

CIRCADIAN RHYTHMICITY OF RAT NEUROENDOTHELIAL CELLS AT THE BLOOD-
BRAIN INTERFACE

BY

AMANDA C WEISS

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Molecular and Integrative Physiology
in the Graduate College of the
University of Illinois Urbana-Champaign, 2024

Urbana, Illinois

Doctoral Committee:

Professor Martha Gillette, Chair
Professor Hyun Joon Kong
Associate Professor Hee Jung Chung
Professor Jonathan Sweedler

ABSTRACT

The Blood-Brain Interface (BBI) facilitates neurological protection from pathogens, the removal of waste products, brain homeostasis, and the permeation of nutrients and drugs. Its dysfunction contributes to the onset of a variety of neurological diseases, including strokes and dementia. Research has shown that both BBI permeability and human susceptibility to strokes fluctuate according to time of day. However, more in vitro studies are required to isolate the effects of circadian rhythms from the effects of sleep and other confounding factors on BBI permeability. The role of neuroendothelial cells, a key component of the BBI, has also not been fully elucidated. We have determined that barrier tightness and permeability oscillate according to time of day in rat neuroendothelial cells. These findings extended to permeability to the blood clotting factor fibrinogen. We also determined that the addition of Carbachol, a pan-acetylcholine receptor agonist, could shift these rhythms.

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CHAPTER 1: INTRODUCTION

Background and Significance

The Blood-Brain Interface (BBI) provides protection from toxic compounds and pathogens in the blood, while allowing the brain to remove waste such as excess glutamate or amyloid-beta plaques¹. It also facilitates neurological homeostasis by protecting the brain from fluctuations of non-toxic compounds in blood, such as glucose¹. Heightened permeability of the BBI can cause strokes, neurodegeneration, and worsened pathology in dementia and strokes². This is because when the BBI becomes excessively permeable, blood can leak into the interstitial areas around neurons and glia². This can result in microhemorrhages and hemorrhagic strokes². Furthermore, the accumulation of fibrin and other blood-derived factors can cause inflammation and cellular damage². Heightened BBI permeability is a particularly pronounced threat for people who are older, have metabolic diseases, or have undergone traumatic brain injury².

The BBI is formed by the contact between neuroendothelial cells of blood vessels and the end feet of astrocytes¹. Small, non-polar chemicals can diffuse through passively, but many compounds traverse only through transporters with selective permeability¹. Permeability to specific compounds can be regulated by the surface expression of these transporters¹, whereas overall BBI permeability is regulated by tight junctions and other cell adhesion complexes³.

Circadian rhythms work to synchronize the activity of cells throughout the body. Recent research has demonstrated that this extends to the BBI, where permeability fluctuated according to time of day⁴⁻⁵. Circadian rhythms regulate a variety of animal behaviors and functions, including sleeping and digestion⁶. They also regulate a variety of hormones and metabolic activities from the level of the cell to the level of the organism⁷⁻¹⁰. Many of the functions known to be under circadian control have demonstrated the ability to influence BBI permeability¹¹⁻¹². However, further research is required to determine how and why BBI permeability changes with time-of-day.

Circadian rhythms are highly conserved amongst different species, suggesting that they have had a high degree of evolutionary importance¹³. All invertebrates share the same clock genes: *Cycle*, *Clock*, *Tim*, and *Per*. Likewise, the homologous mammalian clock genes are shared by all mammals and are, respectively, *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, *Per2*, and *Per3*. Since the invertebrate and mammalian clock genes are homologs of each other, each invertebrate clock gene has a mammalian equivalent with the same role. In fact, it has been shown that circadian rhythmicity in a *Per1* knockout fly could be restored by the substitution of mouse *Per1* and mouse *Per2*¹³. In addition, plants¹⁴, bacteria¹⁵ and fungi¹⁶ express their own sets of clock genes and exhibit circadian rhythms. This high degree of evolutionary conservation of clock genes suggests that circadian rhythms are important for survival and evolutionary fitness.

It is not fully understood why circadian rhythms have this high degree of evolutionary conservation. However, it is well established that they coordinate various behaviors and biological functions with the time of day. As a result, an animal's circadian rhythm predisposes it to a nocturnal or diurnal behavioral pattern. Both nocturnal and diurnal animals display similar patterns of neural activity in the brain region that primarily coordinates circadian rhythms, the Suprachiasmatic Nucleus (SCN). However, the way that these outputs are coupled to other brain areas and other parts of the body differs¹⁷. This results in a general inverse relationship between the circadian rhythmicity of biological processes for nocturnal and diurnal animals. Compared with diurnal animals, nocturnal animals have typically evolved night vision, which confers an advantage for hunting or escaping predators in darkness. In a similar manner, plant behavior is synchronized with daily sunlight patterns. Flowers open more during the day and close more at night due to the cyclic expression of Auxin, which is regulated by plant clock genes¹⁴. In both plants and animals, circadian rhythms promote the synchronization of behavioral patterns with the time of day where those behaviors may be more conducive to survival. In keeping with this idea. Research has demonstrated that BBI permeability peaks

when animals are most active⁴. Researchers have speculated that this may serve the purpose of allowing the brain to remove toxins more readily during the times of day when they are generated in the greatest amounts⁴.

Circadian rhythms provide a variety of daily cues that promote changes in sleep and wakefulness according to time of day. This includes changes in neural activity¹⁸, metabolism^{6,10}, cortisol levels⁷, and insulin secretion⁸⁻⁹, but one of the most influential cues is melatonin. When the SCN detects a decrease in light, it stimulates the pineal gland of the brain to secrete melatonin, resulting in melatonin secretion at night¹⁰. Its secretion is also inhibited by light¹⁹. Research has demonstrated that of all the known cues that can adjust circadian rhythms (known as Zeitgebers), light tends to be the most influential²⁰, followed by melatonin²¹. Together, light and melatonin secretion help coordinate sleep with the time of day. Research has also shown that they play a role in circadian disruption,²² which occurs when circadian rhythms are not properly in sync with the environment.

There are various other factors that contribute to circadian rhythms which may also contribute to the rhythmicity of BBI permeability, including metabolism. Another key function of circadian rhythms is their ability to coordinate metabolic processes with time-of-day. This includes macroscopic processes, such as digestion, as well as cellular metabolism. Studies have shown that insulin, which stimulates glycolysis and lipogenesis, while inhibiting glucose production, fluctuates with the day-night cycle⁸⁻⁹. Likewise, research has shown a daily rhythm for levels of cortisol²³, which serves many functions, including the generation of glucose. Research has found that it peaks around waking²⁴. This occurs around sunrise for diurnal animals and around sunset for nocturnal animals²⁴. In addition, for both nocturnal and diurnal animals, studies have found that body temperatures tend to be lower at times of day which are more conducive to sleep²⁵. Studies have also shown that circadian rhythms influence the expression of genes required to degrade toxins in the mouse liver²⁶. One study found that electrolyte absorption in rats was under circadian control and mediated by changes in ion

channel localization²⁷. It has also been shown that cell differentiation in mice changes with time of day and that this effect is mediated by clock genes²⁸. Overall, circadian rhythms work to increase metabolic rates during times that are more conducive to wakefulness and decrease them, thus conserving energy, at times more conducive to sleep. This may affect BBI permeability, as research has shown that metabolic overload can weaken BBI integrity¹¹.

Circadian disruption and studies involving clock gene mutants have provided further insight into the importance of circadian rhythms. Animal models of circadian disruption and circadian knockout models have implemented altered light-dark cycles, clock gene mutations, and SCN lesions²⁹. By contrast, most human studies have featured alterations in sleep-wake cycles and feeding schedules²⁹. In human studies, circadian disruption has been implicated in metabolic disorders, such as obesity and Diabetes Mellitus³⁰. Studies have also shown that it plays a role in cardiovascular diseases³¹. Similarly, research has shown that circadian disruption can disrupt BBI integrity³². Research also supports the possibility that many of the adverse effects of circadian disruption may be mediated by BBI disruption.

Studies have shown that circadian disruption may contribute to cancer development. For instance, in a chronic jetlag mouse model, circadian disruption was associated with an increase in the number of liver tumors³³. Another study found that mice lacking functional genes for *Per1*, *Per2*, *Cry1*, *Cry2*, or *Bmal1* had more tumors, including spontaneously occurring ones³⁴. Human studies have demonstrated epidemiological links between incidences of circadian disruption and cancer, although they do not prove causation. There are, however, several factors which may cause circadian disruption to contribute to cancer progression. This may be caused in part by the circadian regulation of cell proliferation²⁸. Another possible explanation is increased inflammation due to circadian disruption. Elevated levels of pro-inflammatory cytokines have been found in human patients with a naturally occurring mutation in the Period 3 clock gene¹². In addition, research has shown that clock genes regulate DNA repair³⁵. Research has also demonstrated that circadian disruption in mice decreases the activity of the pro-apoptotic protein

p53³³. Inflammation and apoptosis can contribute to BBI disruption³⁶⁻³⁷. In addition, research has demonstrated that the ensuing BBI disruption may worsen inflammation and apoptosis³⁸. This implies a role for BBI disruption in mediating the heightened risk of cancer that accompanies circadian disruption.

One misconception of circadian rhythms is that their sole purpose is to ensure that animals receive adequate amounts of sleep. However, it has also been shown that circadian disruption can exert its effects independently of sleep deprivation. In fact, one study found that one chief effect of circadian disruption was a decrease in sleep quality. Phillips *et al.* (2015) demonstrated this when they modeled circadian disruption by housing the mice in 10 h of light and 10 h of darkness for a 20 h light-dark cycle³⁹. They found that instead of sleep deprivation, the mice were trending towards spending more time sleeping, likely due to a loss in quality of sleep³⁹. Unlike the control group, the mice in the circadian disruption model did not display significant day-night differences in the portions of time spent awake³⁹, in REM sleep³⁹, or in non-REM sleep³⁹. The mice in the circadian disruption group also experienced significantly more transitions between wakefulness and non-REM sleep³⁹. Since deep sleep and REM sleep are more restorative than the other sleep stages, the lack of time the mice spent in these stages signified a decline in sleep quality. This was also indicated by the increased number of awakenings. Research has shown that a decline in sleep quality can result in BBI disruption⁴⁰. This may explain the finding that circadian disruption can contribute to BBI disruption³².

There are several known avenues through which circadian rhythms may promote enhanced sleep quality. Many of the factors regulated by circadian rhythms influence sleep and wakefulness. For instance, circadian rhythms regulate melatonin secretion, which is influential in determining when animals sleep⁴¹⁻⁴². Circadian rhythms also regulate cortisol levels⁷, insulin secretion⁸⁻⁹, and neural activity¹⁸, all of which have been shown to influence animals' predispositions to sleep⁴³⁻⁴⁵. By synchronizing cues for sleep for one time of day and synchronizing cues for wakefulness with a different time, circadian rhythms may promote better

sleep quality. During circadian disruption, animals may simultaneously experience cues for sleep and wakefulness. While this lack of synchronization may not prevent them from sleeping, it may be responsible for the losses in sleep quality. For instance, one clinical trial involved the use of Dexamethasone, a glucocorticoid receptor agonist⁴⁶. They found when administered at nighttime, it led to changes in sleep stages which may have decreased sleep quality⁴⁶. More specifically, while the subjects were sleeping, they spent less of that time in the slow-wave and Rapid Eye Movement (REM) stages⁴⁶. Research has demonstrated that a decline in sleep quality can contribute to increased accumulation of Reactive Oxygen Species (ROS)⁴⁷. This may, at least in part, explain the finding that reduced sleep quality can reduce BBI integrity⁴⁰.

Circadian disruption and impairments in sleep quality are of particular concern for older populations. In a study of over 9,000 participants, 28% of elderly people reported symptoms of insomnia, and 12.5% reported that they did not typically feel well-rested upon waking⁴⁸. It has also been shown that the portion of sleep spent in slow-wave sleep, the deepest sleep stage, decreases for humans as age increases⁴⁹. Since slow-wave sleep is considered the most biologically restorative sleep stage⁵⁰, its decline may contribute to age-related sleep impairments.

Several factors have been discovered which may contribute to insomnia and decreased sleep quality amongst the elderly population. For instance, one study found that *Per1* and *Per2* expression in the cortex was significantly lower in people older than 60, compared with adults younger than 40⁵¹. The older population also displayed a phase advance in *Per1* and *Per2* expression of approximately 5 h, as well as a loss of rhythmicity in *Cry1* expression⁵¹. In addition, human studies have demonstrated that melatonin secretion tends to decrease as age increases in adults⁵². It has also been shown that cortisol levels are significantly lower in elderly populations⁵³. Research has not yet investigated whether these factors may contribute to BBI dysfunction in older populations. Studies have also yet to determine whether BBI dysfunction may contribute to any of these problems.

Research supports the possibility that when circadian disruption occurs, it may cause BBI disruption. This BBI disruption may, in turn, exacerbate the effects of circadian disruption. For instance, studies have found that circadian disruption may increase susceptibility to cancer³³⁻³⁴. This effect may be mediated by circadian disruption increasing inflammation and apoptosis^{12,33}, both of which can contribute to BBI disruption³⁶⁻³⁷. Then, this BBI disruption may worsen inflammation and apoptosis³⁸. Thus, BBI disruption may contribute to the increased susceptibility to cancer which may result from circadian disruption. Research has also demonstrated that circadian disruption can contribute to BBI disruption³², and that this effect may be mediated by a decline in sleep quality⁴⁰. Taken together, these findings demonstrate that proper functioning circadian rhythms are necessary for proper BBI functioning. These findings also demonstrate that BBI disruption, at least in part, mediates circadian disruption.

Circadian fluctuations in BBI permeability have several implications for human health. It has been shown that susceptibility to strokes in humans varies by time of day², and circadian fluctuations in BBI permeability may contribute to this pattern. Heightened BBI permeability has also been implicated in hypertension, epilepsy, Alzheimer's Disease and other dementias⁵⁴⁻⁵⁵. In addition, BBI permeability influences the ability of drugs to enter the brain, and an understanding of its circadian rhythmicity can be used to optimize drug delivery. Moreover, it also may be more beneficial for patients to take medications which promote BBI tightness at certain times of day.

New research has demonstrated that BBI permeability fluctuates according to circadian rhythmicity⁴⁻⁵. This has been established *in vitro* for mouse⁴ and human cells⁴. This finding has also been replicated *in vivo* in flies⁵ and mice⁵. Together, these studies have discovered a 24-h pattern, whereby peak BBI permeability occurs during the day for diurnal animals and during the night for nocturnal animals⁴⁻⁵. In both of these studies, the genetic knock-out of circadian clock genes was able to abolish this 24-h pattern, demonstrating that it is under circadian control⁴⁻⁵.

Summary of this Study

Previous research has demonstrated that BBI permeability fluctuates with circadian rhythmicity *in vivo* and *in vitro*⁴⁻⁵. However, the published studies in this new area of research have yet to address several key questions. For instance, this limited number of published studies has not yet examined intercellular permeability, which is more relevant for drug delivery than intracellular permeability⁵⁶. There has also been a lack of *in vitro* studies parsing out the role of neuroendothelial cells, as well as disentangling the influences of circadian rhythms from other factors. Studying circadian rhythms *in vitro* requires synchronizing the individual circadian rhythms of cells. One way to accomplish this is by activating glucocorticoid receptors with Dexamethasone⁵⁷. However, no previous research has featured the use of Dexamethasone to synchronize neuroendothelial cells, a key component of the BBI. Another common technique used in circadian rhythm research is the ability to induce phase shifting of the circadian rhythmicity. Previous studies have not attempted this for BBI permeability, nor have they examined the potential of acetylcholine (ACh) receptors in initiating this phase shift. ACh is one of the key neurotransmitters in neuroendothelial cells, given its roles in blood vessel constriction and contraction in response to sympathetic and parasympathetic stimulation⁵⁸⁻⁶¹.

The work presented here has been the first to examine the circadian rhythmicity of BBI permeability in monolayers of neuroendothelial cells *in vitro*. As an *in vitro* study, it has not been confounded by the *in vivo* influences of sleep and locomotor activity, which also exhibit rhythmic diurnal fluctuations. It is the first *in vitro* study to examine the influence of circadian rhythmicity on intercellular, rather than only intracellular, permeability. The study presented here is also one of the first studies to measure general permeability of the BBI, rather than permeability to a specific compound, in the context of circadian rhythms. It is also the first study to examine the ability of an acetylcholine agonist, Carbachol, to induce a phase shift in BBI permeability.

The work presented here has provided a more complete picture of the circadian rhythmicity of BBI permeability. It has been the first study to examine the roles of

neuroendothelial cells, intercellular permeability, phase-shifting, and acetylcholine agonism in this context. Neuroendothelial cells form a substantial portion of the BBI. Moreover, neuroendothelial-cell permeability has particular significance in the context of strokes. One of the two main types of strokes, hemorrhagic ischemia, is caused by leakage of blood into the brain. The work presented here provides insight into how circadian rhythmicity influences neuroendothelial cell permeability, as well as which times of day this permeability exhibits peaks and troughs. In addition, intercellular interactions influence a substantial portion of BBI permeability. They are particularly relevant for the delivery of therapeutic drugs, most of which rely on intercellular transport to enter the brain⁵⁶. Moreover, the use of a circadian phase-resetting cue to induce a phase shift in BBI permeability was a novel approach to demonstrate its susceptibility to circadian influence. Phase-resetting cues also have notable influences on circadian disruption. When administered at inopportune times, they can contribute to circadian disruption. However, they also have therapeutic potential in treating this condition.

I have tested the overarching hypothesis that permeability of neuroendothelial cell monolayers fluctuates according to time of day and is susceptible to Zeitgeber-induced phase shifts. I isolated and cultured primary neuroendothelial cells from the rat cerebral cortex. Then, I synchronized the circadian rhythms of the individual cells with the addition of the glucocorticoid receptor agonist, Dexamethasone. For Aim I, I performed qPCR for circadian-clock genes to assess the successful synchronization of the endothelial cells' circadian rhythms. For Aim II, I performed assays to measure permeability and barrier tightness of the neuroendothelial cell monolayers. Then, for Aim III, I administered Carbachol, an acetylcholine (ACh) agonist and proven cue for shifting circadian rhythms⁶². Through these aims, I examine the circadian rhythmicity of neuroendothelial cell permeability and barrier tightness, as well the ability of these patterns to undergo a circadian phase shift. The results of these studies comprise my doctoral thesis.

Specific Aims

Permeability of the Blood-Brain Interface (BBI) has been implicated in a variety of diseases. As a result, its susceptibility to circadian changes has many potential ramifications for health. It also may be more beneficial for patients to take medications at times of day when BBI permeability is greatest. Prior to conducting the work presented here, the circadian rhythmicity of BBI permeability had not been determined in neuroendothelial cells, a key component of the BBI. I have hypothesized that permeability of neuroendothelial cell monolayers fluctuates according to time of day and is susceptible to circadian phase shifts. Several aspects of this study had never been performed in neuroendothelial cells. This is the first study to examine the circadian rhythmicity of BBI permeability across monolayers, thus factoring in cell-cell interactions *in vitro*. One additional novel factor is examining the influence of a cue that could shift circadian rhythms.

Aim I: Determine circadian rhythmicity of primary rat neuroendothelial cortical cell culture.

Cells cultured *in vitro* in the absence of the suprachiasmatic nucleus (SCN), the brain region which primarily coordinates circadian rhythms, require a treatment with an agent that has the ability to synchronize the circadian rhythm of each cell. Treatment with Dexamethasone, a glucocorticoid receptor agonist, is a well-established method for circadian synchronization. However, few studies have featured Dexamethasone synchronization in endothelial cells. Moreover, this treatment has never been performed in neuroendothelial cells. In this aim, I isolate primary neuroendothelial cells from the rat cerebral cortex and administer Dexamethasone to synchronize their circadian rhythms. Then, I perform qPCR for the key clock genes *Per1* and *Bmal1* at 4-h intervals for 4 days. I hypothesize that the clock-gene expression exhibits circadian rhythmicity in the neuroendothelial tissue treated with Dexamethasone but not in the Sham-treated control group.

Aim II: Evaluate the permeability of an *in vitro* BBI on Transwells across the circadian cycle.

In vitro circadian studies of BBI permeability offer the advantage of isolating circadian rhythms from variables, such as sleep. The study presented here is the first to examine the circadian rhythmicity of intercellular BBI permeability of neuroendothelial cells. For this aim, I culture neuroendothelial cells in monolayers in a Transwell model of the BBI, consisting of a semipermeable membrane forming 2 compartments. I perform Dexamethasone synchronization, as described in Aim I. Then I perform Transepithelial/Transendothelial Electrical Resistance (TEER) assays to quantify barrier tightness. I also quantify permeability of the monolayer to fluorescent microspheres and fluorescent fibrinogen. I hypothesize that permeability of the neuroendothelial cell monolayer fluctuates according to time of day.

Aim III: Evaluate the ability of a cholinergic agonist to induce phase shifts in neuroendothelial cell permeability.

Knowing the impacts of phase-shifting cues on BBI permeability can help establish its susceptibility to circadian rhythm changes. Acetylcholine (ACh) is the primary neurotransmitter in the parasympathetic nervous system, and it exerts various effects on endothelial cells. The pan-ACh agonist, Carbachol, is a phase-shifting cue which had never been studied in this context in neuroendothelial cells prior to my research. For this aim, I treat neuroendothelial cells with Carbachol and subsequently conduct TEER assays, fluorescent-microsphere assays, and fluorescent-fibrinogen assays at 4-h intervals. I hypothesize that Carbachol treatment 14 h post-synchronization will produce a phase delay in BBI permeability and that Carbachol treatment at 20 h will produce a phase advance. I also test the influences of antagonists for nicotinic and muscarinic ACh receptors on permeability, barrier tightness, and the expression of *Per1* and *Bmal1*.

Figures

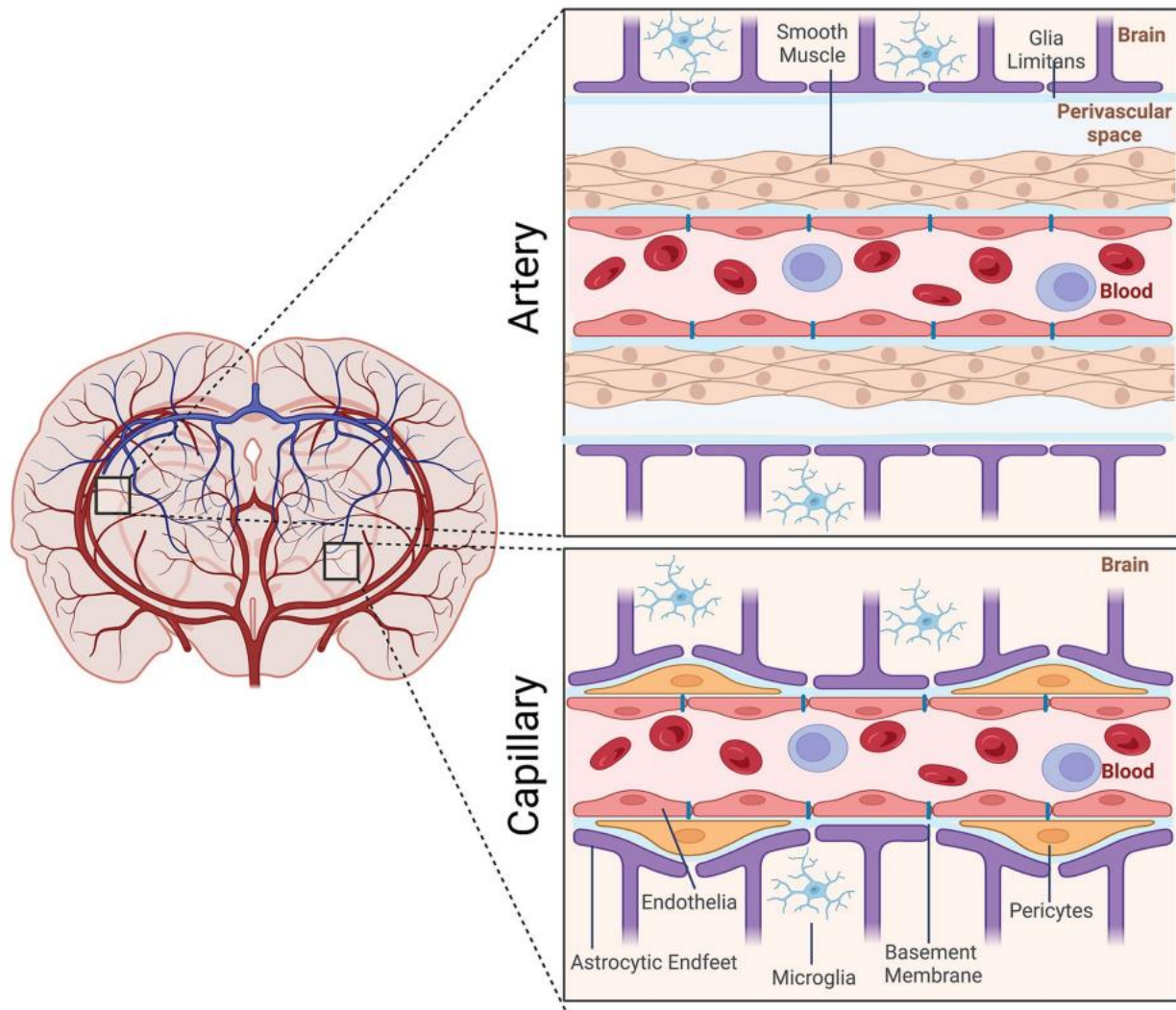


Figure 1: The BBB is comprised of multiple cell types which interact with each other. The BBB is formed throughout the brain. The blood vessels within the brain parenchyma are comprised of neuroendothelial cells and lined with pericytes. Astrocytes interface with the neuroendothelium, and neurons and microglia interface with the astrocytes.

*This figure is from Mitchell and Gillette (2023)⁶³.

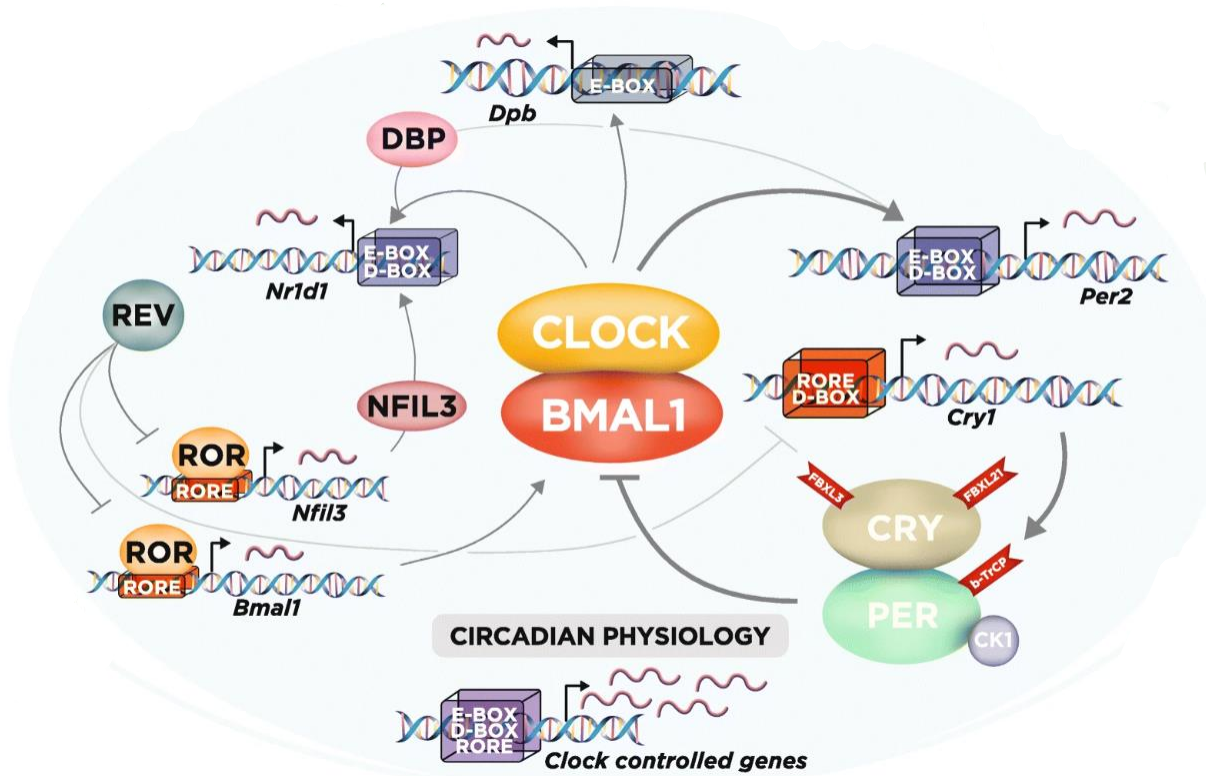


Figure 2: Clock gene expression involves positive and negative feedback.

Various clock genes work in concert to create a transcriptional-translational loop of approximately 24 h. In mammals, this happens primarily by CLOCK and BMAL1 initiating the transcription of *Per* and *Cry* genes. The PER and CRY protein products then inhibit *Clock* and *Bmal1* expression.

*This figure is from Rijo-Ferreira & Takahashi (2019)⁶⁴.

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CHAPTER 2: CIRCADIAN RHYTHMICITY OF PRIMARY RAT NEUROENDOTHELIAL CORTICAL CELLS

Abstract

Circadian rhythms emerge from cellular processes, from cycles of transcription and translation each of which takes approximately 24 h. When cells are cultured *in vitro*, they retain their individual circadian rhythms; however, they are not synchronized to each other. Individual circadian rhythms of *in vitro* cells can be synchronized by the administration of Dexamethasone, a glucocorticoid receptor agonist that binds to Glucocorticoid Response Elements in clock gene promoters. We isolated primary neuroendothelial cells from the rat cerebral cortex and synchronized them with Dexamethasone. We then examined its effects on the expression of the key circadian clock genes *Per1* and *Bmal1*. We found that with Dexamethasone synchronization, significant oscillations in *Per1* and *Bmal1* expression with a near-24-h rhythm. This established the time course of *Per1* and *Bmal1* expression in rat neuroendothelial cells after Dexamethasone synchronization. This may serve as a reference for future experiments by relating different time points after Dexamethasone synchronization to the time course of clock gene expression.

Introduction

At the cellular level, circadian rhythms are orchestrated by a cycle of transcription and translation that takes approximately 24 h. This cycle has two main components: positive regulators and negative regulators. The positive regulators, such as Brain and Muscle Arnt-Like protein-1 (*Bmal1*), initiate transcription of the negative regulators, such as Period 1 (*Per1*). The negative regulators inhibit expression of the positive regulators, and they also inhibit their own transcription. This leads to a point in the cycle where the expression of the negative regulators is at a low, allowing for uninhibited expression of the positive regulators. The cycle then continues, as the positive regulators re-initiate transcription of the negative regulators, causing the levels of positive regulators to decrease¹.

The suprachiasmatic nucleus (SCN) in the brain is deemed the master clock, synchronizing the circadian rhythms of cells throughout the body. It is well established that in the absence of a functional SCN, circadian rhythms of tissues are abolished²⁻⁴. Thus, individual cells cultured *in vitro* without an SCN do not display circadian rhythms which are synchronized to each other. Rather, they have individual circadian rhythms that are unsynchronized with each other.

It is possible to synchronize the circadian rhythms of individual cells cultured *in vitro* by adding cues which can mimic the *in vivo* signals from the SCN. One such cue is Dexamethasone, a glucocorticoid receptor agonist which has been shown to act as a signal for day-like patterns. Research has demonstrated that Dexamethasone administration may significantly increase expression of *Per1*⁵ as well as shift circadian rhythms⁵⁻⁶. Treatment with Dexamethasone has become one of the most common methods of *in vitro* synchronization of circadian rhythms^{5,7-8}.

Glucocorticoids get their name from their actions on glucose, synthesis in the adrenal cortex, and steroidal nature⁹. Their main endogenous agonist is cortisol, which is necessary for survival⁹. As a hormone, it can spread rapidly throughout the body, and glucocorticoid receptors are found in almost every cell type⁹. One of the primary functions of cortisol is understood to be the synchronization of circadian rhythms throughout the body⁹. It also helps synchronize the body's response to various stressors, such as psychological stress, glucose deprivation, or an over-active immune system⁹. Glucocorticoid receptors exert their main functions through their roles as transcription factors. When activated, they translocate to the nucleus, where they can activate or repress transcription⁹. They also bind to other transcription factors, either stimulating or inhibiting their activity⁹. Glucocorticoid agonists, such as Dexamethasone, bind to Glucocorticoid Response Elements in clock gene promoters⁹. In the central nervous system, glucocorticoids including cortisol are primarily excitatory and stimulate norepinephrine

secretion⁹. In diurnal animals, cortisol levels peak during the day and reach their lowest levels at night⁹. For nocturnal animals, the opposite is true, with cortisol levels peaking at night⁹.

In addition to Dexamethasone, a common method for circadian synchronization is a serum shock. During a serum shock, cells are first cultured in media containing low concentrations of serum. They are then exposed to high concentrations of serum. Cell-culture serums are rife with proteins as well as growth factors and hormones. One drawback of a serum shock is that it may induce apoptosis during the serum starvation phase. Serum starvation is a standard method for inducing apoptosis¹⁰, oxidative stress¹¹, and protein degradation¹². Another disadvantage of serum shocks is the general lack of transparency from the companies regarding the contents of the serum. The specific formulations of commercially purchased serums also vary from one lot to another.

Review of Previous Literature

Studies have demonstrated that treatment with Dexamethasone can synchronize the circadian rhythms of cells cultured *in vitro* in the absence of an SCN¹³. Dexamethasone's ability to reset circadian rhythms has also been evidenced by its ability to act as a Zeitgeber¹³⁻¹⁴. In addition, cells cultured *in vitro* after Dexamethasone synchronization have demonstrated patterns of clock gene expression similar to that observed *in vivo*. The moment of its administration, Zeitgeber Time 0 (ZT 0), is considered most similar to Circadian Time 0 (CT 0) or dawn¹³⁻¹⁶. These effects are mediated through glucocorticoid receptors and Dexamethasone treatment may increase *Per1* expression in rat peripheral tissues¹³. Dexamethasone synchronization has been utilized in a multitude of studies, and it has been performed in a variety of cell types, including endothelial cells¹⁵⁻¹⁶.

Even in the absence of SCN cells, peripheral cells cultured *in vitro* can be treated to synchronize their circadian rhythms^{13,15-17}. In 2000, Balsalobre *et al.* found that rat fibroblasts treated with Dexamethasone displayed circadian rhythmicity of clock gene expression¹³. By

contrast, this apparent circadian synchronization was absent in the vehicle control group¹³. These findings have been replicated in rodent brain explants⁵ and endothelial cells¹⁵⁻¹⁶.

Research supports the possibility that Dexamethasone treatment increases the expression of *Per* clock genes¹³⁻¹⁴. Balsalobre *et al.* (2000) found that in rat fibroblasts, a 1-h Dexamethasone treatment appeared to increase *Per1* expression¹³. The researchers also found that Dexamethasone treatment could induce a shift in the phasing of the oscillation in clock gene expression in the rat liver, kidney, and heart¹³. However, they found that for SCN neurons, Dexamethasone was not able to induce a phase shift¹³. These findings indicate that in tissues outside of the SCN, a glucocorticoid receptor agonist may serve as a Zeitgeber; however, this effect does not extend to the SCN. This difference may be explained by their finding that glucocorticoid receptors are expressed in minute levels in the SCN¹³. In keeping with this explanation, they found that in mice with genetically disrupted glucocorticoid receptors, Dexamethasone treatment failed to produce an increase in *Per1* expression¹³. In support of these findings, research has also found that Dexamethasone treatment increased the expression of *Per1* and *Per2* in mouse fibroblasts⁷.

The ability of glucocorticoid receptors to reset circadian rhythms in peripheral tissues has also been established *in vivo*. Kamagata *et al.* (2017) injected Dexamethasone into mice with disrupted *Clock* genes and found that these injections were able to restore the rhythmicity of PER2 expression¹⁴. In addition, when the *Clock* double mutants were injected with Dexamethasone at 30 h intervals, their PER2 expression adopted a pattern with a period of 30 h¹⁴. The researchers did not observe these effects in the wild-type mice¹⁴. The authors speculated that in the absence of a fully functional central circadian clock, the mutants had a higher sensitivity to circadian synchronization¹⁴. These findings are consistent with the observations from Balsalobre *et al.* (2000) that Dexamethasone treatment could phase shift clock gene expression in the rat liver, kidney, and heart¹³. While there have been few *in vivo*

Dexamethasone synchronization studies, these two have supported the idea that Dexamethasone can be a powerful cue for circadian resetting.

Dexamethasone synchronization has also been performed in endothelial cells and validated through subsequent gene expression profiling. Nakai *et al.* (2022) examined human endothelial cells post-synchronization and found that *Bmal1* and over 300 other genes demonstrated circadian expression¹⁵. *Bmal1* expression increased during the time points representing the day, from about ZT 0 to ZT 12¹⁵. Since humans are diurnal, this is consistent with previous literature¹⁷. Inversely, expression of the negative regulators *Per1* and *Per2* decreased at time points representing the night¹⁵. The antiphase relationship between *Bmal1* and the *Period* clock genes was well maintained¹⁵.

In addition, Wang *et al.* (2014) used Dexamethasone to synchronize the circadian rhythms of retinal endothelial cells *in vitro*. They found that the control group displayed circadian rhythmicity of the clock genes *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2*¹⁶. Consistent with other literature^{15,17}, they found *Bmal1* expression increased from 0 to 12 h post-synchronization, whereas the negative regulators decreased in expression from approximately 12 to 24 h post-synchronization¹⁶. By contrast, the cells from diabetic patients expressed lower levels of *Per1*, *Bmal1*, and *Cry2*¹⁶. These findings are consistent with the impaired circadian rhythmicity that the researchers observed in diabetic rats *in vivo*¹⁶.

Research has shown that Dexamethasone can synchronize cells *in vitro*^{13,15-17}. Studies have also demonstrated the ability of Dexamethasone to act as a Zeitgeber in peripheral tissues, thus resetting the circadian clock and inducing phase shifts¹³⁻¹⁴. Dexamethasone synchronization is mediated by glucocorticoid receptors, such that their disruption prevents synchronization¹³. Along these lines, glucocorticoids have demonstrated an ability for circadian synchronization *in vivo* in the peripheral tissues of clock gene mutants¹⁴. Dexamethasone synchronization has been validated through clock gene expression¹³.

Novelty and Significance of this Study

Previous studies have implemented Dexamethasone synchronization of cells in peripheral tissues, including endothelial cells. However, there are only two published papers which feature this synchronization on endothelial cells¹⁵⁻¹⁶. One of them examined endothelial cells in the human cornea¹⁵, and the other study examined endothelial cells of the human retina¹⁶. The study presented here has been the first to use Dexamethasone for the synchronization of cells from brain neuroendothelial cells. It is the first study to implement Dexamethasone synchronization in rat endothelial cells. This work also helped establish the presence of a circadian clock in neuroendothelial cells *in vitro*.

Compared with endothelial cells from other tissues, neuroendothelial cells are more relevant for BBI permeability. The primary cells we have isolated are from the rat cortex, which forms much of the BBI¹⁸. We verified that these neuroendothelial cells display circadian rhythms with regard to clock gene expression. We also determined that Dexamethasone is a viable means of synchronizing clock genes in this specific cell type. Moreover, it has also provided more insight into which circadian times coincided with time points after Dexamethasone treatment.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign approved all animal use protocols. We followed the National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals. We used Long-Evans/Bluegill rats and C57BL/6J mice (Jackson Laboratory), which were maintained at the University of Illinois at Urbana-Champaign. We performed cerebral neuroendothelial cell isolations for rats 3-7 weeks old. We used the minimum number of animals necessary to test the significance of our findings.

Neuroendothelial Cell Isolation and Culture

For neuroendothelial cell isolation, we followed an established protocol¹⁹. For each dissection, we removed brains from at least 5 Long Evans rats, 3-7 weeks old. We placed the brains in 70% ethanol and removed the surface vessels with a swab applicator. We then transferred the brains to Standard Isolation Medium (SIM), consisting of Medium 199 (Gibco), 20 mM HEPES (Gibco), 25 mM sodium bicarbonate (Gibco), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 µg/mL amphotericin B (1% v/v, Gibco), and 5% v/v Fetal Bovine Serum (FBS, Gibco). Before use, we adjusted the pH of the medium to 7.4.

We minced the brains in SIM, centrifuged, and resuspended the pellet in 0.02% w/v Dispase (Sigma) in SIM in the incubator for 1 h for dissociation. After centrifugation, we resuspended the pellet in 15% w/v Dextran (Sigma) in SIM which bound to the fat pad. We then removed the Dextran/fat pad solution through additional centrifugation. For further dissociation, we resuspended the tissue pellet in 0.1% w/v Collagenase/Dispase (Sigma) in SIM in the incubator for 30 min.

After washing with SIM via centrifugation, we utilized a Percoll gradient for the isolation of the neuroendothelial cells. We used an isotonic solution of Percoll Plus (Sigma) consisting of 50% v/v Percoll and 50% v/v 0.9% w/v NaCl (Sigma). Then we formed a Percoll gradient by centrifuging the solution at 25,000 g at 4°C for 70 min. We gently pipetted the cell suspension onto the Percoll gradient and then centrifuged at 1,650 g at 4°C for 10 min. We removed the neuroendothelial cells from the middle layer and washed them via centrifugation with SIM.

Prior to seeding, we coated flasks and Transwells (Nunc) with filter-sterilized 0.1% w/v porcine gelatin (Sigma) for 1 h in the incubator. For cell expansion, we used T-25 and T-75 flasks (Thermo Fisher). For TEER assays and fluorescent-microsphere assays, we seeded the cells on Transwell inserts in 24-well plates (Nunc) with a pore size of 0.4 µm. We plated the cells with a seeding density of 200,000 cells/cm² and kept them at 37 °C and 5% CO₂ in a CO₂ water-jacketed incubator (Forma Scientific, model 3130). We used neuroendothelial cell

maintenance medium, consisting of DMEM (Gibco), 20 mM sodium bicarbonate (Gibco), 1% v/v Glutamax (Gibco), 150 µg/mL Endothelial Cell Growth Factor (Sigma), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 µg/mL Amphotericin B (Gibco, 1% v/v), 1% v/v Sodium Pyruvate (Gibco) and 20% v/v FBS (Gibco).

Once the cells reached 90% confluency, we washed them 3 times with DPBS without Ca^{2+} and Mg^{2+} (Gibco) and permitted them to detach in the incubator for 5 min. using 0.25% Trypsin in EDTA (Gibco). We centrifuged the cell suspension at 3,000 rpm for 3 min. For passaging, we resuspended the pellet in maintenance medium. For freezing, we used medium containing DMEM (Gibco), 20 mM sodium bicarbonate (Gibco), 1% v/v Glutamax (Gibco), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 µg/mL amphotericin B (Gibco, 1% v/v), 20% v/v Dimethyl Sulfoxide (DMSO, Sigma), and 40% v/v FBS (Gibco). We stored the neuroendothelial cells in cryotubes (Nunc) in liquid N_2 .

Immunofluorescence and Microscopy

Prior to immunostaining, we cultured cells on glass coverslips (Esco Optics). For the cells that were treated with Propidium Iodide (Abcam), we administered 1:1000 Propidium Iodide for 2 hr in the incubator prior to fixation. We fixed the neuroendothelial cells in 4% v/v Paraformaldehyde in PBS for a minimum of 30 min prior to blocking and permeabilization. We permeabilized with 0.3% Triton-X (EMD Millipore) in PBS during blocking and incubations with primaries and secondaries. We blocked the samples for 1 h [be consistent...h is the accepted abbreviation for hour/s] with 5% v/v Normal Goat Serum (Abcam) in PBS with 0.3% v/v Triton-X at room temperature. We diluted the primary and secondary antibodies in 2% v/v Normal Goat Serum in PBS with 0.3% Triton-X. Each incubation with primary antibodies was for 2 days at 4°C with gentle shaking. Each incubation with secondaries lasted 1 day at 4°C with gentle shaking in darkness.

In between incubations, we removed excess primary antibodies, secondary antibodies, and DAPI by washing the samples at least 3 times, totaling at least 30 min with PBS. For

primary antibodies, we used CD31 anti-mouse (1:1000, Dako), and ZO-1 anti-rabbit (1:500, Invitrogen). We detected primary antibody binding with Alexa Fluor 488 goat anti-mouse IgG (1:2000, Invitrogen) and Alexa Fluor 568 goat anti-rabbit IgG (1:2000, Invitrogen). In order to identify the cell nucleus, we incubated the cells with DAPI (1:25,000, Invitrogen) in PBS for 1 h at room temperature in darkness. To mount the samples and protect them from photobleaching, we used ProLong Antifade Diamond mountant (Invitrogen).

We performed fluorescent imaging of the neuroendothelial cells with an LSM 700 inverted confocal microscope (Zeiss) operated with Zen software. We used lasers with wavelengths of 405 nm, 488 nm, and 555 nm. These corresponded respectively to the excitation wavelengths of DAPI, Alexa Fluor 488, and Alexa Fluor 568. We used 10X and 20X objective lenses.

Dexamethasone Synchronization

For circadian synchronization, we treated cells with 100 nM Dexamethasone (Thermo Fisher), diluted 1:1000 from a 100 mM stock solution dissolved in DMSO (Sigma). We collected half of the media, added the Dexamethasone to it, and then gently pipetted the solution back onto the cells. After a 2-h incubation at 37 °C and 5% CO₂, we removed the Dexamethasone by moving the Transwell inserts to new wells containing fresh media. We denoted the time of Dexamethasone removal as 0 h post-synchronization. We then replaced half of the media with fresh media every 24 h post-synchronization. The control groups which did not undergo Dexamethasone synchronization were still subjected to treatment with the equivalent volumes of DMSO. After 2 h, we moved the inserts to fresh media which did not contain Dexamethasone or DMSO.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (PCR)

We implemented an established protocol for Quantitative Reverse Transcriptase quantitative PCR (RT-qPCR)²⁰. It began with the isolation of RNA throughout the cells in a sample. The RNA was then reverse-transcribed into complementary DNA (cDNA). During this

process, primers which were specific for target RNA facilitated the production of cDNA from the target RNA. The quantity of cDNA produced was proportionate to the quantity of RNA expressed by the cells. The cDNA was then amplified along via a Polymerase Chain Reaction (PCR) and was quantified with the fluorescent dye SYBR Green. Compared with other methods, such as Western Blotting or immunostaining, qPCR is more quantitative and high-throughput²¹⁻²². It is also highly sensitive and measures RNA expression rather than protein expression, removing the variable of protein modifications²¹⁻²².

We isolated RNA with Trizol (Invitrogen) and alcohol precipitation. We performed reverse transcription with 1 µg of isolated RNA and 200 ng Random Hexamers (Invitrogen), and DEPC water (Invitrogen) to 12 µL per conical tube. We denatured the secondary structures in this solution by heating at 85°C for 10 min. Then we added 5X reverse transcription buffer, 10 mM DTT, 0.5 mM dNTPs, and Superscript IV RNase H Reverse Transcriptase (all from Invitrogen). We heated the solution at 42°C for 1 h and terminated the reaction by heating at 92°C for 10 min. We diluted the samples with RNase-free water (Invitrogen) and stored them at -80°C.

We then performed qPCR with SYBR Green (Lonza), 0.25 mM dNTPs (Denville), and Taq polymerase. For rat *Per1*, we used a Forward primer with the sequence: AGGAGGCCCGGAAGTAGT and a reverse primer with the sequence: AGCCTGAAAGTGCATCCTGATT). For rat *Bmal1*, we used a forward primer with the sequence: TCTATCCGATGACGAACTGAA and a reverse primer with the sequence: CCCTCGGTCACATCCTACAG. For our loading control, we used Acidic ribosomal protein PO with the forward primer sequence: CGTGATGCCAGGGAAGA and the reverse primer sequence: TCCCACAATGAAGCATTTTGG.

There were challenges that we had to take into account when conducting qPCR. For instance, we ensured that RNase-free reagents were used to safeguard against RNA degradation. Another consideration was the choice of Trizol for protein denaturation and RNA

isolation. Trizol is one of the more potent reagents for these processes, and, thus, it is conducive to higher RNA yields as well as purity²³. However, Trizol also contains guanidine salts, which inactivate RNases but also inhibit reverse transcription enzymes²³. We removed these salts through washes with ethanol and isopropanol, followed by centrifugation, resulting in the precipitation of these salts. Before reverse transcription and then again before qPCR, we measured the RNA and DNA concentrations with a NanoDrop Spectrophotometer to ensure that they were present in sufficient quantities. If we had experienced contamination with genomic DNA, we could have used DNA removal kits or column purification of RNA with DNase treatment. If the reverse transcription step ever failed to yield cDNA, we could have replaced the random hexamers used during priming with gene-specific primers or oligo-dTs, which have a higher specificity. Since these 2 alternatives have a higher specificity, they are more conducive to producing cDNA, but we did not find the need to incorporate these alternatives.

Results and Discussion

Neuroendothelial cells cultured *in vitro* require synchronization in order to study their circadian rhythms. The most certain way to verify the success of this synchronization is to measure clock gene expression. Dexamethasone treatment is a well-established method for circadian synchronization. However, few studies have featured Dexamethasone synchronization in endothelial cells. Moreover, this treatment has never been performed in neuroendothelial cells, which are a key component of the BBI.

We isolated primary neuroendothelial cells from the rat cerebral cortex which display a laminar morphology indicative of health. They also expressed the endothelial cell marker CD31, validating the purity of the cell population (Figure 3). We synchronized their circadian rhythms with a 2-h pulse of 100 nM Dexamethasone. These metrics have been used previously for Dexamethasone synchronization studies²⁴, including two featuring endothelial cells¹⁵⁻¹⁶. After the 2-h pulse, the cells were washed with media that did not contain Dexamethasone, and they were cultured without it. There was also one negative control group which never underwent

Dexamethasone treatment. It did, however, undergo the same mechanical perturbations and receive the same volume of DMSO, the solvent used for dissolving the Dexamethasone. Then, we performed RT-qPCR for the *Per1* and *Bmal1* clock genes at 4 h intervals from 0 to 36 h after Dexamethasone was administered. We hypothesized that clock gene expression would exhibit circadian rhythmicity in the group treated with Dexamethasone but not in the control group.

To determine the purity of the isolated neuroendothelial cells, we performed immunostaining for neuroendothelial cells, astrocytes, neurons, and pericytes. We found high expression of CD31, an endothelial cell marker, with low expression of the markers for astrocytes, neurons, and pericytes (ALDH1L1, β -III tubulin, and Desmin, respectively) (Figure 3). This confirmed the presence of endothelial cells and the absence of astrocytes, neurons, and pericytes in the culture.

We also used immunostaining to monitor the health of the neuroendothelial cells for consecutive days after Dexamethasone treatment. Before fixation, we treated the cells for 2 h with Propidium Iodide (PI), which penetrates damaged cell membranes and fluorescently labels their nuclei²⁵. We found low expression of PI, indicating that the cell membranes were intact, for the cells that were treated with Dexamethasone as well as the control group which was not (Figure 3). The neuroendothelial cells displayed a flat morphology (Figure 3), indicative of cellular health²⁶. They also expressed CD31 primarily as apical puncta (Figure 3), which is the standard form of CD31 expression under healthy conditions²⁶. We observed a high level of cellular confluence, as well as apical expression of the tight junction proteins Zonula Occludens-1 (ZO-1) (Figure 3). This expression pattern, which persisted for the 4 days we observed them, is also standard under conditions of cell health and high confluence²⁶. These results provided several indications that the rat cerebral neuroendothelial cells were healthy.

The neuroendothelial cells displayed robust expression of *Per1* and *Bmal1* according to qPCR (Figure 4). We conducted qPCR at 4-h intervals for 36 h. Both genes oscillated significantly with time of day according to one-way, repeated-measures ANOVA, (Figure 4,

$p < 0.0001$, $n=3$ for *Per1* and $p < 0.0001$, $n=3$ for *Bmal1*). In the unsynchronized control group, we observed no significant oscillation, ($p=0.8$ for *Per1* and $p=0.6$ for *Bmal1*). This indicates that the observed rhythmicity was due to Dexamethasone synchronization. *Per1* expression generally decreased from 0 h to 12 h post-synchronization, increased from 12 h to 24 h, and then decreased from 24 h to 36 h (Figure 4). In general, *Bmal1* expression increased from 0 h to 12 h, decreased from 12 h to 24 h, and increased from 24 h to 36 h (Figure 4). These findings are consistent with previous literature, including the well-established antiphase relationship between *Per1* and *Bmal1*¹⁵⁻¹⁷. *Bmal1* expression was greatest at the 12 h and 36 h time points and lowest at the 0 hr and 24 h time points. *Per1* expression peaked at 4 h and 28 h post-synchronization and displayed troughs at the 12, 16, and 36 h time points.

We took several considerations into account to reduce potential confounding variables. Any future research may benefit from an understanding of these potential issues and some possible solutions. For instance, studies have shown that incubation with high concentrations of Dexamethasone can tighten the BBI, if it is not removed after 2 h²⁷⁻²⁹. Fortunately, this issue did not occur (Chapter 3). If Dexamethasone treatments had resulted in an increase in barrier tightness, this would have been reflected in increased TEER values or a decrease in permeability to fluorescent beads or fibrinogen. This would have likely been the result of an excessively high concentration of Dexamethasone or ineffective washes. We removed the Dexamethasone by pipetting off the media in the Transwell inserts and then placing them in new wells containing fresh media. This quick process ensured that the cells would always be in contact with media and thus prevented a serum shock, which is a distinct method for circadian synchronization³⁰. If an unintentional serum shock had resulted, we could have reduced the concentration of serum in the media and used KnockOut Serum Replacement in combination with endothelial-specific growth factors.

The neuroendothelial cells displayed robust expression of *Per1* and *Bmal1* according to qPCR (Figure 4). We conducted qPCR at 4 h intervals for 36 h. Both genes oscillated

significantly with time of day according to one-way, repeated-measures ANOVA, (Figure 4, $p < 0.0001$, $n = 3$ for *Per1* and $p < 0.0001$, $n = 3$ for *Bmal1*). In the unsynchronized control group, we observed no significant oscillation, ($p = 0.8$ for *Per1* and $p = 0.6$ for *Bmal1*). This indicated that the rhythmicity was measurable due to Dexamethasone synchronization. *Per1* expression generally increased from 0 h to 12 h post-synchronization, decreased from 12 h to 24 h, and then increased from 24 h to 36 h (Figure 4). In general, *Bmal1* expression decreased from 0 h to 12 h, increased from 12 h to 24 h, and decreased from 24 h to 36 h (Figure 4). These findings are consistent with previous literature, including the well-established antiphase relationship between *Per1* and *Bmal1*¹⁵⁻¹⁷. *Per1* expression was greatest at the 12 h and 36 h time points and lowest at the 0 h and 24 h time points. *Bmal1* expression peaked at 24 h post-synchronization and displayed troughs at the 12 and 36 h time points.

If the control group had expressed *Per1* or *Bmal1* rhythmically according to time of day, this would have indicated that the cells were unintentionally synchronized by other means. This could have been due to an unintended serum shock during media changes. In addition, research has found that temperature changes can affect circadian rhythms³¹, and they occur to some extent during media changes. It is also well established that changes in redox states influence circadian rhythms³². These can occur during media changes when cells are exposed to air, which contains lower levels of CO₂ and higher levels of oxygen than standard incubator conditions. In addition, mechanical perturbations, which can occur during media removal and replacement, may influence gene expression³³. In sufficient concentrations, DMSO may lengthen circadian rhythms³⁴. Moreover, the dissociation of cells during passaging may disrupt the rhythmicity of *Bmal1* expression³⁵. However, the control group, which was not synchronized with Dexamethasone, did not display significant changes in *Per1* or *Bmal1* expression due to time of day. We controlled for any mechanical perturbations, exposure to ambient air during media changes, trypsinization, and the addition of DMSO. Therefore, we concluded that the

Dexamethasone was inducing the synchronization independently of any significant contributions of other factors.

We found that *Per1* expression peaked at 12 h and 36 h post-synchronization and displayed a trough at 24 h (Figure 4). Previous research has shown that *Per1* expression peaks at 12 h in the mouse liver and displays a trough at ZT 14¹⁷. *Per1* expression is at a trough at ZT 12 *in vitro* in human corneal cells¹⁵. These findings are consistent with each other, since for peripheral tissues, nocturnal animals and diurnal animals express clock genes in a manner antiphase to each other¹. Our results and those previously published also found a 24 h rhythm for *Per1* expression^{15,17}. Compared with these studies, our findings at 12 h post-synchronization correlate with ZT 12, 24 h post-synchronization correlates with ZT 24, and 36 h correlates with ZT 36^{15,17}. This implies that the time point 0 h post-synchronization is equivalent to ZT 0.

Bmal1 expression peaked at 24 h post-synchronization and displayed troughs at 12 h and 36 h (Figure 4). *Bmal1* expression has been shown to peak at ZT 24 in the mouse liver, where it displays troughs at ZT 12 and ZT 36¹⁷. Previous research has shown that *Bmal1* expression is at a trough at ZT 24 *in vitro* in human corneal cells¹⁵ and human retinal cells¹⁶. These findings are consistent given the antiphase relationship between clock gene expression in peripheral tissues for nocturnal and diurnal animals¹. Our results and the previous studies also found a 24 h rhythm for *Bmal1* expression¹⁵⁻¹⁷. As with *Per1*, our findings correlated such that our time points in hours post-synchronization matched best with the ZT times in the previous literature¹⁵⁻¹⁷. According to previous research, these times have also correlated most with the CT times from *ex vivo* studies with the rat vasculature (Figure 5)³⁶.

Conclusions

The work presented here has been the first study to examine clock gene expression in neuroendothelial cells after Dexamethasone synchronization. Neuroendothelial cells are of particular relevance to the BBI compared with endothelial cells outside of the brain. They have defining properties which distinguish them from other endothelial cell types. For example,

neuroendothelial cells experience significantly less transcytosis, the process whereby solutes can move through a cell³⁷. They also tend to be flatter than other types of endothelial cells³⁷. Multiple studies have found that a greater portion of their cell membranes tend to express tight junctions³⁸⁻⁴⁰. The overall increased tightness of interconnections among neuroendothelial cells may be necessary for their roles in maintaining BBI integrity.

We isolated primary rat neuroendothelial cells and determined that there were no neurons, astrocytes, or pericytes in the cell population. We also verified their health with CD31 expression and Propidium Iodide staining. We found through qPCR that *Per1* and *Bmal1* expression in the rat neuroendothelial cells oscillated significantly according to time of day. These clock genes displayed 24 h rhythms that were antiphase to each other. *Per1* expression peaked at 24 h after synchronization with Dexamethasone, and *Bmal1* expression peaked 12 h post-synchronization. These findings are consistent with previous literature regarding *Per1* and *Bmal1* expression in endothelial cells¹⁵⁻¹⁷. They also demonstrate that the time points we have measured as hours-post-synchronization are most similar to hours in Zeitgeber Time (ZT)¹⁵⁻¹⁷. Importantly, this study is the first to demonstrate that clock gene expression in rat neuroendothelial cells oscillates according to time of day.

Acknowledgments

This work was supported by the NIH (R61HL159948), NSF (DGE NRT-UtB 1735252 and STC CBET 0939511), and the 2021 Beckman Institute Graduate Fellows Program.

Figures

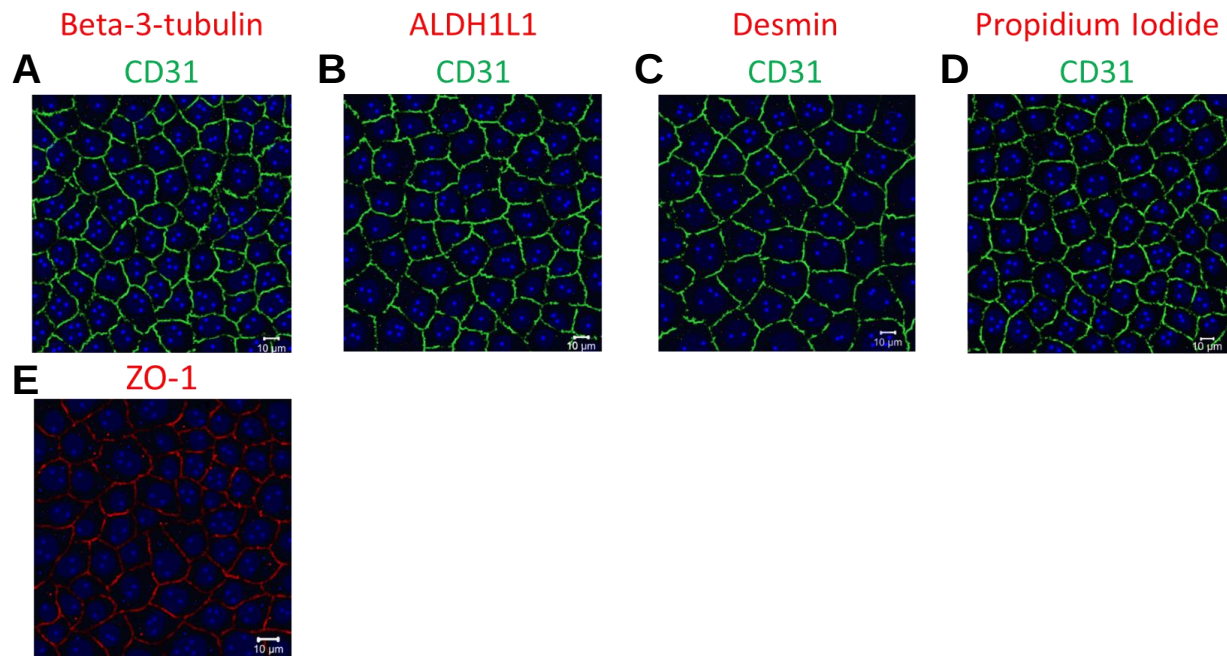


Figure 3: Rat neuroendothelial cells display indications of purity and health after isolation.

A-E. Rat cerebral neuroendothelial cells cultured on Transwell inserts, 20X magnification, 10 µm scalebars. Immunofluorescence for CD31 in green indicates the presence of endothelial cells (**A-D**). In red, fluorescence for beta-3 tubulin indicates neurons (**A**), ALDH1L1 indicates astrocytes (**B**), and Desmin indicates the presence of pericytes (**C**). Propidium Iodide fluorescence in red indicates cells with damaged membranes (**D**). Apical expression of the tight junction Zona Occludens-1 (ZO-1) indicates full confluence (**E**).

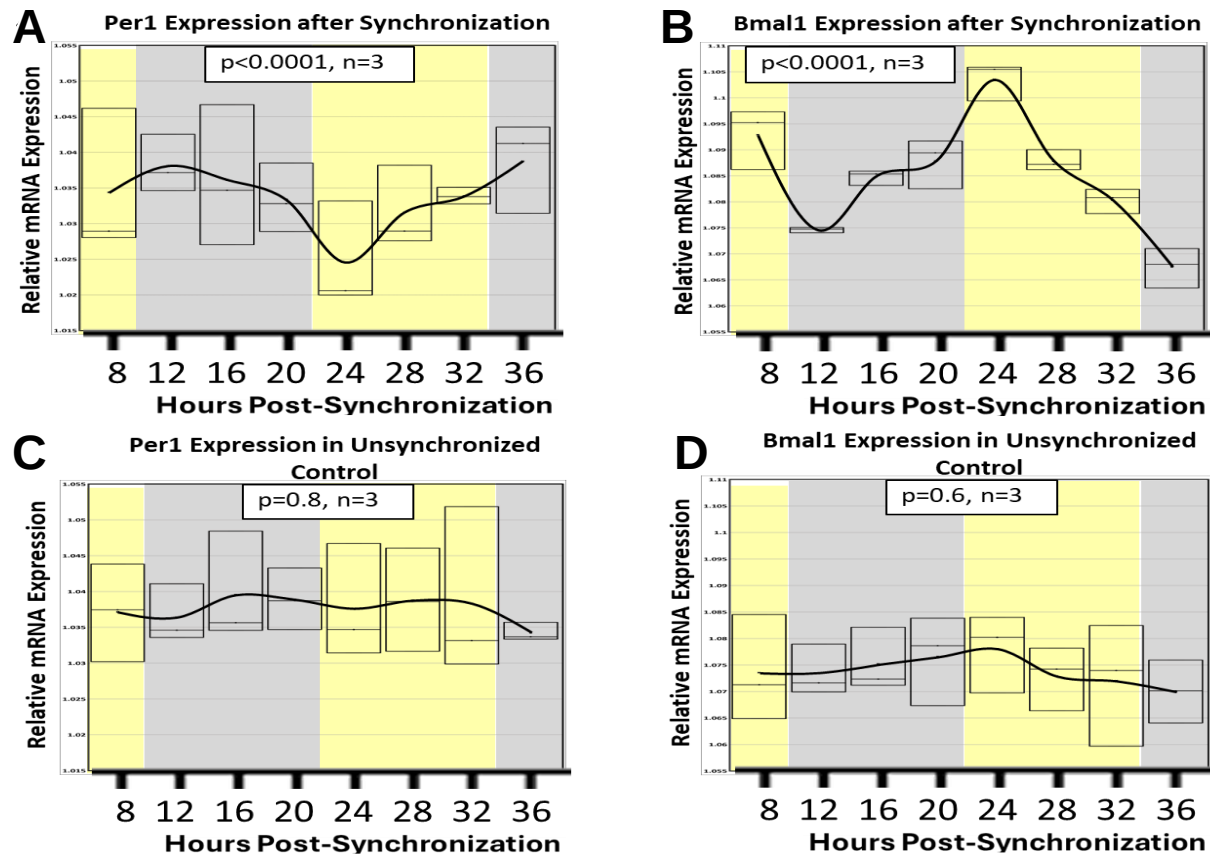


Figure 4: Rat neuroendothelial cell clock gene expression varies with time of day.

B. RT-qPCR showed that *Per1* fluctuated significantly according to time of day after Dexamethasone treatment with a period of approximately 24 h ($p < 0.0001, n=3$, 6 samples per run), but not in the unsynchronized control ($p=0.8, n=3$, 6 samples per run). *Bmal1* expression also fluctuated significantly with time of day with Dexamethasone treatment ($p < 0.0001, n=3$, 6 samples per run) but not in the unsynchronized control ($p=0.6, n=3$, 6 samples per run).

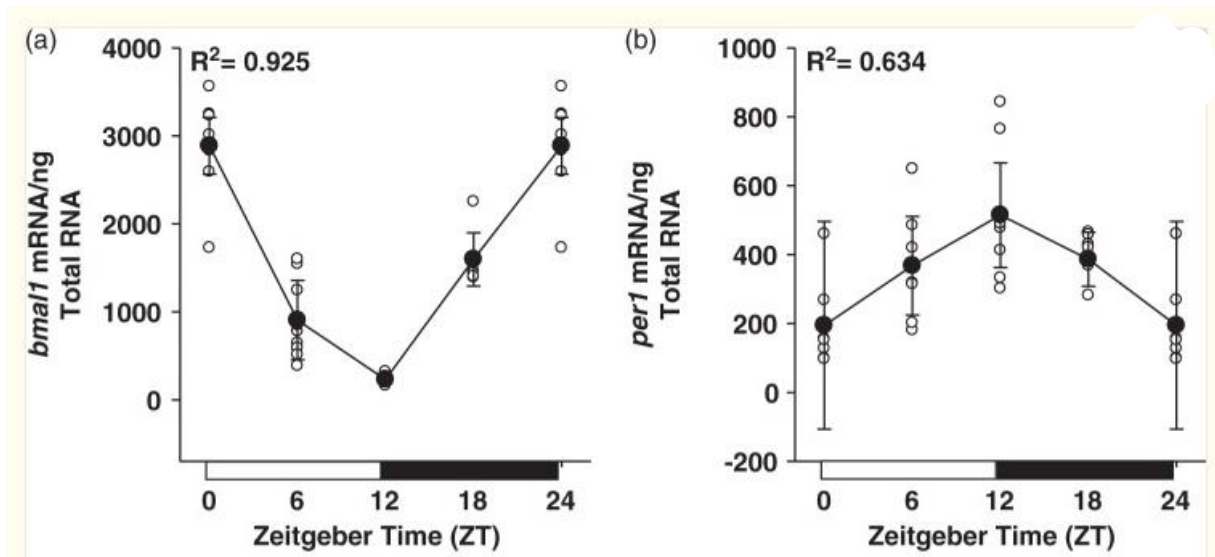


Figure 5: Rat neuroendothelial cells display a similar pattern *ex vivo*.

A-B. In the rat cerebral cortex *ex vivo*, *Bmal1* and *Per1* are expressed in 24 h rhythms that are in antiphase to each other. This is the same general pattern that we observed in the cells we isolated from the rat cerebral cortex and cultured *in vitro*. Durgan *et al.* (2017) referred to the moment that the rats were first exposed to light each day as Zeitgeber Time 0. This implies a similar time course for the time when their rats were exposed to light and the completion of our Dexamethasone synchronization.

*This figure is from the article written by Durgan *et al.* (2017)³⁶.

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CHAPTER 3: CIRCADIAN RHYTHMICITY OF BLOOD-BRAIN INTERFACE PERMEABILITY

IN VITRO

Abstract

The Blood-Brain Interface (BBI) facilitates protection of the brain from pathogens, the removal of waste products, brain homeostasis, and the permeation of nutrients and drugs. Its dysfunction contributes to the onset of neurological diseases, including stroke and dementia. An understanding of daily fluctuations in BBI permeability can also better inform drug administration. Recent research has demonstrated that BBI permeability fluctuates according to circadian rhythmicity. *In vitro* studies are beginning to isolate the effects of circadian rhythms from the effects of sleep and other homeostatic regulators of BBI permeability. Here, we demonstrate the *in vitro* circadian rhythmicity of permeability for neuroendothelial cells, key components of the BBI. This is the first study to examine the circadian rhythmicity of BBI permeability across a monolayer, thus factoring in cell-cell interactions *in vitro*. We show here that permeability and barrier tightness of neuroendothelial cell monolayers fluctuate according to time of day.

Introduction

The Blood-Brain Interface (BBI) provides protection from toxic compounds and other pathogens in the blood¹. It also allows the brain to remove waste particles such as excess glutamate or amyloid-beta plaques¹. In addition, it facilitates neurological homeostasis by protecting the brain from fluctuations of non-toxic compounds in blood, such as glucose¹. BBI permeability is also a key factor for the delivery of drugs to the brain. Heightened permeability of the BBI can cause blood to leak into brain parenchyma, causing strokes, neurodegeneration, and worsened pathology in dementia and stroke². This is because when the BBI becomes excessively permeable, blood can leak into the extracellular micro-environment, bathing neurons and glia. This can result in microhemorrhages and hemorrhagic strokes². Furthermore, the accumulation of fibrin and other blood-derived factors can cause inflammation and cellular

damage². Heightened BBI permeability is a particularly pronounced threat for people who are older, have metabolic diseases, or have undergone traumatic brain injury².

Circadian fluctuations in BBI permeability have several implications for human health. Research has demonstrated that susceptibility to strokes in humans varies by time of day², and circadian fluctuations in BBI permeability may contribute to this pattern. Heightened BBI permeability has also been implicated in hypertension, epilepsy, Alzheimer's Disease and other dementias³⁻⁴. In addition, BBI permeability influences the ability of drugs to enter the brain, and an understanding of its circadian rhythmicity can be used to optimize drug delivery. Moreover, it also may be more beneficial for patients to take medications which promote BBI tightness at certain times of day⁵.

Strokes are one of the leading causes of death in the United States. Every year, approximately 800,000 people in the United States experience strokes, resulting in approximately 140,000 deaths⁶. Stroke is characterized by a loss of patency of cerebral blood vessels, which results in blood flow to the brain. Strokes have two main causes: bleeding (*hemorrhagic*) and loss of oxygen (*ischemic*). Hemorrhagic strokes are caused by leakage of blood into the brain parenchyma, and ischemic strokes are caused by vascular blood clots that prevent the flow of blood to downstream parts of the brain. BBI dysfunction contributes to worsened pathology in strokes, both ischemic and hemorrhagic⁷⁻⁸. In addition, drugs that reduce BBI dysfunction have been shown to alleviate stroke symptoms⁹⁻¹⁰. Strokes are more likely to occur in the morning¹¹, and human cells display peak BBI permeability at this time¹². Vulnerability to stroke also can appear in the early evening, probably by a distinct mechanism.

Fluctuations in circadian rhythms may influence susceptibility of the BBI leakage, although this is not established. For instance, research has shown that strokes are significantly more likely to occur in the early morning¹¹. Furthermore, circadian rhythms regulate blood coagulation. The liver is governed by circadian rhythms, which cause it to produce the clotting factor fibrinogen at levels that oscillate in accordance with a circadian rhythm¹³. The levels of

fibrinogen that the liver releases into the blood are greatest in the early morning¹³. Factors that contribute to the dissolution of blood clots also oscillate in accordance with the time of day¹⁴, and some of them have been shown to be regulated by the same genes that control circadian rhythms¹⁵.

Research has demonstrated that in humans, strokes tend to occur more often in the morning¹¹. One meta-analysis of 31 publications found that strokes were 49% more likely to occur between 6 am and 12 pm¹¹. In another study, researchers obtained data from 800 stroke patients¹⁶. They found overall that stroke incidences varied significantly according to time of day¹⁶. In addition, 74% of strokes occurred while the patients were awake¹⁶. The researchers found that for Cerebral Infarctions and Intra-Cerebral Bleeds, the incidence rates varied significantly according to time of day¹⁶. The higher incidence rate of strokes in the morning for humans is consistent with recent research which has demonstrated that human endothelial cells are more permeable during the day¹². Heightened BBI permeable in the morning may contribute to this heightened risk.

Research has also found that circadian disruption can exacerbate strokes and impair immune systems¹⁷. In one study, ischemic strokes were induced in mice. The group that was subjected to a 6 h phase advance for 4 weeks and then 1 week of a 12:12 light:dark cycle had greater cerebral infarct volumes than control mice¹⁷. Notably, this increase was not observed for the mice that underwent 6 h phase advances for only 1 week or 4 weeks of phase advances followed by 3 weeks of a 12:12 light:dark cycle¹⁷. This suggests that the predisposition to greater infarct volumes was reversed after a standard light:dark cycle was restored. The researchers also injected Lipopolysaccharide to elicit an immune response. They found that the phase-advanced mice responded by displaying greater ratios of pro-inflammatory to anti-inflammatory cytokines compared with controls¹⁷. These findings may be explained by research which has shown that circadian disruption can disrupt BBI integrity¹⁸.

New research has been finding a correlation between BBI dysfunction and the onset of Alzheimer's Disease. It is possible that BBI dysfunction leads to impaired removal of amyloid-beta plaques and as a result increases the predisposition to Alzheimer's Disease. Many of these studies have been correlative, rather than causative. However, one study found that BBI dysfunction preceded cellular, cognitive, and behavioral dysfunction in a mouse model of Alzheimer's Disease¹⁹. This model featured the Apolipoprotein E4 (APOE4) genetic variant¹⁹. Studies have also shown that P-glycoprotein, one component of neuroendothelial cells at the BBI, is influential in removing amyloid-beta particles²⁰. In mice subjected to inhibition or genetic knockouts of P-glycoprotein, the rate of amyloid-beta clearance was halved, and plaque deposition increased²⁰. In addition, studies have demonstrated that P-glycoprotein expression is under circadian control²¹⁻²². It is possible that circadian changes in BBI permeability may cause amyloid-beta plaque accumulation and clearance to oscillate according to time of day.

Understanding when permeability is greatest has the potential to inform more efficient drug delivery. This approach is called chronomedicine. Zhang *et al.* (2018) implemented this approach for administration of the anti-seizure medication phenytoin in a *Drosophila* seizure model. They found that statistically less time was required for its therapeutic effects of when it was administered at nighttime⁵. This was also the time of day that they found efflux from the brain to be lowest⁵. Studies have also found that some types of cancer responded more to treatments at specific times of day²³⁻²⁴. These effects may be explained by circadian rhythms in hormone secretion, liver enzymes that break down drugs²⁵, or in the symptoms being treated. Chronomedicine may allow patients to experience heightened efficacy from their medications, leading to more successful outcomes. Another advantage of this approach is that if drugs are administered more efficiently, they may garner the same effects through lower doses. This may result in the reduction of off-target effects as well as costs.

Several drugs have demonstrated the potential to decrease BBI permeability. This may be beneficial for a variety of conditions in which increased BBI permeability contributes to

symptoms, such as strokes, Traumatic Brain Injury, Multiple Sclerosis, and dementias. Research on the circadian rhythmicity of BBI permeability may help clinicians determine the optimal time of day for patients to take these medications. They generally work by preventing inflammation. One such medication is the anti-inflammatory corticosteroid, methylprednisolone. In a clinical trial, methylprednisolone helped Multiple Sclerosis patients maintain BBI integrity²⁶. In addition, Tumor Necrosis Factor α (TNF α) a pro-inflammatory cytokine, can increase BBI permeability²⁷. A TNF α inhibitor, etanercept, significantly decreases symptoms in patients who have undergone strokes and Traumatic Brain Injuries²⁸. In Multiple Sclerosis patients, Interferon β can prevent BBI leakage²⁹. This may be due in part to its ability to suppress Matrix Metalloproteinase-9, which is pro-inflammatory³⁰. Similarly, Dexamethasone treatment reduces some measurements of inflammation and BBI dysfunction in patients with Tuberculosis Meningitis³¹. In addition, the histamine agonist, hydroxyzine, has shown some promise in reducing BBI permeability, albeit not significantly. This is likely due to its ability to inhibit the pro-inflammatory activity of mast cells³². In keeping with the concept of chronomedicine, it may be more beneficial for patients to receive these medications at times of day which are more conducive to heightened BBI permeability.

By contrast, a multitude of therapies increase BBI permeability, concomitantly enhancing drug delivery. These treatments may be more effective if administered at times of day when BBI permeability is high. One of these therapies is osmotic Blood-Brain Barrier Disruption (BBBD). In clinical trials with humans, BBBD increases the efficacy of chemotherapy for Central Nervous System lymphomas³³ and glial brain tumors³⁴⁻³⁶. This technique works via the injection of a hypertonic solution into the blood, which results in heightened concentration of solutes on the blood side of the BBI, effectively pushing solutes through. Another approach utilizes ultrasonic waves and microbubbles, which are microscopic spheres containing gases. The microbubbles are injected into the blood, and they expand when exposed to ultrasonic waves. In clinical trials with humans, this method has enhanced the efficacy of chemotherapy for glioblastomas³⁷. This

method offers focal control over the site of BBI disruption, as do hyperthermic lasers. In Laser Interstitial Thermal Therapy, lasers delivered to tumors cause necrosis in the tumors, while the area surrounding the tumor is heated to sub-necrotic temperatures³⁸. These warmer temperatures at the tumor's periphery are conducive to increasing BBI permeability, which can be used to enhance delivery of chemotherapy³⁸. In a human trial, this method increased BBI permeability in the areas surrounding glioblastomas³⁸. These three approaches have featured a variety of methods: osmosis, ultrasonic waves, and hyperthermic lasers. However, for each of these methods, the extent of BBI disruption can be scaled. Thus, clinicians administering these treatments may be able to administer them in smaller quantities during the times of day when the BBI is most permeable.

Previous Circadian BBI Research

Levels of BBI permeability fluctuate according to circadian rhythmicity. This has been established *in vitro* for murine and human cells¹². This finding has also been replicated *in vivo* in flies and mice⁵. These studies reported a 24-h pattern, wherein peak BBI permeability occurs during the day for diurnal animals and during the night for nocturnal animals^{5,12}. In both studies, the genetic deletion of a circadian clock gene abolished this 24-h pattern, demonstrating that it is indeed the result of circadian rhythms^{5,12}. However, this is a new area of research, and a multitude of questions remains regarding the circadian rhythmicity of BBI permeability.

Furthermore, clock-gene expression influences tight junction expression. Kyoko *et al.* (2014) found that in wild-type mice, the tight junction proteins, Occludin and Claudin-1 are expressed with a daily rhythm in the mouse large intestine³⁹. This rhythm is abolished in *Per2* mutant mice³⁹. They also expressed higher levels of tight junction proteins, suggesting that *Per2* expression decreases the expression of tight junction proteins³⁹. Of the 7 tight junction proteins they examined at 6-h intervals, Occludin and Claudin-1 fluctuated significantly according to time of day³⁹. For both, the lowest expression occurred at 16-h post-synchronization³⁹. These researchers also found through Chromatin Immunoprecipitation (ChIP) assays that the clock

genes *Clock* and *Bmal1* bound to the promoters of Occludin and Claudin-1³⁹. The finding that the lowest expression of Occludin and Claudin-1 occurred at ZT 16 is consistent with the finding that BBI permeability peaks in the early night (ZT 16) for mice *in vivo*¹².

Novelty of this Study

The study we are presenting here is the first to determine whether neuroendothelial intercellular permeability exhibits circadian rhythmicity. As an *in vitro* study, it has not been confounded by the *in vivo* influences of sleep and locomotor activity, which also exhibit rhythmic diurnal fluctuations. It is the first *in vitro* study to examine the influence of circadian rhythmicity on intercellular, rather than only intracellular, permeability. The study presented here is also one of the first studies to measure general permeability of the BBI, rather than permeability to a specific compound, in the context of circadian rhythms.

Why *in Vitro* Studies Are Needed

One of the challenges in circadian rhythm research is to parse out the influences of circadian rhythms, homeostatic rhythms, and patterns in animal behavior, such as sleep. One solution is *in vitro* studies, which remove several confounding variables that are present *in vivo*. For instance, it has been shown that neural activity can contribute to changes in BBI permeability⁴⁰. It is also possible that BBI permeability may be affected by sleep. Research has demonstrated that sleep affects the exchange between cerebrospinal fluid (CSF) and interstitial fluid⁴¹. Similarly, studies have shown that CSF production in humans *in vivo* oscillates with a 24 h rhythm⁴²⁻⁴³. In addition, the BBI *in vivo* is subject to inflammation, which weakens BBI integrity⁴⁴. Moreover, levels of cortisol, insulin, and melatonin all are regulated by circadian rhythms⁴⁵⁻⁴⁸. Cortisol increases BBI tightness⁴⁹. Further, insulin increases endothelial cell proliferation and preserves the integrity of endothelial cell tight junctions. Melatonin administration may be protective against BBI damage⁵¹. To date, only two circadian BBI permeability studies featuring *in vitro* experiments have been published^{5,12}. Neither study measured intercellular permeability of neuroendothelial cells.

Endothelial Cells are Highly Relevant for BBI Studies

The study presented here is one of only two studies to date that have examined the circadian rhythmicity of endothelial cell permeability¹². As the main component of the blood side of the BBI, endothelial cells play a key role in the function of the BBI. They form the whole interior of blood vessels, and the pericytes and smooth muscle cells which form the blood vessel exteriors are not as specialized for solute transport⁵². Endothelial cells contain membrane proteins that allow for the transportation of several key types of molecules which otherwise would not be able to permeate⁵². For instance, they are the only type of cells in blood vessels that allow for transcytosis⁵², which facilitates the movement of larger molecules directly through a cell. They also express specialized solute carriers for transporting glucose, amino acids, and nucleosides which are not expressed on pericytes or smooth muscles⁵². In blood vessels, endothelial cells are the only cell type that can transport larger solutes via paracellular transport⁵², which is the main way that drugs can cross the BBI, and bulk flow⁵², which occurs during pathological states.

Compromised neuroendothelial cell integrity has been shown to contribute to several lethal diseases. Heightened neuroendothelial cell permeability *in vivo* may cause strokes⁵³. Research has shown that it may contribute to Sepsis-Associated Encephalopathy⁵⁴ and may also have a role in Alzheimer's Disease¹⁹. Leakage of blood from the brain can cause hemorrhagic strokes, and leakage of blood into the brain can cause blood clots that lead to ischemic strokes. Human brains have also been shown to have damaged neuroendothelial cells following strokes⁵³. Research has shown that damage to neuroendothelial cells can result in Sepsis-Associated Encephalopathy (SAE)⁵⁴. Sepsis occurs when a severe infection causes a dangerously heightened immune response. Sepsis allows immune cells to enter the brain, resulting in an increased production of cytokines which cause neuroinflammation, damage to endothelial cells, and destruction of the BBI⁵⁴. The loss of BBI integrity impairs neurotransmission and results in SAE as widespread apoptosis occurs⁵⁴.

The study presented here is the first to determine whether intercellular neuroendothelial cell permeability exhibits circadian rhythmicity. This study is also one of the first to measure general permeability, rather than permeability to a specific compound, in the context of circadian rhythms. Neuroendothelial cell permeability has particular significance in the context of strokes which are often caused by leakage of blood into the brain. In addition, intercellular interactions influence a substantial portion of BBI permeability. They are particularly relevant for the delivery of therapeutic drugs, most of which rely on intercellular transport to enter the brain⁵⁵.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign approved all animal use protocols. We followed the National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals. We used Long-Evans/Bluegill rats and C57BL/6J mice (Jackson Laboratory), which were maintained at the University of Illinois at Urbana-Champaign. We performed cerebral neuroendothelial cell isolations for rats 3-7 weeks old. We used the minimum number of animals necessary for sound experimental design.

Neuroendothelial Cell Isolation and Culture

For neuroendothelial cell isolation, we followed an established protocol⁵⁶. For each dissection, we removed brains from at least 5 Long Evans rats, 3-7 weeks old. We placed the brains in 70% ethanol and removed the surface vessels with a swab applicator. We then transferred the brains to Standard Isolation Medium (SIM), consisting of Medium 199 (Gibco), 20 mM HEPES (Gibco), 25 mM sodium bicarbonate (Gibco), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 µg/mL amphotericin B (1% v/v, Gibco), and 5% v/v Fetal Bovine Serum (FBS, Gibco). Before use, we adjusted the pH of the medium to 7.4.

We minced the brains in the SIM, centrifuged, and resuspended the pellet in 0.02% w/v Dispase (Sigma) in SIM in the incubator for 1 h for dissociation. After centrifugation, we

resuspended the pellet in 15% w/v Dextran (Sigma) in SIM which bound to the fat pad. We then removed the Dextran/fat pad solution through additional centrifugation. For further dissociation, we resuspended the tissue pellet in 0.1% w/v Collagenase/Dispase (Sigma) in SIM in the incubator for 30 min.

After washing with SIM via centrifugation, we utilized a Percoll gradient for the isolation of the neuroendothelial cells. We used an isotonic solution of Percoll Plus (Sigma) consisting of 50% v/v Percoll and 50% v/v 0.9% w/v NaCl (Sigma). Then we formed a Percoll gradient by centrifuging the solution at 25,000 g at 4°C for 70 min. We gently pipetted the cell suspension onto the Percoll gradient and then centrifuged at 1,650 g at 4°C for 10 min. We removed the neuroendothelial cells from the middle layer and washed them via centrifugation with SIM.

Prior to seeding, we coated flasks and Transwells (Nunc) with filter-sterilized 0.1% w/v porcine gelatin (Sigma) for 1 h in the incubator. For cell expansion, we used T-25 and T-75 flasks (Thermo Fisher). For TEER assays and fluorescent microsphere assays, we seeded the cells on Transwell inserts in 24-well plates (Nunc) with a pore size of 0.4 μm . We plated the cells with a seeding density of 200,000 cells/cm² and kept them at 37 °C and 5% CO₂ in a CO₂ water-jacketed incubator (Forma Scientific, model 3130). We used neuroendothelial cell maintenance medium, consisting of DMEM (Gibco), 20 mM sodium bicarbonate (Gibco), 1% v/v Glutamax (Gibco), 150 $\mu\text{g/mL}$ Endothelial Cell Growth Factor (Sigma), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 $\mu\text{g/mL}$ Amphotericin B (Gibco, 1% v/v), 1% v/v Sodium Pyruvate (Gibco) and 20% v/v FBS (Gibco).

Once the cells reached 90% confluency, we washed them 3 times with DPBS without Ca²⁺ and Mg²⁺ (Gibco) and detached them in the incubator for 5 min. using 0.25% Trypsin in EDTA (Gibco). We centrifuged the cell suspension at 3,000 rpm for 3 min. For passaging, we resuspended the pellet in maintenance medium. For freezing, we used medium containing DMEM (Gibco), 20 mM sodium bicarbonate (Gibco), 1% v/v Glutamax (Gibco), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 $\mu\text{g/mL}$ amphotericin B (Gibco, 1% v/v), 20% v/v

Dimethyl Sulfoxide (DMSO, Sigma), and 40% v/v FBS (Gibco). We stored the neuroendothelial cells in cryotubes (Nunc) in liquid N₂.

Immunofluorescence and Microscopy

We fixed the neuroendothelial cells in 4% v/v Paraformaldehyde in PBS for a minimum of 30 min prior to blocking and permeabilization. For the cells that were treated with Propidium Iodide (Abcam), we administered 1:1000 Propidium Iodide for 2 h in the incubator prior to fixation. We permeabilized with 0.3% Triton-X (EMD Millipore) in PBS during blocking and incubations with primary and secondary antibodies. We blocked the samples for 1 h with 5% v/v Normal Goat Serum (Abcam) in PBS with 0.3% v/v Triton-X at room temperature. We diluted the primary and secondary antibodies in 2% v/v Normal Goat Serum in PBS with 0.3% Triton-X. Each incubation with primaries was for 2 days at 4°C with gentle shaking. Each incubation with secondary antibodies lasted 1 day at 4°C with gentle shaking in darkness.

In between incubations, we removed excess primary antibodies, secondary antibodies, and DAPI by washing the samples at least 3 times, totaling at least 30 min with PBS. For primary antibodies, we used CD31 anti-mouse (1:1000, Dako), and ZO-1 anti-rabbit (1:500, Invitrogen). We detected them with Alexa Fluor 488 goat anti-mouse IgG (1:2000, Invitrogen) and Alexa Fluor 568 goat anti-rabbit IgG (1:2000, Invitrogen). For nuclear staining, we incubated the cells with DAPI (1:25,000, Invitrogen) in PBS for 1 h at room temperature in darkness. To mount the samples and protect them from photobleaching, we used ProLong Antifade Diamond mountant (Invitrogen). The cells were immunostained on 0.4 µm polycarbonate Transwell inserts (Millipore). These inserts were translucent, which allowed for imaging the cells directly on the inserts with confocal microscopy.

We performed fluorescent imaging of the neuroendothelial cells with an LSM 700 inverted confocal microscope (Zeiss), which operated with Zen software. We used lasers with wavelengths of 405 nm, 488 nm, and 555 nm. These corresponded respectively to the

excitation wavelengths of DAPI, Alexa Fluor 488, and Alexa Fluor 568. We used 10X and 20X objective lenses.

Dexamethasone Synchronization

For circadian synchronization, we treated cells with 100 nM Dexamethasone (Thermo Fisher), diluted 1:1000 from a 100 mM stock solution dissolved in DMSO (Sigma). We collected half of the media, added the Dexamethasone to it, and then gently pipetted the solution back onto the cells. After a 2 h incubation at 37 °C and 5% CO₂, we removed the Dexamethasone by moving the Transwell inserts to new wells containing fresh media. We denoted the time of Dexamethasone removal as 0 h post-synchronization. We then replaced half of the media with fresh media every 24 h post-synchronization. The control groups, which did not undergo Dexamethasone synchronization, were also subjected to treatment with the equivalent volumes of DMSO. After 2 h, we moved the inserts to fresh media which did not contain Dexamethasone or DMSO.

TEER Assays

For TEER assays, we seeded the cells at a density of 200,000 cells/cm² in growth medium onto inserts in 24-well Transwell plates (Nunc). The inserts had polycarbonate membranes with 0.4 µm pores. Before plating, we coated the inserts with filter-sterilized 0.1% w/v gelatin for 1 h in the incubator. We performed TEER assays using the EVOM3, an Epithelial Volt/Ohm Meter from World Precision Instruments after observing that the cells were fully confluent. We placed the short electrode in the top compartment of the Transwell and the long electrode in the bottom compartment. An electrical current was applied through the electrodes, and the resistance was indicated. These values were inversely proportionate to the permeability of the monolayer. We obtained subsequent readings from the same wells at 4-h intervals. At each time point, the electrodes were cleaned gently after use with 70% ethanol. Before measurements, the electrode was re-chlorided with bleach for 15 min.

For normalization, the readings from blank wells were subtracted from the TEER values obtained from the wells containing cells. The blank wells consisted of Transwell inserts which were gelatin-coated and contained media but no neuroendothelial cells. The readings from each well were then normalized to the 0-h time point from that well. Each experiment consisted of 12 wells per group. We calculated the mean of the readings from all 12 wells.

Fluorescent Microsphere and Fluorescent Fibrinogen Permeability Assays

We used filter-sterilized 0.1% w/v gelatin to coat polycarbonate Transwell inserts (Nunc, pore size 0.4 μm) in the incubator for 1 h. We then plated the cells at a density of 200,000 cells/ cm^2 in growth medium without phenol red. We performed both types of permeability assays after ensuring that the cells were fully confluent by microscopic examination. We removed the neuroendothelial cell medium from the top compartment of each Transwell and replaced it with growth medium containing either fluorescent microspheres or fibrinogen. For fluorescent microspheres, we used carboxylate-modified TransFluoSpheres (Fisher, T8864) with a diameter of 0.04 μm and 488/560 absorbance/emission in a 1:1000 dilution. For fibrinogen permeability assays, we used 488-conjugated fibrinogen (Fisher) in a 1:1000 dilution. We then moved each Transwell insert to a blank well. We allowed the medium containing the microspheres or fibrinogen to permeate into the bottom compartment for 30 min in the incubator in darkness. Then, we transferred the medium from the receiver well to a 96-well plate for microplate reader analysis.

We used the Infinite F200 Pro microplate reader (Tecan) to quantify the fluorescence. We subtracted values obtained from wells containing only deionized water. Then, we further normalized the readings to the 0-h time points for each experimental group. Each replicate per experimental group consisted of the mean of 4 readings per time point.

Results and Discussion

Isolated Neuroendothelial Cells Display High Purity and Indications of Health

To determine the purity of the isolated neuroendothelial cells, we performed immunostaining for neuroendothelial cells, astrocytes, neurons, and pericytes. We found high expression of CD31, an endothelial cell marker, with low expression of the markers for astrocytes, neurons, and pericytes (ALDH1L1, β -III tubulin, and Desmin respectively) (Figure 6). This confirmed the presence of the neuroendothelial cells and the absence of astrocytes, neurons, and pericytes in the culture.

We also used immunostaining to monitor the health of the neuroendothelial cells for consecutive days after Dexamethasone treatment. Before fixation, we treated the cells with Propidium Iodide (PI), which penetrates damaged cell membranes and fluorescently labels their nuclei⁵⁷. We found low expression of PI, indicating that the cell membranes were intact, for the cells that were treated with Dexamethasone as well as the control group which was not (Figure 6). The neuroendothelial cells displayed a flat morphology (Figure 6), indicative of cell health⁵⁸. They also expressed CD31 primarily as apical puncta (Figure 6), which is the standard form of CD31 expression⁵⁸. We observed a high level of cellular confluence, as well as apical expression of the tight junction proteins Zonula Occludens-1 (ZO-1) and Claudin-5 (Figure 6). This expression pattern is also standard under conditions of cell health and high confluence⁵⁸. In addition, we found low PI staining, cellular flatness, apical CD31 puncta, high confluence, and apical expression of ZO-1 and Claudin-5 throughout all 4 days of culture on the Transwell inserts (Figure 6).

Barrier Tightness Changes According to Time of Day

We measured the tightness of the neuroendothelial cell monolayer by performing Transepithelial/Transendothelial Electrical Resistance (TEER) assays. We cultured the cells on Transwell inserts, semipermeable membranes which allowed a well on a culture dish to be separated into 2 compartments (Figure 7). In a TEER assay, an electrical current is applied

through a cellular monolayer (Figure 7). The electrical resistance is measured by electrodes and is directly proportionate to the tightness of the cells in the monolayer (Figure 7).

We cultured primary rat cerebral endothelial cells on the Transwells until they were visibly confluent under the microscope. Then we synchronized their circadian rhythms by adding 100 nM Dexamethasone, a glucocorticoid receptor agonist, for 2 h. This is a standard approach which is necessary to assess coherent *in vitro* circadian rhythms⁵⁹⁻⁶². *In vivo*, the suprachiasmatic nucleus in the brain synchronizes cellular circadian rhythms⁶³⁻⁶⁵. While cells *in vitro* have been shown to display circadian rhythms, they are largely out of synchrony with each other⁶⁰, which flattens individual cellular rhythms so that the rhythm of the population is obscured. Therefore, a synchronization step is required before circadian rhythm assays can be conducted. For comparison, we also performed these experiments in an unsynchronized control group, which underwent the same mechanical perturbations and received the same amount of Dimethyl Sulfoxide (DMSO), the solvent used in the Dexamethasone solution.

We conducted TEER assays for 4 days at 4-h intervals (Figure 8). The TEER values fluctuated significantly according to time of day with a period of approximately 12 h ($p=0.0132$, $n=3$, Figure 8, one-way repeated-measures ANOVA). The pattern was characterized by intervals of approximately 12 h in which a decrease from peak to trough was followed by an increase from trough to peak. The peaks in TEER values, coinciding with maximum barrier tightness, occurred approximately every 12 h, starting at the 0 h time point (Figure 8). No significant time-of-day difference was observed in the unsynchronized control group ($p=0.8708$, $n=3$, Figure 8). We observed the lowest TEER values at 8 h and 16 h post-synchronization in the first 24 h, and this pattern repeated every consecutive 24 h (Figure 8).

Previous research has demonstrated *in vivo* that permeability of the rat BBI is lowest during the subjective daytime and greatest during the subjective nighttime^{5,12}. In human cells, the opposite pattern has been observed, with BBI permeability decreasing during the day and increasing at night¹². This is consistent with the antiphase relationship between diurnal and

nocturnal animals for cells outside of the SCN⁶⁶. The results from our TEER assays (Figure 8) do demonstrate a trend in which peaks in barrier tightness are greater during times representing the day. However, this pattern differs from previous studies in that it has 1 peak every 12 h, rather than 1 peak every 24 h, as has been measured in *in vivo* studies with rodents and flies^{5,12}. It is possible that this 12-h vs. 24 h rhythm represents a difference between *in vitro* studies and *in vivo* studies, which factor in other variables such as sleep-wake patterns and locomotor activity. However, our findings (Figure 8) are consistent with one study which found that blood plasma levels of delta sleep-inducing peptide (DSIP) peaked in 12-h intervals in rats⁶⁷. It is possible that the rat neuroendothelial cells we isolated consist of two distinct populations which respond differently to cues for circadian-mediated changes in permeability.

Barrier Permeability Changes with Time of Day

In addition to the TEER assays, which measured barrier tightness/resistance to flow, we also measured barrier permeability with fluorescent microspheres. These carboxylate-modified TransFluoSphere beads were 0.04 μm in diameter and were loaded with a fluorescent dye that had a 488/560 absorbance/emission. We added the microspheres to the upper compartments of Transwell inserts for 30 min. We then used a microplate reader to quantify the amount of fluorescence that was able to permeate into the bottom compartments. These experiments were conducted in primary rat neuroendothelial cells synchronized with Dexamethasone. For comparison, we included a control group consisting of unsynchronized neuroendothelial cells subjected to the same mechanical perturbations and treated with the same volume of DMSO, the solvent used in the Dexamethasone. We conducted these assays at 4-h intervals for 36 h (Figure 9).

The permeability to the fluorescent microspheres fluctuated significantly according to time of day (one-way repeated-measures ANOVA, $p=0.0378$, $n=3$). The permeability peaked at the 8h, 16 h, and 32 h time points. It was lowest at the 0 h, 12 h, 24 h, and 36 h time points. This pattern was inversely related to the pattern we observed with our TEER assays (Figure 8),

which is consistent with the inverse relationship between barrier tightness and permeability. In the unsynchronized control group, there was no significant oscillation ($p=0.7158$, $n=3$, Figure 9).

Permeability to Fibrinogen Changes According to Time of Day

In vivo, fibrinogen is a soluble clotting factor that circulates in the blood. When the blood clotting process is initiated, the fibrinogen is converted to its insoluble form, fibrin. The most common type of stroke, an ischemic stroke, occurs when a blood clot blocks an artery and thereby the supply of blood to the brain. Starved of oxygen and glucose, the brain areas no longer receiving blood can undergo cell death and a loss of function. In addition, research has established that fibrinogen can trigger neuroinflammation, thus furthering pathogenesis in strokes⁶⁸⁻⁷⁰.

Research suggests that the leakage of fibrinogen across the BBI may contribute to ischemic stroke pathology^{68,71-73}. Fibrinogen production may oscillate with circadian rhythmicity¹³. Furthermore, factors that contribute to the dissolution of blood clots have been found to oscillate in accordance with the time of day¹⁴. Some of them, tPA and PAI-1, are regulated by genes that control circadian rhythms such as *Bmal1*, *Clock*, and the *Cry* genes¹⁵. Vulnerability of humans to stroke changes according to time of day^{2,16}, and it is possible that oscillations in BBI permeability contribute to this.

We measured the permeability of the neuroendothelial cell monolayer to fibrinogen by conducting permeability assays for fluorescence-tagged fibrinogen (Figure 9). We added fluorescence-tagged fibrinogen to the upper compartments of Transwell inserts for 30 min. We then used a microplate reader to quantify the amount of fluorescence that was able to permeate into the bottom compartments. We conducted these experiments at 4-h intervals for 36 h. We found that permeability to fluorescent fibrinogen peaked at the 8 h, 16 h, and 32 h time points. It was lowest at the 0 h, 12 h, 24 h, and 36 h time points. Permeability fluctuated significantly according to time of day for the synchronized experimental group ($p=0.0135$, $n=3$) but not the unsynchronized control group ($p=0.8142$, $n=3$). We obtained these results with one-way

repeated-measures ANOVA. This pattern was similar to what we observed in our fluorescent microsphere permeability assays (Figure 9). It is also inversely related to the pattern we observed with our TEER assays (Figure 8), which measured barrier tightness, the inverse of permeability.

Conclusions

Our key finding in this study is that neuroendothelial cells isolated from rat cortex self-organize in the TEER assay substrate so that they control movement of ions and small molecules across polycarbonate membranes with 0.4 μm pores in a time-of-day fashion. Strikingly, the peaks in barrier tightness and troughs in permeability occurred at 12 h intervals. Our Aim I data demonstrate that the clock genes *Per1* and *Bmal1* are expressed with 24 h rhythms under these conditions. This implies that a 12-h clock gene rhythm is not causing the 12 h rhythms in barrier tightness and permeability. However, factors other than clock genes can determine the circadian rhythmicity of biological processes⁷⁴. Twelve-hour rhythms have been reported in a variety of tissues⁷⁵⁻⁷⁷. Researchers have proposed models whereby 12 h biological rhythms may be caused by 24 h circadian rhythms that are 180° out of synchrony with each other⁷⁸.

Permeability in the glymphatic system is greatest during sleep⁴¹, while other studies have found BBI permeability *in vivo* to be greatest during active hours^{5,12}. It is possible that these two phenomena are related to the 12-h fluctuations in barrier tightness we observed. Twelve-hour rhythms have been reported for a variety of biological processes, including hormones⁷⁹, body temperature⁸⁰, and blood pressure⁸¹. One study identified about 3,000 genes that are expressed with 12-h rhythms, many of which are related to the endoplasmic reticulum and mitochondria⁸². They referred to these genes as circatidal, since tides have a 12-h rhythm⁸². The researchers speculated that animals including humans may have inherited these 12-h rhythms from aquatic ancestors as an adaptation to tidal rhythms⁸².

We isolated rat cerebral neuroendothelial cells and mouse blood microvessels (Figure 6). Through TEER assays and permeability assays, we verified that barrier tightness and permeability changed significantly according to time of day (Figures 8-9). Permeability to fluorescent microspheres or fibrinogen peaked at the 8 h, 16 h, and 32 h time points (Figure 9). They were both lowest at the 0-h, 12-h, 24-h, and 36-h time points (Figure 9). This is consistent with our TEER findings, which indicated that barrier tightness is lowest at 8-h, 16-h, and 32-h post-synchronization (Figure 8). Our TEER data also indicated that barrier integrity peaked at 12-h intervals starting at 0 h (Figure 8). This study is the first to demonstrate that neuroendothelial cells display intercellular fluctuations in permeability according to circadian rhythmicity.

Acknowledgments

Jennifer W. Mitchell and Canran Deng obtained a portion of the measurements for the TEER assays. This work was supported by the NIH (R61HL159948), NSF (DGE NRT-UtB 1735252 and STC CBET 0939511), and the 2021 Beckman Institute Graduate Fellows Program.

Figures

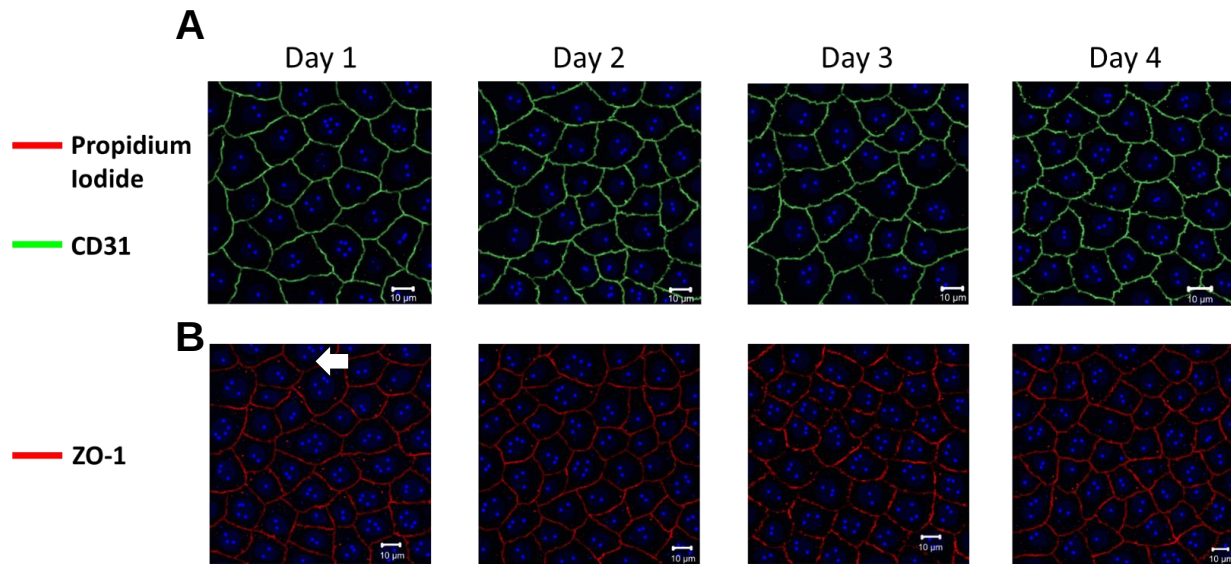


Figure 6: Neuroendothelial cells display high confluence and continued health when maintained on Transwells for multiple days.

A-B. Rat cerebral neuroendothelial cells cultured on Transwell inserts with polycarbonate membranes with 0.4 μm pores for 4 days after 2-h Dexamethasone treatment, 20X magnification, 10 μm scale bars.

A. Immunofluorescence for CD31 (green), an extracellular marker of junctional complexes, indicates the presence of endothelial cells. Propidium Iodide fluorescence in red indicates cells with damaged membranes.

B. Apical expression (indicated by white arrow) of the tight junction protein Zona Occludens-1 (ZO-1) indicates full confluence.

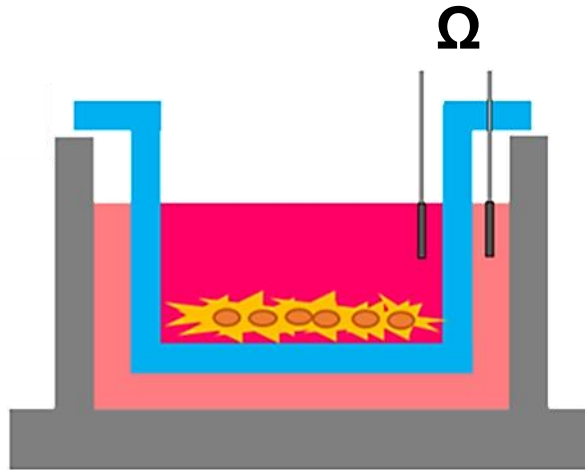


Figure 7: TEER assays measure barrier tightness, the inverse of permeability.

For Trans-Epithelial/Endothelial Resistance (TEER) assays, cells are cultured on Transwell inserts. These are semi-permeable membranes that separate the environment into 2 compartments. One electrode is placed in each compartment. An electrical current travels from one electrode to the other. In doing so, it must travel through the barrier of cells on the Transwell membrane. Greater barrier tightness results in more electrical resistance, which is factored into TEER values.

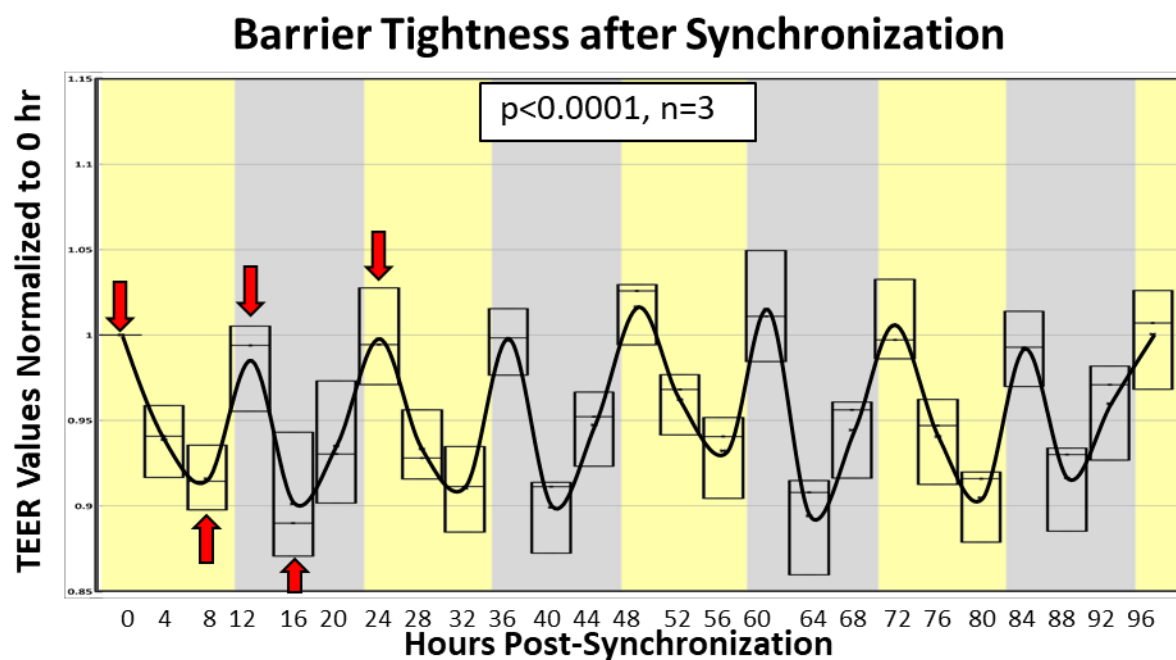


Figure 8: Barrier tightness of synchronized neuroendothelial cells varies with time of day.

Barrier tightness, as measured by TEER assays, oscillated significantly according to time of day ($p < 0.0001$, 5-12 measurements/timepoint, $n = 3$ independent experiments), but not in the unsynchronized control ($p = 0.9$, 5-12 measurements/timepoint, $n = 3$ independent experiments). Arrows denote peaks and troughs in barrier tightness. Repeated measurements were made in cultures post-Dexamethasone synchronization at the initiation of each experiment.

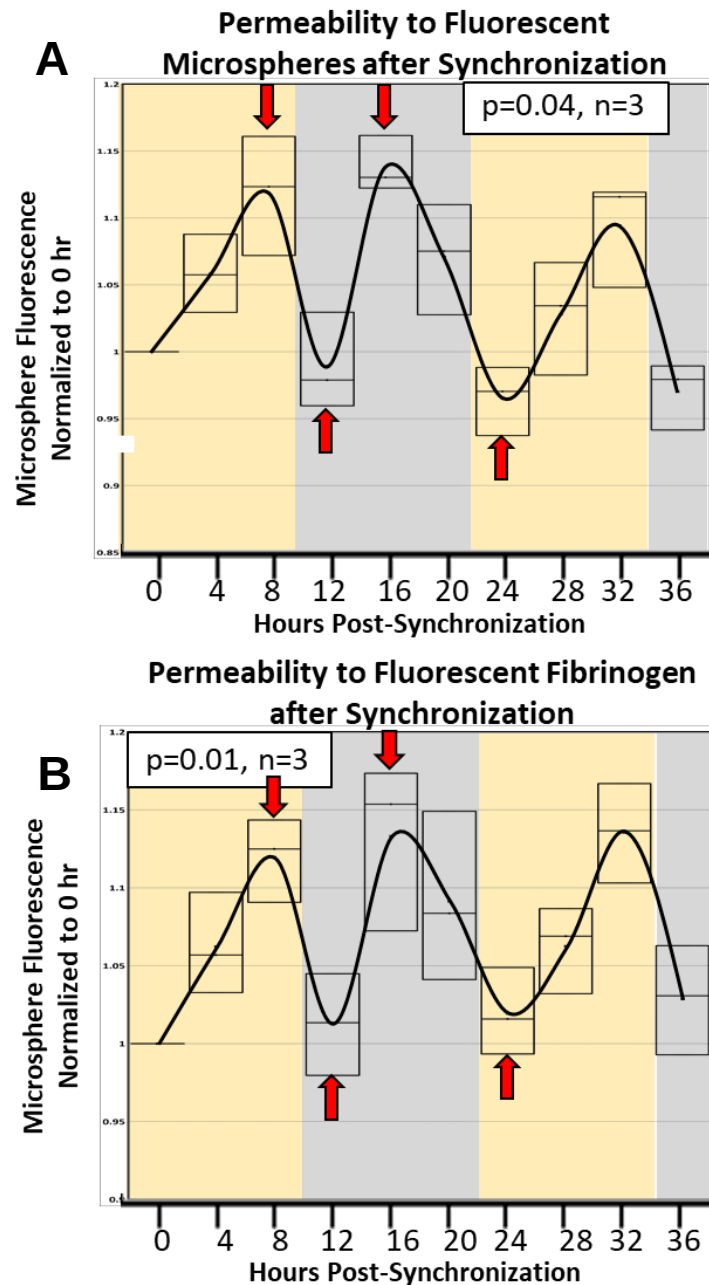


Figure 9: Neuroendothelial cell permeability varies with time of day and in anti-phase to the state of barrier tightness.

A. Permeability to fluorescent microspheres, 0.04 μm in diameter, fluctuates significantly according to time of day after Dexamethasone synchronization ($p=0.04, n=3$) but not in the unsynchronized control ($p=0.7, n=3$). There is a 12-h period between the troughs, an 8-h period between the first and second peaks, and a 16-h period between the second and third peaks. **B.** Permeability to 488-conjugated fluorescent fibrinogen also oscillates significantly with time of day after Dexamethasone treatment ($p=0.01, n=3$) but not in the unsynchronized control ($p=0.8, n=3$). On average, 15 fluorescent molecules are attached to each molecule of fibrinogen. **A-B.** Arrows denote peaks and troughs in permeability.

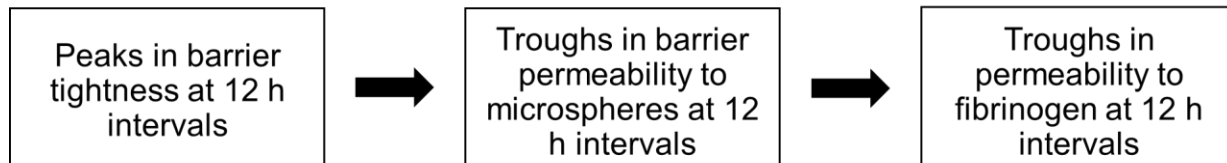


Figure 10: Summary of findings.

Barrier tightness peaks at 12 h intervals, starting at 0 h post-synchronization. It displays troughs 8 h and 16 h post-synchronization each day. Permeability to fluorescent microspheres and fluorescent fibrinogen demonstrate patterns inverse to this. They display troughs at 12 h, starting at 0-h post-synchronization. They also display peaks at 8 h and 16 h post-synchronization.

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CHAPTER 4: PHASE-SHIFTING OF NEUROENDOTHELIAL CELL PERMEABILITY BY A CHOLINERGIC AGONIST

Abstract

Phase shifts in circadian rhythms occur when the timing of a biological pattern under circadian control is moved forward or backwards in time. Determining the impacts of phase shifting cues on BBI permeability is a novel way to establish its susceptibility to circadian influence. The study presented here is the first to examine phase shifting in the circadian rhythmicity of BBI permeability, as well as in neuroendothelial cells. We have demonstrated that Carbachol, a pan-acetylcholine agonist, can induce a phase shift in the permeability of rat neuroendothelial cells through measurements of barrier integrity, permeability to fluorescent microspheres, and permeability to fluorescence-tagged fibrinogen. We have thus demonstrated that neuroendothelial cell permeability can undergo circadian phase shifts and that acetylcholine can regulate them.

Introduction

A common aspect of circadian rhythms is that the phase of these rhythms can be reset, inducing phase shifts. This occurs when the pattern of the rhythm is moved earlier or later than it was previously. One *in vivo* example of this in humans is jet lag. Phase shifts can be represented by graphing a biological pattern which is under circadian control and then demonstrating that this pattern has been shifted forward or backward in time. Phase shifts are a useful tool for determining the role that a given variable has in influencing the timing of circadian rhythms. They can also be used to parse out biological pathways that are controlled by circadian rhythms¹.

Studies have shown that *in vivo*, the most powerful tool for shifting circadian rhythms is light². Another significant signal is melatonin³. In an animal not experiencing circadian disruption, light during the day⁴ and melatonin secretion at night help to coordinate circadian rhythms with time of day⁵. This is known as entrainment. This is true for both diurnal (not

nocturnal) animals and nocturnal animals. However, light exposure predisposes nocturnal animals to sleep⁴, whereas melatonin does not have this effect on them⁵. Studies have also shown that exposure to light at night or melatonin administration during the day can shift circadian rhythms⁶⁻⁷. Cues such as these which can shift circadian rhythms are often referred to as Zeitgebers, which translates directly from German as “time giver”.

Depending on the type of Zeitgeber and the time of day it is administered, a phase advance or phase delay can occur. For instance, light administered in the early night has been shown to produce phase delays⁸, as though the brain perceives the daytime as having lasted longer. Light administered late at night has been shown to produce phase advances⁸, as though the brain perceives the daytime as having occurred earlier. These phase shifts are mediated by the neuronal response to light. In mammals, there are separate signaling pathways for how the brain responds to light during the day and the night. This also differs for nocturnal and diurnal animals⁹⁻¹⁰. There are also various biological cues that have demonstrated the ability to induce phase shifts in both nocturnal and diurnal animals.

Due to the three main mechanisms of oscillations and their myriads of biological pathways, there is a variety of potential influences on circadian rhythms. Transcriptional/translational loops and cyclical fluctuations in redox states and membrane excitability all confer cells with circadian rhythms. Studies have shown that there are many biological cues which can either entrain or shift circadian rhythms. While cells *in vitro* usually cannot be entrained to light, many of these biological cues can still be used to adjust the timing of their circadian rhythms. For instance, research has shown that melatonin can be used to adjust cellular circadian rhythms *in vitro*³. Studies have also shown that adding a shock of high serum¹¹, insulin, insulin-like growth factor 1¹², or forskolin¹³ (an activator of adenylyl cyclase) can synchronize circadian rhythms of cells *in vitro*.

The neurotransmitter Acetylcholine (ACh) exerts a variety of influences throughout the body. In the brain, it helps facilitate memory formation and recall¹⁴. It is also necessary for the

contraction or relaxation of skeletal and smooth muscles in mammals¹⁴. It acts on Nicotinic ACh Receptors (nAChRs), which are generally excitatory, and Muscarinic ACh Receptors (mAChRs), which are generally inhibitory. This leads to a wide variety of effects, depending on the receptor types to which ACh binds. Its combination of ubiquitousness throughout the body and versatility of function affords ACh its key roles in both the sympathetic and parasympathetic nervous systems¹⁴.

A variety of hormones, neurotransmitters, and neuromodulators are involved in the sympathetic and parasympathetic nervous systems. Of these, ACh is the main neurotransmitter in the parasympathetic nervous system. It also plays a role in the sympathetic nervous system. Norepinephrine is the primary neurotransmitter in the sympathetic nervous system, and it is released by most post-ganglionic neurons¹⁴. However, pre-ganglionic neurons release ACh, and there are post-ganglionic neurons that are cholinergic and innervate sweat glands¹⁴. In the parasympathetic nervous system, ACh is the primary neurotransmitter which neurons use when relaying messages to each other and to target tissues¹⁴. This results in a variety of effects, including blood vessel dilation, a reduction in heart rate, and the resumption of digestion and secretions¹⁴.

Studies featuring genetic knockouts of ACh receptors have provided further mechanisms into the various roles they have in organisms. These studies have typically targeted specific subunits of either nicotinic or muscarinic receptors¹⁵. One reason for this has been to elucidate the role of specific subunits. Research has also shown that mutations in cholinergic receptors can be embryonic lethal¹⁶. nAChR knockout mice have displayed restricted overall body growth, enlarged bladders, memory impairments, chronically dilated pupils, and heightened pain sensitivity¹⁷. One study found mortality in mice before weaning when only one subunit (alpha 3) was deleted¹⁷. Studies have shown that mice with various subtype-specific knockouts of mAChRs have a multitude of deficits. Depending on the receptor subtype, these knockouts

resulted in impairments in memory, smooth muscle contractions, and vasodilation, as well as hypothermia and heightened pain sensitivity¹⁵.

Neuroendothelial cells express ACh receptors¹⁸, and studies have shown that ACh exerts a variety of effects on them. ACh can be vasoconstrictive or vasodilating for neuroendothelial cells, depending on which type of receptor it binds to. Research has shown that ACh induces blood vessel contraction when binding to muscarinic ACh receptors (mAChRs) in neuroendothelial cells¹⁹. By contrast, ACh has been shown to induce dilation when binding to nicotinic ACh receptors (nAChRs)²⁰. Research has also demonstrated that activation of ACh receptors in neuroendothelial cells can also affect several biochemical pathways, leading to changes in angiogenesis and cell migration^{18,21}.

Novelty and Significance of this Study

The study presented here marks the first instance of phase shifting in the circadian rhythmicity of BBI permeability. This provides a new type of evidence for BBI permeability being under circadian control. This is also the first study to demonstrate that neuroendothelial cells can undergo circadian phase shifts. More specifically, this study found that in isolated neuroendothelial cells, activation of ACh receptors could induce a phase shift which could affect BBI permeability. The study presented here also provides further evidence of the role of circadian rhythms in neuroendothelial cells, which has yet to be thoroughly established in the literature. This also demonstrates a new potential role for ACh in the regulation of neuroendothelial circadian rhythms.

Knowing the impacts of phase shifting cues on BBI permeability may help establish its susceptibility to circadian rhythm changes. Phase shifting is highly relevant for people who are adjusting to new circadian schedules. This can include people who have traveled to a new time zone or undergone night shift work²². This can also include people who inadvertently expose themselves to Zeitgebers, such as light at night²². In keeping with the findings of this Aim, it is possible that people who experience activation of neural acetylcholine receptors in the early

night may undergo a shift in their circadian rhythms. In particular, the circadian rhythmicity of their BBI permeability may be shifted. In addition, there are also various medications which work by activating acetylcholine receptors. Taking these medications at earlier times may help patients avoid unintentionally shifting the circadian rhythmicity of their BBI permeability.

Review of Previous Literature

One of the earliest studies on Carbachol's phase shifting abilities was conducted by Zatz and Herkenham at the National Institute of Health in 1981²³. They injected Carbachol into mice intravenously. Carbachol injections at CT 15 produced phase delays in wheel-running activity rhythms of about 1.5 h²³. When administered at CT 22, they found phase advances²³. The authors did note that the phase advances and phase delays were not observed in every mouse that they studied²³. Prior to this study, they had injected Carbachol into the rat pineal gland in the late subjective day²⁴. They found that as a result, a step in melatonin synthesis was slower at 4 pm and faster at midnight²⁴. The authors had interpreted these findings as proof that ACh mediated the phase-shifting properties as light²³. Although this interpretation was later disproven^{1,25}, Zatz and Herkenham demonstrated that Carbachol can induce phase shifts²³ and that it is possible that melatonin synthesis plays a role²⁴.

In 1989, Wee and Turek explored the role of the cerebral cortex in mediating the phase-shifting effects of Carbachol²⁶. They administered Carbachol into the ventricles of the cortex of the Djungarian hamster and measured its effects on the circadian rhythmicity of locomotor activity. When administered at CT 12-16, Carbachol caused significant phase delays in locomotor activity²⁶, and when administered at CT 8, it caused significant phase advances²⁶. The researchers also compared these effects in blind and sighted animals to determine whether they were mediated by light. They found that the phase shifts, whether at CT 8 or at CT 12, were similar in magnitude for both the blind and sighted animals²⁶. Thus, they determined that light was not required to induce these phase shifts²⁶.

In 1992, the Turek lab injected Carbachol into the SCN as well as the cortex of Djungarian hamsters and found a phase-shifting pattern that was partially similar for both²⁷. When Wee *et al.* (1992) injected Carbachol into the lateral ventricles of the cortex at CT 14, a phase delay in locomotor activity resulted²⁷, consistent with their previous paper²⁶. However, Wee *et al.* (1992) found that Carbachol injection at CT 22 caused a phase advance²⁷, whereas in 1989, the injection at CT 22 failed to produce a significant phase advance²⁶. When injected into the SCN at CT 22, a phase advance in locomotor activity resulted²⁷. However, they did not find that a CT 14 injection produced a phase shift in the SCN²⁷.

In summary, both papers found that Carbachol administration into the cortex at CT 14 induced a phase delay²⁶⁻²⁷, and only one paper found a phase shift after administration at CT 22²⁷. In the SCN, the researchers did not observe a phase shift at CT 14 but did find that a phase advance occurred after administration at CT 22²⁷. When interpreting this data, it is important to consider that in nocturnal animals, circadian neuronal activity in the SCN is typically antiphase to circadian activity outside of the SCN. This makes it unlikely that the same pattern of phase shifts would be observed in the cortex and the SCN. The findings which are consistent with both papers and this antiphase relationship are that Carbachol administration causes a phase delay at CT 14 in the cortex and a phase advance at CT 22 in the SCN.

In previous decades, it was thought that light-induced phase shifts were mediated by ACh^{23,36}. This was due to the similar phase shifts induced by light and cholinergic agonists²⁶. However, subsequent studies have demonstrated that the pathway for Carbachol-induced phase shifts is separate from the pathway for light-induced phase shifts²⁵. One of the first papers to demonstrate this blocked ACh activity and found that light-induced phase shifts of locomotor activity were unaffected²⁵. Pauly and Horseman (1985) administered Hemicholinium-3 to the rat SCN. This compound blocks the reuptake of choline, thus preventing a necessary step in the synthesis of ACh²⁵. In addition, they treated the SCN with atropine, an ACh antagonist²⁵. In both experiments, they found that a light pulse at the late subjective night

induced a phase advance, and a light pulse in the early subjective night induced a phase delay²⁵. These light-induced phase shifts were consistent with previous literature and demonstrated that they were not mediated by ACh²⁵.

However, the field of circadian research was once not in agreement about whether ACh or Glu was most responsible for mediating the effects of light in mammals. This was known as the Carbachol paradox¹. Carbachol (a pan-ACh agonist) had been shown to evoke a light-like response, although the retinohypothalamic tracts which transmit light signals to the SCN are glutamatergic and PACAPergic, rather than cholinergic¹. Buchanan and Gillette (2005) resolved this paradox by demonstrating that the light-like effects of Carbachol in rodents occurred only when it was injected outside of the SCN¹. Moreover, light has been shown to exert its effects via the retinohypothalamic tracts which lead directly to the SCN. Therefore, the extra-SCN light-like effects of Carbachol were attributable to a pathway separate from the transduction of light¹. This study found that when Carbachol was injected into the ventricles, a phase advance or phase delay could occur, depending on the time of injection¹. However, when Carbachol was injected directly into the SCN at the same time points in the circadian cycle, the result each time was only a phase advance¹. This pattern was replicated *in vitro* as well as *in vivo*¹.

Knowing the impacts of Zeitgebers on BBI permeability can help establish its susceptibility to circadian rhythm entrainment. This would provide further evidence for the circadian rhythmicity of BBI permeability. In addition, this has potential relevance for human health, since Zeitgebers, both endogenous and exogenous, may either interfere with or remedy BBI permeability. ACh is the main neurotransmitter in both the sympathetic and parasympathetic nervous systems. As such, it exerts a variety of effects on neuroendothelial cells. The pan-ACh agonist Carbachol is a proven phase-shifting cue^{1,26-27}. However, no published studies have featured Carbachol as a phase-shifting cue for neuroendothelial cells, which are a key component of the BBI. For this aim, we have tested the ability of Carbachol, an ACh agonist, to act as a Zeitgeber for endothelial cell permeability. We have treated primary

neuroendothelial cells from the rat cerebral cortex with 100 μ M Carbachol for 1 h. We hypothesized that treatment with Carbachol would produce a phase delay when administered at 14 h post-synchronization and a phase advance at 20 h. The control group was treated with deionized water of the same volume and underwent the same mechanical perturbations.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign approved all animal use protocols. We followed the National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals. We used Long-Evans/Bluegill rats which were maintained at the University of Illinois at Urbana-Champaign. We performed cerebral neuroendothelial cell isolations for rats 3-7 weeks old. We used the minimum number of animals necessary for experiments.

Neuroendothelial Cell Isolation and Culture

For neuroendothelial cell isolation, we followed an established protocol²⁸. For each dissection, we removed brains from at least 5 Long Evans rats, 3-7 weeks old. We placed the brains in 70% ethanol and removed the surface vessels with a swab applicator. We then transferred the brains to Standard Isolation Medium (SIM), consisting of Medium 199 (Gibco), 20 mM HEPES (Gibco), 25 mM sodium bicarbonate (Gibco), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 μ g/mL amphotericin B (1% v/v, Gibco), and 5% v/v Fetal Bovine Serum (FBS, Gibco). Before use, we adjusted the pH of the medium to 7.4.

We minced the brains in the SIM, centrifuged, and resuspended the pellet in 0.02% w/v Dispase (Sigma) in SIM in the incubator for 1 h for dissociation. After centrifugation, we resuspended the pellet in 15% w/v Dextran (Sigma) in SIM which separated out the fat pad. We then removed the Dextran/fat pad solution through additional centrifugation. For further dissociation, we resuspended the tissue pellet in 0.1% w/v Collagenase/Dispase (Sigma) in SIM in the incubator for 30 min.

After washing with SIM via centrifugation, we utilized a Percoll gradient for the isolation of the neuroendothelial cells. We used an isotonic solution of Percoll Plus (Sigma) consisting of 50% v/v Percoll and 50% v/v 0.9% w/v NaCl (Sigma). Then we formed a Percoll gradient by centrifuging the solution at 25,000 g at 4°C for 70 min. We gently pipetted the cell suspension onto the Percoll gradient and then centrifuged at 1,650 g at 4°C for 10 min. We removed the neuroendothelial cells from the middle layer and washed them via centrifugation with SIM.

Prior to seeding, we coated flasks and Transwells (Nunc) with filter-sterilized 0.1% w/v porcine gelatin (Sigma) for 1 h in the incubator. For cell expansion, we used T-25 and T-75 flasks (Thermo Fisher). For TEER assays and fluorescent microsphere assays, we seeded the cells on Transwell inserts in 24-well plates (Nunc) with a pore size of 0.4 μm . We plated the cells with a seeding density of 200,000 cells/cm² and kept them at 37 °C and 5% CO₂ in a CO₂ water jacketed incubator (Forma Scientific, model 3130). We used neuroendothelial cell maintenance medium, consisting of DMEM (Gibco), 20 mM sodium bicarbonate (Gibco), 1% v/v Glutamax (Gibco), 150 $\mu\text{g/mL}$ Endothelial Cell Growth Factor (Sigma), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 $\mu\text{g/mL}$ Amphotericin B (Gibco, 1% v/v), 1% v/v Sodium Pyruvate (Gibco) and 20% v/v FBS (Gibco).

Once the cells reached 90% confluence, we washed them 3 times with DPBS without Ca²⁺ and Mg²⁺ (Gibco) and detached them in the incubator for 5 min. using 0.25% Trypsin in EDTA (Gibco). We centrifuged the cell suspension at 3,000 rpm for 3 min. For passaging, we resuspended the pellet in maintenance medium. For freezing, we used medium containing DMEM (Gibco), 20 mM sodium bicarbonate (Gibco), 1% v/v Glutamax (Gibco), 1% v/v Penicillin/Streptomycin mixture (Corning), 2.5 $\mu\text{g/mL}$ amphotericin B (Gibco, 1% v/v), 20% v/v Dimethyl Sulfoxide (DMSO, Sigma), and 40% v/v FBS (Gibco). We stored the neuroendothelial cells in cryotubes (Nunc) in liquid N₂.

Immunofluorescence and Microscopy

We fixed neuroendothelial cells in 4% v/v Paraformaldehyde in PBS for a minimum of 30 min prior to blocking and permeabilization. For the cells that were treated with Propidium Iodide (Abcam), we administered 1:1000 Propidium Iodide for 2 hr in the incubator prior to fixation. We permeabilized with 0.3% Triton-X (EMD Millipore) in PBS during blocking and incubations with primaries and secondaries. We blocked the samples for 1 hr with 5% v/v Normal Goat Serum (Abcam) in PBS with 0.3% v/v Triton-X at room temperature. We diluted the primary and secondary antibodies in 2% v/v Normal Goat Serum in PBS with 0.3% Triton-X. Each incubation with primaries was for 2 days at 4°C with gentle shaking. Each incubation with secondaries lasted 1 day at 4°C with gentle shaking in darkness.

In between incubations, we removed excess primary antibodies, secondary antibodies, and DAPI by washing the samples at least 3 times, totaling at least 30 min with PBS. For primary antibodies, we used CD31 anti-mouse (1:1000, Dako), and ZO-1 anti-rabbit (1:500, Invitrogen). We detected them with Alexa Fluor 488 goat anti-mouse IgG (1:2000, Invitrogen) and Alexa Fluor 568 goat anti-rabbit IgG (1:2000, Invitrogen). For nuclei staining, we incubated the cells with DAPI (1:25,000, Invitrogen) in PBS for 1 h at room temperature in darkness. To mount the samples and protect them from photobleaching, we used ProLong Antifade Diamond mountant (Invitrogen). Some cells were immunostained on glass coverslips (Esco Optics), and other cells were immunostained on 0.4 µm polycarbonate Transwell inserts (Millipore). These inserts were translucent, which allowed for imaging the cells directly on them with confocal microscopy.

We performed fluorescent imaging of the neuroendothelial cells with an LSM 700 inverted confocal microscope (Zeiss), which operated with Zen software. We used lasers with wavelengths of 405 nm, 488 nm, and 555 nm. These corresponded respectively to the excitation wavelengths of DAPI, Alexa Fluor 488, and Alexa Fluor 568. We used 10X and 20X objective lenses.

Dexamethasone Synchronization

For circadian synchronization, we treated cells with 100 nM Dexamethasone (Thermo Fisher), diluted 1:1000 from a 100 mM stock solution dissolved in DMSO (Sigma). We collected half of the media, added the Dexamethasone to it, and then gently pipetted the solution back onto the cells. After a 2 h incubation at 37 °C and 5% CO₂, we removed the Dexamethasone by moving the Transwell inserts to new wells containing fresh media. We denoted the time of Dexamethasone removal as 0 h post-synchronization. We then replaced half of the media with fresh media every 24 h post-synchronization. The control groups which did not undergo Dexamethasone synchronization were still subjected to treatment with the equivalent volumes of DMSO. After 2 h, we moved the inserts to fresh media which did not contain Dexamethasone or DMSO.

Studies have shown that incubation with high concentrations of Dexamethasone can tighten the BBI²⁹⁻³¹. To avoid this issue, we treated the neuroendothelial cells with 100 nM Dexamethasone for 2 hr. These were standard concentrations that had been validated by the literature³²⁻³⁴. We also removed the Dexamethasone by pipetting off the media in the Transwell inserts and then quickly placing them in new wells containing fresh media. If Dexamethasone treatments had resulted in an increase in barrier tightness, this would have been reflected in increased TEER values or a decrease in permeability to fluorescent beads or fibrinogen. However, the group synchronized with Dexamethasone and the control group which was not Dexamethasone-treated displayed similar ranges in both barrier tightness as well as permeability.

Another potential confounding variable would have been an unintentional serum shock, whereby cells are deprived of serum and then high amounts are introduced. This is a distinct method for circadian synchronization¹¹. By moving the Transwell inserts to wells containing Dexamethasone-free media, we ensured that the process of Dexamethasone removal was quick. This maximized the amount of time that the cells were in contact with media. The

Dexamethasone-free media in the new wells also contained the same concentrations of serum as the media containing the Dexamethasone. After Carbachol treatment, we performed the same procedure for moving the inserts to new wells containing media. Throughout the 4-day TEER assays, we changed the media by replacing only half, so that the cells were never deprived of media. We also performed the media changes at the same time of day that the Transwell inserts were placed in the non-Dexamethasone media after treatment. An unintentional serum shock could have resulted from allowing the media to become deprived of serum before media changes. We prevented this by replacing half of the media each day. If an unintentional serum shock had occurred, we could have used KnockOut Serum Replacement in combination with endothelial-specific growth factors.

Carbachol Administration

There were several traits of Carbachol which reduced potential confounding variables during its administration. For instance, Carbachol can be dissolved in deionized water, which lowers the likelihood of its solvent having off-target effects. Since Carbachol is hydrophilic, it does not tend to form aggregates in solution³⁵. In addition, we diluted it from a stock solution one-thousand-fold, resulting in minute amounts of water being added to the Carbachol treatment groups. Research has also shown that it can remain stable in media for consecutive days³⁵. Cells have also displayed functionality after concentrated and prolonged Carbachol treatment. For instance, one study demonstrated that cells could undergo 1 mM Carbachol for up to 24 h before washes with no statistically significant effect on general transcription³⁶. Moreover, Carbachol has been featured in a variety of clinical trials with human patients³⁷⁻³⁹.

To date, there is little published literature regarding the effects of Carbachol on BBI permeability. This research has also not focused on neuroendothelial cells cultured *in vitro*. Furthermore, the available studies have found Carbachol to have somewhat conflicting effects. One study found that Carbachol prevented BBI disruption in rat endothelial cells by inhibiting Tumor Necrosis Factor alpha⁴⁰. This pro-inflammatory factor is also not commonly present in

healthy cells. By contrast, another study found that Carbachol had a pro-inflammatory effect in the mouse lungs⁴¹. It is possible that *in vivo*, Carbachol might have multiple indirect means of influencing BBI permeability. However, our research has shown that Carbachol treatment of neuroendothelial cells *in vitro* does not alter their permeability beyond shifting its circadian rhythmicity. Compared with the control group, the Carbachol-treated group displayed values in the same ranges for the TEER assays and both permeability assays. If we had observed a change in permeability or barrier tightness resulting from Carbachol administration, we would have tested the phase-shifting properties of a different chemical. There are various other compounds which have demonstrated phase-shifting capabilities¹²⁻¹³.

We determined the concentration and duration of Carbachol treatment by referencing previous studies. Most of the studies featuring Carbachol as a Zeitgeber have been performed *in vivo*. However, there are several *in vitro* studies which demonstrated the ability of Carbachol to induce phase shifts. Most of these studies have utilized 100 μ M microdrops of Carbachol, which were applied directly to the surface of SCN slices for 5-10 min^{1,42-43}. A slight majority of the remaining *in vitro* studies also used 100 μ M Carbachol but did so through bath application, in which Carbachol is diluted in larger amounts of cell culture media and the cells were immersed in it. As a result, these studies required longer durations of Carbachol treatment. They demonstrated phase shifts after treating cells with 100 μ M Carbachol for 1 h⁴⁴, 50 μ M for an unspecified duration⁴⁵, and 10 μ M for 1 h⁴⁶. While microdrops are an option for tissue slices, they are typically not used for primary cells, which are at a risk of detaching due to mechanical stress. In keeping with these studies, we treated the neuroendothelial cells with a 100 μ M Carbachol for 1 h.

Several articles helped us determine when to administer Carbachol. For instance, rodent tissues outside of the SCN have been shown to display a phase delay when treated with Carbachol at CT 14²⁶⁻²⁷. In addition, one study found that Carbachol caused a phase advance when administered at CT 8²⁶. Liu and Gillette (1996) found that in the SCN, Carbachol only

induced a significant phase shift when administered during the subjective night⁴². However, it is established that the SCN and peripheral tissues respond differently to Carbachol as a phase-shifting cue^{1,25-26}. Research has also shown that cells outside of the SCN can demonstrate a greater sensitivity to circadian resetting⁴⁷. Based on these studies, we hypothesized that treatment with Carbachol 14 h post-synchronization would produce a phase delay.

We had to consider that treating the neuroendothelial cells with Carbachol 14 h post-synchronization might not have produced a phase shift. If this had occurred, we would have implemented a concentration gradient, testing different concentrations of Carbachol. We also would have tried administering it at additional time points. Studies have typically administered Carbachol at time points that were several hours apart. For instance, Wee and Turek (1989) administered Carbachol at CT 8, 12, 14, and 20²⁶. Another option would have been to administer Carbachol for longer intervals before its removal. If an inability to induce phase shifts persisted after these approaches, we would have administered other ACh receptor agonists. For instance, research has shown that ACh⁴³ and nicotine (an agonist of nicotinic ACh receptors)⁴⁸ can function as cues for phase-shifting. Studies have also shown that agonists of muscarinic ACh receptors, such as muscarine and McN-A-343 are able to induce phase shifts in circadian rhythms⁴².

We designed the control group to account for any confounding variables that could occur during media changes or Carbachol administration. During Carbachol administration, the control group received deionized water in the same volumes as the Carbachol-treated group. This served as a vehicle control, since the Carbachol stock solution was dissolved in deionized water. The control group also underwent the same volumes of media removal and replacement as the Carbachol-treated group. This controlled for the potential confounding variable of a serum shock occurring during a media change¹¹. The control group also underwent the same mechanical movements as the Carbachol-treated group. Research has shown that mechanical stress can influence fundamental cellular processes including gene expression in cells⁴⁹. In

particular, neuroendothelial cells have been shown to be susceptible to the force applied by flowing solutions, known as shear flow⁴⁹. We also kept the control group in ambient air and ambient temperature in the tissue culture biosafety cabinet for the same duration as the experimental group during Carbachol treatment. This allowed us to control for the exposure to ambient air, which contains higher CO₂ levels, and ambient temperatures, which are lower outside of the incubator.

TEER Assays

For TEER assays, we seeded the cells at a density of 200,000 cells/cm² in growth medium onto inserts in 24-well Transwell plates (Nunc). The inserts had polycarbonate membranes with 0.4 µm pores. Before plating, we coated the inserts with filter-sterilized 0.1% w/v gelatin for 1 h in the incubator. We performed TEER assays using the EVOM3 from World Precision Instruments after observing that the cells were fully confluent. We placed the short electrode in the top compartment of the Transwell and the long electrode in the bottom compartment. An electrical current was applied through the electrodes, and the resistance was indicated. These values were inversely proportionate to the permeability of the monolayer. We obtained subsequent readings from the same wells at 4 h intervals. At each time point, the electrodes were cleaned gently after use with 70% ethanol. Before measurements, the electrode was re-chlorided with bleach for 15 min.

For normalization, the readings from blank wells were subtracted from the TEER values obtained from the wells containing cells. The blank wells consisted of Transwell inserts which were gelatin-coated and contained media but no neuroendothelial cells. The readings from each well were then normalized to the 0 h time point from that well. Each experiment consisted of 12 wells per group. We calculated the mean of the readings from all 12 wells.

Fluorescent Microsphere and Fluorescent Fibrinogen Permeability Assays

We used filter-sterilized 0.1% w/v gelatin to coat polycarbonate Transwell inserts (Nunc, pore size 0.4 µm) in the incubator for 1 h. We then plated the cells at a density of 200,000

cells/cm² in growth medium without phenol red. We performed both types of permeability assays after ensuring that the cells were fully confluent by microscopic examination. We removed the neuroendothelial cell medium from the top compartment of each Transwell and replaced it with growth medium containing either fluorescent microspheres or fibrinogen. For fluorescent microspheres, we used carboxylate-modified TransFluoSpheres (Fisher, T8864) with a diameter of 0.04 μ m and 488/560 absorbance/emission in a 1:1000 dilution. For fibrinogen permeability assays, we used 488-conjugated fibrinogen (Fisher) in a 1:1000 dilution. We then moved each Transwell insert to a blank well. We allowed the medium containing the microspheres or fibrinogen to permeate into the bottom compartment for 30 min in the incubator in darkness. Then, we transferred the medium from the receiver well to a 96-well plate for microplate reader analysis.

We used the Infinite F200 Pro microplate reader (Tecan) to quantify the fluorescence. We subtracted values obtained from wells containing only deionized water. Then we further normalized the readings to the 0 h time points for each experimental group. Each replicate per experimental group consisted of the mean of 4 readings per time point.

Quantitative Real-Time Reverse Transcriptase PCR

We isolated RNA with Trizol (Invitrogen) and alcohol precipitation. We performed reverse transcription with 1 μ g of isolated RNA and 200 ng Random Hexamers (Invitrogen), and DEPC water (Invitrogen) to 12 μ L per conical tube. We denatured the secondary structures in this solution by heating at 85°C for 10 min. Then we added 5X reverse transcription buffer, 10 mM DTT, 0.5 mM dNTPs, and Superscript IV RNase H Reverse Transcriptase (all from Invitrogen). We heated the solution at 42°C for 1 h and terminated the reaction by heating at 92°C for 10 min. We diluted the samples with RNase-free water (Invitrogen) and stored them at -80°C.

We then performed qPCR with SYBR Green (Lonza), 0.25 mM dNTPs (Denville), and Taq polymerase. For rat *Per1*, we used a Forward primer with the sequence:

AGGAGGCCCCGGAAGTAGT and a reverse primer with the sequence: AGCCTGAAAGTGCATCCTGATT). For rat *Bmal1*, we used a forward primer with the sequence: TCTATCCGATGACGAACTGAA and a reverse primer with the sequence: CCCTCGGTCACATCCTACAG. For our loading control, we used Acidic ribosomal protein PO with the forward primer sequence: CGTGATGCCCAGGGAAGA and the reverse primer sequence: TCCCACAATGAAGCATTTTGG.

Results and Discussion

To determine the purity of the isolated neuroendothelial cells, we performed immunostaining for neuroendothelial cells, astrocytes, neurons, and pericytes. We found high expression of CD31, an endothelial cell marker, with low expression of the markers for astrocytes, neurons, and pericytes (ALDH1L1, β -III tubulin, and Desmin respectively) (Figure 11). This confirmed the presence of endothelial cells and the absence of astrocytes, neurons, and pericytes in the culture.

We also used immunostaining to monitor the health of the neuroendothelial cells for consecutive days after treatment with Dexamethasone and Carbachol. Before fixation, we treated the cells for 2 h with Propidium Iodide (PI), which penetrates damaged cell membranes and fluorescently labels their nuclei⁵⁰. We found low expression of PI, indicating that the cell membranes were intact, for the cells that were treated with Dexamethasone and the cells that were treated with Carbachol (Figure 11). In both of these groups and the untreated control group, the neuroendothelial cells displayed a flat morphology (Figure 11), indicative of cell health⁵¹. In all 3 groups, they also expressed CD31 primarily as apical puncta (Figure 11), which is the standard form of CD31 expression⁵¹. We also observed a high level of cellular confluence, as well as apical expression of the tight junction proteins Zonula Occludens-1 (ZO-1) and Claudin-5 in all 3 groups (Figure 11). This expression pattern is also standard under conditions of cell health and high confluence⁵¹. In addition, we found low PI staining, cellular flatness,

apical CD31 puncta, high confluence, and apical expression of ZO-1 and Claudin-5 throughout all 4 days of culture on the Transwell inserts (Figure 11).

We conducted TEER assays after administering 100 μ M Carbachol for 1 h at 14 h post-synchronization (Figure 12). In addition, a control group received deionized water in equal volumes and underwent the same mechanical perturbations as the Carbachol-treated group. Initially, before Carbachol treatment, we observed a similar pattern for both groups, with barrier tightness peaking in 12 h intervals starting at 0 h post-synchronization (Figure 12). Then, after Carbachol administration, we observed a phase delay in alternating peaks (Figure 12). These phase delays occurred only during the time points consistent with subjective nighttime. For instance, in the control group, barrier tightness peaked at 36 h post-synchronization, whereas in the Carbachol-treated group, barrier tightness peaked at 40 h. In addition, the peak observed at 60 h post-synchronization in the control group was also present at 64 h, and the control group peaked at 84 h, whereas the Carbachol-treated group peaked at 88 h (Figure 12). The overall finding that Carbachol administration at 14 h post-synchronization can cause phase delays is consistent with previous literature demonstrating shift in locomotor activity during early night²⁶⁻²⁷.

The effects of Carbachol appeared to differ depending on whether the oscillation peak is during subjective daytime or nighttime (Figure 12). It is possible that there are two 24-h oscillators which are 12 hr apart. This is one possible explanation for the 12 hr rhythms we observed for permeability and barrier tightness in the synchronized cells that were not treated with Carbachol (Figure 12). Of these two potential oscillators, it may be that only one responds to Carbachol, and this occurs during the subjective nighttime. Another possibility is that the effects of Carbachol we observed are more influential during the subjective nighttime. There may also be multiple types of neuroendothelial cells present, only a portion of which respond to Carbachol or which respond to it in different ways. These potential different types may respond in ways which are additive only during the subjective nighttime, thus making the effects of Carbachol more influential during this time.

We observed similar phase delays for the fluorescent microsphere and fluorescence-tagged fibrinogen permeability assays (Figures 13-14). In the absence of Carbachol, they displayed their lowest permeability at 0 h, 12 h, 24 h, and 36 h post-synchronization (Figures 13-14). Their permeability peaked at 8 h, 16 h, and 32 h (Figures 13-14). After the addition of Carbachol at 14 h post-synchronization, this pattern remained consistent for the trough at 24 h and the peak at 32 h, which were more consistent with daytime. However, during the time period that was more consistent with nighttime, the peak in permeability occurred at 20 h, instead of 16 h (Figures 13-14). This indicated that a 4 h phase delay occurred but only during the time points more consistent with nighttime.

Our qPCR results are also consistent with a phase delay after the addition of Carbachol at 14 h post-synchronization. In the absence of Carbachol, the Dexamethasone-synchronized cells displayed peaks in *Per1* expression at 12 h and 36 h (Figure 15). After Carbachol was added 14 h post-synchronization, the peak occurred at 40 h instead of 36 h (Figure 15). This result is consistent with a phase delay of approximately 4 h. Similarly, in the absence of Carbachol, *Bmal1* expression displayed troughs at 12 h and 36 h (Figure 15). However, after Carbachol at 14 h post-synchronization, the trough also occurred at 40 h instead of 36 h (Figure 15). Since the 36 h and 40 h time points mimic nighttime more than daytime, it is possible that this shifting only occurred during the time points which represent nighttime.

We administered Atropine, a mAChR inhibitor, and Mecamylamine, a nAChR inhibitor to examine the effects of each receptor type on the phase shifts we observed. We found that when we added Carbachol at 14 h and blocked mAChRs, the peaks and troughs occurred at the same time points as when no Carbachol was added (Figures 12-14). This effect applied to our measurements of barrier tightness and permeability assays with fluorescent microspheres and fluorescent fibrinogen (Figures 12-14). These results indicated that the phase shifting was mediated through mAChRs, such that blocking them was sufficient to prevent it. By contrast, when we antagonized nAChRs, the addition of Carbachol at 14 h produced the same phase

shifts in barrier tightness and permeability as without the inhibitor (Figures 12-14). This indicated that these results were not mediated through nAChRs. When mAChRs or nAChRs were inhibited in the absence of Carbachol, the inhibitors alone did not induce phase shifts (Appendix A).

We also observed phase shifts when we administered Carbachol at 20 h post-synchronization. Our TEER assays and permeability assays with fluorescent microspheres and fluorescent fibrinogen indicated phase advances (Figure 16). These phase advances also occurred during the time points representing the nighttime but not the daytime (Figure 16). Without Carbachol, barrier tightness peaked at 0 h and then repeatedly every 12 h, and it displayed troughs at 8 h and 16 h each day (Figure 16). After Carbachol administration at 20 h, instead of a peak occurring at 36 h, a trough occurred (Figure 16). This made the barrier tightness most similar to that observed at 32 h or at 40 h, presenting a challenge for determining whether a phase advance or phase delay had occurred. However, at 44 h, a peak appeared which was closer to that observed at 48 h than 40 h (Figure 16). This indicated that a phase advance had occurred.

We observed a similar pattern with the permeability to fluorescent microspheres and fluorescent fibrinogen. In the absence of Carbachol, they peaked at 8 h and 16 h (Figures 17-18). They displayed troughs at 0 h, 12 h, 24 h, 36 h, and 48 h post-synchronization (Figures 17-18). When we administered Carbachol at 20 h post-synchronization, we found that the 36 h time point did not display the trough that it did in the absence of Carbachol (Figures 17-18). This result was the inverse of the lowered TEER values at 36 h which resulted from Carbachol administration at 20 h. This reflected a possible phase advance at the 36 h time point, similar to that observed with barrier tightness (Figures 17-18).

Our results with qPCR also indicated possible phase advances after Carbachol was administered 20 h post-synchronization (Figure 19). In the control group which was only synchronized with Dexamethasone, *Per1* expression peaked at 12 h and 36 h (Figure 19). It

displayed troughs at 24 h and 48 h (Figure 19). After the addition of Carbachol at 20 h post-synchronization, the peak at 48 h was preceded by a peak at 44 h (Figure 19). The 44 h time point was closer in magnitude to the 48 h time point than any other time point in an apparent phase advance of about 4 h, resulting in 2 back-to-back peaks (Figure 19). We observed a similar pattern with *Bmal1*. In the absence of Carbachol, *Bmal1* expression displayed troughs at 12 h and 36 h and peaks at 24 h and 48 h (Figure 19). After the addition of Carbachol at 20 h post-synchronization, we observed 2 back-to-back peaks at 44 h and 48 h (Figure 19). This appeared to indicate a phase advance of approximately 4 h. For both *Per1* and *Bmal1*, the time points before the addition of Carbachol and those which most mimicked the daytime did not display phase shifts. This is consistent with our data on barrier tightness and permeability, which have demonstrated phase advances occurring only during the time points which most mimic nighttime (Figure 19).

We administered Atropine, a mAChR inhibitor, and Mecamylamine, a nAChR inhibitor to examine the effects of each receptor type on the phase shifts we observed. We found that when we added Carbachol at 20 h and blocked mAChRs, the peaks and troughs occurred at the same time points as when no Carbachol was added (Figures 16-18). This effect applied to our measurements of barrier tightness and permeability assays with fluorescent microspheres and fluorescent fibrinogen (Figures 16-18). These results indicated that the phase shifting was mediated through mAChRs, such that blocking them was sufficient to prevent it. By contrast, when we antagonized nAChRs, the addition of Carbachol at 20 h produced the same phase shifts in barrier tightness and permeability as without the inhibitor (Figures 16-18). This indicated that these results were not mediated through nAChRs.

Our experiments blocking mAChRs and nAChRs produced similar results when Carbachol was administered at 20 h compared with 14 h (Figures 12-14 and Figures 16-18). When we blocked mAChRs with Atropine, we found that the addition of Carbachol at 20 h no longer produced phase shifts (Figures 16-18). However, when we used Mecamylamine to block

nAChRs, the pattern of phase shifting was the same for Carbachol at 20 h without the inhibitor (Figures 16-18). These results indicated that the phase advance induced by Carbachol at 20 h was also mediated by mAChRs but not nAChRs.

Studies have demonstrated that mAChR agonism in the rodent SCN results in phase shifts but that this only occurs at night^{42-43,52}. This nocturnal gating supports the possibility that mAChR activation in the rat neuroendothelial cells is causing phase delays to occur only during the time points consistent with the subjective night. In one study, Liu *et al.* (1997) probed the mechanism for this nocturnal gating in the rodent SCN. Their evidence supported a mechanism whereby mAChR activation causes an increase in cGMP levels, which leads to an increase in cGMP-dependent protein kinase (PKG)⁴³. Through a combination of agonists, antagonists, and measurements of cGMP levels, they demonstrated that this pathway mediated the nocturnally-gated Carbachol-induced phase shifts⁴³. It is possible that this mechanism may have mediated the nocturnally-gated phase shifts we observed. It is worth noting that these studies which found the role for mAChR in inducing the nocturnally-gated phase shifts were performed in the rodent SCN^{42-43,52-53}.

Research has found that certain biochemical signaling pathways can only occur during specific time frames in the circadian cycle⁵⁴. This may explain the ability of mAChR activation to induce phase shifts only during the subjective night. Research has found that the availability of second messengers changes as levels oscillate throughout the day⁵⁵. This includes cAMP, and PKG-II, both of which can mediate circadian phase shifting⁵⁴. The ratio of these second messengers to each other and to Glutamate can determine whether a phase shift occurs and whether they are advances or delays⁵⁵. Since these ratios change according to time of day, the results of phase shifting cues can also vary depending on the time of day⁵⁵. These findings may explain why in our experiments, Carbachol only induced phase delays during the time points representing the night (Figures 12-20).

There is a lack of phase-shifting studies of Carbachol administration in the late subjective night which were conducted *in vitro* outside of the SCN. However, some of the findings from these studies might help explain our results. For instance, Liu *et al.* (1997) found that Carbachol's ability to induce phase shifting in the SCN was mediated by mAChRs⁴³. They also found that this effect was mediated by guanylyl cyclase, cGMP, and cGMP-dependent protein kinase⁴³. While the SCN and neuroendothelial cells are different cell types, neuroendothelial cells do also contain guanylyl cyclase, cGMP, and cGMP-dependent protein kinase⁵⁶. Research has also found that agonism of mAChRs activates calcium signaling but that activation of nAChRs does not⁵⁷. Second messengers such as cGMP and calcium can have a variety of effects on cells⁵⁶. They also are involved in a multitude of pathways⁵⁶, meaning that elevating them may contribute to other signaling cascades. Thus, there is a variety of ways that mAChRs might mediate our findings regarding Carbachol's phase-shifting effects.

Since we observed peaks for barrier tightness 12 h apart, this is consistent with 2 circadian oscillations which are 24 h rhythms but 12 h out of phase. When Carbachol was administered, either at 14 h or 20 h, the phase shifts occurred only during the time points which represented nighttime. We found that the shifts in these peaks were mediated by mAChRs. However, we do not know which receptors might mediate the peaks in barrier tightness which occurred at the time points representing daytime. We do know that since Carbachol was unable to shift these peaks, it seems they are not mediated by ACh receptors.

Conclusions

We determined that Carbachol, a pan-AChR agonist, could induce phase shifts in the permeability and barrier tightness of rat neuroendothelial cells. When administered 14 h post-synchronization, Carbachol induced a 4 h phase delay but only during the time points representing the nighttime. When administered 20 h post-synchronization, Carbachol induced a 4 h phase advance, an effect which also occurred only during the time points representing nighttime. Our experiments with mAChR and nAChR inhibitors indicated that these effects were

mediated by mAChRs, which is consistent with previous literature^{42-43,52}. This study marks the first instance of phase shifting of barrier tightness and permeability in neuroendothelial cells or any other component of the BBI. We also demonstrated for the first time the ability of ACh agonism to mediate this phase shifting.

Acknowledgments

Jennifer W. Mitchell and Canran Deng obtained a portion of the measurements for the TEER assays. This work was supported by the NIH (R61HL159948), NSF (DGE NRT-UtB 1735252 and STC CBET 0939511), and the 2021 Beckman Institute Graduate Fellows Program.

Figures

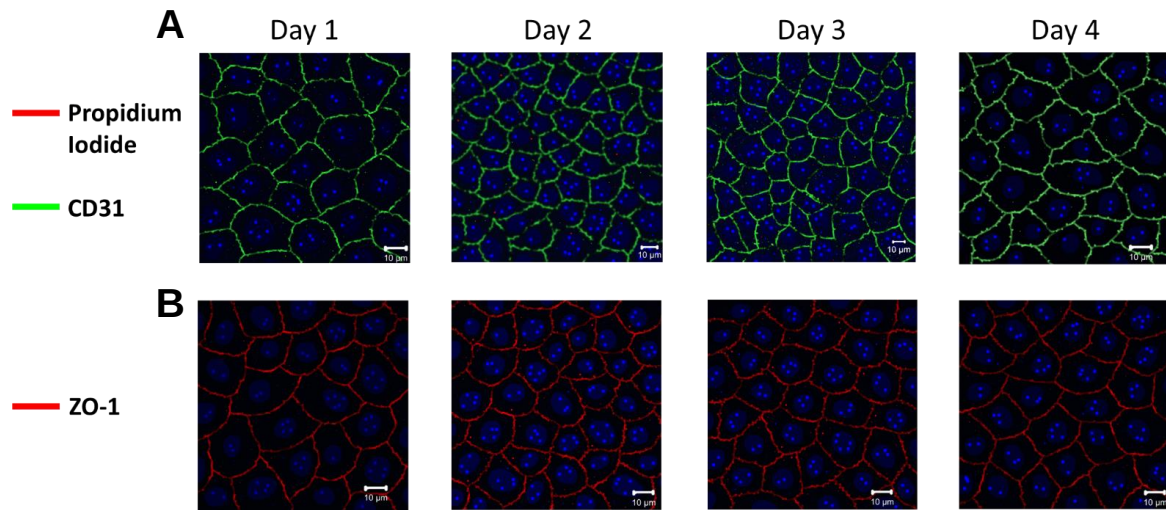


Figure 11: Neuroendothelial cells display high confluence and continued health on Transwells after carbachol treatment.

A-B. Rat cerebral neuroendothelial cells cultured on Transwells for 4 subsequent days after treatment with Dexamethasone and Carbachol, 20X magnification, 10 µm scalebars.

A. Immunofluorescence for CD31 in green indicates the presence of endothelial cells.

Propidium Iodide fluorescence in red indicates cells with damaged membranes.

B. Apical expression of the tight junction Zona Occludens-1 (ZO-1) indicates full confluence.

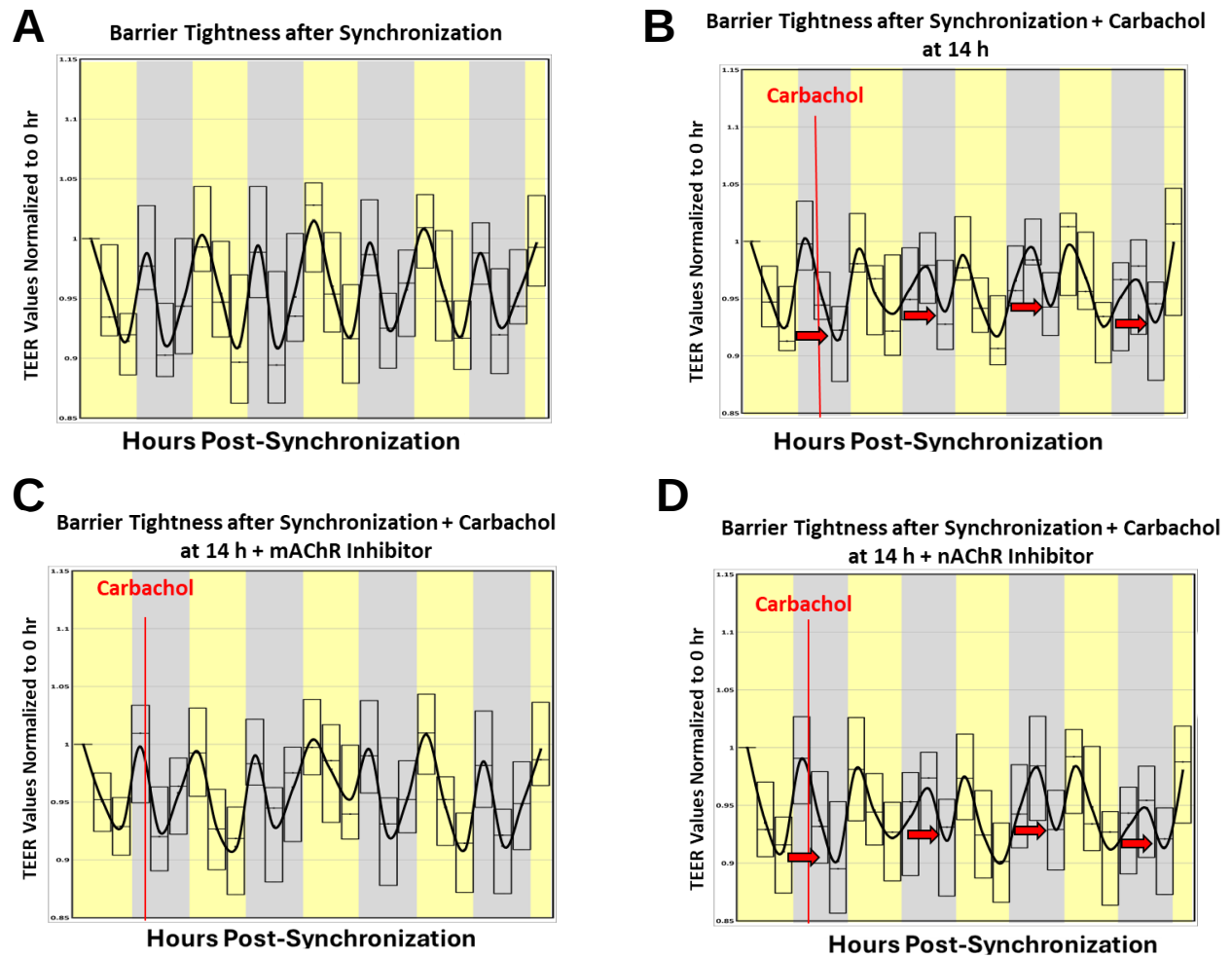
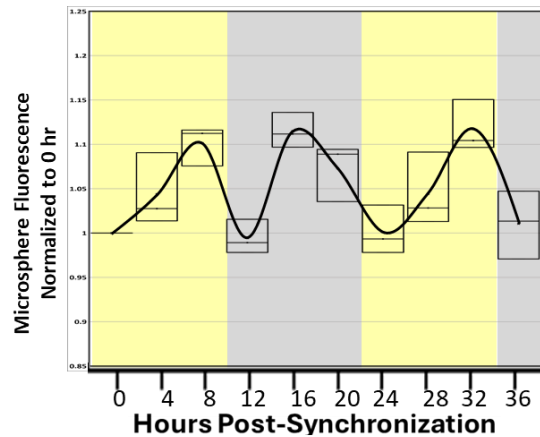
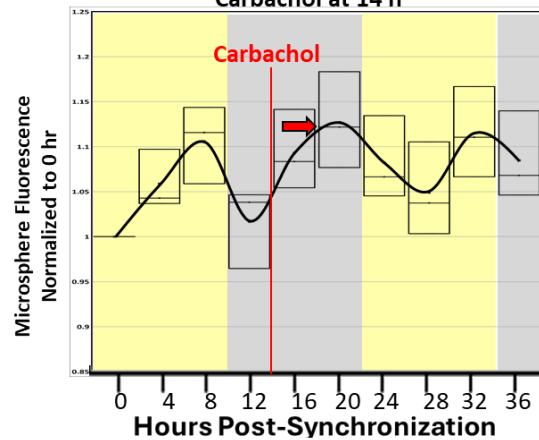
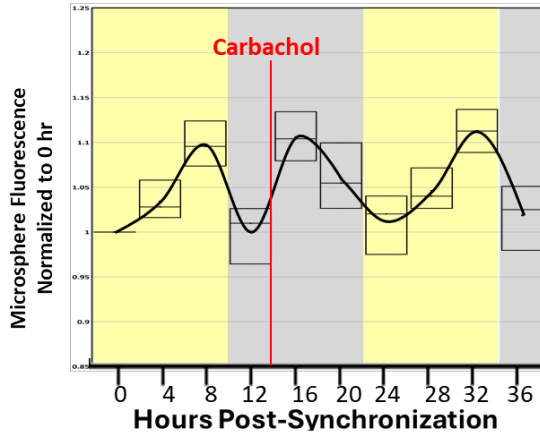
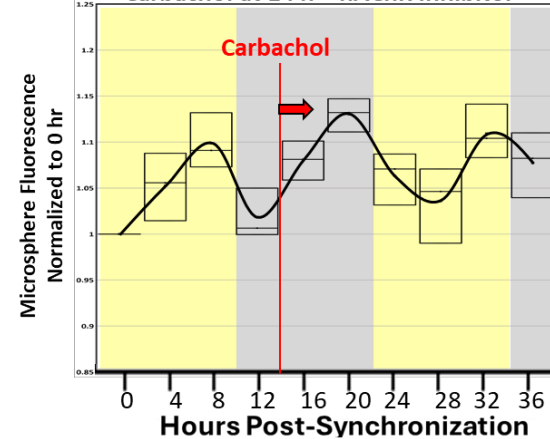


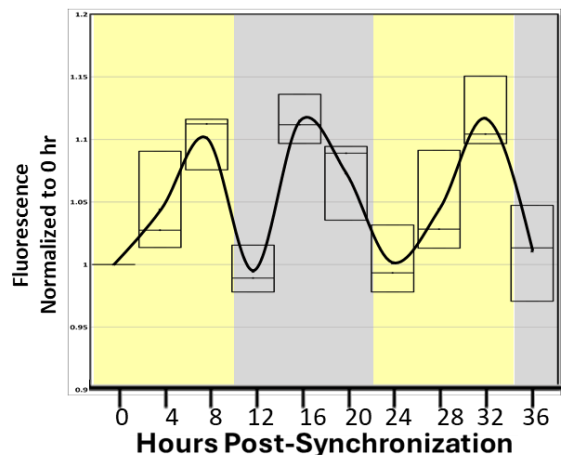
Figure 12: Neuroendothelial cell tightness is phase-delayed after Carbachol administration 14 h post-synchronization.

A-D: Red lines at the 14 h time point indicate when Carbachol was administered in the experimental groups that received Carbachol treatment. Barrier tightness in groups treated with only Dexamethasone; Dexamethasone and Carbachol at 14 h post-synchronization; Dexamethasone, Carbachol at 14 h, and a mACHR inhibitor; or Dexamethasone, Carbachol at 14 h, and a nAChR inhibitor (n=3 for each experimental group).

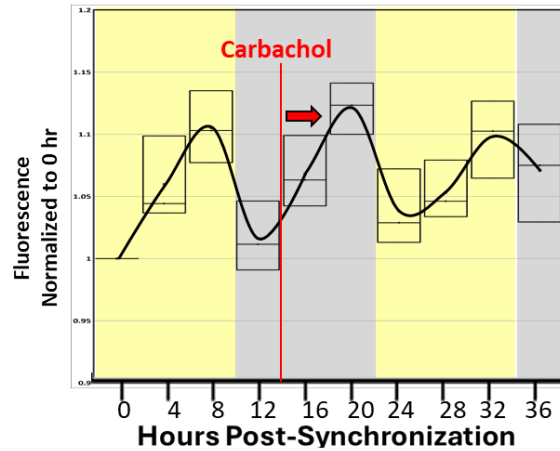
A Microsphere Permeability after Synchronization**B** Microsphere Permeability after Synchronization + Carbachol at 14 h**C** Microsphere Permeability after Synchronization + Carbachol at 14 h + mAChR Inhibitor**D** Microsphere Permeability after Synchronization + Carbachol at 14 h + nAChR Inhibitor**Figure 13: Neuroendothelial cell permeability is phase-delayed after Carbachol administration 14 h post-synchronization.**

A-D: Red lines at the 14 h time point indicate when Carbachol was administered in the experimental groups that received Carbachol treatment. Permeability to fluorescent microspheres in cells treated with only Dexamethasone; Dexamethasone and Carbachol at 14 h post-synchronization; Dexamethasone, Carbachol at 14 h, and a mAChR inhibitor; or Dexamethasone, Carbachol at 14 h, and a nAChR inhibitor (n=3 for each experimental group). Red arrows denote peaks that were phase-delayed.

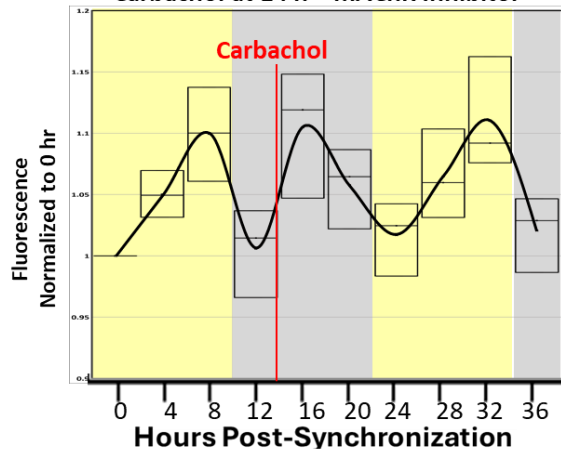
A Fibrinogen Permeability after Synchronization



B Fibrinogen Permeability after Synchronization + Carbachol at 14 h



C Fibrinogen Permeability after Synchronization + Carbachol at 14 h + mAChR Inhibitor



D Fibrinogen Permeability after Synchronization + Carbachol at 14 h + nAChR Inhibitor

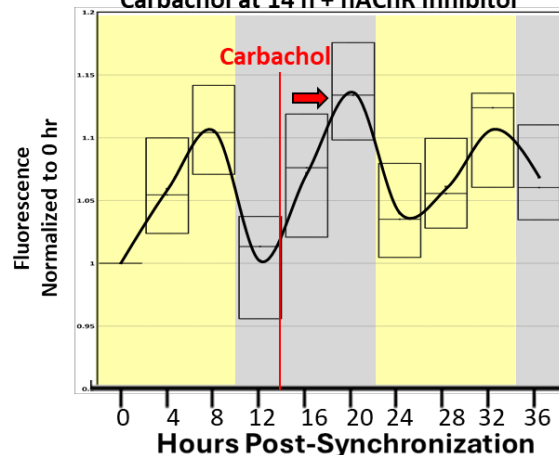


Figure 14: Neuroendothelial cell permeability to fibrinogen is phase-delayed after Carbachol administration 14 h post-synchronization.

A-D: Red lines at the 14 h time point indicate when Carbachol was administered in the experimental groups that received Carbachol treatment. Permeability to fluorescent fibrinogen in cells treated with only Dexamethasone; Dexamethasone and Carbachol at 14 h post-synchronization; Dexamethasone, Carbachol at 14 h, and a mAChR inhibitor; or Dexamethasone, Carbachol at 14 h, and a nAChR inhibitor (n=3 for each experimental group). Red arrows denote peaks that were phase-delayed.

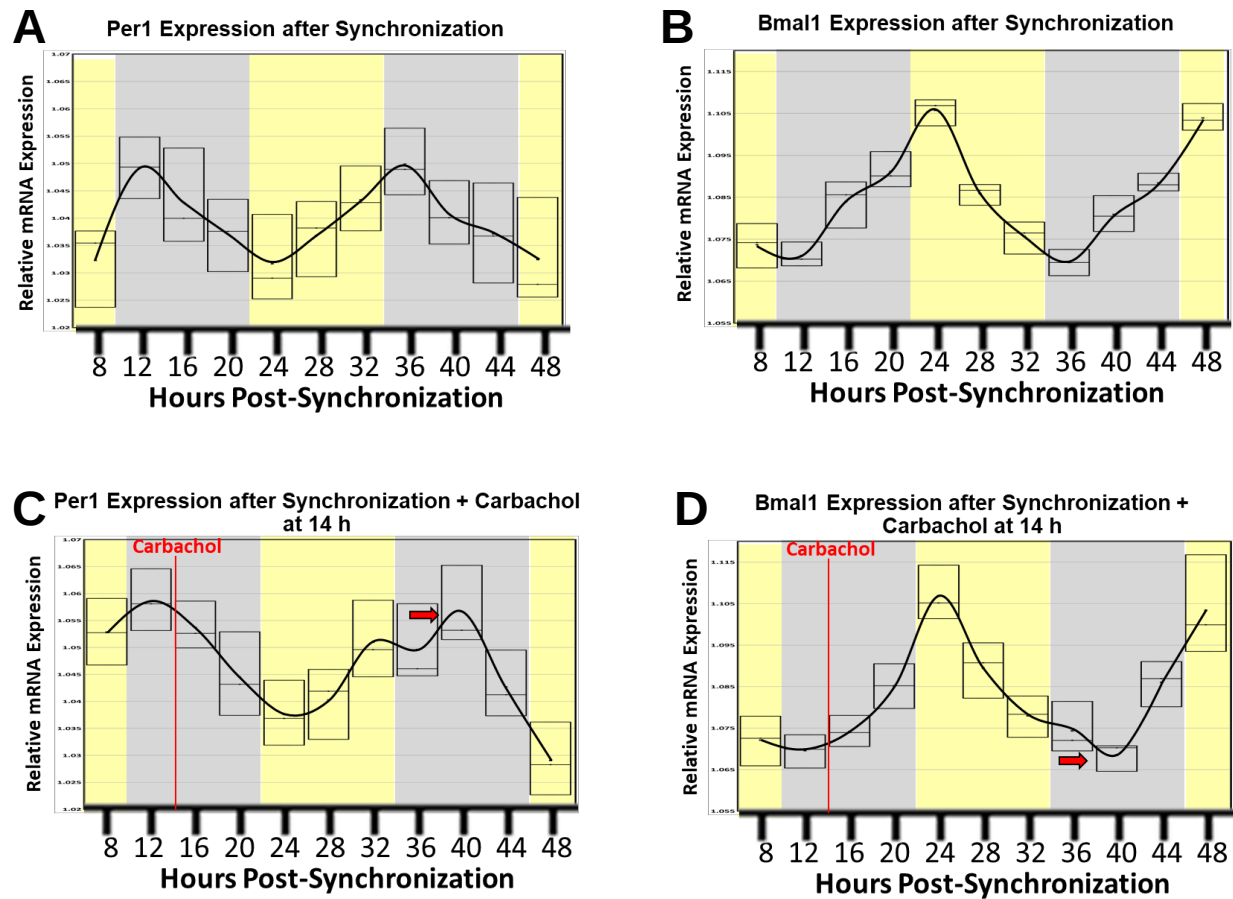


Figure 15: Clock gene expression is phase-delayed after Carbachol administration 14 h post-synchronization.

A-D: Red lines at the 14 h time point indicate when Carbachol was administered in the experimental groups that received Carbachol treatment. RT-qPCR for *Per1* and *Bmal1* after Dexamethasone synchronization, with and without Carbachol 14 h post-synchronization (n=3 for each experimental group). Red arrows denote peaks and troughs that have been phase-advanced.

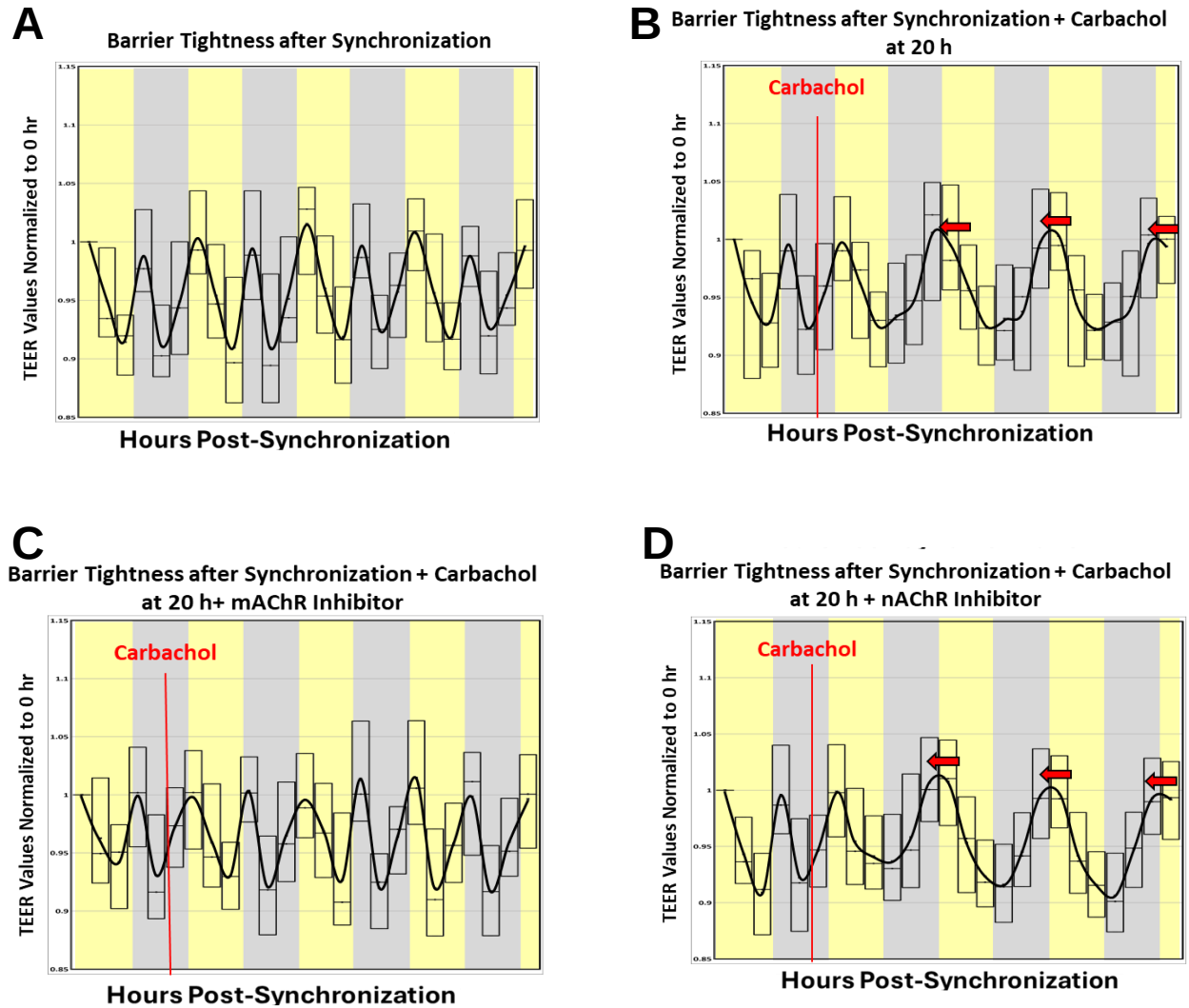


Figure 16: Neuroendothelial cell tightness is phase-advanced after Carbachol administration 20 h post-synchronization.

A-D: Red lines at the 20 h time point indicate when Carbachol was administered in the experimental groups that received Carbachol treatment. Barrier tightness in groups treated with only Dexamethasone; Dexamethasone and Carbachol at 20 h post-synchronization; Dexamethasone, Carbachol at 20 h, and a mAChR inhibitor; or Dexamethasone, Carbachol at 20 h, and a nAChR inhibitor (n=3 for each experimental group).

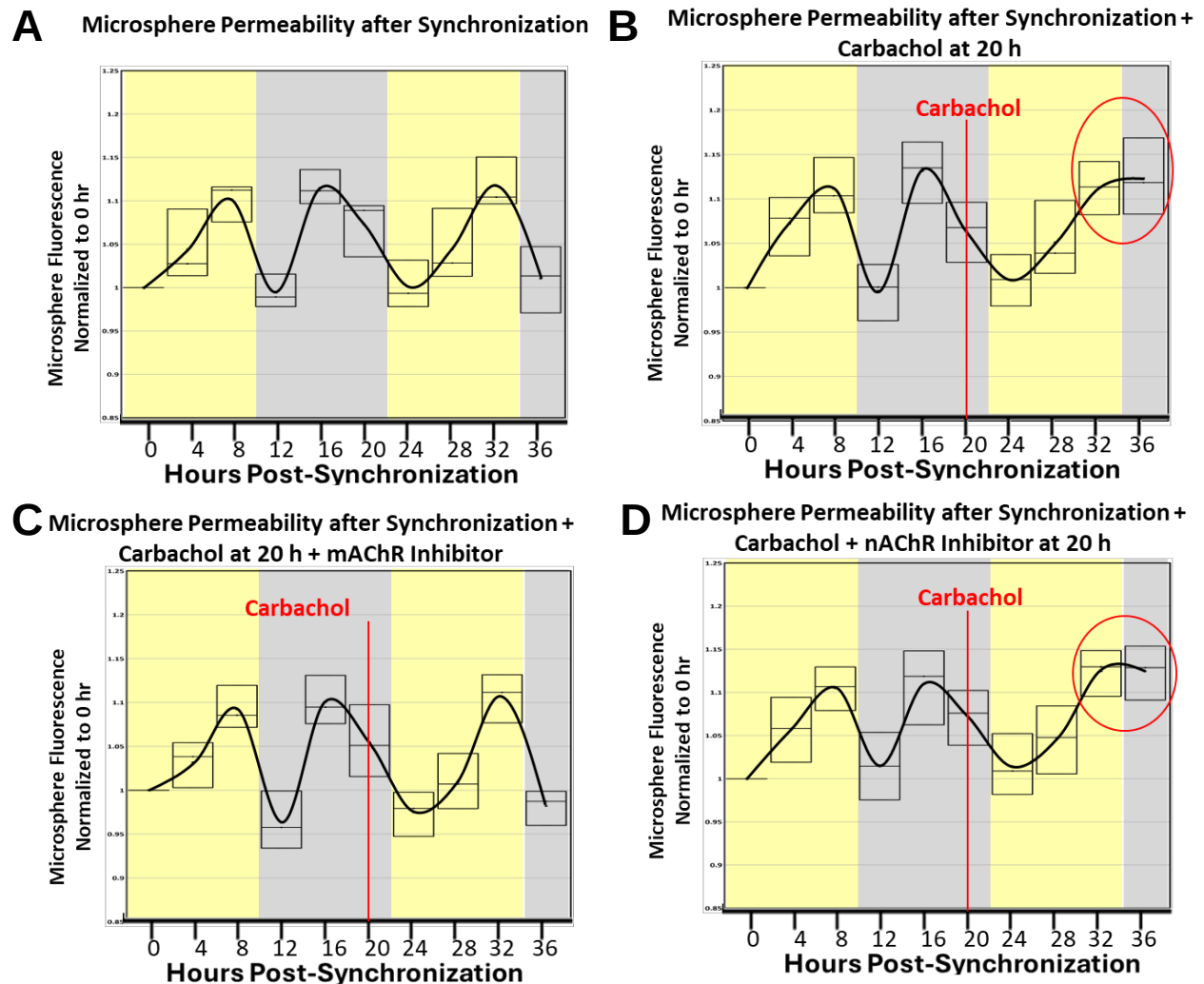


Figure 17: Neuroendothelial cell permeability is phase-shifted after Carbachol administration 20 h post-synchronization.

A-D: Red lines at the 20 h time point indicate when Carbachol was administered in the experimental groups that received Carbachol treatment. Permeability to fluorescent microspheres in cells treated with only Dexamethasone; Dexamethasone and Carbachol at 20 h post-synchronization; Dexamethasone, Carbachol at 20 h, and a mAChR inhibitor; or Dexamethasone, Carbachol at 20 h, and a nAChR inhibitor (n=3 for each experimental group). Red circles denote time points where an apparent phase shift has occurred.

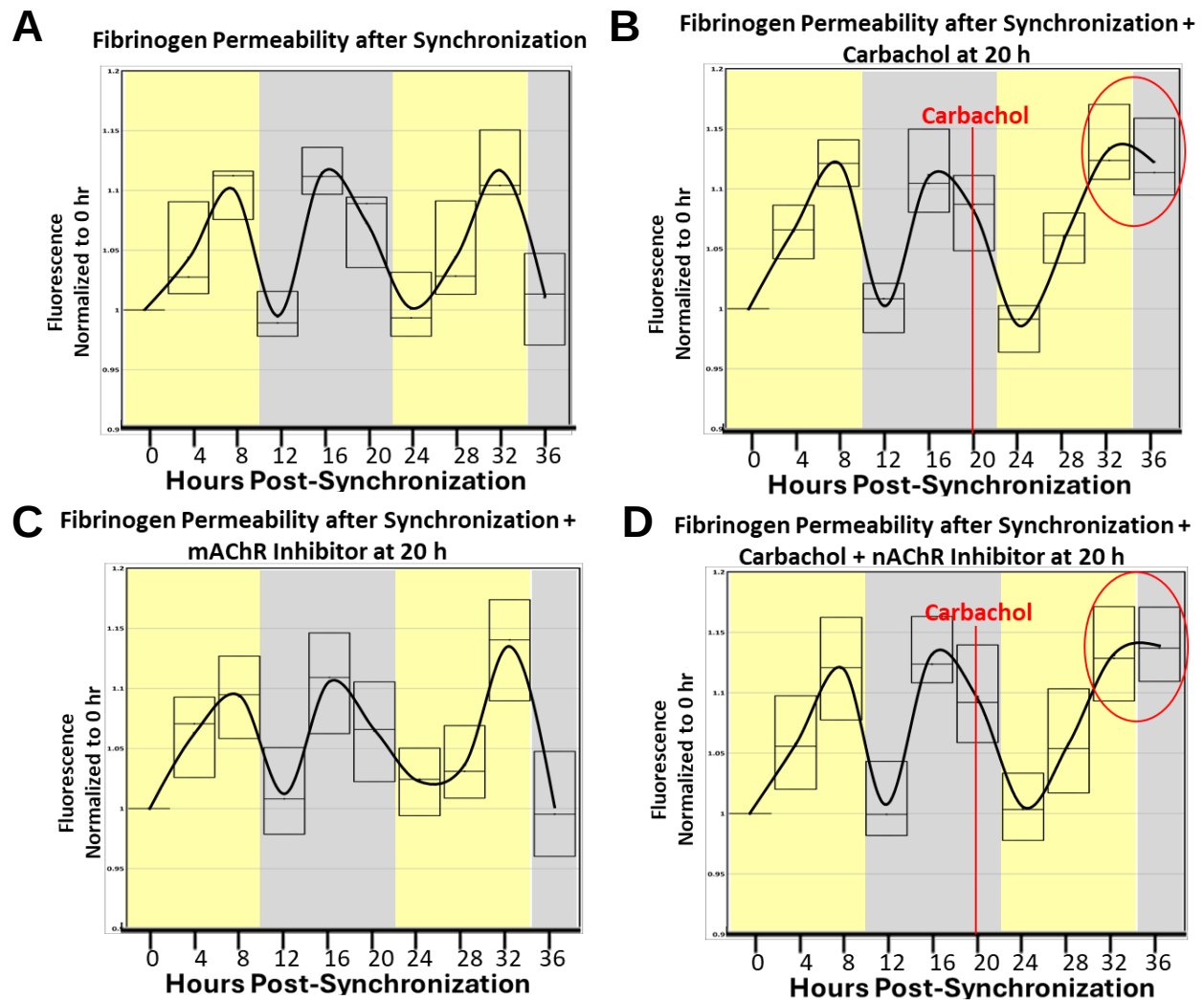


Figure 18: Neuroendothelial cell permeability to fibrinogen is phase-shifted after Carbachol administration 20 h post-synchronization.

A-D: Red lines at the 20 h time point indicate when Carbachol was administered in the experimental groups that received Carbachol treatment. Permeability to fluorescent fibrinogen in cells treated with only Dexamethasone; Dexamethasone and Carbachol at 20 h post-synchronization; Dexamethasone, Carbachol at 20 h, and a mAChR inhibitor; or Dexamethasone, Carbachol at 20 h, and a nAChR inhibitor (n=3 for each experimental group). Red circles denote time points where an apparent phase shift has occurred.

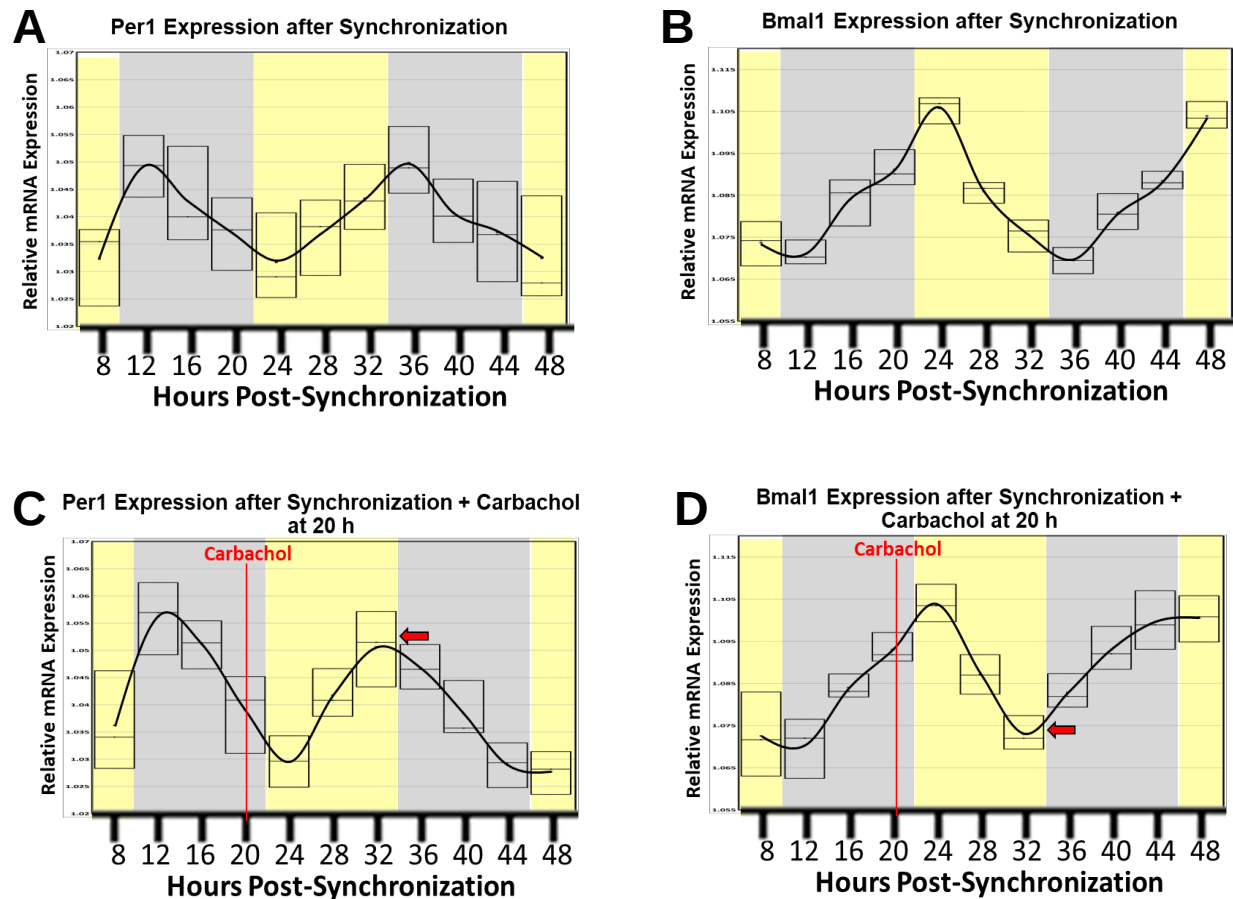


Figure 19: Clock gene expression is phase-advanced after Carbachol administration 20 h post-synchronization.

A-D: Red lines at the 20 h time point indicate when Carbachol was administered in the experimental groups that received Carbachol treatment. RT-qPCR for *Per1* and *Bmal1* was performed after Dexamethasone synchronization, with and without Carbachol 20 h post-synchronization (n=3 for each experimental group). Red arrows denote peaks and troughs that have been phase-advanced.

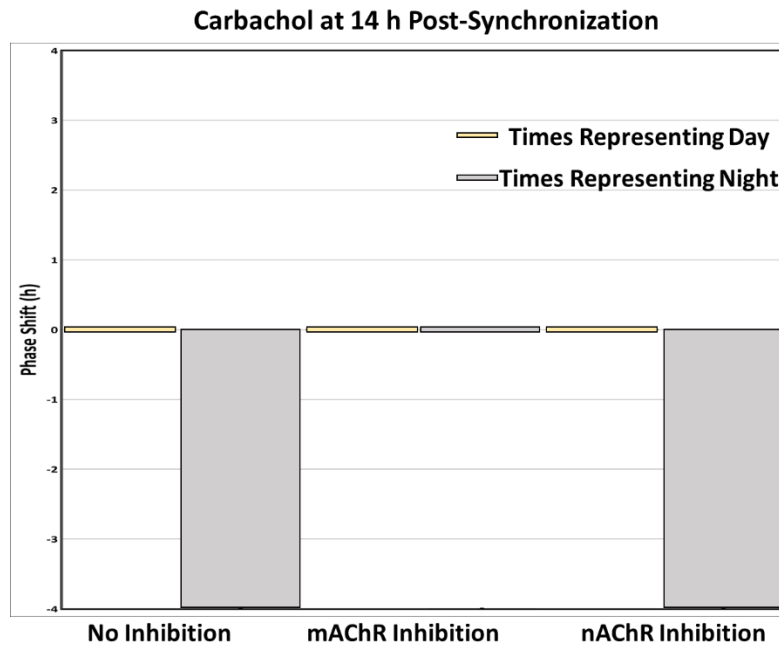
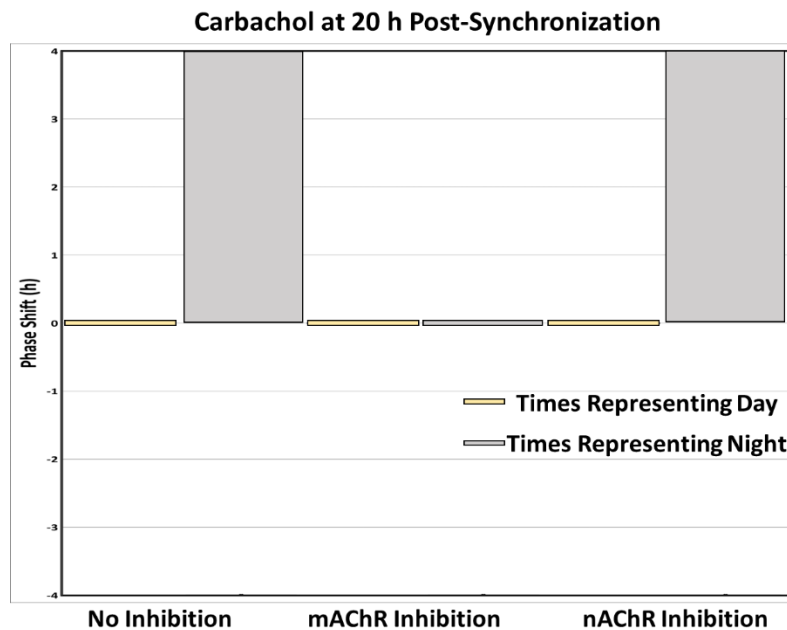
A**B**

Figure 20: Summary of effects of carbachol on phase shifts.

When administered at 14 h post-synchronization, Carbachol induced 4 h phase delays during the time points representing the night (**A**). When administered at 20 h post-synchronization, Carbachol instead induced 4 h phase advances during the time points representing the night (**B**). However, these phase shifts did not occur during the time points representing daytime (**A-B**). We observed the phase shifts in the absence of inhibitors and in the presence of nAChR inhibition (**A-B**). However, the phase shifts were absent in the presence of mAChR inhibition, indicating that they were mediated by mAChRs (**A-B**).

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The study presented here is one of the first to examine the effects of time of day on clock gene expression, barrier tightness, and permeability across the blood-brain interface (BBB). We find that clock gene expression, barrier tightness, and permeability change in rat neuroendothelial cells maintained *in vitro* according to time of day for four consecutive days. This finding also extends to permeability to the nano-beads and the blood clotting factor fibrinogen. We culture the neuroendothelial cells as a monolayer and demonstrate that these measurements of permeability and barrier tightness are intercellular, rather than intracellular. As a result, this became the first study to demonstrate that intercellular permeability and barrier tightness change with time of day in neuroendothelial cells.

This also is the first study to investigate how intercellular permeability and barrier tightness change with time of day *in vitro*. One advantage of *in vitro* studies is that they are not confounded by systems- and behavioral-level factors such as sleep, which can affect permeability¹ or feeding and hormones, which can affect circadian rhythms²⁻³. We performed qPCR for clock genes to verify that the circadian rhythms of the individual cells were synchronized. This allowed us to determine how the time course of the fluctuations in permeability and barrier tightness coincided with the time course of clock gene expression. This has also been the first study which demonstrated the ability of Carbachol, a pan-acetylcholine (ACh) receptor agonist, to induce a phase shift in the permeability and barrier tightness.

We chose to perform experiments in neuroendothelial cells, since they form much of the BBB⁴. No previous studies had examined how the permeability between neuroendothelial cells might change with time of day. We isolated primary cerebral rat neuroendothelial cells and verified their identity through immunostaining. We determined that the cells expressed CD31, an extracellular adhesion protein and established endothelial cell marker. They did not express

markers specific to other cells in the brain and endothelium: astrocytes, neurons, and pericytes (ALDH1L1, β -III tubulin, and Desmin respectively).

Isolated neuroendothelial cells display indications of health, purity, and confluence

We evaluated the health of the neuroendothelial cells by performing immunostaining. The neuroendothelial cells displayed a flat morphology, indicative of cell health⁵. They also expressed CD31 primarily as apical puncta, which is the standard form of CD31 expression⁵. We also exposed the cells to Propidium Iodide (PI), which penetrates damaged cell membranes and fluorescently labels their nuclei⁶. We found that after exposure to PI, its fluorescence was low. This indicated that it was largely unable to enter the cell membranes, because they were intact. The neuroendothelial cells also displayed low levels of PI staining after treatment with Dexamethasone to synchronize their individual circadian rhythms. Similarly, we found low levels of PI fluorescence in the cells after they were treated with Carbachol.

We determined that the cells displayed a high level of cellular confluence, even after being cultured for 4 days on the Transwell inserts. The neuroendothelial cells also expressed the tight junction proteins Zonula Occludens-1 (ZO-1) and Claudin-5 apically. This expression pattern is also indicative of cell health and high confluence⁵.

Neuroendothelial cells display rhythmic clock gene expression

We investigated whether clock-gene expression in the neuroendothelial cells maintained *in vitro* changes according to time of day. We performed qPCR for *Per1* and *Bmal1* at 4-h intervals for 36 h. *Per1* and *Bmal1* oscillated significantly with time of day according to one-way, repeated measures ANOVA, $p < 0.0001$. In the unsynchronized control group, we observed no significant oscillation in the cell population, $p < 0.0001$. This indicates that the measured rhythmicity is due to Dexamethasone synchronization. *Per1* expression decreases from 0 h to 12 h and increased from 12 h to 24 h. It then decreases from 24 h to 36 h post-synchronization, before increasing by 48 h. *Bmal1* expression increases from 0 h to 12 h, decreases from 12 h to 24 h, increases from 24 h to 36 h, and then decreases from 36 h to 48 h.

This antiphase relationship between *Per1* and *Bmal1* is consistent with previous literature⁷⁻¹⁰. The 24 h and 48 h time points exhibit the greatest expression of *Per1* and the lowest expression of *Bmal1*. By contrast, we observe the lowest *Per1* expression and greatest *Bmal1* expression at 12 h and 36 h. Again, these findings are consistent with previous studies⁷⁻¹⁰. However, this is the first study to demonstrate that rat neuroendothelial cells display rhythmic clock gene expression according to time of day.

Barrier tightness fluctuates according to time of day

We measured the tightness of the neuroendothelial cell monolayer by performing TEER assays for 4 days at 4-h intervals. The TEER values fluctuate significantly according to time of day ($p < 0.0001$ with one-way, repeated measures ANOVA, $n=3$). The peaks were 12 h apart, suggesting a 12-h rhythm. We observed the first peak 0 h after Dexamethasone synchronization, and the last peak occurred at 96 h. There was no significant time-of-day difference in the unsynchronized control group ($p=0.8708$, $n=3$). We observed the lowest TEER values at 8 h and 16 h post-synchronization in the first 24 h, and this pattern repeated every consecutive 24 h.

Barrier permeability, including permeability to fibrinogen, oscillates with a daily rhythm

We found that barrier permeability, the inverse of barrier tightness, fluctuates according to time of day. We demonstrate this by conducting permeability assays with fluorescent microspheres and fluorescent fibrinogen at 4-h intervals for 36 h. According to one-way repeated-measures ANOVA, the fluorescent microsphere permeability changes significantly according to time of day ($p=0.0378$, $n=3$). Permeability to fluorescent fibrinogen also oscillated significantly according to time of day ($p=0.0135$, $n=3$). The permeability peaks at the 8h, 16 h, and 32 h time points. It is lowest at the 0 h, 12 h, 24 h, and 36 h time points.

This pattern is inversely related to the pattern we observe with our TEER assays. This is consistent with the inverse relationship between barrier tightness and permeability. In the unsynchronized control groups, there are no significant oscillations due to time of day for the

fluorescent microspheres ($p=0.7158$, $n=3$) or fibrinogen ($p=0.8142$, $n=3$). This indicates that the results we observe are due to the Dexamethasone synchronization and not any unintended *in vitro* synchronization.

ACh agonism can induce phase shifts in barrier permeability and tightness

While investigating the effect of cholinergic stimulation, which would be mediated by parasympathetic innervation *in vivo*, we find that Carbachol, an ACh agonist, appears to induce phase shifts in barrier tightness and permeability. When administered at 14 h post-synchronization, we observe possible 4-h phase delays, but only at the time points more consistent with nighttime. For instance, we still observed peaks in barrier tightness at 24 h, 48 h, 72 h, and 96 h. However, in the control group, barrier tightness peaks at 36 h post-synchronization, and in the Carbachol-treated group, barrier tightness peaks at 40 h. In addition, the peak observed at 60 h post-synchronization in the control group is present at 64 h, and the control group peaked at 84 h, whereas the Carbachol-treated group peaked at 88 h.

We observe similar phase delays for the fluorescent microsphere and fluorescence-tagged fibrinogen in permeability assays. In the absence of Carbachol, they display their lowest permeability at 0 h, 12 h, 24 h, and 36 h post-synchronization. Their permeability peaks at 8 h, 16 h, and 32 h. After the addition of Carbachol at 14 h post-synchronization, this pattern remains consistent for the trough at 24 h and the peak at 32 h, which are more consistent with daytime. However, during the time period that is more consistent with nighttime, the peak in permeability occurs at 20 h, instead of 16 h. This indicates that a 4 h phase delay has occurred, but only in response to stimulation during the time points consistent with nighttime.

We administered Atropine, a mAChR inhibitor, or Mecamylamine, a nAChR inhibitor to examine the involvement of each receptor type on the phase shifts we observe. We found that when we add Carbachol at 14 h while mAChRs are blocked, the peaks and troughs occur at the same time points as when no Carbachol was added. This effect applied to our measurements of barrier tightness and permeability assays with fluorescent microspheres and fluorescent

fibrinogen. These results indicated that the phase shifting was mediated through mAChRs, such that blocking them was sufficient to prevent the change in phase. By contrast, when we antagonize nAChRs, the addition of Carbachol at 14 h produces the same phase shifts in barrier tightness and permeability as without the inhibitor. This indicates that these results are not mediated through nAChRs.

The overall finding that Carbachol administration at 14 h post-synchronization can cause phase delays is consistent with previous literature¹¹⁻¹². The ability of the barrier tightness and permeability to undergo a phase shift may provide further evidence that their rhythmicity is under circadian control. These findings demonstrate for the first time that AChR activation can induce phase shifting in neuroendothelial cells. The finding that Carbachol can induce phase shifts during the time points most similar to nighttime but not daytime is consistent with previous literature¹³⁻¹⁵. These studies also found that this effect is mediated through mAChRs but not nAChRs, which is consistent with our findings regarding mAChR and nAChR inhibition¹³⁻¹⁵. As the previous studies found, it is possible that our findings may be explained by the ability of mAChR signaling to respond differently to phase shifts during the daytime and the nighttime¹³⁻¹⁵.

We observed phase shifts when we administer Carbachol at 20 h post-synchronization. Our TEER assays and permeability assays with fluorescent microspheres and fluorescent fibrinogen indicate that timing of the system is advanced by 4 h. These advances also occur during the time points representing the nighttime but not the daytime. Without Carbachol, barrier tightness peaks at 0 h and then repeatedly every 12 h, and it displayed troughs at 8 h and 16 h each day. After Carbachol administration at 20 h, instead of a peak occurring at 36 h, a trough occurs. This pattern of changes in barrier tightness is most similar to that observed at 32 h or at 40 h, presenting a challenge for determining whether a phase advance or phase delay has occurred. However, at 44 h, a peak appears which is closer to that observed at 48 h than 40 h. We interpret this as evidence that a phase advance has occurred.

We observed a similar pattern with the permeability to fluorescent microspheres and fluorescent fibrinogen. In the absence of Carbachol, they both peak at 8 h and 16 h. They display troughs at 0 h, 12 h, 24 h, 36 h, and 48 h post-synchronization. When we administered Carbachol at 20 h post-synchronization, we find that the 36-h time point does not display the trough that it did in the absence of Carbachol. This result is the inverse of the lowered TEER values at 36 h which from Carbachol administration at 20 h. This reflects a possible phase advance at the 36 h time point, similar to that observed with barrier tightness.

Our results with qPCR also indicate possible phase advances after Carbachol administration at 20 h post-synchronization. In the control group, which was only synchronized with Dexamethasone, *Per1* expression peaks at 12 h and 36 h. It displays troughs at 24 h and 48 h. After the addition of Carbachol at 20 h post-synchronization, the peak at 48 h is preceded by a peak at 44 h. The 44 h time-point is closer in magnitude to the 48 h time-point than any other time point in an apparent phase advance of about 4 h, resulting in 2 back-to-back peaks. We observe a similar pattern with *Bmal1*. In the absence of Carbachol, *Bmal1* expression displays troughs at 12 h and 36 h and peaks at 24 h and 48 h. After the addition of Carbachol at 20 h post-synchronization, we observe two back-to-back peaks at 44 h and 48 h. This appears to indicate a phase advance of approximately 4 h. For both *Per1* and *Bmal1*, the time points before the addition of Carbachol and those which most mimic the daytime do not display phase shifts. This is consistent with our data on barrier tightness and permeability, which demonstrate phase advances occurring only during the time points which most mimic nighttime.

We have determined that the intercellular permeability of rat neuroendothelial cells fluctuates according to time of day. This finding applies to barrier tightness, as measured by TEER assays, as well as permeability to fluorescent beads and fibrinogen. We conducted these measurements for 4 consecutive days on neuroendothelial cells cultured in monolayers. For all 4 days, the primary rat neuroendothelial cells display high purity and indications of health. This is also true after Carbachol treatment. We find that clock gene expression for the cells changes

with time of day after the cells are synchronized with Dexamethasone. We also demonstrate that the addition of Carbachol, an ACh agonist, at 14 h post-synchronization induces a shift in the phasing of rhythms in permeability and barrier tightness of the neuroendothelial cells. In addition, we find that Carbachol administration at 20 h post-synchronization causes phase advances.

This study is the first to demonstrate that the intercellular permeability and barrier tightness of neuroendothelial cells fluctuates according to time of day. This study is also the first to demonstrate that neuroendothelial cell permeability to the blood-clotting factor fibrinogen fluctuates according to time of day. Prior to this study, circadian BBI studies were limited to *in vivo* studies and intracellular permeability of neuroendothelial cells¹⁶⁻¹⁷. *In vivo* studies can be confounded by variables other than circadian rhythms, and intracellular permeability accounts for less than half of all permeability, particularly regarding drug delivery¹⁸. We find that after Dexamethasone treatment, clock gene expression displays a daily rhythmicity in rat neuroendothelial cell culture. This study has also been the first to demonstrate that neuroendothelial cell permeability can undergo circadian phase-shifting. We determine that this effect could occur through ACh receptor activation and that it may be mediated through the activation of muscarinic ACh receptors.

Future Directions

The work presented here discovered novel insights on the circadian regulation of the neuroendothelium and establishes a new level of understanding and need for investigating the regulation of circadian rhythmicity of BBI permeability. For instance, it is not yet clear why we observe 12-h rhythms in permeability and barrier tightness or why Carbachol phase-shifts these rhythms only when applied during the subjective night. The circadian rhythmicity of permeability has not been examined in human neuroendothelial cells, and this now is possible with the advent and availability of human induced pluripotent stem cells (hiPSCs). It is not well established how the intracellular permeability might change with circadian rhythmicity in

neuroendothelial cells. Circadian rhythmicity of intercellular permeability has not been studied in an *in vitro* BBI model featuring neurons, astrocytes, microglia, and pericytes. Moreover, there is a multitude of proven circadian modulators that have not been studied in this context.

Another potential avenue before future studies would be furthering research on the expression of BBI permeability genes. This is a new area of research that could benefit from further studies examining how their expression changes according to time of day and in circadian knock-out models. Further research could also examine how the circadian rhythmicity of BBI permeability changes in the contexts of redox states, neuronal excitation, circadian disruption, sleep deprivation, aging, and numerous pathologies. Another relevant and new area of research is how other functions of neuroendothelial cells and astrocytes change with circadian rhythmicity.

The underpinnings for circadian regulation of BBI permeability have only begun to be established, but there are many possibilities. Our research has shown that this effect is mediated by clock genes in neuroendothelial cells. It is well established that there are three key components of circadian rhythms, which function as oscillators and influence each other¹⁹. One oscillator is transcriptional and translational loops, particularly regarding clock genes¹⁹. Another is the changes in cellular metabolism and consequently redox states¹⁹. Thirdly, circadian rhythms are affected by ion channel expression and membrane excitability¹⁹. It may be that a change in any of these three oscillators is sufficient to directly alter BBI permeability. For instance, clock genes may alter the expression of tight junctions in neuroendothelial cells. A highly oxidized redox state also may result in the destruction of neuroendothelial tight junctions. It is possible that neuroendothelial cells may undergo circadian changes in ion channel expression which alter their permeability.

There are other factors which may contribute to the circadian rhythmicity of BBI permeability. In fact, circadian rhythms have been shown to occur in the absence of rhythmic clock gene expression. For instance, one study found that mouse embryonic stem cells display

24-h rhythms in the absence of clock gene rhythms²⁰. These cells expressed the clock genes *Bmal1*, *Per1*, *Per2*, and *Ror-a* arrhythmically, in contrast to the rhythmic expression observed in differentiated mouse cells²⁰. However, like the cells differentiated from them, the embryonic stem cells still displayed daily rhythmicity in glucose uptake and expression of the glucose transporter mGlut8²⁰. Along similar lines, another study found that human red blood cells displayed circadian rhythms for redox states, even though their lack of DNA precluded any transcriptional circadian rhythms²¹. These two studies demonstrated that mechanisms other than clock gene expression can be sufficient for circadian rhythmicity. Therefore, there may be factors in addition to clock gene expression which have caused the circadian rhythmicity and phase shifting we observed for BBI permeability.

Research has demonstrated that cellular redox states are under circadian control²²⁻²³. For instance, Wang *et al.* (2012) established that the rodent SCN displays a significant oscillation in redox state according to time of day²². These researchers found that the SCN was more reduced during the day and more oxidized at night, particularly in the early night²². The SCN displayed greater electrical activity during the day²². They were also able to heighten electrical activity by administering reducing agents and decrease it by adding oxidizing agents²². Potassium channel currents were enhanced when oxidizing agents were administered, and they were diminished when reducing agents were administered²². Taken together, their findings suggest a mechanism whereby during the day, the rodent SCN is more reduced, which results in decreased potassium currents. This, in turn, results increased electrical activity.

Whether changes in redox states affect endothelial cell permeability remains to be determined. One target which mediates this process has been identified—vascular NADPH oxidase (Nox4)²³. Anea *et al.* (2013) found that *Bmal1* knockout mice displayed significantly greater expression of NOX4 in their aortas compared with wildtype controls²³. NOX4 expression oscillated significantly according to time of day *in vitro* and in wild-type mice but not the *Bmal1* knockout mice²³. They also showed significantly elevated levels of H₂O₂, a key ROS, in their

aortas²³. The researchers also found that the Nox4 gene promoter is activated by *Bmal1* and *Clock*²³. When these cells were subjected to a *Bmal1* knockout, they demonstrated significantly greater levels of H₂O₂²³. These findings suggest a mechanism whereby clock genes influence Nox4 expression through transcriptional regulation, resulting in greater cellular levels of H₂O₂. Another study found that administering H₂O₂ at concentrations of 100 µm or greater decreased tight junction expression, increased apoptosis, and increased BBI permeability²⁴. However, at lower concentrations, H₂O₂ administration appeared to increase angiogenesis²⁴. This finding may have been a compensatory mechanism. It implies a certain degree of complexity for the role of redox states in influencing endothelial cell permeability at the BBI.

Research has shown that clock genes influence inflammation. For instance, Sugimoto *et al.* (2014) found that *Per1* mediates inflammation in rat spinal cord astrocytes²⁵. RNA silencing of *Per1* significantly activated several key apoptotic proteins and increased the expression of pro-inflammatory factors. The researchers also found that inhibition of the pro-apoptotic proteins (p38, JNK, and NF-κB) decreased expression of the pro-inflammatory factors (CCL2 and IL-6). They concluded that *Per1* silencing led to activation of an apoptosis signaling pathway, which led to the production of CCL2 and IL-6. This conclusion was strengthened when they overexpressed *Per1* and found significant reductions in CCL2 and IL-6 expression as well as significantly reduced activation of p38, JNK, and NF-κB. However, the effects of clock-gene expression on BBI inflammation may be more complex than a potential anti-inflammatory effect of *Per1*. Gog *et al.* (2021) found that overexpression of *Bmal1* alleviated inflammation after hemorrhagic strokes²⁶.

Previous research has also demonstrated that clock gene expression influences tight junction expression. Kyoko *et al.* (2014) found that in wild-type mice, the tight junctions Occludin and Claudin-1 are expressed with a daily rhythm in the mouse large intestine²⁷. This rhythm is abolished in *Per2* mutant mice²⁷. They also expressed tight junctions more highly, suggesting that *Per2* expression decreases the expression of tight junctions²⁷. Of the tight junction proteins

they examined, Occludin and Claudin-1 fluctuated significantly according to time of day²⁷. For both, the lowest expression occurred at 16 h post-synchronization²⁷. These researchers also found through Chromatin Immunoprecipitation (ChIP) assays that the clock genes *Clock* and *Bmal1* bound to the promoters of Occludin and Claudin-1²⁷. The finding that the lowest expression of Occludin and Claudin-1 occurred at ZT 16 is consistent with the finding that BBI permeability peaks in the early night (ZT 16) for mice *in vivo*¹⁶.

Another area of research which is currently underdeveloped is the role of various hormones in the circadian rhythmicity of BBI permeability. For instance, research has been finding that cortisol, insulin, and melatonin may play complex roles which would seem to contradict previous research. Recent studies have found that BBI permeability peaks during the night for nocturnal animals and during the day for diurnal animals *in vivo* and *in vitro*¹⁶⁻¹⁷. However, cortisol, insulin, and melatonin rhythms appear to counteract this. It is possible that cortisol, insulin, and melatonin work to balance BBI permeability by preventing it from becoming excessive. Research has demonstrated that cortisol levels peak at approximately 8:30 am in humans²⁸. Since cortisol increases BBI integrity by upregulating the tight junction protein Claudin-5²⁹, this may contribute to heightened BBI integrity in diurnal animals at this time. Similarly, research has demonstrated that insulin promotes BBI integrity by upregulating the tight junctions ZO-1 and Claudin-5³⁰. Research has shown that insulin secretion in humans peaks between noon and 6 pm³⁰. Melatonin secretion peaks at night for both nocturnal and diurnal animals³¹, which react to it along different time frames. Research has shown that melatonin increases BBI integrity by inhibiting matrix metalloproteinase activity which otherwise would degrade tight junctions³².

Future experiments could also feature other methods of circadian rhythm synchronization for comparison. Studies have shown that adding melatonin³³, a shock of high serum⁷, insulin, insulin-like growth factor 1², or forskolin³⁴ (an activator of adenylyl cyclase) can synchronize circadian rhythms of cells *in vitro*. Of these, studies have demonstrated that

melatonin is one of the strongest cues for circadian synchronization or resetting³³. It also plays an important role for circadian synchronization *in vivo*³³. Using multiple means of circadian synchronization could further validate the findings from the current study. It is also possible that different synchronization cues could produce slightly different results.

Currently, no published studies have featured an *in vitro* BBI model featuring neurons, astrocytes, microglia, and pericytes in addition to neuroendothelial cells. *In vitro* studies serve an important role in BBI circadian rhythm research, because they are not confounded by other factors such as sleep, which can affect permeability¹ or feeding and hormones, which can affect circadian rhythms²⁻³. At the same time, studies have demonstrated that neurons, astrocytes, microglia, and pericytes are key components of the BBI which may influence neuroendothelial cell permeability. Research has shown that neuronal excitation may influence BBI permeability³⁵ and that astrocytes influence neuronal excitation³⁶. Research has also demonstrated that during inflammation, microglia may mediate changes in BBI permeability through changes in tight junction expression³⁸. Research has also found that pericytes influence BBI permeability through several types of mechanisms and that a BBI lacking pericytes is characterized by reduced barrier integrity³⁹. In addition to the creation of a BBI featuring all of these cell types, future research could explore the potential contributions of each cell type in the circadian rhythmicity of BBI permeability.

Research has demonstrated that neuronal excitation may contribute to changes in BBI permeability³⁵. In one study, researchers activated glutamate receptors and found that this influenced gene expression in neuroendothelial cells³⁵. After glutamate receptor stimulation, the neuroendothelial cells displayed decreased expression of the clock genes *Bmal1* and albumin D site-binding protein (DBP)³⁵. They also displayed decreased expression of ABC transporter genes³⁵.

During pathological states such as high inflammation, astrocytes have been implicated in impaired BBI integrity, taking the form of reactive astrocytes⁴⁰. In addition, recent research has

shown that astrocytes express chloride channels which may contribute to neuronal inhibition³⁶. When chloride enters neurons, it serves an inhibitory purpose. Untiet *et al.* (2023) found that mouse astrocytic chloride channels released chloride into the synaptic cleft during locomotion and in response to sensory cues that evoked neuronal excitation³⁶. In particular, they found a positive correlation between the chloride released by astrocytes and neuronal calcium concentrations³⁶. The authors reasoned that this may be a compensatory mechanism; neurons may use the chloride released by astrocytes to prevent excessive excitation³⁶. The researchers also found that significantly more chloride was stored in astrocytes when the mice were asleep³⁶. It is not known whether this newly discovered function of astrocytes alters BBI permeability.

Recent research has found that microglia can either increase or decrease BBI permeability³⁷⁻³⁸. The presence of microglia in co-culture with endothelial cells *in vitro* can promote barrier tightness through augmented tight junction expression³⁷. However, the same study also found that under pro-inflammatory conditions, microglia decreased tight-junction expression, impairing barrier integrity³⁷. In addition, one study found that during the first few days of inflammation, microglia mediated the upregulation of Claudin-5, contributing to an increase in permeability³⁸. However, during the next few days of inflammation, the microglia initiated autophagy of astrocytic end-feet, resulting in BBI disruption³⁸.

Pericytes, multi-functional mural cells of the microcirculation that wrap around the endothelial cells that line the capillaries throughout the body, play a role in regulating BBI integrity³⁹. One study found that mutant mice who were lacking pericytes displayed heightened BBI permeability³⁹. This effect is mediated by gene expression in endothelial cells and the polarization of astrocyte end-feet which contact blood vessels³⁹. Thus, pericytes are thought to influence BBI permeability through changes in endothelial gene expression and astrocyte end-feet polarization which promote barrier tightness³⁹.

We are just beginning to discover the factors that contribute to the circadian rhythmicity of BBI permeability. This field of research could benefit from understanding how factors such as redox states, tight junctions, inflammation, and hormones all influence BBI permeability. In addition, research has yet to fully determine how circadian rhythmicity influences these factors. Moreover, few studies have examined the potential roles of neurons, astrocytes, microglia, and pericytes in the circadian rhythmicity of BBI permeability³⁵⁻³⁹. Further research into these topics may provide insights which can help researchers understand the circadian rhythmicity of permeability at the BBI. This understanding may be utilized for optimizing drug delivery according to time of day or treating BBI disruption in cases of circadian disruption⁴². Therefore, a greater understanding of how BBI permeability changes according to time of day will doubtless lead to improvements in health.

Figure

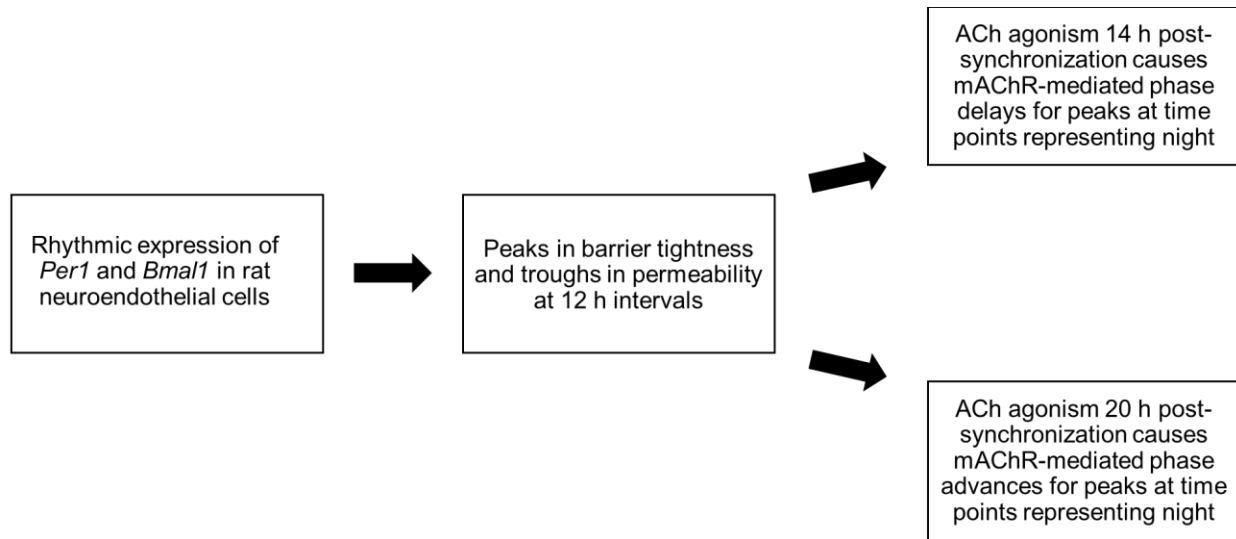


Figure 21: Summary of findings for circadian rhythmicity of BBI permeability in rat neuroendothelial cells.

Primary rat neuroendothelial cells displayed rhythmic expression of *Per1* and *Bmal1* after synchronization with Dexamethasone. They also displayed peaks in barrier tightness coinciding with troughs in permeability every 12 h starting with synchronization. When the ACh agonist Carbachol is administered at 14 h post-synchronization, 4 h phase delays resulted for the peaks during times representing the night. When Carbachol is administered at 20 h, 4 h phase advances resulted for the peaks during times representing the night. These phase advances and phase delays are blocked by mAChR inhibition but not nAChR inhibition. This indicates that they are mediated by mAChRs.

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APPENDIX A: SUPPLEMENTARY FIGURES FOR CIRCADIAN RHYTHMICITY OF RAT NEUROENDOTHELIAL CELLS AT THE BLOOD-BRAIN INTERFACE

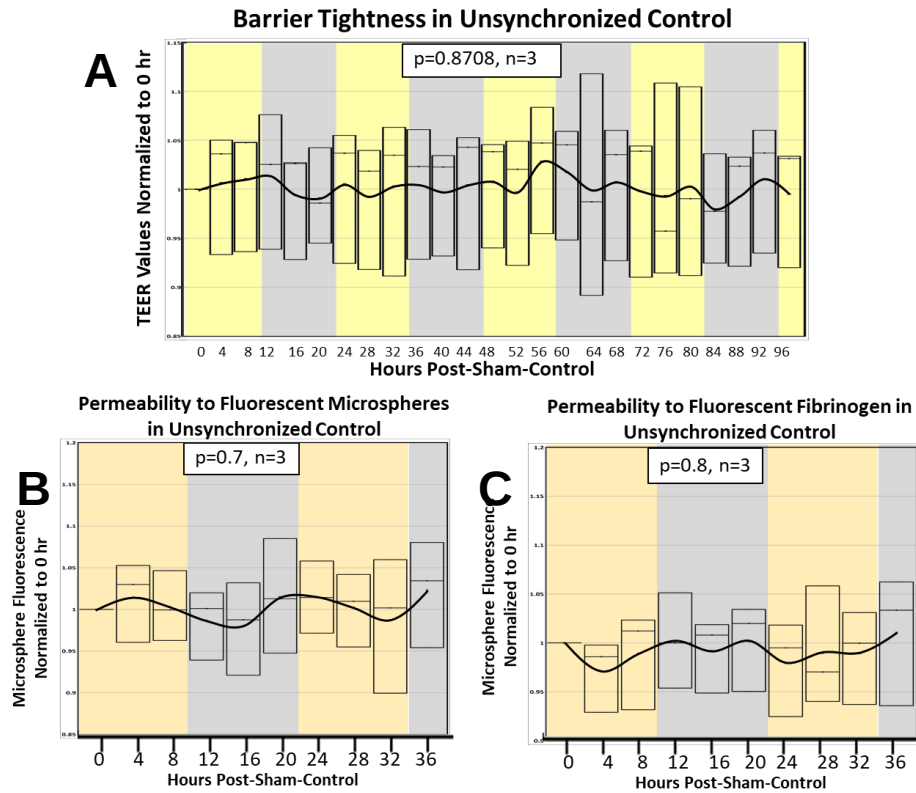


Figure 22: Neuroendothelial cells display a lack of rhythmicity for barrier tightness and permeability in the absence of synchronization.

A-C: Rat neuroendothelial cells with no Dexamethasone synchronization or other means of *in vitro* synchronization. We observed an absence of rhythmicity for barrier tightness (**A**), permeability to fluorescent microspheres (**B**), and permeability to fluorescent fibrinogen (**C**).

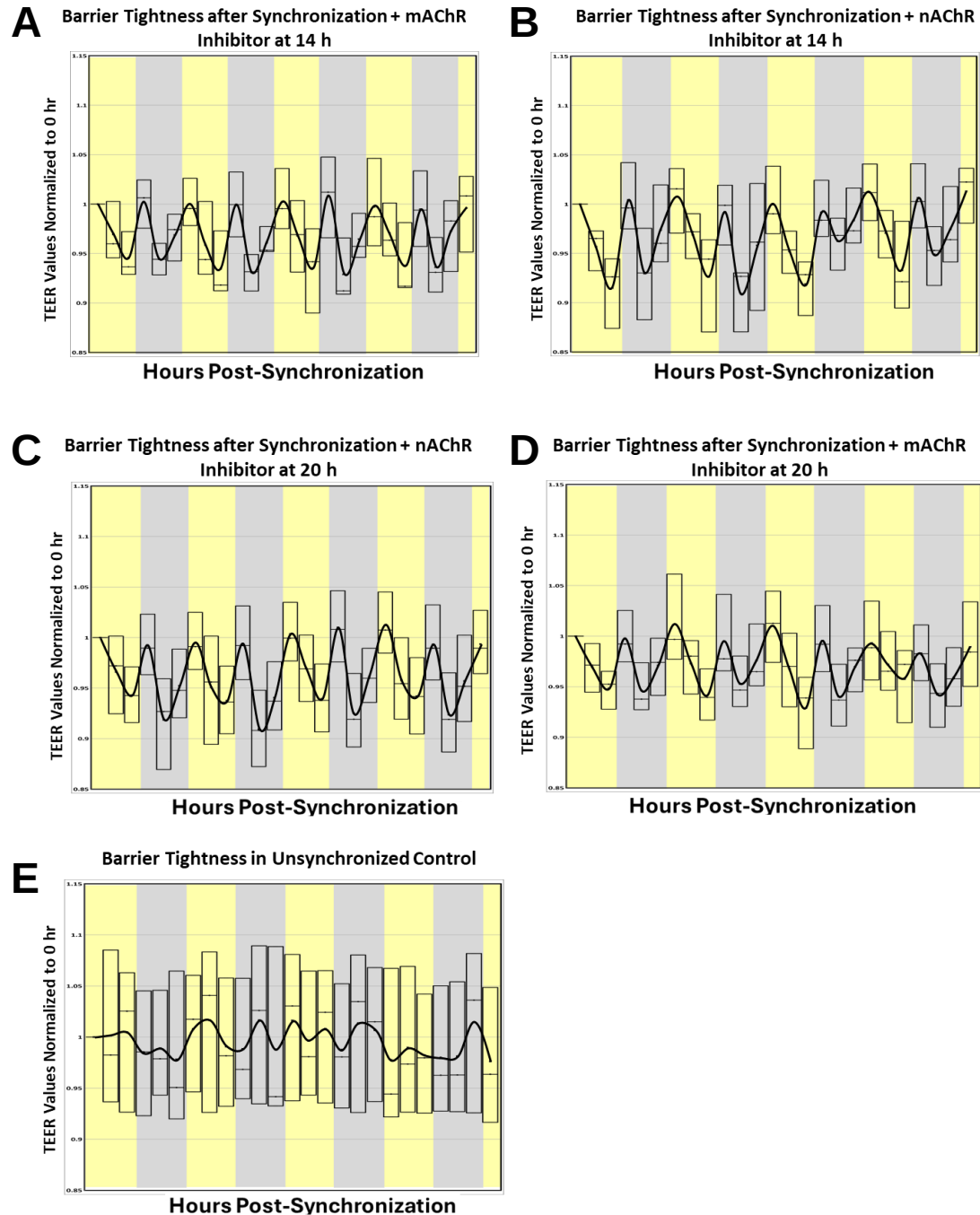


Figure 23: Neuroendothelial cells display a lack of phase shifting for barrier tightness when nAChRs or mAChRs are inhibited in the absence of carbachol.

We observed a lack of phase shifting after only nAChR inhibition or mAChR inhibition in the absence of Carbachol (A-D). We administered the inhibitors either 14 h or 20 h post-synchronization (A-D). This indicated that in instances where phase shifting occurred, the inhibitors were not inducing it. We also observed a lack of rhythmicity in the unsynchronized control (E).

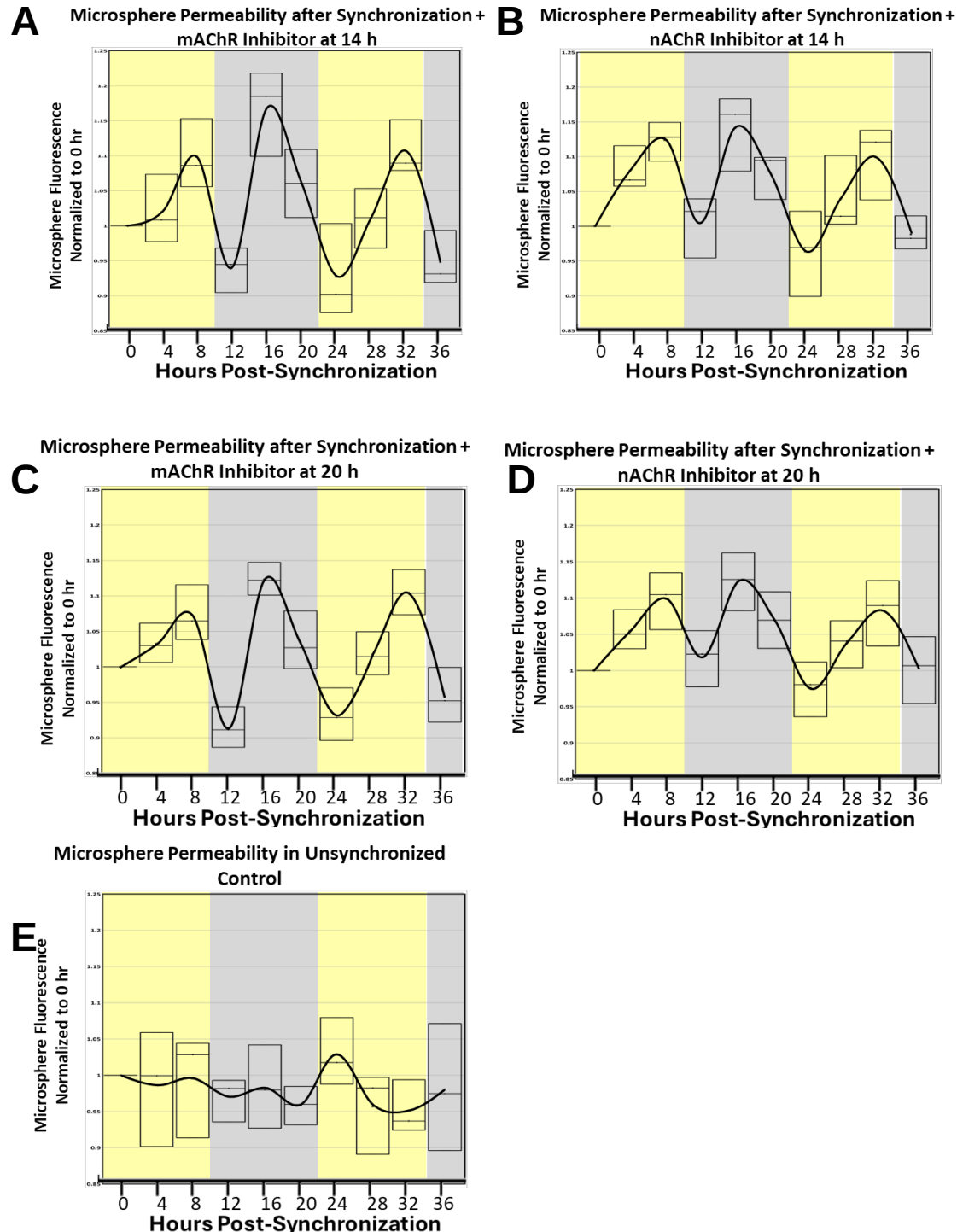
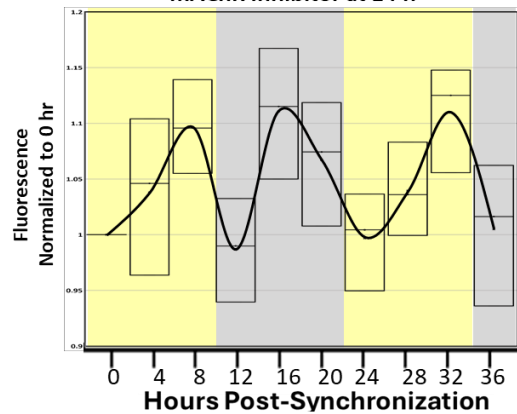


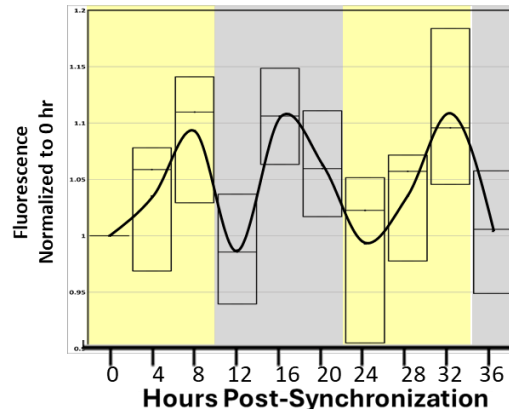
Figure 24: Neuroendothelial cells display a lack of phase shifting for permeability to microspheres when nAChRs or mAChRs are inhibited in the absence of carbachol.

We observed a lack of phase shifting after only nAChR inhibition or mAChR inhibition in the absence of Carbachol (A-D). We administered the inhibitors either 14 h or 20 h post-synchronization (A-D). This indicated that in instances where phase shifting occurred, the inhibitors were not inducing it. We also observed a lack of rhythmicity in the unsynchronized control (E).

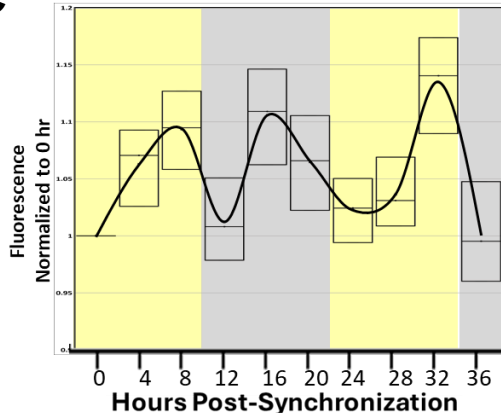
A Fibrinogen Permeability after Synchronization + mAChR Inhibitor at 14 h



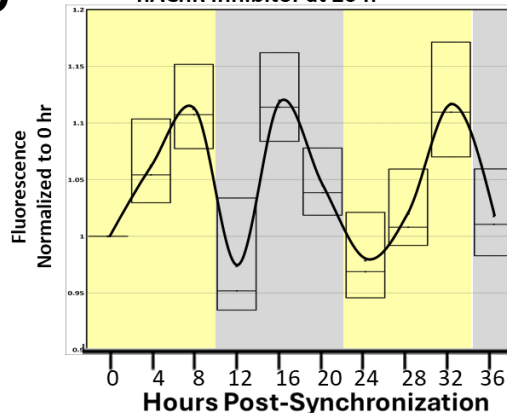
B Fibrinogen Permeability after Synchronization + nAChR Inhibitor at 14 h



C Fibrinogen Permeability after Synchronization + mAChR Inhibitor at 20 h



D Fibrinogen Permeability after Synchronization + nAChR Inhibitor at 20 h



Fibrinogen Permeability in Unsynchronized Control

E

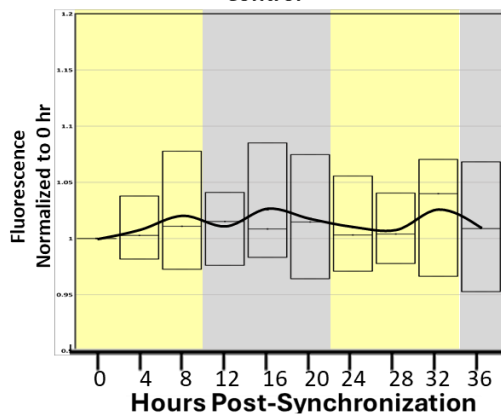


Figure 25: Neuroendothelial cells display a lack of phase shifting for permeability to fibrinogen when nAChRs or mAChRs are inhibited in the absence of carbachol.

We observed a lack of phase shifting after only nAChR inhibition or mAChR inhibition in the absence of Carbachol (A-D). We added the inhibitors at 14 h or 20 h post-synchronization (A-D). This indicated that in instances where phase shifting occurred, the inhibitors were not inducing it. We also observed a lack of rhythmicity in the unsynchronized control (E).

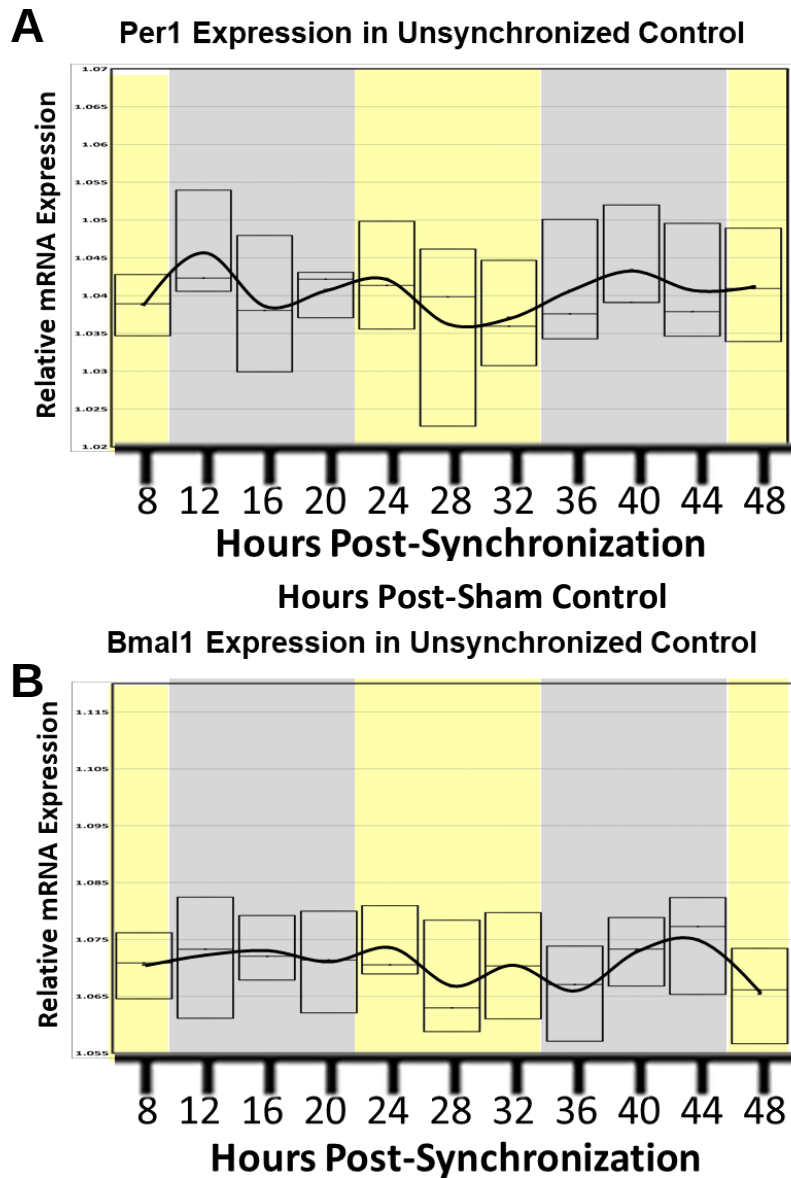


Figure 26: Neuroendothelial cells display a lack of rhythmicity of qPCR expression in the absence of synchronization.

We treated rat neuroendothelial cells with deionized water as a sham control for the Carbachol-treated groups. The cells also underwent the same mechanical perturbations as the cells which underwent Carbachol treatment. After conducting RT-qPCR, we found a lack of rhythmicity in the expression of *Per1* and *Bmal1*.

APPENDIX B: NEUROCHEMICAL AND MORPHOLOGICAL COMPARISONS OF MOTOR NERVE ORGANOIDS AND SPINAL CORD EXPLANTS

Abstract

Organoids are 3D masses of cells differentiated from stem cells *in vitro* that can recapitulate several structural and functional aspects of organs and tissues. They offer unique opportunities to model and treat diseases. We have cultured organoids that express markers of spinal cord motor neurons as well as motor neurons found within the peripheral nervous system. The morphology and neurotransmitter content of these organoids were examined over time to compare the motor nerve organoids to spinal cord explants. Organoids were cultured at different time points and compared to the spinal cord explants throughout the first week of postnatal development. Additional timepoints at two and three weeks postnatal for the spinal cord explants and a 2-month timepoint for the organoids were also compared. We found indications of maturation in the organoids over time, mirrored by similar trends in the spinal cord explants. The levels of the neurotransmitters acetylcholine, gamma aminobutyric acid (GABA), glutamate, and aspartic acid were measured and compared across the developmental timepoints in both the spinal cord explants and the organoid samples. The transmitters increased in level in the spinal cord explants until p14 and then decreased. Although the organoids contained the same transmitters as the spinal cord explants, the developmental changes in transmitters were less marked in organoids. Our chemical measurements support that the organoids contain the appropriate chemicals to model parent tissue and therefore appear accurate *in vitro* models for modeling development and disease.

Introduction

Organoids are 3D masses of cells differentiated from stem cells *in vitro*, from which structural and functional aspects of tissues and organs emerge¹. A key therapeutic advantage of organoids is their unique ability to be cultured from induced pluripotent stem cells (iPSCs) derived from patients². These stem cells can originate from cell types such as skin or blood,

making them an accessible source of stem cells from patients². Since organoids generated from patient iPSCs maintain the patient's genotype, they have been used to model various diseases with a genetic basis^{3,4}. Their 3D nature also allows them to model aspects of tissues that are beyond the scope of 2D tissues⁵. This may confer an advantage in both disease modeling and potential utility in therapeutic transplantation⁵.

A multitude of studies have benefitted from the use of organoids as models of biological phenomena. Compared with 2D cultures, the 3D structure of organoids confers more realistic modeling of biophysical properties and cell-cell interactions⁴⁻⁵. The creation of organoids has also led to findings in human development that otherwise would have been precluded by ethical restrictions^{2,6}. They have also been used to model the effects of viruses and toxic compounds with tissue derived from humans². This has proven particularly useful for testing the effects of viruses that only infect cells with human DNA, including enteroviruses⁴. Organoids from patient iPSCs have been used to test medicinal drugs², implement new strategies for gene therapy², and perform diagnostics². They have recently been used for transplantation in rodents, and the possibility of culturing them from patient iPSCs minimizes the likelihood of tissue rejection after transplantation².

Motor nerve organoids have a particular utility in modeling neurodegenerative diseases that affect spinal cords, including Spinal Muscular Atrophy (SMA) and Amyotrophic Lateral Sclerosis (ALS)⁴. The 3D structure of organoids makes them especially well-suited for modeling ALS⁷⁻⁹. This disease can affect the various regions of the spinal cord differently⁸, and its animal models have been hampered by interspecies differences⁸. In fact, ALS has been modeled with spinal cord organoids generated from ALS patients, further establishing the role of inflammation⁷. Motor nerve organoids have also recently been used for transplant studies in rodents to repair spinal cord lesions¹⁰. There is a growing interest in spinal organoid research,¹¹ as stem cells have recently been transplanted for recovery after spinal cord injury¹².

In using organoids to study disease and development, it is important to understand how organoids model their natural tissue. This information can provide insight into how their rates of maturation compare, which is a factor in the reliability of motor nerve organoids in modeling diseases and development. While the morphology of motor nerve organoids has been characterized³⁻¹⁰, there are few studies examining the changes in their morphology over time as well as a lack of studies in which the organoids contain components of both the central and peripheral nervous systems. In addition, no studies have examined how select neurotransmitter levels change throughout motor nerve organoid development and how this compares with developing spinal cords. Answering these questions will also help establish which time points best align for motor nerve organoids and spinal cords.

While organoids differentiate into the various cell types found in the organ, organoids tend to be smaller, typically less than 1 millimeter in diameter¹³⁻¹⁴. The small size of organoids poses a difficult analytical challenge in measuring their chemical contents¹⁴⁻¹⁵. Additionally, in order to measure the chemical contents, the wide concentration range of molecular classes, such as the neurotransmitters, is problematic, especially as the total volume of an organoid is in the nanoliter or less range. Capillary electrophoresis-mass spectrometry (CE-MS) is an advantageous technique because of its ability to measure many compounds simultaneously without analyte preselection, low volume requirements, and low limits of detection. CE-MS also has a large dynamic range, making it well suited for analyzing small spinal cord organoids and larger spinal cord explants¹⁶⁻²⁰. This study appears to be the first to implement CE-MS for the analysis of neurotransmitters in motor nerve organoids. In this work, motor nerve organoids that model the interface of the spinal cord and peripheral nervous system were generated. This type of organoid should be beneficial for modeling motor neuron diseases, such as ALS, which involves an interplay between the central and peripheral nervous systems, as well as the effects of inflammation on the NMJ in aging^{7,21-22}. The changes in the morphology and select

neurotransmitter content of these organoids were examined over time to compare the development of motor nerve organoids to spinal cords.

Materials and Methods

Chemicals

We obtained standards from Sigma-Aldrich. They were reagent grade or better unless otherwise indicated. Methanol, isopropanol, acetonitrile, and acetic acid were LC-MS grade and obtained from Fisher Chemical. Water was LC-MS grade and obtained from Honeywell Research Chemicals and Fisher Chemical. Formic acid was LC-MS grade and obtained from Thermo Scientific. The background electrolyte solution was prepared using the metabolites reagent kit from 908 devices by adding 1 mL of acid for every 25 mL of metabolite background electrolyte (908 Devices, Metabolites Reagent Kit Part # 810-00033). The metabolite diluent solution was also prepared using the metabolites reagent kit by adding 200 μ L of acid for every 5 mL of diluent (908 Devices, Metabolites Reagent Kit Part # 810-00033). Extraction solution was prepared by adding 0.5% of acetic acid (v/v) to 50/50 water/methanol (v/v). Sodium formate solution was dissolved in 50/50 water methanol (v/v) or 50/50 water/methanol (v/v) plus 0.1% formic acid (vol/vol). Quinine, acetylcholine, GABA, aspartic acid, and glutamic acid (Fluka Biochemika) standards were dissolved in 1% formic acid (v/v) in water and sonicated for 5-10 min.

Organoid Culture

We cultured the mouse embryonic stem cell line ChR2H134R-HBG3 Hb9-GFP, which was a generous gift from Professor Roger Kamm's lab at the Massachusetts Institute of Technology²³. We plated them at a density of 50,000 cells/cm² on top of feeder layers consisting of rat embryonic fibroblasts (ScienCell Research Laboratories). The fibroblasts were plated at a density of 30,000 cells/cm² and inactivated by treatment with Mitomycin-C (Abcam). We cultured them in growth media, consisting of EmbryoMax DMEM (EMD Millipore), 15% v/v stem-cell certified FBS (Stem Cell Technologies), 1X Nonessential Amino Acids (Corning), 1X

EmbryoMax nucleosides (EMD Millipore), 1X Pen-Strep (Gibco), 2 mM L-glutamine (Corning), 0.1 mM beta-mercaptoethanol (Sigma), and 103 units/ml Leukemia Inhibitory Factor (EMD Millipore).

We followed an established protocol for culturing the organoids from stem cells²⁴. We allowed the stem cells to form 3-dimensional spherical aggregates on top of the feeder layer for 9 days. Then we transferred them to droplets of Matrigel (Corning), and we cultured them in differentiation media. This consisted of 45% v/v Advanced DMEM/F-12 (Gibco), 45% v/v Neurobasal medium (Gibco), 10% v/v Knockout Serum Replacement (Gibco), 2 mM L-glutamine (Corning), 1X Pen-Strep (Gibco), 0.1 mM Beta-mercaptoethanol (Sigma), 1 μ M retinoic acid (Santa-Cruz), and 1 μ M purmorphamine (Sigma).

Immunofluorescence

We fixed the organoids and spinal cords in 4% v/v Paraformaldehyde in PBS for a minimum of 30 min prior to blocking and permeabilization. We permeabilized with 0.3% Triton-X (EMD Millipore) in PBS during blocking and incubations with primary and secondary antibodies. We blocked the samples for 1 h with 5% v/v Normal Goat Serum (Abcam) in PBS with 0.3% v/v Triton-X at RT. We diluted the primary and secondary antibodies in 2% v/v Normal Goat Serum in PBS with 0.3% Triton-X. Each incubation with primaries was for 2 days at 4°C with gentle agitation. Each incubation with secondaries lasted 1 day at 4°C with gentle agitation in darkness.

In between incubations, we removed excess primary antibodies, secondary antibodies, and DAPI by washing the samples at least 3 times, totaling at least 30 min with PBS. When washing the organoids, we used 40 μ m cell strainers (Corning). For primary antibodies, we used beta-3-tubulin anti-chicken (1:1000, Abcam), beta-3-tubulin anti-mouse (1:1000, Abcam), ALDH1L1 anti-rabbit (1:1000, Abcam), Myelin Protein Zero anti-chicken (1:500, Abcam), and Hb9 anti-rabbit (1:1000, Invitrogen). We detected them with Alexa Fluor 488 goat anti-chicken IgG (1:1000, Invitrogen), Alexa Fluor 568 goat anti-mouse IgG (1:1000, Invitrogen), Alexa Fluor 568

goat anti-rabbit IgG (1:1000, Invitrogen), and Alexa Fluor 568 goat anti-chicken IgG (1:1000, Invitrogen). For nuclei staining, we incubated with DAPI (Invitrogen) in PBS for 1 h at RT in darkness. To mount the samples and protect them from photobleaching, we used ProLong Antifade Gold mountant (Invitrogen).

Microscopy and Image Analysis

We performed brightfield imaging and measurement of the organoids using an AxioCam MR microscope, which uses AxioVision software. We performed fluorescent imaging with LSM 700 and LSM 710 inverted confocal microscopes (Zeiss), which operated with Zen software. We used lasers with wavelengths of 405 nm, 488 nm, and 555 nm. These corresponded respectively to the excitation wavelengths of DAPI, Alexa Fluor 488, and Alexa Fluor 568. We used 10X and 20X objective lenses.

Animals

The Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign approved all animal use protocols. We followed the National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals. We used C57BL/6J mice (Rhodes Laboratory), which were maintained at the University of Illinois at Urbana-Champaign. Spinal cords were extruded whole by gentle pressure with a syringe²⁵. We performed spinal cord extractions post-mortem at postnatal day 0, day 7, day 14, and day 21. We used the minimum number of animals necessary for experimental significance.

Biological Sample Preparation for CE-MS

We followed an established protocol to remove the Matrigel from the organoids²⁶. We cooled the organoids to a temperature of 4 °C to liquefy the Matrigel. We washed them three times in 1X PBS that was cooled to 4 °C and then centrifuged them at 1000 rpm for 5 min. We repeated the centrifugation step three times, removing the supernatant each time. We then imaged the organoids using an Axio Imager 2 fluorescence microscope (Zeiss) to determine their size. We isolated individual organoids by placing droplets of PBS onto a microscope slide, finding a

droplet with a single organoid in it, and transferring that droplet with the organoid into a sample tube. We performed this procedure with the smallest droplets possible to minimize the amount of PBS solution that was transferred into the sample tubes with the individual organoids.

We placed the samples in a SpeedVac to remove the PBS in each sample tube. We added extraction solution consisting of 50/50 methanol/water with 0.5% acetic acid to each tube. Then we sonicated them to facilitate the extraction of neurotransmitters of interest. We placed the samples in a SpeedVac to dry and concentrate their contents. We performed a sample cleaning step by adding 100 μ L of 4:1 isopropanol:acetonitrile to the dried sample tubes. We then centrifuged the samples to allow the salts from the PBS solution to pellet at the bottom of the tube²⁷. After centrifuging, we transferred 95 μ L of each sample into a new sample tube, taking care to not disturb the pellet at the bottom of the sample. We dried the samples to concentrate using a SpeedVac and stored them at -80 °C until CE-MS analysis²⁸. We placed a membrane filter (Breathe-Easier Sealing Film, Diversified Biotech) over the sample tubes each time they were dried in the SpeedVac to minimize any possible sample contamination from bumping.

We measured the wet weight of each spinal cord sample. We added extraction solution consisting of 50/50 methanol/water with 0.5% acetic acid such that 10 μ L of solution was added for every mg of tissue weight. We sonicated and homogenized the samples with a handheld homogenizer when necessary to break up the tissue. We then centrifuged the samples and transferred an aliquot of the supernatant into a new tube. We dried the samples to concentrate using a SpeedVac and stored them at -80 °C until CE-MS analysis²⁸.

CE-MS Analysis

CE-MS analysis was performed using a ZipChip microfluidic CE system (908 Devices, Boston MA) attached to the front end of a Q-TOF ESI-MS instrument (maXis 4G, Bruker Corp., Bellerica, MA)²⁹. High speed chips (908 Devices, Part # 810-00195) and metabolite background electrolytes from 908 Devices (908 Devices, Metabolites Reagent Kit Part # 810-00033) were used to analyze samples. Samples were redissolved in a 1 μ M quinine internal standard

solution made with metabolite diluent solution from the metabolites reagent kit provided by 908 Devices (908 Devices, Metabolites Reagent kit, Part #850-00033). CE separations were performed with a field strength of 1000 V/cm, an injection volume of 5 nL, and 3-minute run times.

Samples were centrifuged for 1.5 min at 10,000 rcf before analysis. All samples were run in triplicate. To ensure that no contamination to samples occurred, negative controls of the solutions used during the sample preparation procedure were also analyzed. The mass spectrometer was calibrated regularly using the mass range of m/z 70–1000 by infusing sodium formate (10 mM) via the electrospray ionization source.

Data Analysis

For image analysis, we used ImageJ and Graph Pad Prism. We performed one-way ANOVA for the comparisons of Hb9 expression. For CE-MS, we performed data analysis using Compass Data Analysis 4.4 (Bruker Corp., Bellerica, MA). We smoothed the extracted ion chromatograms using a Gaussian smoothing function. We identified the peaks by matching migration time and mass to charge ratio to an analytical standard. We integrated all of the peaks manually. To account for instrument variation, we normalized the peak intensities to the intensity of the quinine internal standard for each run. We performed statistical analysis using a rank sum test (Mann-Whitney U test) with a two-tailed hypothesis. We used a Q test with a confidence level of 95% to test for outliers.

Results and Discussion

We cultured the organoids from mouse embryonic stem cells (mESCs) in growth media on feeder layers of rat embryonic fibroblasts for the first 9 days *in vitro* (DIV), (Figure B1). Before their use in the feeder layer, the fibroblasts were inactivated with Mitomycin-C, to prevent their own proliferation while allowing them to secrete growth factors for the mESCs. Culture on the feeder layer was conducive to the formation of 3-dimensional spherical aggregates of mESCs known as neurospheres (Figure B1). Then, at DIV 9, we cultured the neurospheres in

differentiation media and transferred them to droplets of Matrigel (Figure B1), which provided further 3-dimensionality of growth as well as nutrients.

We cultured the organoids and extracted the spinal cord explants at different time points to compare maturation rates, using the onset of neurogenesis as a benchmark. Mouse spinal cord neurogenesis begins on gestational day 11³⁰, and mouse gestation lasts for an additional 10 days³⁰. Therefore, at postnatal day 0 (P0), neurogenesis has occurred in the mouse spinal cord for 10 days. It has been shown that in organoids differentiated with retinoic acid and purmorphamine, neurogenesis occurred at day 6²⁵. Thus, by DIV 16, the organoids would be expected to have undergone neurogenesis for 10 days. We also examined spinal cord explants and organoids one week later at P7 and DIV 23. Since delayed maturation in the organoids was possible, we also included a 2-month time point for comparison. We also analyzed spinal cord explants at P14 and P21 for comparison.

Mouse spinal cords and motor nerve organoids contained neurons and astrocytes.

When we cultured organoids and developing spinal cords, we observed that both exhibited comparable markers of differentiation, but with slightly different time courses. We performed immunostaining to determine the cellular composition of the organoids and spinal cord explants. We determined that, like the spinal cord explants, the organoids were comprised of both neurons and astrocytes (Figure B2). Having multiple cell types is requisite for a cluster of cells to be an organoid¹. Moreover, mouse spinal cords also consist of multiple cell types, including neurons and astrocytes³¹. We found that neurons were present in the spinal cord explants at P0, P7, P14, and P21 (Figure B2). This is consistent with literature that has shown that neurogenesis begins in mouse spinal cords before birth³⁰. Neurons were also present in the organoids at DIV 16, DIV 23, and DIV 55 (Figure B2). This is consistent with the finding that neurogenesis begins by DIV 6 in organoids differentiated via retinoic acid and purmorphamine²⁵. These findings are consistent with previous studies, which have demonstrated that astrocytes are expressed by P0³² and continue to be expressed into mouse adulthood³¹.

Hb9 expression in spinal cords and organoids decreased with maturation.

In order to determine whether the organoids were derived from the motor-neuron lineage, we performed immunostaining for the gene Homeobox-9 (Hb9), which is expressed in the nuclei of motor neurons in the spinal cord³³. The organoids expressed Hb9 at DIV 16, DIV 23, and DIV 55 (Figure B3). The spinal cord explants also expressed Hb9 at P0, P7, P14, and P21 (Figure B3). Studies have shown that in the mouse spinal cord, motor neurons are present before birth at embryonic day 10.5³⁰⁻³¹. Research has also shown that when retinoic acid and activation of the Hedgehog signaling pathway are used for the differentiation of motor organoids, motor neurons express Hb9 at DIV 6²⁴.

From P0 to P21, the portion of neurons expressing Hb9 decreased (Figure B3). This was indicated by the presence of beta-III tubulin and the absence of Hb9 (Figure B3). Similarly, from DIV 16 to DIV 55, there was a decrease in Hb9 expression in neurons (Figure B3). These decreases were significant for the spinal cords ($p=0.002$), as well as the organoids ($p=0.003$). This is consistent with previous research on motor organoids as the percentage of Hb9 positive cells decreases over the course of 45 DIV for motor organoids differentiated via retinoic acid and purmorphamine³⁴ and Hb9 expression decreases in postnatal spinal motor neurons as they mature³³. Thus, in both the spinal cord explants and the motor organoids, this decrease in Hb9 expression may reflect changes due to maturation.

MPZ was present in spinal cord explants and motor nerve organoids.

Myelin Protein Zero (MPZ) is found in motor neurons in the peripheral nervous system³⁵. We found that MPZ was present in the spinal cord explants at P0, P7, P14, and P21 (Figure B4). MPZ was also present in the organoids at DIV 16, DIV 23, and DIV 55. Since MPZ is not expressed in the central nervous system (CNS) of mammals³⁵, it is likely that the spinal cord explants had peripheral nerves connected to them. Hb9 expression is specific to motor neurons of the central nervous system³³, and its presence suggests that the organoids contain CNS motor neurons (Figure B3). However, the presence of MPZ (Figure B4) suggests that the

organoids also contain peripheral motor neurons. This indicates that the organoids represent an interface between the spinal cord and the peripheral nervous system. Notably, these findings do not indicate the presence of interneurons—which do not express Hb9 or MPZ³⁶. This type of organoid could be beneficial for modeling motor neuron diseases, such as Amyotrophic Lateral Sclerosis (ALS), which involves an interplay between the central and peripheral nervous systems^{7,10,20-21}.

ACh, GABA, Glu, and Asp were present in spinal cords and organoids.

We measured the levels of glutamate (Glu), γ aminobutyric acid (GABA), acetylcholine (ACh), and aspartic acid (Asp) for the organoids and spinal cords at several time points. These determinations enabled us to evaluate the changes in levels of these neurotransmitter over time for both the organoids and spinal cord explants. Glu and GABA are, respectively, the main excitatory and inhibitory neurotransmitters in neurons. In mammals, ACh is the neurotransmitter most directly associated with the activation of muscles and the formation of neuromuscular junctions which allow for their activation²². Asp has also been shown to be abundant throughout rodent spinal cords³³, where it functions as an excitatory neurotransmitter³³.

Neurotransmitter levels were measured using CE-MS. In CE-MS, compounds are separated according to their size, charge, and shape. After separation, the analytes are electrosprayed and characterized via mass spectrometry (Figure B5). Using this technique, ACh, GABA, Glu, and Asp were detected in both spinal cord explants and the organoids (Figure B6). The neurotransmitter/neurotransmitter total ratio normalization approach was used to compare neurotransmitter levels across the different timepoints.

During early development, most neurotransmitters throughout the body increase as synapses begin to form³⁸. The mouse spinal cord becomes more innervated from P0 to P7^{30,39}, which is also a key time for synaptogenesis throughout the central and peripheral nervous systems³⁹. Starting at P0, several motor functions begin, which are further developed at P14⁴⁰. Then, neurotransmitter levels may decrease as synaptic pruning occurs, peaking in the early

postnatal period⁴¹. During this process, some synapses are removed to strengthen the neuronal pathways that are used the most⁴¹. This may explain some of the decreases we observed in neurotransmitter levels at later time points. For the organoids, no statistically significant changes in neurotransmitter levels were present over time, which may reflect variability between organoids.

ACh levels increased during the first 2 weeks postnatally in spinal cord explants and trend towards increasing from DIV 16 to 23 for organoids.

ACh levels in spinal cord explants increased significantly from P0 to P7 (Figure B6A, $p < 0.001$) and then again from P7 to P14 (Figure B6A, $p < 0.05$). This is consistent with previous research on animal physiology, which showed that rodents gain various reflexes between the P0 and P7 timepoints⁴¹⁻⁴⁴. These increases in ACh levels support the idea that the neurotransmitter levels between P0 and P7 increase as the animals gain reflexes and motor control. In addition, exercise has been shown to increase surface expression of ACh receptors⁴⁵⁻⁴⁶ and elevated ACh signaling leads to elevated ACh concentrations⁴⁷⁻⁴⁸. A similar but nonsignificant trend was observed in the organoids with an increase in the average from DIV 16 to DIV 23 (Figure B6B). For the spinal cord explants, from P14 to P21, there was a significant decrease in ACh levels (Figure B6A, $p < 0.05$). This may be due to synaptic pruning in the mouse spinal cord at this time⁴¹. ACh levels were lower overall in the organoids compared with the spinal cord explants (Figure B6B). This may be explained by the absence of muscle in the organoids. Studies have shown that the presence of muscle increases levels of ACh receptors⁴⁸ and the expression of acetylcholinesterase, the enzyme which catabolizes ACh⁴⁹⁻⁵¹. This feedback enhancement of the cholinergic system by active muscle could be effectively examined in a chimeric system that included motor nerve organoids and stem-cell generated muscle.

GABA levels increased at P14 in spinal cord explants, but no difference was detected over time for organoid samples.

From P0 to P7, relative GABA levels almost doubled, although given the extent of sample-to-sample variation, this change was not significant in spinal cord explants (Figure B6A). This increase may be consistent with research that has shown that the amount of GABA in mouse spinal cords increases during this period⁵³. There was a statistically significant increase in GABA levels from P7 to P14 (Figure B6A, $p < 0.01$). This period corresponds to heightened synaptogenesis³⁹ and the development of motor reflexes^{40,44}. There was also a significant decrease in GABA levels from P14 to P21 (Figure B6A, $p < 0.05$), perhaps reflecting synaptic pruning. For the organoids, GABA levels were similar at DIV 16 and DIV 55, but GABA was undetectable in DIV 23 (Figure B6B). Levels were also relatively low at DIV 16 and DIV 55 (Figure B6B), and they were lower overall compared with the mouse spinal cord explants (Figure B6A). No studies have examined whether GABA increases in motor neurons from function / use (e.g., as a result of exercise), which could be one possible explanation for the low levels in organoids. However, GABA has several key roles in neural development⁵⁴. The lack of change in explants, which have no interactions with muscles, suggests that GABA may be regulated by muscle-neuron feedback.

Glu levels decreased from P7 to P14 in spinal cord explants, no difference was observed for organoid samples.

Glu levels in spinal cord explants fluctuated throughout the first 21 postnatal days. From P7 to P14, there was a significant decrease in Glu levels (Figure B6A, $p < 0.01$). This may have been due to synaptic pruning, which research has shown occurs in the mouse spinal cord within this timeframe⁴¹. This is consistent with research which has shown that a decrease in use of glutamatergic spinal cord motor neurons leads to their decline⁵⁵. For the organoids, Glu levels did not change significantly between DIV 16, DIV 23, or DIV 55, and the ranges at these time points overlapped (Figure B6B).

Asp levels in spinal cord explants increased from P7 to P14 and Asp levels trend towards being decreased in organoids from DIV 23 to 55.

As with the other neurotransmitters, these are the first reports of Asp levels in motor nerve organoids. In mouse spinal cord explants, Asp was present in greater levels than ACh, GABA, and Glu. Although not statistically significant, the mean relative levels of Asp were approximately halved from P0 to P7 (Figure B6A). This may have reflected synaptic pruning⁴¹. This was followed by a significant increase in Asp levels from P7 to P14 (Figure B6A, $p < 0.01$). Research has shown that P14 is a key developmental time point in the spinal cord for synaptogenesis³⁹ and motor reflexes^{40,44}. Studies have shown that exercise can significantly increase the levels of aspartate aminotransferase, the enzyme that synthesizes aspartate⁵⁶. It has also been shown that decreased neural activity in the spinal cord can lead to a 6-8-fold decrease in aspartate synthesis⁵⁷. In the organoids, there was a nonsignificant but more than 3-fold decrease in Asp levels from DIV 23 to DIV 55 (Figure B6B). It is not clear whether this was due to synaptic pruning or variation between organoids.

Conclusions

We formed a motor neuron organoid by differentiating mouse embryonic stem cells expressing both central and peripheral motor neuron markers. We confirmed that the motor nerve organoids expressed neurons and astrocytes (Figure B2). They also expressed both the central motor neuron marker Hb9 (Figure B3) and the peripheral motor neuron marker MPZ (Figure B4). Similarly, neurons, astrocytes, Hb9, and MPZ were also present in the mouse spinal cord explants (Figure B2-4). For both the spinal cord explants and organoids, Hb9 expression in neurons decreased significantly over time (Figure B3). This reflected a trait in maturation which was shared by both the spinal cord explants and organoids.

This study is the first to use CE-MS to measure neurotransmitter levels in organoids containing motor neurons. In the spinal cord explants, ACh, GABA, Glu, and Asp all were detectable with significant increases in ACh levels from P0 to P7 and in GABA, Glu, and Asp

from P7 to P14 (Figure B6A). Significant decreases were found for Glu levels from P7 to P14 and in ACh and GABA levels from P14 to P21 which could be attributed to synaptic pruning. For the organoids, all four neurotransmitters were detected, but no significant changes in levels between the different time points were observed (Figure B6B). This may have been due to variability and the lack of muscle and its interaction with motor neurons. Similar trends for ACh were observed between spinal cord explants and the organoids where there was a significant increase in ACh between P0 and P7 for the spinal cord explants and a trend towards an increase between DIV 16 and DIV 23 for the organoids. No other trends were observed for GABA, Glu, and Asp between the spinal cord explants and the organoids.

These results suggest that while organoids contain similar neurotransmitters as spinal cord explants, they do not completely recapitulate the transmitter trends found in the spinal cord during development. Due to the variability in measured neurotransmitter levels from pooled organoid samples, analyzing the chemical contents of single organoids may be advantageous, just as has been done with single cell measurements⁵⁹⁻⁶⁰. This would aid in understanding the chemical variation between organoids and may help in determining how accurately organoids chemically model the spinal cord. Current methods used to create organoids have demonstrated that there can be a high amount of heterogeneity between organoids grown in the same batch, leading to high inter-experimental variation⁶¹. This work helps pave the way for making chemical measurements in organoids to determine if they contain the correct chemical contents to model native tissue and therefore are accurate *in vitro* models for modeling development and disease.

Acknowledgments

Chen Huang and Shannon Murphy conducted the CE-MS measurements. Stanislav Rubakhin removed the mouse spinal cords.

Figures

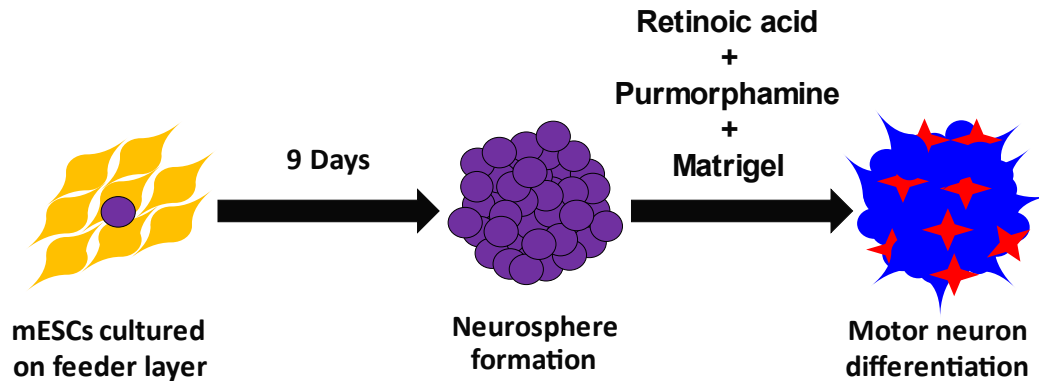


Figure 27: Schematic of organoid culture.

Mouse embryonic stem cells (mESCs) were cultured on feeder layers of rat embryonic fibroblasts in growth media for 9 days. The resulting neurospheres were then transferred to Matrigel droplets and cultured in differentiation media containing retinoic acid and purmorphamine.

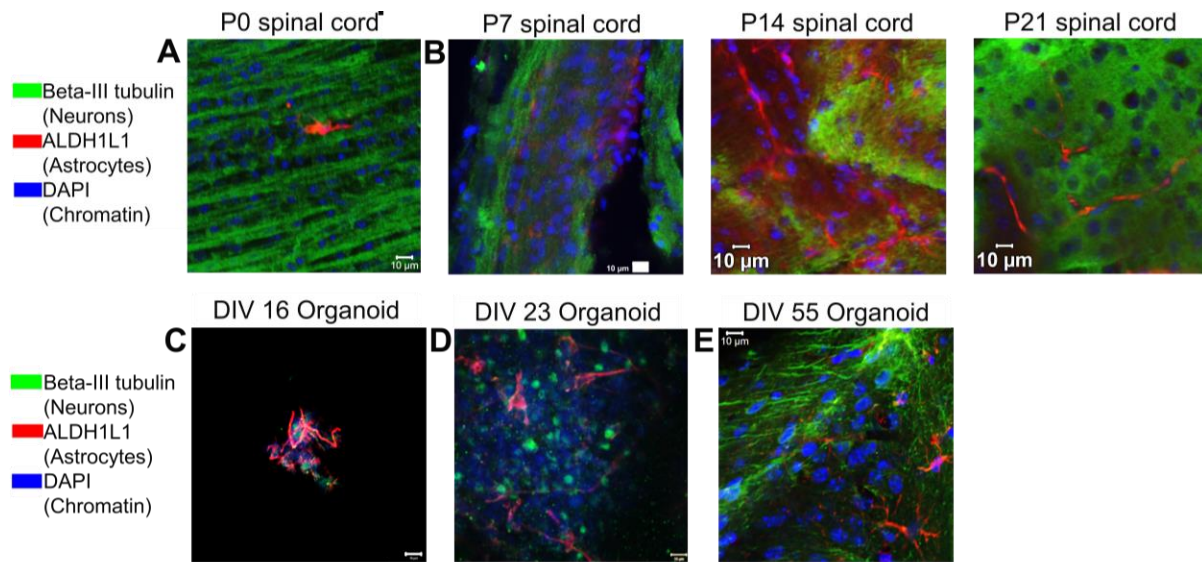
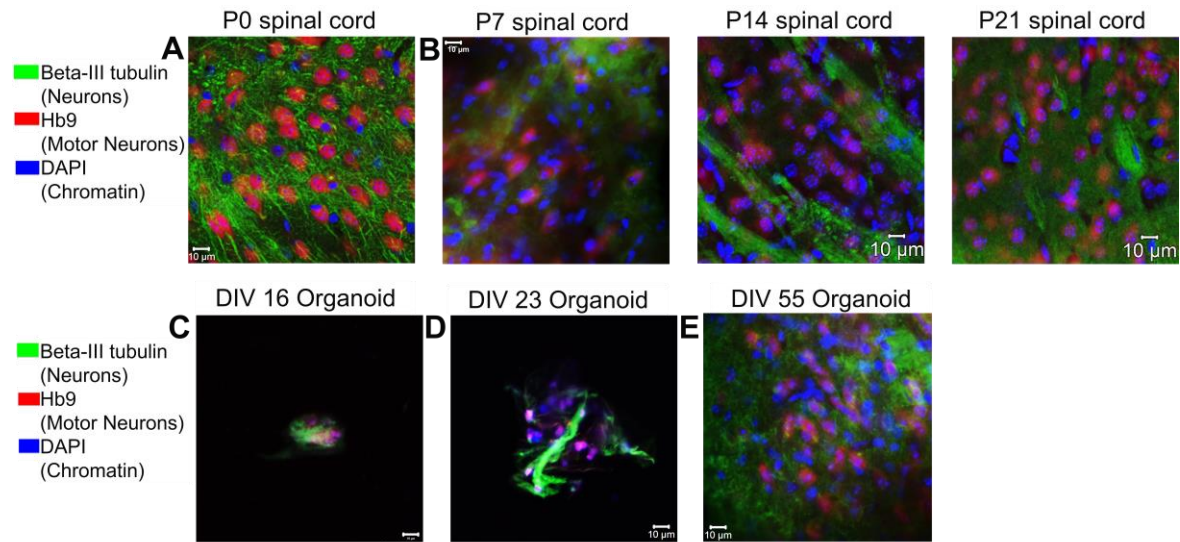


Figure 28: Neurons and astrocytes comprise motor organoids.

A-E: Green: Beta-III Tubulin, expressed in neurons. Red: Aldehyde Dehydrogenase 1 Family Member 1 (ALDH1L1), expressed in astrocytes. Blue: 4',6-diamidino-2-phenylindole (DAPI), a chromatin marker. Scalebars 10 μm. **A-B**: Spinal cord explants at Postnatal days 0, 7, 14, and 21 (P0, P7, P14, and P21). **C-E**: Organoids at 16 Days *In Vitro* (DIV), 23 DIV, and 55 DIV.



F Percent of Neurons Expressing Hb9

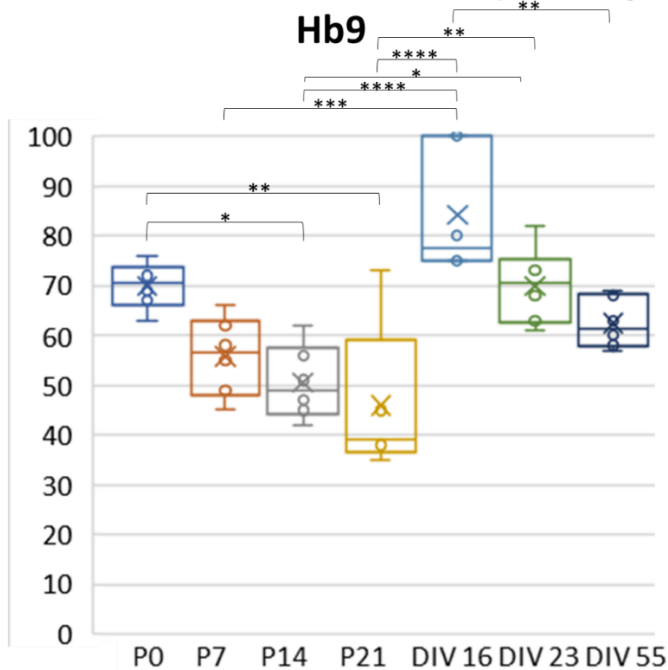


Figure 29: Motor neuron gene Hb9 is expressed in motor organoids and spinal cord explants.

A-E: Green: Beta-III tubulin, expressed in neurons. **Red:** Homeobox-9 (Hb9), expressed in nuclei of motor neurons. **Blue:** 4',6-diamidino-2-phenylindole (DAPI), a chromatin marker. Scalebars 10 μ m. **A-B:** Spinal cord explants at Postnatal days 0, 7, 14, and 21 (P0, P7, P14, and P21). **C-E:** Motor organoids at Day *In Vitro* (DIV) 16, DIV 23, and DIV 55. **F:** Hb9 expression decreased significantly over time for spinal cords ($p=0.002$) and organoids ($p=0.003$), one-way ANOVA. * Represents $p<0.05$; ** represents $p<0.01$; *** represents $p<0.001$.

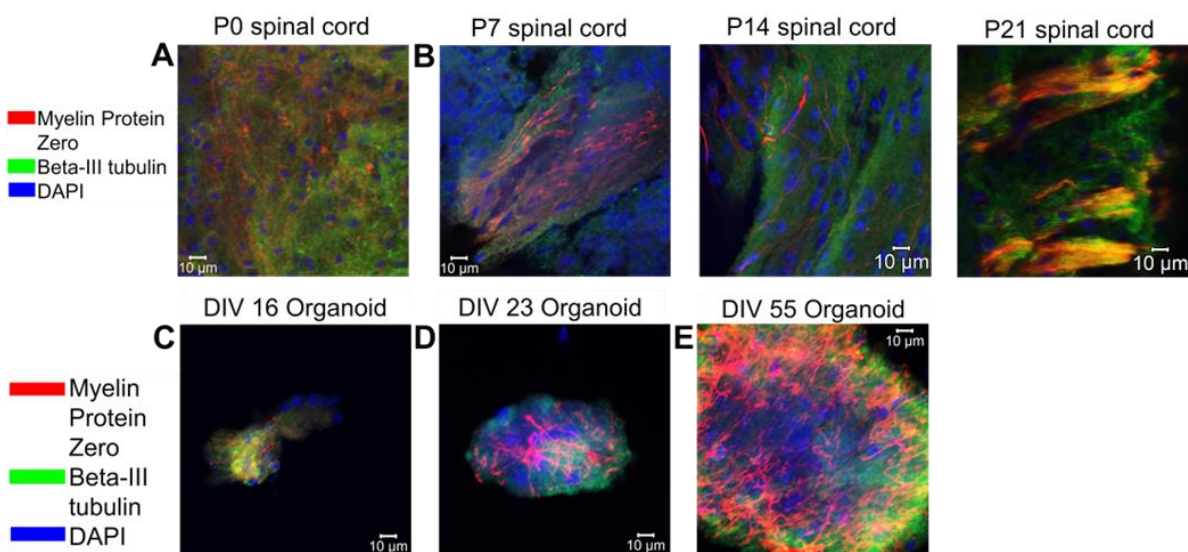


Figure 30: Myelin Protein Zero is present in organoids and spinal cord explants.

A-E: Green: Beta-III Tubulin, expressed in neurons. **Red:** Myelin Protein Zero, expressed in motor neurons. **Blue:** 4',6-diamidino-2-phenylindole (DAPI), a chromatin marker. Scalebars 10 μm. **A-B:** Spinal cord explants at Postnatal days 0, 7, 14, and 21 (P0, P7, P14, and P21). **C-E:** Organoids at 16 Days *In Vitro* (DIV), 23 DIV, and 55 DIV.

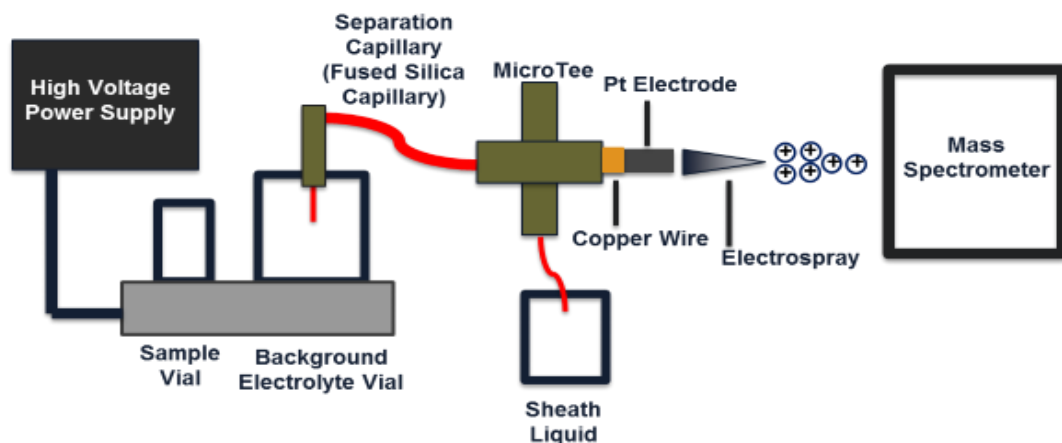
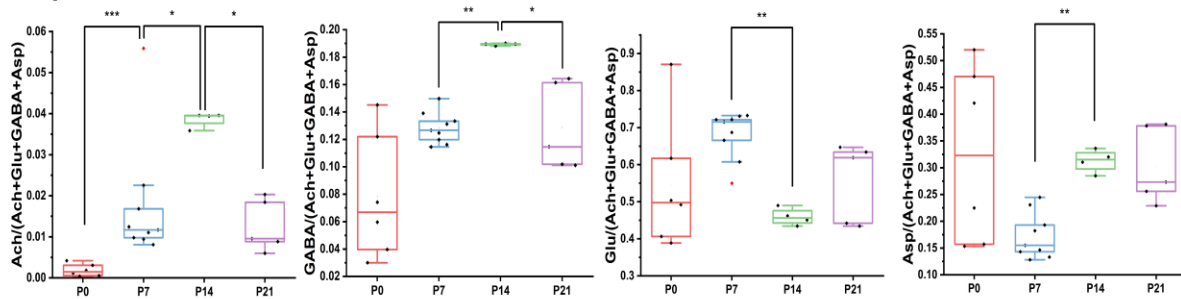


Figure 31: Schematic of Capillary Electrophoresis-Mass Spectrometry.

In Capillary Electrophoresis-Mass Spectrometry (CE-MS), capillary electrophoresis separates compounds by size, charge, and shape. The compounds are then ionized and undergo mass spectrometry, which allows for identification by their mass to charge ratios.

A. Spinal Cords



B. Organoids

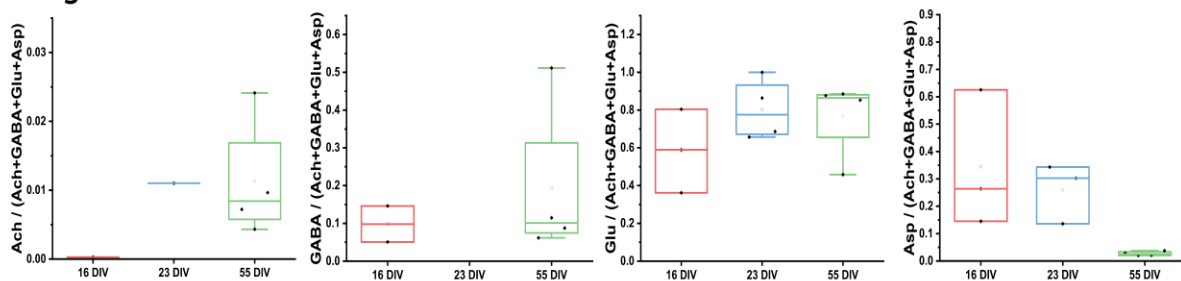


Figure 32: Acetylcholine, GABA, aspartic acid, and glutamate levels quantified in spinal cord explants.

A-D: Comparison of acetylcholine (**A**), gamma-amino butyric acid (GABA) (**B**), aspartic acid (**C**), and glutamic acid (**D**) levels in P0 vs. P7 mouse spinal cord explants, measured by Capillary Electrophoresis-Mass Spectrometry (CE-MS). Preliminary data shows a possible neurotransmitter increase in P7 vs. P0 mice. Mann-Whitney U test was used, two-tailed hypothesis, 0.05 significance level. **A:** p-value=0.1 **B:** p-value=0.12 **C:** p-value=0.2 **D:** p-value=0.1 **A-D:** n=6 for P0 timepoint and n=5 for P7 timepoint. Red data points represent outliers with Grubb's test with $\alpha=0.05$. Mann-Whitney U test was used with a two-tailed hypothesis and assuming 0.05 significance level. * p<0.05; ** p<0.01; *** p<0.001.

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