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Maestría y Doctorado en Ciencias Bioquímicas

Efecto anticonvulsivo de la restricción alimentaria en un modelo murino de crisis convulsivas agudas: Implicaciones metabólicas y epigenéticas.

TESIS

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Índice.

1. Abreviaciones.

(En orden de aparición)

SE- Status epilepticus.

AL- Ad libitum.

TRF- Restricción alimentaria.

EEG- Electroencefalografía.

AMPK- Cinasa dependiente de AMP cíclico.

B-HB- Beta hidroxibutirato.

HDACs- Desacetilasas de histonas.

H3- Histona 3.

SNC- Sistema nervioso central.

EROs- Especies reactivas de oxígeno.

OMS- Organización mundial de la salud.

ILAE- Liga internacional en contra de la epilepsia.

GABA- Acido gaba-aminobutirico.

UCPs- Proteinas desacoplantes.

ATP- Adenosin trifosfato.

IGF-1- Factor de crecimiento insulínico tipo 1.

mTOR- Diana mecanística de rapamicina.

SIRTs- Sirtuinas.

mTORC1- Complejo uno de la diana mecanística de rapamicina.

mTORC2- Complejo dos de la diana mecanística de rapamicina.

mLST8- Proteína ocho con dominio sec13 letal en mamíferos.

DEPTOR- Proteína con dominio DEP que interacciona con mTOR.

PRAS40- Sustrato rico en prolinas de Akt.

Raptor- Proteína regulatoria asociada a mTOR.

Rictor- Proteína regulatoria de mTOR insensible a la rapamicina.

mSIN1- Proteína asociada a las cinasas activadas por mitogenos.

PROTOR1- Proteína rica en prolinas asociada a mTOR tipo 1.

PROTOR2- Proteína rica en prolinas asociada a mTOR tipo 2.

PI3K- Cinasa de fosfatidil inositol 3 fosfato.

HMGSC2- Sintasa mitocondrial de hidroximetilglutaril-CoA tipo dos.

HMGCL- Liasa de hidroximetilglutaril-CoA

BDH1- Deshidrogenasa de B-HB

SLC16A6- Miembro seis del la familia 16 de acarreador de solutos y transportadores de monocarboxilatos.

MCT1/2- Transportadores de monocarboxilatos uno y dos.

OXCT1- Transferasa de succinil-coA tipo uno.

GPCRs- Receptores membranales acoplados a proteínas G.

HCAR2- Receptor de ácidos hidrocarboxílicos de tipo 2.

FFAR3- Receptor de ácidos grasos de tipo 3.

DNA- Acido desoxirribonucleico.

H2- Histona dos.

H2A- Histona dos a.

H4- Histona cuatro.

HATs- Acetil-transferasas de histonas.

Rpd3- Dominio de las desacetilasas de histonas en levadura.

Hda1- Desacetilasa de histonas uno.

Sirts- Sirtuinas.

Sir2- Regulador de la información silenciada dos.

NAD- Nicotina adenina dinucleotido.

2. Glosario.

(Orden alfabético)

Acidósis metabólica- Termino dado para una de las alteraciones en el equilibrio acido-base de un organismo, esta se caracteriza por una acidez en el plasma sanguíneo.

Ayuno- Periodo de tiempo que abarca desde 12 horas hasta tres semanas, durante el cual, los organismos no ingieren ningún alimento o bebida, calórica.

Cetoacidosis- Estado metabólico que se encuentra asociado a un desbalance en la regulación de la cetogénesis, se caracteriza por un incremento exacerbado de los cuerpos cetónicos y tiene como consecuencia la acidez del plasma sanguíneo.

Cetogénesis- Conjunto de reacciones que a partir del catabolismo de ácidos grasos producen los cuerpos cetónicos.

Convulsión- Evento transitorio de signos y síntomas que se deben principalmente a una actividad neuronal anormal y excesiva.

Cuerpos cetónicos- Termino común por el que se conoce a tres moléculas que se forman a partir de la beta oxidación de ácidos grasos, el acetato, el acetoacetato y el beta hidroxibutirato.

Epigenética- Definida por Conrad Waddington en 1940, como la rama de la biología que estudia todas las interacciones causales entre los genes y sus productos las cuales se manifiestan como el fenotipo de un organismo.

Epilepsia- Enfermedad crónica neurológica que se caracteriza principalmente por la aparición de crisis convulsivas las cuales se presentan de manera recurrente y espontánea.

Rapamicina- Fármaco inmunosupresor el cual fue el primer inhibidor descrito de la cinasa mTOR.

Restricción alimentaria- Reto nutricional, en el que se limita la disponibilidad de la comida por distintos periodos de tiempo durante la fase activa de los organismos.

Restricción calórica- Régimen nutricional en el cual la ingesta diaria de un organismo es reducida entre un 20 y un 40% de la ingesta diaria, no se reduce la frecuencia alimenticia y no importa la fuente calórica

Status epilepticus- Condición de emergencia medica, en la cual, se observan convulsiones recurrentes que pueden permanecer hasta por 24 horas.

3. Resumen.

Aproximadamente un tercio de los pacientes con epilepsia no responden a los fármacos antiepilépticos actuales para controlar las crisis convulsivas características de este padecimiento. De manera interesante, terapias basadas en el metabolismo como las dietas cetogénicas han mostrado ser efectivas en el tratamiento de los pacientes farmacorresistentes. Desafortunadamente, el uso clínico de este tipo de dietas y sus variantes es muy controversial debido al gran numero de efectos adversos que puede provocar. Es por esta razón que en el siguiente estudio se planteo el objetivo de investigar si la restricción alimentaria, otra terapia basada en el metabolismo, que podría generar un aumento en los cuerpos cetónicos, es capaz de ejercer un efecto anticonvulsivo en un modelo de *status epilepticus* (SE). Para resolver esta hipótesis, se usaron dos grupos experimentales, un grupo de ratas tuvo acceso *ad libitum* (AL) al alimento, por otro lado, otro grupo siguió un régimen de restricción alimentaria (TRF, por sus siglas en ingles). Después de esto, se uso el modelo de litio-pilocarpina para inducir SE e inmediatamente después de la inyección, los animales fueron monitoreados para realizar pruebas conductuales asociadas a las convulsiones agudas. Además, se realizaron análisis electroencefalográficos (EEG) para verificar la actividad eléctrica cortical después de la inyección de pilocarpina. Para estudiar algunos cambios bioquímicos, se extrajo tejido del sistema nervioso central (hipocampo) y tejido asociado al metabolismo energético (hígado), 24 horas después del SE. En estos se evaluaron proteínas asociadas a cambios en el metabolismo energético y con modificaciones epigenéticas. Nuestros resultados muestran que la restricción alimentaria es capaz de ejercer un efecto anticonvulsivo; disminuyendo la duración de

las crisis convulsivas, aumentando el periodo de latencia al primer clonus unilateral, disminuyendo la severidad de las crisis y además disminuyendo el numero de animales que alcanzan el SE. En relación a estos efectos, se encontró que los registros de EEG de la corteza de los animales que siguieron la restricción alimentaria presentan una disminución significativa en el poder de las descargas eléctricas de esta región. Por otro lado, se encontró que esta dieta es capaz de aumentar significativamente la fosforilación del residuo de treonina 172 de la cinasa dependiente de AMP (AMPK) y de disminuir significativamente la fosforilación del residuo de serina 403 de la cinasa Akt tanto en hipocampo como en el hígado, estas proteínas actúan como sensores energéticos de la célula y son capaces de modular el metabolismo energético. Además, esta dieta aumento de manera significativa la concentración sanguínea de beta hidroxibutirato (B-HB), un cuerpo cetónico capaz de disminuir la actividad de las desacetilasas de histonas (HDACs). Finalmente, se observó que en extractos enriquecidos en cromatina de hipocampo, la restricción alimentaria es capaz de disminuir significativamente la actividad de las HDACs y aumenta la acetilación de los residuos de lisina 9 y 14 de la histona 3 (H3). Estos hallazgos sugieren que la restricción alimentaria es capaz de modular la actividad de componentes involucrados en la regulación del metabolismo energético, aumentando así la concentración de B-HB, disminuyendo de este modo la actividad de las HDACs y probablemente facilitando la transcripción de genes involucrados en los efectos anticonvulsivos de esta dieta.

4. Abstract.

A new generation of antiepileptic drugs has emerged; however, one-third of epilepsy patients do not properly respond to pharmacological treatments. The purpose of the present study was to investigate whether time-restricted feeding (TRF) has an anticonvulsant effect and whether this restrictive diet promotes changes in energy metabolism and epigenetic modifications in a pilocarpine-induced seizure model. To resolve our hypothesis, one group of rats had free access to food and water ad libitum (AL) and a second group underwent a TRF schedule. We used the lithium-pilocarpine model to induce status epilepticus (SE), and behavioral seizure monitoring was analyzed. Additionally, an electroencephalography (EEG) recording was performed to verify the effect of TRF on cortical electrical activity after a pilocarpine injection. For biochemical analysis, animals were sacrificed 24 h after SE and hippocampal homogenates were used to evaluate the proteins related to metabolism and chromatin structure. Our results showed that TRF had an anticonvulsant effect as measured by the prolonged latency of forelimb clonus seizure, a decrease in the seizure severity score and fewer animals reaching SE. Additionally, the power of the late phase EEG recordings in the AL group was significantly higher than the TRF group. Moreover, we found that TRF is capable of inducing alterations in signaling pathways that regulate energy metabolism, including an increase in the phosphorylation of AMP dependent kinase (AMPK) and a decrease in the phosphorylation of Akt kinase. Furthermore, we found that TRF was able to significantly increase the beta hydroxybutyrate (β-HB) concentration, an endogenous inhibitor of histone deacetylases (HDACs). Finally, we

found a significant decrease in HDAC activity as well as an increase in acetylation on histone 3 (H3) in hippocampal homogenates from the TRF group. These findings suggest that alterations in energy metabolism and the increase in β-HB mediated by TRF may inhibit HDAC activity, thus increasing histone acetylation and producing changes in the chromatin structure, which likely facilitates the transcription of a subset of genes that confer anticonvulsant activity.

5.Introducción.

5.1. Epilepsia.

La epilepsia es un desorden neurológico crónico que afecta a personas de todas las edades (Aroniadou-Anderjaska et al. 2007). Clínicamente se describe como un desorden del sistema nervioso central (SNC), caracterizado por una predisposición a generar convulsiones recurrentes y espontáneas (Fisher et al. 2005) (Fisher et al. 2014). Así mismo, una convulsión, es definida clínicamente como un evento transitorio de signos y síntomas específicos que se deben a una actividad neuronal anormal y excesiva en el cerebro (Fisher et al. 2005).

Aunque la definición mas reciente de la epilepsia toma como principal componente, el desbalance en la neurotransmisión (Fisher et al. 2014). Existen diversas teorías, que señalan otros factores relevantes en la patofisiología de esta enfermedad, entre los que se encuentran, el desbalance de los mecanismos pro-inflamatorios, el daño mediado por especies reactivas de oxigeno (EROs) y alteraciones en la dinámica mitocondrial (Waldbaum & Patel 2010; Yuen & Sander 2014; Staley 2015).

Además de esto, también se ha descrito que las alteraciones de estos procesos celulares, son capaces de promover distintos cambios morfológicos, como por ejemplo; durante las fases agudas de la epilepsia, se ha observado principalmente, la aparición de gliósis reactiva y muerte celular en distintas zonas del hipocampo (Figura 1A) (Heck et al. 2004).

Por otro lado, durante la fase crónica, es común observar, la aparición de una condición conocida como esclerosis hipocampal, esta es reflejo del daño continuo producido por las crisis convulsivas (Heck et al. 2004; Devinsky et al. 2013) (Figura 1B).

Desde una perspectiva de salud pública, la Organización Mundial de Salud (OMS) ha registrado que alrededor de 50 millones de personas podrían tener algún tipo de epilepsia, siendo la epilepsia de lóbulo temporal la más frecuente (Engel Jr 2006; Fisher et al. 2014; Mercado-Gómez et al. 2014).

Entre las principales causas que generan esta condición, se encuentran, los traumatismos craneales; los infartos cerebrales, infecciones neurológicas, tumores cerebrales y daños cerebrales por causas prenatales y perinatales (Devinsky et al. 2013) (Figura 2). Por otro lado, solo una pequeña fracción de los pacientes presentan como principal componente de origen, mutaciones genéticas (Staley 2015).

En cuanto al diagnostico clínico de esta enfermedad, este continua siendo muy complicado, esto podría ser debido principalmente a la gran cantidad de factores que se encuentran involucrados en esta enfermedad (Fisher et al. 2014). Es por esta razón que la liga en contra de la epilepsia (ILAE, por sus siglas en ingles) ha hecho un esfuerzo clasificando y definiendo las principales fases de esta condición (Trinka et al. 2015).

Entre estas se encuentra el SE, esta condición es una de las emergencias medicas mas comunes y se encuentra asociada al desarrollo y patofisiología de la epilepsia (Trinka et

al. 2015).

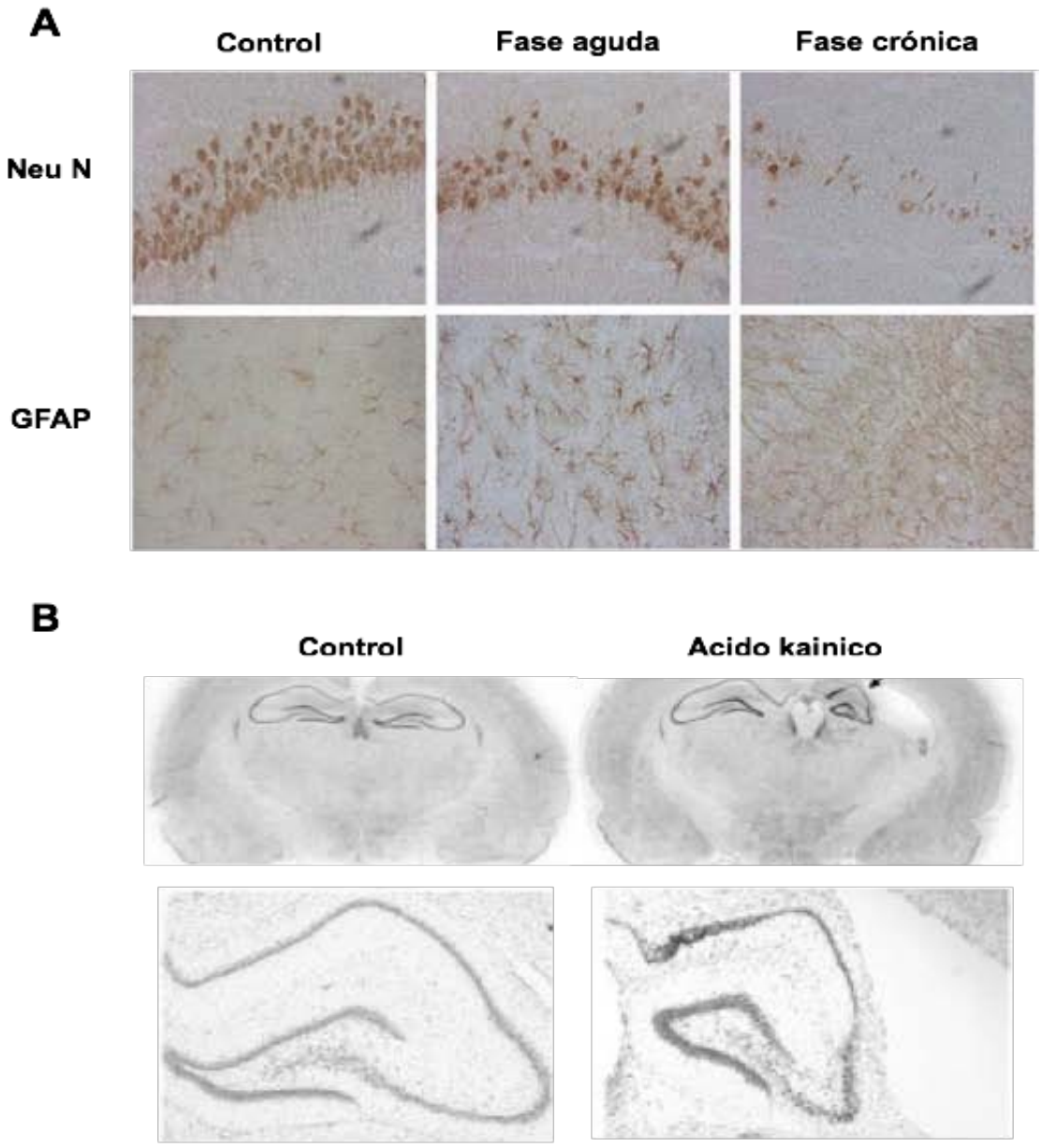

Figura 1. Alteraciones neuroanatómicas de la epilepsia. Durante las distintas fases de la epilepsia es común observar alteraciones anatómicas del algunas estructuras del SNC como el hipocampo. Durante la fase aguda se observa principalmente la muerte neuronal y un proceso conocido como glíosis reactiva **(A).** Por otro lado, durante la fase crónica se observa una disminución en el tamaño de ésta estructura, probablemente debido al daño producido durante la fase aguda y se conoce como esclerosis hipocampal **(B)**. (Modificado de Faissner et al., 2013).

5.2. Status epilepticus.

En la clínica, el SE ha sido definido principalmente como una condición de emergencia medica, en la cual, se observan convulsiones recurrentes que pueden permanecer hasta por 24 horas (Fisher et al. 2005). Esta definición es poco clara y ha provocado confusión en la comunidad científica (Trinka et al. 2015).

Es principalmente por esta razón que la ILAE se ha dado a la tarea de generar una definición mas clara. En este nuevo concepto, se describe al SE, como una condición que resulta del fallo de los mecanismos responsables del inicio o del termino de las convulsiones (Trinka et al. 2015). Además esta definición; también contempla que dependiendo del tipo y duración de las crisis, este estado, es capaz de generar efectos a largo plazo, los cuales incluyen, la alteración de los circuitos neuronales y el daño y muerte de las células del SNC (Trinka et al. 2015).

Ya que durante el SE se alteran distintos procesos que se encuentran involucrados en la epilepsia, el estudio de esta condición, ha sido clave para el estudio de los mecanismos precisos que se encuentran involucrados en la patofisiología de la epilepsia (Scorza et al. 2009). Es debido a esto que la comunidad científica ha descrito distintos modelos animales, los cuales recapitulan la etiología del SE (Scorza et al. 2009; Grone & Baraban 2015).

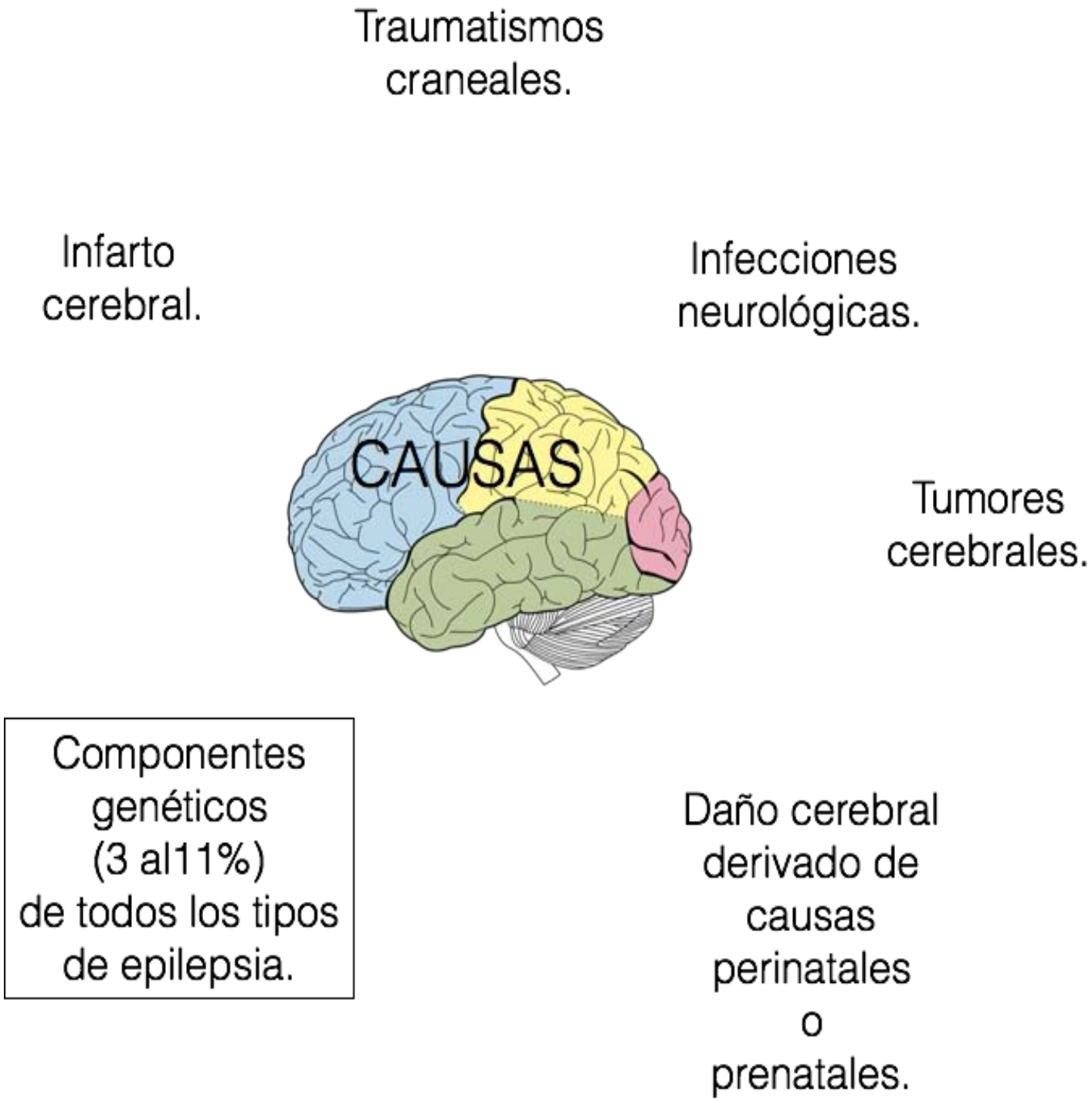

Figura 2. Principales causas que pueden desencadenar epilepsia. Esquema representativo que resume los principales eventos que son capaces de generar epilepsia. Es importante mencionar que aproximadamente el 90% de estos eventos involucran un componente ambiental. Por otro lado, solo el 10% de los casos, presentan como origen un componente genético.

5.3. Modelos animales de epilepsia.

Debido a las implicaciones sociales que involucra estudiar la epilepsia, la mayor parte del trabajo científico y experimental se realiza principalmente en modelos animales (Grone & Baraban 2015). Estos se generan al aplicar distintos agentes farmacológicos (p.e. pentilentetrazol, pilocarpina, ácido kaínico, ácido domoico) o estímulos eléctricos (kindling) (Grone & Baraban 2015)(Scorza et al., 2009).

Uno de los más utilizados en la investigación científica, es el inducido mediante inyecciones de distintas dosis de pilocarpina (Scorza et al. 2009). Este agente farmacológico es un alcaloide extraído de las plantas del género *Pilocarpus sp.* y actúa como un agonista no específico de los receptores muscarínicos en el sistema parasimpático (Grone & Baraban 2015). La administración sistémica de este agente promueve cambios conductuales y electroencefalográficos que pueden dividirse en tres distintos períodos:

a) Un período agudo caracterizado por crisis convulsivas recurrentes que permanecen por lo menos durante 24 horas. En este periodo se recapitula la etiología del SE.

b) Un período con una normalización progresiva de los electroencefalogramas y la conducta, el cual varía de 4 a 44 días. Este periodo es conocido como fase de latencia.

c) Un período crónico con convulsiones recurrentes y espontáneas, las cuales pueden durar años. Las principales características de las convulsiones del último

periodo son muy similares a las crisis parciales complejas en humanos y se repiten de dos a tres veces por semana en cada animal (Scorza et al. 2009).

Ya que este modelo farmacológico es capaz de recapitular distintas fases que se observan en la epilepsia, este es una herramienta valiosa, no sólo para estudiar los procesos de esta patología, sino también para evaluar los posibles tratamientos de esta enfermedad (Scorza et al. 2009; Grone & Baraban 2015).

5.4. Dieta cetogénica.

Gracias al esfuerzo de la comunidad clínica y científica. Y en parte debido al uso de modelos animales de epilepsia para estudiar esta enfermedad, se han logrado desarrollar distintos fármacos anticonvulsivos (Loscher et al. 2013). Este tipo de medicamentos logran controlar los principales signos y síntomas del 70% de los pacientes que sufren epilepsia (Loscher et al. 2013; Landgrave-Gómez et al. 2016).

Desafortunadamente, estos medicamentos no son capaces de curar la epilepsia, solo controlan su principal síntoma, las convulsiones (Loscher et al. 2013). Además, aproximadamente el 30% de los pacientes que padecen esta condición, no responden a este tipo de tratamientos, presentando una condición que en la clínica se conoce como farmacorresistencia (Loscher et al. 2013).

De manera interesante, aproximadamente la mitad de los pacientes que presentan farmacorresistencia han sido tratados exitosamente mediante la practica de dietas

cetogénicas (Bough & Rho 2007).

Estas dietas fueron diseñadas en base a la observación de que los pacientes que realizan un ayuno prolongado (2-3dias), presentan un incremento significativo de la acidosis metabólica en suero, condición que coincidía con una disminución en las crisis convulsivas (Lennox & Cobb 1928). Sin embargo, en la clínica no es viable un tratamiento en donde se priva a los pacientes de alimento durante periodos tan prolongados (Carl & Kristopher 2003). Es principalmente por esta razón que en 1940 los doctores Lennox, Cobb y Wilder en la universidad de Harvard, diseñaron esta dieta (Waldbaum & Patel 2010). En este régimen alimenticio, al priorizar el consumo de los lípidos, sobre los carbohidratos y proteína, en un cociente de 4:1, se es capaz, de inducir un estado energético similar al que se produce al realizar un ayuno prolongado (Newman & Verdin 2014).

Debido a la efectividad de este tipo de dietas en la reducción de las crisis convulsivas, la comunidad científica se dio a la tarea de tratar de descifrar los mecanismos mediante los cuales estas ejercen sus beneficios (Appelberg et al. 2009; Bough & Rho 2007; Bough et al. 2003; Hallböök et al. 2007; Linard et al. 2010; Nei et al. 2014).

Algunos estudios señalan que las dietas cetogénicas actúan aumentando la concentración de cuerpos cetónicos; entre ellos el B-HB, el cual se encuentra involucrado en la biosíntesis del ácido gamma-aminobutírico (GABA) (Yudkoff et al. 2008; Yudkoff et al. 2007). Además tanto el B-HB, como el acetoacetato, son capaces de

disminuir la absorción de glutamato en las vesículas sinápticas, ya que estos metabolitos son capaces de inhibir competitivamente a los transportadores de glutamato (Juge et al. 2010; Newman & Verdin 2014).

Por último, algunos estudios señalan que las dietas altas en grasa, al incrementar la síntesis de adenosín trifosfato (ATP), producen un balance bioenergético favorable, el cual, permite la estabilización del potencial de membrana mediante la activación de la ATPasa de Na^+ / K^+ (Bough & Rho 2007).

A pesar de estos hallazgos y aunque las dietas cetogénicas han demostrado tener un efecto anticonvulsivo en el tratamiento de algunos pacientes con distintos tipos de epilepsia (Bough et al. 2003; Carl & Kristopher 2003). En la actualidad el uso clínico de este tipo de terapias es muy controversial, tal vez, porque estas requieren de atención médica especializada y además pueden tener distintos efectos adversos; entre los que se encuentran la hipoglicemia, la hiperlipidemia, la cetoacidosis y la pérdida de peso corporal, razones por las cuales el uso clínico de estas terapias se encuentra en discusión (Bielohuby et al. 2013; Nei et al. 2014).

Por otro lado, en los últimos años, se ha sugerido que la restricción de alimento por periodos intermitentes podría provocar un estado energético similar al que producen las dietas cetogénicas (Yuen & Sander 2014). Estas evidencias sugieren que la práctica de este reto nutricional podría ser capaz de ejercer un efecto anticonvulsivo.

5.5. Restricción alimentaria.

La restricción alimentaria es un reto nutricional, en el que se limita la disponibilidad de la comida por distintos periodos de tiempo durante la fase activa de los organismos (Bellet & Sassone-Corsi 2010). Este modelo restrictivo, mediante la inclusión de ayunos, es capaz de inducir diversos cambios en la regulación del metabolismo energético (Figura 3). Principalmente, se ha descrito que este tipo de dietas restrictivas producen una diminución del peso corporal; de la concentración total de colesterol y lípidos, del ritmo cardiaco y la presión arterial (Figura 3), condiciones que se han asociado con beneficios en el tratamiento de distintas enfermedades como el cáncer y la diabetes tipo dos, solo por mencionar algunas. (Ahmet et al. 2005; Belkacemi et al. 2012; Kroeger et al. 2012; Parinejad et al. 2009; Vasconcelos et al. 2014; Wegman et al. 2014; Hatori et al. 2012; Chaix et al. 2014).

A pesar de estos estudios, en la actualidad, el patrón más común de alimentación en la sociedad moderna es realizar tres comidas abundantes a lo largo del día y colaciones o "snacks" entre estas comidas. Un patrón alimentario parecido al "ad libitum" utilizado como "grupo control" en distintos modelos experimentales (Martin et al. 2010; Mattson et al. 2014). Apoyando esta aseveración, un estudio realizado este año señala que la mayoría de la población podría pasar aproximadamente de 8 a 12 horas de su periodo de actividad comiendo (Gill & Panda).

De manera interesante, se ha sugerido, que los modelos de alimentación que incluyen ayunos de manera intermitente, podrían restaurar el desbalance del metabolismo

energético que se produce por tener un estilo de vida sedentario (Mattson et al. 2014). Sin embargo, los mecanismos y procesos celulares mediante los cuales este tipo de modelos restrictivos corrigen este desbalance aun no se conoce.

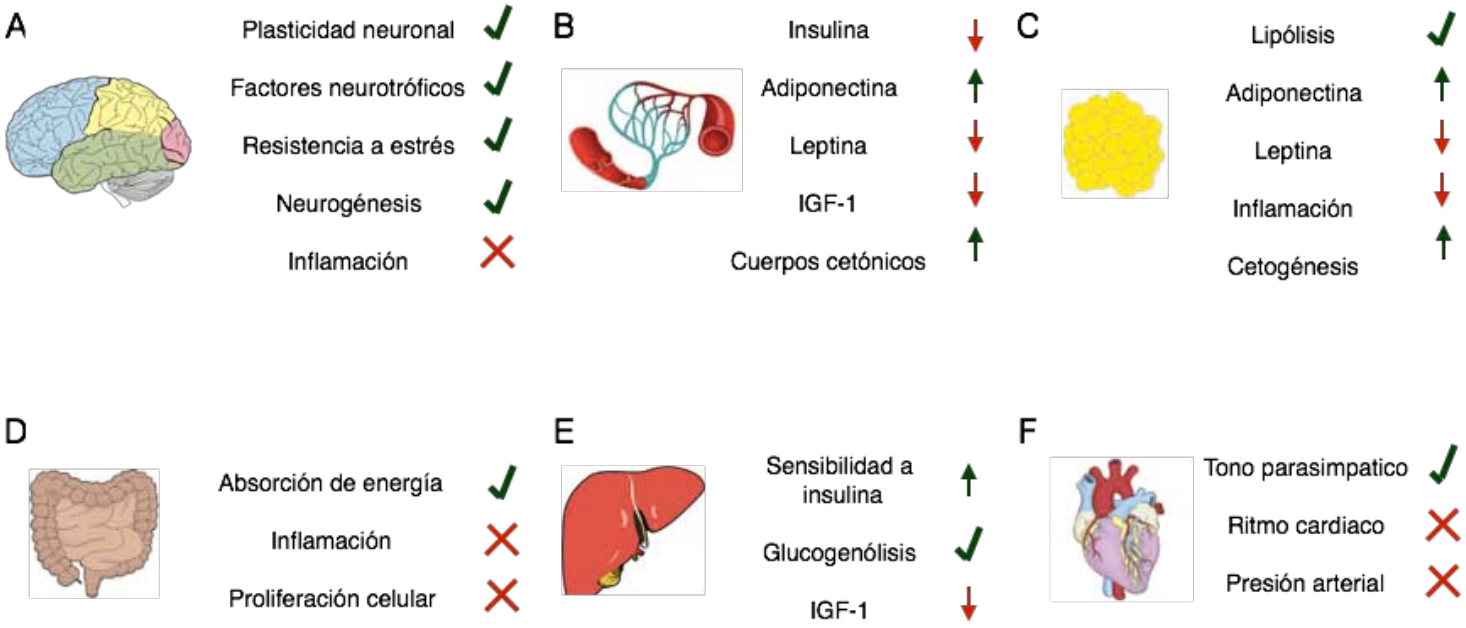

Figura 3. Efectos metabólicos y fisiológicos de la restricción alimentaria. Ejemplificación que resume algunos del efectos que produce la restricción alimentaria. Las flechas verdes denotan un aumento en la concentración de la molécula o el proceso a su lado. Por otro lado, las flechas rojas denotan una disminución en la molécula o el proceso a su lado. Las tildes verdes o las cruces rojas denotan la activación o inhibición respectivamente del proceso a su lado.

Actualmente es bien reconocido que el desbalance del metabolismo energético es un factor patofisiológico clave no solo en el desarrollo sino que también durante la cronicidad de varias enfermedades del SNC (Waldbaum & Patel 2010). Es por esta razón que describir los mecanismos precisos mediante el cual el ayuno ejerce sus beneficios continua siendo un reto para el campo emergente del neurometabolismo.

5.6. Ayuno.

El ayuno se define como un periodo de tiempo que abarca desde 12 horas hasta tres semanas, durante el cual, los organismos no ingieren ningún alimento o bebida, calórica (Longo & Mattson 2014).

En la actualidad, es bien reconocido que el ayuno es capaz de cambiar el contexto de distintos tipos celulares (Stanley et al. 2014), ya que es capaz de alterar la actividad de un gran número de vías señalización, las cuales regulan el metabolismo energético (Mattson et al. 2014). Aunque estos mecanismos aún no se entienden de manera precisa, existe información preliminar que sugiere cuatro vías de señalización principales mediante las cuales el ayuno genera un estado bioenergético favorable. Estas vías de señalización son la activación de AMPK; la disminución en la concentración del factor de crecimiento insulínico (IGF-1), una disminución en la activación de la diana mecanística de rapamicina (mTOR) y por ultimo un incremento en la concentración de las desacetilasas de histonas de tipo III, también llamadas Sirtuinas (SIRTs) (Yuen & Sander 2014; Longo & Mattson 2014).

De entre estas vías de señalización la activación de AMPK y la inhibición de mTOR se han asociado en diversos estudios con los procesos de epileptogénesis e ictogénesis (Baybis et al. 2004; Han et al. 2011). Es por esta razón que el diseño racional de terapias metabólicas y fármacos capaces de regular estas vías podrían representar una alternativa en la lucha contra la epilepsia.

5.7. AMPK.

Entre las numerosas vías de señalización que se encuentran involucradas en la regulación del metabolismo energético, se encuentra, la vía de AMPK. Esta proteína se encuentra altamente conservada a lo largo de la ontogenia y sirve como sensor energético celular (Hardie et al. 2012). Cuando el cociente ATP/ADP en las células es bajo, esta cinasa es activada y en este estado desencadena un gran número de procesos metabólicos, entre los que se encuentran la activación de la beta oxidación de ácidos grasos; la activación de la biogénesis mitocondrial, un aumento en la autofagia y un aumento en la captación de glucosa (Figura 4). Estos procesos generalmente culminan en un cambio general que favorece la producción de energía y coordina una disminución en el uso de ATP (Burkewitz et al. 2014) (Figura 4).

Una de las modificaciones posttraduccionales que aumentan la actividad de esta cinasa es la fosforilación del residuo de treonina 172 de la sub-unidad alfa de esta cinasa (Hawley et al. 1996). Este cambio conformacional promueve un aumento en su actividad de por lo menos 100 veces (Hawley et al. 1996). De manera interesante alteraciones en la regulación de esta proteína han sido asociadas a distintas condiciones neuropatológicas, entre las que se encuentran, la enfermedad de Alzheimer o la enfermedad de Parkinson, donde se ha observado que la activación de esta proteína es capaz de ejercer efectos neuroprotectores en modelos animales de ambas enfermedades (Vingtdeux et al. 2010).

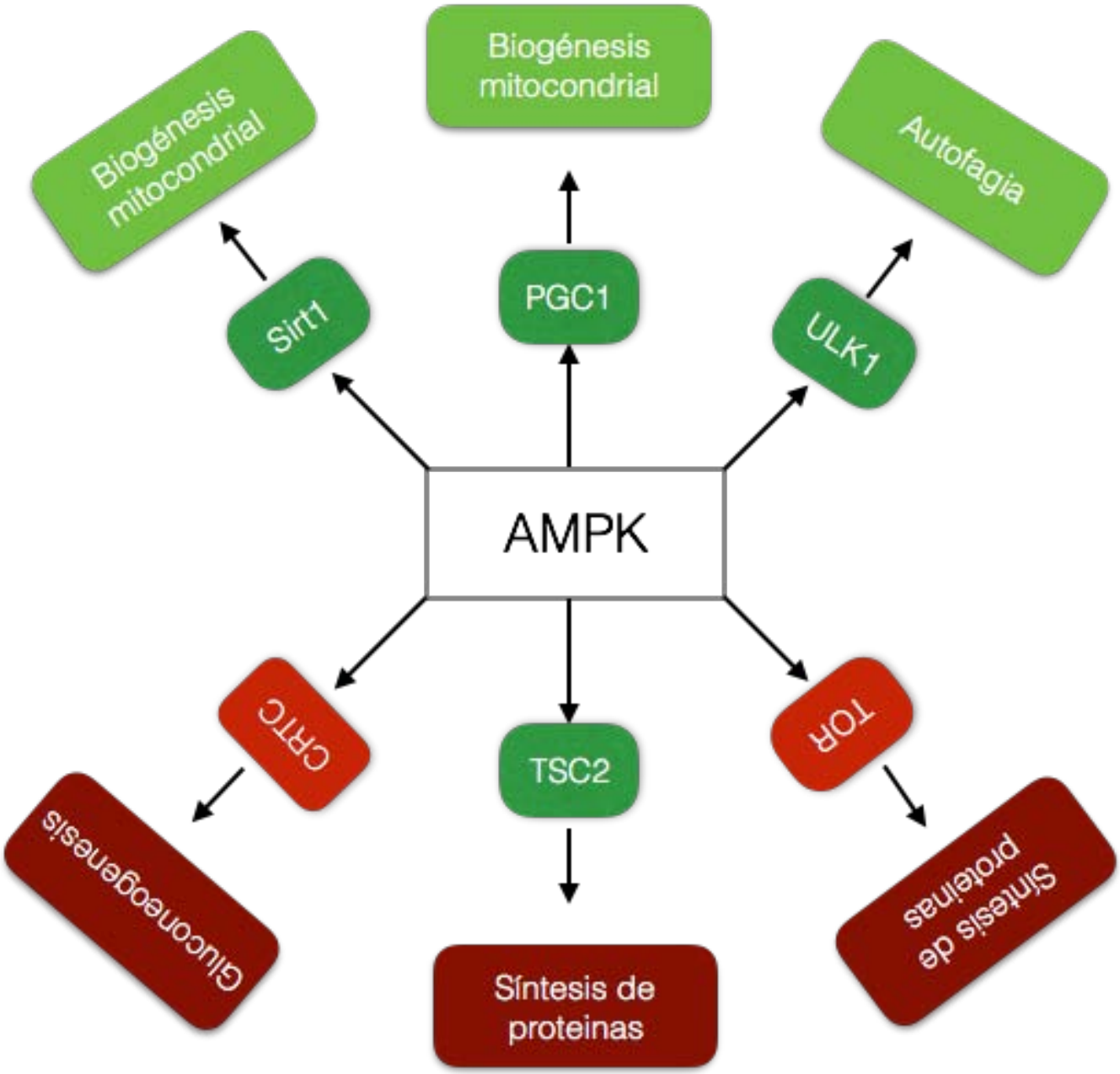

Figura 4. Procesos metabólicos regulados por la activación del sensor energético AMPK. Cuando el radio ATP/ADP en las células es bajo, AMPK es activada y en este estado regula un gran numero de procesos metabólicos entre los que se encuentran: la activación de la beta oxidación de ácidos grasos, la activación de la biogénesis mitocondrial, el aumento en la autofagia y un aumento en la captación de glucosa, los cuales culminan en un cambio general que favorece la producción de energía y coordina una disminución en el uso de ATP.

Por otro lado, en enfermedades neurológicas como la epilepsia se ha sugerido que alteraciones en la dinámica y la estructura mitocondrial son capaces de afectar la producción de ATP, este proceso, ha sido considerado como uno de los factores involucrados en el desarrollo de esta patología. Por lo que en este sentido, la activación de AMPK podría ser capaz de disminuir la frecuencia de las crisis convulsivas (Yuen & Sander 2011). De acuerdo con esta hipótesis, un estudio interesante demostró que la activación de la vía de AMPK confiere efectos neuroprotectores en un modelo de SE (Han et al. 2011).

Esta información en conjunto, sugiere que un desbalance en la regulación de esta vía podría estar involucrado en los procesos ictogénicos y de manera más importante aún, la activación de esta proteína podría ejercer efectos neuroprotectores en distintas enfermedades neurológicas incluyendo la epilepsia. Además de esto la activación de AMPK se encuentra ínter-relacionada con otra vía de señalización importante en la epilepsia, la vía de mTOR (Figura 4 y 5).

5.8. mTOR.

La vía de señalización de Akt y mTOR es una de las vías metabólicas más conservada en distintos organismos, probablemente, debido a su capacidad de integrar las interacciones celulares con el ambiente (Figura 5) (Lipton & Sahin 2014). Uno de los componentes centrales de esta vía es mTOR, una cinasa de gran tamaño (259kD), que se expresa de manera ubicua, incluyendo a células neurales (Sabatini et al. 1999).

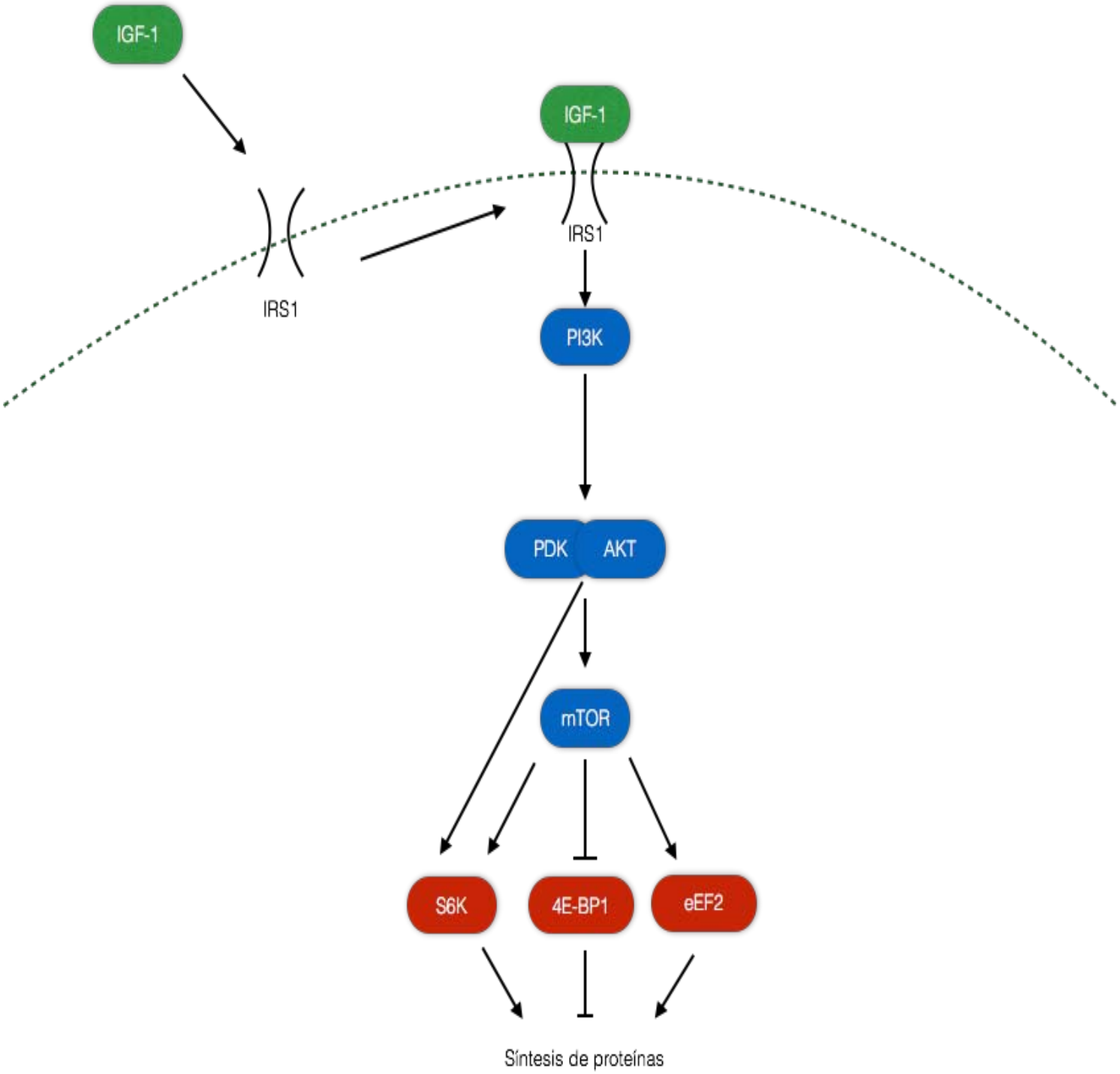

Figura 5. Esquematización de la vía de señalización Akt/mTOR. De manera breve, cuando el estado energético de una célula es alto, el factor de crecimiento parecido a la insulina tipo uno (IGF-1), se une a su receptor en la membrana celular (IRS1). Esta unión, promueve un cambio conformacional de este receptor, provocando la producción de moléculas que son capaces de activar a la cinasa activada por fosfatidil tres fosfato (PI3K), esta cinasa fosforila a Akt, a su vez en este estado esta cinasa es capaz de promover la activación de mTOR. Cuando mTOR es activado, ésta promueve la síntesis de proteínas ya que activa a la cinasa S6K y al factor de elongación de eucariontes tipo dos (eEF2).

Las funciones de esta proteína se modulan mediante dos grandes complejos proteicos (mTORC1 y mTORC2) los cuales se definen por las distintas proteínas que los conforman (Laplante & Sabatini 2012). Ambos complejos tienen en común a la cinasa mTOR, la proteína letal de mamíferos con sec13 (mLST8) y un dominio que interactúa con mTOR llamado (DEPTOR) (Baybis et al. 2004).

Las diferencias entre estos complejos proteicos es que mTORC1 contiene específicamente al sustrato de Akt (PRAS40) y un factor asociado a mTOR (Raptor), el cual es esencial para su actividad (Kim et al. 2002). Por otro lado el complejo mTORC2 incluye específicamente a la proteína (Rictor), la cinasa (mSIN1) y dos proteínas que interactúan con Rictor, (PROTOR 1 y 2). De entre estas, Rictor y mSIN1, son indispensables para la actividad de este complejo (Dos et al. 2004; Jacinto et al. 2004; Jacinto et al. 2006).

Es importante mencionar, que mediante estas interacciones, estos complejos son capaces de regular la homeostasis, influyendo directamente sobre un gran número de procesos entre los que se encuentran; la síntesis y transcripción de proteínas, la autofagia y el mantenimiento y la biogénesis de una amplia variedad de orgánelos (Figura 5) (Lipton & Sahin 2014).

En patologías como la epilepsia se ha señalado que esta vía es de gran importancia, bajo la premisa de que en algunos estudios se ha encontrado una hiperactivación de la cinasa mTOR, la cual ha sido asociada al desarrollo de displasias corticales (alteración

neuroanatomica que se presenta en un gran numero de pacientes con epilepsia) (Baybis et al. 2004; Miyata et al. 2004). Recientemente, distintos grupos de investigación han encontrado que mutaciones *de novo* en los genes que codifican para las proteínas PI3K, AKT3, y mTOR se encuentran en personas con hemimegalencefalia, otra condición, que se encuentra relacionada con convulsiones severas (Poduri et al. 2012; Riviere et al. 2012).

Sin embargo, aunque existen estudios interesantes que relacionan esta vía en modelos genéticos de epilepsia (Nguyen et al. 2015), lamentablemente, aún no se conocen bien los mecanismos precisos mediante los cuales estos complejos proteicos son capaces de influenciar los procesos ictogénicos y epileptogénicos.

Como se menciono anteriormente, la vía de AMPK y mTOR se encuentran ínter-relacionadas (Burkewitz et al. 2014; Chantranupong et al.). En este sentido, se ha demostrado que la activación de AMPK y la inhibición de mTOR convergen en la regulación de un proceso metabólico, la cetogénesis (Sengupta et al. 2010). Este proceso metabólico es el encargado de sintetizar tres metabolitos, el acetato, el acetoacetato y el B-HB, los cuales, son comúnmente conocidos como cuerpos cetónicos (Newman & Verdin 2014).

Las concentraciones elevadas de B-HB contribuyen a la generación de un proceso conocido como acidosis metabólica (Lennox & Cobb 1928). Como se menciono anteriormente, se ha sugerido que los niveles de acidosis en suero correlacionan con

una disminución significativa de las crisis convulsivas (Lennox & Cobb 1928). Es por esta razón, que estudiar las funciones de este acido carboxílico, en el contexto de la epilepsia o del SE, es importante para el diseño de nuevas terapias metabólicas (Huttenlocher 1976; Bough et al. 2003).

5.9. B-HB.

Cuando el contexto energético de un organismo es bajo, el B-HB, se utiliza como fuente alternativa de energía para tejidos con alta demanda energética, como el corazón y el cerebro (Figura 6) (Newman & Verdin 2014). La síntesis de esta molécula ocurre principalmente en el hígado. Sin embargo, también puede sintetizarse, pero en menor concentración, en tejidos como el intestino o el cerebro (Guzmán & Blázquez 2004).

La producción de B-HB en el hígado sucede de la siguiente manera; cuando los niveles energéticos son bajos, el organismo empezara a producir acetil-CoA a partir de los ácidos grasos (Figura 6) (Youm et al. 2015b; Newman & Verdin 2014). Inmediatamente después, la sintasa mitocondrial de hidroximetilglutaril-CoA (HMGSC2), condensara acetoacetil-CoA con el acetil-CoA, formando hidroximetilglutaril-CoA (Figura 6) (Newman & Verdin 2014; Fukao et al. 2004). Este compuesto a su vez puede formar acetoacetato, mediante la acción de la liasa de hidroximetilglutaril-CoA (HMGCL) (Figura 6) (Newman & Verdin 2014; Fukao et al. 2004). Esta molécula es el precursor mas común del B-HB, el cual se produce principalmente por la acción de la deshidrogenasa de B-HB (BDH1) (Figura 6) (Newman & Verdin 2014).

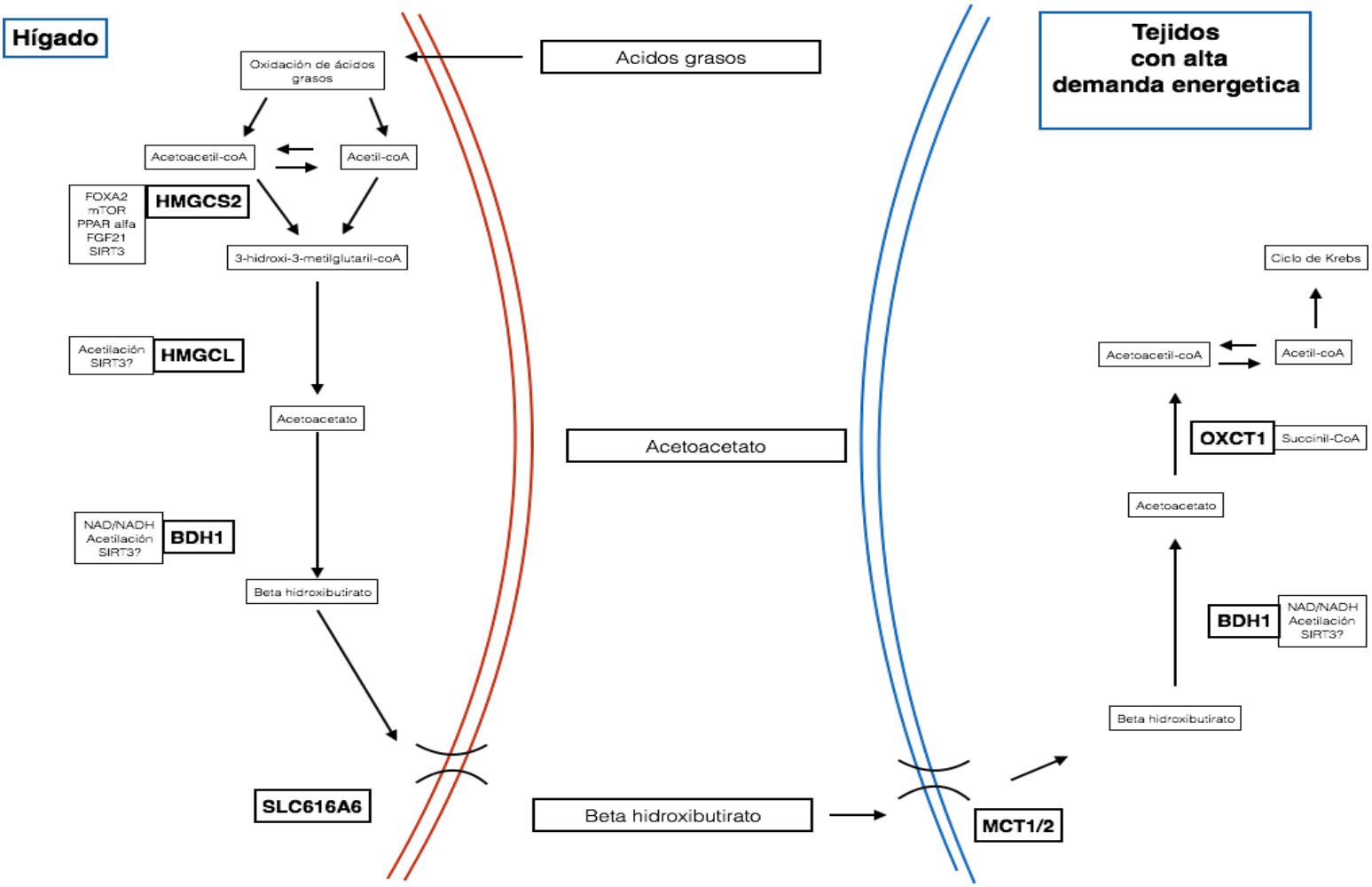

Figura 6. Metabolismo, transporte y regulación de la producción de los cuerpos cetónicos. De manera breve, la síntesis de los cuerpos cetónicos involucra una serie de reacciones enzimáticas que culminan en la producción de B-HB en el hígado. Este acido carboxílico será transportado al torrente sanguíneo mediante el acarreador de solutos y transportador de monocarboxilatos (SLC61A6). Después este puede ser transportado al cerebro mediante los transportadores de monocarboxilatos 1 y 2 (MCT1/2). Es importante mencionar que la enzima limitante en este proceso (HMGCS2) es regulada por FOXA2, mTOR, PPAR alfa, FGF2-1 y la SIRT 3. Algunas otras enzimas de este proceso, como la BDH1, son reguladas en base al radio de NAD/NADH. Además, de manera interesante todas las proteínas que regulan este proceso son blancos de acetilación.

Después de su síntesis en el hígado, el B-HB es transportado al torrente sanguíneo mediante el acarreador de solutos y transportador de monocarboxilatos (SLC16A6) (Figura 6) (Hugo et al. 2012). Mediante esta vía, este acido carboxílico, es llevado a los tejidos con alta demanda energética, como el cerebro, en donde entrara mediante

transportadores de monocarboxilatos (MCT1/2) (Figura 6) (Pellerin et al. 2005).

Dentro de estos tejidos, el B-HB puede ser interconvertido de nuevo a acetoacetato, mediante la acción de la BDH1 (Figura 6) (Newman & Verdin 2014). Después, este precursor, tomará una ruta metabólica distinta. Mediante la acción de la transferasa de succinil-coA (OXCT1), se formara aceto-acetil-CoA (Figura 6) (Fukao et al. 1997). Finalmente, este precursor podrá ser utilizado para generar dos moléculas de acetil-CoA y generar ATP mediante el ciclo de Krebs (Figura 6) (Fukao et al. 2004; Newman & Verdin 2014).

Además de la funciones que se han mencionado anteriormente en el texto. De manera interesante, se ha reportado que el B-HB puede actuar como una molécula de señalización (Newman & Verdin 2014). En este contexto, se ha señalado que esta molécula es uno de los ligandos de dos receptores membranales acoplados a proteínas G (GPCRs, por sus siglas en ingles) (Newman & Verdin 2014). Uno de ellos, es el receptor de ácidos hidrocarboxílicos de tipo 2 (HCAR2), cuya activación se encuentra asociada a una reducción de la lipolisis en los adipocitos, posiblemente como un mecanismo de retroalimentación negativa involucrado en el metabolismo de los cuerpos cetónicos (Taggart et al. 2005; Offermanns et al. 2011). Por otro lado, el B-HB también es capaz de unirse a un GPCR expresado principalmente en los ganglios simpáticos, el receptor de ácidos grasos de tipo 3 (FFAR3) (Kimura et al. 2011). Esta unión antagoniza la función de este receptor y provoca una disminución en el tono simpático (Kimura et al. 2011) .

Además de esto se reporto que el B-HB es el primer inhibidor endógeno de las HDACs de clase 1 (Shimazu et al. 2013). En el contexto de la epilepsia este hallazgo es sumamente interesante ya que es bien reconocido que una gran variedad de fármacos anticonvulsivos como el valproato de potasio, el topiramato y el levetiracetam presentan una actividad inhibitoria de las HDACs (Roopra et al. 2012; Landgrave-Gómez et al. 2015; Eyal et al. 2004).

5.10. HDACs.

Dentro del núcleo celular se encuentra la molécula que contiene la información necesaria para codificar todas las proteínas que conforman un organismo (Strahl & Allis 2000). Esta molécula es el acido desoxirribonucleico (DNA, por sus siglas en ingles) y comúnmente se encuentra enrollada alrededor de un octámero de proteínas, el cual se conforma por dos copias de las histonas (H2, H2A, H3 y H4) (Strahl & Allis 2000). Este complejo de DNA-proteína se conoce como nucleosoma y es la unidad fundamental de la cromatina (Strahl & Allis 2000).

La estructura de la cromatina tiene un papel crucial en el establecimiento, mantenimiento y propagación de los mecanismos que regulan la transcripción génica (Sassone-Corsi 2013). Uno de los mecanismos que regulan la estructura de la cromatina son las modificaciones posttraduccionales de las histonas (Landgrave-Gómez et al. 2015). Estas cambian de manera dinámica en respuesta al ambiente y entre ellas se encuentran la metilación, la fosforilación y la acetilación, solo por nombrar algunas (Sassone-Corsi 2013) (Figura 7). De entre estas la acetilación es la que ocurre de

manera mas abundante en los eucariontes; esta modificación se encuentra asociada a regiones transcripcionalmente activas y es resultado de la actividad enzimática de dos proteínas las acetiltransferasas de histonas (HATs) y de las HDACs (Figura 8) (Fischle et al. 2001).

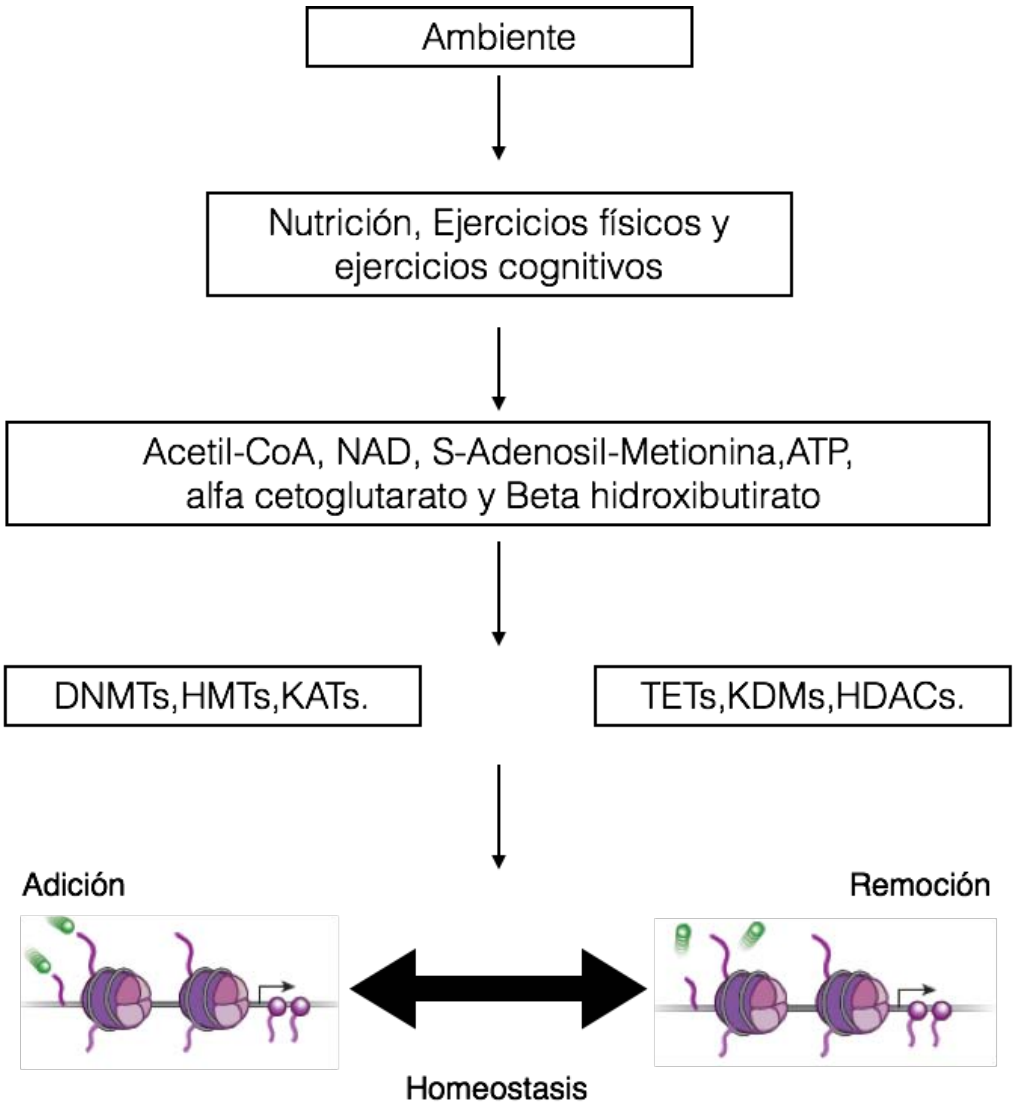

Figura 7. Esquema representativo de la interacción entre las señales ambientales y la regulación de la transcripción genética. Distintas señales ambientales como la nutrición, el ejercicio físico y los ejercicios cognitivos son capaces de alterar la concentración de distintos metabolitos. Estos pueden actuar como sustratos o cofactores de enzimas que modulan distintas modificaciones epigenéticas. De este modo se regula la transcripción de distintos genes, integrando así algunas señales ambientales

Hasta el momento las HDACs se han clasificado en cuatro clases. La sub-familia o clase I (HDAC 1, 2, 3 y 8) generalmente se encuentra en el núcleo celular y presentan homología con el regulador transcripcional de *Sacharomyces Cerevisae* (RPD3) (Tabla 1) (Fischle et al. 2001). Las HDACs de clase II (HDAC 4, 5, 6, 7 y 9) pueden encontrarse en el citoplasma o en el núcleo y presentan dominios similares a los de la proteína de levadura (HDA1) (Tabla 1) (Fischle et al. 2002). Además de esto, esta clase se subdivide en dos sub-familias (a y b) (Tabla 1) (Fischle et al. 2002). La principal diferencia entre estas dos sub-familias es que los miembros de la sub-familia a (HDAC 4, 5, 7 y 9) presentan una mutación en su dominio catalítico (Tabla 1) (Mielcarek et al. 2015). Las HDACs de clase III se conforman por siete enzimas y también son conocidas como Sirtuinas (Sirts 1-7) (Tabla 1) (Guarente 2007). Estas proteínas son homologas al factor de transcripción en levaduras (Sir2) y tienen como principal característica el uso de la nicotinamida adenina dinucleotido (NAD) como cofactor enzimático (Guarente 2007). Por ultimo la clase IV se encuentra formada por una sola proteína la HDAC 11, esta se caracteriza principalmente porque contiene dos sitios catalíticos (Tabla 1) (Mielcarek et al. 2015).

Aunque aun no se conoce bien el papel de estas proteínas en la epilepsia (Landgrave-Gómez et al. 2015; Sweatt 2013). En los últimos años, se ha sugerido que varios de los síntomas característicos de la epilepsia, podrían ser mediados por cambios aberrantes en los patrones de expresión genética (Roopra et al. 2012). En este sentido, se ha propuesto que distintas modificaciones epigenéticas, como la acetilación y la metilación de histonas, podrían estar relacionadas con estos cambios (Kobow et al. 2013; Jagirdar

et al. 2015; Saha & Pahan 2005).

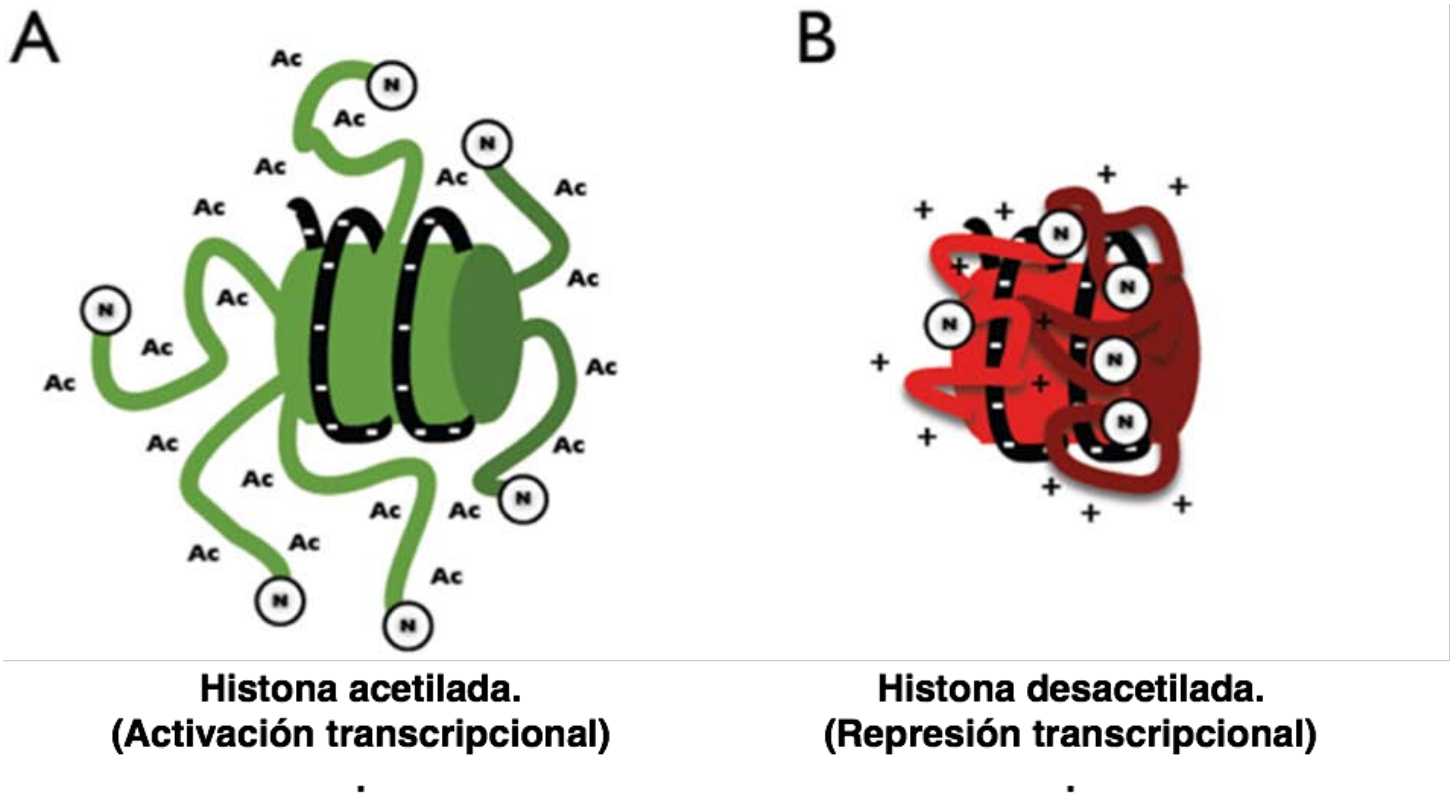

Figura 8. Esquema que ejemplifica el efecto de la acetilación en el dominio N-terminal de las histonas. Cuando el dominio N-terminal se encuentra acetilado, la interacción entre las histonas y el DNA (hebra negra) es débil **(A)**. Por otro lado, cuando el dominio N-terminal de las histonas se encuentra desacetilado esta interacción es mas fuerte **(B).**

Además de esto, como se menciono anteriormente, distintos fármacos y terapias metabólicas que han demostrado su efectividad en el tratamiento de la epilepsia convergen en la inhibición de las HDACs (Eyal et al. 2004; Huberfeld & Vecht 2016). Estas evidencias en su conjunto sugieren que la actividad de estas proteínas así como el proceso de la acetilación de histonas tienen un rol importante en los beneficios ejercidos por distintos tratamientos para controlar la epilepsia.

Es por estas razones que el objetivo del siguiente trabajo es determinar si la practica de

la restricción alimentaria es capaz de ejercer un efecto anticonvulsivo y evaluar si este se debe en parte a cambios en la regulación de la transcripción genética mediados por un aumento en la acetilación de la H3.

Tabla 1.- Descripción breve de la localización y clasificación de las HDACs.

Clase	Proteína	Sitios catalíticos	Localización celular	Presencia en tejidos
I	Hdac 1	1	Núcleo	Ubicua
I	Hdac 2	1	Núcleo	Ubicua
I	Hdac 3	1	Núcleo	Ubicua
I	Hdac 8	1	Núcleo/citoplasma	Ubicua
IIa	Hdac 4	1	Núcleo/citoplasma	Corazón/músculo/cerebro
IIa	Hdac 5	1	Núcleo/citoplasma	Corazón/músculo/cerebro
IIa	Hdac 7	1	Núcleo/citoplasma	Corazón/músculo/pancreas/placenta
IIa	Hdac 9	1	Núcleo/citoplasma	Cerebro/musculo
IIb	Hdac 6	1	Citoplasma	Corazón/hígado/riñón/placenta
IIb	Hdac 10	1	Citoplasma	Hígado/vaso/riñón
III	Sirts (1-7)	-	-	-
IV	Hdac 11	2	Núcleo/citoplasma	Cerebro/corazón/músculo/riñón

6. Justificación.

La epilepsia es un desorden neurológico que afecta a un gran número de personas de todas las edades alrededor del mundo, en el cual, la mayor frecuencia se presenta en países en vías de desarrollo como México. Si consideramos que la epidemiología va en aumento y que un gran porcentaje de los pacientes que viven en estos países no tienen

acceso a la atención médica adecuada o a fármacos antiepilépticos, probar terapias basadas en el metabolismo para disminuir las crisis epilépticas sería un gran logro desde un punto de vista de salud pública. Redundando de manera considerable en el abatimiento del costo de los medicamentos que se tendrían que utilizar.

Es por ello importante, el uso de modelos animales para comprender si estos tratamientos son factibles para uso humano (con sus limitaciones) y así generar nuevas alternativas más accesibles para los pacientes que se encuentran en países en vías de desarrollo.

7. Hipótesis.

La restricción alimentaria incrementa los niveles de acetilación de la H3 y regula la transcripción de genes asociados a un efecto anticonvulsivo, al promover un cambio metabólico global que aumenta la concentración de B-HB.

8. Objetivos.

8.1. Objetivo general.

Determinar si la practica de la restricción alimentaria es capaz de ejercer un efecto anticonvulsivo y evaluar si este se debe en parte a cambios en la regulación de la transcripción genética mediados por un aumento en la acetilación de la H3 .

8.2. Objetivos particulares.

1. Examinar distintos parámetros fisiológicos (i.e. peso corporal, peso neto del cerebro y del hígado, ingesta de comida, ingesta de líquido, niveles de glucosa y B-HB en sangre) en el grupo AL y compararlo con el grupo TRF.

2. Determinar mediante distintos estudios conductuales si la restricción alimentaria es capaz de disminuir la susceptibilidad a las crisis convulsivas durante el SE.

3. Corroborar mediante EEG si la restricción alimentaria es capaz de disminuir la severidad de las crisis convulsivas del SE.

4. Examinar si la restricción alimentaria es capaz de aumentar la actividad de la vía de señalización de AMPK, en tejidos del SNC, como el hipocampo y en tejidos periféricos, como el hígado.

5. Examinar si la restricción alimentaria es capaz de inhibir la vía de señalización de la cinasa Akt, en tejidos del SNC, como el hipocampo y en tejidos periféricos, como el hígado.

6. Analizar la actividad de HDACs en tejido hipocampal de los animales que siguieron distintos regímenes de alimentación previo al SE.

7. Analizar la acetilación de la lisina 9 y la 14 de la H3 en tejido hipocampal de animales que siguieron distintos regímenes de alimentación previo al SE.

9. Material y métodos.

9.1. Regímenes alimentarios y modelo de convulsiones agudas.

Para la realización de nuestro estudio se utilizaron 100 ratas de la cepa Wistar de 8 semanas de edad con un peso aproximado de 230 gramos de los laboratorios Harlan (USA), estos animales se mantuvieron bajo condiciones constantes de temperatura (25ºC) y en un ciclo de 12 horas de luz y 12 horas de obscuridad (Figura 9). Los animales fueron alimentados con una dieta estándar (Dieta 5001, PMI Nutrition International, Inc. Brentwood, MO) y agua *ad libitum* (AL), por otro lado otro grupo de animales siguió el régimen de restricción alimentaria (TRF, por sus siglas en inglés) descrito en el estudio realizado por Rivera-Zabala y colaboradores (Figura 9) (Rivera-Zavala et al. 2011). De manera breve la restricción alimentaria consistió en permitir a los animales comer durante dos horas de su periodo de actividad a lo largo de un periodo de 20 días, después de este periodo de tiempo, se procedió a realizar nuestro modelo de crisis convulsivas agudas; el cual consistió en inyectar cloruro de litio (3 mEq/kg, i.p.), 18 horas después los animales recibieron una inyección de nitrato de escopolamina para minimizar los efectos periféricos de la pilocarpina, 30 minutos después se administró la pilocarpina (60 mg/kg, s.c.) para inducir *status epilepticus,* este se mantuvo durante por lo menos 120 minutos e inmediatamente después de este periodo se inyectaron benzodiacepinas (Valium™, 5 mg/kg i.m.) para detener las convulsiones (Figura 9). El mismo procedimiento se realizó para los animales control intercambiando la inyección de pilocarpina por una inyección de solución salina al 0.9%. Es importante mencionar que para evitar cambios en el metabolismo el *status epilepticus* en los animales AL se indujo después de 6 horas de ayuno, mientras que el

grupo TRF fue inyectado después de un ayuno de ≈ 22 horas y ambos grupos recibieron inyecciones de solución salina para evitar la deshidratación (Figura 9). 24 horas después de la inyección de pilocarpina los animales fueron sacrificados para poder realizar los análisis bioquímicos (Figura 9) . Todos los experimentos del siguiente trabajo fueron aprobados por el comité de ética de la Facultad de Medicina de la U.N.A.M. siguiendo todos los requerimientos necesarios para minimizar el sufrimiento de los animales.

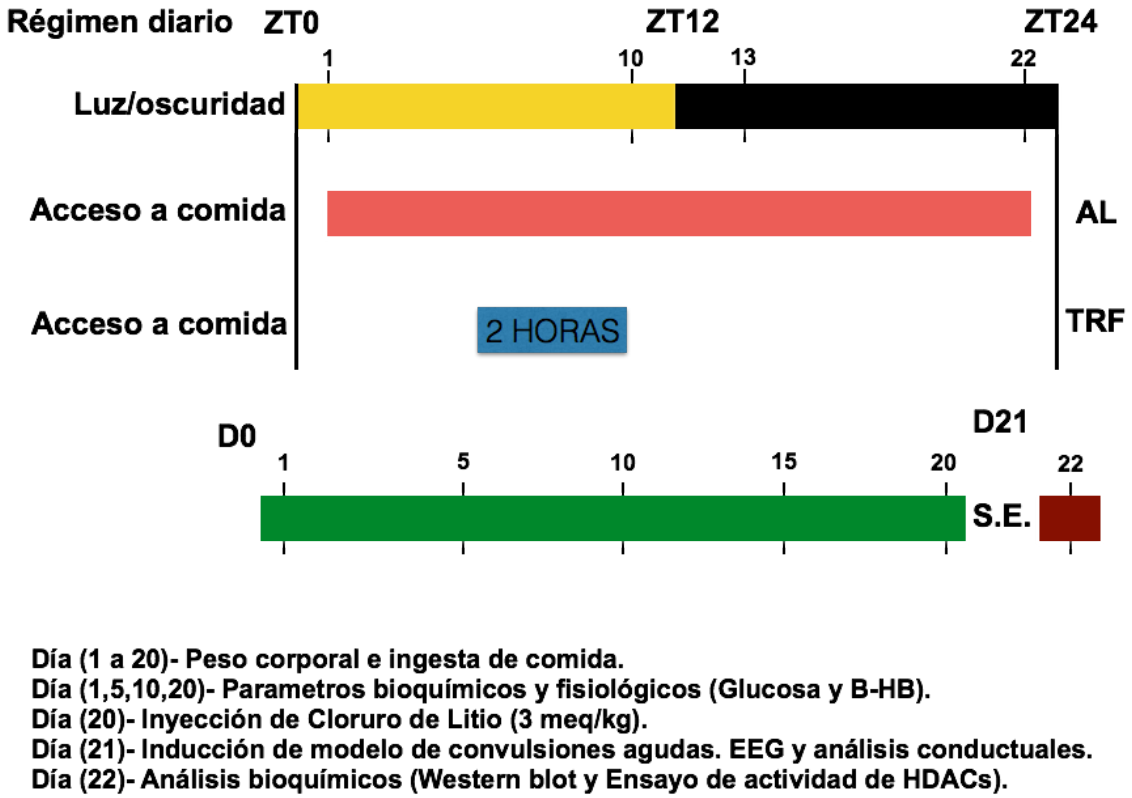

Figura 9. Representación esquemática del procedimiento experimental de los distintos regímenes alimenticios y del modelo de SE. Esquema representativo del procedimiento experimental realizado en este estudio. En este esquema se detalla el numero de días en el que los grupos AL y TRF, siguieron su régimen. Además, también se muestran los días en los que se realizaron distintos análisis, incluyendo el día en que se indujo el SE. Abreviaturas. ZT (Tiempo zeitberg), D (día), S.E. (*status epilepticus*), AL (*ad libitum*), TRF (Restricción alimentaria, por sus siglas en ingles).

9.2. Parámetros fisiológicos y bioquímicos.

La ingesta de comida y el peso corporal de los animales fueron monitoreados de manera diaria y la concentración de glucosa así como la concentración del B-HB sanguíneo se midieron haciendo uso de un monitor digital (Optium Xceed glucometer, Abbott USA) con sus tiras reactivas respectivas en los días 5, 10, 15 y 20 de los distintos regímenes alimentarios (Figura 9). Para los análisis sanguíneos los animales AL ayunaron 6 horas antes del análisis mientras que el grupo TRF ayunó aproximadamente 22 horas antes del análisis. Para los análisis de tolerancia a la glucosa se realizaron experimentos adicionales los cuales consistieron en inyectar de manera intraperitoneal una solución de glucosa (1g/kg de peso) y una hora después se volvió a medir la glucosa. El mismo procedimiento experimental se realizó para el B-HB. Además, para verificar si la restricción alimentaria pudiera tener efectos sobre el crecimiento de los animales se calculó un cociente de la ingesta calórica diaria (kcal/día) y se dividió entre la tasa metabólica basal calculada mediante fórmulas alométricas ($TMB=70M^{0.75}$, en donde la M es igual a masa). Finalmente los cerebros y los hígados fueron perfundidos con solución salina y se pesaron en una balanza para poder determinar si la restricción alimentaria es capaz de alterar el tamaño de cada uno de estos órganos.

9.3. Análisis conductuales.

Inmediatamente después de la administración de pilocarpina, se analizó la actividad convulsiva video-monitoreando a los animales por al menos 5 horas posteriores a, los análisis conductuales se llevaron a cabo por un investigador que no conocía los tratamientos. La latencia a la primera convulsión se midió en minutos hasta

la aparición del primer clonus unilateral, la severidad de las crisis se midió haciendo uso de la escala modificada de Racine (Bough et al. 2003). Finalmente se analizó el número de animales que alcanzaron el estado de SE, el cual se define como un periodo de convulsiones agudas que dura al menos una hora.

9.4. Registros electroencefalográficos.

Diez ratas fueron anestesiadas con una mezcla de ketamina/xilazina (4 ml/kg) y colocadas en un aparato estereotáxico. Para realizar el registro electroencefalográfico se implantaron un par de electrodos de metal en la superficie del cráneo, en las coordenadas 1.5 mm lateral y 2 mm posterior a bregma. Adicionalmente se implantaron dos electrodos uno en el sinus frontal y otro a la altura del cerebelo los cuales sirvieron como electrodos de referencia. Los electrodos fueron fijados al cráneo con acrílico dental. Se dejaron pasar de siete a diez días hasta la recuperación de los animales. Los registros de electroencefalografía fueron amplificados con un preamplificador P15 (Grass Instruments Company, USA), lo que permitía una amplificación de la señal eléctrica de 2000 veces, filtrada en el rango de 0.1-100 Hz y digitalizada a 20 kHz con un convertidor analógico (Micro-1401, CED, Cambridge, UK). Las señales eléctricas fueron grabadas en el disco duro de una computadora para su análisis posterior. Estas señales fueron analizadas utilizando el software (Spike2, CED) y (Matlab, USA).

9.5. Western blot.

Inmediatamente después de los distintos tratamientos se realizó la decapitación

de los organismos, inmediatamente se extrajeron el cerebro y el hígado, en cuanto al cerebro, se hizo una disección más específica que comprendió la región del hipocampo. Inmediatamente después las muestras fueron tratadas con el kit de fraccionamiento celular (Thermoscientific, USA), siguiendo las instrucciones del fabricante. Los extractos de proteína fueron cuantificados haciendo uso de un ensayo de BSA (Pierce, USA), una vez cuantificada la proteína, se cargaron 50 µg en geles SDS-PAGE (10 al 15%). Las proteínas fueron transferidas a una membrana de PVDF durante una hora, mediante un sistema de transferencia semi-húmeda (Biorad, USA). Después, se realizó un lavado de las membranas utilizando TBS para poder bloquearla con leche al 5% diluida en TBS-Tween 20 al 0.1% (TBS-T), durante toda la noche a 4ºC. Después las membranas fueron incubadas con sus respectivos anticuerpos primarios (Tabla 2) con la solución de bloqueo durante toda la noche (18-24 h) a 4ºC, una vez realizado este procedimiento, las membranas fueron lavadas tres veces más con TBS-T durante cinco minutos, para poder incubar las membranas con su respectivo anticuerpo secundario (Tabla 2), durante dos horas a temperatura ambiente. La señal de las membranas se detectó usando un kit de bioluminiscencia (Millipore) en placas de revelado (Amersham). Para los controles de carga se utilizaron los siguientes anticuerpos; anti-AMPK (Cell Signaling Technology), anti-Akt (Cell Signaling Technology) y anti-H3 (Cell Signaling Technology). Para la obtención de imágenes electrónicas de las placas de revelado se utilizó una cámara CCD (DNR Bio-Imaging Systems) y el análisis densitométrico de estas imágenes se realizó mediante el software para análisis de imágenes MCID (InterFocus Imaging Ltd., Cambridge UK). Los resultados se muestran en valores de densidad óptica relativa.

Tabla 2. Anticuerpos y condiciones en las que se utilizaron para este estudio.

Anticuerpo primario	Dilución	Marca y catalogo	Anticuerpo secundario	Dilución	Marca y catalogo
pAMPK-Thr172	1:1000	Cell signaling (4089)	Anticonejo (IgG)	1:2500	Cell signaling (7074)
pAkt-Ser473	1:1000	Cell signaling (9271)	Anticonejo (IgG)	1:2500	Cell signaling (7074)
acH3K9	1:1000	Cell signaling (C5B11)	Anticonejo (IgG)	1:2500	Cell signaling (7074)
acH3K14	1:1000	Cell signaling (D4B9)	Anticonejo (IgG)	1:2500	Cell signaling (7074)
AMPK	1:1000	Cell signaling (2532)	Anticonejo (IgG)	1:10000	Cell signaling (7074)
H3	1:1000	Cell signaling (D1H2)	Anticonejo (IgG)	1:2500	Cell signaling (7074)
Akt	1:1000	Cell signaling	Anticonejo (IgG)	1:10000	Cell signaling (7074)

9.6. Ensayo de actividad de las HDACs.

Los extractos de cromatina fraccionada de homogenados de hipocampo se cuantificaron mediante un ensayo de BCA (Pierce, USA) y de estos se utilizaron 50 µg de proteína para medir la actividad de las HDACs (específicamente las de clase I) haciendo uso de un kit para medir su actividad (Sigma-Aldrich, USA) siguiendo las instrucciones del fabricante. Todas las muestras se analizaron en duplicado y la actividad se calculó haciendo uso de la siguiente formula, (Actividad (ng/h/ug)=[RFU(control-blanco)-RFU(muestra-blanco]/pendiente x h x cantidad de proteína), en donde RFU significa (unidades de fluorescencia relativa), pendiente (concentración de HDACs) y h (tiempo de incubación del ensayo).

9.7. Estadística.

Los valores obtenidos en los distintos experimentos fueron examinados para determinar su tipo de distribución. Los datos que presentaron una distribución normal se analizaron mediante las pruebas de análisis de varianza (ANOVA) de una vía o mediante la de prueba t-Student. Para los datos que no presentaron una distribución normal, se analizaron mediante el análisis de Mann-Whitney. Los datos cualitativos fueron analizados mediante una prueba de Chi cuadrada y el análisis de Fisher. Estos análisis se realizaron usando la quinta versión del software Prism Graphpad (GraphPad Software, San Diego, CA). Una $p<0.05$ fue considerada como estadísticamente significativa.

10.Resultados.

10.1. Influencia de la restricción alimentaria sobre el peso corporal, la ingesta de comida y la concentración de glucosa y BH-B en sangre.

Para poder establecer si nuestro modelo de restricción alimentaria era capaz de inducir un cambio metabólico general, se midió el peso corporal, la ingesta de comida y las concentraciones en sangre, de glucosa y B-HB (Figura 10). Como se puede observar, desde el día dos del tratamiento, hasta el final del régimen, les grupo TRF, presentó una pérdida significativa en el peso corporal, comparado con el grupo AL (Figura 10A, $p<0.001$). De manera interesante, el grupo TRF, empezó a ganar peso en el día 15, recuperando casi su peso inicial al final del régimen (Figura 10A). En cuanto a la ingesta de comida, el grupo TRF, consumió una cantidad significativamente menor a lo largo de todo el tratamiento (Figura 10B, $p<0.001$). Por otro lado, no se observaron cambios

significativos en las concentraciones de glucosa en ayuno entre ambos grupos (Figura 10).

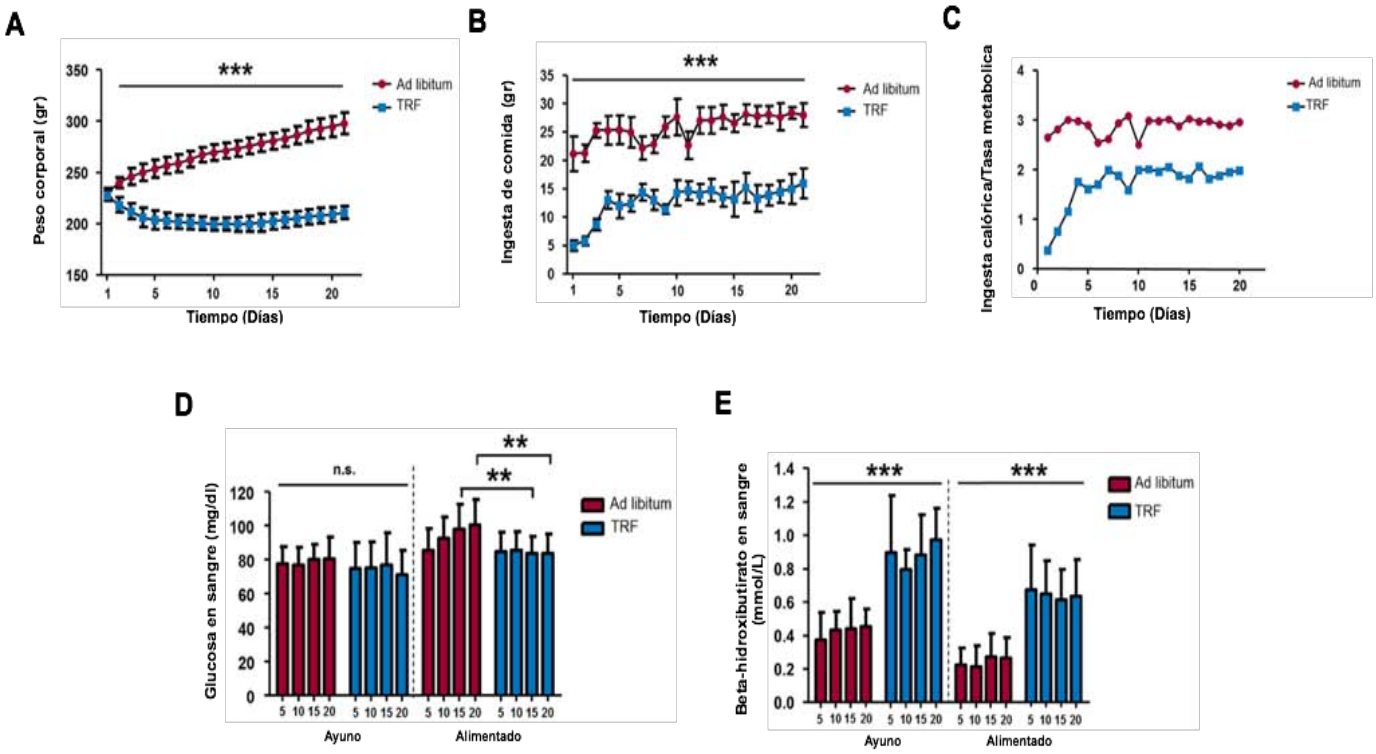

Figura 10. Efectos fisiológicos de la restricción alimentaria. Análisis del peso corporal del grupo AL y TRF a lo largo de 21 días de tratamiento **(A)**. Comparación de la ingesta de comida entre los grupos AL y TRF a lo largo de los 21 días del régimen alimenticio **(B)**. Análisis del cociente de ingesta calórica/tasa metabolica basal a lo largo de los 21 días del tratamiento **(C).** Comparación de los niveles de concentración de glucosa entre los grupos AL y TRF en dos estados (Ayuno y Alimentado) **(D).** Comparación de los niveles de concentración de B-HB entre los grupos AL y TRF en dos estados (Ayuno y Alimentado) **(E)**. Los datos se expresan como el promedio ± la desviación estándar de cada parámetro. (n=30, $^{**}p$<0.01; $^{***}p$<0.001).

Adicionalmente, se estudió la posibilidad de que a lo largo del régimen, existieran cambios en la tolerancia a la glucosa entre ambos grupos. Nuestros análisis mostraron que a partir del día 15 del régimen, los niveles de concentración de glucosa en sangre del grupo AL, fue significativamente mayor, comparándolo con el grupo TRF (Figura 10D, p<0.01). Además de esto, nuestros análisis de concentración de B-HB, muestran

que a lo largo de todo el régimen, la concentración sanguínea de B-HB del grupo TRF, es significativamente mayor, en comparación del grupo AL (Figura 10E, p<0.001).

10.2. Influencia de la restricción alimentaria sobre el peso del hígado.

Así mismo, se investigó el efecto de estas dietas en el tamaño de dos órganos, el cerebro y el hígado (Figura 11). Por un lado, no se encontraron diferencias significativas en el tamaño del cerebro, entre ambos grupos (Figura 11 A y B).

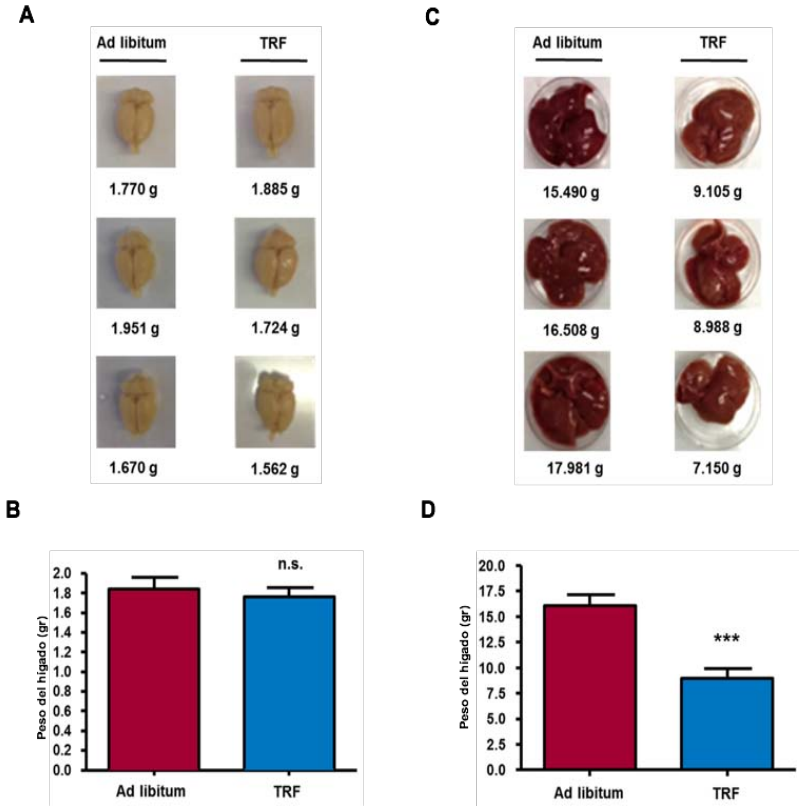

Figura 11. La restricción alimentaria disminuye el peso del hígado y no tiene ningún efecto en el peso del cerebro. Al final de los distintos regímenes alimenticios, se observo que el grupo TRF presento una disminución significativa en el peso del hígado en comparación del grupo AL **(B,D)**; por otro lado, no se observo ningún cambio en el peso del cerebro al comparar los grupos con distintos regímenes alimenticios **(A,C)**. Los datos se expresan como el promedio ± la desviación estándar de cada parámetro (n=10, ***p<0.001).

Por el contrario, se encontró una disminución significativa en el tamaño del hígado del grupo TRF (8.98 ±0.92 g, n= 8,p<0.001), en comparación del grupo AL (16.08 ± 1.05) (Figura 11 C y D) .

10.3. La restricción alimentaria altera la activación de vías de señalización asociadas a la regulación del metabolismo energético.

Es bien reconocido que la fosforilación de cinasas como AMPK y Akt pueden servir como marcadores de un cambio energético en las células (Burkewitz et al. 2014; Guo et al. 2014).

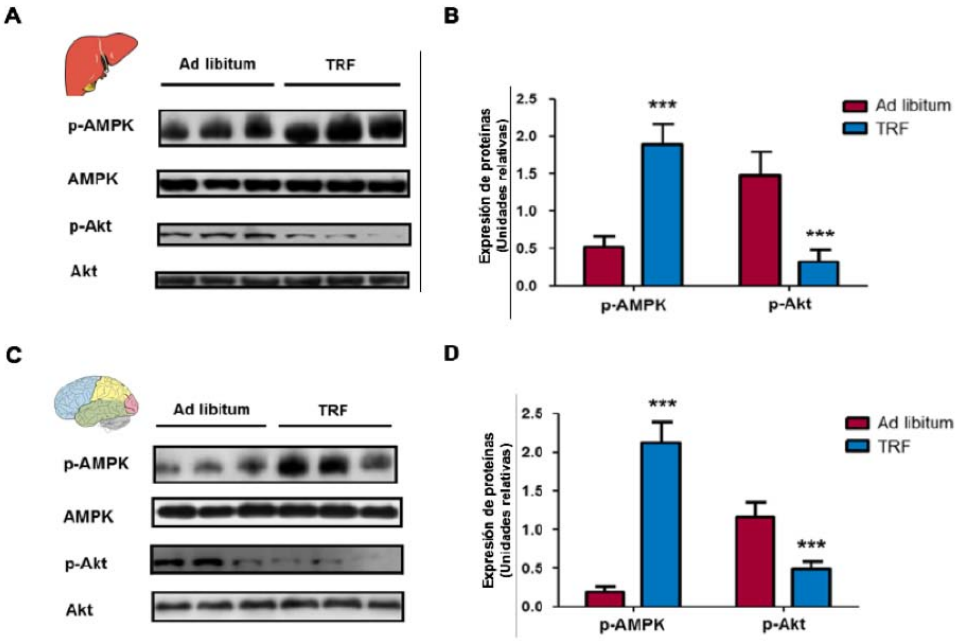

Figura 12. Efecto de la restricción alimentaria en vías de señalización que regulan el metabolismo energético en el hígado y en el cerebro. Se muestran imágenes representativas de los inmunoblots de homogenados de hígado e hipocampo **(A y C, respectivamente)**. En ambos tejidos se observa un aumento significativo en la fosforilación del residuo de treonina 172 de la cinasa AMPK **(B y D)**. Por otro lado, en ambos tejidos, se observo una disminución significativa en la fosforilación del residuo de serina 473 de la cinasa Akt **(B y D)**. Los datos se expresan como el promedio ± la desviación estándar de cada parámetro. (n=8, ***p<0.001).

En este sentido, se observó que en los homogenados de hipocampo e hígado del grupo TRF, presentan un aumento significativo en la fosforilación del residuo de treonina 172 de la proteína AMPK (Figura 12, n=9, $p<0.001$). De manera contraria, se encontró en ambos órganos, una disminución significativa en la fosforilación del residuo de serina 473 de la cinasa Akt, comparado con el grupo AL (Figura 12, n=9, $p<0.001$).

10.4. Efecto anticonvulsivo de la restricción alimentaria.

Para poder evaluar si la restricción alimentaria es capaz de ejercer un efecto anticonvulsivo en un modelo de SE, se realizaron registros electroencefalográficos de ambos grupos de animales después de haber recibido la inyección de pilocarpina (Figura 13 A y B). Unos minutos después de la inyección, ambos grupos, presentaron un incremento significativo en el espectro total medido en μV2/H (Figura 13 C y D).

Además de esto el curso temporal del poder del espectro en ambos grupos mostró un pico máximo de frecuencia después de un periodo de latencia de 21 a 45 minutos, característico del SE (Figura 13 C y D).

De manera interesante, se encontró que el poder del espectro total en una frecuencia de 1-50 Hz, fue significativamente mayor en el grupo AL, en comparación del grupo TRF (Figura 13C, $p<0.05$). Además de esto, se observó que 90 minutos después de la inyección, en la frecuencia de 51-100 Hz, el grupo TRF presenta una disminución significativa en el poder de las descargas corticales, en comparación del grupo AL (Figura 13D, $p<0.05$).

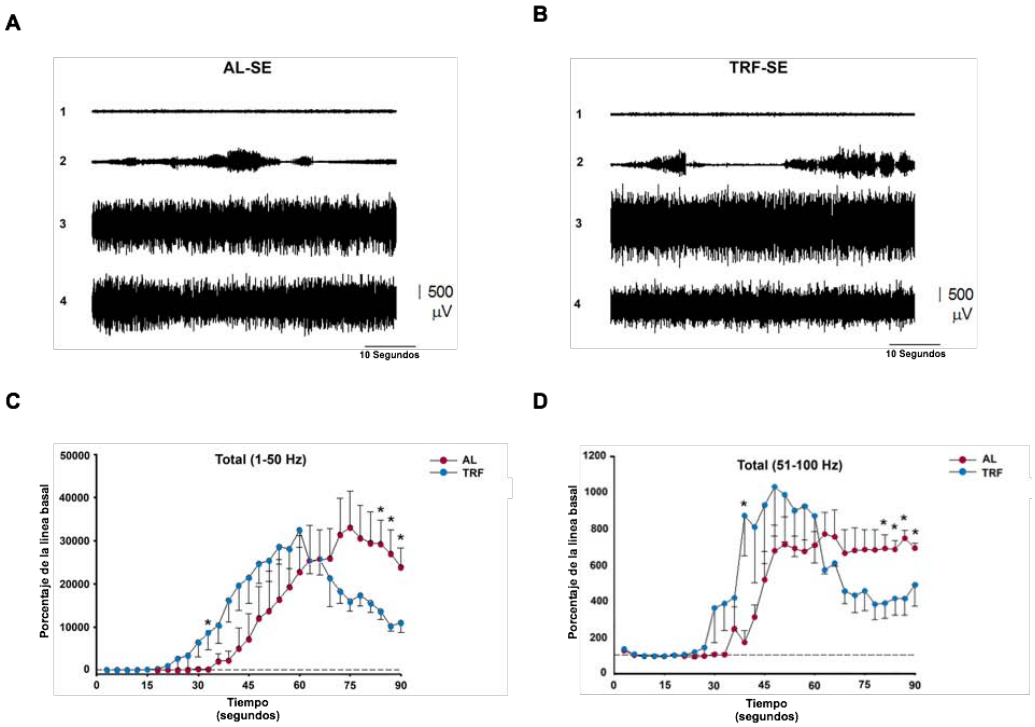

Figura 13. EEGs representativos de animales con SE que siguieron distintos regímenes de alimentación. Fragmentos representativos de los registros de EEG; el trazo numero uno, es un análisis típico de un animal antes de la administración de pilocarpina, del grupo AL **(A)** y del grupo TRF **(B)** (n=5, para cada grupo). Los números a la izquierda de los trazos representan el tiempo después de la inyección de pilocarpina; **(1)** Periodo de registro de la línea base, **(2)** 32 minutos post-inyección, **(3)** 65 minutos post-inyección y **(4)** 75 minutos post-inyección. Los valores de las graficas se calcularon como el porcentaje de la línea base, la cual se midió 45 minutos antes de la inyección con pilocarpina, al poder de este espectro se le asigno un valor del 100%. En las graficas se muestra el análisis de las transformadas de Fourier (FFT) en dos rangos distintos de frecuencia, de 1 a 50 Hz **(C)** y de 51 a 100 Hz **(D).** Los círculos rojos representan los datos del grupo que siguió el régimen *ad libitum*, mientras que los círculos azules representan los datos de los animales que siguieron el régimen de restricción alimentaria. Los datos se expresan como el promedio ± el error medio estándar de cada parámetro (n=10, *p<0.05)

Además de esto se realizaron distintas evaluaciones conductuales para determinar de una manera robusta el efecto anticonvulsivo de la restricción alimentaria (Figura 14). En estos análisis se observo que la duración de las crisis convulsivas del grupo TRF son significativamente menores a las del grupo AL (Figura 14A). Adicionalmente, nuestros análisis de latencia a la primera convulsión, revelaron que el grupo TRF, presenta una

latencia significativamente mayor en comparación a la del grupo AL (Figura 14B).

También se evaluó la severidad de las crisis haciendo uso de la escala modificada de Racine (Figura 14C) . En este análisis conductual se observo que durante el SE, la severidad de las crisis convulsivas del grupo TRF, son significativamente menores a las del grupo AL (Figura 14C).

Finalmente se evaluó la posibilidad de que la restricción alimentaria fuera capaz de interferir con los mecanismos epileptogénicos. Para evaluar esto, se analizo el numero de organismos que entraron en SE, después de haber sido inyectados con pilocarpina. Mediante este análisis se observo que 27 de los 30 animales del grupo AL presentaron el SE, por otro lado, solo 19 de los 30 animales del grupo TRF presentaron esta condición (Figura 14D).

10.5. El B-HB es un factor que contribuye al efecto anticonvulsivo de la restricción alimentaria.

Para determinar si los cambios en la concentración sanguínea de B-HB se encuentran involucrados en el efecto anticonvulsivo de la restricción alimentaria, se analizó la concentración de este acido carboxílico justo antes de la inyección de pilocarpina (Figura 14 E y F). Nuestros análisis muestran que la latencia a la primera convulsión tónico clónica, correlaciona de manera positiva, con la concentración sanguínea de B-HB (Figura 13E, R^2=0.772, $p<0.05$). Por otro lado, se encontró que la severidad de las crisis, se encuentra correlacionada negativamente con la concentración

de B-HB (Figura 13F, R= 0.6407, p<0.01).

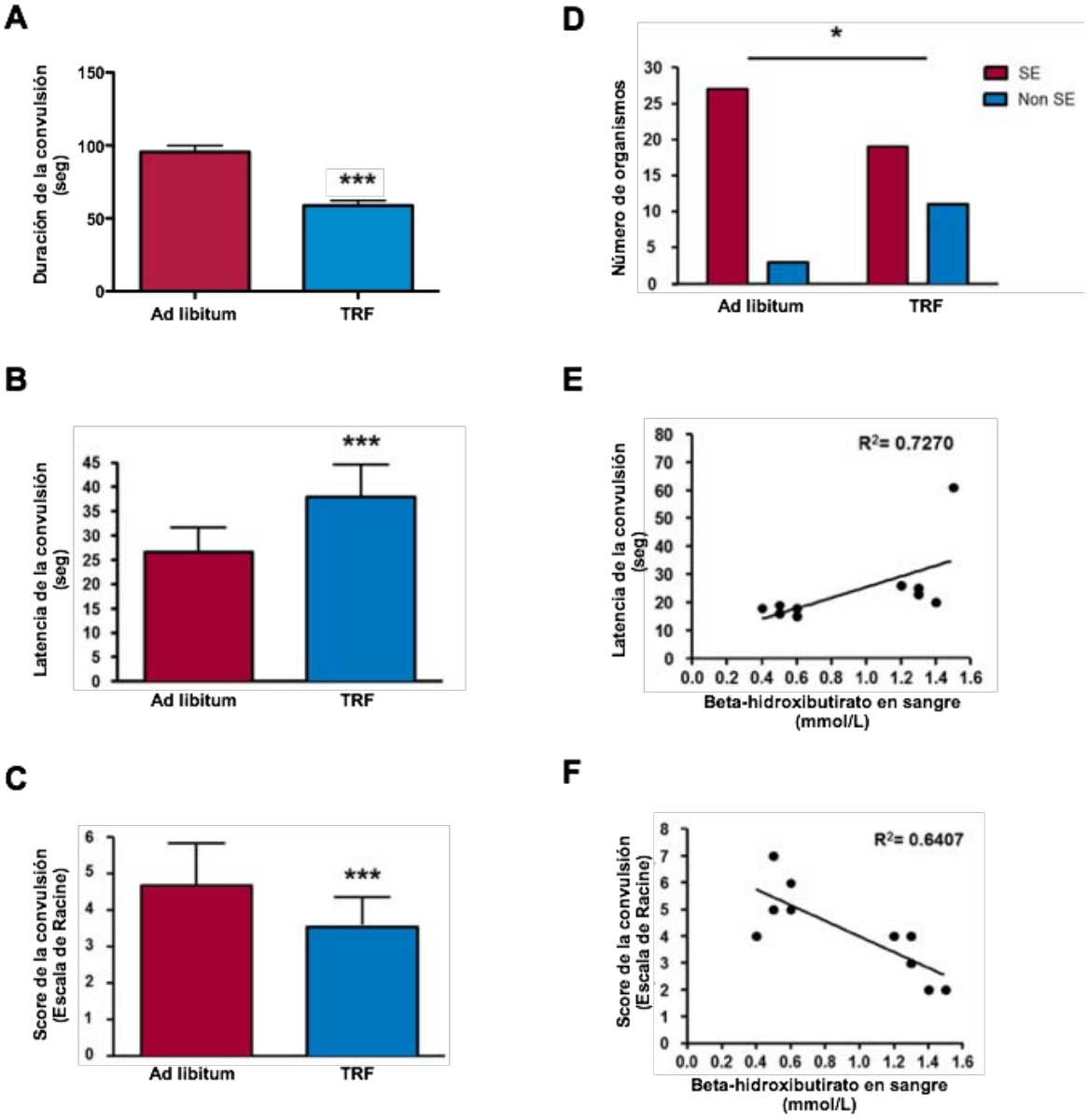

Figura 14. La restricción alimentaria inhibe la susceptibilidad a las crisis convulsivas y su posible relación con el B-HB. Los animales del grupo TRF mostraron una menor duración de la primera convulsión **(A)**, una mayor latencia a la primera convulsión **(B)**, un menor score de severidad a las crisis **(C)** y un menor numero de animales presentaron una condición conocida como SE **(D)**, en comparación con el grupo AL. Además, se encontró que existe una correlación positiva entre la concentración sanguínea de B-HB y la latencia a la primera convulsión **(E)**. Por otro lado, también se encontró una correlación negativa entre la concentración de este cuerpo cetónico en sangre y el score de las crisis convulsivas **(F).** Los datos se expresan como el promedio ± la desviación estándar de cada parámetro (Para los análisis conductuales, n=30, p<0.05*; ***p<0.001, n=10 para los análisis de correlación de Pearson y Spearman p<0.05*; ***p<0.001 respectivamente).

10.6 La restricción alimentaria inhibe la actividad de las HDACs de clase I en extractos nucleares de hipocampo.

Reportes previos han descrito que el B-HB es capaz de actuar como un inhibidor endógeno de las HDACs de clase I (Shimazu et al. 2013). Para poder determinar si la restricción alimentaria es capaz de ejercer una acción similar en homogenados de hipocampo, se realizó un ensayo de actividad de las HDACs de clase I en este tejido (Figura 15 A y D).

Como se puede observar en la figura, los animales del grupo AL obtuvieron un valor de actividad total de 0.1043 ± 0.036 ng/h/ug, por otro lado los animales TRF presentaron un valor significativamente menor (Figura 15A., n=6, $p<0.05$), presentando una reducción en la actividad del 29.48%.

Además, se observó que este efecto permanece aún después del SE, ya que el grupo AL+SE, presentó un valor similar al grupo AL. Por otro lado, los animales del grupo TRF+SE presentaron un valor significativamente menor al grupo AL+SE (0.03070 ± 0.030 ng/h/ug), el cual corresponde a una reducción del 29.66% (Figura 15D, n=6, $p<0.05$).

10.7. La restricción alimentaria incrementa la acH3K9/14 el hipocampo aun después del SE.

Para verificar si el efecto de la restricción alimentaria sobre las HDACs es capaz de alterar los niveles de acetilación de la H3 en hipocampo, se analizó por medio de

Western blot los niveles relativos de acetilación de dos residuos de lisina de la H3 (acH3K9/14), en los mismos extractos donde se medio la actividad de las HDACs.

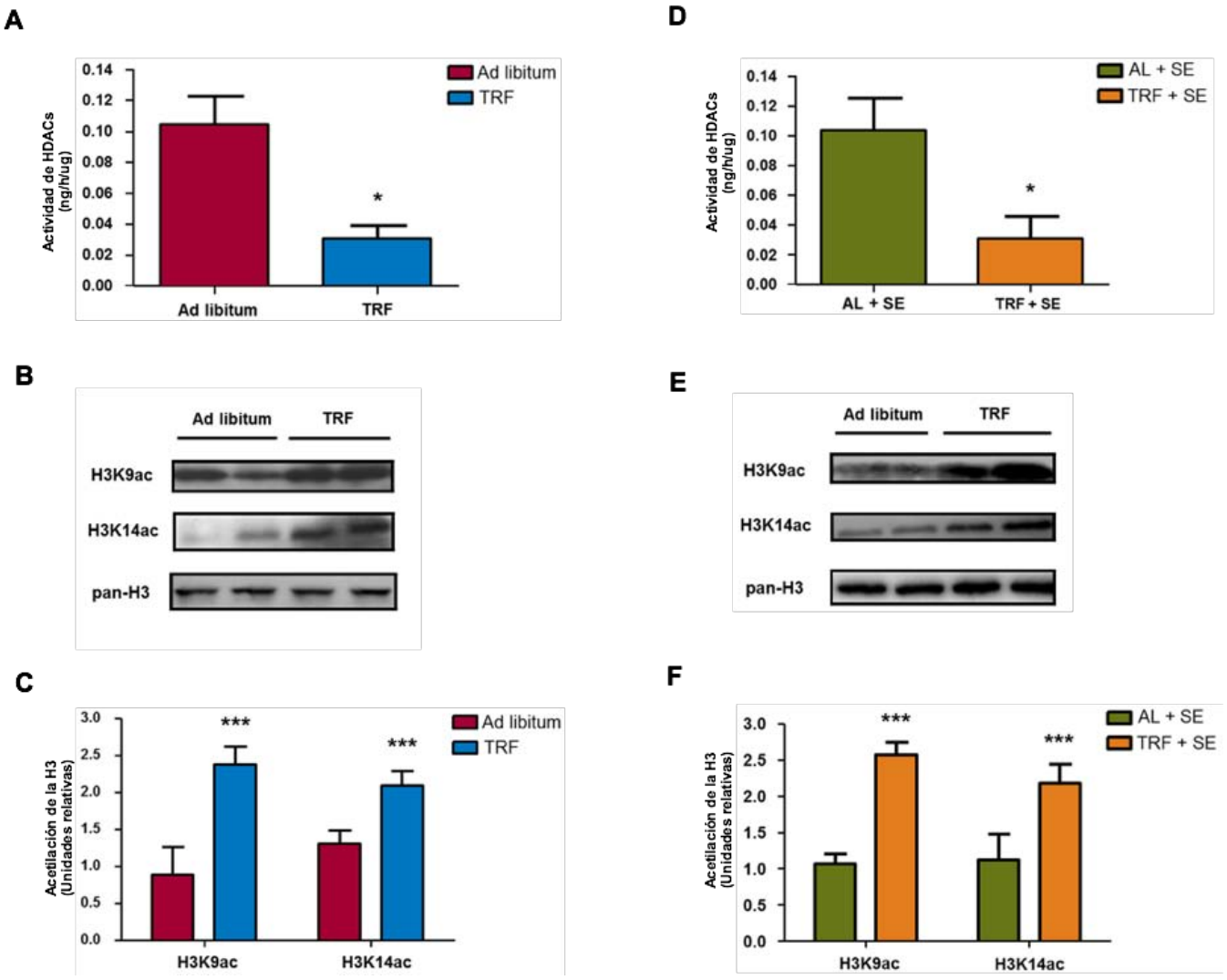

Figura 15. La restricción alimentaria es capaz de producir en el hipocampo una inhibición en la actividad de las HDACs de clase I y promueve un aumento en la acetilación de la H3. Se midió la actividad total de las HDACs en cuatro grupos (AL, TRF, AL-SE y TRF-SE). En este sentido, se observó una disminución significativa en el grupo TRF comparado con el grupo AL **(A)**; además, se encontró un efecto similar comparando los grupos que además de haber seguido los distintos regímenes alimenticios presentaron SE **(D).** Western blots representativos de extractos de cromatina fraccionada de hipocampo, los cuales muestran un aumento significativo en la acetilación de dos residuos de lisina de la H3 (9 y 14) en los grupos que siguieron la restricción alimentaria en comparación de su contraparte que siguió el régimen *ad libitum* **(B y E).** Se utilizo como control de carga a la proteína H3. Las graficas de barras muestran el análisis semi-cuantitativo de la densidad óptica de las bandas de los Western blots **(C y F)**. Los datos se expresan como el promedio ± la desviación estándar de cada parámetro (Para la actividad de HDACs; n=6, *p<0.05, para los western blots, n=8, ***p<0.001).

En este inmunoblot se observó que los extractos de hipocampo del grupo TRF, muestran un aumento significativo en la acetilación de estos dos residuos de la H3, en comparación con el grupo AL. Así mismo, los niveles de acetilación de ambos residuos de las H3 del grupo TRF+SE, fueron significativamente mayores a los niveles del grupo AL+SE (Figura 15., n=9, $p<0.001$).

11. Discusión.

En este trabajo se demostró de manera robusta que la restricción alimentaria es capaz de ejercer un efecto anticonvulsivo en un modelo de SE (Figuras 13 y 14). A la fecha, no es de nuestro conocimiento, que existan reportes que indiquen que esta dieta pueda tener un efecto anticonvulsivo. Sin embargo, existen otros estudios que sugieren que otras terapias basadas en el metabolismo, como la restricción calórica, son capaces de disminuir la excitabilidad neuronal en distintos modelos de convulsiones agudas (Bough et al. 2003; Phillips-Farfan et al. 2015). Acorde a estas evidencias, nuestros análisis de ingesta de comida sugieren que nuestro modelo restrictivo podría también incluir una restricción calórica, ya que el grupo TRF consume una cantidad significativamente menor de comida a comparación del grupo AL (Figura 10B). De acuerdo con estos reportes nuestros resultados sugieren que nuestro modelo restrictivo induce efectos anticonvulsivos similares a los ejercidos por dietas que involucran una restricción calórica (Phillips-Farfan et al. 2015; Bough et al. 2003).

Desde la década de 1920, se ha sugerido que los estados energéticos bajos, en donde se favorece la producción de energía y la disminución en el uso del ATP, correlacionan con

la disminución de las crisis convulsivas en pacientes con epilepsia (Lennox & Cobb 1928; Huttenlocher 1976). Nuestros análisis bioquímicos demuestran que la restricción alimentaria es capaz de ejercer un cambio metabólico global en donde se favorecen estos procesos (Figura 10 y 12). Es importante mencionar que prácticamente todas las terapias metabólicas que han mostrado disminuir la susceptibilidad a las crisis convulsivas convergen en la producción de este estado, ya que aumentan la actividad de la proteína AMPK y disminuyen la actividad de la cinasa mTOR (Lutas & Yellen 2013). Estas evidencias en su conjuntom sugieren que estas vías de señalización se encuentran involucradas en la disminución de las crisis convulsivas y por lo tanto son blancos racionales para el diseño de fármacos para el control de las convulsiones.

Además del estado energético, otro de los procesos metabólicos que se encuentra asociado a una reducción de las crisis convulsivas, es la producción de cuerpos cetónicos (Waldbaum & Patel 2010; Newman & Verdin 2014, Netzahualcoyotzi & Tapia 2014). Sin embargo, existe gran controversia en cuanto al papel de la cetonemia en la epilepsia, ya que, aunque existen numerosos estudios que señalan que este es un factor determinante para la reducción de la crisis, existen también, algunos otros en donde se señala que este proceso no se encuentra relacionado con este efecto (Carl & Kristopher 2003; Huttenlocher 1976). En cuanto a este aspecto, nuestros análisis no solo muestran que la restricción alimentaria es capaz de aumentar la concentración de B-HB (Figura 10E). Sino que también sugieren que la concentración de este acido carboxílico, tiene un papel muy importante en el efecto anticonvulsivo de esta dieta (Figura 13 E y F). Esta aseveración se hace en base a la observación de que la concentración de este metabolito

correlaciona de manera positiva con la latencia a la primera convulsión y de manera negativa con la severidad de las crisis convulsivas (Figura 13 E y F). Sin embargo, también es importante mencionar que es muy probable que este no sea el único factor involucrado en el efecto anticonvulsivo ejercido por este modelo restrictivo.

Es debido a estas asociaciones que se han sugerido distintos mecanismos mediante los cuales el B-HB podría estar involucrado en la reducción de crisis convulsivas (Lutas & Yellen 2013). La mayoría de estos estudios sugieren que este acido carboxílico ejerce su función restaurando el balance de la neurotransmisión (Juge et al. 2010; Yudkoff et al. 2007; Yudkoff et al. 2008; Suzuki et al. 2009). Algunos de estos señalan que este cuerpo cetónico aumenta la concentración de GABA, mediante distintos mecanismos, sin embargo, resultados preliminares de nuestro laboratorio sugieren que esta dieta restrictiva no ejerce sus efectos mediante este proceso (Datos no mostrados). Esta hipótesis se hace en base resultados preliminares de nuestro grupo de trabajo, en donde, no se encontraron diferencias significativas en la concentración de este neurotransmisor al comparar animales con una dieta AL, contra animales que siguieron el régimen restrictivo (Datos no mostrados).

Otro de los mecanismos plausibles mediante los cuales el B-HB podría restaurar el balance en la neurotransmisión es mediante la disminución de la absorción de glutamato en las vesículas sinápticas, ya que se ha demostrado que el B-HB es capaz de inhibir competitivamente a los transportadores de glutamato (Juge et al. 2010). Sin embargo, la concentración a la cual este acido carboxílico inhibe estos transportadores

es muy alta (10-20 mM) (Juge et al. 2010). Nuestros análisis sugieren que la restricción alimentaria no es capaz de aumentar a esos niveles la concentración de B-HB en el cerebro por lo que probablemente este no es el mecanismo de acción por la cual este régimen restrictivo tiene un efecto anticonvulsivo (Figura 10E).

Además de estos mecanismos, un estudio reciente demostró que el B-HB también es capaz de disminuir los procesos pro-inflamatorios, ya que previene la formación del inflamasoma (Youm et al. 2015). Estudios en donde se hacen uso de distintos modelos animales de epilepsia, han sugerido, que la desregulación de los procesos pro-inflamatorios es uno de los procesos patofisiológicos de la epilepsia (Devinsky et al. 2013). Es posible que el B-HB, en parte, contribuya a los efectos benéficos de la restricción alimentaria, mediante este mecanismo. Sin embargo, la concentración a la cual se encontró una inhibición de la formación del inflamasoma es muy alta (10-20 mM) (Youm et al. 2015). Es por esto que hacen falta mas estudios para determinar los mecanismos precisos mediante los cuales el B-HB contribuye al efecto benéfico de distintas dietas.

Por ultimo, también se ha reportado que el B-HB funciona como una molécula señal, ya que entre otras funciones, es capaz de disminuir la actividad de las HDACs de clase I (Shimazu et al. 2013). En este estudio, se demostró que la restricción alimentaria es capaz de producir un aumento en dos de los residuos de lisina de la H3 (9 y 14) en el hipocampo (Figura 15). Este proceso podría estar siendo mediado debido a la disminución de la actividad de las HDACs por el B-HB (Figura 14 A, B, C y D). Sin

embargo, es probable que este no sea el único mecanismo involucrado en aumentar la acetilación, esta aseveración se hace en base a que el B-HB también es un precursor de acetil-CoA, este producto, es el principal sustrato de las HATs, las cuales podrían estar aumentando su actividad (Saha & Pahan 2005; Takahashi et al. 2006). Existe evidencia que señala que la disminución de la actividad de las HDACs de clase I mediada por el B-HB, se encuentra correlacionada con cambios globales en la transcripción, entre estos se encuentra un aumento en la transcripción de genes que codifican para factores de resistencia al estrés oxidativo como FOXO3A y Mt2 (Shimazu et al. 2013). Estudios previos al nuestro han demostrado que el estrés oxidativo es uno de los procesos patofisiológicos que se encuentran involucrados en el SE y en la epilepsia (Waldbaum & Patel 2010). En este sentido, estudios preliminares en nuestro laboratorio sugieren que la restricción alimentaria es capaz de disminuir este proceso durante el SE (Datos no mostrados). Estas evidencias en su conjunto sugieren que la restricción alimentaria podría ejercer sus efectos benéficos, en parte, al promover la transcripción de genes asociados a la resistencia al estrés oxidativo. Sin embargo es importante mencionar que hacen falta mas experimentos para determinar si esto es cierto.

Tomando en cuenta nuestros resultados así como los estudios que se han mencionado anteriormente, proponemos un escenario plausible, por el cual, la restricción alimentaria es capaz de ejercer su efecto anticonvulsivo (Figura 16). Esta hipótesis contempla que al modular la actividad de las vías de señalización que regulan el metabolismo energético (Figura 12), se favorecen procesos celulares que favorecen la producción de energía y coordinan una disminución en el uso de ATP (Burkewitz et al.

2014), entre los que se encuentra un aumento en la cetogénesis (Sengupta et al. 2010). Este proceso favorece un aumento en la concentración sanguínea de B-HB (Figura 10E). Al haber un aumento en la concentración de este cuerpo cetónico este será transportado al cerebro (Newman & Verdin 2014). En este órgano, éste acido carboxílico ejerce su función como molécula señal, disminuyendo la actividad de las HDACs de clase I y promoviendo un aumento en la acetilación de la H3 (Figura 15) (Shimazu et al. 2013). Así bien, este proceso podría estar involucrado en un aumento de la transcripción de genes involucrados en disminuir la susceptibilidad a las crisis convulsivas.

Finalmente, es importante mencionar que hacen falta mas estudios para comprobar esta nueva hipótesis. Sin embargo, los resultados de este trabajo proveen evidencia racional de algunos de los posibles mecanismos mediante los cuales las dietas restrictivas promueven sus efectos benéficos en un contexto de SE.

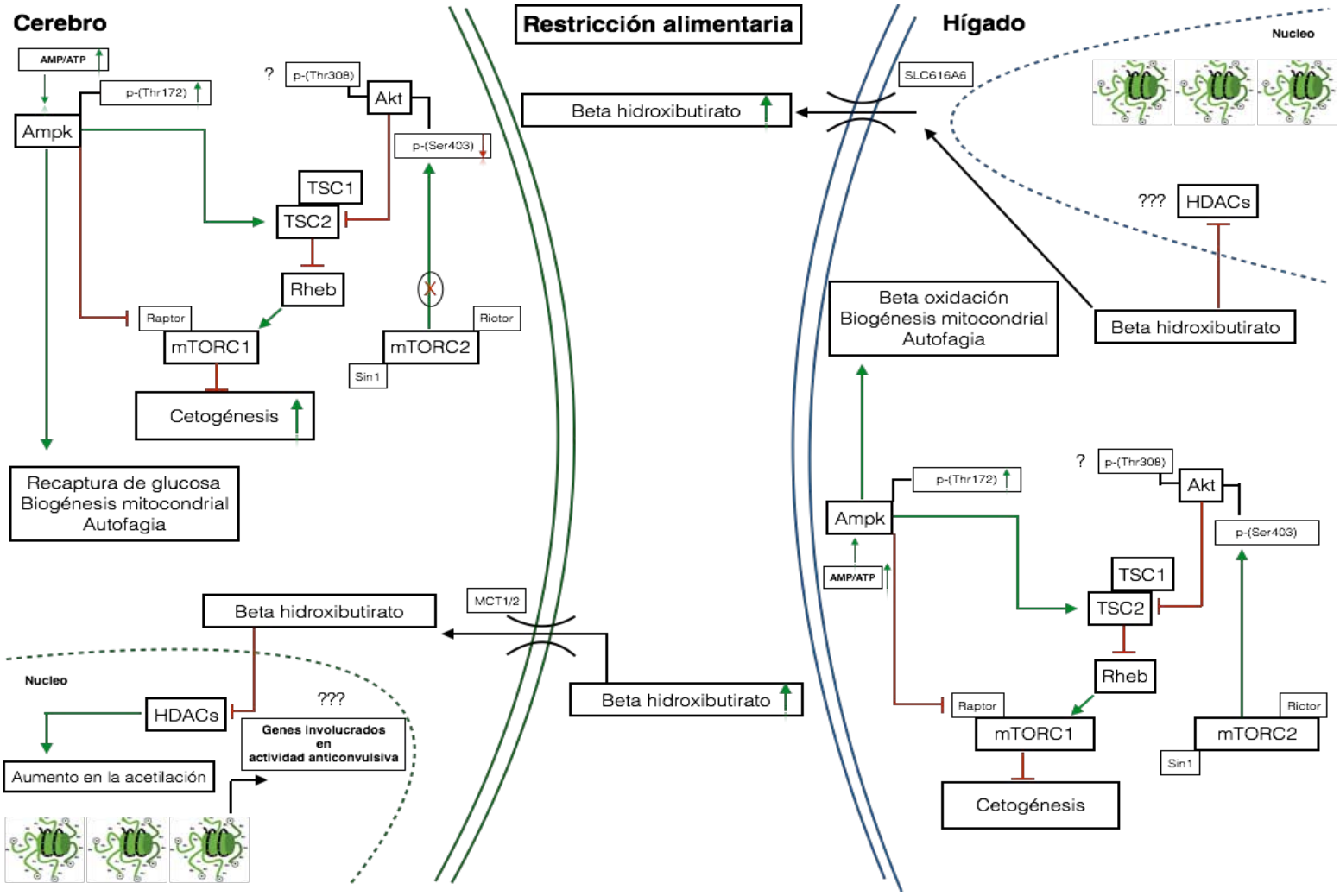

Figura 16. Posible escenario de las vías de señalización que promueven los efectos anticonvulsivos observados bajo el régimen de restricción alimentaria. Al seguir un régimen alimenticio restrictivo se produce un estado energético bajo en el organismo; lo que es capaz de activar la vía se señalización de la cinasa AMPK, en este estado esta cinasa es capaz de inhibir el complejo mTOR. En el hígado esto favorece la producción de cuerpos cetónicos. Estos cuerpos cetónicos, son transportados al torrente sanguíneo vía transportadores de monocarboxilatos (SLC616A6), después serán enviados a órganos como el cerebro en donde la demanda energética es alta. En este órgano se demostró que en este estado; los cuerpos cetónicos son capaces de disminuir significativamente la actividad de las HDACs, esto contribuye a una mayor acetilación de la H3, lo que podría estar regulando la expresión de genes que contribuyen a la disminución de la susceptibilidad a las crisis convulsivas.

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14. Anexo (Publicaciones).

Como parte de esta tesis y durante los estudios de maestría en el posgrado de ciencias bioquímicas, se publicaron los siguientes artículos.

Landgrave-Gómez, J., O. F. Mercado-Gómez, & R. Guevara-Guzman. 2015. Epigenetic mechanisms in neurological and neurodegenerative diseases. *Frontiers in Cellular Neuroscience* 9.

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Epigenetic mechanisms in neurological and neurodegenerative diseases

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The role of epigenetic mechanisms in the function and homeostasis of the central nervous system (CNS) and its regulation in diseases is one of the most interesting processes of contemporary neuroscience. In the last decade, a growing body of literature suggests that long-term changes in gene transcription associated with CNS's regulation and neurological disorders are mediated via modulation of chromatin structure. "Epigenetics", introduced for the first time by Waddington in the early 1940s, has been traditionally referred to a variety of mechanisms that allow heritable changes in gene expression even in the absence of DNA mutation. However, new definitions acknowledge that many of these mechanisms used to perpetuate epigenetic traits in dividing cells are used by neurons to control a variety of functions dependent on gene expression. Indeed, in the recent years these mechanisms have shown their importance in the maintenance of a healthy CNS. Moreover, environmental inputs that have shown effects in CNS diseases, such as nutrition, that can modulate the concentration of a variety of metabolites such as acetyl-coenzyme A (acetyl-coA), nicotinamide adenine dinucleotide (NAD^+) and beta hydroxybutyrate (β-HB), regulates some of these epigenetic modifications, linking in a precise way environment with gene expression. This manuscript will portray what is currently understood about the role of epigenetic mechanisms in the function and homeostasis of the CNS and their participation in a variety of neurological disorders. We will discuss how the machinery that controls these modifications plays an important role in processes involved in neurological disorders such as neurogenesis and cell growth. Moreover, we will discuss how environmental inputs modulate these modifications producing metabolic and physiological alterations that could exert beneficial effects on neurological diseases. Finally, we will highlight possible future directions in the field of epigenetics and neurological disorders.

Keywords: epigenetics, neurodegeneration, DNA methylation, postranslational modification, Parkinson disease, epilepsy

EPIGENETICS

The term epigenetics is derived from the theoretical and experimental work of Conrad Waddington. He coined the term to describe a conceptual solution to a phenomenon that arises as a fundamental consideration of developmental biology (Waddington, 1942). All of the different cells in the body of one individual have exactly the same genome, that is, exactly the same DNA nucleotide sequence, with only a few exceptions in the reproductive, immune and nervous systems. Thus, in the vast majority of instances, one's liver cells have exactly the same DNA as neurons. However, those two types of cells are clearly vastly different in terms of the gene products that they produce. Some level of mechanism must exist, Waddington reasoned, that is "above" the levels of genes encoded by the DNA sequence, which controls the DNA readout. For this reason, he defined the term *epigenetics* in the early 1940s as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" (Waddington, 1968). In the original sense of this definition, epigenetics is referred to all molecular pathways modulating the expression of a genotype into a particular phenotype.

However, and with the fast expansion in this field, epigenetics has been redefined and accepted today as "the study of changes in gene function that are mitotically and/or meiotically heritable and that does not entail a change in DNA sequence." In this way, recent advances have evolved our understanding of classical epigenetic mechanisms and the broader landscape of molecular interactions and cellular functions that are inextricably linked to these processes. The current view of epigenetics includes the dynamic nature of DNA methylation, active mechanisms for DNA demethylation, differential functions of 5-methylcytosine and its oxidized derivatives, the intricate regulatory logic of histone post-translational modifications, the incorporation of histone variants into chromatin, nucleosome occupancy and dynamics. Nevertheless, of all these modifications, the mechanisms better

described in literature generally comprise histone variants, posttranslational modifications of amino acids on the amino-terminal tail of histones, and covalent modifications of DNA bases.

In this chapter, we will discuss some of these epigenetic modifications and how these modifications are associated with neurologic homeostasis and diseases.

LINKING THE ENVIRONMENT, NUTRITION AND EPIGENETIC MODIFICATIONS

Although many aspects of nutrition and different kinds of lifestyles influence metabolic status and disease trajectory throughout our life, emerging findings suggest that changing our metabolism with exercise or different dietary regimens such as ketogenic diets, low-carbohydrate diets, intermittent fasting or physical exercise can alter the concentration of a variety of metabolites, some of them capable of modulating the activity of proteins that elicit epigenetic modifications (**Figure 1**; Shimazu et al., 2013; Shyh-Chang et al., 2013).

These epigenetic modifications seem to regulate important networks of genes mediating physiological processes associated with the beneficial effect of these diets, providing a rationale and simple way to prevent or even treat these diseases. Some reports have shown the efficacy of exercise and diet in cancer; cardiovascular disease, diabetes, obesity, rheumatoid arthritis and even in some neurological/neurodegenerative diseases such as Alzheimer and epilepsy (Müller et al., 2001; Ahmet et al., 2005; Belkacemi et al., 2012; Kroeger et al., 2012; Lee et al., 2012; Varady et al., 2013; Colman et al., 2014).

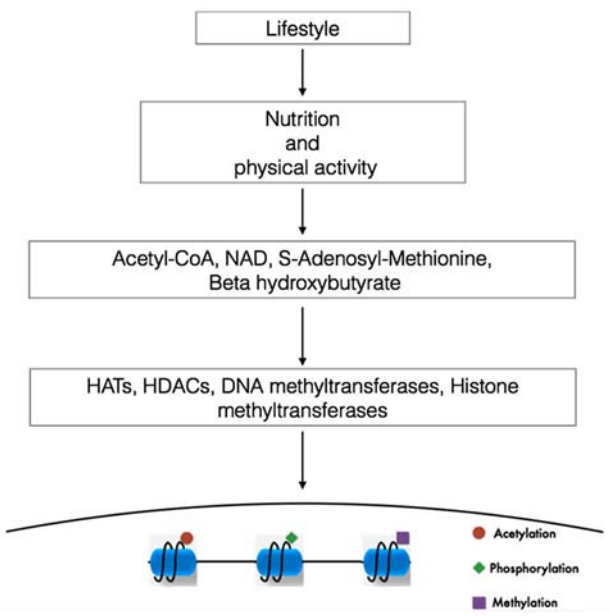

FIGURE 1 | Linking lifestyle with genome expression. DNA and the proteins that provides chromatin structure are targets of multiple modifications. In this way, changes in our lifestyle (diets or physical activity) via the modulation of the metabolism alters the concentration ratio of different metabolites. The availability and cellular compartamentalization of these metabolites alters the activity of proteins capable to elicit epigenetic modifications, contributing to the specificity of the genome expression. NAD, nicotine adenine dinucleotide (Modified from Sassone-Corsi, 2013).

Consistently, some reports have shown that aging it's a process that may be altered through some diets, such as calorie restriction (Colman et al., 2014). The precise mechanisms of how environment mediates epigenetic modifications are not clearly understood, however in this manuscript we will portray some studies that aim to epitomize the relationship between environment, metabolism, epigenetics and neuro -logical/neurodegenerative diseases.

EPIGENETIC MODIFICATIONS

Within cell nucleus, the fundamental units of chromatin are named nucleosomes. Each nucleosome is formed by 147 DNA base pairs wrapped tightly around an octamer of histone proteins, which is assembled by two copies of each of the four core histones (H2A, H2B, H3 and H4). The linker histone H1 binds to the DNA between the nucleosomal core particles, and their function is to stabilize higher order chromatin structures. Moreover, each histone protein consists of a central globular domain and N-terminal tail that contains multiple sites for potential modifications (Wang et al., 2013).

In this regard, a variety of different modifications on amino acid residues of histones have been described. Histone posttranslational modifications include acetylation, methylation, phosphorylation, ubiquitination and sumoylation (**Table 1**; Sassone-Corsi, 2013).

The principal residues that are substrates of these modifications are lysine, arginine, serine and threonine amino acids (Rothbart and Strahl, 2014). These modifications have been associated to repression or activation of gene transcription depending on the site of the modification, strongly suggesting the existence of a histone code. This hypothesis proposes that specific modifications of histones induce to the interaction with proteins associated with the chromatin, producing a differential regulatory response of gene expression (Strahl and Allis, 2000; **Table 1** and **Figure 2**). These modifications are dynamic in the way that they are actively added and removed by histone-modifying enzymes in a site-specific manner, which is essential for coordinated transcriptional control.

Table 1 | Histone posttranslational modifications and their role on transcription.

Modification	Role in transcription	Modification site
Acetylation	Activation	H3(K9, K14, K18, K56). H4(K5, K8, K12, K16). H2B(K6, K7, K16, K17) (Strahl and Allis, 2000).
Methylation	Activation	H3(K4me2, K4me3, K36me3, K79me2) (Strahl and Allis, 2000)
Methylation	Repression	H3(K9me3, K27me3) and H4(K20me3) (Balazs, 2014).
Phosphorylation	Activation	H3(S10) (Strahl and Allis, 2000)

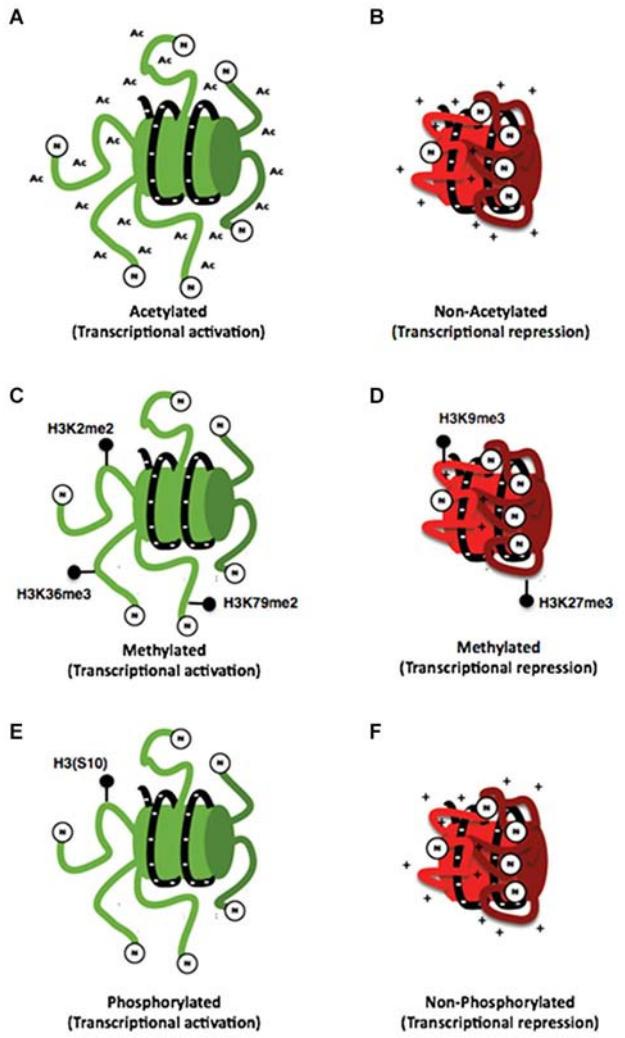

FIGURE 2 | Histone posttranslational modifications. (A) Schemes representing the interaction of the N-terminal dominium of acetylated histones with the DNA strand **(A)** and the interaction of a non-acetylated histone with the DNA strand **(B)**. It can be noticed that acetylated histones have a minor interaction with DNA strand compared with that of non-acetylated histones whose positive charges are attracted to negative charges of DNA. **(C)** On the other hand different specific marks of methylation of histone 3 are associated with both transcriptional activation **(C)** and repression **(D)**. Also a specific mark of phosphorylation on the (S10) amino acid of histone 3 has been associated with transcriptional activation **(E)** so the lack of this mark may be associated with transcriptional repression **(F)**.

HISTONE ACETYLATION

The acetylation of histones is a modification associated generally to transcriptional activity that indicates access of the transcription machinery to the genes and thus active mechanisms (Strahl and Allis, 2000; Balazs, 2014). This effect could be explained by the chemistry of this modification in which an acetyl group (−COCH3) is incorporated to an amino terminal residue and thus, the positive charge of histones is reduced, inducing a minor interaction with DNA resulting in a decrease of the chromatin compaction (**Figures 2A,B**; Shahbazian and Grunstein, 2007).

HISTONE METHYLATION

Histone methylation is currently associated with multiple processes such as transcriptional activation and repression, depending on the modified amino acid residue (**Figures 2C,D**). This modification occurs mainly on arginine and lysine residues. Additionally, these residues could be methylated multiple times giving different signals depending on how many times the residue is methylated, making its analysis difficult. In this regard, current literature has shown that lysine residues can be methylated even three times; meanwhile, arginine residues can only be methylated twice (Strahl and Allis, 2000). Furthermore, there have been some studies associating some processes with these types of modifications for example H3K4, H3K36 and H3K79 are associated with chromatin aperture. Nevertheless, the methylation of these residues has been also associated with other specific functions. On the other hand, H3K4 trimethylation has been associated with promoter regions. The monomethylathion of this same residue recruits regulatory elements that potentiate the promoter activity; such elements are known as *enhancers*. Dimethylation of H3K36 has been related to RNA POL II elongation during transcription (Li et al., 2007). Also, the dimethylation of H3K79 is particular of promoter regions stimulating a permissive chromatin for local transcription (Jacinto et al., 2009). Conversely, the modifications associated with transcriptional repression are performed on H3K9 and H3K27 residues (Baylin and Jones, 2011).

DNA METHYLATION

In mammalians, DNA methylation is the covalent union of methyl groups of cytosines that are found mainly in the context of dinucleotide 5′-CpG-3′ (**Figure 3A**; Klose and Bird, 2006). The addition of methyl groups protrudes above the major groove and when DNA is symmetrically methylated, the methyl groups promote a conformational change of DNA structure. The main consequence of methyl modification is that a variety of transcription factors cannot recognize the DNA and thus induce repressional transcription (Prokhortchouk and Defossez, 2008).

DNA methylation generates patterns that are established during embryonic development and such patterns are maintained by a mechanism when DNA replicates (**Figure 3**). Interestingly, these patterns change over time, principally due to environmental factors (i.e., nutrition, metabolites, exercise, chemical agents) (Fraga et al., 2005). The mechanism of DNA methylation is carried out by a set of proteins named DNA methyltransferases (DNMTs). There are two groups of these proteins; (1) one for *de novo* methylation; and (2), one for methylation maintenance. Both enzymes differ depending on the DNA substrate: for example, maintenance of DNA methylation is accomplished by DNA methyl transferase 1 (DNMT1). These proteins add methyl groups to pre-existing methyl patterns on a new strand of DNA during replication (**Figure 3B**; Jeltsch, 2006). On the other hand, *de novo* DNA methylation are carried out by DNMT3a and DNMT3b. Such proteins are responsible for the addition of new methyl groups to cytosines that have not been methylated previously (Goll and Bestor, 2005).

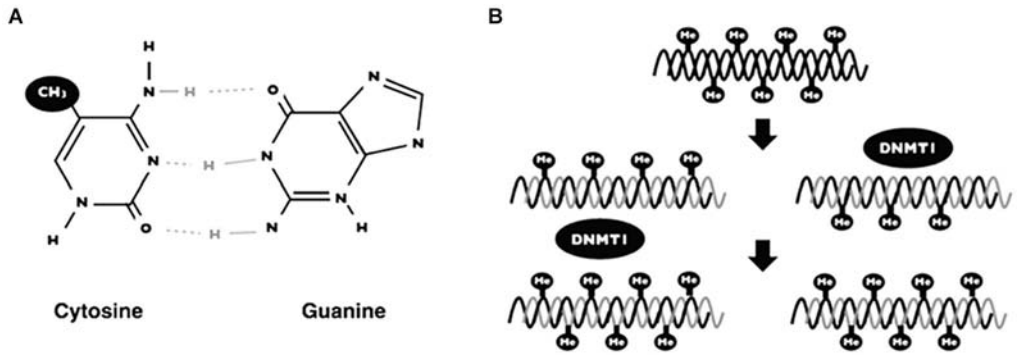

FIGURE 3 | DNA methylation. Scheme showing the addition of a methyl group on the 5 carbon of cytosine in the context of 5′-CpG-3′ dinucleotide **(A)**. The maintenance of DNA methylation is accomplished by DNA methyl-transferases (DNMT1) when DNA replication occurs **(B)**.

HISTONE VARIANTS

Histone variants such as H2A and H3.3 have been known since several decades ago and recently, a lot of evidence has been accumulated about their role in their participation on the differential structure of chromatin (Henikoff et al., 2004). Among them, H2A.Z has been located on DNA regions associated with transcriptional activation, mainly, on promoter regions. This variant is important because it induces a less stable structure of chromatin compared with that of the canonical histone H2 (Draker and Cheung, 2009). Another histone variant associated with promoter regions is H3.3. This variant as well as H2A.Z, is mainly found on promoter regions suggesting that their structure promotes the formation of a more permissive chromatin (Jin et al., 2009).

NEUROEPIGENETICS AND THEIR ROLE IN NEURONAL FUNCTION

Over the last two decades, the field of epigenetics, particularly the emerging field of neuroepigenetics, has begun to have a great impact in different areas such as the study of the CNS development, learned behavior, neurotoxicology, cognition, addiction and lately neurological and neurodegenerative pathology (Sweatt, 2013). In this regard, epigenetics has undergone an exponential expansion. A quick search of the PubMed database reveals that about 98% of all the research work on epigenetics was published within the last 15 years (Sweatt, 2013). Thanks to these studies, nowadays we know that either maternal behavior, environmental toxins, nutrition, physociological or physical stress, learning, drug exposure or psychotrauma, leads to active regulation of the chemical and three-dimensional structure of DNA and thus, regulates epigenetics modifications in the CNS linking environmental *stimuli* and gene expression regulation (Tsankova et al., 2007; Borrelli et al., 2008; Renthal and Nestler, 2008; Champagne and Curley, 2009; Day and Sweatt, 2010; Dulac, 2010).

These epigenenomic changes allow perpetual alterations in gene readout in cells in the CNS affecting neuronal function and physiology. For example, a central regulator of homeostasis in the brain, the brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of proteins that plays crucial roles in the development, maintenance, and plasticity of the CNS (Chao et al., 2006) have been demonstrated to play an important role on different psychiatric disorders associated with early-life adversity, including depression; schizophrenia, bipolar disorder and autism. Even when the underlying mechanisms of the alterations over the expression of BDNF are unknown in these conditions, epigenetic modifications seem as a plausible candidate, as early-life exposures, chronic emotional *stimuli*, or even emotional behavior, disrupts epigenetic programming in the brain with lasting consequences for gene expression and behavior (Renthal et al., 2007; LaPlant et al., 2010; Kundakovic et al., 2014).

However, epigenetics is such a new field of science that in most of the cases, its impact has not been fully demonstrated. Even though, it is now clear that there is a dynamic interplay between genes and experience, a clearly delineated and biochemically driven mechanistic interface between genes and environment, this interface is epigenetics (Sweatt, 2013).

ALZHEIMER'S DISEASE AND EPIGENETICS

Alzheimer's disease (AD) is an age-related and slowly neurodegenerative disorder of the brain and the most common form of dementia in the elderly (Sezgin and Dincer, 2014). The disease is clinically characterized by progressive memory loss and cognitive impairment. Moreover, the histopathological features of AD are senile plaques composed of amyloid beta (Aβ) fibrils and neurofibrillary tangles composed of microtubule-associated protein tau, combined with massive cholinergic neuronal loss, mainly in the hippocampus and association regions of neocortex (Hardy, 2006; Ballatore et al., 2007). This disease currently affects approximately 2% of the population in industrialized countries and its incidence will increase dramatically over the time (Sezgin and Dincer, 2014).

AD is a multifactorial disease involving; genetic, metabolic, nutritional, environmental and social factors that are associated with onset and progression of the pathology. For this reason, and considering that the main risk factor of this disorder is aging, it is reasonable to think that life history such hypertension, diabetes, inflammation, obesity or head injury are closely related with AD (Marques et al., 2011). However, how these factors induce

epigenetic changes that mediate the network genes involved in this disease is a question that remains to be answered.

At present, studies of epigenetic changes in AD are starting to emerge. As we mentioned before, aging is the most important risk factor for AD an epigenetic changes have been observed in aging tissues. Recently, it has been observed that environmental factors even transient ones in early life can induce AD-like pathogenesis in association with aging (Wu et al., 2008a). Furthermore, a difference in DNA methylation patterns typical of brain region and aging has been identified in this context (Balazs, 2014). In this regard, a recent study by Hernandez et al. examined the DNA methylation patterns in >27,000 CpG sites from donors ranging in age 4 months to 102 years and a strong relationship was found between DNA methylation and aging. Moreover, in the temporal and frontal cortices pons and cerebellum regions, more than 1,000 associations were found between DNA methylation at CpG sites and age and some associations were significant in all four regions. Interestingly, the majority of the association sites were in CpG islands and the pattern was similar in the frontal cortex, temporal cortex and pons, but different in cerebellum. These results suggest that and age-dependent increase in DNA methylation may be important for maintaining gene expression with age (Hernandez et al., 2011).

As it has been reported in many studies, memory can be compromised during aging. Preclinical and basic studies have shown that epigenetic mechanisms are involved in formation and maintenance of memory (for reviews, see Levenson and Sweatt, 2005; Zovkic et al., 2013; Jarome et al., 2014). For example, inhibition of DNA methylation has deleterious effects on neuronal plasticity together with histone modifications (Day and Sweatt, 2011; Zovkic et al., 2013). Moreover, it has been observed that associative learning was impaired in 16-month-old mice compared with that of 3-month-old mice which was associated with specific reduction in acetylation of H4K12 (Peleg et al., 2010).

Until now, most of the studies have analyzed DNA methylation in the brain of AD patients (Balazs, 2014). In this regard, a variety of studies suggest a genome-wide decrease in DNA methylation present in aging and AD patients (**Table 2**; Mastroeni et al., 2011). Interestingly, the folate/methionine metabolism is critically linked with DNA methylation mechanisms, consistently with this fact; studies show that folate and S-adenosyl methionine are significantly decreased in AD (Bottiglieri et al., 1990; Morrison et al., 1996). All this data indicates that AD patients produce a hypomethylation across the DNA genome. Recently, Bakulski et al. provided a semi-unbiased, quantitative, genome-wide localization of DNA epigenetic differences in frontal cortex of control and AD cases. These authors determined DNA methylation of 27, 587 CpG sites spanning 14,475 genes. Interestingly, they found that in control samples, the methylation state is markedly affected by age, with about the same number of sites being hypermethylated as hypomethylated with age. Compared with controls, 6% of genes featured on the array were differentially methylated in AD samples, but the mean difference was relatively modest (2.9%). Gene ontology analysis revealed a relationship between the main disease-specific methylation loci and several molecular functions and biological processes, including hypermethylation of genes involved in transcription and DNA replication, while membrane transporters were hypomethylated (Bakulski et al., 2012).

Table 2 | Epigenetic modifications implicated in Alzheimer's disease.

Observation	Sample
APP promoter hypomethylation in Alzheimer's disease patients (Miller, 2003).	Human brains.
Hypomethylation of promoters of ribosomal genes with aging (Decottignies and d'Adda di Fagagna, 2011).	Human lymphocytes.
Decrements in DNA methylation (Al-Mahdawi et al., 2014).	Human prefrontal cortex.
Differences in DNA methylation in a twin pair discordant for Alzheimer's disease (Al-Mahdawi et al., 2014).	Human temporal neocortex.
APP promoter methylation influenced by sex steroids and aging (Maloney et al., 2012).	Intact and gonadectomized mice brains.
PSEN1 is regulated by DNA methylation in response to metabolic stimuli (Zetzsche et al., 2010).	Non-human primate cortical areas of mice brains.

Also, some reports have focused on research DNA methylation at the 5′ promoter regions of candidate genes according to the basis of hypothesis concerning the molecular mechanisms of AD as microtubule-associated protein tau, amyloid precursor protein (APP) and presenilin-1 genes in the frontal cortex and hippocampus of both control and AD cases at different Braak stages. Interestingly, there wasn't any significant difference on CpG methylation between the control and AD samples (Barrachina and Ferrer, 2009). Other studies have reported hypomethylation of APP in the promoter region of normal 70 year-old human brain (Tohgi et al., 1999). However, as mentioned above, no difference was found in methylation of selected regions of the APP gene in various stages of AD progression (Barrachina and Ferrer, 2009). Also, it has been found that the change in methylation status differed among transcription factor binding sites of tau promoter (Wang et al., 2013).

Additionally to DNA methylation, histone modifications have been studied in recent years. Francis et al. investigated histone acetylation in mouse models of AD. In APP/presenilin1 double mutant transgenic mice, associative learning was impaired and this was linked to a marked reduction in H4K14 histone acetylation (Francis et al., 2009). Furthermore, studies *in vitro* have shown that exposure of cortical and hippocampal cultures to Aβ oligomers resulted in increased levels of acetylated H3K14 and a loss of dendritic spines, which was prevented by inhibition of histone acetyl transferase. Also, in young pre-plaque AD transgenic mice, these authors observed markedly increased levels of H3K14 and H3K9me2 compared with those of wild-type non-transgenic mice. Most importantly, similar changes were observed in histone transcription activating and repressive marks in the occipital cortex of AD samples (Lithner et al., 2013).

Although there are now treatments against AD, these are only palliatives and the pathology is currently incurable, whereby,

there is an intense interest in the development of new potential therapies. Epigenetic therapies have achieved some progress in the field of cancer, thus, several inhibitors of HDACs and DNA methylation are approved for hematological malignances by the US Food and Drug Administration and have been in clinical use for several years (Wu et al., 2008a). HDAC inhibitors (HDACIs) are the most thoroughly studied and have shown acceptable results in AD models. The inhibitors widely used in clinical research include trichostatin A (TSA), valproic acid (VPA), sodium 4-phenylbutyrate (4-PBA) and vorinostat (SAHA) (Wang et al., 2013).

In a study conducted by Su et al., VPA showed to inhibit Aβ production in HEK293 cell transfected with a plasmid carrying the Swedish APP751 mutation. Interestingly, using the APPV717F transgenic model of AD, VPA was able to inhibit Aβ production in the brain of mice at biologically relevant doses of 400 mg/kg (Su et al., 2004). In another study, VPA showed to decrease Aβ production and alleviate behavioral deficits by inhibiting GSK-3β-mediated γ-secretase cleavage of APP in APP23 transgenic mice (Qing et al., 2008). These results give us the idea about the possible contribution of epigenetic modifications in AD, which suggests that the drugs targeting epigenetic process may be of future therapeutic value (Wang et al., 2013).

As mentioned widely in scientific literature, the interaction between diet and epigenetics is the best documented in cancer pathology (Ho et al., 2009; Shu et al., 2010). Furthermore, based on evidence in support of epigenomics in regulating gene expression in stress-mediated AD risk factors, and the pathophysiology of AD, there has been growing interest in examining whether diet and nutraceuticals targeting epigenomics may prevent, delay, or reverse the course of AD (Chiu et al., 2014). In this regard, the Mediterranean diet rich in vegetables, fruits and nuts, legumes, olive oil and fish with relative low intakes of red meat has been suggested to reduce the risk for AD onset (Scarmeas et al., 2009; Frisardi et al., 2010). Other studies appoint that anti-oxidant-rich diets and consumption of dietary phytochemical such as caffeic acid, epigallocatechin-3-gallate, *Gingko biloba*, resveratrol and phenolic compounds present in red wine slowed down disease progression by inhibiting Aβ production or amyloid aggregation in animal models (Kolosova et al., 2006).

It is well known that DNA methylation occurs within folate/methionine/homocysteine (HCY) metabolism which uses micronutrients such as folate, methionine, choline and betaine enzyme's cofactors (Chouliaras et al., 2010; Wang et al., 2013). Diverse reactions occur and methionine is converted to S-adenosyl-methionine (SAM) and then converted to S-adenosyl-homocysteine (SAH), which in turn is converted to HYC in a reversible reaction. Most important, SAM is the common methyl donor for DNA methylation that regulates gene expression and determines the chromosome conformation (Sezgin and Dincer, 2014). An early study showed that SAM levels have been found to be decreased in post-mortem AD patients (Morrison et al., 1996). Also, lower bioavailability of SAM causes changes in the expression of genes involved in APP metabolism because this metabolite maintains the appropriate methylation of genes involved in APP processing (Sezgin and Dincer, 2014). Fuso et al. recently reported that reduction of folate and Vitamin B12 in culture medium of neuroblastoma cell lines cause a reduction in SAM levels resulting in an increase of PSEN1 and BACE levels together with Aβ production. Conversely, the simultaneous administration of SAM to the deficient medium restored the normal gene expression and reduced the Aβ levels (Fuso et al., 2007). Interestingly, the same group demonstrated that Vitamin B deficient-animals have shown that SAM inhibits the increase in progression of Alzheimer-like features (Fuso et al., 2012). This data suggests that folate or Vitamin B12-rich diets could be beneficial as therapy for AD patients; however, more studies are needed.

PARKINSON'S DISEASE AND EPIGENETICS

Parkinson's disease (PD) is the second most common neurodegenerative disorder after AD affecting approximately 1–2% of the population over the age of 65 and reaching a prevalence of almost 4% in those aged above 85. Resting tremor, bradykinesia, rigidity, and postural instability are the main clinical symptoms of the disease often accompanied by non-motor symptoms including autonomic insufficiency, cognitive impairment, and sleep disorders (Thomas and Beal, 2011; Coppedè, 2014). The brain of PD individuals is pathologically characterized by a progressive loss of neuromelanin containing dopaminergic neurons in the *substantia nigra* with the presence of eosinophilic, intracytoplasmic inclusions termed as Lewy bodies (structures containing aggregates of α-synuclein as well as other substances) and Lewy neurites in surviving neurons. Unfortunately, only some improvements of the symptoms are offered by current treatments based on levodopa and dopaminergic therapy, but there is no currently available treatment to avoid the progression of the disease (Thomas and Beal, 2011; Coppedè, 2014).

The vast majority of PD cases are idiopathic forms, likely resulting from a combination of polygenic inheritance, environmental exposures, and complex gene-environment interactions imposed on slow and sustained neuronal dysfunction due to aging (Migliore and Coppedè, 2009). In a minority of the cases, PD is inherited as Mendelian trail, and studies in PD families allowed the identification of at least 15 PD loci (PARK1-15) and several causative genes (Nuytemans et al., 2010). In addition, there are genes such as LRRK2, SNCA, MAPT and GBA that are associated with sporadic PD without family history (**Table 3;** Coppedè, 2012).

Most of the studies evaluating the role of epigenetic in pathogenesis have focused on the analysis of promoter methylation of causative PD genes in post-mortem brains and peripheral blood; however, the role of DNA methylation and its links to PD pathogenesis is currently unclear (Coppedè, 2012). Recent studies have shown that methylation of SNCA gene (the gene coding for α-synuclein) may be involved in disease via structural changes or overexpression of the protein, leading to protein aggregation or via impaired gene expression (Ammal Kaidery et al., 2013). In this regard, methylation of SNCA intron 1 has been demonstrated to be associated with decreased SNCA transcription, whereas reduced methylation at this site was found to be decreased in several brain regions, including

Table 3 | Epigenetic modifications of Parkinson's disease related genes.

Gene	Observation
SNCA	Reduced SNCA methylation in the substantia nigra of PD patients. SNCA gene silencing mediated by histone methylation (Nalls et al., 2014). Histone deacetylases inhibitors are neuroprotective against α-synuclein mediated neurotoxicity in PD animal models (IPDGC, 2011).
LRRK2	*Mutant LRKK2 antagonizes miR-184 in Drosophila melanogaster Parkinson's disease models* (IPDGC, 2011).
Parkin	*let-7 family miRNAs were under-expressed in parkin transgenic C.elegans* (Asikainen et al., 2010)
PARK16/Iq32, GPNMB	*Aberrant gene methylation in post-mortem Parkinson's disease brains* (IPDGC, 2011)

the *subtancia nigra* of sporadic patients, causing the increased expression of the SNCA gene (Jowaed et al., 2010). These results raise the possibility that the increased α-synuclein production that is associated with PD may result from increased SNCA expression, as a consequence of a decreased methylation state of its gene (Ammal Kaidery et al., 2013). Additionally, it has been demonstrated that α-synuclein sequesters DNMT1 in the cytoplasm, leading to global DNA hypomethylation in PD and dementia with Lewy body in post-mortem brains, as well as in transgenic mouse models (Desplats et al., 2011). Conversely, the overexpression DNMT1 in both transgenic mouse models and cellular cultures restore the nuclear level of the enzyme (Ammal Kaidery et al., 2013).

The regulation of SNCA by epigenetic histone modifications is yet to be studied in human PD brains. Studies in cell cultures and animal models of the disease, such as those induced by mitochondrial toxins, including 1-methyl-4-phenylpyridinium (MPP^+), paraquat, rotenone, or those overexpressing human α-synuclein, have revealed that α-synuclein translocates into the nucleus interacting with histones and inhibiting histone acetylation (Goers et al., 2003). Furthermore, in Drosophila models, nuclear-targeted α-synuclein has been shown to bind to histones and reduce histone 3 acetylation through its association with HDAC1 and SIRT2 (Kontopoulos et al., 2006).

In recent years, there has been considerable progress in the development of epigenetic-based drugs for the treatment of neurodegenerative disorders such as PD. Such inhibitors of HDACs and DNMTs are currently approved and available for clinical investigation (Xu et al., 2012). In this regard, the targeted downregulation of SIRT2 has been shown to ameliorate α-synuclein toxicity and dopaminergic loss in flies and in primary mesencephalic culture. Moreover, toxicity associated with nuclear-targeted α-synuclein in both SH-SY5Y neuroblastoma cells and flies can be rescued by using HDACIs (Outeiro et al., 2007), thus, HDACIs have been theorized to be efficacious in neurodegenerative diseases (Harrison and Dexter, 2013). In this regard, Wu et al. demonstrated that trichostain A (a well-known HDAC inhibitor), protects dopaminergic neurons from MPP^+ toxicity in primary neuron-glia co-cultures in a dose dependent manner (Wu et al., 2008b). Moreover, Kid and Schneider demonstrated that vorinostat (another HDAC inhibitor) protected two different dopaminergic neuronal cell lines from apoptosis induced by MPP^+ (Kidd and Schneider, 2010), thus, the above results give us an idea about the alternative therapy by inhibiting HDACs in PD patients.

Although the etiology of PD is still unknown, multiple lines of evidence support oxidative stress and mitochondrial dysfunction as part of the pathogenic cascade. It would be interesting to know whether antioxidants-rich diets that have a helpful effect in other degenerative disease such as AD (Kolosova et al., 2006), could have the same effect in PD patients. To this regard, therapy focusing on nutrition, neutraceutical and antioxidants as part of a healthy lifestyle might protect against cell death and thus delay or halt disease progress; however, clinical and basic studies are needed to prove such hypothesis (Bega et al., 2014).

EPILEPSY AND EPIGENETICS

Epilepsy is the third most common chronic brain disorder affecting 50 million of people worldwide (Aroniadou-Anderjaska et al., 2008). In this disorder, a variety of structures of the central nervous system such as the hippocampus, the amygdala and the piriform cortex are susceptible to trigger electrical discharges that contribute to brain damage and to the epileptogenic mechanism (Houser, 1990; Blümcke et al., 1999). These discharges promote some morphological changes in the hippocampus such as, cellular death in the CA1 and mossy fiber sprouting and dispersion of the granule cell layer, alterations that are thought to be involved in the formation of recurrent excitatory circuits that contributes to seizure susceptibility (Heck et al., 2004).

In this regard, it is well known that seizures can give rise to enduring changes that reflect alterations in gene expression patterns, contributing in this way to the hallmarks of epilepsy (Roopra et al., 2012). Moreover, some studies suggest that these long-term changes mediated by seizures are mediated via modulation of chromatin structure. One transcription factor in particular, the repressor element 1-silencing transcription factor (REST/NRSF) has received a lot of attention due to its association with a great sub-set of genes associated with important processes involved in neuronal homeostasis and because it may seem to recruit a variety of proteins that elicit epigenetic modifications such as histone deacetylases and histone methyltransferases (Bruce et al., 2004; Ballas and Mandel, 2005; Ballas et al., 2005; Johnson et al., 2006; Pozzi et al., 2013). Some reports have shown that the induction of seizures in animal models induce an overexpression in both REST/NRSF protein and mRNA levels (Formisano et al., 2007; Noh et al., 2012), suggesting that seizures may cause an unbalance in the epigenetic modifications that control important processes of neuronal homeostasis. In contrast, recent studies have shown that REST/NRSF is induced in the aging human brain regulating a network of genes associated with stress resistance (Lu et al., 2014). This evidence suggests that REST/NRSF regulates important processes in embryonic and adult neuronal homeostasis and that the dysregulation of this transcription factor may impair epigenetic modifications that regulate precisely an important network of genes contributing

to distinct neurological/ neurodegenerative disorders such as epilepsy or AD.

From a public health perspective, an alternative for the treatment of epilepsy is a change of lifestyle or diet. These methods have probably been used for over 2000 years and actually metabolic regulation of neuronal excitability is increasingly recognized as a factor in seizure pathologies and control (Stafstrom et al., 2008; Yuen and Sander, 2014). In this way, approximately half of the pharmacoresistant patients that have tried metabolism based therapies experience seizure control, opening the possibility of a strong link between the environments, in this case nutrition, with this pathology (Greene et al., 2003; Bough et al., 2006; Marsh et al., 2006; Patel et al., 2010).

These studies suggest that metabolism-based therapies such as ketogenic diets, calorie restriction or intermittent fasting leads to a range of biochemical and metabolic changes that induce a metabolic shift in pathways such as glycolysis, ketogenesis or beta oxidation, modifications that have been shown to increase seizure thresholds and to decrease epileptogenesis in animal models (Marsh et al., 2006; Patel et al., 2010).

Moreover, recent studies have shown that environmental inputs such as nutrition or exercise modulates cell metabolism, and critical links between metabolism and epigenetic control are beginning to emerge (Sassone-Corsi, 2013). For example, the availability of specific metabolites such as acetyl-coenzyme A (acetyl-coA) and nicotinamide adenine dinucleotide (NAD^+) dictates the efficacy of histone deacetylases (Katada et al., 2012).

In this regard, it has been shown that beta hydroxybutyrate (β-HB), a ketone body that rises with ketogenic diets, during strenuous exercise or during fasting (Newman and Verdin, 2014), acts as an endogenous inhibitor of histone deacetylases linking in a precise way metabolism, epigenetics and epilepsy (Shimazu et al., 2013). Thus, these studies strongly suggest that the neuroprotective effects exerted by these kinds of therapies are not only mediated via metabolism alterations but also by epigenetic modifications that may be involved in the expression of an unknown sub-set of genes related to epilepsy.

Other interesting epigenetic modifications involved in epilepsy are methylation of DNA. In this field, Kobow et al. using Methyl-seq, mapped for the first time the global DNA methylation patterns in chronic epileptic rats; they showed that chronic epilepsy in animal models is characterized for a global hypermethylation on DNA. Moreover, this group shows that ketogenic diets diminish this increase of DNA methylation, suggesting that these kinds of therapies exert their effect not only modulating metabolism, but also via epigenetic modifications (Kobow et al., 2013). More importantly, it opened the possibility for the development of new metabolism based therapies designed to regulate these epigenetic modifications contributing to the inhibition of the seizure threshold in epilepsy.

THE ROLE OF REST/NRSF IN NEUROLOGICAL DISORDERS

A growing body of literature suggests that long-term changes in gene transcription associated with a lot of neurological disorders are mediated via modulation of chromatin structure. One transcription factor in particular, REST/NRSF (repressor element 1-silencing transcription factor) (**Figure 4**), has received a lot of attention due to the possibility that it may control the expression of approximately 1,300 genes (Bruce et al., 2004; Johnson et al., 2006) that could be associated with a variety of processes that are important for neuronal homeostasis such as; synaptic transmission, synaptogenesis, excitability or even neurogenesis (Ballas and Mandel, 2005; D'Alessandro et al., 2009). REST modulates these genes in the nervous system recruiting protein complexes that elicit different epigenetic modifications (**Figure 4**; Roopra et al., 2012). Now it has been shown that REST is upregulated in pyramidal and dentate gyrus neurons after *status epilepticus* induced by kainate (Palm et al., 1998) or even by ischaemic insults (Formisano et al., 2007; Noh et al., 2012). Therefore, the upregulation of REST has been previously considered as harmful in mature neurons. In contrast, recent studies have shown that induced expression of REST/NRSF in mature hippocampal neurons is a protective mechanism that modulates the inhibitory homeostatic control of intrinsic excitability (Pozzi et al., 2013). Moreover, it has been shown that REST/NRSF protects neurons from age-related toxic insults in AD and surprisingly these levels seems to be associated with preservation of cognitive function and increased longevity (Lu et al., 2014). These findings suggest that basal levels of REST/NRSF are necessary for a normal physiological condition in the adult brain and that elevated levels of REST/NRSF, characteristic of epilepsy, may not be an epileptogenic factor, rather it seems to be a homeostatic mechanism triggered by repeated hyper-excitability *stimuli*. This is an open issue that needs further investigation.

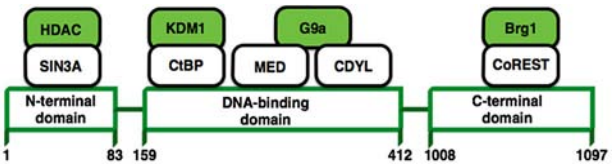

FIGURE 4 | REST structure and their interactions with other proteins. REST modulates the expression of its target genes by recruiting a host of lysine-modifying enzymes. Numbers refer to amino acid residues. Colored molecules possess enzymatic activity.

CONCLUDING REMARKS

As we state in this manuscript, one of the main factors that contributes to a variety of the most common diseases is the environment. Many epigenetic enzymes are potentially susceptible to changes in the levels of a variety of metabolites, and are, hence, poised to respond to changes on environment. In this sense, it has been demonstrated that changing our lifestyle could mediate great beneficial effects regulating a network of genes via the modulation of chromatin structure, providing new alternatives for the prevention of many diseases.

Different questions remain to be answered including which epigenetic modifications are implicated in neurological disorders, how does the environment mediate these changes, could pharmacological inhibitors of these modifications provide an alternative for treating disease, and so on. Increasing evidence on this field had taught us that these modifications are capable

of regulating great networks of genes that can influence a variety of physiological processes important for overall homeostasis and that the disruption of this balance can increase the risk of disease.

From a public health perspective, we need to better understand which alterations in metabolism and in chromatin structure cause disease and, maybe, it will be possible to design rationale metabolism-based therapies that could function as alternative treatments of these kinds of disorders.

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Anticonvulsant Effect of Time-Restricted Feeding in a Pilocarpine-Induced Seizure Model: Metabolic and Epigenetic Implications

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A new generation of antiepileptic drugs has emerged; however, one-third of epilepsy patients do not properly respond to pharmacological treatments. The purpose of the present study was to investigate whether time-restricted feeding (TRF) has an anticonvulsant effect and whether this restrictive diet promotes changes in energy metabolism and epigenetic modifications in a pilocarpine-induced seizure model. To resolve our hypothesis, one group of rats had free access to food and water *ad libitum* (AL) and a second group underwent a TRF schedule. We used the lithium-pilocarpine model to induce *status epilepticus* (SE), and behavioral seizure monitoring was analyzed. Additionally, an electroencephalography (EEG) recording was performed to verify the effect of TRF on cortical electrical activity after a pilocarpine injection. For biochemical analysis, animals were sacrificed 24 h after SE and hippocampal homogenates were used to evaluate the proteins related to metabolism and chromatin structure. Our results showed that TRF had an anticonvulsant effect as measured by the prolonged latency of forelimb clonus seizure, a decrease in the seizure severity score and fewer animals reaching SE. Additionally, the power of the late phase EEG recordings in the AL group was significantly higher than the TRF group. Moreover, we found that TRF is capable of inducing alterations in signaling pathways that regulate energy metabolism, including an increase in the phosphorylation of AMP dependent kinase (AMPK) and a decrease in the phosphorylation of Akt kinase. Furthermore, we found that TRF was able to significantly increase the beta hydroxybutyrate (β-HB) concentration, an endogenous inhibitor of histone deacetylases (HDACs). Finally, we found a significant decrease in HDAC activity as well as an increase in acetylation on histone 3 (H3) in hippocampal homogenates from the TRF group. These findings suggest that alterations in energy metabolism and the increase in β-HB mediated by TRF may inhibit HDAC activity, thus increasing histone acetylation and producing changes in the chromatin structure, which likely facilitates the transcription of a subset of genes that confer anticonvulsant activity.

Keywords: anticonvulsant, pilocarpine, AMP kinase, Akt kinase, histone 3 acetylation, beta-hydroxybutyrate, HDACs inhibition

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INTRODUCTION

Epilepsy is the third most common chronic brain disorder. It affects 50 million people worldwide (Aroniadou-Anderjaska et al., 2008). Although a new generation of antiepileptic drugs has emerged, approximately 30% of epilepsy patients do not respond to classical pharmacological treatment (Löscher et al., 2013). For this reason, it is important to find new alternatives to complement pharmacological therapy in drug-resistant patients. To date, a variety of reports suggest that some metabolism-based therapies, such as ketogenic diet (KD) or calorie restricted (CR) diets, have an anticonvulsant effect (Bough et al., 2003; Stafstrom and Rho, 2012). Recently, it has been suggested that the beneficial effect of these diets may be produced by means of a metabolic shift involving the activation of AMP-activated protein kinase (AMPK), inhibition of the mammalian target of rapamycin (mTOR) and overproduction of ketone bodies (Wong, 2010; McDaniel et al., 2011; Yuen and Sander, 2014).

Time-restricted feeding (TRF) is a nutritional challenge that limits food availability to a brief time during the waking phase in mammals (Belet and Sassone-Corsi, 2010). This restrictive model induces an increase in free fatty acids (FFA) before feeding and an increase in peroxisomal markers, such as PPARα and PPARγ (Rivera-Zavala et al., 2011), suggesting that it may modulate a global metabolic shift that resembles the effects of other metabolism-based therapies.

On the other hand, environmental inputs, such as nutrition, are able to alter cell metabolism. In this sense, functional links between metabolism and epigenetic control are beginning to emerge (Sassone-Corsi, 2013). The regulation of gene expression by epigenetic modifications can occur through a variety of means. To date, the best characterized include DNA methylation, non-coding RNAs and histone posttranslational modifications (Hullar and Fu, 2014).

Histone posttranslational modifications, such as acetylation, occur at specific lysine residues and have been correlated with transcriptional activation (Sassone-Corsi, 2013). Histone deacetylases (HDACs) are enzymes that elicit the induction of repressive chromatin using specific metabolites, such as nicotinamide adenine dinucleotide (NAD^+), whose availability dictates the efficacy of the enzymatic reaction (Katada et al., 2012). Interestingly, it has recently been shown that β-hydroxybutyrate (β-HB), a ketone body produced during fasting or starvation conditions, act as an endogenous inhibitor of HDACs, thus linking metabolism with gene expression (Shimazu et al., 2013).

In spite of these findings, there are no reports showing that TRF may produce beneficial effects, such as those of KD and CR, in an acute seizure model. For this reason, the purpose of this study was to determine whether TRF induces a metabolic shift by activating the energy sensor AMPK, inhibiting the Akt signaling pathway and producing epigenetic modifications that are capable of diminishing seizure susceptibility. Here, we report that TRF had anticonvulsant effects observed as prolonged latency to first seizure, a decrease in the seizure score, and a diminished number of animals that reached *status epilepticus* (SE). Additionally, a reduction in the power of the late phase electroencephalography (EEG) recordings in the TRF group was significantly greater than that in the AL group. Furthermore, TRF produced an increase in the β-HB concentration, activation of AMPK, inhibition of Akt kinase and increased histone 3 (H3) acetylation. These findings suggest that activation of the AMPK signaling pathway together with an increase in ketone bodies could mediate the acetylation of H3, thus contributing to the transcription of a subset of genes conferring anticonvulsant activity.

MATERIALS AND METHODS

Time-Restricted Feeding Schedule and Pilocarpine-Induced Seizure Model

Ninety-five young adult male Wistar rats (8 weeks of age) weighing approximately 220 g obtained from Harlan laboratories (USA) were used and maintained under constant temperature conditions (25°C) and a 12 h light/12 h dark cycle. Animals were fed with a standard diet of Lab Diet Rodent Laboratory Diet 5001 pellets (PMI Nutrition International, Inc., Brentwood MO) and water *ad libitum* (AL, control and pilocarpine groups) or underwent the time-restricted feeding (TRF and TRF plus pilocarpine groups) schedule described by Rivera-Zavala et al. (2011). Briefly, TRF consisted of allowing rats to feed freely for only 2 h daily for 20 days, and after this period of time, we proceeded to perform the acute seizure model at day 21. For the acute seizure model, we chose the lithium-pilocarpine model because it is one of the most widely used models to induce SE and is an excellent model that resembles human temporal lobe epilepsy (Lemos and Cavalheiro, 1995). Animals were first injected with lithium-chloride (3 mEq/kg, i.p.) at day 20; 18 h later, animals received a scopolamine methyl nitrate injection (1 mg/kg, s.c.) 30 min prior to minimize the peripheral cholinergic effects of pilocarpine. Pilocarpine was administered (60 mg/kg, s.c.) to induce SE, and the latter was maintained for 90 min; immediately afterwards, animals received an injection of diazepam (Valium^{TM}, 5 mg/kg i.m.) to stop the seizures. The same procedure was performed for control animals that did not receive a pilocarpine injection but rather received a saline (0.9%) injection (**Figure 1A**). It is important to mention that SE was induced after 6 h of fasting in AL-pilocarpine rats and ≈ 22 h fasting in TRF-pilocarpine rats to avoid any changes in metabolism; both AL- and TRF-pilocarpine animals received a saline solution injection to avoid dehydration. Twenty-four hours after the pilocarpine injection, experimental animals were sacrificed with an overdose of sodium pentobarbital (26 mg/kg) to perform biochemical analyses. All experiments from the present study were approved by the Ethical Committee of the School of Medicine at UNAM following all of their statements to minimize animal suffering.

Physical/Biochemical, Food Intake, Glucose Tolerance, Brain and Liver Weight Measurements

The food intake and body weight from each animal were monitored daily, and both blood glucose and the β-HB

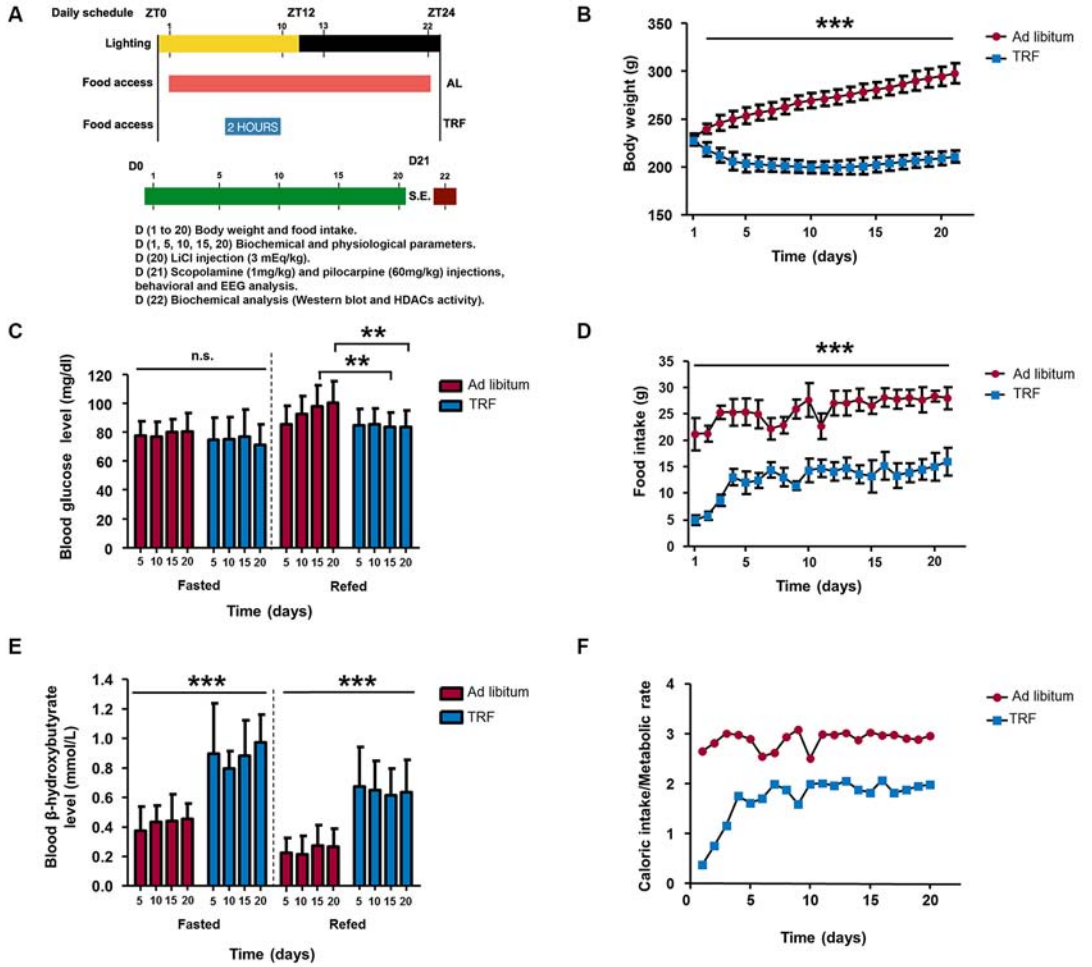

FIGURE 1 | Influence of the time-restricted feeding (TRF) model on body weight, food intake, glucose, and β-hydroxybutyrate in the fasted/refed state and the ratio among kilocalorie consumption and basal metabolic rate (BMR) in rats. (A) Schematic representation of the experimental procedure of the dietary schedule and *status epilepticus* (SE) induction. Body weight of TRF rats showed a significant decrease at all time points measured **(B)**; moreover, there is a reduction in food intake and the ratio of caloric intake/metabolic rate compared with that of *ad libitum* (AL)-fed animals **(D,F)**. Regarding biochemical parameters, the blood glucose concentration showed no significant difference in the fasted state; however, AL-fed rats showed an inability to metabolize glucose at 15 and 20 days, which does not occur in TRF animals **(C)**. Interestingly, the blood β-hydroxybutyrate concentration was high during the TRF schedule and it was maintained even in the refed state, even though it is lower than during the fasted state **(E)**. Data are expressed as the mean ± SD from each determination ($n = 30$, $^{**}p < 0.01$; $^{***}p < 0.001$).

concentration were measured using a digital monitor system (Optium Xceed glucometer, Abbott USA) with specific glucose and β-HB strips at 5, 10, 15 and 20 days. For blood sampling, animals from the AL group were fasted for 6 h before measurement and the TRF group was measured before feeding with chow (≈ 22 h fasting). For glucose tolerance, we performed an additional experiment consisting of measuring the blood glucose in fasted rats, and then, animals were injected intraperitoneally with a glucose solution (refed rats, 1 g/kg body weight) and their blood glucose was measured again after 60 min. The same experimental procedure was carried out for the β-HB measurement to verify whether the β-HB levels remained or decayed after receiving a glucose injection. In addition, we calculated the ratio of the daily caloric intake (kcal/day) consumed for each animal from the AL and TRF groups and divided by basal metabolic rate (BMR) using an allometric scaling formula ($BMR = 70M^{0.75}$, where M means mass) to verify whether the TRF schedule could have an effect on growth in animals. Finally, the brains and livers from the AL and TRF groups were perfused with saline, dissected and weighed to examine whether dietary restriction could affect the size of each organ.

Latency to the First Seizure, Seizure Score and Number of Animals that Reached *Status Epilepticus*

After administration of pilocarpine, animals were continuously video monitored for seizure activity for at least 5 h by an investigator-blinded to the treatment who then scored the

behavioral seizures. Latency to the first seizure was measured in minutes until the first forelimb clonus appeared, and the seizure score and the number of rats that reached SE were also analyzed. The behavioral seizures induced by pilocarpine were scored according to a modified version of the Racine scale (Bough et al., 2002). The latency to first seizure, seizure severity score, and individuals with SE were averaged across animals in each group.

Western Blot Analysis

Experimental animals were sacrificed with an overdose of anesthesia and rat brains were dissected as quickly as possible. Immediately afterwards, hippocampi were obtained and fractioned into cytoplasmic and nuclear extracts using a subcellular fractionation kit (Thermo Scientific, USA) following the manufacturer's instructions. Protein extracts were quantified by a BCA assay kit (Pierce, USA), and 60 μg of protein was loaded on 12 or 15% SDS-PAGE gels. Proteins were transferred to a PVDF membrane in a semi-dry electrophoretic transfer system (BioRad, USA). Then, membranes were rinsed with Tris-buffered saline (TBS) and blocked with a solution containing 5% non-fat dry milk in TBS-Tween 20 0.1% (TBST) overnight at 4°C. Blots were probed with rabbit polyclonal anti-pAMPK (Thr172; 1:1000, Cell Signaling Technology, AB 2535), polyclonal anti-pAkt (Ser473; 1:1000 Cell Signaling Technology, AB 4060) and the H3 acetylation at lysine residues 9 and 14 (H3K9ac and H3K14ac; 1:1000, Cell Signaling Technology, AB 9649 and AB 7627, respectively) in TBST overnight at 4°C. After three rinses with TBST for 5 min each, membranes were incubated with a goat anti-rabbit IgG secondary antibody (1:2500 Cell Signaling Technology, AB7074) for 2 h at room temperature followed by three rinses with TBST for 5 min each. The band signal was detected by a chemiluminescence kit (Millipore, USA) on Amersham Hyperfilm. For loading controls, rabbit polyclonal antibodies against total AMPK, Akt, and H3 (1:1000, Cell Signaling Technology, AB 5831, AB 4691 and AB 4499, respectively) were used. Densitometry measurements of the detected bands images were taken with a CCD camera (DNR Bio-Imaging Systems), and the analysis was carried out with MCID image analysis software (InterFocus Imaging Ltd., Cambridge, UK).

Histone Deacetylase Activity Assay

Protein nuclear extracts from hippocampal homogenates were quantified by a BCA assay kit (Pierce, USA), and 80 μg of protein was used to measure HDAC activity (specifically class I type) with a histone deacetylase assay kit (Sigma-Aldrich, USA) following the manufacturer's instructions. All samples were analyzed in duplicate, and the total HDAC activity was calculated using the following formula:

$$\text{Activity(ng/h/mg)} = \frac{[\text{RFU (control} - \text{blank)} - \text{RFU (sample} - \text{blank)}]}{\text{slope} \times \text{h} \times \text{protein amount added}}$$

where RFU indicates (relative fluorescence units) the slope (HDAC concentration) and h (initial incubation time).

Electroencephalography (EEG) Recording and Frequency Analysis

Ten rats (n = 5 each) were chronically implanted under ketamine/xylazine mixture anesthesia (4 ml/kg). For EEG recording, a stainless steel screw electrode was threaded into the bone epidurally, 1.5 mm lateral and 2 mm posterior to the bregma. Two additional stainless steel screws driven into the bone above the frontal sinus, and the cerebellum served as the reference to ground electrodes. All three screws were secured to the skull with dental acrylic. Seven to ten days were allowed for recovery. EEG signals were recorded monopolarly in freely moving rats from inside a shielded chamber. The EEG recordings were measured with a P15 preamplifier (Grass Instruments Company, USA), amplified 2000 times, filtered (0.1–100 Hz) and digitized at 20 kHz with an analog-to-converter (Micro-1401, CED, Cambridge, UK) and then saved onto the hard drive of a computer. The EEG signals were analyzed off-line with Spike2 (CED) and MatLab software; the data were first band-pass filtered (FIR filter, 1–50 Hz and 51–100 Hz). Subsequently, a Hanning window was applied on blocks of 8192 sample points (bin size 0.12 Hz) of EEG data before the power spectra were calculated using Fast Fourier Transformations (FFT). The total spectral power between 1 and 50 Hz and between 51 and 100 Hz analyses were assessed from artifact-free EEG segments (3 min). The power spectra obtained by using FFT were divided into 1.25–4.5 Hz, 4.75–6.75 Hz, 7.0–9.5 Hz, 9.75–12.5 Hz, 12.75–18.5 Hz, 18.75–35.0 Hz, 35.5–42.0 Hz, and 51.0–100 Hz frequency bands. The normalized power bands below 42 Hz exhibited similar temporal patterns, but were significantly different compared to the normalized power spectrum of 51.0–100 Hz. Therefore, power analysis was conducted in the bands between 1–50 Hz and 51–100 Hz.

Statistics

The values of relative optical units were examined to test the normality of a data set. Then, a two-tailed unpaired Student's t-test and Mann-Whitney U test were used for the body weight, food intake, brain and liver tissue weight, behavioral analysis and EEG recording frequency analysis. One-way ANOVA with Tukey's *post hoc* test were used for data from western blots and HDAC activity tests, while repeated measures ANOVA with Bonferroni's *post hoc* test was used for the blood glucose and β-HB levels. Pearson's or Spearman's correlation test were used for correlation among the seizure latency/score vs. β-HB levels. In addition, Chi squared and Fisher's exact test were applied for the qualitative results (number of animals that reach SE or not). All statistical tests were performed using GraphPad Prism statistics software version 5 (GraphPad Software, San Diego, CA, USA) and $p < 0.05$ was considered statistically significant.

RESULTS

Metabolic Shift Induced by a Time-Restricted Feeding Model

To establish whether the TRF model induces a general metabolic shift, we measured the body weight, food intake, blood glucose

and β-HB concentration. As we observed, animals that were subjected to the TRF schedule significantly lost weight at day 2 and continued up to day 11. Surprisingly, animals began to gain weight at day 15 and recovered to almost their starting weight at the end of the dietary schedule; however, compared with the control fed AL, all weights were significantly lower (**Figure 1B**, $p < 0.001$). According to food intake, rats subjected to TRF consumed less food (5.8 gr) within the first 3 days compared to AL animals (21.3 gr; **Figure 1D**, $p < 0.001$), and this result correlates with dramatic weight loss (**Figure 1B**). At day 4, animals started to consume more food (13 gr) and began to stabilize their weight. However, food consumption was significantly lower compared to the AL rats throughout the dietary schedule (**Figure 1D**). To verify whether the TRF schedule could have an effect on animal growth, we calculated the ratio of the daily caloric intake (kcal/day) consumed by each animal from the AL and TRF groups and divided this by the BMR. In this regard, we observed that in the first 2 days, the animals that followed the TRF schedule had a caloric intake below their metabolic rate; however, ≈ at day 5, these animals increased their caloric intake and maintained this ratio throughout the dietary schedule. Conversely, the AL group tended to eat three times more calories needed to maintain their BMR (**Figure 1F**).

Interestingly, even though a slight decrease was observed in the blood glucose concentration of TRF animals after 5, 10, 15 and 20 days, there was no significant difference compared with AL-fed animals in a fasting condition (**Figure 1C**). In addition, we studied possible alterations in glucose tolerance after an injection of a glucose solution (re-fed condition) in fasted rats in both experimental groups. In this regard, glucose from the AL-fed animals was significantly higher at day 15 and 20 compared with that of the group that followed the TRF schedule. Furthermore, the blood β-HB concentration was significantly higher in the TRF group at all-time points of the dietary schedule compared with that of AL-fed animals (**Figure 1E**, $p < 0.001$). Surprisingly, after refed conditions, β-HB levels remained higher in TRF rats compared with that of AL-fed animals. Nevertheless, the levels of this ketone body were lower compared to TRF rats in a fasted state.

Ultimately, to investigate the effect on organ weight, such as the brain and liver in animals submitted to the TRF schedule, we weighed both organs in each group of animals. We found a slight decrease in the brain weight of TRF rats (1.76 ± 0.09 g, $n = 8$) compared with AL animals (1.84 ± 0.11 g); however, such a decrease was not significant (**Figures 2A,C**). Conversely, livers from TRF rats showed a statistically significant decrease (8.98 ± 0.92 g, $n = 8$, $p < 0.001$) compared with those of AL-fed rats (16.08 ± 1.05; **Figures 2B,D**). These data demonstrate that the TRF schedule has an effect on weight loss and food intake and that it influences the overproduction of ketone bodies, as measured by β-HB. Moreover, high β-HB levels remained in TRF animals after glucose injection indicating that dietary schedule can modulate alternative energy metabolic pathways and maintain the production of ketone bodies despite glucose availability.

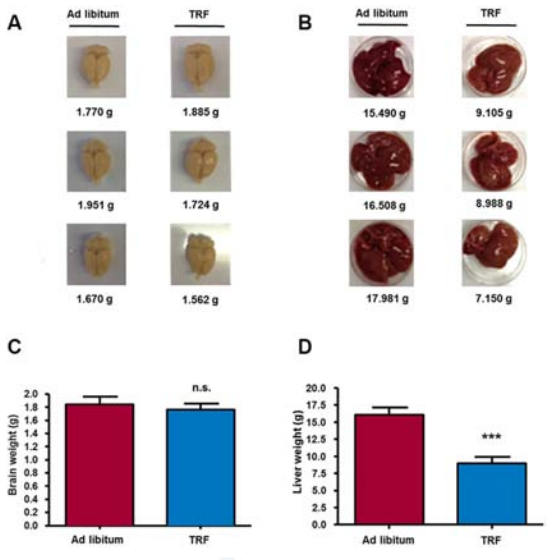

FIGURE 2 | TRF model decreases liver weight, but not brain weight. At the end of the dietary schedule, the mean liver weight of TRF rats showed a significant decrease compared with that of AL-fed animals **(B,D)**; on the other hand, brain weight was not influenced by the time restricted feeding model **(A,C)**. Data are expressed as the mean ± SD from each determination ($n = 10$, $^{***}p < 0.001$).

Time-Restricted Feeding Induces Changes in Metabolism-Related Signaling Pathway Components

It is well known that AMPK and Akt kinases are the main components of the signaling pathways involved in metabolic regulation and cell growth (Burkewitz et al., 2014; Guo, 2014). In this regard, homogenates from the TRF hippocampus showed a statistical increase in AMPK phosphorylation ($n = 8$, $p < 0.001$) and a significant decrease in Akt phosphorylation compared with that of AL-fed animals homogenates ($n = 8$, $p < 0.001$; **Figures 3A,B**), indicating that TRF regulates the activity of metabolism-related signaling pathway components. To corroborate similar changes in peripheral tissues, we performed western blot analysis in liver homogenates. Our results showed that there was a statistically significant increase in AMPK phosphorylation and a significant decrease in Akt phosphorylation in liver homogenates from TRF rats compared with those of AL-fed animals (**Figures 3C,D**). These results indicate that the TRF schedule can induce changes in metabolism-related signaling pathways in the central nervous system and peripheral organs, such as liver tissue.

Anticonvulsant Effect of Time-Restricted Feeding in the Pilocarpine-Induced Seizure Model

To examine the anticonvulsant activity of the TRF schedule in an acute seizure model, we performed behavioral and EEG recordings. All of the control animals fed AL treated with pilocarpine had generalized seizures lasting at least 2 h. The

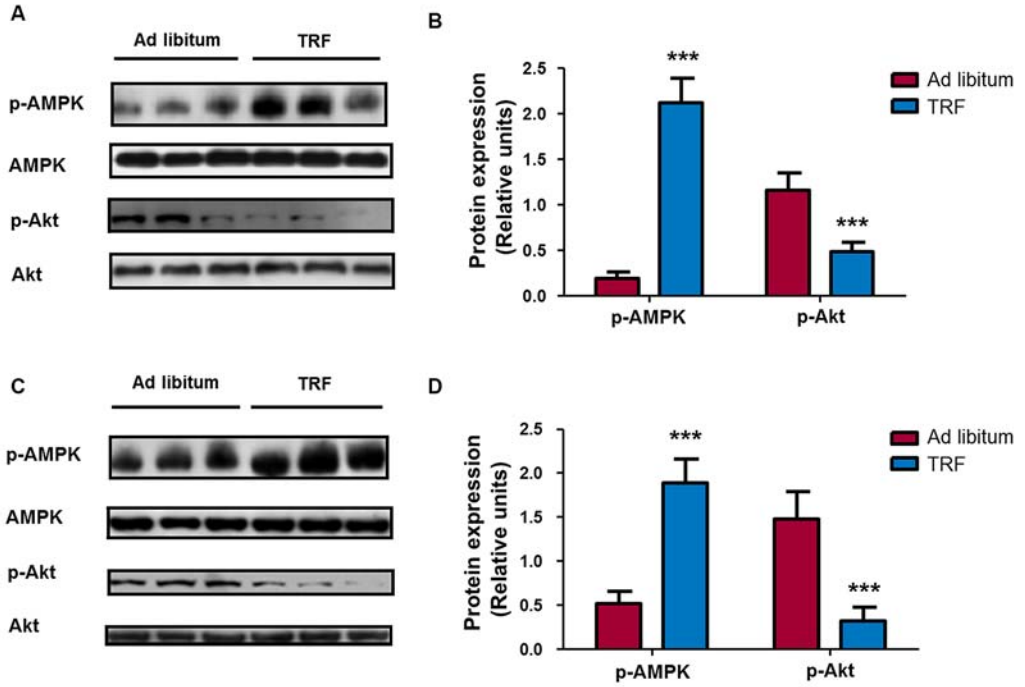

FIGURE 3 | Effects on metabolic related signaling pathways during TRF schedule in brain and liver tissues. Immunoblots from hippocampal rat brain homogenates that followed dietary restriction revealed a significant increase in AMP dependent kinase (AMPK) phosphorylation at threonine Thr172 content compared with that of their respective AL-fed control animals. Conversely, homogenates from TRF rats showed a significant decrease in Akt phosphorylation at serine 473 compared with that of their AL-fed controls **(A,B)**. Additionally, similar results were obtained in liver homogenates, where there was an increase in AMPK phosphorylation and a decrease in Akt phosphorylation **(C,D)**. Samples were normalized with total AMPK or Akt. Bar charts are the semiquantitative optical densities of immunostained bands **(B)**. Data are expressed as the mean ± SD from each determination ($n = 8$, $^{***}p < 0.001$).

mean latency to the first forelimb clonus was 26.53 ± 5.02 min (**Figure 4A**); the mean seizure score was 4.66 ± 1.15 (**Figure 4B**), and 27 of 30 animals reached SE (**Figure 4C**, $n = 30$). In addition, seven of these animals died within 24 h. Conversely, the TRF group showed a significant increase in latency to forelimb clonus seizure of 37.93 ± 6.60 min ($n = 30$, $p < 0.001$; **Figure 4A**) and a significant decrease in the mean of seizure score (3.53 ± 0.82, $n = 30$, $p < 0.001$; **Figure 4B**). Most importantly, only 19 out of 30 animals reached SE and only two out of 30 died within 24 h, thus improving survival ($n = 30$, $p < 0.05$; **Figure 4C**).

A High Level of β-Hydroxybutyrate Positively Correlates with Seizure Latency and Negatively in the Seizure Severity Scale

Additionally, to determine whether changes in β-HB concentrations are involved in anticonvulsant activity, we measured the β-HB concentration in the blood before seizure induction (see above). In this regard, we found that an increase in seizure latency is positively correlated with the blood β-HB concentration ($n = 10$, $R^2 = 0.772$, $p < 0.05$). Conversely, we found that the seizure severity score was negatively correlated with high β-HB levels ($n = 10$, $R = 0.6407$, $p < 0.01$), suggesting that the blood β-HB concentration has an important role in the anticonvulsant activity mediated by TRF (**Figure 4D**).

Time-Restricted Feeding Decreases the Power of EEG Seizures in the Pilocarpine-Induced Seizure Model

In addition, we recorded EEGs from 10 rats ($n = 5$ each) to study the power spectra of the seizures. The mean power differences between 3 min intervals were tested, and one from each data point in the AL and TRF groups were compared for 90 min after pilocarpine injection (**Figures 5C,D**). Fast Fourier Transform analysis (FFT) of EEG recordings revealed a significant increase in the total spectrum, measured as the total power in $\mu V^2/H$ in both groups after pilocarpine administration. The time course of the power spectrum in both groups (AL and TRF) showed that after a latency period (from 21–48 min), an increase until a maximum peak power for several thousand times occurred and then gradually declined to a nonzero level. There were no significant differences between the mean latency of EEG seizure in the data from the spectral power obtained with a broadband of 1–50 Hz (**Figure 5C**) and 51–100 Hz (**Figure 5D**) filtering. However, the time to the peak of the power spectrum of the broadband 1–50 Hz in the AL group was significantly longer than that in the TRF group ($p < 0.05$). At approximately

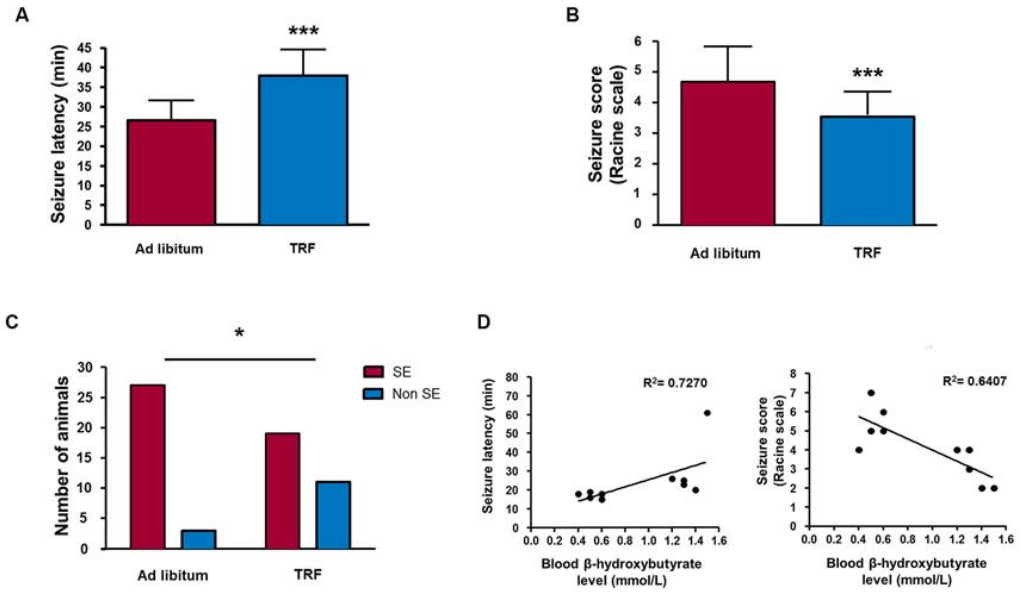

FIGURE 4 | TRF inhibits seizure susceptibility measured by behavioral analysis. TRF animals showed an increased latency **(A)**, a significant decrease in the mean seizure score **(B)** and a smaller number of animals reaching *status epilepticus* (SE) **(C)** compared with that of AL-fed rats. Moreover, there was a positive statistically significant correlation among high β-HB levels and decreased latency to the first seizure and a negative correlation between high β-HB levels and a reduction in seizure score in rats subjected to TRF **(D)**. Data are expressed as the mean ± SD from each determination (n = 30 for behavioral analysis, *$p < 0.05$; ***$p < 0.001$; n = 10 for Pearson's or Spearman's correlation test, $p < 0.05$ and $p < 0.01$, respectively).

90 min after the application of pilocarpine, the power of the TRF group was significantly lower than that of the AL group (**Figures 5C,D**). An illustration of a representative EEG of AL and TRF after pilocarpine injection is shown (**Figures 5A,B**, respectively).

Time-Restricted Feeding Inhibits Histone Deacetylase Activity in Hippocampal Nuclear Extracts

Previous reports described that β-HB could act as an endogenous histone deacetylases class I inhibitor (Shimazu et al., 2013). To determinate whether the β-HB levels produced by TRF may exert a similar action in hippocampal homogenates, we performed a total HDAC activity assay. As seen in **Figure 6A**, animals fed AL had a total activity value of 0.104 ± 0.036 ng/h/μg; however, animals that followed the TRF schedule presented a reduced of activity value of 0.030 ± 0.017 ng/h/μg (n = 4, $p < 0.05$), indicating an important reduction of HDAC activity compared with that of AL-control animals. On the other hand, SE-induced animals fed with AL had a similar activity value (0.103 ± 0.043 ng/h/μg, n = 4, $p < 0.05$) as control animals. In addition, pilocarpine-injected animals that followed the TRF schedule had a decrease in the activity value (0.030 ± 0.030 ng/h/μg) corresponding to a reduction in HDAC activity compared with AL-pilocarpine-injected animals (**Figure 6D**). These results are compatible with the hypothesis that the β-HB levels produced by TRF are able to regulate HDAC activity in hippocampal homogenates and indicated that feeding restriction may exert an inhibitory effect, as previously described in other *in vitro* and *in vivo* models (Shimazu et al., 2013).

Dietary Restriction Induces Epigenetics Changes by Increasing H3 Acetylation in the Hippocampal Nuclear Fraction

To demonstrate whether the TRF model induces epigenetic modifications (principally by an inhibitory effect on HDAC activity), we measured the H3 acetylation protein levels in the hippocampal nuclear fraction by immunoblotting using antibodies that recognize acetylated H3 at lysine 9 and lysine 14 (H3K9ac and H3K14ac, respectively). As observed in **Figures 6B,C**, the content of H3 acetylation in hippocampus homogenates from animals that followed the TRF schedule before seizure induction showed a statistically significant increase in both H3K9ac and H3K14ac epitopes compared with that of AL-fed animals plus pilocarpine or AL-fed control animals (n = 8, $p < 0.001$; **Figures 6E,F**). These results confirm that the TRF schedule might be able to induce epigenetic tags by increasing the β-HB concentration via their inhibitory action on HDAC activity.

DISCUSSION

Dietary treatment for epilepsy has likely been used since ancient times (Yuen and Sander, 2014). The use of metabolism-based diets for the treatment of refractory epilepsy, mainly the classical KD and its variants, is relatively common (Patel et al., 2010). Unfortunately, these diets can produce early onset complications,

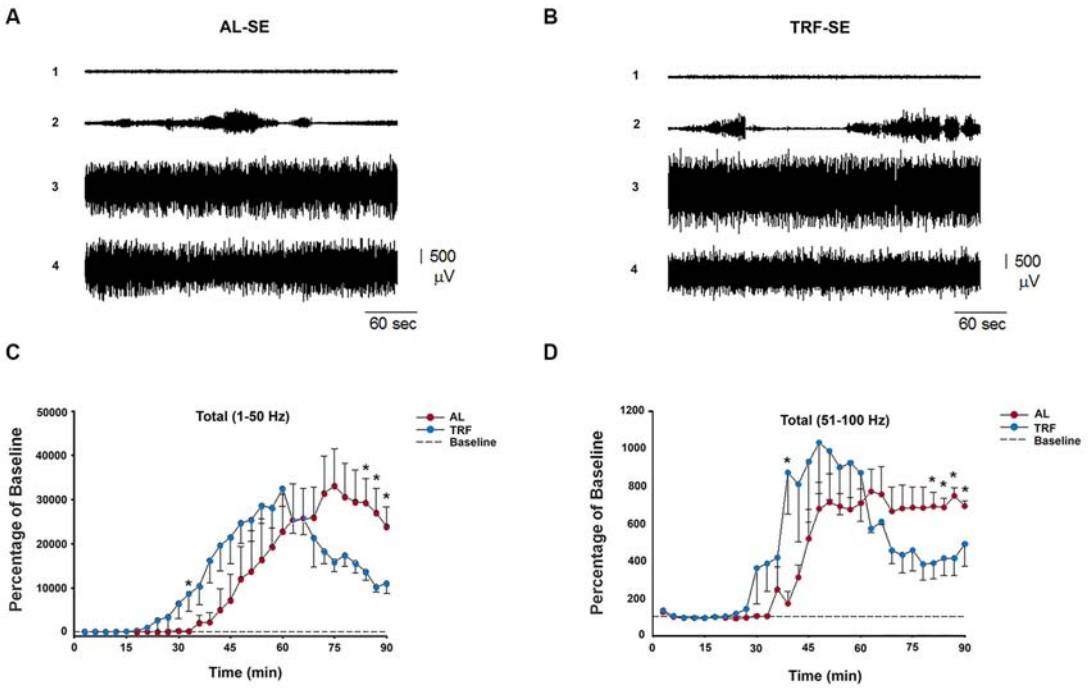

FIGURE 5 | Representative electroencephalography (EEG) signal and FTT analysis from AL and TRF pilocarpine-injected rats. The upper tracings show typical EEG recordings before administration of pilocarpine in AL rats **(A)** and TRF rats **(B)** (n = 5 each). The numbers at the left of each record indicate the time after the application of pilocarpine. Plots of the mean power spectra as a function of time for the EEG recording taken from rats injected with pilocarpine. Power values were calculated as the percent of the 45 min baseline power recorded before pilocarpine injection, assigned as a value of 100% (data not shown). **(C)** Fast fourier transformations (FFT) of the 1–50 Hz frequency spectrum. **(D)** FFT of the 50–100 Hz frequency spectrum. The red symbol represents the mean data from AL rats and the blue symbol represents the mean values from TRF rats. AL: (1) Baseline period, (2) 32, (3) 65, and (4) 75 min, time after the application of pilocarpine and TRF: (1) Baseline period, (2) 15, (3) 40, and (4) 75 min, time after the application of pilocarpine. Data are expressed as the mean ± SEM (*$p < 0.05$).

such as hypoglycemia, loss of body weight, ketosis, metabolic acidosis and late-onset complications, including renal stones and hypocarnitinemia (Kang et al., 2004). Nevertheless, the KD has been widely used to control seizures in children with epilepsy and with relative success in adolescents and adult patients (Hallböök et al., 2007; Nei et al., 2014).

Caloric restriction (CR) produces a range of metabolic and biochemical changes, including reduced glucose concentration, formation of ketone bodies and increased AMP-activated protein kinase activity (Maalouf et al., 2009; Burkewitz et al., 2014; Longo and Mattson, 2014; Yuen and Sander, 2014). In this regard, the present study demonstrated that TRF produces similar metabolic changes, such as an increase in the blood β-HB concentration. Furthermore, we found that after 2 days on the TRF schedule, animals presented with a significant reduction in body weight compared with that of AL-fed animals. Most importantly, at day 5, animals that followed the TRF schedule had a reduction of 10% of their body weight; however, this group tended to recover their weight at day 15, confirming that this model induces body weight loss, as has been previously described (Amigo and Kowaltowski, 2014). Interestingly, a similar result in body weight loss was observed in young rats subjected to a 15% of CR schedule for 30 days (Phillips-Farfán et al., 2015), suggesting that both diets may have similar effects. Such a drastic weight reduction can be correlated with a decrease in food intake, as observed in the TRF model during the early days of the study. Surprisingly, TRF animals began to increase their food consumption and gain weight as the study progressed. Nevertheless, food intake and body weight were lower compared to AL-fed rats. Regarding blood glucose levels, there were no significant changes between the AL and TRF groups, which showed similar results to other dietary models (Phillips-Farfán et al., 2015). However, in glucose tolerance experiments, TRF-subjected animals seemed to better metabolize glucose than AL-fed animals at 15 and 20 days, suggesting that glucose metabolism in TRF animals is more efficient or is not altered as in AL-fed animals. In this regard, it has been described that a short-term CR (21 days) improves glucose homeostasis in 1 and 2 years-old rats and that this beneficial effect involves AMP-activated protein kinase (Pires et al., 2014).

Regarding the evaluation of seizure behavior, the TRF schedule produced a prolonged latency as well as a decrease in the seizure severity score, and fewer animals reached SE after pilocarpine injection. Indeed, such results were consistent with a previous report where intermittent fasting had positive effects, such as a greater number of surviving animals, a fewer number of animals that reached SE and a reduction of the number of animals that became epileptic (Parinejad et al., 2009).

Electrophysiologically, the initial part of the EEG response and the onset/peak of the EEG between TRF and AL animals

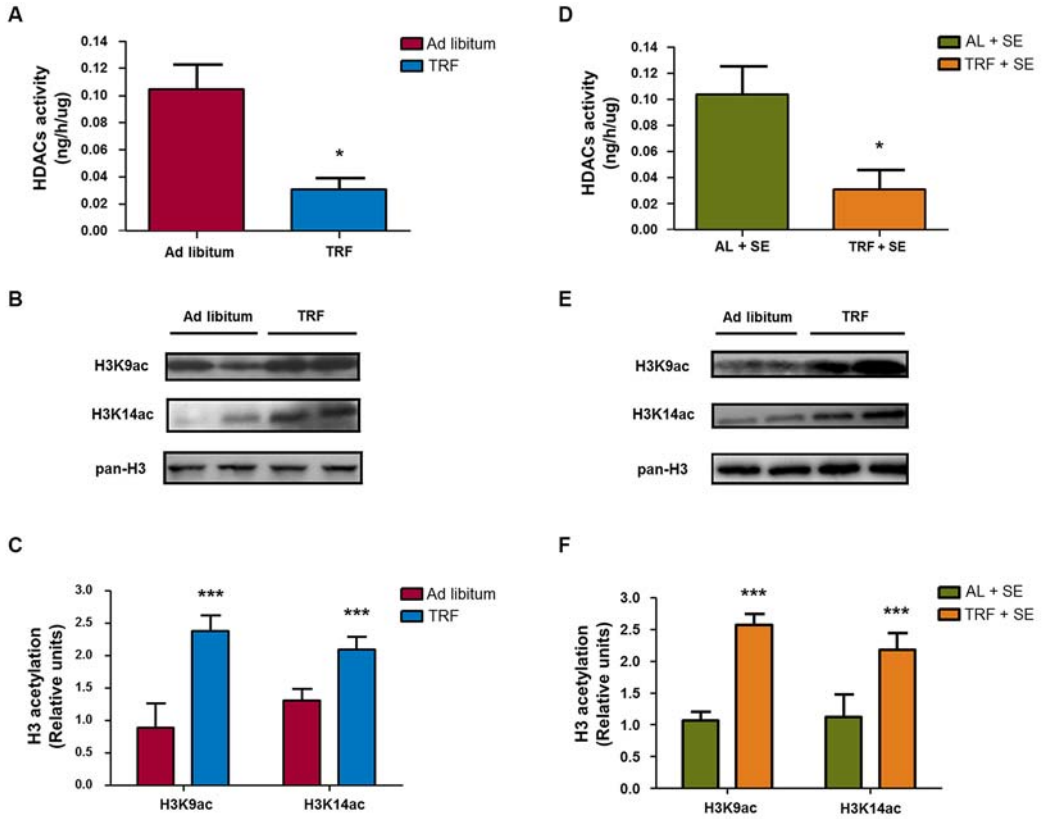

FIGURE 6 | TRF promotes epigenetic changes in the hippocampus through inhibition of HDAC activity and posttranslational modifications on histone 3 (H3). Total HDAC activity was measured in four groups (AL, TRF, AL-SE and TRF-SE). There was a statistically significant decrease in HDAC activity in TRF-subjected animals compared with that of AL-fed animals **(A)**; additionally, the same result was observed in TRF-SE animals compared with that of AL-SE rats **(D)**. Representative immunoblots show that there was a significant increase in the acetylation of H3K9ac and H3K14ac in the nuclear extract from TRF rats compared with that of AL-fed rats **(B,C)**. Interestingly, the same results were observed in pilocarpine injected animals subjected to TRF compared with that of pilocarpine-injected animals fed AL **(E,F)**. Nuclear fraction samples were normalized with total H3 protein. Bar charts are the semiquantitative optical densities of immunostained bands **(B)**. Data are expressed as the mean ± SD from each determination (*$p < 0.05$; ***$p < 0.001$). The total activity assay is from four independent animals and representative blots are from eight independent animals.

are similar; however, there is a significant difference at one point of the early phase. In this regard, the early phase of the analysis did not match with our behavioral studies, perhaps because of intrinsic variations among individuals. Nevertheless, it will be interesting to study this phenomenon more exhaustively. Conversely, the TRF schedule produced a statistical reduction in the power of the EEG recordings during the late phase compared with AL-fed animals. In this sense, these analyses may explain the reduction in seizure score found in the TRF group.

To date, there is no evidence showing that TRF might have an anticonvulsant effect; however, it has been shown that CR diminishes neuronal excitability and increases the after-discharged threshold in an electrical stimulation seizure model (Bough et al., 2003; Phillips-Farfán et al., 2015). In accordance with this assumption, our results suggest that TRF may inhibit seizure susceptibility and induce similar anticonvulsant effects in the pilocarpine-induced SE.

As previously mentioned, dietary restriction (i.e., caloric restriction) leads to a broad range of biochemical and metabolic changes (Yuen and Sander, 2014). In the present work, we demonstrated that TRF induces similar metabolic changes in the hippocampus, as evidenced as an increase in the phosphorylation of AMPK and a decrease in Akt kinase phosphorylation, which might be relevant to inhibit or interfere with the mechanisms involved in seizure generation. One possible explanation for the anticonvulsant effect of TRF could be through the activation of AMPK, which regulates mTOR (Laplante and Sabatini, 2012), a protein kinase that is closely related to the process of epileptogenesis (Zeng et al., 2009; Nguyen et al., 2015). In accordance, Phillips-Farfán et al. (2015) recently showed an increase in AMPK phosphorylation and a decrease in phospho-PKB (p-Akt), together with a decrease in phospho-S6 ribosomal protein in hippocampal homogenates in an electrical kindling model. Interestingly, similar effects on mTOR kinase downstream targets in the hippocampus were found using a KD in a kainic acid-induced seizure model (McDaniel et al., 2011). Although we did not study the activity of mTOR, changes in the phosphorylation of both AMPK and Akt protein kinases

measured in our study strongly suggest that the mTOR signaling pathway could be inhibited during TRF, as seen in the CR model. Interestingly, the same metabolic-related components were found in peripheral organs, which correlated with a decrease in liver weight with the production of ketone bodies (Sengupta et al., 2010).

The role of ketonemia-induced by KDs in seizure control is still controversial because high β-HB levels may or may not confer seizure protection depending on the seizure-induction model and rodent species (for a review, see Schoeler et al., 2013). Nevertheless, there are many reports that have demonstrated that β-HB correlated with improved seizure control, which is considered to be a key feature of successful KD treatment (Huttenlocher, 1976; Bough et al., 2003). In this regard, we found a strong positive correlation between a high β-HB concentration in the blood with prolonged seizure latency and a negative correlation with the seizure severity score, supporting the hypothesis that this ketone body has a significant role in the anticonvulsant properties of metabolism-based therapies (Yum et al., 2012; Yuen and Sander, 2014). However, it is clear that this metabolite may not be the only factor involved in the beneficial effects of metabolism-based diets shown in the present work and others (Nguyen et al., 2015). It is possible that other signaling components involved in metabolic regulation, such as, insulin-like growth factor 1 (IGF-1), sirtuins (SIRTs), AMPK and mTOR, may have an important role in these effects and thus should be further investigated (Yuen and Sander, 2014).

As we mentioned before, TRF raises blood β-HB levels similar to other metabolism-based diets. It is important to note that the concentration of ketosis was not as high as that found in the KD (0.88 mmol/L in TRF compared with 1.9 mmol/L produced by the KD; Linard et al., 2010), suggesting that even though this model induced moderate ketosis, it did not produce the side effects found in other diets (Nei et al., 2014). According to the literature, we hypothesize that the concentration of β-HB induced by TRF might have an inhibitory effect on seizure susceptibility. In this regard, it has recently been described that β-HB has anticonvulsant effects on pilocarpine-induced seizures in mice (Yum et al., 2012). A possible mechanism by which β-HB exerts its effect could be mediated by increasing a shift in the equilibrium of the aspartate-glutamate aminotransferase reaction towards glutamate, thus allowing more glutamate to become accessible to the glutamate decarboxylase enzyme and favoring the synthesis of GABA (Daikhin and Yudkoff, 1998). In addition, β-HB also decreases GABA-transaminase and GABA transporter (GAT-1) gene expression in cultured astrocytes whereby it could be another additional antiepileptic mechanism by suppressing astrocytic GABA degradation (Suzuki et al., 2009).

On the other hand, under pathological conditions, epigenetic dynamics are thought to represent wide-scale alterations in the expression of multiple genes (Grote et al., 2015). Furthermore, it is well known that seizures can give rise to enduring changes that reflect alterations in gene expression patterns that contribute to the hallmarks of epilepsy (Roopra et al., 2012). Currently, a few reports have studied specific markers of epigenetic mechanisms in both acute seizures and epilepsy models *per se*, and even more studies have used a metabolism-based diet. In this regard, Kobow et al. (2013) recently demonstrated that there is an increase in DNA methylation during chronic rat epilepsy and that these aberrant methylation patterns were inversely correlated with gene expression changes. Moreover, the KD attenuated seizure progression and ameliorated DNA methylation mediated changes in gene expression.

In the present study, we describe for the first time that a TRF schedule can produce additional epigenetic modifications, such as acetylation, on two lysine residues (9 and 14) of H3, which are epigenetic tags associated with the activation of gene transcription (Landgrave-Gómez et al., 2015). Such an increase in these posttranslational modifications may be mainly mediated by inhibiting histone deacetylase activity throughout β-HB, an endogenous metabolite that is capable of inhibiting HDAC (Shimazu et al., 2013), which is increased during dietary restriction. The specific mechanism by which modifications on H3 acetylation decrease the seizure susceptibility observed in our results is still unknown. However, one possible explanation could be through global changes in transcription, including genes associated with the resistance to oxidative stress (Shimazu et al., 2013), a pathological event that has been described to contribute to epilepsy (Waldbaum and Patel, 2010). Nevertheless, more studies are needed to elucidate this assumption in the TRF model.

There is substantial research on the pros and cons of continuous CR or intermittent fasting for the improvement of health (Skaznik-Wikiel and Polotsky, 2014) which may be used as a treatment in epilepsy refractory patients. However, our data provide some rational evidence for using this type of diet as an alternative therapy. This assumption may be considered because it has been shown that intermittent fasting induces a transient improvement in seizure control in children with an incomplete response to a KD in a pilot trial (Hartman et al., 2013).

In summary, our study demonstrates that time restricted feeding has an anticonvulsant effect in the pilocarpine-seizure model. Furthermore, we suggest a possible scenario in which restrictive diets may modulate the activity of the main components of signaling pathways involved in energy metabolism (i.e., AMPK and Akt signaling pathways) and therefore increase the concentration of secondary metabolites (such as β-HB), which may decrease the activity of proteins capable of altering the chromatin structure (HDACs), in turn, contributing in part to the beneficial effects of restrictive diets. In this regard, our results support the interesting hypothesis that some endogenous metabolites, such as β-HB, function as a link between environmental cues with transcriptional regulation and deepen our understanding of the molecular mechanisms involved in the beneficial effects of restrictive diets (calorie restriction or intermittent fasting) contributing to seizure control. Finally, we hypothesize that TRF might be considered in the future as an alternative therapeutic tool together with antiepileptic drugs to control seizures in pharmacoresistant epilepsy patients and accordingly improve their quality of life.

AUTHOR CONTRIBUTIONS

JLG and OFMG, have designed the study, made all the experiments and wrote the manuscript. MVG and VRM, contributed with EEG experiments and analysis. LCD, VAA and AMM have provided technical assistance and revised the manuscript. RGG, supervised the work and wrote the manuscript.

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