UNIVERSIDAD AUTÓNOMA METROPOLITANA
Unidad Iztapalapa

Alterations in the junctions between brain endothelial cells and pericytes during chronic sleep restriction

T E S I S

Que para tener el grado de
Maestra en Biología Experimental

P R E S E N T A

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Te amo, oh Jehová, fortaleza mía.
Jehová, roca mía y castillo mío,
en él confiaré; Mi escudo, y la fuerza
de mi salvación, mi alto refugio.
Invocaré a Jehová, quien es digno
de ser alabado.

Salmos 18:1-3

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Bienaventurado el hombre que halla
La sabiduría.
Y que obtiene inteligencia;
Porque su ganancia es mejor que la
Ganancia de la plata,
Y sus frutos más que el oro fino.
Más preciosa es que las piedras
Preciosas.
Y todo lo que puedes desear, no se
Puede comparar a ella.
Ella es árbol de vida a los que de ella
Echan mano,
Y bienaventurados son los que la retienen.

Proverbios 3:13-18

RESUMEN EJECUTIVO

La barrera hematoencefálica (BHE) es una barrera altamente selectiva y semipermeable que regula el paso de moléculas solubles y potencialmente tóxicas desde el lumen del capilar hacia el parénquima cerebral; mientras que sustratos específicos como iones, glucosa y aminoácidos cruzan el endotelio cerebral a través del transporte mediado por acarreadores para garantizar la función neuronal (Keaney y Campbell 2015). La BHE se localiza a nivel de los capilares encefálicos y está constituida por una monocapa de células endoteliales que forman la pared de los vasos sanguíneos. Las células endoteliales cerebrales adquieren su fenotipo de barrera (i.e expresión de uniones estrechas, bajo tránsito por pinocitosis) en función de factores solubles liberados por los astrocitos, la microglia y las neuronas, y de manera importante por las interacciones célula-célula presentes entre el endotelio y los pericitos (Harder et al., 2002; Thomsen et al., 2017).

Los pericitos son células perivasculares que se localizan en la pared externa de los capilares sanguíneos. Debido a la cercana localización espacial entre las células endoteliales cerebrales y los pericitos, y al estar embebidos en la misma lámina basal, ambos tipos celulares mantienen una íntima interacción al establecer puntos de contacto específicos del tipo *peg-socket.* Durante el proceso de angiogénesis las células endoteliales secretan factor de crecimiento derivado de plaquetas B (PDGFB, por sus siglas en inglés), que es sustrato del receptor tirosina-cinasa PDGFR-β expresado en la superficie de los pericitos. La formación del complejo PDGFB/PDGFR-β activa las vías de señalización que promueven el reclutamiento de los pericitos hacia el vaso sanguíneo en formación (Helding y

Westermark, 1999). En el cerebro adulto, esta comunicación es crucial para mantener las uniones estrechas entre las células endoteliales, regular el flujo sanguíneo cerebral y controlar la entrada de células inmunes al sistema nervioso central (Shimizu et al., 2008; Hamilton, 2010; Armulik et al., 2015; Török et al., 2019).

El pericito es un componente integral de la BHE, por lo que su pérdida o ausencia, así como la deficiente interacción entre los pericitos y las células endoteliales del cerebro conducen a una disfunción neurovascular. Los ratones *knockout* para PDGFR-β y PDGFB presentan aberraciones vasculares como consecuencia del déficit de pericitos y por lo tanto mueren perinatalmente (Leveen et al., 1994). Por otra parte, los ratones con una mutación que produce la activación constitutiva de PDGFR-β (PDGFR-$\beta^{ret/ret}$) son viables, pero muestran una disfunción microvascular (Lindblom et al., 2013). En el modelo de ratones PDGFR-$\beta^{ret/ret}$ se observa una densidad disminuida de pericitos, y un aumento en la permeabilidad de la barrera hematoencefálica a azul de Evans, inmunoglobulina G y a fibrinógeno. Estos cambios coinciden con una reducción en la expresión de las proteínas de unión ocluyente ocludina, claudina-5 y zónula occludens-1 (Armulik et al., 2010, Bell et al., 2010).

Pese a que las alteraciones en la entrada de moléculas exógenas al cerebro son características de patologías neurodegenerativas, nuestro grupo ha descrito previamente, que durante la pérdida de sueño, se altera la homeostasis y funcionalidad de la BHE. En roedores, la deficiencia de sueño incrementa la permeabilidad de la BHE a trazadores de bajo y alto peso molecular (e.g. Na-fluoresceína, azul de Evans y dextranos de 10 KDa y 70 KDa), disminuye la

expresión de mRNA y de las proteínas de uniones ocluyentes claudina-5, ocludina y ZO-1 (Gómez-González et al., 2013; He et al., 2014; Hurtado-Alvarado et al., 2017, 2018). De interés particular, Hurtado-Alvarado y colaboradores (2017) presentaron evidencia de que la restricción de sueño podría inducir el desprendimiento de los pericitos de la pared de los capilares encefálicos (Hurtado-Alvarado et al., 2017), por ello en este proyecto caracterizamos el efecto de la restricción crónica de sueño sobre las interacciones entre el pericito y la célula endotelial cerebral.

Hipótesis

La restricción crónica de sueño disminuirá las interacciones entre las células endoteliales cerebrales y los pericitos, lo que inducirá una alteración en el metabolismo energético de la célula endotelial que conducirá a la disfunción de la barrera hematoencefálica.

Objetivo general

Evaluar los cambios en las interacciones entre las células endoteliales cerebrales y los pericitos y su efecto sobre el metabolismo de las células endoteliales cerebrales y las funciones de barrera durante la restricción crónica de sueño.

Objetivos específicos

1.- Evaluar el efecto de la restricción crónica de sueño sobre las interacciones entre las células endoteliales cerebrales y pericitos.

2.- Determinar la relación entre los cambios en las interacciones entre la célula endotelial cerebral y el pericito y el metabolismo de las células endoteliales cerebrales durante la restricción crónica de sueño.

3.- Determinar la relación entre los cambios en las interacciones entre la célula endotelial cerebral y el pericito sobre la función de barrera durante la restricción crónica de sueño.

Metodología

Ratas macho de la cepa Wistar se sometieron a restricción crónica de sueño empleando la técnica modificada de plataformas múltiples durante 20 horas diarias con una oportunidad para dormir durante las últimas 4 horas de la fase de luz, en un periodo de 10 días consecutivos. Al concluir los 10 días de restricción de sueño, las ratas se sacrificaron y se obtuvo el cerebro para aislar los microvasos cerebrales de la corteza cerebral y el hipocampo. Las muestras se emplearon para evaluar por Western blot la expresión de PDGFRβ, conexina-43, claudina-5, ocludina, metaloproteinasa de matriz-9, NFκB, p-NFκB, receptor A_{2A} de adenosina y CD43. Se realizó un ensayo de inmunofluorescencia para PDGFRβ o conexina-43 en microvasos cerebrales aislados de la corteza cerebral e hipocampo. Se empleó un grupo de ratas para realizar ensayos de permeabilidad a Na-fluoresceína, azul de Evans y rodamina 123.

Resultados

La restricción crónica del sueño disminuye las interacciones celulares entre el pericito y la célula endotelial cerebral

La restricción crónica de sueño disminuyó la expresión de PDGFRβ en microvasos aislados de la corteza cerebral (t=2.623, p=0.0173) e hipocampo (t=5.219, p=0.0001) (Figura 4 y figura 5 respectivamente). A través de microscopía confocal, en el grupo control intacto se detectó la inmunoreactividad de PDGFRβ alrededor de los capilares, la cual mostró el patrón alargado del

pericito unido a la pared del capilar. En los animales restringidos de sueño, la inmunoreactividad de PDGFRβ fue menos intensa y separada de la pared del capilar, lo que indica un posible desprendimiento del pericito de la pared capilar.

La restricción crónica de sueño disminuyó la expresión de conexina-43 en microvasos cerebrales aislados de la corteza cerebral (t=1.038, p=0.0063) pero no así en el hipocampo (Figura 6 y 7 respectivamente). Las imágenes de microscopía confocal indican que en la corteza cerebral del grupo control, la inmunoreactividad de Cx-43 fue continua a lo largo del capilar; por el contrario, en las ratas restringidas de sueño, la inmunodetección de Cx-43 fue menor y mostró un patrón discontinuo a lo largo de los capilares (Figura 6). La microscopía electrónica de barrido mostró a los pericitos estrechamente unidos a la pared del capilar en ratas control, mientras que en las ratas restringidas de sueño, se observaron células con forma globular en la cercanía de la pared del capilar en ambas regiones (Figura 8).

Las alteraciones en las interacciones célula endotelial cerebral y pericito se asocian con la disminución de proteínas de uniones ocluyentes en microvasos aislados

La restricción crónica de sueño disminuyó la expresión de claudina-5 (t=3.369, p=0.0276) en vasos aislados de la corteza cerebral, en comparación con el grupo control intacto (Figura 9). En el hipocampo, los vasos aislados de ratas restringidas de sueño mostraron una disminución en la expresión de ocludina (t=1.918, p=0.0305), mientras que la expresión de claudina-5 no cambió entre los grupos (Figura 9).

La disminución en las interacciones célula endotelial y pericito se asocia con un aumento en la permeabilidad de la barrera hematoencefálica

Las ratas restringidas de sueño presentaron un aumento en la permeabilidad de la BHE (Figura 10). La corteza cerebral (t=3.096, p=0.0044) e hipocampo (t=2.157, p=0.0007) de ratas restringidas de sueño mostraron un aumento en la permeabilidad de la BHE a Na-fluoresceína con respecto al grupo control. La corteza cerebral (t=4.375, p=0.0060) e hipocampo (t=4.626, p=0.0049) del grupo restringido de sueño presentaron un aumento en la permeabilidad de la BHE a azul de Evans en comparación con el grupo que durmió libremente. En ratas restringidas de sueño se observó un aumento en la permeabilidad de la BHE a rodamina 123 en la corteza cerebral (t=1.949, p=0.0436) e hipocampo (t=2.184, p=0.0303) *versus* el grupo control intacto.

La restricción de sueño promueve un estado inflamatorio de bajo grado en vasos cerebrales aislados

La pérdida de sueño aumentó la expresión de MMP-9 en microvasos cerebrales aislados de corteza cerebral (t=2.752, p=0.0131) e hipocampo (t=2.452, p=0.0341) en comparación con el grupo control (Figura 12). En la corteza cerebral, los microvasos cerebrales aislados de ratas restringidas de sueño mostraron un aumento en la expresión de p-NFκB (t=6.758, p=0.0212) en comparación con el grupo control (Figura 13). En los microvasos cerebrales aislados del hipocampo la restricción de sueño aumentó la expresión del receptor A_{2A} de adenosina (t=2.483, p=0.0324) en comparación con el grupo que durmió *ad libitum.* La expresión de la ectonucleotidasa CD73 se mantuvo sin cambios en ambas regiones entre los grupos experimentales.

Conclusión

La restricción crónica de sueño disminuye las interacciones entre el pericito y la célula endotelial cerebral. La pérdida de las interacciones entre el pericito y la célula endotelial se relaciona con una alteración de las propiedades de la BHE al disminuir la expresión de las proteínas de uniones ocluyentes y un aumento en la permeabilidad de la barrera hematoencefálica a trazadores exógenos. Las alteraciones en las interacciones celulares entre el pericito y la célula endotelial podrían depender de un estado inflamatorio de bajo grado durante la restricción de sueño.

ABSTRACT

Sleep loss produces blood-brain barrier (BBB) hyperpermeability by increasing endocytosis and promoting tight junction disassemble. Similar effects have been reported in mice with pericyte deficiency. In previous studies, we showed that pericytes seem to detach from the capillary wall in sleep-restricted animals. Thus, we aimed to evaluate the changes in brain endothelial cell-pericyte interactions and its consequences on barrier function during sleep restriction.

Male Wistar rats were subjected to sleep loss using the multiple platform technique for 20 h daily during 10 consecutive days. After 10 days of sleep loss, animals were euthanized, and the brain was removed to isolate brain microvessels from the cerebral cortex and hippocampus. The samples were used to evaluate the expression of PDGFRβ, connexin-43, claudin-5, occludin, MMP-9, NFkB, p-NFkB, A_{2A} adenosine receptor and CD73 by Western blot. An immunofluorescent assay was made for PDGFRβ or connexin-43 in isolated brain microvessels from the cerebral cortex and hippocampus. Another group of rats was used to perform BBB permeability assays to Na-fluorescein, Evans blue and rhodamine 123.

In isolated blood-microvessels of the cerebral cortex and hippocampus, sleep restriction reduced PDGFRβ expression in comparison with the control group. Likely, sleep restriction reduced the expression of connexin-43 in the cerebral cortex; meanwhile, in the hippocampus there was a trend to reduce connexin-43 expression compared to the control group. Sleep loss decreased the expression of claudin-5 in the isolated microvessels only in the cerebral cortex, while it decreased occludin expression in the hippocampus but not in the cerebral cortex as compared to the intact group**.** Sleep restriction increased MMP-9 expression in

isolated blood-vessels of the cerebral cortex and hippocampus. In addition, p-NFkB was increased only in the cerebral cortex *versus* the intact group. Sleep loss increased the expression of the A_{2A} adenosine receptor only in isolated microvessels of the hippocampus. Both regions presented an increase in BBB permeability to Na-fluorescein, Evans blue and rhodamine 123. In conclusion, sleep loss induces a pericyte detachment from the capillary wall, which is related to a decrease in the expression of tight junction proteins and an increase in the BBB permeability. The mechanism that may modulate the interactions between brain endothelial cells and pericytes after sleep restriction seems to be a low-grade inflammatory status.

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INTRODUCTION

The brain is the most energy-intensive organ in the human body, it uses nearly 50% of body's glucose and requests high concentrations of oxygen and other nutrients such as amino acids, vitamins and nucleic acids to maintain the cerebral functions (Zlokovic, 2008; Chandra et al., 2017). Therefore, brain vascular integrity and the optimal microcirculation of nutrients into the brain are crucial for proper function. The specialized structure blood-brain barrier (BBB), which will be described below, has a central role in controlling brain microenvironment homeostasis (Hermann and ElAli et al., 2012).

Paul Ehrlich described the BBB in a series of experiments with mice, using a vital dye that was injected intraperitoneally; Ehrlich observed that all the organs of the body were dyed except the brain (Ehrlich, 1885). Later, Edwin Goldmann injected the dye into the cerebrospinal fluid of animals and found that the brains did become dyed, but the rest of the body did not (Goldmann, 1913). These pioneer works demonstrated the existence of a *"limiting membrane"* that physically separates the brain circulation from the rest of the body.

It is now know that the BBB is a highly selective and semipermeable barrier that controls the passage of soluble and potentially toxic molecules from the lumen of the capillary to the brain parenchyma; while it allows the easy diffusion of oxygen, carbon dioxide and small lipophilic molecules (Keaney and Campbell 2015). Structurally, the BBB is composed of brain endothelial cells lining the cerebral microvasculature; endothelial cells form interactions with different cell types, including pericytes, astrocytes, microglia and neurons. Together, they are called

the neurovascular unit (Figure 1). The interaction and secretion of trophic factors among these cell types maintain the BBB physiology.

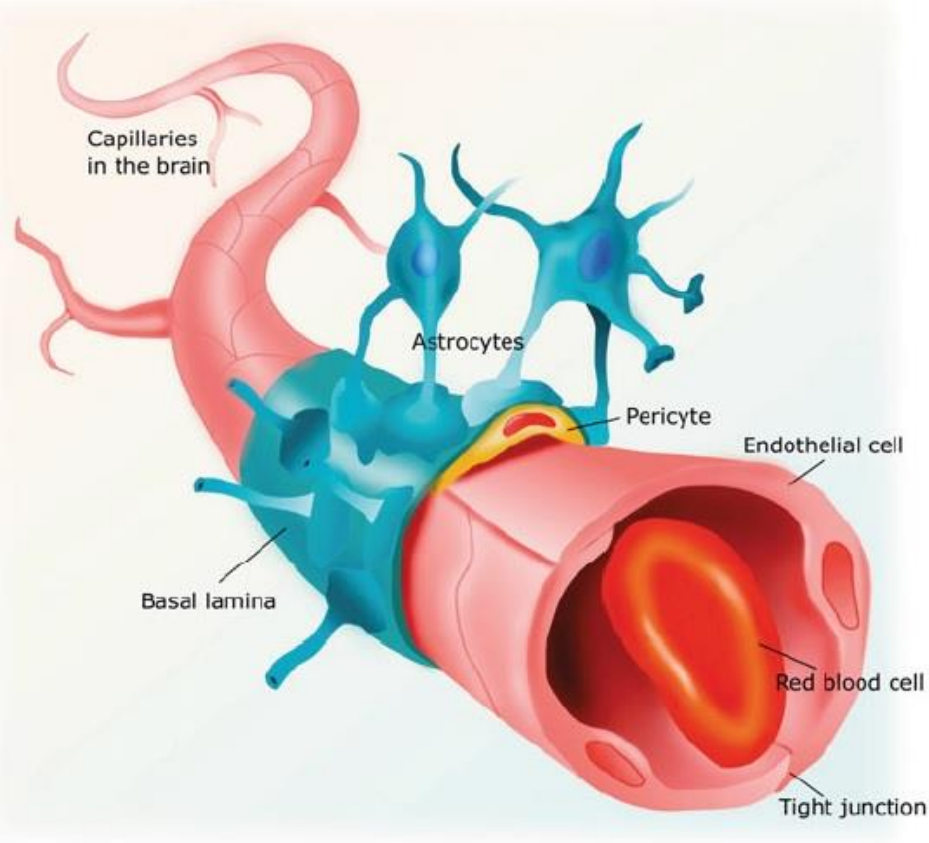


Figure 1. General structure of the blood-brain barrier. The image shows the organization of the cells forming the blood-brain barrier. (Taken from Tornabene and Brodin et al., 2016)

Brain endothelial cells

The main cellular component of the BBB is a monolayer of endothelial cells that form the wall of cerebral microvessels and it is separated from the neurons and glial cells by the basal lamina. This structure provides support to the cerebral endothelium for the formation and maintenance of the BBB, and it works like a barrier for the diapedesis of leukocytes to the brain during inflammation (Abbot et

al., 2002; Morris et al., 2016). The basal lamina that surrounds the endothelial cells is a highly organized protein sheet with a thickness of 50-100 nm (Vracko, 1970). Its biochemical composition consists of extracellular matrix proteins synthesized predominantly by endothelial cells: collagen IV, laminin, nidogen or entactin and perlecan or heparan sulfate proteoglycan 2 (HSPG2) (Yurchenco, 2011).

Brain endothelial cells present a specialized phenotype characterized by the presence of tight junctions and adherent junctions between adjacent cells. The integrity of the tight junctions regulates the paracellular diffusion of soluble molecules across the BBB. Tight junctions are formed by the integral transmembrane proteins claudins, occludin and junctional adhesion molecules (JAMs). These proteins interact with the actin cytoskeleton through the scaffold proteins zonula occludens (ZO), ZO-1, ZO-2, ZO-3, which are essential to the proper assembly of claudins, occludin and JAMs proteins (Wolburg *et al*., 1994; Liebner *et a*l., 2000; Urich *et al*., 2012; Roux *et al*., 2005).

In addition to tight junctions, adherens junctions allow the attachment between membranes of neighboring cells, via homophilic interactions between the transmembrane proteins: vascular endothelial cadherin (VE-cadherin) and epithelial cadherin (E-cadherin). It has been established that before tight junction formation, adherens junctions initiate the cell-to-cell contacts and promote their maturation, maintenance and plasticity (Vorbrodt and Dobrogowska, 2003).

Another set of proteins that are involved in the BBB maintenance are gap junction proteins (Nagasawa et al., 2006). Gap junction is formed by two hemichannels or connexons located on opposite cell membranes; each one is composed of 6

transmembrane proteins called connexins (Cxs). The connexin superfamily comprises 21 isoforms in humans and 20 in mice, which are named according to the molecular weight (Dobrowolski and Willecke, 2009). Connexin channels mediate the cytoplasmic communication by the interchange of ions and small metabolites, in order to maintain metabolic cooperation and the propagation of electrical signals and calcium waves between adjacent cells (Bruzzone et al., 1996; Laird, 2006). In rodents, Cx-32 and Cx-43 are highly expressed in the brain; however, both connexins present a differential pattern of expression during brain development (Kumar and Gilula et al., 1996; Nadarajah et al., 1997). Connexin-32 expression predominates at the late stage of cortical maturation, while connexin-43 expression persists during postnatal development and in the adult cortex (Nadarajah et al., 1997). The interactions between endothelial cells and smooth muscle cells via gap junctions contribute to modulate vessel diameter due to changes in blood pressure, blood flow and shear stress (Schuster et al., 2001; de Wit and Griffith, 2010). Brain endothelial cells have a unique junctional architecture that contributes to the BBB maturation and maintains CNS homeostasis (Figure 2) (Alvarez et al., 2011).

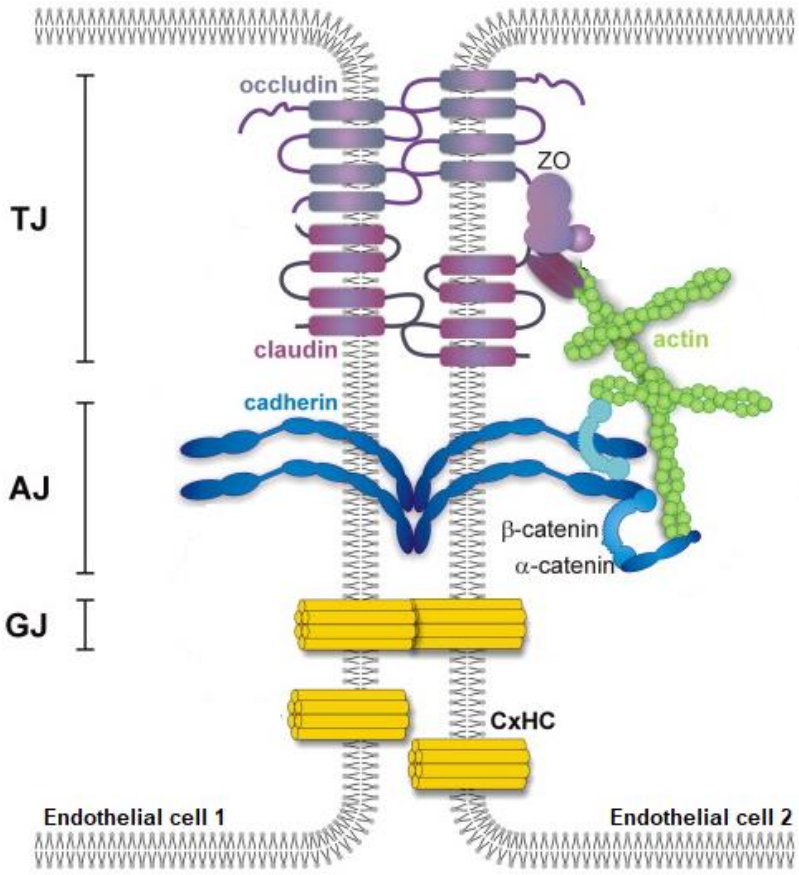


Figure 2. Main junctions between brain endothelial cells. The image shows the tight junction (TJ), adherens junction (AJ) and gap junction (GJ) organization along brain endothelial cell membrane (Modified from De Bock et al., 2014).

The cytoplasm of brain endothelial cells has an uniform thickness with numerous mitochondria, few pinocytic vesicles and lack of fenestrations (Roux *et al*., 2005; Urich *et al*., 2012). In addition to the specialized structural elements that ensure the BBB tightness, there is a wide array of specific transporters in the luminal and abluminal membranes, which allow nutrient entry to the brain (Pardridge et al., 1988). The principal transport mechanisms present in the BBB are:

1) Carrier-mediated transport: small hydrophilic molecules use this mechanism to move from the blood to the brain. The glucose transporter GLUT-1 is a protein that carries extracellular glucose into the cell (Dwyer et al., 2002). Other types of specific transporters include the protein LAT-1, that carries large neutral amino acids like tyrosine, tryptophan, and leucine; and the monocarboxylate transporter (MCT-1), which performs influx transport of pyruvate, lactate, and ketone bodies.

2) Adsorptive endocytosis: the mechanism consists of the invagination of the plasma membrane to introduce solutes from the extracellular space. Vesicles are enriched by caveolin-1.

3) Receptor-mediated transport: this occurs when the substrate binds to its membrane receptor and then internalization of the complex ligand-receptor is achieved in clathrin-coated vesicles (Smith and Gumbleton, 2006).

4) ABC (ATP-binding cassette) transporters: a superfamily of energy-dependent active transporters formed by 48 members classified into 7 families (ABC-A, -B, -C, -D, -E. -F and -G); of which the P-glycoprotein (P-gp) has been widely studied. P-gp is an ATP-dependent efflux bomb of 170 kDa located in the apical side of brain endothelial cells (Schinkel, 1999); it restricts the passage of H1 histamine receptors antagonists, verapamil and other hydrophobic molecules. Jodoin and colleagues (2003) reported that in the cerebral endothelium, P-gp co-localizes with caveolin-1 and caveolin-2 to form a molecular complex of 669 KDa. It has been suggested that this interaction improves the activity of P-gp by facilitating its oligomerization and accelerating drug metabolism (Demeule et al., 2000; Barakat et al., 2007; Guo et al., 2010).

Pericytes

Pericytes were firstly described by Eberth and Rouget in the 1870s and were named *"Rouget cells"* (Rouget, 1873). Later, Zimmermann (1923) coined the term *"pericytes"* (surrounding -peri, cells -cytes) due to their close location to the brain vessels. Recently, pericytes have been defined as perivascular cells that surround blood vessels and are embedded within the basal lamina with the cerebral microvasculature (Armulik et al., 2005). The pericytes morphology consists of a cell body with a prominent nucleus and a small amount of cytoplasm with long processes that embrace the endothelium.

The pericytes origin depends on the vascular bed and their differentiation stage. During angiogenesis nascent endothelial tubes invade the embryonic neural tube and migrate toward the ventricles forming the primitive network of brain vessels. During this process, endothelial cells secrete a wide range of bioactive molecules that specifically trigger the attraction, mobilization, and recruitment of pericytes at the abluminal side of the newly formed vascular network (Bautch and James, 2009). Subsequently, vascular smooth muscle cells or pericyte progenitors proliferate and migrate along with new angiogenic sprouts (Hellström et al., 1999). During brain development, pericytes destinated for the forebrain originate from neural crest cells, while brainstem- and spinal cord-pericytes derive from the mesoderm (Etchevers et al., 2001; Korn et al., 2002; Reyahi et al., 2015). (Etchevers et al., 2001).

The central nervous system has the highest density and coverage of pericytes (in a ratio of 1 pericyte per each 4 endothelial cells) compared to peripheral blood vessels (Shepro and Morel et al., 1993). Pericyte identification is achieved through

different molecular surface markers, whose expression varies according to the type of vascular bed, stage of development, pathologies and even whether the conditions are *in vitro* or *in vivo* (Armulik et alk, 2005; Hill et al., 2015; Smyth et al., 2018). Pericytes on arterioles and venules typically express alpha-smooth muscle actin (α-SMA), whereas pericytes on capillaries predominantly express platelet-derived growth factor receptor beta (PDGFR-β), nerve/glial antigen-2 (NG2) proteoglycan and desmin (Nehls et al., 1991); however, in the presence of brain tumors, cerebral pericytes express α-SMA (Morikawa et al., 2002).

Platelet-derived growth factor subunit B has gained insight due to its importance for pericyte physiology. During angiogenesis, endothelial cells secrete PDGFB (platelet-derived growth factor B), which binds to its tyrosine-kinase receptor PDGFR-β expressed on the surface of the pericytes, resulting in PDGFR-β dimerization. Subsequently, the autophosphorylation of cytoplasmic tyrosine residues and binding of SH2 domain proteins, activate signal transduction pathways that promote proliferation, recruitment and migration of pericytes into the newly developed blood vessel, allowing the establishment of specific *"peg-socket"* contact points with the endothelium. The name *"peg-socket"* comes from the apparent pericyte cell membrane protrusion-like structures (pegs) that are inserted into endothelial cell membrane invaginations (pockets) (Hellström et al., 1999; Helding and Westermark, 1999; Lebrin et al., 2010; Armulik et al., 2011). In addition, ultrastructural studies showed that in some areas, the basal lamina entirely separates pericytes from endothelial cells and the interaction between both cell types is through adhesion plaques made of fibronectin-rich monofilaments. Besides that, pericytes express sets of integrins, allowing them to

attach to the extracellular matrix proteins of the basal lamina and endothelial cells (Armulik et al., 2011; ElAli et al., 2014). In the healthy adult brain, these interactions allow pericytes to perform their physiological functions, such as providing mechanic support to the endothelium to maintain the tight junctions between adjacent cells, controlling the capillary diameter, regulating the microvascular blood flow and controlling the immune cell entry to the central nervous system (Shimizu et al., 2008; Hamilton, 2010; Armulik et al., 2015; Saeed et al., 2017; Török et al., 2019).

The communication between the pericyte and the brain endothelial cell is carried out by paracrine signaling mediated by transforming growth factor β (TGF-β), platelet-derived growth factor B (PDGF-B), angiopoietin, sphingosine-1-phosphate, and vasoactive molecules such as vasopressin (Armulik et al., 2005; Armulik et al., 2011; Winkler et al., 2011). Due to their contractile nature, pericytes have receptors that recognize vasoactive signaling molecules and neurotransmitters that control the capillary diameter and regulate blood flow at the capillary level in response to changes in neural activity or local bioenergetic requirements (Balabanov and Dore-Duffy, 1998; Yamanishi et al., 2006). Pericyte contraction or relaxation is triggered by different vasoconstrictor and vasodilator mediators such as protons, glutamate or adenosine, that active their respective ion channels or receptors (Matsugi et al., 1997). Signs of contraction and dilation can spread from one pericyte to another. This signal propagation can occur through gap junctions between the processes of the pericytes themselves or gap junctions with endothelial cells (Attwell et al., 2010). The pericyte participation in controlling cerebral blood flow in response to neural activity is named neurovascular coupling

(Peppiatt et al., 2006; Zehendner et al., 2013). The pericyte is an integral, dynamic and active component of the BBB, so its loss or absence compromises the homeostasis of the central nervous system (Sweeney et al., 2018).

Sleep regulates blood-brain barrier function

Sleep is a physiological state that guarantees the survival of most mammals, characterized by the reduction of alertness, the acquisition of a typical posture for sleeping, a decrease of voluntary body movements and the presence of a characteristic pattern of brain electrical activity. Polysomnography studies determinate that during sleep, there is a typical cerebral electrical activity and a characteristic pattern of ocular movements and muscular activity. In most of the species, sleep can be classified in two states according to changes in the cerebral electrical activity: non-rapid eye movement (Non-REM) and rapid eye movement (REM), which follow a cyclic pattern (from 90 to 120 minutes in humans) along the night. In humans, NREM sleep is divided into 3 stages: stages 1 and 2 are considered light sleep, and stage 3 corresponds to the slow-wave sleep, also known as delta sleep (Saper et al., 2010). In the rest of the mammals, the non-REM sleep can be subdivided into slow-wave sleep stages 1 and 2. During non-REM sleep, the electroencephalographic activity is characterized by the presence of slow-waves of high amplitude; in addition, there is a decrease in muscle tone and slow eye movements. In REM sleep, the electroencephalographic activity is of high frequency and low amplitude, like the observed during wakefulness, eye movements are rapid and muscular atony is present (Staunton, 2005). In addition to changes in brain electrical activity, each sleep phase is accompanied by

variations in some physiological parameters. During non-REM sleep, there is a decrease in blood pressure, heart rate, respiratory rate and body temperature; whereas, in REM sleep there are stochastic variations in respiratory rate, blood pressure, and heart rate, also, peripheral thermoregulation is reduced (Wehr, 1992; Porkka-Heiskanen et al., 2013).

Animal models and clinical observations in humans have been used to study the function of sleep. Experimental procedures generally involve continuous periods of sleep loss (a procedure called sleep deprivation); periods in which the number of sleep hours *per* night is reduced (called sleep restriction); and periods in which each night the subject is awakened on numerous occasions to induce sleep fragmentation. After sleep loss, subjects can freely sleep during different periods (from hours to days) to study the restoration of the physiological functions with sleep recovery. Several studies have shown that sleep is necessary for body restoration, clearance of toxic brain metabolites and memory consolidation (Xie et al., 2013; Rasch and Born, 2013). On the other hand, continuous sleep loss impairs health status and eventually leads to death in experimental animal models (Rechtschaffen et al. 1989; He et al., 2014; Hurtado-Alvarado et al., 2018). Multiple studies showed central and peripheral effects of sleep loss ranging from alterations in the circulating levels of hormones to pathological changes in various organs and tissues (Gómez-González et al., 2012). In general, it has been described that sleep loss induces a low-grade pro-inflammatory state at the systemic level, characterized by the increase in circulating levels of pro-inflammatory molecules, such as C-reactive protein, pro-inflammatory cytokines

IL-1, TNF-α, IFN-γ and by the variation in subtypes of circulating immune cells (He et al., 2014; Hurtado-Alvarado et al., 2018).

In the central nervous system, sleep loss alters brain neurochemistry by increasing levels of excitatory amino acids, such as glutamate and aspartate, in the cerebral cortex and hippocampus (Mohammed et al., 2011). Sleep loss also decreases the hippocampal volume (Novati et al., 2011) and neurogenesis (Guzman-Marin et al., 2008), which results in deterioration in the execution of learning and memory deficits (Novati et al., 2011). Sleep is, therefore, necessary to maintain the homeostasis of the central nervous system.

ANTECEDENTS

Pericytes are cells with heterogeneous functions that maintain neurovascular stability and their absence compromises the integrity of the BBB (Bastide et al., 2007; Kovács et al., 2012). The knockout mice for PDGFR-β and PDGFB have vascular aberrations as a result of pericyte deficits resulting in perinatal death (Leveen et al., 1994). On the other hand, mice with a mutation that produces constitutive activation of PDGFR-β (PDGFR-$\beta^{ret/ret}$) are viable but show microvascular dysfunction (Lindblom et al., 2013). In the PDGFR-$\beta^{ret/ret}$ mouse model, a decreased density of pericytes is observed accompanied by an increase in the permeability of the BBB to Evans blue, immunoglobulin G and fibrinogen in the cerebral cortex, basal nuclei and hippocampus (Armulik et al., 2010; Villaseñor et al., 2017). These changes coincide with a reduction in the expression of the

tight junction proteins claudin-5 and zonula occludens-1 (Armulik et al., 2010; Bell et al., 2010).

Our laboratory has proved that during sleep loss, central nervous system homeostasis and BBB function are altered. In rodents, chronic sleep restriction increases the permeability of the BBB to Evans blue (Gómez-González et al., 2013), 10 kDa- and 70 kDa-dextrans (Hurtado-Alvarado et al., 2016), and sodium-fluorescein (Hurtado-Alvarado et al., 2017) due to a decrease in the expression of claudin-5 (Hurtado-Alvarado et al., 2018). In addition, these observations are accompanied by an increase in the density of endocytic vesicles, cytoplasmic projections oriented towards the lumen of the capillary and an increase in the thickness of the basal lamina (Gómez-González et al., 2013; Hurtado-Alvarado et al., 2017). Similar results were reported by He and collaborators (2014), they showed that 6 days sleep deprivation decreased mRNA expression of claudin-1, claudin-5, and ZO-2, thus increasing paracellular permeability in the cerebral cortex, brainstem and cerebellum (He et al., 2014). However, it was observed that short periods of sleep recovery, ranging from 40 to 120 minutes, can restore the basal permeability of the BBB in almost all the studied brain regions (Gómez-González et al., 2013). This observation indicates that some mechanisms and molecules restore the function of the BBB. Adenosine is a ubiquitous nucleoside that modulates the cellular activity, and its concentration increases throughout the waking time and decreases during the sleep period (Basheer et al., 2004). During sleep restriction, there may be high concentrations of adenosine in the extracellular space that contributes to the increased permeability of the BBB. Hurtado-Alvarado et al. (2016) conducted an experiment in which a selective A_{2A}

adenosine receptor antagonist was administered immediately after concluding chronic sleep restriction. Subsequently, the hippocampus showed a decrease in the permeability to 10 KDa dextrans and the restoration of claudin-5 protein expression, as well as a decrease in the concentration of pro-inflammatory markers such as Iba-1, glial fibrillar acidic protein (GFAP) (Hurtado-Alvarado et al., 2016). These results indicate that adenosine and its receptor significantly modulate BBB permeability.

Hurtado-Alvarado et al. (2017) presented ultrastructural evidence of a possible pericyte detachment from the capillary wall in sleep-restricted animals. We propose that an inflammatory status could be involved in this alteration in the communication between endothelial cells and pericytes because sleep loss increases COX-2 levels (He et al., 2014), cellular markers of inflammation such as Iba1 and GFAP (Hurtado-Alvarado et al., 2016, 2018), and the expression of C-reactive protein and plasmatic inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, IL-17, Interferon (IFNɣ) and Tumor necrosis factor (TNF-α) (Yehuda et al., 2009; He et al., 2014; Hurtado-Alvarado et al., 2018).

JUSTIFICATION

Sleep is a physiological event necessary to maintain the homeostasis of the central nervous system; nevertheless, sleep restriction is a common phenomenon in modern society. According to the Encuesta Nacional de Salud y Nutrición (ENSANUT), 28% of the Mexican population sleep less than 6.5 hours daily, while the World Health Organization (WHO) reported that poor sleep quality should be considered as a global epidemy, because between 20% and 48% of the adult population reduces sleeping hours voluntarily due to the use of computers, phones or tablets during the night. A vast body of evidence showed that sleep deprivation induces a deleterious effect on cognitive performance, mood and motor function. However, the amount of information has not been integrated to fully understand the effect of sleeplessness on central nervous system function.

Previous research has shown that there is a relationship between sleep loss and BBB function, making the organism prone to neurotoxic insults and neurodegenerative diseases. On the other hand, by understanding the consequences of sleep restriction and the mechanisms by which it modifies the BBB function, interesting windows of opportunity may be generated for the development of highly specific therapeutic strategies that will allow to treat diseases of the central nervous system and it will help us to understand the function of sleep itself. Therefore, the present study is aimed to elucidate the role of pericyte-endothelial cell interactions in the changes in BBB function induced by sleep loss.

HYPOTHESIS

Chronic sleep restriction decreases the interactions between brain endothelial cells and pericytes, which will induce an alteration in the energy metabolism of the endothelial cell that will lead to the dysfunction of the blood-brain barrier.

GENERAL OBJECTIVE

To evaluate the changes in brain endothelial cell-pericyte interactions and their effect on brain endothelial cell metabolism and barrier functions during chronic sleep loss.

SPECIFIC OBJECTIVES

1.- To evaluate the effect of chronic sleep loss on brain endothelial cell-pericyte interactions.

2.- To determine the relationship between the changes in brain endothelial cell-pericyte interactions and metabolism of brain endothelial cells during chronic sleep loss.

3.- To determine the relationship between changes in brain endothelial cell-pericyte interactions and barrier function during chronic sleep loss.

MATERIALS AND METHODS

Experimental subjects

Three-month-old male Wistar rats were used. The animals remained under a 12 h light/dark cycle (lights on at 23:30 hours), at room temperature of 20-23°C and they were fed with commercial rat chow and water *ad libitum.* The rats were randomly assigned for the intact control and chronic sleep restriction groups. All procedures with animals were strictly carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Principles of the Mexican Official Ethics Standard 062-ZOO-1999 and the Standard for the disposal of biological waste (NOM-087-ECOL-1995).

Chronic sleep restriction

Male rats were subjected to sleep restriction using the multiple platform technique, which completely suppresses rapid eye movement sleep and slightly reduces non-REM sleep (Gómez-González et al., 2013). Rats were placed in a sleep deprivation chamber (82 x 59 x 48 cm) with platforms of 7cm diameter surrounded by water for 20h daily with an opportunity to sleep in their home cages for the last 4h of the light phase during 10 consecutive days (Gómez-González et al., 2013). To reduce social stress, the rats remained in their same social group during the sleep deprivation and sleep recovery periods, and to reduce restraint stress an extra platform was added to the deprivation chamber to facilitate the movement of the animals. During the whole experiment, rats had water and food *ad libitum*.

Brain microvessel isolation

Brain microvessels were isolated immediately after removing the rats from the sleep deprivation chamber at 10th day of sleep restriction. The animals were euthanized and the brain was removed to isolate microvessels from the cerebral cortex and hippocampus (Deli et al., 1997; Nakagawa et al., 2009). The regions of interest were placed on a filter paper to eliminate blood residues. Subsequently, the tissue was homogenized with 1mL of cold sucrose buffer (BS, 10.95g sucrose, 0.071493g HEPES, 0.1g BSA 1%, 100mL filtered PBS, pH: 7.4) and centrifuged at 1000xg for 10 minutes at 4°C. The supernatant was decanted, the pellet was homogenized with 1mL BS and centrifuged at the conditions mentioned above. The supernatant was recovered. Supernatants were then centrifuged at 200xg for 5 minutes and the pellets were preserved, the pellets were washed with 1mL BS (x2) and then 1mL PBS 1x at 100xg for 2 minutes each. The pellet contained brain microvessels from each brain region and was frozen until use.

Western blot

Protein expression from isolated brain microvessels was analyzed in sleep-restricted and intact control groups. The isolated brain blood microvessels from cerebral cortex and hippocampus were dissected, homogenized and centrifuged with 200µl RIPA at 13500 rpm for 10 minutes at 4°C. The supernatants were collected and stored to -20°C until use. The concentration of proteins was determinate by the method of Bradford assay (BioRad, #500-0006, 1:5). The proteins (30 µg) were separated by electrophoresis in a 10% polyacrylamide gel and then transferred to membranes of poly-vinylidene fluoride (PVDF). After

transfer, the PVDF membrane was blocked with a solution of 5% non-fat dry milk in PBST for 30 minutes and then incubated with the primary antibodies diluted in PBST overnight at 4°C: PDGFRβ (ThermoFisher, #G.290.3, 1:1000), connexin-43 (Invitrogen, # 71-0700, 1:1000), anti-Claudin-5 (Biorbyt, #orb160461, 1:1000), anti-Occludin (Invitrogen, #40-4700, 1:500), GLUT-1 (Invitrogen, PA5-16793, 1:1000), MMP9 (Abcam, #ab38898, 1:1000), A_{2A} adenosine receptor (Abcam, #ab3461, 1:1000), adenosine synthetizing ectonucleotidase (CD73) (Abcam, #ab175396, 1:1000) and Phospho-NF-kB p65 (Cell signaling, 3033S, 1:1000). Controls of procedure included the analysis of the expression of the neural protein NeuN (Millipore, #MAB377, 1:1000), and the astroglial protein GFAP (Abcam, #ab4648, 1:1000). Later, the PVDF membranes were incubated with secondary antibodies Biotinylated anti-Rabbit IgG (Vector Laboratories, #BA-1000, 1:2500) and Biotinylated anti-Mouse IgG (Vector Laboratories, #BA-2000, 1:2500) for 2 hours in constant agitation and revealed with the chemiluminescence detection system (Amersham, RPN2232). Images were acquired using the C-DiGit image generation and analysis system (LI-COR iS image studio, version 3.1). Proteins were normalized with a 220 KDa band marked with red Ponceau.

Immunofluorescence confocal microscopy

An immunofluorescence procedure was used to corroborate the localization of PDGFRβ and connexin 43 in isolated blood vessels. Evans blue was used to mark blood vessels. Immediately, at the end of the 10^{th} day of sleep restriction, intact controls and sleep-restricted rats were ip. anesthetized with sodium pentobarbital and a thoracic incision was made to expose the heart. Evans blue (1mg/mL) was

administrated in the left heart ventricle (0.2mL/100g of body weight) and it was left to circulate for 5 minutes. Afterward, animals were transcardially perfused with 0.09% saline solution. The brain was removed, and the cerebral cortex and hippocampus were obtained to isolate blood vessels as previously described. The isolated brain blood vessels were resuspended with 1mL 4% paraformaldehyde for 1 hour and centrifuged at 13500 rpm for 10 minutes, the supernatants were eliminated by decanting and the pellets were resuspended with 1mL PBS. Next, 200µl of isolated resuspended blood vessels were placed on gelatinized slides and carefully distributed, after 1 hour of adherence, the blood vessels were washed with 100µl 0.1% PBST and blocked with Normal Horse Serum blocking solution (Vector Labs, #S-2000, 1:1000) for 15 minutes. Slides were then incubated with PDGFRβ (ThermoFisher, #G.290.3, 1:1000) or connexin-43 (Invitrogen, # 71-0700, 1:1000) for 4h under dark and humid conditions, they were washed with PBS and incubated with the secondary antibody Alexa Fluor® 488 donkey anti-rabbit IgG (ThermoFisher, #A21206, 1:250) for 4 hours. The samples were mounted in ImmunoHistoMount mounting medium (Sigma, #I1161-30ML). Confocal images were acquired at 488nm excitation with a detection range of 505-535nm and analyzed with an Axioscop 2 mot plus confocal fluorescence microscope (Carl Zeiss, México) at EC Plan-Neofluar 40x/0.5.

Scanning Electron Microscopy

Sleep-restricted and intact control rats (n=2 *per* group) were sacrificed to isolate brain microvessels from the cerebral cortex and hippocampus, as described above. Isolated brain microvessels were fixed with 4% paraformaldehyde and 2%

glutaraldehyde for 24h at 4°C. Samples were rinsed with PBS 1x and placed on gelatinized coverslips. The samples were post-fixed with 1% OsO_4 for 2h at room temperature and then dehydrated in graded ethanol series. The samples were dried at the critical point in liquid CO_2, followed by gold coating. Scanning electron microscopy micrographs were acquired at 13kV with a scanning electron microscope (JEOL JSM-5900 LV).

Blood-brain barrier permeability assay

Immediately at the end of the 10^{th} day of sleep restriction, rats were ip. anesthetized with sodium pentobarbital and a thoracic incision was done. A cocktail containing Na-Fluorescein (Sigma-Aldrich, #F6377, 10mg/mL), and Evans Blue (Sigma-Aldrich, #E2129, 1mg/mL) tracers was administrated in the left heart ventricle (0.2 mL/100 gr. of weight) and left to circulate for 5 minutes (n=5 per group); tracers were diluted in phosphate-buffered saline solution (1X, pH=7.4). An independent group of rats (n=5 per group) received rhodamine 123 (Sigma-Aldrich, #83702, 1mg/mL) intracardially under deep anesthesia to test the changes in BBB permeability to a P-gp substrate in sleep-restricted rats. After tracer circulation, animals were transcardially perfused with 0.09% saline solution and the brain was removed to obtain the cerebral cortex and hippocampus. The samples were weighed, homogenized with 200 µl of ice PBS (1X) and centrifuged at 13500 rpm/10 minutes at 4°C. The supernatant was collected, 200 µl of methanol was added, mixed and centrifuged at 13500 rpm/10 minutes at 4°C. Thereafter, 100 µl of the supernatant of each sample were placed in a 96-well plate and absorbance values were measured in ELISA plate reader HLAB (H

Reader 1) at 485 nm excitation and 535 nm emission for Na-fluorescein and a 535 nm excitation and 595 nm emission for Evans blue and Rhodamine 123. Each sample was quantified in duplicate.

Mitochondrial oxygen consumption in isolated brain microvessels

Sleep-restricted and intact control rats were sacrificed, the brain was excised, and the hippocampus and cerebral cortex dissected to evaluate the mitochondrial function at the isolated brain microvessels. Isolated blood vessels from the hippocampus and cerebral cortex were pooled for the same group animals (n=10 *per* group). Pools of isolated blood vessels were resuspended in 20mL ice-cold 250mM sucrose, 10mM EDTA, pH=7.4, homogenized and centrifuged at 3500 rpm for 15 minutes. The supernatant was recovered and centrifuged at 12000 rpm for 15 minutes. The supernatant was removed and the pellet of isolated mitochondria from the cerebral cortex was carefully resuspended with 1mL of the same solution, while isolated mitochondria from the hippocampus were resuspended with 300µl of the same solution and stored at 4°C. The Lowry method was used to determine the concentration of the proteins.

Oxygen uptake was measured polarographically using a Clark type electrode in a solution containing 250mM sucrose, 10mM $MgCl_2$, 10mM succinate, 10mM H_3PO_4, 1mM EGTA, 0.05% bovine serum albumin pH= 7.3 and 2mg/mL of mitochondrial protein at 37°C. Oxygen consumption was promoted by adding 300-500nmol of ADP (state 3 of respiration). Respiratory control was defined as oxygen consumption rate of state 3/oxygen consumption rate of state 4 (ADP was converted to ATP and respiration slowed down). Thereafter, 10µM of carbonyl

cyanide *m*-chlorophenyl hydrazine (CCCP) was added to stimulate maximal oxygen consumption.

Statistical analysis

Data obtained from control and sleep-restricted groups were compared with one-tail *t*-student analysis. All results are presented as mean ± standard error of the mean (s.e.m). Statistically significant difference was considered at $p<0.05$. Statistical analyses were conducted using GraphPad Prims 8.0 software.

RESULTS

To verify that the isolated brain microvessels were free of other cell types as neurons and astrocytes, a Western blot against neuronal and gliar markers was performed. A sample of the whole brain was used as a positive control. Figure 3 shows that the brain microvessels isolated from the cerebral cortex (Figure 3A) present low expression of GFAP (Figure 3B, $t=4.309$, $p=0.0499$) and NeuN (Figure 3B, $t=12.69$, $p=0.0061$). Similarly, the isolated brain microvessels from the hippocampus (Figure 3C), present low expression levels of GFAP (Figure 3D, $t=12.84$, $p=0.0060$) and NeuN (Figure 3D, $t=4.868$, $p=0.0397$).

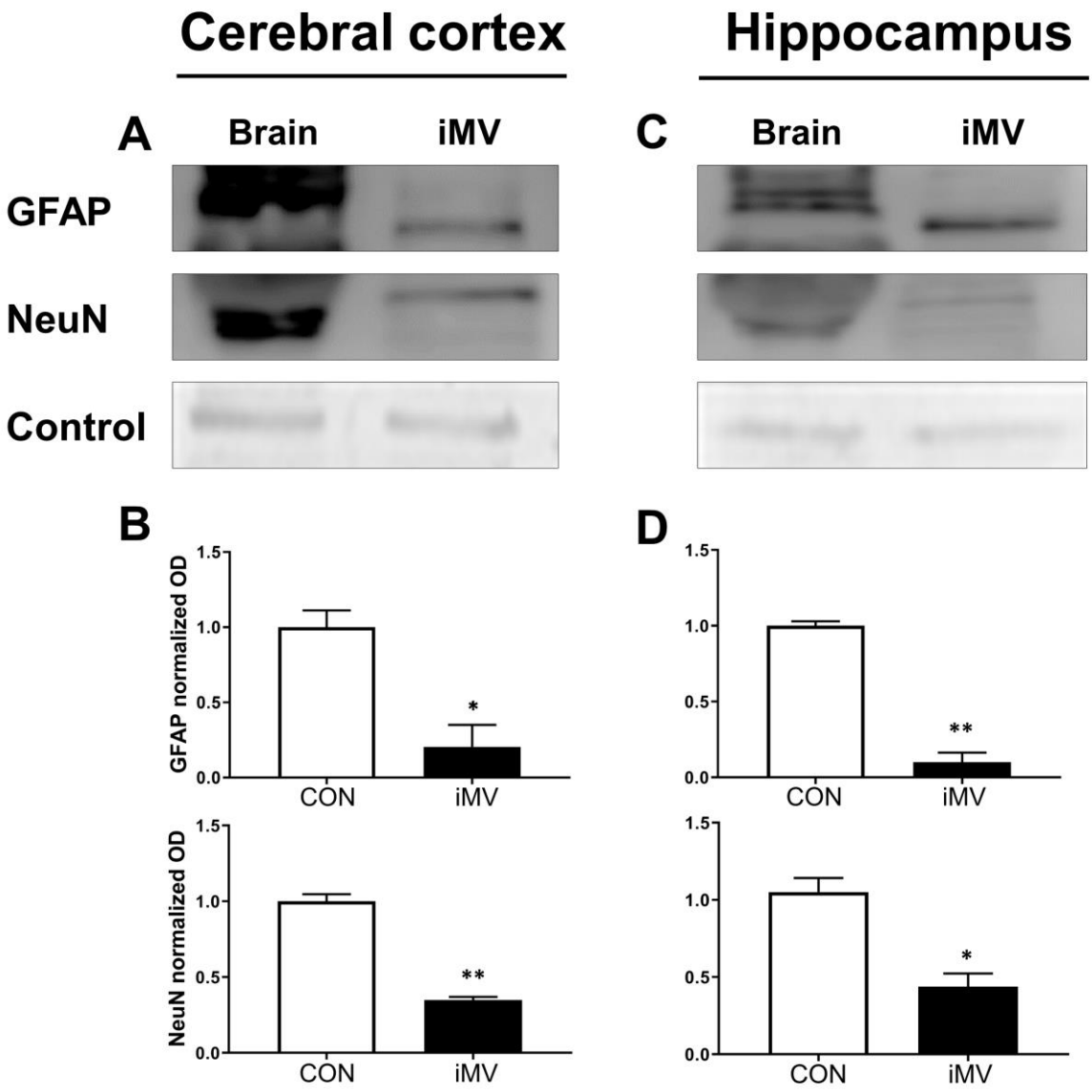


Figure 3. Purity of isolated brain microvessels. Western blot images from the cerebral cortex (A) and hippocampus (C) show that isolated brain microvessels (iMV) had a diminished presence of neuronals and astrocytic markers. A significant reduction in the expression of GFAP and NeuN in comparison with the whole brain (Brain) was observed in the cerebral cortex (B) and hippocampus (D). Protein expression of GFAP and NeuN was normalized with a band of 220 kDa marked with Ponceau red. Mean ± S.E.M. *p<0.01, **p<0.001 as compared to the control group.

Sleep loss reduces pericyte-brain endothelial cells interactions

Chronic sleep restriction reduced the interactions between brain endothelial cells and pericytes in the cerebral cortex and hippocampus. Western blot analysis showed that sleep restriction decreased PDGFRβ expression in isolated brain microvessels from cerebral cortex (t=2.623, p=0.0173) (Figure 4A & 4B) and hippocampus (t=5.219, p=0.0001) (Figure 5A & 5B). The observed changes in protein expression were corroborated by confocal microscopy (Figure 5C & 5C). Figure 4C shows that PDGFRβ immunoreactivity in the isolated microvessels from the cerebral cortex of the control group was detected surrounding capillaries with putative ramifications and the characteristic elongated pattern of a pericyte attached to the capillary wall. However, in sleep-restricted males, the PDGFRβ immunoreactivity in the isolated capillaries from the cerebral cortex was less intense and, interestingly, PDGFRβ immunostaining set apart from the capillary wall and present a globular shape, indicating potential pericyte detachment from the capillary wall (Figure 4C). Likely, in the hippocampus of sleep-restricted rats, low immunoreactivity for PDGFRβ was shown surrounding capillaries (Figure 5C).

Sleep restriction reduced the expression of connexin-43 in isolated brain microvessels from the cerebral cortex (t=1,038, p=0.0063) (Figure 6); while in the hippocampus, there was a trend toward reduction in the expression of connexin-43 in comparison with the control group sleeping *ad libitum* (Figure 7). As shown in the confocal microscopy micrograph in the control group, connexin-43 immunoreactivity was continuously distributed along the capillaries and the signal intensity was higher in specific points, suggesting specific contact points between pericytes and endothelial cells in both the cerebral cortex (Figure 6C) and the

hippocampus (Figure 7C). By the contrary, in sleep-restricted-rats, the immunoreactivity for connexin-43 was lower and showed a discontinuous pattern along the capillaries in the isolated microvessels from the cerebral cortex (Figure 6C) and hippocampus (Figure 7C).

Scanning electron microscopy showed that in the intact controls sleeping *ad libitum* pericytes are closely attached to the capillary wall in the isolated microvessels from the cerebral cortex and hippocampus (Figure 8). As shown in Figure 8, pericytes in the microvessels from intact controls seem embedded in the same basal lamina as the endothelial cells (arrow). In the isolated microvessels from the cerebral cortex and hippocampus of the sleep-restricted animals, globular shaped cells were observed in the proximity of the capillary wall (arrowhead), which is consistent with the globular-like immunoreactivity for PDGFRβ observed in confocal microscopy images.

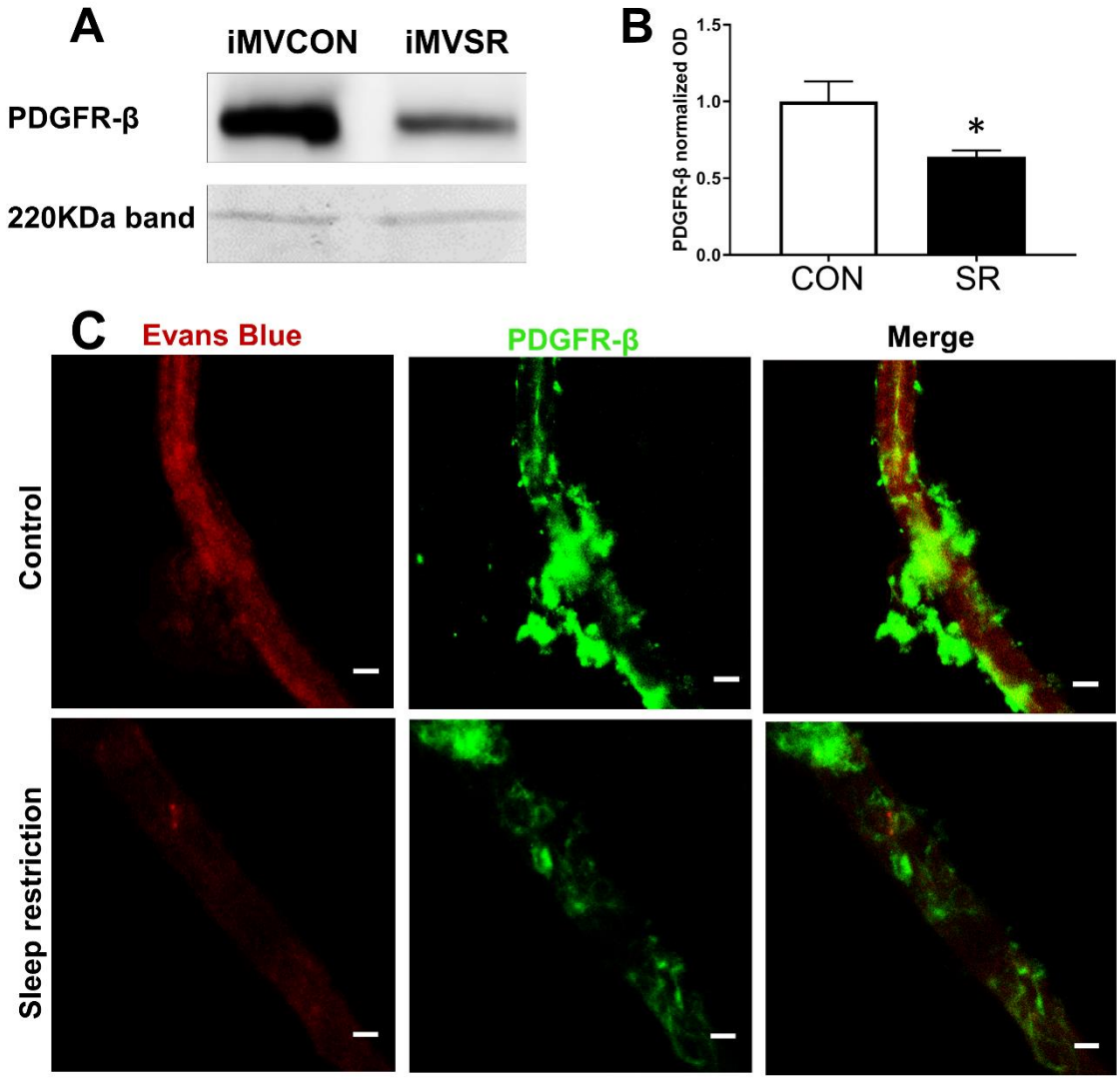


Figure 4. Chronic sleep restriction reduces PDGFRβ expression in the cerebral cortex. A) Representative western blot of isolated microvessels from the control (iMVCON, n=6) and isolated microvessels from the sleep-restricted (iMVSR, n=6) groups. B) Normalized levels of PDGFRβ expression in the isolated brain microvessels from the cerebral cortex. The band of 220 kDa marked with Ponceau red was used for normalization. Samples were analyzed in duplicate in separate PVDF membranes. Mean ± standard error of the mean (SEM). *$p<0.01$ as compared to the control group. C) Confocal microscopy images of isolated brain microvessels of intact control (upper panel) and sleep-restricted rats (lower panel). A reduced immunoreactivity of PDGFRβ (green) in sleep restriction group in comparison with the intact control group is noted. Brain microvessels were marked with Evans blue (red). Scale bar: 20 µm.

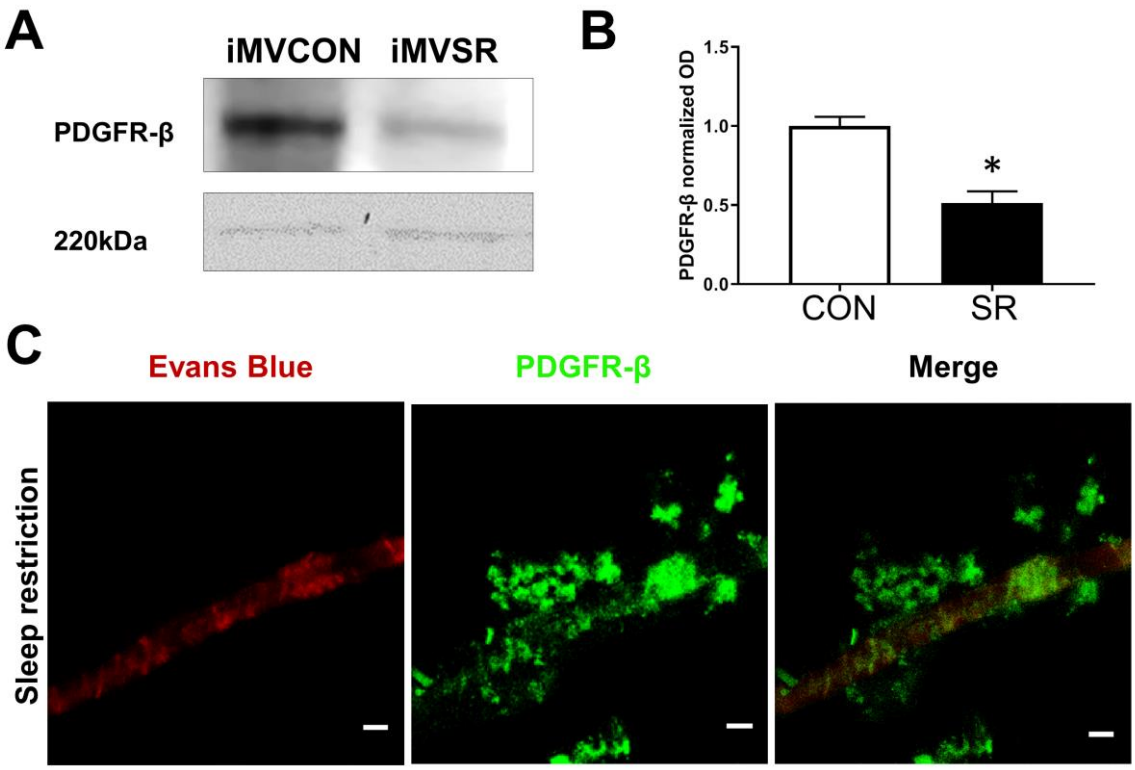


Figure 5. Chronic sleep restriction reduces PDGFRβ expression in the hippocampus. A) Representative western blot of the isolated microvessels from control (iMVCON, n=6) and sleep-restricted (iMVSR, n=6) groups. B) The graph depicts the normalized expression levels of PDGFRβ using a band of 220 kDa marked with Ponceau red. Samples were analyzed in duplicate in separate PVDF membranes. Mean ± standard error of the mean (SEM). *p<0.0001 as compared to the control group. C) Confocal microscopy images of isolated brain microvessels from hippocampus showing PDGFRβ immunoreactivity (green) characterized by a globular-like distribution in the sleep restriction group. Brain microvessels were marked with Evans blue (red). Scale bar: 20µm.

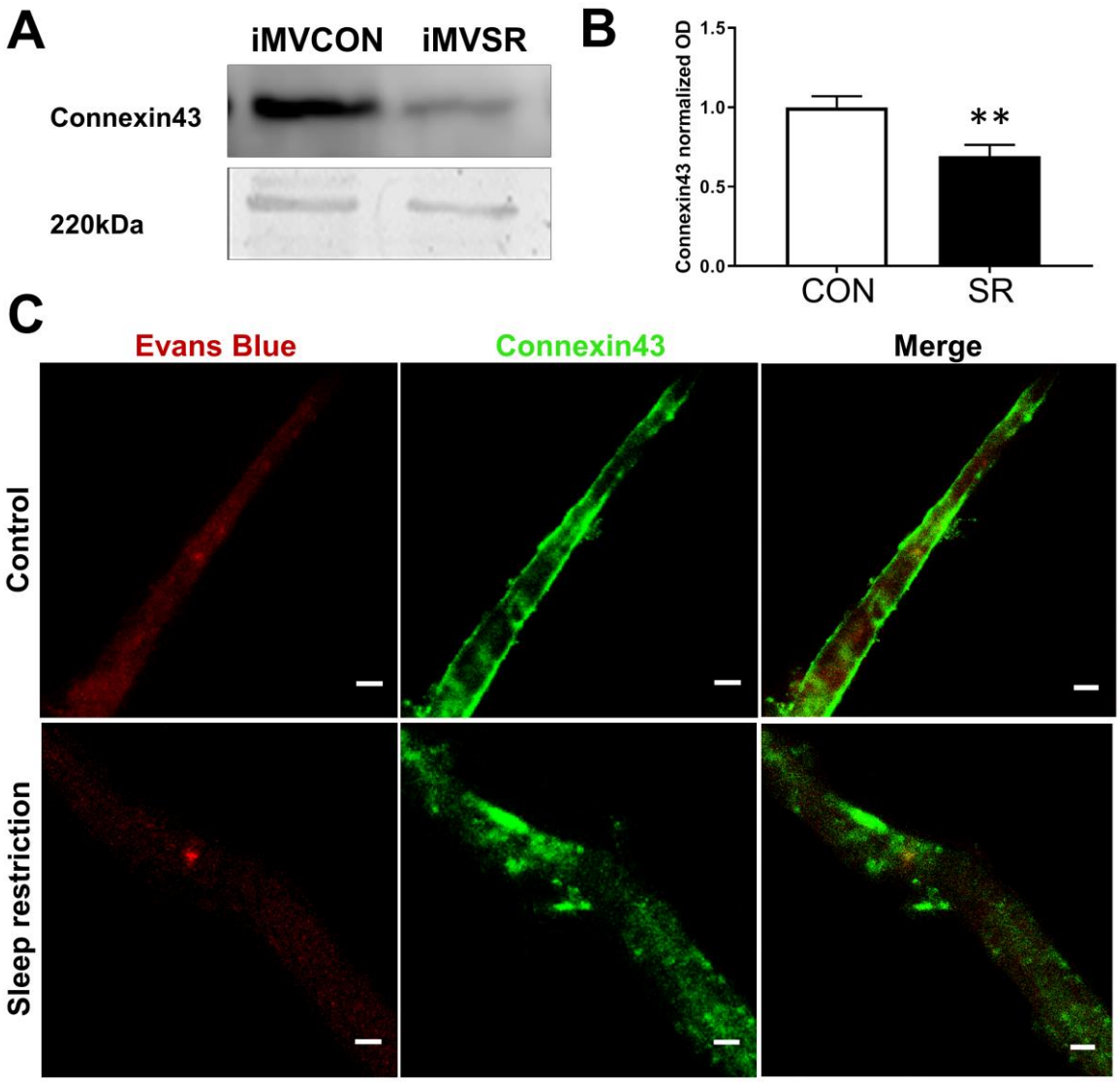


Figure 6. Chronic sleep restriction reduces connexin-43 expression in the cerebral cortex. A) Western blot of connexin-43 in isolated brain microvessels from the sleep restriction (iMVSR) and intact control (iMVCON) groups. B) The graph depicts the expression levels of normalized connexin 43 using a band of 220 kDa marked with Ponceau red. Samples were analyzed in duplicate in separate PVDF membranes. Mean ± standard error of the mean (SEM). **p<0.001 as compared to the control group. C) Confocal microscopy images of isolated brain microvessels from the cerebral cortex illustrate a diminished immunoreactivity of connexin-43 (green) in sleep-restricted rats (lower panel) in comparison with the group sleeping *ad libitum* (upper panel). Brain microvessels were marked with Evans blue (red). Scale bar: 20 µm.

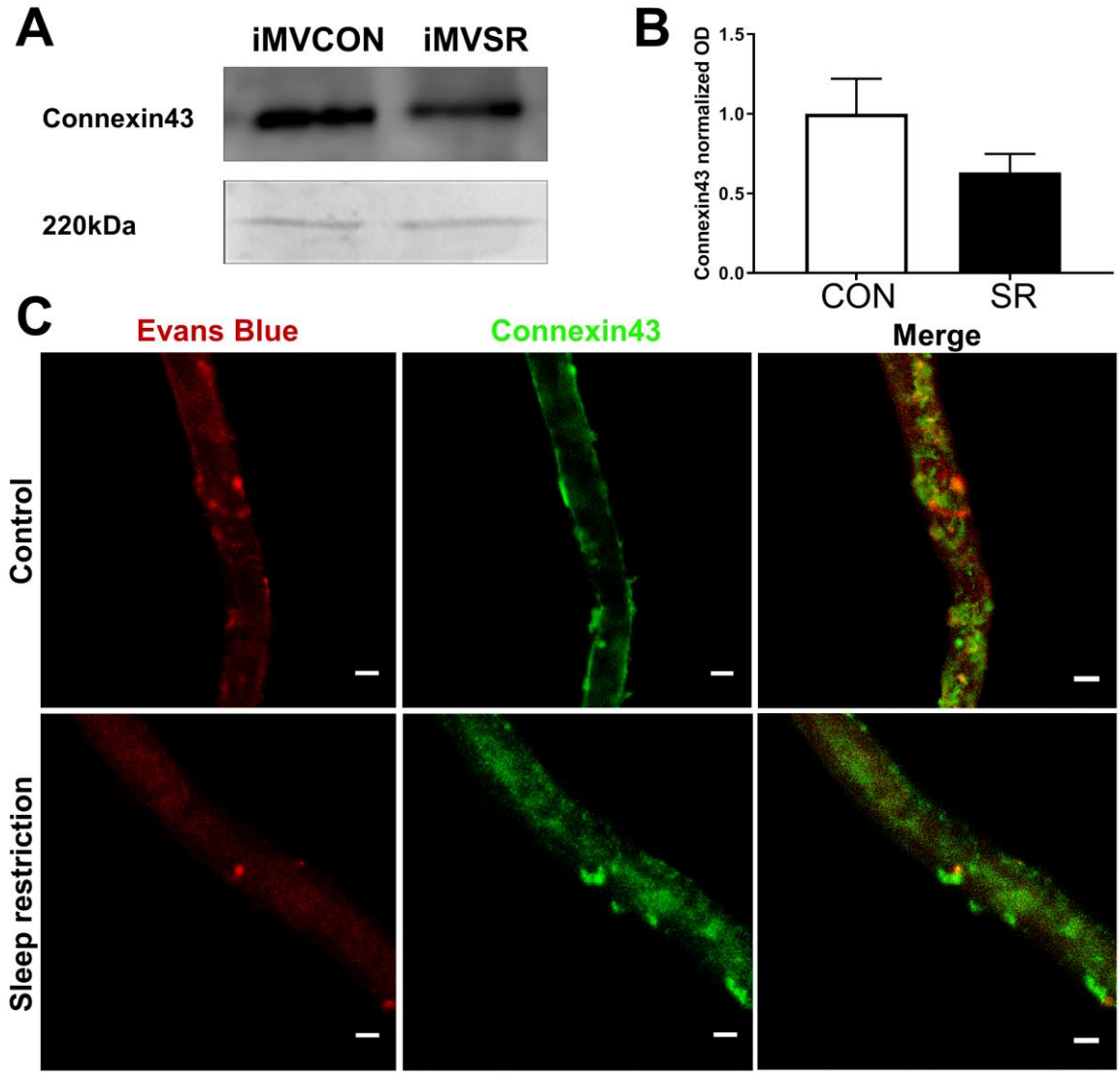


Figure 7. Chronic sleep restriction reduces connexin-43 expression in the hippocampus. A) Western blot showing a trend toward reduction in connexin-43 expression in isolated brain microvessels from sleep-restricted rats (iMVSR) in comparison with the rats sleeping *ad libitum* (iMVCON). B) Graph depicts the expression levels of normalized connexin-43 using a band of 220 kDa marked with Ponceau red. Samples were analyzed in duplicate in separate PVDF membranes. Mean ± standard error of the mean (SEM). $p>0.05$ as compared to the control group. C) Immunofluorescent staining for connexin-43 in isolated brain microvessels from the control (upper panel) and sleep restriction (lower panel) groups. Note the linear expression of connexin-43 along the capillary in the isolated microvessels from the intact control group and the loss of linearity in the isolated microvessel from the sleep-restricted group. Brain microvessels were marked with Evans blue (red). Scale bar: 20 µm.

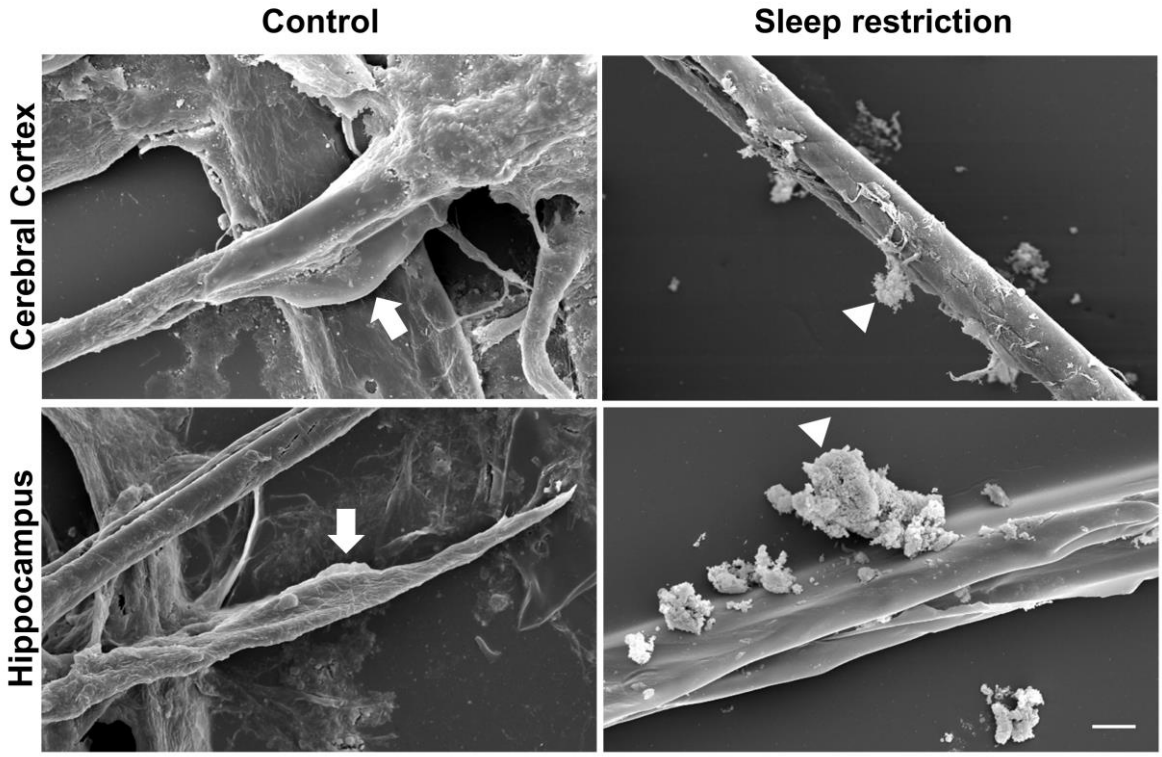


Figure 8. Sleep loss promotes brain pericyte detachment from the capillary wall in the cerebral cortex and hippocampus. Scanning electron micrographs of isolated brain microvessels showing pericyte detachment from the capillary wall in the cerebral cortex and hippocampus. Images also show a phenotype change in pericytes after 10 days of sleep restriction (left panels), in comparison with the group sleeping *ad libitum* (right panels) in cerebral cortex (upper panel) and hippocampus (lower panel). Note the pericyte abutting brain microvessels in the control group (arrow) and the globular-shaped cells located in the vicinity of brain microvessels (arrowhead) in the sleep-restricted group. Scale bar: 10 µm.

Disruption of endothelial cell-pericyte interactions associates with decreased expression of tight junction proteins in isolated microvessels

Chronic sleep restriction modified the expression levels of the proteins that form the tight junctions in isolated brain capillaries (Figure 9). As shown in figure 9, isolated microvessels from the cerebral cortex of the chronic sleep restriction group presented a decrease in the expression of claudin-5 (Figures 9A & 9B, t=3.369, p=0.0276), but not of occludin (Figure 9A, p=0.1973) as compared to the intact control sleeping *ad libitum*. On the contrary, in the hippocampus, only the expression of occludin decreased in the isolated brain blood vessels of the sleep-restricted group (Figure 9C & 9D, t=1,918, p=0.0305) as compared to the intact controls, while claudin-5 did not change (Figure 9C, p=0.9275)**.**

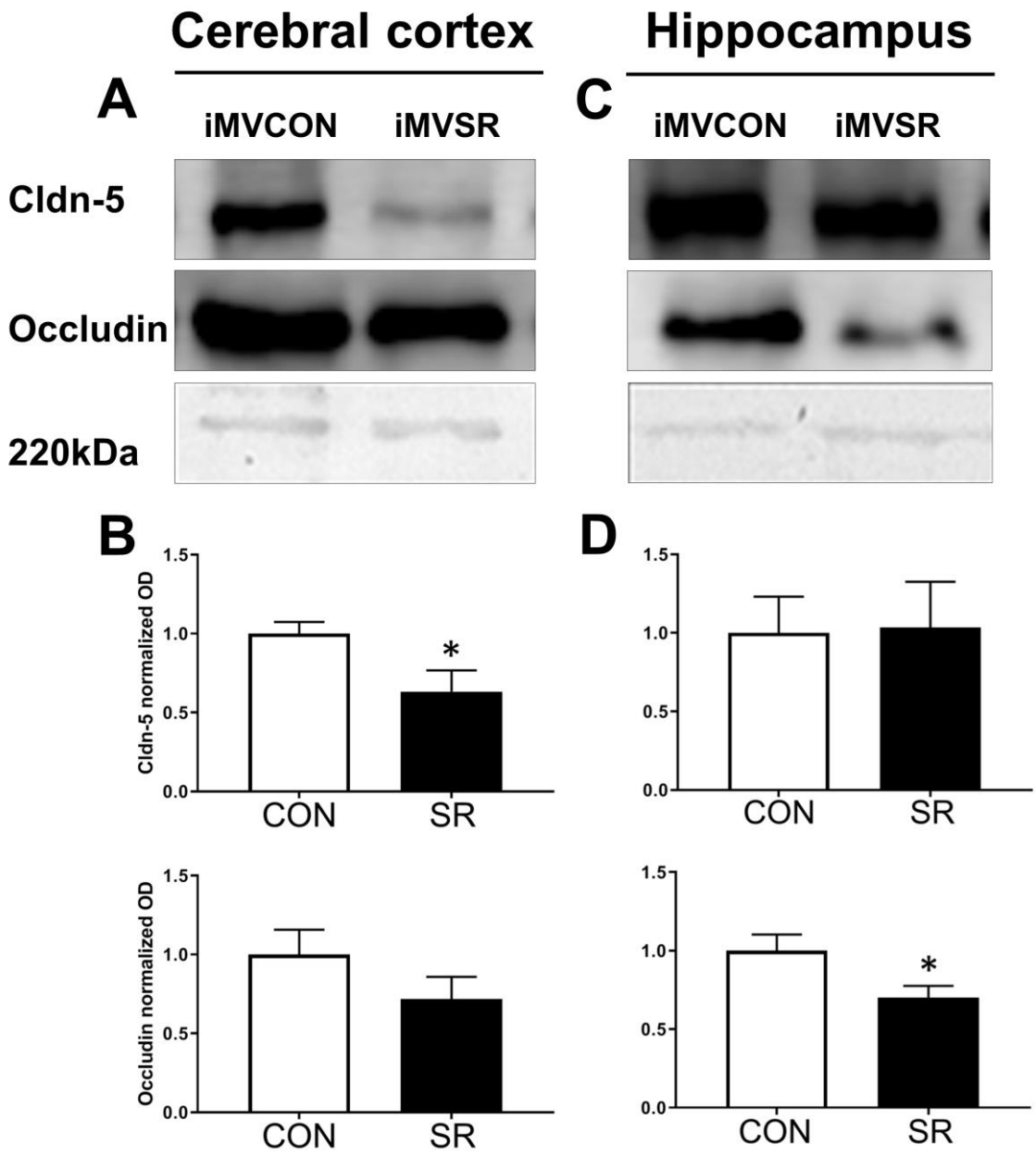


Figure 9. The disruption of normal pericyte-endothelial cell interaction deranges tight junctions in chronic sleep-restricted males. A) Representative western blot of the intact control sleeping *ad libitum* (iMVCON, n=6) and sleep-restricted (iMVSR, n=6) groups. Normalized levels of claudin-5 and occludin expression in the isolated blood vessels of the cerebral cortex (B) and hippocampus (D). The band of 220kDa marked with Ponceau red was used for normalization. Samples were analyzed in duplicate in separate PVDF membranes. Mean ± standard error. *$p<0.01$ as compared to the control group.

Disruption of endothelial cell-pericyte interactions associates with increased blood-brain barrier permeability

To corroborate that the decrease in the expression levels of the tight junction and gap junction proteins modified the function of the BBB, *in vivo* permeability assays were performed using Evans blue, sodium-fluorescein and rhodamine 123 tracers. As shown in figure 10, the cerebral cortex (t=3.096, p=0.0044) and hippocampus (t=2.157, p=0.0007) from sleep-restricted rats presented an increase in BBB permeability to Na-fluorescein compared to the control group. The cerebral cortex (t=4.375, p=0.0060) and hippocampus (t=4.626, p=0.0049) of sleep-restricted rats also presented an increased BBB permeability to Evans blue compared to the control group. A substrate of P-glycoprotein was also used to test the effects of the disruption of pericyte-endothelial cell interactions on a selective carrier system at the BBB. Figure 10 shows that in the sleep restriction group, the cerebral cortex (t=1.949, p=0.0436) and hippocampus (t=2.184, p=0.0303) presented an increase in BBB permeability to rhodamine 123 *versus* the intact control group, depicting dysfunction in P-gp carrier system under sleep restriction conditions.

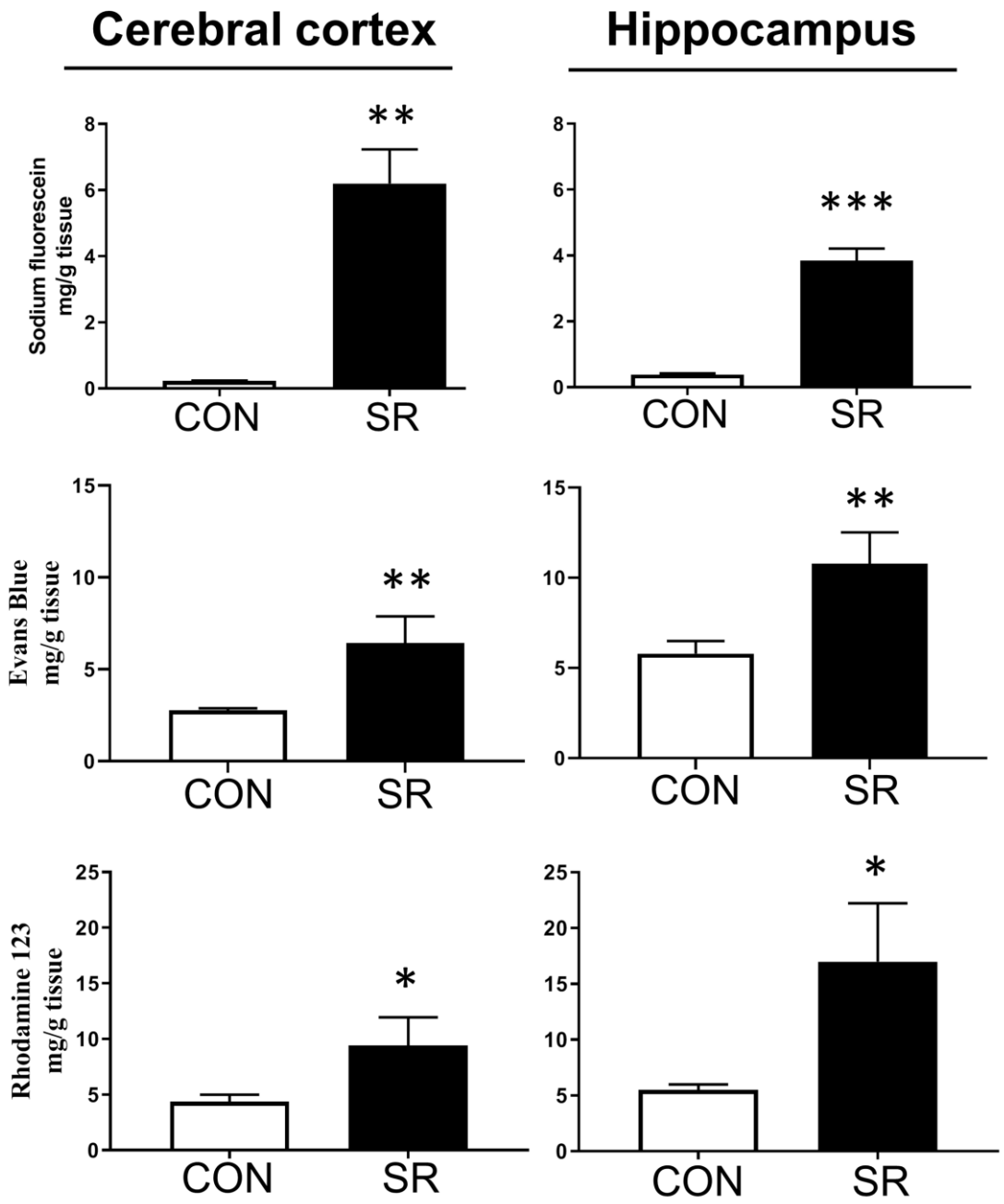


Figure 10. Chronic sleep restriction increases the permeability of the BBB. The graphs depict the BBB permeability to Na-fluorescein, Evans blue and rhodamine 123 in the cerebral cortex and hippocampus of the intact control (CON, n=5) and sleep-restricted (SR, n=5) groups. Quantification was performed in duplicate for each sample. Mean ± standard error. *p<0.05, **p<0.01, ***p<0.001 as compared to the intact control.

Chronic sleep restriction does not alter the metabolic activity of isolated brain microvessels

To test whether sleep loss modified the metabolic activity in isolated brain microvessels GLUT-1 protein expression and an oximetry assay in isolated mitochondria were performed. As shown in figure 11, the expression of GLUT-1 transporter in isolated brain microvessels was similar in both groups, intact control and sleep-restricted in the cerebral cortex and hippocampus ($p>0.05$). The respiratory control was calculated from oxygen consumption experiments, using succinate as a substrate to determine the mitochondrial function from isolated brain microvessels. As shown in Table 1, in the cerebral cortex, the respiratory control was similar in sleep-restricted and intact control groups.

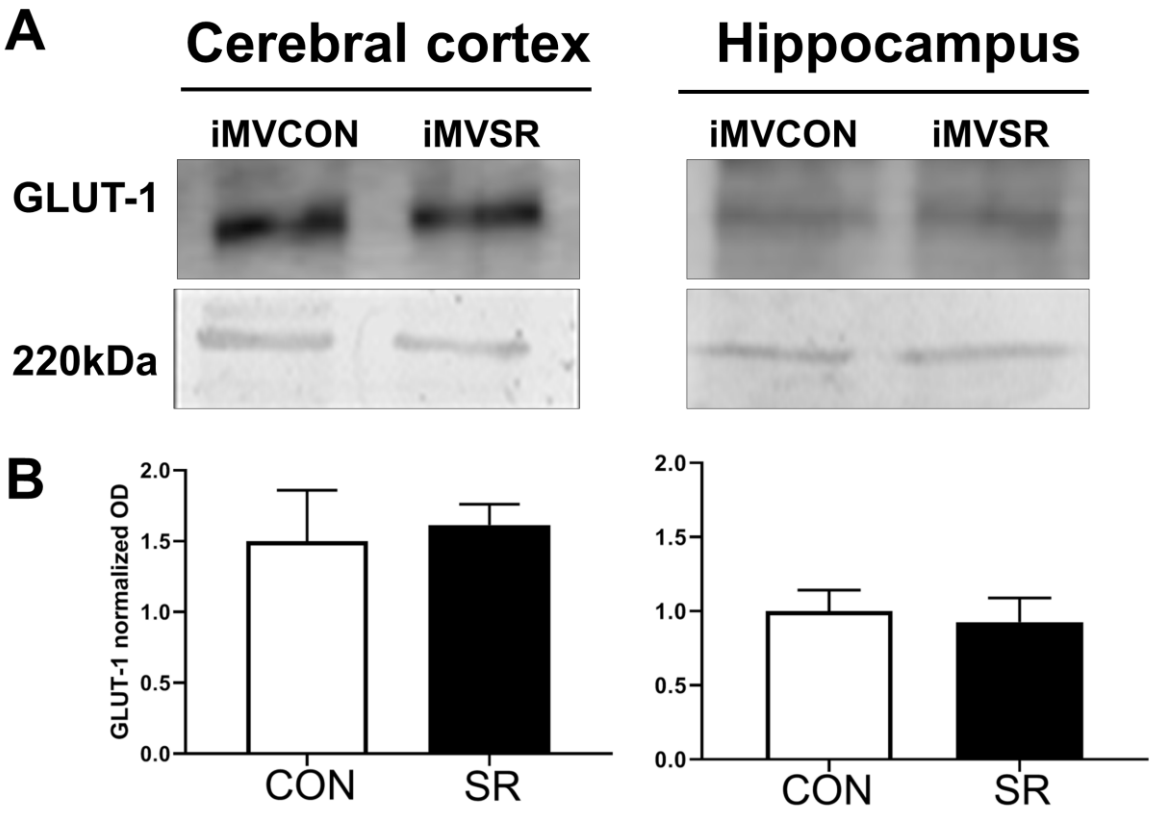


Figure 11. Chronic sleep restriction does not alter metabolic activity in isolated brain microvessels. A) Western blot images illustrate the expression of GLUT-1 in isolated brain microvessels from sleep-restricted (iMVSR) and intact control (iMVCON) groups. B) The graphs depict normalized expression levels of GLUT-1 using a band of 220 kDa marked with Ponceau red. Samples were evaluated in duplicate in separate PVDF membranes. Mean ± standard error of the mean (SEM). $p>0.05$ as compared to the control group.

Table 1. Oximetry in isolated mitochondria from the cerebral cortex brain microvessels.

Oxygen consumption	CON	SR
State 3	198 ng atom O_2/min	222 atom O_2/min
State 4	156 ng atom O_2/min	180 atom O_2/min
Respiratory Control	1.3	1.23

Table 1 shows the mitochondria respiratory control of isolated brain microvessels from the cerebral cortex in the sleep-restricted (SR) and intact control (CON) groups. Mean ± standard error of the mean. Data were obtained from pooled samples of n=10 rats *per* each group.

Sleep restriction promotes a low-grade inflammatory status in isolated cerebral blood vessels

Sleep loss triggered an inflammatory status in the cerebral cortex and hippocampus. Figure 12 shows that sleep restriction increased the expression of MMP-9 in isolated brain microvessels from cerebral cortex (Figure 12A & 12B, t= 2.752, p=0.0131) and hippocampus (Figure 12C & 12D, t=2.452, p=0.0341) in comparison with the intact control group.

The effect of chronic sleep restriction on the interactions between brain endothelial cells and pericytes may depend on pro-inflammatory mediators. Representative western blot images of isolated brain microvessels (Figure 13) show that in cerebral cortex, sleep loss increased the expression of phosphorylated NFκB

(Figure 13A & 13B, t= 6.758, p= 0.0212) in comparison with the control group. On the contrary, in the hippocampus, the expression of p-NFκB (Figure 13C & 13D, p=0.6073) did not show differences compared to the group sleeping *ad libitum*.

Figure 13 shows that the effects of chronic sleep restriction may also depend on local effects of adenosine, as was previously suggested by Hurtado-Alvarado et al. (2016). The isolated brain endothelial cells from the cerebral cortex of sleep-restricted rats did not present differences in the expression of the A_{2A} receptor (p=0.3941) nor of CD73 compared to the intact control group (Figure 13A & 13B, p=0.7151).

In the case of the hippocampus, the sleep-restricted group presented an increase in the expression of the A_{2A} adenosine receptor (Figure 13C & 13D, t= 2.483, p=0.0324) in comparison with the control group. However, the expression levels of the CD73 adenosine-synthesizing ectonucleotidase remained similar to intact controls (p=0.6341).

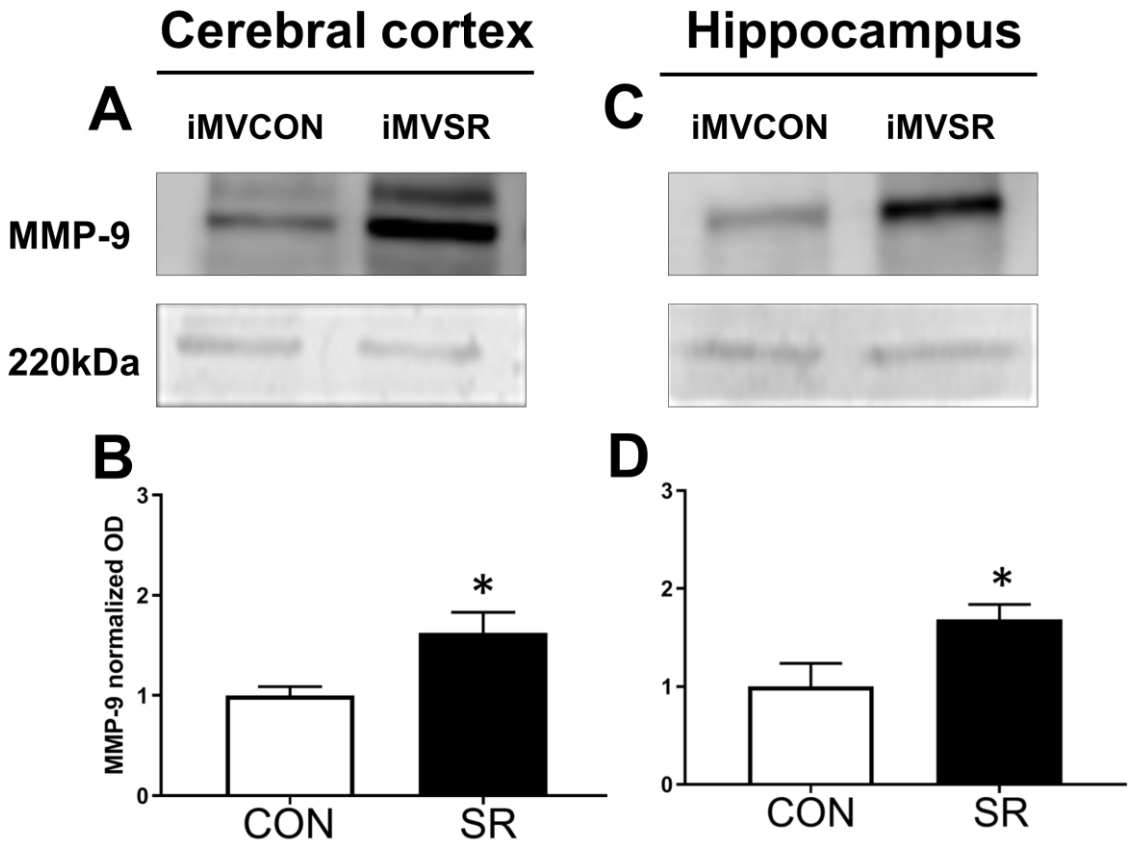


Figure 12. Chronic sleep restriction increases MMP-9 expression in isolated brain microvessels from the cerebral cortex and hippocampus. Representative western blot images of isolated brain microvessels show a significant increase in the expression of MMP-9 in the cerebral cortex (A) and hippocampus (C) after 10 days of sleep restriction (iMVSR) in comparison with the control group (iMVCON). B, D) The graphs depict the expression levels of MMP-9 using a band of 220 kDa marked with Ponceau red for normalization. Samples were analyzed in duplicate in separate PVDF membranes. Mean ± standard error of the mean (SEM). *$p<0.01$ as compared to the control group.

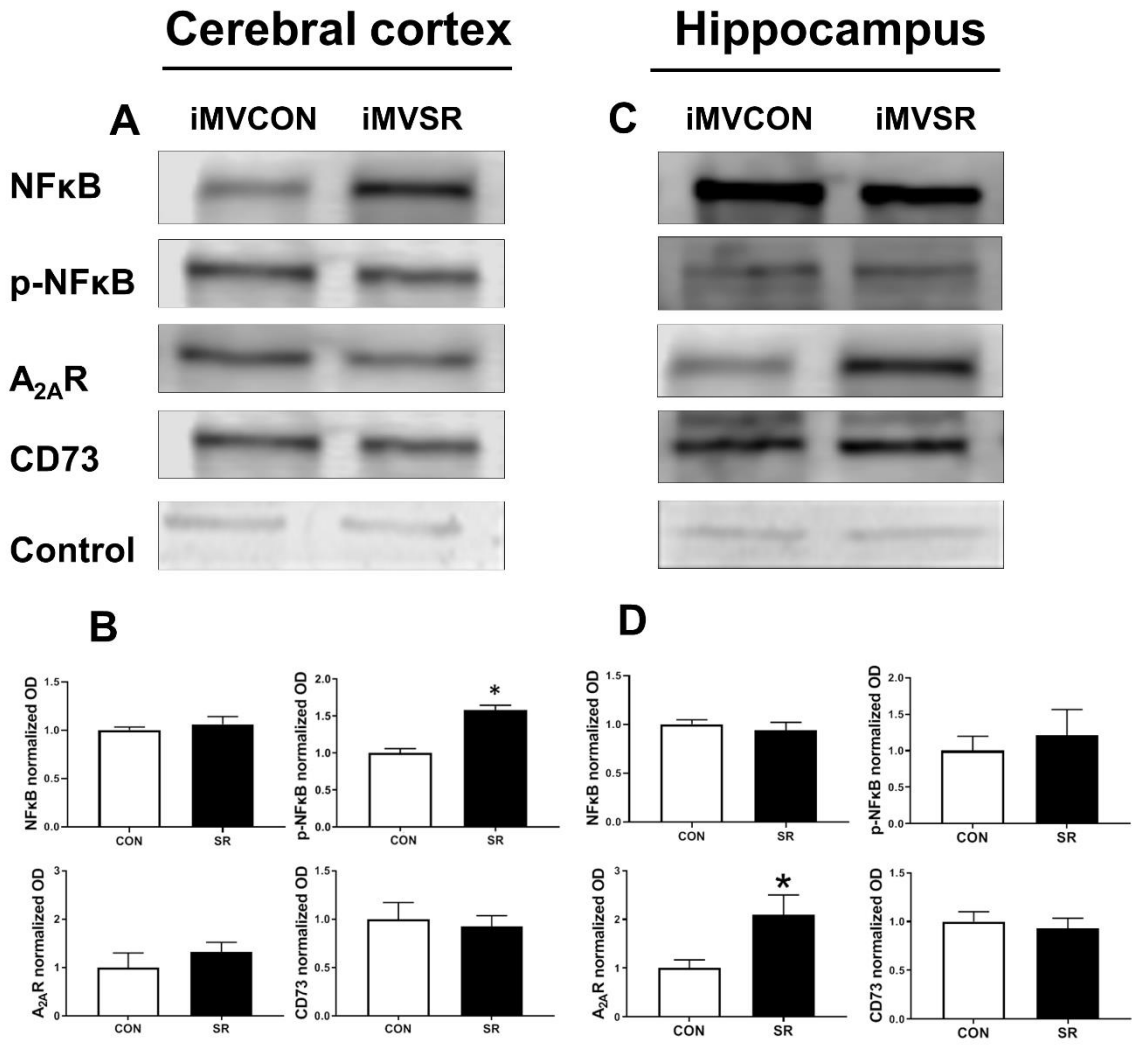


Figure 13. Chronic sleep restriction modulates the expression of pro-inflammatory mediators in isolated microvessels from the cerebral cortex and hippocampus. Representative western blot of isolated microvessels from control (iMVCON) and sleep-restricted (iMVSR) groups (A, C). The graphs depict the expression levels of NFκB, p-NFκB, A_{2A} adenosine receptor and CD73 in isolated microvessels from the cerebral cortex (C) and hippocampus (D) of intact controls and sleep-restricted groups. The expression levels of the proteins of interests were normalized using a band of 220 KDa marked with red Ponceau. Quantification was performed in duplicate for each sample. Mean ± standard error of the mean (SEM). *p<0.01 as compared to the control group.

DISCUSSION

Given the importance of pericytes-brain endothelium interaction to maintain the BBB properties and function (Bell et al., 2010; Rustenhoven et al., 2017), this study aimed to evaluate the changes in pericyte-brain endothelial cell interactions during sleep loss and characterized the effects of the pericyte detachment on BBB physiology in sleep-restricted rats. In this study, we found that sleep loss leads to pericyte detachment from the capillary wall, as indicated by the significant reduction in the expression of PDGFRβ and connexin-43. In addition, we found that isolated brain microvessels from the sleep-restricted rats are characterized by the presence of globular shaped cells abutted to the capillary wall, again indicating pericyte detachment from the capillary wall. The reduced pericyte coverage of brain microvessels was concurrent with an increased BBB permeability to low- and high- molecular weight tracers as well as a defect in the P-glycoprotein efflux pump, as depicted by the increased BBB permeability to rhodamine-123. The disruption of pericyte-endothelial cell interactions in sleep-restricted rats was also accompanied by a reduced expression of the interendothelial tight junction proteins claudin-5 and occludin. Here we found that the loss of cell-cell contacts may be a consequence of an up-regulation of the proteolytic enzyme MMP-9, as well as the activation of NFkB and the adenosine signaling pathways, as indicated by the increased expression of phosphorylated NFkB and the A_{2A} adenosine receptor.

Platelet-derived growth factor receptor β (PDGFRβ) is a marker of pericytes widely used to study pericyte coverage of brain microvessels (Winkler et al., 2010). The importance of PDGFRβ signaling in the maintenance of the barrier phenotype in

brain endothelial cells has been clearly established in the $Pdgfb^{ret/ret}$ mutant mice, a model where the pericytes are partially dissociated from brain endothelial cells as a result of an impaired PDGFB/PDGFRβ signaling pathway. Tallquist et al. (2003) generated in mice PDGFRβ allelic series with mutations at known phosphorylated tyrosine residues, including $Pdgfrb^{F7/-}$ and $Pdgfrb^{F7/F7}$, which present 40% and 50% of pericyte coverage respectively. The results demonstrate that the number of pericytes correlates with PDGFRβ activity, in other words, not only the presence or absence of pericytes are important but also pericyte:endothelial cells ratios determine the strength of the BBB (Tallquist et al., 2003; Daneman et al., 2010).

$Pdgfb^{ret/ret}$ mice presented endothelial cell membrane protrusions into the vessel lumen, increased number of cytoplasmic vesicles and increased BBB permeability to circulating tracers such as Evans blue and biotin via transcytosis. Also, with electron microscopy and using streptavidin-horseradish peroxidase, it was observed that the tracer was endocytosed into endothelial cell vesicles (Daneman et al., 2010). A similar change in phenotype in brain endothelial cells has been observed in chronically sleep-restricted rats; Gómez-González et al. (2013) found increased BBB permeability to Evans blue along with an increased number of cytoplasmic vesicles in hippocampal endothelial cells in 10-day sleep-restricted rats. In addition, Hurtado-Alvarado et al. (2017) found higher frequency of cytoplasmic protrusions into the vessel lumen in hippocampal capillaries from sleep-restricted rats concurrent with increased BBB permeability to Na-fluorescein. Hurtado-Alvarado et al. (2017) also present ultrastructural evidence of potential pericyte detachment from the capillary wall after chronic sleep loss. Our data are

in accordance with the previous findings indicating that PDGFRβ reduction induces pericyte loss and compromises the BBB integrity. Similarly, PDGFRβ loss and pericyte detachment have been observed in neurological diseases such Alzheimer's disease, cerebral ischemia, epilepsy, and stroke (Sagare et al., 2013; Duz et al., 2007; Arango-Lievano et al., 2018; Vates et al., 2010).

We also found that sleep loss reduced the expression of connexin-43 in isolated brain microvessels from the cerebral cortex, connexin-43 is the major connexin expressed in the microvasculature of the central nervous system, indicating that connexin-43 is a major regulator of vascular permeability (Figueroa and Duling, 2009). In physiological conditions, the expression of connexins leads to the interchange of cytoplasm components that allow the proper intercellular signaling pathway and electric coupling among adjacent cells; on the contrary, a lack of connexins reduce the intercellular communication between the cells, furthermore, the deficiency of connexin-43 results in a dysfunctional channel. According to Ivanova et al. (2017), the decreased expression of connexin-43 in mice, evidenced by injecting meclofenamic acid (MFA, 25μM), a selective gap-junction blocker, may compromise pericyte-to-pericyte signaling and functionality at the early stages of diabetic retinopathy. Connexin-43 reduced expression diminished propagation of vasomotor response and impaired mechanism for vascular diameter regulation; because of that pericyte processes encircling the capillaries were sparse, and their density was reduced in the diabetic retina (Ivanova et al., 2017; Figueroa and Duling et al., 2009). In addition, knockdown of Cx43 expression in $Cx43^{+/-}$ mice induces pericyte loss, apoptosis of vascular cells and reduces the expression of the tight junction proteins occludin and ZO-1 and increase the permeability to

FITC-dextran in rat retinal endothelial cells during diabetic retinopathy (Bobbie et al., 2010; Tien et al., 2013). Xie et al. (2013) demonstrated in glomerular mesangial cells of male Sprague-Dawley rats that downregulation of connexin-43 induced by high glucose administration, enhanced nuclear translocation pf NFκB, by the contrary, the restoration of connexin-43 expression by transfection with GFP-connexin-43 attenuated NFκB nuclear translocation, which suggests that decreased connexin-43 expression modulate NFκB activation. This observation correlates with our results because we found that sleep restriction reduced the expression of connexin-43 and increased the expression of p-NFκB in isolated brain microvessels from the cerebral cortex; however, this mechanism remains to be explored. Our data suggest that connexin-43 plays an important role in regulating pericyte-endothelial cell interactions, and its downregulation contributes to BBB defects in sleep-restricted rats.

The loss of interactions between pericyte and brain endothelial cells increased the permeability to rhodamine 123, indicating a change in P-glycoprotein functionality in isolated brain microvessels from cerebral cortex and hippocampus in the sleep-restricted rats. P-glycoprotein (P-gp) is the main efflux transporter expressed in the brain endothelial cells and its dysfunction increases the accumulation of toxic molecules in the brain parenchyma (Kamiie et al., 2008). Vandenhaute and co-workers (2011) showed that the function of P-gp was enhanced in co-cultures of endothelial cells with pericytes, indicating that pericytes influence barrier properties directly. On the contrary, the upregulation of pro-inflammatory cytokines led to P-gp transport dysfunction (Ho and Piquette-Miller 2006). Rats injected with *Klebsiella pneumoniae* endotoxin presented a reduced biliary, renal, and tubular

secretory clearances of rhodamine 123, indicating an impairment of P-glycoprotein transport; and the effects were related with increased levels of TNF-α in plasma (Ando et al., 2001). In addition, rats treated with LPS, which increases the expression of TNF-α, IL-β and IL-6, presented downregulation of P-gp function as depicted by the accumulation of 3H-digoxin in the brain; again, indicating that inflammation modulates P-glycoprotein function (Goralski et al., 2003; Uchida et al., 2014). In a study *in vitro*, brain endothelial cells were exposed to IL-1β, IL-6, or TNF-α, resulting in the inhibition of P-gp function, which indicates that pro-inflammatory cytokines modulate the function of P-gp (Poller et al., 2010).

Here we found a BBB hyperpermeability to small- and large-molecular weight tracers in two brain regions, the cerebral cortex and the hippocampus. Similar changes in BBB permeability were found after 6-day sleep loss in C57/BL6 mice (He et al., 2014); and after 10-day sleep restriction in both Wistar rats (Gómez-González et al., 2013; Hurtado-Alvarado et al., 2017) and C57/BL6 mice (Hurtado-Alvarado et al., 2018). In pathological conditions as Alzheimer's disease, approximately 60% of pericyte coverage of brain capillaries is reduced in cortex and hippocampus from post-mortem human tissue, which promotes an extravascular deposition of IgG, fibrin as well as an increase in the Aβ deposition into both regions. This observation suggests a strong correlation between the reduction of pericytes and the BBB leakage (Sengillo et al., 2013).

We observed that the permeability changes occurred simultaneously with decreased tight junction protein expression in the sleep-restricted rats, which is consistent with previous reports (Hurtado-Alvarado et al., 2017, 2018). Indeed, increased BBB permeability secondary to pericyte loss associated with a

downregulation of claudin-5, occludin and the scaffold protein ZO-1 (Armulik et al., 2010; Villaseñor et al., 2017). These results highlight the importance of intercellular interactions to maintain BBB properties.

The reduced expression of the tight junction proteins was related to an overexpression of matrix metalloproteinase 9 (MMP9) in isolated brain microvessels from the cerebral cortex and hippocampus. Under neuropathological conditions, such as cerebral ischemia, the pericyte detaches from the capillary wall and acquires proteolytic activity (Dave et al., 2018); indeed, previous evidence indicates that the pericyte becomes the primary cell releasing MMP-9 as compared with astrocytes, microglia, and neurons (Takata et al., 2011). Although we did not evaluate in our isolated brain microvessels which cellular type is producing and releasing MMP-9, we may propose that in sleep-restricted rats the detachment of pericytes from the capillary wall is accompanied by a change in their morphology that will drive the release of endopeptidases like MMP-9 contributing to the degradation of the basal lamina. Those effects, pericyte transformation and its acquisition of proteolytic activity, may be related to the low-grade inflammatory environment at the central nervous system, as previously observed in other animal models with inflammation, such as in Lipopolysaccharide (LPS)-treated mice (Nishioku et al., 2009).

Neuroinflammation is the cause of BBB dysfunction in many diseases, and one of the signaling pathways involved is the activation of the transcriptional factor NFκB. Interestingly, increased expression of phosphorylated NFκB was observed only in the cerebral cortex, which correlates with the decreased expression of the claudin-5 protein in our sleep-restricted rats. Chiu and Lai (2013) described in an

eosinophilic meningoencephalitis model increased levels of p-NFκB that correlated with decreased claudin-5 expression and increased BBB permeability to Evans blue. In the case of the hippocampus, there was an increased expression of the A_{2A} adenosine receptor in our sleep-restricted rats. This result is consistent with our previously reported experiments in which sleep restriction increased A_{2A} receptor expression in the hippocampus and basal nuclei but not in the cerebral cortex (Hurtado-Alvarado et al., 2016). *In vitro* studies have reported that A_{2A} stimulation decreases TEER values and increases BBB permeability to chemotherapeutic drugs and T cells (Kim and Bynoe, 2015). Moreover, Kim et al. (2012) reported that sleep deprivation increased mRNA levels of A_{2A} adenosine receptor in the hippocampus but not in other brain regions. Together, these findings suggest that A_{2A} adenosine receptor performs a regulatory role in BBB physiology, which may depend on its distribution and expression in each brain region.

Although sleep loss altered pericyte-brain endothelial cell interactions, there was no change in metabolic activity of brain endothelial cells, as depicted by the absence of changes in the expression of GLUT-1 and the normal mitochondrial respiratory control. Our results differ from previously published studies, He et al. (2014) shown a downregulation of GLUT-1 expression in 6-day sleep-restricted C57/Bl6 mice and Petit et al. (2010) found in mice, increased levels of GLUT-1 mRNA after 6h of sleep deprivation. These discrepancies may be due to the different techniques used to induce sleep loss, as well as the time the animals were in this condition. The absence of change in mitochondrial respiratory control between sleep-restricted and intact control groups indicates a preserved functional

coupling of respiration and ATP synthesis in the mitochondria of isolated brain microvessels despite sleep loss. Our findings suggest that 10 days of sleep loss does not compromise the metabolic activity of the vascular cells.

According to our findings in this study, we propose that during sleep loss the pericyte changes its phenotype and may acquire proteolytic functions by releasing proteolytic enzymes, as result of an inflammatory status mediated by the activation of the transcription factor NFκB in the cerebral cortex and A_{2A} adenosine receptor upregulation in the hippocampus. The different signaling pathways used by both regions suggest heterogeneity of pathways that regulate pericyte-endothelial cell interactions during pathological conditions. Our study provides the possibility of considering different mechanisms that can be used as targets to treat neurodegenerative diseases such as Alzheimer's disease, ischemia, epilepsy and stroke, where the interactions between pericytes/endothelial cells and inflammation seem to have a key role in the pathology development.

CONCLUSION

Chronic sleep restriction decreases the interactions between brain endothelial cells and pericytes by decreasing the expression of the proteins that maintain the junctions between them, such as PDGFR-β and connexin 43. The loss of pericyte-endothelial cell interactions concurs with a disruption of the barrier properties by reducing tight junction proteins between endothelial cells and increasing BBB permeability to exogenous tracers in sleep-restricted rats. The impairment in the cellular interactions between pericytes and endothelial cells seem to depend on a low-grade inflammatory status after chronic sleep restriction.

REFERENCES

Abbott, N. J. (2002). Astrocyte-endothelial interactions and blood-brain barrier permeability*. *Journal of Anatomy*, *200*(6), 629–638. https://doi.org/10.1046/j.1469-7580.2002.00064.x

Alvarez, J. I., Cayrol, R., & Prat, A. (2011). Disruption of central nervous system barriers in multiple sclerosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1812*(2), 252–264. https://doi.org/10.1016/j.bbadis.2010.06.017

Alvarez, J. I., Katayama, T., & Prat, A. (2013). Glial influence on the blood brain barrier: Glial Influence on the Blood Brain Barrier. *Glia*, *61*(12), 1939–1958. https://doi.org/10.1002/glia.22575

Argaw, A. T., Gurfein, B. T., Zhang, Y., Zameer, A., & John, G. R. (2009). VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. *Proceedings of the National Academy of Sciences*, *106*(6), 1977–1982. https://doi.org/10.1073/pnas.0808698106

Armulik, A., Abramsson, A., & Betsholtz, C. (2005). Endothelial/Pericyte Interactions. *Circulation Research*, *97*(6), 512–523. https://doi.org/10.1161/01.RES.0000182903.16652.d7

Armulik, A., Genové, G., & Betsholtz, C. (2011). Pericytes: Developmental, Physiological, and Pathological Perspectives, Problems, and Promises. *Developmental Cell*, *21*(2), 193–215. https://doi.org/10.1016/j.devcel.2011.07.001

Armulik, A., Genové, G., Mäe, M., Nisancioglu, M. H., Wallgard, E., Niaudet, C., … Betsholtz, C. (2010). Pericytes regulate the blood–brain barrier. *Nature*, *468*(7323), 557–561. https://doi.org/10.1038/nature09522

Barakat, S., Demeule, M., Pilorget, A., Régina, A., Gingras, D., Baggetto, L. G., & Béliveau, R. (2006). Modulation of p-glycoprotein function by caveolin-1 phosphorylation: Phosphocaveolin-1 inhibits p-gp function. *Journal of Neurochemistry*, *101*(1), 1–8. https://doi.org/10.1111/j.1471-4159.2006.04410.x

Barakat, S., Turcotte, S., Demeule, M., Lachambre, M.-P., Régina, A., Baggetto, L.

G., & Béliveau, R. (2008). Regulation of brain endothelial cells migration and angiogenesis by P-glycoprotein/caveolin-1 interaction. *Biochemical and Biophysical Research Communications*, *372*(3), 440–446. https://doi.org/10.1016/j.bbrc.2008.05.012

Basheer, R., Strecker, R. E., Thakkar, M. M., & McCarley, R. W. (2004). Adenosine and sleep–wake regulation. *Progress in Neurobiology*, *73*(6), 379–396. https://doi.org/10.1016/j.pneurobio.2004.06.004

Bautch, V. L., & James, J. M. (2009). Neurovascular development: The beginning of a beautiful friendship. *Cell Adhesion & Migration*, *3*(2), 199–204. https://doi.org/10.4161/cam.3.2.8397

Bell, R. D., Winkler, E. A., Sagare, A. P., Singh, I., LaRue, B., Deane, R., & Zlokovic, B. V. (2010). Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. *Neuron*, *68*(3), 409–427. https://doi.org/10.1016/j.neuron.2010.09.043

Benakis, C., Garcia-Bonilla, L., Iadecola, C., & Anrather, J. (2015). The role of microglia and myeloid immune cells in acute cerebral ischemia. *Frontiers in Cellular Neuroscience*, *8*. https://doi.org/10.3389/fncel.2014.00461

Bruzzone, R., White, T. W., & Paul, D. L. (1996). Connections with Connexins: The Molecular Basis of Direct Intercellular Signaling. *European Journal of Biochemistry*, *238*(1), 1–27. https://doi.org/10.1111/j.1432-1033.1996.0001q.x

Chandra, A., Li, W. A., Stone, C. R., Geng, X., & Ding, Y. (2017). The cerebral circulation and cerebrovascular disease I: Anatomy. *Brain Circulation*, *3*(2), 45–56. https://doi.org/10.4103/bc.bc_10_17

Chen, F., Ohashi, N., Li, W., Eckman, C., & Nguyen, J. H. (2009). Disruptions of occludin and claudin-5 in brain endothelial cells in vitro and in brains of mice with acute liver failure. *Hepatology*, *50*(6), 1914–1923. https://doi.org/10.1002/hep.23203

De Bock, M., Vandenbroucke, R. E., Decrock, E., Culot, M., Cecchelli, R., & Leybaert, L. (2014). A new angle on blood-CNS interfaces: a role for connexins? *FEBS Letters*, *588*(8), 1259–1270. https://doi.org/10.1016/j.febslet.2014.02.060

de Wit, C., & Griffith, T. M. (2010). Connexins and gap junctions in the EDHF phenomenon and conducted vasomotor responses. *Pflügers Archiv - European Journal of Physiology*, *459*(6), 897–914. https://doi.org/10.1007/s00424-010-0830-4

Demeule, M., Jodoin, J., Gingras, D., & Béliveau, R. (2000). P-glycoprotein is localized in caveolae in resistant cells and in brain capillaries. *FEBS Letters*, *466*(2–3), 219–224. https://doi.org/10.1016/S0014-5793(00)01087-5

Dobrowolski, R., & Willecke, K. (2009). Connexin-Caused Genetic Diseases and Corresponding Mouse Models. *Antioxidants & Redox Signaling*, *11*(2), 283–296. https://doi.org/10.1089/ars.2008.2128

Drewes, L. R. (2012). Making Connexons in the Neurovascular Unit. *Journal of Cerebral Blood Flow & Metabolism*, *32*(8), 1455–1456. https://doi.org/10.1038/jcbfm.2012.44

Dwyer, D. S., Vannucci, S. J., & Simpson, I. A. (2002). Expression, regulation, and functional role of glucose transporters (GLUTs) in brain. *International Review of Neurobiology*, *51*, 159–188.

ElAli, A., Thériault, P., & Rivest, S. (2014). The Role of Pericytes in Neurovascular Unit Remodeling in Brain Disorders. *International Journal of Molecular Sciences*, *15*(4), 6453–6474. https://doi.org/10.3390/ijms15046453

Ehrlich P. (1885). Das sauerstufbudurfnis des organismus, in Eine Farbenanalytishe studie. Hirschwald, Berlin.

Etchevers, H. C., Vincent, C., Le Douarin, N. M., & Couly, G. F. (2001). The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. *Development (Cambridge, England)*, *128*(7), 1059–1068.

Feng, J., Wang, Q., Wu, Q., & Sun, X. (2013). Obstructive sleep apnea and endothelial progenitor cells. *Patient Preference and Adherence*, 1077. https://doi.org/10.2147/PPA.S51562

Figueroa, X. F., & Duling, B. R. (2009). Gap Junctions in the Control of Vascular Function. *Antioxidants & Redox Signaling*, *11*(2), 251–266. https://doi.org/10.1089/ars.2008.2117

Goldmann EE. (1913). Vitalfarbung am zentralnervesystem. Abhandl Konigl preuss Akad Wiss 1:1-60

Gómez-González, B., Domínguez-Salazar, E., Hurtado-Alvarado, G., Esqueda-Leon, E., Santana-Miranda, R., Rojas-Zamorano, J. A., & Velázquez-Moctezuma, J. (2012). Role of sleep in the regulation of the immune system and the pituitary hormones: Sleep, hormones, and cytokines. *Annals of the New York Academy of Sciences*, *1261*(1), 97–106. https://doi.org/10.1111/j.1749-6632.2012.06616.x

Gómez-González, B., Hurtado-Alvarado, G., Esqueda-León, E., Santana-Miranda, R., Rojas-Zamorano, J. Á., & Velázquez-Moctezuma, J. (2013). REM sleep loss and recovery regulates blood-brain barrier function. *Current Neurovascular Research*, *10*(3), 197–207.

Guo, Z., Zhu, J., Zhao, L., Luo, Q., & Jin, X. (2010). Expression and clinical significance of multidrug resistance proteins in brain tumors. *Journal of Experimental & Clinical Cancer Research*, *29*(1), 122. https://doi.org/10.1186/1756-9966-29-122

Guruswamy, R., & ElAli, A. (2017). Complex Roles of Microglial Cells in Ischemic Stroke Pathobiology: New Insights and Future Directions. *International Journal of Molecular Sciences*, *18*(3), 496. https://doi.org/10.3390/ijms18030496

Guzman-Marin, R., Suntsova, N., Bashir, T., Nienhuis, R., Szymusiak, R., & McGinty, D. (2008). Rapid Eye Movement Sleep Deprivation Contributes to Reduction of Neurogenesis in the Hippocampal Dentate Gyrus of the Adult Rat. *Sleep*, *31*(2), 167–175. https://doi.org/10.1093/sleep/31.2.167

Hamilton, N. B. (2010). Pericyte-mediated regulation of capillary diameter: A component of neurovascular coupling in health and disease. *Frontiers in Neuroenergetics*, *2*. https://doi.org/10.3389/fnene.2010.00005

Harder, D. R., Zhang, C., & Gebremedhin, D. (2002). Astrocytes Function in Matching Blood Flow to Metabolic Activity. *Physiology*, *17*(1), 27–31. https://doi.org/10.1152/physiologyonline.2002.17.1.27

Hawkins, B. T., & Davis, T. P. (2005). The Blood-Brain Barrier/Neurovascular Unit in Health and Disease. *Pharmacological Reviews*, *57*(2), 173–185.

https://doi.org/10.1124/pr.57.2.4

He, J., Hsuchou, H., He, Y., Kastin, A. J., Wang, Y., & Pan, W. (2014). Sleep Restriction Impairs Blood-Brain Barrier Function. *Journal of Neuroscience*, *34*(44), 14697–14706. https://doi.org/10.1523/JNEUROSCI.2111-14.2014

Hellström, M., Kalén, M., Lindahl, P., Abramsson, A., & Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development (Cambridge, England)*, *126*(14), 3047–3055.

Hermann, D. M., & ElAli, A. (2012). The Abluminal Endothelial Membrane in Neurovascular Remodeling in Health and Disease. *Science Signaling*, *5*(236), re4–re4. https://doi.org/10.1126/scisignal.2002886

Hill, R. A., Tong, L., Yuan, P., Murikinati, S., Gupta, S., & Grutzendler, J. (2015). Regional Blood Flow in the Normal and Ischemic Brain Is Controlled by Arteriolar Smooth Muscle Cell Contractility and Not by Capillary Pericytes. *Neuron*, *87*(1), 95–110. https://doi.org/10.1016/j.neuron.2015.06.001

Ho, E., & Piquette-Miller, M. (2006). Regulation of Multidrug Resistance by Pro-Inflammatory Cytokines. *Current Cancer Drug Targets*, *6*(4), 295–311. https://doi.org/10.2174/156800906777441753

Hurtado-Alvarado, G., Becerril-Villanueva, E., Contis-Montes de Oca, A., Domínguez-Salazar, E., Salinas-Jazmín, N., Pérez-Tapia, S. M., … Gómez-González, B. (2018). The yin/yang of inflammatory status: Blood-brain barrier regulation during sleep. *Brain, Behavior, and Immunity*, *69*, 154–166. https://doi.org/10.1016/j.bbi.2017.11.009

Hurtado-Alvarado, G., Velázquez-Moctezuma, J., & Gómez-González, B. (2017). Chronic sleep restriction disrupts interendothelial junctions in the hippocampus and increases blood-brain barrier permeability: CHRONIC SLEEP RESTRICTION DISRUPTS INTERENDOTHELIAL JUNCTIONS. *Journal of Microscopy*, *268*(1), 28–38. https://doi.org/10.1111/jmi.12583

Hurtado-Alvarado, Gabriela, Domínguez-Salazar, E., Velázquez-Moctezuma, J., & Gómez-González, B. (2016). A2A Adenosine Receptor Antagonism Reverts the Blood-Brain Barrier Dysfunction Induced by Sleep Restriction. *PLOS ONE*, *11*(11), e0167236. https://doi.org/10.1371/journal.pone.0167236

Itoh, Y., Takaoka, R., Ohira, M., Abe, T., Tanahashi, N., & Suzuki, N. (2006). Reactive oxygen species generated by mitochondrial injury in human brain microvessel endothelial cells. *Clinical Hemorheology and Microcirculation*, *34*(1–2), 163–168.

Ivanova, E., Kovacs-Oller, T., & Sagdullaev, B. T. (2017). Vascular Pericyte Impairment and Connexin43 Gap Junction Deficit Contribute to Vasomotor Decline in Diabetic Retinopathy. *The Journal of Neuroscience*, *37*(32), 7580–7594. https://doi.org/10.1523/JNEUROSCI.0187-17.2017

Jelic, S., & Le Jemtel, T. H. (2008). Inflammation, Oxidative Stress, and the Vascular Endothelium in Obstructive Sleep Apnea. *Trends in Cardiovascular Medicine*, *18*(7), 253–260. https://doi.org/10.1016/j.tcm.2008.11.008

Jodoin, J., Demeule, M., Fenart, L., Cecchelli, R., Farmer, S., Linton, K. J., … Béliveau, R. (2004). P-glycoprotein in blood-brain barrier endothelial cells: Interaction and oligomerization with caveolins: P-gp in caveolae of the BBB. *Journal of Neurochemistry*, *87*(4), 1010–1023. https://doi.org/10.1046/j.1471-4159.2003.02081.x

Kamiie, J., Ohtsuki, S., Iwase, R., Ohmine, K., Katsukura, Y., Yanai, K., … Terasaki, T. (2008). Quantitative Atlas of Membrane Transporter Proteins: Development and Application of a Highly Sensitive Simultaneous LC/MS/MS Method Combined with Novel In-silico Peptide Selection Criteria. *Pharmaceutical Research*, *25*(6), 1469–1483. https://doi.org/10.1007/s11095-008-9532-4

Keaney, J., & Campbell, M. (2015). The dynamic blood-brain barrier. *FEBS Journal*, *282*(21), 4067–4079. https://doi.org/10.1111/febs.13412

Kim, D.-G., & Bynoe, M. S. (2015). A2A Adenosine Receptor Regulates the Human Blood-Brain Barrier Permeability. *Molecular Neurobiology*, *52*(1), 664–678. https://doi.org/10.1007/s12035-014-8879-2

Korn, J., Christ, B., & Kurz, H. (2002). Neuroectodermal origin of brain pericytes and vascular smooth muscle cells. *The Journal of Comparative Neurology*, *442*(1), 78–88. https://doi.org/10.1002/cne.1423

Kumar, N. M., & Gilula, N. B. (1996). The Gap Junction Communication Channel.

Cell, *84*(3), 381–388. https://doi.org/10.1016/S0092-8674(00)81282-9

Laird, D. W. (2006). Life cycle of connexins in health and disease. *Biochemical Journal*, *394*(3), 527–543. https://doi.org/10.1042/BJ20051922

Lemmon, M. A. (2008). Membrane recognition by phospholipid-binding domains. *Nature Reviews Molecular Cell Biology*, *9*(2), 99–111. https://doi.org/10.1038/nrm2328

Leveen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E., & Betsholtz, C. (1994). Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes & Development*, *8*(16), 1875–1887. https://doi.org/10.1101/gad.8.16.1875

Liebner, S. (2000). Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells. *European Journal of Cell Biology*, *79*(10), 707–717. https://doi.org/10.1078/0171-9335-00101

Lim, D. C., & Pack, A. I. (2014). Obstructive sleep apnea and cognitive impairment: Addressing the blood–brain barrier. *Sleep Medicine Reviews*, *18*(1), 35–48. https://doi.org/10.1016/j.smrv.2012.12.003

Lull, M. E., & Block, M. L. (2010). Microglial activation and chronic neurodegeneration. *Neurotherapeutics*, *7*(4), 354–365. https://doi.org/10.1016/j.nurt.2010.05.014

Mathiisen, T. M., Lehre, K. P., Danbolt, N. C., & Ottersen, O. P. (2010). The perivascular astroglial sheath provides a complete covering of the brain microvessels: An electron microscopic 3D reconstruction. *Glia*, *58*(9), 1094–1103. https://doi.org/10.1002/glia.20990

Matsugi, T., Chen, Q., & Anderson, D. R. (1997). Adenosine-induced relaxation of cultured bovine retinal pericytes. *Investigative Ophthalmology & Visual Science*, *38*(13), 2695–2701.

McConnell, H. L., Kersch, C. N., Woltjer, R. L., & Neuwelt, E. A. (2017). The Translational Significance of the Neurovascular Unit. *Journal of Biological Chemistry*, *292*(3), 762–770. https://doi.org/10.1074/jbc.R116.760215

Mills, J. H., Alabanza, L., Weksler, B. B., Couraud, P.-O., Romero, I. A., & Bynoe, M. S. (2011). Human brain endothelial cells are responsive to adenosine

receptor activation. *Purinergic Signalling*, *7*(2), 265–273. https://doi.org/10.1007/s11302-011-9222-2

Mohammed, H. S., Aboul Ezz, H. S., Khadrawy, Y. A., & Noor, N. A. (2011). Neurochemical and electrophysiological changes induced by paradoxical sleep deprivation in rats. *Behavioural Brain Research*, *225*(1), 39–46. https://doi.org/10.1016/j.bbr.2011.06.018

Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R. K., & McDonald, D. M. (2002). Abnormalities in Pericytes on Blood Vessels and Endothelial Sprouts in Tumors. *The American Journal of Pathology*, *160*(3), 985–1000. https://doi.org/10.1016/S0002-9440(10)64920-6

Moriyama, Y., Takagi, N., Itokawa, C., & Tanonaka, K. (2013). Injection of neural progenitor cells attenuates decrease in level of connexin 43 in brain capillaries after cerebral ischemia. *Neuroscience Letters*, *543*, 152–156. https://doi.org/10.1016/j.neulet.2013.03.053

Morris, A. W. J., Sharp, M. M., Albargothy, N. J., Fernandes, R., Hawkes, C. A., Verma, A., … Carare, R. O. (2016). Vascular basement membranes as pathways for the passage of fluid into and out of the brain. *Acta Neuropathologica*, *131*(5), 725–736. https://doi.org/10.1007/s00401-016-1555-z

Mullington, J. M., Simpson, N. S., Meier-Ewert, H. K., & Haack, M. (2010). Sleep loss and inflammation. *Best Practice & Research Clinical Endocrinology & Metabolism*, *24*(5), 775–784. https://doi.org/10.1016/j.beem.2010.08.014

Nadarajah, B., Jones, A. M., Evans, W. H., & Parnavelas, J. G. (1997). Differential expression of connexins during neocortical development and neuronal circuit formation. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *17*(9), 3096–3111.

Nagasawa, K., Chiba, H., Fujita, H., Kojima, T., Saito, T., Endo, T., & Sawada, N. (2006). Possible involvement of gap junctions in the barrier function of tight junctions of brain and lung endothelial cells. *Journal of Cellular Physiology*, *208*(1), 123–132. https://doi.org/10.1002/jcp.20647

Nakagawa, S., Deli, M. A., Kawaguchi, H., Shimizudani, T., Shimono, T., Kittel, Á., … Niwa, M. (2009). A new blood–brain barrier model using primary rat brain

endothelial cells, pericytes and astrocytes. *Neurochemistry International*, *54*(3–4), 253–263. https://doi.org/10.1016/j.neuint.2008.12.002

Nehls, V. (1991). Heterogeneity of microvascular pericytes for smooth muscle type alpha- actin. *The Journal of Cell Biology*, *113*(1), 147–154. https://doi.org/10.1083/jcb.113.1.147

Novati, A., Hulshof, H. J., Koolhaas, J. M., Lucassen, P. J., & Meerlo, P. (2011). Chronic sleep restriction causes a decrease in hippocampal volume in adolescent rats, which is not explained by changes in glucocorticoid levels or neurogenesis. *Neuroscience*, *190*, 145–155. https://doi.org/10.1016/j.neuroscience.2011.06.027

Pardridge, W M. (1988). Recent Advances in Blood-Brain Barrier Transport. *Annual Review of Pharmacology and Toxicology*, *28*(1), 25–39. https://doi.org/10.1146/annurev.pa.28.040188.000325

Pardridge, William M. (2002). Drug and Gene Delivery to the Brain. *Neuron*, *36*(4), 555–558. https://doi.org/10.1016/S0896-6273(02)01054-1

Parton, R. G., & del Pozo, M. A. (2013). Caveolae as plasma membrane sensors, protectors and organizers. *Nature Reviews Molecular Cell Biology*, *14*(2), 98–112. https://doi.org/10.1038/nrm3512

Peppiatt, C. M., Howarth, C., Mobbs, P., & Attwell, D. (2006). Bidirectional control of CNS capillary diameter by pericytes. *Nature*, *443*(7112), 700–704. https://doi.org/10.1038/nature05193

Perea, G., Navarrete, M., & Araque, A. (2009). Tripartite synapses: Astrocytes process and control synaptic information. *Trends in Neurosciences*, *32*(8), 421–431. https://doi.org/10.1016/j.tins.2009.05.001

Poller, B., Drewe, J., Krähenbühl, S., Huwyler, J., & Gutmann, H. (2010). Regulation of BCRP (ABCG2) and P-Glycoprotein (ABCB1) by Cytokines in a Model of the Human Blood–Brain Barrier. *Cellular and Molecular Neurobiology*, *30*(1), 63–70. https://doi.org/10.1007/s10571-009-9431-1

Porkka-Heiskanen, T., Zitting, K.-M., & Wigren, H.-K. (2013). Sleep, its regulation and possible mechanisms of sleep disturbances. *Acta Physiologica*, *208*(4), 311–328. https://doi.org/10.1111/apha.12134

Rasch, B., & Born, J. (2013). About Sleep's Role in Memory. *Physiological*

Reviews, *93*(2), 681–766. https://doi.org/10.1152/physrev.00032.2012

Rechtschaffen, A., Bergmann, B. M., Everson, C. A., Kushida, C. A., & Carol, A. E. (1989). Sleep Deprivation in the Rat: I. Conceptual Issues. *Sleep*, *12*(1), 1–4. https://doi.org/10.1093/sleep/12.1.1

Reyahi, A., Nik, A. M., Ghiami, M., Gritli-Linde, A., Pontén, F., Johansson, B. R., & Carlsson, P. (2015). Foxf2 Is Required for Brain Pericyte Differentiation and Development and Maintenance of the Blood-Brain Barrier. *Developmental Cell*, *34*(1), 19–32. https://doi.org/10.1016/j.devcel.2015.05.008

Rouget C. (1873). Memoire sur le developpement, la structures et les proprieties des capillaires sanguins et lymphatiques. Archs Physiol Norm Pathol, 5: 603-633.

Roux, F., & Couraud, P.-O. (2005). Rat brain endothelial cell lines for the study of blood-brain barrier permeability and transport functions. *Cellular and Molecular Neurobiology*, *25*(1), 41–58.

Rustenhoven, J., Jansson, D., Smyth, L. C., & Dragunow, M. (2017). Brain Pericytes As Mediators of Neuroinflammation. *Trends in Pharmacological Sciences*, *38*(3), 291–304. https://doi.org/10.1016/j.tips.2016.12.001

Saeed, A. A., Genové, G., Li, T., Lütjohann, D., Olin, M., Mast, N., … Björkhem, I. (2014). Effects of a Disrupted Blood-Brain Barrier on Cholesterol Homeostasis in the Brain. *Journal of Biological Chemistry*, *289*(34), 23712–23722. https://doi.org/10.1074/jbc.M114.556159

Saper, C. B., Fuller, P. M., Pedersen, N. P., Lu, J., & Scammell, T. E. (2010). Sleep State Switching. *Neuron*, *68*(6), 1023–1042. https://doi.org/10.1016/j.neuron.2010.11.032

Schinkel, null. (1999). P-Glycoprotein, a gatekeeper in the blood-brain barrier. *Advanced Drug Delivery Reviews*, *36*(2–3), 179–194.

Schuster, A., Oishi, H., Bény, J. L., Stergiopulos, N., & Meister, J. J. (2001). Simultaneous arterial calcium dynamics and diameter measurements: Application to myoendothelial communication. *American Journal of Physiology. Heart and Circulatory Physiology*, *280*(3), H1088-1096. https://doi.org/10.1152/ajpheart.2001.280.3.H1088

Sengillo, J. D., Winkler, E. A., Walker, C. T., Sullivan, J. S., Johnson, M., &

Zlokovic, B. V. (2013). Deficiency in Mural Vascular Cells Coincides with Blood-Brain Barrier Disruption in Alzheimer's Disease: Pericytes in Alzheimer's Disease. *Brain Pathology*, *23*(3), 303–310. https://doi.org/10.1111/bpa.12004

Shepro, D., & Morel, N. M. (1993). Pericyte physiology. *The FASEB Journal*, *7*(11), 1031–1038. https://doi.org/10.1096/fasebj.7.11.8370472

Smith, M. W., & Gumbleton, M. (2006). Endocytosis at the blood–brain barrier: From basic understanding to drug delivery strategies. *Journal of Drug Targeting*, *14*(4), 191–214. https://doi.org/10.1080/10611860600650086

Smyth, L. C. D., Rustenhoven, J., Scotter, E. L., Schweder, P., Faull, R. L. M., Park, T. I. H., & Dragunow, M. (2018). Markers for human brain pericytes and smooth muscle cells. *Journal of Chemical Neuroanatomy*, *92*, 48–60. https://doi.org/10.1016/j.jchemneu.2018.06.001

Staunton, H. (2005). Mammalian sleep. *Naturwissenschaften*, *92*(5), 203–220. https://doi.org/10.1007/s00114-005-0618-0

Sweeney, M. D., Sagare, A. P., & Zlokovic, B. V. (2018). Blood–brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nature Reviews Neurology*, *14*(3), 133–150. https://doi.org/10.1038/nrneurol.2017.188

Tallquist, M. D., French, W. J., & Soriano, P. (2003). Additive Effects of PDGF Receptor β Signaling Pathways in Vascular Smooth Muscle Cell Development. *PLoS Biology*, *1*(2), e52. https://doi.org/10.1371/journal.pbio.0000052

Thomsen, M. S., Routhe, L. J., & Moos, T. (2017). The vascular basement membrane in the healthy and pathological brain. *Journal of Cerebral Blood Flow & Metabolism*, *37*(10), 3300–3317. https://doi.org/10.1177/0271678X17722436

Tien, T., Barrette, K. F., Chronopoulos, A., & Roy, S. (2013). Effects of High Glucose-Induced Cx43 Downregulation on Occludin and ZO-1 Expression and Tight Junction Barrier Function in Retinal Endothelial Cells. *Investigative Opthalmology & Visual Science*, *54*(10), 6518. https://doi.org/10.1167/iovs.13-11763

Török, O., Schreiner, B., Tsai, H.-C., Utz, S., Schaffenrath, J., Nassiri, S., … Keller, A. (2019). *Pericytes regulate vascular immune homeostasis in the CNS* [Preprint]. https://doi.org/10.1101/644120

Tornabene, E., & Brodin, B. (2016). Stroke and Drug Delivery--In Vitro Models of the Ischemic Blood-Brain Barrier. *Journal of Pharmaceutical Sciences*, *105*(2), 398–405. https://doi.org/10.1016/j.xphs.2015.11.041

Urich, E., Lazic, S. E., Molnos, J., Wells, I., & Freskgård, P.-O. (2012). Transcriptional Profiling of Human Brain Endothelial Cells Reveals Key Properties Crucial for Predictive In Vitro Blood-Brain Barrier Models. *PLoS ONE*, *7*(5), e38149. https://doi.org/10.1371/journal.pone.0038149

Vandenhaute, E., Dehouck, L., Boucau, M.-C., Sevin, E., Uzbekov, R., Tardivel, M., … Dehouck, M.-P. (2011). Modelling the Neurovascular Unit and the Blood-Brain Barrier with the Unique Function of Pericytes. *Current Neurovascular Research*, *8*(4), 258–269. https://doi.org/10.2174/156720211798121016

Villaseñor, R., Kuennecke, B., Ozmen, L., Ammann, M., Kugler, C., Grüninger, F., … Collin, L. (2017). Region-specific permeability of the blood–brain barrier upon pericyte loss. *Journal of Cerebral Blood Flow & Metabolism*, *37*(12), 3683–3694. https://doi.org/10.1177/0271678X17697340

Vorbrodt, A. W., & Dobrogowska, D. H. (2003). Molecular anatomy of intercellular junctions in brain endothelial and epithelial barriers: Electron microscopist's view. *Brain Research Reviews*, *42*(3), 221–242. https://doi.org/10.1016/S0165-0173(03)00177-2

Vracko, R. (1970). CAPILLARY BASAL LAMINA THICKENING: Its Relationship to Endothelial Cell Death and Replacement. *The Journal of Cell Biology*, *47*(1), 281–285. https://doi.org/10.1083/jcb.47.1.281

Wehr, T. A. (1992). In short photoperiods, human sleep is biphasic. *Journal of Sleep Research*, *1*(2), 103–107. https://doi.org/10.1111/j.1365-2869.1992.tb00019.x

Winkler, E. A., Bell, R. D., & Zlokovic, B. V. (2011). Central nervous system pericytes in health and disease. *Nature Neuroscience*, *14*(11), 1398–1405. https://doi.org/10.1038/nn.2946

Wolburg, H., Neuhaus, J., Kniesel, U., Krauss, B., Schmid, E. M., Ocalan, M., … Risau, W. (1994). Modulation of tight junction structure in blood-brain barrier endothelial cells. Effects of tissue culture, second messengers and cocultured astrocytes. *Journal of Cell Science*, *107 (Pt 5)*, 1347–1357.

Xie, L., Kang, H., Xu, Q., Chen, M. J., Liao, Y., Thiyagarajan, M., … Nedergaard, M. (2013). Sleep Drives Metabolite Clearance from the Adult Brain. *Science*, *342*(6156), 373–377. https://doi.org/10.1126/science.1241224

Xie, X., Lan, T., Chang, X., Huang, K., Huang, J., Wang, S., … Huang, H. (2013). Connexin43 mediates NF-κB signalling activation induced by high glucose in GMCs: Involvement of c-Src. *Cell Communication and Signaling*, *11*(1), 38. https://doi.org/10.1186/1478-811X-11-38

Yao, Y., Hong, S., Zhou, H., Yuan, T., Zeng, R., & Liao, K. (2009). The differential protein and lipid compositions of noncaveolar lipid microdomains and caveolae. *Cell Research*, *19*(4), 497–506. https://doi.org/10.1038/cr.2009.27

Yurchenco, P. D. (2011). Basement Membranes: Cell Scaffoldings and Signaling Platforms. *Cold Spring Harbor Perspectives in Biology*, *3*(2), a004911–a004911. https://doi.org/10.1101/cshperspect.a004911

Zehendner, C. M., Wedler, H. E., & Luhmann, H. J. (2013). A Novel In Vitro Model to Study Pericytes in the Neurovascular Unit of the Developing Cortex. *PLoS ONE*, *8*(11), e81637. https://doi.org/10.1371/journal.pone.0081637

Zimmermann, K. W. (1923). Der feinere Bau der Blutcapillaren. *Zeitschrift für Anatomie und Entwicklungsgeschichte*, *68*(1), 29–109. https://doi.org/10.1007/BF02593544

Zlokovic, B. V. (2008). The Blood-Brain Barrier in Health and Chronic Neurodegenerative Disorders. *Neuron*, *57*(2), 178–201. https://doi.org/10.1016/j.neuron.2008.01.003

CERTIFICATE *of* ATTENDANCE

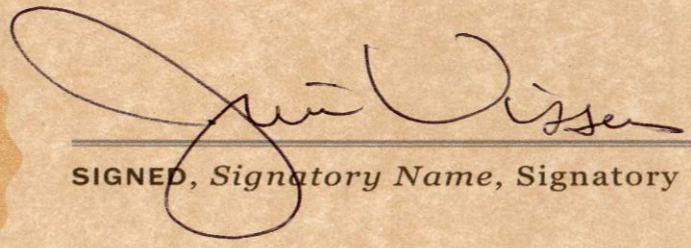
THIS ACKNOWLEDGES THAT

Fernanda Medina Flores

ATTENDED

CVB 2019
In Miami, FL USA

JUNE 25-28
2019



SIGNED, *Signatory Name*, Signatory Title

CEREBRAL VASCULAR BIOLOGY
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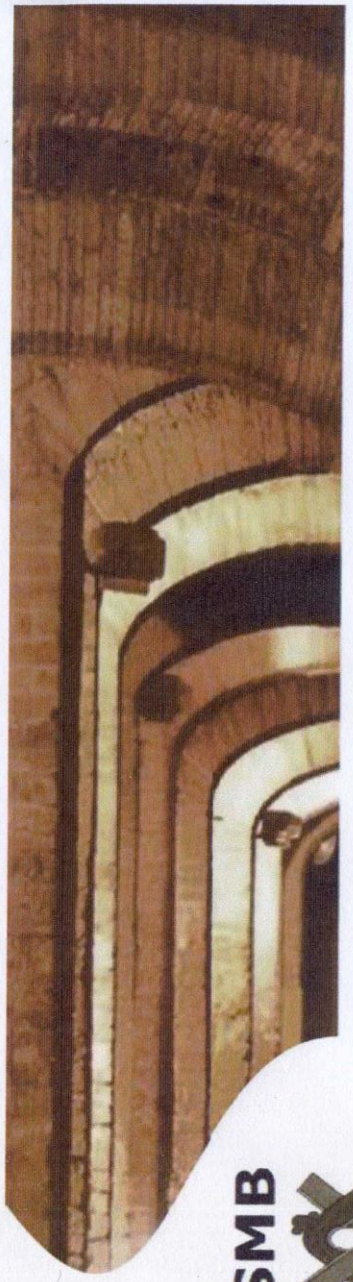

Sociedad Mexicana de Bioquímica
Neurobiología

Se otorga la presente

CONSTANCIA a:

María Fernanda Medina Flores

Quien asistió y presentó el trabajo:

Pericyte detachment during sleep loss disrupts blood-brain barrier

Por:
Fernanda Medina-Flores, Gabriela Hurtado-Alvarado, Maria A. Deli Beatriz Gómez-González

En la modalidad de presentación cartel durante el
III Congreso de Neurobiología, 22 -26 de septiembre 2019, Guanajuato, Gto.

Atentamente
Comité Organizador

Dra. Susana Castro Obregón

Dra. Aliesha González Arenas

Dr. Ignacio Camacho Arroyo

Dr. Octavio García

Dr. Hugo Merchant Nancy

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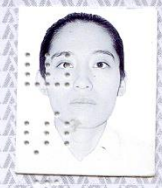
Casa abierta al tiempo
UNIVERSIDAD AUTÓNOMA METROPOLITANA

ACTA DE EXAMEN DE GRADO

No. 00205
Matrícula: 2173801719

Alterations in the junctions between brain endothelial cells and pericytes during chronic sleep restriction



Ma. Fernanda M.T.

MARIA FERNANDA MEDINA FLORES
ALUMNA

En la Ciudad de México, se presentaron a las 12:00 horas del dia 28 del mes de noviembre del año 2019 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DR. JAVIER VELAZQUEZ MOCTEZUMA
DRA. MINA KONIGSBERG FAINSTEIN
DRA. GABRIELA HURTADO ALVARADO
DRA. ANAHI CHAVARRIA KRAUSER

Bajo la Presidencia del primero y con carácter de Secretaria la última, se reunieron para proceder al Examen de Grado cuya denominación aparece al margen, para la obtención del grado de:

MAESTRA EN BIOLOGIA EXPERIMENTAL

DE: MARIA FERNANDA MEDINA FLORES

y de acuerdo con el artículo 78 fracción III del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

APROBAR

Acto continuo, el presidente del jurado comunicó a la interesada el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.

REVISÓ

MTRA. ROSALIA SERRANO DE LA PAZ
DIRECTORA DE SISTEMAS ESCOLARES

DIRECTORA DE LA DIVISIÓN DE CBS

DRA. SARA LUCIA CAMARGO RICALDE

PRESIDENTE

DR. JAVIER VELAZQUEZ MOCTEZUMA

VOCAL

DRA. MINA KONIGSBERG FAINSTEIN

VOCAL

DRA. GABRIELA HURTADO ALVARADO

SECRETARIA

DRA. ANAHI CHAVARRIA KRAUSER