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Participación de mediadores inflamatorios en la alteración estructural y funcional de la barrera hematoencefálica inducida por la restricción de sueño

T E S I S

Que para obtener el grado de

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PRESENTA

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RESUMEN

La pérdida de sueño es un problema de salud pública que altera funciones cognitivas, metabólicas e inmunológicas. La barrera hematoencefálica está alterada en animales restringidos de sueño y los mecanismos moleculares por los cuales la pérdida de sueño regula la integridad de la barrera hematoencefálica son desconocidos. La participación de mediadores inflamatorios que incrementan durante la pérdida de sueño (e.g. adenosina y citocinas pro-inflamatorias) puede ser crucial en la modulación de la barrera hematoencefálica. En el presente trabajo describimos el papel de la adenosina y del estatus inflamatorio sistémico en la regulación de la integridad de la barrera hematoencefálica durante la restricción de sueño. Encontramos que el uso de los antagonistas de receptores de adenosina revirtió el incremento de la permeabilidad de la barrera hematoencefálica a trazadores exógenos y restauró la expresión de las proteínas de las uniones ocluyentes de manera dependiente de la región cerebral. La restricción de sueño incrementó la expresión del receptor A_{2A} de adenosina, de Iba-1 y de GFAP lo cual fue revertido con la administración del antagonista de receptores de adenosina.

Adicionalmente evaluamos la participación del componente inflamatorio en la regulación de la permeabilidad de la barrera hematoencefálica ante la restricción de sueño en dos cepas de ratones con respuesta inflamatoria diferencial: ratones C57BL/6 (respuesta inmunológica predominantemente proinflamatoria) y ratones BALB/c (respuesta inmunológica predominantemente anti-inflamatoria). La restricción de sueño incrementó la permeabilidad de la barrera hematoencefálica en ratones C57BL/6 y el nivel sérico de citocinas proinflamatorias mientras que en los BALB/c no hubo cambios en los niveles de citocinas ni en la permeabilidad de la barrera hematoencefálica. La restricción de sueño modificó la expresión de las proteínas de la unión ocluyente e incrementó la expresión de un marcador cellular para microglia, MMP-9 y del receptor A2A de adenosina en ratones C57BL/6 mientras que los disminuyó en ratones BALB/c. Los datos indican que la participación directa (receptores A_{2A} de adenosina) o indirecta (perfil proinflamatorio sistémico) de mediadores inflamatorios está involucrada en la disfunción de la barrera hematoencefálica inducida por la restricción de sueño.

SUMMARY

Sleep is a common problem in the modern society and is highly related to cognitive impairment, metabolic and immune alterations. Blood-brain barrier is responsive to inflammatory mediators such as adenosine and proinflammatory cytokines increased during sleep loss. We investigated the role of adenosine and intrinsic inflammatory profile in the blood-brain barrier regulation in a model of sleep restriction. First, we reported that the blockade of adenosine receptors reverted the blood-brain barrier disruption and the overexpression of astroglia and microglia markers induced by sleep restriction. In addition, sleep restriction increased the A_{2A} adenosine receptor expression. In the other hand, we investigate the influence of systemic inflammatory status on blood-brain barrier dysfunction induced by sleep restriction using an two strains of mice with different immunological backgrounds: C57BL/6 mice that have a predominant proinflammatory response and BALB/c mice that have a predominant anti-inflammatory response. We found that serum levels of proinflammatory cytokines and blood-brain barrier permeability increased in sleep-restricted C57BL/6 but not in BALB/c mice. In the hippocampus of sleep-restricted C57BL/6 mice exhibited an increase in the protein expression of Iba-1, A_{2A} adenosine receptor and MMP-9 in comparison to control groups; meanwhile in the hippocampus of sleep-restricted BALB/c mice the expression of these markers was lesser than controls group. Together, these data suggest that the intrinsic inflammatory response is implicated in blood-brain barrier dysfunction induced by sleep restriction. In addition, events modulated by MMP-9, Iba-1 and A_{2A} Adenosine receptor could be crucial in blood- brain barrier dysfunction induced by sleep loss.

INDÍCE GENERAL

1. INTRODUCCIÓN

1.1 El sueño y la pérdida de sueño

El sueño es un proceso vital caracterizado por la disminución progresiva y reversible de la actividad motora y la percepción a estímulos ambientales (Siegel, 2009). En mamíferos, el sueño se divide en sueño sin movimientos oculares rápidos (no MOR) y sueño de movimientos oculares rápidos (MOR), cada estadio se pueden distinguir con facilidad empleando conjuntamente un electroencefalograma (EEG), un electrooculograma (EOG) y un electromiograma (EMG). Ambas fases de sueño alternan en el tiempo total que el individuo duerme teniendo ciclos de aproximadamente 4 a 1 de sueño no MOR sobre sueño MOR en humanos (Hsieh et al., 2008). Durante el sueño no MOR el EEG presenta un patrón de baja frecuencia y alta amplitud que correlaciona con una disminución en el tono muscular y no se presentan movimientos oculares. Por otro lado, durante el sueño MOR el patrón de actividad eléctrica cerebral es similar a la vigilia (ondas de baja amplitud y alta frecuencia) pero con la presencia de atonía muscular y de movimientos oculares rápidos (Siegel, 1990).

A pesar de que el sueño constituye la tercera parte del tiempo de vida en los mamíferos no se ha descrito una función especifica, aunque son aceptadas la reparación de tejidos, el mantenimiento del balance energético, modulación de procesos de termorregulación, consolidación de la memoria y plasticidad neuronal (Krueger et al., 1999). El sueño promueve una adecuada función cognitiva mientras que la privación o la disminución en el tiempo total de sueño tienen efectos contrarios, es decir, alteran procesos de atención, aprendizaje, toma de decisiones y diversos tipos de memoria (Kreutzmann et al., 2015). Una de las

regiones más importantes en estos procesos es el hipocampo, que es particularmente sensible a la pérdida de sueño. Los mecanismos propuestos por los cuales la pérdida de sueño altera las funciones cognitivas en modelos animales incluyen cambios moleculares en las células hipocámpicas como: incremento en los niveles de glutamato y adenosina, subsecuente activación de segundos mensajeros que podrían alterar la excitabilidad neuronal, la plasticidad sináptica, la neurogénesis y el volumen hipocámpico (Kreutzmann et al., 2015).

Diversas moléculas potencialmente tóxicas se acumulan en el espacio intersticial en el cerebro durante el periodo de vigilia y son eliminadas durante el sueño a través del sistema glinfático. La vía glinfática está constituida por cambios en el volumen de líquido cefalorraquídeo (CSF por sus siglas en inglés) en el espacio intersticial. Durante el sueño el espacio intersticial en la corteza de ratones es 60% mayor que en comparación con la vigilia resultando en un incremento en eficiencia para eliminar por la vía CSF moléculas relacionadas con procesos inflamatorios y neurodegenerativos (e.g. adenosina y el péptido amiloide β), esto sugiere que la función restaurativa del sueño podría estar asociada a cambios en el sistema glinfático promoviendo la eliminación y degradación de productos neurales acumulados durante la vigilia (Xie et al., 2013).

A nivel sistémico, la pérdida de sueño induce alteraciones metabólicas y desregulación del sistema endocrino e inmunológico (Banks and Dinges, 2007; Faraut et al., 2012). Por ejemplo, la pérdida de sueño incrementa los niveles de citocinas proinflamatorias que son potentes inductores de somnolencia (Vgontzas et al., 2004), altera la secreción normal de glucocorticoides que a su vez puede ser regulada por citocinas proinflamatorias generando una comunicación cruzada

entre el sistema nervioso central, el inmunológico y el endocrino (Papanicolaou et al., 1998). El sueño es entonces, un proceso fisiológico fundamental para mantener la integridad de la interacción neuro-inmuno-endocrina, que asegura el óptimo funcionamiento del organismo (Gómez-González et al., 2012a).

En el presente trabajo se considera el papel de mediadores inflamatorios en condiciones de pérdida de sueño que podrían regular la estructura y función de la interfaz entre la periferia y el cerebro: la barrera hematoencefálica.

1.2 La barrera hematoencefálica en condiciones fisiológicas

La barrera hematoencefálica es un sistema altamente regulado que mantiene la alostasis del sistema nervioso central: 1) al proveer al cerebro de nutrientes necesarios para su óptimo funcionamiento, 2) restringiendo el paso de moléculas potencialmente tóxicas y células circulantes en sangre, 3) degradando o eliminando productos de desecho de la actividad neural y 4) promoviendo la comunicación bidireccional entre el cerebro y la periferia (Zlokovic, 2008; Abbott et al., 2010;).

La barrera hematoencefálica es un complejo localizado en la microvasculatura encefálica que consta de células endoteliales que forman dichos capilares, astrocitos, pericitos, microglia y sus derivados como lámina basal y glicocalix. Los componentes de la barrera hematoencefálica y las neuronas liberan factores solubles que se encargan de regular la permeabilidad a moléculas circulantes así como eventos de vasoconstricción y vasodilatación (Saunders et al., 2014).

1.2.1 Células endoteliales

La microvasculatura cerebral está formada por una monocapa de células endoteliales con un grosor de 500nm y un diámetro menor de 10μm (Zlokovic,

2008; Abbot et al., 2010). Las células endoteliales de la microvasculatura cerebral tienen una tasa de transporte inespecífico muy baja, ausencia de fenestraciones, complejos de proteínas que forman uniones ocluyentes (también llamadas estrechas) y un bajo transporte mediado por vesículas pinocíticas (Ballabh et al., 2004). Las uniones ocluyentes limitan el transporte paracelular a prácticamente todas las moléculas hidrofílicas y están formadas por un sistema de proteínas que incluyen claudinas, ocludinas y proteínas *Zonula occludens* (ZO).

Las proteínas ocludina y claudina son proteínas transmembranales. Las primeras en ser descubiertas fueron las ocludinas (65kDa), estas fosfoproteínas tienen cuatro dominios transmembranales, el grupo carboxilo terminal localizado en el citoplasma interactúa con ZO-1, -2 y -3 para el anclaje al citoesqueleto. Las asas extracelulares interactúan con sus homólogos de las células adyacentes formando parte de la barrera paracelular de las células endoteliales. Si bien la deficiencia de ocludinas no altera dramáticamente la permeabilidad de la barrera hematoencefálica, tiene funciones reguladoras que pueden modificar la permeabilidad paracelular (Furuse et al., 1999). Las claudinas son componentes fundamentales de las uniones ocluyentes, se han descrito más de 24 miembros en la familia de las claudinas tanto en humanos como en roedores. Estás fosfoproteínas de alrededor de 22 kDa tienen cuatro dominios transmembranales pero no tienen una secuencia de aminoácidos similar a las ocludinas. Las claudinas de una pared de la célula endotelial interactúan homólogamente con la célula adyacente. El carboxilo terminal de las claudinas se asocia a proteínas accesorias en el citoplasma incluyendo ZO-1, -2 y -3. En el cerebro, la claudina-5 y -1 se encuentran formando parte de las uniones ocluyentes de la barrera

hematoencefálica y la pérdida de claudina-5 en el cerebro incrementa la permeabilidad de la barrera hematoencefálica (Liebner et al., 2000). Ocludinas y claudinas están ensambladas en heteropolímeros que forman balsas membranales que se ha sugerido contienen canales fluctuantes que permiten el paso de iones y de algunas moléculas hidrofílicas (Matter & Balda, 2003).

Otras proteínas transmembranales que se han relacionado con la función de barrera hematoencefálica son las moléculas de adhesión de la unión (JAM). Estas proteínas (40kDa) sólo tienen un paso transmembranal, son parte de la familia de las inmunoglobulinas, contribuyen a la adhesión célula-célula y participan en procesos de transmigración de monocitos a través de la barrera hematoencefálica (Ballabh et al., 2004).

Las proteínas accesorias de las uniones ocluyentes más descritas son las ZO (220kDa) que son miembros de la familia de proteínas parecidas a guanilato cinasas asociadas a membranas (membrane-associated guanylate kinase-like protein, MAGUKs) y contienen 3 dominios PDZ, un dominio SH3 y uno parecido a guanilato cinasa. Estos dominios sirven como anclaje al citoesqueleto de proteínas transmembranales, de hecho, la actina está unida a los dominios carboxilo terminal de ZO y este complejo entre las proteínas transmembranales, accesorias y el citoesqueleto proporcionan la estabilidad estructural de las células endoteliales (Haskins et al., 1998; Ballabh et al., 2004). Adicionalmente, la presencia de uniones adherentes es fundamental para la formación y mantenimiento de las uniones ocluyentes. La disminución en la expresión o los cambios en la localización celular de las proteínas que forman las uniones interendoteliales puede asociarse a cambios en la permeabilidad de la barrera

hematoencefálica (Abdul-Muneer et al., 2013; Ballabh et al., 2004; Hawkins et al., 2005).

Para asegurar el mantenimiento de la homeostasis cerebral, las células endoteliales proveen de nutrientes necesarios para el metabolismo celular en el parénquima cerebral. Los nutrientes como los aminoácidos y la glucosa tienen transportadores específicos en las células endoteliales, adicionalmente moléculas como insulina, transferrina, y leptina pueden ser transportadas, a través de endocitosis mediada por receptores (Ballabh et al., 2004). Las células endoteliales de la microvasculatura cerebral expresan proteínas asociadas a trifosfato de adenosina (ATP) que sirven como mecanismo de extrusión para diversas moléculas liposolubles que puedan atravesar la membrana de las células endoteliales, estas proteínas se conocen como proteínas de resistencia a multidrogas, entre ellas la que más se ha descrito en la barrera hematoencefálica es la glicoproteína P (Pgp por sus siglas en inglés), la cual extrude diversas moléculas liposolubles incluyendo fármacos antiepilépticos, antidepresivos y pesticidas por mencionar algunos (Schinkel et al., 1994).

Si bien son las células endoteliales las que confieren la característica de barrera, otros tipos celulares son cruciales para el desarrollo y mantenimiento de estas propiedades (Ballabh et al., 2004; Cardoso et al., 2010). Adicionalmente, las interacciones entre los componentes celulares de la barrera hematoencefálica: células endoteliales, astrocitos, microglia y pericitos, están mediadas por elementos extracelulares. Tanto el glicocalix como la membrana basal permiten la comunicación entre un tipo celular y otro a través de la difusión de moléculas de señalización solubles (Ueno, 2009).

Particularmente la membrana basal (también llamada lámina basal) rodea las células endoteliales y los pericitos produciendo una capa de entre 0.5 y 1 micrometro de espesor. Las moléculas que constituyen la membrana basal son collagena IV, elastina y fibronectina que son reguladas por las metaloproteinasas de matriz (MMP) -2 y -9 (Cardoso et al., 2010). Entre las funciones de la membrana basal se encuentran la adhesión celular, soporte mecánico para anclaje celular, separación de tejidos adyacentes y regulación del paso de macromoléculas que permiten la comunicación entre los componentes celulares de la barrera hematoencefálica (Cardoso et al., 2010; Zlokovic, 2008)

1.2.2 Astrocitos

Los astrocitos son fundamentales para el funcionamiento neural y forman, junto con la microglia, la glía limitante que recubre casi en su totalidad la barrera hematoencefálica. Entre sus funciones se encuentran la secreción de factores tróficos, la recaptura de neurotransmisores, el mantenimiento de los niveles de iones en el espacio intersticial y el mantenimiento de la barrera hematoencefálica. Los astrocitos producen factores solubles que son necesarios para el establecimiento de las propiedades de barrera en las células endoteliales, como el Factor de crecimiento neurotrófico derivado de células gliales (GDNF por sus siglas en inglés) y el Factor de crecimiento básico de fibroblastos, además de la comunicación mediada por señales de calcio que promueven la integridad de las uniones ocluyentes (Wolburg et al., 1994; Ballabh et al., 2004). En humanos, los astrocitos están presentes a partir de la semana 9 de gestación y los contactos entre las células endoteliales y los astrocitos se observa 8 semanas después. Aunque los astrocitos están separados de los pericitos que rodean las células

endoteliales, se ha descrito que los astrocitos son responsables de la inducción de la posición relativa de los pericitos en las células endoteliales (Wilkinson et al., 1990; Lécuyer et al., 2016). Los cambios asociados en la fisiología del astrocito pueden tener un efecto benéfico sobre las neuronas y otros tipos celulares. Por ejemplo, los astrocitos reactivos pueden proteger a las neuronas a través de mecanismos que incluyen la recaptura de neurotransmisores excitatorios (e.g. glutamato), facilitando la reparación de la barrera hematoencefálica, reduciendo el edema perivascular y evitando la expansión de agentes infecciosos o de células de sistema inmunológico (Fitch & Silver, 2008; Sofroniew & Vinters, 2010).

1.2.3 Microglia

La microglia es considerada la primera línea de defensa en el sistema nervioso central y es altamente susceptible a mediadores inflamatorios. Además de su participación en procesos de neuroinflamación la microglia contribuye a la angiogénesis en el sistema nervioso central durante el desarrollo promoviendo la migración celular. La microglia también contribuye al mantenimiento de la barrera hematoencefálica pero la mayoría de los estudios se enfocan en estudiar su papel en la ruptura de la barrera hematoencefálica debido a su capacidad de secretar una alta cantidad de mediadores inflamatorios (Lécuyer et al., 2015).

1.2.4 Pericitos

Los pericitos son células murales de tipo músculo liso que rodean a los capilares encefálicos, entre sus funciones se encuentran la regulación de la permeabilidad de la barrera hematoencefálica, procesos de angiogénesis, regulación del flujo sanguíneo cerebral, la regulación de procesos neuroinflamatorios además de presentar actividad de células troncales (Sweeney et al., 2016).

1.3 La barrera hematoencefálica durante procesos inflamatorios

La ruptura de la barrera hematoencefálica es común en patologías relacionadas con la presencia de procesos inflamatorios a nivel sistémico y en el sistema nervioso central, lo cual modifica la morfología y fisiología de los pericitos, los astrocitos y la microglia siendo esto causa o consecuencia de los síntomas que conciernen al cerebro en cada enfermedad (Figura 1). Particularmente la reactividad de la microglia y la astroglia generan un proceso conocido como gliosis, esto es, exhiben cambios morfológicos de hiperramificación o en el caso de la microglia propiedades fagocíticas asociadas la secreción de mediadores inflamatorios (e.g. citocinas y quimiocinas) (Banks et al., 2015) que inducen o contribuyen a que se altere la integridad de la barrera hematoencefálica (Abbott, 2010). La muerte celular de la astroglia y la microglia induce cambios en la expresión de Pgp en células endoteliales en un modelo *in vitro* los cuales regresan a sus niveles basales con la repoblación de astrocitos a pesar de que estos exhiben gliosis reactiva prolongada (Willis et al., 2007). Los astrocitos y la microglia son fundamentales para los procesos de migración de leucocitos circulantes hacia el cerebro en condiciones patológicas como la esclerosis múltiple (Kebir et al., 2007). Como ejemplos de la relación entre procesos inflamatorios mediados por la glía y las alteraciones de la barrera hematoencefálica encontramos que en el caso de los astrocitos, la ausencia de expresión en conexina-43 en los astrocitos contribuye al reclutamiento de leucocitos a través de las células endoteliales de la microvasculatura cerebral. Adicionalmente, deficiencia de acuaporina (AQP)-4 en los astrocitos se relaciona con la ruptura de la barrera hematoencefálica, la generación de edema perivascular y la alteración

funcional del sistema glinfático (Ezan et al., 2012; Boulay et al., 2015; Jessen et al., 2015). También se ha descrito que en un microambiente proinflamatorio, los astrocitos internalizan glutamato, producen antioxidantes, expresan receptores tipo Toll-3 (TLR3) que median la producción de citocinas anti-inflamatorias y angiotensinógeno (un promotor de mantenimiento de la barrera hematoencefálica). El angiotensinógeno puede ser regulado a la baja por citocinas proinflamatorias en procesos de neuroinflamación lo cual correlaciona con la disminución en la expresión de proteínas de la unión ocluyente en células endoteliales (e.g. ocludina) (Bsibsi et al., 2006).

En un ambiente proinflamatorio, la microglia reactiva secreta altas cantidades de IL-1β que puede estimular a los astrocitos para liberar el factor de crecimiento endotelial vascular (VEGF por sus siglas en inglés). Tanto IL-1β como VEGF alteran la integridad de la barrera hematoencefálica. En procesos neuroinflamatorios se ha descrito que a microglia libera un arreglo de citocinas proinflamatorias incluyendo IL-1β, IL-6 y IL-17A, además de especies reactivas de oxígeno (ROS) que incrementan la permeabilidad de la barrera hematoencefálica (Huppert et al., 2010; Lécuyer et al., 2015). Se han descrito otros mediadores inflamatorios (IL-6, IL-10, IL-22, G-CSF, RANTES, MCP-1, MIP- 1α) que modifican la permeabilidad de las células endoteliales en modelos *in vivo* e *in vitro* y que pueden ser producidas y secretadas por neuronas, astrocitos, microglia, pericitos o por las mismas células endoteliales pero también pueden actuar desde la periferia como son: glutamato, aspartato, taurina, ATP, adenosina, endotelina-1, óxido nítrico (NO), bradicinina, histamina, serotonina, sustancia P, ácido quinolínico, factor activador de plaquetas, VEGF y radicales libres (Abbott, 2000; Ballabh et al.,

2004). Las consecuencias de la modificación de la integridad de la barrera hematoencefálica van desde la alteración en la neuroquímica cerebral (e.g. altas concentraciones de glutamato) que promueven eventos de excitotoxicidad, la exacerbación de procesos inflamatorios, la producción descontrolada de radicales libres, inducción de muerte neuronal hasta la subsecuente pérdida de funciones reguladas por la región afectada (Zlokovic, 2008).

La ruptura de la barrera hematoencefálica (incremento en la permeabilidad y alteración de mecanismos inespecíficos de transporte) es un fenómeno común en patologías con un componente inflamatorio (e.g. demencia asociada a diabetes mellitus tipo 2, enfermedad de Alzheimer, esclerosis múltiple, infección por virus de inmunodeficiencia adquirida) (Abbott, 2000; Ballabh et al., 2004; Zlokovic, 2008) y en condiciones aversivas como el estrés y la pérdida de sueño (Kiyatkin et al., 2009; Gómez-González et al., 2013).

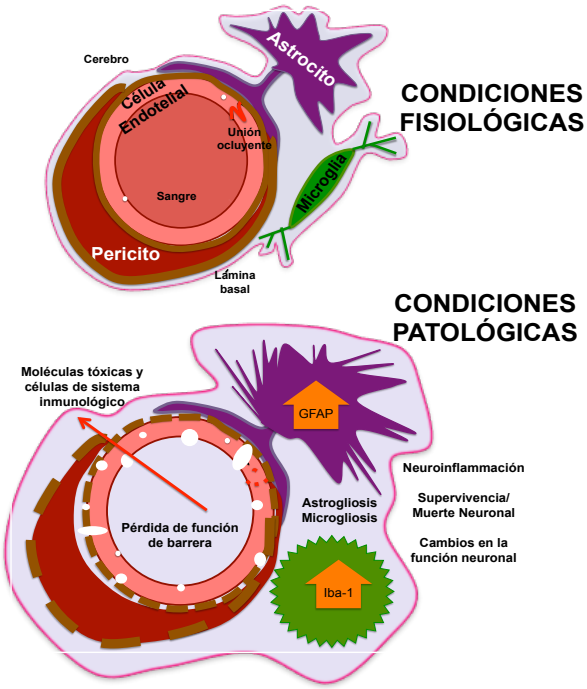

Figura 1. Modelo de la barrera hematoencefálica en condiciones fisiológicas y patológicas. En la parte superior se representan a los componentes celulares de la barrera hematoencefálica: células endoteliales, astrocitos, pericitos y microglia así como la lámina basal. Abajo se representan los cambios morfológicos de las células en condiciones patológicas en donde hay procesos inflamatorios, que de no ser controlados derivan en edema y muerte celular.

2. ANTECEDENTES

2.1 La pérdida y la recuperación de sueño regulan la permeabilidad de la barrera hematoencefálica

La disminución voluntaria y laboral del tiempo total de sueño es un problema de salud pública actual (ENSANUT, Encuesta Nacional de Salud y Nutrición 2016). Nuestro grupo de trabajo, ha desarrollado un modelo de restricción de sueño que es semejante a lo que ocurre en los humanos, en donde se disminuye el tiempo total de sueño, en la mayor parte de los casos durmiendo durante las últimas horas del periodo natural de inactividad (oscuridad). En el caso de las ratas, éstas pasan aproximadamente 12 horas durmiendo, siendo la fase de inactividad (luz) en donde más cantidad de sueño tienen.

El modelo que empleamos es el método modificado de plataformas múltiples, en donde las ratas son colocadas en un grupo socialmente estable en una caja de acrílico con diversas plataformas pequeñas rodeadas de agua. Las ratas se pueden mover, convivir, beber y comer sin mayor dificultad e incluso tener sueño no MOR pero al entrar en la fase de sueño MOR, la pérdida de tono muscular promueve que caigan al agua y despierten. Las ratas caen al agua sólo las primeras 12 horas del procedimiento, después de esto despiertan antes de caer. Este método abole por completo el sueño MOR y es ampliamente usado ya que es durante esta fase de sueño que se consolidan procesos fundamentales para el aprendizaje y la memoria (Machado et al., 2004). Este método suprime por completo el sueño MOR y disminuye en un 30% el sueño no MOR, durante el periodo de oportunidad para dormir se observa el rebote de sueño MOR (Machado et al., 2004; Gómez-González et al., 2013). En nuestro modelo, las ratas pasan 20

horas en la caja de pérdida de sueño y tienen 4 horas de oportunidad para dormir diariamente durante 10 días. El primer estudio exploratorio sobre los efectos de la deficiencia de sueño sobre la barrera hematoencefálica consistió en la administración sistémica de un colorante vital (Azul de Evans) que normalmente no atraviesa la barrera hematoencefálica. La restricción crónica de sueño MOR incrementó la permeabilidad del colorante en todas las regiones cerebrales evaluadas (Figura 2) incluyendo corteza cingular, retrosplenial, orbitofrontal, frontal, insular, rhinal, parietal, somatosensorial, visual, auditiva y motora, el hipocampo, la amígdala, los núcleos de la base, el área septal, el tálamo, el área preóptica, el hipotálamo, el cerebelo y el tallo cerebral (Gómez-González et al., 2013).

Si bien los efectos de la pérdida de sueño sobre la barrera hematoencefálica parecen dramáticos, breves periodos de oportunidad para dormir (40-120 minutos) inducen una recuperación de la integridad de la barrera hematoencefálica dependiente de la región cerebral (Gómez-González et al., 2013). Por ejemplo, hay regiones que se recuperan por completo después de 2 horas de oportunidad para dormir (e.g. los núcleos de la base y la corteza insular), regiones con una recuperación lenta (e.g. la corteza somatosensorial, el hipocampo) y regiones en las que 2 horas no son suficientes para que la función de barrera hematoencefálica se restaure por completo (e.g. el vermis del cerebelo) (Gómez-González et al., 2013). En el hipocampo de ratas con restricción de sueño y oportunidad para dormir, encontramos que la permeabilidad de la barrera hematoencefálica a moléculas de bajo peso molecular (sodio acoplado a fluoresceína, 325 Da) incrementó y a diferencia de moléculas de alto peso

molecular (azul de Evans, 69kDa), la recuperación de sueño por 2 horas restableció la permeabilidad normal de la barrera hematoencefálica. En este estudio, también describimos que las uniones interendoteliales se encuentran alteradas en condiciones de restricción de sueño, lo cual correlaciona con la disminución en la expresión de proteínas de la unión ocluyente, incremento en el grosor de la lámina basal y presencia de edema perivascular (Hurtado-Alvarado et al., 2017) (ANEXO 1).

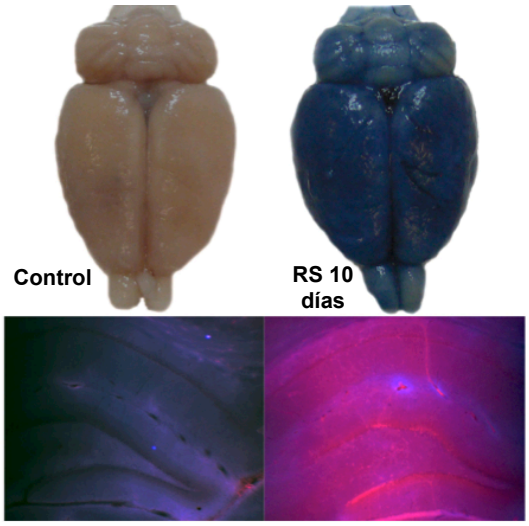

Figura 2. Efecto de la restricción crónica de sueño (RS) en la permeabilidad de la barrera hematoencefálica en ratas. Cerebro de rata control y restringida de sueño inyectadas sistémicamente con azul de Evans. Abajo, imágenes representativas del hipocampo, el colorante se observa en el parénquima cerebral (rojo) sólo en ratas con restricción de sueño (Amplificación X40).

En ratones C57BL/6 la restricción de sueño empleando el método de plataforma rotatoria (18 horas de pérdida de sueño y 6 horas de oportunidad para dormir) durante 6 días, la permeabilidad de la barrera hematoencefálica a sodio acoplado a fluoresceína en la corteza, la regiones subcorticales, el tallo cerebral y la médula espinal incrementó. Después de 24 horas de oportunidad para dormir la

permeabilidad de la barrera hematoencefálica fue restaurada por completo en todas las regiones cerebrales. El incremento en la permeabilidad de la barrera hematoencefálica fue concomitante a la disminución en la expresión del mRNA de proteinas ocludina, claudina-1, claudina-5 y ZO, y un incremento en la expresión de mRNA del marcador inflamatorio ciclooxigenasa (COX)-2 en la microvasculatura cerebral. Adicionalmente, la restricción de sueño en ratones incrementó los niveles circulantes de la proteína C reactiva (CRP por sus siglas en inglés), lo cual ha sido previamente reportado como parte de la inflamación sistémica de bajo grado inducida por la pérdida de sueño (He et al., 2014).

La alteración de la barrera hematoencefálica en animales restringidos de sueño podría estar mediada por moléculas de acción rápida y de fácil degradación que podrían acumularse durante el periodo de pérdida de sueño. En este sentido, la participación de la adenosina podría ser clave en este fenómeno; sin embargo, considerando que la pérdida de sueño induce un estado proinflamatorio sistémico de bajo grado consideramos que éste por si mismo, también podría contribuir a la desregulación de la barrera hematoencefálica.

2.2 Papel de la adenosina en la regulación de la barrera hematoencefálica y la pérdida de sueño

En el sistema nervioso central la adenosina es naturalmente producida por neuronas y células gliales. La adenosina ejerce su acción biológica a través de sus cuatro tipos de receptores acoplados a proteínas G (A_1, A_{2A}, A_{2B} y A_3). Entre las múltiples funciones que la adenosina regula se encuentran la regulación del flujo cerebral, la modulación de procesos inflamatorios locales y la regulación de sueño (Dunwiddie & Masino, 2001). Los receptores que han sido asociados con la

regulación del sueño son el A_1 y el A_{2A}. Estos receptores tienen una expresión diferencial dependiendo de la región cerebral. Por ejemplo, los núcleos de la base tienen mayor expresión de receptor A_{2A}, mientras que el receptor A_1 parece estar distribuido homogéneamente en todas las regiones cerebrales (Dixon et al., 1996, DeMet & Chicz-DeMet, 2002). La adenosina es un potente inductor de sueño ya que se acumula en el espacio intersticial durante el periodo de vigilia asociado al incremento en la tasa metabólica cerebral. En regiones que promueven el sueño como el prosencefálo basal, los niveles de adenosina incrementan en un 140% después de 6 horas de privación de sueño y durante las subsecuentes 3 horas de recuperación de sueño se observa una disminución progresiva pero lenta de los niveles de adenosina (Porkka-Heiskanen, 1999). Durante la pérdida de sueño la expresión de receptores de adenosina se modifica dependiendo de la región cerebral, en cerebro de ratones restringidos de sueño, la expresión del mRNA del receptor A_1 incrementa sólo en el prosencefálo basal el primer día de restricción de sueño mientras que la expresión del receptor A_{2A} incrementa sólo en el hipocampo en el día 5 de la restricción de sueño (Kim et al., 2012).

En condiciones patológicas que involucran procesos inflamatorios (e.g. esclerosis múltiple) los niveles centrales y sistémicos de adenosina se incrementan y contribuyen al reclutamiento de leucocitos modificando la permeabilidad de la barrera hematoencefálica, mientras que el uso de antagonistas de receptores de adenosina revierten este efecto, además de disminuir la intensidad de los eventos de neuroinflamación (Mills et al., 2008, Carman et al., 2011, Byone et al., 2015; Kim & Byone, 2015). Adicionalmente, las enzimas que catalizan la formación de adenosina a partir de ATP-ADP y AMP (CD39/CD73 respectivamente) están

involucradas en el proceso de migración de células de sistema inmunológico hacia el cerebro (Byone et al., 2015).

Las células endoteliales de la microvasculatura cerebral de ratón y humano expresan el receptor A_1 y A_{2A}. El uso de un agonista no selectivo de receptores de adenosina (NECA) incrementa 3 veces la permeabilidad de la barrera hematoencefálica a moléculas de alto peso molecular en ratones, mientras que el uso de una dosis 100 veces menor del agonista selectivo de los receptores A_{2A} de adenosina (Regadenoson, Lexiscan®) incrementó 15 veces la permeabilidad de la barrera hematoencefálica a moléculas de alto peso molecular (70kDa) (Carman et al., 2011). La administración del agonista selectivo de los receptores A_{2A} de adenosina incrementa la permeabilidad de la barrera hematoencefálica hasta por 180 minutos después de la administración del fármaco, siendo 30 minutos el punto máximo de permeación. Está ruptura transitoria de la barrera hematoencefálica se acompaña de una disminución en la expresión de proteínas de la unión ocluyente (claudina-5, ocludina y ZO-1) (Carman et al., 2011). Debido a esta pronta recuperación en la integridad de la barrera hematoencefálica, se han realizado estudios en los que se administra el agonista selectivo de los receptores A_{2A} de adenosina conjuntamente con fármacos para tratar neuropatologías como tumores cerebrales aunque no se ha tenido el éxito esperado en humanos (Jackson et al., 2016). En contraste, el uso del antagonista selectivo del receptor A_{2A} de adenosina (SCH58261) revierte el incremento en la permeabilidad de la barrera hematoencefálica en un modelo de esclerosis múltiple, además de atenuar los síntomas y evitar el infiltrado de células de sistema inmunológico en el cerebro (Carman et al., 2011).

Es importante mencionar que además de las células endoteliales, pericitos, astrocitos y microglia expresan receptores A_1, A_{2A}, A_{2B} de adenosina pero su nivel de expresión es relativamente baja en condiciones fisiológicas; sin embargo el rol del receptor A_{2A} es clave para el establecimiento de procesos neuroinflamatorios ya que su expresión incrementa rápidamente después de un daño cerebral como la administración local o sistémica de lipopolisacárido (LPS) o la intoxicación por 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Boison et al., 2010) y en patologías como enfermedad de Parkinson, enfermedad de Huntington, enfermedad de Alzheimer y esquizofrenia (Cunha et al, 2005). Los animales knock out para el receptor A_{2A} de adenosina o animales tratados con un antagonista selectivo de los receptores A_{2A} de adenosina (SCH58261), tienen una robusta neuroprotección en condiciones como isquemia o excitotoxicidad, donde se sugiere que el bloqueo de estos receptores disminuye las concentraciones intersticiales de glutamato, la concentración de mediadores inflamatorios y la subsecuente astrogliosis (Cunha et al., 2005). Adicionalmente, la acción neuroprotectora reportada para el antagonista no selectivo de adenosina, cafeína, parece estar mediada por el bloqueo de los receptores A_{2A} (Leite et al., 2010).

La sobrexpresión de receptores A_{2A} de adenosina es concomitante al incremento de los niveles locales de mediadores inflamatorios como IL-1β y TNF-α (Boison et al., 2010) y las citocinas proinflamatorias regulan a la alta la expresión del receptor A_{2A} (Trincavelli et al., 2002), sugiriendo una estrecha relación entre la expresión/activación del receptor A_{2A} y la producción/acción de citocinas proinflamatorias.

2.3 La pérdida de sueño induce inflamación de bajo grado: el impacto de las citocinas sobre la barrera hematoencefálica

Se ha descrito que diversas citocinas pueden afectar o modular el patrón de sueño, entre las descritas se encuentran IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-15, IL-18, TNF-α, TNF-β, Interferón (IFN)-α, IFN-β, IFN-γ y la proteína inhibidora de macrófagos (MIP)-1β (Imeri and Opp, 2009). Por otro lado, la pérdida de sueño puede modificar la secreción de componentes humorales de la respuesta inmunológica. El perfil de secreción de citocinas exhibe un ritmo circadiano y particularmente los niveles de citocinas proinflamatorias tienen su pico máximo en la primera parte del sueño en correlación con la acumulación de moléculas como adenosina que promueven la síntesis de citocinas proinflamatorias. La predominancia de la respuesta a citocinas proinflamatorias cambia durante la fase tardía de sueño, cuando hay sueño MOR, promoviendo la producción de citocinas anti inflamatorias (Dimitrov et al., 2004).

En humanos sanos restringidos de sueño durante 5 días (4 horas de sueño al día) se modifica la expresión de genes asociados a procesos inflamatorios en células polimorfonucleares circulantes. Este estudio encontró que hay un incremento en la expresión de más de 2000 genes relacionados con la regulación positiva de procesos biosintéticos de citocinas, activación de leucocitos y regulación de la respuesta inmune innata (Aho et al., 2013).

El incremento en la expresión génica de mediadores inflamatorios circulantes se considera un riesgo para desarrollar enfermedades metabólicas y cardiovasculares. La restricción de sueño en humanos (5 días, 4 horas de sueño por día) incrementa el riesgo de desarrollar enfermedades cardiovasculares lo cual

correlaciona con un aumento en los niveles circulantes de IL-1β, IL-6, IL-17A y CRP (van Leeuwen et al., 2009). La inflamación sistémica de bajo grado inducida por la pérdida de sueño es ampliamente reportada en roedores y humanos y consta de un incremento sutil pero persistente en los niveles circulantes de mediadores inflamatorios como IL-1β, IL-6, IL-17A, TNF-α, y CRP (Yehuda et al., 2007). Los datos del porcentaje de cambio de los niveles circulantes de citocinas proinflamatorias son controversiales ya que depende del método empleado para inducir pérdida de sueño, de la forma en que se toma la muestra e incluso de la población/cepa estudiada (Mills et al., 2007; Hurtado-Alvarado et al., 2013) (ANEXO 2).

Los cambios en la expresión de moléculas inflamatorias también ha sido descrito a nivel cerebral en modelos animales de pérdida de sueño. Por ejemplo, la mRNA y de la proteína TNF-α incrementa en el cerebro en un modelo de fragmentación crónica de sueño (15 días) en ratones C57BL/6 (Ramesh et al., 2012).

Los mediadores inflamatorios que incrementan durante la pérdida de sueño como adenosina, TNF-α e IL-17A también inducen ruptura de la permeabilidad de la barrera hematoencefálica (Hurtado-Alvarado et al., 2016a) (ANEXO 3). Existen algunos estudios que ponen en evidencia que el perfil inflamatorio del organismo es fundamental para los cambios en la permeabilidad de la barrera hematoencefálica en condiciones patológicas, por ejemplo se ha descrito que en procesos infecciosos mediados por virus los animales que presentan una respuesta proinflamatoria exacerbada (e.g. ratones C57BL/6) tienen incremento en la permeabilidad de la barrera hematoencefálica pero que los animales infectados que tienen deficiencia de la respuesta inflamatoria (e.g. BALB/c) no exhiben

cambios en la permeabilidad de la barrera hematoencefálica (Morrey et al., 2008; Silva et al., 2010). Sin embargo, no hay evidencia que indique que el estado proinflamatorio inducido por la pérdida de sueño regule la permeabilidad de la barrera hematoencefálica, pero se sabe que condiciones que presentan un estado proinflamatorio de bajo grado y las alteraciones en el patrón de sueño como obesidad, diabetes mellitus tipo 2, y envejecimiento exhiben cambios en la permeabilidad de la barrera hematoencefálica (Zlokovic, 2005; Gustafson et al., 2007; Kanoski et al., 2010; Mogi & Horiuchi, 2011; Montagne et al., 2015).

3. JUSTIFICACIÓN

Las disminución voluntaria en el tiempo total de sueño, también conocida como restricción de sueño, se considera un problema de salud pública que incrementa significativamente el riesgo de presentar alteraciones cognitivas y de desarrollar enfermedades metabólicas, cardiovasculares, y neurodegenerativas. El estudio de los cambios en la integridad de la barrera hematoencefálica durante la restricción de sueño es clave para entender las alteraciones neuroquímicas que promueven las deficiencias cognitivas asociadas a este evento. Dilucidar la participación de la adenosina y las citocinas proinflamatorias en la regulación de la barrera hematoencefálica en condiciones de restricción de sueño generará información relevante que permitirá 1) generar conocimiento básico que sustente como la falta de sueño promueve el desarrollo o la exacerbación de patologías y 2) desarrollar estrategias basadas en la restricción de sueño para incrementar la permeabilidad de la barrera hematoencefálica a fármacos que normalmente no cruzan la barrera hematoencefálica.

4. HIPÓTESIS

La adenosina a través de sus receptores A_{2A} y el estado inflamatorio sistémico están involucrados en la ruptura de la barrera hematoencefálica inducida por la pérdida de sueño.

5. OBJETIVO GENERAL

Determinar la participación de los receptores A_{2A} de adenosina y del estado proinflamatorio sistémico en la ruptura de la barrera hematoencefálica inducida por la restricción crónica de sueño.

6. OBJETIVOS PARTICULARES

- Evaluar el efecto de un antagonista selectivo de los receptores A_{2A} de adenosina en los cambios de la permeabilidad de la barrera hematoencefálica en ratas restringidas de sueño
- Evaluar el efecto de un antagonista selectivo de los receptores A_{2A} de adenosina en la expresión de proteínas de unión ocluyente y adherente en ratas restringidas de sueño
- Evaluar el efecto de un antagonista selectivo de los receptores A_{2A} de adenosina en la expresión de marcadores de neuroinflamación en cerebro de ratas restringidas de sueño
- Evaluar el efecto de la restricción de sueño sobre la expresión de los receptores A_{2A} y la enzima CD73 en el cerebro de rata
- Caracterizar el perfil inflamatorio sistémico en ratones C57BL/6 y BALB/c restringidos de sueño
- Evaluar la permeabilidad de la barrera hematoencefálica a moléculas de bajo y alto peso molecular en ratones C57BL/6 y BALB/c restringidos de sueño

- Evaluar la expresión de proteínas de la unión ocluyente en ratones C57BL/6 y BALB/c restringidos de sueño
- Evaluar la expresión de marcadores de neuroinflamación en ratones C57BL/6 y BALB/c restringidos de sueño
- Evaluar la expresión de los receptores A_{2A} en ratones C57BL/6 y BALB/c restringidos de sueño

7. MATERIAL Y MÉTODOS

Evaluación del efecto del bloqueo de receptores A_{2A} de adenosina en la integridad de la barrera hematoencefálica ratas restringidas de sueño

Para determinar la participación del receptor A_{2A} de adenosina sobre la regulación de la integridad de la barrera hematoencefálica en ratas restringidas de sueño empleamos un antagonista no selectivo (cafeína) y un antagonista selectivo (SCH58261) de los receptores A_{2A} de adenosina y realizamos la determinación de los niveles séricos de corticosterona, ensayos de permeabilidad de la barrera hematoencefálica a moléculas de bajo y alto peso molecular; western blot para determinar la expresión de proteínas de unión ocluyente, del receptor A_{2A}, y de marcadores de neuroinflamación además de inmunohistoquímica para el receptor A_{2A}, Iba-1 y GFAP.

7.1 Ratas

Para este estudio se emplearon ratas Wistar de tres meses de edad (n=71). Las ratas fueron alojadas en grupos de 4-8 individuos en el bioterio en un ciclo luz-oscuridad de 12 horas (luces apagadas 11am) a temperatura ambiente de 20-25ºC con comida (dieta estándar) y agua *ad libitum*. Las ratas fueron asignadas al azar a las diferentes condiciones experimentales, tratando en todo momento que

las condiciones fueran las apropiadas para mantener bajos niveles de estrés. Los experimentos fueron realizados siguiendo las normas establecidas de cuidado y uso de animales en investigación de neurociencias (National Research Council, 2010), de las normas internacionales de investigación en animales (ARRIVE, Animal Research: Reporting *In Vivo* Experiments. www.nc3rs.org.uk/arrive-guidelines) y contó con la aprobación del Comité de ética de la División de Ciencias Biológicas (CAE.CBS.03.16) y el consejo Divisional de Ciencias Biológicas y de la Salud (CD.CBS.285.13) de la Universidad Autónoma Metropolitana, Unidad Iztapalapa.

7.2 Método de plataformas múltiples

Para el procedimiento de restricción de sueño, se empleó una caja de acrílico (82cm x 59cm x 48cm) y plataformas de 7cm de diámetro. La restricción de sueño se realizó empleando la técnica modificada de plataforma múltiple. Las ratas permanecieron en la caja de acrílico sobre las plataformas rodeadas de agua (1-2cm de altura) 20 horas diarias por 10 días consecutivos. Diariamente al término de las 20 horas en la caja de acrílico, las ratas fueron colocadas en su caja-habitación durante las últimas 4 horas de la fase de luz. Todo el tiempo las ratas tuvieron comida y agua ad libitum. Los controles intactos permanecieron en su caja habitación durante los 10 días del experimento donde pudieron dormir adecuadamente.

La técnica modificada de plataformas múltiples es un protocolo que deriva del método de florero invertido. La técnica de florero invertido está basada en las características del sueño MOR, en donde hay pérdida del tono muscular al entrar a esta fase de sueño, las plataformas pequeñas evitan que el animal entre a

sueño MOR al caerse al agua, clásicamente se coloca una rata sobre una plataforma, lo cual además de privar de sueño MOR, también induce estrés por inmovilización y por aislamiento social. La técnica modificada de plataformas múltiples fue diseña para disminuir los factores estresantes y abolir por completo el sueño MOR, en está técnica las ratas se colocan en una tina de acrílico en la cual hay más plataformas que animales, evitando así el estrés por inmovilización; adicionalmente, el grupo de ratas empleadas deben ser del mismo grupo social o camada inicial (Machado et al., 2004). En este estudio, las ratas asignadas a cada grupo experimental eran del mismo grupo social y permanecían juntas en la caja de privación y durante las 4 horas de oportunidad para dormir. Se ha descrito que este método elimina la fase de sueño MOR y disminuye en un 30% la cantidad de sueño no MOR (Gómez-González et al., 2013) (Figura 3).

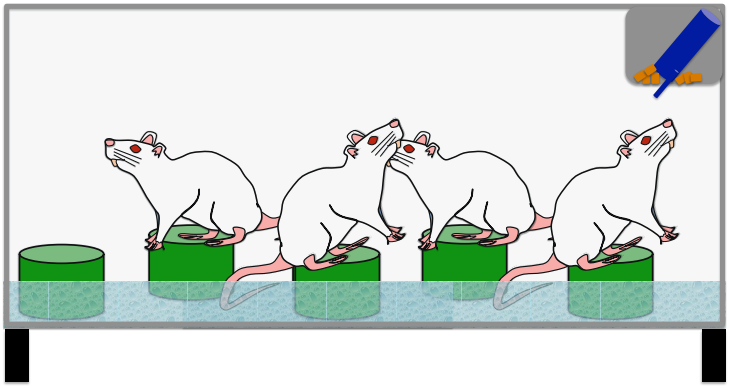

Figura 3. Método Modificado de Plataforma Múltiple. Un grupo de ratas socialmente estable, es alojado en una tina de acrílico en la que hay plataformas pequeñas rodeadas de agua. El número de plataformas es mayor que el número de ratas para eliminar el estrés por inmovilización. Los animales tienen acceso a agua y alimento *ad libitum*.

7.3 Administración de fármacos

Para evaluar el efecto de un antagonista no selectivo de los receptores de adenosina en ratas restringidas de sueño utilizamos cafeína (Sigma C0750); la

cafeína fue disuelta en solución salina y administrada por vía ip en una dosis única de 0.3mg/kg de peso corporal al final del periodo de 20 horas de restricción de sueño en el día 10. Para evaluar la participación del receptor A_{2A} de adenosina en ratas restringidas de sueño, empleamos un antagonista selectivo de los receptores A2A de adenosina, SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-tria-zolo(1,5-c)-pyrimidine) (Sigma S4568) (Figura 4). Este fármaco es un potente antagonista de los receptores A_{2A} de adenosina (48-1561 más afinidad al receptor A_{2A} de adenosina que al A_1 o A_{2B}) y una rápida biodisponibilidad después de la administración ip (Zocchi et al., 1996; Yang et al., 2007). El SCH58261 fue diluido en dimetil sulfóxido (DMSO) y administrado i.p. 3 veces cada 30 minutos a dosis de 0.01, 0.1 o 0.5 mg/kg de peso corporal al final de la restricción de sueño en el día 10. El vehículo fue administrado bajo el mismo esquema. Las ratas permanecieron en la tina de privación durante la administración de los fármacos y fueron sacrificadas 30 minutos después de la ultima administración de SCH58261 (Figura 5).

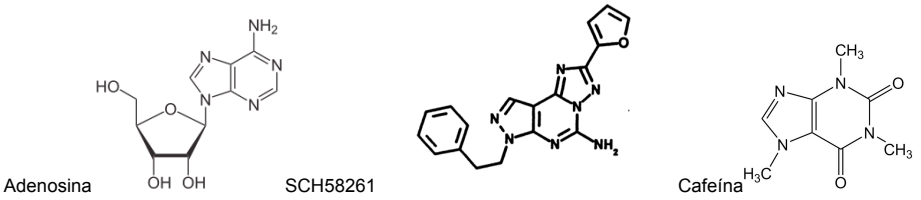

Figura 4. Estructura química de la adenosina, de antagonista selectivo de los receptores A_{2A} de adenosina SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-tria-zolo(1,5-c)-pyrimidine) y cafeína. Tomado de Zocchi et al., 1996.

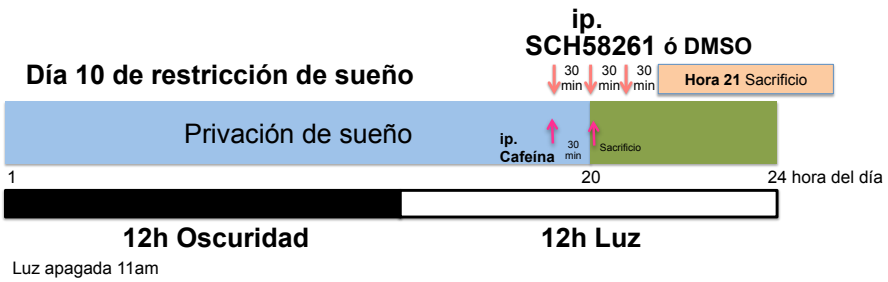

Figura 5. Esquema de administración del antagonista selectivo de los receptores A_{2A} de adenosina, cafeína y vehículo en ratas restringidas de sueño

7.4 Medición de corticosterona

Se realizó la cuantificación de corticosterona en suero de ratas por considerarlo un biomarcador de estrés. La sangre se obtuvo al día 10 de restricción de sueño por decapitación en las dos últimas horas de la fase de luz en los siguientes grupos: restricción de sueño 10 días (RS), restricción de sueño durante 10 días con SCH58261 0.1mg/kg de peso (RS+SCH) y controles intactos (Con) (n=3 por grupo). Las muestras de sangre se centrifugaron a una velocidad de 3000g/10 minutos, el sobrenadante fue colectado y guardado a -80ºC hasta su procesamiento. La corticosterona en suero fue cuantificada por duplicado empleando un kit de ELISA (Enzyme-Linked ImmunoSorbent Assay, Abcam, ab108821) siguiendo el protocolo dado por el proveedor.

7.5 Determinación de dosis óptima de SCH58261 para ensayos de permeabilidad

Antes de realizar el diseño experimental que se describe a continuación se realizó un estudio piloto para determinar las dosis de SCH58261 que serían estudiadas siendo 1mg/kg la dosis mínima que no induce alteraciones motoras. En este estudio las ratas fueron restringidas de sueño por 10 días y se realizó un ensayo de permeabilidad de la barrera hematoencefálica a azul de Evans. Las ratas

fueron sacrificadas entre las 9 y las 11am (fase de luz) con una sobredosis de pentobarbital sódico (ip. 0.8mL/300g de peso corporal). El azul de Evans se administró al final del periodo de pérdida de sueño en el día 10. Los grupos evaluados fueron: restricción del sueño más DMSO (n=1), restricción del sueño más 0,1 mg/kg (x3) de SCH58261 (n=1), restricción del sueño más 0,5 mg/kg (x3) de SCH58261 (n=1) y restricción del sueño más 1 mg/kg (x3) de SCH58261 (RS+SCH, n=1). El colorante se administró vía intracardiaca 0.2mL/100g de peso. Después de 10 minutos de circulación, las ratas fueron perfundidas con una solución salina (0.9%) durante 5 minutos y después con paraformaldehído al 4% durante 5 minutos a una velocidad de 15mL/min. Se removió el cerebro y se post-fijó 24 horas. Los cerebros fueron seccionados en cortes coronales de 2mm. Se tomaron fotografías de las rebanadas del cerebro con la misma intensidad de luz con una cámara digital (Lumix, Panasonic). Con base en los datos obtenidos se determinó el uso de 0.01, 0.1 y 0.5 mg/kg de SCH58261 para los siguientes estudios.

7.6 Ensayo permeabilidad de la barrera hematoencefálica a moléculas de bajo peso molecular

Los dextranos de 10kDa acoplados a isotiocianato de fluoresceína (FITC) (Sigma Cat. FD10S) se suspendieron en PBS a una concentración de 3 mg/ml. Para el experimento del efecto dosis-respuesta del SCH58261, las ratas se dividieron en los siguientes grupos: control intacto más DMSO (Con, n=4); restricción de sueño 10 días más DMSO (RS, n=3); restricción de sueño 10 días más 0.3 mg/kg de cafeína (RS Caf, n=3); restricción de sueño 10 días más 0.01 mg/kg (x3) de SCH58261 (RS+SCH 0.01, n=3); restricción de sueño 10 días más 0.1 mg/kg (x3)

de SCH58261 (RS+SCH 0.1, n=3); y restricción de sueño 10 días más de 0.5 mg/kg (x3) de SCH58261 (RS+SCH 0.5, n=3). Las ratas fueron anestesiadas con una sobredosis de pentobarbital sódico (ip. 0,063 g/kg de peso corporal). La administración de dextranos-FITC de 10 kDa se realizó por vía intracardiaca entre las 9-11 am (fase de luz). Se realizó una incisión torácica de 5 mm en el lado izquierdo de la caja torácica, el corazón fue parcialmente expuesto, y se administraron 0.2 ml/100g de peso corporal de dextranos-FITC en el ventrículo izquierdo del corazón. Después de 10 minutos de circulación de los dextranos-FITC, las ratas se perfundieron durante 5 minutos con solución salina (0,9% w/v) a una velocidad de 15mL/min. El cerebro fue extraído y se disectarón las siguientes regiones cerebrales: el hipocampo, la corteza, los núcleos de la base y el vermis. El tejido fue pesado, protegido de la luz y colocado a -20ºC. Las muestras fueron procesadas el mismo día de su obtención. Las muestras se homogeneizaron y se centrifugaron a una velocidad de 13500 rpm/10min. El sobrenadante se colectó y se determinó la absorbancia en el espectrofotómetro (Genesys20, Thermo Spectronic) a 520 nm. La concentración de 10 kDa de dextranos-FITC se calculó utilizando una curva estándar. Los datos de las absorbancias en las muestras, fueron transformados usando una ecuación polinomial de segundo grado resultante de los datos de la curva patrón. Una vez calculada la concentración de dextranos este valor fue dividido entre el peso de tejido. Los resultados se muestran como la concentración de dextranos-FITC por peso de tejido cerebral (mg/g).

7.7 Ensayo de permeabilidad de la barrera hematoencefálica a moléculas de alto peso molecular

Los dextranos-FITC de 70kDa (Sigma Cat. FD10S) fueron resuspendidos en PBS a una concentración de 3mg/ml. Las ratas fueron divididas en los siguientes grupos: grupo control más DMSO (Con, n=3); restricción del sueño más DMSO (RS, n=3), restricción de sueño más 0.1 mg/kg (x3) de SCH58261 (RS+SCH, n=3); restricción de sueño más 0.3mg/kg de cafeína (RS+Caf, n=4). Las ratas se anestesiaron con pentobarbital sódico (ip. 0,063 g / kg de peso corporal). Los dextranos-FITC de 70 kDa se administraron por vía intracardiaca entre las 9-11 am. Se realizó una incisión torácica de 5mm en el lado izquierdo de la caja torácica, el corazón fue parcialmente expuestos, y se administraron 0,2 ml/100g de peso corporal del dextranos-FITC en el ventrículo izquierdo del corazón. Después de 10 minutos de circulación del dextranos-FITC, las ratas se perfundieron durante 5 minutos con solución salina (0,9% w/v) a una velocidad de 15mL/min. Se extrajo el cerebro y se disectó; la concentración de dextranos-FITC de 70 kDa se calculó en el hipocampo, corteza, ganglios basales y vermis. El procesamiento de las muestras y la concentración de dextranos fue calculada del mismo modo descrito para los dextranos-FITC de 10kDa.

7.8 Ensayo de permeabilidad de la barrera hematoencefálica a azul de Evans

El azul de Evans se administró al final del periodo de pérdida de sueño en el día 10 como se describe en la sección 7.5. Los grupos evaluados fueron: grupo control más DMSO (Con, n=3); restricción del sueño más DMSO (RS, n=3) y restricción del sueño más 0,1 mg/kg (x3) de SCH58261 (RS+SCH, n=3). Se administró el colorante bajo el mismo protocolo que los dextranos a una dosis de

0.2mL/100g de peso corporal. Después de 10 minutos de circulación, las ratas fueron perfundidas con una solución salina (0.9%) durante 5 minutos y después con paraformaldehído al 4% durante 5 minutos a una velocidad de 15mL/min. Se removió el cerebro y se post-fijó 24 horas. Los cerebros fueron seccionados en cortes coronales de 2mm. Se tomaron fotografías de las rebanadas del cerebro con la misma intensidad de luz con una cámara digital (Lumix, Panasonic). Las imágenes fueron transformadas a 8 bits (blanco y negro) en el programa ImageJ y se calculó la densidad óptica utilizando la fórmula Gray=0.299(red)+0.587(green)+0.114(blue) y empleando una tabla calibrada de densidad óptica (Rodbard) como está reportado previamente (Gómez-González et al., 2013).

7.9 Determinación de expresión de proteínas de barrera hematoencefálica y marcadores celulares

El cerebro fue obtenido por decapitación y se disectó en frio para obtener las regiones de interés (de corteza, hipocampo, núcleos de la base y vermis). Las muestras correspondieron a los siguientes grupos: control más DMSO (Con, n=3); restricción del sueño más DMSO (RS, n=3) restricción del sueño más 0,1 mg/kg (x3) de SCH58261 (n=3). Las muestras se homogeneizaron con 200µL de amortiguador con inhibidores de proteasas en frío usando un taladro de mano. Las muestras fueron centrifugadas a 13500rpm durante 10 minutos a 4ºC. El sobrenadante fue recolectado y alicuotado. La concentración total de proteínas se determinó tomando 2µL de la muestra en 18µL del mismo buffer, este ensayo se realizó por triplicado. Una vez preparadas todas las muestras se agregó 1mL de reactivo de Bradford previamente diluido en una concentración 1:5 en agua

destilada (Biorad, Hercules, CA BioRad, cat. 500-0006). Se obtuvo la absorbancia de cada muestra por espectrofotometría a una longitud de onda de 595nm (Genesys20, Thermo Spectronic) y con base en una curva patrón realizada con concentraciones conocidas de inmunoglobulina G se calculó el volumen requerido para tener 100µg de proteína de cada muestra con la cual se realizó la electroforesis en membrana de SDS-acrilamida 30% bis. La electroforesis se realizó dejando correr las proteínas a 60V durante 30 minutos y después se cambió el voltaje a 120V durante 1 hora en frío. Las proteínas fueron transferidas a una membrana de PVDF en una cámara de transferencia durante 1 hora a 70V en frío. Las membranas se bloquearon con leche sin grasa al 5% en PBS/Tween0.1%. Posteriormente las membranas se incubaron con anticuerpos contra ocludina (Invitrogen, 40–4700, 1:1000), claudina-5 (Abcam, ab53765, 1:1000), ZO-1 (Invitrogen, 40–2200, 1:1000), E-cadherin (Santa Cruz Biotechnology, sc-21791, 1:1000), GFAP (Abcam, ab4648, 1:1000), Iba-1 (Abcam, ab48004, 1:1000), A2A AR (Abcam, ab3461, 1:1000), CD73 (Abcam, ab175396, 1:500), y GAPDH (Abcam, ab8245, 1:1000) durante toda la noche a 4ºC. Después de 3 lavados con leche sin grasa al 1% en PBS/Tween 0.1% las membranas fueron incubadas durante 2 horas con el anticuerpo secundario correspondiente. Los 3 lavados con leche sin grasa al 1% en PBS/Tween 0.1% se repitieron y la membrana fue entonces incubada con solución AB que contenía peroxidasa de rábano y avidina (ABC kit, Vector Labs, PK6100). Las membranas fueron nuevamente lavadas 3 veces con leche sin grasa al % en PBS/Tween 0.1% y 1 vez con PBS. Las membranas fueron reveladas con un kit de luminiscencia (Amersham, RPN2232) y las bandas fueron determinadas con el programa C-

digitTM usando un lector de geles (Versión 3.1, Licor®). Se empleó GAPDH como control de carga y los datos fueron normalizados con respecto al grupo control.

7.10 Localización y expresión de marcadores celulares

El proceso de inmunohistoquímica para Iba-1 y GFAP se realizó por flotación en secciones de cerebro de los siguientes grupos: control más DMSO (Con, n=1); restricción del sueño más DMSO (RS, n=1) restricción del sueño más 0.1 mg/kg (x3) de SCH58261 (n=1). Para la evaluación del receptor A_{2A} de adenosina se emplearon los grupos control más DMSO (Con, n=1) y restricción del sueño más DMSO (RS, n=1).

Las ratas fueron anestesiadas con pentobarbital sódico (ip. 0.063 g/kg) y perfundidas con solución salina 0.9% (w/v) durante 10 minutos seguido de una mezcla de 4% paraformaldehído y 1% glutaraldheído en 0.1M de PBS. El cerebro fue removido y post-fijado en la misma solución fijadora durante 48 horas a 4ºC, seguido de una solución de 30% dextrosa para crioprotección. Se obtuvieron cortes coronales de 35 µm de secciones en donde se observará el hipocampo, los núcleos de la base, la corteza y el cerebelo usando un criostato (Leica, CM1850). Los cortes de cerebro fueron preincubadas en 1% H_2O_2 durante 10 minutos para bloquear la actividad endógena de la peroxidasa. Se realizó un lavado con Triton 1% en PBS, las rebanadas de cerebro fueron incubadas con el anticuerpo para GFAP (Abcam, ab4648, 1:2000), Iba-1 (Abcam, ab48004, 1:1500) o el receptor A_{2A} de adenosina (Abcam, ab3461, 1:2000). El anticuerpo fue diluido en Triton1% en PBS con suero normal de cabra toda la noche a 4ºC. Las rebanadas de cerebro fueron incubadas con los anticuerpos secundarios y después con el complejo de avidina-biotina (ABC kit, Vector Labs, PK6100). Al final, las rebanadas

de cerebro fueron tratadas con el kit de diaminobenzidina que contiene el substrato para la peroxidasa (Vector Labs, SK4100). Las rebanadas fueron deshidratadas y montadas para observarlas en el microscopio (Olympus, BX60) y obtener las imágenes utilizando un CCD (MediaCybernetics, Evolution VF) y el software de imágenes Image-Pro Plus software. Las imágenes obtenidas se analizaron sólo cualitativamente.

7.11 Análisis estadístico

Todos los grupos fueron comparados usando un análisis de varianza de dos vías (ANOVA), empleando como factor inter-sujeto el grupo y como factor intra-sujetos las regiones de interés. La prueba de ANOVA fue seguida con un test *post hoc* por códigos de contraste ortogonal. Para las comparaciones referentes al efecto de la restricción de sueño en los niveles de corticosterona se realizó una prueba t. Los valores de $p<0.05$ fueron considerados como diferencias significativas. Todos los valores se presentan como la media ± error estándar de la media. Para realizar los análisis estadísticos se empleó el software JMP (SAS Institute Inc., version 12.2.0).

Evaluación de la susceptibilidad del estado inflamatorio sistémico en la ruptura de la barrera hematoencefálica inducida por la restricción de sueño

Después de determinar el efecto del bloqueo de los receptores A_{2A} de adenosina en la integridad de la barrera hematoencefálica de ratas restringidas de sueño, se prosiguió a determinar si el efecto del estatus inflamatorio del individuo influía en los cambios morfológicos y funcionales de la barrera hematoencefálica asociados a la pérdida de sueño.

Con tal propósito se caracterizó el perfil de citocinas en ratones con respuesta inmunológica predominantemente inflamatoria (C57BL/6) y ratones con una respuesta inmunológica anti-inflamatoria deficiente (BALB/c) que estuvieron sometidos a restricción de sueño, también se realizaron ensayos de permeabilidad de barrera hematoencefálica, western blot para determinación de la expresión de proteínas de la unión ocluyente y de marcadores de neuroinflamación.

7.12 Ratones

Se emplearon ratones machos adultos (18-24g) de la cepa C57BL/c (n=29) y BALB/c (n=29). Los ratones BALB/c fueron proporcionados por el Instituto Nacional de Psiquiatría “Ramón de la Fuente” y los ratones C57BL/6 del Instituto de Neurobiología de la UNAM en Querétaro. Los animales fueron alojados en grupos de 3 o 6 individuos en la sala de animales del Área de Neurociencias en la Universidad Autónoma Metropolitana, Unidad Iztapalapa. Los ratones permanecieron en ciclo de luz oscuridad de 12/12 (luces apagadas a las 11 am) a una temperatura ambiente de entre 20ºC y 25ºC y tuvieron comida y agua *ad libitum* durante todo el periodo del experimento. Los ratones fueron azarosamente asignados a las condiciones experimentales y siempre se tuvo cuidado de proveer un ambiente adecuado para evitar condiciones estresantes. Todos los experimentos fueron realizados siguiendo la guía de cuidado y uso de Mamíferos en Neurociencias e Investigación Conductual, (National Research Council, 2010) y la guía ARRIVE (Animal Research: Reporting In Vivo Experiments, www.nc3rs. org.uk/arrive-guidelines). Adicionalmente, el proyecto fue aprobado por el Comité de Ética de la División de Ciencias Biológicas de la UAM Iztapalapa.

7.13 Restricción de sueño

Dada la naturaleza agresiva de los ratones C57BL/6 y la alta tasa de mortalidad de los ratones BALB/c en experimentos piloto, la restricción de sueño se realizó empleando el método de florero invertido. Los ratones fueron colocados de manera individual en cajas de acrílico con plataformas de 2.5cm de diámetro rodeadas de agua. Para evitar el estrés por inmovilización se colocaron 3 plataformas por cada animal. Los ratones permanecieron 20 horas en la tina de privación de sueño y tuvieron 4 horas de oportunidad para dormir (ultimas 4 horas de la fase de luz) en un periodo de 10 días. Durante el periodo de oportunidad para dormir los ratones fueron colocados con su grupo social estable para evitar el estrés por aislamiento social. Adicionalmente se realizó un control de procedimiento empleando plataformas grandes (10cm) en donde los ratones podían dormir, los animales estuvieron 20 horas en la tina y tuvieron 4 horas de oportunidad para dormir durante 10 días (Figura 6). Los animales fueron sacrificados al día 10 del procedimiento experimental, los controles fueron mantenidos en su caja habitación durante los 10 días de experimento.

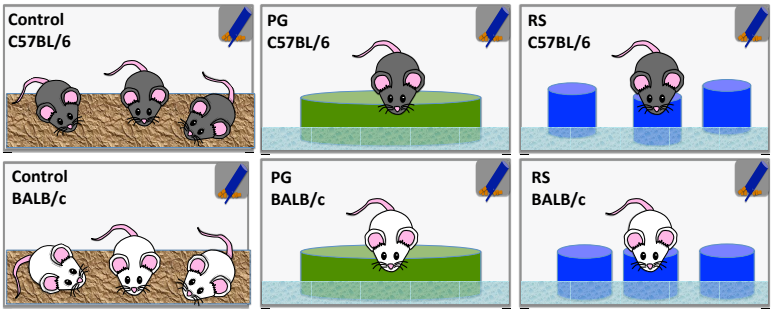

Figura 6. Grupos experimentales en ratones C57BL/6 y BALB/c. Control intacto, control de procedimiento de plataforma grande (PG) y grupo de restricción de sueño (RS).

7.14 Determinación del estado inflamatorio en suero de ratones restringidos de sueño

El kit “BD CBA (Cytometric Bead Array) Mouse Th1/Th2/Th17 Cytokine” (BD Biosciences, Catalogo No. 560485) permite la medición simultanea de citocinas características de la respuesta Th1/Th2/Th17 (IL-2, IL-6, IFN-γ, TNF-α, IL-4, IL-10, e IL-17A) en una sola muestra de suero. El ensayo de CBA es un método de captura para un conjunto de analitos solubles con perlas de peso y fluorescencia conocidos, haciendo posible la detección de los analitos usando un citometría de flujo. Cada perla de captura en el kit ha sido acoplada a un anticuerpo específico. El agente de detección proporcionado en el kit, es una mezcla de anticuerpos acoplados a ficoeritrina que proporciona una intensidad de señal proporcional a la cantidad del analito asociado. Cuando las perlas de captura y el reagente detector son incubados con la muestra de concentraciones desconocidas de los analitos que se pueden reconocer, se forma un complejo tipo sándwich perla de captura+analito+reagente de detección. Este kit provee siete poblaciones de perlas con distintas intensidades de fluorescencia que están acopladas a anticuerpos específicos para IL-2, IL-6, IFN-γ, TNF-α, IL-4, IL-10, e IL-17A. Las poblaciones de perlas están mezcladas para formar el ensayo de perlas, “bead Array”, lo cual puede ser determinado en un canal rojo (FL3 o FL4) del citómetro de flujo. Se obtuvo sangre de ratones C57BL/6 y BALB/c por decapitación en los grupos control (C), plataforma grande (PG) y restricción de sueño (RS) (n=11 por grupo en cada cepa). El suero fue centrifugado 5 minutos a 7000rpm. El sobrenadante (suero) fue colectado y tratado de acuerdo al kit “BD CBA Mouse Th1/Th2/Th17 Cytokine” (BD Biosciences, Catalogo No. 560485). Dado que no se

está evaluando una condición patológica se emplearon las muestras de suero sin diluir. Los datos fueron obtenidos utilizando el citómetro de flujo (FACS Aria III) para detectar las emisiones de fluorescencia entre 576 y 670 nm. Se empleó la curva estándar para determinar la concentración de cada citocina empleando el software FCAP Array™ v3.0.1.

7.15 Efecto de la restricción de sueño en la permeabilidad de la barrera hematoencefálica a azul de Evans en ratones C57BL/6 y BALB/c

Los ratones fueron anestesiados con pentobarbital sódico (ip. 0.1mL/30g de peso corporal) entre las 9 y las 11 am. La administración de azul de Evans fue realizada al final del periodo de restricción de sueño en el día 10 en los grupos C57BL/6 control (n=3), C57BL/6 PG (n=3), C57BL/6 RS (n=3), BALB/c control (n=3), BALB/c PG (n=3) y BALB/c RS (n=3). El colorante se administró por vía intracardiaca 0.1mL por 20g de peso corporal, el colorante circuló 5 minutos y los animales fueron perfundidos con solución salina 0.9% durante seguido de paraformaldehído 4% en PBS1X (5 minutos cada uno). Los cerebros fueron post fijados en la solución de paraformaldehído a 4ºC y fueron seccionados en cortes coronales de 2mm. La tinción del colorante en las rebanadas fue fotografiado con una cámara digital (Panasonic, Lumix). Las imágenes fueron transformadas a 8 bits (blanco y negro) en el programa ImageJ y se calculó la densidad óptica utilizando la fórmula Gray=0.299(red)+0.587(green)+0.114(blue) y empleando una tabla calibrada de densidad óptica (Rodbard) como está reportado previamente (Gómez-González et al., 2013).

7.16 Efecto de la restricción de sueño en la permeabilidad de la barrera hematoencefálica a moléculas de bajo peso molecular en ratones C57BL/6 y BALB/c

Los dextranos-FITC de 10kDa (Sigma Cat. FD10S) se suspendieron en PBS a una concentración de 3 mg/ml. El ensayo de permeabilidad se realizó en los siguientes grupos: C57BL/6 control (n=3), C57BL/6 PG (n=3), C57BL/6 RS (n=3), BALB/c control (n=3), BALB/c PG (n=3) y BALB/c RS (n=3). Los ratones fueron anestesiados con una sobredosis de pentobarbital sódico (ip. 0,063 g/kg de peso corporal). La administración de dextranos-FITC de 10 kDa se realizó por vía intracardiaca entre las 9-11 am (fase de luz). se administraron 0.1mL de dextranos-FITC por 20g. Después de 5 minutos de circulación de los dextranos-FITC, los ratones se perfundieron durante 5 minutos con solución salina (0,9% w/v) a una velocidad de 15mL/min. El cerebro fue extraído y disectado. El tejido fue pesado, protegido de la luz y colocado a -20ºC. Las muestras fueron procesadas el mismo día de su obtención. Las muestras se homogeneizaron con 150uL de PBS1X y se centrifugaron a una velocidad de 13500 rpm/10min. El sobrenadante se colectó y se determinó la absorbancia en un lector de ELISA (H Reader 1) a 495nm. La concentración de 10 kDa de dextranos-FITC se calculó utilizando una curva estándar. Los datos de las absorbancias en las muestras fueron transformados usando una ecuación polinomial de segundo grado resultante de los datos de la curva patrón. Una vez calculada la concentración de dextranos este valor fue dividido entre el peso de tejido. Los resultados se muestran como la concentración de dextranos-FITC por peso de tejido cerebral (mg/g).

7.17 Determinación de expresión de proteínas de barrera hematoencefálica y marcadores celulares

Para evaluar la expresión de proteínas de interés en el hipocampo, se obtuvo el cerebro por decapitación al día 10 del experimento de ratones en los siguientes grupos: C57BL/6 control (n=3), C57BL/6 PG (n=3), C57BL/6 RS (n=3), BALB/c control (n=3), BALB/c PG (n=3) y BALB/c RS (n=3). El hipocampo fue disectado y mantenido a -80ºC hasta su procesamiento. El hipocampo fue homogeneizado con 150uL de amortiguador RIPA que contenía inhibidores de proteasas y centrifugado a 13500rpm durante 10 minutos a 4ºC. El sobrenadante fue recolectado y alicuotado. La concentración total de proteínas se determinó tomando 2µL de la muestra en 18µL del mismo buffer, este ensayo se realizó por triplicado. Una vez preparadas todas las muestras se agregó 1mL de reactivo de Bradford previamente diluido en una concentración 1:5 en agua destilada (Biorad, Hercules, CA BioRad, cat. 500-0006). Se obtuvo la absorbancia de cada muestra por espectrofotometría a una longitud de onda de 595nm (Genesys20, Thermo Spectronic) y con base en una curva patrón realizada con concentraciones conocidas de inmunoglobulina G, se calculó el volumen requerido para tener 100µg de proteína de cada muestra, con la cual se realizó la electroforesis en membrana de SDS-acrilamida 30% bis. La electroforesis se realizó dejando correr las proteínas a 60V durante 30 minutos y después se cambió el voltaje a 120V durante 1 hora en frío. Las proteínas fueron transferidas a una membrana de PVDF en una cámara de transferencia durante 1 hora a 70V en frío. Las membranas se bloquearon con leche sin grasa al 5% en PBS/Tween0.1%. Posteriormente las membranas se incubaron con anticuerpos contra ocludina

(Invitrogen, 40–4700, 1:1000), claudina-5 (Abcam, ab53765, 1:1000), ZO-1 (Invitrogen, 40–2200, 1:1000), Iba-1 (Abcam, ab48004, 1:1000), A_{2A} AR (Abcam, ab3461, 1:1000), MMP-9 (Abcam, ab38898, 1:1000), y GAPDH (Abcam, ab8245, 1:1000) durante toda la noche a 4ºC. Después de 3 lavados con leche sin grasa al 1% en PBS/Tween 0.1% las membranas fueron incubadas durante 2 horas con el anticuerpo secundario correspondiente. Los 3 lavados con leche sin grasa al 1% en PBS/Tween 0.1% se repitieron y la membrana fue entonces incubada con solución AB que contenía peroxidasa de rábano y avidina (ABC kit, Vector Labs, PK6100). Las membranas fueron nuevamente lavadas 3 veces con leche sin grasa al % en PBS/Tween 0.1% y 1 vez con PBS. Las membranas fueron reveladas con un kit de luminiscencia (Amersham, RPN2232) y las bandas fueron determinadas con el programa C-digitTM usando un lector de geles (Versión 3.1, Licor®). Se empleó GAPDH como control de carga y los datos fueron normalizados con respecto al grupo control.

7.18 Análisis estadístico

Se realizó un análisis de varianza de dos vías (ANOVA) para comparar las diferencias entre grupos utilizando la cepa y la condición experimental como factores independientes. Se emplearon códigos de contraste ortogonal como prueba *post hoc* para comparar las diferencias entre grupos. El nivel de significancia estadística fue de $p<0.05$ para la prueba de ANOVA y la prueba *post hoc*. Los análisis estadísticos se realizaron utilizando el software JMP (SAS Institute Inc., versión 12.2.0). Los datos se presentan como la media ± el error estándar de la media.

8. RESULTADOS

Evaluación del efecto del bloqueo de receptores A_{2A} de adenosina en la integridad de la barrera hematoencefálica ratas restringidas de sueño.

8.1 La restricción de sueño no modifica los niveles periféricos de corticosterona en ratas

No se presentaron diferencias en los niveles séricos de corticosterona entre los grupos restringidos de sueño y el grupo control, así mismo, la administración de SCH58261 no modificó los niveles de corticosterona sérica en animales restringidos de sueño (Figura 7).

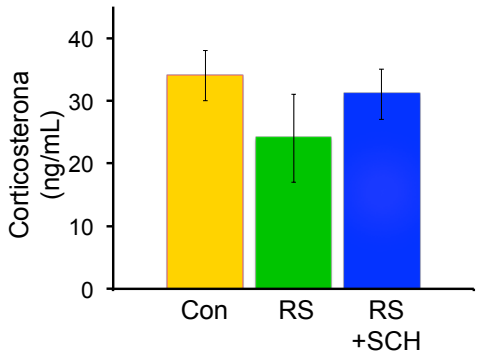

Figura 7. Efecto de la restricción de sueño sobre los niveles séricos de corticosterona. La gráfica muestra los niveles séricos de corticosterona en el grupo control (Con), restringido de sueño (RS) y de restricción de sueño con la administración de SCH58261 a una dosis de 0.1mg/kg (RS+SCH). Datos mostrados como la media+/- error estándar de la media.

8.2 El antagonista de los receptores A_{2A} de adenosina revierte la hiperpermeabilidad de la barrera hematoencefálica inducida por la restricción de sueño en ratas

En el estudio piloto se determinó que las dosis de 0.1, 0.5 y 1 mg/kg de peso de SCH58261 revertían el incremento en la permeabilidad de la barrera hematoencefálica al colorante azul de Evans (n=1 por grupo) (Figura 8) por lo que

se determinó que las dosis evaluadas serían 0.01, 0.1 y 0.5 mg/kg de peso de SCH58261.

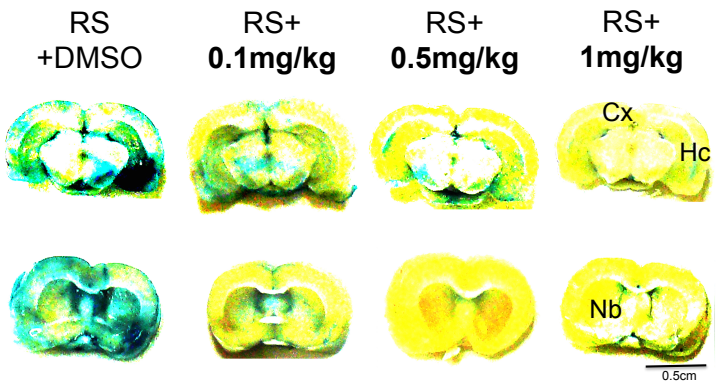

Figura 8. Ensayo de dosis respuesta del efecto de SCH58261 en la permeabilidad de la barrera hematoencefálica a azul de Evans en ratas restringidas de sueño. Cortes coronales de cerebro de rata en condiciones de restricción de sueño (10 días) con la administración de vehículo (DMSO) y 3 distintas dosis del antagonista selectivo de los receptores A_{2A} de adenosina, SCH58261, 0.1, 0.5 y 1mg/kg de peso corporal administradas cada 30 minutos (x3) al final del periodo de restricción de sueño. Cx: Corteza, Hc: Hipocampo, Nb: Núcleos de la base.

La restricción de sueño incrementó la permeabilidad de la barrera hematoencefálica a dextranos-FITC y la administración del antagonista del receptor A_{2A} de adenosina revirtió este efecto en casi todas las regiones evaluadas (Efecto de grupo, F_5= 16.756, $p<0.001$, Poder=0.869). Como se puede observar en la Figura 9, la restricción de sueño incremento la concentración de dextranos-FITC de 10kDa en la corteza ($p<0.001$), los núcleos de la base ($p= 0.003$), el hipocampo ($p=0.010$) y el vermis ($p<0.001$) con respecto al grupo control con vehículo. La dosis empleada de cafeína (0.3mg/kg de peso corporal) disminuyó la hiperpermeabilidad de la barrera hematoencefálica a dextranos-FITC de 10kDa inducida por la restricción de sueño en la corteza ($p =0.003$), núcleos

de la base (p=0.03) y vermis (p=0.01). Por otro lado, la administración de SCH58261 redujo la permeabilidad de la barrera hematoencefálica a dextranos-FITC de 10kDa en ratas restringidas de sueño de manera dependiente de la dosis en la corteza. La dosis 0.1mg/kg de peso corporal de SCH58261 mostró diferencias estadísticamente significativas con respecto al grupo de restricción de sueño en la corteza ($p<0.001$), núcleos de la base ($p=0.002$), hipocampo ($p=0.022$), y vermis ($p<0.001$) (Figura 9). La restricción de sueño también incrementó la permeabilidad de la barrera hematoencefálica a dextranos de alto peso molecular (70kDa) y el antagonista de los receptores A_{2A} de adenosina revirtió este efecto en todas las regiones evaluadas (Efecto Grupo x Región, $F_{3\text{-}9}= 3.409$, $p=0.004$, Poder=0.958). En la figura 9 se puede observar que la restricción de sueño incremento concentración de dextranos-FITC de 70kDa en la corteza ($p<0.001$), hipocampo ($p<0.001$), núcleos de la base ($p<0.001$) y vermis ($p<0.001$) en comparación con el grupo control con vehículo. El tratamiento con una sola dosis de cafeína revirtió el incremento en la concentración de dextranos-FITC de 70kDa solamente en el hipocampo ($p<0.001$), mientras que la administración del SCH58261 (0.1mg/kg (x3)) revirtió el incremento en la concentración de dextranos-FITC de 70kDa inducido por la restricción de sueño en la corteza ($p<0.001$), hipocampo ($p<0.001$), núcleos de la base ($p<0.001$) y vermis ($p = 0.025$) (Figura 10). La administración de SCH58261 (0.1mg/kg (x3)) también revirtió en incremento de la permeabilidad de la barrera hematoencefálica al colorante azul de Evans en ratas restringidas de sueño (Efecto de grupo, $F_2= 56.462$, $p<0.001$, Poder=0.999). Los valores de densidad óptica que indirectamente indican incremento en la concentración de azul de Evans en el

cerebro fueron mayores en todas las regiones cerebrales de sujetos restringidos de sueño ($p<0.001$) mientras que la administración de SCH58261 0.1mg/kg (x3) normalizó la permeabilidad de la barrera hematoencefálica ($p<0.01$) (Figura 11).

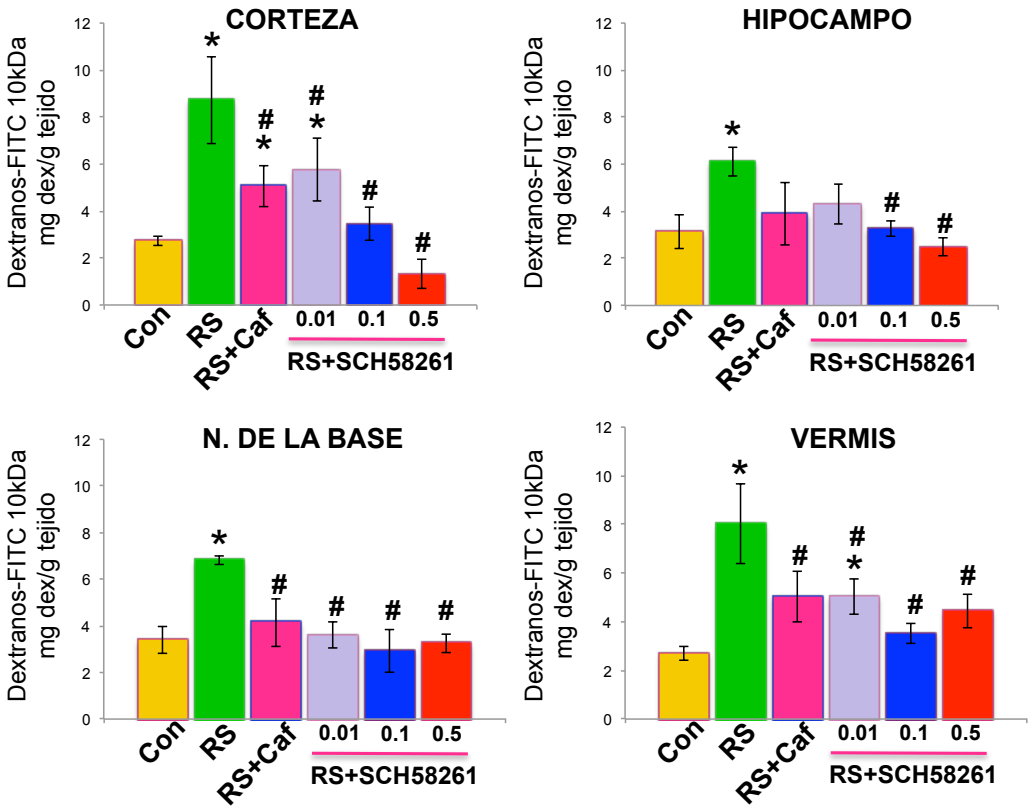

Figura 9. Efectos de la restricción de sueño y la administración de antagonistas de los receptores de adenosina en la permeabilidad de la barrera hematoencefálica a dextranos-FITC de bajo peso molecular (10kDa). Grupo control más DMSO (Con, n=4); restricción de sueño más DMSO (RS, n=3); restricción de sueño más 0.3 mg/kg de cafeína (Cafeína 0.3, n=3); restricción de sueño más 0.01 mg/kg (x3) de SCH58261 (0.01, n=3); restricción de sueño más 0.1 mg/kg (x3) de SCH58261 (0.1, n=3); restricción de sueño más de 0.5 mg/kg (x3) de SCH58261 (0.5, n=3). Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales.*$p<0.05$ con respecto al grupo control, #$p<0.05$ con respecto al grupo RS.

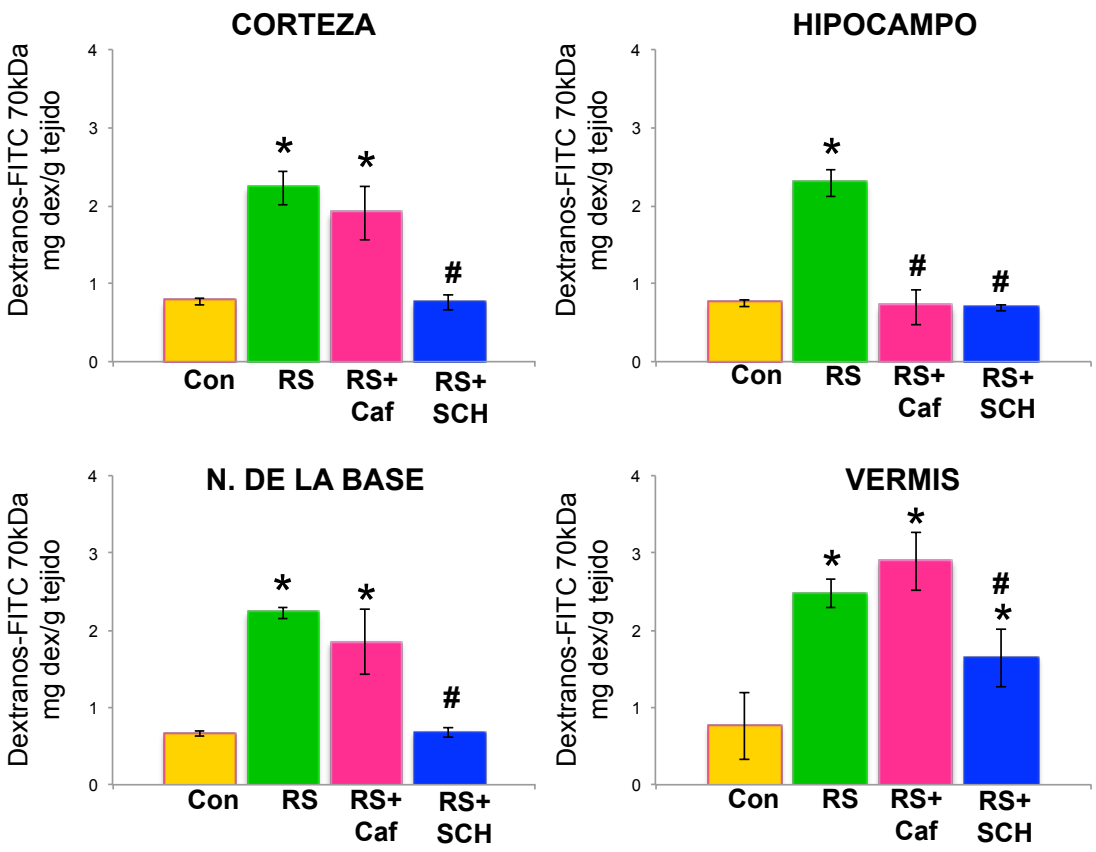

Figura 10. Efectos de la restricción de sueño y la administración de antagonistas de los receptores de adenosina en la permeabilidad de la barrera hematoencefálica a dextranos-FITC de alto peso molecular (70kDa). Grupo control más DMSO (Con, n=4); restricción de sueño más DMSO (RS, n=3); restricción de sueño, más 0.3 mg/kg de cafeína (RS+Caf, n=3); restricción de sueño, más 0.1 mg/kg (x3) de SCH58261 (RS+SCH, n=3). ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. Datos mostrados como la media +/- error estándar de la media. *p<0.05 con respecto al grupo control, #p<0.05 con respecto al grupo RS.

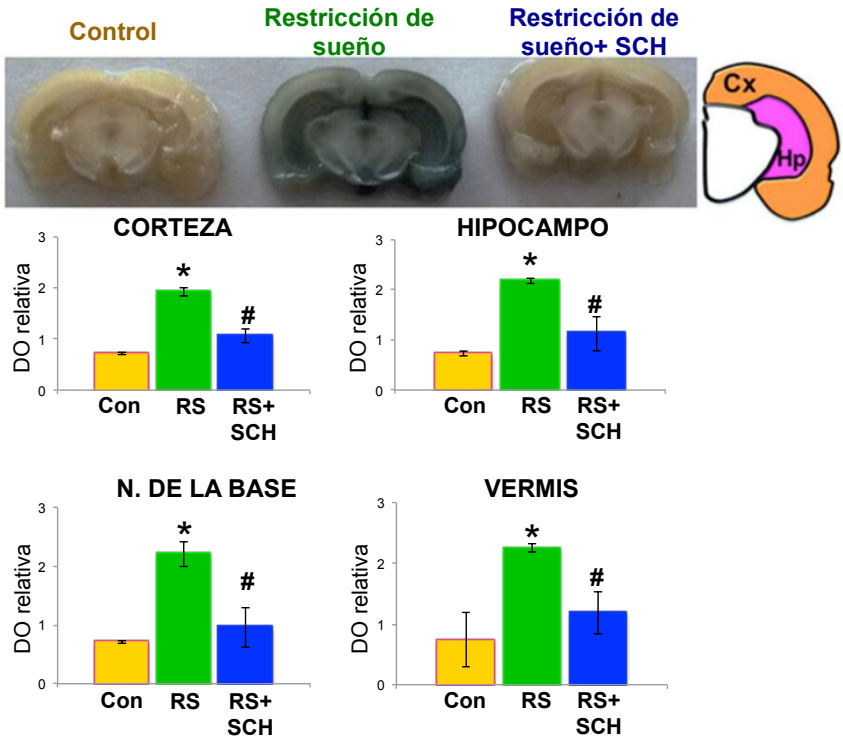

Figura 11. Efectos de la restricción de sueño y la administración de SCH59261 en la permeabilidad de la barrera hematoencefálica a azul de Evans. Grupo control más DMSO (Control+DMSO, n=3); restricción de sueño más DMSO (RS, n=3); restricción de sueño, más 0.1 mg/kg (x3) de SCH58261 (RS+SCH, n=3). Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales.*p<0.05 con respecto al grupo control, #p<0.05 con respecto al grupo RS.

8.3 El antagonista selectivo de los receptores A_{2A} de adenosina revierte la disminución en la expresión de proteínas de las uniones ocluyente inducida por la restricción de sueño en ratas

La dosis de 0.1mg/kg de SCH58261 tuvo un efecto consistente en la modulación de la permeabilidad de la barrera hematoencefálica en ratas restringidas de sueño, por ende, ésta fue la dosis óptima empleada para nuestro grupo con SCH58261. Encontramos que la expresión relativa de claudina-5 (23kDa) fue menor en la corteza (-50%, p=0.001), el hipocampo (-30%, p=0.011) y los núcleos de la base (-20%, p<0.05) en ratas restringidas de sueño con DMSO en comparación con los

controles intactos con DMSO y los restringidos de sueño con SCH58261 (Efecto por grupo x región, $F_{2\text{-}6} = 2.792$, $p = 0.018$, Poder =0.896). No se encontraron diferencias en la expresión de claudina-5 entre los grupos experimentales en el vermis del cerebelo (Figura 12). La expresión relativa de ZO-1 y ocludina fue dependiente de la región cerebral en ratas restringidas de sueño y restringidas de sueño con la administración de SCH58261 (Ocludina: Efecto de Grupo x Región, $F_{2\text{-}6} = 6.382$, $p<0.001$, Poder = 0.997; ZO-1: Efecto de Grupo x Región, $F_{2\text{-}6} = 5.551$, $p<0.001$, Poder = 0.993). Los niveles relativos de ocludina (63kDa) en la corteza disminuyeron en ratas restringidas de sueño en comparación con el grupo control ($p=0.001$) pero el tratamiento con SCH58261 no revirtió este efecto. En el caso de ZO-1 (225kDa), la restricción de sueño redujo la expresión relativa de ZO-1 en la corteza en un 46% en comparación con el grupo control ($p=0.001$), el tratamiento con SCH58261 restauró la expresión de ZO-1 a los niveles basales. En el hipocampo la expresión de ocludina disminuyó significativamente en el grupo de restricción de sueño ($p=0.001$) en comparación con el grupo control al igual que ZO-1 ($p=0.002$), el tratamiento con SCH58261 revirtió el efecto para ocludina pero no para ZO-1. No hubo diferencias en la expresión relativa de ocludina y ZO-1 en el grupo de restricción de sueño en los núcleos de la base; sin embargo, la administración de SCH58261 incrementó en un 64% los niveles de ocludina ($p<0.001$) y en un 54% ZO-1 con respecto al grupo de restricción de sueño con vehículo. No se observaron cambios en la expresión de ocludina y ZO-1 en el vermis (Figura 12).

El análisis para evaluar la expresión relativa de E-cadherina no mostró cambios en ninguno de los grupos experimentales. Se observa una tendencia de incremento

en la expresión relativa de esta proteína sólo en el hipocampo en el grupo de restricción de sueño pero sin diferencias estadísticamente significativas. El tratamiento con SCH58261 tampoco modificó los niveles de expresión de E-cadherina en ratas restringidas de sueño (Figura 13).

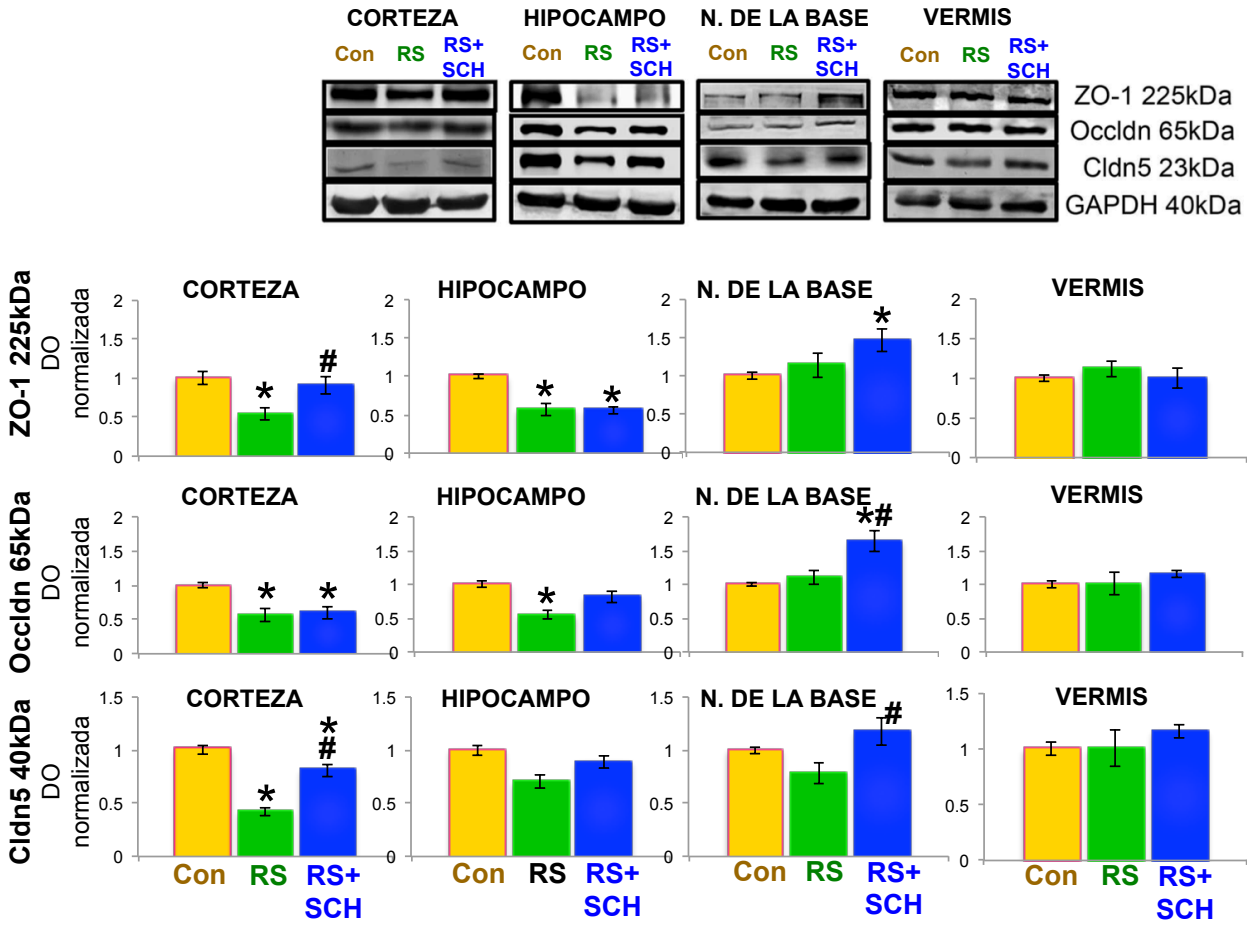

Figura 12. Efectos de la restricción de sueño y la administración de SCH59261 en la expresión relativa de proteínas de la unión ocluyente. Grupo control más DMSO (Con, n=3); restricción de sueño más DMSO (RS, n=3); restricción de sueño, más 0.1 mg/kg (x3) de SCH58261 (RS+SCH, n=3). Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales.*p<0.05 con respecto al grupo control, #p<0.05 con respecto al grupo RS.

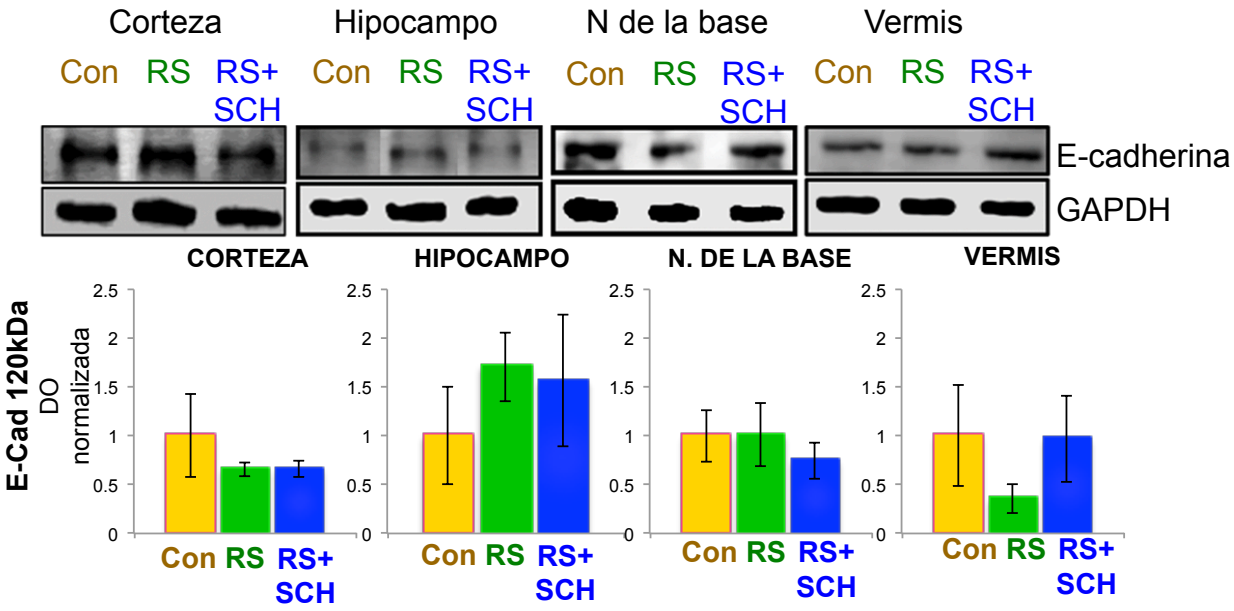

Figura 13. Efectos de la restricción de sueño y la administración de SCH59261 en la expresión relativa de E-cadherina. Grupo control más DMSO (Con, n=3); restricción de sueño más DMSO (RS, n=3); restricción de sueño, más 0.1 mg/kg (x3) de SCH58261 (RS+SCH, n=3). Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales.

8.4 El antagonista selectivo de los receptores A_{2A} de adenosina atenúa la sobreexpresión de marcadores celulares

La restricción de sueño incrementó los niveles de expresión relativa de Iba-1 (17kDa), un marcador de microglia reactiva, en corteza ($p<0.001$), hipocampo ($p<0.001$) y núcleos de la base ($p<0.001$) en comparación con el grupo control, este efecto fue revertido por la administración de SCH58261 (Efecto de Grupo x Región, $F_{2\text{-}6} = 10.621$, $p<0.001$, Poder = 0.862) (Figura 14). No hubo cambios en la expresión de Iba-1 en el vermis. La sobreexpresión de Iba-1 en los núcleos de la base fue corroborada por inmunohistoquímica como se puede observar en la Figura 15.

En el mismo sentido, la restricción de sueño incrementó la expresión relativa de GFAP (55kDa), un marcado de astroglia, y la administración de SCH58261 también revirtió este efecto (Efecto de Grupo x Región, $F_{2\text{-}6} = 4.538$, $p = 0.003$, Poder = 0.952). La restricción de sueño incrementó la expresión relativa de GFAP en corteza ($p=0.003$), hipocampo ($p=0.010$) y núcleos de la base ($p<0.001$), mientras que no tuvo ningún efecto en el vermis (Figura 16). Como se observa en la figura 17, las ramificaciones de la astroglia fueron más intensas en el grupo de restricción de sueño en la corteza, el hipocampo y los núcleos de la base.

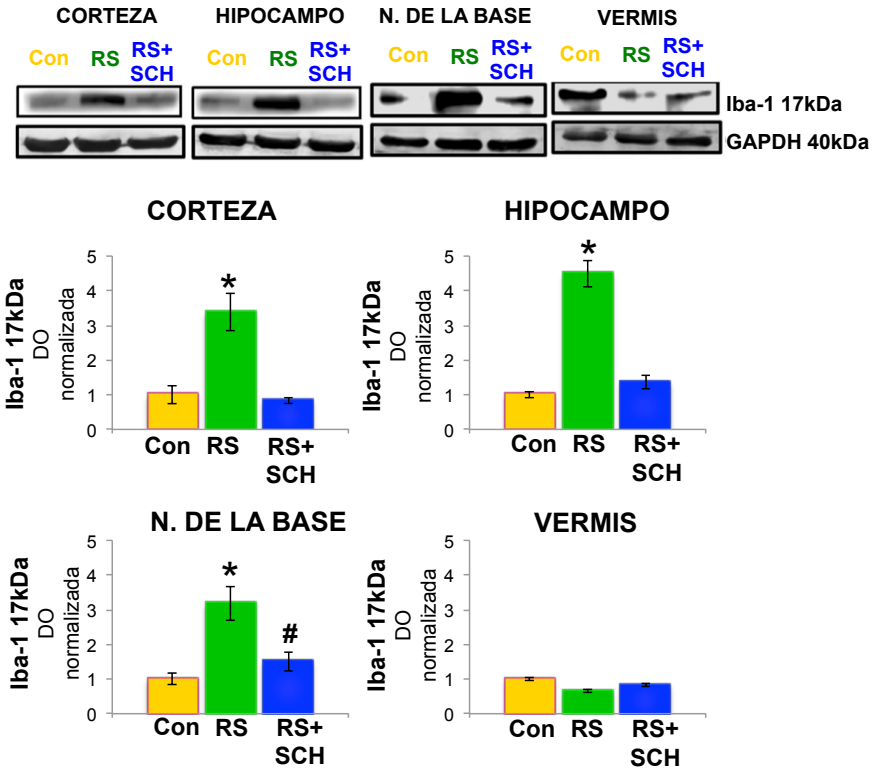

Figura 14. Efectos de la restricción de sueño y la administración de SCH59261 en la expresión relativa de Iba-1. Grupo control más DMSO (Con, n=3); restricción de sueño más DMSO (RS, n=3); restricción de sueño, más 0.1 mg/kg (x3) de SCH58261 (RS+SCH, n=3). Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control, #p<0.05 con respecto al grupo RS.

Figura 15. El antagonismo del receptor A_{2A} de adenosina atenúa la sobreexpresión de Iba-1 inducida por la restricción de sueño. Microfotografías que muestran la diferencia en el número de microglia reactiva en los núcleos de la base en el grupo control con vehículo DMSO (Control): Restricción de sueño y Restricción de sueño más 0.1mg/kg (x3) de SCH58261. X400.

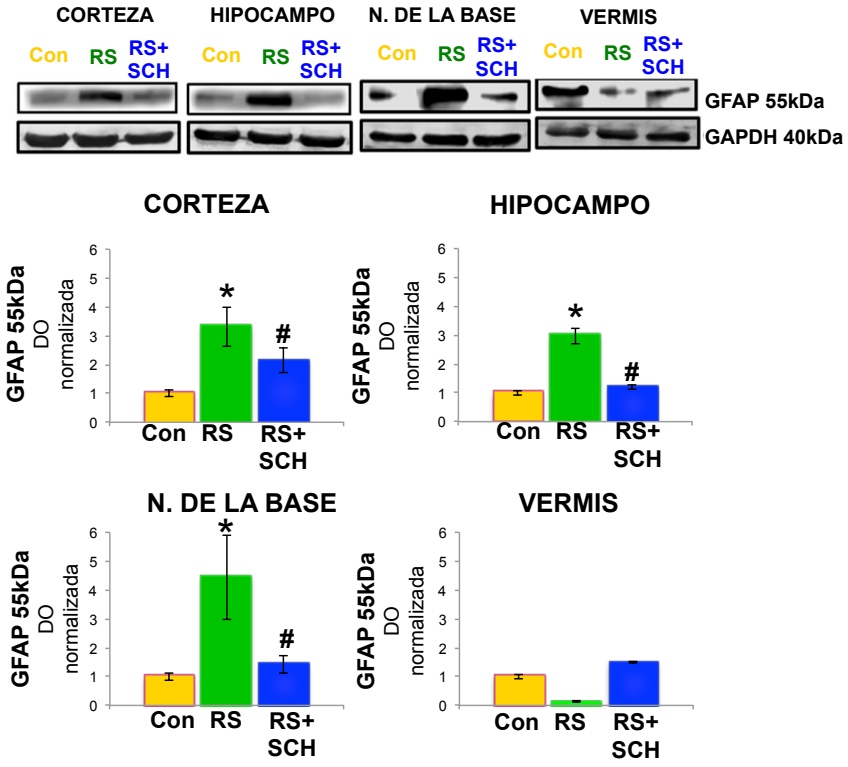

Figura 16. Efectos de la restricción de sueño y la administración de SCH59261 en la expresión relativa de GFAP. Grupo control más DMSO (Con, n=3); restricción de sueño más DMSO (RS, n=3); restricción de sueño, más 0.1 mg/kg (x3) de SCH58261 (RS+SCH, n=3). Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control, #p<0.05 con respecto al grupo RS.

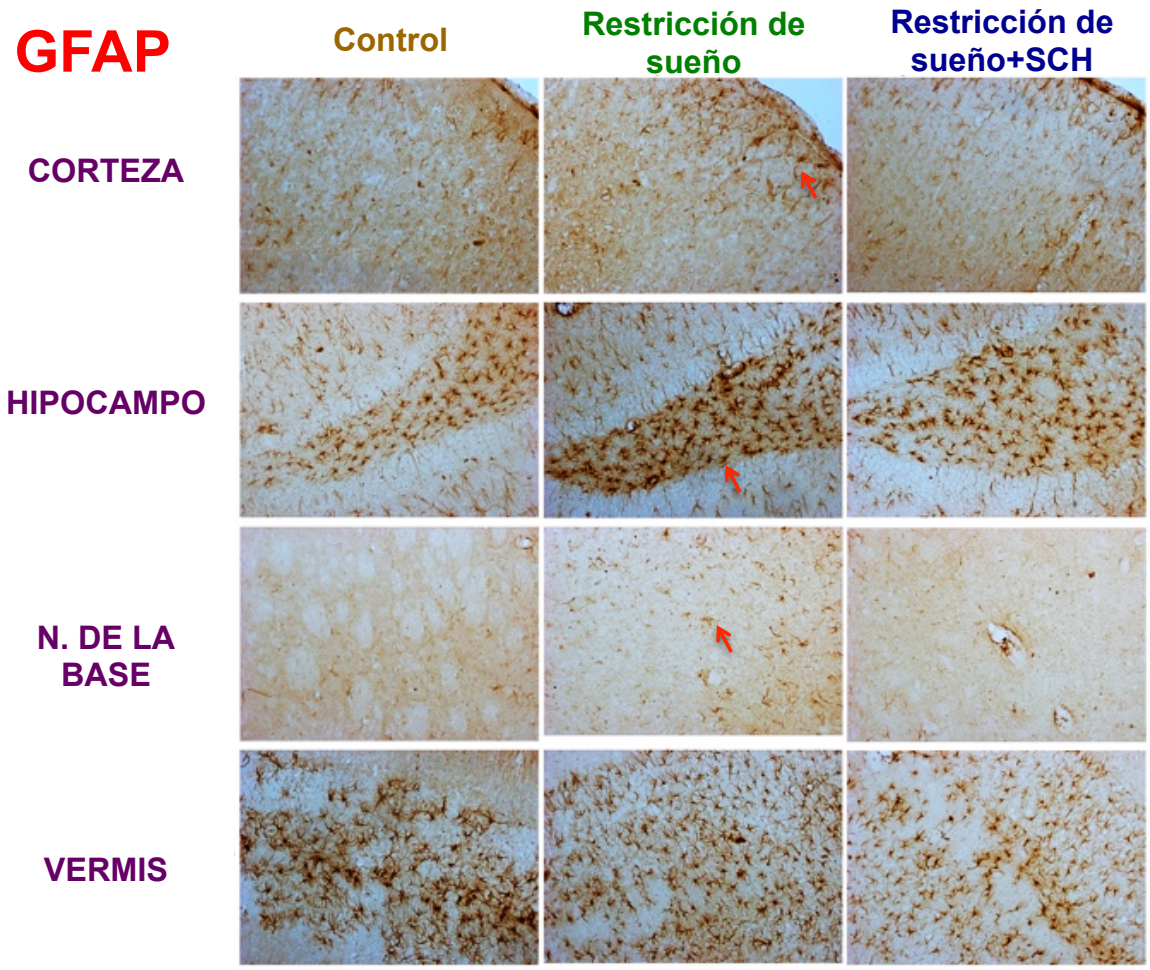

Figura 17. El antagonismo del receptor A_{2A} de adenosina revierte la hiperramificación de la astroglia inducida por la restricción de sueño. Las fotomicrografías muestran la morfología de la astroglia. Inmunohistoquímica realizada con GFAP. Imágenes de corteza, hipocampo, núcleos de la base y vermis en los siguientes grupos: control con vehículo DMSO (Control): Restricción de sueño y Restricción de sueño más 0.1mg/kg (x3) de SCH58261. X100.

8.5 La restricción de sueño modula la expresión del receptor A_{2A} de adenosina pero no la de la enzima CD73

Debido a que el efecto de la restricción de sueño y el tratamiento con SCH58261 sobre los parámetros evaluados fue dependiente de la región cerebral estudiada, la expresión del receptor A_{2A} de adenosina y de la enzima que convierte el AMP

en adenosina fue determinada en condiciones de restricción de sueño. Encontramos que la restricción de sueño modificó la expresión relativa del receptor A_{2A} de adenosina de manera diferencial en cada región evaluada (Efecto de Grupo x Región, $F_{1\text{-}3}$= 94.724, p<0.001, Poder = 0.999). El hipocampo y los núcleos de la base, la restricción de sueño incrementó al doble la expresión relativa del receptor A_{2A} de adenosina (p<0.001); no se encontraron cambios en la expresión del receptor en la corteza. La expresión del receptor A_{2A} de adenosina fue modulado a la baja por la restricción de sueño en el vermis (p<0.001) (Figura 18). El efecto de la restricción de sueño sobre la expresión del receptor A_{2A} de adenosina fue confirmado por las imágenes obtenidas usando inmunohistoquímica, en donde se puede observar que el receptor se encuentra en el parénquima cerebral (Figure 19).

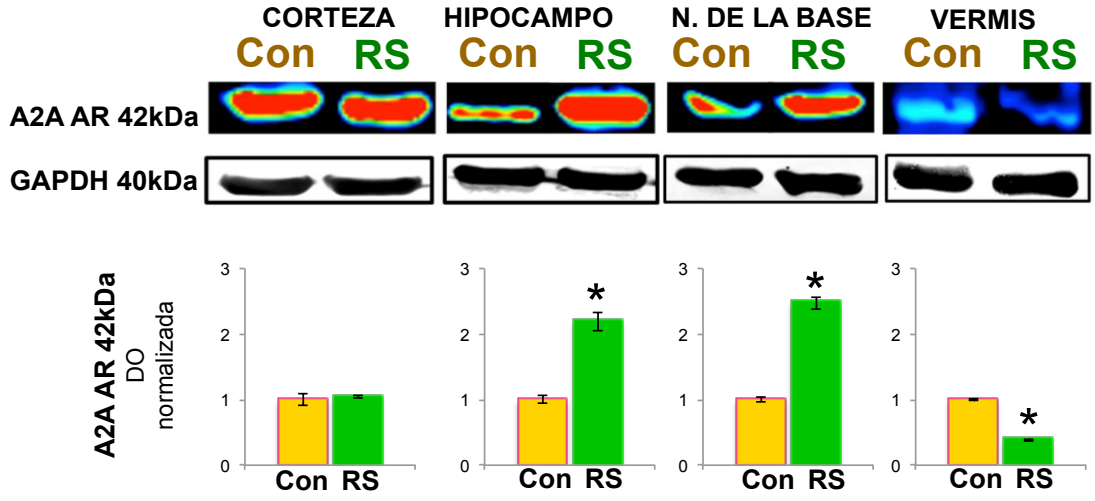

Figura 18. Efectos de la restricción de sueño en la expre8ión relativa del receptor A_{2A} de adenosina. Western blot representativo de la expresión del receptor A_{2A} de adenosina; abajo, gráficas de densidad óptica relativa del receptor A_{2A} de adenosina. Grupo control más DMSO (Con, n=3); restricción de sueño más DMSO (RS, n=3). Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control, #p<0.05 con respecto al grupo RS.

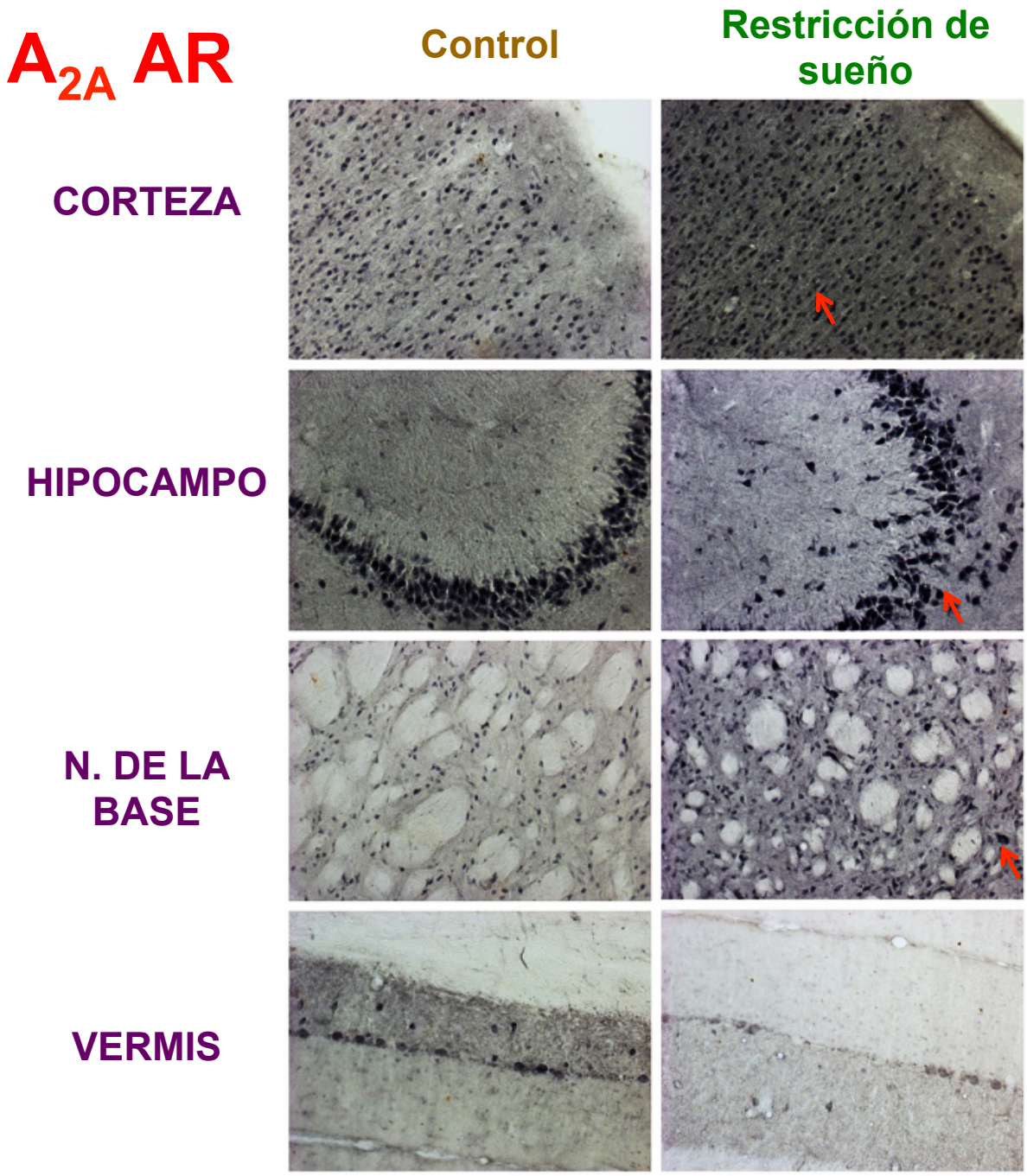

Figura 19. La restricción de sueño modifica la expresión del receptor A_{2A} de adenosina en el parénquima cerebral. Las fotomicrografías muestran la distribución del receptor A_{2A} de adenosina en la corteza, el hipocampo, núcleos de la base y vermis en los siguientes grupos, control con vehículo DMSO (Control) y el grupo de Restricción de sueño. X100. Nótese el incremento en la expresión del receptor en la corteza, hipocampo y núcleos de la base (flechas en rojo).

Al evaluar por western blot la expresión de CD73, encontramos que la restricción de sueño no modifica significativamente la expresión de esta enzima en las regiones evaluadas (Figura 20).

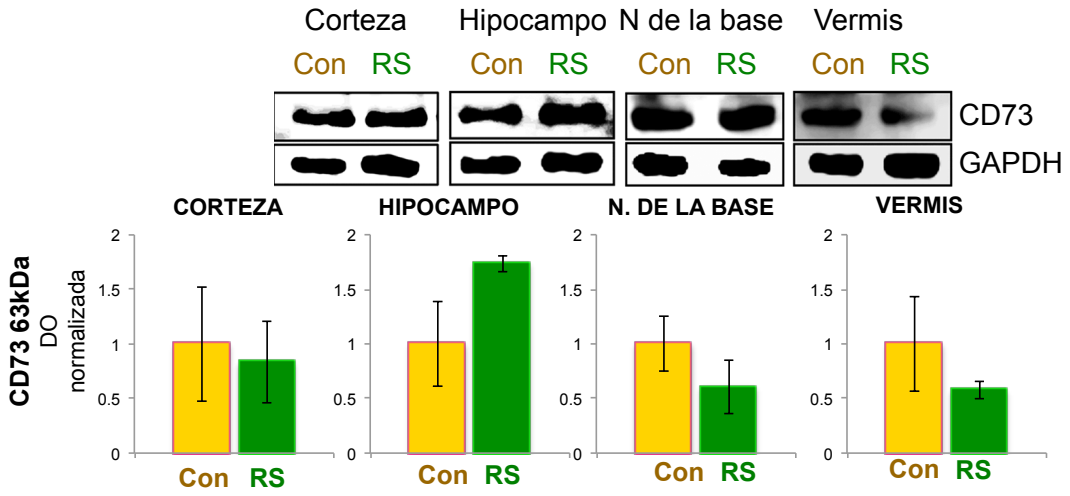

Figura 20. Efectos de la restricción de sueño en la expresión relativa de CD73. Grupo control más DMSO (Con, n=3); restricción de sueño más DMSO (RS, n=3). Datos mostrados como la media +/- error estándar de la media.

Evaluación de la susceptibilidad del estado inflamatorio sistémico en la ruptura de la barrera hematoencefálica inducida por la restricción de sueño

8.6 La restricción de sueño induce un estado inflamatorio sistémico de bajo grado en ratones C57BL/6 pero no en ratones BALB/c

La restricción de sueño indujo efectos diferenciales en ratones C57BL/6 y BALB/c (Figura 21) en las citocinas proinflamatorias TNF-α (F_{3-33}=5.774 p=0.008) e IFN-γ (F_{3-33}=10.148 p<0.001) (Figura 21A). La restricción de sueño incrementó en un 454% los niveles séricos de TNF-α en ratones C57BL/c en comparación con ratones control de la misma cepa (control=0.65+/-0.31pg/mL *versus* RS=2.97+/-0.82pg/mL) (p=0.015) e incrementó 418% en comparación con el grupo de plataforma grande de la misma cepa (PG=0.71+/-0.29pg/mL) (p=0.018). La restricción de sueño no modificó los niveles de TNF-α en ratones BALB/c en comparación con su grupo control (Figure 21A). En el mismo sentido, los niveles séricos de IFN-γ en ratones C57BL/6 restringidos de sueño fue mayor que el grupo control (control=0.37+/-0.16 pg/mL *versus* RS=4.58+/-1.17 pg/mL) (p=0.001) y el grupo de plataforma grande (PG=1.15+/-0.28 pg/mL) (p=0.05); nuevamente, la restricción de sueño no modificó los niveles de IFN-γ en ratones BALB/c restringidos de sueño en comparación con el grupo control (Figure 21A). El análisis de los resultados no mostró diferencias en los niveles séricos de IL-2, IL-4, IL-6, IL-10, e IL-17A entre los grupos de restricción de sueño y los controles en ratones C57BL/6 y BALB/c (Figure 21).

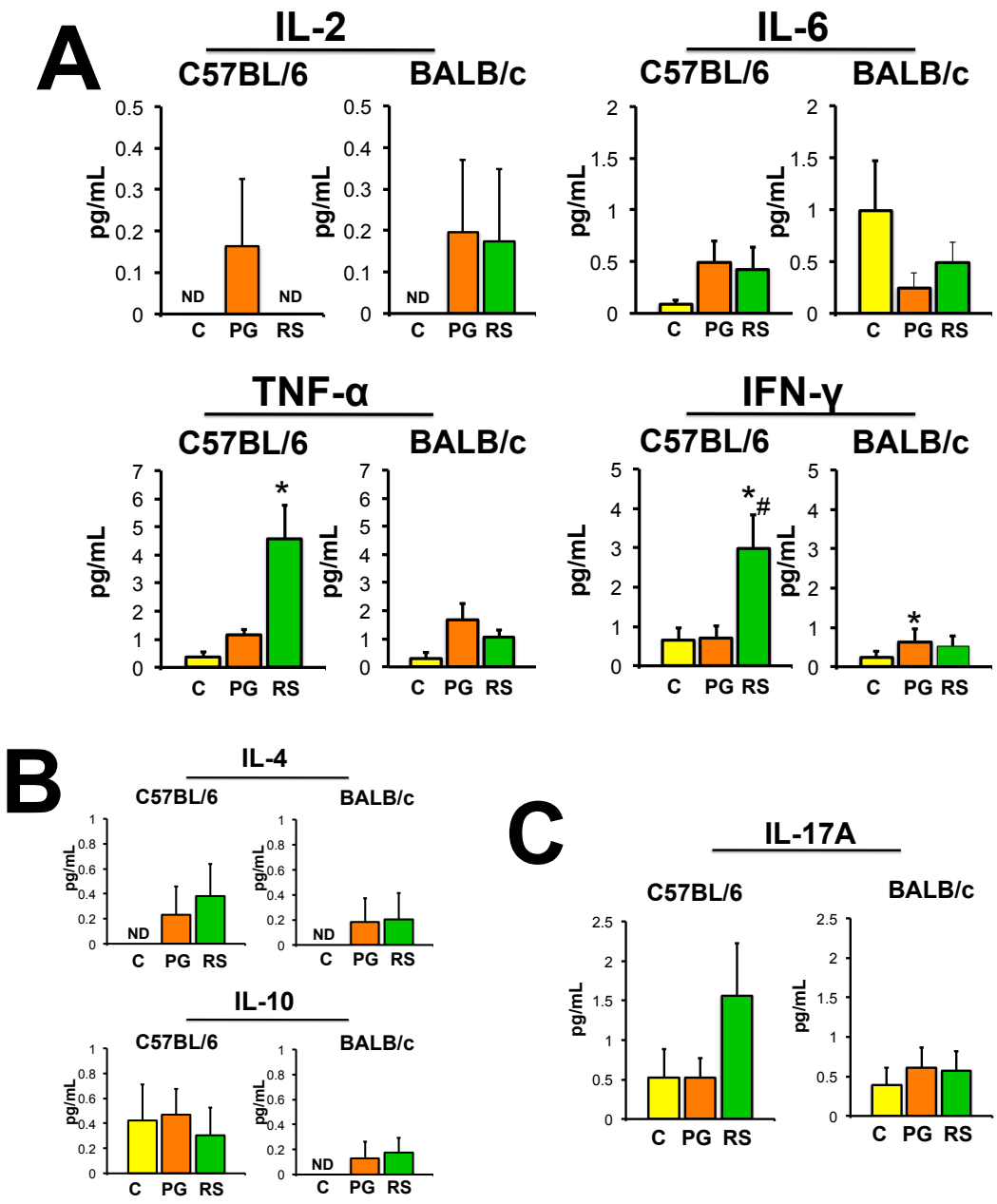

Figura 21. Perfil de citocinas en ratones C57BL/6 y BALB/c al día 10 de la restricción de sueño. Las gráficas muestran los niveles séricos (pg/mL) de citocinas características de la respuesta Th1 (A), Th2 (B) y Th17 (C) en ratones C57BL/6 y BALB/c del grupo control intacto (C), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media+/- el error estándar de la media (n=11 por grupo en cada cepa). Los datos se muestran como la media+/- el error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control, #p<0.05 con respecto al grupo RS.

8.7 La restricción de sueño incrementa la permeabilidad de la barrera hematoencefálica en ratones C57BL/6 pero no en ratones BALB/c

La restricción de sueño modificó de manera diferencial la permeabilidad de la barrera hematoencefálica a moléculas de bajo peso molecular (dextranos de 10kDa) y de alto peso molecular (azul de Evans). La restricción de sueño incrementó la permeabilidad dela barrera hematoencefálica a azul de Evans en el hipocampo, la corteza, los núcleos de la base y el cerebelo ($p<0.01$) sólo en ratones C57BL/6 pero no en ratones BALB/c (Figura 22 y 23). En el caso de dextranos de 10kDa, la restricción de sueño incrementó la permeabilidad de la barrera hematoencefálica en el hipocampo ($F_{1\text{-}2}=7.144$ $p=0.009$) y en la corteza ($F_{1\text{-}2}=3.786$ $p=0.053$) de ratones C57BL/6 en comparación con los grupos control pero no en ratones BALB/c (Figura 24)

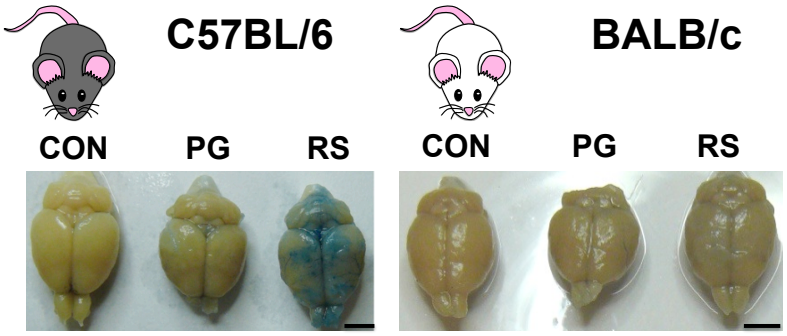

Figura 22. La restricción de sueño modifica la permeabilidad de la barrera hematoencefálica a moléculas de alto peso molecular en ratones C57BL/6 pero no en ratones BALB/c. Imagen representativa de cerebros obtenidos de ratones a los que se les administró por vía sistémica azul de Evans (>69kDa). Control intacto (CON), plataforma grande (PG) y restricción de sueño (RS).

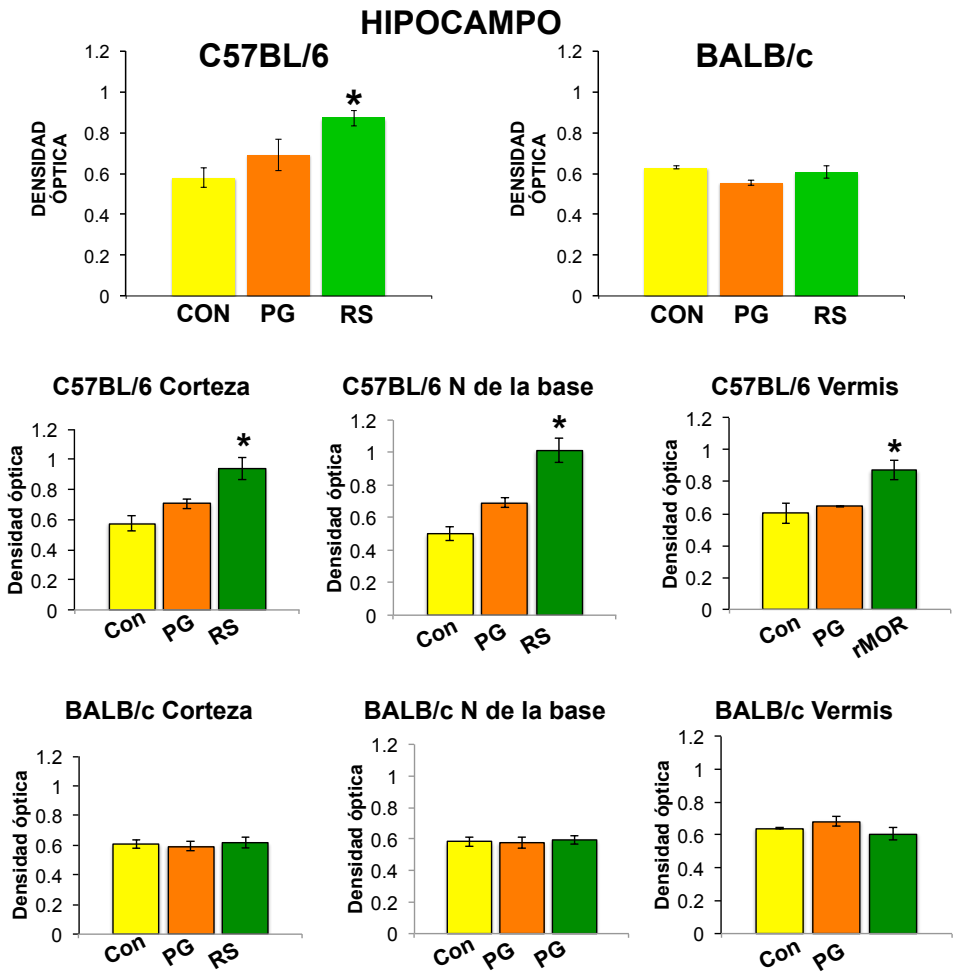

Figura 23 La restricción de sueño modifica la permeabilidad de la barrera hematoencefálica a moléculas de alto peso molecular en ratones C57BL/6 pero no en ratones BALB/c. Las gráficas muestran los niveles de densidad óptica relativa en ratones C57BL/6 y BALB/c del grupo control intacto (CON), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media+/- el error estándar de la media. Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control.

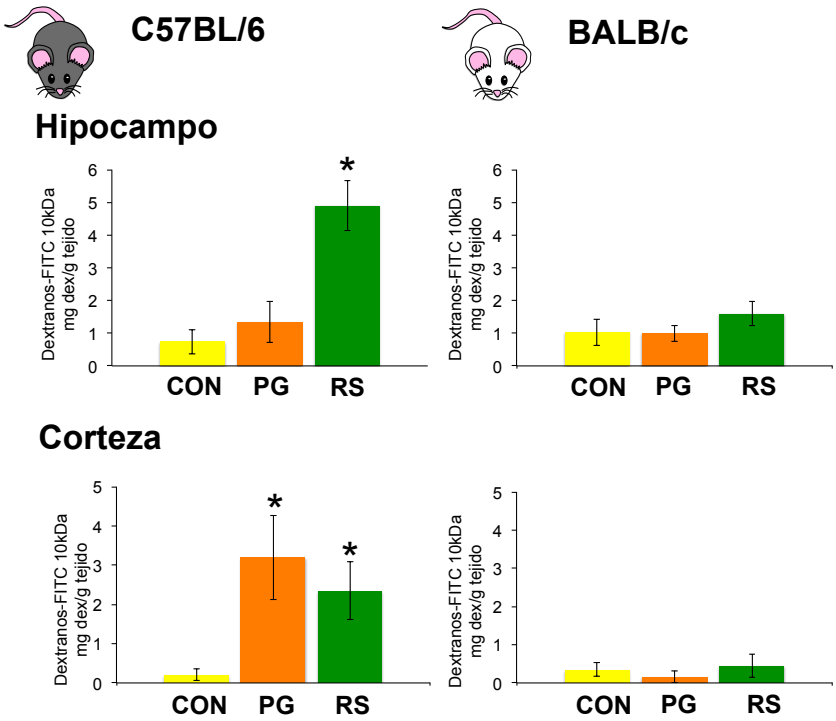

Figura 24. La restricción de sueño modifica la permeabilidad de la barrera hematoencefálica a moléculas de bajo peso molecular en ratones C57BL/6 pero no en ratones BALB/c. Las gráficas muestran la concentración en la corteza y en el hipocampo de dextranos-FITC de 10kDa en ratones C57BL/6 y BALB/c del grupo control intacto (CON), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media+/- el error estándar de la media. Datos mostrados como la media +/- error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control.

8.8 La restricción de sueño modifica la expresión de proteínas de la unión ocluyente en ratones C57BL/6 y BALB/c

En el hipocampo, la expresión relativa de ZO-1 incrementó sólo en el grupo de plataforma grande en los ratones C57BL/6 en comparación con el grupo control (p=0.031) y el de RS (p=0.027) ($F_{3\text{-}9}$=8.258 p=0.019). No hubo cambios en la expresión relativa de ZO-1 en ratones BALB/c. En cuanto a la expresión de ocludina, ésta sólo incrementó en el grupo de restricción de sueño de ratones BALB/c en comparación con el grupo control (p=0.0.048) ($F_{3\text{-}9}$=6.150 p=0.035) y

no hubo variaciones en ratones C57BL/6. La proteína fundamental para el mantenimiento de las uniones ocluyentes, claudina-5, incrementó en el grupo de restricción de sueño en los ratones BALB/c en comparación con el grupo control (p=0.0.021) ($F_{3\text{-}9}$=7.411 p=0.024) y no se modificó en los grupos experimentales de ratones C57BL/6 (Figura 25). Como un experimento exploratorio, evaluamos la expresión de proteínas de la unión ocluyente en corteza, núcleos de la base y vermis en el grupo control y en el grupo de restricción de sueño. En la corteza y los núcleos de la base, la restricción de sueño en ratones C57BL/6 disminuyó la expresión de ocludina y ZO-1 mientras que en ratones BALB/c incrementó la expresión de ZO-1 pero no de ocludina en la corteza y no hubo cambios en la expresión de estas proteínas en los núcleos de la base. La disminución de ZO-1 pero no de ocludina también se presentó en el vermis de ratones C57BL/6 restringidos de sueño, no hubo cambios en la expresión de estas proteínas en ratones BALB/c restringidos de sueño (Figura 26).

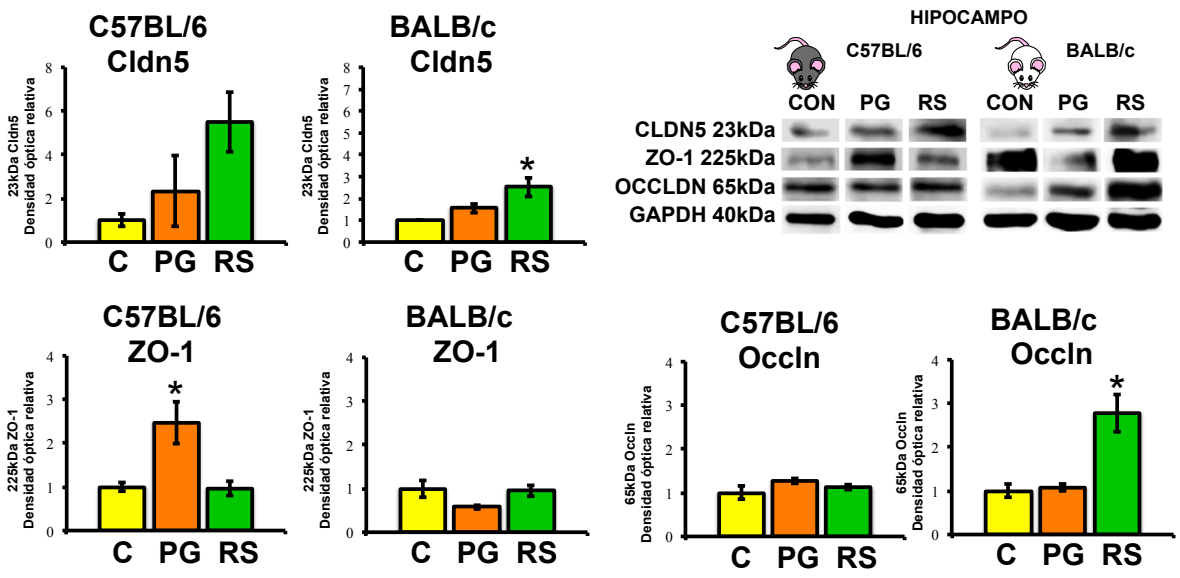

Figura 25. Efecto de la restricción de sueño sobre la expresión de proteínas de la unión ocluyente en ratones C57BL/6 y BALB/c. Las gráficas muestran la expresión relativa de proteínas de la unión ocluyente claudina-5 (CLDN5), ocludina (OCCLDN) y ZO-1 en hipocampo de ratones C57BL/6 y BALB/c del grupo control intacto (C), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media+/- el error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control.

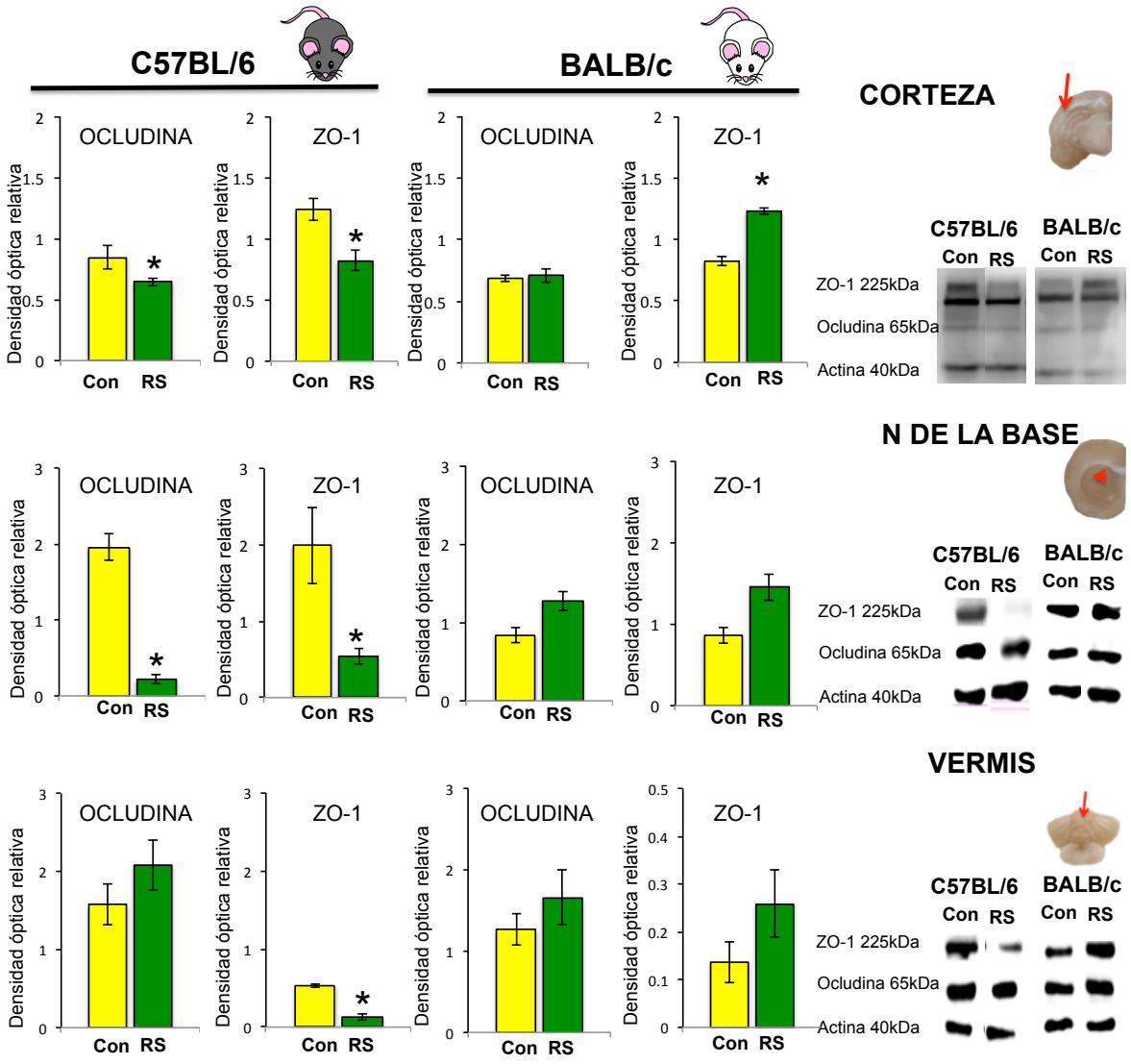

Figura 26. Efecto de la restricción de sueño en la expresión de proteínas de la unión ocluyente en corteza, vermis y núcleos de la base de ratones C57BL/6 y BALB/c. Las gráficas muestran los niveles de densidad óptica relativa en ratones C57BL/6 y BALB/c del grupo control intacto (CON), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media+/- el error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control.

La expresión de glicoproteína p (Pgp) sólo se modificó en el grupo de plataforma grande de los ratones C57BL/6 en comparación con el grupo control (p=0.0.04) ($F_{3\text{-}9}$=6.41 p=0.034), no se observaron cambios en la expresión de esta proteína en ratones BALB/c (Figura 27).

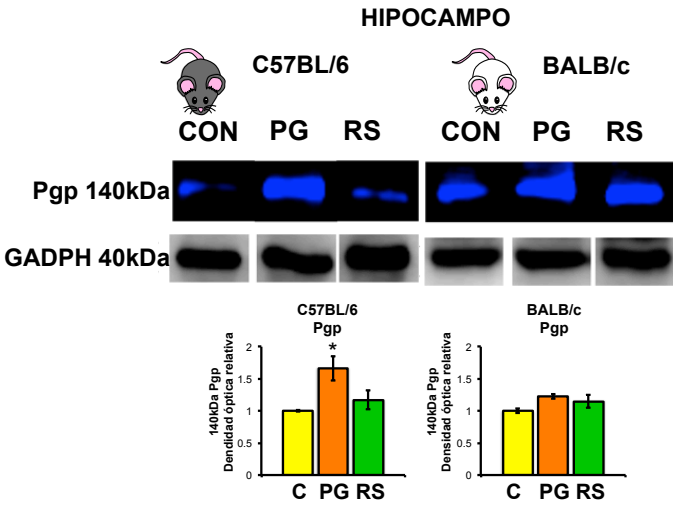

Figura 27. Efecto de la restricción de sueño sobre la expresión de Pgp en ratones C57BL/6 y BALB/c. Las gráficas muestran la expresión relativa de glicoproteína p (Pgp) en hipocampo de ratones C57BL/6 y BALB/c del grupo control intacto (C), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media+/- el error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control.

8.9 La restricción de sueño modifica diferencialmente la expresión del receptor A_{2A} de adenosina y MMP-9 en el hipocampo de ratones C57BL/6 y BALB/c

Dado que se observó un estado proinflamatorio de bajo grado en ratones C57BL/6 pero no en ratones BALB/c restringidos de sueño, determinamos la expresión de marcadores neuroinflamatorios en el hipocampo de ambas cepas. Como marcador de microglia reactiva se empleó Iba-1 y el receptor A_{2A} de adenosina como un modulador clave de la barrera hematoencefálica durante la pérdida de sueño (Hurtado-Alvarado et al., 2016a). La restricción de sueño modificó la expresión de los receptores A_{2A} de adenosina ($F_{1\text{-}2=}5.946$ p=0.016) e Iba-1 ($F_{1\text{-}2}$=22.272 p<0.0001) en el hipocampo de ratones C57BL/6 y BALB/c. En ratones C57BL/6 la expresión de Iba-1 incrementó en comparación con el grupo control (3.839 p=0.0024) y con el grupo de plataforma grande (t=2.998 p=0.011) (Figura 28). Por

el contrario, en ratones BALB/c la restricción de sueño (t=5.407 p=0.002) y la plataforma grande (5.424 p=0.002) disminuyeron la expresión de Iba-1 en comparación con el grupo control. En cuanto a la expresión del receptor A_{2A} de adenosina, la restricción de sueño incrementó la expresión de este receptor en ratones C57BL/6 en comparación con el grupo control de la misma cepa (t=5.325 p=0.001) mientras que en ratones BALB/c la expresión del receptor A_{2A} de adenosina disminuyó en comparación con el grupo control (t=2.359 p=0.036) y el control de plataforma grande (t=2.410 p=0.032) (Figura 29).

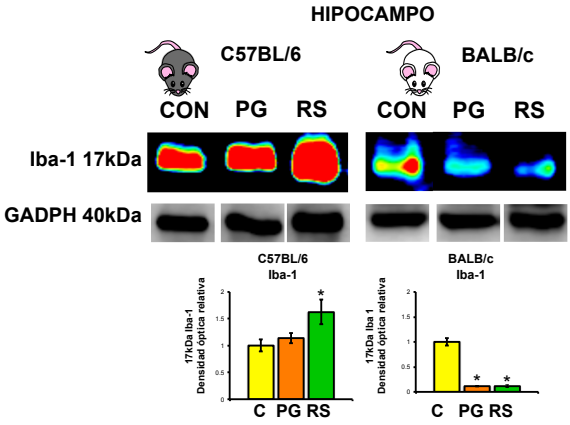

Figura 27. Efecto de la restricción de sueño sobre la expresión de Iba-1 en ratones C57BL/6 y BALB/c. Las gráficas muestran la expresión relativa de Iba-1 en hipocampo de ratones C57BL/6 y BALB/c del grupo control intacto (C), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media +/- el error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control.

Finalmente, dado que la restricción de sueño altera la ultraestructura de las uniones ocluyentes (Hurtado-Alvarado et al., 2017) evaluamos el efecto de la restricción de sueño sobre la expresión de la metaloproteínasa de matriz 9 (MMP-9) que se ha descrito está involucrada en procesos de degradación de uniones ocluyentes. Nuevamente encontramos efectos diferenciales de la restricción de sueño en ambas cepas de ratones. En el hipocampo de ratones C57BL/6

restringidos de sueño se incrementó la expresión de MMP-9 en comparación con el grupo control (t=2.559 p=0.025) y el grupo de plataforma grande (t=2.751 p=0.017). Los niveles de expresión de MMP-9 de ratones BALB/c no fueron diferentes al grupo control (Figura 29)

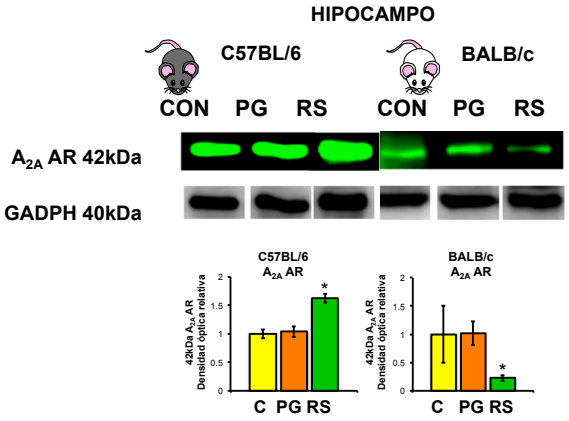

Figura 28. Efecto de la restricción de sueño sobre la expresión del receptor A_{2A} de adenosina en ratones C57BL/6 y BALB/c. Las gráficas muestran la expresión relativa del receptor A_{2A} de adenosina en hipocampo de ratones C57BL/6 y BALB/c del grupo control intacto (C), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media+/- el error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control.

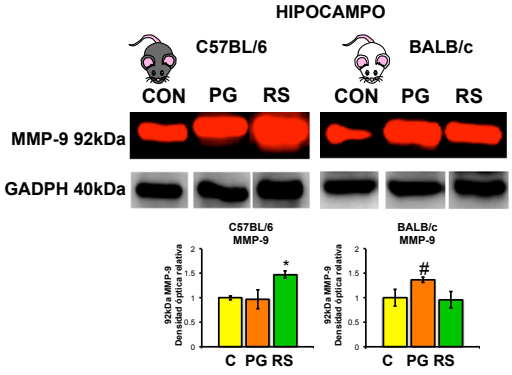

Figura 29. Efecto de la restricción de sueño sobre la expresión de MMP-9 en ratones C57BL/6 y BALB/c. Las gráficas muestran la expresión relativa de la metaloproteínasa de matriz-9 (MMP-9) en hipocampo de ratones C57BL/6 y BALB/c del grupo control intacto (C), plataforma grande (PG) y restricción de sueño (RS). Los datos se muestran como la media+/- el error estándar de la media. ANOVA de dos vías, prueba *post hoc* códigos de contraste ortogonales. *p<0.05 con respecto al grupo control.

9. DISCUSIÓN

La restricción de sueño incrementó la permeabilidad de la barrera hematoencefálica a dextranos-FITC y al colorante azul de Evans. Los cambios en la permeabilidad de la barrera hematoencefálica fueron observado para moléculas de bajo y alto peso molecular (10 y 70kDa) en la corteza, el hipocampo, los núcleos de la base y el cerebelo de ratas restringidas de sueño en comparación con las ratas que durmieron *ad libitum*. La administración de un antagonista no selectivo de los receptores de adenosina, cafeína, revirtió la hiperpermeabilidad a trazadores de bajo peso molecular en todas las regiones cerebrales evaluadas pero lo hizo parcialmente a moléculas de alto peso molecular. De hecho, la cafeína revirtió la ruptura de la barrera hematoencefálica inducida por la restricción de sueño sólo en el hipocampo mientras que la administración de un antagonista selectivo de los receptores A_{2A} de adenosina (SCH58261) restauró por completo la integridad de la barrera hematoencefálica en la corteza, el hipocampo y los núcleos de la base. A pesar de que la administración de la dosis seleccionada (0.1mg/kg) revirtió el incremento de la barrera hematoencefálica con respecto al grupo de restricción de sueño, no se restauró por completo la función normal de la barrera hematoencefálica en el vermis. En incremento en la permeabilidad de la barrera hematoencefálica en el hipocampo, la corteza y los núcleos de la base correlacionan con la disminución de proteínas de la unión ocluyente. La restricción de sueño no modificó la expresión de E-cadherina, una proteína que forma parte de las uniones adherentes. El uso del antagonista selectivo de los receptores A_{2A} de adenosina revirtió la ruptura de la barrera hematoencefálica inducida por la pérdida de sueño y restauró la expresión basal de proteínas de la unión ocluyente

en el cerebro de ratas restringidas de sueño. Las diferencias regionales en la permeabilidad de la barrera hematoencefálica asociadas a la restricción de sueño así como la respuesta al SCH58261 puede estar relacionado con las diferencias de expresión del receptor A_{2A} de adenosina así como con su localización especifica en glía o células endoteliales ya que no encontramos diferencias en la expresión de CD73 que es la enzima encargada de sintetizar adenosina a partir de AMP.

El paso de moléculas pequeñas (5-40 kDa) de la sangre hacia el cerebro es un marcador de permeabilidad de la barrera hematoencefálica a solutos pequeños y a iones mientras que el complejo de Azul de Evans y albúmina (65 kDa) es un marcador de permeabilidad a proteinas (Hoffmann et al., 2011). En ese sentido, los resultados indican que la restricción de sueño puede modificar la permeabilidad de la barrera hematoencefálica a iones y a proteínas y que el bloqueo de los receptores A_{2A} de adenosina revierten ambos efectos. Resultados similares han sido encontrados en animales con encefalomielitis autoinmune experimental en donde el incremento en la permeabilidad de la barrera hematoencefálica es revertido por el uso del antagonista de los receptores A_{2A} de adenosina (Carman et al., 2011).

Los receptores de adenosina están ampliamente distribuidos en el cerebro, particularmente el receptor A_{2A} de adenosina está expresado en mayor cantidad en los núcleos de la base seguido de hipocampo, corteza y una baja expresión en cerebelo (Dixon et al., 1995; DeMet et al., 2002). La sobreexpresión de A_{2A} en el hipocampo en animales restringidos de sueño pero no en otras regiones

cerebrales explica la reducción de la hiperpermeabilidad a dextranos de 70kDa por la administración de cafeína sólo en el hipocampo. Estos datos concuerdan con el hallazgo de un incremento en los niveles de mRNA de receptores A_{2A} en hipocampo de ratones restringidos de sueño pero no en otras regiones cerebrales (Kim et al., 2012). Además es importante mencionar que la cafeína puede revertir el deterioro en el aprendizaje y la memoria en animales privados de sueño (Killgore et al., 2014) y que es un potente neuroprotector regulando procesos asociados a estrés oxidante en ratas viejas a través de su acción sobre los receptores A_{2A} de adenosina (Leite et al., 2010). El papel de la regulación de la barrera hematoencefálica durante la perdida de sueño es sustentado porque el fármaco empleado es 48, 581 y 1561 veces más especifico a los receptores A_{2A} de adenosina que a A_1, A_{2B} y A_3 respectivamente (Zocchi et al., 1996; Yang et al., 2007). Además la dosis empleada es similar a experimentos *in vivo* que muestran que bajas dosis de SCH58261 tienen efectos neuroprotectores (0.01–0.5 mg/kg) (Popoli et al., 1998).

El hecho de que el fármaco SCH58261 revierta los efectos de la restricción de sueño sólo en áreas que expresan más receptor A_{2A} de adenosina sustenta la posible acción diferencial de la adenosina y el receptor A_{2A} sobre la barrera hematoencefálica en condiciones de restricción y recuperación de sueño.

En cuanto a los mecanismos moleculares por los cuales la restricción de sueño puede modificar la permeabilidad de la barrera hematoencefálica, en este estudio describimos que la restricción de sueño disminuye la expresión de proteínas de la unión ocluyente incluyendo claudina-5 y ZO-1. La disminución en la expresión de

estas proteínas induce hiperpermeabilidad en células endoteliales bajo condiciones patológicas (Stamatovic et al., 2008). Principalmente claudina-5 es necesaria para la integridad de la barrera hematoencefálica, en células endoteliales cerebrales humanas, la activación del receptor A_{2A} de adenosina usando un agonista selectivo (Lexiscan ®) disminuye gradualmente la expresión de claudina-5 hasta 30 minutos después de tratamiento mientras que un agonista no selectivo disminuye la expresión de claudina-5 después de 2 horas de tratamiento (Kim et al., 2015). Esto sustenta la hipótesis de que la acción rápida de la adenosina sobre los receptores A_{2A} puede regular la permeabilidad de la barrera hematoencefálica en ratas restringidas crónicamente de sueño. Breves periodos de oportunidad para dormir inducen una recuperación de la expresión de claudina-5 y de la permeabilidad de la barrera hematoencefálica (Hurtado-Alvarado et al., 2017) sugiriendo que la degradación de adenosina durante la recuperación de sueño previene la activación del receptor A_{2A} y restaura la integridad de la barrera hematoencefálica. En este sentido, el bloqueo de los receptores A_{2A} de adenosina restaura la expresión de claudina-5 con lo cual se recupera la permeabilidad basal de la barrera hematoencefálica.

La disminución en la expresión de ZO-1 y ocludina se mantiene incluso con la administración de SCH58261, sugiriendo que hay otras vías involucradas que podrían seguir activadas, por ejemplo una de las características de la pérdida de sueño es el estado inflamatorio de bajo grado. La disminución de ZO-1y ocludina puede asociarse a cambios sutiles en la permeabilidad de la barrera hematoencefálica relacionados con inflamación periférica (Elahy et al., 2015).

El mantenimiento de la expresión relativa de proteínas de unión ocluyente en los núcleos de la base sugiere que esta región tiene mecanismos de disfunción de la barrera hematoencefálica diferentes a los cambios en la permeabilidad paracelular como la pinocitosis o que las proteínas de la unión ocluyente tienen diferente localización celular. Los núcleos de la base son los que más expresan el receptor A_{2A} de adenosina, en caso de que las proteínas de la unión ocluyente tuvieran una localización diferente en animales restringidos de sueño, la administración de SCH58261, podría revertir este efecto, de hecho se observó que los niveles de claudina-5 y ocludina incrementan con la administración de este antagonista en comparación con la restricción de sueño con vehículo. En el caso del vermis, la densidad basal inferior de los receptores de A_{2A} de adenosina y su reducción durante la restricción del sueño pueden explicar la hiperpermeabilidad de la barrera hematoencefálica restante a los dextranos incluso con el tratamiento con el antagonista selectivo del receptor de adenosina A_{2A}. Sugerimos nuevamente que pueden participar otros mecanismos, independientes de la adenosina, como la regulación de la barrera hematoencefálica por citocinas proinflamatorias ya que el cerebelo parece ser propenso a eventos de neuroinflamación en comparación con la corteza cerebral (Guillemot-Legris et al., 2016). Otro ejemplo es el caso de infección por el virus de la rabia CVS-F3, en el cual se ha demostrado que el aumento de la permeabilidad cerebelar de la barrera hematoencefálica se acompaña de una mayor síntesis local de TNF-α, IFN-γ y de la molécula de adhesión intercelular-1 (ICAM-1) que se producen primero en el cerebelo y después en la corteza cerebral (Phares et al., 2006). Además, la ausencia de efectos de la restricción de sueño en la expresión proteínas de la unión ocluyente

en el vermis apunta a un mecanismo diferente de incremento en la permeabilidad de la barrera hematoencefálica en esa región, por ejemplo pinocitosis. El aumento de los niveles de adenosina en el cerebro después de la pérdida del sueño podrían inducir la activación de A_{2A} receptores de adenosina en las células endoteliales, glía y neuronas (Fields & Burnstock, 2006) lo cual concuerda con que la restricción del sueño indujo sobreexpresión de los marcadores de microglia reactiva (Iba-1) y astroglia (GFAP) y que la administración de SCH58261 revirtió este efecto. La microglia participa en la inmunidad innata y en varios procesos fisiológicos como en el neurodesarrollo y la plasticidad neuronal (Santiago et al., 2014). La administración crónica de un inhibidor de la activación microglia, minociclina, previene la acumulación de necesidad de sueño en roedores, lo que sugiere que microglia podría tener un papel en la regulación del sueño (Wisor et al., 2011). Las microglia expresa todos los subtipos de los receptores de adenosina, A_1, A_{2A}, A_{2B} y A_3; sin embargo, el A_{2A} tiene un papel central en procesos de neuroinflamación (Cunha et al., 2005). El aumento en la expresión de Iba-1 puede relacionarse con la activación de la microglia por activación el receptor A_{2A} durante la restricción del sueño. Por otro lado, la adenosina derivada de astrocitos está involucrada en los déficits cognitivos inducidos por la pérdida del sueño, particularmente a nivel del hipocampo (Halassa et al., 2009; Florian et al., 2011), y puede tener un papel importante en la regulación de la permeabilidad de la barrera hematoencefálica. La activación del receptor A_{2A} en astrocitos se relaciona con la activación del estado inflamatorio en varias neuropatologías (Ng et al., 2015; Orr et al., 2015). La gliosis se asocia con la ruptura de la barrera hematoencefálica debido a la liberación de varios mediadores inflamatorios

(Cabezas et al., 2014; da Fonseca et al., 2014; Preti et al., 2015); también es posible que la activación de las células gliales pueda preceder e incluso modificar la permeabilidad de la barrera hematoencefálica, como se muestra después de la administración de LPS (Sumi et al., 2010). Este estudio es el primero en investigar el papel de los receptores de adenosina A_{2A} en la regulación de la barrera hematoencefálica durante la restricción del sueño (Hurtado-Alvarado et al., 2016b, ANEXO 4). Proponemos que los cambios en la permeabilidad de la barrera hematoencefálica contribuyen a muchos procesos fisiopatológicos en el cerebro de sujetos con restricción del sueño (Figura 30). Esos efectos pueden estar mediados por la señalización de la adenosina que implica la activación del receptor A_{2A} y las diferencias regionales en la susceptibilidad pueden depender de la distribución del receptor de la adenosina y de la regulación hacia arriba y hacia abajo durante la pérdida del sueño. Se necesitan más estudios para corroborar esta hipótesis o explicar este fenómeno teniendo en cuenta que hemos evaluado la expresión del receptor de adenosina A_{2A} en regiones específicas del cerebro pero no en tipos celulares específicos. Es probable que cada elemento celular de la barrera hematoencefálica, es decir, células endoteliales, pericitos o astrocitos, pueda contribuir a este fenómeno debido a su potencial de señalización mediada por la adenosina (Byone et al., 2015). Por ejemplo, se ha descrito que durante 12 horas de privación de sueño la adenosina derivada de astrocitos es esencial para alterar los patrones de red de la actividad eléctrica en la corteza frontal (Hines et al., 2013). Por lo tanto, los astrocitos pueden desempeñar un papel clave en la regulación de la permeabilidad de la barrera hematoencefálica durante la pérdida del sueño a través de la liberación de adenosina y la activación de los receptores

de adenosina A_{2A} en las células endoteliales cerebrales. Menos conocido es el papel de los pericitos en la regulación de la permeabilidad de la barrera hematoencefálica. Estas células son fundamentales para el establecimiento y mantenimiento de la barrera hematoencefálica, son altamente responsivas a mediadores inflamatorios y son capaces de adquirir fenotipo de macrófago en condiciones patológicas a través de mecanismos que implican el desacople con las células endoteliales y migración celular empleando estrategias como la liberación masiva de metaloproteinasas de matriz (Sweeney et al., 2016). Empleando microscopia electrónica de transmisión hemos descrito que la alteración en la ultraestructura de las células endoteliales de ratas restringidas de sueño parece estar acompañada de la separación de los pericitos (Hurtado-Alvarado et al., 2017) sugiriendo que los pericitos pueden tener un papel fundamental en la ruptura de la barrera hematoencefálica a través de mecanismos proinflamatorios (Hurtado-Alvarado et al., 2014, ANEXO 5).

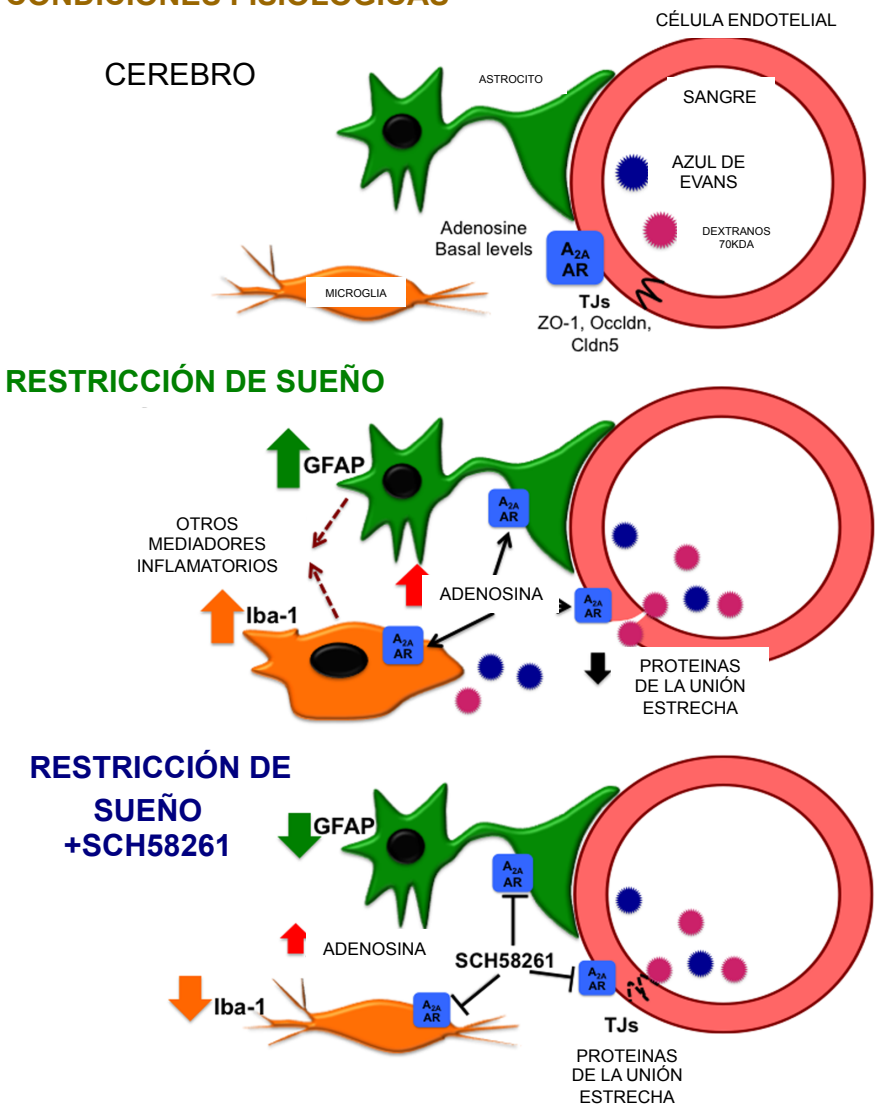

Figura 30. Modelo integrativo del efecto de la administración de un antagonista selectivo de los receptores A_{2A} de adenosina en la ruptura de la barrera hematoencefálica inducida por la restricción de sueño. En condiciones de restricción de sueño hay alteración de las Uniones ocluyentes (TJs) y un incremento en la expresión de los marcadores celulares, Iba-1 y GFAP que son revertidos con el uso de SCH58261. Modificado de Hurtado-Alvarado et al., 2016b.

La pérdida del sueño promueve cambios en la inmunidad humoral induciendo un estado inflamatorio de bajo grado caracterizado por un aumento sutil (3 veces superior a los controles) pero sostenido en los niveles periféricos de mediadores inflamatorios (Yehuda et al., 2009). El primer hallazgo de este estudio fue que los ratones C57BL/6 restringidos de sueño tienen niveles séricos elevados de dos mediadores inflamatorios importantes: TNF-α e IFN-γ en comparación con el grupo control. Por el contrario, en ratones BALB/c la restricción del sueño no modificó los niveles séricos de citocinas proinflamatorias o anti-inflamatorias determinadas. Estudios previos muestran que la privación total de sueño en ratones BALB/c no induce un incremento en los niveles periféricos de mediadores inflamatorios caracterizado en ratones C57BL/6 (Hu et al., 2003; Hirotsu et al., 2012). En otras condiciones proinflamatorias como la administración sistémica de LPS, se sabe que los macrófagos de ratones C57BL/6 producen mayores niveles de TNF-α e IFN-γ que los de ratones BALB/c (Watanabe et al., 2004).

Observamos que sólo los ratones C57BL/6 restringidos de sueño exhibieron un incremento en la permeabilidad de la barrera hematoencefálica a dextranos-FITC de 10kDa y al colorante azul de Evans en comparación con el grupo control. La restricción de sueño no modificó la permeabilidad de la barrera hematoencefálica en ratones BALB/c. Los datos obtenidos de ratones C57BL/6 replican reportes previos que indican que la restricción del sueño aumenta la permeabilidad de la barrera hematoencefálica en el hipocampo de ratas sometidas a pérdida de sueño durante 10 días (Gómez-González et al., 2013; Hurtado-Alvarado et al. , 2016) y en ratones C57BL/6 con restricción de sueño (He et al., 2014). No observamos cambios en la expresión de proteínas de unión estrecha en el hipocampo de

ratones C57BL/6 con restricción de sueño al día 10 en el hipocampo pero si en la corteza y en los núcleos de la base. Esto sugiere una regulación dependiente de la región cerebral pero también podría estar asociado al metabolismo de los ratones en comparación con el de las ratas, es decir, que a los 10 días de restricción de sueño en ratones podrían echarse a andar mecanismos de compensación que incluyan la restauración de la expresión de proteínas de la unión ocluyente en el hipocampo ya que hay incremento en la permeabilidad de la barrera hematoencefálica que también podría estar asociado a un incremento en el transporte mediado por pinocitosis. El aumento de la ZO-1 en el grupo de la plataforma grande podría ser un mecanismo compensatorio que proteja la integridad de la barrera hematoencefálica en condiciones de estrés o disminución del tiempo total de sueño reportado en ratas usando este método (Gómez-González et al. 2013). Se obtuvieron datos relevantes de ratones BALB/c con restricción de sueño, este grupo mostró un aumento significativo en la expresión de claudina-5 y ocludina en el hipocampo, y de ZO-1 en la corteza, esto podría explicar parcialmente la razón por la cual la restricción del sueño no modifica la permeabilidad de la barrera hematoencefálica. Suponemos que a nivel del hipocampo los ratones BALB/c con restricción de sueño podrían tener niveles más altos de moléculas que promueven la sobreexpresión de claudina-5 y ocludina como IL-4 (Dalmasso et al., 2014) pero son necesarios más estudios para corroborarlo.

Con respecto al posible papel del TNF-α en la disrupción de la barrera hematoencefálica inducida por la restricción del sueño, se sabe que el TNF-α es una proteína sintetizada principalmente por monocitos y macrófagos que tienen un

papel crucial en la activación inicial del sistema inmunológico y que también es un potente regulador de la permeabilidad de la barrera hematoencefálica (Varatharaj et al., 2016). La pérdida de sueño incrementa los niveles plasmáticos de TNF-α, el ARNm del TNF-α en el cerebro, los linfocitos y el tejido adiposo peritoneal (Hurtado-Alvarado et al., 2016a). Durante otros eventos de inflamación periférica (por ejemplo, pancreatitis aguda en ratas), los niveles de TNF-α aumentan y al mismo tiempo aumenta la permeabilidad de la barrera hematoencefálica a sodio acoplado a fluoresceína en el hipocampo (Farkas et al., 1998) y la estimulación directa de células endoteliales cerebrales microvasculares a dosis menores (1 ng/ml) disminuye la resistencia eléctrica transendotelial (TEER) en estudios *in vitro* (Varatharaj et al., 2016). Por otro lado, el papel del IFN-γ como regulador de la permeabilidad de la barrera hematoencefálica se describe en varias infecciones incluyendo la infección por *Trypanosoma brucei brucei* o el virus de la encefalitis japonesa (Masocha et al., 2004, Li et al., 2015) y la acción de TNF-α e IFN-γ sobre las células endoteliales de la microvasculatura cerebral está mediada por la expresión de sus receptores. En modelos *in vitro* TNF-α solo o en combinación con IFN-γ aumenta la permeabilidad de la barrera hematoencefálica (López-Ramírez et al., 2012). Se necesitan más experimentos para identificar el papel específico de estas citocinas en la permeabilidad de la barrera hematoencefálica bajo restricción de sueño así como en las vías moleculares involucradas; sin embargo, la literatura sugiere que el TNF-α y el IFN-γ podrían tener un papel potencial como moduladores de la barrera hematoencefálica durante la pérdida del sueño.

En este trabajo sólo se determinó el estado de inmunidad humoral en el día 10 de restricción de sueño por lo que se necesitan más datos para aclarar el papel específico del TNF-α y el IFN-γ y la presencia de otras citocinas en la regulación de la barrera hematoencefálica a lo largo de la restricción del sueño; sin embargo, está claro que la disfunción de la barrera hematoencefálica inducida por la restricción del sueño está presente sólo en ratones con un estado proinflamatorio sistémico de bajo grado también inducido por la deficiencia de sueño. La propuesta de que el sueño es un fenómeno que conduce a la eliminación de residuos cerebrales es bien aceptada (Xie et al., 2013), en este caso podríamos hipotetizar que la disfunción de la barrera hematoencefálica también podría ser una forma en que el cerebro busca eliminar moléculas potencialmente tóxicas durante la pérdida de sueño. Por lo tanto, los ratones BALB/c no presentan la capacidad de regular la permeabilidad de la barrera hematoencefálica bajo restricción de sueño debido a que no produjeron moléculas inflamatorias tales como TNF-α e IFN-γ y esto podría inducir acumulación de moléculas neurotóxicas.
La respuesta al desafío inmunológico entre ratones C57BL/6 y BALB/c es diferente, por ejemplo, los ratones C57BL/6 son susceptibles a la encefalomielitis hemorrágica letal del tipo 1 de adenovirus de ratón, con una dosis letal del 50% mientras que los ratones BALB/c son resistentes a cualquier signo de esta enfermedad neurológica o mortalidad (Guida et al., 1995). En cuanto al efecto del desafío inmunológico sobre la integridad de la barrera hematoencefálica se ha descrito una respuesta diferencial en la permeabilidad de la barrera hematoencefálica asociada al estado inflamatorio en modelos experimentales. Por ejemplo, los ratones C57BL/6 infectados con *Toxoplasma gondii* son más

susceptibles que los ratones BALB/c a la infección porque los ratones C57BL/6 desarrollan una respuesta inmune inflamatoria en el sistema nervioso central más temprana y más grave en comparación con los ratones BALB/c. Por otra parte, esta respuesta inflamatoria exacerbada de ratones C57BL/6 está relacionada con una mayor entrada de las células inflamatorias en el sistema nervioso central, y una mayor permeabilidad de la barrera hematoencefálica en comparación con ratones BALB/c (Silva et al., 2010). Los ratones C57BL/6 infectados con el virus del Nilo Occidental también muestran un aumento de la permeabilidad de la barrera hematoencefálica que está ausente en los ratones BALB/c infectados. Sin embargo, este estudio sugiere que un cambio en la permeabilidad de la barrera hematoencefálica no es un requisito para la supervivencia de los ratones ya que las curvas de supervivencia son similares entre los ratones C57BL/6 y BALB/c (Morrey et al., 2008). De esta manera, la disfunción de la barrera hematoencefálica podría ser una clave en el desarrollo de la neuroinflamación y la presencia de síntomas fisiopatológicos en ratones C57BL/6, pero el hecho de que la permeabilidad de la barrera hematoencefálica no cambia en ratones BALB/c debe considerarse cuidadosamente ya que también podrían tener alteraciones cognitivas.

El mecanismo por el cual la restricción del sueño podría modificar la permeabilidad de la barrera hematoencefálica incluye procesos proteolíticos extracelulares, en los que los componentes de la lámina basal se degradan. La MMP-9 es una colagenasa tipo IV de gelatinasa B (92 kDa), implicada en eventos neuroinflamatorios relacionados con la disrupción de la barrera hematoencefálica. Observamos aumento en la expresión de MMP-9 en el hipocampo de C57BL/6

ratones restringidos de sueño que mostraron mayor permeabilidad de la barrera hematoencefálica que sus controles mientras que no hubo cambio en la expresión de MMP-9 en ratones BALB/c restringidos de sueño. MMP-9 es crucial para la alteración de la barrera hematoencefálica en varias enfermedades y es también un importante modulador de la respuesta inmune mediada por citocinas (Koistinaho et al., 2005). Por ejemplo, la producción de MMP-9 podría inducirse de una manera dependiente de las concentraciones de TNF-α (Prato et al., 2005) el cual incrementa en suero de ratones proinflamatorios restringidos al sueño.

En un modelo de infección de malaria, los ratones BALB/c no producen MMP-9 en el cerebro en comparación con el grupo control mientras que en el cerebro de ratones C57BL/6 infectados hay una alta concentración de MMP-9 activa (Van Den Steen et al., 2006), este aumento en las metaloproteinasas de matriz se ha relacionado con la activación microglial (Koistinaho et al., 2005). En ese sentido, el aumento de la MMP-9 en el hipocampo podría explicar la tendencia al aumento de la microgliosis y la ruptura de la barrera hematoencefálica en los ratones C57BL/6 con restricción del sueño y pero no en ratones BALB/c.

La activación de la microglia se ha asociado a eventos neuroinflamatorios y la restricción del sueño aumenta la expresión de Iba-1 en ratas, algunos estudios indican que las poblaciones de microglia inician una respuesta inflamatoria con marcados cambios en la expresión de moléculas pro y anti-inflamatorias y que estos mediadores regulan la expresión de proteínas de unión ocluyente regulando la integridad de la barrera hematoencefálica (Camire et al., 2015). Observamos un aumento en la expresión de Iba-1 en ratones C57BL/6 con restricción de sueño, debido a que esta cepa es proinflamatoria, los mediadores inflamatorios

previamente reportados (por ejemplo COX-2) podrían activar la microglia antes de la disfunción de la barrera hematoencefálica ya que la expresión de Iba-1 no presenta cambios significativos a los 10 días de restricción de sueño. Es importante considerar que la microglia reactiva secreta varias citocinas proinflamatorias que podrían modular la barrera hematoencefálica en los ratones C57BL/6 pero no en ratones BALB/c debido a su perfil anti-inflamatorio (Figura 31). Por ende, se necesitan más experimentos para identificar el perfil de citoquinas en el cerebro de ratones C57BL/6 y BALB/c.

Finalmente, la acción de los receptores A_{2A} de adenosina sobre la barrera hematoencefálica durante la restricción del sueño podría ser crucial para la hiperpermeabilidad y los eventos neuroinflamatorios ya que el bloqueo de A_{2A} AR revierte la disrupción de la barrera hematoencefálica y el incremento en marcadores de neuroinflamación inducidos por la restricción de sueño en el hipocampo de ratas. La restricción de sueño aumentó la A_{2A} AR expresión en el hipocampo de ratas restringidas de sueño lo cual se observó en ratones proinflamatorios restringidos de sueño mientras que la expresión de estos receptores tiende a disminuir en ratones anti-inflamatorios restringidos al sueño sugiriendo que A_{2A} AR son clave en la regulación de la barrera hematoencefálica mediada por la pérdida del sueño (Hurtado-Alvarado et al., 2017b, en revisión) (ANEXO 6).

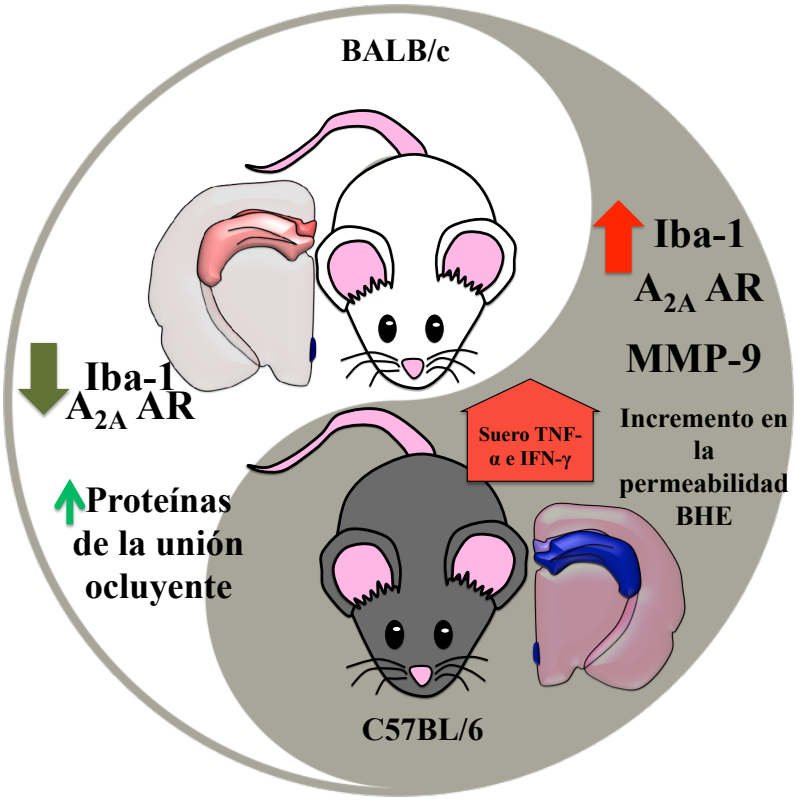

Figura 31. La restricción de sueño regula de manera diferencial la integridad de la barrera hematoencefálica (BHE) y la expresión de mediadores neuroinflamatorios como iba-1 y los receptores A_{2A} de adenosina (A_{2A} AR) en ratones con respuesta predominantemente inflamatoria (C57BL/6) y anti-inflamatoria (BALB/c).

10. CONCLUSIÓN

No dormir bien es un problema que afecta a la mayoría de la población en la sociedad actual. Los riesgos la deficiencia de sueño incluyen una amplia gama de patologías como enfermedades con un alto índice de prevalencia tales como la diabetes tipo 2 y la enfermedad de Alzheimer en donde el componente inflamatorio tiene un papel crucial. El objetivo del presente trabajo fue determinar la participación del componente inflamatorio en los cambios de la integridad de la barrera hematoencefálica asociados a la restricción de sueño. Presentamos evidencia de que activación del receptor A_{2A} participa en la modulación de la permeabilidad de la barrera hematoencefálica de la rata en condiciones de

restricción de sueño y además describimos por primera vez que la ruptura de la barrera hematoencefálica inducida por la pérdida de sueño está relacionada con el estatus inflamatorio del individuo. Se requieren más estudios para determinar si la acción de la adenosina en eventos de restricción de sueño puede contribuir a la generación o exacerbación de diversas patologías; sin embargo, el uso de antagonistas selectivos para el receptor A_{2A} e incluso dosis controladas de cafeína pueden actuar como neuroprotectores durante la pérdida de sueño. Por otro lado, la predisposición a desarrollar estas patologías por no dormir el tiempo necesario podría depender de la susceptibilidad de la respuesta inmunológica de cada individuo que conlleva, en base a nuestros resultados, a relacionar un estado proinflamatorio inducido por la restricción de sueño con la susceptibilidad de ruptura de la barrera hematoencefálica. Estos hallazgos son cruciales para comenzar a considerar la deficiencia de sueño en pacientes con distinto perfil inmunológico y tomarlo en cuenta para el diagnostico y el tratamiento de enfermedades. Finalmente, los hallazgos aquí descritos también puede contribuir a generar conocimiento sobre la regulación de la permeabilidad de la barrera hematoencefálica utilizando la restricción del sueño como modelo no patológico. De esta manera, la pérdida del sueño puede promover la apertura temporal de la barrera hematoencefálica para permitir el paso de sangre a cerebro de moléculas con efectos terapéuticos potenciales con una alta especificidad regional.

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ANEXO 1

Chronic sleep restriction disrupts interendothelial junctions in the hippocampus and increases blood–brain barrier permeability

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Key words. Blood–brain barrier, claudin-5, endothelial cell, fluorescein-sodium, hippocampus, interendothelial junction, sleep restriction, tight junction.

Summary

Chronic sleep loss in the rat increases blood–brain barrier permeability to Evans blue and FITC-dextrans in almost the whole brain and sleep recovery during short periods restores normal blood–brain barrier permeability. Sleep loss increases vesicle density in hippocampal endothelial cells and decreases tight junction protein expression. However, at the ultrastructural level the effect of chronic sleep loss on interendothelial junctions is unknown. In this study we characterised the ultrastructure of interendothelial junctions in the hippocampus, the expression of tight junction proteins, and quantified blood–brain barrier permeability to fluorescein-sodium after chronic sleep restriction. Male Wistar rats were sleep restricted using the modified multiple platform method during 10 days, with a daily schedule of 20-h sleep deprivation plus 4-h sleep recovery at their home-cages. At the 10th day hippocampal samples were obtained immediately at the end of the 20-h sleep deprivation period, and after 40 and 120 min of sleep recovery. Samples were processed for transmission electron microscopy and western blot. Chronic sleep restriction increased blood–brain barrier permeability to fluorescein-sodium, and decreased interendothelial junction complexity by increasing the frequency of less mature end-to-end and simply overlap junctions, even after sleep recovery, as compared to intact controls. Chronic sleep loss also induced the formation of clefts between narrow zones of adjacent endothelial cell membranes in the hippocampus. The expression of claudin-5 and actin decreased after chronic sleep loss as compared to intact animals. Therefore, it seems that chronic sleep loss disrupts interendothelial junctions that leads to blood–brain barrier hyperpermeability in the hippocampus.

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Introduction

The blood–brain barrier is a specialised vascular system that plays a major role in maintaining brain homeostasis by regulating the passage of endogenous and exogenous circulating molecules (Rubin & Staddon, 1998; Zlokovic, 2008). Brain endothelial cells confer to the blood–brain barrier the physical and chemical barrier properties because their characteristic phenotype. Brain endothelial cells exhibit low vesicle-mediated transport, a continuous basal lamina, absence of fenestrations, and present interendothelial junctional complexes formed by tight junctions at the apical membrane and adherens and gap junctions at the basolateral membrane (Nag, 2003). Tight junctions seal the interendothelial space and restrict paracellular diffusion of blood-borne molecules; they are composed of the tetraspan proteins occludin and claudins (Nag, 2011). Tight junctions anchor to the actin cytoskeleton through cytoplasmic accessory proteins, such as zonula occludens (ZO) (Furuse *et al.*, 1993; Tsukita *et al.*, 2001; Schneeberger & Lynch, 2004). Through its interaction with tight junction molecules, the actin cytoskeleton plays an active role in maintaining tight junction integrity and blood–brain barrier function (Wittchen *et al.*, 1999).

Previously it was shown that 6-day sleep restriction in mice increased blood–brain barrier permeability to a low molecular weight tracer, fluorescein-sodium, and decreased mRNA expression of tight junction proteins; while 24 h of sleep recovery reverted blood–brain barrier permeability to control levels (He *et al.*, 2014). Ten-day sleep restriction in the rat increased blood–brain barrier permeability to Evans blue and FITC-dextrans (Gómez-González *et al.*, 2013; Hurtado-Alvarado *et al.*, 2016a); and that effect was related to increased vesicle density in brain endothelial cells (Gómez-González *et al.*, 2013) and decreased tight junction protein expression (Hurtado-Alvarado *et al.*, 2016a). A regional specific pattern of blood–brain barrier breakdown and amendment was observed with sleep restriction and different periods of sleep recovery. The hippocampus and cerebellar vermis presented a prolonged blood–brain barrier hyper-permeability induced by sleep loss

that remained high even after 120 min of sleep recovery, as compared to regions like the basal nuclei, amygdala, and cingulate cortex, which by 120 min have fully recovered normal blood–brain barrier permeability (Gómez-González *et al.*, 2013). Additionally, it was found that A_{2A} adenosine receptor antagonism fully reverted the effects of chronic sleep loss on blood–brain barrier permeability and tight junction protein expression in the hippocampus but not in other brain regions such as the cerebellum and cerebral cortex (Hurtado-Alvarado *et al.*, 2016a). Despite those findings, the dynamic of tight junction protein expression and ultrastructure of interendothelial junctions after chronic sleep loss and recovery is still unknown. Therefore we aimed to study the effect of chronic sleep loss on interendothelial junction ultrastructure and tight junction protein expression in the hippocampus and related those effects with changes in blood–brain barrier permeability to fluorescein-sodium.

Material and methods

Animals

Three-month-old male Wistar rats ($n = 38$) were used. Rats were caged in groups of 5–8 in our laboratory vivarium under a 12-h light/dark cycle (lights on at 11 pm), at room temperature of 20–25 °C. Commercial rat chow and tap water were available *ad libitum* to all rats throughout the experiment. Rats were randomly assigned to the experimental conditions. Ten-day sleep restricted rats were allowed to sleep for 0 min (recovery time 0, RT0), 40 min (RT40) and 120 min (RT120) in their home-cages; controls included rats that slept normally in their home-cages during 10 consecutive days. Experiments were performed following the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2010) and were approved by the Institutional Ethic Committee.

Chronic sleep restriction

For the sleep restriction procedure an acrylic water tank (82 cm × 59 cm × 48 cm) and 7cm diameter platforms were used. Sleep restriction was performed by the multiple platform technique as previously reported (Gómez-González *et al.*, 2013). Rats were kept in the conditions of the multiple platform technique during 20 h for 10 consecutive days; each day they were allowed to sleep 4 h in their home-cages during the last 4 h of the light phase. It was previously reported that the same sleep restriction procedure abolishes rapid eye movement (REM) sleep and reduces 30% slow wave sleep along the 10 days of sleep restriction (Gómez-González *et al.*, 2013). Intact controls slept *ad libitum* in their home-cages during the 10 days of the experiment.

Blood–brain barrier permeability assay

Blood–brain barrier permeability assay was performed in intact controls ($n = 4$), and sleep restricted rats at recovery time 0 (RT0, $n = 4$), RT40 ($n = 4$), and RT120 ($n = 4$). Fluorescein-Na (Sigma, F6377) was used to evaluate blood–brain barrier permeability. Fluorescein-Na was suspended in phosphate buffered saline (PBS) to achieve a concentration of 50 mg mL^{-1} and a bolus of 0.2 mL/100 g of body weight was administered ic. under sodium pentobarbital anaesthesia (0.063 g kg^{-1} body weight) between 8 and 11 am. Fluorescein-Na was left in circulation during 10 min; thereafter rats were perfused during 5 min with saline solution (0.9% w/v). Brains were removed and the hippocampus dissected, weighed, homogenised and centrifuged at 13 500 rpm per 10 min. Supernatant was collected and absorbance was obtained in a spectrophotometer (Genesys20, Thermo Spectronic, Mexico City, Mexico) at 520 nm. Fluorescein-Na concentration per weight of wet brain tissue (mg g^{-1}) was obtained using a standard curve and normalised permeability coefficient was calculated taking into account plasma fluorescein-Na concentration using the following formula:

$$Pc = \frac{C_f/C_i}{V/t},$$

where Pc, normalised permeability coefficient; C_f, fluorescein-Na concentration in tissue (mg mL^{-1}); C_i, fluorescein-Na concentration in blood (mg mL^{-1}); V, volume quantified in brain supernatant (0.05 mL) and t, time of fluorescein-Na in circulation.

Ultrastructure of the blood–brain barrier

Transmission electron micrographs were obtained from intact controls ($n = 2$), and sleep restricted rats at RT0 ($n = 2$), RT40 ($n = 2$) and RT120 ($n = 2$). Between 8 and 11 am, rats were anesthetised with sodium pentobarbital (ip. 0.063 g kg^{-1} body weight). Thereafter, rats were transcardially perfused with saline solution (0.9%) during 5 min, followed by a solution of 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 during 10 min. Brains were removed, hand-sectioned in 2 mm thick coronal slices, and fixed by immersion in the same fixative during 1 h at 4 °C. After that time, slices containing the hippocampus were rinsed in sodium phosphate buffer (PBS) and a ~1 mm^3 block containing the CA3 region was obtained. The block was postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.4, and dehydrated in a graded series of ethanol; briefly the block was immersed in ethanol at 30%, 50%, 70%, 80% and 100% two times during 15 min each. Afterwards, the block was immersed in acetonitrile (two times, 15 min each) and embedded in Epon 812 overnight at room temperature. The Epon-embedded block was polymerised at 60 °C during 48 h. Silver ultrathin sections were obtained with a diamond

knife using an ultramicrotome (Ultracut, Leica, Mexico City, Mexico) and placed on a copper grid. Ultrathin sections were poststained with uranyl acetate and lead citrate. Acquisition of micrographs of brain capillaries was performed blindly in a JEOL electron microscope (JEM1200ex II) coupled to a CCD (GATA, JEOL). All the capillaries (less than 10 μm in diameter) displayed in a section were photographed at X2500 for general appearance; after that, capillaries were observed at X4500 in search of junctional complexes. Micrographs of interendothelial junctions were acquired only for those junctions in which membrane profiles fully ran from luminal to abluminal sides of endothelial cells; micrographs of interendothelial junctions were taken at X7000. Interendothelial junction length was quantified using ImageJ software. Interendothelial clefts were defined as the zones in the interendothelial junctions between two narrow zones (tight junctions) in which a clear separation of the apposed endothelial membranes was observed; it was calculated the length of the cleft in X7000 micrographs, therefore the percentage of the interendothelial junction occupied by the cleft was obtained. The width of interendothelial clefts was calculated by measuring the distance between the membranes of the adjacent endothelial cells at the wider point between two narrow zones in X7000 micrographs. The density of cytoplasmic projections from endothelial cells into the luminal capillary was obtained by counting the number of projections per area of endothelial cell cytosol, taking into consideration that all the observed projections contained both membranes and cytosol. Interendothelial junction complexity was classified in accordance with maturation criteria developed by Schwartz & Benditt (1972) and extensively applied by Huang *et al.* (2001) into four types, namely: type 1 (end-to-end), type 2 (simple overlap), type 3 (mortise) and type 4 (complex overlap). Interendothelial junction classification reflects degrees of maturation of tight junctions, with simple end-to-end junctions (type 1) as the less mature interendothelial junctions and type 4 complex overlap as the interendothelial junctions with higher apposition and interfolding between adjacent endothelial cells and longer and numerous narrow zones. Types 2 and 3 interendothelial junctions reflect intermediate states of junction maturation with progressive tightening of the junctional complex and concomitant reduction in paracellular diffusion (Schwartz & Benditt, 1972; Huang *et al.*, 2001). Basal lamina thickness was obtained by randomly measuring the basal lamina at three different points in X7000 micrographs.

Western blot

Hippocampus was obtained from intact controls ($n = 3$), and sleep restricted rats at RT0 ($n = 3$), RT40 min ($n = 3$), and RT120 min ($n = 3$) between 8 and 11 am by decapitation. Hippocampal samples were frozen and stored at –80 °C until processing. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA, USA). Proteins (100 μg) were resolved using a denaturing 10% SDS-PAGE electrophoresis and transferred to PVDF membranes. Membranes were blocked with 5% w/v nonfat milk in Tris-buffered saline for 2 h and incubated overnight at 4 °C with occludin (Invitrogen, cat. 40–4700, 1:1000, Mexico City, Mexico), claudin-5 (Abcam, cat. Ab53765, 1:1000, Mexico City, Mexico), ZO-1 (Invitrogen, cat. 40–2200, 1:1000, Mexico City, Mexico), actin (Abcam, cat. Ab3280, 1:2000, Mexico City, Mexico), or NeuN (Millipore, cat. MAB77, 1:1000, Mexico City, Mexico) antibodies. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and revealed with chemiluminescence detection system (Amersham, cat. RPN2232, Mexico City, Mexico). Semiquantitative analysis was performed using C-digit program (LI-COR iS image studio, version 3.1). NeuN was used for normalisation of protein expression levels.

Statistical analysis

Data from all groups fulfilled normality and independence requirements to perform statistical parametric analysis, therefore a one-way ANOVA test was performed to test group differences in blood–brain barrier permeability assays, in the ultrastructure of hippocampal interendothelial junctions and in the protein expression levels, using as between subjects factor the experimental condition. The alpha level was maintained at 0.05 in all the ANOVA tests. To reduce the probability of type I statistical error associated with multiple comparisons, pair wise differences between sleep restricted groups and controls were tested on using orthogonal contrasts codes (C_1); contrast coefficients of 1 and –1 were assigned to experimental groups (sleep restricted with sleep recovery times of 0, 40 and 120 min) and control group, respectively. SPSS statistical software was used for statistical analyses.

Results

Chronic sleep loss increases blood–brain barrier permeability to fluorescein-Na

As it is shown in Figure 1(A) chronic sleep restriction significantly increased blood–brain barrier permeability to fluorescein-Na in the hippocampus as compared to intact controls sleeping *ad libitum* ($F_{4-16} = 11.201$, $p = 0.02$), and sleep recovery promoted a progressive restoration of normal blood–brain barrier permeability. Fluorescein-Na plasma concentration was similar between the groups (Fig. 1B); therefore the increased brain concentration of fluorescein-Na was independent of variations in fluorescein-Na plasma concentration.

Chronic sleep loss alters interendothelial junction ultrastructure in the hippocampus

Transmission electron microscopy was used for analysis of ultrastructure of interendothelial junctions in the hippocampus.

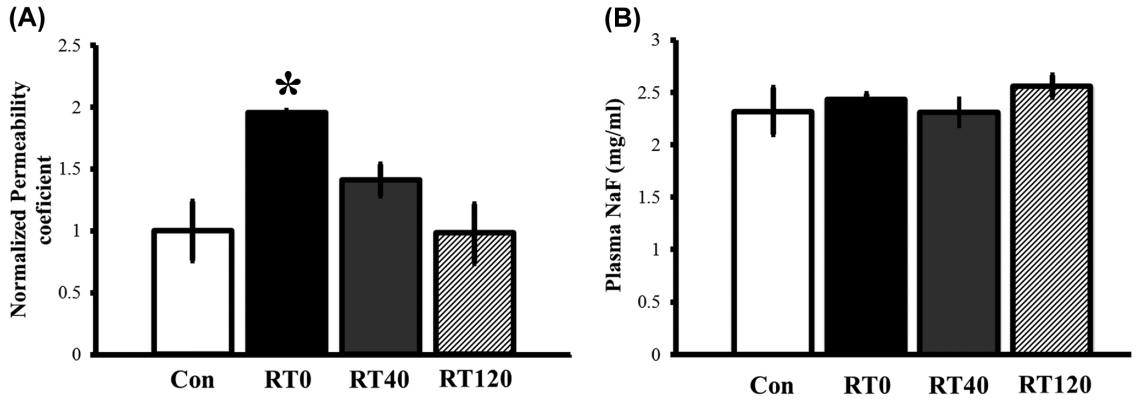

Fig. 1. Chronic sleep restriction increases blood–brain barrier permeability to fluorescein-Na. (A) The graph depicts the blood–brain barrier permeability index in the hippocampus of intact controls (Con) and chronic sleep restricted rats at recovery times of 0 min (RT0), 40 min (RT40) and 120 min (RT120) ($n = 4$ per group). (B) Graph depicts the fluorescein-Na plasma concentration in the Con, RT0, RT40 and RT120 groups ($n = 4$ per group). Mean ± SEM, $^*p < 0.05$ as compared to intact controls.

According to the classification of interendothelial junctions into four types in order of complexity, from simple end-to-end (type 1) to complex overlap (type 4) (Schwartz & Benditt, 1972; Huang *et al.*, 2001), we found that chronic sleep loss modified the percentage of each junction type found in hippocampal endothelial cells. As it is shown in Figure 2, the 43% of interendothelial junctions found in intact controls were type 4 and 43% were type 3. Only 14% of the interendothelial junctions found in intact controls were type 2. Chronic sleep restriction reduced the complexity of interendothelial junctions, in all the three studied recovery times (0, 40 and 120 min of sleep recovery), type 4 complex overlap interendothelial junctions disappeared and the vast majority of observed junctions were types 2 and 1. Sleep restricted rats without sleep recovery (RT0) presented predominance of type 2 interendothelial junctions (56%), followed by type 1 junctions (38%) and only slight appearance of type 3 junctions (Fig. 2). On the other hand, in rats with 40 min of sleep recovery, type 2 interendothelial junctions predominated (83%). By 120 min of sleep recovery, the percentage of type 3 interendothelial junctions was similar to the intact controls; however, types 1 and 2 interendothelial junctions were still present and type 4 junctions were absent.

In addition, the presence of clefts between narrow zones in interendothelial junctions was observed in the hippocampus of sleep-restricted rats (Fig. 3). As it is shown in Figure 3, 17% of the interendothelial junction length was occupied by clefts between the membranes of adjacent endothelial cells in sleep restricted rats after 40–120 min of sleep recovery, indicating a derangement in the ultrastructure of the junctional complex after chronic sleep loss. Interendothelial clefts in sleep-restricted rats, especially in the subjects with 120 min of sleep opportunity, were wider than those occasionally found in complex interendothelial junctions of the control group ($F_{3–35} = 3.758$, $p = 0.019$).

Sleep restriction decreases the expression of tight junction proteins in the hippocampus

To elucidate whether interendothelial junction derangement related to decreased expression of tight junction proteins, western blots were performed using antibodies to occludin, claudin-5, ZO-1 and actin. As it is shown in Figure 4 sleep restriction reduced the relative expression of hippocampal actin (42 kDa, $F_{3–11} = 7.104$, $p = 0.01$) in comparison with intact controls, and tended to reduce hippocampal levels of claudin-5 (23 kDa, $F_{3–11} = 3.216$, $p = 0.08$). Hippocampal occludin (59 kDa, $p > 0.05$) and ZO-1 (225 kDa, $p > 0.05$) expression was maintained in the same levels in the sleep restricted rats as compared to the intact controls (Fig. 4). Despite sleep recovery, the expression levels of tight junction proteins remained without change in sleep restricted rats at RT0, RT40 and RT120.

Chronic sleep loss alters blood–brain barrier ultrastructure in the hippocampus

Concomitant to the changes in interendothelial junctions, sleep restriction also modified the general ultrastructure of hippocampal endothelial cells. As it is shown in Table 1, sleep restriction increased the frequency of cytoplasmic projections

Table 1. Sleep restriction increases cytoplasmic projections in hippocampal endothelial cells.

Experimental group	Cytoplasmic projections (μm^2)
Intact control $n = 20$ vessel profiles	0.02 ± 0.02
RT0 $n = 19$ vessel profiles	$1.49 \pm 0.43^*$
RT40 $n = 15$ vessel profiles	$1.84 \pm 0.60^*$
RT120 $n = 12$ vessel profiles	0.33 ± 0.41

RT0 = recovery time 0, RT40 = recovery time 40, RT120 = recovery time 120. Mean ± SEM, $^*p \leq 0.05$.

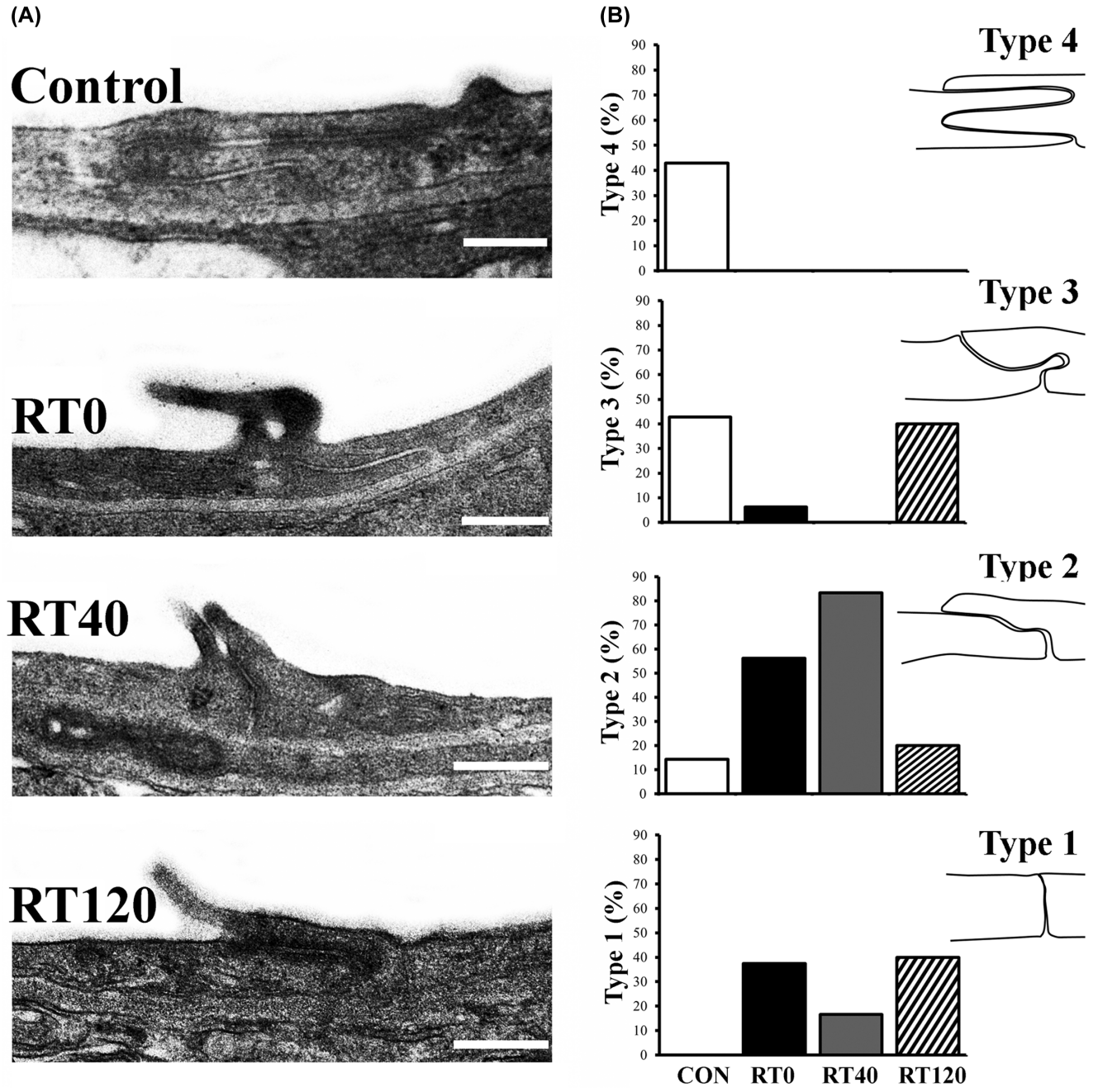

Fig. 2. Chronic sleep restriction decreases interendothelial junction complexity. (A) Microelectrographs show changes in interendothelial junction complexity and the formation of cytoplasmic projections after sleep restriction at recovery times of 0 min (RT0), 40 min (RT40) and 120 min (RT120) as compared to intact controls (Con). Scale bar: 200 nm. (B) Graphs depict percentage of each interendothelial junction type in the hippocampus of control (Con, $n = 7$ microvessels), sleep restricted rats at RT0 ($n = 16$ microvessels), RT40 ($n = 6$ microvessels) and RT120 ($n = 10$ microvessels).

to the vessel lumen per capillary area as compared to control capillaries, which presented a smooth appearance of the luminal membrane; the increased density of cytoplasmic projections was maintained in sleep restricted rats even with 40 min of sleep recovery. Cytoplasmic projections in sleep restricted rats had a mean length of 300 nm and were mainly located close to tight junctions (Fig. 2) or associated to the sites of vesicle formation in the membrane of hippocampal endothelial cells ($F_{3–62} = 4.819$, $p = 0.004$). By 120 min of sleep recovery (RT120), the number of cytoplasmic projections in hippocampal endothelial cells of sleep restricted rats reached similar levels to those observed in the intact controls ($p \geq 0.05$).

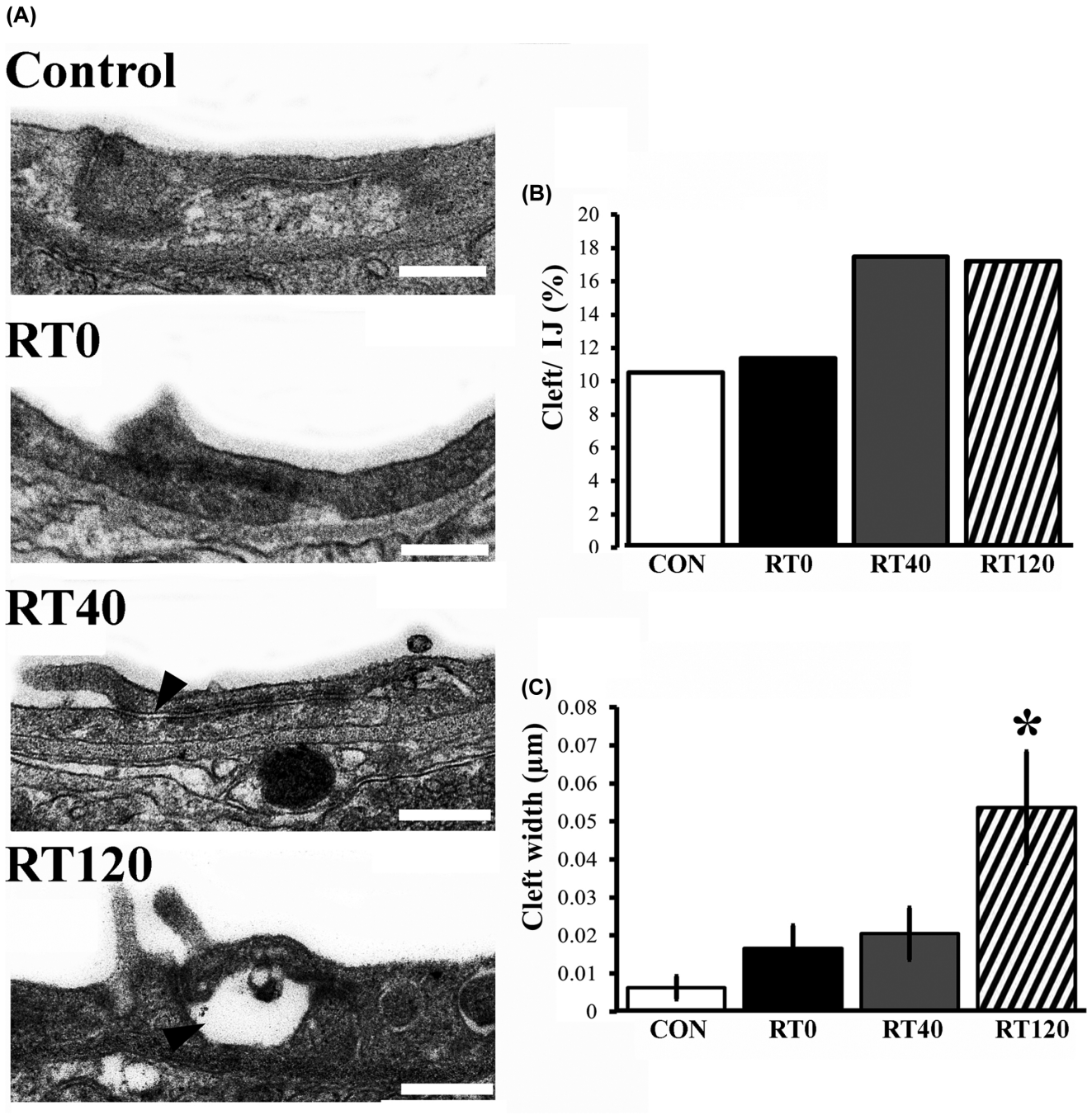

Fig. 3. Sleep restriction promotes the formation of clefts between adjacent endothelial cell membranes in the hippocampus. (A) Representative micrographs show the presence of clefts between two narrow zones of the membrane of hippocampal endothelial cells in the sleep restricted rats at recovery times of 0 min (RT0), 40 min (RT40), and 120 min (RT120) (arrowheads) as compared to intact controls. Scale bar: 200 nm. (B) Graph indicates the percentage of interendothelial junction (IJ) length occupied by clefts in intact controls (Con, $n = 7$ microvessels), sleep restricted rats at RT0 ($n = 16$ microvessels), RT40 ($n = 6$ microvessels) and RT120 ($n = 10$ microvessels). (C) The graph depicts the cleft width in hippocampal endothelial cells of controls, and sleep restricted rats with RT0, RT40 and RT120. Mean ± SEM, $^*p \leq 0.05$ versus intact controls.

Sleep restricted rats also presented thickening of the basal lamina (Fig. 5) and the formation of progressive oedema in the perivascular space (Fig. 6). As it is shown in Figure 5, basal lamina thickness in sleep restricted rats with 0 min of sleep recovery was significantly higher than the observed in intact controls and, sleep recovery reduced the thickness of basal lamina but it never reached intact control levels ($F_{3–62} = 8.10$, $p = 0.0001$). As it is shown in Figure 6, the

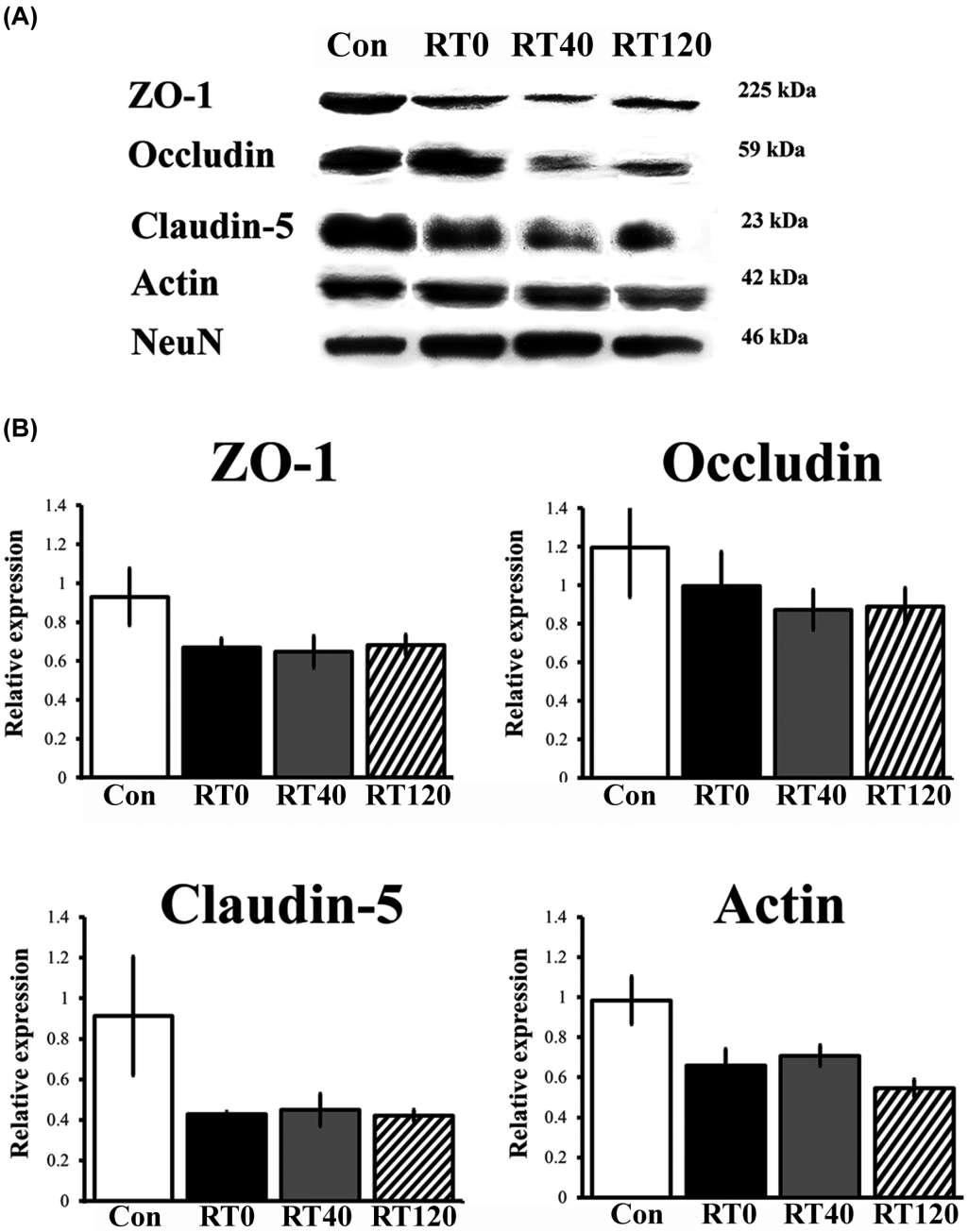

Fig. 4. Chronic sleep restriction decreases the expression of tight junction proteins. (A) Representative western blots of the expression of tight junction proteins ZO-1, occludin, claudin-5 and the cytoskeletal protein actin in intact controls and sleep restricted rats with sleep recovery times of 0 min (RT0), 40 min (RT40) and 120 min (RT120). (B) The graph shows the expression of tight junction and cytoskeleton proteins in Con, RT0, RT40 and RT120 groups. Data were normalised against NeuN and are expressed as the mean ± SEM of three independent experiments.

neuropil surrounding intact control capillaries showed compact appearance; meanwhile, in sleep restricted rats, perivascular oedema was observed after 0, 40 and 120 min of sleep recovery (see arrowheads in Fig. 6). Associated to perivascular oedema, it was also observed pericyte detachment from the capillary wall in sleep-restricted rats (see the asterisk in Fig. 6).

Discussion

In this study, we evaluated the ultrastructural and molecular changes of interendothelial junctions in the hippocampus as well as blood–brain barrier permeability in a model of chronic sleep restriction. Sleep restriction during 10 consecutive days increased blood–brain barrier permeability to fluorescein-Na and concomitantly induced ultrastructural changes in

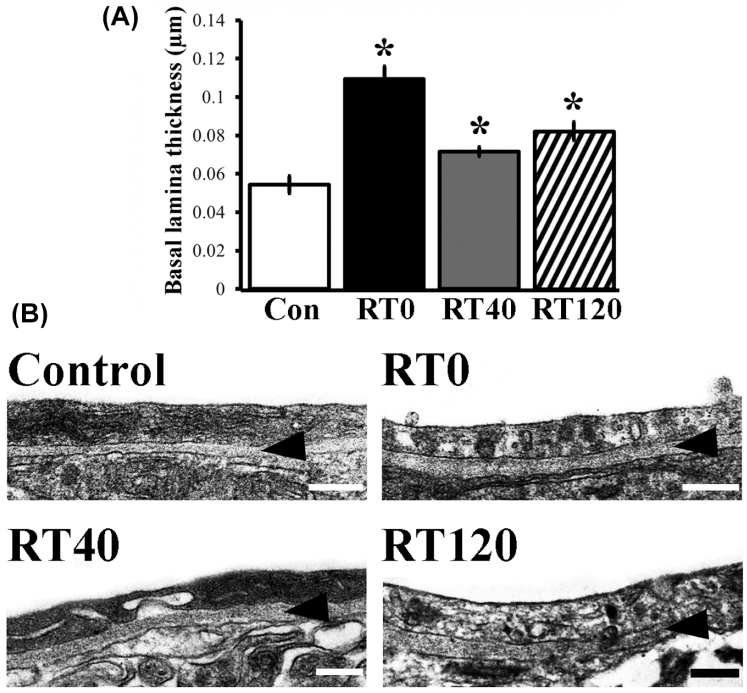

Fig. 5. Chronic sleep restriction increases basal lamina thickness. (A) The graph shows the basal lamina thickness in sleep restricted rats with sleep recovery times of 0 min (RT0, $n = 19$ microvessels), 40 min (RT40, $n = 15$ microvessels) and 120 min (RT120, $n = 12$ microvessels), as compared to intact controls (Con, $n = 20$ microvessels). Mean ± SEM, $^{*}p \leq 0.05$ versus intact controls. (B) Representative microelectrographs show the basal lamina thickness in intact controls and sleep restricted rats (see arrowheads). Scale bar: 200 nm.

hippocampal endothelial cells; these alterations included a decrease in interendothelial junction complexity, the formation of clefts between the membranes of adjacent endothelial cells and of cytoplasmic projections to the vessel lumen, and an increase of basal lamina thickness concomitant to perivascular oedema formation. Interendothelial junction derangement in the hippocampus may be explained by the decrease in the expression of the tight junction protein claudin-5. In fact, our data suggest that subtle changes in the expression of tight junction proteins are enough to disrupt the blood–brain barrier. The increased paracellular flux from blood to brain may be associated with the increase of the basal lamina thickness, the formation of perivascular oedema and pericyte detachment of vascular walls.

Previous reports showed that chronic sleep restriction increased blood–brain barrier permeability to large molecules such as Evans blue, 10 kDa and 70 kDa FITC-dextrans (Gómez-González *et al.*, 2013; Hurtado-Alvarado *et al.*, 2016a) as well as small molecules such as fluorescein-Na (He *et al.*, 2014). Particularly, the hippocampus showed a slow reduction in the permeability to Evans blue along the time of sleep recovery in the 10th day of sleep restriction; however, permeability remained higher than the control group even after 120 min of sleep recovery (Gómez-González *et al.*, 2013). He *et al.* (2014) found that blood–brain barrier permeability to fluorescein-Na in 6-day sleep-restricted mice was fully normalised at 24 h of sleep recovery; however based on our present findings, normal blood–brain barrier permeability to fluorescein-Na is fully achieved at 120 min of sleep recovery.

Similar to the findings reported here, Hurtado-Alvarado *et al.* (2016a) found that 10-day sleep restriction decreased tight junction protein expression in the hippocampus, and the effect was rapidly reverted by the administration of a selective antagonist of A_{2A} adenosine receptors. Likely, He *et al.* (2014) found that 6-day sleep restriction decreased mRNA expression of tight junction proteins. Here we found that a longer period of sleep restriction altered not only tight junction protein expression but also interendothelial junction ultrastructure, inducing simpler interendothelial junctions and the formation of clefts between adjacent endothelial cells in the hippocampus. Previously it was shown that type 4 complex overlap junctions are the maturest form of interendothelial junctions that fully preclude the paracellular diffusion of molecules from blood-to-brain, while type 1 end-to-end junctions are deemed as the less mature interendothelial junction (Schwartz & Benditt, 1972; Huang *et al.*, 2001). Therefore, both changes, the presence of simpler interendothelial junctions and the presence of wide clefts between the membranes of adjacent endothelial cells in the sleep restricted rats, are related to the increased

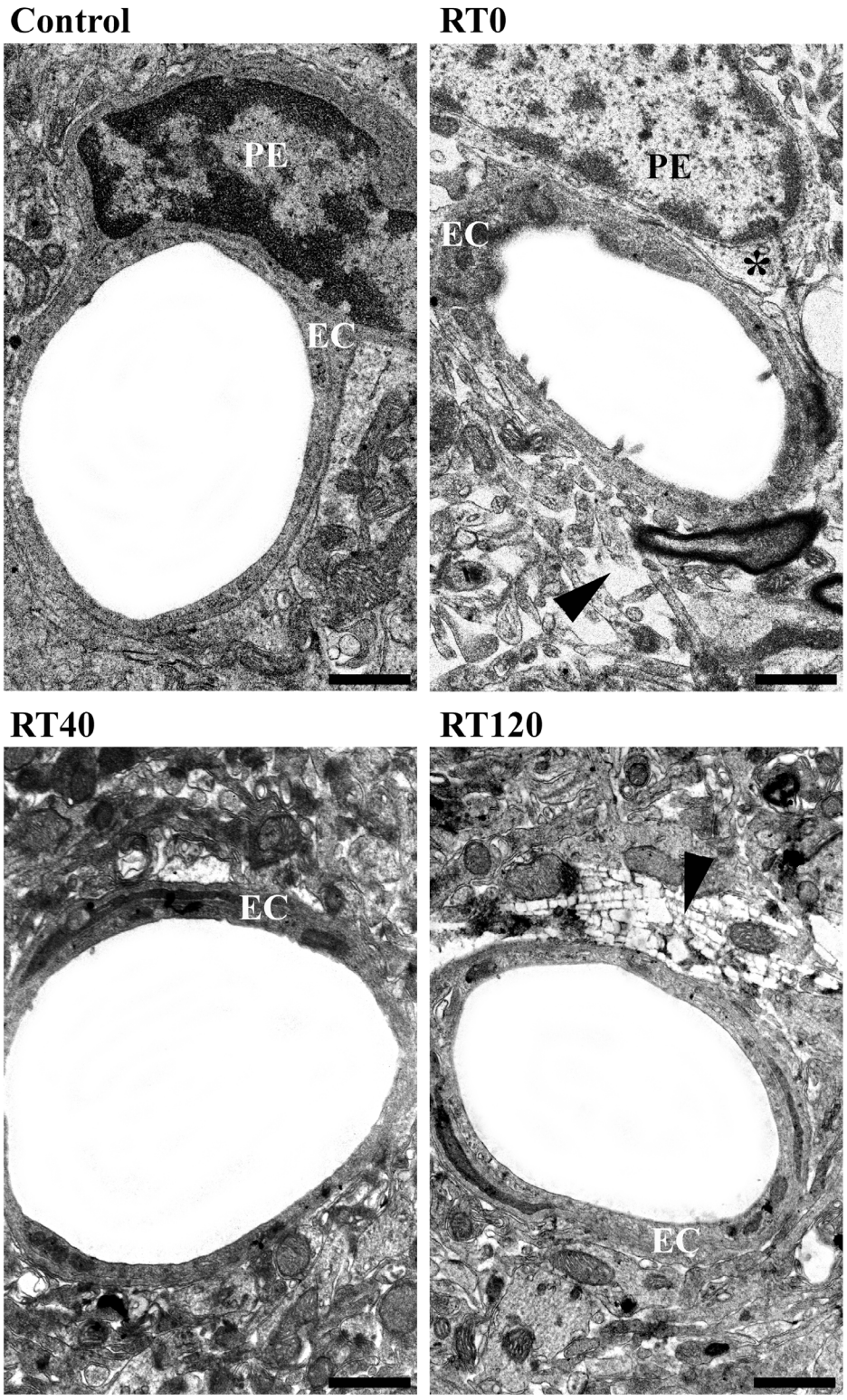

Fig. 6. Chronic sleep restriction alters the ultrastructure of hippocampal endothelial cells and induces perivascular oedema. Representative microelectrographs showing the general ultrastructure of microvessels under normal conditions and after 10 days of sleep restriction. Chronic sleep restriction induces the formation of cytoplasmic projections (arrow) and pericyte detachment (*). In addition, perivascular oedema is observed in sleep-restricted rats with sleep recovery times of 0 min (RT0), 40 min (RT40) and 120 min (RT120) (see arrowheads). Abbreviations are as follow: endothelial cells (EC), pericytes (PE). Scale bar: 1 μm.

blood–brain barrier permeability to both fluorescein-Na in the present study and the previously reported FITC-dextrans and Evans blue (Gómez-González *et al.*, 2013).

The mechanism underlying blood–brain barrier changes during sleep loss and recovery seems to be related to the low-grade inflammatory status induced by chronic sleep loss (Hurtado-Alvarado *et al.*, 2013). Indeed the fact that He *et al.* (2014) found that sleep restricted animals presented increased plasma levels of C-reactive protein and increased mRNA expression of COX-2 in the brain, as well as the report by Hurtado-Alvarado *et al.* (2016a) showing that A_{2A} adenosine receptor antagonism reverted the blood–brain barrier hyperpermeability and the tight junction protein changes induced by chronic sleep loss, point to a role of inflammatory mediators in regulating blood–brain barrier permeability during sleep loss as previously hypothesised by Hurtado-Alvarado *et al.* (2016b).

The increased blood–brain barrier permeability during sleep loss may explain the findings by Benedict *et al.* (2013), regarding the presence of neuronal and glial proteins in plasma after acute sleep deprivation in humans (Benedict *et al.*, 2013). The prolonged blood–brain barrier hyperpermeability after sleep loss might underlie the effects of sleep loss in hippocampal physiology, such as decreased neurogenesis (Guzmán-Marín *et al.*, 2008: Mueller *et al.*, 2008), altered hippocampal neurochemistry (Mohammed *et al.*, 2011) and reduced hippocampal volume (Meerlo *et al.*, 2009; Novati *et al.*, 2011). Indeed, the role of blood–brain barrier in generating cognitive impairments during sleep loss has been considered for obstructive sleep apnoea (Lim & Pack, 2014). During obstructive sleep apnoea, sleep fragmentation occurs concomitant to intermittent hypoxia; in those patients, oxidative stress and a systemic pro-inflammatory state is generally reported; both of them may alter microvessel permeability (Lim & Pack, 2014).

The phenotype of hippocampal endothelial cells in sleep restricted rats is similar to that observed in several pathologies and aversive conditions, such as diabetes (Bouchard *et al.*, 2002), stress (Sharma *et al.*, 1995; Gómez-González & Escobar, 2009), pericyte deficiency (Armulik *et al.*, 2010), hyperthermia (Kiyatkin & Sharma, 2009) and infections (Craig *et al.*, 1997). In the same way, perivascular oedema is present in stroke, ischemia and seizure-related brain damage (Nelson & Olson, 1987). Blood–brain barrier disruption is also considered to be a prime participant in diabetes mellitus (Hawkins *et al.*, 2006; Acharya *et al.*, 2013), chronic inflammatory pain (Brooks *et al.*, 2005) and Alzheimer disease (Levine *et al.*, 2010). Our findings suggest that the neurochemical changes associated to blood–brain barrier disruption may underlie the cognitive impairments reported after variable periods of sleep loss. Sleep restriction might be an interesting model to elucidate mechanisms involved in the onset of pathophysiological changes observed in obesity, diabetes and a wide range of neuropathologies.

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Conflict of interest

The authors declare that there are not conflicts of interest.

Author contributorship

Hurtado-Alvarado G: executed the experiments and wrote the manuscript.
Velázquez-Moctezuma J: designed the experiments and drafted the manuscript.
Gómez-González B: designed and executed the experiments and wrote the manuscript.

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ANEXO 2

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Review Article

Sleep Loss as a Factor to Induce Cellular and Molecular Inflammatory Variations

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A reduction in the amount of time spent sleeping occurs chronically in modern society. Clinical and experimental studies in humans and animal models have shown that immune function is impaired when sleep loss is experienced. Sleep loss exerts a strong regulatory influence on peripheral levels of inflammatory mediators of the immune response. An increasing number of research projects support the existence of reciprocal regulation between sleep and low-intensity inflammatory response. Recent studies show that sleep deficient humans and rodents exhibit a proinflammatory component; therefore, sleep loss is considered as a risk factor for developing cardiovascular, metabolic, and neurodegenerative diseases (e.g., diabetes, Alzheimer's disease, and multiple sclerosis). Circulating levels of proinflammatory mediators depend on the intensity and duration of the method employed to induce sleep loss. Recognizing the fact that the concentration of proinflammatory mediators is different between acute and chronic sleep-loss may expand the understanding of the relationship between sleep and the immune response. The aim of this review is to integrate data from recent published reports (2002–2013) on the effects of sleep loss on the immune response. This review may allow readers to have an integrated view of the mechanisms involved in central and peripheral deficits induced by sleep loss.

1. Introduction

Sleep is a vital phenomenon, classically divided into two distinct phases: sleep with rapid eye movements (REM) and sleep without rapid eye movements (non-REM) [1]. In humans, three stages of non-REM sleep have been characterized by electroencephalography (EEG); these include low-frequency slow wave sleep (SWS) with EEG synchronization, light sleep, and an intermediate sleep stage 2. In contrast, REM sleep is characterized by EEG activity similar to that of waking and by the loss of muscle tone [2, 3]. Both phases, REM sleep and non-REM sleep, alternate throughout total sleep time [2, 3]. REM sleep is amply studied because it is considered important for learning, memory consolidation, neurogenesis, and regulation of the blood-brain barrier function [4–8], while non-REM sleep is related to hormonal release (e.g., growth hormone secretion), the decline in the thermal set point, and is characterized by a reduction of cardiovascular parameters (e.g., lowering of blood pressure) [9, 10]. Although sleep constitutes a considerable portion of the mammalian lifetime [2], specific sleep function still remains controversial. Many hypotheses have been proposed, including tissue repair, thermoregulation, homeostatic restoration, memory consolidation processes, and preservation of neuro-immune-endocrine integrity [10, 11].

The paramount role of sleep in the physiology of animal models and humans is evident by the effects of sleep loss. Serious physiological consequences of sleep loss include

emotional reactivity, cognitive dysfunction (deficits in learning, memory, and decision making), decreased neurogenesis, and metabolic disturbances that may result in the death of experimental animals [1, 7, 12–14]. Sleep loss effects can be evaluated by several methodologies, including acute total or selective sleep deprivation and sleep restriction (also called partial sleep deprivation) or sleep fragmentation. In some cases, deprivation devices connected to the electroencephalograph have been used to selectively deprive a specific sleep phase. In humans, total sleep deprivation is common in individuals working more than 24 hours continuously, while sleep restriction is defined as diminution of time spent asleep. Sleep restriction is linked to lifestyle including longer work hours and shift-work and increased accessibility to media of all sorts, or medical conditions such as insomnia [15, 16]. Pathological conditions (e.g., obstructive sleep apnea (OSA), drug addiction) and aging have a common pattern of sleep fragmentation (also called sleep disruption) characterized by numerous awakenings despite normal time spent asleep [16]. Most of the current knowledge on the effects of sleep loss in humans comes from studies of total sleep deprivation applied for brief time periods or partial sleep deprivation (2-3 hours less than normal sleep time) for one night or even for chronic periods [15, 16]. The majority of animal models used to study the physiological effects of sleep loss are based primarily on total sleep deprivation [16]. Although this method does not resemble human conditions, it still provides valuable information on sleep loss effects.

To study the relationship between sleep and the immune system, researchers have relied on two basic approaches; in the first approach, human volunteers or animals (mainly rodents) are subjected to the administration of immune-stimulating substances (or pathogen administration in animals), and the effects of these manipulations on sleep are evaluated. In the second approach, human volunteers or animals are subjected to sleep loss protocols (sleep deprivation, sleep restriction, or sleep fragmentation) and immunological products such as cells and/or soluble mediators are measured. Here, we present a compilation of recent evidence about the effects of sleep loss on the immune system in both humans and rodents, under acute and chronic sleep loss. Additionally, we propose how sleep recovery might restore the normal balance between proinflammatory and anti-inflammatory molecules at the systemic level and how immune mediators might be in direct contact with the central nervous system via blood-brain barrier disruption, modifying neural activity and the possible pathway for neurological impairments.

2. Sleep Loss as a Stressful Factor

Sleep loss has been deemed a stressor [17, 18]; however, sleep and stress differ in the profile of circulating molecules and in their effects on the immune system. Stress is the response of the organism to any stimulus that alters the homeostasis [19]. The adverse stimuli generating stress, either physical or psychological, also vary in their temporal dimension. Acute stress occurs when stressors appear once and remain for a short period of time (some minutes or hours); while, chronic stress occurs when stressors are repetitive and long lasting (appearing for weeks or months) [19]. Since the initial description of the phenomenon [20], it has been shown that stressors induce activation of the hypothalamus-pituitary-adrenal (HPA) axis and of the sympathetic nervous system [19, 21]. At the beginning of the stress response, there is a large sympathetic activation, followed by glucocorticoid release from the adrenal cortex. Over a prolonged stress period, the adrenaline response is rapidly habituated; however, glucocorticoids remain elevated only when stressors are unpredictable and uncontrollable. If the subject is capable of predicting the appearance of chronic stressors and has control over them, the glucocorticoid response also disappears [21].

Regarding the effect of stress on the immune system, it has been shown that acute stress has an immunostimulatory effect; adrenaline increases the circulating numbers of neutrophils, macrophages, natural killers, and lymphocytes, while glucocorticoids promote traffic of leukocytes to the skin, mucosal lining of the gastrointestinal and urinary-genital tracts, the lung, and liver, both in humans and in experimental animal models [22–27]. Therefore, acute stress seems to prepare the immune system to cope with the damage induced by the noxious agent. On the contrary, chronic stress suppresses the immune function by modifying the levels of both proinflammatory (e.g., interleukin (IL)-6 and tumor necrosis factor (TNF)-α) and anti-inflammatory cytokines (e.g., IL-10, IL-4) [28], by reducing the numbers and traffic of leukocytes [27], and by up-/downregulating T cell number and function [29]. Specifically, glucocorticoids act on antigen-presenting cells (APCs) and T helper 1 (T_h1) cells, inhibiting their production of IL-12, interferon (IFN)-α, IFN-β, and TNF-α, but upregulating the production of anti-inflammatory cytokines (IL-4, IL-10, and IL-13) by T_h2 cells [30].

Since the pioneer studies, sleep loss has been tightly linked to stress; in the first studies it was shown that sleep deprived animals had larger adrenals than their counterpart controls [1, 31]. In animal models, the classical methods for sleep deprivation consist of highly aversive environments (e.g., water surrounding small platforms); therefore, additional animals subjected to the aversive environment but without any sleep loss are constantly included as controls for the procedure. The measurement of circulating levels of glucocorticoids is the main stress index; nevertheless, depending on the intensity and duration of sleep loss, cortisol/corticosterone levels may increase [32–35], not change [33, 36], or even decrease [37] (see Table 1). It is known that the chronic increase in cortisol/corticosterone levels desensitize glucocorticoid receptors, promoting an altered control of the HPA axis [38]; this may explain the maintenance or even the decrease in glucocorticoid levels after sleep deprivation (e.g., >40 h in humans) [39] or chronic sleep restriction (e.g., 21 days in rats) [33].

The role of glucocorticoids in sleep homeostasis has been carefully studied; glucocorticoid administration in both humans and animal models induces waking EEG activity (e.g., [42, 43]); in addition, glucocorticoid administration decreases REM sleep and promotes SWS in humans [42] and decreases SWS and increases sleep latency in animal

Table 1: Differential effect of sleep loss time upon glucocorticoid levels.

Human	Cortisol	Reference
TSD 1 night	↑	[34]
TSD 40 hours	= or ↓	[40]
TSD 40 hours	=	[39]
SR 2 hours TIB/1 night	=	[36]
SR 3 hours TIB/4 days	↓	[37]
SR 6 hours TIB/6 days	=	[41]
Rodents	Costicosterone	Reference
RSD 72 hours	↑	[32]
RSD 96 hours	↑	[33]
RSR with 6 hours of SO/21 days	=	[33]

The table illustrates the differential effect of acute sleep deprivation and sleep restriction upon glucocorticoid levels. Representative samples present in this table were measured within the first four hours after wakefulness in humans or at the beginning of the light phase in rodents.
Abbreviations: TSD: total sleep deprivation; SR: sleep restriction; TIB: time in bed; RSD: REM sleep deprivation; SO: sleep opportunity; ↑: increase; =: not change; ↓: decrease.

models [42]. When they occur, increased corticosterone levels secondary to sleep deprivation are unnecessary for sleep recovery; in animal models, a large sleep rebound was observed after acute sleep deprivation, despite adrenalectomy [44]. Moreover, under chronic REM sleep deprivation in rats, where corticosterone levels are similar to basal levels [33], a tendency to REM sleep rebound is also observed [45].

In the vast majority of studied phenomena (e.g., studies of sleep loss effects on hippocampal neurogenesis), it has also been found that sleep loss effects are maintained even in animals subjected to adrenalectomy [46]. Additionally, chronic administration of an inhibitor of corticosterone secretion (metyrapone) in REM sleep deprived animals did not revert memory deficits; hence glucocorticoids are not responsible for the memory impairments associated to REM sleep loss [47]. These data show that sleep loss may cause more functional deficits than those caused by stress only. It is very likely that the effects of REM sleep deprivation on neural, endocrine, and immune systems accumulate throughout the experimental procedure without any opportunity to restore homeostasis by adequate sleep recovery. Notwithstanding, some authors still consider that sleep loss is a stressful event [18], while the vast majority of sleep researchers deem sleep deprivation and stress as independent events [42–44, 46–48].

3. Sleep and the Immune Response

It is well known that sleep loss makes an individual more susceptible to disease and, conversely, that sleep is important for recovery from illness. Specific immunological active peptides or neuroendocrine hormones influence the sleeping-waking brain, and sleep disturbances may affect inflammatory components. Cellular (macrophages, neutrophils, eosinophils, basophils, natural killer, and T and B lymphocytes) and molecular (proinflammatory cytokines and acute phase proteins) inflammatory components that act as mediators of the acute phase response in inflammatory diseases, additionally, play a role as modulators of metabolic functions that involve the central nervous system, including sleep.

3.1. Effects of Inflammatory Components on Sleep. Cytokines that affect sleep in both humans and laboratory animals include IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-15, IL-18, TNF-α, TNF-β, IFN-α, IFN-β, IFN-γ, and macrophage inhibitory protein (MIP)-1β (MIP-1β) [49]. Immune signaling molecules such as cytokines are present in the healthy brain, where they interact with neurochemical systems (e.g., serotoninergic, cholinergic, and glutamatergic systems) [49, 50] to regulate normal sleep. Particularly, IL-1α, IL-1β, and TNF-α have been widely investigated to state that they are involved in the regulation of physiological sleep. Signaling receptors for both IL-1 (α and β) and TNF-α are present in brain areas involved in sleep physiology including the hypothalamus, brainstem, hippocampus, and cerebral cortex [49]. The brain interacts with peripheral inflammatory mediators through the innervation of lymphoid tissues or the transport or action of these molecules on the blood-brain barrier [51]. In addition, glial cells such as microglia and astroglia, as well as pericytes are capable of releasing proinflammatory mediators in response to peripheral signals (chemokines, acute phase proteins, nitric oxide, and adenosine) contributing to the action of inflammatory mediators upon neuronal function [52, 53]. Because IL-1α, IL-1β, and TNF-α are the most studied cytokines involved in sleep regulation, we focus mainly on these three cytokines; however, the role of IL-6 will also be reviewed because this proinflammatory cytokine is highly related to the interaction between sleep loss and the immune response.

3.1.1. Effect of Proinflammatory Cytokine on Sleep in Humans. Interleukin-1 is a key mediator of the acute phase response in an infected host [54]. IL-1α and IL-1β together with TNF-α have many physiological roles, such as in cognition, synaptic plasticity, and immune function. Both IL-1β and TNF-α are also well-characterized as to their actions on sleep regulation [55]. For instance, IL-1β is a potent enhancer of non-REM

sleep that induces symptoms associated with sleep loss such as sleepiness, fatigue, and poor cognition [56].

Under pathological conditions (e.g., cancer, multiple sclerosis) cytokine administration is used as a treatment [57, 58] and sleep patterns are altered [59, 60]; in patients with multiple sclerosis numerous sleep pathologies (e.g., insomnia, hypersomnia, circadian rhythm sleep disorders, and movement- and breathing-related sleep disorders) have been described [59], while in cancer patients complaints about sleep fragmentation and insomnia are frequent [60]. Although sleep disturbances are frequently reported in autoimmune pathologies and mood disorders with an inflammatory component [59–61], the aetiology of sleep alterations remains unclear. To cite a few instances, it has been reported that autoimmune diseases that exhibit autoantibodies against neuronal voltage-gated potassium channel (VGKC) complexes such as limbic encephalitis or Morvan syndrome present sleep disturbances like insomnia, REM sleep behavior disorder, hypersomnia, and somniloquy [62, 63]. Interestingly, immunotherapy in patients with autoimmune diseases promotes significant sleep improvement in 80% of patients [62]. Also, infections with a proinflammatory component induce sleep disorders, up to 70% of persons living with human immunodeficiency virus (HIV) experience sleep disturbances including insomnia and obstructive sleep apnoea (OSA) syndrome [64], and in people affected by leprosy the prevalence of restless leg syndrome is higher than the general population [65]. In the same way, inhibition of proinflammatory cytokine signalling has been proposed as a viable strategy for targeting sleep disturbances in patients with evidence of proinflammatory activity [66]. For instance, in alcohol-dependent males, inflammatory markers correlated with REM sleep increase [66], but the pharmacological neutralization of TNF-α by etanercept (a decoy receptor that binds to TNF-α) reduced REM sleep until normal values [67]. In addition, both IL-1 (α and β) and TNF-α are present in a variety of clinical conditions involving sleep disorders, such as chronic insomnia and OSA (reviewed in [68]).

3.1.2. Effect of Proinflammatory Cytokines on Sleep in Animal Models. It has been known for over 50 years that mammalian cerebrospinal fluid contains sleep-promoting substances that accumulate during wakefulness [10]. The common criteria to consider any substance a somnogenic molecule include (1) whether the substance injected enhances sleep, (2) whether sleep is reduced if the substance is inhibited, and (3) whether the substance is altered in pathological states associated with sleep disorders. All of these criteria have been met by IL-1α, IL-1β, IL-6, and TNF-α [68].

The effects of IL-1α, IL-1β, and TNF-α on sleep was reported in several animal species including rodents, monkeys, cats, and sheep. Induction of non-REM sleep by IL-1α, IL-1β, and TNF-α is independent of the route of administration (e.g., intracerebroventricular (ICV), intraperitoneal, subcutaneous) and its effect is dose-dependent [68, 69]. In rodents, classical studies show that low doses of IL-1β, through ICV administration, increase non-REM sleep when it is administered during the light phase [69]. However, IL-1α or IL-1β also induce non-REM sleep fragmentation [49], and high doses of IL-1β, administered during the dark phase, suppress non-REM sleep [68].

In addition to the pioneer studies on sleep regulation by cytokines, recent studies focus on the molecular pathways involved in physiological sleep regulation. Recently, mice lacking the TNF 55kDa receptor (TNFR-KO) present a decrease in the amount of non-REM and REM sleep [70]. Furthermore, experimental studies in rodents show that proinflammatory cytokine-induced sleep disturbances can be reversed by administration of anti-inflammatory cytokines or specific cytokine antagonists (e.g., IL-1 receptor antagonist, IL-1ra) [68]. The strong relationship between sleep and its modulation by proinflammatory cytokines provides a key to understand how sleep loss is capable of altering the immune system and subsequently promotes metabolic, cardiovascular, and neurodegenerative impairments [15].

3.2. Effect of Sleep Loss on Immunological Response in Humans

3.2.1. Effects of Sleep Loss on Cellular Immune Components. Circadian rhythms have been described for white blood cells (WBC) in humans; numbers of circulating natural killers (NK) and neutrophils peak at midday and show a nadir during the night; while, monocytes, T and B lymphocytes peak during the first half of the night and present the lowest values during the day hours [71]. Sleep loss shifts the normal circadian rhythm of WBC. In 24-hour total sleep deprived humans, monocytes, T and B lymphocytes presented a delay in the zenith of the rhythm with attainment of peak values between 3 and 6 hours later than in normal sleep conditions [71]; while the rhythm of NK flattened with a net increase in the NK number during the sleep deprived night as compared to normal sleep conditions [71, 72]. However, only few human studies have repeatedly drawn blood samples from sleep deprived subjects to measure circadian effects of sleep deprivation on WBC counts; the majority of reported studies quantify circulating WBC only once, on the morning after sleep deprivation and compare those values with normally sleeping subjects. Generally, in those studies leukocyte population increases after acute sleep deprivation, mainly by rises in circulating numbers of monocytes and neutrophils; in contrast, circulating numbers of B and T lymphocytes remain stable immediately after sleep loss, but exhibit changes after sleep recovery (see Table 2) [73, 74]. Sleep restriction to 4 hours in bed during 5 consecutive nights decreased the number of circulating NK and increased the number of B lymphocytes, maintaining stable the numbers of other WBC [75]. Differences among these studies may be explained by the different techniques to draw blood samples, such as sex, race, or age of the participants.

3.2.2. Effects of Sleep Loss on Molecular Inflammatory Component

Effect of Sleep Loss on Antibodies. Few studies have examined the consequences of sleep loss on the immune response to

Table 2: Sleep loss effects on immune cellular components in humans.

Sleep loss condition	Subject's characteristics	Cells		Reference country
Sleep deprivation	11 males	Leukocyte ↑*	B lymphocytes =	[73]
2 nights	19–29 years	Neutrophil ↑**	T lymphocytes =	Brazil
Sleep restriction	10 females	WBC ↑**	B lymphocytes =	[74]
4 hours time in bed	PM-RT	Monocytes ↑*	T lymphocytes =	Belgium
3 nights	55–65 years	Neutrophils ↑*		
Sleep restriction	7 females, 7 males	NK =	B lymphocytes =	[76]
4.5 hours time in bed	39–61 years	Monocytes =	T lymphocytes =	USA
1 night				
Sleep restriction	8 males	Neutrophils =	Lymphocytes =	[77]
4 hours time in bed	22–29 years			Belgium
3 nights				
Sleep restriction	13 males	Monocytes =	B lymphocytes ↑**	[75]
4 hours time in bed	19–29 years	NK-cells↓**	T lymphocytes =	Finland
5 nights				

The table illustrates the differences between sleep deprivation and sleep restriction upon cellular components of the immune system in humans.
Abbreviations: NK: natural killers; PM-RT: postmenopausal with replacement therapy; ↑: increase; =: not change; ↓: decrease; *significant differences with $P < 0.05$; **significant differences with $P < 0.01$.

vaccination in healthy individuals; highly variable findings have been reported [78–80]. Total sleep deprivation during one night prior to hepatitis A vaccination reduced specific antibody titters in the long-term (28 days postvaccination) in both males and females [78]. However, the same 24 hours of total sleep deprivation reduced specific antibody titters to influenza AH1N1 virus vaccine only in males in the short term (5 days post-vaccination), while sleep deprived females did not have a significant difference as compared to normal sleeping subjects [79]. In another study, short sleep durations during the week of hepatitis B vaccination decreased viral specific antibody titters in both male and female volunteers; while the contrary was true, higher levels of antibody titters were observed in participants with long sleep durations during the week of vaccination [80]. Although few, those studies suggest that sleep plays an important role in humoral immunity, especially in antibody production; however, more studies are necessary to elucidate how sleep loss may induce changes in cellular immune components and subsequently induce antigen-specific immune impairments, such as insufficient antibody production.

IL-1α, IL-1β, IL-6, and TNF-α: The Most Studied Cytokines under Sleep Loss Conditions. Human studies that evaluate sleep loss effects have focused on the correlation among inflammatory markers and metabolic and cardiovascular diseases. For instance, in a study with 124 healthy volunteers, inflammatory markers, such as endothelin-1 (ET-1) and IL-6, were associated with an increase in total sleep time and REM sleep latency [81]. These results show that poor sleep is directly associated with inflammatory status. In the same way, shorter sleep duration is also related to obesity and cardiovascular diseases [82]. It is known that obesity, diabetes, and cardiovascular diseases share a common mechanism characterized by the inflammatory process. If sleep loss induces low-intensity inflammation, we may consider that sleep loss is associated with metabolic and cardiovascular disease generation through immunological deregulation [15].

Similar to immune cells, cytokine production presents circadian rhythms; proinflammatory cytokines present a peak in early nocturnal sleep in correlation with the accumulation of molecules such as adenosine or reactive oxygen species that promote proinflammatory cytokine release; however the dominance of the proinflammatory response shifts during late sleep, when REM sleep is present, promoting the production of anti-inflammatory cytokines [51, 83]. The different periods of exposure to proinflammatory mediators might explain the reported differences between cytokine plasma levels in sleep loss protocols.

Sleep deprivation protocols, lasting 40–88 hours in humans, induce controversial changes in plasma levels of IL-1α, IL-1β, IL-6, and TNF-α, with reported findings of increases, decreases, or absence of measurable changes in cytokine levels [34, 39–41, 83, 84] (see Table 3). For example, IL-6 plasma levels increased after one week of sleep restriction in healthy males [41]. In contrast, a study with 40 hours of continuous total sleep deprivation found decreased IL-6 levels in healthy men [40]. These discrepancies may be attributed to the method employed to obtain blood samples; intravenous catheters used for repetitive blood sampling increase local IL-6 production, which might confound the sleep-dependent changes in plasma concentrations of this cytokine [85]. In addition, all the cellular sources of proinflammatory cytokines are not known, although monocytes, which make up about 5% of circulating leukocytes, are major contributors

Table 3: Sleep loss effects on immune molecular inflammatory mediators.

Sleep loss condition	Subject's characteristics	Cytokines (pg/mL)	C-reactive protein	Reference
Total sleep deprivation 1 night	16 controls 11 females, 5 males BMI 20.7–24.1 kg/m^2 15 unmedicated depressed patients 10 females, 5 males BMI 18.8–26.4 kg/m^2	IL-6 ↑ SL Control Basal 1.50 ± 1.10 TSD 2.56 ± 1.63* Recovery 2.82 ± 1.94* Depressed Basal 1.14 ± 0.69 TSD 2.38 ± 1.87*	ND ND ND ND ND	[34] Germany
Total sleep deprivation 1 night	9 females, 1 male Bipolar disorder 36–53 years	IL-6 ? SL Basal ND TSD 3.15 ± 5.14	ND ND	[87] Italy
Total sleep deprivation 40 hours	9 females, 10 males 20–36 years BMI 18.5–24.5 kg/m^2	IL-1β ↑ PL Basal ~0.20 TSD ~0.45* IL-6 ↑ PL Basal ~1.6 TSD ~1.9*	CRP ↑ PL mg/L Basal ~0.20 TSD ~0.50*	[40] USA
Total sleep deprivation 40 hours	12 healthy males 29.1 ± 3.3 years BMI 23.4 ± 1.5 kg/m^2	IL-6 = Basal 0.60 ± 0.13 TSD 0.62 ± 0.10 Recovery 1.20 ± 0.23* TNF-α = Basal 0.88 ± 0.32TSD 1.05 ± 0.30	CRP = μg/mL Basal 1.22 ± 0.46 TSD 0.55 ± 0.13 Recovery 0.61 ± 0.14	[88] France
Total sleep deprivation 40 hours	12 healthy males 26–32 years BMI 21.9–24.9 kg/m^2	IL-6 = PL Basal ~3.5 TSD ~3.6 TNF-α ↑ PL Basal 0.66 ± 0.19 TSD 1.29 ± 0.33*	ND ND	[39] France
Total sleep deprivation 88 hours	10 healthy males 22–37 years	ND	CRP ↑ PL mg/L Basal 0.39 ± 0.13 Day 1: 0.48 ± 0.16* Day 2: 0.50 ± 0.20* Day 3: 0.65 ± 0.23* Recovery 0.66 ± 0.24*	[89] USA
Sleep restriction 5 hours time in bed (1 night)	20 males 20–22 years 71–75 kg	IL-6 ↑ PL Basal 1.89 ± 0.06 SR 3.9 ± 0.70*	ND ND	[90] Tunisia
Sleep restriction 4.2 hours time in bed (2 nights)	15 males 20–40 years BMI 20.5–24.9 kg/m^2	IL-6 = PL Basal 2.0 ± 0.0 SR 2.2 ± 0.02	ND ND	[84] Germany

Table 3: Continued.

Sleep loss condition	Subject's characteristics	Cytokines (pg/mL)	C-reactive protein	Reference
Sleep restriction 4 hours time in bed (4 days)	25 control males 25 alcoholic males	IL-6 ↑ PL Basal ~2.9 SR ~2.8 SR + Alc ~4.1 TNF-α ↑ PL Basal ~1.2 SR ~1.0 SR + Alc ~3.0	ND ND ND	[91] USA
Sleep restriction 1 hour time in bed (7 nights)	8 males 25.8 ± 0.9 years BMI 80 ± 3.7 kg/m^2 Demanding physical challenges and SR	IL-1β ↑ PL Basal 8.9 ± 2.8 SR day 7: 45.2 ± 6.3*	CRP↑ PL mg/L Basal 1.38 ± 0.89 SR Day 7: 11.38 ± 3.05*	[92] Norway
Sleep restriction 4.2 hours time in bed (10 nights)	4 females, 6 males 26–38 years BMI 21–31 kg/m^2	ND	CRP ↑ PL mg/L Basal 0.51 ± 0.20 SR 2.65 ± 1.31*	[89] USA
Sleep restriction 4 hours time in bed (10 nights)	6 females, 12 males 21–40 years BMI 20–26 kg/m^2	IL-6↑ PL Basal 1.88 ± 0.85 SR D10: 3.04 ± 2.83* Recovery 2.36 ± 1.36*	CRP = SL mg/L Basal 0.34 ± 0.27 SR Day 10: 0.69 ± 0.76	[85] USA
Sleep fragmentation OSA patients	22 females, 136 males BMI < 30 kg/m^2 28 females, 136 males BMI 30.1–34.9 kg/m^2 25 females, 107 males BMI > 35 kg/m^2	IL-6 ↑ SL 1.3 ± 0.1 1.6 ± 0.2** 2.2 ± 0.2**	CRP ↑ SL mg/L 1.8 ± 0.2 4.1 ± 0.5** 2.6 ± 0.3**	[93] Iceland
Sleep fragmentation OSA patients	148 children 6–12 years	TNF-α ↑ PL AHI ≤ 1: 3.30 ± 0.4 AHI ≥ 10: 10.02 ± 1.36*	ND	[94] Spain
Sleep fragmentation veterans	Good sleep 7 males Poor sleep 58 males	IL-1β = PL Good sleep ~1.7 Poor sleep ~3.2 IL-6 = PL Good sleep ~37.6 Poor sleep ~34.2 TNF-α = PL Good sleep ~0.8 Poor sleep ~1.2	ND ND	[95] USA

Table 3: Continued.

Sleep loss condition	Subject's characteristics	Cytokines (pg/mL)	C-reactive protein	Reference
Sleep fragmentation OSA patients	Sleep durations <6 hours, 249 males 6-7 hours, 227 males >7 hours, 135 males	IL-6 = PL <6 hours: 2.08–2.54 6-7 hours: 1.96–2.39 >7 hours: 2.00–2.59 TNF-α = PL <6 hours: 2.86–3.73 6-7 hours: 2.52–3.28 >7 hours: 2.19–3.10	CRP ↑ SL mg/L <6 hours: 1.79–2.47 6-7 hours: 1.71–2.35 >7 hours: 1.71–2.56	[96] USA

Abbreviations: AHI: apnea-hypopnea index (expressed as the number of events per hour of total sleep time); BMI: body mass index; ND: nondetermined; OSA: obstructive sleep apnea; PL: plasma levels; SL: serum levels; SR: sleep restriction; TSD: total sleep deprivation; ~: approximate values obtained from report tables; ↑: increase; =: not change; ↓: decrease; ?: without basal data; * significant differences with $P < 0.05$; ** significant differences with $P < 0.01$. Mean ± standard deviation.

to proinflammatory cytokine production in peripheral blood [71]. Interestingly, studies reported differences in proinflammatory cytokine levels independent of WBC number or activity. This may be explained by considering other sources of cytokines (e.g., macrophages in adipose tissue, epithelium, and endothelium) [86], which may also be affected by sleep loss.

In addition to modifying IL-1α, IL-1β, IL-6, TNF-α, and IL-17A levels, five nights of sleep restriction are accompanied by increased heart rate; both proinflammatory cytokines and hypertension are important risk factors for development of cardiovascular disease [75, 97]. IL-17A plays a key role in sustaining tissue damage in the brain, heart, and intestine, sometimes promoting the development of autoimmune diseases [75]. Helper T cells producing IL-17A require activation by IL-6 [98]. Interestingly, IL-17A is a potent inducer of C-reactive protein (CRP) expression in hepatocytes and in coronary artery smooth muscle cells [99] (see next section). The combination of circulating cytokines with other inflammatory mediators achieves a low-grade inflammatory status induced by sleep loss.

Effect of Sleep Loss on Acute Phase Proteins. The effects of sleep loss on acute phase proteins are poorly studied. For instance, acute total sleep deprivation (one night) results in elevated high-sensitivity C-reactive protein (hsCRP) concentrations, which is a stable marker of inflammation that has been shown to be predictive of cardiovascular morbidity [89]. CRP production in the liver is stimulated by proinflammatory cytokines such as IL-6 or IL-17, which are highly expressed after sleep loss periods [75]. CRP is an important inflammatory marker because this protein lacks diurnal variations [15, 100]. In contrast, total sleep deprivation for 40 hours in young adults decreased CRP levels while increasing other inflammatory markers such as E-selectin and the intracellular adhesion molecule (ICAM)-1 [81]. Several methodological differences among the studies may contribute to the inconsistent findings for CRP (see Table 3), including the sleep deprivation period, blood sampling frequency, nutrition, and all effects and differences in subject's characteristics such as body mass index (BMI), because obesity increases proinflammatory markers [101]. In addition to voluntary sleep loss, several health conditions (e.g., pregnancy, depression) may contribute to deregulation of the immune system [102].

3.2.3. Sleep Loss and Depression. Recently, it has been suggested that one of the functions of sleep may be to regulate the neuro-immune-endocrine network [11]. In this regard, an excellent example of the interaction between the neuro-endocrine-immune network and sleep disorders is major depressive disorder, which is characterized by high levels of cortisol and TNF-α, increased NK percentages, diminished B lymphocyte counts, and no significant variations in T lymphocytes [103]; these changes are similar to the effects observed after sleep deprivation (see previous sections). In depressed patients, sleep disturbances include intermittent awakenings, prolonged sleep latency, and shortened REM sleep latency, which represent sleep fragmentation or sleep restriction (in the case of insomnia) [104, 105]. All antidepressants affect sleep architecture and quality [104], and the immune system might be altered in long-term treatment periods. For instance, depressed patients treated with selective serotonin-reuptake inhibitors for 20 weeks showed an increase in B lymphocytes [106]. The role of both major depressive disorder and sleep disturbances on the increased risk to develop metabolic disturbances is discussed in another recent review (please see [107]).

3.3. Effect of Sleep Loss on the Immune System in Animal Models

3.3.1. Effects of Sleep Loss on Cellular Immune Components. As in humans, the circadian oscillation of immune cells and molecules in rodents has been described. In mice, Ly6C^{hi} inflammatory monocyte traffic is regulated by the circadian gene Bmal1, and is higher during the resting phase and decreases during the active phase [108, 109]. Macrophages and NK contain a cell-autonomous circadian clock [110, 111]. In addition, T lymphocytes exhibit clock gene regulation,

mice immunized during the light phase show a stronger specific T lymphocyte response than those immunized during the dark phase [112]. These data suggest that a disruption of circadian rhythms might be related with changes in the WBC count after sleep loss. In rodents subjected to selective REM sleep deprivation for 24 and 240 hours, the number of T lymphocytes decreases and of B lymphocytes does not change. In the same experiment, an increase in NK percentage was observed [25]. Similarly, REM sleep deprivation for 96 hours does not promote changes in number of lymphocytes but it does increase the number of monocytes and neutrophils [33]. Controversially, REM sleep restriction promotes a decrease of leukocyte number [33]. These contradictory findings might be explained by the alteration in clock genes involved in the circadian oscillation of WBC.

3.3.2. Effects of Sleep Loss on the Molecular Inflammatory Component. Similar to humans, rodents subjected to sleep loss exhibit a proinflammatory component characterized by increase in proinflammatory cytokines, namely IL-1, IL-6, IL-17, and TNF-α as compared to control animals [32, 33]. The proinflammatory status after sleep loss may be explained, in part, because the alteration in clock genes of monocytes is associated with the upregulation of proinflammatory cytokines via NF-κB activation [76, 113]. Exposure to proinflammatory cytokines in chronic sleep restriction may promote tissue damage and subsequent loss of function; however, acute sleep deprivation may exert beneficial effects on the immune system. For instance, acute sleep deprivation is associated with a reduction in ischemia-induced IL-1β gene expression and attenuation of neuronal damage in the hippocampus. This finding may be explained by increased gene expression of IL-6 and the anti-inflammatory cytokine IL-10 after sleep deprivation [114].

4. Impact of Sleep Recovery on Sleep Loss-Induced Inflammation

Usually, the modification of cellular immune components and molecular inflammatory markers by sleep loss returns to basal levels after sleep recovery periods [34, 76]. However, depending on sleep loss time, some immune components may remain altered after sleep recovery or may even present alterations only after sleep recovery [32, 33, 100, 115]. For instance, monocyte and neutrophil numbers do not change after REM sleep deprivation in rats for 96 hours; however, after 24 hours of sleep recovery, monocyte and neutrophil numbers increase in comparison to control animals [33]. Levels of other WBC in rats decrease immediately after sleep restriction, but 24 hours of uninterrupted sleep restores the basal levels [33]. Like cellular components, molecular inflammatory mediators are altered after sleep recovery. Plasma levels of complement protein C3 were higher than controls after sleep deprivation in rats and remained elevated after sleep recovery [33]. REM sleep deprivation in rats (72 hours) increases plasma levels of IL-1, IL-6, IL-17A, and TNF-α. Proinflammatory cytokines IL-1α, IL-1β, and IL-6 return to basal levels after sleep recovery, whereas IL-17A and TNF-α remain higher than controls even after one week of normal sleep [32]. In addition, in the same study anti-inflammatory cytokines, such as IL-10, do not increase. Within the same context, in humans, increased sleepiness after sleep restriction was better reversed with a nap or with extended sleep recovery conditions (10 hours of uninterrupted sleep) [36]. In addition, other parameters associated with sleep loss were restored; for example, cortisol decreased immediately after a nap [36]. A midday nap prior to recovery sleep or an extended night of sleep can return leukocyte counts to baseline values [36]. Although long periods of sleep appear to be the solution to restore immune function, it has been reported that sleeping more than 9 hours is related with greater physical decline than midrange or short periods of sleep and also is related with increased risk of mortality associated with cardiovascular impairments [116].

5. Sleep Loss Alters the Blood-Brain Barrier

Up to this time, we have only discussed the effect of sleep loss on immune mediators at the peripheral level. Nevertheless, brain-immune system communication is very complex and it includes the direct action of proinflammatory cytokines synthesized in the brain [52, 117, 118] on neuronal systems, or the effect of peripheral cytokines on blood-brain barrier components [51]. We reported that chronic REM sleep restriction in rats induces blood-brain barrier disruption and that brief sleep recovery periods lessened these effects in several brain regions. Nevertheless, in the hippocampus hyperpermeability remained even after sleep opportunity [8]. These findings suggest that if sleep restriction increases the unselective transportation across the blood-brain barrier, proinflammatory mediators and toxic blood-borne molecules might enter the brain promoting neurochemical changes or excitotoxicity events that may explain cognitive and emotional impairments associated with sleep deficits.

6. Conclusion and Future Directions

Recent studies focus on evaluating the correlation between inflammatory markers and sleep disorders. Conditions such as obesity or infections may exacerbate the inflammatory condition contributing to systemic impairments and susceptibility to pathogens. Although sleep recovery may restore immune system alterations, when sleep loss is prolonged the proinflammatory status may remain and promote neuro-immune-endocrine axis disruption. Constant systemic inflammatory status after prolonged wakefulness may be the source of metabolic, cardiovascular, and cognitive impairments. The immune system is altered by sleep loss; however, more studies are necessary to elucidate how sleep loss promotes the release of inflammatory mediators and how these molecules act on the brain promoting local and systemic alterations that exacerbate the proinflammatory status and contribute to sleep disorders, fostering a vicious circle between inflammation and sleep disturbances (see Figure 1).

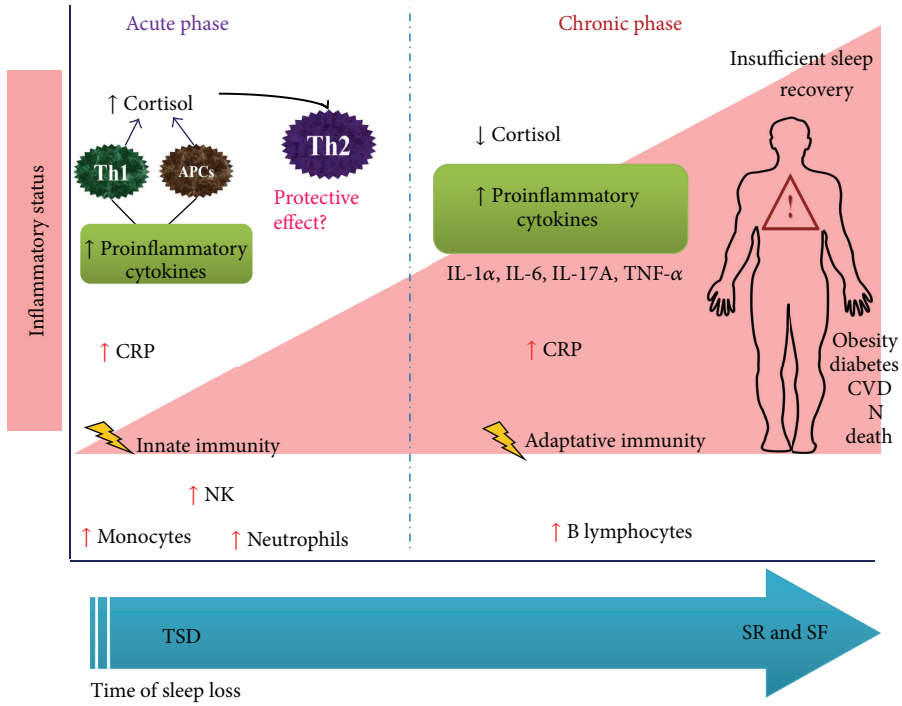

Figure 1: Sleep loss promotes a low-grade proinflammatory status. Sleep loss is characterized by an increase in circulating proinflammatory cytokines (IL-1β, IL-6, IL-17A, TNF-α) and CRP. Image shows the differential effect of sleep loss on the immune system after acute total sleep deprivation and prolonged sleep restriction and or sleep fragmentation. The acute and chronic events of sleep loss correlate with the temporal immune response (innate and adaptive). Prolonged sleep loss plus insufficient sleep recovery are considered an important risk factor to develop metabolic, cardiovascular, and neurodegenerative diseases related with the deregulation of the neuro-endocrine-immune network. Abbreviations: APCs; antigen-presenting cells; CRP, C-reactive protein; CVD, cardiovascular disease; N, neurodegenerative diseases; NK, natural killer; SR, sleep restriction; SF, sleep fragmentation; TSD, total sleep deprivation.

In the last few years, several reviews on sleep and immunity have been written. A review of some of their conclusions could be relevant. Some of them conclude that sleep modulates and is modulated by inflammation [15, 119], or that sleep deprivation impairs immune function, particularly the immune memory/humoral immune response [15, 51]. Also, some of them work with the hypothesis that sleep deprivation is a type of stress and that glucocorticoids are responsible for modifying the immune response [51]. With respect to the hypothesis that inflammation induces sleep changes, one review suggests that IL-6 is the key factor [120]; however, we need to consider that IL-6 has been proposed as a putative sleep factor and is produced by nonimmune cells [121]. We agree that there is enough evidence to conclude that inflammation modifies sleep and that sleep loss modifies circulating cytokines. If we work with the hypothesis that proinflammatory cytokines induce sleep, then we may have found a natural condition in which there is a very high level of inflammation (e.g., sepsis) and test whether sleep is changed. There are some reviews on sepsis and sleep that show that patients with sepsis present increased non-REM sleep and decreased REM sleep, with high levels of cytokines, such as TNF and IL-1β, and show an altered EEG with low-voltage, mixed-frequency waves with variable theta and delta ("septic encephalopathy") and also loss of normal circadian melatonin secretion [122]. Then, we could conclude that proinflammatory cytokines induce non-REM sleep. However, septic encephalopathy is not sleep, it is a sleep disorder, and melatonin has been successfully used in septic patients (reviewed in [122]). Thus, we come back to our hypothesis: the function of sleep is to maintain the integrity of the neuro-immune-endocrine system [11]. In this review we observe how diseases or inflammation can disrupt that integrity, and the organism will respond by modulating sleep to restore the homeostasis and also how sleep loss induces a disruption of the integrity of neuro-immuno-endocrine system causing an inadequate immune response.

Abbreviations

APCs: Antigen-presenting cells
BMI: Body mass index
CRP: C-reactive protein
EEG: Electroencephalography
ET-1: Endothelin-1
HIV: Human immunodeficiency virus
HPA: Hypothalamus-pituitary-adrenal axis
hsCRP: High-sensitivity CRP
IFN: Interferon
ICAM: Intracellular adhesion molecule

ICV: Intracerebroventricular
IL: Interleukin
MIP: Macrophage inhibitory protein
NK: Natural killer
Non-REM: Nonrapid eye movement
OSA: Obstructive sleep apnea
REM: Rapid eye movement sleep
SWS: Slow-wave sleep
T_h: T helper
TNF: Tumor necrosis factor
VGKC: Voltage-gated potassium channels
WBC: White blood cells.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contributions

Gabriela Hurtado-Alvarado, Stephanie Ariadne Castillo-García, and Beatriz Gómez-González drafted the paper. All authors reviewed the paper, and approved the final version.

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Review Article

Blood-Brain Barrier Disruption Induced by Chronic Sleep Loss: Low-Grade Inflammation May Be the Link

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Sleep is a vital phenomenon related to immunomodulation at the central and peripheral level. Sleep deficient in duration and/or quality is a common problem in the modern society and is considered a risk factor to develop neurodegenerative diseases. Sleep loss in rodents induces blood-brain barrier disruption and the underlying mechanism is still unknown. Several reports indicate that sleep loss induces a systemic low-grade inflammation characterized by the release of several molecules, such as cytokines, chemokines, and acute-phase proteins; all of them may promote changes in cellular components of the blood-brain barrier, particularly on brain endothelial cells. In the present review we discuss the role of inflammatory mediators that increase during sleep loss and their association with general disturbances in peripheral endothelium and epithelium and how those inflammatory mediators may alter the blood-brain barrier. Finally, this manuscript proposes a hypothetical mechanism by which sleep loss may induce blood-brain barrier disruption, emphasizing the regulatory effect of inflammatory molecules on tight junction proteins.

1. Introduction

Almost all of our knowledge about the effect of inflammatory events on blood-brain barrier is related to chronic diseases or acute events, in which exacerbated responses to pathogens are present. The role of low-grade inflammation in the generation or exacerbation of neuropathologies is recently explored because several conditions such as obesity and diabetes concur with this inflammatory status during long-term periods and, perhaps, it may be related to systemic and central comorbidities. Most, if not all, pathologies are associated with sleep disturbances. Sleep loss *per se*, including sleep deprivation, sleep restriction, or sleep fragmentation (see Table 1 for a full differentiation between the concepts), generates a pathogen-independent low-grade inflammatory status. Here, we will review (1) the inflammatory mediators that increase during periods of sleep loss and their association with general disturbances in peripheral endothelium and epithelium and (2) how those inflammatory mediators might alter the blood-brain barrier during sleep loss. With the evidence presented in this review, we propose a hypothetical mechanism by which sleep restriction could induce blood-brain barrier disruption, emphasizing the effect of inflammatory molecules on tight junction maintenance.

2. Sleep Loss as an Inflammatory Event

Sleep is one of the most widely observed phenomena in mammals and is recognized to play a vital regulatory role in a number of physiological and psychological systems [1, 2]. The paramount role of sleep in the physiology of animal models and humans is evident by the effects of sleep loss. Serious physiological consequences of sleep loss include decreased neurogenesis, cognitive dysfunction (deficits in learning, memory, and decision-making), metabolic alterations, cardiovascular diseases, immune disturbances, and blood-brain barrier disruption [1–8]. Both chronic and acute sleep loss associate with energy balance disturbances [9] and changes

Table 1: Sleep loss procedures.

	Human procedures	Duration	Animal models	Duration
Sleep deprivation (SD)	(i) Shift working [117] (ii) Voluntary SD [117]	(i) Several days (ii) 12–90 h	(i) Modified multiple platform method (REM SD) [118] (ii) Gentle SD (total SD) [119] (iii) Disk-over-water method (total and selective SD) [120]	(i) 3–96 h (ii) 3–96 h (iii) 3–96 h
Sleep restriction (SR)	(i) Voluntarily SR [117]	(i) 3–5 h	(i) Modified multiple platform method [7] (ii) Rotating bar at the bottom of the house-cage [8]	(i) 20 h of SD plus 4 h of daily sleep recovery (ii) 18 h of SD plus 6 h of daily sleep recovery
Sleep fragmentation (SF)	(i) Obstructive apnoea patients [117] (ii) The elderly [117]	(i) Several days (ii) Several days	(i) Gentle manipulation coupled to EEG recording [119] (ii) Disk-over-water method [120]	(i) 1 to several days (ii) 1 to several days

Sleep deprivation consists of sleep loss without sleep opportunity along a short period; sleep restriction consists of a reduction in total sleep time with short periods of sleep opportunity; and sleep fragmentation consists of multiple awakenings during sleep time.

in cellular and humoral immunity [10, 11]; however, the direct mechanism by which sleep induces a low-grade inflammatory status is unclear. Experimental research has demonstrated that acute and chronic sleep loss result in impairments in the immune response, characterized by deficits in the cellular component (both in number and in function) and increased levels of proinflammatory mediators, such as tumor necrosis factor-α (TFN-α), interleukin-1β (IL-1β), IL-6, IL-17A, and C-reactive protein (CRP) (for details of the cytokine levels related to varying periods of sleep loss see [12]). In addition to immune-derived inflammatory mediators, sleep loss also increases the levels of other inflammatory molecules such as cyclooxygenase-2 (COX-2) [8], nitric oxide synthase (NOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1) [8, 13].

The major aim of this review is to discuss the role of low-grade inflammation in the blood-brain barrier disruption induced by sleep loss; nevertheless, because endothelial cells form the blood-brain barrier we considered it relevant also to discuss the effect of sleep loss on peripheral endothelial and epithelial cells as early markers of inflammation.

3. Peripheral Endothelial and Epithelial Disturbances Induced by Sleep Loss

Endothelial and epithelial cells form protecting barriers in the central nervous system but also in the periphery. Several pathological states are known to target peripheral epithelial and/or endothelial barriers; therefore the knowledge of regulatory mechanisms in those peripheral barriers may contribute to improving the understanding of central barriers. Among the pathologies affecting body barriers, those involving infections and also diabetes, cardiovascular diseases, psoriasis, and cancer are associated with sleep disturbances [14–16]. Here, we present evidence regarding the disrupting effect of sleep loss on peripheral epithelial and endothelial cells.

3.1. Humans. When fluid compartmentalization goes awry, homeostasis is altered and the possibility exists of induction of inflammation by microorganism invasion and even of tumor microenvironment induction [14]. In humans sleep restriction increases sympathetic activity and, concomitantly, causes endothelial dysfunction at the venous level [17]; the effect may be mediated via endothelin-1 (ET-1) because ET-1-mediated vasoconstriction is greater in adults with short sleep duration (less than 7 h per night) than in those with normal sleep duration (7–9 h per night) [18]. ET-1 is the most potent vasoconstrictor peptide released by the endothelium. The link between sleep restriction and increased ET-1 activity is not clear, but the role of the inflammatory status induced by sleep loss may partially explain this association. In this way, inflammatory cytokines, insulin, and epinephrine altered during sleep loss have each been shown to increase ET-1 in hypertensive subjects [19]. The cytokines that may increase in sleep-deprived humans (e.g., TNF-α, IL-1β, and IL-6) raise arterial vascular tone via endothelin receptors [20]. Several reports indicate that sleep loss induces vascular alterations related to inflammatory markers (for a review see [21]). Some studies have tried to clarify the underlying mechanism; for instance, sleep deprivation in humans induced magnesium deficiency [22], which produces arterial constriction, and is a possible cause of myocardial damage [22]. Other barriers are not yet studied in sleep-deprived or sleep-restricted humans, but some studies indicate that sleep deficiency alters skin conductance [23].

3.2. Animal Models. Animal models currently used in sleep research include those that model shift work by totally sleep depriving rodents; human sleep deficiency by sleep restricting; and sleep loss-associated with pathologies, such as obstructive apnoea, by promoting sleep fragmentation. Contrary to the human studies, in the case of animal models, several studies have identified negative effects of sleep loss on peripheral endothelia and epithelia. For instance, sleep fragmentation in mice (20 weeks) induces vascular endothelial dysfunction and mild blood pressure increases. Those physiological effects are accompanied by morphological vessel changes characterized by elastic fiber disruption and disorganization, increased recruitment of inflammatory cells to the vessel wall, and increased plasma levels of IL-6 [24]. In rats, total sleep deprivation reduces endothelial-dependent cutaneous vasodilation. This endothelial dysfunction is independent of blood pressure and sympathetic activity but is associated with changes in NOS and COX pathways [25].

The effect of sleep loss on physical barriers such as the intestinal barrier or blood-testis barrier is not reported; however, gut bacteria are present in blood after sleep deprivation [26] and both sleep-deprived and sleep-restricted rats exhibit lower sperm viabilities associated with an increase in endothelial NOS expression [27]. Those data suggest that sleep loss also might alter the physiology of the above-mentioned barriers with the ensuing tissue damage.

4. Blood-Brain Barrier Impairment Induced by Sleep Loss

We reported for the first time that sleep restriction induces blood-brain barrier hyperpermeability in rats [7]. We used a procedure consisting of 20-hour sleep deprivation plus 4 hours of sleep opportunity during 10 consecutive days; because a reduction in total sleep time is observed, it is named sleep restriction. In our conditions, rapid eye movement (REM) sleep is fully suppressed and non-REM sleep is 30% reduced since the first day of sleep restriction. In those conditions we showed a widespread breakdown of the blood-brain barrier [7]. We described that brief periods of sleep opportunity (40 to 120 minutes) induced a progressive recovery of blood-brain barrier permeability to Evans blue (>60 000 Da) in the majority of brain regions studied, with exception of the hippocampus and cerebellum [7]. We also observed that in the hippocampus the number of pinocytic vesicles increased threefold. In a subsequent study, mice were subjected to sleep restriction for 6 days in a rotatory bar for 12 hours per day. Sleep restriction by this method induced REM

sleep loss in the first 3 days with partial REM sleep recovery afterwards; at the end of the 6th day of sleep restriction, there was 13.3% increase of wakefulness, 10.2% reduction of non-REM sleep, and 2.1% reduction of REM sleep [8]. Under these conditions, increased blood-brain barrier permeability to sodium fluorescein, a low molecular-weight tracer, was observed; sleep recovery by 24 hours fully reverted the effect. In the same way, sleep restriction decreased the mRNA levels of the tight junction proteins claudin-5, zonula occludens-2 (ZO-2), and occludin [8]. In the first study [7] a yoked control was included to avoid any potential confounding effects of stress on blood-brain barrier permeability; rats were placed on large platforms during the same period of time as sleep-restricted subjects and despite being in the same stressful conditions as the sleep-restricted subjects they have a fully functional blood-brain barrier [7]. The second study did not include a yoked control, a newly developed sleep deprivation method was used that involves a rotating bar at the bottom of the house-cage with random changes of direction; this method may certainly be stressful to the rodents due to the presence of forced exercise; however, our recent results replicate their findings (Hurtado-Alvarado et al. personal communication). Therefore, the evidence of changes in the blood-brain barrier integrity induced by sleep loss is substantial and inflammatory molecules appear to play a key role in the mechanism subjacent to this phenomenon.

5. Role of Inflammatory Mediators Released during Sleep Loss in Blood-Brain Barrier Physiology

The increase in the levels of inflammatory mediators during chronic sleep loss may be related to blood-brain barrier disruption because several previous reports show that *per se* those inflammatory molecules affect the integrity of the blood-brain barrier (see Table 2 for a summary).

5.1. Proinflammatory Cytokines Involved in Sleep and Blood-Brain Barrier Modulation

5.1.1. Tumor Necrosis Factor-α. Tumor necrosis factor-α (TNF-α) is a protein synthesized mainly by monocytes and macrophages that plays an essential role in the initial activation of the immune system. In the central nervous system TNF-α is a multipotent cytokine produced by neurons, glia, and microvascular endothelial cells that is implicated in several physiological events, such as memory consolidation and sleep regulation. TNF-α is also a potent regulator of blood-brain barrier permeability. The role of TNF-α as an inductor of blood-brain barrier disruption includes its overexpression in microglia, astrocytes, and microvascular endothelial cells [28].

Several reports indicate that sleep loss increases the plasma and brain levels of TNF-α [29–33], the mRNA expression of TNF-α in the brain [33, 34], the spontaneous production of TNF-α in lymphocytes [35], and the mRNA expression of TNF-α in peritoneal and epididymal adipose tissue [36, 37]. Despite the fact that the changes in TNF-α induced by sleep loss are 2 to 5 times higher compared to rats sleeping *ad libitum*, the levels are below those reported in the case of infectious diseases; however, the chronic exposure to this inflammatory mediator may underlie the sleep-induced blood-brain barrier dysfunction.

The effect of TNF-α in endothelial cells is well studied. *In vivo* and *in vitro* studies report an increase in the permeability of microvascular endothelial cells after the administration of TNF-α in both animal models and human cell lines [38–41]. Nonetheless, the TNF-α levels used in those studies are 100,000 times higher compared to concentrations reported under sleep loss conditions. The lower dose of TNF-α used in *in vitro* studies (1 ng/mL) results in a transendothelial electric resistance (TEER) reduction at 60 minutes after treatment with TEER recovery at 210 minutes after administration, which is similar to the results observed using higher doses of TNF-α (50, 100 ng/mL), suggesting that the effect mediated by TNF-α receptors is saturable [42].

While we can infer that peripheral changes mediate the main effect of TNF-α on blood-brain barrier, we must not ignore the fact that TNF-α levels also increase in the brain. In this way, it is known that after the administration of TNF-α (250 ng) in the lateral ventricle an increase in the transport from cerebrospinal fluid (CSF) to blood of ^{125}I-human serum albumin is observed in rats, which demonstrates that TNF-α promotes the clearance of macromolecules from the CSF to the venous blood [43]. Taking into consideration that the restorative function of non-REM sleep may be a consequence of the enhanced removal of waste products accumulated in the awaking brain via the glymphatic system [44], the TNF-α increase during sleep loss may contribute to the clearance of toxins by efflux of potentially neurotoxic waste products via the blood-brain barrier. Interestingly, in the brain, sleep restriction increases the mRNA expression of TNF-α in a region-dependent manner in the mouse [45], suggesting that if TNF-α regulates the microvascular brain endothelial cells from inside the brain, it may do it in specific areas, such as the somatosensory and frontal cortices, which indicates that blood-brain barrier regulation by inflammatory molecules is heterogeneous (a finding reported by us in the case of blood-brain barrier changes induced by sleep loss and recovery; see [7]).

Another example of TNF-α role in blood-brain barrier regulation during peripheral inflammation occurs after the induction of acute pancreatitis in rats, where an increase in TNF-α levels is observed as early as 6 hours after pancreatitis induction and at the same time increases the blood-brain barrier permeability to sodium fluorescein (365 Da) in the hippocampus and cerebellum as well as to Evans blue in the hippocampus, basal nuclei, and cerebellum. In the case of the low molecular-weight tracer the normal blood-brain barrier permeability reestablishes at 24 hours after induction, while, for Evans blue, reestablishment occurs 48 hours after induction [46]. We also observed region-dependent effects of sleep loss and recovery on blood-brain barrier integrity; for instance, in the cerebellum the hyperpermeability remained even after sleep opportunity periods of 40–120 minutes; meanwhile the cortex recovered the normal blood-brain barrier permeability at the same time points [7]. Therefore,

Table 2: Inflammatory mediators released during sleep loss that may potentially regulate blood-brain barrier integrity.

Inflammatory mediator	General changes during sleep loss	General effects on blood-brain barrier
TNF-α	↑ circulating levels in human and rodents [29–32] ↑ mRNA expression in mice brain [33]	↑ blood-brain barrier permeability in *in vivo* and *in vitro* models (rodent and human brain endothelial cells) [38–40] ↑ efflux of albumin from brain to blood [43] ↓ ZO-1 expression [103] ↑ MMP-9 protein expression [106]
IL-1β	↑ circulating levels in human and rodents [3, 4, 29, 49] ↑ mRNA expression in mice brain [45]	↑ blood-brain barrier permeability in *in vivo* and *in vitro* models (rodent and human brain endothelial cells) [42, 54] ↓ TEER of primary cultures of brain endothelial cells and human brain endothelial cells [42, 54] ↑ production of PGE and COX [57] ↓ ZO-1 expression [103]
IL-6	↑ circulating levels in human after chronic sleep loss [64, 65] ↓ circulating levels in humans [62] ↑ circulating levels during sleep recovery in humans [69] ↑ mRNA expression in human PBMC [50, 67]	↓ TEER in cerebrovascular endothelial cells from rats at higher doses but not at lower doses [42] ↓ blood-brain barrier permeability in ischemic brain in rodents [71]
IL-17A	↑ circulating levels in rodents [29] ↑ mRNA expression in human PBMC [50]	↑ blood-brain barrier permeability in *in vivo* and *in vitro* models (rodent and human brain endothelial cells) [78, 79]
CRP	↑ circulating levels in humans and rodents [4, 8, 50, 67, 81–83]	↑ blood-brain barrier permeability in *in vivo* and *in vitro* models (rodent and human brain endothelial cells) [85] ↑ ROS production in brain endothelial cells [86]

TNF: tumor necrosis factor; IL: interleukin; CRP: C-reactive protein; ZO: zonula occludens; MMP-9: matrix metalloproteinase-9; PBMC: peripheral blood mononuclear cells; ROS: reactive oxygen species; COX; cyclooxygenase; and TEER: transendothelial electric resistance.

the cerebellum could be considered as a highly susceptible region to inflammatory mediators such as TNF-α [47] in comparison with other brain regions (e.g., the hippocampus and cortex). The differential distribution of TNF-α receptors in the brain may explain why TNF-α regulates blood-brain barrier function in a region-dependent manner; however, is it also possible that other molecules may have synergic effects with TNF-α to regulate blood-brain barrier physiology.

5.1.2. Interleukin-1 Beta. IL-1β is the prototypical signal molecule for neuroimmune communication. Classically, phagocytic cells in response to inflammatory stimuli release IL-1β; in the brain IL-1β activates the regions involved in the generation of hyperthermia [48]. Similar to the effect of TNF-α, IL-1β administration promotes sleep in mammals [1] and sleep deprivation has been shown to increase serum IL-1β levels both in humans and in animal models [3, 4, 29, 49]. In addition, sleep loss induces IL-1β gene expression in the brain [34, 45], cardiac muscle, and adipose tissue [36] and on phytohaemagglutinin (PHA) activated peripheral blood mononuclear cells (PBMC) [50]. In the case of the brain, several reports indicate that the expression of the IL-1 receptor-1 (IL-1R1) in endothelial cells is high in the preoptic area, subfornical organ, and supraoptic hypothalamus, while a lesser expression is found in the paraventricular hypothalamus, cerebral cortex, nucleus of the solitary tract, ventrolateral medulla, trigeminal and hypoglossal motor nuclei, and the area postrema [51–53].

In *in vitro* models of blood-brain barrier, IL-1β (in doses of 5, 100, and 1000 ng/mL) decreases the TEER similar to the levels observed after TNF-α administration [42, 54]. IL-1β also promotes the release of IL-6 and prostaglandin E (PGE_2) in rat brain endothelial cells [55]. Likely, *in vivo* studies have shown that IL-1 induces sickness behaviour mediated by endothelial IL-1R1 activation in rats [56]; the probable mechanism may be the induction of COX-2 in brain endothelial cells after IL-1R1 activation with the concomitant increase in the synthesis of PGE_2 [57].

IL-1β may have a key role in blood-brain barrier dysfunction during sleep loss because it has been reported that sleep loss increases IL-1β gene expression in the cerebral cortex, hippocampus, and basal forebrain [45]. In addition, IL-1β released from activated microglia increases blood-brain barrier permeability; this effect may depend on the suppression of astrocyte-derived signals that maintain blood-brain barrier integrity (e.g., sonic hedgehog, SHH) [58]. IL-1β action on blood-brain barrier may induce the expression of other inflammatory mediators produced by microglia and astroglia. For instance, the lack of IL-1R1 specifically in endothelial cells precluded the brain increase of IL-1β, TNF-α, and IL-6 in stressed rats despite the presence of reactive microglia [59, 60], which places IL-1β and its receptor on endothelial cells as central mediators of brain inflammatory responses. Hence, the role of IL-1β in blood-brain barrier could be mainly related to endothelial-glial interactions [61].

5.1.3. Interleukin-6. Sleep onset is associated with an increase in circulating levels of IL-6 [62]; nevertheless, the potential role of IL-6 in sleep regulation is controversial, and it may take a secondary role as compared to its primary role in the acute-phase response [63]. Some studies indicate an increase of IL-6 circulating levels in sleep-deprived subjects [64–66] and also in gene expression in immune cells [35, 50, 67], whereas others report a delay in the sleep-related peak of plasma IL-6 in sleep-restricted subjects [62]. Even some authors report that plasma levels of IL-6 are maintained without change despite sleep loss [30, 68]. Some studies also show that sleep recovery after total sleep deprivation increases plasma levels of IL-6 [69]; however, others found that in immune cells IL-6 levels remain unchanged during sleep recovery [50]. IL-6 is a pleiotropic cytokine key for immune regulation and if secreted during sleep loss and recovery may have neuroprotective effects; indeed, it has been reported that IL-6 appears to be neuroprotective and is involved in endothelial survival after shear stress [70]. However, given the high variability of IL-6 after sleep loss and recovery, the role of IL-6 as a possible modulator of blood-brain barrier during sleep is unclear. It is necessary to elucidate the precise changes in IL-6 levels both centrally and peripherally to clarify the role of IL-6 in blood-brain barrier modulation during sleep.

IL-6 has pyrogenic effects when endogenously released during systemic inflammation; it achieves this function by its binding to IL-6 receptor α (IL-6 Rα) on brain endothelial cells and the subsequent induction of PGE synthesis. However, those effects require high levels of IL-6 (>1 ng/mL). In humans, IL-6 serum levels were less than 100 pg/mL and the normal levels for IL-6 in CSF are around 10 pg/mL, significantly lesser than those measured in several *in vitro* and *in vivo* experiments [70]. For instance, treatment with 50 or 500 ng of IL-6 reduced the infarct volumes and symptoms of neurological deficit in a rat model of cerebral ischemia [71]. In addition, the administration of IL-6 decreased the blood-brain barrier permeability to Evans blue by suppressing the expression of matrix metalloproteinase-9 (MMP-9) [71]. The role of IL-6 as well as TNF-α and IL-1β may depend on the brain region, for example, the stimulation with lipopolysaccharide (LPS) induces in the brain the expression of the IL-6 receptor (IL-6R) in the cortex and hippocampus but not in the cerebellum [72]. Therefore, considering IL-6 a proinflammatory cytokine it is possible to suggest that its role in blood-brain barrier physiology during sleep loss may be related to the modulation of the expression of other proinflammatory cytokines.

5.1.4. Interleukin-17A. Th17 cells have been identified as a subset of T helper lymphocytes characterized by the production of a number of cytokines including IL-17A, IL-17F, and IL-22. Th17 cells have emerged as a key factor in the pathogenesis of autoimmune disorders. For instance, high expression of IL-17A is associated with autoimmune inflammatory diseases including multiple sclerosis [73], rheumatoid arthritis [74], inflammatory bowel disease [75], and systemic lupus erythematosus [76]. During sleep loss a subtle increase of IL-17A is reported (from 0.5 to 3 ng/mL in rat) [29]. IL-17A high levels were found in plasma even after 24 hours of sleep recovery in sleep-restricted rats [29]. Sleep loss also

increases the mRNA and protein expression of IL-17A on PHA activated PBMC in humans [50].

Particularly, the receptor for IL-17A is expressed in epithelial and endothelial cells and promotes the expression of inflammatory mediators such as IL-6 and chemokines [77]. IL-17A induces epithelial and endothelial dysfunction; it decreases the TEER and concomitantly increases tracer permeability; the mechanism is mediated through tight junction disruption [77]. Finally, from *in vitro* experiments it is known that IL-17A increases endothelial cell permeability at 10 or 100 ng/mL doses [78, 79]. These data suggest that IL-17A might be involved in blood-brain barrier disruption during sleep loss.

5.2. Other Inflammatory Molecules Altered during Sleep Loss and Their Role in Blood-Brain Barrier Regulation

5.2.1. C-Reactive Protein. C-reactive protein (CRP) is the major acute-phase protein involved in the resistance to microbes and autoimmune diseases and is an important risk marker of cardiovascular and cerebrovascular disorders. The plasma levels of CRP increase faster and at higher magnitude than other acute-phase proteins [80]. Sleep loss increases the circulating levels of CRP (0.5 μg/mL), which is associated with increased risk of cardiovascular disease and stroke [4, 8, 50, 67, 81, 81–83].

The synthesis of CRP in the liver is controlled by proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-17A [82, 84]. CRP (10–20 μg/mL) induces blood-brain barrier disruption [85] because brain endothelial cells express high levels of CRP receptors (CD16 and CD32) and also because brain endothelial cells express high levels of the p22phox subunit of the NAD(P)H-oxidase. The high expression of both exacerbates the generation of reactive oxygen species (ROS) with the resultant oxidation of tight junction proteins [86].

5.2.2. Intercellular Adhesion Molecule-1 (ICAM-1). The expression of ICAM-1 in endothelial cells is pivotal in supporting lymphocyte migration across the vascular endothelium [87]. ICAM-1 associates with an endothelial cytoskeleton fraction, suggesting that ICAM-1 redistribution is an early event in the signalling cascade during inflammatory events, particularly in lymphocyte transmigration [87]. The expression of endothelial cell adhesion molecules increases in the central nervous system during inflammation secondary to pathogen intracerebral administration (e.g., *Corynebacterium parvum*). Brain vessels located in the centre of the cellular infiltrate began to express markers of fenestrate endothelium such as the endothelial-specific expression of MECA32 suggesting an altered functional status of the endothelial cell [88]. Abundant ICAM-1 expression has been observed after IL-1 or TNF-α stimulation of cultured heart endothelial cells [89].

Elevated levels of ICAM-1 may contribute to cardiovascular disease and are associated with obstructive sleep apnoea (OSA) and obesity, in which sleep deficiency is present [90]. In the same way, it has been shown that patients with diabetes mellitus type 2 and poor sleep present higher morbidity of cardiovascular diseases than diabetes mellitus patients sleeping normally; those patients also present higher plasma levels of ICAM-1 [91]. ICAM-1 higher serum levels were also found during the sleep recovery period after 40 hours of total sleep deprivation in healthy men [69]. Therefore it seems that the mediator between poor sleep (with bad quality and poor sleep recovery) and higher risk for cardiovascular diseases is ICAM-1.

5.2.3. Vascular Endothelial Growth Factor. Inflammation is characterized by upregulation of vascular endothelial growth factor (VEGF). In *in vivo* experiments, increases in VEGF during neuroinflammation (e.g., in experimental autoimmune encephalomyelitis (EAE)) are accompanied with increased blood-brain barrier permeability and decreased expression of tight junction proteins (e.g., claudin-5 and occludin). Likely, VEGF administration to human brain endothelial cells increases permeability of the monolayer and downregulates claudin-5 and occludin, but not junctional adhesion molecule-1 (JAM-1), cingulin, peripheral plasma membrane protein (CASK), or ZO-1 [92].

Given the role of VEGF in regulating blood-brain barrier during neuroinflammation, it may participate in generating the vascular changes associated with sleep loss. Indeed, it has been shown that VEGF is overexpressed in OSA patients and it is generally considered that VEGF increases are associated with hypoxia events [93]. However, OSA patients also have severe sleep fragmentation; therefore, in addition to chronic intermittent hypoxia, VEGF changes may be related to sleep loss [94]. In fact, in a study with major depressive disorder patients, sleep deprivation increased VEGF plasma levels [95].

5.2.4. Insulin-Like Growth Factor-1. Sleep deprivation decreases IGF-1 levels in rats and humans and one night of sleep recovery is sufficient to restore its basal levels [96]. The neuroprotective effects of IGF-1 are unclear but it is known that IGF-1 receptors are present in brain endothelial cells, microglia, and astroglia and even in neurons [97]. Indeed, it has been suggested that IGF-1 may promote neuroprotection by acting on the blood-brain barrier; in an experimental model of ischemic stroke IGF-1 reduced the inflammatory infiltrate in the brain [97]. In an *in vitro* experiment with brain endothelial cells IGF-1 reverted the hyperpermeability to bovine serum albumin induced by oxygen-glucose deprivation (an *in vitro* model of ischemic stroke) [97].

Changes on inflammatory molecules during sleep loss are well described but we do not know what the source of those alterations is. In this way the role of microbiota could appear a good candidate to induce the low-grade proinflammatory status during sleep loss.

6. A Brief View of the Microbiota and Barriers Dysfunction as a Possible Source of Inflammatory Mediators in Sleep-Deficient Subjects

The source of inflammatory mediators during sleep loss remains unclear; however, microbiota may play a key role

in this event. In other conditions that exhibit low-grade systemic inflammation, such as chronic depression, obesity, and diabetes, evidence from murine models initially suggested a role for the gut microbiota in the generation of low-grade inflammation, with the consequent increased risk of endothelial and epithelial dysfunction [98, 99]. For instance, changes in gut microbiota composition increase intestinal permeability [100]. In the same way, during sleep deprivation gut microbiota has been detected in blood, suggesting the induction of systemic inflammation and deficits in gut epithelial permeability [26]. In addition, preclinical evidence from germ-free mice suggests that the microbiota can also modulate the blood-brain barrier; exposure of germ-free adult mice to the faecal microbiota from pathogen-free donors decreased the blood-brain barrier permeability and increased the expression of tight junction proteins in brain endothelial cells [101], therefore strengthening the hypothesis that the blood-brain barrier may also be sensible to changes in the gut microbiota composition [100]. The candidate pathways to induce barriers dysfunction under altered gut microbiota composition include serotonin, cytokines, toll-like receptor activation, and short chain fatty acids [100]. Moreover, the inflammatory response subsequent to microbiota-induced barriers disruption may underlie the sleep loss-related cognitive deficits and the exacerbation of neurological disorders such as depression [100].

These data might support the theory of a coevolution between sleep and blood-brain barrier proposed by Korth in 1995 [102]. Because the brain and blood-brain barrier react sensitively to the exposure to bacterial cell wall constituents and sleep is regulated by gut microbiota products, Korth proposed that low amounts of bacterial cell wall constituents that induce sleep under sleep loss conditions, by themselves or by cytokine production, increase the blood-brain barrier permeability ensuing their passage into the brain [102].

7. Molecular Mechanisms by Which Inflammatory Mediators Might Induce Blood-Brain Barrier Disruption during Sleep Loss

Cytokines and other inflammatory mediators induce blood-brain barrier disruption through mechanisms involving signalling pathways that converge in the disorganization of tight junctions (Figure 1). For instance, it has been reported that proinflammatory cytokines, including TNF-α and IL-1β, decreased ZO-1 expression and ZO-1-occludin coassociation, concomitant to increased ZO-1 phosphorylation in tyrosine and threonine residues [103]. Those effects are presumably mediated by ROS [103]. ZO-1 phosphorylation in tyrosine residues is also observed after VEGF administration [104]. In this way, VEGF-A also promotes disruption of blood-brain barrier by downregulating the expression of claudin-5 and occludin [92]. Low cytokine concentrations (>1 ng/mL) led to activation of effector caspases via c-Jun N-terminal kinases (JNK) and protein kinase C (PKC) signalling pathways, increased paracellular flux, and redistribution of ZO-1 and VE-cadherin but failed to induce apoptosis [105]. In addition to caspase-3, TNF-α activates the production of MMP-9 [106], which is also associated with high levels of IL-1β in brain parenchyma [107].

TNF-α activates the NFκB signalling pathway, leading to increased PGE levels via COX-2 [108]. COX-2 plays a crucial role in the inflammatory response of the blood-brain barrier (for review see [109]); particularly COX-2 derived PGE_2 increases blood-brain barrier permeability [110]. Other cytokines, such as IL-1, use other signalling pathways that finally converge in COX-2 induction; particularly, the IL-1 receptor-1 (IL-1R1) signals via the p38 mitogen-activated protein kinase (MAPK) and the c-Jun pathway to induce COX-2 synthesis, whereas activation of the IL-6 receptor leads to COX-2 expression through activation of signal transducer and activator of transcription-3 (STAT-3) [111]. The activation of NFκB by TNF-α and IL-1β is also correlated with COX-2 expression in microvascular endothelial cells. Indeed, both IκBα and COX-2 are expressed within the same endothelial cells, suggesting a potential interaction between the transcription factor and COX-2 expression in the cerebral endothelium of animals with systemic inflammation [112].

TNF-α and IL-1β promote the release of CRP. The putative mechanism by which CRP increases blood-brain barrier permeability is by its action on CD16/CD32 receptors present in the cell membrane of brain endothelial cells [85]. This association activates the Myosin Light Chain (MLC) phosphorylation by MLC-kinase (MLCK) and the activation of p38-MAPK, with the subsequent formation of actin stress fibers [85]. Brain endothelial cells express the p22phox subunit located in the cell membrane; this enzyme uses NADH or NADPH as the electron donor for the single electron reduction of oxygen to produce ROS during CRP stimulation [86]. The assembly of active NADPH oxidase requires translocation of cytosolic subunits, p47phox, p67phox, and Rac1 (a cytosolic GTPase), to the plasma membrane, where they interact with gp91phox and p22phox and associate with other membrane cofactors to form a functional enzyme complex [113]. In addition, CRP stimulation also disorganizes ZO-1 via MLCK and ROS production [85]. In this way, IL-17A also induces NADPH oxidase- or xanthine oxidase-dependent ROS production and downregulates the expression of occludin by activation of MLCK [79].

The signalling of inflammatory mediators and particularly NADPH oxidase may promote the upregulation of adhesion molecules such as ICAM-1 via JAK/epidermal growth factor receptor (EGFR) signalling [113] contributing to a possible leukocyte infiltration. Therefore, these changes may be deemed as the mechanisms involved in brain endothelial cell dysfunction during sleep loss.

8. Conclusion and Future Directions

We propose that inflammatory mediators increased during chronic sleep loss might promote blood-brain barrier disruption (Figures 1 and 2). For aims of clarity the hypothesis does not explicitly distinguish between REM and non-REM sleep and we know that other molecules altered during sleep loss also should be studied because they may have a potent role in the blood-brain barrier disruption such as adenosine

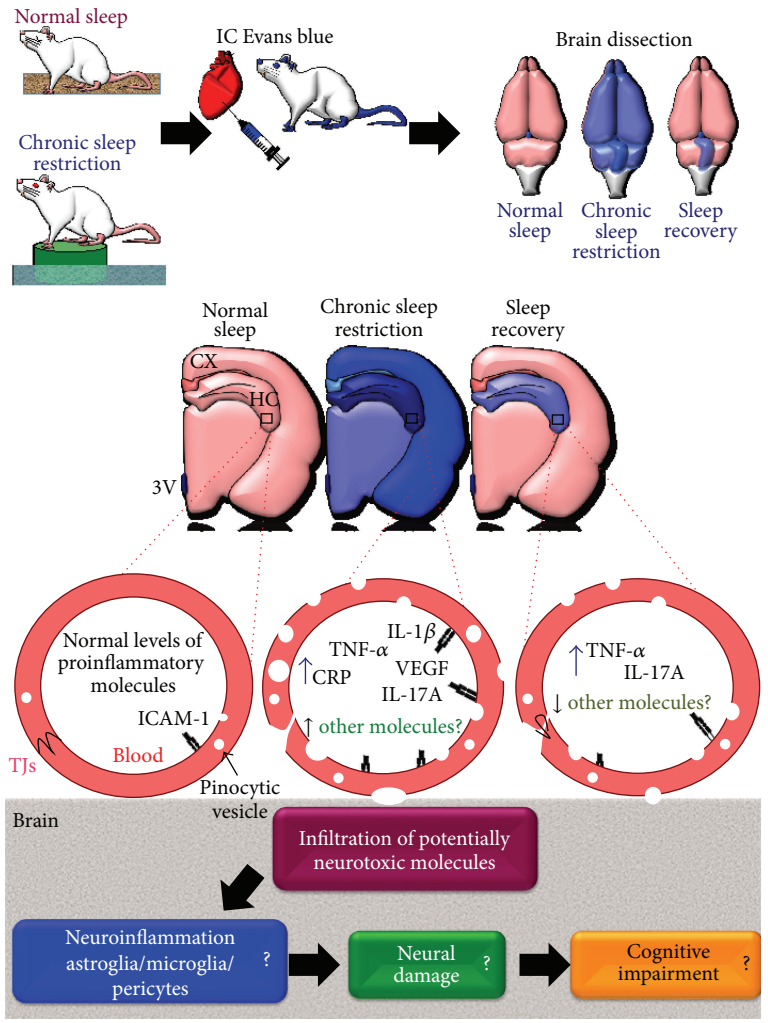

Figure 1: Potential inflammatory mediators participating in the regulation of blood-brain barrier permeability during sleep loss. The figure shows the platform method to induce sleep loss in the rat. Chronic sleep restriction increases blood-brain barrier permeability to circulating molecules (e.g., Evans blue) and sleep recovery promotes restoration of normal blood-brain barrier permeability. Inflammatory mediators with barrier regulation properties, such as tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), interleukin-1β (IL-1β), and IL-17A, are released during sleep loss conditions and some of them return to basal levels during sleep recovery; others, like IL-17A and TNF-α, are maintained at high levels despite sleep recovery. The barrier changes induced by inflammatory mediators may lead to neuroinflammation and potentially may underlie the cognitive impairments induced by sleep loss.

[114] and hormones [115]. In interpreting these data, a number of factors need to be considered. For instance, the cellular components of the blood-brain barrier that promote inflammation in the brain, such as microglia and astroglia, in addition to regulating blood-brain barrier may also be affecting several brain functions during sleep and sleep loss. On the other hand, pericytes have a unique synergistic relationship with brain endothelial cells in the regulation of capillary permeability through secretion of inflammatory mediators including cytokines, chemokines, nitric oxide, and matrix metalloproteinases. Those inflammatory mediators released during sleep restriction may directly induce pericyte detachment from the vessel wall ensuing blood-brain barrier disruption (for review see Hurtado-Alvarado, 2014 [116]).

Summarizing, chronic sleep loss induces systemic low-grade inflammation that may be related to epithelial and endothelial disturbances both at the systemic and at the central level. Particularly, the role of inflammatory mediators in the blood-brain barrier disruption induced by sleep loss might explain the cognitive impairment associated with sleep loss. The systemic and local effect of inflammatory molecules accumulated during chronic sleep loss should be taken into account for the study of general consequences of sleep deficiency including the risk of developing neurologic and neurodegenerative diseases.

Abbreviations

CSF: Cerebrospinal fluid
COX: Cyclooxygenase
CRP: C-reactive protein
CXCL-1: Chemokine (C-X-C motif) ligand 1

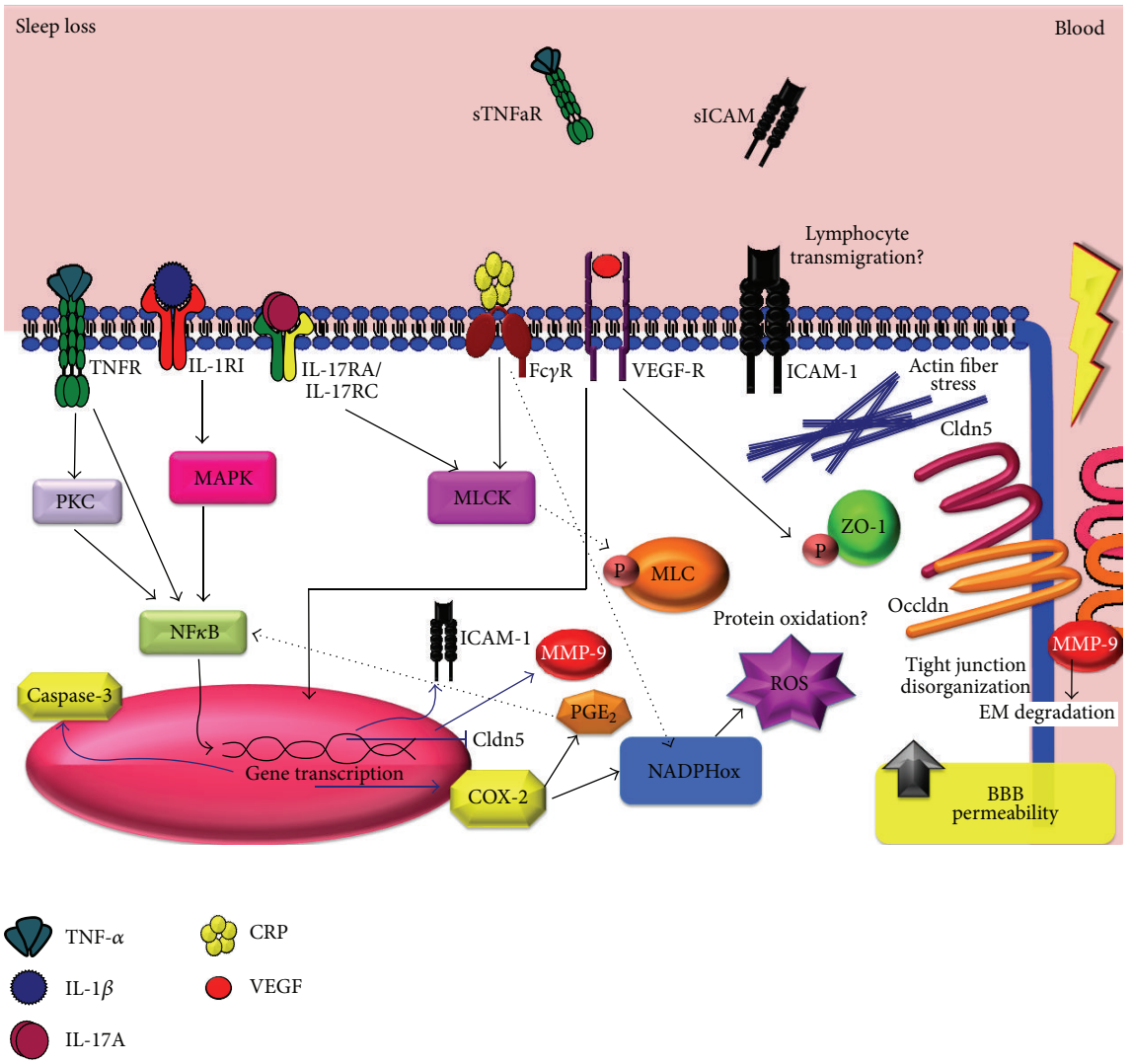

Figure 2: Hypothetical molecular mechanisms mediating sleep loss effect on blood-brain barrier permeability. During sleep loss the increase of soluble inflammatory mediators such as TNF-α, IL-1β, IL-17A, CRP, and VEGF activates several membrane receptors that converge in cellular pathways hallmark of inflammation, for example, the NFκB pathway. The final outcome involves the phosphorylation of tight junction proteins and the generation of actin fiber stress. But also other pathways are potentially activated, such as the NADPH oxidase pathway, leading to ROS generation and the subsequent lipoxidation and protein oxidation. The activation of transcription factors (eg., NFκB) and their translocation to the nuclei may promote the transcription of inflammatory-related genes (e.g., ICAM-1, prostaglandins, and matrix metalloproteinases (MMP)) as well as death-related genes (e.g., caspase 3) and the repression of genes involved in the maintenance of the barrier properties (e.g., claudin-5). Conjointly, all those pathways could lead to increased blood-brain barrier permeability during chronic sleep loss. Cldn5: claudin-5, COX: cyclooxygenase, CRP: C-reactive protein, Fcγ receptor: fragment crystallizable region, ICAM-1: intracellular adhesion molecule-1, IL: interleukin, NADPHox: nicotinamide adenine dinucleotide phosphate oxidase, NFκB: nuclear factor kappa-light-chain-enhancer, MMP: matrix metalloproteinase, MLC: myosin light chain, MLCK: myosin light chain kinase, PGE: prostaglandin, PKC: protein kinase C, sICAM: soluble ICAM, sTNFaR: soluble TNF-α receptor, VEGF: vascular endothelial growth factor, TNF: tumor necrosis factor, and ZO: zonula occludens.

EGFR: Epidermal growth factor receptor
ET-1: Endothelin-1
ICAM-1: Intracellular adhesion molecule-1
IGF-1: Insulin-like growth factor-1
IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL: Interleukin
JNK: c-Jun N-terminal kinase
NADPH: Nicotinamide adenine dinucleotide phosphate
NFκB: Nuclear factor kappa-light-chain-enhancer
NOS: Nitric oxide synthase
MMP: Matrix metalloproteinase
MLC: Myosin light chain
MLCK: Myosin light chain kinase
PBMC: Peripheral blood mononuclear cells
PGE: Prostaglandin

PHA: Phytohaemagglutinin
PKC: Protein kinase C
REM: Rapid eye movement
STAT: Signal transducer and activator of transcription
TEER: Transendothelial resistance
TNF: Tumor necrosis factor
VEGF: Vascular endothelial growth factor
ZO: Zonula occludens.

Competing Interests

The authors declare that there are no competing interests.

Authors' Contributions

G. Hurtado-Alvarado took part in the conception and design of the review, critically revised the manuscript, and took primary responsibility of writing the manuscript. B. Gómez-González took part in the conception of the review and drafted and critically revised the manuscript. L. Pavon, E. Domínguez-Salazar, and J. Velázquez-Moctezuma drafted and critically revised the paper. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

A_{2A} Adenosine Receptor Antagonism Reverts the Blood-Brain Barrier Dysfunction Induced by Sleep Restriction

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Abstract

Chronic sleep restriction induces blood-brain barrier disruption and increases pro-inflammatory mediators in rodents. Those inflammatory mediators may modulate the blood-brain barrier and constitute a link between sleep loss and blood-brain barrier physiology. We propose that adenosine action on its A_{2A} receptor may be modulating the blood-brain barrier dynamics in sleep-restricted rats. We administrated a selective A_{2A} adenosine receptor antagonist (SCH58261) in sleep-restricted rats at the 10^{th} day of sleep restriction and evaluated the blood-brain barrier permeability to dextrans coupled to fluorescein (FITC-dextrans) and Evans blue. In addition, we evaluated by western blot the expression of tight junction proteins (claudin-5, occludin, ZO-1), adherens junction protein (E-cadherin), A_{2A} adenosine receptor, adenosine-synthesizing enzyme (CD73), and neuroinflammatory markers (Iba-1 and GFAP) in the cerebral cortex, hippocampus, basal nuclei and cerebellar vermis. Sleep restriction increased blood-brain barrier permeability to FITC-dextrans and Evans blue, and the effect was reverted by the administration of SCH58261 in almost all brain regions, excluding the cerebellum. Sleep restriction increased the expression of A_{2A} adenosine receptor only in the hippocampus and basal nuclei without changing the expression of CD73 in all brain regions. Sleep restriction reduced the expression of tight junction proteins in all brain regions, except in the cerebellum; and SCH58261 restored the levels of tight junction proteins in the cortex, hippocampus and basal nuclei. Finally, sleep restriction induced GFAP and Iba-1 overexpression that was attenuated with the administration of SCH58261. These data suggest that the action of adenosine on its A_{2A} receptor may have a crucial role in blood-brain barrier dysfunction during sleep loss probably by direct modulation of brain endothelial cell permeability or through a mechanism that involves gliosis with subsequent inflammation and increased blood-brain barrier permeability.

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Introduction

Long-term insufficient sleep has adverse effects on overall health and impairments in cognition [1, 2]. Cognitive deficits during sleep loss may be related to alterations in the interface between the periphery and the central nervous system, the blood-brain barrier. This barrier restricts the entry of toxic blood-borne molecules into the brain. In this way, chronic sleep restriction induces blood-brain barrier hyperpermeability to Evans blue and sodium fluorescein, increases pinocytosis, and decreases mRNA expression of tight junction proteins in rodents [3, 4]. Interestingly, after brief sleep opportunity periods of 40 and 120 minutes, the blood-brain barrier permeability to Evans blue in almost all brain regions decreases to basal levels excluding cerebellar vermis [3]. Hence, levels of potential molecules involved in blood-brain barrier disruption during sleep loss may increase during waking and are rapidly cleared during sleep restoration. In the central nervous system the nucleotide adenosine has a crucial role as a modulator of neural transmission, as a sleep inductor [5], and a potent regulator of cerebral blood flow [6]. Adenosine concentration in the basal forebrain, hippocampus and cortex increases progressively with prolonged waking [7] or during sleep deprivation, and decreases during sleep [8].

High levels of circulating adenosine or adenosine accumulated in the extracellular space increase the permeability of microvascular endothelial cells that form the blood-brain barrier by its action on A_1 and A_{2A} adenosine receptors [9–12]. General agonists of adenosine receptors increase 3-fold the blood-brain barrier permeability to 70-kDa dextran coupled to fluorescein (FITC-dextran); however, the administration of a selective A_{2A} receptor agonist (Lexiscan) increases 20-fold the blood-brain barrier permeability to the above mentioned tracer [10]. Selective A_{2A} receptor antagonists are recently used in the treatment of several conditions such as ischemia [13], Parkinson's disease [14], and multiple sclerosis [9, 10]; in those diseases they are deemed as neuroprotectors by their ability to reduce neuroinflammation. In the present study, we evaluated the effect of an unselective adenosine receptor antagonist (caffeine) and a selective A_{2A} receptor antagonist (SCH58261) on blood-brain barrier integrity in the cortex, hippocampus, basal nuclei and cerebellar vermis of sleep-restricted rats.

Materials and Methods

Animals

Three month-old male Wistar rats (n = 71: FITC-Dextrans assay n = 32; Evans blue assay n = 9; Western blot assay n = 27, Immunohistochemistry n = 3) were used. Rats were caged in groups of 4–8 in our laboratory vivarium under a 12-hour light/dark cycle (lights on at 11 pm), at room temperature of 20–25˚C. Commercial rat chow and tap water were available *ad libitum* to all rats throughout the experiment. Rats were randomly assigned to the experimental conditions. Care was taken to reduce to the minimum the stress and discomfort in the experimental animals as well as to optimize the number of animals used in the experiments described. Experiments were performed following the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2010) and with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (www.nc3rs.org.uk/arrive-guidelines) and were approved by the Academic Ethic Committee of the Biological Sciences Division of the Universidad Autonoma Metropolitana, Unidad Iztapalapa.

Sleep Restriction

For the sleep restriction procedure an acrylic water tank (82cm x 59cm x 48cm) and 7cm diameter platforms were used. Sleep restriction was performed by the multiple platform

technique, which abolishes rapid eye movement (REM) sleep and decreases 30% of non-REM sleep, by placing rats over small platforms surrounded by water as previously reported [3]. Rats were kept in the conditions of the multiple platform technique during 20 hours for 10 consecutive days; every day they were allowed to sleep 4 hours in their home-cages during the last 4 hours of the light phase. Intact controls slept *ad libitum* in their home-cages during the 10 days of the experiment.

Administration of drugs

To evaluate the effect of an unselective adenosine receptor antagonist in sleep-restricted rats caffeine (Sigma C0750) was used; caffeine was dissolved in saline solution and administrated ip at a unique dose of 0.3mg/kg of body weight at the end of the sleep loss period in the 10^{th} day of sleep restriction. To evaluate the effect of a selective A_{2A} receptor antagonist in sleep restricted rats, SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)-pyrimidine) (Sigma S4568) was used. SCH58261 is a potent A_{2A} adenosine receptor antagonist with good selectivity (48–1561 fold) over A_1, A_{2B}, and A_3 adenosine receptors, and rapid bioavailability after ip administration [15, 16]. SCH58261 was dissolved in dimethyl sulfoxide (DMSO) and administrated ip at 0.01, 0.1, or 0.5mg/kg of body weight at the end of the sleep loss period in the 10^{th} day of sleep restriction (3 times each 30 minutes). Rats remained in the acrylic tank during the drug administration to prevent sleep and were sacrificed 30 minutes after the last SCH58261 administration.

Corticosterone quantification

An independent experiment was performed to quantify serum corticosterone concentration as a stress biomarker in the sleep restricted rats. Trunk blood was collected between 9–11 am (at the end of the light phase) from controls sleeping *ad libitum* plus DMSO, from 10-day sleep-restricted rats plus DMSO and from 10-day sleep restricted rats plus SCH58261 (n = 3 *per* group). Blood samples were centrifuged at 3000g/10minutes, supernatant was collected and stored at -80˚C until processing. Serum corticosterone was quantified by duplicate using corticosterone Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (Abcam, ab108821) following the protocol suggested by the supplier. The kit has an overall intra-assay CV of 5% and inter-assay of 7.1% with a sensitivity of 0.3 ng/mL.

Blood-brain barrier permeability assays

Quantification of blood-brain barrier permeability to 10-kDa FITC-dextrans. Dextrans labeled with Fluorescein isothiocyanate (FITC) (Sigma, FD10S) were suspended in phosphate buffered saline (PBS) to achieve a concentration of 3mg/ml. In a dose—response experiment, rats were divided into control group plus DMSO (Con, n = 4); sleep restriction plus DMSO (SR, n = 3); sleep restriction plus 0.3mg/kg of caffeine (SR+caffeine, n = 3); sleep restriction plus 0.01mg/kg (x3) of SCH58261 (SR+0.01, n = 3); sleep restriction plus 0.1mg/kg (x3) of SCH58261 (SR+0.1, n = 3); sleep restriction plus 0.5mg/kg (x3) of SCH58261 (SR+0.5, n = 3). Rats were anesthetized with sodium pentobarbital (ip. 0.063 g/kg body weight), and 10-kDa FITC-dextran was administrated ic between 9-11am. A 5 mm thoracic incision was done on the left side of each subject, the heart was partially exposed, and 0.2mL/100g body weight of FITC-dextran was administrated in the left heart ventricle. After 10 minutes of FITC-dextran circulation, rats were perfused during 5 minutes with saline solution (0.9%w/v). The brain was removed and dissected; the concentration of 10-kDa FITC-dextran was calculated in hippocampus, basal nuclei, cerebellar vermis and cerebral cortex. Samples were weighed, homogenized and centrifuged at 13500 rpm/10

minutes. Supernatant was collected and absorbance was obtained in a spectrophotometer (Genesys20, Thermo Spectronic) at 520nm. The concentration of 10-kDa FITC-dextran was calculated using a standard curve. Results are showed as the concentration of FITC-dextran per weight of brain tissue (mg/g).

Quantification of blood-brain barrier permeability to 70-kDa FITC-dextrans. Dextrans labeled with FITC (Sigma, FD70S) were suspended in PBS to a concentration of 3mg/ml. Rats were divided into control group plus DMSO (Con, n = 3); sleep restriction plus DMSO (SR, n = 3); sleep restriction plus 0.3mg/kg of caffeine (SR+caffeine n = 4); and sleep restriction plus 0.1mg/kg (x3) of SCH58261 (SR+0.1 n = 3). Seventy-kDa FITC-dextran administration procedure and determination of concentration were performed as described above for 10 kDa FITC-dextran.

Quantification of blood-brain barrier permeability to Evans Blue. Rats were sacrificed between 9–11 am. Rats were anesthetized with sodium pentobarbital (ip. 0.063g/kg body weight). Evans blue administration was performed at the end of the 10^{th} day of sleep restriction in the control group plus DMSO (Con, n = 3); sleep restriction plus DMSO (SR, n = 3); and sleep restriction plus 0.1mg/kg (x3) of SCH58261 (SR+0.1 n = 3 per group). Evans blue was administrated as previously described [3]; briefly, 0.2mL/100g body weight of Evans blue was administrated in the left heart ventricle. Evans blue circulated during 10 minutes; at the end of that period subjects were perfused with saline solution (0.9%), followed by 4% paraformaldehyde in 0.1 M saline-phosphate buffer (5 minutes each). Brains were post-fixed 24 hours by immersion in the same fixative at 4˚C and hand-sectioned into 2 mm coronal sections. Evans blue-stained slices were photographed without magnification with a digital camera (Panasonic, Lumix). Evans blue extravasation was quantitatively analyzed by measuring mean optical density of cortex, hippocampus, basal nuclei, and cerebellar vermis using ImageJ software. Optical density was quantified using the calibrated optical density step tablet and the Rodbard function provided by ImageJ software [3].

Western blot

To evaluate the expression of tight and adherens junction proteins and the neuroinflammatory markers glial-fibrilar acidic protein (GFAP) and Iba-1, brains were obtained from control group plus DMSO (Con); sleep restriction plus DMSO (SR); and sleep restriction plus 0.1mg/kg (x3) of SCH58261 (SR+0.1). To determine the expression of A_{2A} AR and CD73, brains were obtained from control group plus DMSO (Con) and sleep restriction plus DMSO (SR). The brain was obtained by decapitation and the cerebral cortex, hippocampus, basal nuclei, and cerebellar vermis were dissected, frozen, and stored at -80˚C until processing. Protein concentrations were determined using the Bradford assay (BioRad, 500–0006). Proteins (100μg) were resolved using a denaturing 10% SDS-PAGE electrophoresis and transferred to PVDF membranes. Membranes were blocked with 5% w/v non-fat milk in Tris-buffered saline for 2 hours and incubated overnight at 4˚C with occludin (Invitrogen, 40–4700, 1:1000), claudin-5 (Abcam, ab53765, 1:1000), ZO-1 (Invitrogen, 40–2200, 1:1000), E-cadherin (Santa Cruz Biotechnology, sc-21791, 1:1000), GFAP (Abcam, ab4648, 1:1000), Iba-1 (Abcam, ab48004, 1:1000), A_{2A} AR (Abcam, ab3461, 1:1000), CD73 (Abcam, ab175396, 1:500), and GAPDH (Abcam, ab8245, 1:1000) antibodies. PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and revealed with a chemiluminescence detection system (Amersham, RPN2232). Semi-quantitative analysis was performed using C-Digit program (LI-COR iS image studio. Version 3.1). GAPDH was used for normalization of junction proteins, neuroinflammatory markers, CD73 and A_{2A} AR.

Immunohistochemistry

To corroborate the possible neuroinflammation associated to sleep restriction an immunohistochemistry to detect the astroglial protein GFAP and the microglial protein Iba-1 was performed in free floating brain sections from the following groups: control plus DMSO, sleep restriction plus DMSO, and sleep restriction plus 0.1mg/kg (x3) of SCH58261 (n = 3). To determine the distribution of the A_{2A} adenosine receptor in the studied brain regions, an immunohistochemistry using the A_{2A} antibody (Abcam, ab3461) was also performed in the control plus DMSO and the sleep restricted plus DMSO groups.

Rats were anesthetized with sodium pentobarbital (ip. 0.063 g/kg body weight) and transcardially perfused with normal saline solution followed by a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS. The brains were removed and post-fixed in the same fixative during 48 hours at 4˚C, followed by cryoprotection in 30% dextrose solution. Thirty-five μm thick coronal sections containing the hippocampus, basal nuclei, and cerebellum were obtained in a cryostat (Leica, CM1850). Sections were preincubated in 1% H_2O_2 for 10 min to block endogenous peroxidase activity. After a brief washing with 1% Triton-PBS, sections were incubated in the GFAP (Abcam, ab4648, 1:2000), Iba-1 (Abcam, ab48004, 1:1500), and A_{2A} adenosine receptor (Abcam, ab3461, 1:2000) antibodies diluted in 1% Triton-Normal goat serum-PBS overnight at 4˚C. The sections were then washed and incubated in the secondary antibodies, followed by incubation in avidin-biotin complex (ABC kit, Vector Labs, PK6100). Finally the sections were reacted with the peroxidase substrate diaminobenzidine kit (Vector Labs, SK4100), collected on slides, dehydrated and coverslipped. Representative photomicrographs were obtained with a microscope (Olympus, BX60) using a CCD (MediaCybernetics, Evolution VF) and the Image-Pro Plus software.

Statistical analysis

All of the groups were compared using two-way analysis of variance (ANOVA), using as between-subjects factor the group and as within-subjects factor the regions of interests. The ANOVA tests were followed by orthogonal contrast codes as a *post hoc* test where appropriate. For the comparisons regarding the effect of sleep restriction on serum corticosterone quantification a *t* test for independent samples was performed. P values <0.05 were considered significant; a *post-hoc* power test was performed for all the ANOVA tests. All values were represented as mean ± standard error of the mean (s.e.m.). Statistical analyses were conducted using JMP software (SAS Institute Inc., version 12.2.0).

Results

As shown in Fig 1 the protocol used to induce sleep restriction did not modify serum corticosterone levels; moreover, SCH58261 did not affect corticosterone levels in the sleep-restricted rats.

Adenosine receptor antagonism reverted the blood-brain barrier hyperpermeability induced by sleep restriction

Sleep restriction increased blood-brain barrier permeability to 10-kDa FITC-dextran and adenosine receptor antagonism reverted the effect of sleep restriction in almost all brain regions studied (Group effect, $F_5 = 16.756$, $p<0.001$, Power = 0.869). As shown in Fig 2 sleep restriction increased the concentration of 10-kDa FITC-dextran in the cortex ($p<0.001$), basal nuclei ($p = 0.003$), hippocampus ($p = 0.010$), and vermis ($p<0.001$) in comparison with the control group. A single dose of caffeine (0.3mg/kg of body weight) decreased the blood-brain

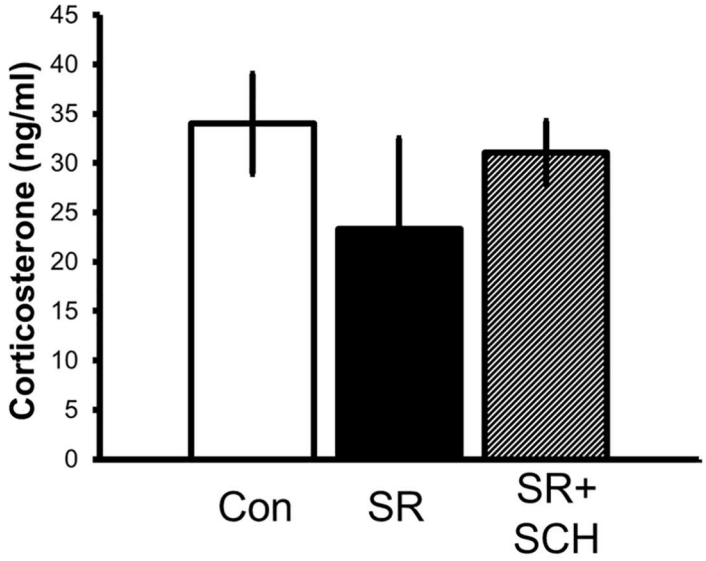

Fig 1. Corticosterone concentration in control and sleep-restricted groups. Control plus vehicle DMSO (Control), sleep restriction plus DMSO (SR), and sleep restriction plus SCH58261 at 0.1mg/kg (SR+SCH) (n = 3 *per* group). Mean ± s.e.m.

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barrier hyperpermeability induced by sleep restriction to 10-kDa FITC-dextran in the cortex (p = 0.003), basal nuclei (p = 0.03), and vermis (p = 0.01) (Fig 2). The administration of the A_{2A} selective antagonist, SCH58261, reduced the permeability to 10-kDa FITC-dextran in sleep-restricted rats in a dose response manner in the cortex. The dose of 0.1mg/kg of body weight of SCH58261 had statistical significant differences with respect to sleep restriction group in the cortex ($p<0.001$), basal nuclei (p = 0.002), hippocampus (p = 0.022), and cerebellar vermis ($p<0.001$) (Fig 2).

Sleep restriction increased blood-brain barrier permeability to 70-kDa FITC-dextran and the antagonism of adenosine receptors reverted the blood-brain barrier hyperpermeability (Group x Region effect, $F_{3\text{-}9} = 3.409$, p = 0.004, Power = 0.958). As shown in Fig 3A, sleep restriction increased the 70-kDa FITC-dextran concentration in the cortex ($p<0.001$), hippocampus ($p<0.001$), basal nuclei ($p<0.001$), and cerebellar vermis ($p<0.001$) as compared to controls sleeping *ad libitum*. Caffeine treatment reverted the increase of 70-kDa FITC-dextran concentration induced by sleep restriction only in the hippocampus ($p<0.001$). The administration of SCH58261 (0.1mg/kg (x3)) reverted the increase of the concentration of 70-kDa FITC-dextran induced by sleep restriction in the cortex ($p<0.001$), hippocampus ($p<0.001$), basal nuclei ($p<0.001$), and cerebellum (p = 0.025) (Fig 3A).

Sleep restriction increased the blood-brain barrier permeability to Evans blue and the administration of SCH58261 at 0.1mg/kg (x3) in sleep-restricted rats restored normal blood-brain barrier permeability (Group effect, $F_2 = 56.462$, $p<0.001$, Power = 0.999) (Fig 3A). Sleep restriction increased the blood-brain barrier permeability to Evans blue in all the brain regions ($p<0.001$) and the administration of SCH58261 at 0.1mg/kg (x3) in sleep-restricted rats restored normal blood-brain barrier permeability to Evans blue in those brain regions ($p<0.01$) (Fig 3B).

Selective A_{2A} adenosine receptor antagonism reverted the sleep restriction-dependent decrease in tight junction protein expression

Because the main and consistent effect on blood-brain barrier permeability in sleep-restricted rats was observed with the SCH58261 treatment at a dose of 0.1mg/kg, tight junction protein

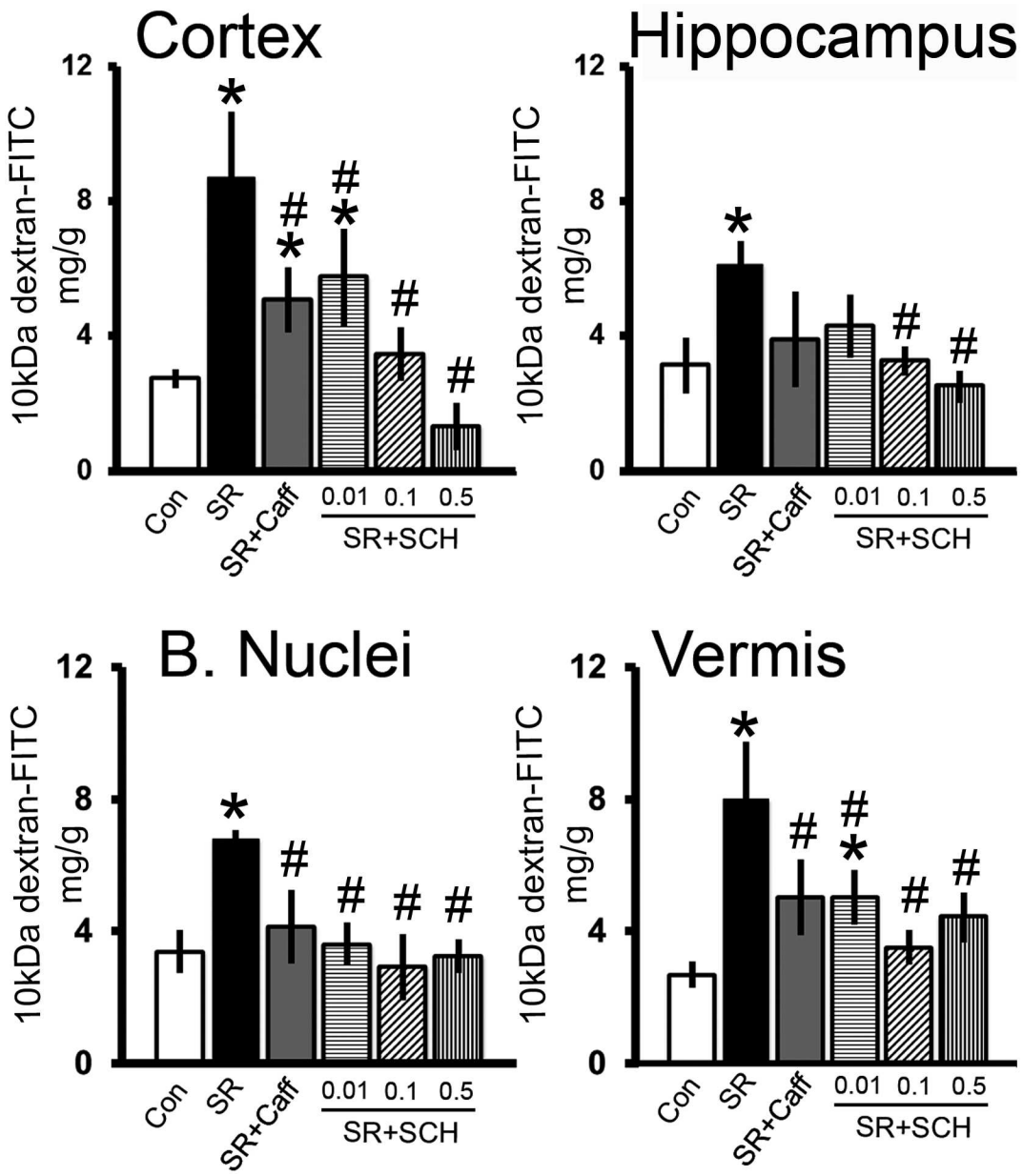

Fig 2. Adenosine receptor antagonism reverts the effect of sleep restriction on blood-brain barrier permeability to 10-kDa FITC-dextran. Graphs show the concentration (mg/g of tissue) of 10-kDa FITC-dextran in the cortex, hippocampus, basal nuclei, and cerebellar vermis of the following groups: control plus DMSO (Con), sleep restriction plus DMSO (SR), sleep restriction plus caffeine at 0.3mg/kg (SR+Caff), sleep restriction plus SCH58261 at 0.01mg/kg (SR+SCH 0.01), sleep restriction plus SCH58261 at 0.1mg/kg (SR+SCH 0.1), and sleep restriction plus SCH58261 at 0.5mg/kg (SR+SCH 0.5) (n = 3–4 per group). Mean ± s.e.m. Two-way ANOVA test, *post hoc* test orthogonal contrast codes, *p<0.05 with respect to the control group. #p<0.05 with respect to the sleep restriction group.

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expression experiment was performed only in that group as compared to intact controls and sleep restricted rats treated with the vehicle (DMSO). We found that the relative expression of claudin-5 (23kDa) decreased in the cortex, hippocampus, and basal nuclei of sleep-restricted rats as compared to control rats sleeping *ad libitum* and SCH58261 normalized claudin-5 expression in sleep restricted rats (Group x Region effect, $F_{2\text{-}6} = 2.792$, $p = 0.018$,

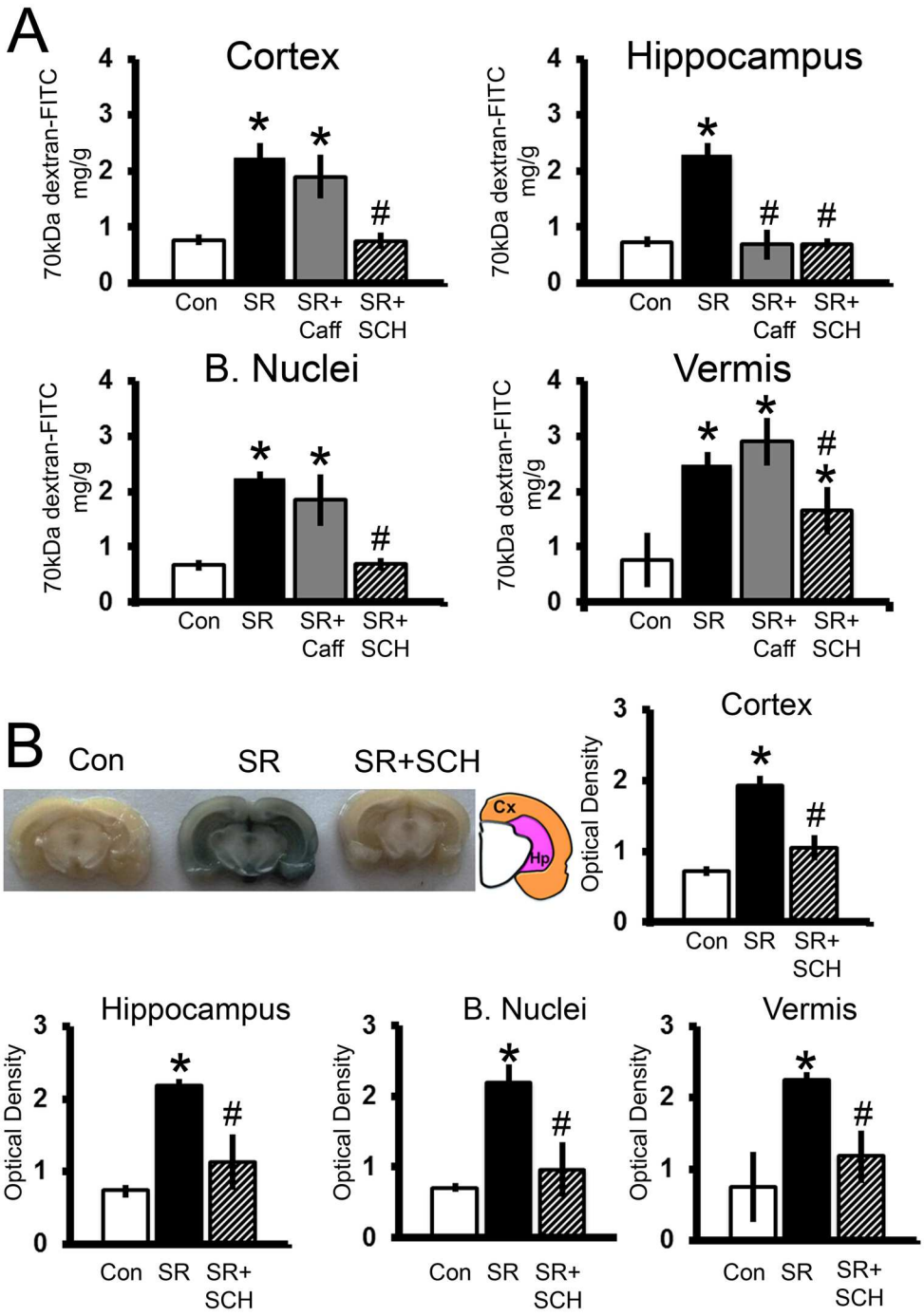

Fig 3. Adenosine receptor antagonism reverts the effect of sleep restriction on blood-brain barrier permeability to 70-kDa FITC-dextran and Evans blue. In A, graphs show the concentration (mg/g of tissue) of 70-kDa FITC-dextran in the cortex, hippocampus, basal nuclei, and vermis of the following groups: control plus vehicle DMSO (Con), sleep restriction plus DMSO (SR), sleep restriction plus caffeine at 0.3mg/kg (SR +Caff), and sleep restriction plus SCH58261 at 0.1mg/kg (SR+SCH). In B are depicted representative coronal slices of Evans blue extravasation in the cortex (Cx) and hippocampus (Hp) of the control (Con), sleep restricted (SR) and sleep restricted plus SCH58261 at 0.1mg/kg (SR+SCH) groups. Quantification of Evans blue deposition is shown as relative units of optical density in the cortex, hippocampus, basal nuclei, and vermis of the following groups: control plus vehicle DMSO (Con), sleep restriction plus DMSO (SR), and sleep restriction plus SCH58261 at 0.1mg/kg (SR+ SCH) (n = 3–4 per group). Mean ± s.e.m. Two-way ANOVA test, *post hoc* test orthogonal contrast codes, *p<0.05 as compared to the control group, #p<0.05 with respect to the sleep restriction group.

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Power = 0.896). In comparison to the control group, sleep restriction induced a 50% decrease in the relative expression of claudin-5 in the cortex ($p<0.001$). SCH58261 induced a recovery of claudin-5 expression in the cortex ($p = 0.001$) (Fig 4). In the hippocampus the reduction of claudin-5 expression was 30% ($p = 0.011$), and the administration of SCH58261 restored to basal levels the relative expression of claudin-5 (Fig 4). In the basal nuclei, the relative expression of claudin-5 tend to decrease in the sleep restriction group (approximately 20%), nevertheless we did not find significant differences. In that region, the group of sleep-restricted rats administrated with SCH58261 had statistically significant differences with the sleep restriction group; those SCH58261 treated animals presented an increase in the relative expression of claudin-5 as compared to sleep-restricted rats ($p = 0.001$) (Fig 4). As shown in Fig 4 no differences were observed in the relative expression of claudin-5 in the vermis under any experimental condition in comparison to the control group.

The relative expression of the tight junction proteins occludin and ZO-1 varied as a function of the region studied in the sleep restricted rats and also in the animals treated with the selective A_{2A} adenosine receptor antagonist SCH58261 (Occludin: Group x Region effect, $F_{2\text{-}6} = 6.382$, $p<0.001$, Power = 0.997; ZO-1: Group x Region effect, $F_{2\text{-}6} = 5.551$, $p<0.001$, Power = 0.993). Western blot analysis showed that the relative levels of occludin (65kDa) in the cortex decreased 45% in the sleep-restricted rats as compared to intact controls ($p = 0.001$) but the treatment with SCH58261 did not restore the basal relative expression of occludin ($p = 0.003$ as compared to intact controls). In the case of the relative expression of ZO-1 in the cortex, sleep restriction reduced 46% the expression in comparison to the control group ($p = 0.001$) and the treatment with SCH58261 restored to the basal levels its expression (Fig 4). In the hippocampus, a significant difference in comparison to the control group was detected in the expression of occludin, in which the decrease was 45% ($p = 0.001$), and ZO-1, with a decrease of 44% ($p = 0.002$) in the sleep restriction group. The treatment with SCH58261 restored the basal levels of occludin but not of ZO-1 ($p = 0.002$) (Fig 4). Sleep restriction did not induce significant changes in occludin and ZO-1 relative expression in the basal nuclei, however we found that the treatment with SCH58261 increased 64% the levels of occludin with respect to the control group ($p<0.001$) and 54% with respect to the sleep-restriction group ($p<0.001$) (Fig 4). No changes were observed in the expression of occludin and ZO-1 in the cerebellar vermis (Fig 4). Regarding the adherens junction protein E-cadherin, no clear pattern of change was observed in any of the groups; sleep restriction increased E-cadherin protein expression only in the hippocampus and SCH58261 did not modify the levels of the protein in the sleep restricted rats (see S1 Fig).

SCH58261 attenuates the overexpression of neuroinflammatory markers induced by sleep restriction

As shown in Fig 5, sleep restriction increased the relative expression of Iba-1 (17 kDa), a marker of reactive microglia, in almost all brain regions as compared to control rats sleeping *ad libitum*, and SCH58261 restored to normal levels the Iba-1 expression in sleep restricted rats (Group x Region effect, $F_{2\text{-}6} = 10.621$, $p<0.001$, Power = 0.862). Sleep restriction increased Iba-1 expression in the cortex ($p<0.001$), hippocampus ($p<0.001$), and basal nuclei ($p<0.001$) (Fig 5), but not in the cerebellar vermis. As shown in the lower panel of Fig 5, Iba-1 overexpression seen in western blot was related to a higher number of Iba-1 immunoreactive cells at least in the basal nuclei.

In the same way, sleep restriction increased the relative expression of the astroglial marker GFAP (55 kDa), this effect was also reverted with SCH58261 administration (Group x Region effect, $F_{2\text{-}6} = 4.538$, $p = 0.003$, Power = 0.952) (Figs 6 and 7). Sleep restriction increased GFAP

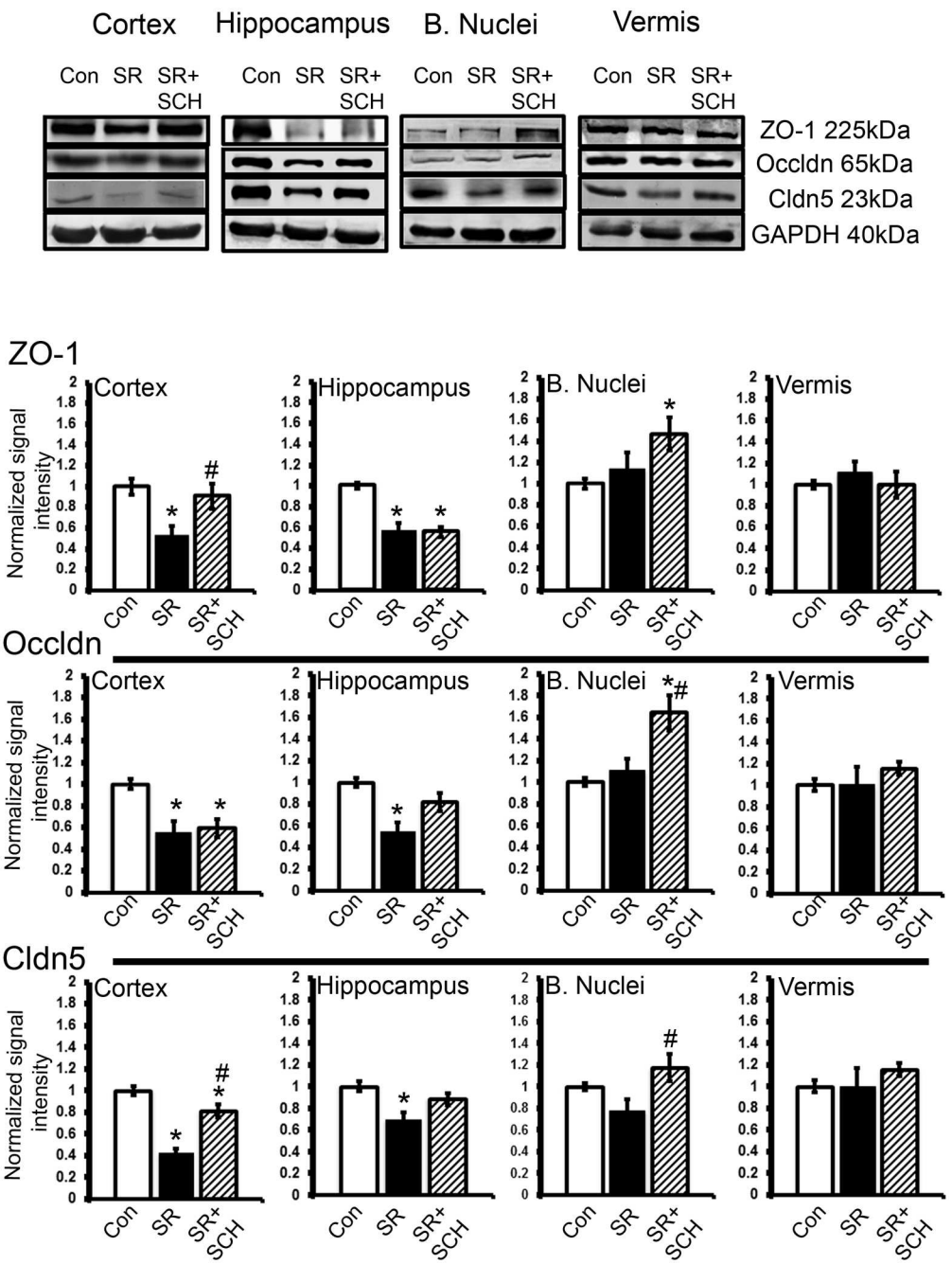

Fig 4. A_{2A} adenosine receptor antagonism reverts the effect of sleep restriction on tight junction protein expression. At the top are depicted representative western blots of tight junction protein expression in the cortex, hippocampus, basal nuclei and vermis. Graphs show the relative optical density of tight junction proteins on the following groups: control plus DMSO (Con), sleep restriction plus DMSO (SR), and sleep restriction plus SCH58261 at 0.1mg/kg (SR+SCH) (n = 6 per group). GAPDH was used for normalization. Abbreviations are as follow: Cldn5: claudin-5; Occldn: occludin; ZO-1: Zonula occludens-1. Mean ± s.e.m. Two-way ANOVA test, *post hoc* test orthogonal contrast codes, *p<0.05 as compared to the control group. #p<0.05 with respect to the sleep restriction group.

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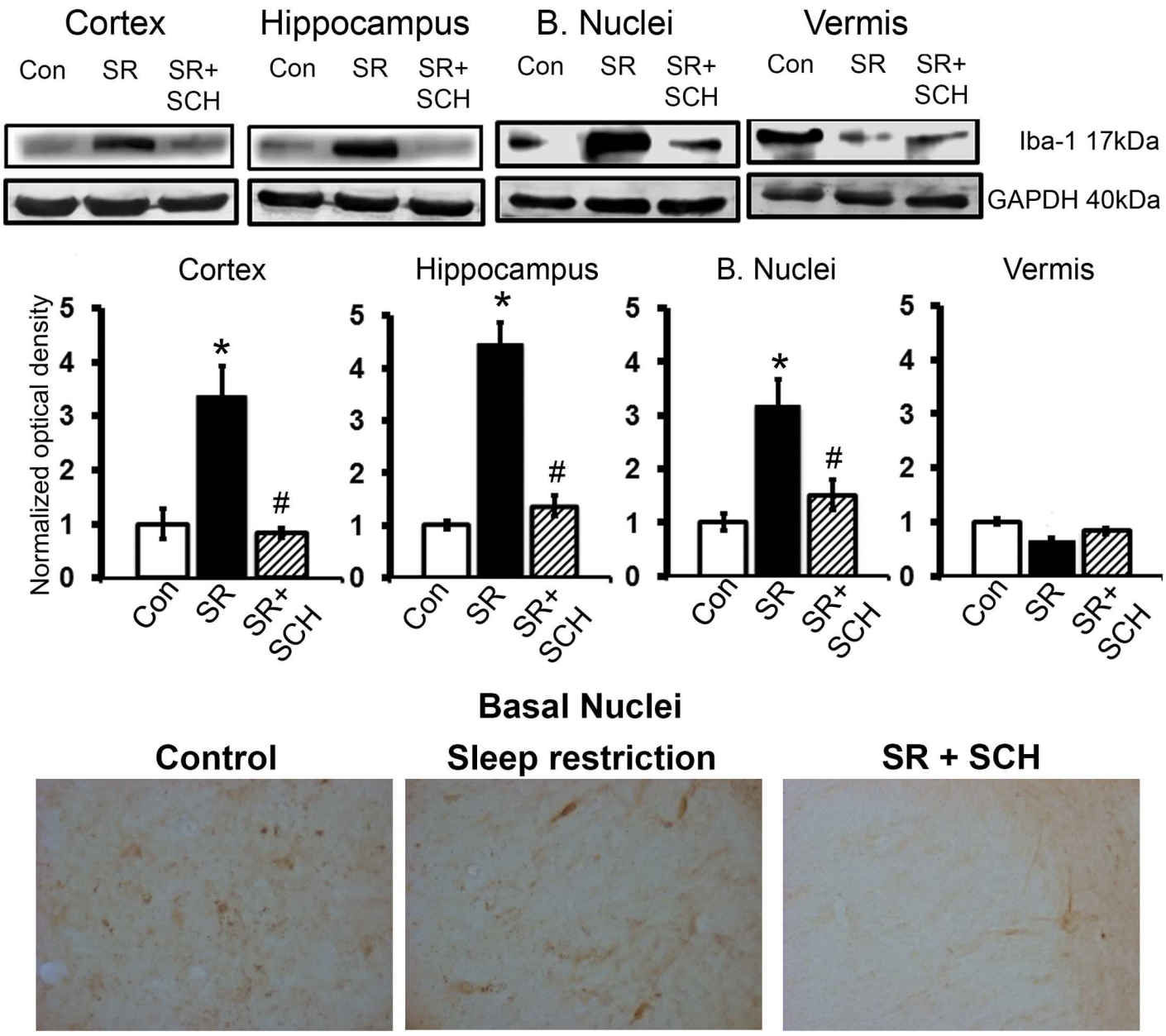

Fig 5. A_{2A} adenosine receptor antagonism attenuates Iba-1 overexpression induced by sleep restriction. Representative western blot images of expression of Iba-1 *per* region (cortex, hippocampus, basal nuclei, and vermis) are shown, as well as graphs depicting the relative optical density of Iba-1 in the following groups: control plus DMSO (Con), sleep restriction plus DMSO (SR), and sleep restriction plus SCH58261 at 0.1mg/kg (SR+SCH) (n = 3 *per* group). GAPDH was used for normalization. Mean ± s.e.m. Two-way ANOVA test, *post hoc* orthogonal contrast codes, *p<0.05 as compared to the control group; #p<0.05 with respect to the sleep restriction group. In the lower panel, microphotographs show the differences in the number of Iba-1 immunoreactive cells in the basal nuclei of control, sleep restricted and sleep restricted plus SCH groups. X400.

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expression in the cortex (p = 0.003), hippocampus (p = 0.010), and basal nuclei (p<0.001), while did not modify GFAP expression in the cerebellar vermis. A_{2A} adenosine receptor antagonism reverted to basal levels the sleep-related GFAP overexpression in all the three regions. As shown in Fig 7 the GFAP changes in sleep restricted rats were related to increased astroglial ramifications rather than apparent changes in astroglial density in the cortex, hippocampus and basal nuclei.

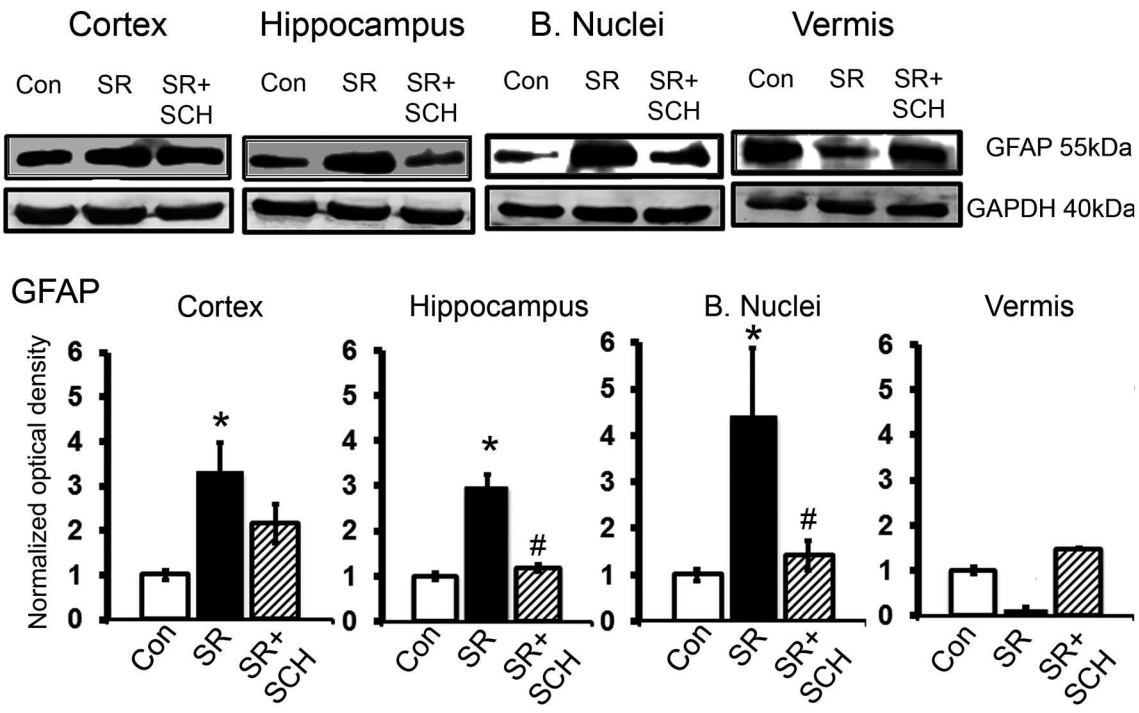

Fig 6. A_{2A} adenosine receptor antagonism attenuates GFAP overexpression induced by sleep restriction. Representative western blot images of expression of GFAP *per* region (cortex, hippocampus, basal nuclei, and vermis) are shown, as well as graphs depicting the relative optical density of GFAP in the following groups: control plus DMSO (Con), sleep restriction plus DMSO (SR), and sleep restriction plus SCH58261 at 0.1mg/kg (SR+SCH) (n = 3 *per* group). GAPDH was used for normalization. Mean ± s.e.m. Two-way ANOVA test, *post hoc* orthogonal contrast codes, *p<0.05 as compared to the control group; #p<0.05 with respect to the sleep restriction group.

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Sleep restriction modulates the expression of A_{2A} adenosine receptor but not of the adenosine-synthesizing enzyme CD73

Because the differential effect of sleep restriction and SCH58261 treatment on blood-brain barrier permeability, tight junction and neuroinflammatory markers expression may be associated to the differential density of A_{2A} adenosine receptors in each brain region; we tested the

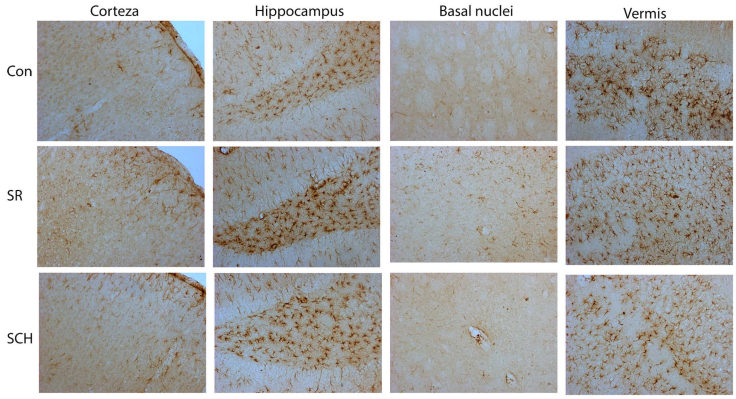

Fig 7. A_{2A} adenosine receptor antagonism reverts the astroglial hyper-ramification induced by sleep restriction. Photomicrographs show the astroglial morphology in the controls plus DMSO, sleep restricted plus DMSO, and sleep restriction plus SCH58261 at 0.1mg/kg in the cortex, hippocampus, basal nuclei, and cerebellar vermis. X100.

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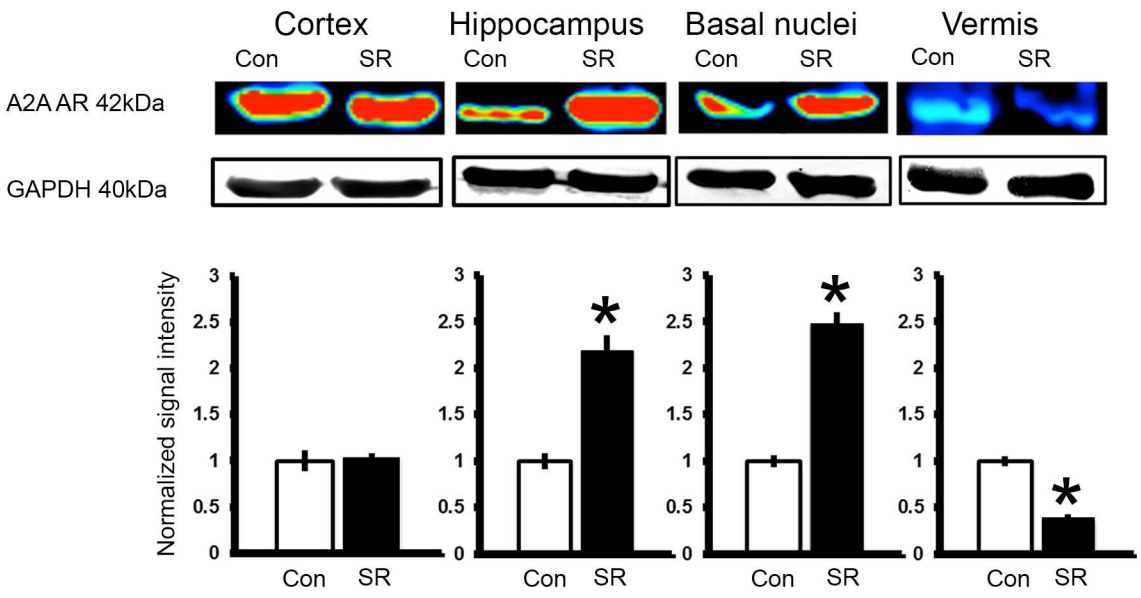

Fig 8. Sleep restriction modulates A_{2A} adenosine receptor expression. At the top, representative western blot of the expression for A_{2A} adenosine receptor in the cortex, hippocampus, basal nuclei, and vermis using chemiluminescent signal. Graphs show the relative optical density of A_{2A} adenosine receptor in the following groups: control plus DMSO (Con) and sleep restriction plus DMSO (SR) (n = 3 *per* group). GAPDH was used for normalization. Mean ± s.e.m. Two-way ANOVA test, *post hoc* orthogonal contrast codes, *p<0.05 as compared to the control group.

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expression of this receptor in the cortex, hippocampus, basal nuclei, and vermis of sleep-restricted rats and intact controls. We found that sleep restriction modified the A_{2A} adenosine receptor expression in a region dependent way (Group x Region effect, $F_{1\text{-}3} = 94.724$, $p<0.001$, Power = 0.999) (Figs 8 and 9). In the hippocampus sleep restriction increased 2-fold the expression levels of A_{2A} adenosine receptors as compared to the control group ($p<0.001$). In the same way, the relative expression of A_{2A} adenosine receptors in the basal nuclei increased 2-fold than the control group ($p<0.001$); however, in the cerebral cortex sleep restriction did not modify the relative expression of A_{2A} adenosine receptors (as shown in Fig 8). By the contrary, in the cerebellar vermis the relative expression of A_{2A} adenosine receptors decreased 50% in the group of sleep restriction as compared to intact controls ($p<0.001$) (Fig 8). As shown in Fig 9, A_{2A} adenosine receptors expressed in brain parenchymal cells, mainly in neurons and endothelial cells.

Besides A_{2A} adenosine receptor differential distribution along brain regions, other possible mechanism involved in the differential regional effects of sleep restriction on blood-brain barrier function implied the differential expression of the adenosine-synthesizing enzyme, the ecto-5'-nucleotidase (CD73), in the studied brain regions. As shown in S2 Fig western blot analysis showed that the expression of CD73 remained without change in all brain regions of the sleep restricted rats as compared to controls sleeping *ad libitum*.

Discussion

Sleep restriction increased blood-brain barrier permeability to FITC-dextran and Evans blue; the changes in blood-brain barrier permeability were observed for both low- and high-molecular weight tracers in the cortex, hippocampus, basal nuclei and cerebellar vermis of sleep-restricted rats as compared to controls sleeping *ad libitum*. The use of an unselective adenosine receptor antagonist, caffeine, reverted the blood-brain barrier

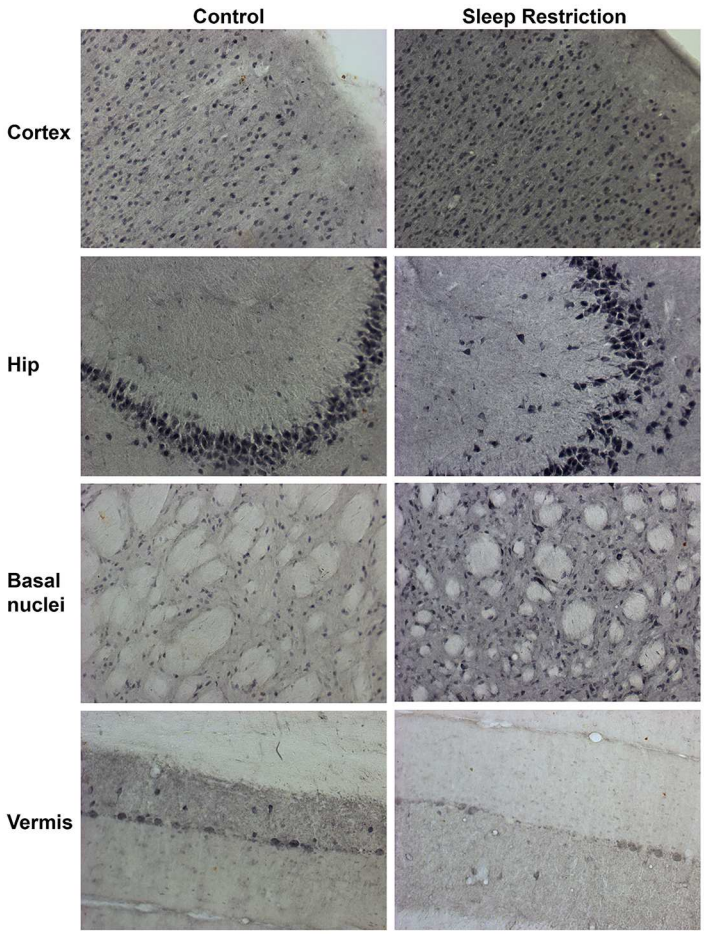

Fig 9. Sleep restriction modifies A_{2A} adenosine receptor expression in brain parenchymal cells. Photomicrographs show the distribution of A_{2A} adenosine receptor in the cortex, hippocampus, basal nuclei, and cerebellar vermis of the following groups: controls plus DMSO, sleep restriction plus DMSO, and sleep restriction plus SCH58261 at 0.1mg/kg. Note the expression of A_{2A} adenosine receptors in cerebellar capillaries. X100.

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hyperpermeability to a low molecular weight tracer in all brain regions, but only partially for large molecules; caffeine reverted the sleep restriction-related hyperpermeability in the hippocampus but failed to do that in the cortex, basal nuclei and cerebellar vermis. Meanwhile, the administration of a selective A_{2A} adenosine receptor antagonist (SCH58261) fully restored the normal permeability of the blood-brain barrier to both low- and high-molecular weight tracers in the cortex, hippocampus and basal nuclei; however, only the dose of 0.1mg/kg had consistent significant differences with respect to the sleep restriction group. The changes in blood-brain barrier permeability were accompanied by corresponding changes in tight junction protein expression; sleep restriction decreased the expression of claudin-5, occludin, and ZO-1 in almost all brain regions studied, while did not affect the expression of the adherens junction protein E-cadherin. The selective antagonism of A_{2A} adenosine receptor was able to revert the changes in tight junction protein expression in sleep-restricted rats. Regional differences in blood-brain barrier permeability associated to chronic sleep loss may have arisen from differences in the expression of A_{2A} adenosine receptors as well as on its selective localization on glial or endothelial cells, because there

were not differences among groups in the expression of the adenosine-synthesizing enzyme CD73 in the studied brain regions.

Sleep loss effects on physiology may potentially be confounded with unspecific stress effects associated to the sleep restriction procedures [17], here we reported that sleep restriction during 10 days using the multiple platform technique did not alter the serum corticosterone concentration. Serum corticosterone was quantified at the end of the light phase, near the normal circadian peak of plasma corticosterone, other studies using a similar sleep restriction protocol have shown that rises in corticosterone concentration occur only during the 20 hours of sleep deprivation *per* day since the day 1 until day 7 of sleep restriction [18]. In addition, similar to our findings, previous reports have shown that during the hours of sleep opportunity corticosterone returns to basal levels [17, 18]. The fact that we found low levels of serum corticosterone near the peak of the corticosterone circadian rhythm indicates a potential deregulation of the hypothalamus-pituitary-adrenal axis during sleep loss, therefore more experiments are needed to test potential changes in the rhythmicity of corticosterone associated to chronic sleep loss, as has been shown for chronic stress [19].

Generally, the passage of small molecules (5–40 kDa) from blood-to-brain is considered a marker of blood-brain barrier permeability to small solutes and ions, while Evans blue dye (~65kDa), which binds to albumin, is a marker for protein permeability [20]. Therefore our results indicate that sleep restriction could modify both ion and protein permeability in the blood-brain barrier and that A_{2A} adenosine receptor antagonism reverted both effects. Similar results have been described in animal models of brain diseases in which an initial increase in blood-brain barrier permeability is reverted to basal levels after adenosine receptor antagonism, eg. in experimental autoimmune encephalomyelitis [10].

The role of adenosine receptors in regulating blood-brain barrier permeability is amply described [9–12]. However a recent report showed that stimulation of adenosine receptors modified blood-brain barrier permeability to low molecular weight FITC-dextrans by a mechanism independent of blood-brain barrier regulation *per se*, by increasing FITC-dextrans plasma availability secondary to impaired renal function [21]; those findings impel a thoughtful review of the role of adenosine in regulating blood-brain barrier function. We antagonized physiological levels of adenosine, particularly adenosine acting on A_{2A} receptors; A_{2A} receptors are involved in the maintenance of renal medullary blood-flow through vasodilation and are not involved in fluid or ion reabsorption (reviewed in [22]), therefore it is possible that A_{2A} adenosine receptor antagonism may have had little effect on dextran-FITC clearance from the blood. In addition, the lack of kidney damage during sleep loss [23], which guarantees a normal clearance of FITC-dextrans through urine, and the fact that we used Evans blue, an albumin-binding dye with little renal clearance [21], allow us to affirm that A_{2A} adenosine receptors are playing a central role in regulating blood-brain barrier permeability during sleep loss by directly acting on blood-brain barrier cellular components (eg. endothelial cells, pericytes, and astrocytes) or on microglial cells.

It is known that adenosine receptors are amply distributed in the rat brain; particularly A_{2A} receptor is highly expressed in the basal nuclei, hippocampus and cortex, but its expression is low in the cerebellum [24, 25]; A_{2B} and A_3 adenosine receptors are poorly expressed in the brain, and A_1 adenosine receptor is highly expressed in the cerebellum and widespread distributed in the rest of the brain but with low levels [24]. In this way, the overexpression of A_{2A} adenosine receptor in the hippocampus of sleep restricted rats but not in the cortex, basal nuclei or vermis, explains the reduction of hyperpermeability to 70-kDa FITC-dextran by caffeine administration in the hippocampus but not in other brain regions. These data agree with a previous report that showed that sleep restriction in mice increase the mRNA levels of A_{2A} adenosine receptor in the hippocampus but not in other brain regions [26]. The hippocampus

is predominantly affected by insufficient sleep; hence, sleep loss negatively impacts memory, causing deficits in memory processes, which can be attenuated with caffeine administration [27]. Therefore, caffeine is considered as a potent neuroprotector and this property has been associated to caffeine action on A_{2A} adenosine receptor [28], then it is very likely that the effect of caffeine on the restoration of normal blood-brain barrier permeability after sleep restriction is mediated by A_{2A} adenosine receptor.

The role of A_{2A} adenosine receptor in regulating the blood-brain barrier during sleep loss is supported by the fact that the used antagonist, SCH58261, is 48, 581, and 1561 fold more selective to A_{2A} adenosine receptor than to A_1, A_{2B}, and A_3 adenosine receptors, respectively [15, 16]. Moreover, the low dose used in this experiment is similar to the dose used in previous *in vivo* studies that showed that SCH58261 neuroprotective effects are only observed at very low doses (in the range of 0.01–0.5 mg/kg) [29]. The fact that SCH58261 modified blood-brain barrier permeability only in the brain regions enriched in A_{2A} adenosine receptors pinpoints to a regional highly selective regulation of blood-brain barrier function during sleep loss and recovery by adenosine acting on its A_{2A} receptors.

Regarding the mechanism by which sleep loss increases blood-brain barrier permeability, here, we report that sleep restriction decreased the expression of tight junction proteins, including claudin-5, ZO-1, and occludin in the hippocampus and cortex. The decrease in the expression of these proteins is related to the induction of hyperpermeability in brain endothelial cells under pathological conditions [30]. Claudin-5 is necessary for endothelial barrier integrity, it has been reported that in human brain endothelial cells, a selective A_{2A} adenosine receptor agonist (Lexiscan) decreased gradually the expression of claudin-5 up to 30 minutes after treatment; meanwhile, the treatment with an unselective adenosine receptor agonist decreased the expression of claudin-5 after 2 hours post treatment [11]. This supports our hypothesis that the rapid action of adenosine and particularly its action on A_{2A} adenosine receptors might regulate the blood-brain barrier permeability in sleep-restricted rats. Indeed, we found that brief sleep opportunity periods (40–120 minutes) induced a recovery of claudin-5 expression (unpublished data) suggesting that the degradation of adenosine during sleep recovery prevents the A_{2A} adenosine receptor activation restoring the blood-brain barrier integrity. In this way, blocking A_{2A} adenosine receptor in sleep-restricted rats may promote the physiological expression of claudin-5 and therefore recover the basal permeability of the blood-brain barrier.

The decrease in the expression of ZO-1 and occludin is maintained even with SCH58261 administration, suggesting that other pathways are yet activated. For instance, one hallmark characteristic of sleep loss is the generation of a low-grade inflammatory status (reviewed in [31]). Several inflammatory mediators, such as the cytokines tumor necrosis factor (TFN)-α, interleukin (IL)-1β, IL-6, and IL-17A [32] as well as molecules such as C-reactive protein (CRP), cyclooxygenase (COX)-2, and endothelin (ET)-1 [4] are released during sleep loss and are able to modify tight junction protein expression both in *in vitro* and in *in vivo* experiments (for a review see [33, 34]). In addition, the decrease of ZO-1 and occludin might be associated to subtle permeability changes related to peripheral inflammation like that occurring in naturally aged rodents [35].

The maintenance of relative expression of tight junction proteins in the basal nuclei suggest that in this region the mechanism of blood-brain barrier dysfunction might be mediated by other unselective transport such as pinocytosis or that these proteins are present but have a different localization, for example in the cytoplasm. Because basal nuclei have more A_{2A} adenosine receptors than other brain regions, we also propose that the possible re-distribution of tight-junction proteins might be restored after SCH58261 administration, indeed, we observed that levels of claudin-5 and occludin increased with the administration of this antagonist in comparison to the sleep restriction.

In the case of vermis, the basal lower density of A_{2A} adenosine receptors and its reduction during sleep restriction can explain the remaining blood-brain barrier hyperpermeability to dextrans even with the treatment with the selective A_{2A} adenosine receptor antagonist. Here, we again suggest that other mechanisms, independent of adenosine may be participating, such as blood-brain barrier regulation by pro-inflammatory cytokines (as hypothesized in [33]). Indeed it has been shown that the cerebellum seems to be prone to high-fat diet induced neuroinflammation mediated by the endogenous synthesis of the inflammatory mediators monocyte chemo attractant protein-1 (MCP-1), interleukin 1β (IL-1β), tumor necrosis factor-α (TNFα), and cyclooxygenase-2 (COX-2) as compared to the cerebral cortex [36]. In the case of the infection with the attenuated rabies virus CVS-F3 it has been shown that increased cerebellar blood-brain barrier permeability is accompanied by increased local synthesis of TNFα, interferon γ (IFNγ), and intercellular adhesion molecule-1 (ICAM-1) and occurs earlier in the cerebellum than in the cerebral cortex [37]. Additionally, the absence of sleep loss effects on tight junction protein expression in the cerebellar vermis point to a different mechanism by which sleep loss increases blood-brain barrier permeability in that region, such as pinocytosis.

The increase of adenosine levels in the brain after sleep loss [7] may induce the activation of A_{2A} adenosine receptors in endothelial cells but also in neural and glial cells [38] here we showed that A_{2A} receptors are present in parenchymal brain cells including neurons and endothelial cells. We also report that sleep restriction induced overexpression of markers of reactive microglia (Iba-1) and astroglia (GFAP) and that the administration of SCH58261 reverted this effect. Microglia participate in innate immunity, and several physiological processes such as in neurodevelopment and structural plasticity [39]. Interestingly, chronic administration of an inhibitor of microglia activation, minocycline, prevents the buildup of sleep need in rodents, suggesting that microglia might have a role in sleep regulation [40]. Microglial cells express all the subtypes of adenosine receptors, A1, A_{2A}, A_{2B}, and A_3; however, particular attention has been paid to A_{2A}, considered to have a central role in neuroinflammation [41]. In this way, the increase in Iba-1 expression may be related to the activation of microglia by adenosine acting on A_{2A} receptor during sleep restriction. On the other hand, astrocyte-derived adenosine is a candidate molecule involved in the cognitive deficits following sleep loss particularly at the hippocampal level [42, 43] and may have an important role in regulating blood-brain barrier permeability. Moreover, activation of A_{2A} receptor in astrocytes is related to activation of inflammatory status in several neuropathologies [44, 45]. In any case, gliosis is associated with blood-brain barrier disruption because the release of several inflammatory mediators [46–49]; however, it is also possible that glial cell activation may precede and even modify blood-brain barrier permeability, as shown after LPS administration [50].

This study is the first to investigate A_{2A} adenosine receptor role in the regulation of blood-brain barrier during sleep restriction. We propose that changes in blood-brain barrier permeability contribute to many pathophysiological processes in the brain of subjects with sleep restriction (Fig 10). Those effects may be mediated by adenosine signaling that involves A_{2A} receptor activation and the regional differences in susceptibility may depend on adenosine receptor distribution and up-/down-regulation during sleep loss. More studies are needed to corroborate this hypothesis or explain this phenomenon taking into account that we evaluated the expression of A_{2A} adenosine receptor in specific brain regions but not in specific cellular types. It is probable that each cellular element of the blood-brain barrier, namely endothelial cells, pericytes, or astrocytes, may contribute to this phenomenon because of its potential adenosine-mediated signaling (for review see [12]). Indeed, it has been shown that during 12 hours of sleep deprivation astrocyte-derived adenosine is essential to alter network patterns of electrical activity in the frontal cortex [51]. Therefore, astrocytes may be playing a key role in

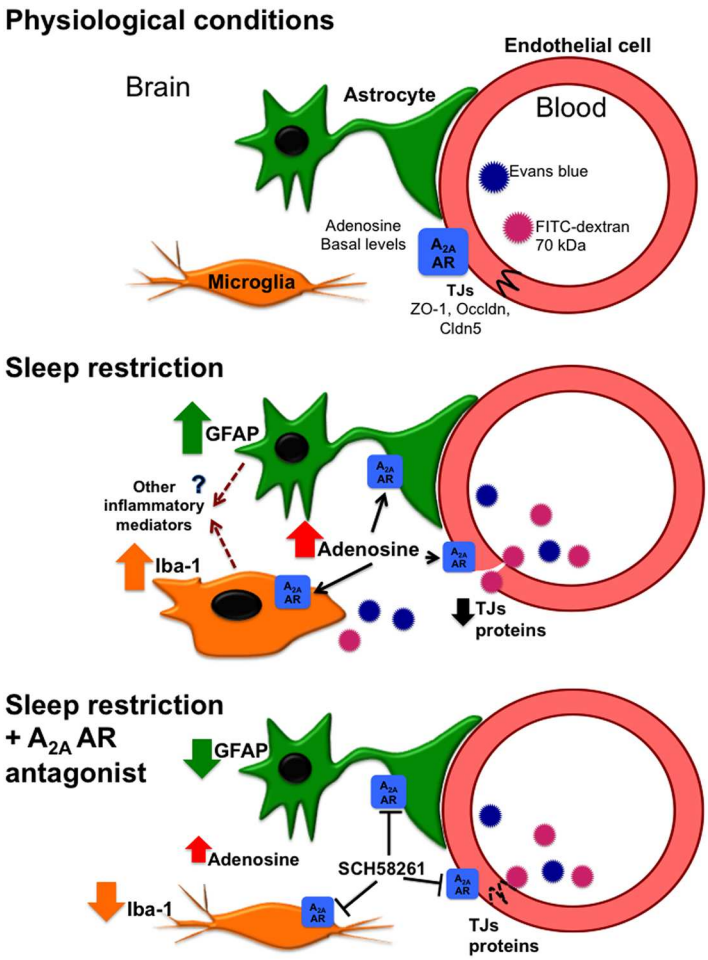

Fig 10. Blood-brain barrier regulation by adenosine during sleep restriction. Under physiological conditions adenosine modulates cerebral blood-flow and is involved in neurophysiological events including sleep regulation. Prolonged waking increases adenosine levels in the central nervous system. Sleep restriction induces gliosis, characterized by the overexpression of Iba-1 and GFAP markers, probably by the activation of A_{2A} adenosine receptor in the microglial and astroglial cells. The activation of glial cells may induce the release of other inflammatory mediators, eg. the pro-inflammatory cytokines IL-6 and TNF-α, which conjointly with adenosine may contribute to blood-brain barrier modulation during sleep loss. In endothelial cells, A_{2A} adenosine receptor activation promotes the decrease in tight junction protein expression, such as claudin-5 (Cldn5), occludin (Occldn) and Zonula occludens (ZO)-1. These changes may ensue blood-brain barrier hyperpermeability to molecules with high molecular weight such as 70-kDa FITC-dextran and Evans blue, and are rapidly reverted using a selective A_{2A} adenosine receptor antagonist such as SCH58261.

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regulating blood-brain barrier permeability during sleep loss through the release of adenosine and activation of A_{2A} adenosine receptors in brain endothelial cells; however *in vitro* assays are needed to clarify the role astrocyte-derived adenosine in regulating blood-brain barrier in sleep-restricted animals.

The findings here reported are relevant to consider the possible impact of chronic low-grade neuroinflammation in the development or exacerbation of neuropathologies associated with sleep deficiency. It may also contribute to generate knowledge about regulation of blood-brain barrier permeability using sleep restriction as a non-pathological model. In this way, sleep loss may promote temporal blood-brain barrier opening to allow the passage from blood-to-brain of molecules with potential therapeutic effects with a high regional specificity.

Supporting Information

S1 Fig. Sleep restriction increases the expression of the adherens junction protein E-cadherin in the hippocampus. At the top, representative western blot of the expression of E-cadherin in the cortex, hippocampus, basal nuclei, and cerebellar vermis. Graphs show the relative optical density of E-cadherin expression in the following groups: control plus DMSO (Con), sleep restriction plus DMSO (SR) and sleep restriction plus SCH58261 at 0.1mg/kg (SR+SCH). GAPDH was used for normalization. Mean ± s.e.m. Two-way ANOVA test $^{*}p<0.05$ as compared to the control group.
(TIF)

S2 Fig. Adenosine-synthesizing enzyme (CD73) expression remains unchanged despite chronic sleep loss. At the top, representative western blot of the expression of CD73 in the cortex, hippocampus, basal nuclei, and cerebellar vermis. Graphs show the relative optical density of CD73 in the following groups: control plus DMSO (Con) and sleep restriction plus DMSO (SR). GAPDH was used for normalization. Mean ± s.e.m.
(TIF)

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Formal analysis: GHA BGG.

Funding acquisition: BGG JVM.

Investigation: GHA EDS.

Methodology: GHA BGG EDS.

Project administration: GHA BGG.

Resources: BGG JVM.

Supervision: BGG.

Validation: GHA EDS.

Visualization: GHA BGG.

Writing – original draft: GHA BGG.

Writing – review & editing: EDS JVM.

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ANEXO 5

Pericytes: brain-immune interface modulators

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The premise that the central nervous system is immune-privileged arose from the fact that direct contact between immune and nervous cells is hindered by the blood–brain barrier. However, the blood–brain barrier also comprises the interface between the immune and nervous systems by secreting chemo-attractant molecules and by modulating immune cell entry into the brain. The majority of published studies on the blood–brain barrier focus on endothelial cells (ECs), which are a critical component, but not the only one; other cellular components include astroglia, microglia, and pericytes. Pericytes are poorly studied in comparison with astrocytes or ECs; they are mesenchymal cells that can modify their ultrastructure and gene expression in response to changes in the central nervous system microenvironment. Pericytes have a unique synergistic relationship with brain ECs in the regulation of capillary permeability through secretion of cytokines, chemokines, nitric oxide, matrix metalloproteinases, and by means of capillary contraction. Those pericyte manifestations are related to changes in blood–brain barrier permeability by an increase in endocytosis-mediated transport and by tight junction disruption. In addition, recent reports demonstrate that pericytes control the migration of leukocytes in response to inflammatory mediators by up-regulating the expression of adhesion molecules and releasing chemo-attractants; however, under physiological conditions they appear to be immune-suppressors. Better understanding of the immune properties of pericytes and their participation in the effects of brain infections, neurodegenerative diseases, and sleep loss will be achieved by analyzing pericyte ultrastructure, capillary coverage, and protein expression. That knowledge may provide a mechanism by which pericytes participate in the maintenance of the proper function of the brain-immune interface.

Keywords: pericytes, blood–brain barrier, immune response, inflammation, cytokines, REM sleep loss, brain endothelial cell, tight junction disruption

INTRODUCTION

The brain must respond to blood-borne signals but has no direct access to them (Persidsky et al., 2006; Saper, 2010). Likewise, the immune system does not contact directly the brain *milieu*; they interact through the brain-immune interface, the blood–brain barrier. The interface is comprised by endothelial cells (ECs), astrocytes, microglia, pericytes, and extracellular matrix components (basal lamina and glycocalyx; Risau, 1991; Ballabh et al., 2004; Ueno, 2007; Gómez-González et al., 2012). ECs limit blood-borne macromolecules or cells from crossing into the brain through junction complexes that fasten together adjacent cell membranes. In addition, transcellular trafficking of molecules is limited by the minimal expression of endocytosis and the presence of specialized carrier systems (Zlokovic, 2008; Abbott et al., 2010). Although ECs provide the physical and chemical barrier function *per se*, all elements are crucial for the development and maintenance of the blood–brain barrier, allowing it to be the interface between peripheral systems and the brain (Zlokovic, 2008).

Pericytes have been increasingly implicated in the regulation of local blood-flow in brain regions with increased synaptic activity, a phenomenon known as neurovascular coupling (reviewed in Hamilton et al., 2010); furthermore, they have also been involved in the regulation of the blood–brain barrier permeability to circulating molecules (Armulik et al., 2010). Better understanding of the immune properties of pericytes and their participation in the changes observed during brain infections and neurodegenerative diseases will provide a mechanism by which pericytes participate in the maintenance of the proper function of the brain-immune interface, the blood–brain barrier. Here we present recent evidence depicting the new roles of pericytes in regulating blood–brain barrier function under normal and pathological conditions and hypothesize its potential role in the regulation of the blood–brain barrier after chronic sleep loss.

PERICYTES AS BLOOD–BRAIN BARRIER COMPONENTS

Pericytes are smooth muscle-derived cells that play a crucial role in keeping brain homeostasis given their presence at the blood–brain barrier and particularly their active role in what is known as the neurovascular unit (Zlokovic, 2008; Gómez-González et al., 2012). Rouget (1874), for the first time, described a population of branched cells with contractile properties that surrounded ECs. Fifty years later, these mesenchymal cells were renamed "pericytes" by Zimmerman in concordance with their anatomical location: abluminal to ECs and luminal to parenchymal cells (Kim et al., 2006; Sá-Pereira et al., 2012). Anatomically, pericytes have projections that wrap around capillaries and are embedded within the basal lamina. The diversity in pericyte

marker expression may be related to vessel size or embryonic origin; the main markers are α-smooth muscle actin (αSMA), desmin, the regulator of G-protein signaling 5 (RGS-5), neuron-glial antigen 2 (NG2), platelet-derived growth factor receptor (PDGFRα and PDGFRβ), and amino-peptidase-N (CD13; Ozerdem et al., 2002; Bergers and Song, 2005). These proteins show different expression patterns under physiological and pathological states (see **Table 1**). Furthermore, pericytes express numerous macrophage markers, namely CD4, CD11b, CD146, and proteins related to immune function such as the fragment crystallizable receptor (FcR) and the major histocompatibility complex (MHC) classes I and II (Bergers and Song, 2005; Kamouchi et al., 2011). Differences in the expression of those markers are based on the local environmental influences on pericytes. For example, it has been reported that CD146 is expressed during embryonic development but not in all freshly isolated pericytes in adulthood. Also, RGS-5 protein expresses during embryonic development, but decreases after birth and is absent in pericytes of the normal adult central nervous system (Dore-Duffy, 2008; Sá-Pereira et al., 2012).

Although pericyte identification is rather difficult owing to the lack of one specific marker (Özen et al., 2012), its ultrastructure was described (Nag, 2003; Sá-Pereira et al., 2012). Two classes of pericytes exist in the brain: granular and agranular; this classification arises from the presence or absence of lysosome-like granules in the cytoplasm (Farrell et al., 1987). In humans, less than 5% of the pericyte population is agranular (Farrell et al., 1987; Nag, 2003). Both, granular and agranular pericytes exhibit an oval cell body and a prominent round nucleus that is different from the elongated nucleus of ECs. Each pericyte may cover 100 μm of capillary length with up to 90 ramifications 300–800 nm wide (Nag, 2003; Sá-Pereira et al., 2012). Pericyte distribution is intermittent along the walls of arterioles, venules and, particularly, in capillaries (Dore-Duffy, 2003). They are crucial for the development and maintenance of the main nervous system barriers, namely, blood–spinal cord barrier, blood–retinal barrier, blood–nerve barrier and blood–brain barrier. In fact, pericyte coverage of brain ECs *in vitro* is approximately 80%, in the capillaries of the retina it is 90%, and in the microvessels of the spinal cord it is less than 60%. Pericyte coverage and

Table 1 | Pericyte markers in health and disease.

Pericyte marker/ location	Main function	Main physiological role	Health	Disease	Reference
PDGFRβ/cell surface protein	Tyrosine-protein kinase; Kinase receptor	Embryonic development, proliferation, chemotaxis, host-virus interaction	+	+/− Fibrosis Tumor Blood–brain barrier disruption	Song et al. (2005), Armulik et al. (2010), Dore-Duffy and Cleary (2011)
αSMA/Filament protein	Contractility	Regulation of blood flow and motility	−	++ Fibrosis Tumor Blood–brain barrier disruption	Song et al. (2005), Dore-Duffy and Cleary (2011)
NG2/cell surface protein	Cell adhesion protein	Vasculo-genesis	+	+ Fibrosis Tumor Blood–brain barrier disruption	Ozerdem et al. (2002), Dore-Duffy and Cleary (2011)
RGS-5/intracellular protein	GTPase-activating protein	Cell motility	+	++ Fibrosis Tumor Blood–brain barrier disruption	Song et al. (2005), Dore-Duffy and Cleary (2011)
Desmin/filament protein	Contractility	Regulation of blood flow and motility	+	+ Fibrosis Tumor Blood–brain barrier disruption	Dore-Duffy and Cleary (2011), Kamouchi et al. (2011)
CD13/cell surface protein	Ectopeptidase	Pericyte differentiation	+	++ Fibrosis Tumor Blood–brain barrier disruption	Armulik et al. (2010), Kamouchi et al. (2011)

Symbols are as follow: (+) Indicates that the marker is present; (–) indicates that the marker is absent; (+/–) indicates a decrease in marker expression and; (++) indicates that the marker is over expressed.

number is related to the permeability of the biological-barriers, higher coverage correlates with lower permeability (Winkler et al., 2012). Specifically, it has been shown that pericytes contribute to regulate capillary structure and diameter (Peppiatt et al., 2006; Armulik et al., 2010; Bell et al., 2010; Daneman et al., 2010). Pericytes express junctional complexes that include gap junctions, tight junctions (Tjs), and focal adhesions with ECs (Zlokovic, 2008). These associations lead to the maintenance of low permeability of the cerebral endothelium (Lai and Kuo, 2005; Nakagawa et al., 2007). Brain pericytes promote a reduction in vesicular transport, (Daneman et al., 2010), and promote endothelial Tj protein expression (Zonula occludens, ZO-1, claudin-5, occludin; **Figure 1**; Armulik et al., 2005, 2010; Daneman et al., 2010). In addition, the morphological pattern of pericyte projections around brain capillaries is linked to their function and intimately correlates with brain health state (normal, angiogenic, or injured; Dore-Duffy and Cleary, 2011). The classic wrapping pattern consists of broad processes with a large continuous surface in the external wall of brain microvessels (Dore-Duffy, 2003; Nag, 2003; Dore-Duffy and Cleary, 2011). Under normal conditions, the wrapping pattern predominates, but in pathological conditions detachment and migrating patterns can be observed with the formation of finger-like projections followed by retraction of projections (**Figure 1**; Dore-Duffy and Cleary, 2011). Different morphological patterns in pericyte processes may appear in response to changes in the microenvironment. For example, the migrating pattern is associated to up-regulation of cell surface proteases in aversive conditions, and also with early stages of angiogenesis, in contrast with the wrapping pattern that predominates in normal capillaries (Dore-Duffy, 2003; Sá-Pereira et al., 2012).

Morphological changes in pericytes vary as a function of exposure to soluble molecules released by blood–brain barrier components such as ECs, neurons, microglia or astrocytes; pericytes can differentiate into fibroblasts, smooth muscle cells or macrophages, depending on the stimulus received (**Figure 1**). The molecules released to the basal lamina that can promote pericyte morphological changes include neurotransmitters, neurohormones and inflammatory mediators (Özen et al., 2012). To illustrate this, it has been shown that adenosine and adenosine triphosphate (ATP) released by neurons and glial cells may modify pericyte status by activating purinergic receptors; in addition, rat brain pericytes express ecto-nucleotidase 1 and 2 (Ceruti et al., 2011; Lecca et al., 2012). After immune challenges such as lipopolysaccharide (LPS) administration, hippocampal brain pericytes present increased ecto-nucleotidase expression and function and also morphological changes (Kittel et al., 2007). Activation of purinergic receptor P2X7 initiates an inflammatory response by inducing interleukin (IL) 1β secretion from ECs, astrocytes, microglia, and also pericytes (Derks and Beaman, 2004; Lecca et al., 2012).

Pericyte versatility is, for the most part, unexplored, but several studies suggest that pericytes may play potential roles in brain repair through contractile, migratory, pro-angiogenic and phagocytic functions but they can also promote brain impairment by uncontrolled immune response (Dore-Duffy et al., 2000; Dore-Duffy et al., 2006; Özen et al., 2012; Sá-Pereira et al., 2012).

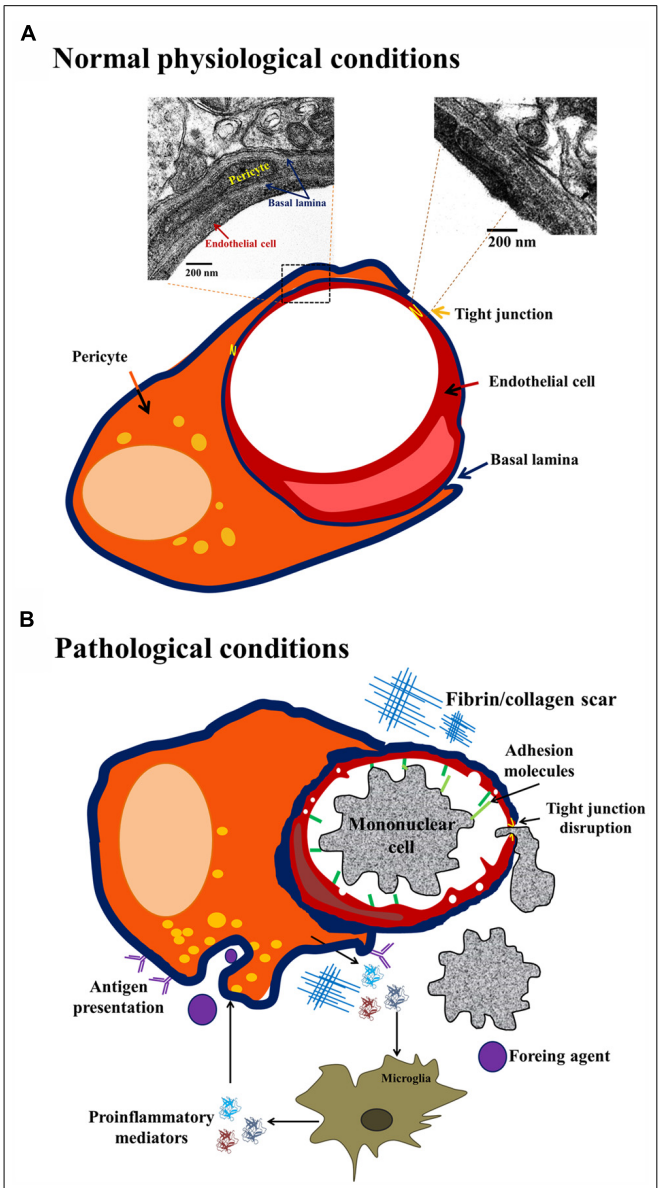

FIGURE 1 | Brain pericyte phenotype in normal and pathological conditions. Under normal physiological conditions **(A)** brain pericytes exhibit tight junctions (Tjs) with endothelial cells (ECs), and are embedded in the basal lamina. Under pathological conditions, such as injury, infection or neurodegeneration **(B)**, pericytes present a migrating phenotype with up-regulation of ICAM expression, pro-inflammatory cytokine release with ensuing recruitment of peripheral mononuclear cells. Additionally, under pathological conditions, the continuity of basal lamina is lost and the existence of fibrin scars contributes to blood–brain barrier impairment.

IMMUNE PROPERTIES OF BRAIN PERICYTES

Mesodermal or neural crest origins of pericytes are generally accepted. Pericytes are considered as "brain macrophages". In fact, for some authors, they represent the first line of defense in the central nervous system due to their antigen presentation properties and because they are directly associated with the microvasculature, in contrast to microglia (**Figure 1**; Balabanov et al., 1999; Guillemin and Brew, 2004). Thomas (1999)

reported pericytes leaving the basal lamina and migrating to the perivascular space where they are indistinguishable from perivascular macrophages and reactive microglia (Guillemin and Brew, 2004). Pericyte de-differentiation into cells presenting antigens may initiate a local pro-inflammatory response. Immune response in the brain induces monocyte and lymphocyte recruitment; this process is mediated by the increased expression of adhesion molecules (e.g., intracellular adhesion molecule 1, ICAM-1) in the luminal region of ECs that correlates with decreases in the number of Tjs (**Figure 1**; Guillemin and Brew, 2004). In addition, pericytes are able to produce chemo-attractants and promote transmigration to the brain of circulating immune cells, starting an inflammatory process. Pericytes may also release inflammatory mediators, such as IL-1β, IL-6, tumor necrosis factor (TNF) α, reactive oxygen species, nitric oxide (NO), and matrix metalloproteinases (MMP-2 and MMP-9), all of which contribute to pericyte detachment and blood–brain barrier disruption (Kovac et al., 2011).

These immunoactive properties of pericytes suggest mechanisms by which they can act as an integral part of the blood–brain barrier during brain inflammatory processes. A pro-inflammatory component is the hallmark of several brain diseases. Vascular damage associated to pericyte deficiency may precede neurodegeneration in brain infections, Alzheimer's or Parkinson's disease, diabetes (Özen et al., 2012), and perhaps in less-explored phenomena that exhibit considerable cognitive impairments, such as sleep loss.

PERICYTES AND BRAIN INFECTIONS

The blood–brain barrier provides a shield against foreign agents that initiate inflammatory responses (Al-Ghananeem et al., 2013). The structural variability and the nature of biotic/abiotic inflammatory agents that may promote neuropathology are reflected in the mechanisms used to access the brain. These mechanisms include receptor-mediated endocytosis, unspecific transport by pinocytotic vesicles, paracellular diffusion, transmigration through infected leukocytes, and crossing after blood–brain barrier breakdown (Alcendor et al., 2012; Nakagawa et al., 2012; Pulzova et al., 2012).

The inflammatory response to a foreign agent may cause irreversible brain damage by continuous exposure to pathogen-derived toxic molecules and immune mediators (Kumar et al., 2009; Hirooka and Kaji, 2012). Factors that promote a pro-inflammatory state in the brain include abiotic agents such as heavy metal ions or viruses, and biotic factors such as bacteria, fungi, and parasites (Gasque et al., 1998; Liou and Hsu, 1998; Alvarez and Teale, 2007; Hirooka and Kaji, 2012; Nakagawa et al., 2012). There is scant knowledge of pericyte function and structure under inflammatory response induced by foreign agents.

Heavy metal ions, such as methyl-mercury, cadmium and inorganic mercury induce a potent inflammatory response in the brain. These metal ions have high affinity to sulfhydryl groups favoring the formation of a methionine-like complex that easily crosses the blood–brain barrier. The methionine-like complex enters the brain by the large neutral amino acid transporter (LAT-1); once inside, the heavy metal ions induce cytokine and growth factor release by blood–brain barrier components. Heavy metal ions associate with the fibroblast growth factor type 2 (FGF-2); this union may cause cell damage because FGF-2 is unable to repair endothelial damage; therefore, heavy metal ions promote less autoregulatory signaling inhibition of EC proliferation (Hirooka and Kaji, 2012).

In the case of viral and bacterial infections, such as congenital human cytomegalovirus (HCMV), human immunodeficiency virus type 1 (HIV-1), Japanese encephalitis (JE) virus and bacterial meningitis, the main transport routes through the blood–brain barrier include endocytosis of blood-circulating vesicles, microvessel wall degradation, and indirect crossing via previous blood–brain barrier disruption. When infectious agents are detected, pericytes begin an inflammatory response through increased expression of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Liou and Hsu, 1998; Alcendor et al., 2012; Nakagawa et al., 2012). In HIV-1 infection, pericytes express the chemokine receptors CXCR4 and CCR5 that are used by infected cells to contribute to the formation of viral reservoirs in the brain (Nakagawa et al., 2012). It is known that 80% of cultured pericytes infected by HCMV generate an inflammatory response; in fact, only 72 h after infection, a huge rise in IL-1β, a medium increase in IL-6, and a minimal increase in TNF-α concentration are observed. However, later on those pro-inflammatory cytokine profiles are reversed by the compensatory effect of anti-inflammatory cytokines (Alcendor et al., 2012). In contrast, bacterial meningitis infection increases expression of receptors C5a and C3a in brain pericytes. These complement molecules are powerful chemo-attractants to recruit polimorfonuclear cells and macrophages to the inflammation site causing cell activation (Gasque et al., 1998). On the other hand, it has been reported that *Taenia solium* infiltrates cause brain inflammation by pericyte release of pro-inflammatory cytokines and MMP-2 and MMP-9, which are associated to blood–brain barrier disruption. Blood–brain barrier breakdown allows infiltration of antigen-presenting cells and specialized immune cells (B cells and T cells), exacerbating the inflammatory condition (Alvarez and Teale, 2007).

These studies illustrate that although each pathogen exhibits a characteristic pathway, the same inflammatory mediators participate in the orchestration of the brain immune response (**Figure 2**). It is known that rises in pro-inflammatory cytokines, particularly IL-1 β, IL-6, and TNF-α, disrupt Tjs by down-regulating occludin and ZO-1 expression (Liou and Hsu, 1998; Alcendor et al., 2012; Nakagawa et al., 2012). Pro-inflammatory cytokines alter Tj integrity by promoting an increase in prostaglandin-E (PGE) receptors in pericytes, which leads to MMP overproduction and release, causing pericyte uncoupling with ECs (Alvarez and Teale, 2007). In fact, ECs are the unique brain cell type that expresses PGE-2 synthase (Yamagata et al., 2001); PGE-2 is produced in response to immune challenges (e.g., IL-1 or LPS administration; Cao et al., 1997; Laflamme et al., 1999) suggesting a relevant role of perivascular cells (astrocytes, interneurons and particularly pericytes) in the response to low doses of immune stimulators (Schiltz and Sawchenko, 2002). Interestingly, perivascular cell response is different for each type of molecule; e.g., pericytes elicit cyclooxygenases in brain ECs in response to low doses of IL-1, but with low doses of LPS perivascular cells

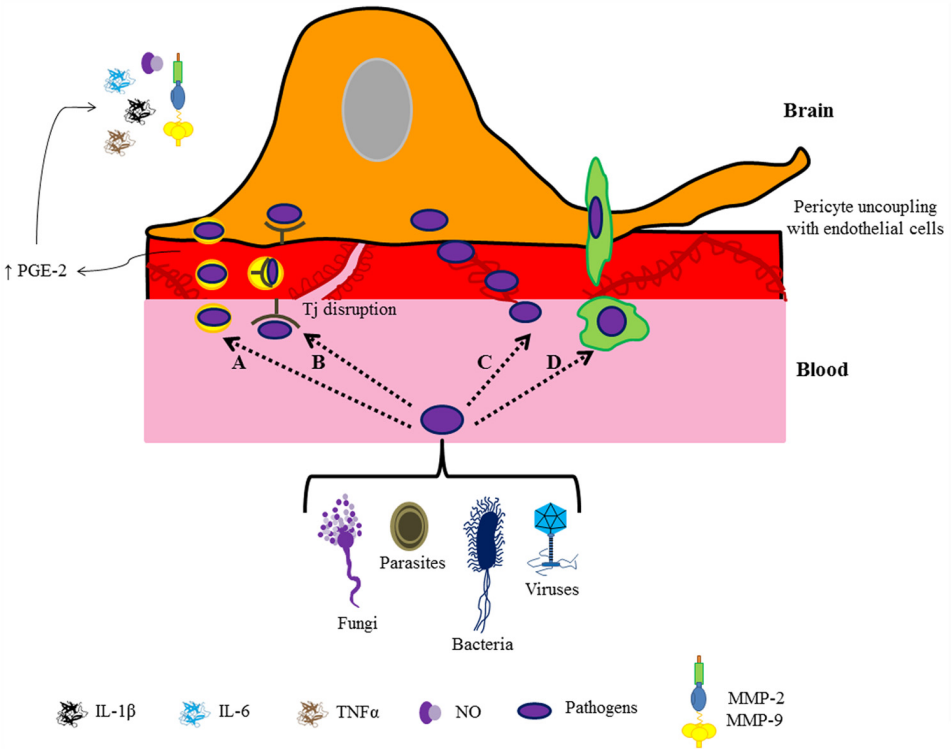

FIGURE 2 | Pathogen entry to the brain. The cartoon depicts different routes for pathogen entry to the brain: caveolae **(A)**, receptor-mediated transport **(B)**, paracellular diffusion **(C)**, and transmigration through infected leukocytes **(D)**. In all cases a pericyte-mediated immune response followed by blood–brain barrier breakdown is observed.

apparently have an inhibitory effect on cyclooxygenase production (Schiltz and Sawchenko, 2002). Some neuro-infections are associated with neurodegenerative diseases, for example, the bacteria *Borrelia burgdorferi* in Alzheimer's disease (Miklossy et al., 2004). So, is the pathogenic action on pericytes a promoter of neurodegenerative disease? Undoubtedly, pericyte function has an important role in the progression of brain pathologies. Although several studies provide relevant information on the immune role of pericytes in the protection of the brain against an infectious threat, the molecular and cellular mechanisms involved in blood–brain barrier disruption are poorly understood.

ROLE OF PERICYTES IN NEURODEGENERATIVE DISORDERS

Similar to infectious processes, during neurodegenerative and cerebrovascular diseases inflammatory phenomena occur, which are characterized by increased release of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), subsequent hyperthermia, and mononuclear cell infiltration (Bleys and Cowen, 2001). In both, neurodegenerative and cerebrovascular diseases, pericyte detachment of ECs and differentiation into fibroblasts or phagocytes correlates with an increase in vesicle number in ECs, Tj disruption and immune cell recruitment (Özen et al., 2012). Additionally, fibrosis-like pathophysiological changes are described (**Figure 3**); pericyte-derived fibrin and collagen form scars, which are involved in cell death by neurotoxicity (Armulik et al., 2010; Fernández et al., 2013). Deposits of extracellular matrix components and organ failure are common after prolonged exposure to pro-inflammatory cytokines, suggesting that the first step leading to cell death relates to the immune response (Lin et al., 2008; Armulik et al., 2010). Furthermore, cytokine production is accompanied by oxidative stress; both, inflammatory mediators and oxidative stress are directly involved in increased blood–brain barrier permeability through the same signaling pathways.

Recent studies revealed the role of NO released by microglia and pericytes in neurodegenerative diseases and neuro-immune interactions; it was shown that amyloid β deposits in Alzheimer's disease promote pericyte constriction despite NO over-production. The role of NO in blood–brain barrier disruption is also related to its high ability to form free radicals such as peroxynitrite (ONOO-•), which may induce cell death (Hamilton et al., 2010; Kovac et al., 2011). In addition, it has been reported that amyloid β deposits promote over-production of reactive oxygen species in pericytes, endothelial, and glial cells (Veszelka et al., 2013). Blood–brain barrier disruption promotes lymphocyte recruitment in neurodegenerative diseases and stroke; hence, after cerebral ischemia, polymorphonuclear leukocytes impede reperfusion leading to generation of free radicals, and promoting pericyte constriction. Indeed, pericyte detachment from the vessel wall occurs following ischemia and reperfusion (Takahashi et al., 1997). Recently, Tigges et al. (2013) reported an increase in fibronectin and collagen I deposits in animal models of Alzheimer's disease, these deposits are related to pericyte

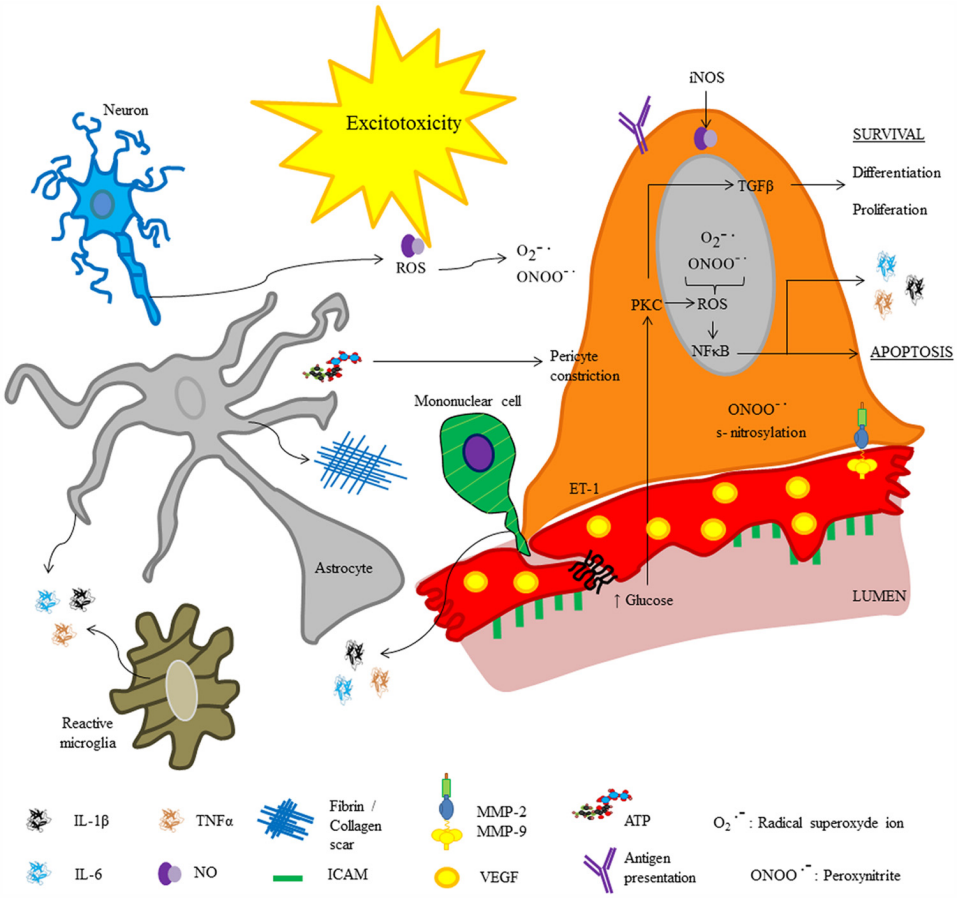

FIGURE 3 | Molecular inflammatory changes in brain pericytes in pathophysiological conditions. The cartoon depicts molecules released by pericytes under altered physiological conditions (e.g., neurodegeneration, infections or brain injury). Excitotoxicity may occur secondary to blood–brain barrier disruption.

differentiation and migration. Tigges et al. (2013) showed that under normal conditions, brain pericytes express high levels of α5 integrin and lower levels of α1, α2, and α6 integrins. This expression pattern has a crucial role in the attachment of pericytes to the vessel wall; in fact, an *in vivo* study shows that TNF-α promotes pericyte proliferation and detachment as well as a switch in integrin expression pattern, with predominance of α2 integrin (Tigges et al., 2013). Interestingly, Tigges et al. (2013) also found that α2 integrin expression strongly correlated with brain vessel remodeling in experimental autoimmune encephalomyelitis. Similarly, in Alzheimer's disease it is reported that fibrin deposition and increased extravascular immunoglobulin G (IgG) correlate with a reduction in pericyte coverage of ECs (Sengillo et al., 2013).

Fibrin deposits are a signal of fibroblast activity and probably represent an index of de-differentiation from pericytes to fibroblasts. Transforming growth factor-β (TGF-β) is the most potent known growth inhibitor for ECs, fibroblasts, neurons, and lymphoid cells. TGF-β inhibits proliferation of T-lymphocytes by down-regulating pro-inflammatory cytokines, e.g., IL-2-mediated proliferative signals (Dohgu et al., 2005). Under diabetic conditions, pericytes release TGF-β, which increases fibronectin levels (Shimizu et al., 2013). Shimizu et al. (2013) suggest that advanced glycation end-products (AGEs) induce blood–brain barrier disruption in diabetic conditions by stimulation of autocrine TGF-β signaling in pericytes, and up-regulation of vascular endothelial growth factor (VEGF) and MMP-2. Both, VEGF and MMP-2 modify trans-endothelial electric resistance (TEER) leading to Tj disruption and increased vesicular transport (Thanabalasundaram et al., 2011). Pericyte deficiency reported in diabetes is attributed to raises in glucose concentration, and production of reactive oxygen species through the NFκB pathway (Hamilton et al., 2010). Interestingly, in diabetic animal models, pericytes are highly immunosuppressive; under early hyperglycemic conditions retinal-derived pericytes inhibit T cell proliferation and protect ECs from inflammation-induced apoptosis (Tu et al., 2011). In addition, it is known that pericytes are especially susceptible to oxidative stress; for example, high glucose levels cause oxidative stress and apoptosis (Shah et al., 2013). In addition to the reactive oxygen species effect, the production of large amounts of NO by inducible-nitric oxide synthase (iNOS) can lead to changes in cerebral blood-flow, nitrosative stress, and subsequent cell death of pericytes, ECs and neurons through toxicity caused by excitatory amino acids and massive entry of toxic

molecules to the brain (Kischer, 1992; Li et al., 1997; Tu et al., 2011; Baloyannis and Baloyannis, 2012). A decrease in pericyte capillary coverage and cell number has been reported in hyperglycemia, early diabetes retinopathy, brain tumors, and Alzheimer's disease. Therefore, brain microvascular alterations seem to reciprocally interact with underlying neurodegeneration in inducing cognitive impairments (Pimentel-Coelho and Rivest, 2012). The role of pericytes in the genesis of neurodegenerative diseases and in brain regeneration is poorly studied; however, pericytes undoubtedly, cause alterations in brain physiology.

PERICYTES AND SLEEP LOSS: AN IMMUNOLOGICAL PERSPECTIVE

Sleep loss is a common problem in modern society (Mills et al., 2007; Yehuda et al., 2009) and a risk factor for the development of obesity, metabolic syndrome, diabetes, and neurodegenerative diseases (Tasali et al., 2009; van Leeuwen et al., 2009; Reynolds et al., 2012). Similar to infections and neurodegenerative diseases, sleep loss has an important pro-inflammatory component (Mills et al., 2007; Zager et al., 2007). Specific sleep function is yet unclear; but it has been proposed that sleep is associated with changes in parameters of host defense (Benca and Quintas, 1997). Sleep is divided into two distinct stages namely; slow wave sleep and rapid eye movement (REM) sleep (Siegel, 2010). Particularly, REM sleep has an important role in biological processes; REM sleep loss decreases neurogenesis in the hippocampus (Guzman-Marin et al., 2008; Mueller et al., 2008), alters the brain neurochemical content (Mohammed et al., 2011), and impairs learning and memory in both rodents and humans (Meerlo et al., 2008). Prolonged wakefulness promotes an increase of inflammatory mediators such as adenosine and NO (Kalinchuk et al., 2011; Cespuglio et al., 2012; Raymond et al., 2012), and increases plasma levels of IL-1β, IL-6, IL-17A, TNF-α (Yehuda et al., 2009), and endothelin-1 (ET-1; Mills et al., 2007). These changes may act directly on the blood–brain barrier components; for example, IL-1, IL-17, and ET-1 disrupt the blood–brain barrier (Banks et al., 1995; Blamire et al., 2000; Didier et al., 2003; Huppert et al., 2010). REM sleep deprivation also increases body temperature (Jaiswal and Mallick, 2009), which also disrupts the blood–brain barrier (Kiyatkin and Sharma, 2009).

Our research group recently found that chronic REM sleep restriction induces a generalized blood–brain barrier breakdown, and subsequent sleep opportunity is capable of restoring blood–brain barrier integrity. In addition, we studied EC ultrastructure and observed alterations in vesicle trafficking (Gómez-González et al., 2013). It is highly likely that pericyte dysfunction may contribute to increases in blood–brain barrier permeability secondary to sleep loss because ultrastructural changes in ECs are similar to those reported in pericyte-deficient mice, e.g., increased caveolae density, and endothelial derangement (Armulik et al., 2010). Chronic exposure to pro-inflammatory cytokines, NO and other inflammatory mediators released during sleep restriction may directly induce pericyte detachment from the vessel wall and subsequent differentiation into migratory and phagocytic phenotypes, mediating blood–brain barrier disruption. It is likely that the synthesis of antioxidants and anti-inflammatory molecules during sleep recovery may restore normal blood–brain barrier permeability through neutralization of free radicals (**Figure 4**).

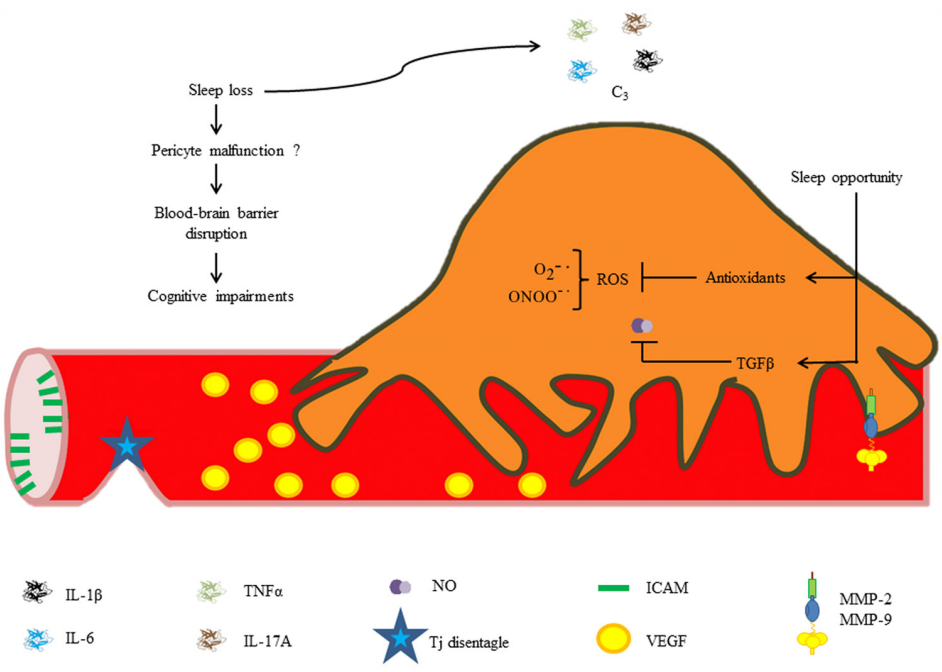

FIGURE 4 | Hypothetical roles of pericytes in REM sleep loss-induced blood–brain barrier disruption. Increased cytokine and chemokine concentrations during sleep loss may disrupt the blood–brain barrier. Brief periods of sleep opportunity may reestablish the blood–brain barrier integrity through synthesis of antioxidants and anti-inflammatory cytokines (e.g., TGF-β).

CONCLUSION

Classically, pericytes have been considered a cell population involved mainly in microvessel contractility. New research on pericyte contribution to optimal blood–brain barrier function and neural pathogenesis shows that they have a substantial influence on the neuro-immune response. The immunoactive properties of pericytes suggest mechanisms by which they could act as an integral component of the blood–brain barrier during inflammatory processes, such as during brain infections, neurodegenerative diseases or sleep loss. Future studies are needed to elucidate pericyte role under inflammatory conditions. Knowledge on pericyte contribution to disease pathogenesis will allow more specific treatment of brain pathologies and perhaps the development of better diagnostic markers. The field study of pericytes is generating frontier knowledge and may be exploited as an example of neuro-integration. Certainly, pericytes are crucial cells in optimal brain function, but their deficit results from molecular interactions between all brain cells.

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