier and Lavialle, with the peak immunostaining during the night and lowest during the day.[8] TEM analysis of vasopressin (AVP) and vasoactive intestinal peptide (VIP) containing cells differed in the dynamics of fine astrocytic processes. VIP, containing cells found primarily in the ventrolateral retinorecipient area, showed diurnal changes while the AVP cells of the SCN did not.[43-45]

Studies that have identified seasonal and hormonal influences on GFAP expression in the SCN. Gerics et al observed seasonality in the rat with higher levels of GFAP at night during the winter but high during the day in the summer.[39] GFAP expression in the SCN can to be regulated by adrenal hormones. Maurel et al found no difference in GFAP staining between times ZT2 and ZT14 but if the animal was adrenalectomized, GFAP expression dropped.[97] Whether there is a circadian rhythm to GFAP expression within the SCN is confusing at best. The failure of agreement between studies, animal models, and methodologies leaves this topic in need of further study.

1.10 Astrocytes and the tripartite synapse

Astrocytes have emerged as important participants in synaptic regulation. The specificity commonly given to synaptic signaling between pre and post synaptic neurons has now been shown in astrocytes. Astrocytes associated with striatopallidal neuronal circuits excite only those neurons associated with each circuit.[93] The associations with neurons and the domains that astrocytes inhabit is specific to each astrocyte and persists from development of the organism through the life-course. One way astrocytes participate in synaptic signaling is through clearance of excitatory neurotransmitters within the synaptic cleft. The most prominent excitatory neurotransmitter is glutamate. Presynaptic neurons release glutamate into the synaptic cleft and if it remains within, can rapidly lead to overstimulation and death of the postsynaptic neuron (excitotoxicity). Astrocytes prevent this by clearing Glu, via Glu transporters GLAST and GLT-1, and recycling Glu to Gln via glutamine synthetase.[3,153] Glu-Gln astrocyte cycling is especially important in SCN physiology since the primary signal of light from the RHT is glutamate with excessive glutamate being excitotoxic. Interestingly, cell lines derived from the SCN are resistant to glutamatergic excitotoxicity.[17] Moreover, Glu uptake and Gln synthetase activity both exhibit circadian variation with higher activity of both during the light phase.[85] The most abundant inhibitory neurotransmitter within the brain is gamma-aminobutyric acid (GABA). Moreover, GABA is the most abundantly expressed neurotransmitter in the SCN, where some reports suggest that all neurons within the SCN are GABA-ergic.[36] Commonly considered to be an inhibitory neurotransmitter, GABA has mixed functions within the SCN depending on the phase of the clock.[48] GABA is cleared from the synapse by astrocytes through the GABA transporters GAT1 and GAT3. Neurons do not express GAT1 or GAT3 in the SCN, while astrocytes express these transporters primarily within the neuro-glial membrane appositions.[107] Thus, clearance of both glutamate and GABA by astrocytes is a function in all areas of the brain but is of critical importance within the SCN.

The second way astrocytes can regulate the excitability of neuronal networks is through the release of gliotransmitters. Gliotransmitters include glutamate, D-serine, adenosine triphosphate (ATP),

and nitric oxide (NO).[137] These factors can modulate the excitability of individual neurons, whole circuits, or serve as a local signal to regional astrocytes or even the astrocyte that released it. Glutamate can be released by astrocytes into the synaptic cleft in response to several stimuli. The context of the stimulus and the brain region determine the end result of the astrocytic glutamate signal. D-serine has been shown to play a key role in plasticity in several brain regions where it is released by astrocytes into the synaptic cleft. Here, D-serine allosterically binds to activate NMDA receptors, facilitating NMDA signaling.[117,139] ATP is released in a circadian manner by astrocyte cultures.[92] ATP serves as an inhibitory astrocytic signal to neurons or can act in a autocrine fashion via the purinergic receptor P2Y1R, causing astrocytic release of glutamate.[31] This evidence clearly shows that astrocytes not only maintain the synapse but actively release regulatory factors, making them essential participants in brain signaling.

A third way astrocytes regulate neuronal excitability is through astrocyte shape changes or morphological plasticity. One way astrocytes can change shape is through cell volume changes, which occur in response to increased synaptic activity.[79,156] Increased neuronal firing results in extracellular fluctuation of ions and neurotransmitters in the synaptic cleft. A critical ion whose concentration rapidly increases in the synaptic cleft during activity is K^+. K^+ is cleared slowly by the ATP-dependent Na^+/K^+ pump located in the membranes of both neurons and astrocytes. However, during high levels of activity, high K^+ levels are cleared by astrocytes through the astrocytic membrane K^+ channel, $K_{ir}4.1$. Cell swelling has not been shown to be dependent on the uptake of K^+ since acute slice cultures in which Na^+/K^+ ATPase and Kir4.1 were inhibited still exhibited cell swelling and extracellular space shrinkage (as measured by TMA+ concentration).[79]

Astrocyte swelling/extracellular space shrinkage is effected by the astrocytic water channel, aquaporin-4 (AQP-4).[160] AQP-4 is enriched in astrocytes, with primary cellular localization within astrocytic process surrounding blood vessels and perisynaptic astrocytic processes (PAPs). AQP-4

enriched astrocytic end-feet help to regulate cerebral fluid flow and levels while AQP-4 in PAPs help regulate smaller, local changes in fluid volumes. In addition to volume swelling, astrocytes have been shown to change the extent of PAP intercalation between the membranes of neurons.[169] PAPs are the fine processes that lack larger cytoskeletal proteins and ensheath the synapse. Many key astrocytic proteins are localized to these fine processes, illustrating their role in astrocyte-neuron interactions. Originally, studies within the hypothalamus utilized immunostaining and electron microscopy to show retraction of PAPs in response to normal physiological stimuli such as osmotic challenge, pulsatile hormonal release, and reductions in blood pressure.[23,38,124] Recent improvements in imaging and molecular techniques have allowed for live imaging of fluorescent markers, showing the extent of PAP movement in response to synaptic activity.[10]

1.11 Disease and pathology

Circadian biology influences all aspects of life, either directly or indirectly. It comes as no surprise that when we are out of phase with respect to environmental cues, disease results. On the extreme end, rodents that are placed in a chronic jet-lag lighting schedule die much earlier than control animals.[28] Most of us won't experience such extreme jet-lag, however we do send daily conflicting circadian signals that our brain and body must interpret through excessive night-time environmental lighting and social activities that disrupt sleep-wake cycles. Night shift workers have been studied extensively with strong correlative rates of cancer, inflammation, and metabolic syndrome.[4,75] The dollar value for the 15-20% of the population that suffer from daily fatigue and chronic sleepiness, loss of labor hours, and road and workplace accidents is estimated at over 70 billion dollars annually.[12,132] Unfortunately, the need to earn is greater than the detriment an individual will suffer working a night shift. However, the more we understand the communication between the environment, brain, and body the more likely we are to develop methods to treat circadian disruption. Moreover, research on the

diurnal plasticity of astrocytes is an important and often overlooked potential contributor to our understanding considering the emerging role of astrocytes in brain function and circadian rhythms.

Currently, Alexander's Disease is the only disease attributed to a mutation in the GFAP gene.[103] This mutation results in over expression of GFAP and plaques that cause pathological synaptic signaling and disruptions of the blood-brain-barrier. Children born with this disease die young and present clinically with symptoms associated with disrupted astrocyte regulation of the blood-brain-barrier, such as hydrocephaly. However, astrocyte pathology, whether the cause or an effect of a disease process, is seen in a plethora of neurological disorders: Alzheimer's Disease, Huntington's Disease, Amyotropic Lateral Sclerosis (ALS), Parkinson's Disease, and epilepsy.[9]

Epilepsy is possibly the best studied neurological disease associated with astrocytic dysfunction. Epilepsy is one of the most prevalent neural disorders, effecting between 1-2% of the world's population, costing more than 15 billion dollars annually.[34,63,159] Epilepsy is caused by a variety of etiologies, from traumatic brain injury, central nervous system infections, brain tumors, stroke, and genetic disorders.[90,127] Epilepsy is characterized by abnormally synchronized neuronal excitability across large spatial domains within the brain. The most common type of epilepsy is medial temporal lobe epilepsy (MLTE) centered in the hippocampus.

Abnormal astrocyte shape and function is seen in epileptic brain regions, caused by focal brain damage, genetic abnormalities, and neuronal death. Normally astrocytes develop and maintain distinct spatial domains with little to no overlap with adjacent astrocytes.[20] However, in the epileptic brain, reactive astrocytes increase their expression of GFAP and glutamate receptors, decrease their expression of glutamine synthetase (GS) and Kir4.1, and increase domain overlap.[146,147] Increases in GFAP result in the formation of a glial scar, physically preventing neuron regrowth following damage.

Although believed to be a protective mechanism, glial scars have been linked to induction of epileptic seizures while removal of the scar is often curative.[134]

The decrease in expression of GS and Kir4.1 both lead to increase excitability in the brain due to loss of GS recycling synaptic Glu to Gln. A loss of this enzyme results in prolonged high concentrations of glutamate in the synapse. Moreover, a lack of GS switches GABA signaling from inhibitory to excitatory, adding even more excitatory signaling. Kir4.1 loss results in higher extracellular potassium, which inhibits neuronal repolarization.[65]

How an increase in domain overlap in epileptic regions of the brain contributes to the over excited phenotype of epilepsy isn't immediately clear. However, one may hypothesize that an increase in domain overlap fails to allow metabolic and neurotransmitter clearance from the synaptic cleft due to an increase in extracellular tortuosity. In the cortex, astrocytes normally reduce their volume during the sleep phase, increasing the extracellular volume and decreasing tortuosity of the glymphatic system, allowing for daily clearance of metabolites.[170]

1.12 Statement of problem and hypothesis

The study of astrocytic roles in neuronal excitability and function is a growing field. Astrocytes are known regulators of synaptic activity through the clearance of the synapse, release of gliotransmitters, and morphological changes. The role of light signaling on SCN astrocytes has yet to be fully understood while astrocytic contributions to circadian biology are still mainly unknown. Understanding the role of circadian rhythms and astrocytes in disease morbidity and mortality has the potential to be far reaching, with astrocytes as therapeutic interventions being possible targets. ***This study's central hypothesis is that diurnal light and the signaling pathways it activates mediate astrocytic morphological plasticity in the SCN.*** To address this hypothesis, the following aims will be explored. 1) Evaluate the role of the environmental lighting schedule on glial morphology in the SCN. 2)

Evaluate the role of optic nerve innervation on glial morphology in the SCN. 3) Evaluate the role of Glu and PACAP on glial morphology in the SCN.

Figures 1.13

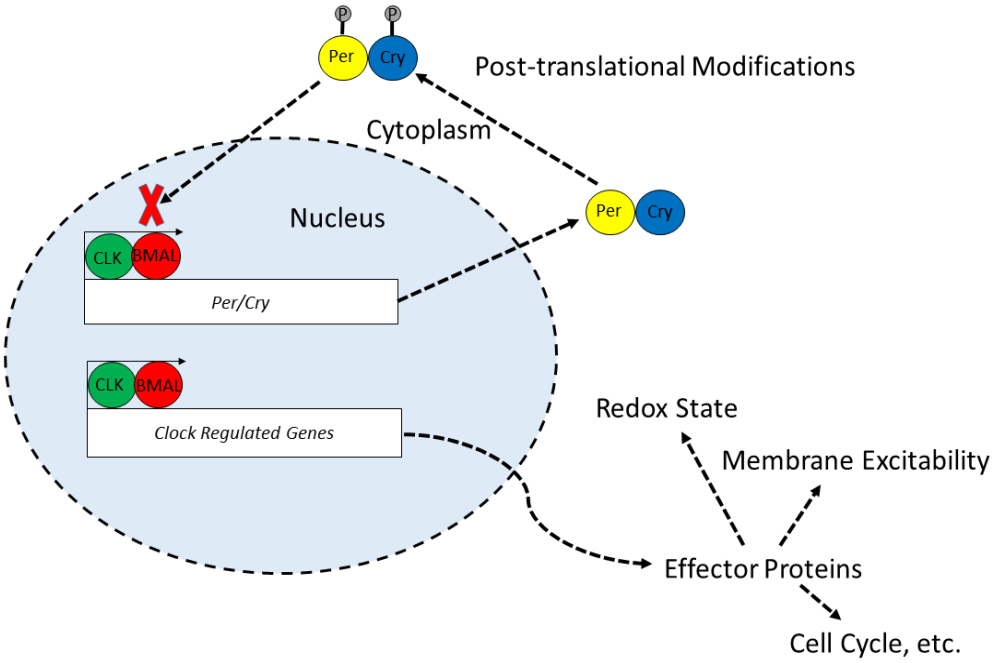

Figure 1.1. The molecular transcription-translation feedback loop. Positive transcription factors CLOCK (CLK) and BMAL increase transcription of the *Period and Cryptochrome* genes as well many other genes known as Clock Regulated Genes. PERIOD and CRYPTOCHROME proteins are phosphorylated in the cytoplasm and translocate to the nucleus where they act as negative transcription factors by inhibiting CLOCK and BMAL binding. This positive-negative transcription-translation feedback loop takes approximately 24-hours, giving rise to a molecular circadian rhythm.

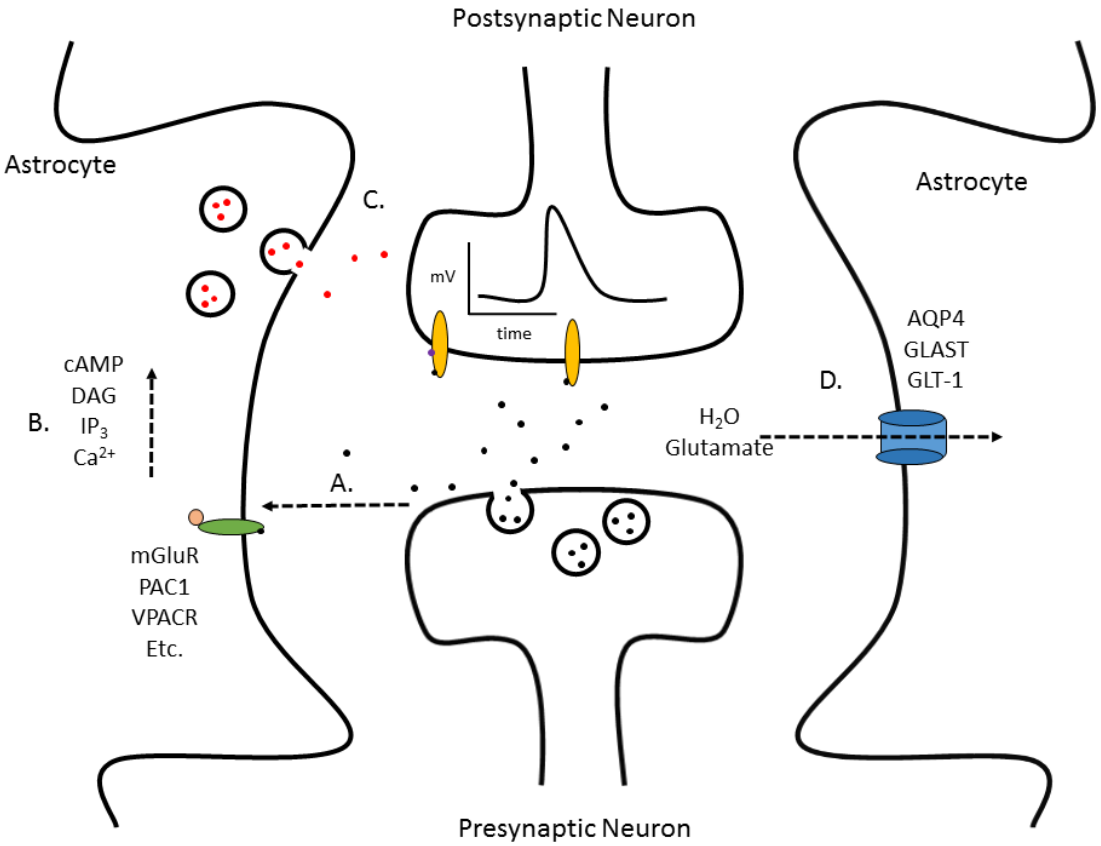

Figure 1.2. Astrocytes respond to excitatory synaptic signaling by glutamate and clear the synapse. A) Neuronal activity releases excitatory neurotransmitters and neuropeptides into the synaptic cleft inducing activation of postsynaptic neurons as well as astrocytes. B) Receptor activation results in an intracellular increase of second messengers. C) Astrocyte activation results in release of gliotransmitters that can modulate synaptic activity. D) Astrocytes are responsible for clearing synaptic glutamate and potassium, and regulating water homeostasis in the synaptic cleft. This is done through channels and transporters for each respective factor. (Modified from Ruby et al.)[136]

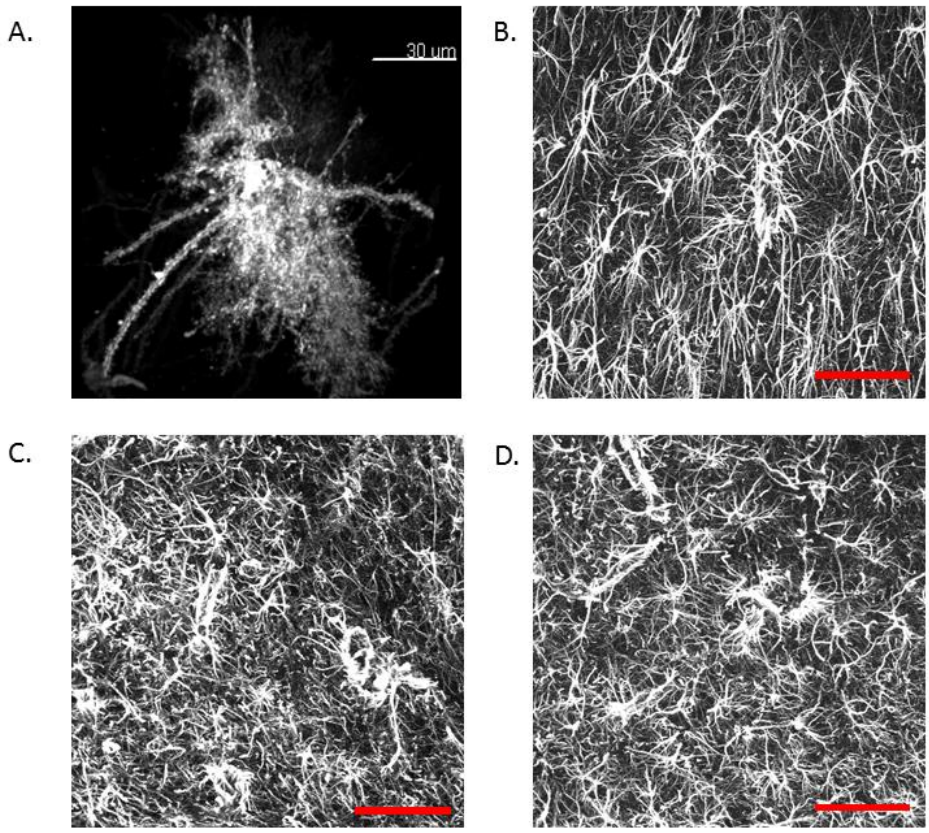

Figure 1.3. Astrocytes are complex cells that differ between regions of the brain. A) A fluorescent lipophilic membrane dye outlines the complexity of an astrocyte in the dentate gyrus region of the hippocampus. B – D) GFAP immunoreactivity in the brains of adult rats allows for visualization of the astrocyte cytoskeleton in the dentate gyrus of the hippocampus (B), the suprachiasmatic nuceus (C), and the paraventricular nucleus (D). Astrocyte shape and organization are visibly different between regions of the brain. Red scale bar equal to 50 µm. (S. Irving)

CHAPTER TWO

DIURNAL ASTROCYTIC CYTOSKELETAL PLASTICITY WITHIN THE RAT SCN

2.1 Abstract

Light is the primary external cue that aligns an organism's internal physiology to the external environment. This cue is passed from the eye via the retinohypothalamic tract (RHT) to the suprachiasmatic nucleus (SCN). It is here in the body's master clock that light information is processed and passed to the rest of the body. The neuronal synchronization and signaling of the SCN has historically been the focus of most circadian biologists. However, the role of astrocytes in SCN function and light signaling remains a mystery. Unfortunately, differing animal models, astrocyte parameters studied, SCN regions analyzed, and methodologies used have resulted in an incomplete picture of astrocyte morphological plasticity within the SCN. In this study, we report a significantly higher level of GFAP immunofluorescence in the ventrolateral region of the mid-SCN at zeitgeber time (ZT) 14 (early night) as compared to ZT2 (early day). Confocal analysis of individual cell GFAP cytoskeletons was not significantly different between early day and early night while Chi-squared analysis of branching distributions was significantly different. This suggests that despite individual astrocyte morphological heterogeneity within the SCN, GFAP branching is less complex in the early day compared to early night. Lastly, Western blot analysis shows a significant difference between filamentous and soluble GFAP, with early day having higher levels of soluble GFAP compared to early night. Taken together, these results demonstrate a diurnal rhythm in the astrocyte cytoskeleton, which has the potential to play a role in SCN physiology.

2.2 Introduction

Diurnal light is the primary signal animals use to anticipate and synchronize internal and external environments. Synchronization requires transmission and centralized integration of the signal, as well as output to the rest of the brain and body. Transmission of photic input travels from the eye to the brain via the retinohypothalamic tract (RHT).[105] Integration, interpretation, and output of this information is done within the body's master clock, the suprachiasmatic nucleus (SCN). The SCN is a bilateral pair of nuclei consisting of about 20,000 neurons immediately dorsal to the optic chiasm.[50] The neurons of the SCN coordinate with each other through synaptic and peptidergic signaling, creating an emergent, cohesive circadian rhythm.[106] The SCN circuit then transmits biological time to the rest of the brain and body by way of synaptic and humoral signals.

The extent to which astrocytes influence this organization and signaling mechanism of circadian rhythms has yet to be elucidated.[130] A recent study by Brancaccio et al. identified astrocytic calcium and glutamate tone in the SCN during the subjective night as being key regulators of inhibitory GABAergic signaling. Interestingly, only astrocytes within the dorsomedial region of the SCN seemed to exhibit this circadian behavior.[18] Astrocytes can influence synaptic activity through clearance of neurotransmitters and ions, release of gliotransmitters, and movement of perisynaptic astrocytic processes.[10,137] Although synaptic clearance and release of gliotransmitters is well studied, the extent to which astrocytic movement can modulate synaptic activity is poorly understood. This lack of knowledge is due in part to the difficulty of visualizing astrocyte morphological dynamics. Several techniques have historically been utilized including electron microscopy, dye filling of whole astrocytes, and staining of the cell cytoskeleton.[8,21,33] High resolution analysis using an electron microscopy give nanometer resolution of a small sample of the astrocytic membrane and requires special sample treatment, in addition to requiring an electron microscope, which many be difficult to access. More recent studies have utilized injection of small fluorescent dyes to visualize the cell's volume. Although this gives more information of the

astrocyte shape it requires live dye-filling of single astrocytes in acute slice culture. A more generalized approach to analyzing astrocyte morphology is through immunohistochemical staining of an astrocyte specific cytoskeletal protein, glial fibrillary acidic protein (GFAP). This approach can yield information about a much larger sample of astrocytes, including whole brain regions. GFAP staining is limited by the fact that GFAP only reveals 13% of the cell volume and does not extend to the cell membrane.[21] However, this technique has been the most utilized approach when analyzing glial morphology in the SCN.

Our current understanding of GFAP's physiological role in the central nervous system (CNS) is limited. Only when inflammation, neurotoxicity, or acute physical damage to the brain occurs do we see changes in GFAP expression and swelling of astrocyte cell bodies creating a physical barrier between healthy and damaged or necrotic tissue, indicative of a scarring event. Dynamic expression or changes in GFAP morphology in normal physiological states is poorly understood at best.

Unfortunately, research is murky in regards to the role environmental signals play in regulation of SCN astrocytic GFAP dynamics. Previous studies have failed to agree on circadian changes in GFAP immunofluorescence or somal apposition between neurons and astrocytes in the SCN around the clock. This is confounded by the variation in animal models, techniques used, and anatomical region studied. For example, Servier and Lavialle's studies within the Syrian hamster reported the first diurnal pattern in GFAP immunofluorescence.[81-83,141] Contrasting this, studies in the rat SCN have shown either no change in GFAP immunostaining or appear to be phased differently to what Servier and Lavialle observed in the hamster.[8,116] Elliot and Nunez used transmission electron microscopy (TEM) to look at the extent of somal apposition of neurons and astrocytes in the rat SCN every 4 h and found no difference across the day. Becquet *et al.* found significant changes with respect to time of day and region studied.[8,15,33,43,45] Interestingly, the ventrolateral area of the SCN, specifically the VIPergic region, appears to be the only area exhibiting astrocyte plasticity in membrane apposition with neurons.[43,44] Synaptic coverage at the

EM level is highest during the night and lowest during the day.[16] This may relate to the research by Xie et al. that observed an increase in the tortuosity of the extracellular space in the cortex of the conscious mouse and decreased while asleep.[170] This diurnal change in tortuosity allows for clearance of metabolites during sleep and seems to be dependent on retraction of astrocyte membranes. Whether this type of morphological plasticity plays a role in the SCN's sensitivity to RHT signaling during day versus night remains to be elucidated.

Here we show that GFAP immunostaining does exhibit a diurnal difference with higher immunostaining in the early night compared to early day. We also show that although there are not significant differences in the branching complexity of the total population of individual astrocytes in the SCN between early day and early night, there is a significant shift in the population distribution of cell branching where early day has fewer cells with more complex GFAP branching compared to early night. Lastly, we show that the total amount of GFAP does not change between early day and early night but the polymerization state of GFAP is dynamic. We found that early day SCN tissue has significantly more soluble GFAP compared to early night. We hypothesize that this shift from the insoluble or filamentous form to a soluble or monomeric form is likely due to a post-translational modification of GFAP that prevents filament polymerization.

2.3 Materials and methods

Animals

All animal experiments were conducted in full compliance with federal guidelines for humane animal research and according to an IACUC approved protocol. Adult Long Evans (LE/Blue Gill) rats were kept on a 12-h:12-h light/dark lighting schedule. Animals were sacrificed at two *zeitgeber* times (ZTs). ZT2 refers to two hours after lights were turned on and ZT14 refers to two hours after lights were turned off. Seven adult male rats were sacrificed and analyzed at ZT2 and eight adult male rats were sacrificed at ZT14. These times were chosen as maximum and minimum of GFAP immunoreactivity and complexity based on previous work in the Gillette Laboratory by Dr. Harry Rosenberg and other published studies.[8,81,82]

Tissue preparation and fixation

ZT2 animals were sacrificed with lights on while animals at ZT14 were sacrificed in a dark room with a hood to avoid light from hitting the retina. Animals from both groups were anesthetized by an intraperitoneal dose of pentobarbital (1 mL per animal) within 20 min of the designated time points. Following anesthetization, animals were transcardially perfused with 300 mL of cold normal saline (0.9% sodium chloride) followed by 300 mL of cold fixative containing 4% paraformaldehyde. After fixation, animals were decapitated and the brains removed carefully to avoid damage to the ventral brain and optic nerves. Brains were then post-fixed for 24-h. After post fixation, brains were then placed into PBS and sectioned using a vibratome to a thickness of 40 µm.

Animals used for Western blots in GFAP solubility analyses were sacrificed without anesthetic. Animals were briefly disoriented by spinning and rapidly decapitated with a guillotine. Brains were then removed and 500 µm thick hypothalamic slices containing SCN (as observed from the characteristic

shape of the optic chiasm) were sectioned. The SCN was then punched out using a 1mm biopsy punch and rapidly frozen on dry ice.

Immunohistochemistry

Brains were stained for GFAP in groups of four, with two brains from each experimental group stained together. This was repeated four times for a total of seven brains at ZT2 and eight brains at ZT14. Following slicing, tissue was placed in a blocking/permeablization (Triton-X with 5% goat serum) solution for 1 h. Tissue was then washed three times in PBS. Slices were then incubated for 48 h at 4˚C in primary antibody. For GFAP immunohistochemistry, the primary antibody used was mouse anti-GFAP (1:10,000 dilution; EMD Millipore, Bellirica, MA). Following primary antibody, tissue was washed three times in PBS. Tissue was incubated with secondary antibody conjugated to a fluorophore for 2 h at room temperature. The excitation wavelength for the fluorophore conjugated to the secondary GFAP associated antibody was 568 nm. Following secondary antibody, tissue was washed three times and mounted on gel-coated coverslips and allowed to dry. Once tissue is dry, ProLong Gold Antifade (ThermoFishcer Scientific, Life Technologies, Grand Island, NY) was applied and the slide is coverslipped. Slides were allowed to cure for 24 h at room temperature in the dark. After curing, clear nail polish was applied to the coverslip-slide edges to seal the ProLong and allowed to dry. Tissue was then taken to the microscopy facility and imaged.

Image acquisition

Rigorous testing of fluorescence intensities at both time points showed that small changes of laser power, gain intensity, or amplifier offset created differences in observed branching complexity by up to 50 percent (data not shown). Because of this, the researcher was blinded to the time point being analyzed, and images were obtained in a reproducible and constant manner. Fluorescent images were obtained using a Nikon Optiphot-2 microscope (Nikon, Japan) with a 4X air (0.13 DL/1 NA) lens with a

Texas Red dichroic mirror. Images were captured using a SPOT RT3 (Model 25.4 2 Mp Slider, Diagnostic Imaging, Sterling Heights, MI) camera and software. Three dimensional mages were acquired on a LSM510 Meta scanning confocal microscope (Zeiss Microscopy, Germany). To image GFAP, a 568 nm laser (30% laser power, 850 master gain, -0.10 digital offset) and a 40x oil lens (1.4 NA) were used. One brain slice containing the middle SCN was chosen from each brain. Two images, one from each bilateral SCN, were then taken of the vetrolateral region of the SCN immediately dorsal to the optic nerve. Three dimensional Z-stacks are taken with a depth resolution of 0.5 µm. Upper and lower vertical limits of tissue imaging is set accordingly to the presence of signal during scanning mode and varied from slice to slice.

Gel electrophoresis and Western blotting

Methods used for gel electrophoresis and Western blotting were modified from Hsiao et al.[64] Flash frozen tissue was lysed with a pestle on ice using 100 µl lysis buffer for 15 min. Lysis buffer consisted of 0.5% Triton-X100 v/v, 2mM Tris-HCl, 2mM EDTA, 2mM phenylmethylsulfonyl fluoride, EDTA-free protease inhibitor cocktail (Roche), protein phosphatase inhibitor cocktails set I and set II (Millipore), at pH 7.0. Following lysis, 30 µl was removed and kept as a total sample. Cells were then spun at 14,000 x g at 4˚C for 10 min. Protein concentrations were calculated using a Pierce BCA protein assay kit (Thermo Scientific). Soluble fraction samples were very dilute, therefore only 10 µg protein from total and supernatant were placed in running buffer (1% SDS w/v, 50mM Tris-HCl, 5% glycerol, 0.05% bromophenol blue w/v, 0.25% betamercaptoethanol v/v, at pH 6.8) and boiled for 5 min.

Samples were separated on a 10% polyacrylamide gel and wet transferred to polyvinyldifluoride (PVDF) membranes and blocked with 5% milk in tris-buffered saline with 5% triton (TBST) for 1 h at 37˚C. Blots were then probed with the same GFAP antibody as used in IHC at a dilution of 1:10,000 overnight in 1% milk in TBST. As a loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was

probed with anti-GAPDH antibody at 1:10,000 dilution overnight. Blots were washed 3 times for 5 min with TBST following primary antibody incubation. Horse radish peroxidase conjugated secondary (goat anti-mouse antibody) was used at a dilution 1:1,000 in 1% milk in TBST for 1 h at room temperature. Following secondary antibody, blots were washed 3 times for 5 min with TBST. Blots were then incubated with SuperSignal Chemiluminescent Substrate for two minutes and imaged using a digital camera. Exposure was set to avoid saturation of signal for any given band. Relative intensities were calculated by dividing the observed intensity of GFAP by the observed intensity of the tubulin signal.

Analysis and statistics

For 4x immunofluorescence studies researchers were blinded to the identification of slices when analysis was being done. Fluorescent images were imported into ImageJ software (ImageJ, NIH). ROIs were drawn around both SCN nuclei. As a staining control, the subparaventricular zone (SPZ), which did not change with respect to time of day, was used as a control region. Average pixel intensity from each SCN was divided by the average pixel intensity from the SPZ from the same brain. The resulting value was then considered a normalized value, which allowed the researchers to control for potential immunostaining variance between brains. Fluorescent values were analyzed using a two-tailed Student's t-test with a 95 % confidence interval.

For confocal images, three dimensional 8-bit Z-stacks were loaded into Imaris software (Bitplane, Concord, MA). The "Filaments" function was used to automap the GFAP cytoskeleton. The maximum width of GFAP filaments was set as 5 µm while the minimum was set at 0.5 µm, which approaches the optical resolution for the system used. Voxel threshold was set at 60 out of a total of 256 grey scale values. Due to the intercalation and varying signal intensities within the SCN, cells were manually chosen based on the center of intensity and observed branching. Total length, area, and volume were calculated while GFAP branch end points were used as a metric of individual cell

complexity. Branch points were defined as the point at which the fluorescent signal fell below the set voxel threshold at the end of a defined branch. Branch terminal points were logarithmically transformed creating a standard distribution required for parametric statistical analysis. Statistical tests were done using the mixed model ANOVA in Statistical Analysis Software (SAS, JMP). Statistical significance was set at 95 % confidence interval. For Chi-squared analysis, individual cell branch end points were binned by 100 branches up to 400 branch end points. Chi-squared analysis was conducted within Prism 6 (GraphPad).

2.4 Results

Fluorescence analysis of GFAP immunoreactivity.

To determine whether the polymerization state of GFAP exhibits diurnal variation, GFAP immunostaining was performed. This analysis allows for the measurement of filamentous levels of GFAP at early day and early night. The region of interest (ROI) for each SCN was centered on the ventrolateral SCN from each brain, which was also had the highest intensity observed for GFAP immunostaining. Areas within the SPZ were used as control regions to standardize immunofluorescence for each SCN analyzed due to potential immunostaining differences between slices (Figure 2.1B-C). Our results show significant differences in GFAP filament immunostaining within the SCN, with higher intensities at ZT14 compared to ZT2 (Fig. 2.1A). These results are consistent with previous studies that examined immunostaining intensity and electron microscopy of the adult rat SCN during the day and night.[8,51]

GFAP branching reconstruction and analysis

Although GFAP fills only a portion of the astrocytic extension, branching of GFAP extends into astrocytic processes and indirectly supports the cell membrane. Because of this, GFAP filament branch end points were used as a measure of astrocyte morphological complexity. In our study, large variation between individual GFAP branch end points was observed. The branch end-point results did not exhibit

a normal distribution and therefor were logarithmically transformed to obtain a normal distribution for statistical analysis. Our results were not significant in any of the metrics analyzed from Imaris software, including GFAP length, area, volume, or branch terminal points within the SCN (Fig 2.3B). However, Chi-squared analysis of the distribution of branch end points binned into groups every 100 branch end points at ZT2 and ZT14 was significantly different ($p < 0.05$), with ZT14 having a greater number of cells with more branch terminal points than compared to ZT2 (Fig 2.3).

Diurnal changes in filamentous and soluble GFAP levels

To confirm our observed changes in GFAP immunofluorescence, we conducted a Western Blot based study probing the differences in solubility of GFAP in the SCN tissue punch at ZT2 and ZT14. We first confirmed that the total amount of GFAP and GAPDH were not changing between early day and early night (Figure 2.4A&B). We then separated the filamentous from soluble GFAP using high speed centrifugation (14,000 rcf). We observed significantly higher levels of soluble GFAP in the early day compared to early night (Figure 2.6). These results agree with the hypothesis where GFAP immunostaining represents the polymerized state. As GFAP becomes soluble, the immunostaining decreases within the astrocytes in the brain slice.

2.5 Discussion

GFAP is a key structural protein in mature astrocytes of the CNS.[98,149] Moreover, GFAP is integral in normal astrocytic function in the tripartite synapse where they are responsible for clearance of glutamate, a potentially exitotoxic neurotransmitter.[2] This role is likely crucial in the SCN, which receives significant glutamatergic input from the RHT when light is present in the environment. Whether diurnal changes in RHT signaling can drive changes in the GFAP cytoskeleton in adult rat SCN is unknown. Our study has identified a significant difference in the intensity of immunofluorescence in the ventrolateral SCN, with higher intensity at ZT14 compared to ZT2, which agrees with previous studies

immunofluorescence and electron microscopy or the rat.[8,43,51] Moreover, utilizing a 3D immunostaining reconstruction method, we have identified a shift in branching of the astrocytic cytoskeleton within the SCN. Lastly, we have identified a previously unknown shift in soluble GFAP levels in SCN tissue. These observed dynamics could be due to changes in GFAP phosphorylation state, which causes depolymerization and solubilization, resulting in decreased immunostaining, fewer branches in the GFAP cytoskeleton, and a shift from filamentous to soluble GFAP.[69,70]

Here we confirm using GFAP immunostaining that GFAP is dynamic in the adult male rat SCN. We showed that GFAP staining is significantly higher in the early night compared to the early day. We then sought to confirm the results observed by Dr. Harry Rosenberg, which stated that GFAP branching complexity was higher in the early day compared to the early night. Here we found that GFAP branching reconstructions were not significantly different between ZT2 and ZT14. The lack of significance may be due to the fact that branch terminal point values were absolute and not compared to any internal control creating a large amount of variation. Branch terminal points had to be logarithmically transformed, creating a normal distribution, which was required for statistical analysis. This large variation of branch terminal points between individual astrocytes within the same brain suggests the astrocytes are not a heterogeneous population with respect to branch complexity. This observation together with the variation between animals makes statistical analysis a nightmare. Without a control or standard to normalize branch terminal points between samples fails to address the potential variation between perfusion, staining, and anatomical differences of each rat, which influences the data collected.

This fails to agree with previous results seen by Dr. Harry Rosenberg in the Gillette Laboratory. His results showed that GFAP branching was significantly higher at ZT2 and lowest at ZT14. The contradicting results between these two studies may be explained by the imaging technique, staining methods, and sex differences in animals utilized by each researcher. The current study was done with a rigorous setting for the image acquisition, which was reproduced for each image obtained. In fact, a

change of amplifier gain by as small as 5 % on image acquisition can result in a 50 % reduction of observable branch terminal points (data not shown). The current study also had the primary researcher blinded to the time point groups until the end of analysis. Taken with the fact that the GFAP cytoskeleton only represents roughly 13 % of the cell volume, the researcher does not feel that this experimental design is a reliable and reproducible way to measure astrocyte GFAP cytoskeletal complexity.[21]

Finally, we show that the solubility of GFAP at ZT 2 is significantly higher than at Z 14 . This observation agrees with the previous immunostaining results in the rat SCN.[43,51] We hypothesize that at ZT 2 GFAP staining is less in immunofluorescence images likely due to the shift from filamentous into a soluble state. Observable diurnal changes in GFAP is exciting, especially since the change from filamentous to soluble GFAP is driven by phosphorylation of the head domain.[69,70] Moreover, phosphorylation of GFAP protects it from degradation.[152] Whether or not this change is a circadian clock-driven phenomenon or is instead driven by an unknown signal to astrocytes in the SCN is currently unknown. However, cytoskeletal changes that underlie the observed differences in immunostaining intensity are still unknown. This diurnal variation is likely due to a change in complexity of the GFAP cytoskeleton although the significance at the time points studied is over shadowed by the heterogeneity of individual astrocyte cytoskeletons. Our Western blot results showing a shift from filamentous to soluble GFAP explains our observed diurnal GFAP immunostaining intensity results and the observed shift in reconstructed branching complexities in the population distributions between early day and early night. Taken together, an increase in filamentous GFAP would lead to an increase in signal intensity measured by immunofluorescence imaging while depolymerization would result in lower imaging intensities.

The role that diurnal astrocytic plasticity of GFAP could be playing in SCN physiology and rhythm generation is still unknown. One hypothesis lies in clearance of glutamate from the RHT-SCN synapse

during the light phase. Not only are these levels of glutamate excitotoxic in most regions of the CNS, but the neuronal rhythms of the SCN fail to respond to glutamatergic signaling during the day despite increasing firing frequency.[102] Moreover, an SCN-derived cell line is resistant to glutamate excitoxicity.[17] Additionally, astrocytic shape change is established in regulation of extracellular space and clearance of the glymphatic system of the cortex. Oberheim et al. observed that adrenergic signaling regulated an increase in extracellular space clearance while mice were asleep (during the subjective day), suggesting that astrocytic volumes were changing to allow metabolic clearance.[170] The connection between GFAP dynamics, astrocyte morphological plasticity, and their role in the tripartite synapse within the SCN are still unknown.

The signals driving the observed change in the astrocyte cytoskeleton are still unknown. One hypothesis is that PACAP and glutamate, which have been shown to activate signaling mechanisms upstream of GFAP phosphorylation and subsequent depolymerization, may be released during the day and act directly on SCN astrocytes.[69,70,95,96,158,163] Glutamate is the retinal signal for the presence of light while PACAP has been shown to colocalize in RHT-SCN synapses with glutamate.[30,54,101] Our future experiments explore RHT signaling in the SCN in order to identify potential mechanisms behind the observed diurnal dynamics of GFAP.

2.6 Figures

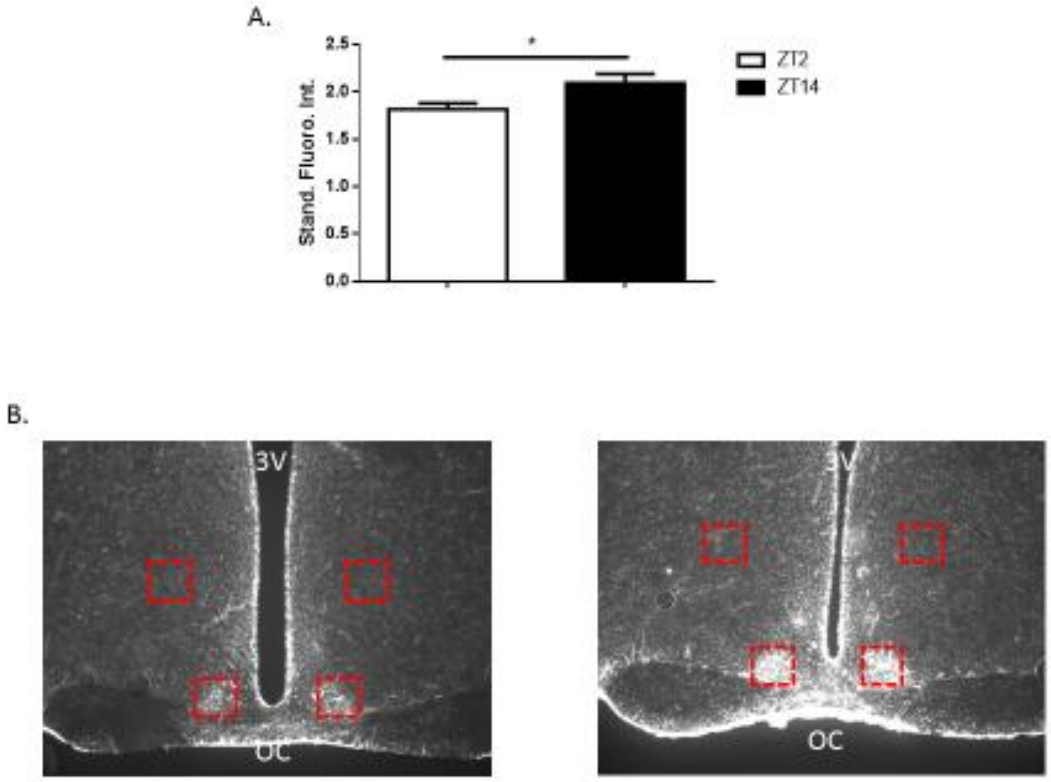

Figure 2.1. Fluorescent intensity of GFAP immunoreactivity is significantly different between ZT2 and ZT14. A.) Fluorescent intensity was measured at ZT2 (n=7) and ZT14 (n=8) and analyzed with an unpaired two tailed Student's t-test. * $p < 0.05$. B.) Representative fluorescent images from ZT2 and ZT14. ROIs in red are 230 μm^2. SPZ ROIs were the same size as SCN ROI. (S. Irving)

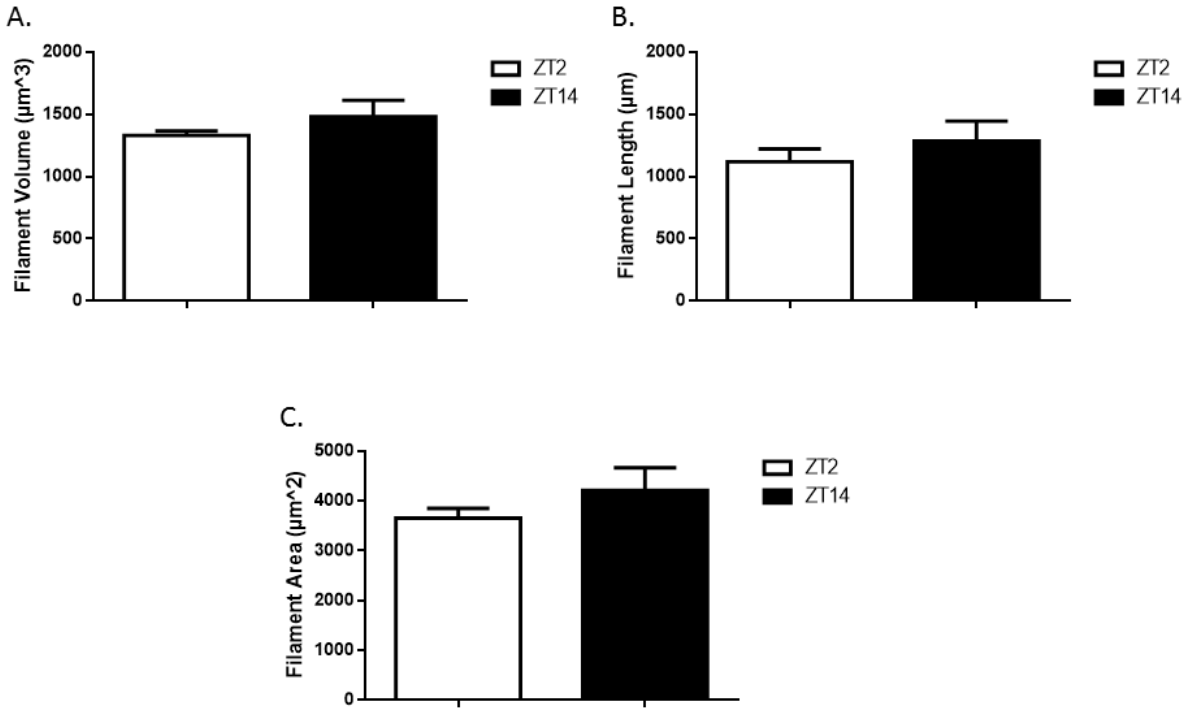

Figure 2.2. GFAP complexity does not change significantly between early day and early night. A-C.) GFAP length, area, and volume do not change significantly between early day and early night. Student's t-test for each measurement. P >0.05. n = 5 animals per time point.

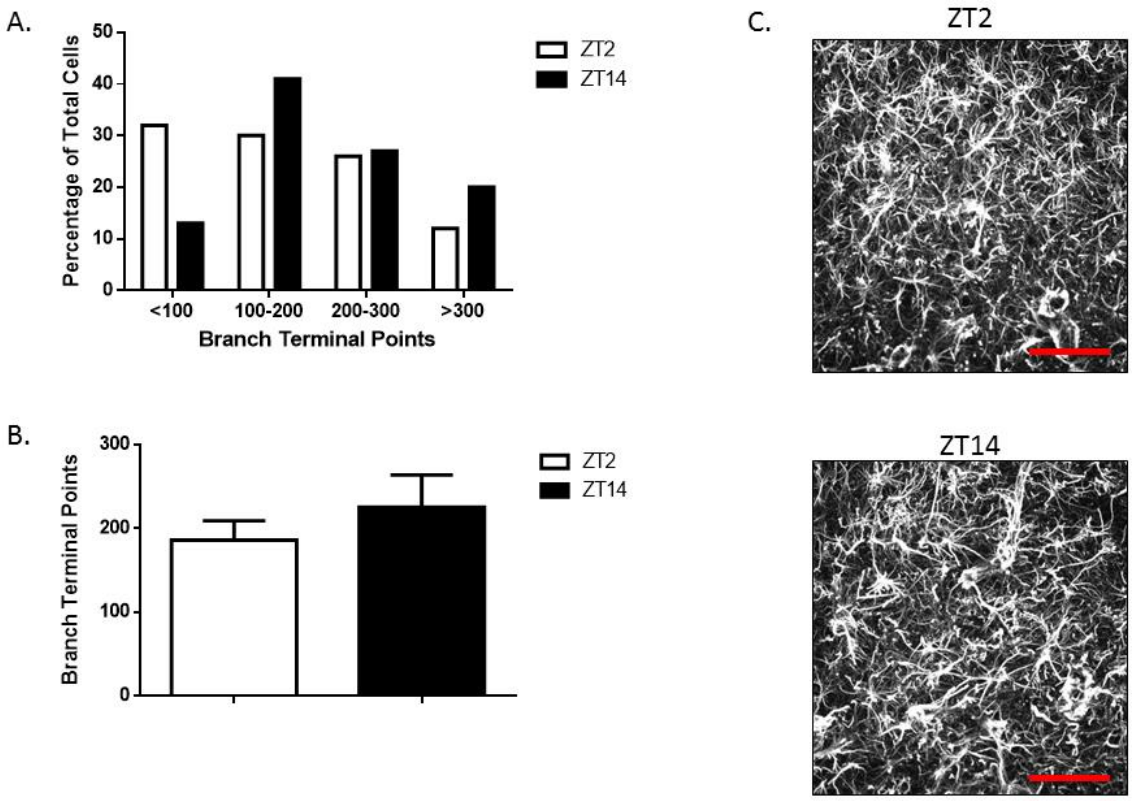

Figure 2.3. Astrocytic population distributions of GFAP complexity changes between early day and early night but individual cell complexity of the population does not. A.) The distribution of astrocytes with larger or smaller branch terminal points changes significantly between ZT2 and ZT14. The population distribution of numbers of cells with different total numbers of branch points are significantly different between ZT 2 and ZT 14. Chi-squared contingency test. * = $P < 0.01$. N=5 animals, 95 cells per time point. B.) Average branch terminal point analysis between ZT 2 and ZT 14 was not significant for the total population of cells. Unpaired Student's t-test. $P > 0.05$, n = 5 animals per time point. Between 16-20 cells per animal. C.) Example images of SCN GFAP branching at ZT2 and ZT14. Scale bar = 50 µm.

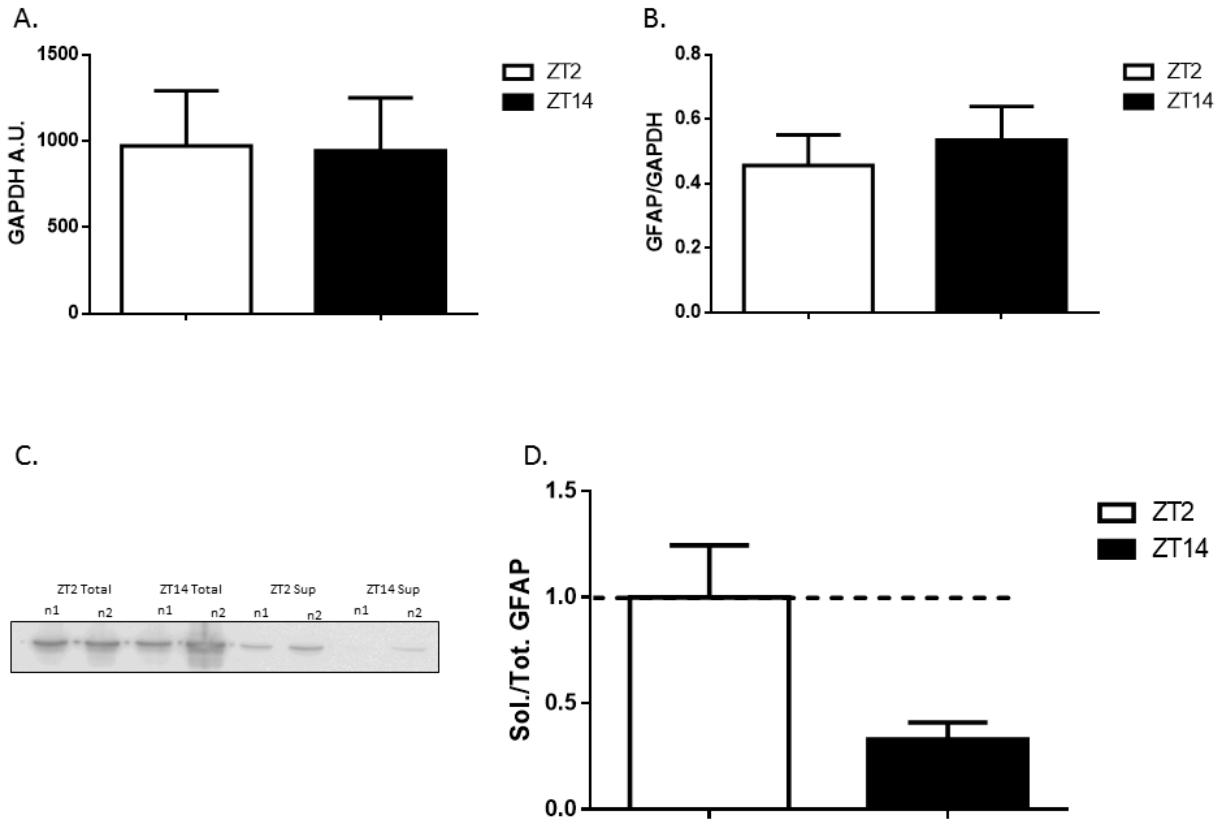

Figure 2.4. Solubility of GFAP in the 500 µm SCN punch is higher at ZT2 compared to ZT14. A.) Total GAPDH was used as a loading control and was confirmed to be not significantly different between ZT2 and ZT14. Student's t-test $p > 0.05$. n = 6 animals per time point. B.) Total GFAP was not significantly different between ZT2 and ZT14. Student's t-test $p > 0.05$. n = 6 animals per time point. C.) Representative Western blot with total samples and supernatant samples from ZT2 and ZT14. D.) Soluble to total GFAP ratios were significantly decreased by 67% at ZT14 compared to ZT2. $p < 0.05$. n = 6 animals per time point.

CHAPTER THREE

INVESTIGATING THE ROLE OF THE OPTIC NERVE ON SCN ASTROCYTIC CYTOSKELETAL PLASTICITY

3.1 Abstract

Circadian rhythms synchronize an organism's internal clock to external timing cues. Perhaps the strongest, most robust timing cue an animal receives from the environment is light. In mammals, this cue is transmitted from the eye to the brain via the optic nerve. Within the retina there reside photosensitive cells that contain melanopsin, which is sensitive to light. The axonal projections these intrinsic photosensitive retinoganglion cells (ipRGCs) send to the suprachiasmatic nucleus (SCN) are known as the retinohypothalamic tract (RHT).[110] These ipRGCs synapse on a region of the SCN known as the retinorecipient area.[56,57] TEM studies in this region have shown changes in the astrocytes surrounding neurons expressing vasoactive intestinal peptide (VIP) while astrocytes surrounding vasopressinergic (AVP) neurons failed to change around the clock.[8] Moreover, proper RHT innervation is in part responsible for the correct development of SCN components, including both neurons and astrocytes.[81,118] Our study focused on the role of the retina in adult rats on expression and complexity of the astrocytic intermediate filament GFAP in the SCN. Here we identify retinal signaling as the primary driver of SCN astrocyte GFAP dynamics.

3.2 Introduction

The primary input for photic information to the brain is via the retinohypothalamic tract (RHT). The RHT synapses on the SCN, the brain's master clock. The SCN interprets this timing information and synchronizes the rest of the brain and body. In fact, the two regions of the brain that receive light information from the retina, the SCN and intergeniculate leaflet (IGL), show increased levels of GFAP staining relative to other regional brain tissue.[115] In addition to relaying light information, the RHT is responsible for proper post-natal development of astrocytic and neuronal elements within the SCN.[68,108] Maturation of cellular elements within the SCN is dependent upon photic stimulation of the retina. Animals who have their eyes removed after birth have significantly reduced numbers of GFAP containing cells along with a reduction in the amount of total GFAP expressed in SCN.[68,116] Animals that have an intact optic nerve but are kept in constant darkness for 50 days also have reduced GFAP staining intensity, but GFAP levels return when the animals are placed in a 12-h light/dark cycle.[68] The extent to which the retina regulates astrocyte activity in the adult rat SCN is unknown. We have previously identified a shift in GFAP polymerization between early day and early night in the adult rat SCN under a 12-h light/dark cycle. However, the extent to which the retina regulates these dynamics has yet to be studied. Glutamine synthetase, the astrocytic enzyme responsible for recycling glutamate into glutamine in the glutamate-glutamine cycle, normally has peak activity while lights are on but in enucleated animals this peak is shifted to the rat subjective night, suggesting a role for the optic nerve in normal SCN astrocyte function.[85] These studies suggest a role for the optic nerve in SCN development and show a previously understated level of astrocytic plasticity within the SCN that may be regulated by retinal signals.

The RHT releases glutamate within the SCN when an animal's eyes are exposed to light. Both neurons and astrocytes within the SCN express receptors for light signals. Although the neuronal response to glutamate in the SCN has been thoroughly studied, astrocytic responses are largely

overlooked. Therefore we may look to astrocytic responses to glutamatergic signaling in other regions of the brain as a starting point for SCN astrocyte experimental design. Glutamate signaling can induce phosphorylation of GFAP in post-natal hippocampal and cerebellar brain slices but fails to induce phosphorylation in older animals.[76,77] However, excitatory signaling within the hippocampus in adult brain slices induces movement of fine perisynaptic astrocytic processes (PAPs) around the synapse. This movement is associated with greater synaptic stability and long term potentiation (LTP).[10] It should be noted that the GFAP cytoskeleton does not reach the cell membrane is not thought to enter the PAP.

Although identified as a regulator of GFAP in the developing SCN, the extent retinal signaling plays in the already developed SCN is largely unknown. The level of plasticity adult astrocytes can express in the SCN is a novel and exciting field of research. In our study we show that environmental light is the primary driver of GFAP polymerization state within the SCN. Additionally, we show that an intact retina is required for basal levels of GFAP solubility and long term enucleation reduces the overall GFAP expression within the SCN relative to a control hypothalamic region. These results suggest a retinal signal (or signals) regulates the solubility state of GFAP within the SCN in a diurnal manner and that the polymerization dynamics we have previously described are primarily regulated at the level of the retina and not by an astrocytic molecular clock or by SCN neuronal signaling.

3.3 Materials and methods

Animals

All animal experiments were conducted according to IACUC approved protocol. Long Evans (LE/Blue Gill) age matched groups were separated into four groups; L:D, D:D, enuc-short, and enuc-long. The L:D group was not enucleated and kept on a 12 h:12 h L:D cycle. This group consisted of 16 animals, 8 for each time point (ZT 2 and ZT 14). The D:D group was also not enucleated and was kept in constant darkness starting at ZT 0 for an additional 48 h. After 48 h, the animals were sacrificed at the

corresponding circadian times (CT), CT 2 and CT 14. Therefore, CT 2 animals were kept in constant darkness for 50 total hours and CT 14 animals for 62 h. The amount of time kept in constant darkness should abolish any light signaling the retina received while keeping the animal on the anticipated circadian time. Two enucleated animal groups were made, enuc-short and enuc-long. Each group consisted of 8 animals. Enuc-short animals were enucleated at ZT0. These animals were allowed to recover for at least 48 h followed by sacrifice at subjective CT 2 and CT 14. Therefore, these animals were sacrificed approximately 50 and 62-h post-enucleation. Enuc-long animals were enucleated while lights were on and allowed to recover for at least 3 months and sacrificed at ZT 2 and ZT 14.

Enucleation procedures

Enucleated group animals were anesthetized with inhaled 10% isoflurane mix and enucleated. Eye sockets were packed with antibiotic swabs and sutured shut. Enucleated animals were allowed to recover for at least 2 months. Age matched control animals were kept in the same room during enucleation and housed in the same room as the experimental group until sacrificed. Control and experimental rats kept in a 12 h:12 h L:D lighting schedule were sacrificed at two *zeitgeber* times, ZT 2 and ZT 14. ZT 2 refers to two hours after lights were turned on and ZT14 refers to two hours after lights were turned off. Enucleated animals were sacrificed at the same time as controls despite being on CT due to the lack of environmental light input to the circadian system. Circadian timing for long enucleated animals was subsequently assessed by PERIOD2 staining. Age-matched animals from control and enucleated groups were sacrificed on the same day.

SCN tissue punch preparation

Animals used for Western blots in GFAP solubility analyses were sacrificed without anesthetic. Animals at ZT2 and all enucleated groups were rapidly sacrificed and decapitated with lights on. Animals at ZT14 and all DD groups were sacrificed with the lights off. Animals were briefly disoriented by

spinning and rapidly decapitated with a guillotine. Brains were then removed and 500 µm thick hypothalamic slices containing SCN (as observed from optic chiasm) were sectioned. The SCN was then punched out using a 1mm biopsy punch and rapidly frozen on dry ice.

Gel electrophoresis and Western blotting

Methods used for gel electrophoresis and Western blotting were modified from Hsiao et al.[64] Flash frozen tissue was lysed with a pestle on ice using 100 µl lysis buffer for 15 min. Lysis buffer consisted of 0.5% Triton-X100 v/v, 2mM Tris-HCl, 2mM EDTA, 2mM phenylmethylsulfonyl fluoride, EDTA-free protease inhibitor cocktail (Roche), protein phosphatase inhibitor cocktails set I and set II (Millipore), at pH 7.0. Following lysis, 30 µl was removed and kept as a total sample. Cells were then spun at 14,000 x g at 4˚C for 10 min. Protein concentrations were calculated using a Pierce BCA protein assay kit (Thermo Scientific). Soluble fraction samples were very dilute, therefore only 10 µg protein from total and supernatant were placed in running buffer (1% SDS w/v, 50mM Tris-HCl, 5% glycerol, 0.05% bromophenol blue w/v, 0.25% betamercaptoethanol v/v, at pH 6.8) and boiled for 5 minutes.

Samples were separated on a 10% polyacrylamide gel and wet transferred to polyvinyldifluoride (PVDF) membranes and blocked with 5% milk in tris-buffered saline with 5% triton (TBST) for 1 h at 37˚C. Blots were then probed with the same GFAP antibody as used in IHC at a dilution of 1:10,000 overnight in 1% milk in TBST. As a loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was probed with anti-GAPDH antibody at 1:10,000 dilution overnight. Blots were washed 3 times for 5 minutes with TBST following primary antibody incubation. Horse radish peroxidase conjugated secondary (goat anti-mouse antibody) was used at a dilution 1:1,000 in 1% milk in TBST for 1 h at room temperature. Following secondary antibody, blots were washed 3 times for 5 minutes with TBST. Blots were then incubated with SuperSignal Chemiluminescent Substrate for two minutes and imaged using a digital camera. Exposure was set to avoid saturation of signal for any given band. Relative intensities

were calculated by dividing the observed intensity of GFAP by the observed intensity of the tubulin signal.

Fixed tissue preparation

Brains from all experimental group animals were removed at ZT/CT 2 and ZT/CT 14 for each group, respectively. ZT2 and all enucleated animals were sacrificed with the lights on. ZT 14, CT 2 D:D, and CT 14 D:D animals were sacrificed in a dark room with a hood covering the head to avoid any potential environmental light hitting the retina. Animals from all groups were anesthetized by an intraperitoneal dose of pentobarbital (1 mL per animal) within 20 min of the designated time points. Following anesthetization, animals were transcardially perfused with 300 mL of cold normal saline (0.9% sodium chloride) followed by 300 mL of cold fixative containing 4% paraformaldehyde. After fixation, animals were decapitated and the brains removed carefully to avoid damage to the ventral brain and optic nerves. Brains were then post-fixed for 24 h. After post fixation, brains were then placed into PBS and sectioned using a vibratome to a thickness of 40 μm. Brains were serially sliced on a vibratome into 40 μm thick slices. Alternating slices were used for GFAP and PERIOD2-DAB staining.

Immunohistochemistry - GFAP

Following slicing, tissue was placed in a blocking/permeablization (Triton-X and 5% secondary host serum) solution for 1 h. Tissue was then washed three times in PBS. Slices were then incubated for 48 h at 4˚C in primary antibody. For GFAP immunohistochemistry, the primary antibody used was mouse anti-GFAP (1:10,000 dilution; Millipore). Following primary antibody, tissue was washed three times in PBS. Tissue was then incubated with a goat anti-mouse secondary antibody conjugated to a 568 nm fluorophore (Molecular Probes) for 2 h at room temperature. Following secondary antibody, tissue was washed three times and mounted on gel-coated coverslips. Tissue was allowed to dry. Once tissue was dry, ProLong Gold was applied and tissue is cover slipped. Slides were allowed to cure for 24 h at room

temperature. After curing, clear nail polish was applied to the coverslip-slide edges to seal the ProLong and allowed to dry. Tissue was then taken to the imaging facility to be imaged.

Immunohistochemistry – PERIOD2 DAB staining

DAB staining protocol used was a modified version from Karen Weis. Slices were washed 3 times with PBS. Slices were then quenched in 1% hydrogen peroxide (1%) for 30 min at room temperature. Slices were then washed 3 times with PBS. Slices were then blocked with 5% normal goat serum in PBS-triton X (0.3%) for 1 h at room temperature. Slices were then incubated with primary PERIOD2 antibody (developed at the University of Illinois at Urbana-Champaign, at 1:25,000 dilution) in 3% normal goat serum and PBS-triton X for 48 h at 4˚C. Following primary antibody incubation, slices were washed 3 times with PBS. Biotin conjugated secondary antibody (1:1,000) was then added for 1 h at room temperature in 3% normal goat serum and PBS-triton X. Following secondary antibody incubation, slices were washed 3 times with PBS. Slices were then incubated with ABC reagent mixture for 1 h at room temperature. Slices were then washed 3 times with PBS. Slices were then incubate with DAB reagent (1 mM DAB, 240 mM NiSO4, 1% H2O2, and 185 mM NaOAc) for 10 min at room temperature. Tissue was then washed 3 times with PBS and mounted on microscope slides and allowed to dry. Once dried, slices were covered with Permount and a coverslip. Microscope slides were sealed with fingernail polish.

Image acquisition

All images were obtained using the same rigorous imaging techniques as discussed in Chapter 2. Images were obtained from the middle SCN, midway between the rostral and caudal tips, in the ventrolateral region. Researchers were also blinded to the condition and the time points for all brains analyzed.

For PERIOD2-DAB imaging, brightfield illumination was used with a Nikon 4X objective microscope. For immunofluorescent intensity imaging was done with a Nikon 4X objective with a Texas

Red dichroic mirror. For GFAP reconstructions, images were acquired on a LSM510 Meta scanning confocal microscope (Zeiss Microscopy, Germany). To image GFAP, a 563 nm laser (30% laser power, 850 master gain, -.1 digital offset) with a 40x oil immersion lens (1.4 NA) was used. Images were taken within the ventrolateral region of the middle SCN, rostral to caudal, immediately dorsal to the optic nerve. Three dimensional Z-stacks were taken with a vertical resolution of 0.5 µm. Upper and lower vertical limits of tissue imaging was set accordingly to the presence of signal during scanning mode.

Analysis and statistics

Analysis and statistics were done as described previously in Chapter 2. For GFAP immunofluorescence at ZT2 and ZT14 a two-tailed Student's t-test was used. At ZT/CT2 and 14 comparison within the time point between experimental groups was done using one way ANOVA in Prism 6 (GraphPad). For GFAP branching complexity, branch terminal point values were logarithmically transformed to allow parametric statistical analysis with a mixed model ANOVA in SAS software. Significance was set at a 95 % confidence interval.

3.4 Results

PERIOD2-DAB staining results

Enucleated animals have intact SCN rhythms and will free run with a near 24-h period. However, each animal has a unique period and without activity monitoring equipment, animals may become misaligned to control groups. Short term enucleated animals were free running for only three days with little to no drift from estimated CT. To ensure that long term enucleated animals were analyzed with the correct control group, animals were stained for the core clock protein, PERIOD2. PERIOD2 protein concentration peaks at ZT 14 in the SCN and is at its minimum at ZT 2.[119,133] As expected, when control brains were stained for PER2, ZT 2 control brains had low PER2 and ZT 14 brains had high PER2 (Figure 3.1). However, long term enucleated animals exhibited variation in the amount of PER2 staining in the

SCN, likely due to individual circadian rhythms that were greater or less than 24 h. Because of this, long term enucleated animals were grouped into either high or low PER2, instead of assigned a specific CT, and compared to the corresponding ZT controls.

Fluorescence analysis of GFAP immunohistochemistry

GFAP immunofluorescence was used as a measure of GFAP polymerization state. Previous studies have identified changes in the immunofluorescence at different times of day. The source of this change is still unknown but may report a change in GFAP state or protein level. GFAP standardized immunofluorescence intensity was significantly different between ZT 2 and ZT 14 (Figure 3.2B, $p < 0.05$, $n = 8$). All experimental groups lost diurnal variation in GFAP immunostaining (Figure 3.2C-E). At CT 2 both enucleated groups showed significantly lower GFAP immunostaining relative to control regions as compared to controls. Interestingly, animals at CT 2 kept in constant darkness did not have significantly lower GFAP immunostaining compared to controls (Figure 3.3A). GFAP immunostaining in experimental groups at CT 14 were all significantly lower compared to controls (Figure 3.3B). The loss of diurnal variation in GFAP immunostaining shows that the polymerization state of the astrocytic cytoskeleton in the SCN is regulated by light signals from the RHT. Previous work has shown that retinal input is required for proper development of GFAP in the rat SCN of the post-natal rat.[118] However, this loss of diurnal GFAP immunofluorescence intensity between enucleated groups of adult rats suggests a role for retinal input in the regulation of GFAP polymerization state in the adult rat SCN.

GFAP branching reconstruction and analysis

GFAP branch end points were used a measure of astrocyte complexity. GFAP does not contact the cell membrane but branches do support astrocyte projections. GFAP branch end points were not significantly different between control animals at ZT 2 and ZT 14. However, branch end points were

significantly different between controls and enucleated animals (3.4 A&B). We hypothesize that this significant reduction in branching is due to an overall decrease in expression of the GFAP cytoskeleton.

GFAP diurnal polymerization changes are lost in constant darkness and short-term enucleated animals

To confirm results from imaging analysis of GFAP immunohistochemistry, we utilized a previously described method to separate soluble GFAP from polymerized GFAP (Chapter 2, page 37). Animals kept in constant darkness and short-term enucleated animals lost the observed diurnal shift in soluble GFAP as seen previously in control animals complimenting the GFAP immunostaining results (Figure 3.7A-C). The overall levels of GFAP were unchanged in DD animals compared to controls at both CT2 and CT14 (Figure 3.6A&B). Interestingly, rats enucleated for three days had significantly higher levels in overall GFAP at both CT2 and CT14 compared to control and DD groups but lost diurnal variation in soluble levels (Figure 3.6A&B, 3.7C). When comparing soluble to total GFAP at CT2, only short-term enucleated rats had significantly lower levels of soluble GFAP compared to controls (Figure 3.8A). Intriguingly, significant differences were only observed between animals in constant darkness and short-term enucleated animals (Figure 3.8B).These results confirm that retinal input regulates the solubility state of GFAP in the SCN through an unidentified mechanism and that GFAP immunostaining and polymerization state are inversely correlated.

3.5 Discussion

The role of the retina in circadian rhythms has been extensively studied. Nearly all of these studies have focused on the neural component of RHT signaling despite the growing evidence of astrocytic regulation of synaptic and circuit behaviors within the brain. Retinal signaling is a regulator of astrocytic development and plasticity in post-natal rodents but the extent to which the retina can influence mature astrocytic plasticity is unclear. Our current study clearly demonstrates that the retina is the primary driver of the observed diurnal variation in GFAP cytoskeletal dynamics. Moreover, RHT

signaling is key for the relative increased levels of GFAP within the SCN compared to other local brain regions as previously observed.

Enucleation with either short or long-term recovery significantly reduced the immunostaining of GFAP in the SCN at both ZT2 and ZT14 (Fig. 3.1). Constant darkness and both short and long-term enucleation also eliminated the diurnal difference between ZT2 and ZT14. This result suggests that diurnal differences observed in GFAP immunofluorescence is driven by an external signal and not driven by an autonomous astrocytic cell clock. Although GFAP was reduced in the enucleated SCN compared to control animals, the SCN immunofluorescence was still greater than the SPZ, demonstrating that SCN GFAP staining is still stronger in enucleated SCN tissue than in control regions of enucleated animals (Fig. 3.1). Additional studies with animals kept in constant dark or enucleated for three days confirmed that the RHT signaling regulates GFAP immunostaining within the SCN. It is unclear whether this shift in polymerization state is driven directly by RHT signals onto astrocytes or indirectly via RHT signals onto SCN neurons.

GFAP solubility state was significantly reduced in short-term enucleated animals compared to controls at CT2, likely due to a loss of RHT signaling, driving GFAP into insoluble filaments. Interestingly, at CT14 short-term enucleated animals were significantly lower compared to animals kept in constant darkness but not compared to controls. This result is likely due to slightly but not significantly higher levels of soluble GFAP in D:D animals from the loss of retinal signaling or possibly trophic signals from the eye in darkness due to the presence of an intact optic nerve. The resulting inflammation from enucleation likely drives GFAP into insoluble filaments through an unknown mechanism, creating the observed difference between CT14 D:D and short-term enucleated animals. We also observed significantly elevated levels of total GFAP in short-term enucleated animals compared to all other groups at both CT2 and CT14. We hypothesize that this observed increase is astrogliosis in response to the

damaged RHT post enucleation. Paradoxically, this increase in total GFAP was not observed in GFAP immunofluorescence.

It is not understood how the RHT and the signals it carries to the circadian system regulate astrocytic GFAP. Moreover, the role of GFAP in astrocytic function is poorly understood within the SCN and the circadian system. Whether changes in GFAP immunoreactivity in enucleated animals are the result of an increase in degradation of GFAP remains unknown. One hypothesis is that signals from the RHT may play a role in the expression and maintenance of GFAP. RHT signals activate kinases known to phosphorylate GFAP.[95] GFAP, when phosphorylated, depolymerizes from filaments to soluble form of the protein.[70] Moreover, phosphorylation of GFAP appears protective from degradative mechanisms.[152] If RHT signals are phosphorylating GFAP in the presence of environmental light, then in the absence of RHT signals, GFAP would be dephosphorylated and subsequently degraded over time. Additionally, this diurnal dance of phosphorylation state may be responsible for the diurnal differences observed in GFAP immunoreactivity in intact animals.

This study is the first to look at the effect of an intact optic nerve on adult astrocytic GFAP immunoreactivity in the rat SCN. The brain receives information from the optic nerve and processes time of day by the SCN. Astrocytes in the SCN express receptors and respond to these signals. However, previous studies have failed to agree on whether a diurnal GFAP fluctuation exists in the SCN. Moreover, to the researcher's knowledge, no study has investigated the role of intact optic nerve signaling on the plasticity of the adult astrocyte cytoskeleton in the SCN. This comes as a surprise considering the potential role that diurnal astrocytic morphological plasticity plays in SCN physiology and the potential for astrocytes as targets for therapeutics. With the current data we hypothesize the model that retinal signals drive polymerization of the astrocyte cytoskeleton (Figure 3.8). Loss of the RHT results in a loss of signaling driving the GFAP cytoskeleton into the polymerized filamentous state. Since phosphorylation and depolymerization likely protects GFAP from degradation, the extended filamentous state results in

progressive degradation of GFAP as seen in the long term enucleated SCN brain slices. Moreover, if PACAP from the RHT drives the increased GFAP levels observed within the SCN, the loss of the RHT would also decrease overall GFAP from this perspective.

3.6 Figures

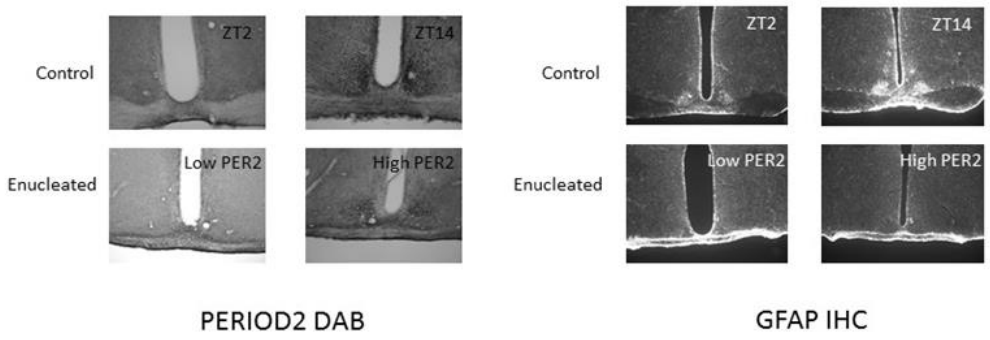

Figure 3.1. To determine correct circadian time in long-enucleated animals, we performed DAB and GFAP staining in control and animals enucleated for at least 3 months. These long-enucleated animals were grouped into low (CT2) or high (CT14) Per2 groups. GFAP immunofluorescence was then compared to the correct control animals.

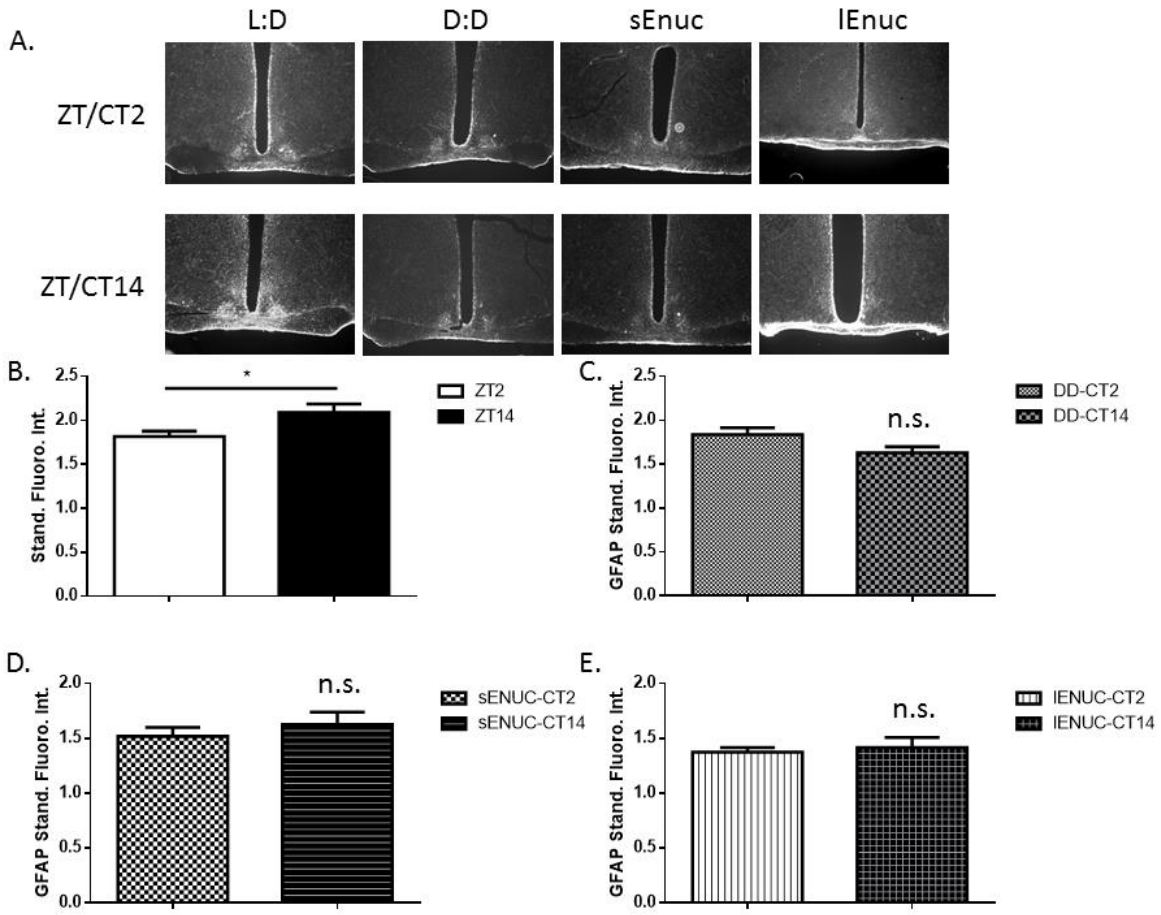

Figure 3.2. To compare the effects of constant darkness and enucleation on SCN astrocytes, we examined GFAP immunofluorescence in control, constant darkness, and both long-term and short-term enucleated animals. A) Representative GFAP staining from all experimental groups. B) Control animals had significantly higher GFAP immunostaining within the SCN at ZT14 compared to ZT2. Unpaired Student's t-test. * $p < 0.05$. n = 8 animals per time point. C-E) Animals kept in constant darkness, short enucleated, and long enucleated lost the previously observed diurnal variation in GFAP immunofluorescence. Unpaired Student's t-test for all treatment groups. DD treatment n = 5 animals per time point. sEnuc treatment n = 5 animals per time point. lEnuc treatment CT2 n = 9, CT14 n = 7.

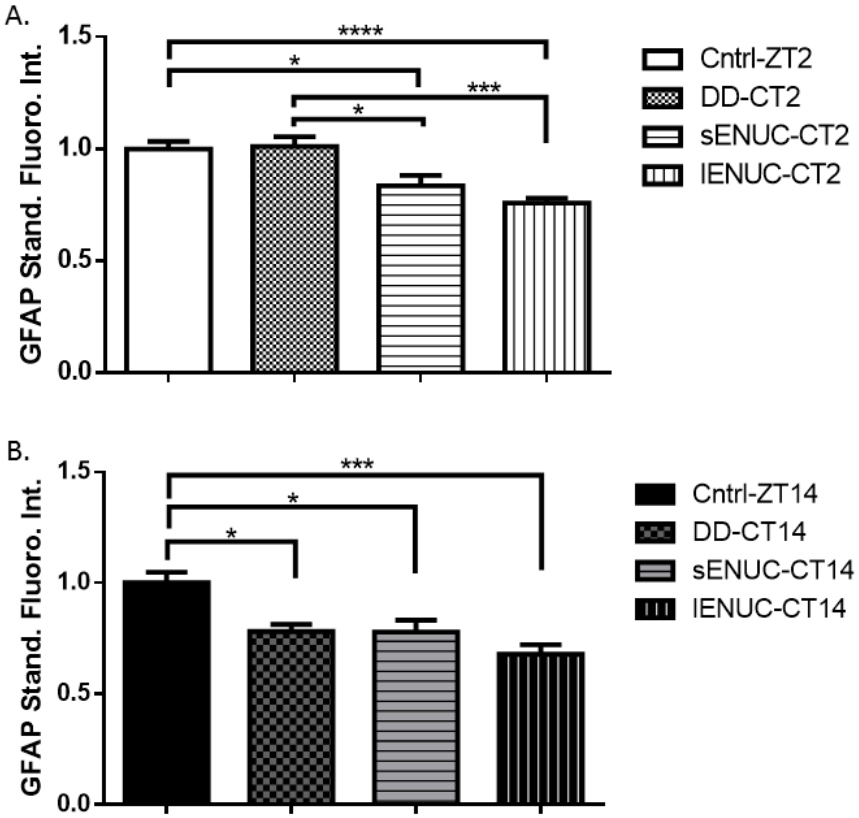

Figure 3.3. GFAP immunofluorescence is decreased at ZT 2 and ZT 14 in the SCN of animals kept in constant darkness and in both short and long term enucleated animals. A) GFAP immunofluorescence at ZT/CT 2 was significantly lower in both sENUC and lENUC groups compared to controls animals and DD animals. One way ANOVA. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. n = 6 animals per time point. B) GFAP immunofluorescence at ZT/CT 14 was significantly lower in DD, sENUC, and lENUC groups compared to control animals. One way ANOVA * $p < 0.05$, *** $p < 0.001$. n = 6 animals per time point.

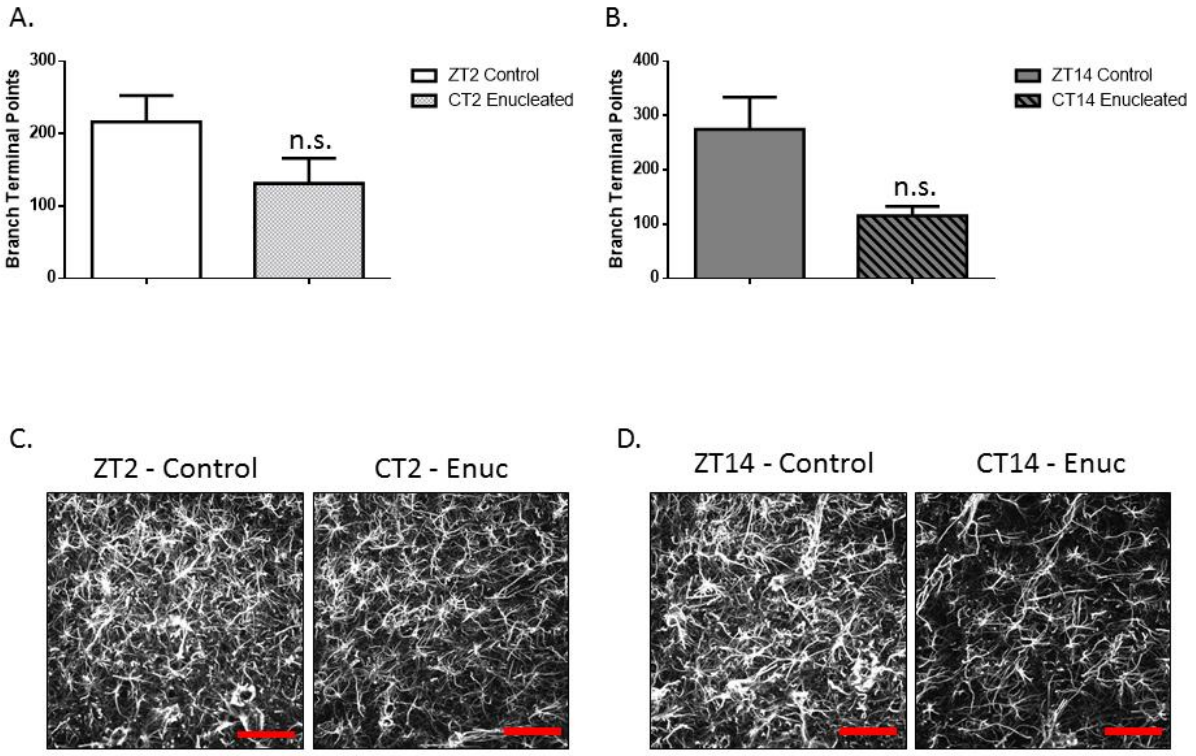

Figure 3.4. Enucleation results in decreased branching complexity in the rat SCN. A. Average branch terminal points were reduced in enucleated rats when compared to controls at ZT 2, although not significant (n=5 for control, n=2 for enucleated; $p>0.05$). B) Average branch terminal points were reduced in enucleated rats when compared to controls at ZT 14, although not significant (n=5 for control, n=4 for enucleated; $p>0.06$). C) Representative images of SCN GFAP reconstructions at ZT 2 for control (left) and enucleated (right) animals. D) Representative images of SCN GFAP reconstructions at ZT 14 for control (left) and enucleated (right). Size bar = 50 µm.

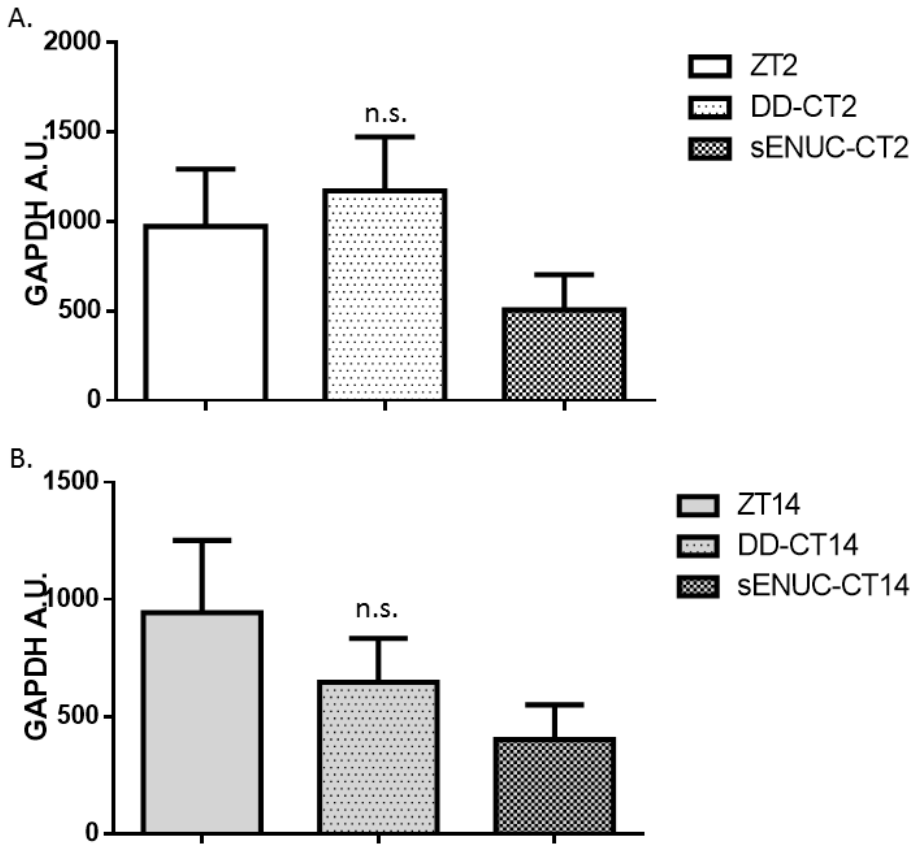

Figure 3.5. Total GAPDH is not significantly different at ZT 2 and ZT 14. A) Mean signal intensity of GAPDH at ZT 2 in control animals and CT2 in DD and short enucleated animals was not significantly different between treatment groups. One way ANOVA. n = 6 animals per time point. B) Mean signal intensity of GAPDH at ZT 14 in control animals and CT 2 in constant dark and short enucleated animals was not significantly different between treatment groups. One way ANOVA. n = 6 animals per time point.

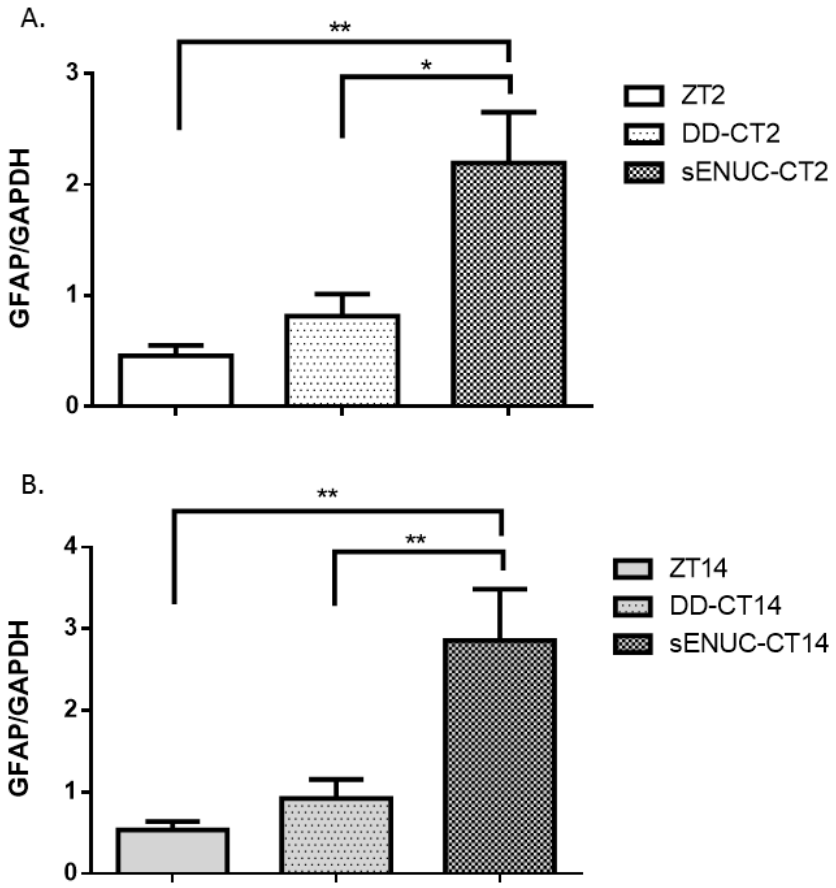

Figure 3.6. Total GFAP is increased in short enucleated animals at both ZT2 and ZT14. A) Total GFAP was significantly elevated in short enucleated animals. One way ANOVA. * $p < 0.05$, ** $p < 0.01$. n = 6 animals per time point. B) Total GFAP was elevated in short enucleated animals compared to controls and DD animals. One way ANOVA. ** $p < 0.01$. n = 6 animals per time point.

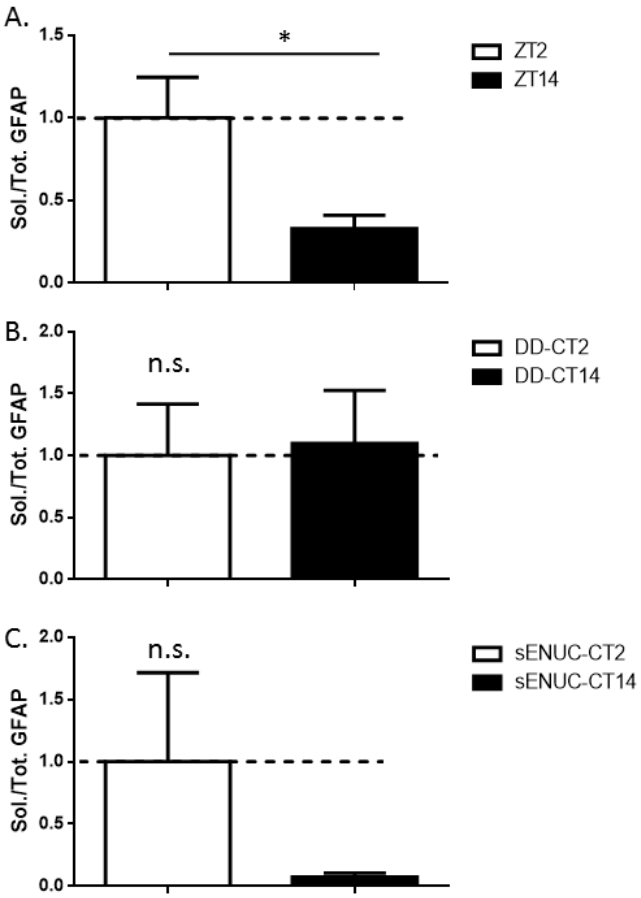

Figure 3.7. Soluble GFAP levels are different between ZT 2 and ZT 14 in control animals but is lost in constant darkness or in short-term enucleteated animals. A.) Soluble GFAP levels are significantly different in animals kept on a 12-h:12-h light-dark cycle. Unpaired Student's t-test. $p < 0.05$. B.) Animals kept in constant darkness for 3 days failed to exhibit significantly different soluble GFAP levels. Unpaired Student's t-test. $p > 0.05$. C.) Animals enucleated and allowed to recover for 3-days also failed to show significantly different soluble GFAP levels. Unpaired Student's t-test. $p > 0.05$. $n = 6$ animals for all time points and treatments.

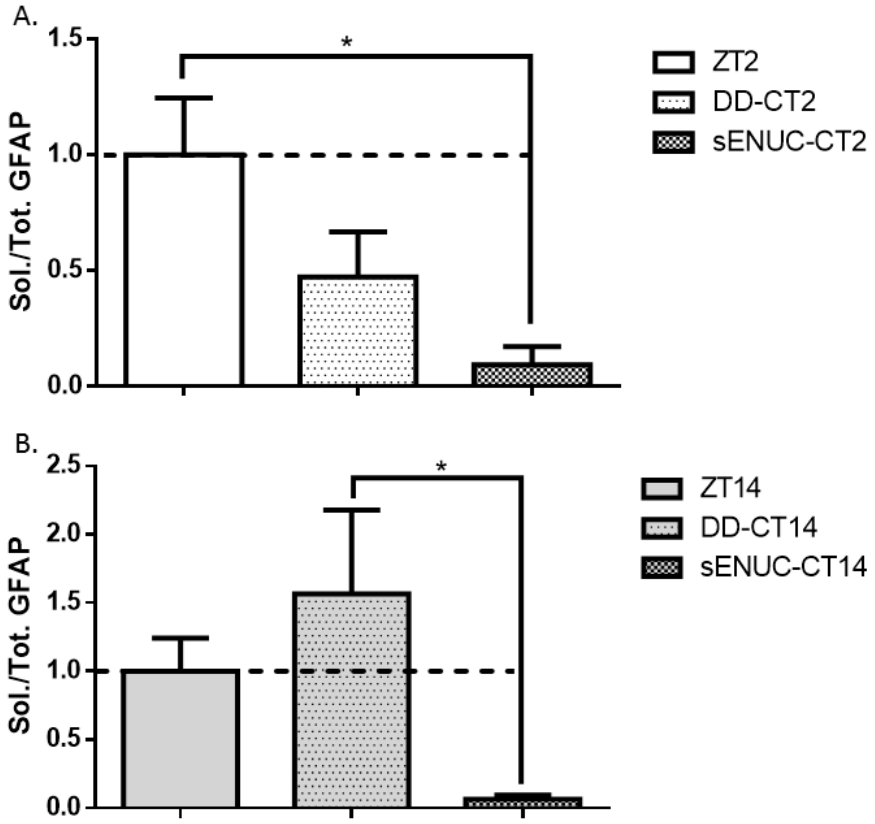

Figure 3.8. GFAP solubility is altered in enucleated animals. A) Analysis of the ratio of soluble to total GFAP at ZT2 in controls and CT2 in DD and short enucleated rats. Analysis shows that DD solubility is not significantly reduced compared to controls however short enucleated rats have significantly lower soluble GFAP. One way ANOVA. * $p < 0.05$. n = 6 animals per time point. B) Analysis of the ratio of soluble to total GFAP at ZT14 and CT14 in DD and short enucleated rats. Analysis shows that both DD and short enucleated rats do not have significantly different soluble GFAP compared to controls. Comparison between constant dark and enucleated rats was significantly lower. One way ANOVA. * $p < 0.05$. n = 6 animals per time point.

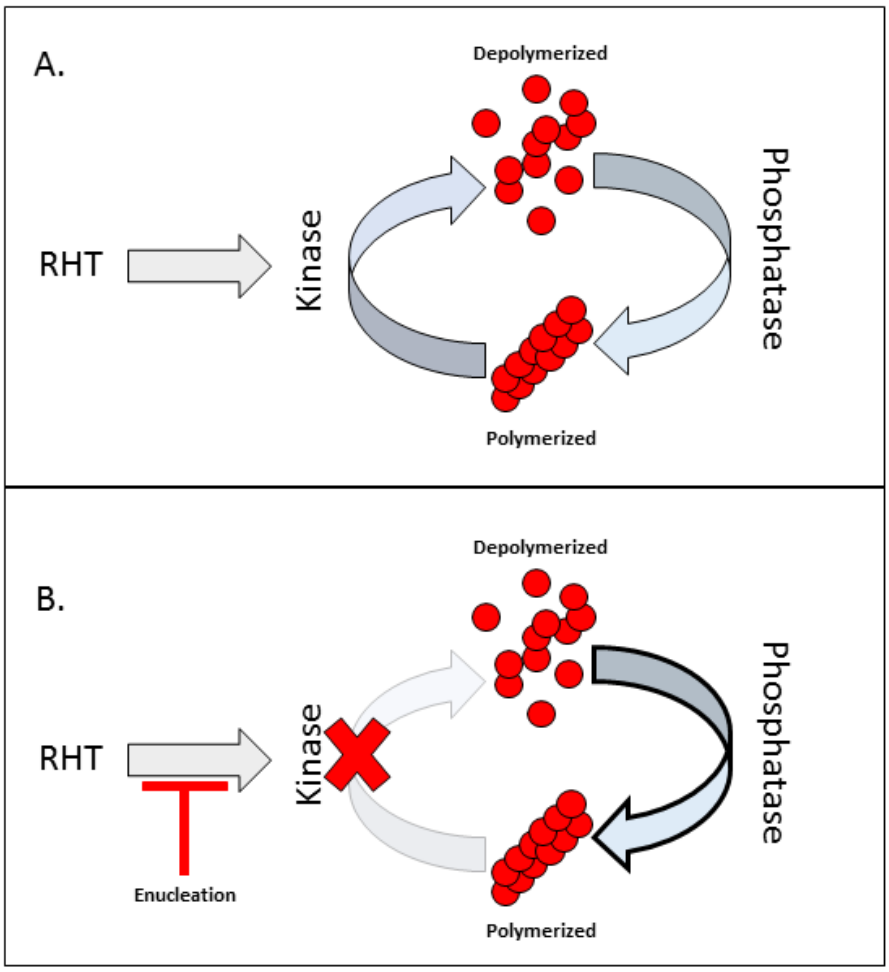

Figure 3.9. Optic nerve innervation and signaling to the SCN plays a role in astrocytic diurnal GFAP plasticity. A) Normal RHT innervation in the adult rat SCN results in diurnal differences between night and day GFAP filament polymerization. B) Enucleation eliminates RHT signals that normally drive GFAP plasticity.

CHAPTER FOUR

INVESTIGATING ASTROCYTIC CYTOSKELETAL POLYMERIZATION STATE WITHIN THE ACUTE SCN BRAIN SLICE

4.1 Abstract

Evidence suggests that light signaling from the RHT to the SCN influences the astrocyte cytoskeleton and subsequently their morphology.[81-83] Moreover, astrocytes within the retinorecipient area of the SCN show circadian variation of GFAP and fine perisynaptic astrocytic process (PAP) intercalation between neurons.[8,43] Axons within the RHT release glutamate in response to light signaling and may release PACAP concomitantly onto the SCN.[25,54,55] Intriguingly, SCN astrocytes express receptors and respond to PACAP from the RHT.[46,95] Additionally, astrocytes express glutamate receptors and glutamate transporters, making them not only targets of glutamatergic signaling but also potentially responsible for clearance of excitotoxic levels of glutamate in the SCN.[161] We have previously shown that the astrocytic cytoskeletal protein GFAP polymerization state differs between early day and early night. Here we have utilized the acute SCN brain slice to determine whether the polymerization state of GFAP is regulated by the retinal signals glutamate and PACAP. Our results show that the GFAP state within the brain slice loses the diurnal variation observed in the intact animal within two hours in the slice environment. Moreover, the pharmacological treatments with glutamate and PACAP failed to elicit a significant response in solubility of the GFAP cytoskeleton as hypothesized.

4.2 Introduction

The RHT plays an important role in the development of the SCN. If the optic nerve is removed by enucleation in postnatal animals, astrocyte proliferation and development fail to occur in the SCN.[118] However, the importance of the optic nerve and the role that the RHT's signal plays in adult astrocyte plasticity is not understood. The RHT releases glutamate onto the SCN in response to retinal stimulation.[22] Normally, constant levels of glutamate is excitotoxic to neurons. However, the SCN is well known to be resistant to glutamate levels that are excitotoxic in many other tissues.[17] The source of this protection is unknown but the local astrocytes are likely to play some role. Astrocytes exhibit neuroprotective qualities through the uptake of glutamate by Na^+ dependent glutamate transporters, by releasing neuroprotective gliotransmitters, and by changing their shape to modulate synapses and the extracellular space.[11,52,161] Moreover, a recent study has shown that the astrocytes within the dorsomedial region of the SCN are key regulators of inhibitory signaling within the SCN during the subjective night. Intriguingly, astrocytic regional heterogeneity within the SCN and the potential physiological roles of these cells is a critical question for circadian biology.

The role of glutamate, often considered the signal of light, has been well established by the Gillette laboratory in circadian biology.[29,30,40,41] Ding et al. showed that the circadian rhythm of SCN neuronal firing rate is non-responsive to glutamate signaling during the day, only shifting neuronal rhythms when glutamate from the RHT is released on the SCN during the subjective night.[30] The Gillette laboratory then went on to establish neuronal signaling cascades responsible for phase-shifting the peak activity in the SCN.[29] However, the role of glutamate in SCN astrocytes within the retinorecipient area *in vivo* has not been well studied. Astrocytes express glutamate transporters, metabotropic glutamate receptors, and ionotropic glutamate receptors, making it likely that they not only clear glutamate but also actively respond to glutamatergic signaling.[3,80,139] Whether or not glutamate can induce structural plasticity of astrocytes within the SCN, similar to the hippocampus, is unknown.[10]

The other signal from the RHT released within the SCN is PACAP.[25,55] PACAP is a well-known neuroprotective and neurodevelopmental signal in addition to modulating photic input to the SCN.[25] PACAP is structurally very similar and exhibits similar binding affinities to receptors of other known neuropeptides of the circadian clock.[59,95] PACAP binds to PAC1 and PAC2 receptors as well as VIP receptors VPAC1 and VPAC2, although affinities are notably different from VIP.[58] SCN astrocytes express receptors to both peptides. The functional relevance of astrocytic PACAP signaling has been hinted at by a study where PACAP induced stellation of cultured SCN astrocytes while glutamate did not.[68] The mechanism by which stellation of cultured astrocytes occurs is largely unknown however it is likely GFAP phosphorylation and cytoskeleton rearrangement is crucial. The PAC receptors are G-protein coupled receptor that increase intracellular cyclic AMP (cAMP), diacylglycerol, and inositol triphosphate (IP3).[94,95] These second messengers induce a plethora of cellular and transcriptional changes including activation of the kinases PKA and PKC. Both of this kinases have been shown to phosphorylate GFAP, resulting in depolymerization or structural reorganization.[60,70] If this holds true for SCN astrocytes *in vivo*, the hypothesis that SCN astrocyte cytoskeletal complexity is more dynamic during the day due to tonic RHT release of PACAP naturally follows. Whether the activity of PACAP is influenced by the co-release of glutamate by the RHT during the day is unknown. PACAP can influence light induced phase shifts in the subjective night of rodents, so there is evidence of crosstalk between the two signaling mechanisms.[7,25,41]

Both PACAP and glutamate bind to receptors that increase second messengers known to activate kinases that phosphorylate GFAP. Moreover, phosphorylation of GFAP regulates filament polymerization and degradation. PACAP increases intracellular cAMP and diacyl glycerol (DAG). These cofactors bind to and activate cAMP dependent kinase A (PKA) and DAG dependent kinase C (PKC). When activated, PKA and PKC can phosphorylate sites within the N-terminus of GFAP filaments. Metabotropic glutamate receptors 3 and 5 (mGluR3 and mGluR5) result in increased cAMP and DAG,

respectively. Taken together, synaptic signaling from the RHT in the form of PACAP and glutamate can induce phosphorylation of GFAP through second messenger signaling. The phosphorylation of GFAP results in depolymerization and a shift from the filamentous form of GFAP to a soluble, monomeric form. This dynamic phosphorylation and structural reorganization of a well-known, astrocytic cytoskeletal protein could directly translate to changes in glial-neuron interactions and communication.

Our current study focused on the regulation of GFAP structural plasticity by released RHT signals in the acute SCN brain slice. Slice treatements of vehicle, glutamate, PACAP, or glutamate and PACAP were administered to the SCN slice at either ZT 2 or ZT 14. Our study found that the observed diurnal variation in GFAP polymerization state previously observed was lost within two hours in slice culture and that the pharmacological treatments failed to drive GFAP into the soluble state.

4.3 Materials and methods

Animals

All animal experiments were conducted according to IACUC approved protocols. Long Evans (LE/BluGill) rats were sacrificed immediately after lights were turned on (ZT0) and immediately before lights were turned off (ZT12). This way brain slices were kept in the microenvironment chamber for approximately the same amount of time. To avoid potential shifts to the SCN clock, animals were rapidly sacrificed by guillotine decapitation without anesthetic.

Tissue preparation and acute slice treatments

After brains were removed, the hypothalamus was isolated, and 500 µm thick coronal SCN slices were obtained by tissue chopper. Acute tissue slices were kept alive in controlled microenvironment chambers through constant perfusion of Hank's buffered salt solution (HBSS) and glucose. Control slices were mock treated for 10 min with vehicle. To address the role of retinal signals on astrocyte GFAP

state, SCN slices were treated with either a 20µm drop of glutamate, 20µm drop of PACAP, or a treatment drop containing both glutamate and PACAP.

Treatments were administered for 10 min starting at CT 2 or CT 14 in a static bath. Following treatment, tissue was flash frozen on dry ice. Flash frozen tissue was treated as described previously (Chapter 2).

4.4 Results

Characterization of the acute SCN brain slice

To determine the state of the astrocytic cytoskeleton within the acute SCN slice, Western blot analysis was conducted on acute SCN brain slices made while room lights were still on at either ZT0 for treatments at CT2 or ZT12 for treatments at CT14 (Figure 4.1A). GAPDH levels were not significantly different between treatments and time points (Figure 4.1C). Total GFAP levels were not significantly different between treatments and time points as well, however CT2 controls and glutamate, and CT14 glutamate and PACAP all trended towards higher levels of total GFAP (Figure 4.1D). Unexpectedly, control SCN slices lacked the significant differences previously observed where ZT2 SCN slices had higher levels of soluble GFAP compared to ZT14 (Figure 4.2A). This result suggests that within two hours within the culture chamber the SCN brain slice appears similar to enucleated animal's SCN tissue.

RHT signals fail to drive depolymerization of the GFAP cytoskeleton

To assess whether RHT signals drive filamentous GFAP into the soluble form glutamate and PACAP were administered to the acute SCN brain slice. At either CT2 or CT14 glutamate, PACAP, or glutamate and PACAP together failed to elicit depolymerization of the GFAP within the SCN slice (Figure 4.3A&B). Although not significant for either time of day or treatment, all manipulations at CT14 trended toward less soluble GFAP (4.3B). This was unexpected since we hypothesized that RHT signals drive the

previously observed diurnal changes in the intact rat at ZT2 and 14. These results taken with the observation that control slices lack the expected diurnal variation in GFAP solubility suggest that the acute SCN slice is not the optimal system to study the role of the RHT in SCN astrocytic cytoskeletal plasticity.

4.5 Discussion

GFAP structural organization is regulated by phosphorylation of the head domain within GFAP monomers, where phosphorylated GFAP dissociates into soluble GFAP. The RHT synaptic signals PACAP and glutamate are known to increase the intracellular second messengers, cAMP and DAG. Moreover, cAMP and DAG are co-activators of PKA and PKC, both of which are kinases that are known to phosphorylate GFAP (Figure 4.2). Therefore, if PACAP and glutamate are released by the RHT during the day, increasing GFAP phosphorylation and decreasing GFAP filament formation, then GFAP immunoreactivity and branching complexity will be lower during the subjective day relative to subjective night. Additionally, if RHT signals are administered to an acute brain slice preparation of the SCN, one may predict that GFAP will become phosphorylated and depolymerize. If administered during the subjective night, RHT signals could potentially increase GFAP phosphorylation, resulting in significant immunoreactivity and average astrocyte GFAP branching complexity reductions, relative to controls. Whether one would see a reduction in GFAP immunoreactivity and branching during the subjective day is not entirely predictable, considering there is constitutive RHT signaling during the day.

The SCN brain slice has been extensively characterized in regards to neuronal function. Unfortunately, very little is known about the how the astrocytes within SCN behave ex vivo. In tissue culture where the brain is sliced, astrocytic scarring appears rapidly. However, multiple imaging techniques on the brain slice culture have shown that astrocytes still function. Additionally, a recent study focused on astrocytes within the SCN showed that astrocytes in the dorsomedial or “shell” region

of the SCN still exhibit calcium oscillations in the organotypic slice. Unfortunately, the localization of the previously observed GFAP cytoskeletal dynamics is unknown.

The understanding of signaling mechanisms that regulate astrocytic cytoskeletal dynamics is a powerful tool in neurobiology. Astrocytes regulate the blood-brain barrier, clear the brain of metabolites during sleep, participate in synaptic signaling, and play pivotal roles in brain immunological function. Identifying a circadian mechanism for this regulation may translate to clinical application through identification of temporal windows for drug delivery to the brain, increasing memory and learning, regulating sleep patterns, and helping clinicians determine better treatment regimens. Unfortunately, the acute SCN slice model utilized here is likely not the best method for studying the role of RHT signaling on astrocyte cytoskeletal plasticity.

4.6 Figures

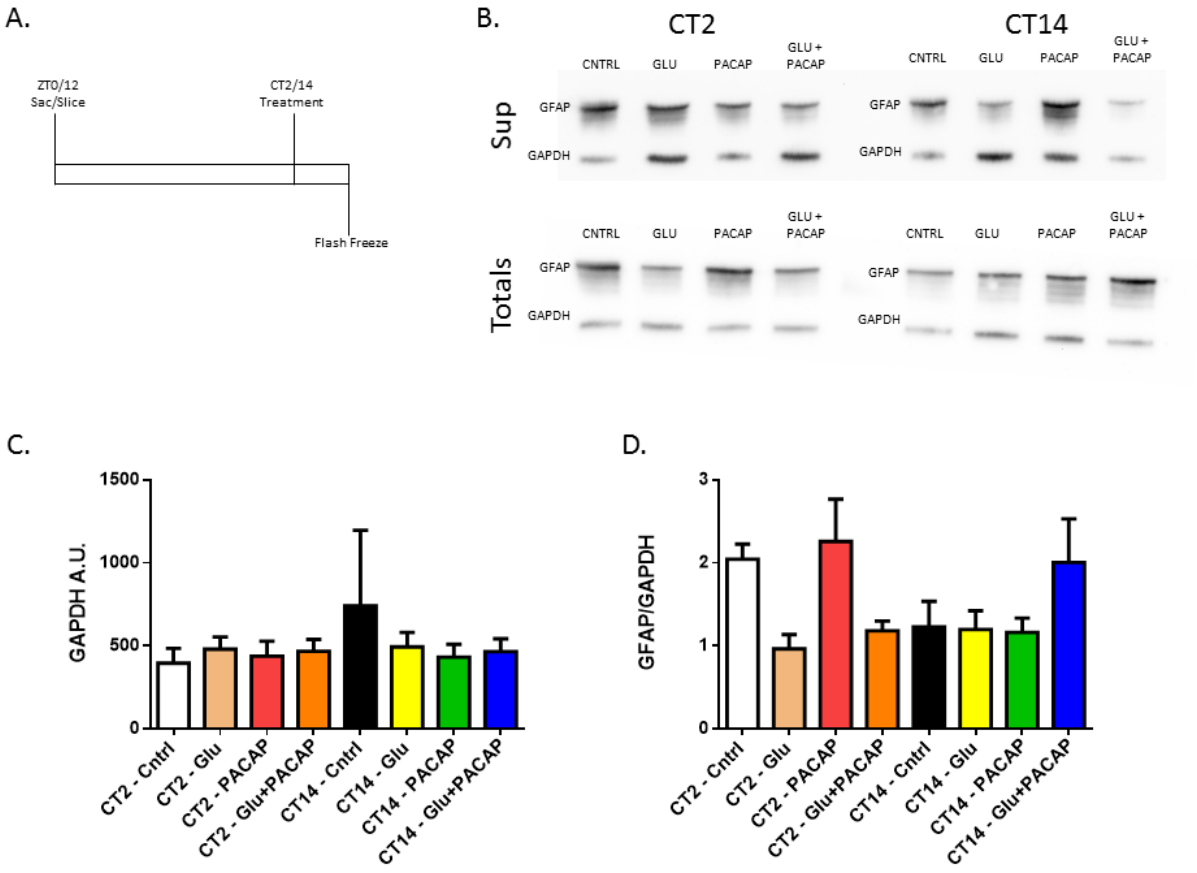

Figure 4.1. Characterization of soluble GFAP within the acute SCN slice. A) Schematic illustrating the experimental design of acute SCN slice culture. SCN slices were made at ZT0/12 and allowed to recover for 2 h. At CT2/14 slices were treated for 10 min in a standing bath. After treatment, tissue was flash frozen and analyzed using techniques described. B) Western blots of supernatant and total GFAP samples with controls and treatments at both CT2 and CT14. C) GAPDH is not significantly different as measured by Western blot probed with α-GAPDH antibody. Two way ANOVA. $p > 0.05$ n= 4 samples per time point. D) Total GFAP was not significantly different between time points and treatments. Two way ANOVA. $p > 0.05$ n= 4 samples per time point.

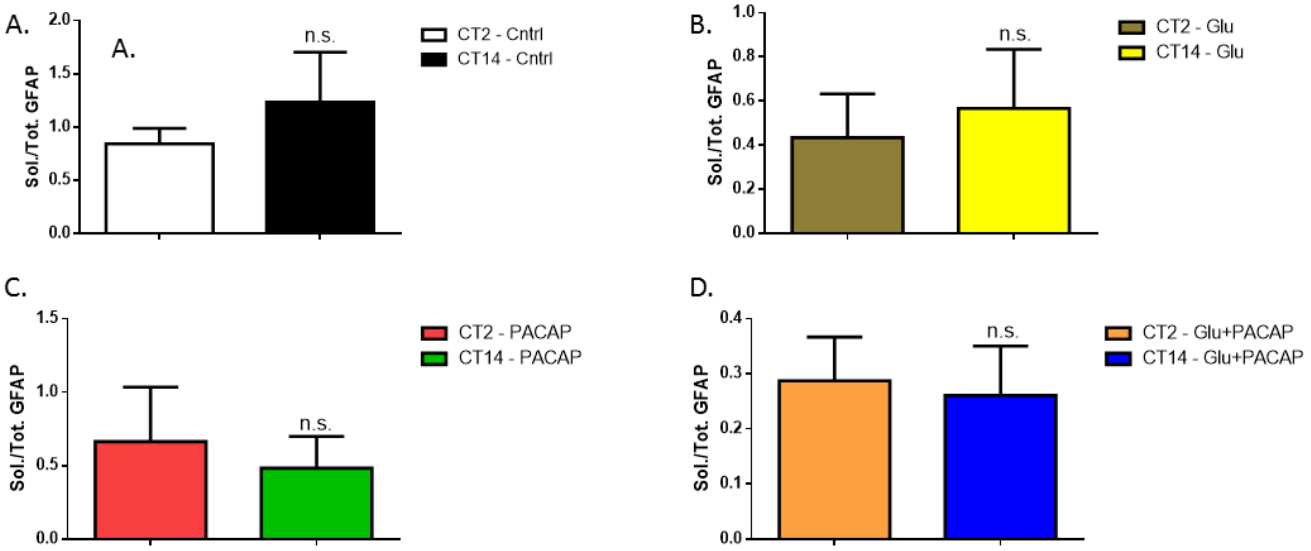

Figure 4.2. Solubility of GFAP is not significantly different within treatments at CT2 and CT14. A-D) Solubility of GFAP is not significantly different at CT2 compared to CT14 in control, glutamate treated, PACAP treated, or glutamate + PACAP treated acute SCN slices. Unpaired Student's t-test for all groups. $p > 0.05$ for all groups. n = 4 samples per treatment and per time point.

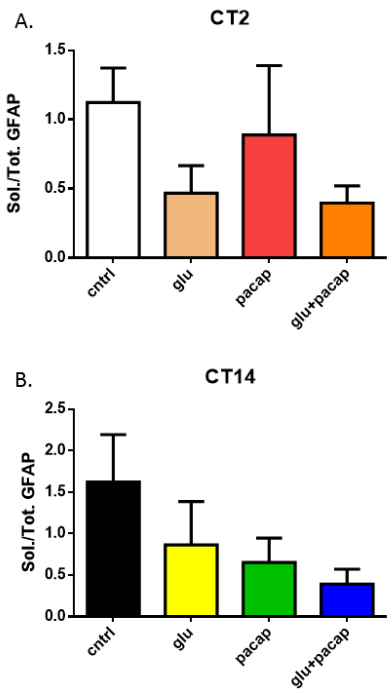

Figure 4.3. GFAP solubility is not significantly different between treatments at CT2 and CT14. A) Solubility of GFAP at CT2 was not significantly different between controls and all treatment groups. One way ANOVA. $p > 0.05$. $n = 4$ samples per treatment. B) Solubility of GFAP at CT14 was not significantly different between controls and all treatment groups. One way ANOVA. $p > 0.05$. $n = 4$ samples per treatment.

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 Abstract

Astrocytes are emerging as pivotal regulators of synaptic signaling and circuit activity. Research has shown that astrocytes can participate in brain activity through clearance of synaptic signals such as glutamate or GABA, regulation of ions critical for neuronal excitability, release of neuroactive signals known as gliotransmitters, and through morophological plasticity.[104] Despite the increasing focus on astrocytes in neurophysiology, morphological plasticity and the potential extent it plays in astrocyte function is poorly understood. Astrocytic perisynaptic endfeet change shape with neuronal activity within the hippocampus, strengthening or weakening the signal as the astrocyte moves closer or further away from the synapse.[10] Moreover, a single astrocyte can ensheath up to 10,000 synapses, all of which may be regulated through morphological movement independent of the others, suggesting an amazing level of complexity and regulation through astrocytic shape changes.[121]

Despite this attractive new field of study, we have yet to understand how astrocyte movement is actualized. Morphological plasticity is facilitated through changes in the astrocyte's cytoskeleton of which GFAP is a key intermediate filament. In addition to playing a role in overall structural stability of astrocytes, GFAP has been implicated in movement of glutamate transporters to the cell surface, and as an inflammatory marker. Moreover, studies have illustrated the marked astrocytic heterogeneity throughout the CNS, suggesting a wide variety of roles astrocytes may play.

5.2 Diurnal variation in GFAP state within the adult rat SCN

It was previously unknown whether GFAP changes between day and night or in a circadian fashion within the adult male rat SCN. Different animal models and study methods have shown conflicting results.[82,86,118] In my first aim I showed a shift in the complexity within the astrocyte population's GFAP cytoskeleton between day and night, where more cells have fewer branches in the early day compared to early night. We then showed that the overall GFAP immunostaining of the rat SCN is lower in the early day compared to early night. Lastly, we have shown that GFAP can be found in the soluble fraction of SCN punch lysates. Moreover, we have shown that there is significantly more GFAP in the soluble state in the early day compared to the early night. This suggests that the GFAP cytoskeleton is less polymerized in early day compared to early night, corroborating our previous observations in both branching complexity and overall immunostaining intensity of GFAP in the SCN.

There are two primary hypotheses that arise from the observed differences in GFAP state between early day and early night. The first states that there is circadian regulation at the level of the SCN or even individual cells, driven by the cell clock that regulates the polymerization state of the GFAP cytoskeleton. If this were the case, then this change in GFAP state should be observable in the absence of external signaling. The second hypothesis states that the observed shift in GFAP is driven by retinal signaling. The SCN receives significant retinal input in the form of glutamate and PACAP. Astrocytes express receptors to both of these signals. Moreover, the secondary messengers associated with these receptors activate PKA, PKC, and CaMKII all of which have been shown to phosphorylate GFAP.[61,69] GFAP phosphorylation depolymerizes filaments into monomers. If this hypothesis were correct then the observed shift in GFAP state would cease in the absence of retinal signaling.

There are three key experiments that still need to be completed to capitalize on these observations. First, it needs to be shown that the shift in GFAP into the soluble fraction is regulated by a

phosphorylation event. There are commercial antibodies against phosphorylated forms of GFAP. There are 4 serine residues and one tyrosine residue that are potential phosphorylation sites all of which are putative regulators of polymerization of GFAP filaments. A screen of soluble fractions of GFAP samples by the various antibodies could rapidly show which site is phosphorylated. Additionally, some GFAP residues are phosphorylated by a limited number of putative kinases, which could aid in the focus on signaling mechanisms. Second, the GFAP cytoskeleton represents a limited fraction of the total astrocyte. It needs to be clearly demonstrated to what extent the GFAP cytoskeleton associates with the cell membrane. In the hippocampus researchers have shown that the GFAP cytoskeleton represents roughly thirteen percent of the total cell volume while failing to extend into the fine perisynaptic astrocytic processes that surround the tripartite synapse.[21] It should be noted however that the SCN astrocyte is morphologically different than a hippocampal astrocyte. These differences suggest a variant relationship between the SCN astrocyte's cytoskeleton and the cell membrane. Lastly, the use of emerging technology in the objective measurement of immune-based studies need to be validated. In our study we utilized the neural reconstructive software Imarus to recreate the GFAP cytoskeleton and measure branch terminal points as a mark of cellular complexity. This experiment from the very beginning is flawed. Absolute reconstructions of immunostaining based analyses potentially suffer from transitive error, meaning that an error during the steps of sample acquisition and analysis will be compounded by further steps in analysis. Variance in staining, image acquisition, and image analysis can change the observed number of GFAP branches dramatically. Without a standard to compare to, such as a control region of the brain, changes in branching complexity due to experimental error could be virtually undetected. For this very reason, we utilized staining controls in our additional immunostaining experimental procedures to confirm that the observed variance was due to an actual change in the GFAP polymerization state rather than experimental error.

5.3 Retinal regulation of GFAP state within the rat SCN

The greatest environmental signal that an organism utilizes to assess time-of-day is the presence or absence of environmental light. The retina sends photic information to the occipital lobe for visual processing but also contains specialized cells that send light information to the SCN, the intrinsically photosensitive retinal ganglion cells. This retinal input is the largest afferent signal that the SCN receives and light information is passed to SCN neurons in the form of the excitatory amino acid glutamate and the peptide PACAP.[25,55] These signals fail to initiate a response during the subjective day in the SCN but can shift firing rhythms earlier or later, depending on when the signal is received. The extent to which astrocytes within the SCN respond to these signals is unknown but their role in retinal signal is suggested by the fact the regions of the brain that receive these signals express higher levels of GFAP relative to other brain regions.

In my first aim I showed that astrocytes exhibit diurnal variation in GFAP state between early day and early night, where early day had higher levels of soluble GFAP. To test whether this observation is dependent on the presence of environmental light signals or is maintained in a circadian manner, animals were kept in constant darkness. Animals kept in constant darkness lost the diurnal variance in GFAP solubility state and immunostaining of the SCN. To determine the importance of an intact retina on GFAP levels within the SCN, we enucleated animals for both short (3 days) or long (3 months) term durations. Short-term enucleated animals lost fluctuations in both solubility state of GFAP as well as immunofluorescence, similar to animals kept in constant darkness. Interestingly, short-term enucleated animals exhibited greatly increased levels of GFAP relative to controls and animals kept in constant darkness. Long-term enucleated animals lost diurnal variations in GFAP and also appeared to have significantly lowered GFAP compared to control regions of the hypothalamus. Moreover, long term enucleated animals had significantly atrophied optic nerves. Unfortunately, there were no samples available from long term enucleated animals for Western blot analysis. Taken together, these results show that variation in GFAP state is regulated by retinal signaling either directly through ipRGC signals

directly at receptors on astrocytes or indirectly through SCN neuronal signals released in response to retinal signaling. Enucleation results in increased levels of GFAP, like due to an inflammatory response to RHT damage.

In order to complete this study, Western blot analysis needs to be conducted on long term enucleated SCN tissue. This experiment should show to what extent an intact retina regulates the observed increase in levels of SCN GFAP compared to other brain regions. PACAP is an established driver of GFAP expression in the developing cortex. The SCN can be identified from other hypothalamic nuclei based on the elevated basal expression of GFAP and PACAP localized to the RHT synapse. Despite this information we still haven't definitively shown that PACAP is driving the elevated GFAP expression in the SCN. Moreover, what physiological benefit does elevated GFAP expression bestow on the SCN? Lastly, it is still unclear whether astrocytes respond directly to retinal signals (glutamate and PACAP) or indirectly through SCN signals released in response to retinal input.

5.4 Retinal signals regulate GFAP state in the SCN brain slice

The retinohypothalamic tract (RHT) projects from the retina to the SCN of the hypothalamus. The presence of environmental light is transmitted to SCN neurons from ipRGCs primarily in the form of glutamate and to a lesser extent, PACAP. Glutamate signaling has been characterized within the SCN where it elicits phase advances or delays during the night (depending on when the signal is given) while having no effect during the day. PACAP plays more of a modulatory role of glutamatergic responses in the SCN during the night while invoking a phase shift if administered during the day. Both glutamate and PACAP can be found within the ipRGC pre-synaptic bouton, suggesting that both are released in response to light hitting the retina. In the first two aims of my thesis I have shown that diurnal variances of GFAP state exist between early day and early night and that this variance is dependent on retinal

signals. In my second aim, I identified light signaling and the presence of an intact optic nerve as key regulators of diurnal GFAP cytoskeleton plasticity.

In chapter 4 we originally intended to study which signals from the optic nerve could shift GFAP polymerization state. To study how SCN astrocyte GFAP state is regulated by retinal signals, we utilized the acute SCN slice model to directly treat SCN astrocytes with glutamate and PACAP. Unexpectedly, we found that SCN astrocytes within control slices lose their diurnal variation in GFAP solubility within two hours. This result suggests that the acute tissue slice is in a state that may or may not represent the intact signaling observed in chapters 2 and 3. These results were frustrating considering the significant body of literature showing the SCN continues to oscillate within the culture chamber. Moreover, Brancacchio et al. recently showed that astrocytic calcium signaling in the "shell" region of the SCN also express circadian calcium fluctuations in organotypic culture. Despite the ability of the SCN brain slice to maintain neuronal and transcriptional circadian rhythms, our results suggest that the GFAP cytoskeleton and likely astrocyte morphology rhythms are not intrinsic and require external stimuli, namely light, to maintain the previously observed rhythm. Our original thrust was to study how retinal signals would drive GFAP into the soluble or filamentous state. At both time points, results were not significant with the four repeats although there seems to be a strong trend towards less soluble GFAP in the early night with treatment of glutamate, PACAP, or both. Future experiments need to be conducted to ascertain the effect of creating the brain slice on the state of astrocytes and the GFAP cytoskeleton. Once the astrocytes within the slice have been characterized, we can begin to manipulate astrocytic receptors and intracellular signals in response to glutamate and PACAP to determine the mechanism of GFAP polymerization state regulation.

In conclusion, the work within this thesis clearly shows that there are diurnal shifts in GFAP state between early day and early night. First, we showed that the GFAP cytoskeleton of individual astrocytes can be reconstructed while variability between astrocytes make computer-based reconstructions a

difficult method to reproduce. However, the extent of morphological heterogeneity, even within the same brain region may have physiological significance. Second, we show that the observed changes in GFAP are likely due to a shift in GFAP polymerization state and not as a result of fluctuations in total GFAP. Third, we clearly establish that this cytoskeletal dynamic is the result of RHT signaling and not a cell clock phenomenon. Lastly, in order to identify the regulator of SCN astrocyte cytoskeletal dynamics we unexpectedly establish that the brain slice culture is not a good model for this analysis. Clearly, work still needs to be conducted to flush out the physiological importance of astrocytic morphological dynamics within the SCN. As it stands, we propose the following model describing our current understanding of how retinal signals regulate diurnal astrocytic morphological dynamics within the adult male SCN.

5.5 Figures

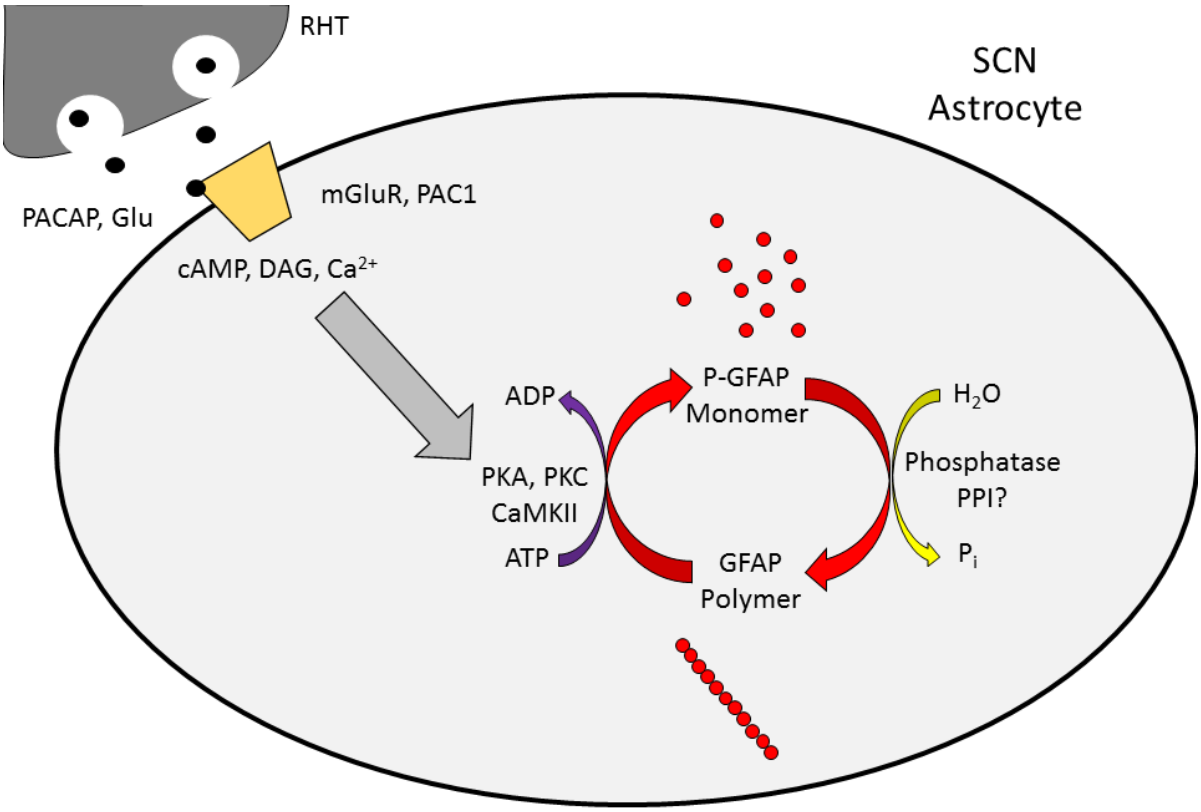

Figure 5.1. Proposed mechanism for GFAP changes in response to light signaling. RHT cells release PACAP and glutamate onto the SCN, which signals to both neurons and astrocytes. Astrocytes express receptors to both signals, which increase the intracellular second messengers cAMP, DAG, and Ca2+. These second messengers activate kinases known to phosphorylate GFAP (PKA, PKC, and CaMKII). These kinases phosphorylate GFAP, which depolymerizes the filaments and protects the protein from degradation. During the night, when light signals are absent from the SCN, an unknown phosphatase (hypothesized protein phosphatase 1) dephosphorlylates GFAP, allowing for repolymerization. Thus, light signaling is proposed to create the diurnal differences observed in GFAP complexity between day and night as well as the significant decrease in GFAP complexity and overall protein levels in the SCN in enucleated animals.

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