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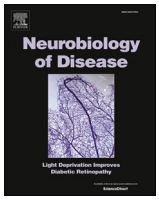

Review

Rhythms in barriers and fluids: Circadian clock regulation in the aging neurovascular unit

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ABSTRACT

The neurovascular unit is where two very distinct physiological systems meet: The central nervous system (CNS) and the blood. The permeability of the barriers separating these systems is regulated by time, including both the 24 h circadian clock and the longer processes of aging. An endogenous circadian rhythm regulates the transport of molecules across the blood-brain barrier and the circulation of the cerebrospinal fluid and the glymphatic system. These fluid dynamics change with time of day, and with age, and especially in the context of neurodegeneration. Factors may differ depending on brain region, as can be highlighted by consideration of circadian regulation of the neurovascular niche in white matter. As an example of a potential target for clinical applications, we highlight chaperone-mediated autophagy as one mechanism at the intersection of circadian dysregulation, aging and neurodegenerative disease. In this review we emphasize key areas for future research.

1. Introduction

While it is generally accepted that the neuron is the functional unit of the central nervous system, and that cognition arises from the networked activity of interconnected neurons, what is often over-looked in neuroscience is the critical role of the neurovascular unit in support of neuronal function. Even though much of the field of cognitive neuroscience measures blood flow as a proxy of brain function (e.g. BOLD response in fMRI), we still often attempt to understand neural circuits without consideration of the multiple other cell types and fluids that support and modify neuronal function. In addition to these "structural blinders" we also often have "temporal blinders". We sometimes will conduct measures of neuronal function at one time of day or at one age, ignoring the well-known circadian and developmental modulation of nearly all biological processes.

The concept of the neurovascular unit was first proposed to expand investigations of central nervous system (CNS) disease beyond a purely neurocentric focus (Hawkins and Davis, 2005; Iadecola, 2017). This basic idea emphasizes that CNS function depends not only on the electrical activity of neurons but also requires coordinated crosstalk between all cell types in the CNS, comprising neuronal, glial and vascular compartments (Lo et al., 2003; Henstridge et al., 2019). Over the past two decades, this concept has been further refined and extended. In the context of CNS injury and disease, it is now accepted that the neurovascular unit is dynamic and plastic, with substrates and signals actively shifting between mechanisms of injury and repair (Lo, 2010; Moskowitz et al., 2010). Furthermore, the neurovascular unit is not isolated, and thus the CNS is intimately connected to systemic biology, including interactions with the immune system, gut microbiome, and cardiovascular physiology (Fung et al., 2017; Powell et al., 2017; Illiano et al., 2020; Morais et al., 2021; Ungvari et al., 2021; Tan et al., 2022; Tiedt et al., 2022). In this way, the CNS is intricately interconnected with changes to the body.

Although the original definition of the neurovascular unit was based on cellular compartments, it is now increasingly appreciated that intracellular mechanisms cannot be understood without consideration of the extracellular milieu. For example, blood-brain barrier function is mediated not only by cell-cell signalling between endothelium and perivascular astrocytes and pericytes, but it also involves complex interactions with the extracellular matrix (Baeten and Akassoglou, 2011;

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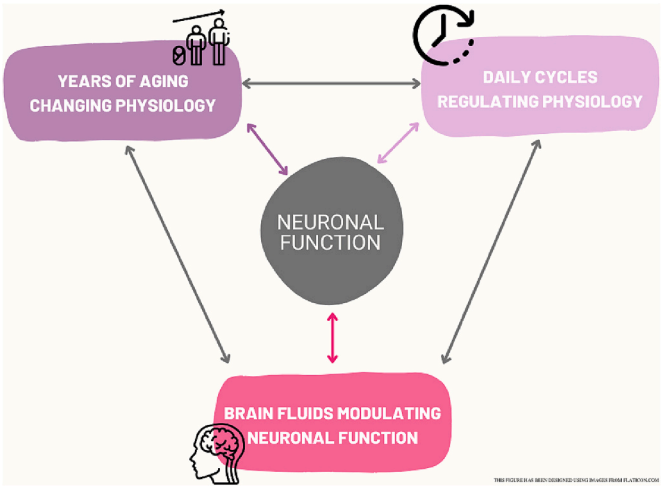

Fig. 1. The interplay of different systems at the neurovascular unit: The circadian clock, aging and the physiology of brain fluids.

Pan et al., 2016; Edwards and Bix, 2019; Yue et al., 2019; Li et al., 2022). Similarly, neurotransmitter kinetics depend not only on neuronal-astrocytic interplay at the synapse, but also on the dynamic flow and distribution of cerebrospinal fluid (CSF) (Rodan et al., 2015). Hence, homeostatic regulation of cerebrospinal fluid and the extracellular milieu is essential for function of the neurovascular unit (MacAulay, 2021; Rasmussen et al., 2022b).

One of the most powerful systemic modifiers of neuronal function is aging (Fig. 1). In almost all CNS disorders, aging plays a central role and the aging brain is inherently more vulnerable to wide spectrum of insults (Chen et al., 2010; Hou et al., 2019; He et al., 2020). Beyond direct effects within the neurovascular unit, aging also profoundly alters almost all aspects of systemic biology, including circadian biology (Buijink and Michel, 2021; Tiedt et al., 2022). The circadian system is an evolutionarily conserved mechanism that provides a temporal organizing framework for almost all biological processes (Sartor et al., 2019; Lee et al., 2021; Rosbash, 2021; Walker et al., 2021; Poole and Ray, 2022). It is possible that the aging brain becomes more susceptible to disease because decreased amplitude of the aging circadian clock alters the amplitude of clock-regulated gene expression and changes phase relationships of clock-regulated processes. If this is so, what candidate mechanisms might be involved? In this review, we survey the accumulating literature and assess the overall framework that pathophysiologic mechanisms in the brain that can be interpreted in the context of how aging affects the circadian clock and changes in CNS fluid homeostasis.

2. Circadian rhythms and effects of aging

2.1. Circadian systems

Continuous rotation of the Earth around the Sun, as well as around its own axis, creates a complex yet highly predictable set of rhythmic changes in the physical environment. These pronounced, daily changes throughout the biosphere have inspired the theory that daily, temporal, biological structures – circadian clocks - are an adaptive response to the external world (Roenneberg and Merrow, 2002; Gerhart-Hines and Lazar, 2015; Jabbur and Johnson, 2021). Circadian clocks are characterised by several key properties (Pittendrigh, 1960). A most remarkable property is the continuation of oscillations in constant conditions. Free-running circadian rhythms have a period of approximately 24 h. Rhythms of exactly 24 h are achieved through entrainment to zeitgebers (see Box 1) – for example, the daily cycles of light and dark or warm and cold. Entrainment leads to a specific phase of expression, meaning that a recurring point of a rhythmic process (peak, trough, etc) is allocated to a specific time of day. Thousands of processes are regulated by the circadian clock, and thus, chronobiology is critical to understand when conceptualising how neuronal function is regulated in time.

The circadian pacemaker in mammals is located in the suprachiasmatic nucleus (SCN). The SCN receives direct retinal input that mediates entrainment to light (Lokshin et al., 2015; Fernandez et al., 2016; Foster et al., 2020). SCN cells function as a tightly coupled neuronal network, with important roles of neuropeptides such as vasopressin and vasoactive intestinal peptide in intercellular coupling (Freeman and Herzog, 2011; Ono et al., 2021). The SCN pacemaker regulates the entrained phase found in many other tissues in the body. Extra-SCN cells have their own, autonomous circadian rhythmicity (Reppert and Weaver, 2002). We do not currently have a good understanding of the mechanisms for the control of phase in peripheral tissues by SCN, but experiments have shown important roles of neuronal signals, humoral signals, and mediating pathways such as driven rhythms in food intake and body temperature (Silver et al., 1996; Schibler et al., 2015; Vujovic et al., 2015).

In mammals, the molecular circadian clock in each cell can be characterised by following expression levels within transcriptional feedback loops (Takahashi, 2017). The activators BMAL1 and CLOCK heterodimerize and induce the transcription of thousands of clock-regulated genes via binding to *E*-boxes. PERIOD (Per) 1, 2 and 3 and CRYPTOCHROME (Cry) 1 and 2 are among these and they function as repressors of BMAL1:CLOCK transcriptional activity. This is subsequently followed by reduced expression of Per and Cry themselves. This is thought to be *the* central regulatory feedback loop. Knockout of just one of these components (BMAL1) leads to profound loss of circadian rhythms (Bunger et al., 2000). A systems biology approach highlights key roles of other components (Ueda, 2007). Phosphorylation, acetylation and ubiquitination all play an important role in the circadian clock mechanism (Crosby and Partch, 2020). The circadian system therefore is organised bottom up as feedback loops within each individual cell and top down as zeitgeber sensitive organs integrating signals from the environment. The 'problem' of circadian organisation, whereby how entrainment of various tissues is achieved and measured, remains a massive problem in the field of circadian biology. Multiple experimental approaches are in common use (see Box 1).

How is the circadian clock relevant for medicine? There are a number of reports demonstrating that circadian disruption increases risk for pathologies including cancer and metabolic disorders (Lee et al., 2021; Poole and Ray, 2022). The term circadian disruption is not formally defined but it generally refers to a state of unstable entrainment of the circadian clock. This could happen from the imposition of environmental conditions, such as shift work, that are inconsistent with stable entrainment of the circadian clock. Even the regular, weekday use of an alarm clock leads to a chronic, low-level misalignment of internal clocks with the solar cycle. The regular practice of shortened sleep is called social jetlag, in a reference to the mismatch between internal and external time that occurs with travel across time zones (Wittmann et al., 2006). It is not known in which tissues disruption of normal circadian clock physiology occurs, representing a gap in our knowledge and in the ability to translate chronobiology to the clinic.

A key element of translational chronobiology would thus be the availability of validated biomarkers that reveal endogenous circadian phase. Traditionally, melatonin – found in serum or in saliva – has been used for this purpose. The time of melatonin onset in the evening reflects the chronotype of an individual: it can be early, late or, most often, somewhere in between. This is an expensive and time-consuming measurement. In recent years, at least four groups have developed biomarkers for determining circadian phase (Laing et al., 2017; Braun et al., 2018; Wittenbrink et al., 2018). They use various approaches (cellular substrates (whole blood, purified monocytes), temporal protocols (single versus multiple sampling times)), all using mRNA as the target molecules to quantify. They have various accuracies (Münch and Kramer, 2019; Dijk and Duffy, 2020). These studies are all in the

Box 1
Background on sleep and circadian rhythms.

The Two Process Model of Sleep Regulation.

The two process model comprises the S-process and the C-process (Borbély and Achermann, 1999). The S-process, also known as the sleep homeostatic process, is responsible for regulating the drive to sleep, based on the amount of time that has elapsed since the last sleep period. The longer an individual is awake, the stronger the drive to sleep becomes. This process is thought to be related to the accumulation of certain substances in the brain, such as adenosine, that promote sleep. The C-process, also known as the circadian process, is responsible for regulating the timing of sleep (see Section 2.1). The circadian clock places sleep at certain times of day (entrained phase). Together, the S-process and C-process are hypothesised to create the appropriate amount of sleep at the right time.

Zeitgebers.

Zeitgebers are environmental cues that are used by organisms for entrainment. The term "zeitgeber" comes from the German words "zeit," meaning time, and "geber," meaning giver. The most common zeitgeber is the light-dark cycle, but other cues such as meal timing, social cues, and temperature can act as zeitgebers (Aschoff, 1965; Roenneberg and Merrow, 2007).

Experimental approaches for studying Circadian Rhythms.

To better understand the role of endogenous circadian clocks in sculpting various functions, researchers use varied approaches, each of which has advantages and disadvantages.

Studies conducted in a Light:Dark Cycle: In this approach, animals are exposed to a consistent light:dark cycle, often 12 h dark: 12 h light. Physiological and behavioural variables such as gene expression, hormone levels, body temperature, and/or activity levels are monitored. Experiments in light:dark cycles can help us understand how organisms respond to light or dark as a zeitgeber. The sleep-wake cycle, hormone production, and metabolism, for instance, entrain to the light-dark cycle. We can conclude from such experiments that we measure "daily" rhythms, but not "circadian" rhythms. This is because, as long as the light:dark cycle is present, we do not know if the apparently rhythmic processes are driven by an internal clock or if they are responses to the light:dark cycle, or indeed, some combination of the two.

Studies conducted in Constant Light or Constant Dark: In this approach, animals are placed in a constant light or dark environment to study the expression of the endogenous circadian rhythms. Experiments in constant conditions can help us understand the intrinsic properties of the circadian clock. These experiments involve isolating organisms from zeitgebers and observing the rhythms of their biological processes in the absence of external synchronisation. These experiments allow us to determine if circadian rhythms are endogenously generated and persist even in the absence of external cues.

Studies using arrhythmic animals: Researcher might include a group of animals that are arrhythmic, in that their circadian pacemaker in the brain (the suprachiasmatic nucleus (SCN)) is ablated or expression of a key circadian clock gene (commonly, Bmal1 in mammals) is removed. Genetic approaches allow researchers to ablate Bmal1 in a select group of tissues in some cases. Use of an arrhythmic animal or tissue allows us to surmise what the adaptive role of a circadian clock might be in the process under study. Each model includes specific limitations in our conclusions; for example, a genetic disruption from early life might invoke some compensations from other, non-clock regulatedf systems.

Studies conducted in vitro: In many cases, circadian rhythms can be measured from tissues or cells maintained in culture. Synchronisation of cellular rhythms out of the body can be achieved with a number of zeitgebers such as 24 h temperature cycles, or pulses of dexamethasone or forskolin. Various assays are used to measure rhythmic outputs. For instance:

1. Luciferase Assay: In this design, cells or tissues are genetically engineered to express a circadian clock-regulated reporter gene, such as luciferase. Promoters of clock genes are most often used for this method. The level of luciferase activity can be measured over time to track the circadian rhythm of the cells or tissues.
2. Real-Time PCR: In this design, cells or tissues are harvested at different time points and the expression levels of circadian clock genes are measured using real-time PCR. This allows researchers to track the changes in gene expression over time and determine the endogenous circadian rhythm. More high throughput methods such as microarrays are also appropriate here.
3. Microelectrode Recording: In this design, cells or tissues are placed on a microelectrode array and the electrical activity of the cells is recorded over time. This allows researchers to study the circadian rhythm of electrical activity in the cells.

Studies conducted in vitro come with a wide range of caveats, given the many artificialities of the preparation (Nicholls et al., 2019). On the other hand, tissues are accessible to multiple manipulations and they represent building blocks of circadian systems. They are thus a powerful tool in our repertoire.

development phase. Additional translational hurdles include adapting a biomarker assay for the field and quantifying molecules other than RNA. For instance, using hair follicles as a tissue source would enable much more extensive experimentation and thus a better understanding of endogenous phase. Eventually measuring proteins or metabolites would in addition target effector molecules thus potentially yielding information more relevant for clock-regulated function in addition to clock-determined phase of expression (Hancox et al., 2021).

2.2. Effects of aging on circadian rhythms and sleep

Aging can occur without disease, and two aspects of healthy aging are a gradual decrease of circadian rhythm amplitude and fragmentation of sleep (Dijk et al., 1999). EEG recordings show less slow wave (deep) sleep. In addition, the timing of sleep becomes earlier (Roenneberg et al., 2004). Similar changes are seen in other rhythms, such as the diurnal rhythm of core body temperature or rhythms of various hormones (Kim et al., 2022). See Box 1 for an explanation of a model of the impact of the circadian system on sleep.

Age-related changes in the circadian system might occur either upstream from the SCN (in the light input pathways), within the SCN (cell coupling mechanisms) or downstream (in SCN outputs) (Farajnia et al., 2014). For example, studies in rodents have shown that the aged SCN is less responsive to photic input and shows changes in glutamate receptors that may mediate these changes (Zhang et al., 1996; Biello et al., 2018). Some studies show changes in molecular rhythms in the SCN with age

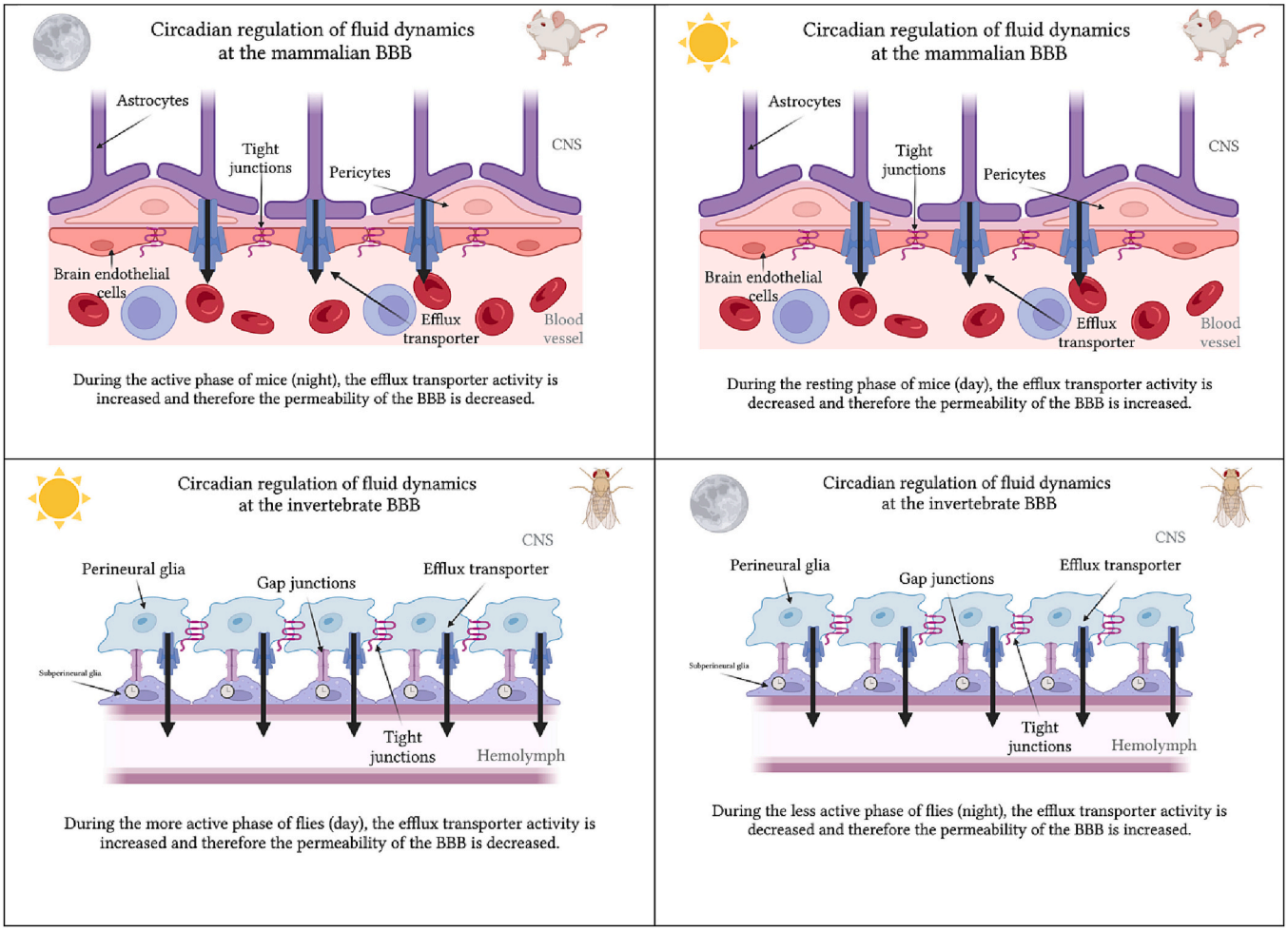

Fig. 2. The circadian regulation of fluid dynamics in mouse and fly BBB. The BBB in mice (upper panels). The anatomical structure of the BBB in humans is similar to that of mice, with brain microvascular endothelial cells forming the physical barrier, and pericytes and astrocytes playing supportive roles. However, there are some differences between the two species, such as the expression of certain transporters and receptors, which can affect the permeability of the BBB and the transport of specific molecules across the barrier (Abbott et al., 2010). The BBB in fruit flies (lower panels). The anatomical structure of the blood-brain barrier in fruit flies is referred to as the hemolymph-brain interface (HBI). The HBI in fruit flies is composed of two layers of cells: the perineurial glia and the subperineurial glia. The perineurial glia cells are the outermost layer of cells that form a continuous sheet around the brain and are connected to one another through tight junctions. They regulate the exchange of ions and small molecules between the brain and the hemolymph. The subperineurial glia cells are located beneath the perineurial glia and form a honeycomb-like structure that surrounds the blood vessels in the brain. These cells are also connected by tight junctions and play a critical role in regulating the movement of molecules between the hemolymph and the brain. They are thought to act as a selective filter to allow nutrients and signalling molecules to enter the brain while preventing potentially harmful substances from crossing the barrier (Limmer et al., 2014).

(Nakamura et al., 2011; Buijink and Michel, 2021; Wolff et al., 2022). Reduced amplitude output from the SCN leads to massive changes in circadian clock-regulated gene expression throughout the body (Wolff et al., 2022). Reductions in daily cortisol rhythms, nightly melatonin release, and fluctuations in body temperature might all converge to alter the phase of peripheral clocks with age (Nakamura et al., 2011).

Circadian rhythms are generated internally and they entrain to external cues, such as light-dark or temperature cycles. With lower amplitude rhythms arising from the SCN cellular network, and decreased amplitude of rhythms in body temperature and hormones, one way to maintain robust function would be to maintain or even strengthen the entraining input pathways. We might consider if lights can be used to achieve this. Although, as mentioned above, some evidence from laboratory animals points to alterations in photic input pathway glutamate receptor function (Zhang et al., 1996; Biello et al., 2018), and in elderly humans, cataracts often act to diminish light input, it is clear that photic pathways are at least working well enough because light can still effectively adjust circadian phase in older adults (Duffy et al., 2007; Scheuermaier et al., 2019; Kim et al., 2022). Across all ages, effects of light on circadian clocks can be increased using light intensity and duration, although there is significant inter-individual variability (Phillips et al., 2019; Stone et al., 2020). We suggest an opportunity exists to utilize light to maintain and improve rhythms as we age.

Will maintaining robust daily rhythms improve brain health with aging? We can find indirect evidence for this in studies of exercise, as well as daily patterning of food intake. Restricting food intake to the active phase in mice can extend life by 35% (Acosta-Rodríguez et al., 2022). Exercise has been shown to increase amplitude of rhythms in aged mice, even at the level of the SCN (Leise et al., 2013). These effects were associated with more rapid phase resetting and with less disorder of peripheral clocks. Thus, exercise might have a multi-level benefit on the circadian system. Exercise or high levels of locomotor activity can feedback onto the SCN through multiple neural pathways (Hughes and Piggins, 2012) and can have beneficial effects on multiple tissues, as, for example, the muscle clock (Martin and Esser, 2022). To our knowledge, no one as yet has accomplished a direct test of the idea that simply increasing circadian robustness will improve brain health, but studies using exercise or timed food intake offer encouragement for this

direction of investigation. Effects on cell types might vary and thus studies focused on the neurovascular unit might consider if circadian robustness in each specific cell type involved plays distinct roles.

3. Circadian modulation of fluid dynamics

3.1. Daily rhythms in blood-brain barrier permeability and transport mechanisms

The transport of molecules across the blood-brain barrier is a bi-directional, energy-consuming process, and it is highly regulated in order to both protect neurons from harmful substances as well to remove metabolic waste- and by-products (Kadry et al., 2020). These energy-intensive processes vary in a rhythmic manner and have either been found to be regulated by the circadian clock or by the act of sleeping (Cuddapah et al., 2019). Molecules can cross the blood-brain barrier using direct passive, direct active or indirect mechanisms (Kadry et al., 2020).

A wide range of lipid-soluble molecules diffuse passively through the blood-brain barrier and enter the brain. The crossing rate correlates with their lipid solubility (Clark, 2003) and molecular weight (Fischer et al., 1998).

Direct active transport is mediated by several ATP-binding cassette (ABC) proteins that are expressed on the luminal endothelial side of the blood-brain barrier. These ATP-driven efflux pumps limit permeability of multiple toxins. A high efflux transporter activity is therefore associated with a lower permeability of the blood-brain barrier. A major efflux transporter that is highly expressed in the luminal membrane of the blood-brain barrier is the P-glycoprotein (Pgp or Multidrug Resistance Protein ABCB1). Glucose, amino acids, and other essential, polar nutrients are transported over the blood-brain barrier via carrier-mediated transport (CMT), using transporters coded by genes within the Solute Carrier (SLC) Transporter Gene Family. As carrier-mediated transport takes place on the luminal as well as the abluminal side of the blood-brain barrier, the distribution of these transporter proteins should lead to a preferred directional transport of corresponding molecules.

Larger peptides and proteins (e.g., albumin) rely on indirect endocytic mechanisms, namely receptor-mediated transcytosis or adsorptive-mediated transcytosis. Both mechanisms result in endocytosis and subsequent transcytosis (Kadry et al., 2020).

As described above, the blood-brain barrier uses direct passive, direct active, or indirect mechanisms to exchange molecules in between the blood and central nervous system. Some of these molecules show daily oscillations in CNS concentrations. Cytokines and pro-inflammatory neuromodulators, such as TNF-α (Pan et al., 2002), IL-1 α (Banks et al., 1998) and IL-6 (Banks et al., 1994; Agorastos et al., 2014) are in this group. TNF-α is known to be an important modulator in acute neurodegenerative processes, such as stroke, and its receptor-mediated transport system has been found to be upregulated by CNS trauma and inflammation (Pan and Kastin, 2007). This suggests that daily oscillations in TNF-α could play an important role in the outcome of a stroke-event. Other endogenous molecules of which concentrations in the CNS undergo daily oscillations include the appetite-regulating hormone leptin (Pan and Kastin, 2001) and the hormone-like prostaglandin D2 (Pandey et al., 1995; Ram et al., 1997) as well as peptides such as amyloid-β (Cirrito et al., 2005; Kress et al., 2018) and the Delta-sleep inducing peptide (Banks et al., 1985).

We have the most understanding of factors involved in the circadian regulation of direct active transport mechanisms (see Fig. 2). The efflux of xenobiotics by the blood-brain barrier was found to be robustly rhythmic (Zhang et al., 2021). Studies using both nocturnal rats and diurnal *Drosophila melanogaster* demonstrated that Pgp-mediated efflux is greater in the active period (Kervezee et al., 2014; Savolainen et al., 2016; Zhang et al., 2018). One suggested explanation for why this mechanism is needed during wakefulness is the increased exposure to harmful substances that occurs with active exploration of the environment.

Within the neurovascular unit it appears that pericytes play a critical role in regulating circadian rhythms in endothelial cells (Mastrullo et al., 2022). Human umbilical vein endothelial cells (HUVECs) were not rhythmic in culture, but saphenous vein derived pericytes (SVPs) were. Culturing these endothelial cells with pericytes led to the endothelial cells' rhythmic expression of clock genes. The authors propose that lactate, which was suppressed in pericytes lacking the key circadian regulator Bmal1 and is a known mediator of cell-cell interaction, is mediating intercellular communication and synchronisation. As pericytes also possess a pro-angiogenic function, silencing this system could have far-reaching effects not only on the circadian activity of HUVECs but also on angiogenesis. Further studies are needed here to thoroughly investigate cellular interactions and how these can influence daily rhythms in physiology. Other studies point to the daily variation in the magnesium balance in brain endothelial cells mediating rhythms in efflux. Interestingly, the mechanisms underlying this appear to differ between mice and flies, but in both cases the systems appear to regulate intracellular Mg^{2+} (Zhang et al., 2021). Intracellular Mg^{2+} can regulate the period, amplitude, and phase of daily oscillations (Feeney et al., 2016). Daily changes in BBB efflux as assessed with Rhodamine 123 appear to depend on circadian clock gene expression in brain endothelial cells (Pulido et al., 2020) as show in experiments using inducible endothelial-specific Bmal1-floxed knockouts. These studies highlight the complexity of circadian clock regulation of a multi-cellular system.

Interestingly, endocytosis at the Drosophila hemolymph-brain interface is more common during the night, the inactive period. Endocytosis is strongly modulated by sleep history in Drosophila, and interference with the process of endocytosis at the BBB can alter sleep homeostasis (Artiushin et al., 2018).

3.2. Rhythms observed in the CSF and glymphatic systems

The cerebrospinal fluid (CSF) and glymphatic system play a central role in the removal of certain metabolites and molecules that might be considered as "waste products" to be removed from the CNS. CSF is produced by the choroid plexus which consists mainly of epithelial cells that are located in the four brain ventricles, as well as by endothelial cells throughout the blood-brain barrier (Rasmussen et al., 2022b). It circulates in a system of communicating cavities known collectively as the ventricles. Some of the CSF resorption takes place via the arachnoid granulations in the subarachnoid space, which is also filled with CSF, and lymphatic vessels in the meninges (Louveau et al., 2015), while some is resorbed around the spinal cord via pathways around the cranial nerves.

The glymphatic system is a network of perivascular channels for CSF that facilitates the movement of metabolic waste products from the interstitial space (Iliff et al., 2012; Rasmussen et al., 2022b). The term "glymphatic" is derived from the words "glial" and "lymphatic," reflecting the involvement of glial cells (astrocytes) in the system and its similarity to the lymphatic system. Aquaporin-4 water channels on the endfeet of the astrocytes lining these channels create a conduit for CSF to flow into the parenchyma (Iliff et al., 2012). With the CSF, metabolic waste products such as amyloid-beta, tau proteins, and other metabolites are removed from the interstitial fluids (Benveniste et al., 2019). The glymphatic system appears to be most active during sleep (Chong et al., 2022).

A daily pattern in CSF production (Nilsson et al., 1992) and sodium concentration (Harrington et al., 2010) is reported in humans. In vitro, the mouse choroid plexus shows robust rhythms in circadian clock reporters, and the cells appear to be locally coupled, perhaps via gap junctions (Myung et al., 2018). Experiments indicate that choroid plexus rhythms may influence the rhythms of other tissues. Interestingly, co-culture of choroid plexus tissues with the SCN changed the SCN culture rhythm period (~24–25 h) to be closer to that of the choroid plexus

(~22–24 h); these studies await extension by further experiments, including co-culture with genetically non-rhythmic choroid plexus tissue (Myung et al., 2018). Studies using rats confirm choroid plexus rhythms in clock genes with short period (~20–22 h) rhythms (Yamaguchi et al., 2020). Other groups have reported a daily (under a light: dark cycle) pattern in gene expression in the choroid plexus that shows some alterations in a mouse model of Alzheimer's Disease (Furtado et al., 2020, 2023).

Sleep may be associated with an up to 60% increase in interstitial space (Xie et al., 2013), possibly contributing to the increased exchange of fluids. On the other hand, some researchers argue that this reported increase in interstitial space may be an artifact of experimental conditions (Ferris, 2021) and find support from results using diffusion-weighted imaging to estimate extracellular space volume across changes in arousal state (Gakuba et al., 2018; Demiral et al., 2019). A daily rhythm of perivascular clearance measured in awake rats supports an alternative idea that circadian rhythms, instead of sleep, gate changes in glymphatic system function (Cai et al., 2020). Interestingly, circadian rhythm regulation of the glymphatic system persists in mice housed in constant light (a condition leading to arrhythmicity), with lower levels of clearance during the rest phase. Surprisingly, lymph nodes showed opposite phasing, with circadian rhythms of cerebrospinal fluid flow drainage in the cervical lymph nodes peaking during the active phase of mice. Circadian regulation of glymphatic flow depends on aquaporin-4 function (Hablitz et al., 2020).

Studies in mice demonstrate that the glymphatic function is impaired already by middle age, with further loss as mice continue to age (Kress et al., 2014). Studies in healthy humans report a negative correlation of glymphatic function with age (Zhou et al., 2020). These aging-related changes might correlate with changes to the vascular system (Fossel et al., 2022) or the aging of the circadian system (see Section 2.2). We consider this an area that will be rewarding to research further, especially if researchers could develop methods to help clarify what appear to be many bidirectional interactions (see Fig. 1).

The regulation of rhythms observed at the blood-brain barrier and the blood-CSF barrier arise in part from processes modulating permeability and transportation at these barriers. By regulating cell-to-cell communication at a fundamental level, a complex interaction of different physiological systems at the brain-barriers might be ensured. Factors and molecules involved in hormonal, metabolic, cardiovascular and immune processes are likely to be affected by circadian changes as well as brain fluid dynamics, making further research on this interplay essential.

3.3. The effect of sleep homeostasis and neuronal activity on blood-brain barrier functionality

Neuronal activity may impact BBB function, in ways that are just being understood. Induced widespread glutamatergic activity (from the last hour of the dark phase until several hours into the light phase, ZT23–24 until ZT2–3) can alter expression of key ABC transporter genes in brain endothelial cells (Pulido et al., 2020). Similar changes in expression were observed in barrel cortex following exploration of a novel arena during the same phase of the light cycle. It is likely these interventions also induced sleep loss given the timing.

Sleep deprivation has been shown to increase BBB permeability in animal models. For instance, a study conducted in mice demonstrated that sleep deprivation increases BBB permeability to sodium-fluorescein (He et al., 2014). The researchers found that after six days of sleep restriction, the levels of sodium-fluorescein in the brain increased significantly, indicating increased BBB permeability. Another study conducted in rats found that chronic sleep restriction leads to BBB breakdown, resulting in increased infiltration of 70kDa FITC-dextran and Evans Blue into the brain and neuroinflammation (Hurtado-Alvarado et al., 2016).

In contrast, a study conducted in mice found that sleep enhances glymphatic clearance and reduces the accumulation of amyloid-beta in the brain (Xie et al., 2013). Another study conducted in humans found that sleep deprivation leads to increased levels of amyloid-beta in the brain (Shokri-Kojori et al., 2018).

4. The important role for circadian regulation of oligodendrocyte precursor cells

White matter consists primarily of myelinated axons that connect neurons in different regions of the brain. Appropriate myelination by oligodendrocytes in the white matter is required for the development of cognition, memory, motor function, and complex skills. Oligodendrocytes and their precursor cells (oligodendrocyte precursor cells: OPCs) are classified as a subtype of glial cells and are found in the neurovascular module within white matter. Because oligodendrocytes are required for myelination and because oligodendrocytes cannot proliferate, OPCs play an indispensable role in controlling the oligodendrocyte population for myelin renewal. OPCs are active during developmental stages, but some pools of OPCs remain in the adult brain and these cells underlie white matter homeostasis (Hughes et al., 2013). It has been proposed that, in addition to astrocytes and pericytes, a subset of OPCs are found in close proximity to the cerebral endothelium, and in this microenvironment (e.g. so-called oligovascular niche), cerebral endothelial cells and OPCs communicate closely in order to support the cerebrovascular system, including blood-brain barrier integrity and physiological angiogenesis mechanisms (Seo et al., 2014; Niu et al., 2019; Chavali et al., 2020).

Recently, circadian rhythms have been proposed to regulate oligodendrocyte/OPC function (Colwell and Ghiani, 2020). For example, Cirelli et al. demonstrated a sleep-associated increase in oligodendrocyte maturation transcription factor (Cirelli and Tononi, 2000; Cirelli et al., 2004, 2006). In addition, several oligodendrocyte/OPC-enriched genes, including Pdgfra, Mog, Mbp, Cnpase, and Sgk1, are rhythmically regulated in the central nervous system (Colwell and Ghiani, 2020) and OPC proliferation has been reported to exhibit daily rhythms (Matsumoto et al., 2011; Bellesi et al., 2013). Therefore, oligodendrocytes/OPCs may exhibit cell-autonomous circadian rhythms, and disruption of these rhythms may impair the function and homeostasis of oligodendrocyte lineage cells, which would lead to white matter damage (Colwell and Ghiani, 2020). In fact, sleep disorders are associated with multiple sclerosis, which is a demyelinating disease (Caminero and Bartolomé, 2011), and importantly, genetic polymorphisms in circadian clock-related genes, such as Per3, Bmal1/Arntl, and Clock, were reported to be associated with multiple sclerosis (Clark, 2003; Lavtar et al., 2018).

The effects of aging on circadian rhythms in white matter are still largely unknown. However, studies have shown that advanced aging is associated with cognitive decline and structural changes in white matter. Using diffusion tensor imaging, reduced correlations within higher-order brain systems in older adults were confirmed to be associated with disruptions in white matter integrity and poor cognitive performance (Andrews-Hanna et al., 2007). Furthermore, there is a difference in age-related structural changes between white and gray matter, as the reduction in white matter volume with aging is non-linear, with a more rapid change with advancing age, whereas gray matter volume shows a smaller and more linear decrease (Vinke et al., 2018). Interestingly, circadian rhythms may influence the white matter structure. A recent study reported a relationship between white matter structure and rest-activity rhythm stability (McMahon et al., 2021). In addition, circadian rhythms are involved in the pathogenesis of depression, and the gene polymorphisms of Per1, a key circadian clock gene, affect the association between white matter microstructural integrity and depression risk (Zhao et al., 2022). As in all other systems discussed so far, these signalling pathways in white matter are also likely to be multi-directional. For example, OPC renewal mechanisms can be affected by aging, and degradation of OPC-to-endothelial signalling may impair the BBB. Conversely, circadian disruptions may perturb BBB function and

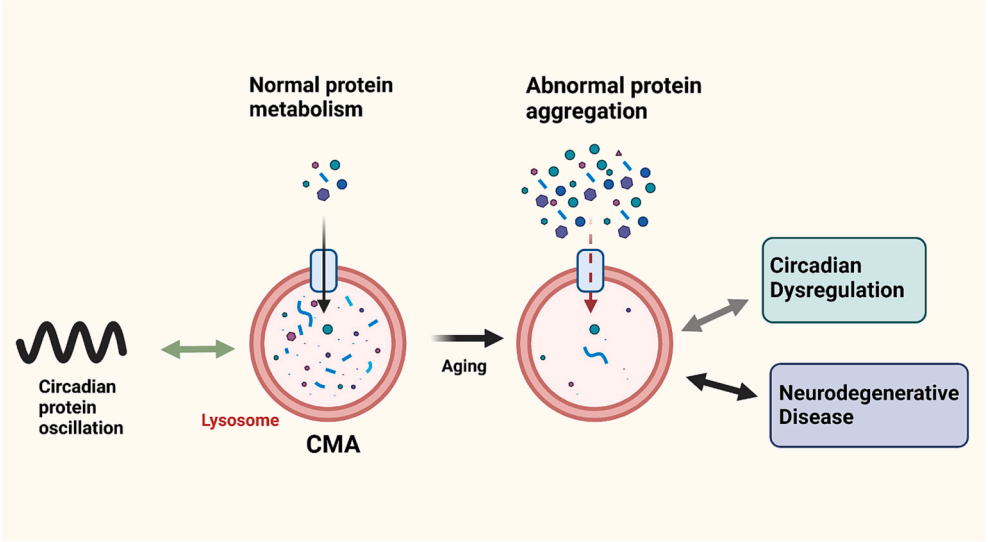

Fig. 3. Chaperone Mediated Autophagy (CMA) modulates circadian gene expression and normal protein metabolism. In the aging brain CMA declines and may play a key role in circadian dysregulation and abnormal protein aggregation in neurodegenerative disease.

damage OPCs, thus contributing to aging-related declines in white matter function. Future studies are warranted to investigate the causal mechanisms that connect the temporal dimensions of aging and circadian clocks with OPC homeostasis, BBB function, and CSF handling. Dissection of these underlying mechanisms may hopefully lead to potential targets for rescuing white matter integrity in the diseased and aging brain.

5. Neurodegeneration

Aging in the context of disease is of course much more complex. We can consider this with respect to Alzheimer's disease or related dementias. People with Alzheimer's disease show additionally decreased amplitudes in daily rhythms, and increased fragmentation of sleep. In contrast to healthy aging, these individuals can show a phase delay in timing of sleep (Satlin et al., 1995; Ancoli-Israel et al., 1997), as well as agitation in the late afternoon ("sundowning"). Some of these changes are also seen with preclinical Alzheimer's disease (Musiek et al., 2018). At the moment, we do not know the mechanism accounting for sundowning. Fragmentation of sleep might arise from weakened regulation by the circadian clock. Degeneration in the SCN has been reported in brains of patients with Alzheimer's disease (Swaab et al., 1985; Wang et al., 2015). Age, sex hormones and circadian rhythms can affect the expression of proteins that clear amyloid-beta from the CSF at the choroid plexus (Duarte et al., 2020). Plasma levels of amyloid beta have been shown to be a potential biomarker of cognitive decline in individuals with preclinical Alzheimer's disease (Lim et al., 2020). The relationship between Alzheimer's disease and sleep or the circadian clock is bidirectional (Musiek and Ju, 2022).

Cardiovascular disease is increasing in our aged population, likely attributable in large part to lifestyle factors such as negative effects from the Western diet. Vascular comorbidities and cerebrovascular insufficiency occur in almost two-thirds of Alzheimer's disease patients. The severity of impairment in cerebral blood flow correlates with the severity of cognitive deficits (Bracko et al., 2021). Vascular impingement will combine with Alzheimer's disease and related dementias in negative ways.

A new therapeutic opportunity arises from our consideration of the bidirectional relationship between circadian rhythms and diseases associated with either age or with disorders of the neurovascular unit. It should be possible to develop compounds to boost the molecular circadian clock at the cellular level, and therefore to treat these changes associated with both healthy aging as well as aging in the context of a disease. Several possibilities were discussed in recent papers (Jagannath et al., 2021; Rasmussen et al., 2022a). Unfortunately, none of these are yet at the point of development to allow clinical trials, although preclinical research is a strong focus of current research. For example, one might focus on altering the "positive" arm of the molecular circadian clock transcriptional feedback loop, namely the transcriptional drive from CLOCK and BMAL1. Two regulators of Bmal1 help maintain daily rhythms: the transcriptional repressor REV-ERBα (Preitner et al., 2002) and the activator RORA (Sato et al., 2004). REV-ERBs show strong oscillation in both mRNA expression and protein degradation and compete with RORs to regulate occupancy and activity at target gene promoters. Antagonists of REV-ERBs (REV-ERBα and REV-ERBβ) are expected to boost rhythmic transcription, as are agonists of ROR. The natural ligand for REV-ERBα is heme, and in our early studies, we used heme to demonstrate damped rhythms in the SCN but not in peripheral tissues in vitro (Guenthner et al., 2009). Although a Rev-Erbα $^{-/-}$ mouse appeared relatively normal (Chomez et al., 2000), they show increased expression of CLOCK and BMAL1, shorter free running periods, and increased phase shifts to light. Combined deletion of both Rev-Erbα and Rev-Erbβ has severe impact on circadian rhythms, leading to loss of rhythms (Cho et al., 2012). Treatment strategies might focus on Rev-Erbα, because increased BMAL1 and CLOCK could in turn increase amplitude of rhythmic gene expression, and increased response to light could stabilize entrainment. Several groups have developed small molecule ligands for REV-ERBα (Grant et al., 2010; Trump et al., 2013). Researchers could use Rev-Erbα $^{-/-}$ mice to study effects of increased amplitude of daily rhythms. This direction is supported by prior studies indicating that suppression of Rev-Erbα enhances clearance of Aβ1–42 in 5xFAD mice (Lee et al., 2020) and a REV-ERBα agonist SR9009 enhanced cognitive deficits in an Alzheimer's disease model mouse (Ni et al., 2019).

Further, researchers might employ these tools to boost or suppress clock regulation in studies of fluid dynamics. We would benefit from greater understanding of clock modulation of the glymphatic system, and clearance of amyloid-Beta, for instance. Another consideration is that processes associated with age might alter fluid dynamics, and this might then alter circadian phenotype.

6. Chaperone-mediated autophagy at the intersection of dysregulation of the circadian clock, aging, and neurodegenerative disease

Aberrant protein aggregation is a prominent feature of both the aging brain and a diverse range of (clinically divergent) neurodegenerative diseases ranging from Alzheimer's disease to frontotemporal dementia (see Fig. 3) (Soto and Pritzkow, 2018; Hou et al., 2019). As the brain ages, its ability to regulate various cellular protein control mechanisms declines, and as a consequence intracellular protein aggregation occurs (Lee et al., 2001; Soto and Pritzkow, 2018; Juste et al., 2021). Among these mechanisms, autophagy constitutes a highly conserved homeostatic pathway responsible for the degradation of cellular components via lysosomes (Parzych and Klionsky, 2014). In mammalian cells, there are three main types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Although all of these pathways result in the degradation of cellular protein and cargo, they are morphologically distinct: macroautophagy relies on the de novo formation of double-membrane vesicles to transport cargo to the lysosome (Yorimitsu and Klionsky, 2005), CMA transports individual unfolded proteins directly across the lysosome membrane (Massey et al., 2004), whereas microautophagy involves the direct uptake of cargo through invaginations of the lysosomal membrane (Mijaljica et al., 2011). The identification of specific autophagy-related genes (ATG genes) has helped to uncover the mechanistic complexities of these pathways (Tsukada and Ohsumi, 1993; Thumm et al., 1994; Harding et al., 1995; Schlumpberger et al., 1997; Straub et al., 1997).

Dysregulated autophagy has emerged as a prominent feature of aging present across diverse species (Aman et al., 2021). Early studies using Drosophila demonstrated that aging is associated with the reduced expression of several core autophagy related genes (*Atg2, Atg8a and bchs*) that are critical for both the initiation and progression of autophagy (Simonsen et al., 2008). In line with these findings, aged mice exhibit decreased expression of the autophagy related genes *Atg5-Atg12* and *Becn1* within the brain (Ott et al., 2016), and decreased rates of autophagolysosomal fusion and impaired delivery of substrates within neuronal cells (Kaushik et al., 2012). Human studies have also demonstrated that the expression of autophagy related genes (*ATG5, ATG7*, and *BECN1*) substantially decrease with age (Lipinski et al., 2010). Taken together these studies highlight that a gradual decline of autophagy-related genes and the reduced delivery of autophagy related cargo occurs with age. In line with these findings, genetically impairing autophagy has been shown to shorten lifespan in various experimental animal models (Simonsen et al., 2008; Tóth et al., 2008; Hansen et al., 2018; Leidal et al., 2018), whereas genetic or pharmacological upregulation of autophagy promotes longevity and lifespan extension in diverse organisms (Ravikumar et al., 2004; Harrison et al., 2009; Fang et al., 2014). These studies begin to establish a central role for autophagy as a critical regulator of aging. The importance of autophagy in the development of neurodegenerative diseases, independently of aging, has been highlighted by studies showing that the loss of autophagy related proteins (ATG7 and ATG5) in mice, results in the development of a neurodegenerative phenotype (Hara et al., 2006; Komatsu et al., 2006), and axonal dystrophy (Komatsu et al., 2007). Autophagy has also been specifically linked to α-synuclein accumulation in Parkinson's disease (Webb et al., 2003), mutant TDP-43 accumulation in *amyotrophic lateral sclerosis* (Barmada et al., 2014), and mutant tau accumulation in various forms of dementia (Berger et al., 2006).

Rhythmicity has been demonstrated for both macroautophagy and CMA within the liver where they function to spatially and temporally regulate protein catabolism during the day (Ryzhikov et al., 2019). Daily changes in the proteome as mediated by CMA have also been demonstrated (Kaushik et al., 2022). Protein abundance, post-translational modifications, and epi-genetic modifications function to reinforce both the strength and adaptability of the circadian clock (Lee et al., 2001). The rhythmic removal of circadian clock proteins has been shown to be important for maintaining both the amplitude and free running period of the circadian clock-regulated proteins through CMA, which functions to drive the degradation of selective proteins into lysosomes (Juste et al., 2021). A model has been proposed in which CMA and clock-regulated proteins impact one another through the formation of a negative feedback loop. In one arm of the loop, CMA degrades clock proteins, thus promoting circadian rhythmicity, and in the other arm of the loop, clock proteins suppress the expression of CMA activating genes, thus regulating circadian changes in CMA activity (He, 2021; Juste et al., 2021). Increasing evidence supports a role for CMA at the intersection of circadian dysregulation, aging, and neurodegenerative disease. A decreased amplitude in behavioural rhythmicity has been observed in CMA deficient mice (Juste et al., 2021), CMA has been shown to decline with age (Cuervo and Dice, 2000; Zhang and Cuervo, 2008; Schneider et al., 2015), CMA deficient mice phenocopy features of aging such as proteostasis failure (Schneider et al., 2015; Bourdenx et al., 2021) and diminished T cell responses (Valdor et al., 2014), and CMA activity has been found to decline in neurodegenerative diseases (Valdor et al., 2014; Dong et al., 2021). Moreover, young CMA deficient mice phenocopy aspects of aging circadian dysregulation, in support of the notion that age-dependent CMA decline potentially contributes to circadian disruption in aged animals (Juste et al., 2021). The demonstration that CMA regulates clock protein levels raises the intriguing possibility that the system could be targeted to help restore normal rhythms in the aging brain.

Tauopathies are marked by the disrupted homeostatic profile of the microtubule binding protein Tau (Morris et al., 2011). The acetylation of Tau has been shown to be an early deleterious post-translational event which occurs in the brains of patients with Alzheimer's disease and other related tauopathies (Cohen et al., 2011; Irwin et al., 2012, 2013; Cook et al., 2014). Interestingly, a large portion of unacetylated neuronal tau undergoes degradation via CMA, however upon acetylation, it is diverted to alternative pathways such as macroautophagy and endosomal microautophagy (Caballero et al., 2021). Acetylated tau was found to exert an inhibitory effect on CMA through a pH dependent mechanism, in its extracellular release into ISF. Experimentally inhibiting CMA, enhances the cell to cell transmission of pathologic tau. Recently, amyloid precursor protein was also identified as a CMA substrate. In the APP/PS1 mouse model of AD, the activation of CMA resulted in a reduction in Aβ plaque levels and consequently led to a reversal of both behavioural and AD phenotypes (Xu et al., 2021).

In the context of Alzheimer's disease, extracellular protein accumulations of amyloid plaques composed of aggregated Aβ peptides and phosphorylated neurofibrillary tau tangles are defining features of disease progression (Ross and Poirier, 2004). Soluble Aβ peptides levels have been found to display prominent daily oscillations within both mouse hippocampal interstitial fluid (ISF) and human cerebrospinal fluid (Kang et al., 2009; Huang et al., 2012; Roh et al., 2012). These oscillations in Aβ exhibit a clear 24 h. rhythm, which persists under dim light conditions and is in phase with activity rhythms (Kang et al., 2009). Although tau is predominately cytoplasmic, it can be released by neurons into the extracellular space and accumulate within the ISF (Chai et al., 2012; Karch et al., 2012). Interestingly, both the extracellular release of Aβ and tau into the ISF have been shown to be increased through excitatory neuronal activity (Pooler et al., 2013; Yamada et al., 2014). Under normal physiological conditions the daily variation of both Aβ levels and tau within ISF peak during the dark period (Kang et al., 2009; Holth et al., 2019).

In nocturnal mice, these ISF rhythms were significantly correlated with the amount of time spent awake during the dark phase (Kang et al., 2009; Holth et al., 2019). Sleep deprivation was found to increase both Aβ pathology and tau seeding/spreading, whereas chemo-genetically induced wakefulness in mice substantially increased both ISF levels of both Aβ and tau (Holth et al., 2019). In humans, one night of sleep deprivation was found to increase CSF levels of Aβ and tau by 30 and 50% respectively (Lucey et al., 2018; Holth et al., 2019). Taken together

these data demonstrate that the sleep-wake cycle regulates brain ISF levels of tau and Aβ in mice and CSF in humans. Moreover, increased wakefulness has been demonstrated to increase ISF and CSF tau, tau spreading, and tau aggregation over longer time periods (Holth et al., 2019). Fragmented sleep/wake cycles can be detected in normal aging as well as in preclinical AD, and become increasingly more apparent in early symptomatic AD (Lim et al., 2013). Therapies targeted at consolidating sleep/wake cycles by normalizing circadian clock-regulated timing rather than altering total sleep time may be beneficial in the treatment of AD.

7. Final thoughts

Two processes that are defined by time have a profound effect on the neurovascular unit: aging and the circadian clock. Aging increases cumulatively over years. The rate of aging is not understood, as there are interindividual differences and the process may be programmed to proceed in a linear or non-linear rate at different stages. In the case of the circadian clock, the timespan is 24 h, with dynamic changes occurring over this cycle length, essentially bringing the system back to its starting point (resembling the concept of homeostasis). The circadian clock is pervasive across nature and also within an organism: each cell has a circadian clock that governs daily molecular oscillations that are often tissue specific. Both time-defined processes impact not only the neurovascular unit but the entire human body. Furthermore, they act on each other: the amplitude of the circadian clock decreases with aging and chronic disruption of the circadian clock can speed the onset of aging-associated disease such as cancers (Farajnia et al., 2014; Wegrzyn et al., 2017).

Multiple questions should be addressed in the future, keeping this reciprocal relationship between aging and the circadian clock in mind. For example, it is necessary to further investigate the effects of aberrant or non-existing circadian rhythms on the transporter systems at the blood-brain barrier as well as cerebrospinal fluid-brain barrier, the brain fluid physiology, and the neurovascular unit. Research needs to be continued and extended on the interaction of tight junction proteins, degradation processes and circadian regulators, specifically in brain endothelial cells. Findings that we have included in this literature review suggest a connection between circadian disruption and neurodegenerative disease progression, but the mechanisms are far from being understood in enough detail.

Moreover, it is essential to address the impact of sleep on the neurovascular unit in this context. Homeostatic processes driven by sleep and wakefulness are sometimes hard to disentangle from circadian clock-regulated mechanisms and, in many cases, both sleep and the circadian clock seem to be key influencing factors. By primarily focusing on the circadian rhythms in brain barriers and fluids, we have neglected the potential role that sleep homeostasis plays in the observed daily rhythms across the neurovascular unit.

Here, we reflect specifically on the role of the circadian clock in the neurovascular unit (see Box 2). Is it possible that the aging neurovascular unit becomes more susceptible to triggers of disease because circadian rhythms with a decreased amplitude perturb the delicate regulation of signalling between the CNS and the periphery? And if so, what candidate mechanisms might be involved? Our survey of the literature is not complete, but offers examples of areas of new research where we find potential for further progress.

Box 2
Open questions and opportunities.

Mechanisms
1) What is the role for central versus peripheral clocks in terms of interactions between circadian rhythms and aging?
2) Are there differences between central versus peripheral aging, and how are these pathways affected in CNS disease?
3) What cell types in the neurovascular unit are affected by altered rhythms in the aging or diseased CNS?
4) How do rhythms in glymphatics modify the immune system within the aging or diseased CNS?
5) How do we disentangle perturbations in sleep versus circadian disruption in the aging of damaged/diseased brain?
6) Can we separate the effects of aging on rhythms versus the effects of rhythms on aging in the damaged/diseased brain?

Targets
1) Can we target circadian genes and modify non-directly-rhythm-regulating pathways to restore BBB and fluid-handling function in the aging CNS?
2) Are there common epigenetic drivers for aging and loss of circadian rhythms?
3) How do we pursue physiologic modifiers (e.g. diet or exercise) that can affect multiple pathways in aging and circadian rhythms?

Translation
1) For circadian mechanisms, how do we translate findings in nocturnal rodent models into meaningful applications in diurnal humans?
2) For BBB and CSF mechanisms, how do we account for differences in spatial dimensions between small rodent brains and large human brains that may affect mathematical considerations for molecular diffusion, fluid flow, and drug delivery?
3) For aging-related mechanisms, how do we correlate time-scales in short-lived rodents with longer-lived humans?

Data availability

No data was used for the research described in the article.

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