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## Participación de mTOR en el transporte de aminoácidos involucrados en la síntesis de glutatión en el sistema nervioso del ratón

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**ABREVIATURAS**

**4EBP-1**, la proteína de unión al factor de inicio de la traducción eucariota elF-4E
**4F2hc**, proteína de unión a membrana la cadena pesada de antígeno 4F2
**γGT**, γ-glutamil transpeptidasa
**AMPK**, proteína cinasa activada por AMP
**AKT o PKB**, el nombre viene de un retrovirus aislado de la cepa Ak llamado "Akt-8". El oncogen codificado en este virus se denominó v-Akt. Proteína cinasa B
**ARE**, elementos de respuesta antioxidante
**ASK1**, cinasa reguladora de la señal de apoptosis
**ATF4**, factor de transcripción 4
**ATP,** adenosín trifosfato
**BCA,** ácido bicinconínico
**BCH,** 2-aminobiciclo-(2,2,1)-heptano-2-ácido carboxílico
**BHE**, barrera hematoencefálica
**BSA**, albúmina de suero bovino
**BSO**, L-butionina-S-R-sulfoximina
**CAT,** catalasa
**COX2**, subunidad 2 de la citocromo c oxidasa
**CPT-1**, carnitina palmitoiltransferasa 1
**$Cu^{2+, 3+}$**, iones de cobre
**Cys**, cisteína
**Cys2**, cistina
**DAPI**, 4 ',6-diamino-2-fenilindol
**DEPTOR**, proteína de interacción con mTOR
**DMEM/F-12**, Dulbecco's Modified Eagle Medium: Nutrient Mixture F- 12
**DTPA,** pentaacetato de dietilentriamina
**EAAT1/2**, transportadores de aminoácidos excitadores 1 y 2
**EAAT3**, transportador de aminoácidos excitadores 3
**EEF2K**, cinasa del factor de elongación eEF2
**eIF4E**, factor de iniciación de traducción 4
**ERK**, cinasa regulada por señales extracelulares
**$Fe^{2+, 3+}$**, iones de hierro
**FOXO1/3a**, proteína forkhead box O1/3a
**G6P-D**, glucosa 6-fosfato deshidrogenasa
**GAP**, GTPasa
**GCL**, cisteína-glutamato ligasa
**GCLc**, subunidad catalítica
**GCLm**, subunidad modulatoria
**Glu**, glutamato
**Gly**, glicina
**GPCR,** receptores asociados a proteínas G
**GR,** glutatión reductasa
**GRB2,** proteína 2 de unión al receptor de factores de crecimiento
**GS,** glutatión sintetasa
**GSH**, glutatión
**GSK3**, glucógeno sintasa cinasa 3
**GSSG**, glutatión disulfuro
**GST,** GSH S-transferasas
**$H_2O_2$**, peróxido de hidrógeno
**HIF1α**, factor inducible de hipoxia 1-alfa
**iNOS**, NOS inducible

**IκB**, inhibidor de kappa B
**IKK,** cinasa de IκB
**ip**, intraperitonial
**IRAK**, cinasa asociada al receptor de interleucina 1
**JNK**, cinasa c-jun N -terminal
**LAT1**, transportador 1 de aminoácidos de tipo-L
**MAPK**, cinasas activadas por mitógenos
**mBCl**, monoclorobimano
**$Mg^{2+}$**, ión de magnesio
**mGluR,** receptores de glutamato metabotrópicos
**MK571,** ácido 5-(3-(2-(7-cloroquinolina-2-il) etenil) fenil)-8-dimetilcarbamil-4,6-ditiaoctanoico
**mLST8**, proteína letal 8 de mamífero con Sec13, también conocida como GbL
**mRNA,** ácido ribonucleico mensajero
**MRP1**, proteína 1 de resistencia a multidroga
**mSINI**, proteína interactiva de la cinasa de mamífero activada por estrés
**mTOR**, blanco funcional de la rapamicina
**mtTFA**; factor de transcripción mitocondrial
**$Na^{+}$**, ión sodio
**NADPH;** nicotinamida-adenina-dinucleótido-fosfato
**NeuN**, núcleos neuronales
**NFκB**, factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas
**NGF**, factor de crecimiento neuronal
**NMDA**, N-metil-D-aspartato
**$NO^{\bullet}$**, óxido nítrico
**$NO_2^{\bullet}$**, radical dióxido de nitrógeno
**NOS,** NO sintasa
**Nrf2**, factor nuclear 2 derivado del factor eritroide 2
**NOX**, NADPH oxidasas
**$O_2$**, oxígeno molecular
**$O_2^{\bullet -}$**, superóxido
**OD**; densidad óptica
**$OH^{\bullet}$**, radical hidroxilo
**$OONO^{-}$**, peroxinitrito
**OPA**, O-ftalaldehído
**p**, fosforilado
**PB**, amortiguador de fosfatos
**PBT**, PB + 0.3% Triton X-100
**PC12**, línea celular de tumores de médula adrenal de ratón
**PDC**, pirrolidina-2,4-ácido dicarboxílico
**PDK1**, piruvato deshidrogenasa cinasa
**PI**, ioduro de propidio
**PI3K**, fosfatidilinositol 3-cinasa
**PIKK**, cinasas relacionadas a PI3K
**PKC**, proteína cinasa c
**PLCγ**, fosfolipasa C gamma
**PRAS40**, sustrato de Akt rico en prolinas de 40 kDa
**Protor-1**, proteína observada con rictor-1
**Prx**, peroxiredoxina
**Raptor**, proteína reguladora asociada a mTOR

**Rheb**, homólogo de Ras enriquecido en el cerebro
**Rictor**, proteína asociada a mTOR insensible a rapamicina
**RIPA,** radioinmunoprecipitación
**RNS**, especies reactivas del nitrógeno
**roGFP,** proteína verde fluorescente redox sensible
**ROS**, especies reactivas del oxígeno
**RPE**, células epiteliales de pigmento retinal
**RSK**, familia de cinasas ribosomales S6
**RTK**, receptores tirosina cinasa
**S6K**, proteína ribosomal S6 cinasa beta-1
**SAS**, sulfasalazina
**SBF**, suero bovino fetal
**SE**, error estándar
**Ser**, serina
**-SH**, tiol
**Shc**, proteína adaptadora 1 Shc
**Sistema xc-**, sistema de transporte de cistina y glutamato sodio-independiente
**SNAT2**, transportador del sistema A tipo 2
**SNC**, sistema nervioso central
**SOD**, superóxido dismutasa
**Sos**, hijo de las proteínas sevenless
**SREBP**, elementos reguladores de esteroles
**TCA**, ácidos tricarboxílicos
**Thr**, treonina
**TNF**, factor de necrosis tumoral
**TrkA**, receptor de tropomiosina tipo cinasa A
**Trx**, tiorredoxina
**TR**, Trx reductasa
**TRAFs**, factores asociados al receptor TNF
**TSC1/2**, complejo de esclerosis tuberosa
**Tyr**, tirosina
**UCPH**, 2-Amino-5,6,7,8-tetrahidro-4-(4-metoxifenil)-7-(naftaleno-1-yl)-5-oxo-4H-cromeno-3-carbonitrilo
**ULK1**, cinasa 1 activadora de la autofagia similar a unc-51
**UK5099**, mitocondrial 2-ciano-3-(1-fenil-1H-indol-3-il)-2-ácido propenóico
**UV**, ultravioleta
**xCT**, subunidad catalítica del sistema xc-

**RESUMEN**

El glutatión (GSH) es un antioxidante que juega un papel primordial en la protección de las células contra el estrés oxidante, mantiene la homeostasis redox y además participa en la desintoxicación de xenobióticos. En el cerebro la síntesis de GSH está limitada por la disponibilidad de cisteína y glutamato. La cistina y la cisteína son transportadas a través de la barrera hematoencefálica (BHE) y hacia los astrocitos y neuronas vía el sistema de transporte de cistina y glutamato sodio ($Na^+$)- independiente (sistema xc-), compuesto por la subunidad catalítica (xCT) y la de unión a membrana, la cadena pesada de antígeno 4F2 (4F2hc), el transportador de aminoácidos tipo L (LAT1) y el transportador de aminoácidos excitadores 3 (EAAT3). Las vías de señalización del blanco funcional de la rapamicina (mTOR) y de las neurotrofinas pueden estar involucradas en la regulación de los transportadores de aminoácidos para la síntesis de GSH. En este trabajo se encontró que la administración sistémica de L-butionina-S-R-sulfoximina (BSO) altera la homeostasis de GSH en el cerebelo una región del cerebro de ratón, y en un modelo *in vitro* de cultivo primario de astrocitos. La inyección ip de 6 mmol/kg de BSO disminuyó los niveles de GSH en el hígado. De las regiones de cerebro evaluadas, el cerebelo fue el único que presentó una respuesta redox, activando las vías de mTOR y del factor de crecimiento neuronal (NGF) y su receptor de tropomiosina tipo cinasa A (TrkA), llevando a un incremento en el transportador EAAT3 y en la concentración de GSH. Por otro lado, en astrocitos la disminución de GSH fue reversible 12 h después de retirar el BSO. La recuperación de los niveles de GSH se previno al inhibir simultáneamente a xCT, LAT1 y EAAT3. La inhibición del transporte de piruvato mitocondrial y la oxidación de ácidos grasos, así como la inhibición de mTOR disminuyeron los niveles de GSH y previnieron la recuperación después del tratamiento con BSO. Además, la inhibición de mTOR exacerbó los niveles de estrés oxidante y participó en la regulación bioenergética celular. Estos resultados sugieren que mTOR y el metabolismo mitocondrial regulan la homeostasis de GSH en los astrocitos.

**ABSTRACT**

Glutathione (GSH) is an essential component of intracellular antioxidant systems that plays a primordial role in the protection of cells against oxidative stress, maintaining redox homeostasis and xenobiotic detoxification. GSH synthesis in the brain is limited by the availability of cysteine and glutamate. Cystine/cysteine are transported through the endothelial cells of the blood-brain barrier (BBB) and into the astrocytes and neurons via the system xc-, which is composed of the catalytic subunit xCT and the heavy chain of 4F2 cell surface antigen (4F2hc), the L-type amino acid transporter 1 (LAT1) and the excitatory amino acid transporter 3 (EAAT3). The mammalian target of rapamycin (mTOR) and neurotrophins can activate signaling pathways that modulate amino acid transporters for GSH synthesis. The present study found that systemic L-buthionine-S-R-sulfoximine (BSO) administration selectively altered GSH homeostasis in the mice cerebellum and in an *in vitro* model of primary cortical astrocytes. Intraperitoneal treatment of mice with 6 mmol/kg of BSO depleted GSH in the liver. The cerebellum, but not other brain regions, exhibited a redox response, activating mTOR and the neuronal growth factor (NGF)/tropomyosin receptor kinase A (TrkA) signaling pathways that lead to an increase in the protein levels of the EAAT3 transporter, and an increase of the GSH concentration. Furthermore, inhibition of *de novo* GSH synthesis with BSO (500 μM) induced GSH depletion in astrocytes. This depletion was reversible after 12 h of BSO withdrawal. Replenishment of the intracellular GSH pool was prevented by the simultaneous inhibition of xCT, EAAT3 and LAT1. Inhibition of mitochondrial pyruvate transport and free fatty acid oxidation as well as the mTOR signaling depleted GSH and prevented the recovery of the intracellular GSH pool after BSO withdrawal. Moreover, mTOR inhibition exacerbated oxidative stress and was found to regulate bioenergetics in astrocytes after BSO withdrawal. Our results suggest that mTOR signaling and mitochondrial metabolism regulate GSH homeostasis in astrocytes.

## INTRODUCCIÓN

### Estrés oxidante y homeostasis redox

Las especies reactivas del oxígeno (ROS) son compuestos derivados del oxígeno que pueden recibir o donar electrones participando en reacciones nucleofílicas, electrofílicas y/o redox (Circu y Aw, 2010; Halliwell y Cross, 1994; Trachootham et al., 2008). Las ROS comprenden radicales libres y no radicales, como el radical hidroxilo ($OH^{\bullet}$), el anión superóxido ($O_2^{\bullet-}$) y el peróxido de hidrógeno ($H_2O_2$) (Olsen et al., 2013). Por otro lado, las especies reactivas del nitrógeno (RNS) derivan de nitrógeno y oxígeno e incluyen al óxido nítrico ($NO^{\bullet}$), al radical dióxido de nitrógeno ($NO_2^{\bullet}$) y el peroxinitrito ($OONO^-$) (Olsen et al., 2013; Ye et al., 2015).

La reducción del oxígeno molecular ($O_2$) es el principal mecanismo para la formación de ROS. En la célula, la mitocondria es la principal fuente generadora de ROS a través de la cadena transportadora de electrones, principalmente con la reducción de $O_2$ a $O_2^{\bullet-}$ (Olsen et al., 2013). El $O_2^{\bullet-}$ generado puede ser dismutado generando $H_2O_2$ a través de la acción de las superóxido dismutasas (SOD) (Finkel, 2011). Además, el $O_2^{\bullet-}$ y el $H_2O_2$ fomentan la formación del $OH^{\bullet}$ a través de las reacciones de Fenton/Haber-Weiss. La reacción de Fenton es un proceso de reducción a partir de $H_2O_2$ y iones metálicos de hierro o cobre ($Fe^{2+}$ y $Cu^{2+}$); posteriormente en la reacción de Haber-Weiss se genera $OH^{\bullet}$ a partir de $H_2O_2$ y $O_2^{\bullet-}$, la reacción es catalizada por $Fe^{3+}$ y $Cu^{3+}$(Forman, 2016; Halliwell y Cross, 1994). Las enzimas nicotinamida-adenina-dinucleótido-fosfato (NADPH) oxidasas (NOX) son las únicas enzimas cuya función es la de generar ROS ($O_2^{\bullet-}$ o $H_2O_2$), a diferencia de las otras fuentes que generan ROS como subproductos, estas enzimas tienen como función primaria la producción de ROS que participan en procesos fisiológicos, éstas representan la segunda fuente importante de formación de ROS en la célula (Coyoy y Moran, 2012; Ye et al., 2015).

Otras fuentes intracelulares de ROS incluyen a la xantina oxidasa, las ciclooxigenasas, las enzimas citocromo p450, y las lipooxigenasas. Los microsomas y peroxisomas también son fuentes de ROS, junto con los neutrófilos y macrófagos que poseen mecanismos dependientes del oxígeno como defensa en contra de microorganismos patógenos (Finkel, 2011; Schrader y Fahimi, 2006; Ye et al., 2015).

La formación de RNS comienza con la síntesis de $NO^{\bullet}$, a partir de arginina, oxígeno y NADPH, catalizada por la $NO^{\bullet}$ sintasa (NOS). En la presencia de $O_2^{\bullet -}$, el $NO^{\bullet}$ se transforma en $OONO^-$, un potente agente oxidante. La mayoría del $OONO^-$ se sintetiza dentro de las células fagocíticas del sistema inmune, a través de la NOS inducible (iNOS) y las NOX (Olsen et al., 2013).

Las ROS/RNS pueden actuar como moléculas de señalización teniendo efecto en la estabilidad, la expresión, función y actividad de múltiples proteínas controlando procesos celulares (Finkel, 2011; Reczek y Chandel, 2015). En condiciones fisiológicas el balance entre la generación y la eliminación de las ROS/RNS mantiene un funcionamiento adecuado dentro de la célula (Trachootham et al., 2008). Sin embargo, el desbalance entre el incremento de los niveles de ROS/RNS y la habilidad de la célula de metabolizar/desintoxicarlas genera un estado en el cual se pierde la homeostasis llamado estrés oxidante. Las ROS/RNS a altas concentraciones son deletéreas generando daño oxidante en las biomoléculas como DNA, lípidos, proteínas, etc. (Circu y Aw, 2010; Ye et al., 2015). La generación de estrés oxidante resulta en modificaciones oxidantes que pueden llevar a la pérdida de función, envejecimiento y hasta muerte celular (Forman, 2016; Olsen et al., 2013).

Para contrarrestar la producción excesiva de ROS/RNS y mantener la homeostasis redox celular, los organismos poseen sistemas antioxidantes que se dividen en enzimáticos y no enzimáticos (Durackova, 2010; Trachootham et al., 2008). Los antioxidantes incluyen

moléculas de bajo peso molecular como el glutatión o γ-L-glutamil-L-cisteinilglicina (GSH), así como diversas proteínas antioxidantes con ubicaciones subcelulares distintas (Finkel, 2011). El sistema de la tiorredoxina (Trx) es otro antioxidante importante compuesto por la Trx y la Trx reductasa (TR), que participan en la reducción de proteínas oxidadas (Trachootham et al., 2008). Las enzimas catalasa (CAT) y las peroxiredoxinas (Prx) se encargan de la desintoxicación de peróxidos, mientras que las SOD catalizan la dismutación del $O_2^{\bullet -}$ (Holmstrom y Finkel, 2014). Otros antioxidantes endógenos son: el ácido úrico, el ácido lipico y el ubiquinol, así como los que se obtienen de la dieta, entre los que se incluyen las vitaminas C y E, los carotenoides y los flavonoides (Durackova, 2010; Ye et al., 2015).
El factor nuclear 2 derivado del factor eritroide 2 (Nrf2) es un factor transcripcional activado por estrés oxidante y electrófilos. Una vez en el núcleo se une a los elementos de respuesta antioxidante (ARE) activando la transcripción de genes que funcionan como antioxidantes, de enzimas de desintoxicación de fase II, así como enzimas involucradas en la síntesis de GSH, todas estas proteínas juegan papeles primordiales en la defensa celular contra el estrés oxidante (Trachootham et al., 2008).

El sistema nervioso central (SNC) es especialmente susceptible al daño oxidante, siendo las neuronas y los oligodendrocitos más sensibles que los astrocitos y la microglía. Esto se debe al alto consumo de $O_2$, a que tiene una alta concentración de lípidos que pueden ser susceptibles a la lipoperoxidación y a que presentan menores niveles de defensas antioxidantes en comparación con otros órganos (Patel, 2016; Salim, 2017).

**Glutatión (GSH)**

El GSH es el tiol (-SH) de bajo peso molecular predominante en los tejidos de los mamíferos con concentraciones que van de 1 a 10 mM, siendo el hígado el órgano con mayor concentración (Janssen-Heininger et al., 2013). Este antioxidante se encarga del mantenimiento de la homeostasis redox en la célula, almacén de cisteína y glutamato,

desintoxicación de xenobióticos y metabolitos a través de las GSH S-transferasas (GST) y de la formación de puentes disulfuro, así como la protección de residuos de cisteína para evitar la oxidación de proteínas. Estos mecanismos son importantes en la regulación de diversas funciones celulares tales como la proliferación y la apoptosis (Aquilano et al., 2014; Lu, 2013; Meister, 1981; Wu et al., 2004).

Dentro de la célula, este -SH es mantenido en su forma reducida por la enzima GSH reductasa (GR) la cual reduce el grupo disulfuro del GSH disulfuro (GSSG) a través de una reacción dependiente de la donación de electrones provenientes del NADPH iniciando un ciclo redox (Lu, 2009; Meister, 1981). El cociente GSH/GSSG es un indicador del ambiente redox, estos dos compuestos son la pareja redox más abundante dentro de la célula (Schafer y Buettner, 2001).

El GSH se sintetiza a partir de los aminoácidos glutamato, cisteína y glicina, en dos reacciones dependientes de adenosín trifosfato (ATP). En la primera, la enzima cisteína-glutamato ligasa (GCL), cataliza la formación del L-γ-glutamil-L-cisteína, mientras que en la segunda reacción la glutatión sintetasa (GS) agrega el aminoácido glicina (Lu, 2013). Bajo condiciones fisiológicas la GCL es regulada por la unión competitiva de GSH, generando un proceso de retroalimentación negativa cuando hay incremento en la concentración de GSH (Richman y Meister, 1975). Además, la cisteína es el aminoácido limitante para la síntesis de GSH. Ésta puede ser obtenida de la ruptura de proteínas obtenidas de la dieta y a partir de la vía de la transsulfuración. También, la actividad de la GCL es un paso limitante en el proceso (Lu, 2013; Vitvitsky et al., 2006). Una vez sintetizado el GSH se exporta al medio extracelular a través de transportadores de membrana. En la porción extracelular de la membrana existen peptidasas como la γ-glutamil transpeptidasa (γGT) que generan γ-glutamil y el dipéptido CysGly que posteriormente es hidrolizado para que

los aminoácidos puedan ser importados nuevamente por las células para continuar el ciclo de síntesis de GSH y/o proteínas (Dringen et al., 1999; Dringen et al., 2001).

Dada la importancia del GSH se han desarrollado diferentes estrategias para modelar la falta de éste y conocer las vías de señalización que participan en su regulación. La más utilizada, es el uso de L-butionina-S-R-sulfoximina (BSO), el inhibidor específico de la GCL más efectivo que bloquea la síntesis *de novo* de GSH. BSO se une reversiblemente a la GCL como un análogo del primer producto de la síntesis. Por acción del BSO, la poza preexistente de GSH es consumida gradualmente, ya sea por reacciones enzimáticas o por su exportación fuera de la célula (Limón-Pacheco y Gonsebatt, 2007).

**Glutatión en el SNC**

El cerebro contiene diferentes concentraciones de GSH (2-3 mM), las regiones con mayores niveles son la corteza y el cerebelo (Kang et al., 1999). El GSH realiza funciones específicas en el SNC; este tripéptido es considerado un neuromodulador/neurotransmisor que puede regular los receptores de glutamato del tipo N-metil-D-aspartato (NMDA) y también participa en la proliferación y diferenciación neuronal (Aoyama et al., 2008; Dringen, 2000; Janaky et al., 1999).

El contenido de GSH varía en los diferentes tipos celulares. Las neuronas tienen menor concentración comparada con los astrocitos (Rice y Russo-Menna, 1998). Los astrocitos participan en el metabolismo antioxidante y la desintoxicación de xenobióticos en el cerebro, además, liberan GSH a través de la proteína 1 de resistencia a multidroga (MRP1) para proveer los precursores necesarios para la síntesis de GSH en neuronas (Dringen et al., 1997; Hirrlinger y Dringen, 2005). Como se mencionó anteriormente, el GSH liberado es procesado vía la γGT generando γ-glutamil y el dipéptido CysGly que es hidrolizado por la dipeptidasa neuronal para transportar cisteína a la neurona y ésta pueda sintetizar su propio GSH (Fig. 1) (Dringen et al., 1999; Dringen et al., 2001).

La exposición a metales, pesticidas, radiación ionizante, luz UV y a fármacos puede afectar los niveles de GSH (Limón-Pacheco y Gonsebatt, 2009). Las alteraciones en el metabolismo de GSH son comunes en el envejecimiento, en desórdenes neuropsiquiátricos y en las enfermedades neurodegenerativas. Los niveles bajos de GSH, alteraciones en las enzimas GSH-dependientes y la alta producción de ROS, han sido asociados a neuropatías provocadas por exposición a tóxicos ambientales y a enfermedades tales como Parkinson, Huntington, Alzheimer y la degeneración cerebelar. Sin embargo, la participación del GSH en el desarrollo de estas enfermedades neurodegenerativas todavía sigue siendo estudiada (Aoyama et al., 2008; Aoyama y Nakaki, 2013; Gu et al., 2015; Johnson et al., 2012). Debido a que el BSO no es capaz de cruzar la barrera hematoencefálica (BHE) fácilmente, su uso constituye un acercamiento para estudiar los efectos en el cerebro ante una depleción sistémica de GSH (Limón-Pacheco y Gonsebatt, 2007).

**Transportadores de aminoácidos asociados a la síntesis de GSH**

Debido a que los aminoácidos no pueden cruzar la membrana plasmática, el transporte de aminoácidos, principalmente cisteína y cistina (su forma oxidada), también es muy importante en la síntesis de GSH (Lu, 2009; Wu et al., 2004). Además, el GSH y la cisteína no pueden atravesar la BHE y llegar al SNC. La cistina es transportada del torrente sanguíneo a las células endoteliales de la BHE vía el sistema de transporte de cistina y glutamato sodio ($Na^+$)- independiente (sistema xc-), compuesto por dos subunidades la subunidad catalítica (xCT) y la de unión a membrana, la cadena pesada de antígeno 4F2 (4F2hc) (Sato et al., 1999; Shih et al., 2006). Posteriormente la cistina es reducida dentro de la célula y la cisteína transportada al cerebro a través del transportador 1 de aminoácidos de tipo-L (LAT1), este transportador también transporta metionina que puede llevar a la síntesis de cisteína a través de la vía de transsulfuración (Killian y Chikhale, 2001; Wade y Brady, 1981). Una vez dentro del cerebro la cistina es transportada a los astrocitos a través

del intercambiador xCT. Debido a la característica excitotóxica del glutamato, los transportadores de aminoácidos excitadores 1 y 2 (EAAT1 y EAAT2) del sistema $x^-_{AG}$ $Na^+$-dependientes, se encargan de remover el glutamato que libera el xCT (Valdovinos-Flores y Gonsebatt, 2012). Las neuronas dependen de la cisteína que le proveen los astrocitos y la transportan a través del transportador EAAT3 también conocido como el acarreador de aminoácidos excitadores 1 (EAAC1) (Fig. 1) (Himi et al., 2003; Zerangue y Kavanaugh, 1996).

Los transportadores de aminoácidos tienen papeles cruciales, no sólo proveen los precursores para la síntesis de GSH sino que también regulan el crecimiento y la proliferación celular por lo tanto, las vías de señalización que regulan estos procesos tienen una importante relación con la expresión de los transportadores de nutrientes (McCracken y Edinger, 2013).

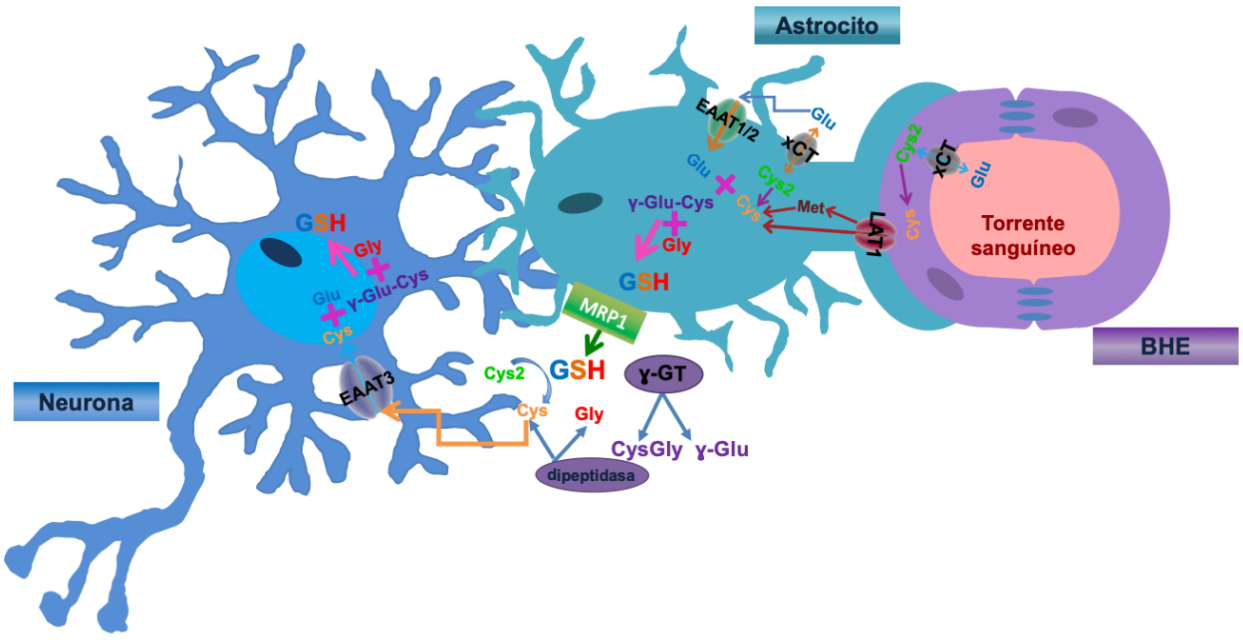


**Figura 1. Transporte de aminoácidos en el astrocito y la neurona para la síntesis de GSH.** La Cys2 es transportada del torrente sanguíneo a las células endoteliales de la BHE vía xCT. La Cys2 es reducida dentro de la célula y la Cys transportada al cerebro a través del transportador LAT1, este transportador también transporta Met que puede llevar a la síntesis de Cys a través de la vía de la transsulfuración. Una vez dentro del cerebro la Cys2 es transportada a los astrocitos a través del intercambiador xCT. Los transportadores EAAT1 y EAAT2 transportan el glutamato que libera el xCT. (continúa)

Posteriormente, los astrocitos liberan GSH a través de la MRP1, éste es procesado vía la γGT generando γ-glutamil y CysGly que es hidrolizado por la dipeptidasa neuronal para que la neurona transporte Cys a través del transportador EAAT3 y pueda sintetizar su propio GSH. Abreviaturas: γGT, γ-glutamil transpeptidasa; BHE, barrera hematoencefálica; Cys, cisteína; Cys2, cistina; EAAT1/2, transportadores de aminoácidos excitadores 1 y 2; EAAT3, transportador de aminoácidos excitadores 3; Glu; glutamato Gly; glicina GSH, glutatión; Met, metionina; MRP1, proteína 1 de resistencia a multidroga; LAT1, transportador 1 de aminoácidos de tipo-L; sistema xc-, sistema de transporte de cistina y glutamato $Na^+$- independiente; xCT, subunidad catalítica del sistema xc-.

**Vías de señalización**

Las células utilizan las vías de señalización para regular su actividad. La mayoría son activadas por estímulos externos que activan receptores presentes en la membrana plasmática como los receptores asociados a proteínas G (GPCRs) y los receptores tirosina cinasas (RTKs). Sin embargo, algunas también responden a información generada dentro de la célula en forma de mensajeros metabólicos. Estas vías de señalización transmiten, procesan, codifican e integran señales, para generar respuestas como por ejemplo cambios en la actividad enzimática, expresión génica o la actividad de canales iónicos (Berridge, 2014; Kholodenko, 2006).

En este trabajo analizamos las vías de señalización que activan al blanco funcional de la rapamicina (mTOR) así como la vía activada por el factor de crecimiento neuronal (NGF).

**Blanco funcional de la rapamicina (mTOR)**

Los factores de crecimiento a través de sus receptores, activan la vía de señalización de la fosfatidilinositol 3-cinasa (PI3K)/AKT/mTOR y aumentan la expresión de los transportadores de nutrientes, permitiendo la entrada de éstos a la célula para el crecimiento y otras funciones importantes (Edinger, 2007). Estas vías de señalización también responden a la disponibilidad y el requerimiento de nutrientes (McCracken y Edinger, 2013).

mTOR es una cinasa de serina (ser) /treonina (thr) de la familia de las cinasas relacionadas a PI3K (PIKK). Esta proteína se encarga de regular el crecimiento celular (el incremento de masa celular, síntesis de proteínas y la proliferación), así como el metabolismo celular en respuesta a diferentes estímulos como el contenido de aminoácidos, cambios en la

bioenergética celular, factores de crecimiento y estrés oxidante, entre otros (Garza-Lombó et al., 2018b). Forma dos complejos diferentes, el complejo sensible a rapamicina y nutrientes o mTORC1 y el complejo insensible a rapamicina o mTORC2 (Saxton y Sabatini, 2017). El primero está compuesto por 5 proteínas: mTOR, la proteína reguladora asociada a mTOR (Raptor), el sustrato de Akt, rico en prolinas de 40 kDa (PRAS40), la proteína letal 8 de mamífero con Sec13 (mLST8, también conocida como GbL), y la proteína de interacción con mTOR (DEPTOR) (Alayev y Holz, 2013). Mientras que el mTORC2; está compuesto por 6 proteínas: mTOR, la proteína asociada a mTOR insensible a rapamicina (Rictor), la proteína interactiva de la cinasa de mamífero activada por estrés (mSINI), la proteína observada con rictor-1 (Protor-1), mLST8 y DEPTOR (Alayev y Holz, 2013; Kim y Guan, 2011).

La activación de la vía PI3K/AKT a través de los receptores de factores de crecimiento inactiva el complejo de esclerosis tuberosa (TSC), un regulador negativo de mTORC1. TSC es un complejo heterotrimérico compuesto por TSC1/TSC2; TSC2 actúa como una proteína aceleradora de la actividad GTPasa (GAP) e inactiva al homólogo de Ras enriquecido en el cerebro (Rheb) un activador de mTORC1. La activación de mTORC1 también requiere que se encuentre cerca de los lisosomas lo cual es regulado por la presencia de aminoácidos y nutrientes vía las proteínas GTPasas Rag, el mTORC1 fosforila a la proteína ribosomal S6 cinasa beta-1 (S6K) y a la proteína de unión al factor de inicio de la traducción eucariota elF-4E (4EBP-1), los cuales regulan la traducción de mRNAs, la biogénesis mitocondrial, la glicólisis y al factor inducible de hipoxia 1-alfa (HIF1$\alpha$) (Garza-Lombó et al., 2018b; Kim y Guan, 2011). mTORC1 también puede regular la síntesis lipídica y de nucleótidos a través de la proteína de unión a elementos reguladores de esteroles (SREBP) y a través de la activación del factor de transcripción 4 (ATF4) respectivamente, así como inhibir la autofagia al fosforilar la cinasa 1 activadora de la autofagia similar a unc-51 (ULK1) (Garza-

Lombó et al., 2018b). Además, un reporte reciente demostró que mTORC1 controla la actividad y la biogénesis mitocondrial, al promover la traducción de mRNAs de genes mitocondriales a través de 4EBP-1 (Fig. 2) (Morita et al., 2013).

Por otro lado, mTORC2 controla la proliferación y sobrevivencia vía la activación de las proteínas cinasas de la familia AGC (nombrada por las proteínas cinasas A, G y C) como la proteína cinasa C (PKC) que se encargan de regular el remodelamiento del citoesqueleto y la migración celular. La fosforilación de AKT por mTORC2 incrementa la actividad de AKT induciendo la fosforilación de sustratos específicos como la proteína forkhead box O1/3a (FOXO1/3a) o la glucógeno sintasa cinasa 3 (GSK3). Además, también se ha demostrado que mTORC2 regula el metabolismo de aminoácidos y de glucosa (Fig. 2) (Garza-Lombó et al., 2018b; Saxton y Sabatini, 2017). En este trabajo se estudió el papel de mTOR en la regulación del transporte de aminoácidos.

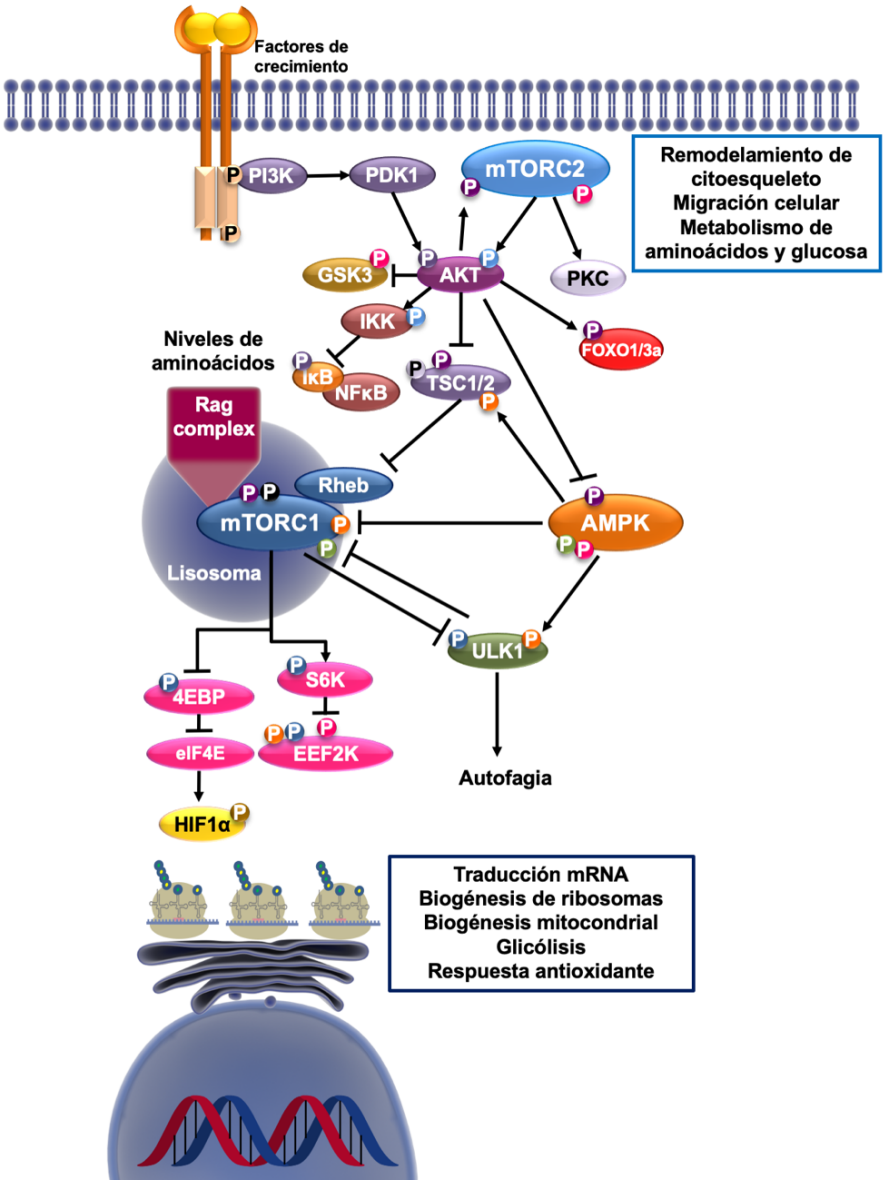


**Figura 2**. **Vía de señalización de mTOR**. La activación de la vía PI3K/AKT a través de los receptores de factores de crecimiento inactivan a TSC1/TSC2 que a su vez inactiva al Rheb un activador de mTORC1. La activación de mTORC1 también requiere que se encuentre cerca de los lisosomas regulado por la presencia de aminoácidos y nutrientes vía las proteínas Rag. mTORC1 fosforila a S6K y a 4EBP-1, los cuales regulan HIF1α, la traducción de mRNAs, la biogénesis mitocondrial, la glicólisis y la respuesta antioxidante. mTORC1 también fosforila a ULK1 inhibiendo la autofagia, que puede ser activada a través de AMPK que activa TSC1/2 y también inhibe a mTORC1. mTORC2 controla la proliferación y sobrevivencia vía PKC y fosforila a AKT que a su vez fosforila sustratos como FOXO1/3a o GSK3 o a IKK que fosforila IκB, resultando en la activación de NFκB; regulando el remodelamiento del citoesqueleto, la migración celular, la sobrevivencia y el metabolismo de aminoácidos y glucosa. Abreviaturas: 4EBP-1, la proteína de unión al factor de inicio de la traducción eucariota eIF-4E; AMPK, proteína cinasa activada por AMP; EEF2K, cinasa del factor de elongación eEF2; eIF4E, factor de iniciación de traducción 4; FOXO1/3a, proteína forkhead box O1/3a; GSK3, glucógeno sintasa cinasa 3; HIF1α, factor inducible de hipoxia 1-alfa; IκB, inhibidor de κB; IKK, cinasa de IκB; mTOR, blanco funcional de la rapamicina; NFκB, factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas; PDK1, piruvato deshidrogenasa cinasa; PI3K, fosfatidilinositol 3-cinasa; PKC, proteína cinasa C; Rheb, homólogo de Ras enriquecido en el cerebro; S6K, proteína ribosomal S6 cinasa beta-1; TSC1/2, complejo de esclerosis tuberosa; ULK1, cinasa 1 activadora de la autofagia similar a unc-51.

**mTOR y los transportadores de aminoácidos**

El mTORC1 puede ser activado cuando los aminoácidos son abundantes. En ausencia de nutrientes e hipoxia, los niveles de ATP disminuyen rápidamente activando a la cinasa activada por AMP (AMPK), quien a su vez fosforila a TSC2 y Raptor, inactivando a mTORC1 (Fig. 2) (Kim y Guan, 2011; Poncet y Taylor, 2013). Se ha sugerido que los transportadores de aminoácidos pueden influenciar la actividad de mTORC1 regulando la concentración intracelular de aminoácidos o al actuar como sensores llamados “transceptores” que inician vías de señalización. Además, los transportadores proveen la fuente primaria de nitrógeno celular y participan en la síntesis de nucleótidos, carbohidratos, proteínas y GSH (Fuchs y Bode, 2005; Goberdhan et al., 2016; Kim y Guan, 2011). Un aumento en la disponibilidad de aminoácidos esenciales aumenta la expresión de transportadores de aminoácidos como el transportador del sistema A tipo 2 (SNAT2) y LAT1, lo que representa una respuesta adaptativa para mejorar la entrega de aminoácidos y, en consecuencia, aumentar la señal de crecimiento mediada por mTORC1 (Poncet y Taylor, 2013). Además, existen estudios que indican que mTOR puede regular el aumento de los transportadores de aminoácidos (Edinger, 2007). Esta doble regulación hace que sea difícil distinguir entre causa y efecto, si es mTOR quien activa a los transportadores o los transportadores a mTOR (McCracken y Edinger, 2013).

**Señalización de mTOR en el SNC**

En el cerebro mTORC1 es parte de las vías de señalización activadas por neurotransmisores y neurotrofinas (Garza-Lombó y Gonsebatt, 2016). mTOR está involucrado en numerosos procesos en el SNC incluyendo la regulación del neurodesarrollo, la plasticidad sináptica, la neurogénesis en adultos, así como la memoria y el aprendizaje. Durante el envejecimiento, el mTOR incrementa la neurogénesis, inhibe la autofagia y también regula cambios epigenéticos (Garza-Lombó y Gonsebatt, 2016).

La desregulación de mTOR se ha relacionado a diversos desórdenes cerebrales asociados a la disfunción neuronal y la muerte celular entre ellos, la neurodegeneración, epilepsia, autismo y alteraciones en el comportamiento (Crino, 2016; Garza-Lombó et al., 2018b).

**Factor de crecimiento neuronal (NGF)**

El NGF es una neurotrofina que se descubrió como una molécula que regula la sobrevivencia y maduración de las neuronas en el desarrollo (Sofroniew et al., 2001). El NGF es sintetizado en la forma de un precursor de 30-35 kDa que debe ser procesado por proteasas para dar origen a la neurotrofina madura de aproximadamente 13 kDa (Friedman y Greene, 1999; Lu et al., 2005).

El NGF puede unirse a dos tipos de receptores transmembranales. El primero es el receptor de tropomiosina tipo cinasa A (TrkA), que es un RTK. Este receptor al unirse con su ligando NGF se dimeriza y transfosforila activando tirosinas (Tyr) dentro y fuera de su dominio de cinasa. Esta transfosforilación genera sitios específicos de unión para proteínas adaptadoras que contienen dominios SH2 como la fosfolipasa C gamma (PLCγ), PI3K y Shc una proteína adaptadora que promueve la activación de Ras a través de la unión del complejo Grb2-Sos. Cuando el NGF se une a su receptor promueve la iniciación de diversas vías de señalización a través de la fosforilación específica de residuos de Tyr (490 y 785) en el receptor. Algunas de las vías de señalización que se activan a través de las fosforilación del residuo Tyr 490, son las cinasas activadas por mitógenos/ cinasa regulada por señales extracelulares (MAPK/ERK) y la de PI3K/AKT que también puede fosforilar a la cinasa de IκB (IKK) que fosforila al inhibidor de κB (IκB), resultando en la activación del factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NFκB); mientras que la Tyr 785 induce la vía de la PLCγ/proteína cinasa C delta (PKCδ) que también puede activar la vía de las MAPKs (Fig. 3) (Huang y Reichardt, 2003; Khwaja, 1999; Reichardt, 2006; Sofroniew et al., 2001). Estas vías están involucradas en las funciones de

sobrevivencia y diferenciación de esta neurotrofina. El segundo tipo son los receptores $p75^{NTR}$ que pertenecen a la superfamilia de receptores del factor de necrosis tumoral (TNF) y son considerados de baja afinidad; su activación está asociada con diferentes vías de señalización (Friedman y Greene, 1999; Lu et al., 2005). La activación de la de la GAP Cdc42 por el receptor, activa a la cinasa reguladora de la señal de apoptosis (ASK1), la cual a su vez activa la cascada de las MAPK/ de la cinasa c-jun N -terminal (JNK) que puede activar una respuesta de muerte celular por apoptosis (Reichardt, 2006). El receptor $p75^{NTR}$ interactúa con los factores asociados al receptor TNF (TRAFs) dando lugar a la activación de NFκB otra vía importante de este receptor. $p75^{NTR}$ se une a TRAF6 que, en un complejo con la cinasa asociada al receptor de interleucina 1 (IRAK) activa a IKK promoviendo la actividad de NFκB el cual activa respuestas de sobrevivencia celular (Reichardt, 2006; Sofroniew et al., 2001) (Fig. 3).

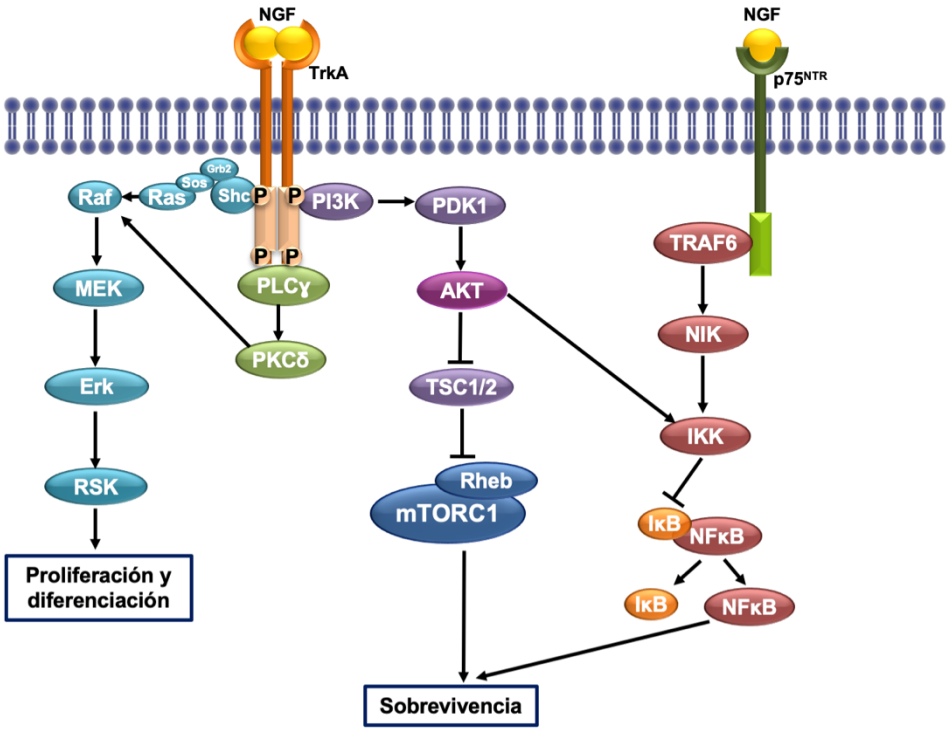


**Figura 3. Vía de señalización del NGF.** NGF se une a dos tipos de receptores transmembranales, el receptor TrkA el cual se dimeriza y transfosforila, activando las siguientes vías de señalización: MAPK/ERK, PI3K/AKT/TSC1/2/mTOR o PI3K/AKT/IKK/ IκB/NFκB y PLCγ/PKCδ que también puede activar la vía de las MAPKs llevando a respuestas de proliferación, diferenciación y sobrevivencia. (continúa)

Los receptores $p75^{NTR}$, activan a la GAP Cdc42 que activa a la cinasa ASK1, la cual a su vez activa la cascada de las MAPK/JNK que puede activar una respuesta de muerte celular por apoptosis. $p75^{NTR}$ también se une a TRAF6 que, en un complejo con IRAK activa a IKK que fosforila a IκB promoviendo la actividad de NFκB el cual activa respuestas de sobrevivencia celular. Abreviaturas: ASK1, a la cinasa reguladora de la señal de apoptosis; ERK, cinasa regulada por señales extracelulares; GAP, GTPasa; GRB2, proteína 2 de unión al receptor de factores de crecimiento; IκB, inhibidor de κB; IKK, cinasa de IκB; IRAK, cinasa asociada al receptor de interleucina 1; JNK, cinasa c-jun N -terminal; mTOR, blanco funcional de la rapamicina; NFκB, factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas; PDK1, piruvato deshidrogenasa cinasa; PI3K, fosfatidilinositol 3-cinasa; PKCδ, proteína cinasa C delta; PLCγ, fosfolipasa C gamma; Rheb, homólogo de Ras enriquecido en el cerebro; RSK, familia de cinasas ribosomales S6; Shc, proteína adaptadora 1 Shc; Sos, hijo de las proteínas sevenless; TRAF6; factor 6 asociado al receptor TNF; TrkA; tropomiosina tipo cinasa A; Tyr, tirosina; TSC1/2, complejo de esclerosis tuberosa.

**Señalización de NGF en la respuesta antioxidante**

Existe evidencia de que el NGF puede inducir respuestas antioxidantes en diferentes condiciones experimentales, no sólo en el SNC, pero en otros tejidos no neuronales, así como regular el transporte de aminoácidos y la síntesis de GSH.

En la línea celular de tumores de médula adrenal de ratón (PC12) el tratamiento con NGF aumentó el consumo de cisteína y cistina llevando a un incremento en los niveles de GSH (Pan y Perez-Polo, 1996). Del mismo modo el NGF incrementa los niveles de las enzimas antioxidantes CAT y la GPx (Sampath et al., 1994). En células PC12 tratadas con $H_2O_2$, el NGF estimuló la síntesis de GSH y la actividad de la GCL, la GPx y la glucosa 6-fosfato deshidrogenasa (G6P-D) (Pan y Perez-Polo, 1993). El NGF también protegió a las células del daño inducido por $H_2O_2$ a través de la inducción de la actividad de la CAT y GPx (Jackson et al., 1990). Todos estos estudios sugieren que esta neurotrofina está involucrada en los procesos de regulación del GSH.

Adicionalmente, en modelos *in vivo*, se ha demostrado que el NGF restaura los niveles de enzimas antioxidantes como la SOD, la GPx y la CAT en diferentes regiones del cerebro de ratas envejecidas (Nistico et al., 1992). En un modelo de Parkinson se demostró que el NGF puede restaurar los niveles de la SOD y la GPx (Ninkovic et al., 2000); en un modelo de Huntington el NGF puede mantener el cociente GSH/GSSG en las regiones del cerebro, estriado e hipocampo (Maksimovic et al., 2001); y en un modelo de Alzheimer el tratamiento con NGF restauró los niveles de la GR y la GPx (Cruz-Aguado et al., 1999). También, los

ratones transgénicos que sobre expresan NGF presentan un incremento en los niveles de GSH en cerebro, hígado y plasma (Arsenijevic et al., 2007). Finalmente, en nuestro grupo de trabajo se demostró que la vía de NGF/TrkA/AKT/IKK/IκB /NFκB es activada en el hígado de ratón después del tratamiento con agentes que consumen GSH, induciendo una respuesta antioxidante y de sobrevivencia (Valdovinos-Flores y Gonsebatt, 2013).

Estos resultados revelan la función neuroprotectora del NGF no sólo en enfermedades neurodegenerativas, envejecimiento y contra estrés oxidante en tejidos y células neuronales; sino también en otros tejidos periféricos.

## JUSTIFICACIÓN

El estudio de la regulación de la homeostasis de GSH y la respuesta a estrés oxidante en el SNC, es de gran interés por las aplicaciones potenciales que puede tener en los tratamientos para prevenir neuropatías, neurodegeneración y el envejecimiento prematuro. Los resultados de este trabajo ayudarán a entender los mecanismos moleculares que participan en la regulación de la homeostasis de GSH con el fin de diseñar estrategias de neuroprotección y prevención.

Asimismo, el mTOR se ha convertido en un blanco terapéutico para inhibir la proliferación tumoral, por lo que el conocimiento de su participación en la regulación de la homeostasis de GSH puede aportar argumentos para tomar con cautela las terapias que utilizan inhibidores de mTOR en otros padecimientos como las enfermedades neurodegenerativas.

## HIPÓTESIS

Los cambios en el estado redox y en la homeostasis de GSH generados por el BSO, inducirán un incremento en el transporte de aminoácidos involucrados en la síntesis de GSH y con las vías de señalización de mTOR y NGF en el SNC.

## OBJETIVOS

### Objetivo modelo *in vivo*

- Investigar los cambios en la homeostasis de GSH en el SNC inducidos por un tratamiento sistémico con BSO; y su asociación con el transporte de aminoácidos involucrados en la síntesis de GSH y la activación de las vías de señalización de mTOR y NGF.

### Objetivo modelo *in vitro*

- Investigar en cultivos primarios de astrocitos corticales, los cambios en la homeostasis de GSH inducidos por el tratamiento con BSO, así como el papel de la vía de señalización de mTOR.

## MATERIAL Y MÉTODOS

*Reactivos*

Los anticuerpos contra fosforilado (p)-p70 S6K (Thr 389, #9234), p-mTOR (Ser 2448, #5536), p-TrkA (Tyr 490, #9141), p70 S6K (#2708), mTOR (#2983), NGF (#2046) p-4EBP1 (Thr 37/46, #2855), 4EBP1 (#9452), p-AKT (Ser 473, #9271), AKT (#9272), el secundario anti-conejo (#7074) y el marcador biotinilado (#7727) se obtuvieron de Cell Signaling Technology (Danvers, MA, EUA). Los anticuerpos contra EAAT3 (ab124802), TrkA (ab76291), xCT (ab37185), NeuN (ab177487) y NGF (ab52918, para inmunofluorescencia) se adquirieron en Abcam (Cambridge, Reino Unido). Los anticuerpos contra LAT1 (sc-134994), mtTFA (sc-376672), GCLc (sc-390811) y GCLm (sc-55586) se obtuvieron de Santa Cruz Biotechnology (Santa Cruz, CA, EUA). El anticuerpo contra β-tubulina (T4026), el BSO, el O-ftalaldehído (OPA, P0657-5G) y las microplacas (Greiner high and medium binding 96 well plates M4686-40EA) fueron comprados en Sigma-Aldrich (Darmstadt, Alemania). El anticuerpo contra β-actina (GT5512) en GeneTex (Irvine, CA, EUA). El anticuerpo secundario anti-ratón (61-6520), el ensayo de cuantificación de proteína Pierce de ácido bicinconínico (BCA), el medio de cultivo Dulbecco's Modified Eagle Medium: Nutrient Mixture F- 12 (DMEM/F-12) y los colorantes para citometría de flujo, ioduro de propidio (PI) y el monoclorobimano (mbCl) se obtuvieron de Thermo Fisher Scientific (Waltham, MA, EUA). Los anticuerpos secundarios para inmunofluorescencia Alexa Fluor 546 anti-conejo, Alexa Fluor 488 anti-ratón y el marcador de núcleos 4',6-diamino-2-fenilindol (DAPI) fueron adquiridos en Invitrogen (Carlsbad, CA, EUA). El kit de quimioluminiscencia para Western blot, ECL (Enhanced Chemiluminescence) Advance Western Blotting y las placas fotográficas Amersham Hyperfilm ECL se compraron en Amersham Biosciences (Little Chalfont, Reino Unido). El suero de caballo y el medio de montaje de fluorescencia se obtuvieron de laboratorios Vector (Burlingame, CA, EUA) y Agilent DAKO (Santa Clara, CA, EUA), respectivamente.

**Modelo *in vivo***

*Protocolo de tratamiento*

Los animales utilizados en este estudio fueron proporcionados por la Unidad de Modelos Biológicos del Instituto de Investigaciones Biomédicas de la UNAM.

En nuestro modelo experimental utilizamos ratones BALB/c de 5 semanas de edad mantenidos a 23-25°C con una humedad relativa de 50-60% y bajo un ciclo de luz/oscuridad de 12 h. Los ratones recibieron una inyección intraperitoneal (ip) de 6 mmol/kg de BSO disuelto en solución salina. Los controles se inyectaron vía ip con solución salina. Los animales se sacrificaron a las 0.5 y 2 h después de la inyección. El sacrificio se realizó por dislocación cervical y los tejidos se procesaron inmediatamente o se guardaron a una temperatura de -70 ºC.

Los experimentos descritos en este trabajo se realizaron siguiendo las normas descritas en "Principles of Laboratory Animal Care" (Institutos Nacionales de Salud, NIH) y la Norma Oficial Mexicana de la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) titulada "Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio" (Clave NOM-062-ZOO-1999, publicado en agosto de 2001).

*Determinación de GSH*

Las muestras de tejido se homogenizaron en 10 volúmenes de amortiguador A (KCl 154 mM, pentaacetato de dietilentriamina (DTPA) 5 mM y amortiguador de fosfatos (PB) 0.1 M, pH 6.8). Después de homogenizar, se agregó un volumen de amortiguador B (HCl 40 mM, DTPA 10 mM, ácido ascórbico 20 mM y ácido tricloroacético 10%) a un volumen del homogenado. La suspensión se centrifugó a 14,000 *g* y el sobrenadante se filtró a través de filtros de 0.45 μm. Las muestras pueden ser almacenadas a -70 ºC por 4 semanas.

Los niveles de GSH y GSSG se determinaron en las muestras de tejido. El homogenado y

las mediciones se realizaron mediante la metodología descrita por Senft et al., 2000. El ensayo fluorométrico de OPA se adaptó a microplaca. La fluorescencia se determinó con filtros de 365 nm de excitación y 430 nm de emisión en un Multidetector de Elisa DTX 800/880 (Beckman Coulter, Fullerton, CA, EUA). Se usó como referencia una curva estándar de GSH y GSSG.

El método de OPA se basa en la formación de un derivado fluorescente, isoindol, para el caso del GSH. Para la determinación del GSSG en un primer paso se inhibe esta reacción y posteriormente el GSSG es reducido a GSH para la posterior derivación del isoindol con OPA.

*Extracción de fracción citoplásmica*

Se disectó el cerebelo y las muestras frescas de tejido se homogenizaron con el amortiguador A (HEPES 10 mM pH 7.9, KCl 10 mM, EDTA 10 mM, DTT 1 mM, IGEPAL 0.4%, $Na_3VO_4$ 1 mM, PMSF 1 mM, aprotinina y leupeptina 10 mg/ml), se incubaron 10 minutos en hielo, y se centrifugaron a 4,000 *g* 15 min a 4 °C. El sobrenadante (fracción citoplásmica) se recolectó y se almacenó a -70 ºC (Basaki et al., 2007).

*Extracción de proteínas de membrana por ultracentrifugación*

También se utilizó un protocolo de extracción de membranas por ultracentrifugación (Schindler et al., 2006). Las muestras de tejido se homogenizaron en 10 volúmenes de amortiguador de extracción BCL (HEPES 10 mM, NaCl 10 mM, $KH_2PO_4$ 1 mM, $NaHCO_3$ 5 mM, EDTA 5 mM, $CaCl_2$1 mM, $MgCl_2$ 0.5 mM, PMSF 1 mM, aprotinina y leupeptina 10 mg/ml) que contenía 10% de sacarosa 2.5 M. El homogenado obtenido se centrifugó en dos ocasiones a 6,300 *g* por 10 minutos a 4 °C. Los sobrenadantes se recolectaron y se ultracentrifugaron a 100,000 *g* por 30 minutos a 4 °C. El precipitado se recuperó y solubilizó en Tris-HCl 40 mM, pH 9.5, urea 8 M y Tritón X-100 4%. La concentración de proteína se determinó usando el ensayo de BCA.

*Western blot*

Las muestras se corrieron en un gel de acrilamida al 10% o 6% en el caso de mTOR, en condiciones desnaturalizantes (SDS-PAGE) posteriormente se electro-transfirieron a una membrana de nitrocelulosa. Para prevenir la unión inespecífica las membranas se bloquearon en TBS/Tween (Tris pH 7.6, NaCl y Tween 20 al 0.1%) con leche Blotto o albúmina de suero bovino (BSA) al 5% a 4 °C toda la noche con agitación. Las membranas se incubaron con el anticuerpo primario correspondiente, después de la incubación las membranas se lavaron 3 veces en TBS/Tween. A continuación, se incubó con el anticuerpo secundario correspondiente acoplado a la peroxidasa de rábano. Finalmente, la presencia de las proteínas se determinó por quimioluminiscencia incubando las membranas con el sistema de ECL y exponiendo una placa fotográfica. Para analizar las diferentes proteínas en la misma membrana después de cada quimioluminiscencia, las membranas se sometieron a un despegado de los anticuerpos con NaOH 0.2 mM para poder repetir el procedimiento anterior con cada uno de los anticuerpos de las proteínas de interés. Se utilizó el marcador de peso molecular biotinilado para identificar las bandas de las proteínas según su peso. Para la detección del marcador de peso molecular se agregó la anti-biotina junto con los anticuerpos secundarios. Los análisis densitométricos se realizaron utilizando el software ImageJ versión 1.50 g (Wayne Rasband, NIH, MD, EUA). Para la normalización de los datos obtenidos se utilizaron la β-tubulina o la β-actina.

*Inmunofluorescencia*

Los ratones se trataron con BSO y se sedaron con 70 mg/kg de pentobarbital sódico y se perfundieron con solución salina, seguido de paraformaldehído al 4% en PB, pH 7.4. Posteriormente los animales se decapitaron y el cerebro completo se removió cuidadosamente, se postfijó en solución crioprotectora de sacarosa al 20% y 30% por 24 h a 4 °C y posteriormente se congelaron con 4-metilbutano. Se cortaron secciones de 50 µm

en criostato y se colectaron en placas de 24 pozos con PB. Los cortes se lavaron tres veces con PB + Triton X-100 0.3% (PBT), y se incubaron con los anticuerpos primarios: anti-NeuN (marcador neuronal; 1:600) y anti-TrkA (1:200) o anti-NGF (1:200) en PBT+ BSA 0.3% y suero de caballo 2%. Los cortes se lavaron tres veces con PBT y se incubaron con los anticuerpos secundarios anti-conejo Alexa Fluor 546 (1:600) anti-ratón Alexa Fluor 488 (1:400) durante 2 h. Finalmente, los núcleos se tiñeron con DAPI siguiendo las instrucciones del fabricante. Los cortes se montaron en medio de montaje de fluorescencia. Se adquirieron imágenes confocales con un confocal laser Nikon A1R$^{+}$ acoplado a un microscopio invertido Eclipse Ti-E (Nikon Corporation, Tokio, Japón). Para analizar las imágenes se utilizó el programa ImageJ 1.50g.

*Análisis estadístico*

Los datos se analizaron con la prueba *t* de Student utilizando el software GraphPad Prism versión 6.00 (GraphPad Software, San Diego, CA, EUA). Los datos se graficaron con los promedios ± error estándar (SE). Los valores de $p < 0.05$ se consideraron significativos.

**Modelo *in vitro***

*Cultivo primario de astrocitos corticales*

Los astrocitos se aislaron de la corteza de ratas Wistar de 1-2 días de edad. Las cortezas se disectaron removiendo las meninges y los vasos sanguíneos. El tejido se desmenuzó en pequeños pedazos y se digirió con 0.25 mg/ml de tripsina a 37 °C por 15 minutos. Posteriormente se añadió solución de DNasa al 0.016% y se incubó en baño maría por 10 minutos, mezclando periódicamente. La digestión se detuvo con medio de cultivo DMEM/F-12 con suero bovino fetal (SBF) 10 %, penicillina (200 unidades/ml)- streptomicina (200 µg/ml). A continuación, las cortezas se trituraron con pipetas Pasteur y se añadió más solución de DNasa con medio de cultivo, después se realizó una centrifugación a 1700 rpm por 5 minutos. Finalmente, las células se filtraron a través de un filtro de 70 µm, se diluyeron

con medio, se contaron y se sembraron. Los astrocitos se mantuvieron en una incubadora a 37 °C con $CO_2$ 5% en una atmósfera húmeda hasta que alcanzaron una confluencia de 90-95%.

Los astrocitos se trataron con BSO 500 μM y con inhibidores de la vía de señalización de mTOR: mTORC1, mTORC1/2 y la proteína S6K, así como de los transportadores de aminoácidos y con inhibidores de la función mitocondrial, en los resultados se indican las diferentes estrategias de tratamiento utilizadas. Por otro lado, las células se transdujeron con vectores de expresión que expresan la proteína verde fluorescente redox sensible (roGFP), tanto en citosol (cito-roGFP) como en mitocondria (mito-roGFP), para determinar la presencia de estrés oxidante en la célula.

*Determinación de la muerte celular (pérdida de la integridad de membrana y depleción de GSH) para determinar la viabilidad celular*

La pérdida en la viabilidad celular se determinó por citometría de flujo en presencia de PI 1 μg/ml como un marcador de pérdida de la integridad de membrana. Además, se midieron los niveles intracelulares de GSH utilizando el marcador mBCl 5 μM. La fluorescencia del PI se detectó usando FL-3 (488 nm excitación, 695/40 nm emisión), y para mBCl, las células se excitaron con un laser ultravioleta (UV) (405 nm excitación, 440/40 nm emisión). Tanto el consumo de PI como la concentración de GSH se determinaron en todas las condiciones experimentales para asegurar que las dosis utilizadas en los tratamientos no resultaran citotóxicas.

*Extracción de proteína y Western blot*

Las muestras se homogenizaron con amortiguador de radioinmunoprecipitación (RIPA) (Tris HCl 50 mM pH 8, NaCl 150 mM, sodio deoxicolato 0.5%, SDS 0.1%, Tritón X-100 1%) con inhibidores de proteasas. Las células se sonicaron y se centrifugaron descartando el precipitado. La concentración de proteína se determinó usando el ensayo de BCA.

Las muestras se corrieron siguiendo el protocolo utilizado en el modelo *in vivo*, con la excepción de que los geles fueron de Bis-Tris acrilamida utilizando el amortiguador 3-N-morfolino y ácido propansulfónico (MOPS) + 5 mM de bisulfito de sodio y las membranas se revelaron en un fotodocumentador VersaDoc (Bio-Rad, Hercules, CA, EUA).

*Determinación de la concentración celular de ATP*

La concentración de ATP en las células se determinó con el ensayo cell titer-Glo luminiscent cell viability, los datos se normalizaron con el marcador de viabilidad celular calceína AM. Este ensayo cuantifica el ATP presente en las células, marcando las células metabólicamente activas; utiliza a la enzima luciferasa para mono-oxigenar a la luciferina en presencia de magnesio ($Mg^{2+}$), ATP y oxígeno molecular. La calceína AM es absorbida por las células vivas donde las enzimas esterasas la transforman en calceína la cual es fluorescente. La concentración de ATP se muestra en función de los valores de calceína y los datos se normalizaron con el grupo control.

*Análisis estadístico*

Los datos se analizaron utilizando un análisis de varianza (ANOVA) de dos vías con la prueba posthoc adecuada utilizando el software GraphPad Prism. Los datos se graficaron con los promedios ± SE. Los valores de $p < 0.05$ se consideraron significativos.

## RESULTADOS

### Modelo *in vivo*

#### Cambios en los niveles de GSH y GSSG en el hígado de ratones tratados con BSO

Para demostrar la eficacia del BSO bloqueando la síntesis *de novo* de GSH, se determinaron los niveles de GSH y GSSG en el hígado. No se observaron cambios en los niveles de GSH hasta 2 h después de la inyección con BSO (Fig. 4a). Pero, sí se encontró una disminución significativa en los niveles de GSSG a las 0.5 h (Fig. 4b). Sin embargo, tampoco se observaron cambios en el cociente GSH/GSSG (Fig. 4a, b), el cual es un indicador del estado redox celular (Schafer y Buettner, 2001), por lo que estos resultados sugieren que las células están exportando o metabolizando GSSG como un mecanismo de desintoxicación para prevenir la generación de estrés oxidante en el hígado (Franco y Cidlowski, 2012).

#### Niveles de GSH y GSSG en diferentes regiones del cerebro de ratones tratados con BSO

En estudios previos de nuestro grupo, se demostró que la inhibición sistémica de la síntesis de GSH con una inyección ip de BSO generó un incremento de los niveles de GSH en homogenado total de cerebro 1-3 h después del tratamiento, aunado a una activación de ERK2 y Nrf2 (Limón-Pacheco et al. 2007). En el presente trabajo se determinaron los niveles de GSH y GSSG en diferentes regiones del cerebro (corteza, hipocampo, estriado y cerebelo). No se encontraron cambios en los niveles de GSH y GSSG de la corteza, hipocampo y estriado (Fig. 5-7), sin embargo, en el cerebelo sí se observó una respuesta redox en respuesta al tratamiento sistémico con BSO (Fig. 8).

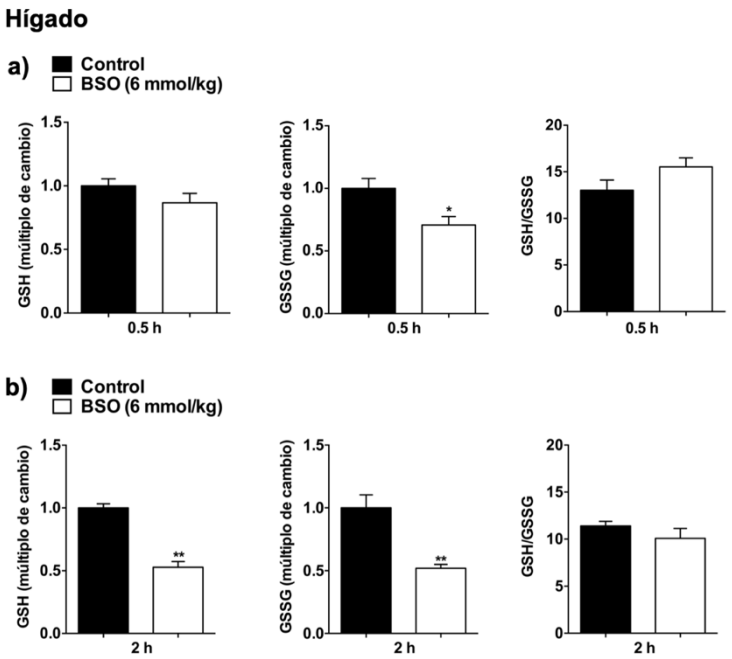


**Figura 4.** Efecto de BSO en los niveles de GSH y GSSG en el hígado de ratones tratados ip con BSO 6 mmol/kg. Niveles normalizados de GSH y GSSG, y cociente entre GSH/GSSG a la 0.5 h (**a**) y 2 h (**b**) después del tratamiento con BSO. Las gráficas representan promedios±SE n=6. Prueba de *t* de Student *p<0.05, **p<0.01 vs Control se consideran significativos. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; GSSG, glutatión disulfuro; SE, error estándar.

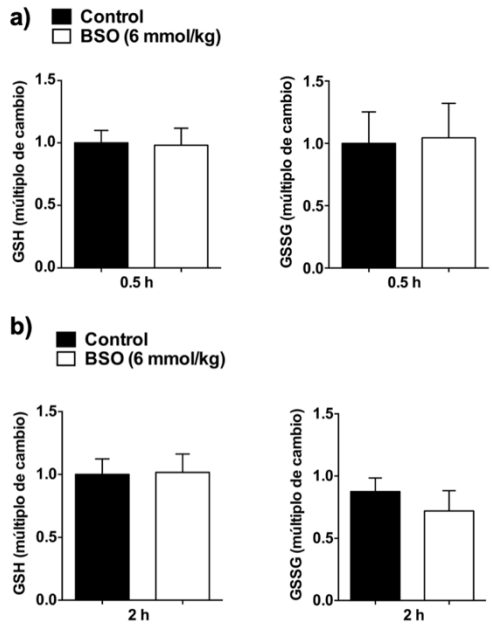


**Figura 5.** Efecto de BSO en los niveles de GSH y GSSG en corteza de ratones tratados ip con BSO 6 mmol/kg. Niveles normalizados de GSH y GSSG a la 0.5 h (**a**) y 2 h (**b**) después del tratamiento con BSO. Las gráficas representan promedios±SE n=6. Prueba de *t* de Student *p<0.05, **p<0.01 vs Control se consideran significativos. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; GSSG, glutatión disulfuro; SE, error estándar.

**El tratamiento con BSO induce un incremento en los niveles de GSH del cerebelo**

En el cerebelo no se observaron cambios significativos en los niveles de GSH y GSSG después de 0.5 h de tratamiento con BSO (Fig. 8a), sin embargo, los pequeños cambios en las concentraciones dieron lugar a un incremento significativo del cociente GSH/GSSG (Fig. 8a). Además, 2 h después de la inyección con BSO se observó un incremento en la concentración de GSH (Fig. 8b). Estos resultados revelan un incremento en el ambiente reductor de la célula a la 0.5 h, así como un aumento en los niveles de GSH a las 2 h después de la administración de BSO. El cerebelo está respondiendo a los cambios en la homeostasis redox de la periferia, aunque el BSO no sea transportado hacia el cerebro (Limón-Pacheco y Gonsebatt, 2007; Steinherz et al., 1990).

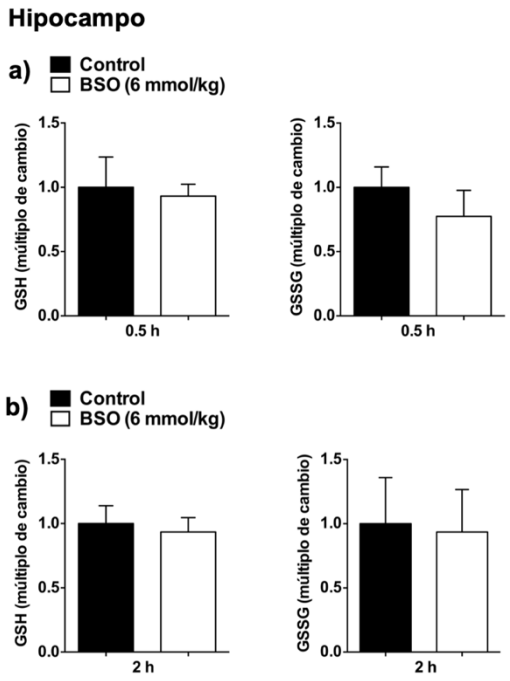


**Figura 6.** Efecto de BSO en los niveles de GSH y GSSG en hipocampo de ratones tratados ip con BSO 6 mmol/kg. Niveles normalizados de GSH y GSSG a la 0.5 h (**a**) y 2 h (**b**) después del tratamiento con BSO. Las gráficas representan promedios±SE n=6. Prueba de t de Student *p<0.05, **p<0.01 vs Control se consideran significativos. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; GSSG, glutatión disulfuro; SE, error estándar.

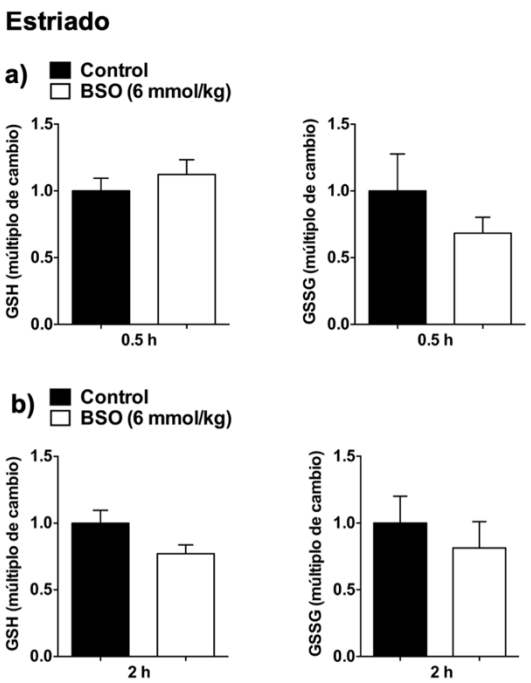


**Figura 7.** Efecto de BSO en los niveles de GSH y GSSG en estriado de ratones tratados ip con BSO 6 mmol/kg. Niveles normalizados de GSH y GSSG a la 0.5 h (**a**) y 2 h (**b**) después del tratamiento con BSO. Las gráficas representan promedios±SE n=6. Prueba de *t* de Student *p<0.05, **p<0.01 vs Control se consideran significativos. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; GSSG, glutatión disulfuro; SE, error estándar.

## Expresión de los transportadores involucrados en la síntesis de GSH (LAT1, xCT, EAAT3) en el cerebelo

La síntesis de GSH en el cerebro se ha asociado con un incremento en la expresión de los transportadores de cistina/cisteína (Limón-Pacheco et al., 2007; Nelson-Mora et al., 2018; Ramos-Chavez et al., 2015). Para determinar si el incremento en los niveles de GSH en el cerebelo estaba relacionada con los niveles de los transportadores involucrados en el transporte de cistina/cisteína, se utilizó la técnica de Western blot de fracciones membranales para determinar los niveles proteicos de los transportadores. No se observaron cambios en los niveles del transportador de aminoácidos neutros LAT1 ni de la subunidad xCT del sistema xc-, después del tratamiento con BSO (Fig. 9a-b, d-e).

Sin embargo, sí se observó un incremento en los niveles de proteína del transportador neuronal de glutamato EAAT3, que también está involucrado en el transporte de cisteína

principalmente en las neuronas, 2 h después de la inyección con BSO (Fig. 9f); pero no a la 0.5 h (Fig. 9c).

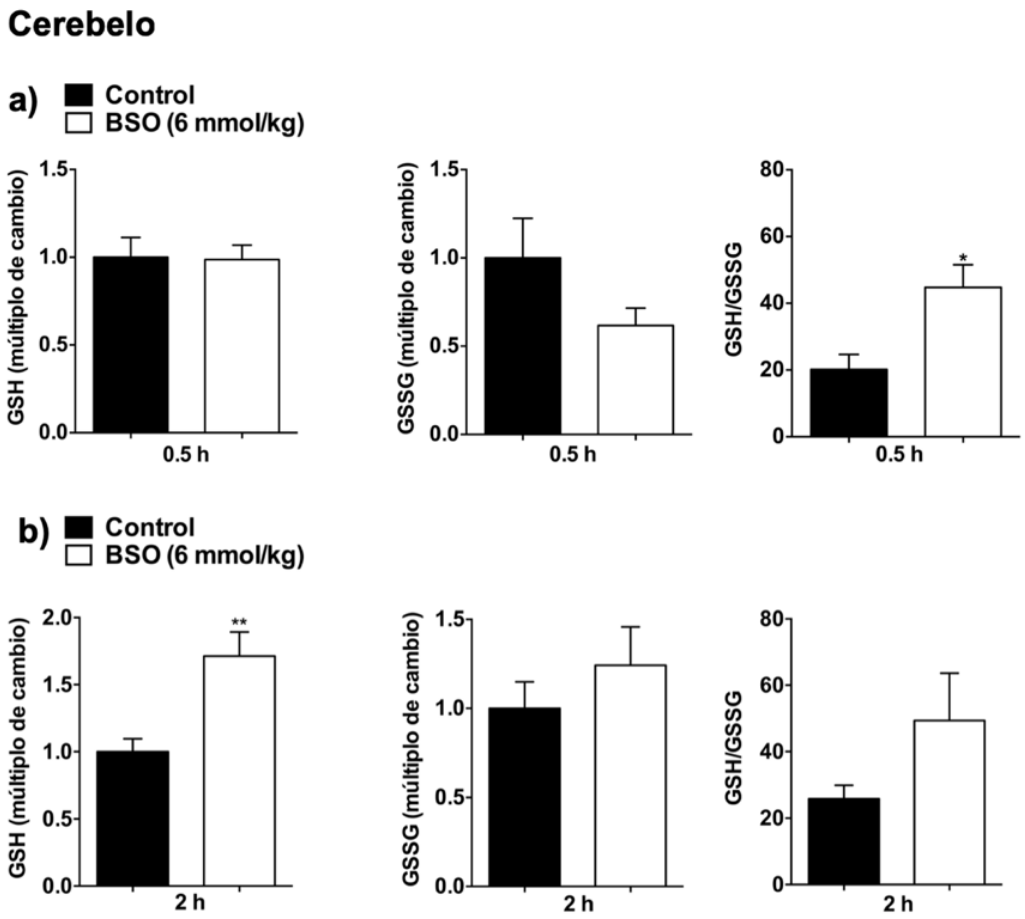


**Figura 8.** Efecto de BSO en los niveles de GSH y GSSG en el cerebelo de ratones tratados ip con BSO 6 mmol/kg. Niveles normalizados de GSH y GSSG, y cociente entre GSH/GSSG a la 0.5 h (**a**) y 2 h (**b**) después del tratamiento con BSO. Las gráficas representan promedios±SE n=6. Prueba de *t* de Student *p<0.05, **p<0.01 vs Control se consideran significativos. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; GSSG, glutatión disulfuro; SE, error estándar.

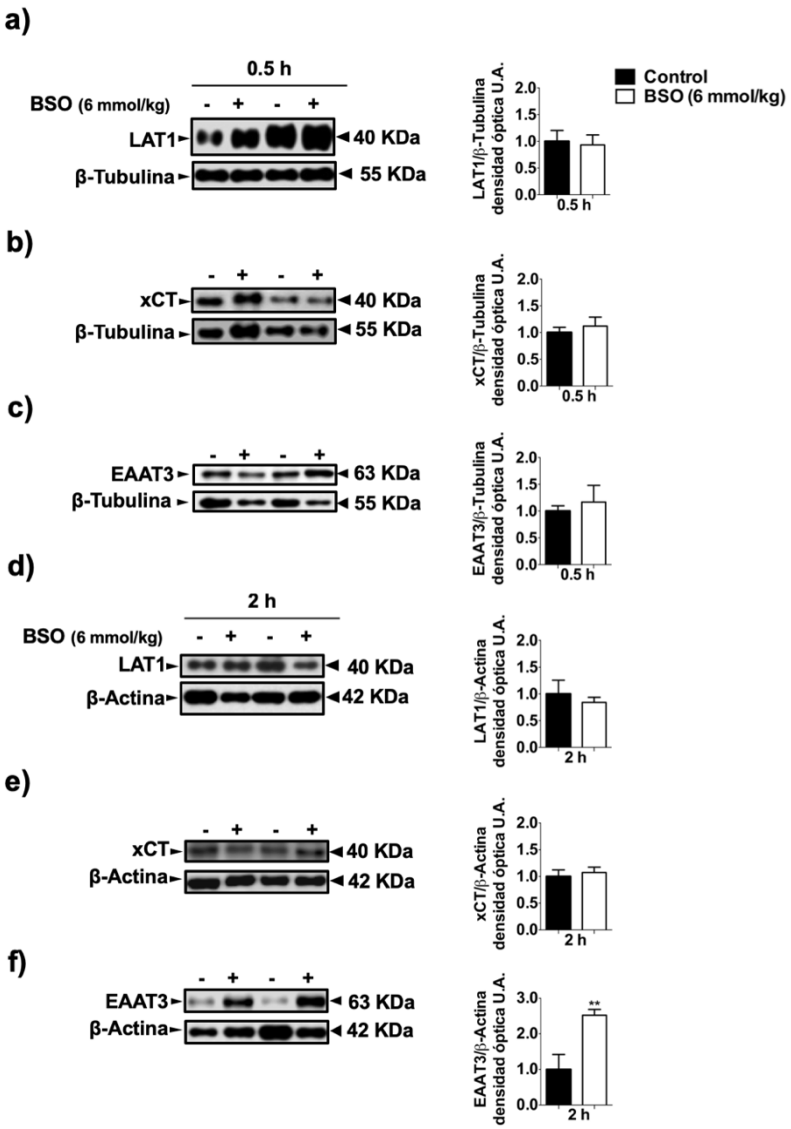


**Figura 9.** Efecto del BSO en los niveles de proteína de los transportadores de aminoácidos, LAT1, xCT y EAAT3, en el cerebelo de ratones tratados ip con BSO 6 mmol/kg. Se evaluaron los cambios en los niveles de proteína de la fracción de proteínas membranales con Western blot, a la 0.5 y 2 h después de la inyección con BSO. Las gráficas representan promedios±SE de los análisis densitométricos de las bandas normalizadas con β-tubulina (**a-c**) o β-actina (**b-d**) n=6. Prueba de *t* de Student *p<0.05, **p<0.01 vs Control se consideran significativos. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; LAT1, transportador de aminoácidos tipo L; xCT, subunidad del sistema xc-; EAAT3, transportador de aminoácidos excitadores 3; U.A.; unidad arbitraria; SE, error estándar.

### El inhibidor BSO activa las vías de señalización de mTOR y NGF en el cerebelo

Posteriormente, se decidió investigar si el tratamiento con BSO estaba activando la vía de señalización de mTOR en el cerebelo debido a la estrecha relación entre mTOR y los transportadores de aminoácidos (Goberdhan et al., 2016; Kim y Guan, 2011). No se encontró un incremento en la fosforilación ni en la expresión de mTOR (Fig. 10a, c). No

obstante, se encontró un aumento significativo en la fosforilación de, la cinasa blanco de mTORC1, S6K a la 0.5 h de tratamiento, lo que sugiere que la vía de mTORC1 está activada y puede estar participando en el aumento del transportador EAAT3.

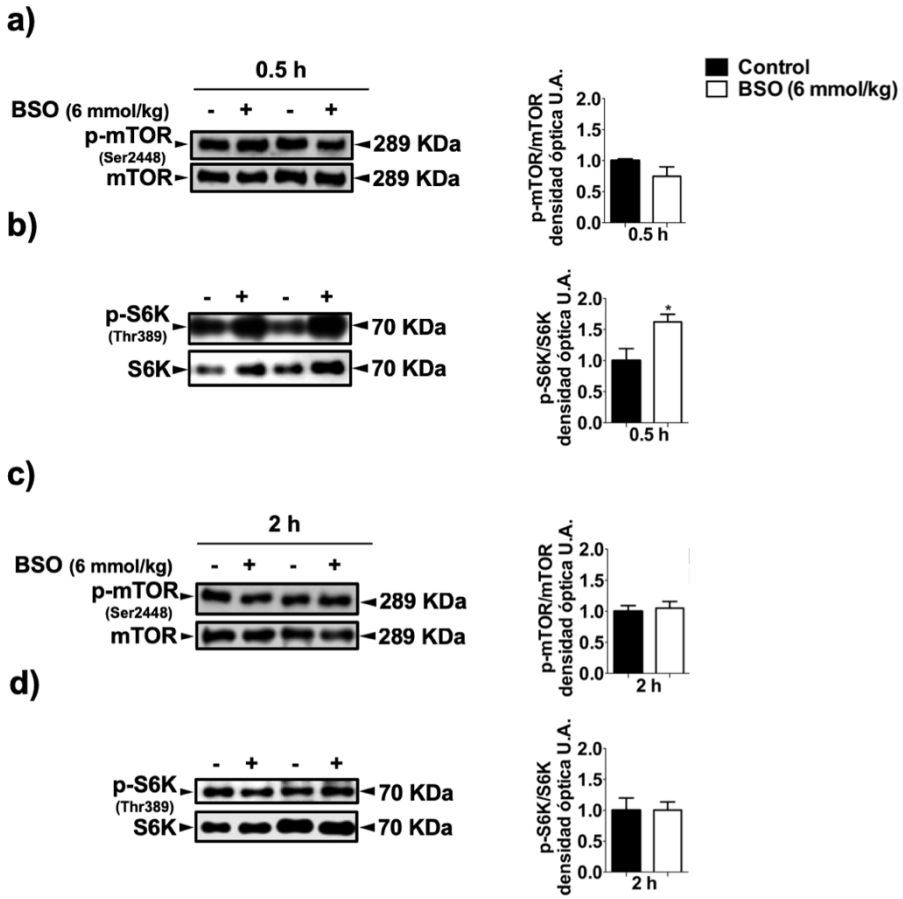


**Figura 10.** Efecto del BSO en los niveles de fosforilación de mTOR y S6K en el cerebelo de ratones tratados ip con BSO 6 mmol/kg. Se evaluaron los cambios en la fosforilación con Western blot, a la 0.5 y 2 h después de la inyección con BSO. p-mTOR (Ser 2448) (**a, c**) y p-S6K (Thr 389) (**b,d**) Las gráficas representan promedios±SE de los análisis densitométricos de las bandas normalizadas con mTOR o S6K respectivamente n=6. Prueba de *t* de Student *p<0.05, **p<0.01 vs Control se consideran significativos. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; mTOR; blanco funcional de la rapamicina; S6K, proteína ribosomal S6 cinasa beta-1; U.A.; unidad arbitraria; SE, error estándar.

Como se mencionó en la introducción, en estudios previos se demostró que la vía de NGF/TrkA/AKT/IKK/IκB /NFκB es activada en el hígado de ratón después del tratamiento con agentes que consumen GSH, induciendo una respuesta antioxidante (Valdovinos-Flores y Gonsebatt, 2013). En este estudio se determinaron los niveles de NGF y la activación de su receptor TrkA, para evaluar si la vía de señalización de NGF/TrkA estaba

activa en el cerebelo en respuesta al BSO. El análisis con Western blot demostró que el tratamiento con BSO indujo la expresión de NGF, así como la fosforilación de su receptor TrkA a la 0.5 h de tratamiento (Fig. 11a, b) pero no a las 2 h (Fig. 11a, b).

Las inmunofluorescencias confirmaron los datos obtenidos con Western blot, se observó un incremento de NGF y TrkA en las neuronas a la 0.5 h después de la inyección con BSO. Además, la expresión de TrkA se distinguió en el soma de las neuronas de la capa granular del cerebelo y en la capa molecular que contiene axones y dendritas (Fig. 12). No se observaron cambios de NGF ni de TrkA a las 2 h después del tratamiento (Fig. 13). Estos resultados sugieren que las vías de señalización de NGF y mTOR están involucradas en la regulación de la homeostasis de GSH, induciendo una respuesta antioxidante asociada a la depleción periférica de GSH. Esta activación podría estar relacionada con el incremento del transportador EAAT3 y de los niveles de GSH en el cerebelo.

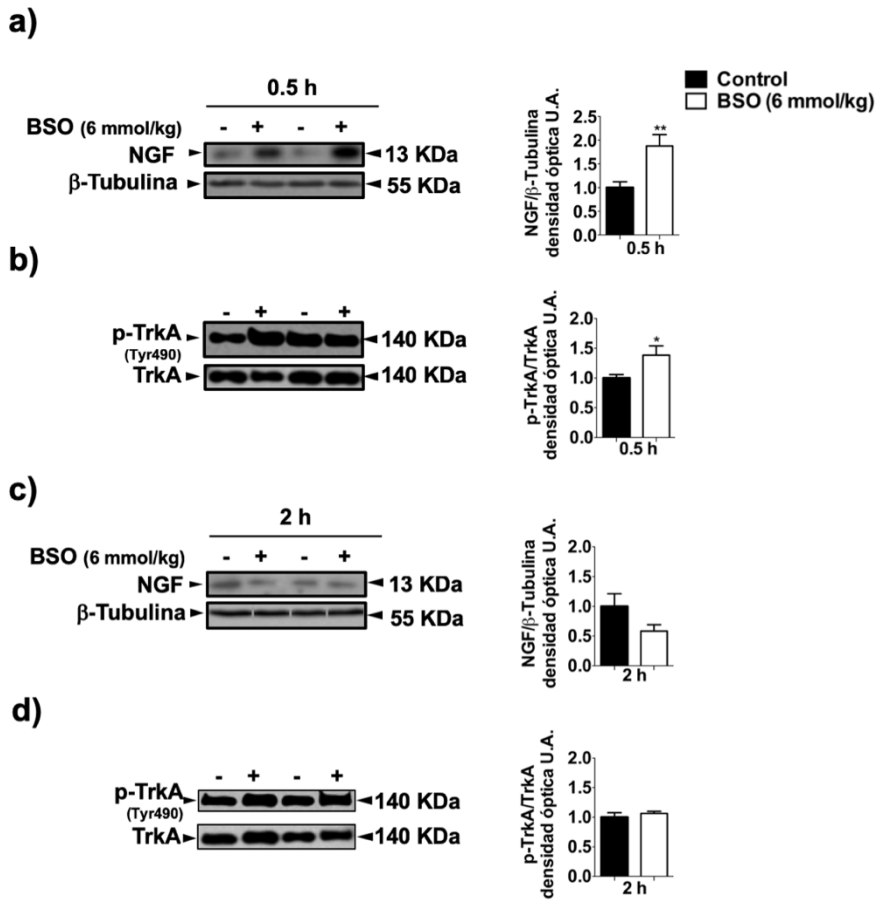


**Figura 11.** Efecto del BSO en los niveles de proteína de NGF y de fosforilación de TrkA en el cerebelo de ratones tratados ip con BSO 6 mmol/kg. Se evaluaron los cambios en los niveles proteicos y en la fosforilación con Western blot, a la 0.5 y 2 h después de la inyección con BSO. NGF (**a,c**) y p-TrkA (Tyr 490) (**b,d**). Las gráficas representan promedios±SE de los análisis densitométricos de las bandas normalizadas con β-tubulina o TrkA respectivamente n=5. Prueba de *t* de Student *p< 0.05, **p<0.01 vs Control se consideran significativos. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; NGF, factor de crecimiento neuronal; TrkA, receptor de tropomiosina tipo cinasa A; U.A.; unidad arbitraria; SE, error estándar.

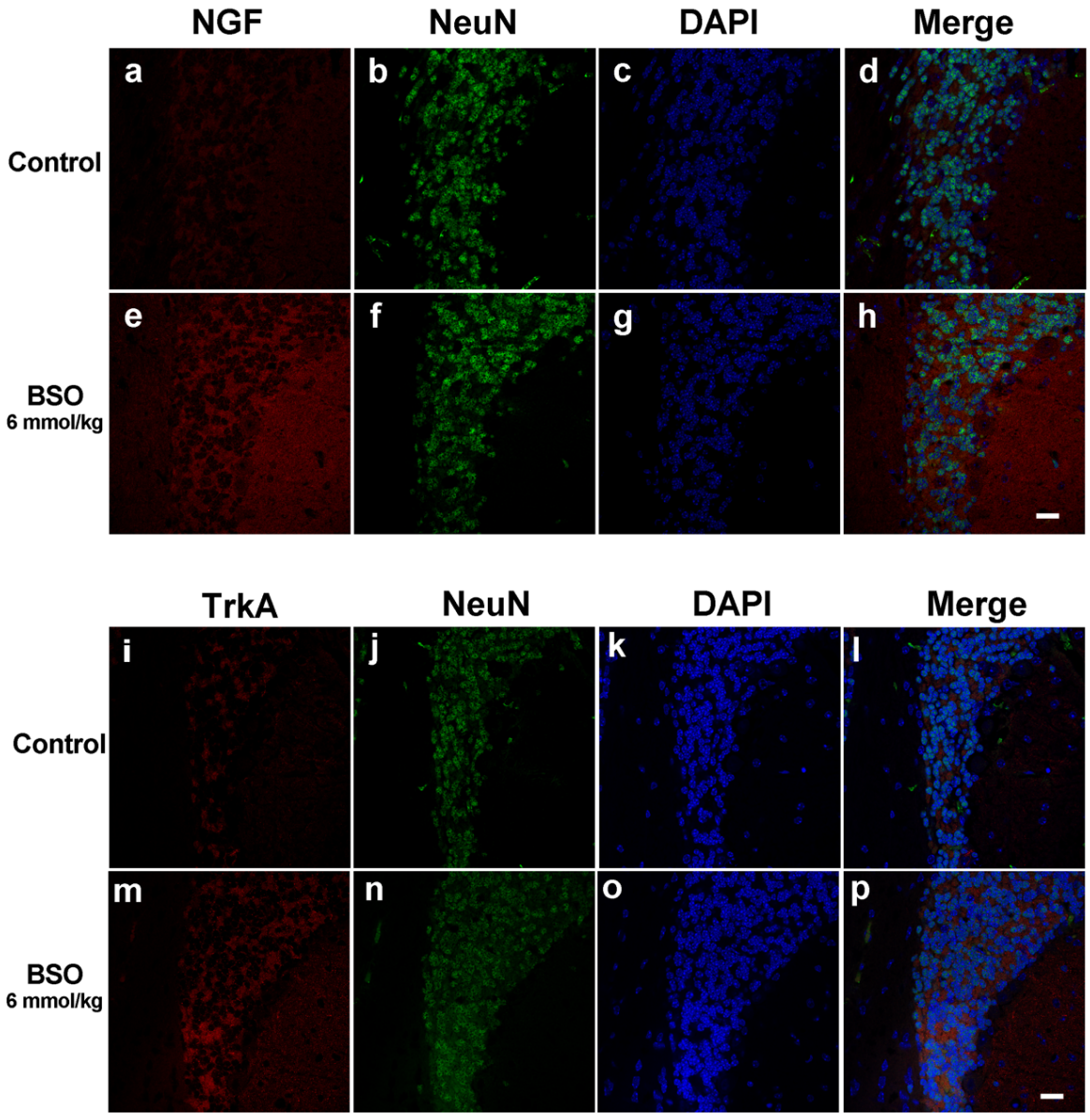


**Figura 12.** Efecto del BSO en los niveles de proteína de NGF y TrkA en el cerebelo de ratones tratados ip con BSO 6 mmol/kg. Se evaluaron los cambios en los niveles proteicos con inmunofluorescencias, a la 0.5 h después de la inyección con BSO. Imágenes confocales de ratones control y tratados. (**a, e**) muestra imágenes inmunofluorescentes de NGF (rojo), (**b, f**) marcador neuronal NeuN (verde), (**c, g**) marcador de núcleos DAPI (azul) y (**d, h**) merge. El segundo panel muestra imágenes inmunofluorescentes de (**i, m**) TrkA (rojo), (**j,n**) NeuN (verde), (**k, o**) DAPI (azul) y (**l, p**) merge. Escala: 20 micrómetros. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; NGF, factor de crecimiento neuronal; TrkA, receptor de tropomiosina tipo cinasa A; NeuN, núcleos neuronales; DAPI, 4',6-diamino-2-fenilindol.

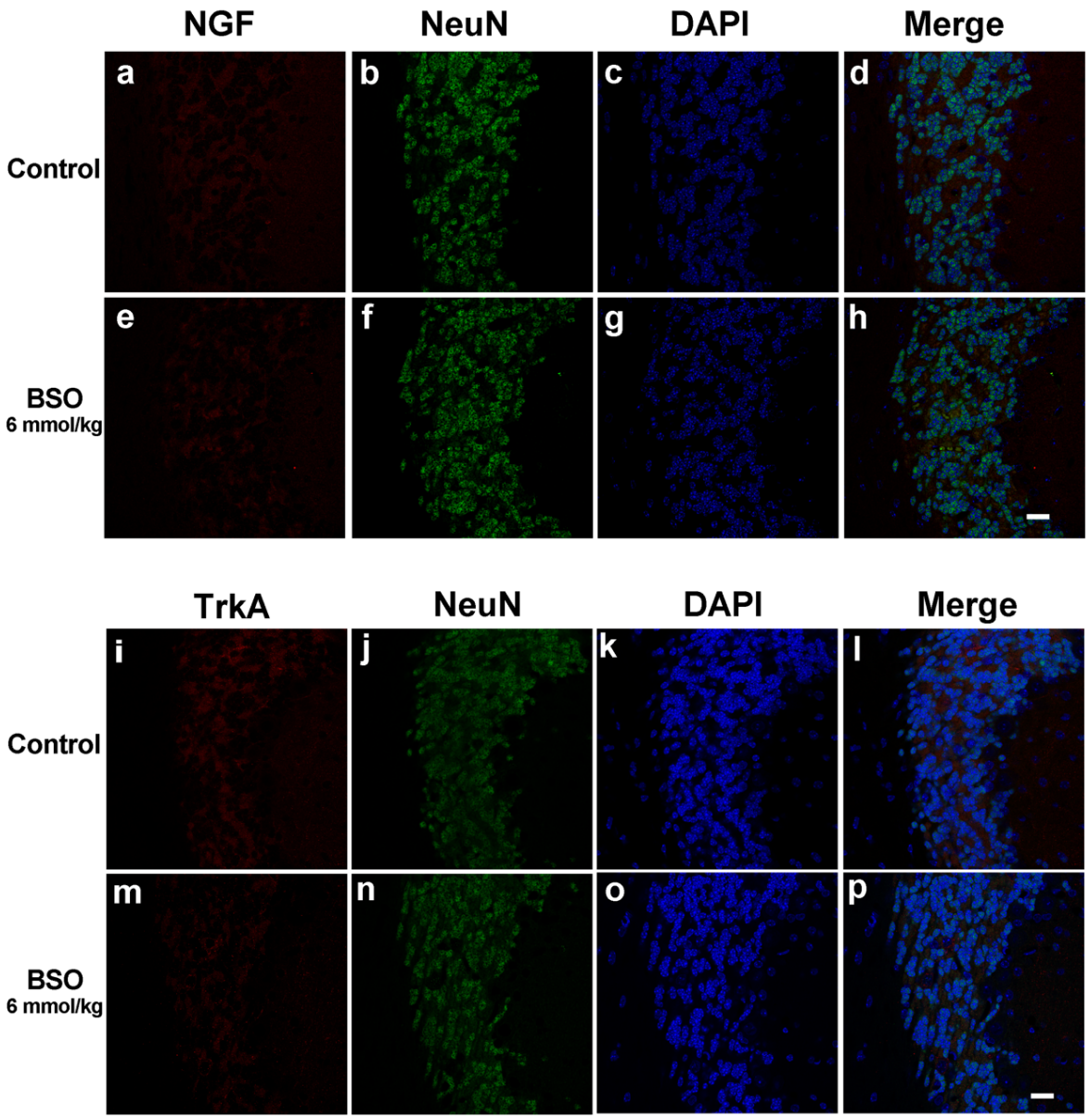


**Figura 13.** Efecto del BSO en los niveles de proteína de NGF y TrkA en el cerebelo de ratones tratados ip con BSO 6 mmol/kg. Se evaluaron los cambios en los niveles protéicos con inmunofluorescencias, a la 2 h después de la inyección con BSO. Imágenes confocales de ratones control y tratados. (**a, e**) muestra imágenes inmunofluorescentes de NGF (rojo), (**b, f**) marcador neuronal NeuN (verde), (**c, g**) marcador de núcleos DAPI (azul) y (**d, h**) merge. El segundo panel muestra imágenes inmunofluorescentes de (**j, m**) TrkA (rojo), (**j, n**) NeuN (verde), (**k, o**) DAPI (azul) y (**l, p**) merge. Escala: 20 micrómetros. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; NGF, factor de crecimiento neuronal; TrkA, receptor de tropomiosina tipo cinasa A; NeuN, núcleos neuronales; DAPI, 4',6-diamino-2-fenilindol.

## Modelo *in vitro*

### Depleción y recuperación de los niveles de GSH en astrocitos

Para tratar de entender los mecanismos que utilizan los astrocitos para mantener la homeostasis de GSH se utilizó el inhibidor BSO. La inhibición de la síntesis *de novo* de GSH con BSO (500 μM) induce la depleción de GSH de forma tiempo dependiente en astrocitos, alcanzando un 80% de disminución a las 12 h de tratamiento (Fig. 14a). Sin embargo, esta depleción no tuvo efecto en la viabilidad celular (Fig. 14b). Posteriormente, se demostró que la disminución en la concentración de GSH no está relacionada a la actividad de la MRP1, ya que el inhibidor de este transportador ácido 5-(3-(2-(7-cloroquinolina-2-il)etenil)fenil)-8-dimetilcarbamil-4,6-ditiaoctanoico (MK571) no previene la pérdida de GSH en las células (Fig. 14c), lo que indica que el GSH no está siendo exportado fuera de la célula a través de la MRP1 en respuesta al BSO. La depleción de GSH crónica (24 h) genera estrés oxidante, determinado por un aumento en la proteína roGFP oxidada en el citoplasma (Fig. 14d). Cuando se retiró el BSO después de 12 h de tratamiento se observó que su efecto es reversible, los resultados muestran que las células pueden reponer el GSH casi a niveles del control 12 h después de retirar el BSO (Fig.14e).

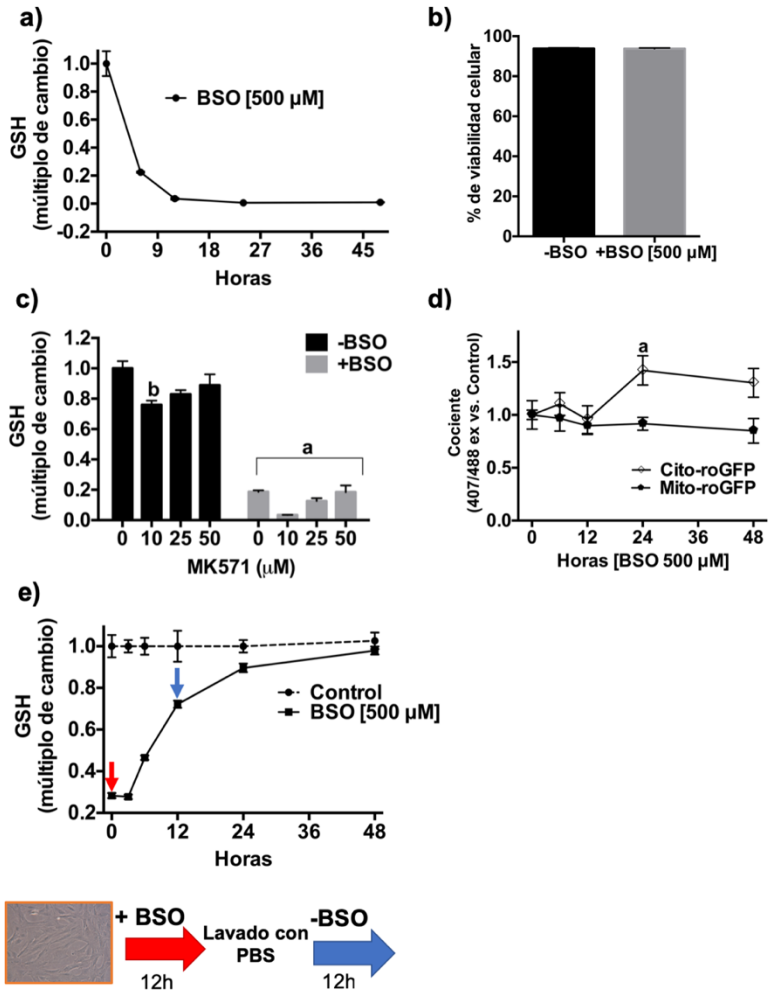


**Figura 14.** Efecto de BSO en los niveles de GSH. (**a**) Curso temporal de depleción de GSH inducida por BSO 500 µM, se evaluaron los niveles de GSH en los astrocitos con citometría de flujo utilizando mBCl. (**b**) Efecto del BSO en la viabilidad celular, la pérdida en la viabilidad celular se determinó usando citometría de flujo midiendo el consumo de PI y los niveles de GSH con mBCl. (**c**) Efecto del inhibidor de MRP1, MK571 en la depleción de GSH inducida por BSO. (**d**) Efecto de BSO en el estado redox citosólico y mitocondrial determinado a través de la oxidación de roGFP. (**e**) Depleción de GSH inducida por BSO y recuperación de GSH después de retirar el BSO. 12 h BSO (flecha roja),12 h sin BSO (flecha azul). Las gráficas representan promedios±SE n=3. ANOVA de dos vías, prueba post hoc Holm-Sidak $^{a}p<0.05$ vs Control; $^{b}p<0.05$ –MK571 vs +MK571. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; PI, ioduro de propidio; mBCl, monoclorobimano; roGFP, proteína verde fluorescente redox sensible; MRP1, proteína 1 de resistencia a multidroga; MK571, ácido 5-(3-(2-(7-Cloroquinolina-2-il)etenil)fenil)-8-dimetilcarbamil-4,6-ditiaoctanoico; SE, error estándar.

### Papel aditivo de los transportadores de aminoácidos en la homeostasis de GSH

Se utilizaron inhibidores de los transportadores de aminoácidos, sulfasalazina (SAS) para inhibir xCT, 2-aminobiciclo-(2,2,1)-heptano-2-ácido carboxílico (BCH) para inhibir LAT1 y pirrolidina-2,4-ácido dicarboxílico (PDC) para inhibir EAAT3. Por separado ninguno de los inhibidores tuvo efecto en la recuperación de los niveles intracelulares de GSH (Fig. 15a-

c). Sin embargo, cuando se utilizaron los 3 inhibidores de manera simultánea la recuperación de GSH se evitó significativamente (Fig. 15d), lo que indica que en los astrocitos los transportadores de cistina/cisteína tienen un papel complementario, si uno es inhibido los otros pueden compensar el efecto. Como en los astrocitos la mayoría del glutamato es transformado en glutamina para las neuronas, éste también puede ser un aminoácido limitante en la síntesis de GSH (Rose et al., 2017); se utilizó el inhibidor del transportador de glutamato EAAT1 2-Amino-5,6,7,8-tetrahidro-4-(4-metoxifenil)-7-(naftaleno-1-yl)-5-oxo-4H-cromeno-3-carbonitrilo (UCPH) pero tampoco tuvo efecto sobre la recuperación de GSH (Fig. 15e).

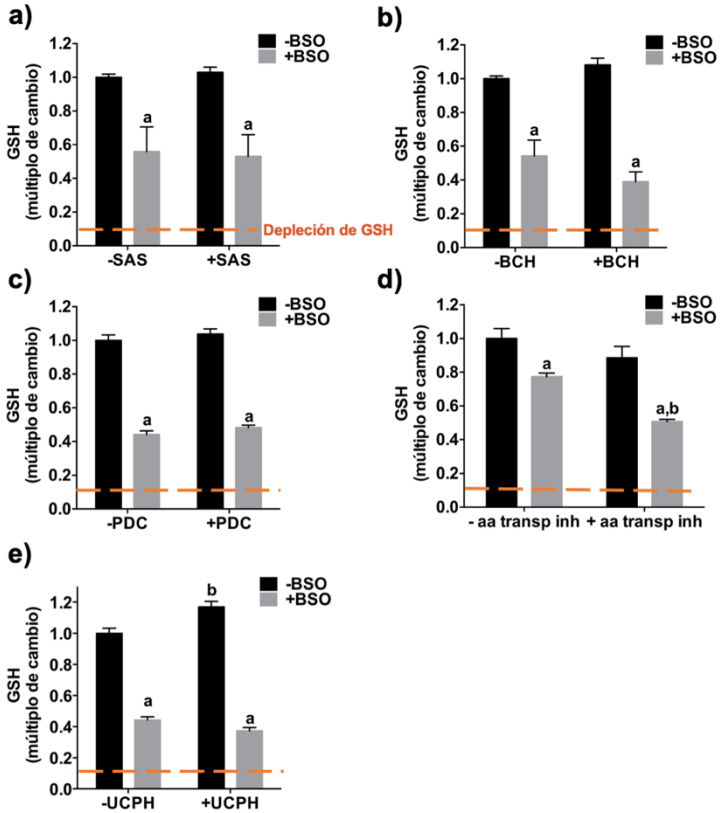


**Figura 15.** Efecto de la inhibición del transporte de aminoácidos en los niveles de GSH. Se evaluaron los niveles de GSH con citometría de flujo utilizando mBCl. (**a**) Los astrocitos se trataron con el inhibidor de xCT SAS 50 µM (**b**) inhibidor de LAT1 BCH 5mM y (**c**) el inhibidor de EAAT3 PDC 100 µM 12 h después del tratamiento con BSO 500 µM. (**d**) Combinación de los tres inhibidores (SAS, BCH y PDC) 12 h después del tratamiento con BSO. (**e**) Los astrocitos también se trataron con el inhibidor de EAAT1 UCPH 20 µM. La línea punteada indica los niveles de GSH después de 12 h BSO (Depleción de GSH). Las gráficas representan promedios±SE n =3. ANOVA de dos vías, prueba post hoc Holm-Sidak [a]p<0.05 vs Control (-BSO); [b]p<0.05 –inhibidores *vs* +inhibidores respectivamente. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; mBCl, monoclorobimano; SAS, sulfasalazina; BCH, 2-aminobiciclo-(2,2,1)-heptano-2-ácido carboxílico; PDC, pirrolidina-2,4-ácido dicarboxílico; UCPH, 2-Amino-5,6,7,8-tetrahidro-4-(4-metoxifenil)-7-(naftaleno-1-yl)-5-oxo-4H-cromeno-3-carbonitrilo; SE, error estándar.

**El piruvato mitocondrial y el metabolismo de los ácidos grasos contribuyen a la homeostasis de GSH.**

Los astrocitos son altamente glicolíticos, sin embargo se ha observado que durante condiciones de estrés o de falta de energía pueden activar el metabolismo mitocondrial (Rose et al., 2017). Para estudiar otros mecanismos que regulan la homeostasis de GSH además del transporte de aminoácidos, se decidió evaluar el papel de la actividad mitocondrial en la recuperación de GSH, mediante la inhibición del transportador de piruvato mitocondrial con 2-ciano-3-(1-fenil-1H-indol-3-il)-2-ácido propenóico (UK5099) (Fig. 16a) y la oxidación de ácidos grasos con etomoxir, inhibidor de la enzima mitocondrial carnitina palmitoiltransferasa 1 (CPT-1) que media el transporte de ácidos grasos de cadena larga a través de la membrana, uniéndolos a moléculas de carnitina (Fig. 16b). Estos dos inhibidores interesantemente redujeron la recuperación de GSH significativamente, lo que sugiere que el metabolismo mitocondrial también es importante en la regulación de la homeostasis del GSH en astrocitos.

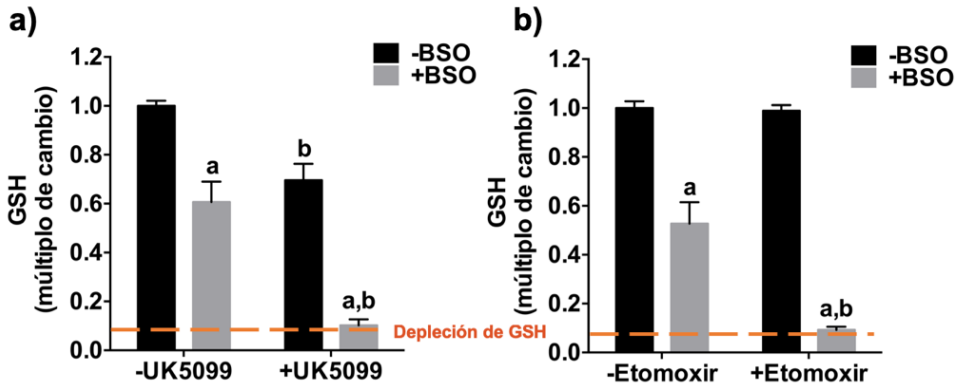


**Figura 16.** Efecto de la inhibición del transporte de piruvato y de la oxidación de ácidos grasos mitocondrial en la homeostasis de GSH. Se evaluaron los niveles de GSH con citometría de flujo utilizando mBCl. (**a**) Los astrocitos se trataron con el inhibidor del transporte de piruvato mitocondrial UK5099 5 µM y con (**b**) el inhibidor de la oxidación de ácidos grasos Etomoxir 200 µM 12 h después del tratamiento con BSO. La línea punteada indica los niveles de GSH después de 12 h BSO (Depleción de GSH). Las gráficas representan promedios±SE n=3. ANOVA de dos vías, prueba post hoc Holm-Sidak [a]p<0.05 vs Control (-BSO); [b]p<0.05 –inhibidores *vs* +inhibidores respectivamente. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; mBCl, monoclorobimano; UK5099, 2-ciano-3-(1-fenil-1H-indol-3-il)-2-ácido propenóico; SE, error estándar.

**La vía de señalización de mTOR regula la homeostasis de GSH en astrocitos**

La vía de señalización de mTOR está involucrada tanto en el transporte de aminoácidos como en la actividad y biogénesis mitocondrial. Se utilizaron los inhibidores de la vía de señalización de mTOR, la rapamicina que sólo inhibe al mTORC1 y torin1 que inhibe a los dos complejos mTORC1/2. También se utilizó el inhibidor de la S6K, LY2584702 o tosilato (Fig. 17a-c). De manera interesante el único inhibidor que tuvo efecto en la recuperación de los niveles de GSH fue torin1, incluso se observa que por sí mismo disminuye los niveles de GSH (Fig. 17a). Existen estudios que sugieren que la rapamicina no inhibe todas las funciones de mTORC1 como la regulación de la traducción CAP-dependiente y la autofagia. Se realizaron Western blots para determinar los niveles de proteína de algunos blancos moleculares de mTORC1/2; se observó que la rapamicina sólo inhibe la fosforilación de la S6K y no la de 4EBP1 (Fig. 17d). Por otro lado, torin1 inhibió tanto los blancos moleculares de mTORC1 (S6K y 4EBP1), como la fosforilación de Akt (Ser 473) blanco de mTORC2 (Fig. 17d). El efecto de mTOR en la homeostasis de GSH en los astrocitos puede estar regulado por 4EBP1 en el caso de mTORC1 o por alguna de las vías donde participa mTORC2.

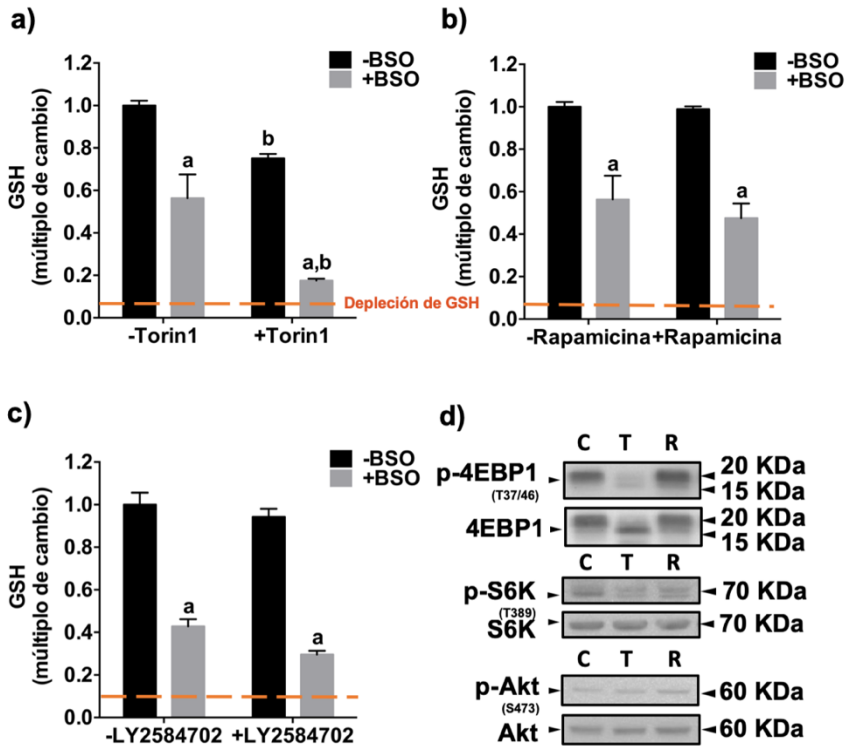


**Figura 17**. Efecto de la inhibición de la vía de señalización de mTOR en la homeostasis de GSH. Se evaluaron los niveles de GSH con citometría de flujo utilizando mBCl. (**a**) Los astrocitos se trataron con el inhibidor de mTORC1/2 torin1 250 nM, (**b**) el inhibidor del mTORC1 rapamicina 1 µM o (**c**) el inhibidor de S6K LY2584702 5 µM 12 h después del tratamiento con BSO. (**d**) Se determinaron los niveles de fosforilación de los blancos de mTORC1/2 (4EBP, S6K y Akt) por Western blot. Las gráficas representan promedios±SE n=3. ANOVA de dos vías, prueba post hoc Holm-Sidak $^{a}p<0.05$ vs Control (-BSO); $^{b}p<0.05$ –inhibidores *vs* +inhibidores respectivamente. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; mBCl, monoclorobimano; mTOR, blanco funcional de la rapamicina; S6K; proteína ribosomal S6 cinasa beta-1, 4EBP1, proteína de unión al factor de inicio de la traducción eucariota eIF-4E; SE, error estándar.

### La inhibición de mTOR exacerba el estrés oxidante en la célula

Se evaluó el estado redox de la célula con las proteínas roGFP citoplásmica y mitocondrial durante la recuperación después de la administración de BSO junto con el tratamiento de torin1 (Fig. 18a y b). El tratamiento con torin1 exacerbó la generación de estrés oxidante en los astrocitos, incrementando la oxidación de las proteínas roGFP en citoplasma y mitocondria.

Por otro lado, se determinaron los niveles de proteína de las subunidades moduladora y catalítica de la enzima GCL (GCLc y GCLm) sin embargo no hubo cambios ni en presencia sólo de BSO o con el co-tratamiento con torin1 (Fig. 18c). Esto indica por un lado que la inhibición por torin1 está induciendo un incremento en el estrés oxidante y que la

recuperación de los niveles intracelulares de GSH después del tratamiento con BSO no está incrementando los niveles de la enzima de síntesis. La inhibición de la recuperación de GSH que se observó con torin1 no se debe a un efecto en los niveles de GCL si no a un efecto en el estado redox celular.

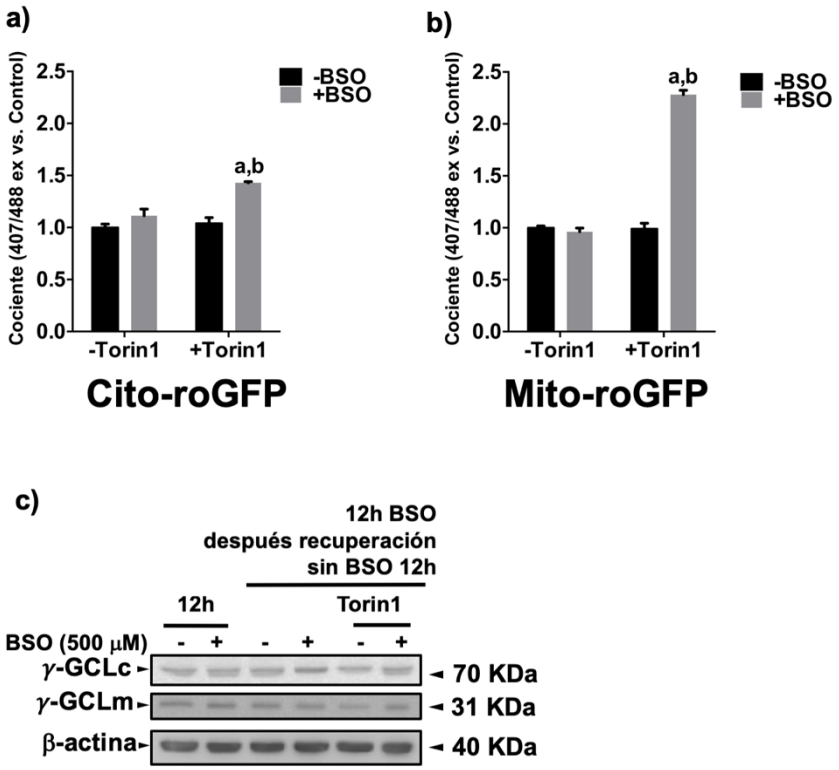


**Figura 18**. Efecto de la inhibición de mTOR en el estado redox de la célula y en los niveles de la enzima GCL. El estado redox (**a**) citosólico y (**b**) mitocondrial se determinaron a través de la oxidación de la proteína roGFP citosólica y mitocondrial en los astrocitos tratados con torin1 250 nM 12 h después del tratamiento con BSO. (**c**) Se determinaron los niveles proteicos de las subunidades GCLm y GCLc de la enzima GCL por Western blot normalizados con β-actina. Las gráficas representan promedios±SE n=3. ANOVA de dos vías, prueba post hoc Holm-Sidak [a]p<0.05 vs Control (-BSO); [b]p<0.05 –Torin1 *vs* +Torin1. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; GSH, glutatión; roGFP, proteína verde fluorescente redox sensible; mTOR, blanco funcional de la rapamicina; GCL cisteína-glutamato ligasa; GCLc, subunidad catalítica; GCLm, subunidad modulatoria; SE, error estándar.

**La vía de señalización de mTOR no regula la biogénesis mitocondrial, pero regula la generación de ATP**

Otra de las vías que podría estar afectando la recuperación de los niveles de GSH a través de la inhibición de mTOR con torin1 es la biogénesis mitocondrial. Se midió la cantidad de mitocondrias con Mitotracker red un colorante que marca mitocondrias, la expresión de

mRNA del gen de la citocromo c oxidasa subunidad 2 (COX2) específico de la mitocondria y los niveles de proteína del factor transcripcional mitocondrial (mtTFA) y no se encontraron cambios (Fig. 19). El mTOR no está regulando la homeostasis de GSH a través de la síntesis de nuevas mitocondrias.

Finalmente, se determinó la concentración de ATP en los astrocitos con el ensayo cell titer-Glo luminiscent cell viability normalizando con el colorante calceína AM (Fig. 19d). Se encontró que tanto el tratamiento con BSO, como la presencia de UK5099 y torin1 disminuyen considerablemente la concentración de ATP en las células vivas. Estos resultados sugieren que el mTOR y la mitocondria regulan la generación de energía en respuesta a la depleción de GSH inducida con BSO.

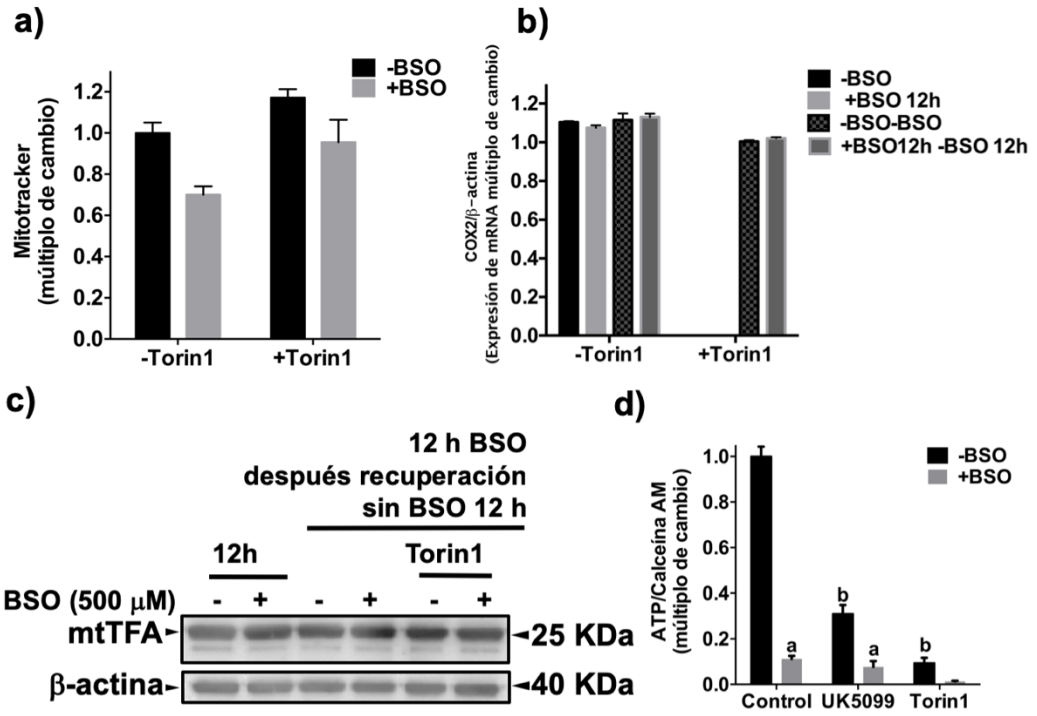


**Figura 19**. Efecto del BSO y la inhibición de mTOR en la biogénesis mitocondrial y la concentración de ATP (**a**) Acumulación mitocondrial en astrocitos tratados con torin1 250 nM 12 h después del tratamiento con BSO determinada por el colorante rojo mitotracker por citometría de flujo. (**b**) Expresión del gen mitocondrial COX2 determinado por qPCR y (**c**) Niveles de expresión de mtTFA determinados por Western blot. (**d**) Concentración de ATP en astrocitos tratados con UK5099 y torin1 12 h después del tratamiento con BSO, la concentración de ATP se determinó con el ensayo cell titer-Glo luminiscent cell viability, los datos fueron normalizados con el colorante calceína AM. Las gráficas representan promedios±SE n=3. ANOVA de dos vías, prueba post hoc Holm-Sidak [a]p<0.05 vs Control (-BSO); [b]p<0.05 –inhibidores *vs* +inhibidores respectivamente. Abreviaturas: BSO, L-butionina-S-R-sulfoximina; COX2, subunidad 2 de la citocromo c oxidasa; mtTFA; factor de transcripción mitocondrial; UK5099, 2-ciano-3-(1-fenil-1H-indol-3-il)-2-ácido propenóico; SE, error estándar.

## DISCUSIÓN

Varios procesos celulares como la reparación del DNA o los antioxidantes tienen vías redundantes que compensan la ausencia o baja actividad de alguno de sus componentes. Sin embargo, el caso de la síntesis del GSH es interesante porque su ausencia no es compatible con la vida. Embriones que no presentan la enzima GCL no pueden realizar la gastrulación y mueren antes del día 8 (Dalton et al., 2000; Shi et al., 2000).

Desde que el BSO fue desarrollado en 1979 como el inhibidor más específico y efectivo de la síntesis de GSH (Griffith y Meister, 1979b) se han realizado diversos estudios donde es utilizado para estudiar los efectos de la depleción del antioxidante. En este estudio primero se decidió medir la concentración de GSH en el hígado para confirmar el efecto inhibidor del BSO (Fig. 4). Los mismos autores que sintetizaron el BSO descubrieron que los órganos responden diferencialmente a la depleción del GSH y demostraron que una sola dosis del inhibidor disminuye rápidamente los niveles de GSH en el hígado, sin embargo presenta una recuperación dentro de las siguientes 12 h, lo que sugiere que en el hígado hay un incremento de las enzimas involucradas en la síntesis de GSH o que tiene una alta capacidad de metabolizar el inhibidor (Griffith y Meister, 1979a). Los resultados de este trabajo coinciden con otros dos estudios que encontraron una baja en la concentración del GSH en el hígado, llegando a valores cercanos al 50% dentro de las primeras 2 h después de la inyección ip con BSO (Drew y Miners, 1984; Minchinton et al., 1984). También se encontró una disminución en la concentración de GSSG después del tratamiento con BSO (Fig. 4a y b), este resultado puede estar relacionado a un incremento en la actividad de la GR como una respuesta para compensar la inhibición de la síntesis de GSH con BSO, reduciendo el GSSG a GSH. La sobreexpresión de la GR eucarionte mejora la resistencia a estrés oxidante en *Escherichia coli* (Yoon et al., 2005). Además, en células de pulmón y fibroblastos el Nrf2 disminuye los niveles de GSSG incrementando la actividad de la GR,

aumentando la sobrevivencia celular en un estado de estrés oxidante (Harvey et al., 2009). Otra explicación de la disminución de los niveles de GSSG puede ser la liberación de éste en respuesta a los cambios redox; el GSSG puede ser exportado fuera de la célula como un mecanismo de desintoxicación y protección (Franco y Cidlowski, 2012).

En el mismo sentido, los estudios previos de nuestro grupo han demostrado que el BSO no sólo es capaz de disminuir los niveles de GSH en el hígado y riñones en donde activa vías de señalización específicas y, al mismo tiempo aumentar los niveles del tripéptido en homogenado de cerebro completo (Limón-Pacheco et al., 2007). El incremento observado en el cerebro se dio a partir de 1 h y duró 2 h después de la inyección con BSO, regresando a los valores control a las 3 h después del tratamiento. Se observó que el aumento en la síntesis de GSH en cerebro se acompaña de un incremento en la activación de ERK2 y de la transcripción de *nrf2, xCT* y *GCL*. Por lo que el objetivo de este trabajo fue investigar la respuesta de las diferentes regiones del cerebro a un tratamiento periférico con BSO, así como las vías de señalización involucradas en esta respuesta, utilizando un modelo experimental similar, que permitiera evaluar la activación de estas vías a la 0.5 h y la respuesta final a las 2 h después del tratamiento.

De las regiones evaluadas en este estudio sólo el cerebelo presentó una respuesta al tratamiento con BSO (Figs. 5-8). El cerebro es particularmente vulnerable al estrés oxidante y la concentración del GSH varía en las diferentes regiones, siendo la corteza y el cerebelo las regiones con mayor concentración (Hjelle et al., 1994; Kang et al., 1999; Kudo et al., 1990). Se ha observado que las distintas regiones del cerebro tienen diferente capacidad antioxidante, también se ha documentado que las diferentes poblaciones de neuronas responden de manera diferencial al estrés y daño durante el envejecimiento y el desarrollo de enfermedades neurodegenerativas (Hussain et al., 1995; Siqueira et al., 2005; Wang y Michaelis, 2010).

Interesantemente en el cerebelo se observó un aumento en la concentración de GSH a las 2 h de exposición con BSO (Fig. 8b). Esta respuesta fue precedida por la activación de las vías de señalización NGF/TrkA y mTOR (Fig. 10b, Fig. 11a-b y Fig. 12) seguido de un incremento en los niveles de proteína del transportador EAAT3 (Fig. 9f), lo que sugiere que el cerebelo responde a los cambios periféricos en los niveles de GSH.

Los resultados observados en el cerebelo sugieren que esta región puede tener una resistencia mayor al estrés oxidante comparado con otras regiones del cerebro. El mecanismo que explica una mayor resistencia del cerebelo al estrés oxidante todavía no se ha dilucidado. En estudios previos se ha demostrado que la sensibilidad del cerebelo a compuestos que inducen estrés oxidante es menor comparado con otras regiones (Son et al., 2016; Vandresen-Filho et al., 2015). Algo que podría ayudar a explicar esta mayor resistencia es la heterogeneidad de los microvasos sanguíneos. Austin y colaboradores demostraron que los microvasos del cerebelo tienen mayor capacidad antioxidante que los del hipocampo y la corteza (Austin et al., 2015).

La homeostasis redox y la regulación del GSH han sido ampliamente estudiadas en el cerebelo. Un balance entre los niveles de GSH y la producción de ROS es requerida para la sobrevivencia en el desarrollo de las neuronas granulares cerebelares (CGN) (Coyoy et al., 2013; Olguin-Albuerne y Moran, 2015). Por otro lado, la concentración del GSH es un factor importante en la vulnerabilidad a un daño excitotóxico inducido por ácido kaínico en CGNs (Ceccon et al., 2000). Además, el cerebelo es capaz de adaptarse enzimáticamente para resistir la depleción de GSH inducida por falla hepática aguda, al disminuir los niveles de la GPx pero manteniendo la actividad de la GR (Singh et al., 2014). El balance entre el GSH y la producción de ROS está altamente regulado en el cerebelo durante el desarrollo, la exposición a xenobióticos y el desarrollo de patologías.

Los transportadores xCT y LAT1 participan en el transporte de la cisteína hacia el cerebro y los astrocitos, sin embargo, no se encontraron cambios en los niveles proteicos después del tratamiento con BSO (Fig. 9a-b, d-e). Los ratones transgénicos con una deleción del gen de xCT no presentan un aumento en el estrés oxidante (De Bundel et al., 2011; Massie et al., 2011), estos resultados sugieren que debe haber otros transportadores de cistina/cisteína compensando la falta de xCT. LAT1 media el transporte de varios aminoácidos y su actividad es importante en el transporte de cisteína de la BHE al cerebro; sin embargo, este transportador parece ser más importante durante el desarrollo mas que en la vida adulta (Boado et al., 2004). A diferencia de los ratones knock-out para xCT, los ratones con una expresión reducida o con una deleción de EAAT3 exhiben alteraciones en la captación de cisteína neuronal, niveles neuronales reducidos de GSH y estrés oxidante crónico, que promueven la muerte celular neuronal y el deterioro cognitivo (Berman et al., 2011; Cao et al., 2012; Wang et al., 2015). La evidencia y nuestros resultados proponen que EAAT3 tiene un papel crucial en el transporte de cisteína a las neuronas y la BHE (Aoyama y Nakaki, 2013; Himi et al., 2003; O'Kane et al., 1999; Shanker y Aschner, 2001). Los resultados de este trabajo sugieren que EAAT3 participa en la respuesta del cerebelo frente a los cambios redox inducidos por el efecto periférico del BSO, a pesar de que el BSO no cruza la BHE, en estos tiempos de tratamiento (Limón-Pacheco y Gonsebatt, 2007; Steinherz et al., 1990). Adicionalmente, la disfunción del transportador EAAT3 se ha asociado a enfermedades como Alzheimer y Parkinson en pacientes y modelos murinos (Aoyama y Nakaki, 2013; Nafia et al., 2008). Los cambios en la expresión de EAAT3 también se han observado en otras condiciones patológicas, como ataques epilépticos y en condiciones de hipoxia/isquemia (Bianchi et al., 2014).

También se observó un aumento en la fosforilación de la S6K a la 0.5 h después del tratamiento con BSO (Fig. 10b), lo que sugiere una activación de la vía de señalización de

mTORC1 en respuesta a BSO. En este trabajo no se encontró un aumento en la fosforilación de mTOR, sin embargo, hay estudios que sugieren que la fosforilación en el residuo Ser 2448 no está relacionada a la activación de esta cinasa, incluso una mutación en este sitio no tiene efecto en su actividad (Mothe-Satney et al., 2004; Sekulic et al., 2000). No obstante, el papel de este sitio todavía no se ha descrito. Su correlación con la actividad de mTOR sigue en controversia pues dependiendo del modelo de estudio y el tipo de estimulación algunos estudios muestran que en respuesta por ejemplo a la insulina, a ácido palmítico o a suero, existe un aumento en la fosforilación de este residuo (Calvo-Ochoa et al., 2017; Rosner et al., 2010). Figueiredo et al. sugieren que un aumento en la fosforilación de este residuo podría reflejar un incremento generalizado de la vía, pero que es preferente utilizar otros blancos moleculares o proteínas efectoras para analizar la activación de mTOR (Figueiredo et al., 2017).

Se ha observado que las vías de señalización de mTOR y ERK modulan la traducción y expresión de EAAT3 después de la activación de receptores de glutamato metabotrópicos (mGluR) 1 y 5 (Ross et al., 2011). Además, se demostró en un modelo de ovocitos de Xenopus coexpresando mTOR y EAAT3, un incremento de EAAT3 en la superficie de la membrana, lo que prueba el papel de mTOR en la regulación positiva de este transportador de aminoácidos (Almilaji et al., 2012).

En el SNC se ha visto que el estrés oxidante activa respuestas antioxidantes mediadas por mTOR (Chong et al., 2012; Ichimura et al., 2013; Zhang et al., 2014; Zhou et al., 2016). La activación de la vía de señalización de PI3K/AKT/mTOR incrementó la expresión de la enzima GCL resultando en un aumento en la síntesis de GSH como una respuesta de protección frente a la generación de estrés oxidante (Kimura et al., 2009; Okouchi et al., 2006). Además, Limón-Pacheco et al. (2007) observaron la transcripción aumentada de GCL y la activación de ERK2 en cerebro.

Finalmente, se encontró un incremento en los niveles proteicos de NGF y la fosforilación de su receptor TrkA 0.5 h después de la inyección de BSO (Fig. 11a y b). Además, en las imágenes de las inmunofluorescencias no sólo se observó un aumento de NGF y TrkA, si no que TrkA se observó tanto en el soma como en las dendritas de las neuronas cerebelares (Fig. 12); estos resultados sugieren que la respuesta a BSO induce un incremento en la síntesis y el tráfico del receptor TrkA. Generalmente, los receptores TrkA recién sintetizados en los somas de la célula son transportados anterógradamente a través de los axones y dendritas donde se pueden unir a NGF e iniciar la señalización (Marlin y Li, 2015; Scott-Solomon y Kuruvilla, 2018).

Como se mencionó en la introducción, el NGF puede inducir respuestas antioxidantes en modelos *in vitro* e *in vivo* (Arsenijevic et al., 2007; Pan y Perez-Polo, 1993; Pan y Perez-Polo, 1996). También se ha observado que el NGF puede inducir la liberación de glutamato en CGNs, lo que sugiere su participación en la modulación de la neurotransmisión excitadora junto con la regulación del transporte de aminoácidos (Numakawa et al., 1999). La activación de la vía de señalización de PI3K/AKT vía NGF/TrkA está relacionada con una respuesta de sobrevivencia celular (Huang y Reichardt, 2003). En células epiteliales de pigmento retinal (RPE), el NGF protege contra la apoptosis generada por $H_2O_2$, estimulando la sobrevivencia celular a través de la activación de mTOR (Cao et al., 2011).

Los astrocitos participan en funciones complejas del SNC, incluyendo la transmisión sináptica, el procesamiento de información, así como el metabolismo y la homeostasis celular (Sofroniew y Vinters, 2010). Además, están involucrados en el desarrollo de patologías, pueden llegar a perder su función, pero también pueden montar una respuesta de defensa contra daño (Verkhratsky et al., 2015). Sus extensiones cubren la superficie de los capilares lo que los hace estar en contacto con la BHE (Fig. 1), esto genera que sean la primera línea de defensa contra la exposición a xenobióticos (Rose et al., 2017). El modelo

de astrocitos primarios ayuda a investigar las propiedades y funciones de estas células en el SNC (Lange et al., 2012). En la segunda parte de este trabajo se decidió evaluar el papel de los astrocitos en la regulación de la homeostasis de GSH. Se planteó un protocolo experimental *in vitro* para estudiar cómo responden después de una exposición aguda a BSO. Este diseño experimental buscó tener una aproximación a lo que se estudió *in vivo* ya que el BSO tiene una baja permeabilidad para cruzar la BHE (Limón-Pacheco y Gonsebatt, 2007; Steinherz et al., 1990), pero genera una respuesta en el cerebelo (Garza-Lombó et al., 2018a). Además, el tratamiento con BSO es una aproximación para estudiar la liberación de GSH de los astrocitos para las neuronas, así como el consumo de éste para el metabolismo de xenobióticos y ROS.

En primer lugar, se encontró que los astrocitos en presencia de BSO no están exportando GSH, ya que al inhibir la proteína MRP1 la depleción continúa (Fig. 14c). Una opción es que el GSH está siendo utilizado para contrarrestar el estrés generado por el BSO (Fig. 14d). Además, se ha observado que la liberación de GSH también puede generar alteraciones en el estado redox que pueden llevar a muerte celular (Franco y Cidlowski, 2012) por lo que los astrocitos pueden estar previniendo esto al reciclar el GSSG a través de la GR.

Una vez que se demostró que los niveles de GSH se recuperan después de la exposición a BSO (Fig. 14e), se decidió estudiar los mecanismos a través de los cuales los astrocitos estaban recuperando los niveles de GSH. En los astrocitos el transporte de cistina/cisteína parece ser complementario, ya que sólo se observó un efecto parcial en la recuperación del GSH cuando se inhibieron al mismo tiempo los tres transportadores, LAT1, xCT y EAAT3 (Fig. 15d).

Posteriormente se decidió evaluar el papel de la mitocondria en la regulación de la homeostasis de GSH. Como se mencionó, los astrocitos son principalmente glicolíticos,

durante condiciones de estrés o de falta de energía pueden activar el metabolismo mitocondrial (Rose et al., 2017). Por lo mismo, los astrocitos son menos susceptibles, que las neuronas, a compuestos que inducen disfunción mitocondrial (Bolanos et al., 1995); sin embargo, se ha observado que la depleción de GSH aumenta el complejo I de la cadena respiratoria (Vasquez et al., 2001), los mecanismos que regulan esta respuesta todavía no son entendidos. En este estudio, la inhibición del transporte de piruvato así como la inhibición del transporte de ácidos grasos a la mitocondria previno la recuperación de los niveles de GSH (Fig. 16). La mitocondria puede estar regulando diversos mecanismos en respuesta a la depleción de GSH. Por un lado, puede estar generando glutamato como precursor para la síntesis de GSH a través del ciclo de los ácidos tricarboxílicos (TCA) o ciclo de Krebs (Rose et al., 2017). En astrocitos, el glutamato también es un aminoácido limitante para la síntesis de GSH, debido a que es convertido a glutamina para su transporte a las neuronas. En la mitocondria este aminoácido puede ser sintetizado a partir de $\alpha$-cetoglutarato, un intermediario del ciclo de los TCA (Schousboe et al., 2014). Otro mecanismo que puede estar regulando la mitocondria durante la recuperación de los niveles de GSH, es la síntesis de ATP a través de la fosforilación oxidativa. Los astrocitos pueden estar aumentando la actividad mitocondrial en respuesta a la depleción de GSH para completar los requerimientos energéticos para la recuperación en los niveles de GSH ya que las enzimas que lo sintetizan requieren energía. Los resultados revelan que tanto el BSO como la inhibición del transporte de piruvato a la mitocondria disminuyen significativamente la concentración de ATP en los astrocitos (Fig. 19d). Por último, la actividad mitocondrial puede estar regulando la homeostasis de GSH al regenerar NADPH como poder reductor para la reducción del GSSG (Blacker y Duchen, 2016). Todos estos mecanismos ayudan a explicar por qué la disfunción mitocondrial afecta la recuperación de los niveles de GSH en este modelo.

Por otra parte, las mitocondrias al igual que otros organelos contienen GSH, que es transportado desde el citoplasma hacia el interior de la mitocondria. Procesos relacionados a la síntesis, degradación, transporte, oxidación y reducción del GSH pueden tener influencia en la homeostasis de este antioxidante en la mitocondria (Calabrese et al., 2017). Se ha demostrado que la poza de GSH en el espacio intermembranal se encuentra en equilibrio con el GSH citoplasmático, sin embargo, el que se encuentra en la matriz mitocondrial parece tener mecanismos homeostáticos diferentes e independientes (Calabrese et al., 2017). Los cambios en los niveles citoplásmicos de GSH pueden también estar regulando la homeostasis de GSH mitocondrial.

Finalmente, los resultados de este trabajo revelan, de manera interesante, que la vía de señalización de mTOR también está involucrada en la recuperación de los niveles de GSH en los astrocitos (Fig. 17). Además, su inhibición con torin1 afecta el estado redox de la célula exacerbando la generación de estrés oxidante (Fig. 18). Como se mencionó en la introducción, el mTOR regula la biogénesis mitocondrial (Morita et al., 2013) pero contrario a lo que se esperaba mTOR no induce la generación de mitocondrias en respuesta al BSO (Fig. 19 a-c).

Los resultados de este trabajo sugieren que mTOR regula la recuperación de los niveles de GSH a través de la modulación de funciones bioenergéticas de la célula. La inhibición de mTOR con torin1 afecta los niveles de ATP (Fig. 19d), una de las opciones es que esté regulando la glicólisis, mecanismo a través del cual los astrocitos obtienen la energía para transportar lactato a las neuronas (Belanger et al., 2011; Bolanos, 2016). El mTOR puede estar regulando el aumento en la función glicolítica al activar a HIF1$\alpha$, un factor de transcripción que regula la expresión de enzimas y transportadores glicolíticos (Brix et al., 2012; Duvel et al., 2010; Semenza et al., 1994).

## CONCLUSIONES

Los resultados de este trabajo ayudan a entender los mecanismos involucrados en la regulación de la homeostasis de GSH.

### Modelo *in vivo*

La inhibición de la síntesis *de novo* de GSH periférica induce una depleción de los niveles de GSH en el hígado. La única región cerebral que respondió al tratamiento con BSO, fue el cerebelo. En el cerebelo la inhibición aguda de la síntesis de GSH, aumenta los niveles de GSH. Esta respuesta está asociada con la activación de las vías de señalización NGF/TrkA y PI3K/AKT/mTOR llevando a un aumento en los niveles del transportador de cisteína EAAT3.

Los resultados del modelo *in vivo* ayudan a explicar los mecanismos involucrados en la regulación de la homeostasis redox y el metabolismo del antioxidante GSH en el cerebelo. Esta inhibición podría presentarse por el tratamiento con fármacos o por la exposición a agentes tóxicos que no atraviesan la BHE pero tienen efectos en el SNC, así como el desarrollo de enfermedades neurodegenerativas.

### Modelo *in vitro*

Los resultados del modelo *in vitro* muestran por primera vez que la vía de señalización de mTOR y el transporte mitocondrial de piruvato y de ácidos grasos tienen un papel significativo en la regulación de la homeostasis de GSH en los astrocitos.

La comprensión de los mecanismos a través de los cuales los astrocitos regulan la homeostasis redox y la defensa antioxidante ayudarán a entender el papel de los astrocitos en la protección del SNC frente a los retos que afecten el estado redox cerebral.

**REFERENCIAS**


Alayev, A., Holz, M.K., 2013. mTOR signaling for biological control and cancer. J Cell Physiol. 228**,** 1658-64.

Almilaji, A., Pakladok, T., Guo, A., Munoz, C., Foller, M., Lang, F., 2012. Regulation of the glutamate transporter EAAT3 by mammalian target of rapamycin mTOR. Biochem Biophys Res Commun. 421**,** 159-63.

Aoyama, K., Watabe, M., Nakaki, T., 2008. Regulation of neuronal glutathione synthesis. J Pharmacol Sci. 108**,** 227-38.

Aoyama, K., Nakaki, T., 2013. Neuroprotective properties of the excitatory amino acid carrier 1 (EAAC1). Amino Acids. 45**,** 133-42.

Aquilano, K., Baldelli, S., Ciriolo, M.R., 2014. Glutathione: new roles in redox signaling for an old antioxidant. Front Pharmacol. 5**,** 196.

Arsenijevic, D., Hernadfalvi, N., von Meyenburg, C., Onteniente, B., Richard, D., Langhans, W., 2007. Role for nerve growth factor in the in vivo regulation of glutathione in response to LPS in mice. Eur Cytokine Netw. 18**,** 93-101.

Austin, S.A., Santhanam, A.V., d'Uscio, L.V., Katusic, Z.S., 2015. Regional Heterogeneity of Cerebral Microvessels and Brain Susceptibility to Oxidative Stress. PLoS One. 10**,** e0144062.

Basaki, Y., Hosoi, F., Oda, Y., Fotovati, A., Maruyama, Y., Oie, S., Ono, M., Izumi, H., Kohno, K., Sakai, K., Shimoyama, T., Nishio, K., Kuwano, M., 2007. Akt-dependent nuclear localization of Y-box-binding protein 1 in acquisition of malignant characteristics by human ovarian cancer cells. Oncogene. 26**,** 2736-46.

Belanger, M., Allaman, I., Magistretti, P.J., 2011. Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. Cell Metab. 14**,** 724-38.

Berman, A.E., Chan, W.Y., Brennan, A.M., Reyes, R.C., Adler, B.L., Suh, S.W., Kauppinen, T.M., Edling, Y., Swanson, R.A., 2011. N-acetylcysteine prevents loss of dopaminergic neurons in the EAAC1-/- mouse. Ann Neurol. 69**,** 509-20.

Berridge, M.J., 2014. Module 2: Cell Signalling Pathways. Cell Signalling Biology. 6.

Bianchi, M.G., Bardelli, D., Chiu, M., Bussolati, O., 2014. Changes in the expression of the glutamate transporter EAAT3/EAAC1 in health and disease. Cell Mol Life Sci. 71**,** 2001-15.

Blacker, T.S., Duchen, M.R., 2016. Investigating mitochondrial redox state using NADH and NADPH autofluorescence. Free Radic Biol Med. 100**,** 53-65.

Boado, R.J., Li, J.Y., Pardridge, W.M., 2004. Developmental regulation of the rabbit blood-brain barrier LAT1 large neutral amino acid transporter mRNA and protein. Pediatr Res. 55, 557-60.

Bolanos, J.P., Heales, S.J., Land, J.M., Clark, J.B., 1995. Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurones and astrocytes in primary culture. J Neurochem. 64, 1965-72.

Bolanos, J.P., 2016. Bioenergetics and redox adaptations of astrocytes to neuronal activity. J Neurochem. 139 Suppl 2, 115-125.

Brix, B., Mesters, J.R., Pellerin, L., Johren, O., 2012. Endothelial cell-derived nitric oxide enhances aerobic glycolysis in astrocytes via HIF-1alpha-mediated target gene activation. J Neurosci. 32, 9727-35.

Calabrese, G., Morgan, B., Riemer, J., 2017. Mitochondrial Glutathione: Regulation and Functions. Antioxid Redox Signal. 27, 1162-1177.

Calvo-Ochoa, E., Sanchez-Alegria, K., Gomez-Inclan, C., Ferrera, P., Arias, C., 2017. Palmitic acid stimulates energy metabolism and inhibits insulin/PI3K/AKT signaling in differentiated human neuroblastoma cells: The role of mTOR activation and mitochondrial ROS production. Neurochem Int. 110, 75-83.

Cao, G.F., Liu, Y., Yang, W., Wan, J., Yao, J., Wan, Y., Jiang, Q., 2011. Rapamycin sensitive mTOR activation mediates nerve growth factor (NGF) induced cell migration and pro-survival effects against hydrogen peroxide in retinal pigment epithelial cells. Biochem Biophys Res Commun. 414, 499-505.

Cao, L., Li, L., Zuo, Z., 2012. N-acetylcysteine reverses existing cognitive impairment and increased oxidative stress in glutamate transporter type 3 deficient mice. Neuroscience. 220, 85-9.

Ceccon, M., Giusti, P., Facci, L., Borin, G., Imbesi, M., Floreani, M., Skaper, S.D., 2000. Intracellular glutathione levels determine cerebellar granule neuron sensitivity to excitotoxic injury by kainic acid. Brain Res. 862, 83-9.

Chong, Z.Z., Shang, Y.C., Wang, S., Maiese, K., 2012. Shedding new light on neurodegenerative diseases through the mammalian target of rapamycin. Prog Neurobiol. 99, 128-48.

Circu, M.L., Aw, T.Y., 2010. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med. 48, 749-62.

Coyoy, A., Moran, J., 2012. Papel de las ERO producidas por las NOX en procesos fisiológicos. Revista de Educación Bioquímica (REB). 31, 100-109.

Coyoy, A., Olguin-Albuerne, M., Martinez-Briseno, P., Moran, J., 2013. Role of reactive oxygen species and NADPH-oxidase in the development of rat cerebellum. Neurochem Int. 62, 998-1011.

Crino, P.B., 2016. The mTOR signalling cascade: paving new roads to cure neurological disease. Nat Rev Neurol. 12, 379-92.

Cruz-Aguado, R., Francis-Turner, L., Diaz-Suarez, C.M., Bergado, J., 1999. NGF prevents changes in rat brain glutathione-related enzymes following transection of the septohippocampal pathway. Neurochem Int. 34, 125-30.

Dalton, T.P., Dieter, M.Z., Yang, Y., Shertzer, H.G., Nebert, D.W., 2000. Knockout of the mouse glutamate cysteine ligase catalytic subunit (Gclc) gene: embryonic lethal when homozygous, and proposed model for moderate glutathione deficiency when heterozygous. Biochem Biophys Res Commun. 279, 324-9.

De Bundel, D., Schallier, A., Loyens, E., Fernando, R., Miyashita, H., Van Liefferinge, J., Vermoesen, K., Bannai, S., Sato, H., Michotte, Y., Smolders, I., Massie, A., 2011. Loss of system x(c)- does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility. J Neurosci. 31, 5792-803.

Drew, R., Miners, J.O., 1984. The effects of buthionine sulphoximine (BSO) on glutathione depletion and xenobiotic biotransformation. Biochem Pharmacol. 33, 2989-94.

Dringen, R., Kranich, O., Hamprecht, B., 1997. The gamma-glutamyl transpeptidase inhibitor acivicin preserves glutathione released by astroglial cells in culture. Neurochem Res. 22, 727-33.

Dringen, R., Pfeiffer, B., Hamprecht, B., 1999. Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. J Neurosci. 19, 562-9.

Dringen, R., 2000. Metabolism and functions of glutathione in brain. Prog Neurobiol. 62, 649-71.

Dringen, R., Gutterer, J.M., Gros, C., Hirrlinger, J., 2001. Aminopeptidase N mediates the utilization of the GSH precursor CysGly by cultured neurons. J Neurosci Res. 66, 1003-8.

Durackova, Z., 2010. Some current insights into oxidative stress. Physiol Res. 59, 459-69.

Duvel, K., Yecies, J.L., Menon, S., Raman, P., Lipovsky, A.I., Souza, A.L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S., Vander Heiden, M.G., MacKeigan, J.P., Finan,

P.M., Clish, C.B., Murphy, L.O., Manning, B.D., 2010. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell. 39, 171-83.

Edinger, A.L., 2007. Controlling cell growth and survival through regulated nutrient transporter expression. Biochem J. 406, 1-12.

Figueiredo, V.C., Markworth, J.F., Cameron-Smith, D., 2017. Considerations on mTOR regulation at serine 2448: implications for muscle metabolism studies. Cell Mol Life Sci. 74, 2537-2545.

Finkel, T., 2011. Signal transduction by reactive oxygen species. J Cell Biol. 194, 7-15.

Forman, H.J., 2016. Redox signaling: An evolution from free radicals to aging. Free Radic Biol Med. 97, 398-407.

Franco, R., Cidlowski, J.A., 2012. Glutathione efflux and cell death. Antioxid Redox Signal. 17, 1694-713.

Friedman, W.J., Greene, L.A., 1999. Neurotrophin signaling via Trks and p75. Exp Cell Res. 253, 131-42.

Fuchs, B.C., Bode, B.P., 2005. Amino acid transporters ASCT2 and LAT1 in cancer: partners in crime? Semin Cancer Biol. 15, 254-66.

Garza-Lombo, C., Gonsebatt, M.E., 2016. Mammalian Target of Rapamycin: Its Role in Early Neural Development and in Adult and Aged Brain Function. Front Cell Neurosci. 10, 157.

Garza-Lombo, C., Petrosyan, P., Tapia-Rodriguez, M., Valdovinos-Flores, C., Gonsebatt, M.E., 2018a. Systemic L-buthionine-S-R-sulfoximine administration modulates glutathione homeostasis via NGF/TrkA and mTOR signaling in the cerebellum. Neurochem Int. 121, 8-18.

Garza-Lombo, C., Schroder, A., Reyes-Reyes, E., Franco, R., 2018b. mTOR/AMPK signaling in the brain: Cell metabolism,
proteostasis and survival. Current Opinion in Toxicology. 8, 102-110.

Goberdhan, D.C., Wilson, C., Harris, A.L., 2016. Amino Acid Sensing by mTORC1: Intracellular Transporters Mark the Spot. Cell Metab. 23, 580-9.

Griffith, O.W., Meister, A., 1979a. Glutathione: interorgan translocation, turnover, and metabolism. Proc Natl Acad Sci U S A. 76, 5606-10.

Griffith, O.W., Meister, A., 1979b. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J Biol Chem. 254, 7558-60.

Gu, F., Chauhan, V., Chauhan, A., 2015. Glutathione redox imbalance in brain disorders. Curr Opin Clin Nutr Metab Care. 18, 89-95.

Halliwell, B., Cross, C.E., 1994. Oxygen-derived species: their relation to human disease and environmental stress. Environ Health Perspect. 102 Suppl 10, 5-12.

Harvey, C.J., Thimmulappa, R.K., Singh, A., Blake, D.J., Ling, G., Wakabayashi, N., Fujii, J., Myers, A., Biswal, S., 2009. Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. Free Radic Biol Med. 46, 443-53.

Himi, T., Ikeda, M., Yasuhara, T., Nishida, M., Morita, I., 2003. Role of neuronal glutamate transporter in the cysteine uptake and intracellular glutathione levels in cultured cortical neurons. J Neural Transm (Vienna). 110, 1337-48.

Hirrlinger, J., Dringen, R., 2005. Multidrug resistance protein 1-mediated export of glutathione and glutathione disulfide from brain astrocytes. Methods Enzymol. 400, 395-409.

Hjelle, O.P., Chaudhry, F.A., Ottersen, O.P., 1994. Antisera to glutathione: characterization and immunocytochemical application to the rat cerebellum. Eur J Neurosci. 6, 793-804.

Holmstrom, K.M., Finkel, T., 2014. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol. 15, 411-21.

Huang, E.J., Reichardt, L.F., 2003. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem. 72, 609-42.

Hussain, S., Slikker, W., Jr., Ali, S.F., 1995. Age-related changes in antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and glutathione in different regions of mouse brain. Int J Dev Neurosci. 13, 811-7.

Ichimura, Y., Waguri, S., Sou, Y.S., Kageyama, S., Hasegawa, J., Ishimura, R., Saito, T., Yang, Y., Kouno, T., Fukutomi, T., Hoshii, T., Hirao, A., Takagi, K., Mizushima, T., Motohashi, H., Lee, M.S., Yoshimori, T., Tanaka, K., Yamamoto, M., Komatsu, M., 2013. Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. Mol Cell. 51, 618-31.

Jackson, G.R., Apffel, L., Werrbach-Perez, K., Perez-Polo, J.R., 1990. Role of nerve growth factor in oxidant-antioxidant balance and neuronal injury. I. Stimulation of hydrogen peroxide resistance. J Neurosci Res. 25, 360-8.

Janaky, R., Ogita, K., Pasqualotto, B.A., Bains, J.S., Oja, S.S., Yoneda, Y., Shaw, C.A., 1999. Glutathione and signal transduction in the mammalian CNS. J Neurochem. 73, 889-902.

Janssen-Heininger, Y.M., Nolin, J.D., Hoffman, S.M., van der Velden, J.L., Tully, J.E., Lahue, K.G., Abdalla, S.T., Chapman, D.G., Reynaert, N.L., van der Vliet, A., Anathy, V., 2013. Emerging mechanisms of glutathione-dependent chemistry in biology and disease. J Cell Biochem. 114, 1962-8.

Johnson, W.M., Wilson-Delfosse, A.L., Mieyal, J.J., 2012. Dysregulation of glutathione homeostasis in neurodegenerative diseases. Nutrients. 4, 1399-440.

Kang, Y., Viswanath, V., Jha, N., Qiao, X., Mo, J.Q., Andersen, J.K., 1999. Brain gamma-glutamyl cysteine synthetase (GCS) mRNA expression patterns correlate with regional-specific enzyme activities and glutathione levels. J Neurosci Res. 58, 436-41.

Kholodenko, B.N., 2006. Cell-signalling dynamics in time and space. Nat Rev Mol Cell Biol. 7, 165-76.

Khwaja, A., 1999. Akt is more than just a Bad kinase. Nature. 401, 33-4.

Killian, D.M., Chikhale, P.J., 2001. Predominant functional activity of the large, neutral amino acid transporter (LAT1) isoform at the cerebrovasculature. Neurosci Lett. 306, 1-4.

Kim, J., Guan, K.L., 2011. Amino acid signaling in TOR activation. Annu Rev Biochem. 80, 1001-32.

Kimura, R., Okouchi, M., Fujioka, H., Ichiyanagi, A., Ryuge, F., Mizuno, T., Imaeda, K., Okayama, N., Kamiya, Y., Asai, K., Joh, T., 2009. Glucagon-like peptide-1 (GLP-1) protects against methylglyoxal-induced PC12 cell apoptosis through the PI3K/Akt/mTOR/GCLc/redox signaling pathway. Neuroscience. 162, 1212-9.

Kudo, H., Kokunai, T., Kondoh, T., Tamaki, N., Matsumoto, S., 1990. Quantitative analysis of glutathione in rat central nervous system: comparison of GSH in infant brain with that in adult brain. Brain Res. 511, 326-8.

Lange, S.C., Bak, L.K., Waagepetersen, H.S., Schousboe, A., Norenberg, M.D., 2012. Primary cultures of astrocytes: their value in understanding astrocytes in health and disease. Neurochem Res. 37, 2569-88.

Limon-Pacheco, J., Gonsebatt, M.E., 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat Res. 674, 137-47.

Limon-Pacheco, J.H., Gonsebatt, M.E., 2007. Glutathione (GSH) synthesis inhibition in the nervous system: A model to comprehend GSH role in neuropathologies. In New Perspectives on Brain Cell Damage, Neurodegeneration and Neuroprotective Strategies. Vol., A.S.a.M.E. Jiménez-Capdeville, ed.^eds. Research Signpost, Kerala, India, pp. 173-188.

Limon-Pacheco, J.H., Hernandez, N.A., Fanjul-Moles, M.L., Gonsebatt, M.E., 2007. Glutathione depletion activates mitogen-activated protein kinase (MAPK) pathways that display organ-specific responses and brain protection in mice. Free Radic Biol Med. 43**,** 1335-47.

Lu, B., Pang, P.T., Woo, N.H., 2005. The yin and yang of neurotrophin action. Nat Rev Neurosci. 6**,** 603-14.

Lu, S.C., 2009. Regulation of glutathione synthesis. Mol Aspects Med. 30**,** 42-59.

Lu, S.C., 2013. Glutathione synthesis. Biochim Biophys Acta. 1830**,** 3143-53.

Maksimovic, I.D., Jovanovic, M.D., Colic, M., Mihajlovic, R., Micic, D., Selakovic, V., Ninkovic, M., Malicevic, Z., Rusic-Stojiljkovic, M., Jovicic, A., 2001. Oxidative damage and metabolic dysfunction in experimental Huntington's disease: selective vulnerability of the striatum and hippocampus. Vojnosanit Pregl. 58**,** 237-42.

Marlin, M.C., Li, G., 2015. Biogenesis and function of the NGF/TrkA signaling endosome. Int Rev Cell Mol Biol. 314**,** 239-57.

Massie, A., Schallier, A., Kim, S.W., Fernando, R., Kobayashi, S., Beck, H., De Bundel, D., Vermoesen, K., Bannai, S., Smolders, I., Conrad, M., Plesnila, N., Sato, H., Michotte, Y., 2011. Dopaminergic neurons of system x(c)(-)-deficient mice are highly protected against 6-hydroxydopamine-induced toxicity. FASEB J. 25**,** 1359-69.

McCracken, A.N., Edinger, A.L., 2013. Nutrient transporters: the Achilles' heel of anabolism. Trends Endocrinol Metab. 24**,** 200-8.

Meister, A., 1981. Metabolism and functions of glutathione. Trends in Biochemical Sciences. 6**,** 231-234.

Minchinton, A.I., Rojas, A., Smith, K.A., Soranson, J.A., Shrieve, D.C., Jones, N.R., Bremner, J.C., 1984. Glutathione depletion in tissues after administration of buthionine sulphoximine. Int J Radiat Oncol Biol Phys. 10**,** 1261-4.

Morita, M., Gravel, S.P., Chenard, V., Sikstrom, K., Zheng, L., Alain, T., Gandin, V., Avizonis, D., Arguello, M., Zakaria, C., McLaughlan, S., Nouet, Y., Pause, A., Pollak, M., Gottlieb, E., Larsson, O., St-Pierre, J., Topisirovic, I., Sonenberg, N.,

2013. mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. Cell Metab. 18, 698-711.

Mothe-Satney, I., Gautier, N., Hinault, C., Lawrence, J.C., Jr., Van Obberghen, E., 2004. In rat hepatocytes glucagon increases mammalian target of rapamycin phosphorylation on serine 2448 but antagonizes the phosphorylation of its downstream targets induced by insulin and amino acids. J Biol Chem. 279, 42628-37.

Nafia, I., Re, D.B., Masmejean, F., Melon, C., Kachidian, P., Kerkerian-Le Goff, L., Nieoullon, A., Had-Aissouni, L., 2008. Preferential vulnerability of mesencephalic dopamine neurons to glutamate transporter dysfunction. J Neurochem. 105, 484-96.

Nelson-Mora, J., Escobar, M.L., Rodriguez-Duran, L., Massieu, L., Montiel, T., Rodriguez, V.M., Hernandez-Mercado, K., Gonsebatt, M.E., 2018. Gestational exposure to inorganic arsenic (iAs3+) alters glutamate disposition in the mouse hippocampus and ionotropic glutamate receptor expression leading to memory impairment. Arch Toxicol. 92, 1037-1048.

Ninkovic, M.B., Jovanovic, M.D., Malicevic, Z., Dukic, M., Jelenkovic, A., Mihajlovic, R., Vasiljevic, I., Jovicic, A., 2000. Effects of nerve growth factor on antioxidative system in the thalamus of MPTP treated Wistar rats. Vojnosanit Pregl. 57, 257-63.

Nistico, G., Ciriolo, M.R., Fiskin, K., Iannone, M., De Martino, A., Rotilio, G., 1992. NGF restores decrease in catalase activity and increases superoxide dismutase and glutathione peroxidase activity in the brain of aged rats. Free Radic Biol Med. 12, 177-81.

Numakawa, T., Takei, N., Yamagishi, S., Sakai, N., Hatanaka, H., 1999. Neurotrophin-elicited short-term glutamate release from cultured cerebellar granule neurons. Brain Res. 842, 431-8.

O'Kane, R.L., Martinez-Lopez, I., DeJoseph, M.R., Vina, J.R., Hawkins, R.A., 1999. Na(+)-dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) of the blood-brain barrier. A mechanism for glutamate removal. J Biol Chem. 274, 31891-5.

Okouchi, M., Okayama, N., Alexander, J.S., Aw, T.Y., 2006. NRF2-dependent glutamate-L-cysteine ligase catalytic subunit expression mediates insulin protection against hyperglycemia- induced brain endothelial cell apoptosis. Curr Neurovasc Res. 3, 249-61.

Olguin-Albuerne, M., Moran, J., 2015. ROS produced by NOX2 control in vitro development of cerebellar granule neurons development. ASN Neuro. 7.

Olsen, L.F., Issinger, O.G., Guerra, B., 2013. The Yin and Yang of redox regulation. Redox Rep. 18, 245-52.

Pan, Z., Perez-Polo, R., 1993. Role of nerve growth factor in oxidant homeostasis: glutathione metabolism. J Neurochem. 61, 1713-21.

Pan, Z., Perez-Polo, R., 1996. Increased uptake of L-cysteine and L-cystine by nerve growth factor in rat pheochromocytoma cells. Brain Res. 740, 21-6.

Patel, M., 2016. Targeting Oxidative Stress in Central Nervous System Disorders. Trends Pharmacol Sci. 37, 768-78.

Poncet, N., Taylor, P.M., 2013. The role of amino acid transporters in nutrition. Curr Opin Clin Nutr Metab Care. 16, 57-65.

Ramos-Chavez, L.A., Rendon-Lopez, C.R., Zepeda, A., Silva-Adaya, D., Del Razo, L.M., Gonsebatt, M.E., 2015. Neurological effects of inorganic arsenic exposure: altered cysteine/glutamate transport, NMDA expression and spatial memory impairment. Front Cell Neurosci. 9, 21.

Reczek, C.R., Chandel, N.S., 2015. ROS-dependent signal transduction. Curr Opin Cell Biol. 33, 8-13.

Reichardt, L.F., 2006. Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci. 361, 1545-64.

Rice, M.E., Russo-Menna, I., 1998. Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. Neuroscience. 82, 1213-23.

Richman, P.G., Meister, A., 1975. Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. J Biol Chem. 250, 1422-6.

Rose, J., Brian, C., Woods, J., Pappa, A., Panayiotidis, M.I., Powers, R., Franco, R., 2017. Mitochondrial dysfunction in glial cells: Implications for neuronal homeostasis and survival. Toxicology. 391, 109-115.

Rosner, M., Siegel, N., Valli, A., Fuchs, C., Hengstschlager, M., 2010. mTOR phosphorylated at S2448 binds to raptor and rictor. Amino Acids. 38, 223-8.

Ross, J.R., Ramakrishnan, H., Porter, B.E., Robinson, M.B., 2011. Group I mGluR-regulated translation of the neuronal glutamate transporter, excitatory amino acid carrier 1. J Neurochem. 117, 812-23.

Salim, S., 2017. Oxidative Stress and the Central Nervous System. J Pharmacol Exp Ther. 360, 201-205.

Sampath, D., Jackson, G.R., Werrbach-Perez, K., Perez-Polo, J.R., 1994. Effects of nerve growth factor on glutathione peroxidase and catalase in PC12 cells. J Neurochem. 62, 2476-9.

Sato, H., Tamba, M., Ishii, T., Bannai, S., 1999. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. J Biol Chem. 274, 11455-8.

Saxton, R.A., Sabatini, D.M., 2017. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 169, 361-371.

Schafer, F.Q., Buettner, G.R., 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 30, 1191-212.

Schindler, J., Jung, S., Niedner-Schatteburg, G., Friauf, E., Nothwang, H.G., 2006. Enrichment of integral membrane proteins from small amounts of brain tissue. J Neural Transm (Vienna). 113, 995-1013.

Schousboe, A., Scafidi, S., Bak, L.K., Waagepetersen, H.S., McKenna, M.C., 2014. Glutamate metabolism in the brain focusing on astrocytes. Adv Neurobiol. 11, 13-30.

Schrader, M., Fahimi, H.D., 2006. Peroxisomes and oxidative stress. Biochim Biophys Acta. 1763, 1755-66.

Scott-Solomon, E., Kuruvilla, R., 2018. Mechanisms of neurotrophin trafficking via Trk receptors. Mol Cell Neurosci.

Sekulic, A., Hudson, C.C., Homme, J.L., Yin, P., Otterness, D.M., Karnitz, L.M., Abraham, R.T., 2000. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res. 60, 3504-13.

Semenza, G.L., Roth, P.H., Fang, H.M., Wang, G.L., 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem. 269, 23757-63.

Senft, A.P., Dalton, T.P., Shertzer, H.G., 2000. Determining glutathione and glutathione disulfide using the fluorescence probe o-phthalaldehyde. Anal Biochem. 280, 80-6.

Shanker, G., Aschner, M., 2001. Identification and characterization of uptake systems for cystine and cysteine in cultured astrocytes and neurons: evidence for methylmercury-targeted disruption of astrocyte transport. J Neurosci Res. 66, 998-1002.

Shi, Z.Z., Osei-Frimpong, J., Kala, G., Kala, S.V., Barrios, R.J., Habib, G.M., Lukin, D.J., Danney, C.M., Matzuk, M.M., Lieberman, M.W., 2000. Glutathione synthesis is essential for mouse development but not for cell growth in culture. Proc Natl Acad Sci U S A. 97, 5101-6.

Shih, A.Y., Erb, H., Sun, X., Toda, S., Kalivas, P.W., Murphy, T.H., 2006. Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. J Neurosci. 26, 10514-23.

Singh, S., Mondal, P., Trigun, S.K., 2014. Acute liver failure in rats activates glutamine-glutamate cycle but declines antioxidant enzymes to induce oxidative stress in cerebral cortex and cerebellum. PLoS One. 9, e95855.

Siqueira, I.R., Fochesatto, C., de Andrade, A., Santos, M., Hagen, M., Bello-Klein, A., Netto, C.A., 2005. Total antioxidant capacity is impaired in different structures from aged rat brain. Int J Dev Neurosci. 23, 663-71.

Sofroniew, M.V., Howe, C.L., Mobley, W.C., 2001. Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci. 24, 1217-81.

Sofroniew, M.V., Vinters, H.V., 2010. Astrocytes: biology and pathology. Acta Neuropathol. 119, 7-35.

Son, S.W., Lee, J.S., Kim, H.G., Kim, D.W., Ahn, Y.C., Son, C.G., 2016. Testosterone depletion increases the susceptibility of brain tissue to oxidative damage in a restraint stress mouse model. J Neurochem. 136, 106-17.

Steinherz, R., Martensson, J., Wellner, D., Meister, A., 1990. Transport into brain of buthionine sulfoximine, an inhibitor of glutathione synthesis, is facilitated by esterification and administration of dimethylsulfoxide. Brain Res. 518, 115-9.

Trachootham, D., Lu, W., Ogasawara, M.A., Nilsa, R.D., Huang, P., 2008. Redox regulation of cell survival. Antioxid Redox Signal. 10, 1343-74.

Valdovinos-Flores, C., Gonsebatt, M.E., 2012. The role of amino acid transporters in GSH synthesis in the blood-brain barrier and central nervous system. Neurochem Int. 61, 405-14.

Valdovinos-Flores, C., Gonsebatt, M.E., 2013. Nerve growth factor exhibits an antioxidant and an autocrine activity in mouse liver that is modulated by buthionine sulfoximine, arsenic, and acetaminophen. Free Radic Res. 47, 404-12.

Vandresen-Filho, S., Martins, W.C., Bertoldo, D.B., Mancini, G., De Bem, A.F., Tasca, C.I., 2015. Cerebral cortex, hippocampus, striatum and cerebellum show differential susceptibility to quinolinic acid-induced oxidative stress. Neurol Sci. 36, 1449-56.

Vasquez, O.L., Almeida, A., Bolanos, J.P., 2001. Depletion of glutathione up-regulates mitochondrial complex I expression in glial cells. J Neurochem. 76, 1593-6.

Verkhratsky, A., Nedergaard, M., Hertz, L., 2015. Why are astrocytes important? Neurochem Res. 40, 389-401.

Vitvitsky, V., Thomas, M., Ghorpade, A., Gendelman, H.E., Banerjee, R., 2006. A functional transsulfuration pathway in the brain links to glutathione homeostasis. J Biol Chem. 281, 35785-93.

Wade, L.A., Brady, H.M., 1981. Cysteine and cystine transport at the blood-brain barrier. J Neurochem. 37, 730-4.

Wang, X., Michaelis, E.K., 2010. Selective neuronal vulnerability to oxidative stress in the brain. Front Aging Neurosci. 2, 12.

Wang, Y.N., Zhou, L., Li, Y.H., Wang, Z., Li, Y.C., Zhang, Y.W., Wang, Y., Liu, G., Shen, Y., 2015. Protein Interacting with C-Kinase 1 Deficiency Impairs Glutathione Synthesis and Increases Oxidative Stress via Reduction of Surface Excitatory Amino Acid Carrier 1. J Neurosci. 35, 6429-43.

Wu, G., Fang, Y.Z., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J Nutr. 134, 489-92.

Ye, Z.W., Zhang, J., Townsend, D.M., Tew, K.D., 2015. Oxidative stress, redox regulation and diseases of cellular differentiation. Biochim Biophys Acta. 1850, 1607-21.

Yoon, H.S., Lee, I.A., Lee, H., Lee, B.H., Jo, J., 2005. Overexpression of a eukaryotic glutathione reductase gene from Brassica campestris improved resistance to oxidative stress in Escherichia coli. Biochem Biophys Res Commun. 326, 618-23.

Zerangue, N., Kavanaugh, M.P., 1996. Interaction of L-cysteine with a human excitatory amino acid transporter. J Physiol. 493 ( Pt 2), 419-23.

Zhang, H., Liu, Y.Y., Jiang, Q., Li, K.R., Zhao, Y.X., Cao, C., Yao, J., 2014. Salvianolic acid A protects RPE cells against oxidative stress through activation of Nrf2/HO-1 signaling. Free Radic Biol Med. 69, 219-28.

Zhou, Q., Chen, B., Wang, X., Wu, L., Yang, Y., Cheng, X., Hu, Z., Cai, X., Yang, J., Sun, X., Lu, W., Yan, H., Chen, J., Ye, J., Shen, J., Cao, P., 2016. Sulforaphane protects against rotenone-induced neurotoxicity in vivo: Involvement of the mTOR, Nrf2, and autophagy pathways. Sci Rep. 6, 32206.

## ANEXOS

Artículos publicados como parte del proyecto de doctorado:

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Garza-Lombó, C., Gonsebatt, M.E., 2016. Mammalian Target of Rapamycin: Its Role in Early Neural Development and in Adult and Aged Brain Function. Front Cell Neurosci. 10, 157.

Garza-Lombo, C., Posadas, Y., Quintanar, L., Gonsebatt, M.E., Franco, R., 2018b. Neurotoxicity Linked to Dysfunctional Metal Ion Homeostasis and Xenobiotic Metal Exposure: Redox Signaling and Oxidative Stress. Antioxid Redox Signal. 28, 1669-1703.

Garza-Lombó, C., Schroder, A., Reyes-Reyes, E., Franco, R., 2018c. mTOR/AMPK signaling in the brain: Cell metabolism, proteostasis and survival. Current Opinion in Toxicology. 8, 102-110.

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# Systemic L-buthionine-S-R-sulfoximine administration modulates glutathione homeostasis via NGF/TrkA and mTOR signaling in the cerebellum

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ABSTRACT

Glutathione (GSH) is an essential component of intracellular antioxidant systems that plays a primordial role in the protection of cells against oxidative stress, maintaining redox homeostasis and xenobiotic detoxification. GSH synthesis in the brain is limited by the availability of cysteine and glutamate. Cystine, the disulfide form of cysteine is transported into endothelial cells of the blood-brain barrier (BBB) and astrocytes via the system $x_c^-$, which is composed of xCT and the heavy chain of 4F2 cell surface antigen (4F2hc). Cystine is reduced inside the cells and the L-type amino acid transporter 1 (LAT1) transports cysteine from the endothelial cells into the brain, cysteine is transported into the neurons through the excitatory amino acid transporter 3 (EAAT3), also known as excitatory amino acid carrier 1 (EAAC1). The mechanistic/mammalian target of rapamycin (mTOR) and neurotrophins can activate signaling pathways that modulate amino acid transporters for GSH synthesis. The present study found that systemic L-buthionine-S-R-sulfoximine (BSO) administration selectively altered GSH homeostasis and EAAT3 levels in the mice cerebellum. Intraperitoneal treatment of mice with 6 mmol/kg of BSO depleted GSH and GSSG in the liver at 2 h of treatment. The cerebellum, but not other brain regions, exhibited a redox response. The mTOR and the neuronal growth factor (NGF)/tropomyosin receptor kinase A (TrkA) signaling pathways were activated and lead to an increase in the protein levels of the EAAT3 transporter, which was linked to an increase in the GSH/GSSG ratio and GSH concentration in the cerebellum at 0.5 and 2 h, respectively. Therefore, the cerebellum responds to peripheral GSH depletion via activation of the mTOR and NGF/TrkA pathways, which increase the transport of cysteine for GSH synthesis.

## 1. Introduction

Glutathione (γ-L-glutamyl-L-cysteinylglycine or GSH) is the predominant low-molecular-weight thiol in mammals. Its concentrations range from 1 to 10 mM in different tissues (Janssen-Heininger et al., 2013). Diverse functions are attributed to GSH, including maintenance of redox homeostasis, detoxification of xenobiotics and/or metabolites via GSH S-transferases (GST), cysteine storage and the regulation of numerous cellular functions, such as proliferation and apoptosis (Aquilano et al., 2014; Lu, 2013). Exposure to metals, pesticides,

*Abbreviations:* 4F2hc, 4F2 cell surface antigen; BBB, blood-brain barrier; BSO, L-buthionine-S-R-sulfoximine; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindole DTPA; diethylenetriamine penta-acetic acid, EAAC1; excitatory amino acid carrier 1, EAAT3; excitatory amino acid transporter 3, ERK; extracellular signal-regulated kinases, GCL; γ-glutamylcysteine ligase, GR; GSH reductase, GPx; GSH peroxidase, GS; GSH synthetase, GSH; glutathione, GSSG; disulfide-oxidized glutathione, GST; GSH S-transferase, $H_2O_2$; hydrogen peroxide, ip; intraperitoneal, LAT1; L-type amino acid transporter 1, mGluR; metabotropic glutamate receptors, mTOR; mechanistic or mammalian target of rapamycin, mTORC1; mTOR Complex 1, NAC; N-acetylcysteine, NeuN; neuronal nuclei NFκB:nuclear factor kappa B, NGF; neuronal growth factor, OPA; o-phthalaldehyde, PI3K; phosphoinositide 3-kinase, PIKK; PI3K-related kinase, PKB/AKT; protein kinase B/AKT, ROS; reactive oxygen species, S6K; S6 kinase beta-1, SE; standard error TrkA, Tropomyosin receptor kinase A; xCT, system $x_c^-$ subunit

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ionizing radiation, UV light and some pharmaceutical drugs compromises GSH levels (Limon-Pacheco and Gonsebatt, 2009). Aging and degenerative and neuropsychiatric diseases, such as Parkinson's, Alzheimer's, autism, schizophrenia, pulmonary and cystic fibrosis, are also associated with disruption in cellular GSH and alterations of GSH-dependent enzymes (Gu et al., 2015; Johnson et al., 2012).

Cells synthetize GSH in two ATP-dependent enzymatic steps: γ-glutamylcysteine ligase (GCL) catalyzes the formation of γ-glutamylcysteine from glutamate and cysteine, and GSH synthetase (GS) synthesizes GSH from γ-glutamylcysteine and glycine. The availability of GSH precursors, primarily cysteine, limits GSH synthesis (Valdovinos-Flores and Gonsebatt, 2012). L-buthionine-S-R-sulfoximine (BSO) binds reversibly to GCL and blocks *de novo* GSH synthesis thus, decreasing intracellular GSH levels (Limón-Pacheco et al., 2007).

GSH and cysteine are unable to cross the blood-brain barrier (BBB), and cystine, the disulfide form of cysteine, is transported across the BBB via the $Na^+$- independent cystine/glutamate exchanger system $x_c^-$, which is composed of xCT and the heavy chain of 4F2 cell surface antigen (4F2hc) subunits (Sato et al., 1999; Shih et al., 2006). Cystine is reduced inside the BBB, and the resulting cysteine is transported out of the endothelial cells into the brain via L-type amino acid transporter 1 (LAT1) (Killian and Chikhale, 2001; Wade and Brady, 1981). Cysteine may be reooxidized once inside the brain and transported through the system $x_c^-$ system into the astrocytes (Shih et al., 2006). Astrocytes release GSH via the multidrug resistance protein 1 (MRP1) as the first step in supplying cysteine for GSH synthesis in neurons (Hirrlinger and Dringen, 2005; Wang and Cynader, 2000). Neurons take up cysteine via excitatory amino acid transporter 3 (EAAT3), which is a glutamate transporter that is part of the $x^-_{AG}$ system in the postsynaptic neurons. It is also known as excitatory amino acid carrier 1 (EAAC1), which also transports cysteine (Himi et al., 2003; Valdovinos-Flores and Gonsebatt, 2012; Zerangue and Kavanaugh, 1996). EAAT3 dysfunction was associated with Alzheimer's and Parkinson's diseases in patients and murine models (Aoyama and Nakaki, 2013; Nafia et al., 2008). Changes in EAAT3 expression were also observed in other pathological conditions, such as epileptic seizures and hypoxia/ischemia conditions (Bianchi et al., 2014).

The mechanistic/mammalian target of rapamycin complex 1 (mTORC1) is a serine/threonine protein kinase of the PI3K-related kinase (PIKK) family that regulates cell growth and metabolism in response to different stimuli, such as variations in the amino acid content, changes in cellular bioenergetics, growth factors and oxidative stress (Saxton and Sabatini, 2017). Therefore, we investigated the role of the mTORC1 pathway in the expression of these amino acid transporters. mTORC1 is also a key downstream molecule of the signaling pathways activated by neurotransmitters and neurotrophin signals in the brain (Garza-Lombo and Gonsebatt, 2016; Garza-Lombo et al., 2018).

Neuronal growth factor (NGF) and its receptor, tropomyosin receptor kinase A (TrkA) are also involved in the regulation of the amino acid transport and GSH synthesis (Pan and Perez-Polo, 1993; Valdovinos-Flores and Gonsebatt, 2013). NGF binds to the TrkA receptor, which results in its dimerization and transphosphorylation (Huang and Reichardt, 2003). Activation of this pathway during the modulation of amino acid transport was also an aim of the present study.

We observed that the systemic inhibition of GSH synthesis and exposure to arsenic, upregulated the expression of amino acid transporters, such as xCT and EAAT3, in the brain in murine models (Limon-Pacheco et al., 2007; Ramos-Chavez et al., 2015) and modulated glutamate availability, which may lead to neurotoxicity (Nelson-Mora et al., 2018). Chen et al. demonstrated that cadmium-induced reactive oxygen species (ROS) activated mTOR, and the GSH analog N-acetyl cysteine (NAC), blocked mTOR activation in cultured neuronal cells (Chen et al., 2011).

To investigate if the peripheral inhibition of GSH synthesis is associated with an upregulation of cystine/cysteine transporters and the activation of the mTORC1 pathway in the brain, we injected mice intraperitoneally (ip) with an inhibitor of the GCL, L-buthionine-S-R-sulfoximine (BSO), which is poorly transported into the brain in adult animals (Limon-Pacheco and Gonsebatt, 2007; Steinherz et al., 1990). We observed a depletion of GSH and GSSG in the liver at 2 h after BSO treatment. At this time point, in the central nervous system (CNS), the cerebellum, but not other brain regions, revealed activation of mTOR and the NGF/TrkA pathways associated with an increase in EAAT3 protein levels (i.e., the cysteine transporter in neurons), the GSH/GSSG ratio and GSH concentration at 0.5 and 2 h respectively. These results suggest that the cerebellum exhibits a higher antioxidant capacity.

## 2. Material and methods

### 2.1. Reagents

Antibodies against phosphorylated (p)-p70 S6K (Thr 389, #9234), p-mTOR (Ser 2448, #5536), p-TrkA (Tyr 490, #9141), p70 S6K (#2708), mTOR (#2983), NGF (#2046), the secondary anti-rabbit (#7074) and the biotinylated protein ladder (#7727) were purchased from Cell Signaling Technology (MA, USA). Antibodies against EAAT3 (ab124802), TrkA (ab76291), xCT (ab37185), NeuN (ab177487) and NGF (ab52918, for immunofluorescence) were purchased from Abcam (MA, USA). LAT1 (sc-134994) was purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against β-tubulin (T4026) and β-actin (GT5512) were purchased from Sigma-Aldrich and GeneTex, respectively. Anti-mouse (61–6520) secondary antibody was from Thermo Fisher Scientific (MA, USA). The secondary Alexa Fluor 546 anti-rabbit and Alexa Fluor 488 anti-mouse antibodies were purchased from Invitrogen (CA, USA).

### 2.2. Animals and treatment protocol

Four-to five-week-old BALB/c male mice were obtained from the Animal Care Facility of the Instituto de Investigaciones Biomédicas of the Universidad Nacional Autónoma de México. Mice were maintained at 23–25 °C at a relative humidity of 50–60% and under a 12 h light/dark cycle. The animals had access to food and water *ad libitum*. Mice received BSO dissolved in saline solution ip at a dose of 6 mmol/kg. Animals were sacrificed at 0.5 and 2 h after treatment, and the livers and cerebella were dissected and processed immediately or stored at −80 °C.

Experiments were performed following the guidelines stated in the "Principles of Laboratory Animal Care" (National Institutes of Health, NIH) and "Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio (Clave NOM-062-ZOO-1999)" of the "Norma Oficial Mexicana de la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA)" (2001).

### 2.3. Determination of GSH and GSSG levels

Tissue GSH and GSSG levels were measured by the fluorometric o-phthalaldehyde (OPA) method (Senft et al., 2000), adapted to opaque microplates (Ramos-Chavez et al., 2015). Briefly, tissues were weighed and homogenized in 10 vol of ice-cold homogenization buffer containing 154 mM KCl, 5 mM diethylenetriamine penta-acetic acid (DTPA), and 0.1 M potassium phosphate buffer, pH 6.8, followed by the addition of 10 vol of cold buffer containing 40 mM HCl, 10 mM DTPA, 20 mM ascorbic acid, and 10% trichloroacetic acid. Samples were centrifuged at 14,000 × *g*, and the supernatant was filtered through a 0.45 μm filter. Samples were stable at −80 °C for at least 4 weeks. The OPA reagent was added, and fluorescence was determined at 365 nm excitation and 430 nm emission using a DTX 800/880 Multimode Detector (Beckman Coulter, CA, USA). The final values were calculated using standard curves after subtracting the background reaction (non-glutathione-dependent fluorescence) and normalized to the tissue

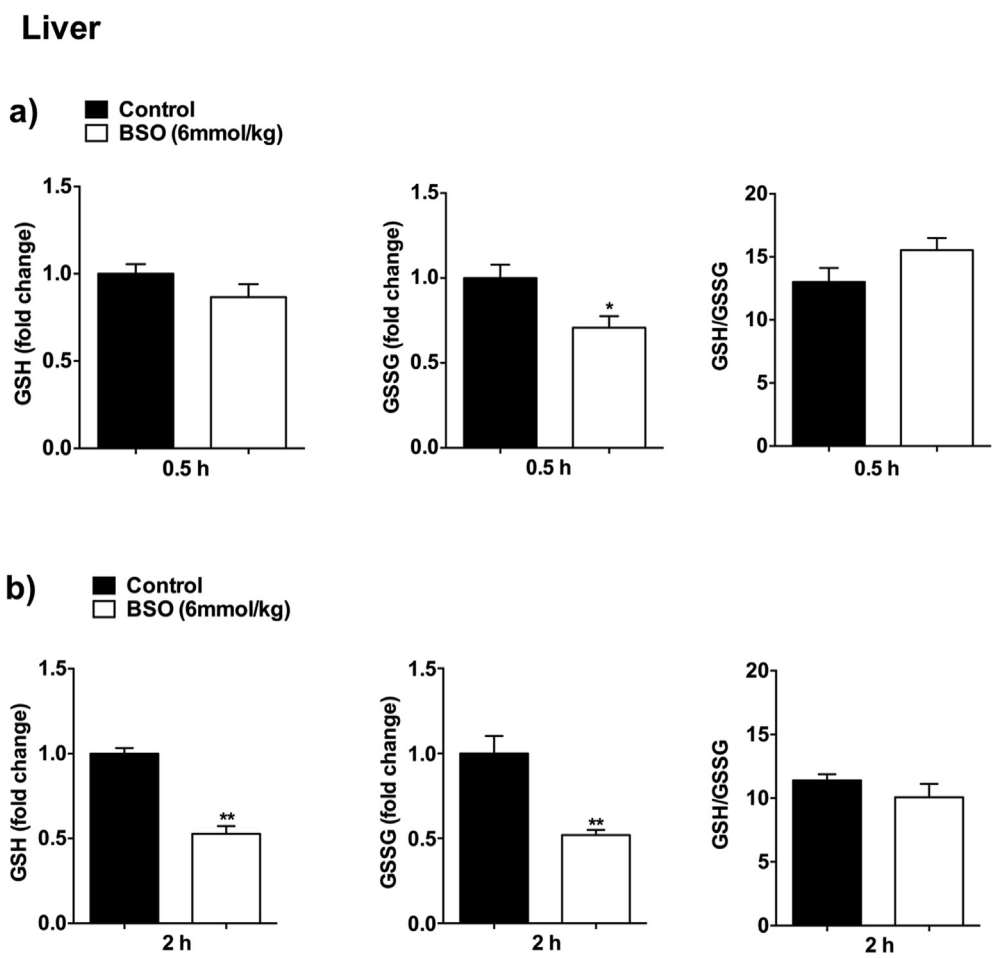


**Fig. 1.** BSO effect on GSH and GSSG levels in the liver of mice treated ip with 6 mmol/kg BSO. Normalized levels of GSH, GSSG, and GSH/GSSG ratio at 0.5 h (**a**) and 2 h (**b**) after BSO administration. Data in graphs represent means ± SE; n = 6. Student's *t*-test. *p < 0.05, **p < 0.01 vs Controls are considered significative. Abbreviations: BSO, L-buthionine-S-R-sulfoximine; GSH, glutathione; GSSG, disulfide-oxidized glutathione; SE, standard error.

weight.

### 2.4. Protein extraction and western immunoblot

Membrane-enriched fractions were obtained from tissue samples as described previously (Schindler et al., 2006) for Western blot determinations of EAAT3, TrkA, xCT, and LAT1. Tissues were homogenized in 10 vol of extraction buffer containing 10 mM HEPES, 10 mM NaCl, 1 mM $KH_2PO_4$, 5 mM $NaHCO_3$, 5 mM EDTA, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM $Na_3VO_4$, 1 mM PMSF, and a protease inhibitor cocktail (Roche, Switzerland). Homogenates were centrifuged at 6300 × *g* for 10 min at 4 °C. Supernatants were recovered and centrifuged at 100,000 × *g* for 30 min at 4 °C. Pellets were resuspended in 40 mM Tris-HCl, pH 9.5, 8 M urea and 4% (w/v) Triton X-100 supplemented with 1 mM $Na_3VO_4$, 1 mM PMSF, and a protease inhibitor cocktail. Protein concentrations were determined using a Pierce BCA Protein Assay kit (Thermo Scientific, MA, USA).

Cytoplasmic fractions were extracted for S6K, mTOR and NGF determinations as previously described (Basaki et al., 2007). Tissues were lysed in Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM DTT, 0.4% v/v IGEPAL, with 1 mM $Na_3VO_4$, 1 mM PMSF, and a protease inhibitor cocktail), incubated for 10 min on ice, and then centrifuged for 15 min at 4000 × *g*. The supernatant fractions containing cytoplasmic soluble proteins were collected.

Samples (5–40 μg protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Germany). The membranes were blocked with 1x Tris-buffered saline (TBS) and 0.1% Tween-20, containing 5% Blotto or bovine serum albumin (BSA) according to the antibody's specifications, Membranes were incubated with the primary antibodies. Mouse anti-β-tubulin or anti-β-actin were used as loading controls. Normalization for p-mTOR was performed against mTOR instead of β-tubulin or β-actin to reflect changes in mTOR phosphorylation, and not mTOR protein levels.

Protein bands were visualized using the appropriate HRP-linked secondary antibodies and the ECL Prime Western blotting detection reagent (GE Healthcare Bio-Sciences, PA, USA). Images were captured, and densitometry analysis was performed using ImageJ version 1.50 g software (Wayne Rasband, NIH, MD, USA).

### 2.5. Immunofluorescence

Mice were treated with BSO and perfused with a cold saline solution followed by ice-buffered paraformaldehyde (4%). The brains were removed, postfixed for 24 h at 4 °C, immersed in 20% and 30% sucrose cryoprotection solutions and frozen in 4-methylbutane. Sections (50 μm) were cut using a cryostat and collected in 24-well culture plates filled with 0.1 M phosphate buffer (PB). Sections were washed three times with PB + 0.3% Triton X-100 (PBT) and incubated overnight at 4 °C with the primary antibodies: mouse anti-NeuN (neuronal nuclei; 1:600) and rabbit anti-TrkA (1:200) or anti-NGF (1:200) in PBT containing 0.3% BSA and 2% normal horse serum (Vector Laboratories, CA, USA). Sections were washed three times with PBT and incubated with the anti-rabbit Alexa Fluor 546 (1:600) and anti-mouse Alexa Fluor 488

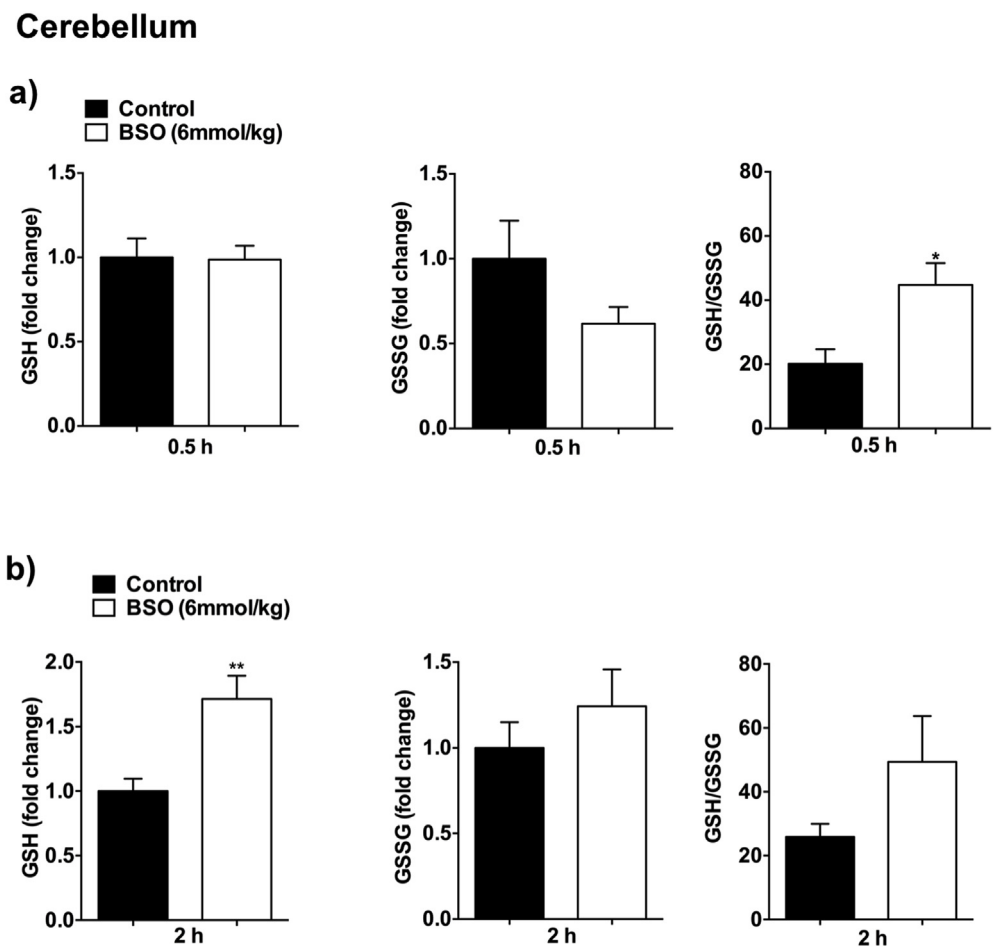


**Fig. 2.** BSO effect on GSH and GSSG levels in the cerebellum of mice treated ip with 6 mmol/kg BSO. Normalized levels of GSH, GSSG, and GSH/GSSG ratio at 0.5 h (**a**) and 2 h (**b**) after BSO administration. Data in graphs represent means ± SE; n = 6. Student's *t*-test. *p < 0.05, **p < 0.01 vs Controls are considered significative. Abbreviations: BSO, L-buthionine-S-R-sulfoximine; GSH, glutathione; GSSG, disulfide-oxidized glutathione; SE, standard error.

(1:400) secondary antibodies for 2 h. Then, the sections were washed in 0.1 M PB, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen), according to the manufacturer's protocol. Brain sections were mounted in Fluorescence Mounting Medium (DAKO, Denmark). Confocal images were sequentially acquired using a Nikon A1R$^+$ laser scanning confocal head coupled to an Eclipse Ti-E inverted microscope (Nikon Corporation, Japan) equipped with a motorized stage (TI-S-E, Nikon). ImageJ version 1.50 g software (Wayne Rasband, NIH; MD, USA) was used to analyze the final images.

### 2.6. Statistical analysis

Collected data were analyzed using Student's *t*-test using GraphPad Prism version 6.00 (GraphPad Software, CA, USA). Data were plotted as the means ± standard error (SE). P values < 0.05 were considered as statistically significant.

## 3. Results

### 3.1. Changes in GSH and GSSG levels in the liver following BSO treatment

GSH and GSSG levels were determined to demonstrate that ip BSO diminished the GSH concentration in mouse liver. No decrease in GSH levels was observed 0.5 h after BSO treatment (Fig. 1a). However, a significant decrease in GSSG levels was found (Fig. 1a). A significant decrease in GSH and GSSG levels was observed 2 h after BSO injection (Fig. 1b) which suggests that BSO decreased intracellular GSH and GSSG content via blockade of *de novo* GSH synthesis. The GSH/GSSG ratio is an indicator of the redox environment status (Schafer and Buettner, 2001), and it was not altered at either time point (Fig. 1a and b). These results suggest that cells export or metabolize GSSG as a detoxifying mechanism to prevent oxidative stress (Franco and Cidlowski, 2012).

### 3.2. BSO induces an increase in GSH levels in the cerebellum

We previously observed that BSO treatment (6 mmol/kg) increased GSH levels in whole brain homogenates (Limon-Pacheco et al., 2007). The present study measured GSH levels in different brain regions (data not shown), however a clear redox response was observed only in the cerebellum. BSO administration produced no significant changes in GSH or GSSG levels at 0.5 h (Fig. 2a), but the slight changes in their concentrations significantly increased the GSH/GSSG ratio (Fig. 2a). A significant increase in GSH levels was observed at 2 h (Fig. 2b). These results demonstrate an increase in the reductive environment and GSH levels in the cerebellum after BSO administration. BSO is poorly transported into the brain (Limon-Pacheco and Gonsebatt, 2007; Steinherz et al., 1990), but the cerebellum responded to the changes in the redox state of the periphery.

### 3.3. Expression of xCT, EAAT3 and LAT1 transporters in the cerebellum

GSH synthesis in the brain is associated with an increased expression of cystine/cysteine transporters (Limon-Pacheco et al., 2007;

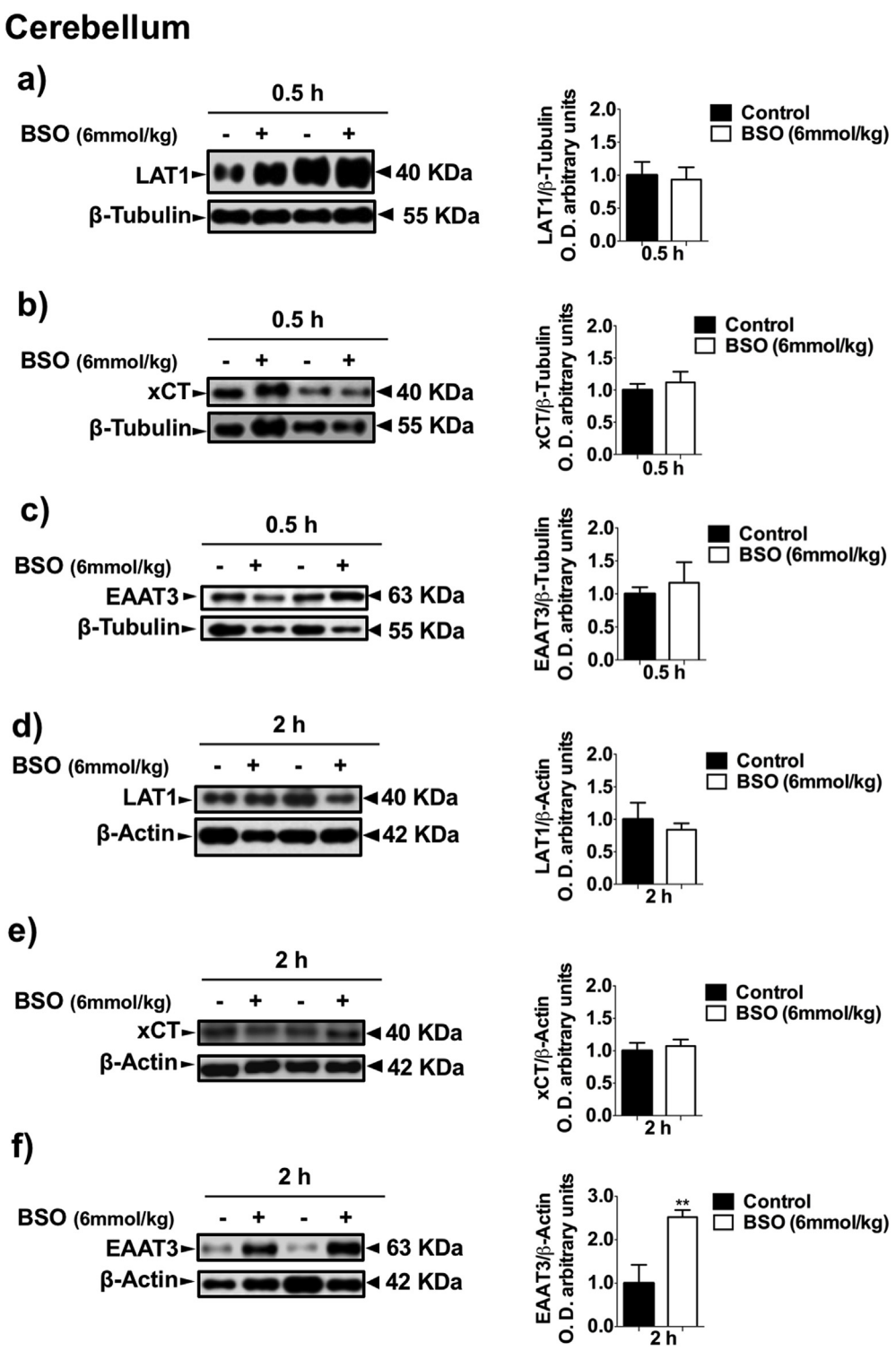


**Fig. 3.** BSO effect on the protein levels of the amino acid transporters, LAT1, xCT, and EAAT3, in the cerebellum of mice, treated ip with 6 mmol/kg BSO. Changes in the protein levels in enriched membrane fraction were evaluated by western blots, at 0.5 h and 2 h after BSO injection. Graphs show the densitometric analysis of the protein bands normalized to β-tubulin (**a-c**) or β-actin (**b-d**), means ± SE; n = 6. Student's *t*-test. **p < 0.01 vs Controls are considered significative. Abbreviations: BSO, L-buthionine-S-R-sulfoximine; LAT1, L-type amino acid transporter; xCT, system xc-subunit; EAAT3, excitatory amino acid transporter 3; SE, standard error.

Nelson-Mora et al., 2018; Ramos-Chavez et al., 2015). Western blot analysis of membrane-enriched fractions was performed to determine the expression of the amino acid transporters involved in the import of cysteine for GSH synthesis. No changes in the expression of the xCT subunit of the $x_c^-$ system, or the transporter of large neutral amino acids, LAT1 were observed after BSO treatment (Fig. 3 a-b, d-e). On the other hand, the expression of the neuronal glutamate transporter EAAT3, which is also involved in the uptake of cysteine primarily in neurons, was significantly upregulated 2 h after BSO treatment (Fig. 3 c and f).

### 3.4. BSO activates the mTOR and NGF pathways in the cerebellum

We investigated whether BSO treatment activated the mTOR

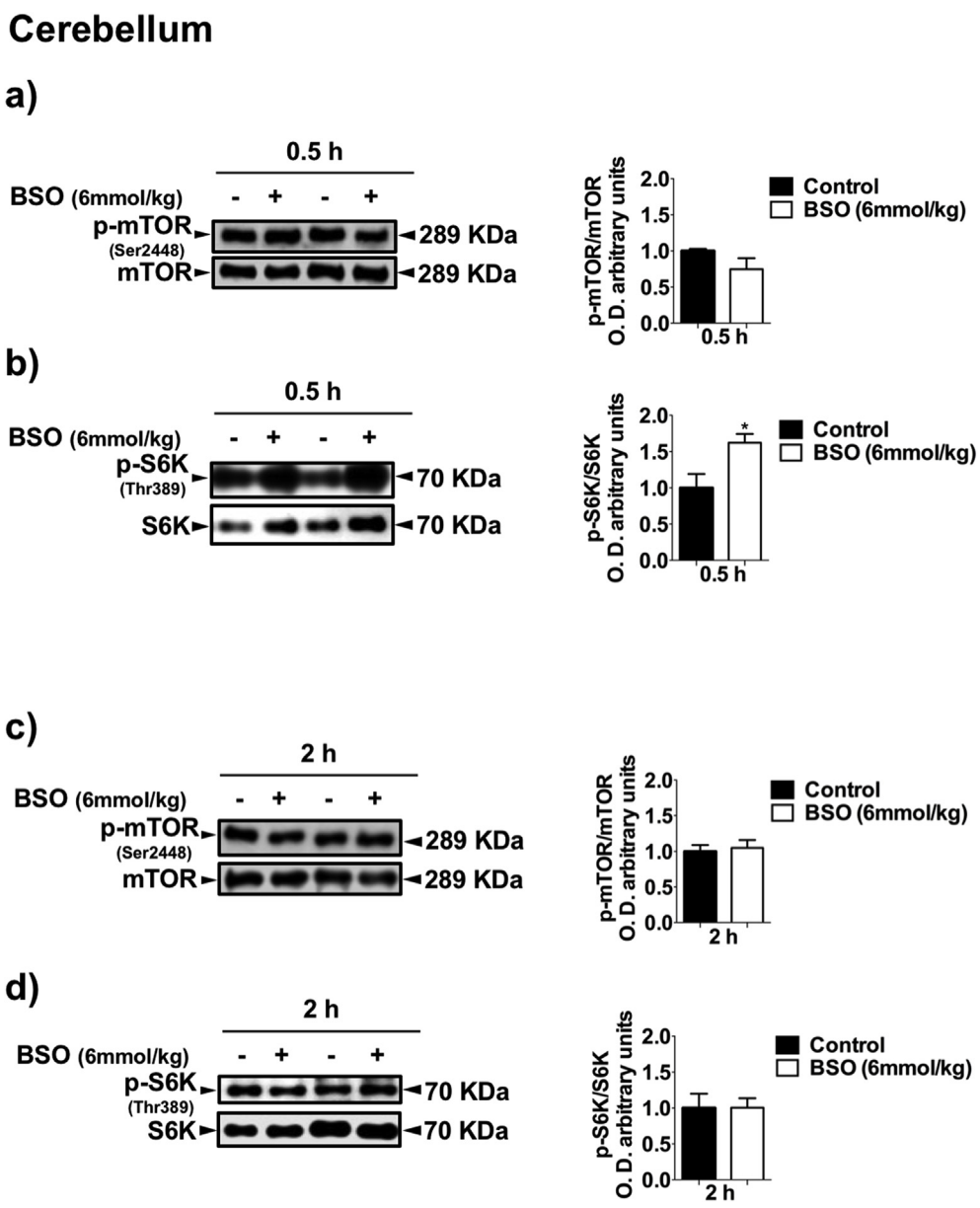


**Fig. 4.** BSO effect on the phosphorylation of mTOR and S6K in the cerebellum of mice treated ip with 6 mmol/kg BSO at 0.5 h and 2 h. Changes in the phosphorylation levels were evaluated by western blots, at 0.5 h and 2 h after BSO injection (**a, c**) p-mTOR (Ser 2448) and (**b, d**) p-S6K (Thr 389). Graphs show densitometric analysis of protein bands normalized with respect to total mTOR and S6K respectively and represent means ± SE;n = 6. Student's t-test. *p < 0.05 vs Controls are considered significative. Abbreviations: BSO, L-buthionine-S-R-sulfoximine; mTOR mechanistic or mammalian target of rapamycin; S6K, S6 kinase beta-1; SE, standard error.

signaling pathway in the cerebellum because of the close relationship between mTOR and the amino acid transporters (Goberdhan et al., 2016; Kim and Guan, 2011). Western blot analysis revealed no increase in the phosphorylation or the expression of mTOR (Fig. 4a, c). However, an increased phosphorylation of its downstream target ribosomal protein S6 kinase beta-1 (S6K) (Garza-Lombo et al., 2018) was found 0.5 h after treatment (Fig. 4b,d), which suggests that S6K could be participating in the activation of EAAT3.

We demonstrated that activation of the NGF/TrkA/phosphoinositide 3-kinase/protein kinase B/AKT/nuclear factor kappa B (PI3K/PKB/AKT/NFκB) pathway in mouse liver induced an antioxidant response after treatment with GSH-consuming agents (Valdovinos-Flores and Gonsebatt, 2013). It has also been shown that NGF is involved in the regulation of cellular GSH levels *in vitro* and *in vivo* (Arsenijevic et al., 2007; Pan and Perez-Polo, 1996). We measured NGF and TrkA expression using Western blot and immunofluorescence analysis to evaluate whether the NGF/TrkA pathway was activated in the cerebellum in response to BSO. Western blot analysis demonstrated that BSO treatment significantly induced NGF expression and TrkA phosphorylation at 0.5 h (Fig. 5a and b) but not at 2 h (Fig. 5c and d). Immunofluorescence labeling revealed increased NGF and TrkA expression in neurons at 0.5 h. TrkA increase was found in the soma of the neurons of the granular cell layer and the molecular layer, which contains axonal and dendritic processes (Fig. 6, Supplementary movies). No change was observed at 2 h (Fig. 7). These results suggest that the NGF signaling pathway and mTOR are involved in GSH homeostasis and induce an antioxidant response that is associated with the depletion of peripheral GSH. We hypothesize that this activation drives the up-regulation of EAAT3 and increase in GSH levels.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.neuint.2018.10.007.

## 4. Discussion

Our previous studies demonstrated that BSO diminished GSH levels in liver and kidneys but surprisingly increased the GSH levels in whole brain homogenates (Limon-Pacheco et al., 2007) despite the limited permeability of BSO across to the BBB (Limon-Pacheco and Gonsebatt, 2007; Steinherz et al., 1990). This increase began 1 h after BSO injection and lasted 2 h. The GSH content returned to the control value at

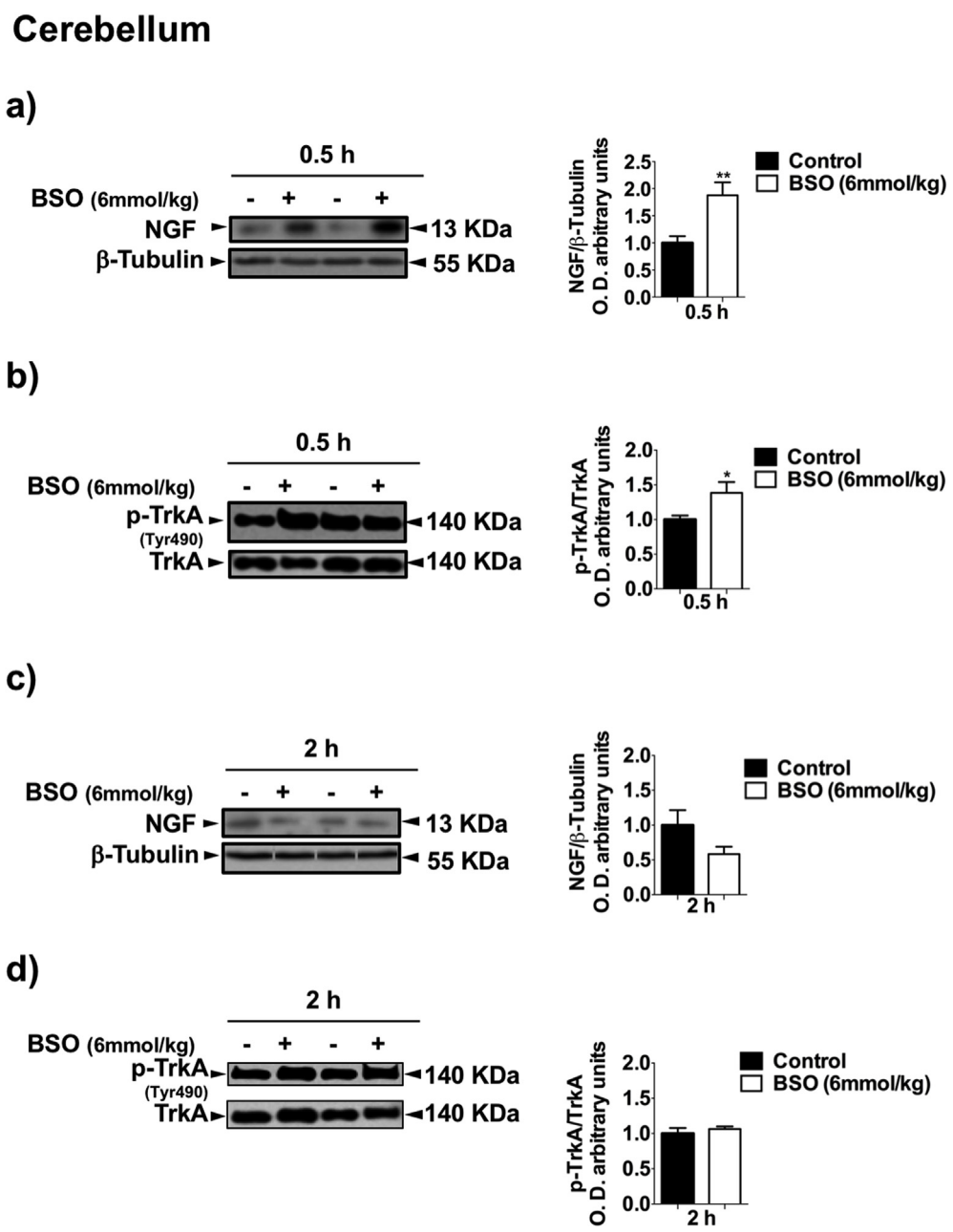


**Fig. 5.** BSO effect on NGF protein levels and the phosphorylation of TrkA in the cerebellum of mice treated ip with 6 mmol/kg BSO at 0.5 h and 2 h. Changes in the protein and phosphorylation levels were evaluated by western blots at 0.5 h and 2 h after BSO injection (**a, c**) NGF; p-TrkA (Tyr 490) (**b, d**). Graphs show densitometric analysis normalized with respect to total TrkA and represent means ± SE; n = 5. Student's *t*-test. *p < 0.05, **p < 0.01 vs Controls are considered significative. Abbreviations: BSO, L-buthionine-S-R-sulfoximine; NGF, neuronal growth factor; TrkA, Tropomyosin receptor kinase A. SE, standard error.

3 h. A single dose of BSO rapidly decreased GSH levels, but GSH content recovered within 12 h in the liver, which suggests an increase rate of enzyme synthesis or a great capacity to metabolize the inhibitor (Griffith and Meister, 1979). The present study investigated the responses of different brain regions to peripheral BSO and the potential signaling pathways involved in this response using an experimental protocol to observe signaling pathways activation at 0.5 h and the final response at 2 h.

We observed an increase in GSH concentration in the cerebellum after BSO exposure (Fig. 2a). This response was preceded by the activation of the NGF/TrkA and mTOR signaling pathways (Figs. 4b and 5a and b and Fig. 6) and increased protein levels of the EAAT3 transporter (Fig. 3f), which suggests that the cerebellum responds to peripheral changes in GSH.

The brain is especially vulnerable to oxidative stress, and different regions contain varying concentrations of GSH. The cortex and the cerebellum contain higher GSH levels (Kang et al., 1999). Our results suggest that the increase in GSH levels (Fig. 2b) and the GSH/GSSG ratio (Fig. 2a) observed in the cerebellum after peripheral BSO exposure might be related to a higher resistance of the cerebellum to oxidative stress compared to other brain regions.

The mechanism of the increased resistance of the cerebellum to oxidative stress is not clear. Previous reports demonstrated that the sensitivity of the cerebellum to compounds that induce oxidative stress was lower than other brain areas (Son et al., 2016; Vandresen-Filho et al., 2015). The cerebellum exhibited higher GSH content (~1.5 mmol/g tissue) compared to other brain regions (Hjelle et al., 1994; Kang et al., 1999; Kudo et al., 1990). Notably, the heterogeneity

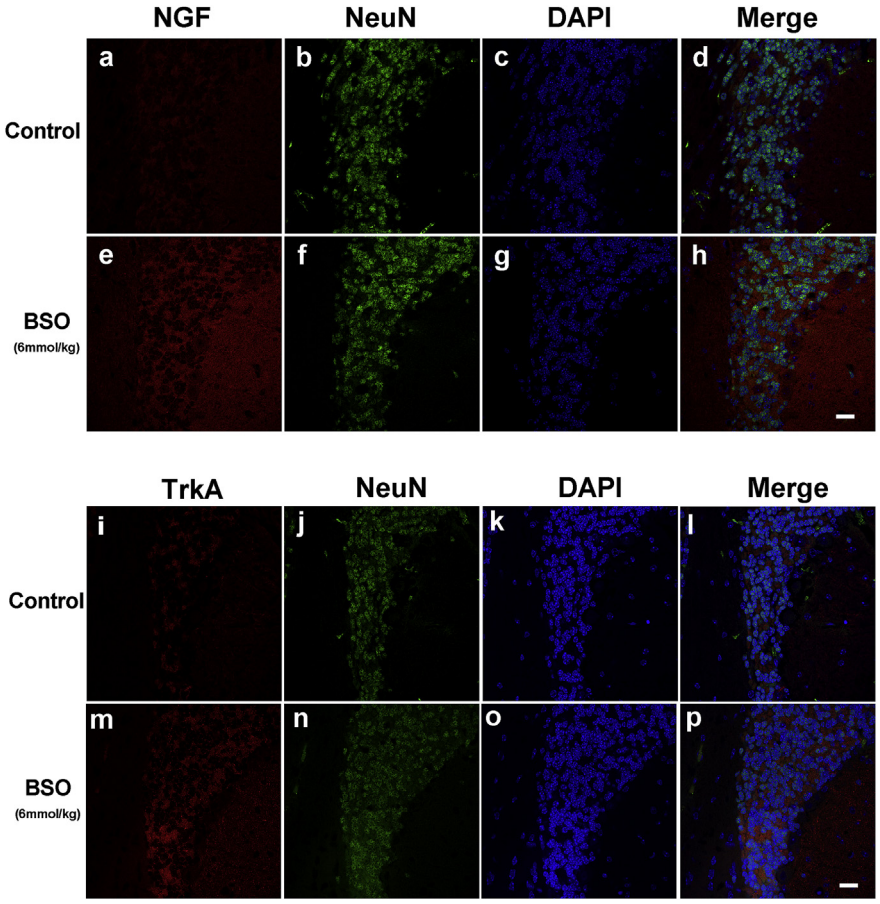


**Fig. 6.** BSO effect on NGF and TrkA protein levels in cerebellum of mice treated ip with 6 mmol/kg BSO at 0.5 h. Confocal images of immunostained sections from the cerebella of control and BSO treated mice. (**a, e**) shows immunofluorescence images of NGF (red), (**b, f**) neuron marker anti-NeuN (green), (**c, g**) nuclei marker DAPI (blue) and (**d, h**) merge, in control and treated mice. The second panel shows immunofluorescence images of (**i, m**) TrkA (red), (**j, n**) NeuN (green), (**k, o**) DAPI (blue) and (**l, p**) merge in control and treated mice. Please note the relative increase of intensity in both NGF and TrkA of BSO-treated animals. Scale bar: 20 μm. Supplementary material shows a 3D reconstruction of the molecular layer of the cerebellum immunostained with TrkA (red), NeuN (green) and DAPI (blue); movie1 (control), movie 2 (6 mmol/kg BSO at 0.5 h). Abbreviations: BSO, L-buthionine-S-R-sulfoximine; NGF, neuronal growth factor; TrkA, Tropomyosin receptor kinase A; NeuN, neuronal nuclei DAPI, 4′,6-diamidino-2-phenylindole.

of cerebral microvessels may help explain the different susceptibility. Austin and colleagues have demonstrated that the microvessels of the cerebellum exhibited greater antioxidant capacity than the hippocampus or cortex (Austin et al., 2015).

Additionally, redox and GSH regulation in the cerebellum has been widely studied. A balance between GSH levels and ROS production is required for cerebellar granule neurons (CGN) survival during early development (Coyoy et al., 2013; Olguin-Albuerne and Moran, 2015). GSH content is also a critical factor in the sensitivity to excitotoxic injury from kainic acid in the CGN (Ceccon et al., 2000). The cerebellum adapts enzymatically to resist GSH depletion induced by acute liver failure via decreasing GSH peroxidase (GPx) levels but not GSH reductase (GR) activity (Singh et al., 2014). The important balance between GSH and ROS production in the cerebellum during development, exposure to xenobiotics and the development of pathologies, is tightly regulated in this brain region.

The increased GSH levels in the cerebellum associated with the liver depletion of GSH was linked to the upregulation of EAAT3 but not xCT or LAT1 (Fig. 3). xCT and LAT1 are crucial for cysteine transport into the brain and astrocytes, but we did not find any significant upregulation in xCT or LAT1 protein levels (Fig. 3a–b, d-e). Transgenic mice with deletion of the xCT gene do not exhibit any signs of increased oxidative stress (De Bundel et al., 2011; Massie et al., 2011), which suggests the participation of additional cystine/cysteine transporters. LAT1 mediates the transport of several amino acids, and its activity is important to transport cysteine from the BBB to the brain. However, LAT1 seems to be more important during development than during adult life (Boado et al., 2004). In contrast to xCT knock-out mice, mice with reduced EAAT3 expression or lacking EAAT3 expression exhibit impaired neuronal cysteine uptake, reduced neuronal GSH levels and chronic oxidative stress, which promote neuronal cell death and cognitive impairment (Berman et al., 2011; Cao et al., 2012; Wang et al., 2015). This evidence suggests that EAAT3 plays a crucial role in cysteine uptake in neurons and the BBB and the regulation of GSH homeostasis (Aoyama and Nakaki, 2013). Our results suggest that EAAT3 participates in the cerebellar response against redox changes induced by the peripheral effect of BSO although BSO is poorly transported through the BBB (Limon-Pacheco and Gonsebatt, 2007; Steinherz et al., 1990).

We also observed an increase in the phosphorylation of the mTORC1 target S6K at 0.5 h after BSO treatment (Fig. 4b), which suggests activation of the mTORC1 pathway in response to BSO. Although we did not find an increase in mTOR phosphorylation, there are diverse studies that suggest that mTOR phosphorylation on Ser 2448 is not directly involved in its activation additionally, its mutation has no effect on mTOR activity (Mothe-Satney et al., 2004; Sekulic et al., 2000).

The mTOR and extracellular signal-regulated kinases (ERK) pathways mediated EAAT3 translation and protein following activation of the metabotropic glutamate receptors (mGluR) 1 and 5 (Ross et al., 2011). Increased EAAT3 protein abundance on the membrane surface, was observed in *Xenopus* oocytes coexpressing mTOR and EAAT3, which demonstrates the role of mTOR in EAAT3 upregulation (Almilaji et al., 2012).

mTOR is protective in the CNS, and it induces antioxidant responses, against oxidative stress (Chong et al., 2012; Ichimura et al., 2013; Zhang et al., 2014; Zhou et al., 2016). In two studies, activation of the PKB/AKT/mTOR pathway clearly increased GCL expression and resulted in GSH synthesis and protection against oxidative stress (Kimura et al., 2009; Okouchi et al., 2006).

We also found an increase in NGF expression and phosphorylation of its receptor TrkA 0.5 h after BSO treatment (Fig. 5a and b). Similar results were observed using immunofluorescence labeling. TrkA upregulation was observed in the soma, axons and dendrites of cerebellar

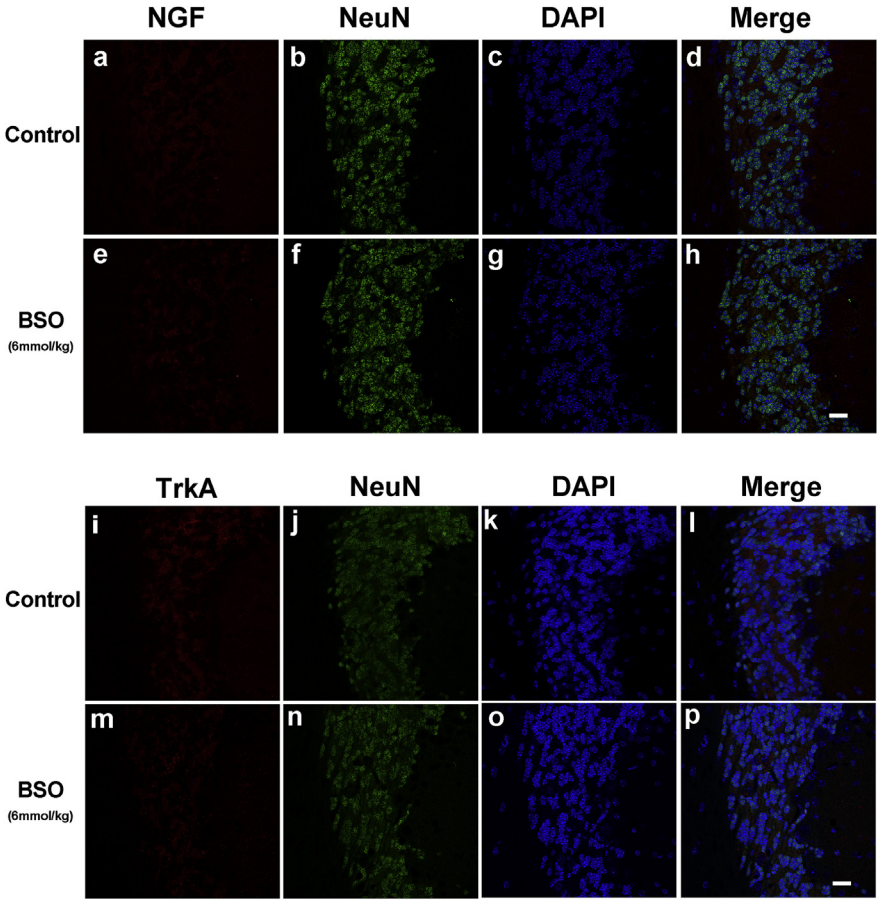


**Fig. 7.** BSO effect on NGF and TrkA protein levels in cerebellum of mice treated ip with 6 mmol/kg BSO at 2 h. Confocal images of immunostained sections from the cerebella of control and BSO treated mice. (**a, e**) shows immunofluorescence images of NGF (red), (**b, f**) neuron marker anti-NeuN (green), (**c, g**) nuclei marker DAPI (blue) and (**d, h**) merge, in control and treated mice. The second panel shows immunofluorescence images of (**i,m**) TrkA (red), (**j,n**) NeuN (green), (**k, o**) DAPI (blue) and (**l, p**) merge in control and treated mice. There are no differences between BSO-treated and control animals at this time point. Scale bar: 20 μm. Abbreviations: BSO, L-buthionine-S-R-sulfoximine; NGF, neuronal growth factor; TrkA, Tropomyosin receptor kinase A; NeuN, neuronal nuclei DAPI, 4′,6-diamidino-2-phenylindole.

neurons (Fig. 6, Supplementary movies), which suggests that BSO induced the synthesis and trafficking of the TrkA receptor. New TrkA receptors synthesized in cell bodies undergo anterograde transport through axons and dendrites, where they bind NGF and initiate signaling (Marlin and Li, 2015; Scott-Solomon and Kuruvilla, 2018).

NGF induced an antioxidant response in *in vitro* and *in vivo* models (Arsenijevic et al., 2007; Pan and Perez-Polo, 1993, 1996). NGF induced glutamate release from cerebellar granule cells, which suggests its involvement in the modulation of excitatory neurotransmission and amino acid transport regulation (Numakawa et al., 1999). Activation of the PI3K/PKB/AKT pathway via NGF/TrkA, is recognized as a pro-survival pathway (Huang and Reichardt, 2003). In human retinal pigment epithelial (RPE) cells, NGF protects against hydrogen peroxide ($H_2O_2$)-induced apoptosis and stimulates cell survival via the activation of the mTOR signaling pathway (Cao et al., 2011).

In summary, our results show a response activated in the cerebellum to remedy the redox changes induced by BSO. The ip administration of BSO induced GSH depletion in the liver and increased GSH levels in the cerebellum. These changes are likely associated with activation of the NGF/TrkA and mTOR signaling pathways and increased expression of the cysteine importer EAAT3. We hypothesize that the changes in the liver and other peripheral organs, such as kidneys (Limon-Pacheco et al., 2007), induce redox responses in the cerebellum. These observations are important because the liver controls the detoxification of most chemicals in the body, including drugs. Exposure to xenobiotic agents that consume antioxidants, including metals, ionizing radiation, pesticides or ozone (Limon-Pacheco and Gonsebatt, 2009), affects GSH pools. The current results help elucidate the mechanisms involved in the regulation of GSH metabolism and cellular redox changes that occur during oxidative stress. Chronic exposure to high levels of these xenobiotic agents may lead to the deregulation of GSH metabolism that is observed in neurodegenerative diseases.

## Authors disclosure statement

The authors declare no conflict of interest.

The movies complement Fig. 6 showing an increase TrkA levels in the molecular layer of the cerebellum.

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## References

Almilaji, A., Pakladok, T., Guo, A., Munoz, C., Foller, M., Lang, F., 2012. Regulation of the glutamate transporter EAAT3 by mammalian target of rapamycin mTOR. Biochem. Biophys. Res. Commun. 421, 159–163.

Aoyama, K., Nakaki, T., 2013. Neuroprotective properties of the excitatory amino acid Carrier 1 (EAAC1). Amino Acids 45, 133–142.

Aquilano, K., Baldelli, S., Ciriolo, M.R., 2014. Glutathione: new roles in redox signaling for an old antioxidant. Front. Pharmacol. 5, 196.

Arsenijevic, D., Hernadfalvi, N., von Meyenburg, C., Onteniente, B., Richard, D., Langhans, W., 2007. Role for nerve growth factor in the in vivo regulation of glutathione in response to LPS in mice. Eur. Cytokine Netw. 18, 93–101.

Austin, S.A., Santhanam, A.V., d'Uscio, L.V., Katusic, Z.S., 2015. Regional heterogeneity of cerebral microvessels and brain susceptibility to oxidative stress. PloS One 10 e0144062.

Basaki, Y., Hosoi, F., Oda, Y., Fotovati, A., Maruyama, Y., Oie, S., Ono, M., Izumi, H., Kohno, K., Sakai, K., Shimoyama, T., Nishio, K., Kuwano, M., 2007. Akt-dependent nuclear localization of Y-box-binding protein 1 in acquisition of malignant

characteristics by human ovarian cancer cells. Oncogene 26, 2736–2746.
Berman, A.E., Chan, W.Y., Brennan, A.M., Reyes, R.C., Adler, B.L., Suh, S.W., Kauppinen, T.M., Edling, Y., Swanson, R.A., 2011. N-acetylcysteine prevents loss of dopaminergic neurons in the EAAC1-/- mouse. Ann. Neurol. 69, 509–520.
Bianchi, M.G., Bardelli, D., Chiu, M., Bussolati, O., 2014. Changes in the expression of the glutamate transporter EAAT3/EAAC1 in health and disease. Cell. Mol. Life Sci. 71, 2001–2015.
Boado, R.J., Li, J.Y., Pardridge, W.M., 2004. Developmental regulation of the rabbit blood-brain barrier LAT1 large neutral amino acid transporter mRNA and protein. Pediatr. Res. 55, 557–560.
Cao, G.F., Liu, Y., Yang, W., Wan, J., Yao, J., Wan, Y., Jiang, Q., 2011. Rapamycin sensitive mTOR activation mediates nerve growth factor (NGF) induced cell migration and pro-survival effects against hydrogen peroxide in retinal pigment epithelial cells. Biochem. Biophys. Res. Commun. 414, 499–505.
Cao, L., Li, L., Zuo, Z., 2012. N-acetylcysteine reverses existing cognitive impairment and increased oxidative stress in glutamate transporter type 3 deficient mice. Neuroscience 220, 85–89.
Ceccon, M., Giusti, P., Facci, L., Borin, G., Imbesi, M., Floreani, M., Skaper, S.D., 2000. Intracellular glutathione levels determine cerebellar granule neuron sensitivity to excitotoxic injury by kainic acid. Brain Res. 862, 83–89.
Chen, L., Xu, B., Liu, L., Luo, Y., Zhou, H., Chen, W., Shen, T., Han, X., Kontos, C.D., Huang, S., 2011. Cadmium induction of reactive oxygen species activates the mTOR pathway, leading to neuronal cell death. Free Radic. Biol. Med. 50, 624–632.
Chong, Z.Z., Shang, Y.C., Wang, S., Maiese, K., 2012. Shedding new light on neurodegenerative diseases through the mammalian target of rapamycin. Prog. Neurobiol. 99, 128–148.
Coyoy, A., Olguin-Albuerne, M., Martinez-Briseno, P., Moran, J., 2013. Role of reactive oxygen species and NADPH-oxidase in the development of rat cerebellum. Neurochem. Int. 62, 998–1011.
De Bundel, D., Schallier, A., Loyens, E., Fernando, R., Miyashita, H., Van Liefferinge, J., Vermoesen, K., Bannai, S., Sato, H., Michotte, Y., Smolders, I., Massie, A., 2011. Loss of system x(c)- does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility. J. Neurosci. 31, 5792–5803.
Franco, R., Cidlowski, J.A., 2012. Glutathione efflux and cell death. Antioxidants Redox Signal. 17, 1694–1713.
Garza-Lombo, C., Gonsebatt, M.E., 2016. Mammalian target of rapamycin: its role in early neural development and in adult and aged brain function. Front. Cell. Neurosci. 10, 157.
Garza-Lombo, C., Schroder, A., Reyes-Reyes, E., Franco, R., 2018. mTOR/AMPK signaling in the brain: cell metabolism, proteostasis and survival. Current Opinion in Toxicology 8, 102–110.
Goberdhan, D.C., Wilson, C., Harris, A.L., 2016. Amino acid sensing by mTORC1: intracellular transporters mark the spot. Cell Metabol. 23, 580–589.
Griffith, O.W., Meister, A., 1979. Glutathione: interorgan translocation, turnover, and metabolism. Proc. Natl. Acad. Sci. U. S. A. 76, 5606–5610.
Gu, F., Chauhan, V., Chauhan, A., 2015. Glutathione redox imbalance in brain disorders. Curr. Opin. Clin. Nutr. Metab. Care 18, 89–95.
Himi, T., Ikeda, M., Yasuhara, T., Nishida, M., Morita, I., 2003. Role of neuronal glutamate transporter in the cysteine uptake and intracellular glutathione levels in cultured cortical neurons. J. Neural. Transm. 110, 1337–1348.
Hirrlinger, J., Dringen, R., 2005. Multidrug resistance protein 1-mediated export of glutathione and glutathione disulfide from brain astrocytes. Methods Enzymol. 400, 395–409.
Hjelle, O.P., Chaudhry, F.A., Ottersen, O.P., 1994. Antisera to glutathione: characterization and immunocytochemical application to the rat cerebellum. Eur. J. Neurosci. 6, 793–804.
Huang, E.J., Reichardt, L.F., 2003. Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. 72, 609–642.
Ichimura, Y., Waguri, S., Sou, Y.S., Kageyama, S., Hasegawa, J., Ishimura, R., Saito, T., Yang, Y., Kouno, T., Fukutomi, T., Hoshii, T., Hirao, A., Takagi, K., Mizushima, T., Motohashi, H., Lee, M.S., Yoshimori, T., Tanaka, K., Yamamoto, M., Komatsu, M., 2013. Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. Mol. Cell. 51, 618–631.
Janssen-Heininger, Y.M., Nolin, J.D., Hoffman, S.M., van der Velden, J.L., Tully, J.E., Lahue, K.G., Abdalla, S.T., Chapman, D.G., Reynaert, N.L., van der Vliet, A., Anathy, V., 2013. Emerging mechanisms of glutathione-dependent chemistry in biology and disease. J. Cell. Biochem. 114, 1962–1968.
Johnson, W.M., Wilson-Delfosse, A.L., Mieyal, J.J., 2012. Dysregulation of glutathione homeostasis in neurodegenerative diseases. Nutrients 4, 1399–1440.
Kang, Y., Viswanath, V., Jha, N., Qiao, X., Mo, J.Q., Andersen, J.K., 1999. Brain gamma-glutamyl cysteine synthetase (GCS) mRNA expression patterns correlate with regional-specific enzyme activities and glutathione levels. J. Neurosci. Res. 58, 436–441.
Killian, D.M., Chikhale, P.J., 2001. Predominant functional activity of the large, neutral amino acid transporter (LAT1) isoform at the cerebrovasculature. Neurosci. Lett. 306, 1–4.
Kim, J., Guan, K.L., 2011. Amino acid signaling in TOR activation. Annu. Rev. Biochem. 80, 1001–1032.
Kimura, R., Okouchi, M., Fujioka, H., Ichiyanagi, A., Ryuge, F., Mizuno, T., Imaeda, K., Okayama, N., Kamiya, Y., Asai, K., Joh, T., 2009. Glucagon-like peptide-1 (GLP-1) protects against methylglyoxal-induced PC12 cell apoptosis through the PI3K/Akt/mTOR/GCLc/redox signaling pathway. Neuroscience 162, 1212–1219.
Kudo, H., Kokunai, T., Kondoh, T., Tamaki, N., Matsumoto, S., 1990. Quantitative analysis of glutathione in rat central nervous system: comparison of GSH in infant brain with that in adult brain. Brain Res. 511, 326–328.
Limon-Pacheco, J., Gonsebatt, M.E., 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat. Res. 674, 137–147.
Limon-Pacheco, J.H., Gonsebatt, M.E., 2007. Glutathione (GSH) synthesis inhibition in the nervous system: a model to comprehend GSH role in neuropathologies. In: Jiménez-Capdeville, A.S.a.M.E. (Ed.), New Perspectives on Brain Cell Damage, Neurodegeneration and Neuroprotective Strategies. Research Signpost, Kerala, India, pp. 173–188.
Limon-Pacheco, J.H., Hernandez, N.A., Fanjul-Moles, M.L., Gonsebatt, M.E., 2007. Glutathione depletion activates mitogen-activated protein kinase (MAPK) pathways that display organ-specific responses and brain protection in mice. Free Radic. Biol. Med. 43, 1335–1347.
Lu, S.C., 2013. Glutathione synthesis. Biochim. Biophys. Acta 1830, 3143–3153.
Marlin, M.C., Li, G., 2015. Biogenesis and function of the NGF/TrkA signaling endosome. Int Rev Cell Mol Biol 314, 239–257.
Massie, A., Schallier, A., Kim, S.W., Fernando, R., Kobayashi, S., Beck, H., De Bundel, D., Vermoesen, K., Bannai, S., Smolders, I., Conrad, M., Plesnila, N., Sato, H., Michotte, Y., 2011. Dopaminergic neurons of system x(c)(-)-deficient mice are highly protected against 6-hydroxydopamine-induced toxicity. Faseb. J. 25, 1359–1369.
Mothe-Satney, I., Gautier, N., Hinault, C., Lawrence Jr., J.C., Van Obberghen, E., 2004. In rat hepatocytes glucagon increases mammalian target of rapamycin phosphorylation on serine 2448 but antagonizes the phosphorylation of its downstream targets induced by insulin and amino acids. J. Biol. Chem. 279, 42628–42637.
Nafia, I., Re, D.B., Masmejean, F., Melon, C., Kachidian, P., Kerkerian-Le Goff, L., Nieoullon, A., Had-Aissouni, L., 2008. Preferential vulnerability of mesencephalic dopamine neurons to glutamate transporter dysfunction. J. Neurochem. 105, 484–496.
Nelson-Mora, J., Escobar, M.L., Rodriguez-Duran, L., Massieu, L., Montiel, T., Rodriguez, V.M., Hernandez-Mercado, K., Gonsebatt, M.E., 2018. Gestational exposure to inorganic arsenic (iAs3+) alters glutamate disposition in the mouse hippocampus and ionotropic glutamate receptor expression leading to memory impairment. Arch. Toxicol. 92, 1037–1048.
Numakawa, T., Takei, N., Yamagishi, S., Sakai, N., Hatanaka, H., 1999. Neurotrophin-elicited short-term glutamate release from cultured cerebellar granule neurons. Brain Res. 842, 431–438.
Okouchi, M., Okayama, N., Alexander, J.S., Aw, T.Y., 2006. NRF2-dependent glutamate-L-cysteine ligase catalytic subunit expression mediates insulin protection against hyperglycemia- induced brain endothelial cell apoptosis. Curr. Neurovascular Res. 3, 249–261.
Olguin-Albuerne, M., Moran, J., 2015. ROS produced by NOX2 control in vitro development of cerebellar granule neurons development. ASN Neuro 7.
Pan, Z., Perez-Polo, R., 1993. Role of nerve growth factor in oxidant homeostasis: glutathione metabolism. J. Neurochem. 61, 1713–1721.
Pan, Z., Perez-Polo, R., 1996. Increased uptake of L-cysteine and L-cystine by nerve growth factor in rat pheochromocytoma cells. Brain Res. 740, 21–26.
Ramos-Chavez, L.A., Rendon-Lopez, C.R., Zepeda, A., Silva-Adaya, D., Del Razo, L.M., Gonsebatt, M.E., 2015. Neurological effects of inorganic arsenic exposure: altered cysteine/glutamate transport, NMDA expression and spatial memory impairment. Front. Cell. Neurosci. 9, 21.
Ross, J.R., Ramakrishnan, H., Porter, B.E., Robinson, M.B., 2011. Group I mGluR-regulated translation of the neuronal glutamate transporter, excitatory amino acid Carrier 1. J. Neurochem. 117, 812–823.
Sato, H., Tamba, M., Ishii, T., Bannai, S., 1999. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. J. Biol. Chem. 274, 11455–11458.
Saxton, R.A., Sabatini, D.M., 2017. mTOR signaling in growth, metabolism, and disease. Cell 169, 361–371.
Schafer, F.Q., Buettner, G.R., 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med. 30, 1191–1212.
Schindler, J., Jung, S., Niedner-Schatteburg, G., Friauf, E., Nothwang, H.G., 2006. Enrichment of integral membrane proteins from small amounts of brain tissue. J. Neural. Transm. 113, 995–1013.
Scott-Solomon, E., Kuruvilla, R., 2018. Mechanisms of neurotrophin trafficking via Trk receptors. Mol. Cell. Neurosci. 91, 25–33.
Sekulic, A., Hudson, C.C., Homme, J.L., Yin, P., Otterness, D.M., Karnitz, L.M., Abraham, R.T., 2000. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res. 60, 3504–3513.
Senft, A.P., Dalton, T.P., Shertzer, H.G., 2000. Determining glutathione and glutathione disulfide using the fluorescence probe o-phthalaldehyde. Anal. Biochem. 280, 80–86.
Shih, A.Y., Erb, H., Sun, X., Toda, S., Kalivas, P.W., Murphy, T.H., 2006. Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. J. Neurosci. 26, 10514–10523.
Singh, S., Mondal, P., Trigun, S.K., 2014. Acute liver failure in rats activates glutamine-glutamate cycle but declines antioxidant enzymes to induce oxidative stress in cerebral cortex and cerebellum. PloS One 9, e95855.
Son, S.W., Lee, J.S., Kim, H.G., Kim, D.W., Ahn, Y.C., Son, C.G., 2016. Testosterone depletion increases the susceptibility of brain tissue to oxidative damage in a restraint stress mouse model. J. Neurochem. 136, 106–117.
Steinherz, R., Martensson, J., Wellner, D., Meister, A., 1990. Transport into brain of buthionine sulfoximine, an inhibitor of glutathione synthesis, is facilitated by esterification and administration of dimethylsulfoxide. Brain Res. 518, 115–119.
Valdovinos-Flores, C., Gonsebatt, M.E., 2012. The role of amino acid transporters in GSH synthesis in the blood-brain barrier and central nervous system. Neurochem. Int. 61, 405–414.

Valdovinos-Flores, C., Gonsebatt, M.E., 2013. Nerve growth factor exhibits an antioxidant and an autocrine activity in mouse liver that is modulated by buthionine sulfoximine, arsenic, and acetaminophen. Free Radic. Res. 47, 404–412.
Vandresen-Filho, S., Martins, W.C., Bertoldo, D.B., Mancini, G., De Bem, A.F., Tasca, C.I., 2015. Cerebral cortex, hippocampus, striatum and cerebellum show differential susceptibility to quinolinic acid-induced oxidative stress. Neurol. Sci. 36, 1449–1456.
Wade, L.A., Brady, H.M., 1981. Cysteine and cystine transport at the blood-brain barrier. J. Neurochem. 37, 730–734.
Wang, X.F., Cynader, M.S., 2000. Astrocytes provide cysteine to neurons by releasing glutathione. J. Neurochem. 74, 1434–1442.
Wang, Y.N., Zhou, L., Li, Y.H., Wang, Z., Li, Y.C., Zhang, Y.W., Wang, Y., Liu, G., Shen, Y., 2015. Protein interacting with C-kinase 1 deficiency impairs glutathione synthesis and increases oxidative stress via reduction of surface excitatory amino acid Carrier 1. J. Neurosci. 35, 6429–6443.
Zerangue, N., Kavanaugh, M.P., 1996. Interaction of L-cysteine with a human excitatory amino acid transporter. J. Physiol. 493 (Pt 2), 419–423.
Zhang, H., Liu, Y.Y., Jiang, Q., Li, K.R., Zhao, Y.X., Cao, C., Yao, J., 2014. Salvianolic acid A protects RPE cells against oxidative stress through activation of Nrf2/HO-1 signaling. Free Radic. Biol. Med. 69, 219–228.
Zhou, Q., Chen, B., Wang, X., Wu, L., Yang, Y., Cheng, X., Hu, Z., Cai, X., Yang, J., Sun, X., Lu, W., Yan, H., Chen, J., Ye, J., Shen, J., Cao, P., 2016. Sulforaphane protects against rotenone-induced neurotoxicity in vivo: involvement of the mTOR, Nrf2, and autophagy pathways. Sci. Rep. 6, 32206.

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REVIEW
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# Mammalian Target of Rapamycin: Its Role in Early Neural Development and in Adult and Aged Brain Function

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The kinase mammalian target of rapamycin (mTOR) integrates signals triggered by energy, stress, oxygen levels, and growth factors. It regulates ribosome biogenesis, mRNA translation, nutrient metabolism, and autophagy. mTOR participates in various functions of the brain, such as synaptic plasticity, adult neurogenesis, memory, and learning. mTOR is present during early neural development and participates in axon and dendrite development, neuron differentiation, and gliogenesis, among other processes. Furthermore, mTOR has been shown to modulate lifespan in multiple organisms. This protein is an important energy sensor that is present throughout our lifetime its role must be precisely described in order to develop therapeutic strategies and prevent diseases of the central nervous system. The aim of this review is to present our current understanding of the functions of mTOR in neural development, the adult brain and aging.

**Keywords: mTOR, central nervous system, CNS development, adult brain, aging CNS**

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## INTRODUCTION

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is involved in the control of cell growth and proliferation. This kinase integrates signals triggered by different stimuli such as variations in the amino acid supply, changes in the cellular energy state, and by receptors for various hormones and growth factors and in the brain by transduction of neurotransmitters and neurotrophin signals (Harris and Lawrence, 2003; Gal-Ben-Ari et al., 2012; Burket et al., 2015). Several studies have shown that mTOR participates in multiple functions of the brain. Its activation contributes to processes involved in synaptic plasticity and metabolic regulation (Garelick and Kennedy, 2011). Additionally, mTOR participates in key processes during neural development, particularly in axon and dendrite development, neuron differentiation, and gliogenesis. Furthermore, consistent with its role as a nutrient and growth factor sensor, decreased mTOR signaling reduces aging and thereby extends lifespan (Cornu et al., 2013). The aim of this

**Abbreviations:** 4E-BP1, eukaryotic initiation factor 4E-binding protein; DEPTOR, DEP domain-containing mTOR-interacting protein; eEF2, eukaryotic elongation factor 2; eEF2K, eukaryotic elongation factor 2 kinase; eIF4E, eukaryotic translation initiation factor 4E; FKBP12, FK506-binding protein; mLST8, mammalian lethal with SEC13 protein 8; mPFC, medial prefrontal cortex; mSIN1, stress-activated MAP kinase-interacting protein 1; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; NSCs, neural stem cells; PRAS40, 40 kDa Pro-rich AKT1 substrate 1; PROTOR, Pro-rich protein 5; RAPTOR, regulatory associated protein of mTOR; Rheb, Ras homolog enriched in brain; RICTOR, rapamycin-insensitive companion of mammalian target of rapamycin; S6K1, S6 kinase; SVZ, subventricular zone; TSC1, tuberous sclerosis complex proteins, hamartin; TSC2, tuberous sclerosis complex proteins, tuberin.

review is to present our current understanding of the functions of mTOR in neural development, the adult brain and aging.

## THE mTOR MULTI-PROTEIN COMPLEX AND SIGNALING PATHWAYS

Metazoans have a single *TOR* gene, whereas yeast has two genes, *TOR1* and *TOR2*. In mammals, the *mTOR* gene (also known as *FRAP*, *RAFT1*, or *SEPT*) encodes a protein of 2549 amino acids with 42 and 45% sequence identity to yeast TOR1 and TOR2, respectively (Harris and Lawrence, 2003).

mTOR exists in two distinct multi-protein complexes; mTOR complex 1 (mTORC1) which is sensitive to rapamycin and consists of the proteins: regulatory associated protein of mTOR (RAPTOR), 40 kDa pro-rich AKT1 substrate 1 (PRAS40), mammalian lethal with SEC13 protein 8 (mLST8), and DEP domain-containing mTOR-interacting protein (DEPTOR). On the other hand, mTOR complex 2 (mTORC2) is relatively insensitive to rapamycin and consists of rapamycin-insensitive companion of mTOR (RICTOR), pro-rich protein 5 (PRR5, also known as PROTOR), DEPTOR, mLST8, and stress-activated MAP kinase-interacting protein 1 (mSIN1). However, prolonged treatment with rapamycin can also inhibit mTORC2, most likely due to the progressive sequestration of mTOR pools by rapamycin-FK506-binding protein (FKBP12; Sarbassov et al., 2005; Weber and Gutmann, 2012).

Rapamycin is an antifungal agent originally isolated from a strain of the soil bacterium *Streptomyces hygroscopicus*. It has potent immunosuppressive and antiproliferative properties and forms an inhibitory complex with its intracellular receptor, FKBP12, which binds to the C-terminus of TOR proteins, thereby inhibiting TOR activity (Weber and Gutmann, 2012). Because of the promising therapeutic potential of rapamycin, several rapamycin analogs have been synthesized to improve its pharmacokinetic properties (Tsang et al., 2007).

Nutrients, energy, stress, oxygen levels, and growth factors are among the major upstream signaling inputs for mTORC1. In this regard, the canonical mTORC1 pathway begins with the activation of tyrosine kinase receptors (Trk) through the induction of phosphoinositide-3-kinase (PI3K) and protein kinase B (PKB, also known as Akt) and includes the phosphorylation and inhibition of tuberous sclerosis complex proteins, hamartin (TSC1) and tuberin (TSC2). The TSC1/TSC2 complex acts as a GTPase-activating protein (GAP) for Ras homolog enriched in brain (Rheb). Subsequently, the increase in GTP-bound Rheb stimulates mTORC1 (Sarbassov et al., 2005; Weber and Gutmann, 2012).

Mitogens can also activate mTOR independently of Akt through phospholipase D (PLD) as well as through the extracellular signal-regulated kinase (ERK)–mitogen-activated protein kinases (MAPK) pathway (Jaworski and Sheng, 2006). The cellular energy levels (amino acid concentration) also require Rheb to activate mTORC1, however, instead of through the tuberous sclerosis complex (TSC), the signal is transduced via the Ras-related GTPase (Rag) complex, a heterodimer small GTPase-containing RagA or B with RagC or D. The increase in the amino acids concentration, induces the movement of mTORC1 to the lysosomal membranes where the Rag complex resides. The complex "Ragulator" consisting of Rag GTPase-interacting proteins (p18, p14, and MP1) has been identified in the interaction with the Rag GTPases and its consequent localization to the lysosome (Sancak and Sabatini, 2009; Kim and Guan, 2011).

Under conditions of energy deprivation that increase the AMP/ATP ratio, AMP-activated protein kinase (AMPK) becomes active and phosphorylates TSC2 to stimulate its GAP activity, therefore inhibiting Rheb and presumably mTORC1. Oxygen sensing by the mTORC1 pathway also requires the TSC1/2 complex, but it functions through a distinct mechanism that involves the hypoxia-inducible factor-dependent expression of regulated in development and DNA damage responses 1 (REDD1) and REDD2, two growth regulators (Sarbassov et al., 2005).

It has long been known that translational machinery operates in neuronal dendrites and synapses, and that local protein synthesis and its regulation is important for diverse processes of the central nervous system (CNS), e.g., memory and synaptic plasticity (Taha et al., 2013). In neurons, neurotransmission modulates the translation machinery, however, all the components of this delicate processes have not been identified yet. mTORC1 is a key downstream molecule in a signaling cascade beginning with the transduction of neurotransmitters and neurotrophin signals. Among the receptors found to activate mTORC1 in neurons, we can mention the muscarinic acetylcholine receptors, AMPA receptors, the glutamate metabotropic receptors (mGlu1/5), the dopaminergic D1 and D3 receptors, the opioid receptor, the amino acid/glutamate T1R1–T1R3 receptors, the serotonin 5-HT6 receptor, the cannabinoid 1 receptor (CB1R), and the GABAB receptors (Gal-Ben-Ari et al., 2012; Bockaert and Marin, 2015). Burket et al. (2015) discusses different articles where *N*-methyl-D-aspartate receptor (NMDAR) activation is downregulating mTOR signaling activity.

The hallmark of mTORC1 activity is the stimulation of ribosome biogenesis, mRNA translation, nutrient metabolism, and autophagy inhibition. In this regard mTORC1 regulates protein synthesis through the phosphorylation and inactivation of a repressor of mRNA translation, eukaryotic initiation factor 4E-binding protein (4E-BP1), and through the phosphorylation and activation of the S6 kinase (S6K1). Thus, S6K1 or 4E-BP1 phosphorylation is often used as an *in vivo* readout of mTOR activity (Hay and Sonenberg, 2004; Sarbassov et al., 2005). Phosphorylation of 4E-BP1 by mTORC1 signals the onset of cap-dependent translation; it allows the binding of several important initiation factors, as well as the positioning of the 40S ribosomal subunit at the 5′ end of the mRNA to begin the process of translation (Harris and Lawrence, 2003).

Active S6K1 stimulates the translation of mRNAs containing unique 5′-terminal oligopyrimidine tracts (TOPs). TOP-containing mRNAs encode ribosomal proteins, elongation factors and other critical components of ribosome production. However, S6K1 activity is not obligatory for this important process: cells lacking S6K1 still actively translate TOP mRNAs in response to growth factor stimulation (Weber and Gutmann,

2012). Furthermore, S6K regulates both the initiation and elongation phases of translation. It phosphorylates and inactivates the eukaryotic elongation factor 2 kinase (eEF2K) resulting in the dephosphorylation of the eukaryotic elongation factor 2 (eEF2) which mediates the translocation step of elongation (Wang et al., 2001; Taha et al., 2013). eEF2K is also named as calcium/calmodulin (CaM)-dependent protein kinase III (CaMKIII) because it can be activated by elevated levels of calcium and binding of CaM. In dendrites, this process depends on glutamate signaling and NMDAR activation, which suggests a complex regulation of the elongation step, dependent on particular cellular conditions (Taha et al., 2013; Heise et al., 2014).

Besides, mTORC2 plays an important role in Akt activation. Akt itself possesses pleiotropic cellular effects, regulating events including metabolism, survival, and proliferation. In addition to Akt, mTORC2 also regulates the actin cytoskeleton through unknown mechanisms that involve protein kinase C alpha (PKCα) and Rho. Evidence also points to mTORC2 as a key player in mRNA translation processes that have been isolated in polysomes and associated with individual ribosomal proteins (Sarbassov et al., 2005; Weber and Gutmann, 2012). Inactivation of mTORC2, but not mTORC1, conditional knockout (CKO) mice disrupted motor coordination early in life. The Purkinje cells using of these mice showed developmental alterations in the processes of climbing fiber elimination and dendritic self-avoidance (Angliker et al., 2015). Little is known about the upstream activating signals for mTORC2. Moreover, most of the activity of this protein complex has been studied in nervous system cancer cells such as glioblastomas where mTORC2 plays a central role in the metabolic reprogramming of this cancer pathology (Masui et al., 2015). The activity of mTORC2 in transformed cells is not discussed in this review (**Figure 1**).

## mTOR AND EARLY CNS DEVELOPMENT

Early CNS development is a complex, dynamic process that relies on a precisely orchestrated interplay of inductive signals and cellular migration. Building this complex and highly organized system involves the generation of a wide variety of specialized neural and non-neural cell types that must be placed at appropriate locations and with the right timing (Budday et al., 2015; Jiang and Nardelli, 2015).

Neurons, oligodendrocytes, astrocytes, and the ependymal lining of the central lumen are generated from a common source, the neuroepithelial cells that are part of the neural tube in early embryos (Kintner, 2002). Later, cell migration and extensive synapse formation are responsible for the initial establishment of the neural circuitry in the brain (Purves, 2012; Bury and Sabo, 2015). The first step in this process is to generate both axons and dendrites in the newly generated neurons. Axons must grow to reach the appropriate target cells and begin to make the synaptic connections that will form neural circuits (Barnes and Polleux, 2009; Purves, 2012).

Clinical evidence from developmental disorders that affect the CNS, including Lhermitte–Duclos disease (LDD), TSC, and neurofibromatosis type 1 (NF1) suggest that mTOR plays a key role in cell differentiation and growth control (Sandsmark et al., 2007; Crino, 2011). These inherited neurodevelopmental disorders and cancer syndromes are caused by mutations in genes that encode negative regulators of the mTOR-signaling pathway. The continued expression of mTOR in TSC affected individuals leads to benign overgrowths in multiple organs including brain. Neurological alterations such as epilepsy, autism, and learning disabilities are frequent among them and the administration of mTOR inhibitors result beneficial (Tee et al., 2016). Also, patients with germinal mutations in phosphatase and tensin homolog (*PTEN*; a negative modulator of mTOR) have been observed in 11 out of 31 LDD patients and associated with granular cell hypertrophy (Abel et al., 2005). Cognitive impairment is the most common neurological impairment in NF1 children which is frequently associated with neuropathological abnormalities (Gipson and Johnston, 2012).

### Neuron Differentiation

During embryonic development, the brain undergoes a dramatic transformation from a simple tubular structure to a highly convoluted shape. Neurulation is the process of forming the neural tube, which will become the brain and spinal cord. Soon after the neural tube forms, the forerunners of the major brain regions become apparent as a result of morphogenetic movements. Eventually, the anterior and posterior regions of the neural tube become the brain and spinal cord, respectively. Morphogenesis of the neural tube occurs in a specific spatiotemporal pattern along the length of the embryo (Sadler, 2005; Purves, 2012; Filas et al., 2013).

Neural stem cells (NSCs) in the early neural plate and tube and subsequently within each nascent brain region must follow instructions to differentiate into nerve cells specific to each region; balanced control of neural progenitor maintenance and neuron production is crucial for the establishment of functional neural circuits (Purves, 2012). *Mtor* null mice die before the differentiation of cortical neural progenitors into neurons (Ka et al., 2014). Thus, conditional mutant mTOR mice were developed to study functions and mechanisms of mTOR in developing brain. Conditional mutant mTOR mice had smaller brains with lower numbers of cells in the proliferating progenitors and cortical layer neurons. According to these authors an abnormal cell cycle progression disrupted the population of neural progenitors and thereby the progenitor self-renewal process (Ka et al., 2014).

Similar disruptive effects were observed in mice with a knock-in mutation in the PH domain of 3-phosphoinositide dependent protein kinase-1 (PDK1) and chick embryos treated with PI3K and TOR inhibitors exhibit deficient neuron production. Furthermore, rapamycin treatment repressed the expression of early neuronal differentiation genes, such as neurogenin 2 (*Ngn2*). Exposure to rapamycin can also disrupt coordination of cell cycle exit and progression of the neuronal differentiation program and interfere with the neuroepithelial organization (Fishwick et al., 2010). Rapamycin treatment in the early days of development resulted in a decrease in the number of progenitor stem cells available in the dentate gyrus of the hippocampus,

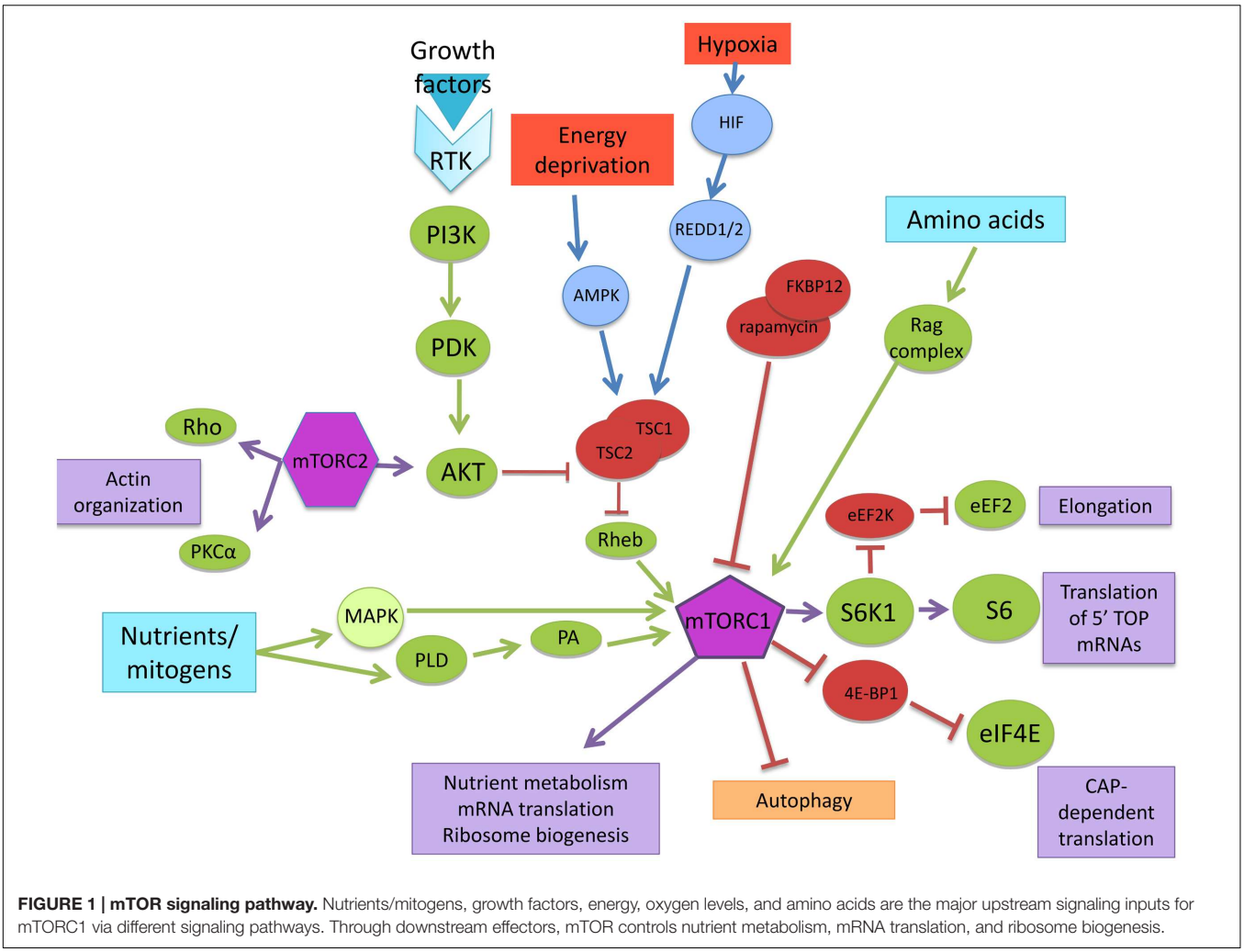


**FIGURE 1 | mTOR signaling pathway.** Nutrients/mitogens, growth factors, energy, oxygen levels, and amino acids are the major upstream signaling inputs for mTORC1 via different signaling pathways. Through downstream effectors, mTOR controls nutrient metabolism, mRNA translation, and ribosome biogenesis.

as well as an impairment in the development of this region (Raman et al., 2013). Thus, TOR signaling levels may function as a checkpoint for the progression of differentiation, and appears to be essential for the production of NSCs and a proper development of the dentate gyrus of the hippocampus.

Decreasing mTORC1 activity with Rheb and RAPTOR knockdowns in neonatal NSCs in the subventricular zone (SVZ) prevented their differentiation resulting in reduced lineage expansion and aborted neuron production (Hartman et al., 2013). Also, the constitutive activation of the translational repressor 4E-BP1 had similar effects: it prevented hyperactive mTORC1 from inducing NSC differentiation and promoted self-renewal. By contrast, mTORC1-activated S6K1/S6K2 regulated NSC size but not differentiation, indicating different functions of 4E-BPs and S6K1/S6K2 in NSCs. These data demonstrated a crucial role of mTORC1 and 4E-BP for switching cap-dependent translation on and off in NSCs (Hartman et al., 2013).

On the other hand, Fu et al. (2012) generated CKO mice with selective deletion of the *Tsc1* gene in GABAergic interneuron progenitor cells, which have impaired growth and decreased survival. In this case, the cortical and hippocampal GABAergic interneurons of CKO mice were enlarged and showed increased mTORC1 signaling. Ectopic clusters of cells with increased mTORC1 signaling are also observed, suggesting impaired interneuron migration. Increased mTORC1 signaling may have negative effects on the CNS development, so regulatory proteins, such as TSC1, play important roles in keeping mTOR in check (Fu et al., 2012). In neuronal cultures from rat cortices, a triiodothyronine (T3)-dependent increase in the size of the soma in GABAergic neurons was mediated mainly by mTOR signaling. Both TrkB and mTOR signaling mediate the T3-dependent reduction of GABAergic axon extension, suggesting the existence of parallel regulatory pathways for T3-dependent changes (Westerholz et al., 2013).

When cortical cells from embryonic stages E13.5 and E16.5 were treated with transforming growth factor β (TGFβ) and insulin growth factor 1 (IGF1), IGF1 induced PI3K, Akt and mTORC1/mTORC2 primarily in E13.5-derived cells, resulting in proliferation, survival, and neuronal differentiation. At E16.5, TGFβ did not directly activate the Akt-mTOR pathway but required active PI3K-mTORC2-signaling to mediate the neuronal differentiation of cortical cells (Wahane et al., 2014).

The data presented in these studies suggest that mTOR plays an important role in the development of neuronal progenitors, controlling proliferation and differentiation in various regions of the CNS. It seems that mTOR may function as a checkpoint for the differentiation progression, while its hyperactivation does not inhibit but can affect interneuron migration and neuron differentiation.

## Axon and Dendrite Development

Once nerve cells have been generated, neurons must become interconnected to form the neural circuits. The first step in this process is to establish axons and dendrites in the newly generated neurons. Differentiation of axons and dendrites is a critical step in neuronal development (Cheng and Poo, 2012; Purves, 2012). Neurons typically form a single axon and multiple dendrites, which underlie the directional flow of information transfer in the CNS. Axons grow to reach appropriate target cells and begin to make the synaptic connections that will form neural circuits. Dendrites integrate synaptic inputs, triggering the generation of action potentials, making presynaptic contacts onto target cells. There are multiple signaling pathways underlying the establishment of axon-dendrite development (Barnes and Polleux, 2009; Purves, 2012). As in neuron differentiation, there are several studies that show that mTOR plays a pivotal role in both dendritic and axonal growth.

The pharmacological stimulation of the serotonin receptor subtype 7 (5-$HT_7$R) using a highly selective agonist, LP-211, enhances neurite outgrowth in primary neuronal cultures derived from embryonic mouse brain, via ERK, cyclin-dependent kinase 5 (Cdk5), mTOR, and cell division control protein 42 homolog (Cdc42). However, the neurite elongation induced by the agonist stimulation of 5-$HT_7$R is dependent on mTOR, because the outgrowth is completely inhibited by both rapamycin and torin 1, a specific ATP-competitive mTOR inhibitor (Speranza et al., 2015).

Methylcobalamin (MeCbl), a vitamin B12 analog, increases mTOR activity via the activation of Akt and promotes neurite outgrowth in cerebellar granule neurons while rapamycin decreased the effect of MeCbl on neurite outgrowth (Okada et al., 2011).

In a shRNA-induced selective knockdown of RAPTOR and RICTOR, it was shown that both mTORCs are needed for the presence of proper dendritic morphology of neurons, whether grown under basal culture conditions and treated with insulin or transfected with constitutively active PI3K. Neurons with knockdown of either RAPTOR or RICTOR produced fewer new dendrites, whereas the number of retracted dendrites remained relatively stable (Urbanska et al., 2012).

However, different outcomes have been reported with respect to the positive role of mTOR in neurite elongation and growth. The overexpression of the mammalian nicotinamide-adenine dinucleotide-dependent deacetylase sirtuin 1 (Sirt1) promoted neurite outgrowth and improved cell viability under both normal and stress conditions, such as nutrient deprivation or neurotoxic insult, in primary culture systems. Enhanced Sirt1 expression in neurons downregulated the protein levels and phosphorylation of mTOR. Correspondingly, rapamycin markedly improved neuronal cell survival in response to nutrient deprivation and significantly enhanced neurite outgrowth in wild-type mouse neurons. Sirt1 is activated in low energy availability promoting tissue repair processes, while mTOR is activated during high energy conditions (Garcia-Rodriguez et al., 2014). In this case, the above results suggest that mTOR could have negative effects on CNS development, so regulatory proteins such as Sirt1 play important roles in keeping mTOR in check (Guo et al., 2011).

Regarding axon and dendrite development, the studies presented here show that mTOR participates in the processes of neurite production, promoting outgrowth and elongation, as well as in maintaining proper dendritic morphology. On the other hand, Guo et al. (2011) showed that rapamycin also enhances neurite outgrowth. These apparently contradictory results could be interpreted in terms of spatiotemporal participation of mTOR in these processes, which is extremely tightly regulated. During embryonic development, the temporary silencing of some proteins has been reported as a switch between stages (Hirabayashi and Gotoh, 2010; Golbabapour et al., 2013). However, we must consider that most of these studies used *in vitro* models where cell type and culture conditions could greatly influence the results mainly in key regulators of cell metabolism such as Sirt1 and mTOR. Further *in vivo* studies are necessary to be able to confirm the role of mTOR in axon and dendrite development.

## Gliogenesis

Glia constitute the majority of the brain cells, they perform key functions vital to CNS physiology, including blood–brain barrier formation and maintenance, synaptogenesis, neurotransmission, and metabolic regulation. Glial cell generation starts late in embryonic stage. Regulation of neuron to glia switch involves complex neuron–glial interactions as well as spatiotemporal interplay of both cell-intrinsic factors and extracellular signals (Jiang and Nardelli, 2015; Molofsky and Deneen, 2015).

mTORC1 signaling pathway has a crucial function in the process of astrocyte differentiation (Lee da, 2015). Evidence from different reports show that the axon inhibitor Nogo-66 promotes the NSCs differentiation into the glial lineage via mTOR and the signal transducer and activator of transcription 3 (STAT3; Rajan et al., 2003; Wang et al., 2008; Cloetta et al., 2013). Similar to mTORC1, in NF1 genetically engineered mice and derivative NSC neurosphere cultures, was shown that hyperactivation of RICTOR containing mTORC2 activation also increases gliogenesis in the brainstem (Lee da et al., 2010).

Oligodendrocytes are the glial cells that generate CNS myelin; myelin is a multilamellar differentiation of the oligodendrocyte plasma membrane that sheaths axons to facilitate electrical conduction. Progression through the oligodendrocyte lineage is tightly regulated by a multitude of intrinsic and extrinsic cues, which control myelination both spatially and temporally during development and after demyelination. These signals include growth factors, protein kinases, and extracellular matrix molecules, all of which influence epigenetic modifications, transcriptional and translational regulation, and the actin cytoskeleton in oligodendrocytes (Zuchero and Barres, 2013; Bercury and Macklin, 2015).

Both *in vivo* and *in vitro* studies have demonstrated that the mTOR pathway is an essential mediator during oligodendrocyte development and that it can be associated to several external growth factors or hormones with known roles in oligodendroglia differentiation and myelination (Wood et al., 2013). For example, conditionally ablation of either RAPTOR or RICTOR in the oligodendrocyte showed that RAPTOR is a positive regulator myelination whereas RICTOR ablation has a modest positive effect on oligodendrocyte differentiation, and very little effect on myelination (Bercury et al., 2014; Bercury and Macklin, 2015). Purified oligodendrocyte progenitor cells (OPCs) treated with CB receptor agonists and antagonists, as well as with PI3K-Akt and mTOR inhibitors. Maximal phosphorylation of mTOR was obtained after 10 min stimulation with the agonist HU210, and this level of phosphorylation was sustained for 60 min. In contrast, incubation with the other agonists ACEA or JWH133 provoked transient mTOR phosphorylation that peaked at 2 min and then fell below the basal level (Gómez et al., 2011).

The roles of Rheb1 and mTORC1 in the mouse oligodendrocyte lineage were examined using separate *Cre* drivers to generate *Rheb1* or *mTor* CKO animals for OPCs as well as for differentiated and mature oligodendrocytes. Deletion of *Rheb1* in OPCs impairs the differentiation to mature oligodendrocytes, which involves mTORC1. This was accompanied by a reduction in OPCs exiting the cell cycle, similarly as during neuron differentiation (Zou et al., 2014).

mTOR is downstream of known activators of PI3K-Akt signaling and upstream of a number of targets important for regulating many aspects of oligodendrocyte differentiation and myelination, including nuclear transcriptional regulators, mediators of cytoskeletal organization and enzymes necessary for lipogenesis (Wood et al., 2013). The finding that both Rheb1 and mTORC1 are essential for early stage differentiation of OPCs to mature oligodendrocytes, which occurs during a narrow time window, reinforced its importance. Additionally, it seems that the endocannabinoids may be the extracellular signals that activate Akt and mTOR during oligodendrocyte differentiation; the activation of mTOR depended on the agonist used and the CB receptors activation.

## mTOR IN THE ADULT CNS

As we mentioned in the previous section, during early postnatal life, the neural circuits process diverse types of information and build the main neural circuits. The sensory systems process information about the state of the organism and the environment, and the motor systems organize and generate actions, and associational systems link the sensory and motor components, providing the substrate for higher brain functions such as perception, attention, cognition, emotions, language, rational thinking, and consciousness (Purves, 2012).

Neurogenesis persists in adult mammals in specific brain areas known as neurogenic niches. Either physiological or pathological conditions can modulate the rate of proliferation of adult NSCs via the differentiation and fate determination of progenitor cells, as well as the survival, maturation, and integration of newborn neurons. Adult neurogenic niches can be conceptualized as remnants of embryonic signaling centers: they are the source of instructive signals that determine the fate of neighboring stem cells. Furthermore, these cells may be required for some learning and memory processes (Zhao et al., 2008; Urban and Guillemot, 2014).

### Learning, Memory, and Synaptic Plasticity

Learning-related changes include modulation of synaptic and non-synaptic ion channels and receptors, dendritic branching, spine density, and plasticity through genetic and epigenetic mechanisms (Sehgal et al., 2013; Stuchlik, 2014). Synaptic connectivity between neurons is a dynamic entity that is constantly changing in response to neural activity and other influences. At the shortest time scales, facilitation, augmentation, potentiation, and depression provide rapid but transient modifications in synaptic transmission (Klann and Dever, 2004; Purves, 2012; Bailey et al., 2015).

The biological processes mediating memory formation involve numerous tightly regulated molecular and cellular events. These include mRNA transcription, protein synthesis, mRNA and protein degradation, mRNA and protein trafficking, post-translational modifications such as phosphorylation and ubiquitination, and epigenetic modulation. Such processes can be brain hemisphere- and brain subregion-specific (Kandel, 2001; Middei et al., 2014; Rosenberg et al., 2014).

Long-lasting synaptic plasticity and memory rely on protein synthesis. Not surprisingly, TOR, one of the major controllers of translation, has been involved in important processes such as synaptic plasticity, memory and diverse types of behavior in various organisms, including mammals (Swiech et al., 2008).

As in early CNS development, mTOR also participates in processes such as synaptic plasticity, adult neurogenesis, learning, and memory. Its role depends on the regions of the CNS studied and the stimuli used. Here we describe the participation of mTOR in signaling pathways in the hippocampus, striatum, amygdala, medial prefrontal and auditory cortices.

#### Hippocampus

The hippocampus is essential for memory formation; in addition, its neuronal connections are highly ordered, and it is easy to identify specific populations of neurons and synapses. Here, we reviewed studies that suggest that mTOR participates in hippocampal neurogenesis, neurite growth and survival, and dendritic spine formation.

We have mentioned that Akt-mTOR participates in dendritic development. This signaling pathway was identified as an unexpected target of the gene disrupted-in-schizophrenia 1 (DISC1) regulating morphogenesis and dendritic development in new neurons in the adult mouse hippocampus (Kim et al., 2009). There is also evidence that insulin receptor (IR) regulates dendritic spine formation and excitatory synapse development in primary cultures of rat hippocampal neurons through the activation of the PI3K-Akt-mTOR signaling pathway, which in turn promotes Rac1-dependent actin cytoskeletal rearrangement and dendritic spine formation (Lee et al., 2011). Moreover,

Ezh2 (methyltransferase of histone H3K27, enhancer of zeste homolog 2) regulates adult neurogenesis and preserves cognitive functions via the Akt-mTOR pathway (Zhang et al., 2014). The methyltransferase suppresses PTEN expression, promoting the activation of Akt-mTOR. The deletion of *Ezh2* in progenitor cells leads to a decrease in neuron production *in vivo*; more importantly, *Ezh2*-null mice showed impairments in spatial learning and memory, contextual fear memory, and pattern separation.

Similarly, the administration of ABG001 (tetradecyl 2,3-dihydroxybenzoate), a neuritogenic substance, enhanced the survival and neurite growth of newborn cells in adult hippocampal via TrkA receptor-triggered ERK and PI3K-Akt-mTOR signaling pathways, that leads to an enhanced preferential spatial cognitive function. The treatment of amyloid beta$_{25-35}$ (A$\beta_{25-35}$)-mice with ABG001 protected the survival and neurite growth of newborn cells by increasing TrkA receptor-induced phosphorylation of Akt and mTOR, which was accompanied by improved spatial cognitive performance (Zhou et al., 2014).

In cultured hippocampal neurons, mTORC1 activity tags synapses, allowing the RNA-stabilizing protein HuD to capture the $Ca^{2+}$ calmodulin-dependent protein kinase II α (CaMKIIα) in a branch-specific manner, promoting site-specific and long-lasting forms of plasticity in the tagged branch. Thus, mTORC1 and HuD are good candidates to target the mRNAs coding for proteins required to strengthen neighboring synapses to facilitate late-stage plasticity (Sosanya et al., 2015).

Most importantly, Su et al. (2015) showed that the activation of mTOR pathway components was lower in the hippocampus of premature rats relative to full-term rats, and this was associated with poorer learning and memory performance. They showed that long-term consumption of a protein-rich diet can restore the impairment in learning and memory in pre-term rats via upregulation of mTOR-S6K1 signaling. In addition, mTOR activation and phosphorylation of S6K1 and 4E-BP1 in rat hippocampus was observed during a spatial learning paradigm. When mTORC1 was inhibited by chronic intracerebral ventricular infusion of rapamycin the phosphorylation of mTOR substrates were also inhibit as well as the learning-induced enhancement of protein synthesis and the acquisition of learning. These results show the activation of mTOR and its downstream targets during spatial learning in hippocampal pyramidal neurons (Qi et al., 2010).

There are several recent studies addressing the participation of mTOR and hormones in memory consolidation and learning. First, three different studies showed that the sex hormones estrogen and progesterone induced memory consolidation in the hippocampus. Estradiol induced the enhancement of object-recognition memory consolidation via PI3K-mTOR activation in the dorsal hippocampus (Fortress et al., 2013). Estradiol also modulated hippocampal synaptic plasticity by activating mTOR through a signaling pathway that involved TrkB activation, ERK phosphorylation, and calpain activation (Briz and Baudry, 2014). In young ovariectomized mice, bilateral dorsal hippocampal infusion of progesterone significantly increased the levels of ERK and S6K1. This activation was essential for the progesterone-induced facilitation of memory consolidation (Orr et al., 2012). These studies showed that rapid protein synthesis is necessary for these hormones to modulate the consolidation of hippocampal LTP and memory.

Also, the neurotrophin brain-derived neurotrophic factor (BDNF) is an upstream activator of mTOR during and after training to regulate GluR1 translation in hippocampal synaptic plasma membranes during IA (inhibitory avoidance) training memory consolidation (Slipczuk et al., 2009).

The markers for initiation of translation, including eukaryotic translation initiation factor 4E (eIF4E), 4E-BP1, ribosomal protein S6, and eIF4F cap-complex formation, undergo diurnal oscillations in the mouse hippocampus. This diurnal oscillation in translation initiation has been associated with increased activity of ERK1/2 MAPK and mTOR, and it was lost in memory-deficient mice lacking calmodulin-stimulated adenylyl cyclases 1 and 8. Disruption of circadian rhythms leads to loss of diurnal translation oscillation and causes memory deficits; moreover, inhibition of protein synthesis during the midday for 4 days post-training impairs memory persistence (Saraf et al., 2014).

The stimulation of the cannabinoid receptor CB1R by endogenous and exogenous cannabinoids can trigger the activation of the mTOR pathway and protein synthesis in the hippocampus. Contrary to what has been observed in other cases, this activation causes long-term memory impairment and amnesic-like effects, probably due to the fact that CB1R is mainly expressed in GABAergic interneurons. These findings suggest that the activation of mTOR through CB1R would contribute to an imbalance between the excitatory and inhibitory inputs in the hippocampus (Puighermanal et al., 2009).

Moreover, disruption of DISC1 function in adult-born dentate granule neurons is sufficient to cause several profound behavioral phenotypes, including pronounced learning and memory deficits, as well as clear anxiety and depression-like phenotypes. The knockdown of *Disc1* leads to increases in mTOR, indicating that this signaling abnormality is responsible for the cognitive and affective deficits. Rapamycin reversed these behavioral deficits even when associated neuroanatomical abnormalities persisted; remarkably, the rapamycin treatment, which rescued memory deficits in shRNA-DISC1 mice, caused memory deficits in control mice (Zhou et al., 2013).

Most studies also showed that mTOR is involved in both memory and learning consolidation processes, as well in the responses of various behavior to hormones, BDNF, and circadian changes. Furthermore, long-term memory deficits can be associated with an overactivation of the mTOR signaling pathway and an imbalance in protein synthesis, suggesting that mTOR requires mechanisms for tight spatiotemporal control of its expression.

## Striatum

For motor control and learning, the basal ganglia, the motor cortex, and the cerebellum cooperate to enable movement; the striatum, part of the basal ganglia, plays a significant role in the learning processes of motor skilled tasks. Bergeron et al. (2014) analyzed whether mTOR activity is influenced or engaged during the execution of motor movement and motor learning. They showed that mTOR activity of the dorsal striatum is an important

molecular step involved in learning consolidation during the acquisition of a complex motor skill in mice, but it is not related to motor abilities. These data are consistent with the studies in the hippocampus showing the role of mTOR in memory and learning consolidation.

Mice with disrupted mTORC2 signaling in the striatum exhibit altered striatal dopamine-dependent behaviors, such as increased basal locomotion, stereotypic counts, etc., by altering the D2R and ERK1/2 pathway (Dadalko et al., 2015).

### Amygdala

The amygdala mediates neural processes that relate sensory experience with emotional significance, and it is also the site where learning about fearful stimuli occurs. It was found that rapamycin increases neuronal activity and anxiety-related behavior, impairs both consolidation and reconsolidation of an auditory fear memory, and produces impairment of IA memory. Given the importance of the amygdala in mood regulation, associative learning, and modulation of cognitive functions, it is important to consider the role of mTOR in this region; rapamycin can be used as a treatment for reducing the emotional strength of established traumatic memories analogous to those observed in acquired anxiety disorders, but it may also induce alterations in mood regulation.

In three different studies, Jobim et al. (2012a,b) and Pedroso et al. (2013) showed that infusion of rapamycin into the basolateral complex of the amygdala and hippocampus before and after a reactivation (retrieval) session produced IA memory impairment. These data showed that non-reinforced fear memory retrieval could lead to memory reconsolidation through a mechanism that requires protein synthesis and mTOR signaling in the amygdala and hippocampus.

The systemic administration of a single low dose of rapamycin led to enhanced neuronal activity in the amygdala and an increase in anxiety-related behaviors. The behavioral alterations correlated to enhanced amygdalar expression of KLK8 and FKBP51, proteins that have been implicated in the development of anxiety and depression (Hadamitzky et al., 2014). Moreover, systemic inhibition by rapamycin administration immediately or 12 h after either training or reactivation for auditory fear conditioning, blocks both consolidation and reconsolidation-like activities that contribute to the formation, retention, and maintenance of long-term memory. These data suggest that biphasic translational control through the mTOR pathway is normally required during the long-term formation and stabilization of memory through recurrent consolidation and reconsolidation-like events (Mac Callum et al., 2014).

### Medial Prefrontal Cortex

The amygdala is connected with the medial prefrontal cortex (mPFC), a brain region that is involved in decision making, task switching, memory consolidation, and the retrieval of remote long-term memory. It was shown that the PI3K-Akt-mTOR signaling pathway is involved in the LTP and mPFC-dependent long-term trace fear memory (Sui et al., 2008). Another study in the mPFC demonstrated that overexpression of S6K1 produces antidepressant effects in the forced swim test without altering locomotor activity. Conversely, expression of dominant-negative S6K in the mPFC resulted in prodepressive behavior in the forced swim test and was sufficient to cause anhedonia in the absence of chronic stress exposure. These data demonstrate a critical role for S6K1 activity in depressive behaviors and suggest that pathways downstream of mTORC1 may underlie the pathophysiology and treatment of major depressive disorder (Dwyer et al., 2015).

These results suggest that fear regulation is mediated by connections from the mPFC to the amygdala, and it seems that mTOR participates in this relationship; in the mPFC, the animals learn to predict aversive events via mTOR, and in the amygdala, the memory is retained. However, mTOR also participates in avoiding anxiety and depression.

### Auditory Cortex

The auditory cortex of mammals mediates particular aspects of auditory stimulus processing, task-specific performance, and learning; one report (Schicknick et al., 2008) showed that the memory required for the discrimination of complex sensory stimuli is controlled by dopaminergic activity via mTOR.

In the same way, the dopaminergic inputs to the gerbil auditory cortex regulate mTOR-mediated, protein-synthesis-dependent mechanisms, thereby controlling the consolidation of memory required for the discrimination of complex auditory stimuli (Schicknick et al., 2008). In a more recent work of this group, Reichenbach et al. (2015) tested the impact of local pharmacological activation of different D1/D5 dopamine receptor signaling modes in the auditory cortex and found differentially regulation of several protein profiles related to rearrangement of cytomatrices, energy metabolism, and synaptic neurotransmission in cortical, hippocampal, and basal brain structures. These results may be mTOR-mediated, which, in turn, might enhance the ability to synthesize plasticity-related proteins locally on demand and facilitate the consolidation of discrimination memory (Reichenbach et al., 2015).

## mTOR AND CNS AGING

During normal aging, the brain suffers both morphological and functional modifications that affect dendritic trees and synapses, neurotransmission, circulation, and metabolism; these changes promote neurodegeneration, impair neurogenesis, and can be considered a cause of cognitive impairment and sensory and motor deficits in the elderly (Mariani et al., 2005; Sarlak et al., 2013). Mounting evidence, however, appears to implicate increased susceptibility to the long-term effects of oxidative stress, mitochondrial dysfunction and inflammatory insults as major contributing factors (Mariani et al., 2005). Understanding the mechanisms and the detailed metabolic interactions involved in the processes of normal and pathological neuronal aging and thus improving health is critical to increasing the quality of life in the elderly population, especially given the dramatic increase in the aging population worldwide (Toescu, 2005; Yang et al., 2014).

In recent years, the manipulation of nutrient-sensing and stress-response pathways has extended the lifespans of organisms from yeast to mammals. Growth-promoting cell programs may

accelerate aging by generating metabolic by-products and by directly inhibiting the clearance of these by-products (Zoncu et al., 2011). mTOR is a prime target in the genetic control of aging, and evidence from genetic studies supports the view that mTOR may be a master determinant of lifespan and aging in yeast (Kaeberlein et al., 2005), worms (Vellai et al., 2003), flies (Bjedov et al., 2010), and mice (Harrison et al., 2009).

There is compelling evidence that cellular mechanisms and signaling pathways regulating brain aging and age-related neurodegenerative disorders are at least partially controlled by mTOR. The complex signaling networks underlying the age-related effects have not been fully elucidated. There are multiple reviews discussing the relationship between mTOR and CNS aging (Maiese et al., 2012; Sarlak et al., 2013; Jenwitheesuk et al., 2014; Perluigi et al., 2015); here, we describe recent studies on this topic.

One of the mechanisms that relate mTOR to aging is its participation in inhibiting autophagy. Autophagy is essential for removing damaged macromolecules and organelles from the cytoplasm and recycling amino acids (Mizushima et al., 2008). Studies suggest that autophagy declines with age and that this leads to an accumulation of damage, such as protein aggregates and degenerated mitochondria, that contribute to age-related cellular dysfunction (Cuervo, 2008).

A recent study using naked mole rats which live for up to 24 years, shows that they maintain high brain autophagy levels during the majority of their lifespan. Additionally, the p-mTOR/mTOR ratio showed a significant increase from the early to intermediate age group and a significant decrease from the intermediate age group to the old and oldest age groups (Triplett et al., 2015).

In mouse retinal pigment epithelium (RPE) explants and cultured human RPE cells, aged RPE cells contained more lysosome-associated mTOR and showed an increased response to amino acid stimulation. Increased mTORC1 activity caused a decreased rate of degradation of internalized photoreceptor outer segments. These data suggest that the Rag–Ragulator complex controls the lysosomal distribution of mTORC1 in RPE cells and may further exacerbate the lysosomal dysfunction of aged RPE (Yu et al., 2014).

The use of caloric restriction (CR) as a strategy to study mechanisms behind aging and age-associated diseases is based on evidence suggesting that CR can delay aging and protect the CNS from neurodegenerative disorders. Yang et al. (2014) used C57BL/6J mice with different diets and found that cognitive function declined with aging, especially after 12 months of age while CR ameliorated the age-dependent cognition deficit by deactivating mTOR and its upstream BDNF-Akt signaling in the hippocampus. Unexpectedly, they found a decline in mTOR signaling with aging, suggesting that other mediators play a more important role in regulating age-dependent autophagy than mTOR by itself in this model.

Dong and colleagues published two other studies using the CR strategy. C57BL/6 mice were randomly assigned to a NC group (standard diet), a CR group or a HC group (high-calorie diet) for 10 months. Activation of the mTOR/S6K1 and p62 signaling pathways was significantly upregulated in hippocampal neurons of mice in the HC and NC groups and ameliorated by the CR treatment. They also found that the HC diet and CR have opposing effects on learning and memory related to age. Different caloric intake may be an important way to accelerate or slow the progression of age-related neurodegenerative disorders (Dong et al., 2015a,b).

Furthermore, when the epigenetic changes in old mice with dietary restriction (DR) were analyzed, the histone methylation levels of H3K27me3, H3R2me2, and H3K79me3 in 22-month-old mouse brain were lower than in 3-month-old animals. However, either DR or rapamycin treatment prevented the age-induced losses of H3K27me3, H3R2me2, and H3K79me3. Moreover, DR and rapamycin each enhanced the levels of H3K18ac and H3K4me2. The level of H3K4me declined with age and was further diminished by either DR or rapamycin. The results from this study suggest that either DR or rapamycin can restore, at least partially, the age-related alterations in histone methylation levels (Gong et al., 2015).

Mild mitochondrial uncoupling is similar to the physiological energetic challenges, including exercise and intermittent fasting, that seem to protect the CNS from the effects of aging. The induction of mild mitochondrial uncoupling using 2,4-dinitrophenol (DNP) triggers a complex integrated cellular response in the brain that includes suppression of mTOR and insulin signaling, enhanced autophagy, and upregulation of cAMP response element-binding (CREB), changes that are known to play important roles in synaptic plasticity. Moreover, DNP treatment improves the performance of mice in a learning and memory task (Liu et al., 2015).

In terms of cognitive function and memory in the elderly, genome-wide screening showed a novel association of a polymorphism in the pro-apoptotic gene *FASTKD2* (fas-activated serine/threonine kinase domains 2) with better memory performance in older adults. Complementary analyses at the gene and pathway levels identified additional genome-wide significant associations with episodic memory and the genes *LARS2* (leucyl-tRNA synthetase 2, mitochondrial) and *mTor*, which along with *FASTKD240*, encode proteins with critical roles in mitochondrial function. These new findings identify potential targets to help improve risk stratification and therapeutic development in normal cognitive aging and dementia (Ramanan et al., 2015).

Adult neurogenesis is another process that decreases with age. This is mainly because of the reduction in the proliferation of active NSCs. As mice age, the activity of the mTOR signaling pathway decreases in the NSCs. Short-term treatment with ketamine significantly restored the proliferation of NSCs in the aged mice via mTOR signaling activity. Stimulating mTOR signaling revitalized the NSCs, restored their proliferation, and enhanced neurogenesis in the hippocampus of the aged brain. These data show the potential of mTOR for restoring neurogenesis in elderly individuals, but it is important to be aware that overstimulation of mTOR may also lead to other unwanted effects, such as stem cell depletion, aging, and carcinogenesis (Romine et al., 2015).

Also Paliouras et al. (2012) showed that mTOR is pivotal in determining proliferation versus quiescence in the adult

forebrain niche. mTOR inhibition yielded a quiescence-like phenotype *in vitro*, while epidermal growth factor (EGF)-induced upregulation of mTOR activity enabled the reactivation of the quiescent SVZ niche within the aging brain. These findings reveal that the mTOR signaling pathway is a key regulator of neurogenesis in the adult and aging brain.

Finally, the deficiency of the core clock protein BMAL1 increased mTORC1 activity. A significant increase in

**TABLE 1 | mTOR role and signaling components in the different stages of the CNS.**

| Stage | Processes | Identified signaling pathway components | Reference |
|---|---|---|---|
| **Early CNS development** | | | |
| | Check point for the progression of differentiation | *Ngn2*, *Pax6* | Fishwick et al., 2010 |
| | NSCs size and switching cap-dependent translation on and off | S6K1/2, 4E-BPs | Hartman et al., 2013 |
| | Neural progenitor development | GSK3, Sox2 | Ka et al., 2014 |
| | Soma size increase and axon extension | T3, BDNF, TrkB | Westerholz et al., 2013 |
| | Impaired growth and interneuron migration and decreased survival | *Tsc1* | Fu et al., 2012 |
| | Proliferation, survival, and neuronal differentiation | IGF1, TGFβ | Wahane et al. (2014) |
| | Neurite outgrowth | 5HT7R, Erk, Cdk5, Cdc42<br>MeCbl<br>Insulin, RAPTOR, RICTOR | Speranza et al., 2015<br>Okada et al., 2011<br>Urbanska et al., 2012 |
| | Impaired neurite outgrowth and cell viability | Sirt1 | Guo et al., 2011 |
| | Oligodendrocyte development | Cannabinoid receptors<br>Myelinating proteins: MBP, Tmem10, MOG, PLP | Gómez et al., 2011<br>Zou et al., 2014 |
| **Adult CNS** | | | |
| • Hippocampus | Neurogenesis | Ezh2 | Zhang et al., 2014 |
| | Dendritic spine formation | IR, Rac<br>*Disc1* | Lee et al., 2011<br>Kim et al., 2009 |
| | Late-stage plasticity | HuD, CaMKIIα | Sosanya et al., 2015 |
| | Enhanced spatial cognitive function | TrkA, ERK | Zhou et al., 2014 |
| | Learning and memory performance | S6K1, 4E-BP1 | Qi et al., 2010; Su et al., 2015 |
| | Memory consolidation | Estrogen and progesterone, TrkB, ERK calpain BDNF, GluR1 | Fortress et al., 2013; Briz and Baudry, 2014; Orr et al., 2012; Slipczuk et al., 2009 |
| | Long-term memory, protein synthesis | Circadian oscillations eIF4E, 4E-BP1, S6, ERK1/2<br>CB1R | Saraf et al., 2014<br>Puighermanal et al., 2009 |
| | Learning and memory deficits | DISC1 | Zhou et al., 2013 |
| • Striatum | Motor learning consolidation | S6K1, 4E-BP | Bergeron et al., 2014 |
| • Amygdala | Memory consolidation of fear memory | KLK8, FKBP51 | Jobim et al., 2012a,b; Pedroso et al., 2013<br>Hadamitzky et al., 2014<br>Mac Callum et al., 2014 |
| • Medial prefrontal cortex | Trace fear memory Antidepressant effects | S6K1 | Sui et al., 2008<br>Dwyer et al., 2015 |
| • Auditory cortex | Memory consolidation for the discrimination of auditory stimuli | Dopaminergic activity | Schicknick et al., 2008 |
| **CNS aging** | | | |
| | Increase lifespan | Decreased p-mTOR/mTOR | Triplett et al., 2015 |
| | Decreased rate of degradation | Rag-Ragulator complex | Yu et al., 2014 |
| | Decreased cognitive function | BDNF, Akt | Yang et al., 2014 |
| | Learning and memory dysfunction | p62, S6K1 | Dong et al., 2015a,b |
| | Alterations in histone methylation levels | H3K27me3, H3R2me2, H3K79me3 H3K4me2 | Gong et al., 2015 |
| | Enhanced autophagy, synaptic plasticity | CREB, decreased mTOR | Liu et al., 2015 |
| | Better memory performance | *LARS2*, *FASTKD40* | Ramanan et al., 2015 |
| | Adult neurogenesis | S6, EGF | Romine et al., 2015<br>Paliouras et al., 2012 |
| | Circadian control of aging | BMAL1 | Khapre et al., 2014 |

phosphorylation of downstream targets of mTORC1 was observed at several time points in different tissues of *Bmal1−/−* mice. Treatment with rapamycin increased the lifespan of *Bmal1−/−* mice. The authors suggest that the circadian clock controls the activity of the mTOR pathway through BMAL1-dependent mechanisms and that this regulation is important for the control of aging and metabolism (Khapre et al., 2014).

The role of mTOR during aging seems to participate in more mechanisms than merely autophagy, and it will be challenging to untangle the role of mTOR in all these processes. The findings also depend on the model and the approach used. mTOR increases with age and inhibits autophagy, an important process in degradation of cellular debris, as well as impairing cognitive functions, and these effects can be ameliorated with a CR diet or with mTOR inhibitors such as rapamycin; in fact, rapamycin has been approved clinically for a variety of uses, including as an immunosuppressant and as an anticancer drug. Yang et al. (2014) report evidence of a decline in mTOR with aging, suggesting that there could be other longevity factors that can also regulate basal autophagy in the mouse hippocampus during normal brain aging.

mTOR is also regulated by the circadian rhythm and by mitochondrial uncoupling and is related to a polymorphism associated with better memory performance in humans. mTOR regulates processes such as epigenetic changes (histone methylation levels), neurogenesis and proliferation in various niches in the aging brain. Therefore, inhibiting mTOR to increase the lifespan should be undertaken with caution because this protein participates in pathways that are essential for health, and it is likely that alterations in mTOR activity could have negative effects throughout the entire system (**Table 1**).

## CONCLUDING REMARKS

In early life mTOR participates in neurogenesis, in neurite outgrowth and elongation and finally in gliogenesis. All of these processes seem to be regulated by mTOR via translation, and it likely functions as a cell-cycle checkpoint. There is need of more *in vivo* studies that show the timing, region and cell specificity of mTOR activation/inhibition. It is also important to identified the signaling pathways and effectors that specifically participate in each these process.

In the adult brain, mTOR participates in key processes such as synaptic plasticity, adult neurogenesis, and learning and memory. Its role depends on many factors, and in some cases, it seems to have opposite actions. We also need to understand which mechanisms spatiotemporally modulate or balance mTOR expression and how their disruption is associated with neurodegenerative diseases. Addressing these gaps would help to generate therapeutic strategies.

Finally, during aging, mTOR seems to increase neurogenesis, decrease autophagy, and regulate epigenetic changes.

This kinase is an important energy sensor that is present throughout our lifespan. Several stimuli and transduction pathways tightly modulate its expression. Its role must be precisely described in order to develop therapeutic strategies and prevent CNS diseases.

## AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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## REFERENCES

Abel, T. W., Baker, S. J., Fraser, M. M., Tihan, T., Nelson, J. S., Yachnis, A. T., et al. (2005). Lhermitte-Duclos disease: a report of 31 cases with immunohistochemical analysis of the PTEN/AKT/mTOR pathway. *J. Neuropathol. Exp. Neurol.* 64, 341–349. doi: 10.1093/jnen/64.4.341

Angliker, N., Burri, M., Zaichuk, M., Fritschy, J. M., and Ruegg, M. A. (2015). mTORC1 and mTORC2 have largely distinct functions in Purkinje cells. *Eur. J. Neurosci.* 42, 2595–2612. doi: 10.1111/ejn.13051

Bailey, C. H., Kandel, E. R., and Harris, K. M. (2015). Structural components of synaptic plasticity and memory consolidation. *Cold Spring Harb. Perspect. Biol.* 7:a021758. doi: 10.1101/cshperspect.a021758

Barnes, A. P., and Polleux, F. (2009). Establishment of axon-dendrite polarity in developing neurons. *Annu. Rev. Neurosci.* 32, 347–381. doi: 10.1146/annurev.neuro.31.060407.125536

Bercury, K. K., Dai, J., Sachs, H. H., Ahrendsen, J. T., Wood, T. L., and Macklin, W. B. (2014). Conditional ablation of raptor or rictor has differential impact on oligodendrocyte differentiation and CNS myelination. *J. Neurosci.* 34, 4466–4480. doi: 10.1523/JNEUROSCI.4314-13.2014

Bercury, K. K., and Macklin, W. B. (2015). Dynamics and mechanisms of CNS myelination. *Dev. Cell* 32, 447–458. doi: 10.1016/j.devcel.2015.01.016

Bergeron, Y., Chagniel, L., Bureau, G., Massicotte, G., and Cyr, M. (2014). mTOR signaling contributes to motor skill learning in mice. *Front. Mol. Neurosci.* 7:26. doi: 10.3389/fnmol.2014.00026

Bjedov, I., Toivonen, J. M., Kerr, F., Slack, C., Jacobson, J., Foley, A., et al. (2010). Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. *Cell Metab.* 11, 35–46. doi: 10.1016/j.cmet.2009.11.010

Bockaert, J., and Marin, P. (2015). mTOR in brain physiology and pathologies. *Physiol. Rev.* 95, 1157–1187. doi: 10.1152/physrev.00038.2014

Briz, V., and Baudry, M. (2014). Estrogen regulates protein synthesis and actin polymerization in Hippocampal neurons through different molecular mechanisms. *Front. Endocrinol. (Lausanne)* 5:22. doi: 10.3389/fendo.2014.00022

Budday, S., Steinmann, P., and Kuhl, E. (2015). Physical biology of human brain development. *Front. Cell. Neurosci.* 9:257. doi: 10.3389/fncel.2015.00257

Burket, J. A., Benson, A. D., Tang, A. H., and Deutsch, S. I. (2015). NMDA receptor activation regulates sociability by its effect on mTOR signaling activity. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 60, 60–65. doi: 10.1016/j.pnpbp.2015.02.009

Bury, L. A., and Sabo, S. L. (2015). Building a terminal: mechanisms of presynaptic development in the CNS. *Neuroscientist*. doi: 10.1177/1073858415596131 [Epub ahead of print]

Cheng, P. L., and Poo, M. M. (2012). Early events in axon/dendrite polarization. *Annu. Rev. Neurosci.* 35, 181–201. doi: 10.1146/annurev-neuro-061010-113618

Cloetta, D., Thomanetz, V., Baranek, C., Lustenberger, R. M., Lin, S., Oliveri, F., et al. (2013). Inactivation of mTORC1 in the developing brain causes microcephaly and affects gliogenesis. *J. Neurosci.* 33, 7799–7810. doi: 10.1523/JNEUROSCI.3294-12.2013

Cornu, M., Albert, V., and Hall, M. N. (2013). mTOR in aging, metabolism, and cancer. *Curr. Opin. Genet. Dev.* 23, 53–62. doi: 10.1016/j.gde.2012.12.005

Crino, P. B. (2011). mTOR: a pathogenic signaling pathway in developmental brain malformations. *Trends Mol. Med.* 17, 734–742. doi: 10.1016/j.molmed.2011.07.008

Cuervo, A. M. (2008). Autophagy and aging: keeping that old broom working. *Trends Genet.* 24, 604–612. doi: 10.1016/j.tig.2008.10.002

Dadalko, O. I., Siuta, M., Poe, A., Erreger, K., Matthies, H. J., Niswender, K., et al. (2015). mTORC2/rictor signaling disrupts dopamine-dependent behaviors via defects in striatal dopamine neurotransmission. *J. Neurosci.* 35, 8843–8854. doi: 10.1523/JNEUROSCI.0887-15.2015

Dong, W., Wang, R., Ma, L. N., Xu, B. L., Zhang, J. S., Zhao, Z. W., et al. (2015a). Autophagy involving age-related cognitive behavior and hippocampus injury is modulated by different caloric intake in mice. *Int. J. Clin. Exp. Med.* 8, 11843–11853

Dong, W., Wang, R., Ma, L. N., Xu, B. L., Zhang, J. S., Zhao, Z. W., et al. (2015b). Influence of age-related learning and memory capacity of mice: different effects of a high and low caloric diet. *Aging Clin. Exp. Res.* 28, 303–311. doi: 10.1007/s40520-015-0398-0

Dwyer, J. M., Maldonado-Aviles, J. G., Lepack, A. E., DiLeone, R. J., and Duman, R. S. (2015). Ribosomal protein S6 kinase 1 signaling in prefrontal cortex controls depressive behavior. *Proc. Natl. Acad. Sci. U.S.A.* 112, 6188–6193. doi: 10.1073/pnas.1505289112

Filas, B. A., Xu, G., and Taber, L. A. (2013). "Mechanisms of brain morphogenesis," in *Computer Models in Biomechanics*, eds G. A. Holzapfel and E. Kuhl (Netherlands: Springer), 337–349.

Fishwick, K. J., Li, R. A., Halley, P., Deng, P., and Storey, K. G. (2010). Initiation of neuronal differentiation requires PI3-kinase/TOR signalling in the vertebrate neural tube. *Dev. Biol.* 338, 215–225. doi: 10.1016/j.ydbio.2009.12.001

Fortress, A. M., Fan, L., Orr, P. T., Zhao, Z., and Frick, K. M. (2013). Estradiol-induced object recognition memory consolidation is dependent on activation of mTOR signaling in the dorsal hippocampus. *Learn. Mem.* 20, 147–155. doi: 10.1101/lm.026732.112

Fu, C., Cawthon, B., Clinkscales, W., Bruce, A., Winzenburger, P., and Ess, K. C. (2012). GABAergic interneuron development and function is modulated by the Tsc1 gene. *Cereb. Cortex* 22, 2111–2119. doi: 10.1093/cercor/bhr300

Gal-Ben-Ari, S., Kenney, J. W., Ounalla-Saad, H., Taha, E., David, O., Levitan, D., et al. (2012). Consolidation and translation regulation. *Learn. Mem.* 19, 410–422. doi: 10.1101/lm.026849.112

Garcia-Rodriguez, J. L., Barbier-Torres, L., Fernandez-Alvarez, S., Gutierrez-de Juan, V., Monte, M. J., Halilbasic, E., et al. (2014). SIRT1 controls liver regeneration by regulating bile acid metabolism through farnesoid X receptor and mammalian target of rapamycin signaling. *Hepatology* 59, 1972–1983. doi: 10.1002/hep.26971

Garelick, M. G., and Kennedy, B. K. (2011). TOR on the brain. *Exp. Gerontol.* 46, 155–163. doi: 10.1016/j.exger.2010.08.030

Gipson, T. T., and Johnston, M. V. (2012). Plasticity and mTOR: towards restoration of impaired synaptic plasticity in mTOR-related neurogenetic disorders. *Neural Plast.* 2012:486402. doi: 10.1155/2012/486402

Golbabapour, S., Majid, N. A., Hassandarvish, P., Hajrezaie, M., Abdulla, M. A., and Hadi, A. H. (2013). Gene silencing and Polycomb group proteins: an overview of their structure, mechanisms and phylogenetics. *OMICS* 17, 283–296. doi: 10.1089/omi.2012.0105

Gómez, O., Sanchez-Rodriguez, A., Le, M., Sanchez-Caro, C., Molina-Holgado, F., and Molina-Holgado, E. (2011). Cannabinoid receptor agonists modulate oligodendrocyte differentiation by activating PI3K/Akt and the mammalian target of rapamycin (mTOR) pathways. *Br. J. Pharmacol.* 163, 1520–1532. doi: 10.1111/j.1476-5381.2011.01414.x

Gong, H., Qian, H., Ertl, R., Astle, C. M., Wang, G. G., Harrison, D. E., et al. (2015). Histone modifications change with age, dietary restriction and rapamycin treatment in mouse brain. *Oncotarget* 6, 15882–15890. doi: 10.18632/oncotarget.4137

Guo, W., Qian, L., Zhang, J., Zhang, W., Morrison, A., Hayes, P., et al. (2011). Sirt1 overexpression in neurons promotes neurite outgrowth and cell survival through inhibition of the mTOR signaling. *J. Neurosci. Res.* 89, 1723–1736. doi: 10.1002/jnr.22725

Hadamitzky, M., Herring, A., Keyvani, K., Doenlen, R., Krugel, U., Bosche, K., et al. (2014). Acute systemic rapamycin induces neurobehavioral alterations in rats. *Behav. Brain Res.* 273, 16–22. doi: 10.1016/j.bbr.2014.06.056

Harris, T. E., and Lawrence, J. C. Jr. (2003). TOR signaling. *Sci. STKE* 2003:re15. doi: 10.1126/stke.2122003re15

Harrison, D. E., Strong, R., Sharp, Z. D., Nelson, J. F., Astle, C. M., Flurkey, K., et al. (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* 460, 392–395. doi: 10.1038/nature08221

Hartman, N. W., Lin, T. V., Zhang, L., Paquelet, G. E., Feliciano, D. M., and Bordey, A. (2013). mTORC1 targets the translational repressor 4E-BP2, but not S6 kinase 1/2, to regulate neural stem cell self-renewal in vivo. *Cell Rep.* 5, 433–444. doi: 10.1016/j.celrep.2013.09.017

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes Dev.* 18, 1926–1945. doi: 10.1101/gad.1212704

Heise, C., Gardoni, F., Culotta, L., di Luca, M., Verpelli, C., and Sala, C. (2014). Elongation factor-2 phosphorylation in dendrites and the regulation of dendritic mRNA translation in neurons. *Front. Cell. Neurosci.* 8:35. doi: 10.3389/fncel.2014.00035

Hirabayashi, Y., and Gotoh, Y. (2010). Epigenetic control of neural precursor cell fate during development. *Nat. Rev. Neurosci.* 11, 377–388. doi: 10.1038/nrn2810

Jaworski, J., and Sheng, M. (2006). The growing role of mTOR in neuronal development and plasticity. *Mol. Neurobiol.* 34, 205–219. doi: 10.1385/MN:34:3:205

Jenwitheesuk, A., Nopparat, C., Mukda, S., Wongchitrat, P., and Govitrapong, P. (2014). Melatonin regulates aging and neurodegeneration through energy metabolism, epigenetics, autophagy and circadian rhythm pathways. *Int. J. Mol. Sci.* 15, 16848–16884. doi: 10.3390/ijms150916848

Jiang, X., and Nardelli, J. (2015). Cellular and molecular introduction to brain development. *Neurobiol. Dis.* 92, 3–17. doi: 10.1016/j.nbd.2015.07.007

Jobim, P. F., Pedroso, T. R., Christoff, R. R., Werenicz, A., Maurmann, N., Reolon, G. K., et al. (2012a). Inhibition of mTOR by rapamycin in the amygdala or hippocampus impairs formation and reconsolidation of inhibitory avoidance memory. *Neurobiol. Learn. Mem.* 97, 105–112. doi: 10.1016/j.nlm.2011.10.002

Jobim, P. F., Pedroso, T. R., Werenicz, A., Christoff, R. R., Maurmann, N., Reolon, G. K., et al. (2012b). Impairment of object recognition memory by rapamycin inhibition of mTOR in the amygdala or hippocampus around the time of learning or reactivation. *Behav. Brain Res.* 228, 151–158. doi: 10.1016/j.bbr.2011.12.004

Ka, M., Condorelli, G., Woodgett, J. R., and Kim, W. Y. (2014). mTOR regulates brain morphogenesis by mediating GSK3 signaling. *Development* 141, 4076–4086. doi: 10.1242/dev.108282

Kaeberlein, M., Powers, R. W. III, Steffen, K. K., Westman, E. A., Hu, D., Dang, N. et al. (2005). Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* 310, 1193–1196. doi: 10.1126/science.1115535

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science* 294, 1030–1038. doi: 10.1126/science.1067020

Khapre, R. V., Kondratova, A. A., Patel, S., Dubrovsky, Y., Wrobel, M., Antoch, M. P., et al. (2014). BMAL1-dependent regulation of the mTOR signaling pathway delays aging. *Aging (Albany NY)* 6, 48–57. doi: 10.18632/aging.100633

Kim, J., and Guan, K. L. (2011). Amino acid signaling in TOR activation. *Annu. Rev. Biochem.* 80, 1001–1032. doi: 10.1146/annurev-biochem-062209-094414

Kim, J. Y., Duan, X., Liu, C. Y., Jang, M. H., Guo, J. U., Pow-anpongkul, N., et al. (2009). DISC1 regulates new neuron development in the adult brain via modulation of AKT-mTOR signaling through KIAA1212. *Neuron* 63, 761–773. doi: 10.1016/j.neuron.2009.08.008

Kintner, C. (2002). Neurogenesis in embryos and in adult neural stem cells. *J. Neurosci.* 22, 639–643.

Klann, E., and Dever, T. E. (2004). Biochemical mechanisms for translational regulation in synaptic plasticity. *Nat. Rev. Neurosci.* 5, 931–942. doi: 10.1038/nrn1557

Lee, C. C., Huang, C. C., and Hsu, K. S. (2011). Insulin promotes dendritic spine and synapse formation by the PI3K/Akt/mTOR and Rac1 signaling pathways. *Neuropharmacology* 61, 867–879. doi: 10.1016/j.neuropharm.2011.06.003

Lee da, Y. (2015). Roles of mTOR signaling in brain development. *Exp. Neurobiol.* 24, 177–185. doi: 10.5607/en.2015.24.3.177

Lee da, Y., Yeh, T. H., Emnett, R. J., White, C. R., and Gutmann, D. H. (2010). Neurofibromatosis-1 regulates neuroglial progenitor proliferation and glial differentiation in a brain region-specific manner. *Genes Dev.* 24, 2317–2329. doi: 10.1101/gad.1957110

Liu, D., Zhang, Y., Gharavi, R., Park, H. R., Lee, J., Siddiqui, S., et al. (2015). The mitochondrial uncoupler DNP triggers brain cell mTOR signaling network reprogramming and CREB pathway up-regulation. *J. Neurochem.* 134, 677–692. doi: 10.1111/jnc.13176

Mac Callum, P. E., Hebert, M., Adamec, R. E., and Blundell, J. (2014). Systemic inhibition of mTOR kinase via rapamycin disrupts consolidation and reconsolidation of auditory fear memory. *Neurobiol. Learn. Mem.* 112, 176–185. doi: 10.1016/j.nlm.2013.08.014

Maiese, K., Chong, Z. Z., Wang, S., and Shang, Y. C. (2012). Oxidant stress and signal transduction in the nervous system with the PI 3-K, Akt, and mTOR cascade. *Int. J. Mol. Sci.* 13, 13830–13866. doi: 10.3390/ijms131113830

Mariani, E., Polidori, M. C., Cherubini, A., and Mecocci, P. (2005). Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 827, 65–75. doi: 10.1016/j.jchromb.2005.04.023

Masui, K., Cavenee, W. K., and Mischel, P. S. (2015). mTORC2 and metabolic reprogramming in GBM: at the interface of genetics and environment. *Brain Pathol.* 25, 755–759. doi: 10.1111/bpa.12307

Middei, S., Ammassari-Teule, M., and Marie, H. (2014). Synaptic plasticity under learning challenge. *Neurobiol. Learn. Mem.* 115, 108–115. doi: 10.1016/j.nlm.2014.08.001

Mizushima, N., Levine, B., Cuervo, A. M., and Klionsky, D. J. (2008). Autophagy fights disease through cellular self-digestion. *Nature* 451, 1069–1075. doi: 10.1038/nature06639

Molofsky, A.V., and Deneen, B. (2015). Astrocyte development: a Guide for the Perplexed. *Glia* 63, 1320–1329. doi: 10.1002/glia.22836

Okada, K., Tanaka, H., Temporin, K., Okamoto, M., Kuroda, Y., Moritomo, H., et al. (2011). Akt/mammalian target of rapamycin signaling pathway regulates neurite outgrowth in cerebellar granule neurons stimulated by methylcobalamin. *Neurosci. Lett.* 495, 201–204. doi: 10.1016/j.neulet.2011.03.065

Orr, P. T., Rubin, A. J., Fan, L., Kent, B. A., and Frick, K. M. (2012). The progesterone-induced enhancement of object recognition memory consolidation involves activation of the extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) pathways in the dorsal hippocampus. *Horm. Behav.* 61, 487–495. doi: 10.1016/j.yhbeh.2012.01.004

Paliouras, G. N., Hamilton, L. K., Aumont, A., Joppe, S. E., Barnabe-Heider, F., and Fernandes, K. J. (2012). Mammalian target of rapamycin signaling is a key regulator of the transit-amplifying progenitor pool in the adult and aging forebrain. *J. Neurosci.* 32, 15012–15026. doi: 10.1523/JNEUROSCI.2248-12.2012

Pedroso, T. R., Jobim, P. F., Carvalho, L. M., Christoff, R. R., Maurmann, N., Reolon, G. K., et al. (2013). Inhibition of protein synthesis or mTOR in the basolateral amygdala blocks retrieval-induced memory strengthening. *J. Neural. Transm.* 120, 1525–1531. doi: 10.1007/s00702-013-1032-y

Perluigi, M., Di Domenico, F., and Butterfield, D. A. (2015). mTOR signaling in aging and neurodegeneration: at the crossroad between metabolism dysfunction and impairment of autophagy. *Neurobiol. Dis.* 84, 39–49. doi: 10.1016/j.nbd.2015.03.014

Puighermanal, E., Marsicano, G., Busquets-Garcia, A., Lutz, B., Maldonado, R., and Ozaita, A. (2009). Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nat. Neurosci.* 12, 1152–1158. doi: 10.1038/nn.2369

Purves, D. (2012). *Neuroscience*. Sunderland, MA: Sinauer Associates.

Qi, S., Mizuno, M., Yonezawa, K., Nawa, H., and Takei, N. (2010). Activation of mammalian target of rapamycin signaling in spatial learning. *Neurosci. Res.* 68, 88–93. doi: 10.1016/j.neures.2010.06.008

Rajan, P., Panchision, D. M., Newell, L. F., and McKay, R. D. (2003). BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells. *J. Cell Biol.* 161, 911–921. doi: 10.1083/jcb.200211021

Raman, L., Kong, X., and Kernie, S. G. (2013). Pharmacological inhibition of the mTOR pathway impairs hippocampal development in mice. *Neurosci. Lett.* 541, 9–14. doi: 10.1016/j.neulet.2013.01.045

Ramanan, V. K., Nho, K., Shen, L., Risacher, S. L., Kim, S., McDonald, B. C., et al. (2015). FASTKD2 is associated with memory and hippocampal structure in older adults. *Mol. Psychiatry* 20, 1197–1204. doi: 10.1038/mp.2014.142

Reichenbach, N., Herrmann, U., Kahne, T., Schicknick, H., Pielot, R., Naumann, M., et al. (2015). Differential effects of dopamine signalling on long-term memory formation and consolidation in rodent brain. *Proteome Sci.* 13:13. doi: 10.1186/s12953-015-0069-2

Romine, J., Gao, X., Xu, X. M., So, K. F., and Chen, J. (2015). The proliferation of amplifying neural progenitor cells is impaired in the aging brain and restored by the mTOR pathway activation. *Neurobiol. Aging* 36, 1716–1726. doi: 10.1016/j.neurobiolaging.2015.01.003

Rosenberg, T., Gal-Ben-Ari, S., Dieterich, D. C., Kreutz, M. R., Ziv, N. E., Gundelfinger, E. D., et al. (2014). The roles of protein expression in synaptic plasticity and memory consolidation. *Front. Mol. Neurosci.* 7:86. doi: 10.3389/fnmol.2014.00086

Sadler, T. W. (2005). Embryology of neural tube development. *Am. J. Med. Genet. C Semin. Med. Genet.* 135C, 2–8. doi: 10.1002/ajmg.c.30049

Sancak, Y., and Sabatini, D. M. (2009). Rag proteins regulate amino-acid-induced mTORC1 signalling. *Biochem. Soc. Trans.* 37, 289–290. doi: 10.1042/BST0370289

Sandsmark, D. K., Pelletier, C., Weber, J. D., and Gutmann, D. H. (2007). Mammalian target of rapamycin: master regulator of cell growth in the nervous system. *Histol. Histopathol.* 22, 895–903.

Saraf, A., Luo, J., Morris, D. R., and Storm, D. R. (2014). Phosphorylation of eukaryotic translation initiation factor 4E and eukaryotic translation initiation factor 4E-binding protein (4EBP) and their upstream signaling components undergo diurnal oscillation in the mouse hippocampus: implications for memory persistence. *J. Biol. Chem.* 289, 20129–20138. doi: 10.1074/jbc.M114.552638

Sarbassov, D. D., Ali, S. M., and Sabatini, D. M. (2005). Growing roles for the mTOR pathway. *Curr. Opin. Cell Biol.* 17, 596–603. doi: 10.1016/j.ceb.2005.09.009

Sarlak, G., Jenwitheesuk, A., Chetsawang, B., and Govitrapong, P. (2013). Effects of melatonin on nervous system aging: neurogenesis and neurodegeneration. *J. Pharmacol. Sci.* 123, 9–24. doi: 10.1254/jphs.13R01SR

Schicknick, H., Schott, B. H., Budinger, E., Smalla, K. H., Riedel, A., Seidenbecher, C. I., et al. (2008). Dopaminergic modulation of auditory cortex-dependent memory consolidation through mTOR. *Cereb. Cortex* 18, 2646–2658. doi: 10.1093/cercor/bhn026

Sehgal, M., Song, C., Ehlers, V. L., and Moyer, J. R. Jr. (2013). Learning to learn - intrinsic plasticity as a metaplasticity mechanism for memory formation. *Neurobiol. Learn. Mem.* 105, 186–199. doi: 10.1016/j.nlm.2013.07.008

Slipczuk, L., Bekinschtein, P., Katche, C., Cammarota, M., Izquierdo, I., and Medina, J. H. (2009). BDNF activates mTOR to regulate GluR1 expression required for memory formation. *PLoS ONE* 4:e6007. doi: 10.1371/journal.pone.0006007

Sosanya, N. M., Cacheaux, L. P., Workman, E. R., Niere, F., Perrone-Bizzozero, N. I., and Raab-Graham, K. F. (2015). Mammalian target of rapamycin (mTOR) tagging promotes dendritic branch variability through the capture of Ca2+/Calmodulin-dependent protein kinase II alpha (CaMKIIalpha) mRNAs by the RNA-binding protein HuD. *J. Biol. Chem.* 290, 16357–16371. doi: 10.1074/jbc.M114.599399

Speranza, L., Giuliano, T., Volpicelli, F., De Stefano, M. E., Lombardi, L., Chambery, A., et al. (2015). Activation of 5-HT7 receptor stimulates neurite

elongation through mTOR, Cdc42 and actin filaments dynamics. *Front. Behav. Neurosci.* 9:62. doi: 10.3389/fnbeh.2015.00062

Stuchlik, A. (2014). Dynamic learning and memory, synaptic plasticity and neurogenesis: an update. *Front. Behav. Neurosci.* 8:106. doi: 10.3389/fnbeh.2014.00106

Su, Z. W., Liao, J. Y., Zhang, H., Zhang, T., Wu, F., Tian, X. H., et al. (2015). Postnatal high-protein diet improves learning and memory in premature rats via activation of mTOR signaling. *Brain Res.* 1611, 1–7. doi: 10.1016/j.brainres.2015.01.052

Sui, L., Wang, J., and Li, B. M. (2008). Role of the phosphoinositide 3-kinase-Akt-mammalian target of the rapamycin signaling pathway in long-term potentiation and trace fear conditioning memory in rat medial prefrontal cortex. *Learn. Mem.* 15, 762–776. doi: 10.1101/lm.1067808

Swiech, L., Perycz, M., Malik, A., and Jaworski, J. (2008). Role of mTOR in physiology and pathology of the nervous system. *Biochim. Biophys. Acta* 1784, 116–132. doi: 10.1016/j.bbapap.2007.08.015

Taha, E., Gildish, I., Gal-Ben-Ari, S., and Rosenblum, K. (2013). The role of eEF2 pathway in learning and synaptic plasticity. *Neurobiol. Learn. Mem.* 105, 100–106. doi: 10.1016/j.nlm.2013.04.015

Tee, A. R., Sampson, J. R., Pal, D. K., and Bateman, J. M. (2016). The role of mTOR signalling in neurogenesis, insights from tuberous sclerosis complex. *Semin. Cell Dev. Biol.* 52, 12–20. doi: 10.1016/j.semcdb.2016.01.040

Toescu, E. C. (2005). Normal brain ageing: models and mechanisms. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 360, 2347–2354. doi: 10.1098/rstb.2005.1771

Triplett, J. C., Tramutola, A., Swomley, A., Kirk, J., Grimes, K., Lewis, K., et al. (2015). Age-related changes in the proteostasis network in the brain of the naked mole-rat: implications promoting healthy longevity. *Biochim. Biophys. Acta* 1852, 2213–2224. doi: 10.1016/j.bbadis.2015.08.002

Tsang, C. K., Qi, H., Liu, L. F., and Zheng, X. F. (2007). Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discov. Today* 12, 112–124. doi: 10.1016/j.drudis.2006.12.008

Urban, N., and Guillemot, F. (2014). Neurogenesis in the embryonic and adult brain: same regulators, different roles. *Front. Cell. Neurosci.* 8:396. doi: 10.3389/fncel.2014.00396

Urbanska, M., Gozdz, A., Swiech, L. J., and Jaworski, J. (2012). Mammalian target of rapamycin complex 1 (mTORC1) and 2 (mTORC2) control the dendritic arbor morphology of hippocampal neurons. *J. Biol. Chem.* 287, 30240–30256. doi: 10.1074/jbc.M112.374405

Vellai, T., Takacs-Vellai, K., Zhang, Y., Kovacs, A. L., Orosz, L., and Muller, F. (2003). Genetics: influence of TOR kinase on lifespan in *C. elegans*. *Nature* 426:620. doi: 10.1038/426620a

Wahane, S. D., Hellbach, N., Prentzell, M. T., Weise, S. C., Vezzali, R., Kreutz, C., et al. (2014). PI3K-p110-alpha-subtype signalling mediates survival, proliferation and neurogenesis of cortical progenitor cells via activation of mTORC2. *J. Neurochem.* 130, 255–267. doi: 10.1111/jnc.12718

Wang, B., Xiao, Z., Chen, B., Han, J., Gao, Y., Zhang, J., et al. (2008). Nogo-66 promotes the differentiation of neural progenitors into astroglial lineage cells through mTOR-STAT3 pathway. *PLoS ONE* 3:e1856. doi: 10.1371/journal.pone.0001856

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D. R., and Proud, C. G. (2001). Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *EMBO J.* 20, 4370–4379. doi: 10.1093/emboj/20.16.4370.

Weber, J. D., and Gutmann, D. H. (2012). Deconvoluting mTOR biology. *Cell Cycle* 11, 236–248. doi: 10.4161/cc.11.2.19022

Westerholz, S., de Lima, A. D., and Voigt, T. (2013). Thyroid hormone-dependent development of early cortical networks: temporal specificity and the contribution of trkB and mTOR pathways. *Front. Cell. Neurosci.* 7:121. doi: 10.3389/fncel.2013.00121

Wood, T. L., Bercury, K. K., Cifelli, S. E., Mursch, L. E., Min, J., Dai, J., et al. (2013). mTOR: a link from the extracellular milieu to transcriptional regulation of oligodendrocyte development. *ASN Neuro* 5:e00108. doi: 10.1042/AN20120092

Yang, F., Chu, X., Yin, M., Liu, X., Yuan, H., Niu, Y., et al. (2014). mTOR and autophagy in normal brain aging and caloric restriction ameliorating age-related cognition deficits. *Behav. Brain Res.* 264, 82–90. doi: 10.1016/j.bbr.2014.02.005

Yu, B., Xu, P., Zhao, Z., Cai, J., Sternberg, P., and Chen, Y. (2014). Subcellular distribution and activity of mechanistic target of rapamycin in aged retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 55, 8638–8650. doi: 10.1167/iovs.14-14758

Zhang, J., Ji, F., Liu, Y., Lei, X., Li, H., Ji, G., et al. (2014). Ezh2 regulates adult hippocampal neurogenesis and memory. *J. Neurosci.* 34, 5184–5199. doi: 10.1523/JNEUROSCI.4129-13.2014

Zhao, C., Deng, W., and Gage, F. H. (2008). Mechanisms and functional implications of adult neurogenesis. *Cell* 132, 645–660. doi: 10.1016/j.cell.2008.01.033.

Zhou, L., Lu, Z., Li, L., Chen, L., Qi, J., and Chen, L. (2014). Pro-neurogenesis and anti-dementia properties of tetradecyl 2,3-dihydroxybenzoate through TrkA receptor-mediated signalling pathways. *Int. J. Neuropsychopharmacol.* 17, 1847–1861. doi: 10.1017/S1461145714000558

Zhou, M., Li, W., Huang, S., Song, J., Kim, J. Y., Tian, X., et al. (2013). mTOR Inhibition ameliorates cognitive and affective deficits caused by Disc1 knockdown in adult-born dentate granule neurons. *Neuron* 77, 647–654. doi: 10.1016/j.neuron.2012.12.033

Zoncu, R., Efeyan, A., and Sabatini, D. M. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat. Rev. Mol. Cell Biol.* 12, 21–35. doi: 10.1038/nrm3025

Zou, Y., Jiang, W., Wang, J., Li, Z., Zhang, J., Bu, J., et al. (2014). Oligodendrocyte precursor cell-intrinsic effect of Rheb1 controls differentiation and mediates mTORC1-dependent myelination in brain. *J. Neurosci.* 34, 15764–15778. doi: 10.1523/JNEUROSCI.2267-14.2014

Zuchero, J. B., and Barres, B. A. (2013). Intrinsic and extrinsic control of oligodendrocyte development. *Curr. Opin. Neurobiol.* 23, 914–920. doi: 10.1016/j.conb.2013.06.005

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FORUM REVIEW ARTICLE

# Neurotoxicity Linked to Dysfunctional Metal Ion Homeostasis and Xenobiotic Metal Exposure: Redox Signaling and Oxidative Stress

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## Abstract

***Significance:*** Essential metals such as copper, iron, manganese, and zinc play a role as cofactors in the activity of a wide range of processes involved in cellular homeostasis and survival, as well as during organ and tissue development. Throughout our life span, humans are also exposed to xenobiotic metals from natural and anthropogenic sources, including aluminum, arsenic, cadmium, lead, and mercury. It is well recognized that alterations in the homeostasis of essential metals and an increased environmental/occupational exposure to xenobiotic metals are linked to several neurological disorders, including neurodegeneration and neurodevelopmental alterations.

***Recent Advances:*** The redox activity of essential metals is key for neuronal homeostasis and brain function. Alterations in redox homeostasis and signaling are central to the pathological consequences of dysfunctional metal ion homeostasis and increased exposure to xenobiotic metals. Both redox-active and redox-inactive metals trigger oxidative stress and damage in the central nervous system, and the exact mechanisms involved are starting to become delineated.

***Critical Issues:*** In this review, we aim to appraise the role of essential metals in determining the redox balance in the brain and the mechanisms by which alterations in the homeostasis of essential metals and exposure to xenobiotic metals disturb the cellular redox balance and signaling. We focus on recent literature regarding their transport, metabolism, and mechanisms of toxicity in neural systems.

***Future Directions:*** Delineating the specific mechanisms by which metals alter redox homeostasis is key to understand the pathological processes that convey chronic neuronal dysfunction in neurodegenerative and neurodevelopmental disorders. *Antioxid. Redox Signal.* 28, 1669–1703.

**Keywords:** neurodegeneration, redox, essential metals, heavy metals, neurotoxicity

## Introduction

MOST ELEMENTS IN the periodic table are considered metals due to their propensity to lose electrons and react with molecular oxygen ($O_2$) to form oxides. Metals in biological systems may be broadly divided into four groups: alkali and alkaline-earth metals, such as sodium (Na), potassium (K), magnesium (Mg), and calcium (Ca); essential transition metals, such as copper (Cu), manganese (Mn), iron (Fe), and zinc (Zn); and xenobiotic heavy metals such as mercury (Hg), lead (Pb), and cadmium (Cd). In addition, metalloids such as arsenic (As) are present in the

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environment and have chemical and physical properties of both metal and nonmetal elements. Some authors include aluminum (Al) and selenium (Se) as metalloids. For simplicity, herein we will refer to metalloids as metals. Importantly, while essential metals participate in normal biological functions, alterations in their handling or their increased accumulation are well reported to exert neurotoxicity (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/ars).

Prospective epidemiological studies have associated cognitive, motor, and behavioral alterations to environmental exposure to metals and metalloids (153, 158, 250, 371), effects that are exacerbated when environmental exposures occur chronically and during development (153, 158, 250). Long-term effects of either environmental metal exposure or alterations in metal homeostasis in the central nervous system (CNS) and peripheral nervous system (PNS) have been proposed to play a role in neurodegenerative disorders (379). Importantly, alterations in the cellular redox environment of the cell are central to the toxic effects of metals.

Previous reviews address the general role of metals in neurodegeneration, or the mechanisms by which metals produce oxidative stress or neurotoxicity (85, 86, 89, 163, 355). In this work, we present an integrated review on recent advances in (a) the metabolism of both essential and xenobiotic metals; (b) the mechanisms by which distinct metals determine or modify the cellular redox homeostasis; (c) the link between metal redox activity and function in neural systems; and (d) how alterations in metal homeostasis or intracellular/extracellular levels participate in neurotoxicity and neurodegeneration.



## Overview of Oxidative Stress and Redox Homeostasis

Reactive species is a term used to describe compounds that can receive or provide a couple of electrons or one electron participating in nucleophilic, electrophilic, or redox metabolic reactions, respectively. Reactive oxygen species (ROS) are molecules derived from $O_2$, an obligate component of aerobic organisms. The reduction of $O_2$ is one of the primary reactions that sustain aerobic life, yet it is also the main source for ROS. ROS include free ($^{\bullet}$) and nonfree radical species such as hydroxyl radicals ($^{\bullet}OH$), superoxide anion radicals ($O_2^{\bullet-}$), and hydrogen peroxide ($H_2O_2$). Reactive nitrogen species (RNS) contain both nitrogen (N) and O (oxygen atom), and thus can be categorized as ROS. RNS include nitric oxide ($NO^{\bullet}$), nitrogen dioxide radical ($NO_2$), and peroxynitrite ($OONO^-$) (120, 252).

A major source of intracellular ROS production are the mitochondrial electron transport complexes, primarily the one-electron reduction of $O_2$ to $O_2^{\bullet-}$ by complex I (ubiquinone: NADH oxidoreductase), by the semiquinone of ubiquinone (coenzyme Q), and by complex III (cytochrome bc1 complex or CoQH2-cytochrome c reductase). $O_2^{\bullet-}$ undergoes rapid dismutation into $H_2O_2$ through the action of superoxide dismutases (SODs). Three types of SODs exist in mammalian cells that use an essential metal as cofactor. Cu/Zn-dependent SOD1 and 3 are localized in the cytosol (SOD1), the extracellular space (SOD3) and to a lesser extent, in the inner membrane space of the mitochondria (SOD1). MnSOD (SOD2) is solely localized in the mitochondrial matrix. The transition metal (Cu or Mn) in the active site of SODs is required for the breakdown of $O_2^{\bullet-}$ by catalyzing both the one-electron oxidation and one-electron reduction of separate $O_2^{\bullet-}$ to give the overall disproportionation reaction that produces $O_2$ and $H_2O_2$. Binding of Zn to SOD1 or 3 is not essential for $O_2^{\bullet-}$ dismutation reaction but confers higher thermal stability to the proteins (64).

One to 2% of the total mitochondrial $O_2$ consumed is leaked and contributes to the formation of ROS. Usually, this occurs at a slow rate and can be counteracted by mitochondrial antioxidant systems, but in damaged or aged mitochondria, increased ROS formation occurs. $O_2^{\bullet-}$ and $H_2O_2$ fuel $^{\bullet}OH$ formation through Fenton/Haber–Weiss reactions, where $H_2O_2$ oxidizes a redox-active metal (Fe or Cu) leading to the formation of $^{\bullet}OH$. Then, the oxidized metal is reduced back by $O_2^{\bullet-}$ or other cellular reductants promoting metal-catalyzed free radical chain reactions (100, 120, 252).

Other sources of ROS are the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidases (NOX), enzymes whose principal function is to generate $O_2^{\bullet-}$ or $H_2O_2$. The formation of RNS begins with the synthesis of $NO^{\bullet}$, catalyzed by nitric oxide synthases (NOSs), which are Fe dependent. Zn is an important structural element of NOS enzymes and is also known to inhibit their activity. $NO^{\bullet}$ reacts with $O_2^{\bullet-}$ to produce $OONO^-$, which is a strong oxidizing agent. Microsomes and peroxisomes are important sources of ROS due to the presence of NOX and NOS (302). In addition, ROS production can be mediated by the activity of enzymes such as xanthine oxidase (that contains an Fe-sulfur [S] cluster), the heme proteins cyclooxygenases, cytochrome P450 enzymes, lipoxygenases, and myeloperoxidases, as well as the protein folding machinery in the endoplasmic reticulum (ER) (96, 120, 252).

ROS/RNS act as signaling molecules affecting the stability, expression, function, and activity of a multiplicity of proteins controlling almost all cellular functions, including proliferation, cell survival, metabolism, and signaling. An adequate balance between the formation and elimination of ROS/RNS facilitates the signaling role of these reactive species. However, an imbalance between an increase in the steady-state levels of ROS/RNS and the ability of the cell to metabolize/detoxify them leads to a nonhomeostatic state referred to as oxidative stress. Oxidative stress results in the irreversible oxidative modification of biomolecules with the concomitant loss of function of proteins, damage to cellular organelles, and eventual cell death (96, 100, 252, 282). Polyunsaturated fatty acids are one of the preferred oxidation targets for ROS; particularly, free radicals that are potent initiators of lipid peroxidative chain reactions. Lipid peroxidation products, including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), can react further with DNA bases and proteins (83). DNA bases are also susceptible to direct oxidation by free radicals that can cause mutations as well as deletions in both nuclear and mitochondrial DNA (328).

Protein oxidation can be a reversible or irreversible phenomenon depending on the type of modification, ROS involved, and the extent of oxidation. Tyrosine (Tyr) nitration, protein carbonylation, and protein crosslinkage generated by adduct formation between oxidized proteins, lipid peroxides, or glycative products are irreversible modifications that promote a loss of function, aggregation, and degradation of the targeted protein, and in some cases, the formation of toxic by-products (71). In contrast, reversible oxidative modifications

in the sulfur-containing amino acids methionine (Met) and cysteine (Cys) act as sensors and transducers of ROS/RNS-mediated signaling. Thiol-based oxidoreductases thioredoxins (Trxs), glutaredoxins (Grxs), and Met sulfoxide reductases reduce such modifications acting as the OFF-switch for redox signaling processes (101, 160). On the contary, both Trxs and peroxiredoxins (Prxs) have been proposed to act as redox sensors, buffers, and relays for $H_2O_2$- and $NO^{\bullet}$-mediated signal transduction (46, 195, 268, 323). Cys are also targeted by electrophiles generating irreversible modifications, which are thought to be a primary mechanism of toxicity by xenobiotics (193).

Cells are equipped with enzymatic and nonenzymatic antioxidant systems to counteract the toxic effects of ROS/RNS and maintain redox homeostasis (96, 100, 120, 252). The reducing power of glutathione (GSH) is essential for the detoxification of peroxides by GSH peroxidases (GPX) with the resultant conversion of GSH to GSH disulfide (GSSG). GSSG is reduced back by GSH reductase (GR) in an NADPH-dependent manner (105). Catalases also detoxify peroxides but their localization is primarily restricted to peroxisomes. Other endogenous nonenzymatic antioxidants include uric acid, lipoic acid, and ubiquinol (or reduced coenzyme Q), and those obtained from the diet, such as vitamins and flavonoids (96, 100, 120, 252).

The CNS is particularly sensitive to oxidative damage, from which neurons and oligodendrocytes seem to be more susceptible than astrocytes and microglia. The basis for this increased sensitivity is linked to the high levels of $O_2$ consumption (and electron leakage as a consequence), the low levels of antioxidant defenses when compared to other cells, and the abundance of lipids or fatty acids (262, 295).

Higher levels of endogenous antioxidants and antioxidant systems in astrocytes are explained by the activation of the nuclear factor erythroid-2-related factor 2 (Nrf2) transcription factor (314). Nrf2 recognizes antioxidant response elements to trigger the transcription of antioxidant systems. The ubiquitin ligase Kelch-like ECH-associated protein 1 (Keap1) negatively regulates Nrf2 signaling by inducing its ubiquitination and degradation. Upon modification of specific Cys residues within Keap1 by oxidants or electrophiles (including metals), Nrf2 is released from Keap1 and translocates to the nucleus to induce gene expression dependent on antioxidant response elements (ARE). Nrf2 signaling in neurons has been reported to be epigenetically silenced (27), and induction of the Nrf2 pathway does not seem to be able to promote antioxidant protection (148). Astrocytes also have higher levels of NADPH and glucose 6-phosphate dehydrogenase (G6PD) (109). In contrast, antioxidant genes in neurons seem to be transcriptionally regulated, independent from Nrf2 by synaptic activity, through the triggering of the activating transcription factor 4 (ATF4) and the activator protein 1 (AP-1) (23, 177). Furthermore, while both neurons and astrocytes can synthesize GSH, neurons depend on the supply of GSH precursors *via* GSH efflux (13, 26).

## Neurotoxicity of Metals and Metalloids

Neurotoxicity is defined as a damaging effect on the nervous system caused by a biological or chemical agent. The neurotoxic effects of chemicals are the result of a series of events that include the following: the entry and/or changes in the distribution of a chemical into the brain, interactions with specific cellular targets (neurons and glia), and the initiation of biochemical changes, resulting in structural and functional changes of the nervous system (270). Environmental neurotoxicants include organic and inorganic chemical compounds, such as heavy metals, organic solvents, and cytotoxic substances that can also contain heavy metal mixtures (*e.g.*, pesticides, cigarette smoke, diesel exhaust particles,). The neurotoxic effect of environmental agents is determined by their chemical composition, metabolic function, and pathologican consequence, differing widely according to the brain region targeted and the mechanism(s) of action (270).

## Essential Metals

Micronutrients are defined by their essentiality and very limited quantity in humans, where their deficiency results in the impairment of biological functions (103, 205). Some metals are essential for the maintenance of cellular homeostasis. Essential metals display important roles as signaling agents or cofactors and, in particular, as activators or redox system components (Supplementary Table S1) (103, 205). Traditionally, cellular osmotic balance and signaling (including synaptic communication and excitability) are associated with nontransition metal ions, such as $Na^+$, $K^+$, and $Ca^{2+}$, which form complexes with proteins using low-affinity binding sites, are found at high concentrations, and move quickly across cellular compartments. On the contrary, transition metal ions are known as catalytic cofactors or structural elements in enzymes. Transition metals are present in lower concentration (''trace elements'') and are usually coordinated to proteins at high-affinity binding sites. In the last decade, a role for $Zn^{2+}$ ion as a second messenger has been recognized; however, the role of redox-active metals, such as Cu, Fe, and Mn, in cellular signaling is less explored.

Cu, Fe, and Mn are cofactors of many enzymes that catalyze redox reactions. Although the high reactivity of these metals is essential for life, they can also be involved in uncontrolled redox reactions associated with oxidative stress and cellular damage. Hence, a highly conserved network of proteins strictly regulates the homeostasis of redox-active metal ions, by controlling their uptake, intracellular distribution, storage, and export (205). In the following sections, we describe how brain homeostasis of redox reactive metal ions requires close communication between the blood-brain barrier (BBB), neurons, astrocytes, oligodendrocytes, and microglia. The cases where metal trafficking is tightly linked to the cellular redox environment are highlighted, while the potential role of metal redox cycling in redox signaling is also discussed. Finally, for each essential metal ion, we review how the disruption of its homeostasis may cause two major features associated with neurodegenerative diseases: dysfunction of metalloproteins and aberrant metal-protein interactions that can lead to protein aggregation and uncontrolled ROS production.

### *Copper*

Cu is present in biological systems as $Cu^+$ (cuprous ion) and $Cu^{2+}$ (cupric ion) (Supplementary Table S1). Cu is a redox-active metal and a cofactor of many enzymes involved in cellular respiration, radical detoxification, as well as

biosynthesis of neurotransmitters, neuropeptides, and hormones. For example, Cu is required as cofactor of several important enzymes in the brain, such as peptidylglycine monooxygenase (PHM), dopamine $\beta$-monooxygenase (DBM), tyrosinase (TYR), and cytochrome C oxidase (COX). Cu can activate $O_2$ for reduction and although its high reactivity with $O_2$ is essential for life, if uncontrolled it can promote oxidative stress and cellular damage. $Cu^+$ can react with $H_2O_2$ to produce highly reactive $^{\bullet}OH$. Cu also induces microglial activation and mitochondrial ROS formation (137).

The control of Cu homeostasis in the brain requires a close interrelationship between the BBB, neurons, and astrocytes (300) (Fig. 1). Astrocytes regulate the properties of the BBB, which is the entry point for Cu into the brain from the blood stream, where Cu is bound to albumin or ceruloplasmin (Cp) (58) (Fig. 1a). At the same time, neurons require Cu as a cofactor and neuromodulator, while astrocytes are key players in synaptic transmission and Cu homeostasis (58). The Cu trafficking machineries of the BBB endothelial cells, neurons, and astrocytes resemble those of other extensively studied mammalian cells (Fig. 1a–c). Extracellular Cu is primarily transported into cells as $Cu^+$ *via* the Cu transporter 1 (CTR1) (58). $Cu^{2+}$ reduction to $Cu^+$, and Cu uptake from Cp *via* CTR1, has been proposed to involve a reduction step but no $Cu^{2+}$ reductase has been identified (281). CTR1-independent mechanisms have also been proposed. The divalent metal transporter 1 (DMT1) seems to play a compensatory role for Cu uptake under certain conditions such as in the absence of CTR1 or under low Fe conditions (147, 231). Interestingly, DMT1 loss promotes brain Cu accumulation and oxidative stress (122). Other potential candidates recently proposed to mediate Cu uptake are the Zrt (Zn-regulated transporter)- or Irt (Fe-regulated transporter)-like protein 4 (ZIP4) (11, 29).

Intracellular Cu distribution depends on the relative concentration and metal affinity of chaperones or chelators (18) (Fig. 1b, c). The antioxidant protein 1 (Atox1), the Cu chaperone for superoxide dismutase 1 (CCS1), and GSH have been proposed to take $Cu^+$ from CTR1 (197). Chaperones not only bind Cu but they also deliver it to specific targets. CCS1 transfers Cu to SOD1, where its reactivity with $O_2$ is required for SOD1 maturation *via* the formation of a disulfide bridge (17). Copper chaperones COX19 and COX17 deliver Cu to the COX assembly proteins (SCO1 and SCO2) and COX11. Finally, Atox1 transports Cu to the ATPase copper transporting alpha (ATP7A) and beta (ATP7B) in the secretory pathway, where cuproenzymes such as SOD3 are metalized (Fig. 1b, c). Upon an excess in cytosolic Cu levels, vesicles in



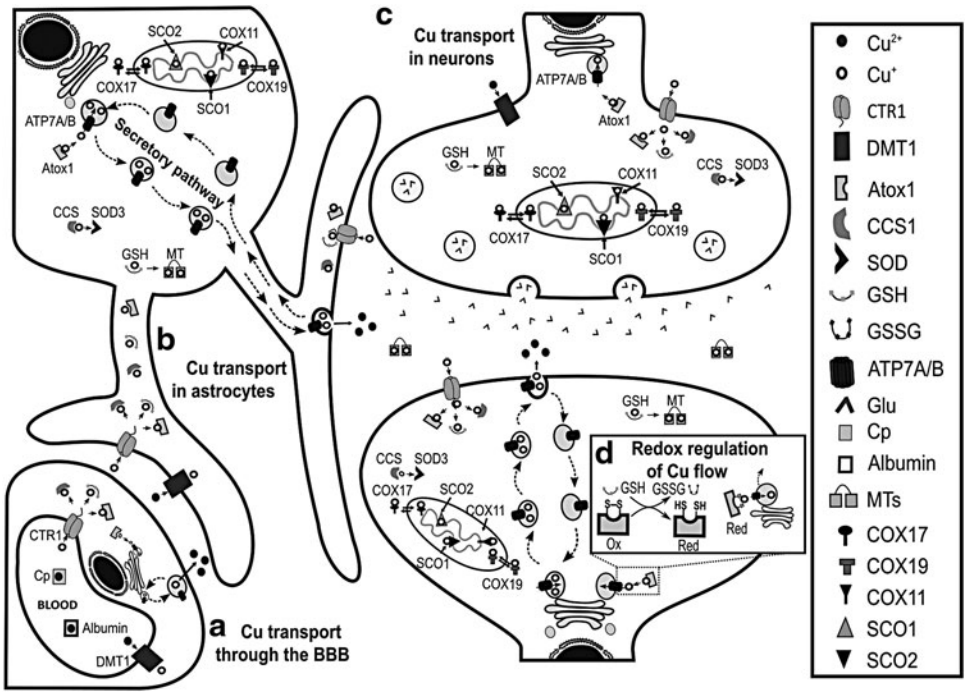


**FIG. 1. Cu homeostasis in the brain and its redox control.** Regulation of Cu homeostasis is a complex process requiring intercommunication between endothelial cells of the BBB, astrocytes, and neurons. These three types of cells have the same protein machinery as other mammalian cells: CTR1 and DMT1 for Cu uptake; Atox1, CCS1, and GSH for Cu delivery to ATP7(A/B), SOD3, and MT, respectively; COX17 and COX19 for Cu delivery to mitochondria, where SCO1, SCO2, and COX11 load Cu into COX; while ATP7 (A/B) participates in Cu export through the secretory pathway **(a–c)**. **(a)** Cp- and albumin- bound Cu enters into the brain by crossing endothelial cells of BBB, which are in tight communication with astrocytes. **(b)** Astrocytes serve as an interface to deliver Cu to **(c)** neurons. **(d)** Recently, a redox control of Cu efflux was elucidated in neurons during differentiation: the redox state of Atox1 controls Cu delivery to the secretory pathway. ATP7A, ATPase copper transporting alpha; ATP7B, ATPase copper transporting beta; Atox1, antioxidant protein 1; BBB, blood-brain barrier; CCS1, copper chaperone for superoxide dismutase 1; COX, cytochrome C oxidase; Cp, ceruloplasmin; CTR1, copper transporter 1; Cu, copper; DMT1, divalent metal transporter 1; Glu, glutamate; GSSG, glutathione disulfide; GSH, glutathione; MT, metallothionein; SCO, cytochrome c oxidase assembly protein; SOD, superoxide dismutase.

the secretory pathway are loaded with Cu and trafficked to the plasma membrane, where Cu is released into the extracellular space (196, 197).

Strikingly, although GSH has a lower affinity for Cu, compared to Atox1 and CCS1, the rate of Cu entry into the cell *via* CTR1 is affected by GSH, but not by Atox1 or CCS1 depletion, likely due to the higher concentration of GSH (208). Thus, GSH is the most important cytosolic first acceptor of Cu from CTR1, providing a tight link between cellular Cu uptake and cellular redox homeostasis. GSH in turn is known to transfer Cu ions to metallothioneins (MTs), small Cys-rich proteins that play a major role as scavengers for metal ions (93) (Fig. 1b, c). Three distinct isoforms of MTs are expressed in the human brain. MT-I, MT-II, and MT-III are found in astrocytes, while MT-III is the main isoform expressed in neurons. MTs can be secreted, playing a crucial role in modulating Cu homeostasis and protecting the cell from oxidative damage (299).

Chaperones use Cys residues to coordinate $Cu^+$ in their reduced state. Thus, Cys oxidation affects Cu dynamics. Atox1 coordinates Cu using a CysXXCys motif that can form a disulfide bond, which can be reduced directly by GSH or by Grx1 in a GSH-dependent manner (Fig. 1d) (41, 127). During neuronal differentiation, the GSH/GSSG ratio increases promoting a more reductive environment, which in turn reduces Cys residues at the Cu binding site of Atox1. These events enable Cu transport from Atox1 to ATP7A/B and enhance Cu availability to load the active sites of newly synthetized cuproenzymes (128) (Fig. 1d). After neuronal differentiation, both Cu and MT-III levels increase (249).

The recent development of fluorescent sensors has revealed new important roles of intracellular Cu in neuronal activity (78); for example, in the spine neck of hippocampal neurons, Cu is essential for the control of the dendritic actin cytoskeleton (269). Cu export by ATP7A has been reported to be triggered by the activation of glutamate (Glu)/N-methyl-D-aspartate receptors (NMDARs) (301) (Fig. 2a). At the synapse, Cu can modulate many neurotransmitter receptors (66). For example, Cu inhibits NMDAR activity by Cys nitros(yl)ation, a neuroprotective mechanism associated with neuronal plasticity that requires the participation of the cellular prion protein ($PrP^C$) (110) (Fig. 2b). Accordingly, selective depletion of ATP7A in neurons and glia increases the susceptibility to NMDA seizures (132).

Cu trafficking at the synapse is complex and involves several Cu binding proteins, such as the membrane-bound $PrP^C$, the amyloid precursor protein (APP), and the amyloid beta (A$\beta$) peptide from neurons, or the neurokinin B (NKB) peptide from astrocytes (Fig. 2). $PrP^C$ has several Cu binding sites and it might be involved in Cu sensing and transport into neurons (Fig. 3a). During synaptic transmission, the extracellular Cu concentration may reach $\sim 100\,\mu M$. Cu binding to $PrP^C$ induces its endocytosis, possibly contributing to Cu delivering into the cytosol (263). APP has been reported to regulate Cu efflux, as the APP knockout mice display higher levels of Cu in the brain and in neurons, while APP overexpression leads to decreased intracellular Cu concentrations (28, 330, 375). In contrast, A$\beta$ peptides produced from the proteolytic cleavage of APP have been proposed to act as Cu scavengers (264), particularly those cleaved to yield 4–40 peptides that have higher affinity for Cu and make up to 50% of the A$\beta$ in plaques (374). NKB has been suggested to compete for Cu from $PrP^C$ and transport it into astrocytes by endocytosis (Fig. 2c) (308). $PrP^C$ and A$\beta$ contain intrinsically disordered regions with Cu binding sites that have the capacity to adopt different Cu coordination modes, some of which have been proposed to activate $O_2$ and produce ROS (Fig. 3a) (184, 350).

A dysfunction in Cu homeostasis is reported to alter neuronal function and lead to disease progression, including neurodegeneration (Supplementary Table S1). Menkes disease and Wilson disease are caused by mutations or partial deletions in *ATP7A* and *ATP7B*, respectively. These Cu transporters have different patterns of expression in the CNS, explaining the distinct pathological features of each disease. ATP7B is found in the visual cortex, anterior cingulate cortex, caudate, putamen, substantia nigra (SN), and cerebellum. ATP7A is detected in astrocytes and neurons from the hippocampus and cerebellum, the BBB and choroid plexus, and during neural development (338). Wilson patients display parkinsonism, underscoring the importance of Cu homeostasis in the motor controlling systems. On the contrary, the ubiquitous expression of ATP7A has challenged mechanistic investigations in Menkes disease. However, a recent study shows that depleting ATP7A in neurons and glia does not lead to neurodegeneration, but to an increased susceptibility to NMDA seizures (132), underscoring its neuroprotective role as described above (Fig. 2a,b). Although neurodegeneration is clearly linked to alterations in Cu homeostasis in Menkes and Wilson diseases, the mechanisms are still unknown.

Neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), Parkinson's disease (PD), and Alzheimer's disease (AD) have been associated with alterations in Cu homeostasis, but a link to specific genetic alterations in Cu transport or handling is missing (Supplementary Table S1). These neurodegenerative diseases are associated with the formation of amyloid aggregates composed of proteins that are either a Cu-dependent antioxidant enzyme, such as SOD1 in ALS, or Cu-binding proteins, such as A$\beta$ and $\alpha$-synuclein in AD and PD, respectively.

ALS is characterized by the degeneration of motor neurons, and mouse models show increased intracellular Cu levels and the formation of protein aggregates composed of SOD1 and Cu transport proteins such as Ctr1, CCS, Atox1, and Cox17 (344, 346). SOD1 aggregation has been associated with an alteration in protein stability, which is impacted by metallation and Cu-dependent dimerization. Although SOD1 plays an important role in clearing $O_2^{\bullet -}$, studies have demonstrated that the mechanism is likely other than the alteration in the antioxidant capacity of SOD1 (312). Increased intracellular Cu levels have been reported in ALS models (346). Consistently, Cu chelators or MT-I overexpression extend the life span and slow disease progression of the *SOD1* (G93A) ALS mouse model (345).

HD is an autosomal dominant genetic disorder caused by polyglutamine (polyQ) repeat expansions near the N-terminus of the huntingtin (Htt) protein. HD is characterized by movement dysfunction, psychiatric and cognitive alterations linked to the degeneration of striatal spiny neurons. Cu accumulation in the striatum of HD transgenic mice has been reported (102). Mutant Htt forms toxic aggregates, but the mechanisms of toxicity are still unclear. A putative Cu binding site in Htt involving Met8 and His82 has

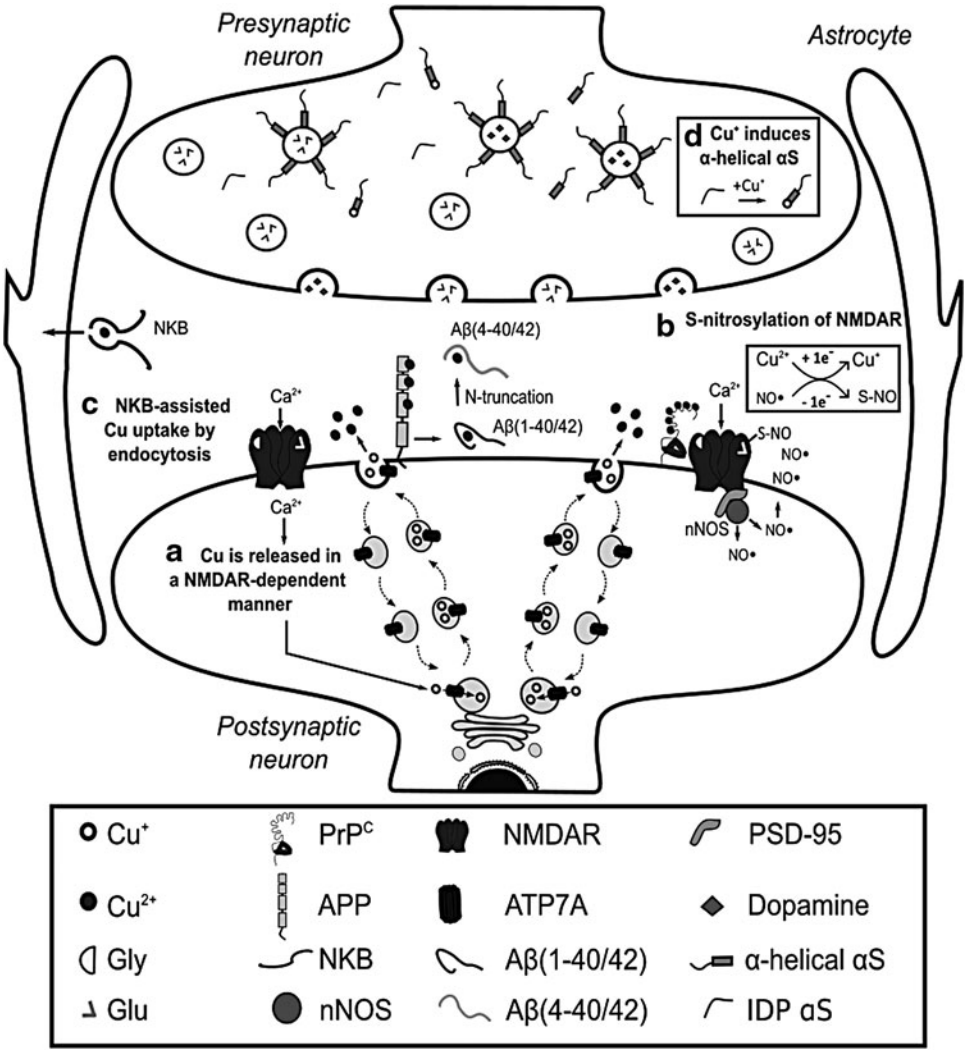


**FIG. 2. Cu at synapse and redox signaling. (a)** After activation of NMDAR, Cu is released at the synapse through the secretory pathway. Several Cu binding proteins are present in the synaptic cleft, including $PrP^C$, APP, A$\beta$ peptides, and NKB. **(b)** The Cu-$PrP^C$ interaction is particularly important for the modulation of NMDAR activity *via* S-nitros(yl)ation. In turn, NO production is dependent on the activation of NMDAR and its interaction with nNOS through the PSD-95. **(c)** While NKB can assist Cu uptake into astrocytes, Cu distribution among Cu-binding proteins at the synapse likely depends on their relative concentration and affinities for Cu. **(d)** On the contrary, α-synuclein is an important presynaptic Cu-binding protein found in neurons; it is an IDP that can interact with membranes when it acquires α-helix structure at N-terminal region. Interestingly, $Cu^+$ binding to the N-Terminal of α-synuclein enhances its α-helical conformation. A$\beta$, amyloid beta; APP, amyloid precursor protein; $Cu^+$, cuprous ion; IDP, intrinsically disordered protein; NKB, neurokinin B; NMDAR, glutamate/N-methyl-D-aspartate receptor; nNOS, neural nitric oxide synthetase; $PrP^C$, cellular prion protein; PSD-95, postsynaptic density protein 95.



been identified, and this interaction promotes polyQ aggregate formation (102, 381), which is reduced by MT-III overexpression (123).

PD is characterized by the degeneration of dopaminergic neurons in the SN, which are rich in Cu. PD patients show decreased Cu content in the SN (70) without changes in Cu levels in serum, plasma, and cerebrospinal fluid (CSF) (203). Accordingly, decreased levels of CTR1 (70) have been reported in the SN, while MT-I and MT-II levels in active astrocytes are increased, reflecting a glial response to the loss of Cu homeostasis in PD (226). In contrast, occupational exposure to Cu has been linked to an increased risk to develop PD (115).

The accumulation of intracellular protein inclusions (Lewy bodies), where α-synuclein is the main protein component, is another hallmark of PD (362). α-Synuclein is a small intrinsically disordered protein (IDP) enriched in presynaptic terminals and nucleus that can interact with cytoskeleton components and lipid membranes (170). IDPs are proteins that can adopt different conformations, and thus respond to changes in their biochemical environment. This property allows them to engage in interactions with multiple protein targets (23). $Cu^{2+}$ and $Cu^+$ ions are capable of binding to α-synuclein at three different sites (20, 33, 228, 361) (Fig. 3a). Interestingly, the H50Q *SNCA*/α-synuclein mutation linked to hereditary PD abolishes one Cu binding site altering Cu-induced α-synuclein aggregation (361). Moreover, Cu binding to the high-affinity Cu-binding site at the N-terminal region of α-synuclein accelerates its amyloid aggregation

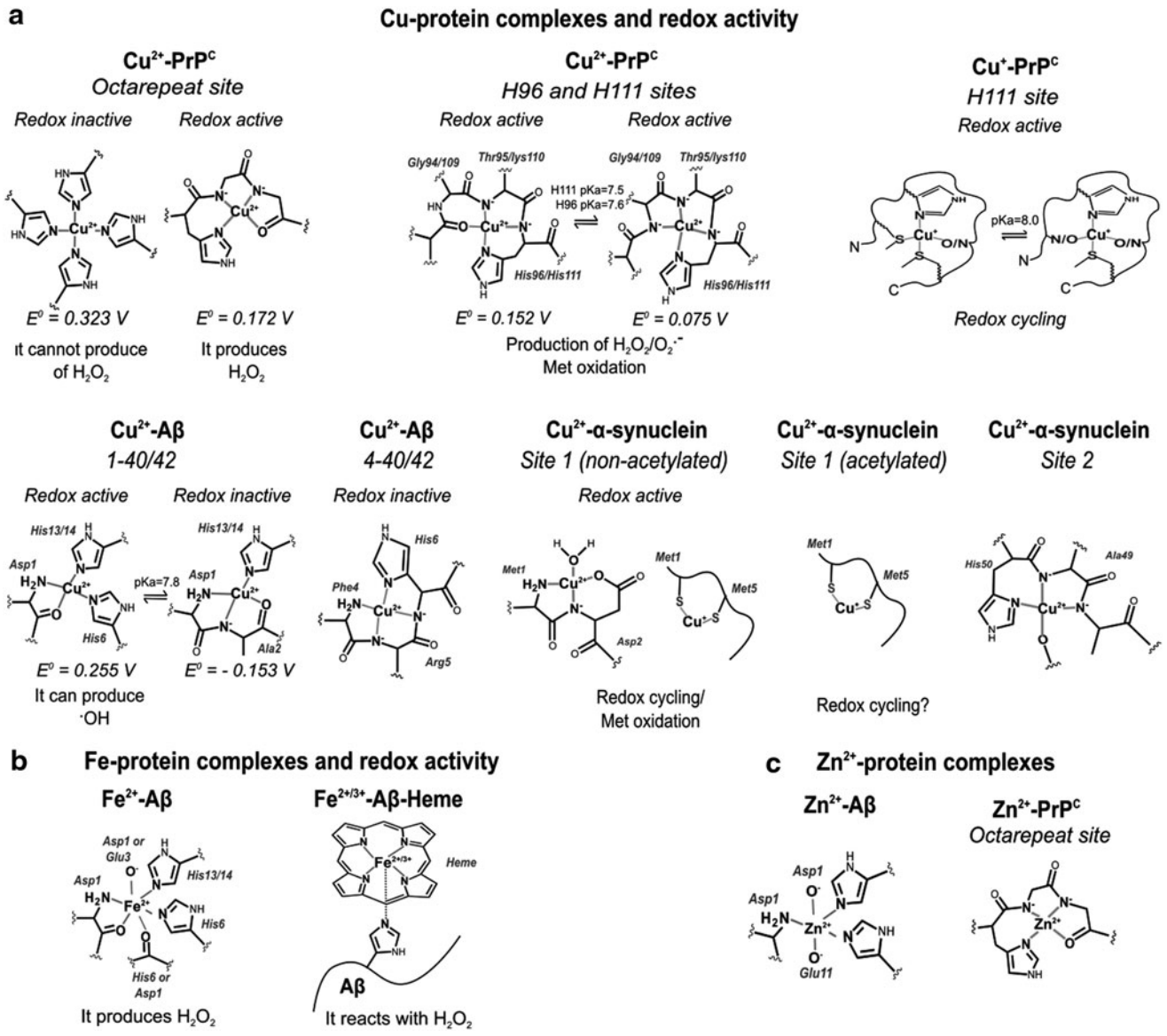


**FIG. 3. Structural features and redox properties of relevant metal-protein complexes in neurodegenerative diseases. (a)** Cu can interact with $PrP^C$, A$\beta$, and α-synuclein. $PrP^C$ has three coordination sites: *histidine (His) His96*, His111, and an octarepeat. $Cu^{2+}$ coordination to His96 and His111 yields very similar complexes different to those generated upon $Cu^+$ binding to His111. The octarepeat region coordinates up to four $Cu^{2+}$ ions depending on the $Cu^{2+}/PrP^C$ stoichiometric ratio. At low Cu:protein ratios, four His residues coordinate $Cu^{2+}$, while at high ratios each His can bind one $Cu^{2+}$ ion. The different coordination chemistry of these Cu-$PrP^C$ complexes is reflected in their redox properties. Similarly, $Cu^{2+}$ coordination to A$\beta$ (1–40/42) yields two different coordination modes that display different redox properties. Recently, coordination to A$\beta$ (4–40/42) has also been described; this site displays higher affinity for $Cu^{2+}$ than A$\beta$ (1–40/42), and it yields a redox silent complex. On the contrary, nonacetylated α-synuclein has two high-affinity binding sites for $Cu^{2+}$: site one involving residues at the N-terminal, and site two at His50. Acetylation of α-synuclein, as described to occur *in vivo*, abolishes $Cu^{2+}$ binding at site 1, although both forms of α-synuclein can bind $Cu^+$. **(b)** $Fe^{2+}$ can interact with A$\beta$, but the redox properties of Fe-A$\beta$ complexes remain unclear. Interestingly, recent studies propose that heme can bind to A$\beta$, yielding a heme (Fe)-A$\beta$ complex with peroxidase activity. **(c)** Finally, $Zn^{2+}$ can bind to the octarepeat region of $PrP^C$ and with A$\beta$. $Cu^{2+}$, cupric ion; Fe, iron; $Fe^{2+}$, ferrous; Zn, zinc.

*in vitro* (20, 33); although this effect is abolished in acetylated α-synuclein (234), which is found in Lewy bodies (10).

While several studies have suggested that Cu-induced aggregation of α-synuclein is directly linked to its neurotoxicity, recent studies suggest a lack of correlation between protein aggregation and cytotoxicity (361); in fact, it has been demonstrated that α-synuclein potentiates the toxicity of Cu in dopaminergic cells in the absence of enhanced accumulation of protein aggregates (8). Clearly, further investigations are needed to completely understand the structural impact of Cu-α-synuclein interactions and their role in PD. On the contrary, acetylation and $Cu^+$ binding to α-synuclein are two synergistic events that turn the intrinsically disordered N-terminal region into an α-helix conformation (228) (Fig. 2d), which displays higher affinity for membranes (75). These observations suggest a potential link between $Cu^+$-α-

synuclein interactions and the proposed function of α-synuclein in vesicle trafficking; a link that might be perturbed in PD.

Different redox modifications of α-synuclein are found in Lewy bodies, including Met oxidation, Tyr nitration (67), and formation of di-Tyr-linked α-synuclein dimers. $Cu^{+}$-α-synuclein complexes have been implicated in these modifications, as they are capable of activating $O_2$, leading to Met oxidation and di-Tyr bond formation (5, 227) (Fig. 3a). In contrast, a recent report suggests that $Cu^{+}$ complexes with oligomeric or fibrillar α-synuclein reduce metal-catalyzed ROS formation (264). Cu has also been shown to potentiate oxidative damage induced by the dopamine (DA) analog 6-hydroxydopamine (63). Cu also interacts with the early-onset PD recessive genes (protein) *PARK7* (DJ-1) and *PARK2* (Parkin). Cu binding to DJ-1 protects against metal toxicity, possibly acting as a chaperone for SOD1 (35, 114); while Parkin mutations have been reported to increase the cytotoxic effect of heavy metals, including Cu (1).

AD is a neurodegenerative disease associated with the degeneration of hippocampal and cortical neurons and eventual loss of memory and progressive dementia (326). Decreased levels of Cu are found in AD brains (42), while the total Cu content and labile nonprotein-bound Cu fraction are increased in the plasma of AD patients (325). Interestingly, polymorphisms in *ATP7B* have been linked to AD (326). AD is associated with the formation of extracellular amyloid plaques composed of Cu bound to Aβ peptides 1–40 and 1–42 fragments produced by cleavage of the APP, as well as N-truncated forms 4–40 and 4–42, and to a lesser extent 11–40/42 (210, 273, 364). The Cu binding features of Aβ 4–40/42 and 11–40/42 are different from those of 1–40/42, leading to the formation of Cu-Aβ complexes with distinct redox properties (Fig. 3a) (229). Different aggregation properties have also been described, as illustrated by the faster fiber assembly rate of Aβ 11–40/42 when compared with 1–40/42 (21). Substoichiometric $Cu^{2+}$ concentrations trigger Aβ aggregation through a different pathway that involves the formation of oligomers more neurotoxic than those generated by the peptide alone (25, 204). On the contrary, the redox activity of Cu-Aβ complexes has also been proposed to lead to the generation of ROS (52, 214), but contradictory results exist as well (264). Interestingly, interaction of Cu with N-truncated 4–40 and 4–42 peptides yields redox-inactive Cu-Aβ complexes (229, 350). A dysfunction in Cu homeostasis in AD is also evidenced by decreased levels of MT-III (390). MTs are capable of exchanging $Cu^{2+}$ with Aβ1–40/42, reducing Cu and stabilizing it. This Cu exchange by MTs has been proposed as a redox-silencing mechanism that prevents ROS formation by Cu-Aβ complexes (223).

The impact of dysfunctional Cu homeostasis in AD might go beyond the neurotoxicity of Cu-Aβ complexes. APP, whose mutations are associated with AD, is a type 1 transmembrane protein that displays three Cu binding sites in its extracellular domain (22, 140). One Cu-binding site is located in the growth factor-like domain, and has been implicated in Cu-induced dimerization of APP, a process that would be important in cell adhesion and signaling (22). Cu was found to induce APP phosphorylation at Thr668 promoting its localization to the axonal membrane, suggesting an important link between Cu and APP functions at the synapse that might be perturbed in AD. Aβ peptides can also interfere with Cu-$PrP^{C}$ interactions implicated in the regulation of NMDAR activity (Fig. 2b) (389). In addition, Cu has been reported to promote the degradation of the low-density lipoprotein receptor-related protein 1 (LRP1) *via* Tyr nitration and proteasomal degradation, which was linked to a decrease of Aβ clearance and its resultant accumulation in brain vasculature (319). Clearly, Cu plays important roles in neuromodulation and signaling processes, which would be perturbed in AD.

### *Iron*

Fe is found in biological systems primarily as ferrous (2+) and ferric (3+) ions (Supplementary Table S1). Fe is a redox-active metal involved in several redox reactions that catalyze the formation of ROS. Fe is tightly bound to Fe storage and transport proteins, while <5% is present as labile redox-active Fe bound to low-affinity molecules. Fe is required as cofactor of several important enzymes for respiration and synthesis of neurotransmitters, including tryptophan hydroxylase (serotonin) and Tyr hydroxylase (norepinephrine and DA), cholesterol, and fatty acids; the latter particularly important for nerve myelination (161, 342). Electron transfer in many Fe enzymes, including the mitochondrial respiratory complexes, is facilitated by heme and Fe/S clusters (291). Fe is also important as cofactor for peroxidases and catalases, which are important for cellular redox homeostasis (6).

Fe is heterogeneously distributed in the brain; it is highly concentrated in the SN, hippocampus, striatum, interpeduncular nuclei (125), and myelin (329) (Supplementary Table S1). Fe homeostasis is regulated by communication between the BBB and astrocytes. In the blood stream, $Fe^{3+}$ is found coordinated to transferrin (Tf) or ferritin (Ft), and as heme. Fe uptake into the BBB can occur by two pathways: (a) by direct transport of $Fe^{2+}$ into the cytosol *via* DMT1; or (b) by endocytosis of Tf-bound $Fe^{3+}$ *via* the Tf receptor (TfR), where the low pH of the endosome causes the release of $Fe^{3+}$ from Tf. In both cases, $Fe^{3+}$ is reduced to $Fe^{2+}$ by the duodenal cytochrome b (Dcytb) or by the six transmembrane epithelial antigen of the prostate 2 (Steap2) ferrireductases and then transported by DMT1 (Fig. 4a) (215, 218).

Fe efflux from endothelial cells to the interstitial space occurs *via* the coordinated activity of the $Fe^{2+}$ transporter ferroportin (Fpn) and the Cu-dependent ferroxidases hephaestin (Hp) and soluble ceruloplasmin (sCp), which oxidize $Fe^{2+}$ to $Fe^{3+}$. Astrocytes regulate the release of Fe from BBB by either secretion of sCp, which stimulates Fe release, or by production of hepcidin, a peptide that induces internalization and ubiquitination of Fpn and thus, decreased Fe efflux (216). Astrocytes also express a glycosylphosphatidylinositol (GPI)-anchored form of Cp, which interacts with Fpn and participates in Fe efflux (Fig. 4a) (144). In general, oligodendrocytes, astrocytes, microglia, and neurons have the same machinery for Fe efflux, involving the concerted action of Fpn with Cp or Hp (60).

The mechanisms of Fe uptake differ between brain cell types. Fe, Tf, and Ft are primarily found in oligodendrocytes (61). Most Tf in the brain is synthesized and secreted by oligodendrocytes as Fe-free Tf or apo-Tf and is required for Fe mobilization within the interstitial fluid brain (87). Although oligodendrocytes and astrocytes can accumulate high levels of Fe, they do not express TfR. Fe uptake into

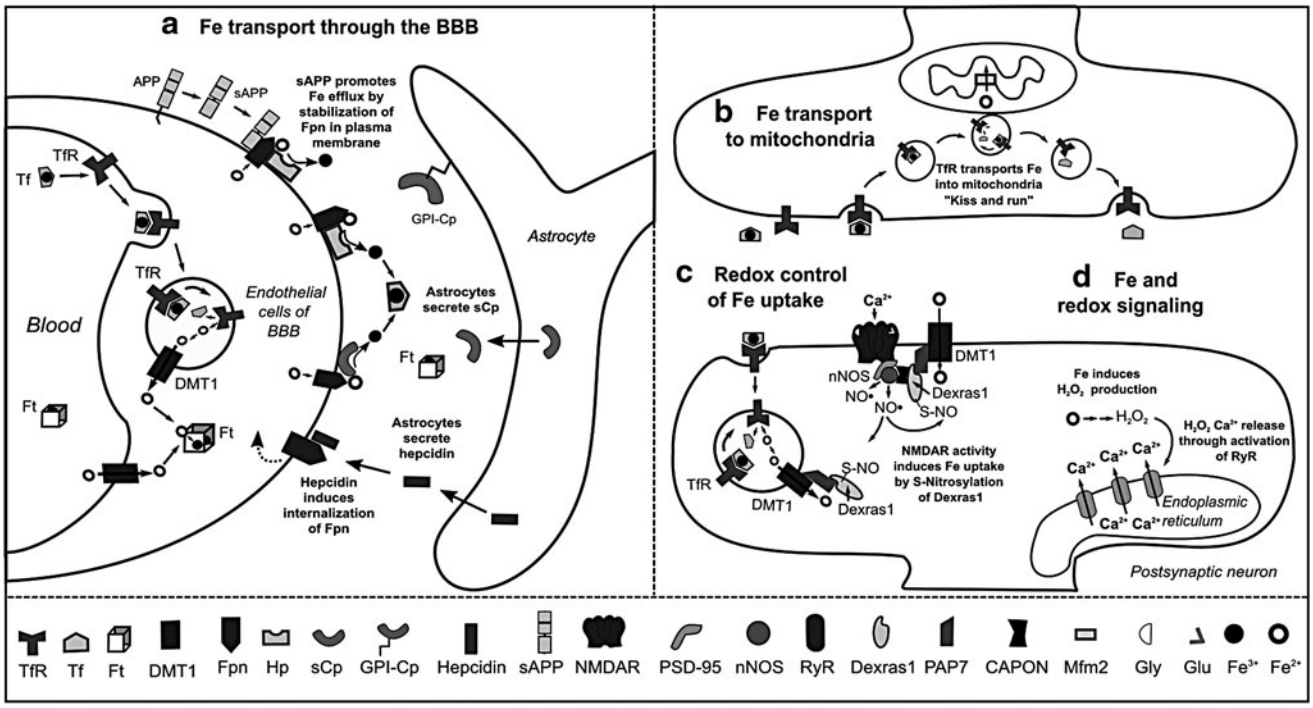


**FIG. 4. Fe transport and redox signaling. (a)** Fe transport across the BBB occurs *via* DMT1 or by endocytosis of Tf- or Ft- bound $Fe^{3+}$; followed by Fe release *via* the concerted action of Fpn with a ferroxidase, either Hp or sCp. Astrocytes modulate Fe transport through the production of sCp, sAPP, and hepcidin. sAPP stabilizes Fpn, while hepcidin induces its internalization. **(b)** Mitochondria are the organelles with highest demand of Fe. Although Fe transport into mitochondria is not fully understood, the recently demonstrated interaction between mitochondria and TfR-containing endosomes supports the ''kiss and run'' hypothesis. Inside the mitochondria, Fe crosses the inner membrane by Mfrn2. **(c)** In hippocampal neurons, NMDAR activation induces $Ca^{2+}$ entry and $NO^{\bullet}$ production by nNOS, causing S-nitros(yl)ation of Dexras1, which in turn induces Fe uptake through DMT1 and TfR. **(d)** Increased intracellular Fe also induces $H_2O_2$ production, inducing $Ca^{2+}$ release from ER *via* RyR, which is important for neuronal plasticity. Dexras1, Ras-related dexamethasone induced 1; ER, endoplasmic reticulum; $Fe^{3+}$, ferric; Fpn, ferroportin; Ft, ferritin; $H_2O_2$, hydrogen peroxide; Hp, hephaestin; Mfrn2, mitoferrin-2; $NO^{\bullet}$, nitric oxide; PAP7, peripheral benzodiazepine receptor-associated protein 7; RyR, ryanodine receptor; sAPP, soluble fragment of APP; sCp, soluble ceruloplasmin; Tf, transferrin; TfR: transferrin receptor.



oligodendrocytes has been recently proposed to involve the internalization of H-Ft by a mucin-domain containing protein (Tim-2) (343). In astrocytes, ascorbate-dependent $Fe^{2+}$ uptake is mediated by DMT1 (167) and transient-receptor potential channels (265).

In neurons, the mechanisms involved in Fe uptake are still unclear. DMT1 is found in human neurons and its expression levels are negatively regulated by Fe exposure *via* ubiquitination and degradation (134). However, DMT1 seems to colocalize in cytoplasmic vesicles with TfR and to primarily contribute to Tf-bound Fe uptake (232, 266). The ZIP8 and ZIP14 members of the ZIP family of metal transporters have also been demonstrated to mediate $Fe^{2+}$ uptake (188) and to be expressed in the brain (113). A recent report demonstrates that ZIP8 at the plasma membrane is the primary transporter involved in non-Tf-bound Fe into neurons (145).

In brain cells, the ferrireductases Dcytb and stromal cell-derived receptor, SDR2, are expressed in astrocytes (192, 351), while SDR2 and Steap2 are found in neurons (145). In all cases, it is important to note that Fe uptake and efflux always require redox cycling between $Fe^{2+}$ and $Fe^{3+}$ oxidation states.

Cytosolic Fe is stored by Ft, which is a protein complex formed by 24 subunits of heavy-ferritin (H-Ft) and light-ferritin (L-Ft) chains. Ft genes are regulated by Nrf2 (272). While H-subunits have ferroxidase activity and participate in Fe uptake, L-subunits are involved in Fe mineralization and long-term storage. Ft can store $\sim$4500 Fe ions in its core. However, the mechanism by which Fe is released from Ft remains unclear (47). Although Fe is mainly stored in the cytosol, mitochondria are the organelles with the highest Fe demand, as they require Fe-S clusters and heme groups for electron transfer during respiration. Similarly, mitochondria are also considered the main sources of ROS under physiological conditions. Therefore, mitochondrial Fe homeostasis must be tightly regulated to prevent uncontrolled ROS production.

The mechanism(s) involved in mitochondrial Fe uptake are still unclear. Fe is delivered by a direct ''kiss and run'' interaction between the endosomes containing Tf-bound Fe and mitochondria (69). Subsequently, the metal crosses the inner membrane *via* the Fe importer mitoferrin-2 (Mfrn2) (Fig. 4b). In the mitochondrial matrix, Fe is either used for the biogenesis of prosthetic groups, Fe-S clusters, or heme, or it is stored by mitochondrial ferritin (FtMt), which is homologous to H-Ft, and it displays the same Fe uptake efficiency but lower ferroxidase activity. Fe distribution between mitochondria and cytosol depends on FtMt expression and the export of Fe prosthetic groups (106, 176).

Heme is a component of globins, a superfamily of heme-containing proteins involved in binding and/or transporting $O_2$, and a cofactor for cytochromes, catalase, NOX, NOS, and myeloperoxidase, which is also found in microglia (59, 151, 198). Hemoglobin (Hb) is involved in $O_2$, NO, and carbon dioxide ($CO_2$) transport in cells of erythroid lineage, but Hb$\alpha$ and $\beta$ transcripts have been found in dopaminergic neurons, oligodendrocytes, and cortical or hippocampal astrocytes as well (32). Brain Hb levels are altered during neurodegeneration (94), but their functional consequences are unclear. Neuroglobin is another globin expressed in the CNS and PNS and found in both neurons and astrocytes. Neuroglobin has a higher affinity for $O_2$ than Hb and exerts a protective effect against oxidative and ischemic insults (7, 43, 81, 179, 331, 368). Oxidative stress has the potential to release heme from Hb, and labile heme can induce oxidative damage *via* Fenton reactions or NOX activation (165, 239). Heme oxygenases (HOs) catalyze heme degradation. HO-1 transcription is induced by oxidative stress, inflammation, hypoxia, and metal exposure, while HO-2 is constitutively expressed. HO activity has both antioxidant and pro-oxidant effects that relate to the ability of the cell to detoxify labile Fe released from heme (286, 369).

Cellular Fe trafficking is controlled by the iron regulatory proteins (IRP) IRP1 and IRP2 which regulate translation of proteins involved in Fe storage (H-Ft and L-Ft), Fe uptake (TfR), and Fe efflux (Fpn). Under conditions of Fe depletion, IRPs can bind to messenger RNAs (mRNAs), decreasing the levels of Ft and Fpn and promoting the translation of TfR *via* the stabilization of its mRNA. Conversely, Fe overload prevents mRNA binding to IRPs, promoting Ft and Fpn translation while reducing TfR levels. The ability of IRP1 and IRP2 to bind mRNAs exerts an important redox control *via* two distinct mechanisms. IRP1 has two conformations: (a) a closed one, triggered *via* an Fe-S cluster that prevents mRNA binding and has aconitase activity; and (b) an open conformation that is favored when $NO^{\bullet}$, $O_2$, and $H_2O_2$ cause dissociation of the Fe-S cluster. In contrast, the ability of IRP2 to bind mRNAs is controlled by proteasomal degradation, involving the ubiquitin ligase F-box/LRR-repeat protein (FBXL5), which is also an Fe and $O_2$ sensing protein. FBXL5 has a binuclear nonheme Fe site, and it is stabilized on Fe and $O_2$ binding, promoting IRP2 degradation. Together, these two mechanisms illustrate the redox control of cellular Fe trafficking (164).

In neurons, Fe uptake is induced by a redox signaling cascade that starts with the activation of the NMDAR (Fig. 4c). Increased intracellular $Ca^{2+}$ induces $NO^{\bullet}$ production by the neuronal (n) NOS, leading to S-nitros(yl)ation of the small GTPase Dexras1 (Ras-related dexamethasone induced 1), which in turn induces Fe uptake through DMT1 and TfR (51). In hippocampal neurons, NMDAR activation increases intracellular Fe and $H_2O_2$ production, which activates the redox-sensitive ryanodine receptor (RyR) and promotes $Ca^{2+}$ release from the ER (237) (Fig. 4d). Thus, Fe uptake is clearly part of a redox signaling mechanism that might be important for neuronal plasticity.

Cell death induced by an increase in the labile Fe pool within cells has been defined as a specific entity named ferroptosis. Ferroptotic cell death is a necrotic-like cell death characterized by Fe-dependent lipid peroxidation due to either the formation of $^{\bullet}OH$ and $H_2O_2$ *via* Fenton-like reactions or the activation of lipoxygenases. As such, ferroptosis is counteracted by Fe-chelators and the GSH/GPX4 system. *GPX4* knockout in neurons induces motor neuron degeneration, paralysis (53), and cognitive impairment (121). During ferroptosis, Tf-dependent Fe uptake and release of Fe from lysosomal compartments have been shown to act as important sources for Fe. Interestingly, lysosomal permeabilization is a common phenomenon observed in a number of neurodegenerative disorders, including PD (40, 73). However, during pathological conditions such as neurodegeneration and hemorrhagic stroke, cell death is likely to involve a combination of different pathways and a complex balance between them, including apoptosis, necrosis, autophagic cell death, and ferroptosis as well (77, 143, 357, 398).

During aging, accumulation of Fe in the frontal lobes and striatum is associated with motor dysfunction, loss of myelin sheaths, and memory decline (2, 327). Inflammation, a common hallmark of many brain disorders, increases the expression of DMT1 and hepcidin (354).

Abnormal accumulation of Fe is a common feature of many neurodegenerative diseases, including a group of twelve diseases known as neurodegeneration with brain iron accumulation (NBIA) (225). The most common clinical features of NBIA are movement disorders, such as ataxia, parkinsonism, and dystonia. Although in NBIA Fe is usually accumulated in the globus pallidus, and in some cases in the SN and cerebellum, only two types of NBIA involve the dysfunction of a protein that participates in Fe trafficking: aceruloplasminemia (lack of Cp) and neuroferritinopathy (loss of function mutations in L-Ft) (65, 261, 290, 360). Another neurodegenerative disease associated with disturbed Fe homeostasis is Friederich ataxia, which is linked to mutations in frataxin, an Fe-chaperone involved in Fe-S cluster biogenesis (55).

Altered Fe homeostasis and mitochondrial dysfunction are also hallmarks of PD. While no significant differences in Fe levels have been found in the blood, serum, and CSF (203), Fe is increased in the SN of PD patients (365). Neuromelanin is an Fe-rich pigment found in the dopaminergic neurons targeted in PD (A9) and it has been suggested that its presence makes this neuronal population vulnerable to oxidative damage (91). Decreased levels of serum Cp or its oxidation has also been proposed to exacerbate Fe accumulation in PD (149, 251). DMT1 is found increased in the SN of PD brains (294). Interestingly, Parkin regulates DMT1 expression levels *via* ubiquitination and proteasomal degradation (289).

Fe accumulation in SN might be associated with a dysfunctional delivery of Fe to mitochondria through the ''kiss and run'' interaction mentioned above (Fig. 4b) (69). Indeed, a Tf/TfR2-dependent mechanism for Fe transport into the mitochondria of dopaminergic neurons has been described (211), while a role for *Tf* and *TfR2* genes in Fe accumulation and mitochondrial dysfunction in PD has been implicated as well (285).

Mitochondrial dysfunction in PD might lead to decreased synthesis of Fe-S clusters, which in turn would activate IRP1 binding to mRNAs, resulting in augmented Fe accumulation (142). Accordingly, knockdown of mitochondrial Grx2 impairs Fe-S cluster biogenesis in dopaminergic cells, decreases the activity of Complex I and aconitase, and increases the activation of IRP1 (171). Loss of PINK1/*PARK6*, another autosomal early-onset PD-related gene, has also been

reported to inactivate Fe-S clusters *via* $O_2^{\bullet-}$ formation. As such, overexpression of FtMt exerts a protective effect on mitochondrial dysfunction and oxidative stress induced by loss of PINK1 (88).

HO-1 protects against neuronal cell death induced by PD-related mitochondrial toxins and α-synuclein, suggesting a role of heme in dopaminergic cell loss. However, HO-1 becomes toxic at high levels, but this effect is counteracted by FtMt (391). Interestingly, the pathogenic PINK1 mutation G309D impairs the induction of HO-1 on oxidative stress (56).

$Fe^{2+}$ can interact with the negatively charged C-terminal region of α-synuclein, accelerating its amyloid aggregation (34). Conversely, α-synuclein overexpression also enables Fe accumulation (254). In PD, oxidative stress has been linked to intracellular Fe levels due to the redox activity of Fe-DA and Fe-neuromelanin complexes (400). Furthermore, overexpression of FtMt protects against neuronal cell death induced by 6-hydroxydopamine (313). Interestingly, a recent report demonstrates that depletion of Fpn has no consequence on the survival of dopaminergic neurons. In contrast, loss of TfR causes Fe deficiency and a PD-like neurodegeneration in mice (212). Previous findings have also suggested a link between Fe deficiency and predisposition to PD, while Fe overload seems to be protective (157, 190, 271). Thus, the exact role of Fe homeostasis in PD is still far from being understood.

A role of Fe in AD has also been proposed based on observations showing that Fe levels are decreased in the serum of AD patients (324), and increased in AD-related brain areas such as the hippocampus, neocortex, and basal ganglia (168). Mitochondrial dysfunction is also observed in AD, which might be related to a disruption in Fe homeostasis. Accordingly, overexpression of FtMt protects against the toxicity of A$\beta$ (380). Fe is accumulated in amyloid plaques in the AD brain (38, 194), consistent with the observation that A$\beta$ is able to bind $Fe^{2+}$ *in vitro* (Fig. 3b) (39). Fe bound to A$\beta$ plaques catalyzes $H_2O_2$ formation (321).

A novel mechanism for controlling Fe efflux from the BBB was recently discovered, involving the soluble fragment of APP (sAPP), which interacts with Fpn, stabilizing its membrane location (219) and counteracting the effects of hepcidin (216). APP expression is also negatively regulated by IRP1 (57), and production of sAPP and A$\beta$ is increased in AD. Thus, the proposed role of sAPP in Fe movement from BBB into the brain is consistent with the accumulation of Fe in AD brains and the observation of higher levels of A$\beta$ plaques surrounding the brain blood vessels. Indeed, BBB damage is a common feature in AD (217).

Heme metabolism also contributes to AD. APP binds and inhibits HO-1 activity and this effect is enhanced by pathogenic APP mutations (334). In addition, A$\beta$ can form a complex with heme that displays peroxidase activity (16, 112). Conversely, neuroglobin has been proposed to protect against the toxicity of 1–42 A$\beta$ peptides (180). Overall, disruption of Fe homeostasis in AD is likely linked to oxidative stress, and it may also impair NMDAR- and Fe- dependent redox signaling mechanisms (Fig. 4d) impacting neuronal plasticity and memory.

### *Manganese*

Mn is an essential metal required for the activity of a plethora of enzymes, including hydrolases, isomerases, ligases, lyases, oxidoreductases, and transferases, involved in diverse metabolic functions such as amino acid (arginase and glutamine synthetase [GS]), lipid, protein, and carbohydrate metabolism (phosphoenolpyruvate decarboxylase), as well as protein glycosylation, energy production, and redox homeostasis (SOD2). The main source for Mn intake is food, but occupational/environmental exposures also occur associated with mining, smelting, welding, alloy, battery, pesticide, and electrical industries. Ingestion and inhalation are the primary routes of Mn exposure. Importantly, inhalation can transfer Mn directly to the brain. The brain is a major target for chronic Mn intoxication (manganism) where Mn is accumulated in nonheme Fe-rich regions. Manganism is defined as a parkinsonism that results in dystonia, hypokinesia, and rigidity as a consequence of impaired neurotransmitter function (133, 352).

Mn can be transported *via* Tfr and DMT1 as it competes with Fe for its binding sites (333). Other proposed Mn transporters include the Zn carriers ZIP-8 and ZIP-14, voltage-regulated, store-operated, and ionotropic Glu receptor $Ca^{2+}$ channels, and the Mn-citrate complex shuttle (Fig. 5a) (352). Mn is efficiently detoxified by Fpn at the plasma membrane. In addition, Mn is also detoxified by sequestration in the Golgi *via* the solute carrier family 30 member 10 or human Zn transporter 1 (*SLC30A10*/hZnT1) transporter whose mutations are directly associated with manganism (178). Alternatively, the $Ca^{2+}/Mn^{2+}$ ATPases SPCA1 and SPCA2 also detoxify Mn *via* the secretory pathway (356). Finally, the autosomal recessive early-onset PD-related gene ATP13A2/*PARK9* mediates sequestration of Mn in lysosomes (Fig. 5b) (336). Recently, direct comparison of different detoxification proteins demonstrated that hZnT1 and SPCA1, but not ATP13A2, are involved in Mn detoxification and resistance (246).

$Mn^{2+}$ is the predominant species found in cells that can be oxidized to the more reactive and toxic species $Mn^{3+}$. Neither $Mn^{2+}$ nor $Mn^{3+}$ can generate free radicals *via* Fenton-type reactions. However, it has been proposed that Mn enhances ROS generation *via* the Mn-catalyzed autoxidation of DA that involves the redox cycling of $Mn^{2+}$ and $Mn^{3+}$ and the generation of ROS and DA-o-quinone (Fig. 5c) (79, 89). Mn accumulates in the mitochondria *via* the mitochondrial $Ca^{2+}$ uniporter (MCU) (111) and increases the accumulation of labile Fe. Both mitochondrial dysfunction and Fe lead to ROS formation and oxidative damage (Fig. 5d) (54, 206). Mn specifically generates $H_2O_2$ but not $O_2^{\bullet-}$ in the mitochondria *via* complex II (92, 186, 322). Furthermore, Mn impairs oxidative phosphorylation and ATP production.

Astrocytes seem to have a high capacity to accumulate Mn (14). Mn-induced neurotoxicity has been linked to a decrease in Glu uptake by astrocytes (156) leading to excitotoxicity in neurons, as well as the induction of inflammation and increased activity of NOS (Fig. 5e) (95, 185).

### *Zinc*

Zn is a redox-inactive transition metal ion with an oxidation state of +2. The majority of intracellular Zn is bound to proteins and is distributed in the cytoplasm (~50%) and nucleus (~40%) (304) (Supplementary Table S1). The function of Zn as enzyme cofactor is limited to structural roles (*e.g.*, SOD1) (312), or as a Lewis acid that activates

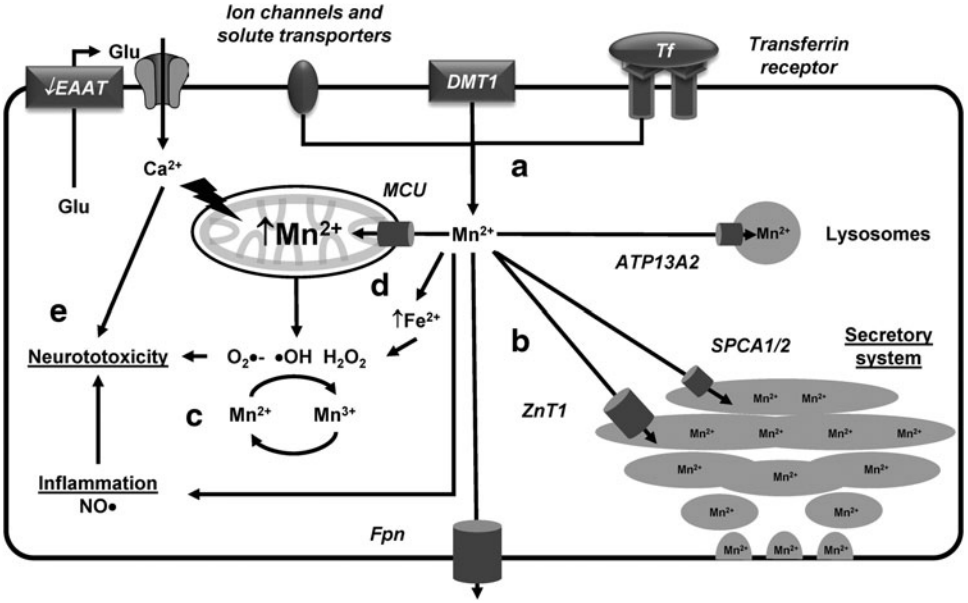


**FIG. 5. Mn homeostasis and toxicity. (a)** Mn can be transported *via* Tf and DMT1, as well as *via* Zn carriers, ion channels, and shuttle systems. **(b)** Mn is detoxified by Fpn at the plasma membrane or by its sequestration in the secretory system *via* the ZnT1 transporter or the $Ca^{2+}/Mn^{2+}$ ATPases SPCA1/2. ATP13A2 mediates sequestration of Mn in lysosomes. **(c)** Mn enhances ROS generation *via* Mn-catalyzed autoxidation of DA that involves the redox cycling of $Mn^{2+}$ and $Mn^{3+}$ and the generation of ROS and DA-o-quinones. **(d)** Mn accumulates in the mitochondria *via* the MCU and increases the accumulation of labile Fe. **(e)** Mn-induced neurotoxicity is linked to oxidative damage and mitochondrial dysfunction, and also to a decrease in Glu uptake by the EAATs in astrocytes (*arrow* in EAAT indicates reversal of Glu transport) leading to excitotoxicity in neurons, as well as the induction of inflammation. DA, dopamine; EAAT, excitatory amino acid transporter; Glu, glutamate; MCU, mitochondrial $Ca^{2+}$ uniporter; Mn, manganese; ROS, reactive oxygen species; SPCA, secretory pathway $Ca^{2+}$ ATPase; ZnT, zinc transporter.



substrates for nucleophilic attack (*e.g.*, carbonic anhydrase, where $Zn^{2+}$ catalyzes the hydration of $CO_2$ to form bicarbonate [$HCO_3^-$]) (138). Enzymes with Zn-dependent catalytic activity control many cellular processes, including DNA synthesis and brain development. Zn also plays an important role in cell signaling associated with development and learning. In the brain, Zn is highly concentrated in the hippocampus and cortex (304).

The key players in Zn homeostasis are the ZIP transporters that mediate Zn uptake into the cytosol, the zinc transporters (ZnT) that participate in Zn efflux, and MTs involved in Zn chelation. ZIP1 is expressed in astrocytes and microglia (303). In neurons Zn uptake is mediated by, voltage-gated $Ca^{2+}$ channels, ZIP1 and 3 transporters, as well as $Ca^{2+}$ and the $Zn^{2+}$ permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR) (146, 278, 335). Interestingly, Zn has been reported to be transported into postsynaptic neurons through a complex formed by $PrP^C$, which is evolutionary linked to ZIP proteins, and the AMPAR (Fig. 6b) (373). ZnT1 is expressed in astrocytes, microglia, and oligodendrocytes, and its expression levels are directly modulated by Zn (247). Hypoxia decreases the levels of ZnT1 in astrocytes inducing the accumulation of cytosolic Zn (257). At the postsynaptic density, the ZnT1 transporter interacts with NMDAR and this complex is regulated during synaptic plasticity (Fig. 6c) (222, 373).

Cytosolic Zn is distributed to different organelles, including synaptic vesicles, Golgi, ER, and mitochondria. Similarly, Zn uptake by these organelles is performed by ZnTs, while ZIPs participate in Zn efflux into the cytosol (155). Zn modulates cellular signaling pathways and acts as a neuromodulator. ZnT3 participates in the transport and accumulation of Zn within synaptic vesicles of glutamatergic terminals (Fig. 6a) (125). During synaptic transmission, Zn is released with Glu, where it inhibits the activity of NMDAR and AMPAR (9, 154), modulating neuronal excitability and long-term synaptic plasticity (long-term potentiation [LTP] and long-term depression). In addition, Zn regulates the activation of the tropomyosin kinase receptor B (TrkB) (304). A metabotropic Zn-sensing receptor (mZnR) has also been reported (31). At the synapse, Zn can also bind to MTs, A$\beta$, and $PrP^C$ (Figs. 3c and 6).

Although $Zn^{2+}$ is a nonredox-active metal ion, it participates in redox signaling through several mechanisms. MTs bind about a fifth of the intracellular Zn with a stoichiometry of 1:7. MTs and proteins containing Zn-finger domains use Cys residues to bind to $Zn^{2+}$ ions. Zn coordination stabilizes the reduced state of Cys thiol groups preventing their oxidation and subsequent formation of disulfide bonds. Transcription of MTs is induced by oxidative stress *via* Nrf2, and by heavy metal exposure *via* metal-response elements (MREs). MREs are recognized by the Zn-finger domain containing protein metal-responsive transcription factor-1 (MTF1, also known as MRE-binding transcription factor-1 or metal regulatory transcription factor-1). MTF1 senses Zn levels. Furthermore, *via* Zn displacement from MTs or Cys oxidation, MTF1 also senses heavy metal toxicity (Cd) and oxidative stress. In addition to MTs, MTF1 regulates the transcription of a number of genes involved in redox homeostasis and metal ion detoxification, including Zn transporters,

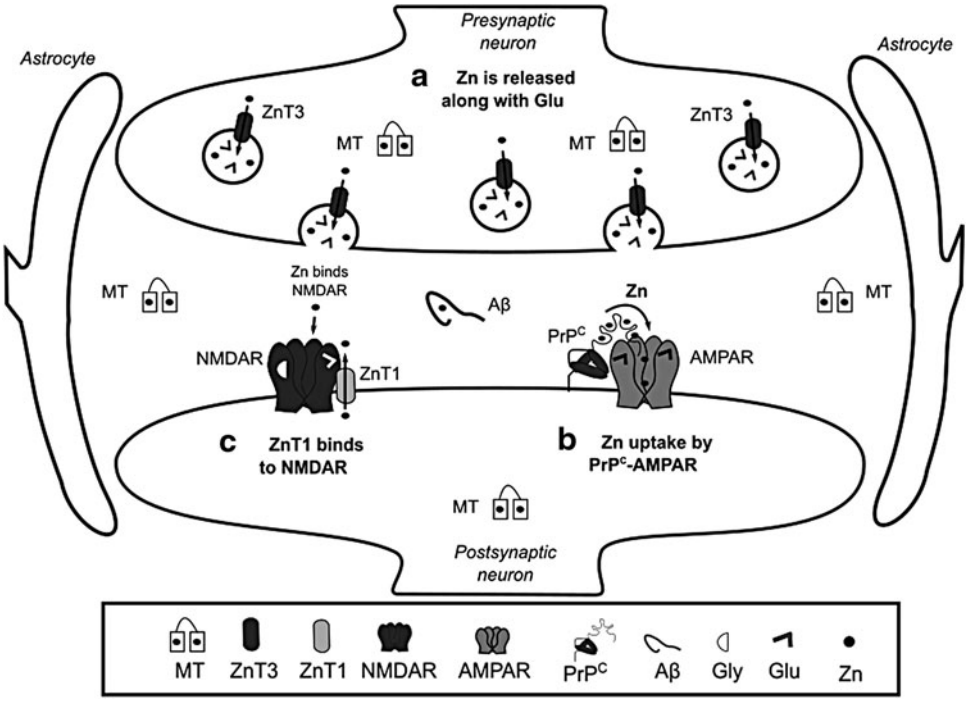


**FIG. 6. Zn trafficking at the synapse. (a)** Zn ions are loaded into neurotransmitter vesicles by ZnT3, and are coreleased with Glu during synaptic transmission. In the synaptic cleft, Zn can bind to MT, A$\beta$, $PrP^C$, and NMDAR. **(b)** Recently, two links between synaptic activity and Zn transport have been discovered: Zn uptake by the postsynaptic terminal occurs *via* a complex formed by $PrP^C$ and AMPAR, **(c)** while ZnT1 interacts with NMDAR and modulates spine morphology. AMPAR, $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor.



Fr, Fpn, ATP7(A/B), Trx, selenoproteins, and $\gamma$-glutamate-cysteine ligase (GCL), a rate-limiting enzyme in GSH synthesis (119). The role of MTF1 in brain function and redox homeostasis is unclear. MTF1 has been shown to regulate the expression of $\beta$-synuclein (220), which is thought to act as a negative regulator of its homologue $\alpha$-synuclein (126). Interestingly, deletion of *MTF1* induces lethality in Parkin-deficient flies (*Drosophila melanogaster*) (293).

Altered Zn levels have been reported to promote neuronal injury. Upon Cys oxidation, Zn is released from MTs (255). Cellular acidification also releases intracellular Zn in neurons (159). In a recent report, AMPA-induced oligodendrocyte cell death was shown to be linked to Zn mobilization from mitochondria and protein-bound pools that were mediated by cytosolic acidification, independently from ROS (213). Exposure of mitochondria to Zn promotes increased ROS formation (305). While no specific mitochondrial Zn transporter(s) has been identified, potential candidates include the MCU, ZIP8, and Znt2 transporters (30, 199, 307). Zn also increases NOX-derived ROS formation and NOS activity (162). High extracellular Zn enhances microglia activation and ROS formation (130). Zn deficiency also induces oxidative stress *via* a reduction in the activity of SOD1 (378), and an impairment in the transcriptional regulation of GCL by Nrf2 (253).

Alterations in Zn homeostasis are associated to neurodegenerative diseases. Serum levels of Zn are decreased in AD patients (358), while Zn is enriched in A$\beta$ plaques (364). Furthermore, a decrease in the levels of Znt3 and MT-III (390) is found in AD. Importantly, the predominant localization of A$\beta$ plaques in Zn-containing glutamatergic synapses might explain why they are primarily found in the neocortex (304). Zn promotes a rapid, but reversible, aggregation of A$\beta$ that is different to the aggregation of A$\beta$ or A$\beta$-Cu complexes (25). Zn also reduces the toxicity of Cu-induced A$\beta$ aggregates (214). *ZnT3* knockout increases soluble A$\beta$ in transgenic APP mice corroborating the role of extracellular Zn in plaque formation (172).

Elevated Zn levels have been found in the SN of PD brains (74), while reduced levels of Zn in serum and plasma have been linked to an increased risk for PD (80). Zn has been shown to potentiate the toxicity of DA as well (189). Furthermore, Zn chelation reduces the toxicity of mitochondrial PD-related toxins (310). Recently, ATP13A2 was identified as a Zn transporter localized to multivesicular bodies. Loss of function mutations of *ATP13A2* induces alterations in Zn homeostasis and mitochondrial dysfunction (259).

### Xenobiotic Metals

Measurable concentrations of xenobiotic metals with no physiological functions are present in humans (Supplementary Table S1) (103). In addition, environmental or occupational exposures to xenobiotic metals may take place by inhalation, ingestion, or skin penetration and are often linked to the development of toxicity and pathological conditions (Supplementary Table S1). Metals can reach the CNS from the vascular lumen affecting neuronal and glial function. Metals hijack transport systems of essential metals to pass through the BBB and enter neuronal tissues (molecular mimicry). Metal toxicity is largely attributable to their physicochemical properties, which mediate their interference with cellular biochemical systems, including redox-related processes (205, 355).

Environmental or occupational exposure to xenobiotic metals has been reported to contribute to neuronal dysfunction (cognitive, motor, and behavioral) and in some cases, neurodegeneration. However, the mechanisms involved are largely unclear. We next review the sources and routes of exposure to xenobiotic metals; the metabolic pathways involved in their transport and activation, and the mechanisms by which they alter cellular redox balance to promote neurotoxicity.

*Arsenic*

As is naturally present in air, water, and soil and is the 20th most abundant element in the earth's crust and 12th in the human body. This metal is named inorganic As (iAs) when found combined with other elements such as $O_2$, chlorine (Cl), and sulfur (S). Combined with carbon (C) and hydrogen (H) is referred to as organic As. In the environment and within the human body, iAs predominantly exists in two oxidation states: arsenite +3 (or AsIII, found as arsenic trioxide [$As_2O_3$], sodium arsenite [$NaAsO_2$], and arsenic trichloride [$AsCl_3$]), and arsenate +5 (or AsV found as arsenic pentoxide [$As_2O_5$], arsenic acid [$H_3AsO_4$], and arsenates [$PbHAsO_4$, $Ca_3(AsO_4)_2$]).

iAs has been widely used as a therapeutic agent to treat leukemia. Currently, iAs compounds are predominantly used in pesticides, herbicides, cotton desiccants, wood preservatives, alloys for batteries, and in semiconductors and light-emitting diodes. Millions of individuals are currently exposed to iAs across the world due to natural groundwater contamination. Fish and crustaceans contain very high levels of organic arsenobetaine but no toxicity has been reported *in vivo* (382). The concentration of iAs in natural surface and groundwater is generally about 1 parts per billion (ppb) of water but it may exceed 1000 ppb in contaminated areas or where iAs soil levels are high (118, 200) (Supplementary Table S1).

While As is considered a carcinogen, in the brain, acute exposure to iAs can induce encephalopathy, with symptoms such as confusion, hallucinations, reduced memory, and emotional lability (exaggerated changes in mood or affect). Long-term exposure to lower levels of iAs can lead to the development of peripheral neuropathies.

There are reports of neurobehavioral alterations (cognitive function, verbal abilities, long-term memory, and motor skills) in children exposed to As concentrations ranging from 5 to 50 ppb in water, in Bangladesh (260), Mexico (45, 287), and in the United States (370). Although scientific understanding of the developmental neurotoxicity of As is still evolving, epidemiological and toxicological studies clearly show that As is a developmental neurotoxicant that affects intellectual function. Moreover, exposures even below current safety guidelines are associated with decrements in full-scale intelligence quotient (IQ) and memory (90, 347). Evidence in experimental models, including mice, rats, *Caenorhabditis elegans* (worm), and *Danio rerio* (zebrafish), has replicated many of the observations in humans supporting the notion that As can lead to cognitive, locomotor, and neurological impairment (85). Gestational exposure to $NaAsO_2$ leads to a significant iAs accumulation in the mice offspring's brain (280). As neurotoxicity has been linked to changes in neurotransmitter metabolism and synaptic transmission (85, 276, 280). However, the mechanisms involved remain unclear.

In the environment, oxygenated water contains iAsV species, while in reducing environments iAsIII species are prevalent. iAsV enters cells through phosphate transporters to be subsequently reduced to iAsIII, while iAsIII is transported *via* aqua(glycerol)porins (AQP), organic anion transporters, and glucose transporters (GLUT) (Fig. 7a) (44, 187, 348). Once in the cytoplasm, iAsIII is methylated by different mechanisms. Oxidative methylation (Fig. 7b) is mediated by arsenite methyltransferase (AS3MT) that uses S-adenosylmethionine (AdoMet) as a cosubstrate. AS3MT methylates iAsIII to monomethylarsonic acid or arsonate (MMAV) that is reduced to monomethylarsonous acid (MMAIII) before being methylated again to dimethylarsinic acid (DMAV) by AS3MT (353, 372). Finally, DMAV is reduced generating dimethylarsinous acid (DMAIII). The reduction of pentavalent arsenicals (iAsV, MMAV, and DMAV) in this pathway is now well recognized to be mediated by the Trx/TR system, but GSH seems to increase the methylation rates by an unknown mechanism (76).

Developmental exposure to As alters the methylation patterns of genes involved in neuroplasticity likely due to changes in AdoMet, but its long-term implications are unclear (207). Recent *in vivo* studies demonstrated that the alterations in synaptic plasticity (LTP), memory, and learning induced by gestational exposure to iAs were associated with an increase in extracellular Glu levels and downregulation of AMPAR subunits (244).

As methylation *via* the GSH conjugation mechanism is based on the formation of GSH complexes with iAsIII resulting in arsenic triglutathione [$As(SG)_3$] (Fig. 7c). Conjugation of iAsIII with GSH has been proposed to occur nonenzymatically, but enzymatically as well by the activity of glutathione-S transferases (GST isoforms GSTO1, GSTM1, or GSTP1) (173, 372). $As(GS)_3$ is subsequently methylated by AS3MT to form monomethylarsinic diglutathione [$MMA(GS)_2$] and then again to generate dimethylarsinic GSH [DMA(GS)]. At low GSH levels, As(GS) conjugates are hydrolyzed and then oxidized to generate MMAV and DMAV (372). A third mechanism for iAsIII methylation has been recently proposed, where instead of As(GS) conjugate formation, iAsIII binds to protein-Cys (thiol) and is methylated while still being conjugated to proteins (Fig. 7d). This hypothesis is supported by the preferential binding of iAsIII to protein-Cys when compared to GSH (284).

Methylated (and maybe unmethylated) As metabolites are exported through the multidrug resistance proteins (MRP1, MRP2, or MRP4) (173, 317, 388) (Fig. 7e). AS3MT is ubiquitously expressed in all brain regions, and animal studies have shown that the iAs that crosses the BBB is methylated and accumulated across the brain, with the highest accumulation observed in the pituitary gland (297). Interestingly, knockout mouse for P-glycoprotein accumulates more As in the brain (183). Endothelial cells and astrocytes feet surrounding capillaries are the first barrier of detoxification of xenobiotics entering from the circulation. We (unpublished data) and others have observed that the resistance of astrocytes to iAsIII is mediated by MRPs (332).

iAs generates ROS and dimethylarsenic or peroxyl radicals that in turn lead to lipid peroxidation and the accumulation of oxidized by-products (MDA and HNE) (Fig. 7f). Importantly, MMAIII and DMAIII are proposed to be more potent toxicants than iAsIII due to their increased ability to generate radicals (392). Oxidative stress has been reported in brain regions of different animal models and in neurons and glial cell cultures exposed to As compounds (48, 107, 108, 243, 394).

Mitochondria have been proposed to be a primary source for ROS formation by iAs (Fig. 7g) (97, 150). Chronic iAs exposure generates mitochondrial oxidative stress in the rat brain by impairment of mitochondrial complexes I, II, and IV

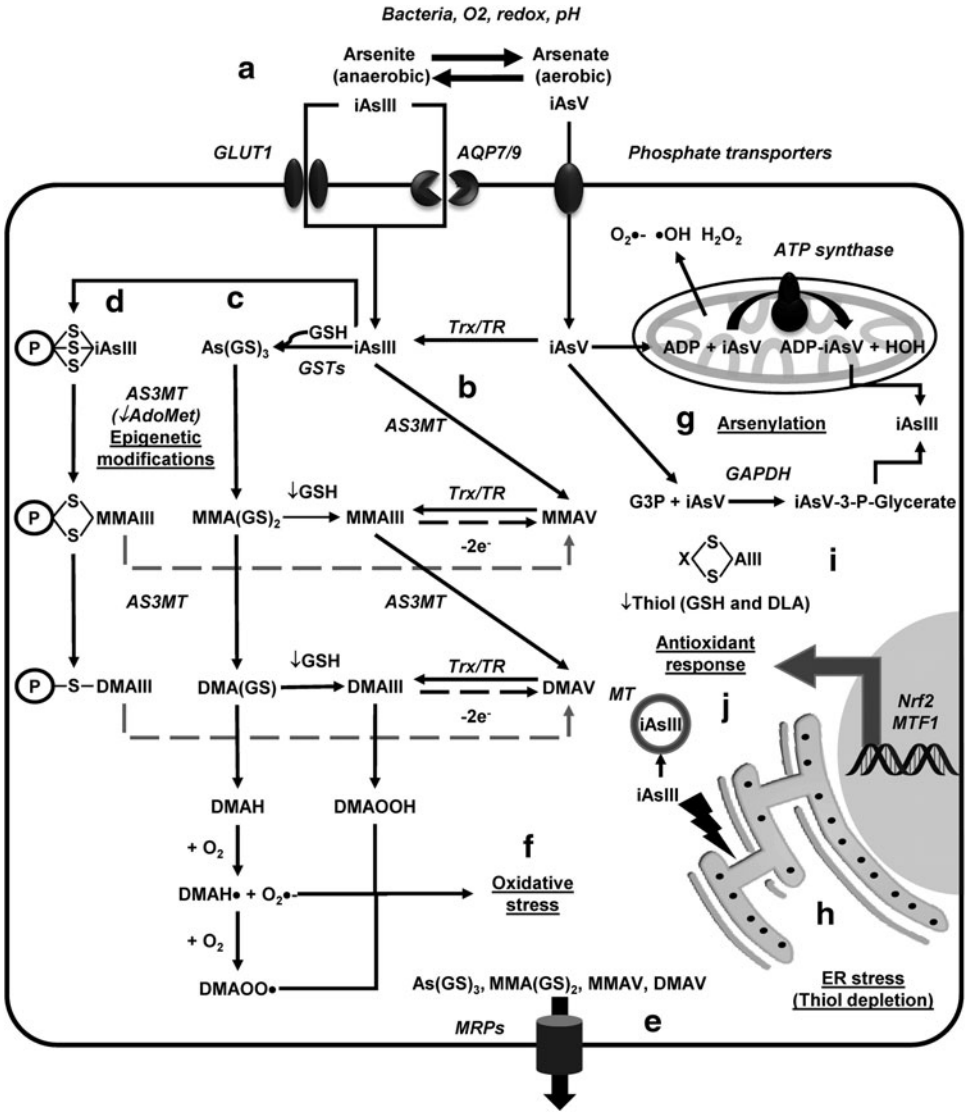


**FIG. 7. iAs biochemistry, redox signaling, and neurotoxicity. (a)** iAsIII species prevail in anaerobic conditions, while aerobic environments contain iAsV species. iAsIII enters cells through AQP9/7 and GLUT, while iAsV uses phosphate transporters. Three mechanisms for iAs methylation have been proposed. **(b)** Oxidative methylation refers to the progressive reduction of AsV to AsIII metabolites by the Trx/TR system and the oxidative methylation of AsIII species by AS3MT. **(c)** The GSH conjugation pathway is based on the formation of $As(GS)_3$ by the GSTs. $As(GS)_3$ is subsequently methylated by AS3MT. **(d)** The protein thiol conjugation mechanism suggests that instead of $As(GS)_3$, iAsIII binds to protein thiols and is methylated while still being conjugated to proteins. iAsIII methylation is likely to alter epigenetic signatures by alterations in AdoMet, a cosubstrate required for methyl group transfer. **(e)** Methylated metabolites and GSH/protein complexes are highly reactive and are exported through the MRPs. **(f)** DMA(GS) can form DMAH and react with $O_2$ to form $DMAH^{\bullet}$ and $DMAOO^{\bullet}$. DMAIII also reacts with $O_2$ and forms DMAOOH. These reactive species lead to lipid peroxidation and protein carbonylation (oxidative stress). **(g)** AsV can replace phosphate in several metabolic pathways (arsenylation) where the end product is reduced AsIII. AsV binds ADP *via* ATP-synthase and uncouples oxidative phosphorylation and ATP formation in the mitochondria. AsV is also arsenylated by GAPDH to generate iAsV-3-P-glycerate from G3P. **(h)** iAsIII toxicity generates ER stress likely *via* thiol depletion and alterations in redox balance. **(i)** AsIII binds to thiol containing molecules (coenzyme A, DLA, GSH) and protein thiols inactivating enzymatic function. **(j)** iAsIII toxicity is counteracted by Nrf2 and MTF1 that mediate the transcriptional-dependent induction of antioxidant systems and MTs. AdoMet, S-adenosylmethionine; AQP, aqua(glycerol)porin; AS3MT, arsenite methyltransferase; AsIII, arsenite +3; $As(SG)_3$, arsenic triglutathione; AsV, arsenate +5; DLA, dihydrolipoamide; DMA(GS), dimethylarsinic GSH; DMAH, dimethylarsine; $DMAH^{\bullet}$, DMAH radical; DMAIII, dimethylarsinous acid; $DMAOO^{\bullet}$, dimethylarsine peroxyl radical; DMAOOH, dimethylated arsenic peroxide; G3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT, glucose transporters; GSTs, glutathione-S transferases; iAs, inorganic As; iAsV-3-P-glycerate, 1-arsenato-3-phospho-D-glycerate; MRPs, multidrug resistance proteins; MTF1, metal-responsive transcription factor-1; Nrf2, nuclear factor erythroid-2-related factor 2; $O_2$, molecular oxygen; Trx, thioredoxin.

activities followed by increased ROS generation, lipid peroxidation, and protein carbonylation (275). Mitochondrial pyruvate dehydrogenase is also directly inhibited by iAs (136). In addition, iAs reduces the levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1$\alpha$), downstream targets Nrf1 and Nrf2, and the mitochondrial transcription factor A (TFAM) decreasing mitochondrial biogenesis (274). ER stress has also been shown to contribute to iAs toxicity, but the mechanisms involved remain unclear (Fig. 7h) (182).

iAs toxicity has also been attributed to the ability of AsV to replace phosphate in several metabolic pathways (arsenylation) where the end product is the reduction of AsV to AsIII, because the arsenylated by-product is more readily reduced than AsV itself (Fig. 7g). AsV uncouples oxidative phosphorylation and ATP formation in the mitochondria by binding to ADP *via* ATP synthase. Replacement of phosphate in glycolysis also impairs carbon flux and ATP production. Reaction of AsV with glucose generates glucose 6-arsenate, an analog of glucose 6-phosphate that is suggested to act as an inhibitor of hexokinase. AsV is also arsenylated by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to produce the unstable product 1-arsenato-3-phospho-D-glycerate (iAsV-3-P-glycerate). Purine nucleoside phosphorylase, glycogen phosphorylase, and mitochondrial ornithine carbamoyl transferase (OCT) have also been shown to arsenylate AsV (139, 245, 340). Thus, energy failure, alterations in central carbon metabolism, and mitochondrial dysfunction are consequences of AsV toxicity.

AsIII binds to thiol containing molecules (coenzyme A, GSH, and dihydrolipoamide also known as dihydrolipoic acid [DLA]) and protein-Cys thiols inactivating enzyme function (Fig. 7d, i). AsIII has higher affinity for dithiols than monothiols as demonstrated by the transfer of AsIII from the GSH-adduct to 2,3-dimercaptosuccinic acid (DMSA) a sulfhydryl-containing metal chelator used to treat heavy metal toxicity. In addition, AsIII conjugated with GSH has the ability to bind protein thiols, which highlights the importance of detoxification of GSH-As adducts from the cell (230). Dithiol molecules such as the cofactor DLA and dithiol oxidoreductases Trxs, Trx reductase (TrxR), Prxs (except for monothiol Prx6), Grx, and GR, as well as proteins with adjacent Cys (MT), have been reported to avidly bind AsIII (Fig. 7d, j) (50, 279, 311, 393, 397). In addition to binding AsIII, Zn finger domains have been shown to be oxidized upon As binding (396). Binding of AsIII to DLA (Fig. 7i) is expected to interfere with the TCA cycle and energy production as DLA is a cofactor for the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes that catalyze the synthesis of acetyl-CoA and succinyl-CoA, respectively. DLA reverses protein oxidation and loss of protein-SHs in the brains of rats exposed to high levels of iAs (82, 296, 315, 316).

As activates the cystine/Glu exchanger system (xCT) in microglia to increase extracellular Glu levels (320), while in astrocytes it decreases the expression levels and activity of GS and Glu transporters (GLAST/excitatory amino acid transporter (EAAT) 1 and GLT-1/EAAT2) (48, 395). Importantly, these effects were linked to an increase in GSH levels and Nrf2 activity (Fig. 7i), but not oxidative stress (48). Accordingly, activation of Nrf2 by As seems to involve a noncanonical pathway where inhibition of autophagy leads to the accumulation of the ubiquitin-binding protein/adaptor p62 that sequesters Keap1 (169). iAsIII toxicity is also counteracted by the transcriptional regulation of MT *via* MTF1 (Fig. 7j) (129).

### *Lead*

Inorganic Pb remains one of the most studied toxic elements due to several reasons. To begin with, human contact with Pb started very early in human civilization, and its toxic effects were also known since then. However, more importantly, Pb is neurotoxic leading to lower IQ even at lower doses than those recommended by the World Health Organization (10 ppb in drinking water). Human exposure to Pb not only occurs occupationally but also environmentally. The presence of Pb in the environment has multiple sources such as gasoline, industrial processes, paint, water pipes, and solder in canned food. It is present in air, household dust, soil, water, and food (Supplementary Table S1). Environmental Pb levels have fortunately decreased, especially in those countries where the Pb addition to gasoline and paints was banned. This prohibition was enforced after several studies associated the presence of high blood levels of Pb with impaired or diminished cognitive functions. Epidemiological studies have clearly shown that exposure to Pb in early stages of development is associated with significant deficits in neurobehavioral performance, including lower IQ, attention deficits, and aggressiveness later in life. Despite all the enforced restrictions, Pb contamination is still a major public health concern. For example, in November 2000 in Washington DC, there was a ''lead drinking water crisis'' triggered by a change in the disinfectant used to clean the water, this contamination affected hundreds of kids for 3 years. The health consequences of the recent crisis of Pb-contaminated water in Flint Michigan (United States, 2015) are still to be revealed in the future (68, 84, 124, 298).

Inhalation and ingestion of Pb and Pb-containing particles or products are the main routes of Pb entry into the body. Young children are especially vulnerable because they show higher gastrointestinal absorption than adults. Inhaled Pb particles are quickly absorbed in alveoli and distributed to other organs through the circulation. Thus, blood lead levels (BLL) are reliable biomarkers of exposure and risk. However, BLL do not reflect the total Pb body burden because Pb is absorbed in bones where it can be stored for several years (68). Currently, the acceptable BLL for children is lower than 10 $\mu$g/dl (0.48 $\mu M$) (49, 376), but due to the devastating effects that might occur later in life, there is a consensus to recommend efficient surveillance methods for children protection to reduce BLL to the lowest possible level (141). Pb binds with high affinity to erythrocytes' $\delta$-aminolevulinic acid dehydratase (ALAD) that catalyzes the second step in the porphyrin and heme biosynthetic pathway, causing the accumulation of aminolevulinic acid (ALA) in both plasma and urine, which is used as a biomarker of exposure (248).

Pb can cross the BBB and cell membrane because of its ability to mimic $Ca^{2+}$ and $Fe^{2+}$ ions (Fig. 8a) (205, 298). In children, due to a more permeable BBB and a lower bone storage capacity for Pb, the amount of Pb passing into the nervous system is higher than in adults. The highest accumulation of Pb has been reported in the hippocampus, amygdala (116), and choroids plexus (201). The PNS may

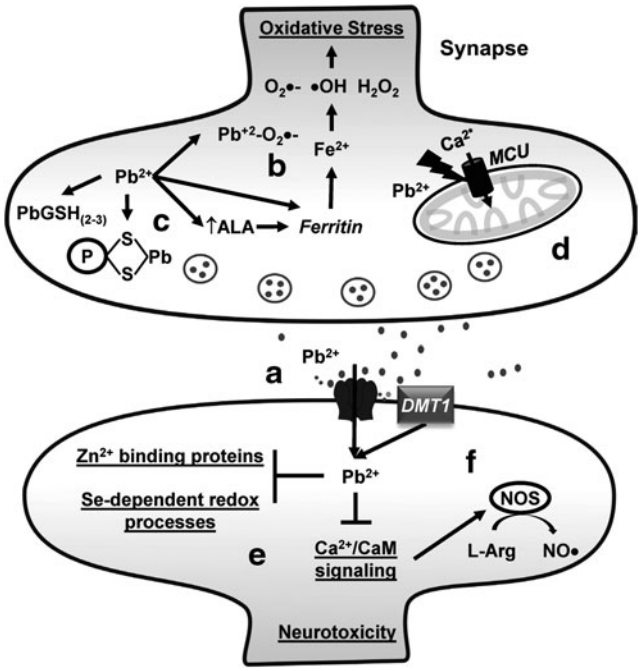


**FIG. 8. Pb neurotoxicity and synaptic signaling. (a)** Pb enters cells due to its ability to mimic $Ca^{2+}$ and $Fe^{2+}$ ions. **(b)** Inside the cell, Pb can trigger oxidative stress by different mechanisms, including the accumulation of ALA; the formation of $Pb^{2+}$–$O_2^{\bullet-}$ complexes; and the release of labile $Fe^{2+}$ from Ft. **(c)** Pb can also form complexes with GSH [$Pb(GSH_{2-3})$] and bind to protein thiols. **(d)** Mitochondrial dysfunction and oxidative stress induced by Pb have also been linked to MCU. **(e)** Pb can displace $Zn^{2+}$ and impair $Zn^{2+}$ binding protein function. Furthermore, Pb interferes with the Se-dependent redox processes. **(f)** Finally, Pb can prevent CaM activation and thus reduce NOS activity. ALA, aminolevulinic acid; CaM, $Ca^{2+}$/calmodulin; NOS, nitric oxide synthase; $O_2^{\bullet-}$, superoxide anion radical; Pb, lead; Se, selenium.



accumulate considerably more Pb than the CNS. Animals chronically exposed to Pb had impaired dendritic spines and synapse formation (306). Developmental exposure to Pb impacts the prefrontal cerebral cortex, hippocampus, and cerebellum regions, which can lead to neurological disorders, mental retardation, behavioral problems, and nerve damage (242). Early life exposure to Pb has also been linked to neurodegenerative diseases such as AD and PD (62, 209).

The mechanisms of Pb toxicity include the ability of Pb to bind SH groups of proteins Cys and to mimic or compete with $Ca^{2+}$, $Fe^{2+}$, and $Zn^{2+}$ (Fig. 8b, c, e, f) (99, 283). Zn deficiency increases the toxicity of Pb (4). The generation of oxidative damage by Pb *in vitro* and *in vivo* suggests that ROS also participate in Pb toxicity. For example, Pb acetate induces the opening of the mitochondrial permeability transition pore in human neuroblastoma SH-SY5Y cells *via* ROS (384). Pb can form a $Pb^{2+}$–$O_2^{\bullet-}$ complex with higher oxidizing capacity than $O_2^{\bullet-}$ (3). In addition, accumulated $\delta$-ALA by Pb-induced ALAD inhibition can be subsequently oxidized to generate $O_2^{\bullet-}$, $^{\bullet}OH$, and $H_2O_2$. Pb *per se* has been reported to stimulate $Fe^{2+}$-initiated lipid peroxidation (Fig. 8b) (337). Early postnatal exposure of rats to Pb leads to a higher accumulation of oxidative DNA damage in the cerebral cortical tissue when compared with aged controls or aged mice exposed acutely to Pb (36).

Perinatal exposure to Pb acetate inhibits the activity of brain acid and alkaline phosphatases, catalase, acetylcholinesterase, and ATPases (12). Similar observations have been made for the activities/levels of SOD1, GPX1, and GPX4 in the hippocampus, and for mitochondrial SOD2 and GSH, both in the cortex and hippocampus (19). Antioxidant nutrients such as vitamin E, vitamin C, vitamin B6, $\beta$-carotene, and DLA, as well as metal chelators such as DMSA, or replenishment of displaced metals has been shown to be beneficial against Pb-induced oxidative stress in the brain (98, 236, 256, 258, 277, 367). Diet supplementation with Zn and Se, which participates in the regulation of the GSH and Trx antioxidant systems, can effectively outcompete Pb binding to Zn- and Se-binding sites (Fig. 8e) (135).

Pb interferes with and disrupts $Ca^{2+}$ signaling and homeostasis leading to excitotoxicity. In addition, Glu potentiates Pb-induced cell death in PC12 cells (267). Recently, oxidative stress induced by Pb has been shown to be linked to changes in the levels of MCU (Fig. 8d) (383). Other important intracellular targets of Pb in the brain are both neural NOS and endothelial NOS due to an impairment in their $Ca^{2+}$/calmodulin (CaM)-dependent activation (Fig. 8f) (241). Importantly, Pb amplifies Glu-induced oxidative stress in a $Ca^{2+}$-independent manner, but neither $Ca^{2+}$ nor ROS seem to be essential for the enhanced cytotoxicity of combined exposure to Glu and Pb (191, 238).

### *Mercury*

Hg is a transition metal that exists as elemental, inorganic, and organic Hg (Fig. 9a). Hg is ubiquitously found in the environment as sulfide compounds generated from volcanic activity and erosion, or released by anthropogenic sources such as fuel combustion, waste disposal, and industrial activities (Supplementary Table S1). Elemental or metallic Hg

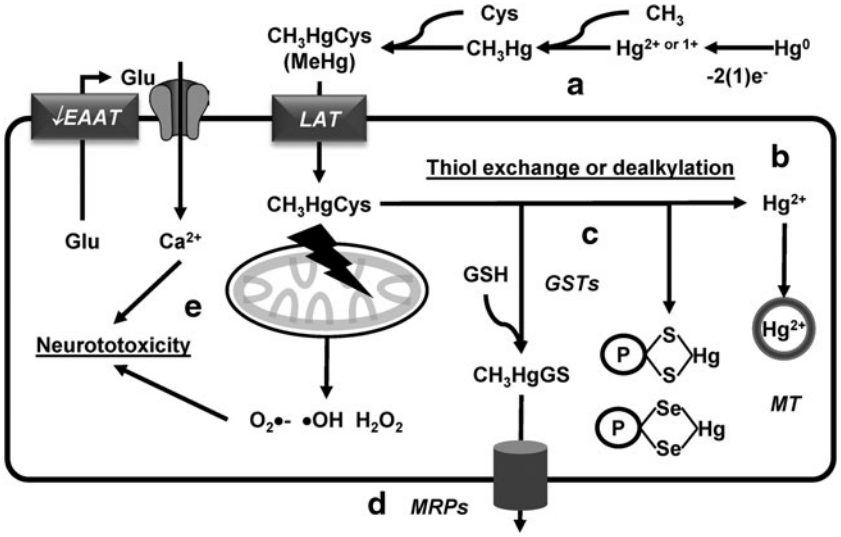


**FIG. 9. Hg redox-related toxicity mechanisms in brain cells**. **(a)** Hg species are highly transformed (oxidation or methylation) before they are absorbed into organisms. MeHg and EtHg form a complex with Cys ($CH_3HgCys$) and are transported across the membranes *via* LAT. **(b)** MeHg suffers dealkylation and thiol exchange to form $Hg^{2+}$. **(c)** Thiol exchange reactions of $CH_3HgCys$ complexes with low-molecular-weight thiols (GSH) and protein thiols alter the cellular redox balance. MeHg also has a strong affinity for selenol groups. **(d)** GSH-MeHg adduct formation ($CH_3HgGS$) is catalyzed by the GSTs and adducts are detoxified by MRPs. **(e)** MeHg also induces mitochondrial ROS and energy failure. Furthermore, MeHg-induced neurotoxicity has been ascribed to a reduction in Glu uptake by astrocytes *via* EAAT triggering neuronal excitotoxicity (*arrow* in EAAT indicates reversal of Glu transport). Cys, cysteine; Hg, mercury; EtHg, ethylmercury; $Hg^{1+}$, inorganic mercurous ions; $Hg^{2+}$, inorganic mercuric ions; LAT, L-type neutral amino acid transporter; MeHg, methylmercury.



($Hg^0$) used in thermometers and amalgams is primarily absorbed *via* inhalation, while inorganic mercury ($Hg^{1+\text{ or }2+}$) used in medicine and everyday life products is partially absorbed through the gut. Organic Hg (ethylmercury [EtHg or $C_2H_5Hg$] and methylmercury [MeHg or $CH_3Hg$]) is originated from atmospheric sources that are deposited in water body surfaces to be biomethylated and magnified in the food chain (Fig. 9a). Around 95% of MeHg is absorbed by the gastrointestinal tract making it the most toxic Hg species. Neurotoxic signs of Hg intoxication are vast and include ataxia, dizziness, insomnia, speech impairment, arthralgia, cognitive and behavioral changes, seizures, fatigue, and sensory disruption. While there has been an association between Hg exposure and neurodegeneration or autism, the neurological effects of chronic exposure to Hg are largely unclear. However, research has clearly demonstrated that Hg impairs neuronal development, communication, and myelination (89).

Most of the studies regarding the mechanisms involved in Hg neurotoxicity have been done using MeHg. MeHg and EtHg are potent electrophiles that form a complex with Cys ($CH_3HgCys$ or $C_2H_5HgCys$), and then transported across the BBB and into neuronal cells *via* L-type neutral amino acid transporters (LAT1 and 2) (Fig. 9a) (318, 385, 399).

A high percentage of Hg in individuals intoxicated with MeHg is found as $Hg^{2+}$, suggesting that dealkylation of MeHg is an important mechanism for the high persistence of Hg in the brain (Fig. 9b) (72). Thiol exchange from $CH_3HgCys$ to low-molecular-weight thiols (GSH) and protein thiols has been proposed to be central mechanisms by which MeHg induces GSH depletion, inhibition of thiol-dependent antioxidant systems, and alters the activity or function of proteins with redox-sensitive Cys (signaling proteins, metabolic enzymes, neurotransmitter receptors, and transporters) (Fig. 9c) (89). MeHg also has a stronger affinity for selenol groups (selenohydryl groups in selenocysteines) compared with thiol groups. As such, selenoproteins are important targets for direct electrophilic attack of MeHg or transfer from thiol adducts ($CH_3HgCys$, $CH_3HgGS$, or $CH_3HgPS$ [protein-Cys adduct]) (Fig. 9c) (104, 221). GSTs have been proposed to mediate the formation of $CH_3HgGS$ adducts, which are detoxified by MRP1-mediated transport (Fig. 9d). GSH synthesis, GST, and MRP1 levels are regulated transcriptionally by the Nrf2 antioxidant system (152, 292, 349).

MeHg induces mitochondrial ROS and energy failure (175, 233). Neurotoxicity induced by MeHg has also been ascribed to its inhibitory effect on Glu uptake by astrocytes, triggering neuronal excitotoxicity (15, 235) (Fig. 9e).

$Hg^0$ absorbed through the respiratory tract is oxidized to inorganic mercurous ($Hg^{1+}$) and mercuric ions ($Hg^{2+}$) (Fig. 9a). While inorganic Hg ions have limited access to the CNS, they induce profound neurotoxic alterations that seem to be mediated as well by their binding to thiol groups (89). Accordingly, MTs exert protective effects against $Hg^0$-induced neurotoxicity (387) (Fig. 9b).

### *Other xenobiotic metals*

Aluminum. Al is one of the most abundant metals in the earth's crust (8.1%). Al has a plethora of uses in industry and manufacturing, as well as in food additives. As such, human exposure is primarily originated from food and drinking water. Importantly, pharmaceuticals have higher levels of Al

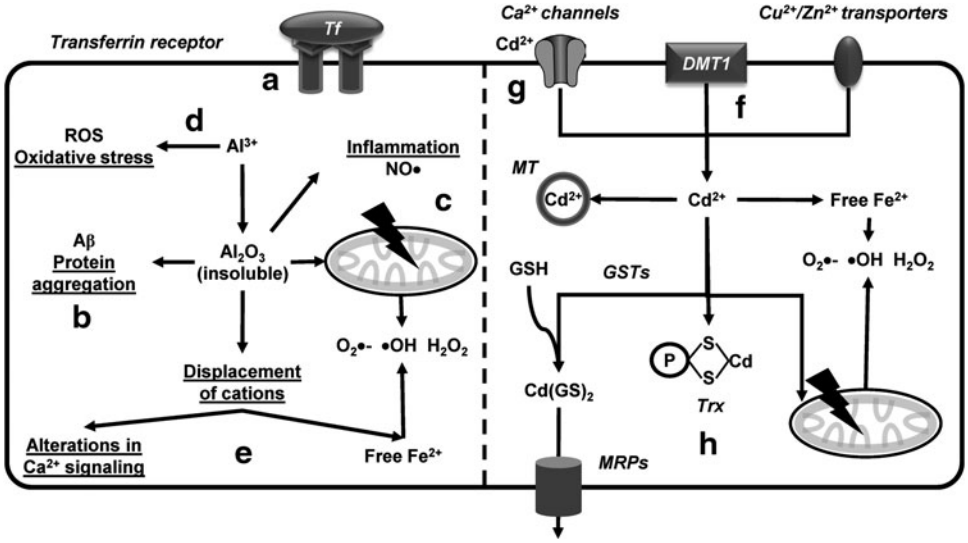


**FIG. 10. Mechanisms of Al and Cd neurotoxicity. (a)** Al is bound to Tf, which mediates its transport across the BBB *via* the Tfr, but a Tf-independent Al transport also exists. **(b)** Al promotes A$\beta$ aggregation and **(c)** mitochondrial dysfunction trigging neuroinflammation and $NO^{\bullet}$ formation. **(d)** $Al^{3+}$ has been proposed to react with $H_2O_2$ to produce $AlO_2^{\bullet-}$ that can promote oxidative damage. **(e)** Al toxicity is also related to its ability to displace other metals (Ca or Fe). **(f)** Cd transport across membranes is through molecular mimicry *via* several transporters such as Cu/Zn transporters, DMT1, and $Ca^{2+}$-channels. **(g)** High concentrations of Cd can block $Ca^{2+}$ currents as well. **(h)** Cd toxicity is linked to its ability to bind thiol containing molecules such GSH, and protein-Cys (Trx and MT), as well as the displacement of redox-active metals such as Fe. Cd detoxification of cells is facilitated by the activity of GST and the detoxification of GSH-Cd adducts *via* MRPs. Al, aluminum; $AlO_2^{\bullet-}$, aluminum superoxide radicals; Ca, calcium; Cd, cadmium.



compared to food. Occupational exposures to Al are related to mining, processing, and welding (359) (Supplementary Table S1). While Al is poorly absorbed in the gut, inhalation mediates direct transfer to the brain *via* the olfactory system (341). Importantly, $\sim$85% of Al in blood is bound to Tf, which is considered to mediate its transport across the BBB (Fig. 10a) (288), but Tf-independent Al transport also exists. Interestingly, monocarboxylate and xCT transporters have also been proposed to mediate the transport of Al-citrate complexes (240, 386). Acute Al toxicity occurs as a result of occupational exposure or chronic renal failure and is known to target the nervous system. Al is neurotoxic in animal models triggering the accumulation of neurofibrillary tangles and impairment of cognitive, behavioral, and motor functions. Al promotes A$\beta$ aggregation, mitochondrial dysfunction, and triggers neuroinflammation (Fig. 10b, c) (24, 202, 309). However, conflicting results exist regarding the association of Al with any human disease, including AD (37, 359, 377).

Al exists primarily in a trivalent state ($Al^{3+}$). While Al has no redox capacity, Al toxicity is linked to oxidative damage. $Al^{3+}$ has been proposed to react with $H_2O_2$ to produce Al superoxide radicals ($AlO_2^{\bullet-}$) that can deplete mitochondrial Fe and promote generation of ROS (Fig. 10d) (166). However, because of its high reactivity, Al is primarily found forming insoluble oxides whose toxicity seem to be related with the displacement of other biological cations ($Ca^{2+}$, $Fe^{2+}$, or $Mg^{2+}$) (Fig. 10e) (377).

Cadmium. Cd is a transition metal whose use in industry has increased dramatically in the recent years. Cd is widely used in batteries, alloys, and pigments, and produced as a by-product from the extraction of other metals from ores. Food is the major source for Cd exposure as both animals and plants accumulate high levels of Cd. Inhalation is the prevalent route of Cd exposure due to industrial emissions and occupational activities (tobacco) (Supplementary Table S1). Cd neurotoxicity seems to occur only during development, before complete BBB formation, or in association with BBB dysfunction. Cd transport across membranes is thought to be mediated by molecular mimicry *via* several transporters and receptors for essential metals such as Cu/Zn transporters, DMT1, and $Ca^{2+}$-channels (Fig. 10f) (117, 131, 174, 224, 339). Importantly, at high concentrations, Cd also has the ability to block $Ca^{2+}$ currents (Fig. 10g).

Cd toxicity is linked to its ability to bind thiol containing molecules such as GSH, and protein-Cys (Trxs and MT) and as a consequence, displacement of redox-active metals and mitochondrial and metabolic dysfunction (Fig. 10h) (363). Cd detoxification of cells is facilitated by the activity of GST and the detoxification of GSH-Cd adducts *via* MRPs (Fig. 10h) (181, 339). Accordingly, resistance to Cd-toxicity is directly associated with the Nrf2-mediated antioxidant response (366).

## Conclusions and Perspectives

Metals are important for brain function and human health. Thus, alterations in their content and/or distribution are expected to exert neurotoxicity. Both alterations in the homeostasis of essential metals and environmental exposure to xenobiotic metals can have silent chronic effects leading to neurodegeneration and neurological dysfunction (behavioral and cognitive alterations). The neurotoxic mechanisms by

which metals impact neuronal or glial function are starting to become elucidated.

In this review, we have summarized how essential metals are trafficked in the brain. It is interesting to note that the mechanisms involved in metal transport and homeostasis are strongly linked to the chemical properties of each metal ion, while their coordination chemistry preferences also allow them to share some metal transport routes. For instance, Cu and Fe go through several redox cycles during their transport, while Mn and Zn remain in the same oxidation state. Metal trafficking in the cell is tightly regulated to control the high reactivity toward $O_2$ of some metal ions, such as $Fe^{2+}$ and $Cu^{+}$, or to keep in solution otherwise insoluble species such as $Cu^{+}$ and $Fe^{3+}$. Moreover, while similar O-based ligand coordination preferences of $Mn^{2+}$, $Fe^{2+}$, and $Fe^{3+}$ allow them to share some transport systems, the affinity of $Zn^{2+}$ and $Cu^{+}$ for Cys ligands makes MTs important players in their homeostasis. In fact, a close relationship between cellular redox environment and metal transport has been recently demonstrated for Cu and Fe (Figs. 1D and 4C).

Since metal trafficking machineries in neurons and astrocytes resemble those of other extensively studied mammalian cells, our understanding of intracellular metal homeostasis has advanced significantly. In contrast, metal trafficking at the synapse and the role in neuromodulation have just begun to be revealed, and point to a close inter-relationship among Zn, Cu, and Fe. For example, the synaptic release of Zn and Glu ultimately leads to the activation of NMDAR and the activation of signaling pathways that lead to postsynaptic Cu release and Fe uptake. Clearly, the close interplay between these metals at the synapse must play an important role in neuromodulation, while disturbed metal trafficking in neurodegenerative diseases would impact these processes.

We have also illustrated the differential alteration of metal homeostasis that occurs in neurodegenerative disorders as well as the key metal-protein interactions that might be involved in protein aggregation and metal-mediated oxidative damage. Understanding these interactions at the molecular level will shed light into the role of essential metals in neurodegenerative diseases.

Metals are transported across the BBB and into brain cells by selective transport systems for essential metals, while xenobiotic metals hijack those transporters *via* molecular mimicry (Supplementary Fig. S1a). The ability of xenobiotic metals to be transported and/or react with cellular targets is strongly determined by their metabolism *via* reduction/oxidation reactions, methylation, or adduct formation ($Mt0 \rightarrow Mt1 \rightarrow Mt2$). Phase II enzyme systems, GSH/GST, and MTs are essential for the detoxification of metals (Supplementary Fig. S1b). Metals induce or enhance ROS and RNS formation leading to oxidative stress (Supplementary Fig. S1c). While oxidative damage is one of the causative mechanisms involved in cellular damage induced by metals, it is now clear that other redox processes participate as well. The intrinsic reactivity of xenobiotic metals with thiol and selenol groups (Supplementary Fig. S1d) and their capacity to displace essential metals (Supplementary Fig. S1e) are also central to their capacity to promote energy failure (mitochondrial dysfunction), protein damage/aggregation, and metabolic alterations that challenge neuro/glial function and survival and trigger excitotoxic and inflammatory processes (Supplementary Fig. S1f). Cells have the capacity to respond by activating redox- or metal-dependent transcriptional regulation of antioxidant and anti-inflammatory responses (Supplementary Fig. S1g).

The aim of this review was to provide an integrated overview of the recent advances regarding how dysfunctional metal ion homeostasis of essential metals and exposure to xenobiotic metals alter cellular function to promote chronic neurodegeneration and neurotoxicity. Although the mechanisms involved in these processes are still being elucidated, the studies highlighted here are a starting point toward a better understanding of the pathological consequences of alterations in metal ion homeostasis, justifying the need of further studies regarding their metabolism and its impact on cellular homeostasis, function, and survival.

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## References

1. Aboud AA, Tidball AM, Kumar KK, Neely MD, Han B, Ess KC, Hong CC, Erikson KM, Hedera P, and Bowman AB. PARK2 patient neuroprogenitors show increased mitochondrial sensitivity to copper. *Neurobiol Dis* 73: 204–212, 2015.
2. Acosta-Cabronero J, Betts MJ, Cardenas-Blanco A, Yang S, and Nestor PJ. In vivo MRI mapping of brain iron deposition across the adult lifespan. *J Neurosci* 36: 364–374, 2016.
3. Adonaylo VN and Oteiza PI. $Pb^{2+}$ promotes lipid oxidation and alterations in membrane physical properties. *Toxicology* 132: 19–32, 1999.
4. Aimo L and Oteiza PI. Zinc deficiency increases the susceptibility of human neuroblastoma cells to lead-induced activator protein-1 activation. *Toxicol Sci* 91: 184–191, 2006.
5. Al-Hilaly YK, Biasetti L, Blakeman BJ, Pollack SJ, Zibaee S, Abdul-Sada A, Thorpe JR, Xue WF, and Serpell LC. The involvement of dityrosine crosslinking in alpha-synuclein assembly and deposition in Lewy bodies in Parkinson's disease. *Sci Rep* 6: 39171, 2016.
6. Alfonso-Prieto M, Vidossich P, and Rovira C. The reaction mechanisms of heme catalases: an atomistic view by ab initio molecular dynamics. *Arch Biochem Biophys* 525: 121–130, 2012.
7. Amri F, Ghouili I, Amri M, Carrier A, and Masmoudi-Kouki O. Neuroglobin protects astroglial cells from hydrogen peroxide-induced oxidative stress and apoptotic cell death. *J Neurochem* 140: 151–169, 2017.
8. Anandhan A, Rodriguez-Rocha H, Bohovych I, Griggs AM, Zavala-Flores L, Reyes-Reyes EM, Seravalli J,

Stanciu LA, Lee J, Rochet JC, Khalimonchuk O, and Franco R. Overexpression of alpha-synuclein at non-toxic levels increases dopaminergic cell death induced by copper exposure via modulation of protein degradation pathways. *Neurobiol Dis* 81: 76–92, 2015.
9. Anderson CT, Radford RJ, Zastrow ML, Zhang DY, Apfel UP, Lippard SJ, and Tzounopoulos T. Modulation of extrasynaptic NMDA receptors by synaptic and tonic zinc. *Proc Natl Acad Sci U S A* 112: E2705–E2714, 2015.
10. Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, Barbour R, Huang J, Kling K, Lee M, Diep L, Keim PS, Shen X, Chataway T, Schlossmacher MG, Seubert P, Schenk D, Sinha S, Gai WP, and Chilcote TJ. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J Biol Chem* 281: 29739–29752, 2006.
11. Antala S and Dempski RE. The human ZIP4 transporter has two distinct binding affinities and mediates transport of multiple transition metals. *Biochemistry* 51: 963–973, 2012.
12. Antonio MT, Corredor L, and Leret ML. Study of the activity of several brain enzymes like markers of the neurotoxicity induced by perinatal exposure to lead and/or cadmium. *Toxicol Lett* 143: 331–340, 2003.
13. Aoyama K, Watabe M, and Nakaki T. Regulation of neuronal glutathione synthesis. *J Pharmacol Sci* 108: 227–238, 2008.
14. Aschner M, Gannon M, and Kimelberg HK. Manganese uptake and efflux in cultured rat astrocytes. *J Neurochem* 58: 730–735, 1992.
15. Aschner M, Yao CP, Allen JW, and Tan KH. Methylmercury alters glutamate transport in astrocytes. *Neurochem Int* 37: 199–206, 2000.
16. Atamna H and Boyle K. Amyloid-beta peptide binds with heme to form a peroxidase: relationship to the cytopathologies of Alzheimer's disease. *Proc Natl Acad Sci U S A* 103: 3381–3386, 2006.
17. Banci L, Bertini I, Cantini F, Kozyreva T, Massagni C, Palumaa P, Rubino JT, and Zovo K. Human superoxide dismutase 1 (hSOD1) maturation through interaction with human copper chaperone for SOD1 (hCCS). *Proc Natl Acad Sci U S A* 109: 13555–13560, 2012.
18. Banci L, Bertini I, Ciofi-Baffoni S, Kozyreva T, Zovo K, and Palumaa P. Affinity gradients drive copper to cellular destinations. *Nature* 465: 645–648, 2010.
19. Baranowska-Bosiacka I, Gutowska I, Marchlewicz M, Marchetti C, Kurzawski M, Dziedziejko V, Kolasa A, Olszewska M, Rybicka M, Safranow K, Nowacki P, Wiszniewska B, and Chlubek D. Disrupted pro- and antioxidative balance as a mechanism of neurotoxicity induced by perinatal exposure to lead. *Brain Res* 1435: 56–71, 2012.
20. Barnes N, Tsivkovskii R, Tsivkovskaia N, and Lutsenko S. The copper-transporting ATPases, Menkes and Wilson disease proteins, have distinct roles in adult and developing cerebellum. *J Biol Chem* 280: 9640–9645, 2005.
21. Barritt JD and Viles JH. Truncated amyloid-beta(11–40/42) from Alzheimer disease binds $Cu^{2+}$ with a femtomolar affinity and influences fiber assembly. *J Biol Chem* 290: 27791–27802, 2015.
22. Baumkotter F, Schmidt N, Vargas C, Schilling S, Weber R, Wagner K, Fiedler S, Klug W, Radzimanowski J, Nickolaus S, Keller S, Eggert S, Wild K, and Kins S. Amyloid precursor protein dimerization and synaptogenic function depend on copper binding to the growth factor-like domain. *J Neurosci* 34: 11159–11172, 2014.
23. Baxter PS and Hardingham GE. Adaptive regulation of the brain's antioxidant defences by neurons and astrocytes. *Free Radic Biol Med* 100: 147–152, 2016.
24. Becaria A, Lahiri DK, Bondy SC, Chen D, Hamadeh A, Li H, Taylor R, and Campbell A. Aluminum and copper in drinking water enhance inflammatory or oxidative events specifically in the brain. *J Neuroimmunol* 176: 16–23, 2006.
25. Beck MW, Oh SB, Kerr RA, Lee HJ, Kim SH, Kim S, Jang M, Ruotolo BT, Lee J-Y, and Lim MH. A rationally designed small molecule for identifying an in vivo link between metal-amyloid-[small beta] complexes and the pathogenesis of Alzheimer's disease. *Chem Sci* 6: 1879–1886, 2015.
26. Belanger M, Allaman I, and Magistretti PJ. Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. *Cell Metab* 14: 724–738, 2011.
27. Bell KF, Al-Mubarak B, Martel MA, McKay S, Wheelan N, Hasel P, Markus NM, Baxter P, Deighton RF, Serio A, Bilican B, Chowdhry S, Meakin PJ, Ashford ML, Wyllie DJ, Scannevin RH, Chandran S, Hayes JD, and Hardingham GE. Neuronal development is promoted by weakened intrinsic antioxidant defences due to epigenetic repression of Nrf2. *Nat Commun* 6: 7066, 2015.
28. Bellingham SA, Ciccotosto GD, Needham BE, Fodero LR, White AR, Masters CL, Cappai R, and Camakaris J. Gene knockout of amyloid precursor protein and amyloid precursor-like protein-2 increases cellular copper levels in primary mouse cortical neurons and embryonic fibroblasts. *J Neurochem* 91: 423–428, 2004.
29. Belloni-Olivi L, Marshall C, Laal B, Andrews GK, and Bressler J. Localization of zip1 and zip4 mRNA in the adult rat brain. *J Neurosci Res* 87: 3221–3230, 2009.
30. Besecker B, Bao S, Bohacova B, Papp A, Sadee W, and Knoell DL. The human zinc transporter SLC39A8 (Zip8) is critical in zinc-mediated cytoprotection in lung epithelia. *Am J Physiol Lung Cell Mol Physiol* 294: L1127–L1136, 2008.
31. Besser L, Chorin E, Sekler I, Silverman WF, Atkin S, Russell JT, and Hershfinkel M. Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus. *J Neurosci* 29: 2890–2901, 2009.
32. Biagioli M, Pinto M, Cesselli D, Zaninello M, Lazarevic D, Roncaglia P, Simone R, Vlachouli C, Plessy C, Bertin N, Beltrami A, Kobayashi K, Gallo V, Santoro C, Ferrer I, Rivella S, Beltrami CA, Carninci P, Raviola E, and Gustincich S. Unexpected expression of alpha- and beta-globin in mesencephalic dopaminergic neurons and glial cells. *Proc Natl Acad Sci U S A* 106: 15454–15459, 2009.
33. Binolfi A, Quintanar L, Bertoncini CW, Griesinger C, and Fernández CO. Bioinorganic chemistry of copper coordination to alpha-synuclein: relevance to Parkinson's disease. *Coord Chem Rev* 256: 2188–2201, 2012.
34. Binolfi A, Rasia RM, Bertoncini CW, Ceolin M, Zweckstetter M, Griesinger C, Jovin TM, and Fernandez CO. Interaction of alpha-synuclein with divalent metal ions reveals key differences: a link between structure, binding specificity and fibrillation enhancement. *J Am Chem Soc* 128: 9893–9901, 2006.

35. Bjorkblom B, Adilbayeva A, Maple-Grodem J, Piston D, Okvist M, Xu XM, Brede C, Larsen JP, and Moller SG. Parkinson disease protein DJ-1 binds metals and protects against metal-induced cytotoxicity. *J Biol Chem* 288: 22809–22820, 2013.
36. Bolin CM, Basha R, Cox D, Zawia NH, Maloney B, Lahiri DK, and Cardozo-Pelaez F. Exposure to lead and the developmental origin of oxidative DNA damage in the aging brain. *FASEB J* 20: 788–790, 2006.
37. Bondy SC. Low levels of aluminum can lead to behavioral and morphological changes associated with Alzheimer's disease and age-related neurodegeneration. *Neurotoxicology* 52: 222–229, 2016.
38. Bourassa MW, Leskovjan AC, Tappero RV, Farquhar ER, Colton CA, Van Nostrand WE, and Miller LM. Elevated copper in the amyloid plaques and iron in the cortex are observed in mouse models of Alzheimer's disease that exhibit neurodegeneration. *Biomed Spectrosc Imaging* 2: 129–139, 2013.
39. Bousejra-ElGarah F, Bijani C, Coppel Y, Faller P, and Hureau C. Iron(II) binding to amyloid-beta, the Alzheimer's peptide. *Inorg Chem* 50: 9024–9030, 2011.
40. Bove J, Martinez-Vicente M, Dehay B, Perier C, Recasens A, Bombrun A, Antonsson B, and Vila M. BAX channel activity mediates lysosomal disruption linked to Parkinson disease. *Autophagy* 10: 889–900, 2014.
41. Brose J, La Fontaine S, Wedd AG, and Xiao Z. Redox sulfur chemistry of the copper chaperone Atox1 is regulated by the enzyme glutaredoxin 1, the reduction potential of the glutathione couple GSSG/2GSH and the availability of Cu(I). *Metallomics* 6: 793–808, 2014.
42. Bucossi S, Ventriglia M, Panetta V, Salustri C, Pasqualetti P, Mariani S, Siotto M, Rossini PM, and Squitti R. Copper in Alzheimer's disease: a meta-analysis of serum,plasma, and cerebrospinal fluid studies. *J Alzheimers Dis* 24: 175–185, 2011.
43. Burmester T, Weich B, Reinhardt S, and Hankeln T. A vertebrate globin expressed in the brain. *Nature* 407: 520–523, 2000.
44. Calatayud M, Barrios JA, Velez D, and Devesa V. In vitro study of transporters involved in intestinal absorption of inorganic arsenic. *Chem Res Toxicol* 25: 446–453, 2012.
45. Calderon J, Navarro ME, Jimenez-Capdeville ME, Santos-Diaz MA, Golden A, Rodriguez-Leyva I, Borja-Aburto V, and Diaz-Barriga F. Exposure to arsenic and lead and neuropsychological development in Mexican children. *Environ Res* 85: 69–76, 2001.
46. Calvo IA, Boronat S, Domenech A, Garcia-Santamarina S, Ayte J, and Hidalgo E. Dissection of a redox relay: $H_2O_2$-dependent activation of the transcription factor Pap1 through the peroxidatic Tpx1-thioredoxin cycle. *Cell Rep* 5: 1413–1424, 2013.
47. Carmona F, Palacios Ò, Gálvez N, Cuesta R, Atrian S, Capdevila M, and Domínguez-Vera JM. Ferritin iron uptake and release in the presence of metals and metalloproteins: chemical implications in the brain. *Coord Chem Rev* 257: 2752–2764, 2013.
48. Castro-Coronel Y, Del Razo LM, Huerta M, Hernandez-Lopez A, Ortega A, and Lopez-Bayghen E. Arsenite exposure downregulates EAAT1/GLAST transporter expression in glial cells. *Toxicol Sci* 122: 539–550, 2011.
49. Chandran L and Cataldo R. Lead poisoning: basics and new developments. *Pediatr Rev* 31: 399–405; quiz 406, 2010.
50. Chang YY, Kuo TC, Hsu CH, Hou DR, Kao YH, and Huang RN. Characterization of the role of protein-cysteine residues in the binding with sodium arsenite. *Arch Toxicol* 86: 911–922, 2012.
51. Cheah JH, Kim SF, Hester LD, Clancy KW, Patterson SE, 3rd, Papadopoulos V, and Snyder SH. NMDA receptor-nitric oxide transmission mediates neuronal iron homeostasis via the GTPase Dexras1. *Neuron* 51: 431–440, 2006.
52. Cheignon C, Jones M, Atrian-Blasco E, Kieffer I, Faller P, Collin F, and Hureau C. Identification of key structural features of the elusive Cu-A[small beta] complex that generates ROS in Alzheimer's disease. *Chem Sci* 8: 5107–5118, 2017.
53. Chen L, Hambright WS, Na R, and Ran Q. Ablation of the ferroptosis inhibitor glutathione peroxidase 4 in neurons results in rapid motor neuron degeneration and paralysis. *J Biol Chem* 290: 28097–28106, 2015.
54. Chen P, Chakraborty S, Mukhopadhyay S, Lee E, Paoliello MM, Bowman AB, and Aschner M. Manganese homeostasis in the nervous system. *J Neurochem* 134: 601–610, 2015.
55. Chiang S, Kovacevic Z, Sahni S, Lane DJ, Merlot AM, Kalinowski DS, Huang ML, and Richardson DR. Frataxin and the molecular mechanism of mitochondrial iron-loading in Friedreich's ataxia. *Clin Sci (Lond)* 130: 853–870, 2016.
56. Chien WL, Lee TR, Hung SY, Kang KH, Lee MJ, and Fu WM. Impairment of oxidative stress-induced heme oxygenase-1 expression by the defect of Parkinson-related gene of PINK1. *J Neurochem* 117: 643–653, 2011.
57. Cho HH, Cahill CM, Vanderburg CR, Scherzer CR, Wang B, Huang X, and Rogers JT. Selective translational control of the Alzheimer amyloid precursor protein transcript by iron regulatory protein-1. *J Biol Chem* 285: 31217–31232, 2010.
58. Choi BS and Zheng W. Copper transport to the brain by the blood-brain barrier and blood-CSF barrier. *Brain Res* 1248: 14–21, 2009.
59. Choi DK, Pennathur S, Perier C, Tieu K, Teismann P, Wu DC, Jackson-Lewis V, Vila M, Vonsattel JP, Heinecke JW, and Przedborski S. Ablation of the inflammatory enzyme myeloperoxidase mitigates features of Parkinson's disease in mice. *J Neurosci* 25: 6594–6600, 2005.
60. Codazzi F, Pelizzoni I, Zacchetti D, and Grohovaz F. Iron entry in neurons and astrocytes: a link with synaptic activity. *Front Mol Neurosci* 8: 18, 2015.
61. Connor JR, Menzies SL, St Martin SM, and Mufson EJ. Cellular distribution of transferrin, ferritin, and iron in normal and aged human brains. *J Neurosci Res* 27: 595–611, 1990.
62. Coon S, Stark A, Peterson E, Gloi A, Kortsha G, Pounds J, Chettle D, and Gorell J. Whole-body lifetime occupational lead exposure and risk of Parkinson's disease. *Environ Health Perspect* 114: 1872–1876, 2006.
63. Cruces-Sande A, Mendez-Alvarez E, and Soto-Otero R. Copper increases the ability of 6-hydroxydopamine to generate oxidative stress and the ability of ascorbate and glutathione to potentiate this effect: potential implications in Parkinson's disease. *J Neurochem* 141: 738–749, 2017.
64. Culotta VC, Yang M, and O'Halloran TV. Activation of superoxide dismutases: putting the metal to the pedal. *Biochim Biophys Acta* 1763: 747–758, 2006.
65. Curtis AR, Fey C, Morris CM, Bindoff LA, Ince PG, Chinnery PF, Coulthard A, Jackson MJ, Jackson AP, McHale DP, Hay D, Barker WA, Markham AF, Bates

D, Curtis A, and Burn J. Mutation in the gene encoding ferritin light polypeptide causes dominant adult-onset basal ganglia disease. *Nat Genet* 28: 350–354, 2001.
66. D'Ambrosi N and Rossi L. Copper at synapse: release, binding and modulation of neurotransmission. *Neurochem Int* 90: 36–45, 2015.
67. Danielson SR and Andersen JK. Oxidative and nitrative protein modifications in Parkinson's disease. *Free Radic Biol Med* 44: 1787–1794, 2008.
68. Dapul H and Laraque D. Lead poisoning in children. *Adv Pediatr* 61: 313–333, 2014.
69. Das A, Nag S, Mason AB, and Barroso MM. Endosome-mitochondria interactions are modulated by iron release from transferrin. *J Cell Biol* 214: 831–845, 2016.
70. Davies KM, Bohic S, Carmona A, Ortega R, Cottam V, Hare DJ, Finberg JP, Reyes S, Halliday GM, Mercer JF, and Double KL. Copper pathology in vulnerable brain regions in Parkinson's disease. *Neurobiol Aging* 35: 858–866, 2014.
71. Davies MJ. Protein oxidation and peroxidation. *Biochem J* 473: 805–825, 2016.
72. Davis LE, Kornfeld M, Mooney HS, Fiedler KJ, Haaland KY, Orrison WW, Cernichiari E, and Clarkson TW. Methylmercury poisoning: long-term clinical, radiological, toxicological, and pathological studies of an affected family. *Ann Neurol* 35: 680–688, 1994.
73. Dehay B, Bove J, Rodriguez-Muela N, Perier C, Recasens A, Boya P, and Vila M. Pathogenic lysosomal depletion in Parkinson's disease. *J Neurosci* 30: 12535–12544, 2010.
74. Dexter DT, Carayon A, Javoy-Agid F, Agid Y, Wells FR, Daniel SE, Lees AJ, Jenner P, and Marsden CD. Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia. *Brain* 114 (Pt 4): 1953–1975, 1991.
75. Dikiy I and Eliezer D. N-terminal acetylation stabilizes N-terminal helicity in lipid- and micelle-bound alpha-synuclein and increases its affinity for physiological membranes. *J Biol Chem* 289: 3652–3665, 2014.
76. Ding L, Saunders RJ, Drobna Z, Walton FS, Xun P, Thomas DJ, and Styblo M. Methylation of arsenic by recombinant human wild-type arsenic (+3 oxidation state) methyltransferase and its methionine 287 threonine (M287T) polymorph: role of glutathione. *Toxicol Appl Pharmacol* 264: 121–130, 2012.
77. Do Van B, Gouel F, Jonneaux A, Timmerman K, Gele P, Petrault M, Bastide M, Laloux C, Moreau C, Bordet R, Devos D, and Devedjian JC. Ferroptosis, a newly characterized form of cell death in Parkinson's disease that is regulated by PKC. *Neurobiol Dis* 94: 169–178, 2016.
78. Dodani SC, Firl A, Chan J, Nam CI, Aron AT, Onak CS, Ramos-Torres KM, Paek J, Webster CM, Feller MB, and Chang CJ. Copper is an endogenous modulator of neural circuit spontaneous activity. *Proc Natl Acad Sci U S A* 111: 16280–16285, 2014.
79. Donaldson J, McGregor D, and LaBella F. Manganese neurotoxicity: a model for free radical mediated neurodegeneration? *Can J Physiol Pharmacol* 60: 1398–1405, 1982.
80. Du K, Liu MY, Zhong X, and Wei MJ. Decreased circulating zinc levels in Parkinson's disease: a meta-analysis study. *Sci Rep* 7: 3902, 2017.
81. Duong TT, Witting PK, Antao ST, Parry SN, Kennerson M, Lai B, Vogt S, Lay PA, and Harris HH. Multiple protective activities of neuroglobin in cultured neuronal cells exposed to hypoxia re-oxygenation injury. *J Neurochem* 108: 1143–1154, 2009.
82. Dwivedi N, Flora G, Kushwaha P, and Flora SJ. Alpha-lipoic acid protects oxidative stress, changes in cholinergic system and tissue histopathology during co-exposure to arsenic-dichlorvos in rats. *Environ Toxicol Pharmacol* 37: 7–23, 2014.
83. Eckl PM and Bresgen N. Genotoxicity of lipid oxidation compounds. *Free Radic Biol Med* 111: 244–252, 2017.
84. Edwards M, Triantafyllidou S, and Best D. Elevated blood lead in young children due to lead-contaminated drinking water: Washington, DC, 2001–2004. *Environ Sci Technol* 43: 1618–1623, 2009.
85. Escudero-Lourdes C. Toxicity mechanisms of arsenic that are shared with neurodegenerative diseases and cognitive impairment: role of oxidative stress and inflammatory responses. *Neurotoxicology* 53: 223–235, 2016.
86. Eskici G and Axelsen PH. Copper and oxidative stress in the pathogenesis of Alzheimer's disease. *Biochemistry* 51: 6289–6311, 2012.
87. Espinosa de los Monteros A, Chiapelli F, Fisher RS, and de Vellis J. Transferrin: an early marker of oligodendrocytes in culture. *Int J Dev Neurosci* 6: 167–175, 1988.
88. Esposito G, Vos M, Vilain S, Swerts J, De Sousa Valadas J, Van Meensel S, Schaap O, and Verstreken P. Aconitase causes iron toxicity in Drosophila pink1 mutants. *PLoS Genet* 9: e1003478, 2013.
89. Farina M, Avila DS, da Rocha JB, and Aschner M. Metals, oxidative stress and neurodegeneration: a focus on iron, manganese and mercury. *Neurochem Int* 62: 575–594, 2013.
90. Farzan SF, Karagas MR, and Chen Y. In utero and early life arsenic exposure in relation to long-term health and disease. *Toxicol Appl Pharmacol* 272: 384–390, 2013.
91. Faucheux BA, Martin ME, Beaumont C, Hauw JJ, Agid Y, and Hirsch EC. Neuromelanin associated redox-active iron is increased in the substantia nigra of patients with Parkinson's disease. *J Neurochem* 86: 1142–1148, 2003.
92. Fernandes J, Hao L, Bijli KM, Chandler JD, Orr M, Hu X, Jones DP, and Go YM. From the cover: manganese stimulates mitochondrial $H_2O_2$ production in SH-SY5Y human neuroblastoma cells over physiologic as well as toxicologic range. *Toxicol Sci* 155: 213–223, 2017.
93. Ferreira AM, Ciriolo MR, Marcocci L, and Rotilio G. Copper(I) transfer into metallothionein mediated by glutathione. *Biochem J* 292 (Pt 3): 673–676, 1993.
94. Ferrer I, Gomez A, Carmona M, Huesa G, Porta S, Riera-Codina M, Biagioli M, Gustincich S, and Aso E. Neuronal hemoglobin is reduced in Alzheimer's disease, argyrophilic grain disease, Parkinson's disease, and dementia with Lewy bodies. *J Alzheimers Dis* 23: 537–550, 2011.
95. Filipov NM, Seegal RF, and Lawrence DA. Manganese potentiates in vitro production of proinflammatory cytokines and nitric oxide by microglia through a nuclear factor kappa B-dependent mechanism. *Toxicol Sci* 84: 139–148, 2005.
96. Finkel T. Signal transduction by reactive oxygen species. *J Cell Biol* 194: 7–15, 2011.
97. Flora SJ. Arsenic-induced oxidative stress and its reversibility. *Free Radic Biol Med* 51: 257–281, 2011.
98. Flora SJ, Pande M, and Mehta A. Beneficial effect of combined administration of some naturally occurring antioxidants (vitamins) and thiol chelators in the treatment

of chronic lead intoxication. *Chem Biol Interact* 145: 267–280, 2003.
99. Flora SJ, Saxena G, and Mehta A. Reversal of lead-induced neuronal apoptosis by chelation treatment in rats: role of reactive oxygen species and intracellular Ca(2+). *J Pharmacol Exp Ther* 322: 108–116, 2007.
100. Forman HJ. Redox signaling: an evolution from free radicals to aging. *Free Radic Biol Med* 97: 398–407, 2016.
101. Forman HJ, Davies MJ, Kramer AC, Miotto G, Zaccarin M, Zhang H, and Ursini F. Protein cysteine oxidation in redox signaling: caveats on sulfenic acid detection and quantification. *Arch Biochem Biophys* 617: 26–37, 2017.
102. Fox JH, Kama JA, Lieberman G, Chopra R, Dorsey K, Chopra V, Volitakis I, Cherny RA, Bush AI, and Hersch S. Mechanisms of copper ion mediated Huntington's disease progression. *PLoS One* 2: e334, 2007.
103. Fraga CG. Relevance, essentiality and toxicity of trace elements in human health. *Mol Aspects Med* 26: 235–244, 2005.
104. Franco JL, Posser T, Dunkley PR, Dickson PW, Mattos JJ, Martins R, Bainy AC, Marques MR, Dafre AL, and Farina M. Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. *Free Radic Biol Med* 47: 449–457, 2009.
105. Franco R and Cidlowski JA. Glutathione efflux and cell death. *Antioxid Redox Signal* 17: 1694–1713, 2012.
106. Gao G and Chang YZ. Mitochondrial ferritin in the regulation of brain iron homeostasis and neurodegenerative diseases. *Front Pharmacol* 5: 19, 2014.
107. Garcia-Chavez E, Jimenez I, Segura B, and Del Razo LM. Lipid oxidative damage and distribution of inorganic arsenic and its metabolites in the rat nervous system after arsenite exposure: influence of alpha tocopherol supplementation. *Neurotoxicology* 27: 1024–1031, 2006.
108. Garcia-Chavez E, Santamaria A, Diaz-Barriga F, Mandeville P, Juarez BI, and Jimenez-Capdeville ME. Arsenite-induced formation of hydroxyl radical in the striatum of awake rats. *Brain Res* 976: 82–89, 2003.
109. Garcia-Nogales P, Almeida A, and Bolanos JP. Peroxynitrite protects neurons against nitric oxide-mediated apoptosis. A key role for glucose-6-phosphate dehydrogenase activity in neuroprotection. *J Biol Chem* 278: 864–874, 2003.
110. Gasperini L, Meneghetti E, Pastore B, Benetti F, and Legname G. Prion protein and copper cooperatively protect neurons by modulating NMDA receptor through S-nitrosylation. *Antioxid Redox Signal* 22: 772–784, 2015.
111. Gavin CE, Gunter KK, and Gunter TE. $Mn^{2+}$ sequestration by mitochondria and inhibition of oxidative phosphorylation. *Toxicol Appl Pharmacol* 115: 1–5, 1992.
112. Ghosh C, Seal M, Mukherjee S, and Ghosh Dey S. Alzheimer's disease: a heme-abeta perspective. *Acc Chem Res* 48: 2556–2564, 2015.
113. Girijashanker K, He L, Soleimani M, Reed JM, Li H, Liu Z, Wang B, Dalton TP, and Nebert DW. Slc39a14 gene encodes ZIP14, a metal/bicarbonate symporter: similarities to the ZIP8 transporter. *Mol Pharmacol* 73: 1413–1423, 2008.
114. Girotto S, Cendron L, Bisaglia M, Tessari I, Mammi S, Zanotti G, and Bubacco L. DJ-1 is a copper chaperone acting on SOD1 activation. *J Biol Chem* 289: 10887–10899, 2014.
115. Gorell JM, Johnson CC, Rybicki BA, Peterson EL, Kortsha GX, Brown GG, and Richardson RJ. Occupational exposure to manganese, copper, lead, iron, mercury and zinc and the risk of Parkinson's disease. *Neurotoxicology* 20: 239–247, 1999.
116. Grandjean P. Regional distribution of lead in human brains. *Toxicol Lett* 2: 65–69, 1978.
117. Gu C, Chen S, Xu X, Zheng L, Li Y, Wu K, Liu J, Qi Z, Han D, Chen G, and Huo X. Lead and cadmium synergistically enhance the expression of divalent metal transporter 1 protein in central nervous system of developing rats. *Neurochem Res* 34: 1150–1156, 2009.
118. Gundert-Remy U, Damm G, Foth H, Freyberger A, Gebel T, Golka K, Rohl C, Schupp T, Wollin KM, and Hengstler JG. High exposure to inorganic arsenic by food: the need for risk reduction. *Arch Toxicol* 89: 2219–2227, 2015.
119. Gunther V, Lindert U, and Schaffner W. The taste of heavy metals: gene regulation by MTF-1. *Biochim Biophys Acta* 1823: 1416–1425, 2012.
120. Halliwell B and Cross CE. Oxygen-derived species: their relation to human disease and environmental stress. *Environ Health Perspect* 102(Suppl 10): 5–12, 1994.
121. Hambright WS, Fonseca RS, Chen L, Na R, and Ran Q. Ablation of ferroptosis regulator glutathione peroxidase 4 in forebrain neurons promotes cognitive impairment and neurodegeneration. *Redox Biol* 12: 8–17, 2017.
122. Han M, Chang J, and Kim J. Loss of divalent metal transporter 1 function promotes brain copper accumulation and increases impulsivity. *J Neurochem* 138: 918–928, 2016.
123. Hands SL, Mason R, Sajjad MU, Giorgini F, and Wyttenbach A. Metallothioneins and copper metabolism are candidate therapeutic targets in Huntington's disease. *Biochem Soc Trans* 38: 552–558, 2010.
124. Hanna-Attisha M and Kuehn BM. Pediatrician sees long road ahead for flint after lead poisoning crisis. *JAMA* 315: 967–969, 2016.
125. Hare DJ, Lee JK, Beavis AD, van Gramberg A, George J, Adlard PA, Finkelstein DI, and Doble PA. Three-dimensional atlas of iron, copper, and zinc in the mouse cerebrum and brainstem. *Anal Chem* 84: 3990–3997, 2012.
126. Hashimoto M, Rockenstein E, Mante M, Mallory M, and Masliah E. beta-Synuclein inhibits alpha-synuclein aggregation: a possible role as an anti-parkinsonian factor. *Neuron* 32: 213–223, 2001.
127. Hatori Y, Clasen S, Hasan NM, Barry AN, and Lutsenko S. Functional partnership of the copper export machinery and glutathione balance in human cells. *J Biol Chem* 287: 26678–26687, 2012.
128. Hatori Y, Yan Y, Schmidt K, Furukawa E, Hasan NM, Yang N, Liu CN, Sockanathan S, and Lutsenko S. Neuronal differentiation is associated with a redox-regulated increase of copper flow to the secretory pathway. *Nat Commun* 7: 10640, 2016.
129. He X and Ma Q. Induction of metallothionein I by arsenic via metal-activated transcription factor 1: critical role of C-terminal cysteine residues in arsenic sensing. *J Biol Chem* 284: 12609–12621, 2009.
130. Higashi Y, Aratake T, Shimizu S, Shimizu T, Nakamura K, Tsuda M, Yawata T, Ueba T, and Saito M. Influence of extracellular zinc on M1 microglial activation. *Sci Rep* 7: 43778, 2017.

131. Hinkle PM, Kinsella PA, and Osterhoudt KC. Cadmium uptake and toxicity via voltage-sensitive calcium channels. *J Biol Chem* 262: 16333–16337, 1987.
132. Hodgkinson VL, Zhu S, Wang Y, Ladomersky E, Nickelson K, Weisman GA, Lee J, Gitlin JD, and Petris MJ. Autonomous requirements of the Menkes disease protein in the nervous system. *Am J Physiol Cell Physiol* 309: C660–C668, 2015.
133. Horning KJ, Caito SW, Tipps KG, Bowman AB, and Aschner M. Manganese is essential for neuronal health. *Annu Rev Nutr* 35: 71–108, 2015.
134. Howitt J, Putz U, Lackovic J, Doan A, Dorstyn L, Cheng H, Yang B, Chan-Ling T, Silke J, Kumar S, and Tan SS. Divalent metal transporter 1 (DMT1) regulation by Ndfip1 prevents metal toxicity in human neurons. *Proc Natl Acad Sci U S A* 106: 15489–15494, 2009.
135. Hsu PC and Guo YL. Antioxidant nutrients and lead toxicity. *Toxicology* 180: 33–44, 2002.
136. Hu Y, Su L, and Snow ET. Arsenic toxicity is enzyme specific and its affects on ligation are not caused by the direct inhibition of DNA repair enzymes. *Mutat Res* 408: 203–218, 1998.
137. Hu Z, Yu F, Gong P, Qiu Y, Zhou W, Cui Y, Li J, and Chen H. Subneurotoxic copper(II)-induced NF-kappaB-dependent microglial activation is associated with mitochondrial ROS. *Toxicol Appl Pharmacol* 276: 95–103, 2014.
138. Huang CC, Lesburg CA, Kiefer LL, Fierke CA, and Christianson DW. Reversal of the hydrogen bond to zinc ligand histidine-119 dramatically diminishes catalysis and enhances metal equilibration kinetics in carbonic anhydrase II. *Biochemistry* 35: 3439–3446, 1996.
139. Hughes MF. Arsenic toxicity and potential mechanisms of action. *Toxicol Lett* 133: 1–16, 2002.
140. Hureau C. Coordination of redox active metal ions to the amyloid precursor protein and to amyloid-$\beta$ peptides involved in Alzheimer disease. Part 1: an overview. *Coord Chem Rev* 256: 2164–2174, 2012.
141. International Lead Management Center. 1999. OECD declaration on lead risk reduction. The LEAD Group, Inc. 2016. www.lead.org.au/lanv7n1/L71–L79.html (November 28 2016).
142. Isaya G. Mitochondrial iron-sulfur cluster dysfunction in neurodegenerative disease. *Front Pharmacol* 5: 29, 2014.
143. Ito K, Eguchi Y, Imagawa Y, Akai S, Mochizuki H, and Tsujimoto Y. MPP+ induces necrostatin-1- and ferrostatin-1-sensitive necrotic death of neuronal SH-SY5Y cells. *Cell Death Discov* 3: 17013, 2017.
144. Jeong SY and David S. Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. *J Biol Chem* 278: 27144–27148, 2003.
145. Ji C and Kosman DJ. Molecular mechanisms of non-transferrin-bound and transferring-bound iron uptake in primary hippocampal neurons. *J Neurochem* 133: 668–683, 2015.
146. Jia Y, Jeng JM, Sensi SL, and Weiss JH. $Zn^{2+}$ currents are mediated by calcium-permeable AMPA/kainate channels in cultured murine hippocampal neurones. *J Physiol* 543: 35–48, 2002.
147. Jiang L, Garrick MD, Garrick LM, Zhao L, and Collins JF. Divalent metal transporter 1 (Dmt1) mediates copper transport in the duodenum of iron-deficient rats and when overexpressed in iron-deprived HEK-293 cells. *J Nutr* 143: 1927–1933, 2013.
148. Jimenez-Blasco D, Santofimia-Castano P, Gonzalez A, Almeida A, and Bolanos JP. Astrocyte NMDA receptors' activity sustains neuronal survival through a Cdk5-Nrf2 pathway. *Cell Death Differ* 22: 1877–1889, 2015.
149. Jin L, Wang J, Zhao L, Jin H, Fei G, Zhang Y, Zeng M, and Zhong C. Decreased serum ceruloplasmin levels characteristically aggravate nigral iron deposition in Parkinson's disease. *Brain* 134: 50–58, 2011.
150. Jomova K, Jenisova Z, Feszterova M, Baros S, Liska J, Hudecova D, Rhodes CJ, and Valko M. Arsenic: toxicity, oxidative stress and human disease. *J Appl Toxicol* 31: 95–107, 2011.
151. Jucaite A, Svenningsson P, Rinne JO, Cselenyi Z, Varnas K, Johnstrom P, Amini N, Kirjavainen A, Helin S, Minkwitz M, Kugler AR, Posener JA, Budd S, Halldin C, Varrone A, and Farde L. Effect of the myeloperoxidase inhibitor AZD3241 on microglia: a PET study in Parkinson's disease. *Brain* 138: 2687–2700, 2015.
152. Julvez J and Grandjean P. Genetic susceptibility to methylmercury developmental neurotoxicity matters. *Front Genet* 4: 278, 2013.
153. Jusko TA, Henderson CR, Lanphear BP, Cory-Slechta DA, Parsons PJ, and Canfield RL. Blood lead concentrations <10 microg/dL and child intelligence at 6 years of age. *Environ Health Perspect* 116: 243–248, 2008.
154. Kalappa BI, Anderson CT, Goldberg JM, Lippard SJ, and Tzounopoulos T. AMPA receptor inhibition by synaptically released zinc. *Proc Natl Acad Sci U S A* 112: 15749–15754, 2015.
155. Kambe T, Tsuji T, Hashimoto A, and Itsumura N. The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism. *Physiol Rev* 95: 749–784, 2015.
156. Karki P, Webb A, Smith K, Johnson J, Jr., Lee K, Son DS, Aschner M, and Lee E. Yin Yang 1 is a repressor of glutamate transporter EAAT2, and it mediates manganese-induced decrease of EAAT2 expression in astrocytes. *Mol Cell Biol* 34: 1280–1289, 2014.
157. Kasten M, Tadic V, Klein C, Rocca WA, Savica R, Eric Ahlskog J, and Grossardt BR. Anemia or low hemoglobin levels preceding Parkinson disease: a case-control study. *Neurology* 74: 1655; author reply 1655–1656, 2010.
158. Khan K, Wasserman GA, Liu X, Ahmed E, Parvez F, Slavkovich V, Levy D, Mey J, van Geen A, Graziano JH, and Factor-Litvak P. Manganese exposure from drinking water and children's academic achievement. *Neurotoxicology* 33: 91–97, 2012.
159. Kiedrowski L. Cytosolic acidification and intracellular zinc release in hippocampal neurons. *J Neurochem* 121: 438–450, 2012.
160. Kim G, Weiss SJ, and Levine RL. Methionine oxidation and reduction in proteins. *Biochim Biophys Acta* 1840: 901–905, 2014.
161. Kim J and Wessling-Resnick M. Iron and mechanisms of emotional behavior. *J Nutr Biochem* 25: 1101–1107, 2014.
162. Kim YH and Koh JY. The role of NADPH oxidase and neuronal nitric oxide synthase in zinc-induced poly(ADP-ribose) polymerase activation and cell death in cortical culture. *Exp Neurol* 177: 407–418, 2002.
163. Koskenkorva-Frank TS, Weiss G, Koppenol WH, and Burckhardt S. The complex interplay of iron metabolism, reactive oxygen species, and reactive nitrogen species: insights into the potential of various iron therapies to in-

duce oxidative and nitrosative stress. *Free Radic Biol Med* 65: 1174–1194, 2013.
164. Kuhn LC. Iron regulatory proteins and their role in controlling iron metabolism. *Metallomics* 7: 232–243, 2015.
165. Kumar S and Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett* 157: 175–188, 2005.
166. Kumar V and Gill KD. Oxidative stress and mitochondrial dysfunction in aluminium neurotoxicity and its amelioration: a review. *Neurotoxicology* 41: 154–166, 2014.
167. Lane DJ, Robinson SR, Czerwinska H, Bishop GM, and Lawen A. Two routes of iron accumulation in astrocytes: ascorbate-dependent ferrous iron uptake via the divalent metal transporter (DMT1) plus an independent route for ferric iron. *Biochem J* 432: 123–132, 2010.
168. Langkammer C, Ropele S, Pirpamer L, Fazekas F, and Schmidt R. MRI for iron mapping in Alzheimer's disease. *Neurodegener Dis* 13: 189–191, 2014.
169. Lau A, Zheng Y, Tao S, Wang H, Whitman SA, White E, and Zhang DD. Arsenic inhibits autophagic flux, activating the Nrf2-Keap1 pathway in a p62-dependent manner. *Mol Cell Biol* 33: 2436–2446, 2013.
170. Lautenschlager J, Kaminski CF, and Kaminski Schierle GS. alpha-Synuclein—regulator of exocytosis, endocytosis, or both? *Trends Cell Biol* 27: 468–479, 2017.
171. Lee DW, Kaur D, Chinta SJ, Rajagopalan S, and Andersen JK. A disruption in iron-sulfur center biogenesis via inhibition of mitochondrial dithiol glutaredoxin 2 may contribute to mitochondrial and cellular iron dysregulation in mammalian glutathione-depleted dopaminergic cells: implications for Parkinson's disease. *Antioxid Redox Signal* 11: 2083–2094, 2009.
172. Lee JY, Cole TB, Palmiter RD, Suh SW, and Koh JY. Contribution by synaptic zinc to the gender-disparate plaque formation in human Swedish mutant APP transgenic mice. *Proc Natl Acad Sci U S A* 99: 7705–7710, 2002.
173. Leslie EM, Haimeur A, and Waalkes MP. Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. *J Biol Chem* 279: 32700–32708, 2004.
174. Leslie EM, Liu J, Klaassen CD, and Waalkes MP. Acquired cadmium resistance in metallothionein-I/II(-/-) knockout cells: role of the T-type calcium channel Cacnalpha1G in cadmium uptake. *Mol Pharmacol* 69: 629–639, 2006.
175. Levesque PC and Atchison WD. Disruption of brain mitochondrial calcium sequestration by methylmercury. *J Pharmacol Exp Ther* 256: 236–242, 1991.
176. Levi S, Corsi B, Bosisio M, Invernizzi R, Volz A, Sanford D, Arosio P, and Drysdale J. A human mitochondrial ferritin encoded by an intronless gene. *J Biol Chem* 276: 24437–24440, 2001.
177. Lewerenz J and Maher P. Basal levels of eIF2alpha phosphorylation determine cellular antioxidant status by regulating ATF4 and xCT expression. *J Biol Chem* 284: 1106–1115, 2009.
178. Leyva-Illades D, Chen P, Zogzas CE, Hutchens S, Mercado JM, Swaim CD, Morrisett RA, Bowman AB, Aschner M, and Mukhopadhyay S. SLC30A10 is a cell surface-localized manganese efflux transporter, and parkinsonism-causing mutations block its intracellular trafficking and efflux activity. *J Neurosci* 34: 14079–14095, 2014.
179. Li RC, Guo SZ, Lee SK, and Gozal D. Neuroglobin protects neurons against oxidative stress in global ischemia. *J Cereb Blood Flow Metab* 30: 1874–1882, 2010.
180. Li RC, Pouranfar F, Lee SK, Morris MW, Wang Y, and Gozal D. Neuroglobin protects PC12 cells against beta-amyloid-induced cell injury. *Neurobiol Aging* 29: 1815–1822, 2008.
181. Li ZS, Szczypka M, Lu YP, Thiele DJ, and Rea PA. The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. *J Biol Chem* 271: 6509–6517, 1996.
182. Lin AM, Chao PL, Fang SF, Chi CW, and Yang CH. Endoplasmic reticulum stress is involved in arsenite-induced oxidative injury in rat brain. *Toxicol Appl Pharmacol* 224: 138–146, 2007.
183. Liu J, Liu Y, Powell DA, Waalkes MP, and Klaassen CD. Multidrug-resistance mdr1a/1b double knockout mice are more sensitive than wild type mice to acute arsenic toxicity, with higher arsenic accumulation in tissues. *Toxicology* 170: 55–62, 2002.
184. Liu L, Jiang D, McDonald A, Hao Y, Millhauser GL, and Zhou F. Copper redox cycling in the prion protein depends critically on binding mode. *J Am Chem Soc* 133: 12229–12237, 2011.
185. Liu X, Sullivan KA, Madl JE, Legare M, and Tjalkens RB. Manganese-induced neurotoxicity: the role of astroglial-derived nitric oxide in striatal interneuron degeneration. *Toxicol Sci* 91: 521–531, 2006.
186. Liu Y, Barber DS, Zhang P, and Liu B. Complex II of the mitochondrial respiratory chain is the key mediator of divalent manganese-induced hydrogen peroxide production in microglia. *Toxicol Sci* 132: 298–306, 2013.
187. Liu Z, Shen J, Carbrey JM, Mukhopadhyay R, Agre P, and Rosen BP. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. *Proc Natl Acad Sci U S A* 99: 6053–6058, 2002.
188. Liuzzi JP, Aydemir F, Nam H, Knutson MD, and Cousins RJ. Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells. *Proc Natl Acad Sci U S A* 103: 13612–13617, 2006.
189. Lo HS, Chiang HC, Lin AM, Chiang HY, Chu YC, and Kao LS. Synergistic effects of dopamine and $Zn^{2+}$ on the induction of PC12 cell death and dopamine depletion in the striatum: possible implication in the pathogenesis of Parkinson's disease. *Neurobiol Dis* 17: 54–61, 2004.
190. Logroscino G, Chen H, Wing A, and Ascherio A. Blood donations, iron stores, and risk of Parkinson's disease. *Mov Disord* 21: 835–838, 2006.
191. Loikkanen JJ, Naarala J, and Savolainen KM. Modification of glutamate-induced oxidative stress by lead: the role of extracellular calcium. *Free Radic Biol Med* 24: 377–384, 1998.
192. Loke SY, Siddiqi NJ, Alhomida AS, Kim HC, and Ong WY. Expression and localization of duodenal cytochrome b in the rat hippocampus after kainate-induced excitotoxicity. *Neuroscience* 245: 179–190, 2013.
193. Lopachin RM and Decaprio AP. Protein adduct formation as a molecular mechanism in neurotoxicity. *Toxicol Sci* 86: 214–225, 2005.
194. Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, and Markesbery WR. Copper, iron and zinc in Alzheimer's disease senile plaques. *J Neurol Sci* 158: 47–52, 1998.

195. Lu J and Holmgren A. The thioredoxin superfamily in oxidative protein folding. *Antioxid Redox Signal* 21: 457–470, 2014.
196. Lutsenko S. Human copper homeostasis: a network of interconnected pathways. *Curr Opin Chem Biol* 14: 211–217, 2010.
197. Lutsenko S. Copper trafficking to the secretory pathway. *Metallomics* 8: 840–852, 2016.
198. Maki RA, Tyurin VA, Lyon RC, Hamilton RL, DeKosky ST, Kagan VE, and Reynolds WF. Aberrant expression of myeloperoxidase in astrocytes promotes phospholipid oxidation and memory deficits in a mouse model of Alzheimer disease. *J Biol Chem* 284: 3158–3169, 2009.
199. Malaiyandi LM, Vergun O, Dineley KE, and Reynolds IJ. Direct visualization of mitochondrial zinc accumulation reveals uniporter-dependent and -independent transport mechanisms. *J Neurochem* 93: 1242–1250, 2005.
200. Mandal BK and Suzuki KT. Arsenic round the world: a review. *Talanta* 58: 201–235, 2002.
201. Manton WI, Kirkpatrick JB, and Cook JD. Does the choroid plexus really protect the brain from lead? *Lancet* 2: 351, 1984.
202. Mantyh PW, Ghilardi JR, Rogers S, DeMaster E, Allen CJ, Stimson ER, and Maggio JE. Aluminum, iron, and zinc ions promote aggregation of physiological concentrations of beta-amyloid peptide. *J Neurochem* 61: 1171–1174, 1993.
203. Mariani S, Ventriglia M, Simonelli I, Donno S, Bucossi S, Vernieri F, Melgari JM, Pasqualetti P, Rossini PM, and Squitti R. Fe and Cu do not differ in Parkinson's disease: a replication study plus meta-analysis. *Neurobiol Aging* 34: 632–633, 2013.
204. Marquez M, Blancas-Mejia LM, Campos A, Rojas L, Castaneda-Hernandez G, and Quintanar L. A bifunctional non-natural tetrapeptide modulates amyloid-beta peptide aggregation in the presence of Cu(ii). *Metallomics* 6: 2189–2192, 2014.
205. Martinez-Finley EJ, Chakraborty S, Fretham SJ, and Aschner M. Cellular transport and homeostasis of essential and nonessential metals. *Metallomics* 4: 593–605, 2012.
206. Martinez-Finley EJ, Gavin CE, Aschner M, and Gunter TE. Manganese neurotoxicity and the role of reactive oxygen species. *Free Radic Biol Med* 62: 65–75, 2013.
207. Martinez L, Jimenez V, Garcia-Sepulveda C, Ceballos F, Delgado JM, Nino-Moreno P, Doniz L, Saavedra-Alanis V, Castillo CG, Santoyo ME, Gonzalez-Amaro R, and Jimenez-Capdeville ME. Impact of early developmental arsenic exposure on promotor CpG-island methylation of genes involved in neuronal plasticity. *Neurochem Int* 58: 574–581, 2011.
208. Maryon EB, Molloy SA, and Kaplan JH. Cellular glutathione plays a key role in copper uptake mediated by human copper transporter 1. *Am J Physiol Cell Physiol* 304: C768–C779, 2013.
209. Masoud AM, Bihaqi SW, Machan JT, Zawia NH, and Renehan WE. Early-life exposure to lead (Pb) alters the expression of microrna that target proteins associated with Alzheimer's disease. *J Alzheimers Dis* 51: 1257–1264, 2016.
210. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, and Beyreuther K. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci U S A* 82: 4245–4249, 1985.
211. Mastroberardino PG, Hoffman EK, Horowitz MP, Betarbet R, Taylor G, Cheng D, Na HM, Gutekunst CA, Gearing M, Trojanowski JQ, Anderson M, Chu CT, Peng J, and Greenamyre JT. A novel transferrin/TfR2-mediated mitochondrial iron transport system is disrupted in Parkinson's disease. *Neurobiol Dis* 34: 417–431, 2009.
212. Matak P, Matak A, Moustafa S, Aryal DK, Benner EJ, Wetsel W, and Andrews NC. Disrupted iron homeostasis causes dopaminergic neurodegeneration in mice. *Proc Natl Acad Sci U S A* 113: 3428–3435, 2016.
213. Mato S, Sanchez-Gomez MV, Bernal-Chico A, and Matute C. Cytosolic zinc accumulation contributes to excitotoxic oligodendroglial death. *Glia* 61: 750–764, 2013.
214. Mayes J, Tinker-Mill C, Kolosov O, Zhang H, Tabner BJ, and Allsop D. beta-Amyloid fibrils in Alzheimer disease are not inert when bound to copper ions but can degrade hydrogen peroxide and generate reactive oxygen species. *J Biol Chem* 289: 12052–12062, 2014.
215. McCarthy RC and Kosman DJ. Mechanistic analysis of iron accumulation by endothelial cells of the BBB. *Biometals* 25: 665–675, 2012.
216. McCarthy RC and Kosman DJ. Glial cell ceruloplasmin and hepcidin differentially regulate iron efflux from brain microvascular endothelial cells. *PLoS One* 9: e89003, 2014.
217. McCarthy RC and Kosman DJ. Iron transport across the blood-brain barrier: development, neurovascular regulation and cerebral amyloid angiopathy. *Cell Mol Life Sci* 72: 709–727, 2015.
218. McCarthy RC and Kosman DJ. Mechanisms and regulation of iron trafficking across the capillary endothelial cells of the blood-brain barrier. *Front Mol Neurosci* 8: 31, 2015.
219. McCarthy RC, Park YH, and Kosman DJ. sAPP modulates iron efflux from brain microvascular endothelial cells by stabilizing the ferrous iron exporter ferroportin. *EMBO Rep* 15: 809–815, 2014.
220. McHugh PC, Wright JA, and Brown DR. Transcriptional regulation of the beta-synuclein 5′-promoter metal response element by metal transcription factor-1. *PLoS One* 6: e17354, 2011.
221. Meinerz DF, Branco V, Aschner M, Carvalho C, and Rocha JB. Diphenyl diselenide protects against methylmercury-induced inhibition of thioredoxin reductase and glutathione peroxidase in human neuroblastoma cells: a comparison with ebselen. *J Appl Toxicol* 37: 1073–1081, 2017.
222. Mellone M, Pelucchi S, Alberti L, Genazzani AA, Di Luca M, and Gardoni F. Zinc transporter-1: a novel NMDA receptor-binding protein at the postsynaptic density. *J Neurochem* 132: 159–168, 2015.
223. Meloni G, Sonois V, Delaine T, Guilloreau L, Gillet A, Teissie J, Faller P, and Vasak M. Metal swap between Zn7-metallothionein-3 and amyloid-beta-Cu protects against amyloid-beta toxicity. *Nat Chem Biol* 4: 366–372, 2008.
224. Menon AV, Chang J, and Kim J. Mechanisms of divalent metal toxicity in affective disorders. *Toxicology* 339: 58–72, 2016.
225. Meyer E, Kurian MA, and Hayflick SJ. Neurodegeneration with brain iron accumulation: genetic diversity and pathophysiological mechanisms. *Annu Rev Genomics Hum Genet* 16: 257–279, 2015.
226. Michael GJ, Esmailzadeh S, Moran LB, Christian L, Pearce RK, and Graeber MB. Up-regulation of me-

tallothionein gene expression in parkinsonian astrocytes. *Neurogenetics* 12: 295–305, 2011.
227. Miotto MC, Rodriguez EE, Valiente-Gabioud AA, Torres-Monserrat V, Binolfi A, Quintanar L, Zweckstetter M, Griesinger C, and Fernandez CO. Site-specific copper-catalyzed oxidation of alpha-synuclein: tightening the link between metal binding and protein oxidative damage in Parkinson's disease. *Inorg Chem* 53: 4350–4358, 2014.
228. Miotto MC, Valiente-Gabioud AA, Rossetti G, Zweckstetter M, Carloni P, Selenko P, Griesinger C, Binolfi A, and Fernandez CO. Copper binding to the N-terminally acetylated, naturally occurring form of alpha-synuclein induces local helical folding. *J Am Chem Soc* 137: 6444–6447, 2015.
229. Mital M, Wezynfeld NE, Fraczyk T, Wiloch MZ, Wawrzyniak UE, Bonna A, Tumpach C, Barnham KJ, Haigh CL, Bal W, and Drew SC. A functional role for abeta in metal homeostasis? N-truncation and high-affinity copper binding. *Angew Chem Int Ed Engl* 54: 10460–10464, 2015.
230. Mizumura A, Watanabe T, Kobayashi Y, and Hirano S. Identification of arsenite-and arsenic diglutathione-binding proteins in human hepatocarcinoma cells. *Toxicol Appl Pharmacol* 242: 119–125, 2010.
231. Monnot AD, Zheng G, and Zheng W. Mechanism of copper transport at the blood-cerebrospinal fluid barrier: influence of iron deficiency in an in vitro model. *Exp Biol Med (Maywood)* 237: 327–333, 2012.
232. Moos T. Immunohistochemical localization of intraneuronal transferrin receptor immunoreactivity in the adult mouse central nervous system. *J Comp Neurol* 375: 675–692, 1996.
233. Mori N, Yasutake A, and Hirayama K. Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity. *Arch Toxicol* 81: 769–776, 2007.
234. Moriarty GM, Minetti CA, Remeta DP, and Baum J. A revised picture of the Cu(II)-alpha-synuclein complex: the role of N-terminal acetylation. *Biochemistry* 53: 2815–2817, 2014.
235. Morken TS, Sonnewald U, Aschner M, and Syversen T. Effects of methylmercury on primary brain cells in mono- and co-culture. *Toxicol Sci* 87: 169–175, 2005.
236. Moshtaghie AA, Ani M, Aghadavod E, and Fazilati M. Protective effects of selenium and zinc on changes in catecholamine levels of brain regions in lead intoxified rat. *Pak J Biol Sci* 10: 2964–2967, 2007.
237. Munoz P, Humeres A, Elgueta C, Kirkwood A, Hidalgo C, and Nunez MT. Iron mediates N-methyl-D-aspartate receptor-dependent stimulation of calcium-induced pathways and hippocampal synaptic plasticity. *J Biol Chem* 286: 13382–13392, 2011.
238. Naarala JT, Loikkanen JJ, Ruotsalainen MH, and Savolainen KM. Lead amplifies glutamate-induced oxidative stress. *Free Radic Biol Med* 19: 689–693, 1995.
239. Nagababu E and Rifkind JM. Heme degradation by reactive oxygen species. *Antioxid Redox Signal* 6: 967–978, 2004.
240. Nagasawa K, Ito S, Kakuda T, Nagai K, Tamai I, Tsuji A, and Fujimoto S. Transport mechanism for aluminum citrate at the blood-brain barrier: kinetic evidence implies involvement of system Xc- in immortalized rat brain endothelial cells. *Toxicol Lett* 155: 289–296, 2005.
241. Nava-Ruiz C, Mendez-Armenta M, and Rios C. Lead neurotoxicity: effects on brain nitric oxide synthase. *J Mol Histol* 43: 553–563, 2012.
242. Neal AP and Guilarte TR. Molecular neurobiology of lead (Pb(2+)): effects on synaptic function. *Mol Neurobiol* 42: 151–160, 2010.
243. Negishi T, Takahashi M, Matsunaga Y, Hirano S, and Tashiro T. Diphenylarsinic acid increased the synthesis and release of neuroactive and vasoactive peptides in rat cerebellar astrocytes. *J Neuropathol Exp Neurol* 71: 468–479, 2012.
244. Nelson-Mora J, Escobar ML, Rodríguez-Durán L, Massieu L, Montiel T, Rodríguez VM, Hernández-Mercado K, and Gonsebatt ME. Gestational exposure to inorganic arsenic (iAs3+) alters glutamate disposition in the mouse hippocampus and ionotropic glutamate receptor expression leading to memory impairment. *Arch Toxicol* 92: 1037–1048, 2018.
245. Nemeti B, Regonesi ME, Tortora P, and Gregus Z. Polynucleotide phosphorylase and mitochondrial ATP synthase mediate reduction of arsenate to the more toxic arsenite by forming arsenylated analogues of ADP and ATP. *Toxicol Sci* 117: 270–281, 2010.
246. Nishito Y, Tsuji N, Fujishiro H, Takeda TA, Yamazaki T, Teranishi F, Okazaki F, Matsunaga A, Tuschl K, Rao R, Kono S, Miyajima H, Narita H, Himeno S, and Kambe T. Direct comparison of manganese detoxification/efflux proteins and molecular characterization of ZnT10 protein as a manganese transporter. *J Biol Chem* 291: 14773–14787, 2016.
247. Nolte C, Gore A, Sekler I, Kresse W, Hershfinkel M, Hoffmann A, Kettenmann H, and Moran A. ZnT-1 expression in astroglial cells protects against zinc toxicity and slows the accumulation of intracellular zinc. *Glia* 48: 145–155, 2004.
248. Nordberg G, Fowler BA, and Nordberg M. *Handbook on the Toxicology of Metals*. Amsterdam: Academic Press, 2014, p. 1 online resource.
249. Ogra Y, Tejima A, Hatakeyama N, Shiraiwa M, Wu S, Ishikawa T, Yawata A, Anan Y, and Suzuki N. Changes in intracellular copper concentration and copper-regulating gene expression after PC12 differentiation into neurons. *Sci Rep* 6: 33007, 2016.
250. Oken E and Bellinger DC. Fish consumption, methylmercury and child neurodevelopment. *Curr Opin Pediatr* 20: 178–183, 2008.
251. Olivieri S, Conti A, Iannaccone S, Cannistraci CV, Campanella A, Barbariga M, Codazzi F, Pelizzoni I, Magnani G, Pesca M, Franciotta D, Cappa SF, and Alessio M. Ceruloplasmin oxidation, a feature of Parkinson's disease CSF, inhibits ferroxidase activity and promotes cellular iron retention. *J Neurosci* 31: 18568–18577, 2011.
252. Olsen LF, Issinger OG, and Guerra B. The Yin and Yang of redox regulation. *Redox Rep* 18: 245–252, 2013.
253. Omata Y, Salvador GA, Supasai S, Keenan AH, and Oteiza PI. Decreased zinc availability affects glutathione metabolism in neuronal cells and in the developing brain. *Toxicol Sci* 133: 90–100, 2013.
254. Ortega R, Carmona A, Roudeau S, Perrin L, Ducic T, Carboni E, Bohic S, Cloetens P, and Lingor P. alpha-Synuclein over-expression induces increased iron accumulation and redistribution in iron-exposed neurons. *Mol Neurobiol* 53: 1925–1934, 2016.

255. Oteiza PI. Zinc and the modulation of redox homeostasis. *Free Radic Biol Med* 53: 1748–1759, 2012.
256. Pachauri V, Saxena G, Mehta A, Mishra D, and Flora SJ. Combinational chelation therapy abrogates lead-induced neurodegeneration in rats. *Toxicol Appl Pharmacol* 240: 255–264, 2009.
257. Pan R and Liu KJ. ZNT-1 expression reduction enhances free zinc accumulation in astrocytes after ischemic stroke. *Acta Neurochir Suppl* 121: 257–261, 2016.
258. Pande M and Flora SJ. Lead induced oxidative damage and its response to combined administration of alpha-lipoic acid and succimers in rats. *Toxicology* 177: 187–196, 2002.
259. Park JS, Koentjoro B, Veivers D, Mackay-Sim A, and Sue CM. Parkinson's disease-associated human ATP13A2 (PARK9) deficiency causes zinc dyshomeostasis and mitochondrial dysfunction. *Hum Mol Genet* 23: 2802–2815, 2014.
260. Parvez F, Wasserman GA, Factor-Litvak P, Liu X, Slavkovich V, Siddique AB, Sultana R, Sultana R, Islam T, Levy D, Mey JL, van Geen A, Khan K, Kline J, Ahsan H, and Graziano JH. Arsenic exposure and motor function among children in Bangladesh. *Environ Health Perspect* 119: 1665–1670, 2011.
261. Patel BN, Dunn RJ, Jeong SY, Zhu Q, Julien JP, and David S. Ceruloplasmin regulates iron levels in the CNS and prevents free radical injury. *J Neurosci* 22: 6578–6586, 2002.
262. Patel M. Targeting oxidative stress in central nervous system disorders. *Trends Pharmacol Sci* 37: 768–778, 2016.
263. Pauly PC and Harris DA. Copper stimulates endocytosis of the prion protein. *J Biol Chem* 273: 33107–33110, 1998.
264. Pedersen JT, Chen SW, Borg CB, Ness S, Bahl JM, Heegaard NH, Dobson CM, Hemmingsen L, Cremades N, and Teilum K. Amyloid-beta and alpha-synuclein decrease the level of metal-catalyzed reactive oxygen species by radical scavenging and redox silencing. *J Am Chem Soc* 138: 3966–3969, 2016.
265. Pelizzoni I, Zacchetti D, Campanella A, Grohovaz F, and Codazzi F. Iron uptake in quiescent and inflammation-activated astrocytes: a potentially neuroprotective control of iron burden. *Biochim Biophys Acta* 1832: 1326–1333, 2013.
266. Pelizzoni I, Zacchetti D, Smith CP, Grohovaz F, and Codazzi F. Expression of divalent metal transporter 1 in primary hippocampal neurons: reconsidering its role in non-transferrin-bound iron influx. *J Neurochem* 120: 269–278, 2012.
267. Penugonda S, Mare S, Lutz P, Banks WA, and Ercal N. Potentiation of lead-induced cell death in PC12 cells by glutamate: protection by N-acetylcysteine amide (NACA), a novel thiol antioxidant. *Toxicol Appl Pharmacol* 216: 197–205, 2006.
268. Perkins A, Nelson KJ, Parsonage D, Poole LB, and Karplus PA. Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. *Trends Biochem Sci* 40: 435–445, 2015.
269. Perrin L, Roudeau S, Carmona A, Domart F, Petersen JD, Bohic S, Yang Y, Cloetens P, and Ortega R. Zinc and copper effects on stability of tubulin and actin networks in dendrites and spines of hippocampal neurons. *ACS Chem Neurosci* 8: 1490–1499, 2017.
270. Philbert MA, Billingsley ML, and Reuhl KR. Mechanisms of injury in the central nervous system. *Toxicol Pathol* 28: 43–53, 2000.
271. Pichler I, Del Greco MF, Gogele M, Lill CM, Bertram L, Do CB, Eriksson N, Foroud T, Myers RH, Nalls M, Keller MF, Benyamin B, Whitfield JB, Pramstaller PP, Hicks AA, Thompson JR, and Minelli C. Serum iron levels and the risk of Parkinson disease: a Mendelian randomization study. *PLoS Med* 10: e1001462, 2013.
272. Pietsch EC, Chan JY, Torti FM, and Torti SV. Nrf2 mediates the induction of ferritin H in response to xenobiotics and cancer chemopreventive dithiolethiones. *J Biol Chem* 278: 2361–2369, 2003.
273. Portelius E, Bogdanovic N, Gustavsson MK, Volkmann I, Brinkmalm G, Zetterberg H, Winblad B, and Blennow K. Mass spectrometric characterization of brain amyloid beta isoform signatures in familial and sporadic Alzheimer's disease. *Acta Neuropathol* 120: 185–193, 2010.
274. Prakash C and Kumar V. Arsenic-induced mitochondrial oxidative damage is mediated by decreased PGC-1alpha expression and its downstream targets in rat brain. *Chem Biol Interact* 256: 228–235, 2016.
275. Prakash C, Soni M, and Kumar V. Biochemical and molecular alterations following arsenic-induced oxidative stress and mitochondrial dysfunction in rat brain. *Biol Trace Elem Res* 167: 121–129, 2015.
276. Prakash C, Soni M, and Kumar V. Mitochondrial oxidative stress and dysfunction in arsenic neurotoxicity: a review. *J Appl Toxicol* 36: 179–188, 2016.
277. Prasanthi RP, Devi CB, Basha DC, Reddy NS, and Reddy GR. Calcium and zinc supplementation protects lead (Pb)-induced perturbations in antioxidant enzymes and lipid peroxidation in developing mouse brain. *Int J Dev Neurosci* 28: 161–167, 2010.
278. Qian J, Xu K, Yoo J, Chen TT, Andrews G, and Noebels JL. Knockout of Zn transporters Zip-1 and Zip-3 attenuates seizure-induced CA1 neurodegeneration. *J Neurosci* 31: 97–104, 2011.
279. Qu W and Waalkes MP. Metallothionein blocks oxidative DNA damage induced by acute inorganic arsenic exposure. *Toxicol Appl Pharmacol* 282: 267–274, 2015.
280. Ramos-Chavez LA, Rendon-Lopez CR, Zepeda A, Silva-Adaya D, Del Razo LM, and Gonsebatt ME. Neurological effects of inorganic arsenic exposure: altered cysteine/glutamate transport, NMDA expression and spatial memory impairment. *Front Cell Neurosci* 9: 21, 2015.
281. Ramos D, Mar D, Ishida M, Vargas R, Gaite M, Montgomery A, and Linder MC. Mechanism of copper uptake from blood plasma ceruloplasmin by mammalian cells. *PLoS One* 11: e0149516, 2016.
282. Reczek CR and Chandel NS. ROS-dependent signal transduction. *Curr Opin Cell Biol* 33: 8–13, 2015.
283. Reddy GR and Zawia NH. Lead exposure alters Egr-1 DNA-binding in the neonatal rat brain. *Int J Dev Neurosci* 18: 791–795, 2000.
284. Rehman K and Naranmandura H. Arsenic metabolism and thioarsenicals. *Metallomics* 4: 881–892, 2012.
285. Rhodes SL, Buchanan DD, Ahmed I, Taylor KD, Loriot MA, Sinsheimer JS, Bronstein JM, Elbaz A, Mellick GD, Rotter JI, and Ritz B. Pooled analysis of iron-related genes in Parkinson's disease: association with transferrin. *Neurobiol Dis* 62: 172–178, 2014.
286. Rogers B, Yakopson V, Teng ZP, Guo Y, and Regan RF. Heme oxygenase-2 knockout neurons are less vulnerable to hemoglobin toxicity. *Free Radic Biol Med* 35: 872–881, 2003.
287. Rosado JL, Ronquillo D, Kordas K, Rojas O, Alatorre J, Lopez P, Garcia-Vargas G, Del Carmen Caamano M,

Cebrian ME, and Stoltzfus RJ. Arsenic exposure and cognitive performance in Mexican schoolchildren. *Environ Health Perspect* 115: 1371–1375, 2007.
288. Roskams AJ and Connor JR. Aluminum access to the brain: a role for transferrin and its receptor. *Proc Natl Acad Sci U S A* 87: 9024–9027, 1990.
289. Roth JA, Singleton S, Feng J, Garrick M, and Paradkar PN. Parkin regulates metal transport via proteasomal degradation of the 1B isoforms of divalent metal transporter 1. *J Neurochem* 113: 454–464, 2010.
290. Rouault TA. Iron metabolism in the CNS: implications for neurodegenerative diseases. *Nat Rev Neurosci* 14: 551–564, 2013.
291. Rouault TA. Mammalian iron-sulphur proteins: novel insights into biogenesis and function. *Nat Rev Mol Cell Biol* 16: 45–55, 2015.
292. Rush T, Liu X, Nowakowski AB, Petering DH, and Lobner D. Glutathione-mediated neuroprotection against methylmercury neurotoxicity in cortical culture is dependent on MRP1. *Neurotoxicology* 33: 476–481, 2012.
293. Saini N, Georgiev O, and Schaffner W. The parkin mutant phenotype in the fly is largely rescued by metal-responsive transcription factor (MTF-1). *Mol Cell Biol* 31: 2151–2161, 2011.
294. Salazar J, Mena N, Hunot S, Prigent A, Alvarez-Fischer D, Arredondo M, Duyckaerts C, Sazdovitch V, Zhao L, Garrick LM, Nunez MT, Garrick MD, Raisman-Vozari R, and Hirsch EC. Divalent metal transporter 1 (DMT1) contributes to neurodegeneration in animal models of Parkinson's disease. *Proc Natl Acad Sci U S A* 105: 18578–18583, 2008.
295. Salim S. Oxidative stress and the central nervous system. *J Pharmacol Exp Ther* 360: 201–205, 2017.
296. Samuel S, Kathirvel R, Jayavelu T, and Chinnakkannu P. Protein oxidative damage in arsenic induced rat brain: influence of DL-alpha-lipoic acid. *Toxicol Lett* 155: 27–34, 2005.
297. Sanchez-Pena LC, Petrosyan P, Morales M, Gonzalez NB, Gutierrez-Ospina G, Del Razo LM, and Gonsebatt ME. Arsenic species, AS3MT amount, and AS3MT gene expression in different brain regions of mouse exposed to arsenite. *Environ Res* 110: 428–434, 2010.
298. Sanders T, Liu Y, Buchner V, and Tchounwou PB. Neurotoxic effects and biomarkers of lead exposure: a review. *Rev Environ Health* 24: 15–45, 2009.
299. Santos CR, Martinho A, Quintela T, and Goncalves I. Neuroprotective and neuroregenerative properties of metallothioneins. *IUBMB Life* 64: 126–135, 2012.
300. Scheiber IF and Dringen R. Astrocyte functions in the copper homeostasis of the brain. *Neurochem Int* 62: 556–565, 2013.
301. Schlief ML, Craig AM, and Gitlin JD. NMDA receptor activation mediates copper homeostasis in hippocampal neurons. *J Neurosci* 25: 239–246, 2005.
302. Schrader M and Fahimi HD. Peroxisomes and oxidative stress. *Biochim Biophys Acta* 1763: 1755–1766, 2006.
303. Segawa S, Tatsumi N, Ohishi A, Nishida K, and Nagasawa K. Characterization of zinc uptake by mouse primary cultured astrocytes and microglia. *Metallomics* 7: 1067–1077, 2015.
304. Sensi SL, Paoletti P, Koh JY, Aizenman E, Bush AI, and Hershfinkel M. The neurophysiology and pathology of brain zinc. *J Neurosci* 31: 16076–16085, 2011.
305. Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, and Weiss JH. Modulation of mitochondrial function by endogenous $Zn^{2+}$ pools. *Proc Natl Acad Sci U S A* 100: 6157–6162, 2003.
306. Senut MC, Cingolani P, Sen A, Kruger A, Shaik A, Hirsch H, Suhr ST, and Ruden D. Epigenetics of early-life lead exposure and effects on brain development. *Epigenomics* 4: 665–674, 2012.
307. Seo YA, Lopez V, and Kelleher SL. A histidine-rich motif mediates mitochondrial localization of ZnT2 to modulate mitochondrial function. *Am J Physiol Cell Physiol* 300: C1479–C1489, 2011.
308. Shahzad R, Jones MR, Viles JH, and Jones CE. Endocytosis of the tachykinin neuropeptide, neurokinin B, in astrocytes and its role in cellular copper uptake. *J Inorg Biochem* 162: 319–325, 2016.
309. Sharma DR, Sunkaria A, Wani WY, Sharma RK, Kandimalla RJ, Bal A, and Gill KD. Aluminium induced oxidative stress results in decreased mitochondrial biogenesis via modulation of PGC-1alpha expression. *Toxicol Appl Pharmacol* 273: 365–380, 2013.
310. Sheline CT, Zhu J, Zhang W, Shi C, and Cai AL. Mitochondrial inhibitor models of Huntington's disease and Parkinson's disease induce zinc accumulation and are attenuated by inhibition of zinc neurotoxicity in vitro or in vivo. *Neurodegener Dis* 11: 49–58, 2013.
311. Shen S, Li XF, Cullen WR, Weinfeld M, and Le XC. Arsenic binding to proteins. *Chem Rev* 113: 7769–7792, 2013.
312. Sheng Y, Abreu IA, Cabelli DE, Maroney MJ, Miller AF, Teixeira M, and Valentine JS. Superoxide dismutases and superoxide reductases. *Chem Rev* 114: 3854–3918, 2014.
313. Shi ZH, Nie G, Duan XL, Rouault T, Wu WS, Ning B, Zhang N, Chang YZ, and Zhao BL. Neuroprotective mechanism of mitochondrial ferritin on 6-hydroxydopamine-induced dopaminergic cell damage: implication for neuroprotection in Parkinson's disease. *Antioxid Redox Signal* 13: 783–796, 2010.
314. Shih AY, Johnson DA, Wong G, Kraft AD, Jiang L, Erb H, Johnson JA, and Murphy TH. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *J Neurosci* 23: 3394–3406, 2003.
315. Shila S, Kokilavani V, Subathra M, and Panneerselvam C. Brain regional responses in antioxidant system to alpha-lipoic acid in arsenic intoxicated rat. *Toxicology* 210: 25–36, 2005.
316. Shila S, Subathra M, Devi MA, and Panneerselvam C. Arsenic intoxication-induced reduction of glutathione level and of the activity of related enzymes in rat brain regions: reversal by DL-alpha-lipoic acid. *Arch Toxicol* 79: 140–146, 2005.
317. Shukalek CB, Swanlund DP, Rousseau RK, Weigl KE, Marensi V, Cole SP, and Leslie EM. Arsenic triglutathione [As(GS)3] transport by multidrug resistance protein 1 (MRP1/ABCC1) is selectively modified by phosphorylation of Tyr920/Ser921 and glycosylation of Asn19/Asn23. *Mol Pharmacol* 90: 127–139, 2016.
318. Simmons-Willis TA, Koh AS, Clarkson TW, and Ballatori N. Transport of a neurotoxicant by molecular mimicry: the methylmercury-L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2. *Biochem J* 367: 239–246, 2002.
319. Singh I, Sagare AP, Coma M, Perlmutter D, Gelein R, Bell RD, Deane RJ, Zhong E, Parisi M, Ciszewski J, Kasper RT, and Deane R. Low levels of copper disrupt

brain amyloid-beta homeostasis by altering its production and clearance. *Proc Natl Acad Sci U S A* 110: 14771–14776, 2013.
320. Singh V, Gera R, Kushwaha R, Sharma AK, Patnaik S, and Ghosh D. Hijacking microglial glutathione by inorganic arsenic impels bystander death of immature neurons through extracellular cystine/glutamate imbalance. *Sci Rep* 6: 30601, 2016.
321. Smith MA, Harris PL, Sayre LM, and Perry G. Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci U S A* 94: 9866–9868, 1997.
322. Smith MR, Fernandes J, Go YM, and Jones DP. Redox dynamics of manganese as a mitochondrial life-death switch. *Biochem Biophys Res Commun* 482: 388–398, 2017.
323. Sobotta MC, Liou W, Stocker S, Talwar D, Oehler M, Ruppert T, Scharf AN, and Dick TP. Peroxiredoxin-2 and STAT3 form a redox relay for $H_2O_2$ signaling. *Nat Chem Biol* 11: 64–70, 2015.
324. Squitti R, Salustri C, Siotto M, Ventriglia M, Vernieri F, Lupoi D, Cassetta E, and Rossini PM. Ceruloplasmin/transferrin ratio changes in Alzheimer's disease. *Int J Alzheimers Dis* 2011: 231595, 2010.
325. Squitti R, Simonelli I, Ventriglia M, Siotto M, Pasqualetti P, Rembach A, Doecke J, and Bush AI. Meta-analysis of serum non-ceruloplasmin copper in Alzheimer's disease. *J Alzheimers Dis* 38: 809–822, 2014.
326. Squitti R, Ventriglia M, Gennarelli M, Colabufo NA, El Idrissi IG, Bucossi S, Mariani S, Rongioletti M, Zanetti O, Congiu C, Rossini PM, and Bonvicini C. Non-ceruloplasmin copper distincts subtypes in Alzheimer's disease: a genetic study of ATP7B frequency. *Mol Neurobiol* 54: 671–681, 2017.
327. Steiger TK, Weiskopf N, and Bunzeck N. Iron level and myelin content in the ventral striatum predict memory performance in the aging brain. *J Neurosci* 36: 3552–3558, 2016.
328. Storr SJ, Woolston CM, Zhang Y, and Martin SG. Redox environment, free radical, and oxidative DNA damage. *Antioxid Redox Signal* 18: 2399–2408, 2013.
329. Stuber C, Morawski M, Schafer A, Labadie C, Wahnert M, Leuze C, Streicher M, Barapatre N, Reimann K, Geyer S, Spemann D, and Turner R. Myelin and iron concentration in the human brain: a quantitative study of MRI contrast. *Neuroimage* 93 (Pt 1): 95–106, 2014.
330. Suazo M, Hodar C, Morgan C, Cerpa W, Cambiazo V, Inestrosa NC, and Gonzalez M. Overexpression of amyloid precursor protein increases copper content in HEK293 cells. *Biochem Biophys Res Commun* 382: 740–744, 2009.
331. Sun Y, Jin K, Mao XO, Zhu Y, and Greenberg DA. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proc Natl Acad Sci U S A* 98: 15306–15311, 2001.
332. Tadepalle N, Koehler Y, Brandmann M, Meyer N, and Dringen R. Arsenite stimulates glutathione export and glycolytic flux in viable primary rat brain astrocytes. *Neurochem Int* 76: 1–11, 2014.
333. Tai YK, Chew KC, Tan BW, Lim KL, and Soong TW. Iron mitigates DMT1-mediated manganese cytotoxicity via the ASK1-JNK signaling axis: implications of iron supplementation for manganese toxicity. *Sci Rep* 6: 21113, 2016.
334. Takahashi M, Dore S, Ferris CD, Tomita T, Sawa A, Wolosker H, Borchelt DR, Iwatsubo T, Kim SH, Thinakaran G, Sisodia SS, and Snyder SH. Amyloid precursor proteins inhibit heme oxygenase activity and augment neurotoxicity in Alzheimer's disease. *Neuron* 28: 461–473, 2000.
335. Takeda A, Fuke S, Minami A, and Oku N. Role of zinc influx via AMPA/kainate receptor activation in metabotropic glutamate receptor-mediated calcium release. *J Neurosci Res* 85: 1310–1317, 2007.
336. Tan J, Zhang T, Jiang L, Chi J, Hu D, Pan Q, Wang D, and Zhang Z. Regulation of intracellular manganese homeostasis by Kufor-Rakeb syndrome-associated ATP13A2 protein. *J Biol Chem* 286: 29654–29662, 2011.
337. Tang L, Zhang Y, Qian Z, and Shen X. The mechanism of Fe(2+)-initiated lipid peroxidation in liposomes: the dual function of ferrous ions, the roles of the pre-existing lipid peroxides and the lipid peroxyl radical. *Biochem J* 352 (Pt 1): 27–36, 2000.
338. Telianidis J, Hung YH, Materia S, and Fontaine SL. Role of the P-Type ATPases, ATP7A and ATP7B in brain copper homeostasis. *Front Aging Neurosci* 5: 44, 2013.
339. Thevenod F. Catch me if you can! Novel aspects of cadmium transport in mammalian cells. *Biometals* 23: 857–875, 2010.
340. Thomas DJ. Arsenolysis and thiol-dependent arsenate reduction. *Toxicol Sci* 117: 249–252, 2010.
341. Tjalve H and Henriksson J. Uptake of metals in the brain via olfactory pathways. *Neurotoxicology* 20: 181–195, 1999.
342. Todorich B, Pasquini JM, Garcia CI, Paez PM, and Connor JR. Oligodendrocytes and myelination: the role of iron. *Glia* 57: 467–478, 2009.
343. Todorich B, Zhang X, Slagle-Webb B, Seaman WE, and Connor JR. Tim-2 is the receptor for H-ferritin on oligodendrocytes. *J Neurochem* 107: 1495–1505, 2008.
344. Tokuda E, Okawa E, and Ono S. Dysregulation of intracellular copper trafficking pathway in a mouse model of mutant copper/zinc superoxide dismutase-linked familial amyotrophic lateral sclerosis. *J Neurochem* 111: 181–191, 2009.
345. Tokuda E, Okawa E, Watanabe S, and Ono S. Overexpression of metallothionein-I, a copper-regulating protein, attenuates intracellular copper dyshomeostasis and extends lifespan in a mouse model of amyotrophic lateral sclerosis caused by mutant superoxide dismutase-1. *Hum Mol Genet* 23: 1271–1285, 2014.
346. Tokuda E, Okawa E, Watanabe S, Ono S, and Marklund SL. Dysregulation of intracellular copper homeostasis is common to transgenic mice expressing human mutant superoxide dismutase-1s regardless of their copper-binding abilities. *Neurobiol Dis* 54: 308–319, 2013.
347. Tolins M, Ruchirawat M, and Landrigan P. The developmental neurotoxicity of arsenic: cognitive and behavioral consequences of early life exposure. *Ann Glob Health* 80: 303–314, 2014.
348. Torres-Avila M, Leal-Galicia P, Sanchez-Pena LC, Del Razo LM, and Gonsebatt ME. Arsenite induces aquaglyceroporin 9 expression in murine livers. *Environ Res* 110: 443–447, 2010.
349. Toyama T, Shinkai Y, Yasutake A, Uchida K, Yamamoto M, and Kumagai Y. Isothiocyanates reduce mercury accumulation via an Nrf2-dependent mechanism during exposure of mice to methylmercury. *Environ Health Perspect* 119: 1117–1122, 2011.

350. Trujano-Ortiz LG, Gonzalez FJ, and Quintanar L. Redox cycling of copper-amyloid beta 1–16 peptide complexes is highly dependent on the coordination mode. *Inorg Chem* 54: 4–6, 2015.
351. Tulpule K, Robinson SR, Bishop GM, and Dringen R. Uptake of ferrous iron by cultured rat astrocytes. *J Neurosci Res* 88: 563–571, 2010.
352. Tuschl K, Mills PB, and Clayton PT. Manganese and the brain. *Int Rev Neurobiol* 110: 277–312, 2013.
353. United States. Agency for Toxic Substances and Disease Registry. *Toxicological Profile for Arsenic*. Atlanta, GA: U.S. Dept. of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, 2007, p. 1 online resource (xx, 500 p.).
354. Urrutia P, Aguirre P, Esparza A, Tapia V, Mena NP, Arredondo M, Gonzalez-Billault C, and Nunez MT. Inflammation alters the expression of DMT1, FPN1 and hepcidin, and it causes iron accumulation in central nervous system cells. *J Neurochem* 126: 541–549, 2013.
355. Valko M, Jomova K, Rhodes CJ, Kuca K, and Musilek K. Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. *Arch Toxicol* 90: 1–37, 2016.
356. Vanoevelen J, Dode L, Van Baelen K, Fairclough RJ, Missiaen L, Raeymaekers L, and Wuytack F. The secretory pathway $Ca^{2+}/Mn^{2+}$-ATPase 2 is a Golgi-localized pump with high affinity for $Ca^{2+}$ ions. *J Biol Chem* 280: 22800–22808, 2005.
357. Venderova K and Park DS. Programmed cell death in Parkinson's disease. *Cold Spring Harb Perspect Med* 2: a009365, 2012.
358. Ventriglia M, Brewer GJ, Simonelli I, Mariani S, Siotto M, Bucossi S, and Squitti R. Zinc in Alzheimer's disease: a meta-analysis of serum, plasma, and cerebrospinal fluid studies. *J Alzheimers Dis* 46: 75–87, 2015.
359. Verstraeten SV, Aimo L, and Oteiza PI. Aluminium and lead: molecular mechanisms of brain toxicity. *Arch Toxicol* 82: 789–802, 2008.
360. Vidal R, Miravalle L, Gao X, Barbeito AG, Baraibar MA, Hekmatyar SK, Widel M, Bansal N, Delisle MB, and Ghetti B. Expression of a mutant form of the ferritin light chain gene induces neurodegeneration and iron overload in transgenic mice. *J Neurosci* 28: 60–67, 2008.
361. Villar-Pique A, Lopes da Fonseca T, Sant'Anna R, Szego EM, Fonseca-Ornelas L, Pinho R, Carija A, Gerhardt E, Masaracchia C, Abad Gonzalez E, Rossetti G, Carloni P, Fernandez CO, Foguel D, Milosevic I, Zweckstetter M, Ventura S, and Outeiro TF. Environmental and genetic factors support the dissociation between alpha-synuclein aggregation and toxicity. *Proc Natl Acad Sci U S A* 113: E6506–E6515, 2016.
362. Wakabayashi K, Tanji K, Mori F, and Takahashi H. The Lewy body in Parkinson's disease: molecules implicated in the formation and degradation of alpha-synuclein aggregates. *Neuropathology* 27: 494–506, 2007.
363. Wang B and Du Y. Cadmium and its neurotoxic effects. *Oxid Med Cell Longev* 2013: 898034, 2013.
364. Wang H, Wang M, Wang B, Li M, Chen H, Yu X, Yang K, Chai Z, Zhao Y, and Feng W. Immunogold labeling and X-ray fluorescence microscopy reveal enrichment ratios of Cu and Zn, metabolism of APP and amyloid-beta plaque formation in a mouse model of Alzheimer's disease. *Metallomics* 4: 1113–1118, 2012.
365. Wang JY, Zhuang QQ, Zhu LB, Zhu H, Li T, Li R, Chen SF, Huang CP, Zhang X, and Zhu JH. Meta-analysis of brain iron levels of Parkinson's disease patients determined by postmortem and MRI measurements. *Sci Rep* 6: 36669, 2016.
366. Wang L and Gallagher EP. Role of Nrf2 antioxidant defense in mitigating cadmium-induced oxidative stress in the olfactory system of zebrafish. *Toxicol Appl Pharmacol* 266: 177–186, 2013.
367. Wang Q, Luo W, Zhang W, Dai Z, Chen Y, and Chen J. Iron supplementation protects against lead-induced apoptosis through MAPK pathway in weanling rat cortex. *Neurotoxicology* 28: 850–859, 2007.
368. Wang X, Liu J, Zhu H, Tejima E, Tsuji K, Murata Y, Atochin DN, Huang PL, Zhang C, and Lo EH. Effects of neuroglobin overexpression on acute brain injury and long-term outcomes after focal cerebral ischemia. *Stroke* 39: 1869–1874, 2008.
369. Wang X, Mori T, Sumii T, and Lo EH. Hemoglobin-induced cytotoxicity in rat cerebral cortical neurons: caspase activation and oxidative stress. *Stroke* 33: 1882–1888, 2002.
370. Wasserman GA, Liu X, Loiacono NJ, Kline J, Factor-Litvak P, van Geen A, Mey JL, Levy D, Abramson R, Schwartz A, and Graziano JH. A cross-sectional study of well water arsenic and child IQ in Maine schoolchildren. *Environ Health* 13: 23, 2014.
371. Wasserman GA, Liu X, Parvez F, Ahsan H, Factor-Litvak P, Kline J, van Geen A, Slavkovich V, Loiacono NJ, Levy D, Cheng Z, and Graziano JH. Water arsenic exposure and intellectual function in 6-year-old children in Araihazar, Bangladesh. *Environ Health Perspect* 115: 285–289, 2007.
372. Watanabe T and Hirano S. Metabolism of arsenic and its toxicological relevance. *Arch Toxicol* 87: 969–979, 2013.
373. Watt NT, Taylor DR, Kerrigan TL, Griffiths HH, Rushworth JV, Whitehouse IJ, and Hooper NM. Prion protein facilitates uptake of zinc into neuronal cells. *Nat Commun* 3: 1134, 2012.
374. Wezynfeld NE, Stefaniak E, Stachucy K, Drozd A, Plonka D, Drew SC, Krezel A, and Bal W. Resistance of Cu(Abeta4-16) to copper capture by metallothionein-3 supports a function for the abeta4-42 peptide as a synaptic Cu(II) scavenger. *Angew Chem Int Ed Engl* 55: 8235–8238, 2016.
375. White AR, Reyes R, Mercer JF, Camakaris J, Zheng H, Bush AI, Multhaup G, Beyreuther K, Masters CL, and Cappai R. Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice. *Brain Res* 842: 439–444, 1999.
376. WHO. *Childhood Lead Poisining*. World Health Organization Press, 2010. http://www.who.int/ceh/publications/leadguidance.pdf
377. Willhite CC, Karyakina NA, Yokel RA, Yenugadhati N, Wisniewski TM, Arnold IM, Momoli F, and Krewski D. Systematic review of potential health risks posed by pharmaceutical, occupational and consumer exposures to metallic and nanoscale aluminum, aluminum oxides, aluminum hydroxide and its soluble salts. *Crit Rev Toxicol* 44 (Suppl 4): 1–80, 2014.
378. Wu CY, Steffen J, and Eide DJ. Cytosolic superoxide dismutase (SOD1) is critical for tolerating the oxidative stress of zinc deficiency in yeast. *PLoS One* 4: e7061, 2009.
379. Wu J, Basha MR, Brock B, Cox DP, Cardozo-Pelaez F, McPherson CA, Harry J, Rice DC, Maloney B, Chen D, Lahiri DK, and Zawia NH. Alzheimer's disease (AD)-like pathology in aged monkeys after infantile exposure to

environmental metal lead (Pb): evidence for a developmental origin and environmental link for AD. *J Neurosci* 28: 3–9, 2008.
380. Wu WS, Zhao YS, Shi ZH, Chang SY, Nie GJ, Duan XL, Zhao SM, Wu Q, Yang ZL, Zhao BL, and Chang YZ. Mitochondrial ferritin attenuates beta-amyloid-induced neurotoxicity: reduction in oxidative damage through the Erk/P38 mitogen-activated protein kinase pathways. *Antioxid Redox Signal* 18: 158–169, 2013.
381. Xiao G, Fan Q, Wang X, and Zhou B. Huntington disease arises from a combinatory toxicity of polyglutamine and copper binding. *Proc Natl Acad Sci U S A* 110: 14995–15000, 2013.
382. Yamauchi H, Kaise T, and Yamamura Y. Metabolism and excretion of orally administered arsenobetaine in the hamster. *Bull Environ Contam Toxicol* 36: 350–355, 1986.
383. Yang X, Wang B, Zeng H, Cai C, Hu Q, Cai S, Xu L, Meng X, and Zou F. Role of the mitochondrial Ca(2)(+) uniporter in Pb(2)(+)-induced oxidative stress in human neuroblastoma cells. *Brain Res* 1575: 12–21, 2014.
384. Ye F, Li X, Li F, Li J, Chang W, Yuan J, and Chen J. Cyclosporin A protects against Lead neurotoxicity through inhibiting mitochondrial permeability transition pore opening in nerve cells. *Neurotoxicology* 57: 203–213, 2016.
385. Yin Z, Jiang H, Syversen T, Rocha JB, Farina M, and Aschner M. The methylmercury-L-cysteine conjugate is a substrate for the L-type large neutral amino acid transporter. *J Neurochem* 107: 1083–1090, 2008.
386. Yokel RA, Wilson M, Harris WR, and Halestrap AP. Aluminum citrate uptake by immortalized brain endothelial cells: implications for its blood-brain barrier transport. *Brain Res* 930: 101–110, 2002.
387. Yoshida M, Watanabe C, Horie K, Satoh M, Sawada M, and Shimada A. Neurobehavioral changes in metallothionein-null mice prenatally exposed to mercury vapor. *Toxicol Lett* 155: 361–368, 2005.
388. Yoshino Y, Yuan B, Kaise T, Takeichi M, Tanaka S, Hirano T, Kroetz DL, and Toyoda H. Contribution of aquaporin 9 and multidrug resistance-associated protein 2 to differential sensitivity to arsenite between primary cultured chorion and amnion cells prepared from human fetal membranes. *Toxicol Appl Pharmacol* 257: 198–208, 2011.
389. You H, Tsutsui S, Hameed S, Kannanayakal TJ, Chen L, Xia P, Engbers JD, Lipton SA, Stys PK, and Zamponi GW. Abeta neurotoxicity depends on interactions between copper ions, prion protein, and N-methyl-D-aspartate receptors. *Proc Natl Acad Sci U S A* 109: 1737–1742, 2012.
390. Yu WH, Lukiw WJ, Bergeron C, Niznik HB, and Fraser PE. Metallothionein III is reduced in Alzheimer's disease. *Brain Res* 894: 37–45, 2001.
391. Yu X, Song N, Guo X, Jiang H, Zhang H, and Xie J. Differences in vulnerability of neurons and astrocytes to heme oxygenase-1 modulation: implications for mitochondrial ferritin. *Sci Rep* 6: 24200, 2016.
392. Zamora PL, Rockenbauer A, and Villamena FA. Radical model of arsenic(III) toxicity: theoretical and EPR spin trapping studies. *Chem Res Toxicol* 27: 765–774, 2014.
393. Zhang HN, Yang L, Ling JY, Czajkowsky DM, Wang JF, Zhang XW, Zhou YM, Ge F, Yang MK, Xiong Q, Guo SJ, Le HY, Wu SF, Yan W, Liu B, Zhu H, Chen Z, and Tao SC. Systematic identification of arsenic-binding proteins reveals that hexokinase-2 is inhibited by arsenic. *Proc Natl Acad Sci U S A* 112: 15084–15089, 2015.
394. Zhang Y, Duan X, Li J, Zhao S, Li W, Zhao L, Li W, Nie H, Sun G, and Li B. Inorganic arsenic induces NRF2-regulated antioxidant defenses in both cerebral cortex and hippocampus in vivo. *Neurochem Res* 41: 2119–2128, 2016.
395. Zhao F, Liao Y, Jin Y, Li G, Lv X, and Sun G. Effects of arsenite on glutamate metabolism in primary cultured astrocytes. *Toxicol In Vitro* 26: 24–31, 2012.
396. Zhou X, Cooper KL, Sun X, Liu KJ, and Hudson LG. Selective sensitization of zinc finger protein oxidation by reactive oxygen species through arsenic binding. *J Biol Chem* 290: 18361–18369, 2015.
397. Zhou X, Sun X, Mobarak C, Gandolfi AJ, Burchiel SW, Hudson LG, and Liu KJ. Differential binding of monomethylarsonous acid compared to arsenite and arsenic trioxide with zinc finger peptides and proteins. *Chem Res Toxicol* 27: 690–698, 2014.
398. Zille M, Karuppagounder SS, Chen Y, Gough PJ, Bertin J, Finger J, Milner TA, Jonas EA, and Ratan RR. Neuronal death after hemorrhagic stroke in vitro and in vivo shares features of ferroptosis and necroptosis. *Stroke* 48: 1033–1043, 2017.
399. Zimmermann LT, Santos DB, Naime AA, Leal RB, Dorea JG, Barbosa F, Jr., Aschner M, Rocha JB, and Farina M. Comparative study on methyl- and ethylmercury-induced toxicity in C6 glioma cells and the potential role of LAT-1 in mediating mercurial-thiol complexes uptake. *Neurotoxicology* 38: 1–8, 2013.
400. Zucca FA, Segura-Aguilar J, Ferrari E, Munoz P, Paris I, Sulzer D, Sarna T, Casella L, and Zecca L. Interactions of iron, dopamine and neuromelanin pathways in brain aging and Parkinson's disease. *Prog Neurobiol* 155: 96–119, 2017.

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**Abbreviations Used**

A$\beta$ = amyloid beta
AD = Alzheimer's disease
AdoMet = S-adenosylmethionine
Al = aluminum
ALA = aminolevulinic acid
ALAD = $\delta$-aminolevulinic acid dehydratase
$AlO_2^{\bullet-}$ = aluminum superoxide radicals
ALS = amyotrophic lateral sclerosis
AMPAR = $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor
APP = amyloid precursor protein
AQP = aqua(glycerol)porin
As = arsenic
AS3MT = arsenite methyltransferase
AsIII = arsenite +3
$As(SG)_3$ = arsenic triglutathione
AsV = arsenate +5
Atox1 = antioxidant protein 1
ATP7A = ATPase copper transporting alpha
ATP7B = ATPase copper transporting beta
BBB = blood-brain barrier
BLL = blood lead levels
Ca = calcium
CaM = $Ca^{2+}$/calmodulin
CCS1 = copper chaperone for superoxide dismutase 1
Cd = cadmium
CNS = central nervous system
$CO_2$ = carbon dioxide
COX = cytochrome C oxidase
Cp = ceruloplasmin
CSF = cerebrospinal fluid
CTR1 = copper transporter 1
Cu = copper
$Cu^+$ = cuprous ion
$Cu^{2+}$ = cupric ion
Cys = cysteine
DA = dopamine
Dcytb = duodenal cytochrome b
Dexras1 = Ras-related dexamethasone induced 1
DLA = dihydrolipoamide or dihydrolipoic acid
DMAIII = dimethylarsinous acid
DMAV = dimethylarsinic acid
DMSA = 2,3-dimercaptosuccinic acid
DMT1 = divalent metal transporter 1
EAAT = excitatory amino acid transporter
ER = endoplasmic reticulum
EtHg or $C_2H_5Hg$ = ethylmercury
FBXL5 = F-box/LRR-repeat protein
Fe = iron
$Fe^{2+}$ = ferrous
$Fe^{3+}$ = ferric
Fpn = ferroportin
Ft = ferritin
FtMt = mitochondrial ferritin
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GCL = $\gamma$-glutamate-cysteine ligase
Glu = glutamate
GLUT = glucose transporters
GPX = glutathione peroxidases
GR = glutathione reductase
Grxs = glutaredoxins
GS = glutamine synthetase
GSH = glutathione
GSSG = glutathione disulfide
GST = glutathione-S transferase
$H_2O_2$ = hydrogen peroxide
Hb = hemoglobin
HD = Huntington's disease
H-Ft = heavy-ferritin
Hg = mercury
$Hg^0$ = elemental or metallic Hg
$Hg^{1+}$ = inorganic mercurous ions
$Hg^{2+}$ = inorganic mercuric ions
HNE = 4-hydroxy-2-nonenal
HO = heme oxygenase
Hp = hephaestin
Htt = huntingtin
iAs = inorganic As
iAsV-3-P-glycerate = 1-arsenato-3-phospho-Dglycerate
IDP = intrinsically disordered protein
IQ = intelligence quotient
IRP = iron regulatory proteins
K = potassium
Keap1 = kelch-like ECH-associated protein 1
LAT = L-type neutral amino acid transporter
L-Ft = light-ferritin
LTP = long-term potentiation
MCU = mitochondrial $Ca^{2+}$ uniporter
MDA = malondialdehyde
MeHg or $CH_3Hg$ = methylmercury
Met = methionine
Mfrn2 = mitoferrin-2
Mg = magnesium
MMAIII = monomethylarsonous acid
MMAV = monomethylarsonic acid or arsonate
Mn = manganese
MRE = metal response element
mRNA = messenger RNA
MRPs = multidrug resistance proteins
MT = metallothionein
MTF1 = metal-responsive transcription factor-1
Na = sodium
$NaAsO_2$ = sodium arsenite
NADPH = nicotinamide adenine dinucleotide phosphate
NBIA = neurodegeneration with brain iron accumulation
NKB = neurokinin B
NMDAR = glutamate/N-methyl-D-aspartate receptor
$NO^{\bullet}$ = nitric oxide
NOS = nitric oxide synthase
NOX = NADPH oxidases
Nrf1/2 = nuclear factor erythroid-2-related factor 1 or 2
$O_2$ = molecular oxygen
$O_2^{\bullet-}$ = superoxide anion radical
$^{\bullet}OH$ = hydroxyl radical
$OONO^-$ = peroxynitrite

**Abbreviations Used (Cont.)**

Pb = lead
PD = Parkinson's disease
PNS = peripheral nervous system
polyQ = polyglutamine
ppb = parts per billion
$PrP^C$ = cellular prion protein
Prxs = peroxiredoxins
RNS = reactive nitrogen species
ROS = reactive oxygen species
RyR = ryanodine receptor
S = sulfur
sAPP = soluble fragment of APP
SCO = cytochrome c oxidase assembly protein
sCp = soluble ceruloplasmin
SDR2 = stromal cell-derived receptor
Se = selenium
SLC30A10/hZnT1 = solute carrier family 30 member 10 or human Zn transporter 1
SN = substantia nigra
SOD = superoxide dismutase
SPCA = secretory pathway $Ca^{2+}$ ATPase
Steap2 = six transmembrane epithelial antigen of the prostate 2
Tf = transferrin
TfR = transferrin receptor
Trxs = thioredoxins
Tyr = tyrosine
xCT = cystine/glutamate exchanger system
ZIP = Zrt-(Zn-regulated transporter)- or Irt (Fe-regulated transporter)-like proteins
Zn = zinc
ZnT = zinc transporters

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Current Opinion in Toxicology

# mTOR/AMPK signaling in the brain: Cell metabolism, proteostasis and survival

Carla Garza-Lombó[1,2,3], Annika Schroder[1,2], Elsa M. Reyes-Reyes[4] and Rodrigo Franco[1,2]

**Abstract**

The mechanistic (or mammalian) target of rapamycin (mTOR) and the adenosine monophosphate-activated protein kinase (AMPK) regulate cell survival and metabolism in response to diverse stimuli such as variations in amino acid content, changes in cellular bioenergetics, oxygen levels, neurotrophic factors and xenobiotics. This Opinion paper aims to discuss the current state of knowledge regarding how mTOR and AMPK regulate the metabolism and survival of brain cells and the close interrelationship between both signaling cascades. It is now clear that both mTOR and AMPK pathways regulate cellular homeostasis at multiple levels. Studies so far demonstrate that dysregulation in these two pathways is associated with neuronal injury, degeneration and neurotoxicity, but the mechanisms involved remain unclear. Most of the work so far has been focused on their antagonistic regulation of autophagy, but recent findings highlight that changes in protein synthesis, metabolism and mitochondrial function are likely to play a role in the regulatory effects of both mTOR and AMPK on neuronal health. Understanding their role and relationship between these two master regulators of cell metabolism is crucial for future therapeutic approaches to counteract alterations in cell metabolism and survival in brain injury and disease.

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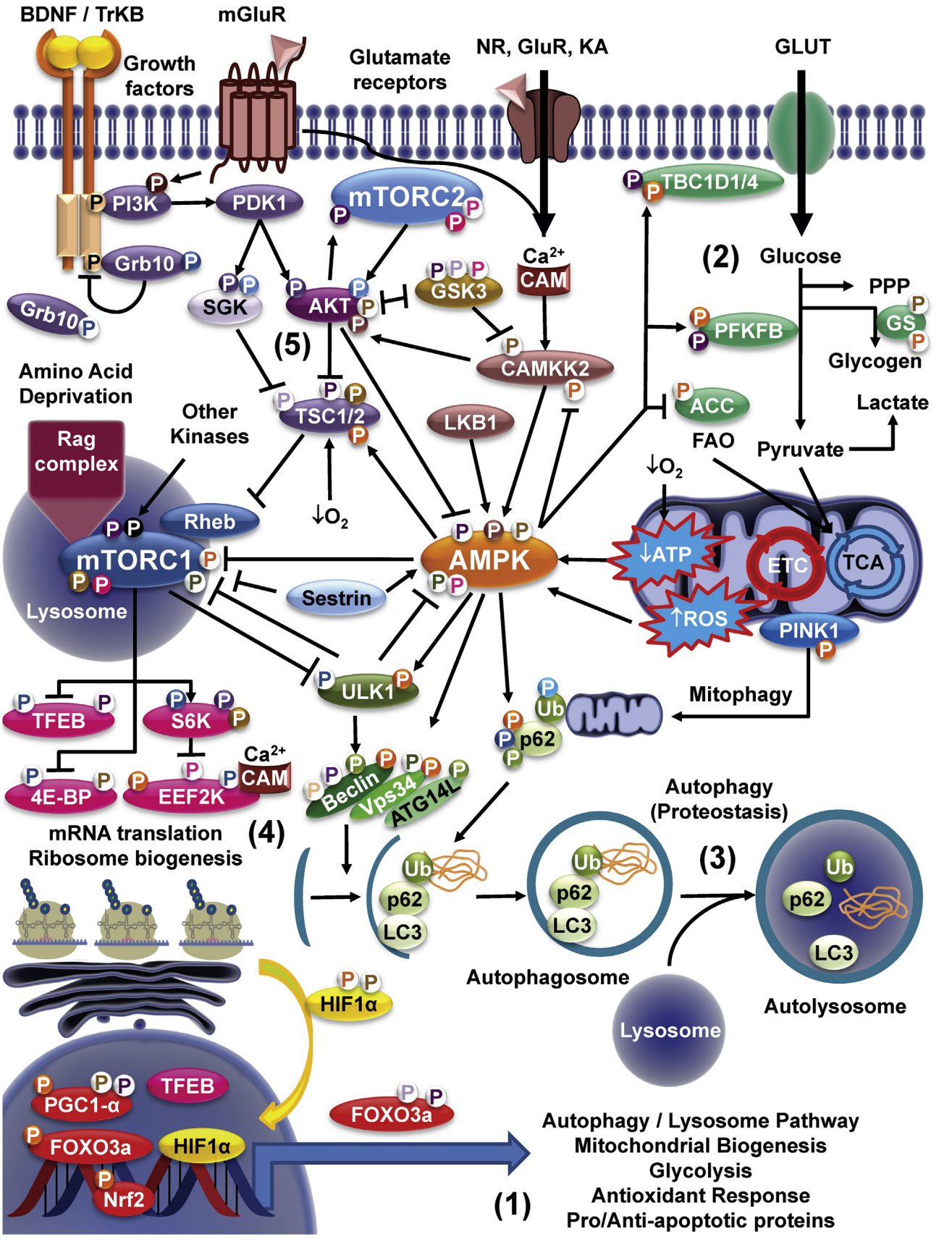
## 1. Introduction

The mechanistic (or mammalian) target of rapamycin (mTOR) and the adenosine monophosphate-activated protein kinase (AMPK) regulate cell growth and metabolism incorporating signals triggered by different stimuli such as variations in the amino acid content, changes in cellular bioenergetics, activation of hormone and growth factor signaling and stress such as oxidative stress, hypoxia or DNA damage. In the brain, AMPK and mTOR signaling are regulated by neurotransmitters and neurotrophin signals. mTOR and AMPK are considered master regulators of cell metabolism. Their activation is directly linked to the regulation of cellular metabolism (mitochondria homeostasis and central carbon metabolism), growth (protein synthesis) and survival (autophagy and cell death pathways). This Opinion paper aims to discuss the current state of knowledge regarding how mTOR and AMPK regulate brain metabolism and survival upon stress and more importantly, the close interrelationship between both signaling molecules (Figure 1 and Supplementary Table 1).

## 2. mTOR signaling in brief and its role in regulating cell metabolism

mTOR is a serine (Ser)/threonine (Thr) protein kinase in the PI3K-related kinase (PIKK) family that forms the catalytic subunit of two distinct protein complexes, known as mTOR Complex 1 (mTORC1) and 2 (mTORC2) (Figure 1). Together with mTOR, the mammalian lethal with sec13 protein 8 (mLST8 or GβL), and the inhibitory DEP domain containing mTOR-interacting protein (DEPTOR) are common to both mTORC1 and mTORC2. Phosphorylation of DEPTOR by mTOR and other kinases promotes its degradation and activation of mTORCs. mLST8 seems to stabilize the kinase activation loop of mTOR but is dispensable for its activity. mTORC1 also includes the regulator-associated protein of the mammalian target of rapamycin (Raptor or RPTOR) and the proline-rich protein kinase B (PKB or AKT) substrate of 40 kDa (PRAS40). Because the interaction between Raptor and mTOR is essential for the stability of the mTORC1 and the recruitment of substrates, post-translational modifications in Raptor (phosphorylation, cleavage or ubiquitination) regulate mTORC1 activity. PRAS40 inhibits the substrate recruitment sites of mTORC1 and its phosphorylation by AKT and mTOR promotes its dissociation from the complex. On the other hand, the mTORC2 includes the rapamycin-insensitive

**Figure 1**


BDNF / TrKB
mGluR
Growth factors
Glutamate receptors
NR, GluR, KA
GLUT
PI3K
PDK1
mTORC2
TBC1D1/4
Grb10
SGK
AKT
GSK3
Ca2+
CAM
(2)
Glucose
PPP
PFKFB
GS
Glycogen
(5)
CAMKK2
Amino Acid Deprivation
Other Kinases
TSC1/2
LKB1
ACC
FAO
Lactate
Pyruvate
Rag complex
Rheb
mTORC1
Lysosome
↓O2
AMPK
↓ATP
ETC
TCA
↑ROS
PINK1
Sestrin
ULK1
Mitophagy
TFEB
S6K
Ub
p62
Beclin
Vps34
ATG14L
4E-BP
EEF2K
mRNA translation
Ribosome biogenesis
(4)
LC3
Autophagy (Proteostasis)
(3)
Autophagosome
Autolysosome
HIF1α
PGC1-α
FOXO3a
Nrf2
(1)
Autophagy / Lysosome Pathway
Mitochondrial Biogenesis
Glycolysis
Antioxidant Response
Pro/Anti-apoptotic proteins

companion of mTOR (Rictor), the mammalian stress-activated MAP kinase-interacting protein 1 (mSIN1), and the proteins observed with rictor 1 and 2 (PROTOR 1 and 2) [1].

The GTP binding protein Ras homolog enriched in brain (Rheb) is an obligate upstream activator of mTORC1 by growth factors and amino acids (Figure 1). Activation of the phosphoinositide-3 kinase (PI3K)/AKT pathway by growth factor receptors inactivates the tuberous sclerosis complex (TSC), a key negative regulator of mTORC1 (Figure 1). TSC is a heterotrimeric complex comprising TSC1, TSC2, and the Tre-2/BUB2/cdc 1 domain family member TBC1D7. TSC2 acts as a GTPase-activating protein (GAP) to inactivate Rheb (Rheb-GDP). mTORC1 activation requires its localization in the lysosomal surface which is regulated by amino acid and nutrient sensing via Rag GTPases (Figure 1). Induction of protein synthesis by mTORC1 involves the phosphorylation of the ribosomal protein S6 kinase beta-1 (S6K) and the eukaryotic translation initiation factor 4 (eIF4E)-binding protein (4E-BP) (Figure 1) [1]. Glucose metabolism has been reported to be regulated by translational regulation of the hypoxia-inducible factor 1-alpha (HIF1α) via 4E-BP and mTORC1 [2]. Via 4E-BP, mTORC1 signaling regulates mitochondrial biogenesis as well [3]. mTORC1 also regulates lipid and nucleotide synthesis through the sterol responsive element binding protein (SREBP) transcription factors and the activation of the transcription factor 4 (ATF4), respectively [4,5].

mTORC2 primarily controls proliferation and survival via the activation of members of the AGC family of protein kinases such as PKC that regulate cytoskeletal remodeling and cell migration. AKT phosphorylation by mTORC2 is required for AKT-mediated phosphorylation of specific substrates such as Forkhead box O1/3a (FOXO1/3a). Reciprocally, AKT has been shown to phosphorylate mSIN1 enhancing mTORC2 kinase activity (Figure 1) [1]. mTORC2 has also been demonstrated to regulate glucose and amino acid metabolism [6–9]. Interestingly, a recent report also demonstrates that mTORC2 has the ability to act as a tyrosine kinase [10].

## 3. AMPK signaling in brief and its role in regulating cell metabolism

AMPK is a Ser/Thr kinase that exists as a heterotrimer composed of catalytic α and regulatory β and γ subunits. In the absence of AMP, the autoinhibitory domain of AMPKα maintains the kinase in an inactive conformation. Upon ATP consumption, AMP/ADP directly bind to AMPKγ promoting the phosphorylation of AMPKα. The liver kinase B1 (LKB1) is the primary kinase that phosphorylates AMPKα at Thr172 (Figure 1). LKB1 is found constitutively active as a complex with Ste20-related adaptor (STRAD) and mouse protein 25 (MO25). A second binding event of AMP/ADP to AMPKγ induces a conformational change that protects AMPKα from dephosphorylation. AMPKα phosphorylation has also been reported to be mediated by the $Ca^{2+}$/calmodulin (CaM)-dependent protein kinase kinase 2 or β (CAMKK2 or β), independent from changes in the AMP/ADP:ATP ratio (Figure 1), or by the transforming growth factor β-activated kinase 1 (TAK-1). AMP binding also upregulates allosterically the activation of AMPK [11]. A new study shows that AMPK can also be activated by sensing the absence of fructose-1,6-bisphosphate (FBP), via aldolase even if there are not changes in the AMP/ADP:ATP ratio [12].

AMPK activation inhibits anabolic processes, stimulates catabolism, and restores ATP levels. Several conditions that either interfere with ATP synthesis or promote ATP consumption can lead to AMPK activation. At least 60 targets have been identified as substrates of AMPK [11], but we will focus on those involved in the regulation of cell metabolism (Figure 1). AMPK inhibits the *de novo* synthesis of fatty acids and activates lipid catabolism via phosphorylation and inactivation of acetyl-CoA carboxylase (ACC). AMPK also stimulates glucose uptake via the phosphorylation of the TBC domain family members TBC1D1 and TBC1D4, and the thioredoxin-interacting protein (TXNIP) that results in an increased plasma membrane translocation of glucose transporters (GLUT) (Figure 1). Phosphorylation of the bifunctional 6-phosphofructo-2-kinase (PFKFB) by AMPK increases fructose-2,6-bisphosphate (F2,6P2), which activates glycolysis via phosphofructokinase 1 (PFK1) (Figure 1). In contrast, glycogen synthesis and hexosamine biosynthesis are inhibited by the phosphorylation and inhibition of glycogen synthase (GS) (Figure 1) and glutamine-fructose-6-phosphate aminotransferase-1 (GFPT1), respectively [13]. AMPK also promotes mitochondrial biogenesis by phosphorylation of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) increasing its transcriptional activity (Figure 1) [14,15], as well as mitochondrial fission and autophagy (mitophagy) [16].

**mTOR/AMPK signaling crosstalk.** Signaling by mTOR and AMPK integrates changes in the cellular environment linked to growth factors, nutrient, oxygen and energy availability, excitotoxicity ($Ca^{2+}$) and stress (oxidative stress). Five major points of convergence include: 1) the transcriptional regulation of genes linked to cell survival/death, proteostasis, redox balance and bioenergetics; 2) metabolism (central carbon and bioenergetics); 3) autophagy; 4) mRNA translation and protein synthesis; and 5) cell survival and growth (AKT). Arrows indicate activation. Stop lines indicate inhibition. Phosphorylation events (P) are color coded according to the kinase involved in phosphorylating the corresponding targeted protein. White background/colored "P" highlights an inhibitory effect; Colored background/white P highlights an stimulatory (activation) effect. A detailed explanation of the phosphorylation events is included in the Supplementary Table 1. Additional crosstalk might involve indirect pathways not highlighted in this figure.

Remarkably, it was recently shown that reactive oxygen species (ROS) activate AMPK triggering a PGC-1α-dependent antioxidant response that limits mitochondrial ROS production [17]. Thus, AMPK is a central regulator of mitochondrial homeostasis.

## 4. mTOR-AMPK signaling crosstalk

While both mTOR and AMPK seem to regulate different pathways, there are key hubs of crosstalk where both converge to regulate homeostatic processes such as autophagy and cell metabolism (Figure 1 and Supplementary Table 1). AMPK utilizes different targets to effectively suppress mTORC1. AMPK inhibits mTORC1 both directly, through the phosphorylation of Raptor [18], as well as indirectly through the phosphorylation and activation of TSC2 [19]. Reciprocally, mTORC1 signaling has also been proposed to regulate AMPK as S6K has been found to phosphorylate and inhibit AMPK (Figure 1) [20].

Autophagy is a proteostatic quality control mechanism that requires the formation of double membraned autophagosomes that deliver cargo for degradation within the lysosome (Figure 1). The unc-51-like autophagy activating kinase 1 (ULK1 or autophagy related protein 1 [Atg 1]) drives autophagosome formation and mTORC1 suppresses autophagy by phosphorylating ULK1. In contrast, phosphorylation of ULK1 by AMPK promotes autophagy (Figure 1). Therefore, the relative activity of mTORC1 and AMPK in different cellular contexts largely determines the extent of autophagy induction [21].

Another point of convergence is that of protein synthesis. Within the polypeptide translation elongation cycle, translocation of the ribosome is mediated by the elongation factor eEF2. eEF2 is inhibited by phosphorylation catalyzed by the eEF2 kinase (EEF2K). EEF2K is phosphorylated and inactivated by S6K [22] and mTORC1 [23], while its phosphorylation by AMPK promotes its activation (Figure 1) [24].

FOXO3a is a transcription factor involved in cell survival whose phosphorylation by AKT is well known to inhibit its activity [25], suggesting a possible feedback loop via mTORC2 [26]. FOXO3a has been reported to inhibit mTORC1 activity via transcriptional control of TSC1 [27]. In contrast, phosphorylation by AMPK activates FOXO3a (Figure 1) [28].

## 5. mTOR/AMPK signaling in the brain

mTOR signaling is involved in a myriad of processes regulating neurodevelopment (proliferation and differentiation) and brain function (neuronal plasticity). Thus, a dysregulation of mTOR signaling has been linked to a number of brain disorders associated with neuronal dysfunction and cell death such as neurodegeneration, epilepsy, autism and neurobehavioral alterations. Previous reviews have focused on the role of mTOR in brain function and development [29,30]. In the brain, energy-sensing neurons in the hypothalamus are involved in the regulation of organismal energy balance via AMPK signaling. AMPK activation in the hypothalamus is modulated by energy-related signals associated with food intake (fasting and feeding), energy expenditure (ATP levels), and body weight (hormones and adipokines) [31]. We will next focus on the role of mTOR and AMPK on the survival and cell death of neuro-glial cells and their link with metabolic stress in brain disorders.

Brain metabolism is characterized by a complex interplay between different cell types. Glucose is the primary energy substrate of the brain and it has been proposed to be metabolized through two complementary metabolic pathways in neurons and astrocytes. Neurons seem to predominantly metabolize glucose through the pentose phosphate pathway (PPP) to produce the NADPH necessary for antioxidant defense. On the other hand, astrocytes primarily metabolize glucose through glycolysis to produce lactate, where lactate is shuttled as an energy substrate for oxidative phosphorylation in neurons [32–34]. While this astrocyte-neuron lactate shuttle seems to act at rest, recent studies demonstrate that during energy-demanding conditions neurons have the capacity to upregulate glycolysis [35].

Very little is known regarding the role of AMPK and mTOR signaling in normal brain metabolism. Deletion of Raptor (mTORC1) or Rictor (mTORC2) in the brain leads to neurodevelopmental alterations [36–39], but whether these effects are related to alterations in cell metabolism is unclear. AMPKα*2* has been reported to be the predominant catalytic subunit in neurons and astrocytes in the brain and spinal cord [40]. In contrast to mTOR, AMPK is not necessary for neuronal development or survival, but its hyperactivation impairs axon growth via inhibition of mTORC1 [41]. Indeed, AMPK antagonizes mTOR activation by neurotrophic factors in neurons [42]. We have observed that mTOR signaling is important for astrocytes' basal glycolytic rate (*unpublished results*). A recent report demonstrates that in astrocytes and neural stem cells, the G-protein-coupled receptor kinase-interacting protein 1 (GIT1) is a novel mTOR-binding protein within a unique mTOR complex regulated by AKT that lacks both Raptor and Rictor [43]. In glioblastoma cells, mTORC2 upregulates glycolysis via acetylation of FOXO1 and FOXO3a transcription factors that result in an increase in c-Myc levels [9].

## 6. mTOR/AMPK in neuronal cell death and survival

Because mTOR and AMPK are essential for the regulation of metabolism, their dysregulation can generate

negative effects leading to cell death and disease progression. There are different examples where the activation of these two kinases has been found to be protective against pathological processes, but it has also been shown that when dysregulated, their increased activity can be detrimental.

### 6.1. Brain ischemia and injury

In ischemic brain injury both mTOR and AMPK have been involved in cell death progression. While this topic has been studied extensively [44,45], there are still contradictory results depending on the experimental conditions used. mTOR protects against neuronal cell death induced by ischemic insults [46,47]. Similarly, mTOR signaling via S6K protects astrocytes from oxygen-glucose deprivation (OGD) and mice from middle artery occlusion (MCAO) [48]. Pharmacological inhibition of AMPK also protects against MCAO and OGD [49], but the opposite is found using a global cerebral ischemia model [50]. AMPK$\alpha 2$, but not $\alpha 1$ knockout in mice has been shown to protect against MCAO [51]. Interestingly, a dual role of AMPK in neuronal cell death in neonatal hypoxia has been reported, where inhibition of AMPK prior to OGD increases cell death, while its CAMKK2-dependent activation upon OGD mediates neuronal cell loss [52]. In traumatic brain injury (TBI), pharmacological stimulation of AMPK ameliorates cognitive dysfunction [53].

Accumulation of extracellular excitatory amino acids (glutamate) is a common event associated with neuronal cell death during ischemia and TBI. Neuronal cell death induced by glutamate excitotoxicity has been reported to involve AMPK activation and an increase in the levels of the pro-apoptotic Bim protein [54]. In contrast, AMPK-dependent translocation of GLUT3 and glucose availability have been reported to exert a protective effect against glutamate excitotoxicity [55]. mTOR signaling has also been proposed to contribute to the toxicity of glutamate receptor agonists (N-methyl-D-aspartate [NMDA]) [56]. Finally, mTOR is well known to mediate axon regeneration and myelination after injury [57–59].

### 6.2. Neurodegeneration

Both mTOR and AMPK signaling modulate the progression of different neurodegenerative disorders, but whether their activation is beneficial or detrimental remains unclear. One of the main mechanisms related to neurodegeneration where mTOR and AMPK converge is autophagy, which can exert protective effects via the degradation of misfolded, damaged or toxic proteins, as well as damaged organelles [60]. Parkinson's (PD) and Huntington's disease (HD) are characterized by the accumulation of intracellular misfolded protein aggregates. Because mTORC1 represses autophagy, its inhibition may represent a plausible therapeutic approach [61]. Accordingly, rapamycin has been reported to protect neurons by promoting the clearance of protein aggregates of $\alpha$-synuclein and mutant huntingtin which are found in PD and HD, respectively [62,63]. A decrease in mTOR activity also reduces Alzheimer's disease-like amyloid-$\beta$ (A$\beta$) deposits *in vivo* via the induction of autophagy [64]. Interestingly, while mTOR activation is usually considered to inhibit autophagy, autophagy can be induced by mTOR stimulation as well. Accordingly, activation of mTORC1 signaling in the striatum stimulates autophagy, ameliorates mitochondrial dysfunction and protects against HD [65]. In contrast, hyperactivation of mTORC1 via striatum-specific deletion of TSC1 has been reported to accelerate motor dysfunction and mortality in HD mouse models [66].

Most of the studies regarding mTOR signaling and neurodegeneration have been focused on autophagy, but alterations in cell metabolism or proteostasis should also bee considered. A recent report demonstrates that inhibition of mTOR with rapamycin preserves neuronal ATP levels upon inhibition of mitochondrial function, and this effect was associated with a decrease in energy consumption by protein synthesis [67]. Rapamycin-induced activation of 4E-BP reduces dopaminergic degeneration and mitochondrial dysfunction linked to mutations in the PD-related genes PTEN-induced putative kinase 1 (PINK1) and E3 ubiquitin-protein ligase Parkin [68]. Conversely, the PD-related protein leucine-rich repeat kinase 2 (LRRK2) has been shown to phosphorylate and inactivate 4E-BP [69]. Together these results suggest that alterations in protein translation via the mTORC1-4E-BP signaling pathway might be involved in PD.

Contradicting results have been reported regarding the role of AMPK in dopaminergic cell death associated with PD [70–75]. A protective role for AMPK against mitochondrial dysfunction and toxicity induced by Parkin-, LRRK2-mutations, $\alpha$-synuclein and mitochondrial toxins has been previously reported [70–72]. In contrast, other reports have shown that AMPK mediates dopaminergic cell death induced by 6-hydroxydopamine (6-OHDA) and mitochondrial toxins as well [73,74]. We have demonstrated that AMPK exerts a protective effect against cell death induced by paraquat (PQ), a pesticide recognized as an important PD risk factor, in combination with the PD-related gene $\alpha$-synuclein [76]. Importantly, while autophagy protects against PQ toxicity [77], the modulatory effects of AMPK seem to be primarily related to metabolic dysfunction [76].

The intracellular aggregation of abnormally and hyperphosphorylated tau proteins generates neurofibrillary tangles (NFTs), the pathological hallmark of neurodegenerative diseases commonly known as tauopathies. A$\beta$ oligomers activate AMPK via CAMKK2, which mediates

tau phosphorylation and neurotoxicity [78,79]. In contrast, short-term exposure to Aβ oligomers transiently decrease AMPK activity and result in a reduction in glucose transport [80]. In HD, activation of AMPK mediates neuronal atrophy downstream of oxidative stress [81]. However, another study suggests that AMPK activation at early stages of HD pathology protects against neuronal cell death [82].

### 6.3. Environmental neurotoxicity

Exposure to environmental toxicants is a risk factor for neurodegenerative, cognitive and neurodevelopmental disorders. Arsenic, a natural occurring neurotoxicant, impairs neurite outgrowth by inhibition of AMPK signaling [83]. We have observed that AMPK signaling and autophagy promote apoptotic cell death in cortical astrocytes exposed to inorganic arsenic *(unpublished results)*. Cadmium (Cd) neurotoxicity has been shown to be mediated via mTOR signaling [84]. Bisphenol-A (BPA), an endocrine disruptor released from the polycarbonate containers, activates AMPK while it downregulates mTOR signaling, resulting in enhanced autophagy. AMPK signaling and autophagy were reported to exert a protective effect against BPA-induced neurotoxicity in hippocampal neurons [85]. In contrast, the neurotoxicity of tributyltin, a water organotin contaminant, has been linked to the activation of AMPK and excitotoxic glutamate release [84].

## 7. Conclusions and perspectives

It is now clear that both mTOR and AMPK pathways regulate cellular homeostasis at multiple levels. By continuously sensing the cellular energy and nutrient state mTOR and AMPK have the capacity to facilitate cellular adaptation to stress. In addition, $Ca^{2+}$, growth factors and oxidative stress have the ability to activate mTOR and AMPK to regulate protein synthesis, autophagy and metabolism. We have aimed to discuss the current state of knowledge regarding how mTOR and AMPK regulate brain metabolism and survival upon stress and the close interrelationship between both signaling pathways. Studies so far have demonstrated that dysregulation in these two pathways is associated with neuronal cell death, but the mechanisms involved remain unclear. Most of the work has been focused on their convergence regulating proteostasis via autophagy, but recent findings highlight that changes in protein synthesis, metabolism and mitochondrial function are likely to play a role in the regulatory effects of mTOR and AMPK on neuronal health. While there is still controversy regarding whether AMPK and mTOR signaling can exert a neuroprotective or neurotoxic effect, some of these controversies might be addressed when considering: 1) the poor specificity of widely used pharmacological approaches to modulate their activity (rapamycin, torin 1, compound C, 5-Aminoimidazole-4-carboxamide ribonucleotide [AICAR] and others); and 2) the time and dose-dependent effect of modulating these signaling pathways. Understanding their roles and the relationship between these two master regulators of cell metabolism is crucial for future therapeutic approaches to counteract alterations in cell metabolism and survival during neuronal cell death and degeneration.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cotox.2018.05.002.

## References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Saxton RA, Sabatini DM: **mTOR signaling in growth, metabolism, and disease**. *Cell* 2017, **168**:960–976.

2. Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, Triantafellow E, Ma Q, Gorski R, Cleaver S, *et al.*: **Activation of a metabolic gene regulatory network downstream of mTOR complex 1**. *Mol Cell* 2010, **39**:171–183.

3. Morita M, Gravel SP, Chenard V, Sikstrom K, Zheng L, Alain T, Gandin V, Avizonis D, Arguello M, Zakaria C, *et al.*: **mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation**. *Cell Metab* 2013, **18**:698–711.

4. Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, Griffiths JR, Chung YL, Schulze A: **SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth**. *Cell Metab* 2008, **8**:224–236.

5. Ben-Sahra I, Hoxhaj G, Ricoult SJH, Asara JM, Manning BD: **mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle**. *Science* 2016, **351**:728–733.

6. Albert V, Svensson K, Shimobayashi M, Colombi M, Munoz S, Jimenez V, Handschin C, Bosch F, Hall MN: **mTORC2 sustains thermogenesis via Akt-induced glucose uptake and glycolysis in brown adipose tissue**. *EMBO Mol Med* 2016, **8**:232–246.

7. * Gu Y, Albuquerque CP, Braas D, Zhang W, Villa GR, Bi J, Ikegami S, Masui K, Gini B, Yang H, *et al.*: **mTORC2 regulates amino acid metabolism in cancer by phosphorylation of the cystine-glutamate antiporter xCT**. *Mol Cell* 2017, **67**:128–138.e127.
Using an unbiased proteomic screen, this work identified mTORC2 as a regulator of amino acid metabolism via phosphorylation and inhibition of the cystine-glutamate antiporter (xCT), which had a direct effect on glutamate release, cystine uptake, and glutathione homeostasis. This study identified a novel target for mTORC2 involved in cellular redox homeostasis.

8. Moloughney JG, Kim PK, Vega-Cotto NM, Wu CC, Zhang S, Adlam M, Lynch T, Chou PC, Rabinowitz JD, Werlen G, *et al.*: **mTORC2 responds to glutamine catabolite levels to modulate**

**the hexosamine biosynthesis enzyme GFAT1**. *Mol Cell* 2016, **63**:811–826.

9. Masui K, Tanaka K, Akhavan D, Babic I, Gini B, Matsutani T, Iwanami A, Liu F, Villa GR, Gu Y, *et al.*: **mTOR complex 2 controls glycolytic metabolism in glioblastoma through FoxO acetylation and upregulation of c-Myc**. *Cell Metab* 2013, **18**: 726–739.

10. Yin Y, Hua H, Li M, Liu S, Kong Q, Shao T, Wang J, Luo Y, Wang Q, Luo T, *et al.*: **mTORC2 promotes type I insulin-like growth factor receptor and insulin receptor activation through the tyrosine kinase activity of mTOR**. *Cell Res* 2016, **26**:46–65.

11. Hardie DG, Lin SC: **AMP-activated protein kinase – not just an energy sensor**. *F1000Res* 2017, **6**:1724.

12. ** Zhang CS, Hawley SA, Zong Y, Li M, Wang Z, Gray A, Ma T, Cui J, Feng JW, Zhu M, *et al.*: **Fructose-1,6-bisphosphate and aldolase mediate glucose sensing by AMPK**. *Nature* 2017, **548**:112–116.

Activation of AMPK has been largely ascribed to changes in AMP or ADP: ATP ratios. This study describes an AMP/ADP-independent mechanism for AMPK activation that involves the sensing of fructose-1,6-bisphosphate (FBP) levels via aldolases. This mechanism seems to depend on the lysosomal complex as FBP disrupts its assembly. These results establish a novel "moonlight" role for aldolase in metabolic signaling.

13. Jeon SM: **Regulation and function of AMPK in physiology and diseases**. *Exp Mol Med* 2016, **48**:e245.

14. Mihaylova MM, Shaw RJ: **The AMPK signalling pathway coordinates cell growth, autophagy and metabolism**. *Nat Cell Biol* 2011, **13**:1016–1023.

15. Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GI: **AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation**. *Proc Natl Acad Sci U S A* 2002, **99**: 15983–15987.

16. * Toyama EQ, Herzig S, Courchet J, Lewis Jr TL, Loson OC, Hellberg K, Young NP, Chen H, Polleux F, Chan DC, *et al.*: **Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress**. *Science* 2016, **351**:275–281.

AMPK has been previously reported to regulate mitochondrial dynamics via mitochondrial biogenesis. Now, in this study, a novel role for AMPK mediating mitochondrial fragmentation via the phosphorylation of the mitochondrial fission factor (MFF) is described.

17. * Rabinovitch RC, Samborska B, Faubert B, Ma EH, Gravel SP, Andrzejewski S, Raissi TC, Pause A, St-Pierre J, Jones RG: **AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species**. *Cell Rep* 2017, **21**:1–9.

This study reports that mitochondrial ROS activate AMPK, which reciprocally limits mitochondrial ROS production via a PGC-1$\alpha$-dependent antioxidant response. Cells lacking AMPK increased mitochondrial ROS, undergo premature senescence, and HIF-1$\alpha$-dependent Warburg effect, highlighting a key function for AMPK in stress resistance and metabolic balance by controlling mitochondrial ROS.

18. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ: **AMPK phosphorylation of raptor mediates a metabolic checkpoint**. *Mol Cell* 2008, **30**: 214–226.

19. Inoki K, Zhu T, Guan KL: **TSC2 mediates cellular energy response to control cell growth and survival**. *Cell* 2003, **115**: 577–590.

20. Dagon Y, Hur E, Zheng B, Wellenstein K, Cantley LC, Kahn BB: **p70S6 kinase phosphorylates AMPK on serine 491 to mediate leptin's effect on food intake**. *Cell Metab* 2012, **16**:104–112.

21. Kim J, Kundu M, Viollet B, Guan KL: **AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1**. *Nat Cell Biol* 2011, **13**:132–141.

22. Wang X, Li W, Williams M, Terada N, Alessi DR, Proud CG: **Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase**. *EMBO J* 2001, **20**:4370–4379.

23. Wang X, Regufe da Mota S, Liu R, Moore CE, Xie J, Lanucara F, Agarwala U, Pyr Dit Ruys S, Vertommen D, Rider MH, *et al.*: **Eukaryotic elongation factor 2 kinase activity is controlled by multiple inputs from oncogenic signaling**. *Mol Cell Biol* 2014, **34**:4088–4103.

24. Browne GJ, Finn SG, Proud CG: **Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398**. *J Biol Chem* 2004, **279**:12220–12231.

25. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME: **Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor**. *Cell* 1999, **96**:857–868.

26. Feehan RP, Shantz LM: **Negative regulation of the FOXO3a transcription factor by mTORC2 induces a pro-survival response following exposure to ultraviolet-B irradiation**. *Cell Signal* 2016, **28**:798–809.

27. Khatri S, Yepiskoposyan H, Gallo CA, Tandon P, Plas DR: **FOXO3a regulates glycolysis via transcriptional control of tumor suppressor TSC1**. *J Biol Chem* 2010, **285**:15960–15965.

28. Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP, Brunet A: **The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor**. *J Biol Chem* 2007, **282**:30107–30119.

29. Garza-Lombo C, Gonsebatt ME: **Mammalian target of rapamycin: its role in early neural development and in adult and aged brain function**. *Front Cell Neurosci* 2016, **10**:157.

30. Crino PB: **The mTOR signalling cascade: paving new roads to cure neurological disease**. *Nat Rev Neurol* 2016, **12**:379–392.

31. Ronnett GV, Ramamurthy S, Kleman AM, Landree LE, Aja S: **AMPK in the brain: its roles in energy balance and neuroprotection**. *J Neurochem* 2009, **109**(Suppl 1):17–23.

32. Magistretti PJ, Allaman I: **A cellular perspective on brain energy metabolism and functional imaging**. *Neuron* 2015, **86**: 883–901.

33. * Machler P, Wyss MT, Elsayed M, Stobart J, Gutierrez R, von Faber-Castell A, Kaelin V, Zuend M, San Martin A, Romero-Gomez I, *et al.*: **In vivo evidence for a lactate gradient from astrocytes to neurons**. *Cell Metab* 2016, **23**:94–102.

Using *in vivo* fluorescence imaging, this study demonstrates the existence of a lactate gradient from astrocytes to neurons, a prerequisite for the astrocyte-neuron lactate shuttle that substantiates its existance. See also reference 35.

34. ** Supplie LM, Duking T, Campbell G, Diaz F, Moraes CT, Gotz M, Hamprecht B, Boretius S, Mahad D, Nave KA: **Respiration-deficient astrocytes survive as glycolytic cells in vivo**. *J Neurosci* 2017, **37**:4231–4242.

This is the first report in vivo demonstrating that astrocytes do not require mitochondrial respiration for survival, at least under non-stressed conditions. This observation is key to our understanding regarding the energetic requirements for astrocytes

35. ** Diaz-Garcia CM, Mongeon R, Lahmann C, Koveal D, Zucker H, Yellen G: **Neuronal stimulation triggers neuronal glycolysis and not lactate uptake**. *Cell Metab* 2017, **26**:361–374. e364.

The exact contribution of glucose vs lactate to neuronal bioenergetics at "rest" or during "stimulation" is still under debate. In this study, using metabolic biosensors, Dr Yellen et al. reported that metabolic responses to neuronal stimulation do not seem to depend on lactate uptake, but instead they require increased glycolysis that provides a rapid response to energy demands.

36. Thomanetz V, Angliker N, Cloetta D, Lustenberger RM, Schweighauser M, Oliveri F, Suzuki N, Ruegg MA: **Ablation of the mTORC2 component rictor in brain or Purkinje cells affects size and neuron morphology**. *J Cell Biol* 2013, **201**: 293–308.

37. Cloetta D, Thomanetz V, Baranek C, Lustenberger RM, Lin S, Oliveri F, Atanasoski S, Ruegg MA: **Inactivation of mTORC1 in the developing brain causes microcephaly and affects gliogenesis**. *J Neurosci* 2013, **33**:7799–7810.

38. Zhang Y, Xu S, Liang KY, Li K, Zou ZP, Yang CL, Tan K, Cao X, Jiang Y, Gao TM, *et al.*: **Neuronal mTORC1 is required for**

**maintaining the nonreactive state of astrocytes**. *J Biol Chem* 2017, **292**:100–111.

39. Carson RP, Fu C, Winzenburger P, Ess KC: **Deletion of Rictor in neural progenitor cells reveals contributions of mTORC2 signaling to tuberous sclerosis complex**. *Hum Mol Genet* 2013, **22**:140–152.

40. Turnley AM, Stapleton D, Mann RJ, Witters LA, Kemp BE, Bartlett PF: **Cellular distribution and developmental expression of AMP-activated protein kinase isoforms in mouse central nervous system**. *J Neurochem* 1999, **72**:1707–1716.

41. Williams T, Courchet J, Viollet B, Brenman JE, Polleux F: **AMP-activated protein kinase (AMPK) activity is not required for neuronal development but regulates axogenesis during metabolic stress**. *Proc Natl Acad Sci U S A* 2011, **108**:5849–5854.

42. Ishizuka Y, Kakiya N, Witters LA, Oshiro N, Shirao T, Nawa H, Takei N: **AMP-activated protein kinase counteracts brain-derived neurotrophic factor-induced mammalian target of rapamycin complex 1 signaling in neurons**. *J Neurochem* 2013, **127**:66–77.

43. ** Smithson LJ, Gutmann DH: **Proteomic analysis reveals GIT1 as a novel mTOR complex component critical for mediating astrocyte survival**. *Genes Dev* 2016, **30**:1383–1388.
This is a very provocative study that suggests that we are still far away from understanding the complexity and tissure/cell type specificity of mTOR signaling. Here, the authors demonstrated that in astrocytes and neural stem cells, the G-protein-coupled receptor kinase-interacting protein 1 (GIT1) acts as as a novel mTOR-binding protein, creating a unique mTOR complex lacking Raptor and Rictor. GIT1 binding to mTOR was found to be regulated by AKT and to be essential for the mTOR-mediated astrocyte survival. The identification of a novel third mTOR complex requires confirmation by other studies.

44. Chong ZZ, Yao Q, Li HH: **The rationale of targeting mammalian target of rapamycin for ischemic stroke**. *Cell Signal* 2013, **25**:1598–1607.

45. Li J, McCullough LD: **Effects of AMP-activated protein kinase in cerebral ischemia**. *J Cereb Blood Flow Metab* 2010, **30**:480–492.

46. Hwang JY, Gertner M, Pontarelli F, Court-Vazquez B, Bennett MV, Ofengeim D, Zukin RS: **Global ischemia induces lysosomal-mediated degradation of mTOR and activation of autophagy in hippocampal neurons destined to die**. *Cell Death Differ* 2017, **24**:317–329.

47. Xie R, Cheng M, Li M, Xiong X, Daadi M, Sapolsky RM, Zhao H: **Akt isoforms differentially protect against stroke-induced neuronal injury by regulating mTOR activities**. *J Cereb Blood Flow Metab* 2013, **33**:1875–1885.

48. Pastor MD, Garcia-Yebenes I, Fradejas N, Perez-Ortiz JM, Mora-Lee S, Tranque P, Moro MA, Pende M, Calvo S: **mTOR/S6 kinase pathway contributes to astrocyte survival during ischemia**. *J Biol Chem* 2009, **284**:22067–22078.

49. McCullough LD, Zeng Z, Li H, Landree LE, McFadden J, Ronnett GV: **Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke**. *J Biol Chem* 2005, **280**:20493–20502.

50. Ashabi G, Khodagholi F, Khalaj L, Goudarzvand M, Nasiri M: **Activation of AMP-activated protein kinase by metformin protects against global cerebral ischemia in male rats: interference of AMPK/PGC-1 alpha pathway**. *Metab Brain Dis* 2014, **29**:47–58.

51. Li J, Zeng Z, Viollet B, Ronnett GV, McCullough LD: **Neuroprotective effects of adenosine monophosphate-activated protein kinase inhibition and gene deletion in stroke**. *Stroke* 2007, **38**:2992–2999.

52. Rousset CI, Leiper FC, Kichev A, Gressens P, Carling D, Hagberg H, Thornton C: **A dual role for AMP-activated protein kinase (AMPK) during neonatal hypoxic-ischaemic brain injury in mice**. *J Neurochem* 2015, **133**:242–252.

53. Hill JL, Kobori N, Zhao J, Rozas NS, Hylin MJ, Moore AN, Dash PK: **Traumatic brain injury decreases AMP-activated protein kinase activity and pharmacological enhancement of its activity improves cognitive outcome**. *J Neurochem* 2016, **139**:106–119.

54. Concannon CG, Tuffy LP, Weisova P, Bonner HP, Davila D, Bonner C, Devocelle MC, Strasser A, Ward MW, Prehn JH: **AMP kinase-mediated activation of the BH3-only protein Bim couples energy depletion to stress-induced apoptosis**. *J Cell Biol* 2010, **189**:83–94.

55. Weisova P, Concannon CG, Devocelle M, Prehn JH, Ward MW: **Regulation of glucose transporter 3 surface expression by the AMP-activated protein kinase mediates tolerance to glutamate excitation in neurons**. *J Neurosci* 2009, **29**:2997–3008.

56. Swiatkowski P, Nikolaeva I, Kumar G, Zucco A, Akum BF, Patel MV, D'Arcangelo G, Firestein BL: **Role of Akt-independent mTORC1 and GSK3beta signaling in sublethal NMDA-induced injury and the recovery of neuronal electrophysiology and survival**. *Sci Rep* 2017, **7**:1539.

57. Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, *et al.*: **Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway**. *Science* 2008, **322**:963–966.

58. Figlia G, Gerber D, Suter U: **Myelination and mTOR**. *Glia* 2018, **66**:693–707.

59. Berry M, Ahmed Z, Morgan-Warren P, Fulton D, Logan A: **Prospects for mTOR-mediated functional repair after central nervous system trauma**. *Neurobiol Dis* 2016, **85**:99–110.

60. Menzies FM, Fleming A, Rubinsztein DC: **Compromised autophagy and neurodegenerative diseases**. *Nat Rev Neurosci* 2015, **16**:345–357.

61. Wong M: **Mammalian target of rapamycin (mTOR) pathways in neurological diseases**. *Biomed J* 2013, **36**:40–50.

62. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, *et al.*: **Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease**. *Nat Genet* 2004, **36**:585–595.

63. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC: **Alpha-Synuclein is degraded by both autophagy and the proteasome**. *J Biol Chem* 2003, **278**:25009–25013.

64. Caccamo A, De Pinto V, Messina A, Branca C, Oddo S: **Genetic reduction of mammalian target of rapamycin ameliorates Alzheimer's disease-like cognitive and pathological deficits by restoring hippocampal gene expression signature**. *J Neurosci* 2014, **34**:7988–7998.

65. Lee JH, Tecedor L, Chen YH, Monteys AM, Sowada MJ, Thompson LM, Davidson BL: **Reinstating aberrant mTORC1 activity in Huntington's disease mice improves disease phenotypes**. *Neuron* 2015, **85**:303–315.

66. Pryor WM, Biagioli M, Shahani N, Swarnkar S, Huang WC, Page DT, MacDonald ME, Subramaniam S: **Huntingtin promotes mTORC1 signaling in the pathogenesis of Huntington's disease**. *Sci Signal* 2014, **7**. ra103.

67. ** Zheng X, Boyer L, Jin M, Kim Y, Fan W, Bardy C, Berggren T, Evans RM, Gage FH, Hunter T: **Alleviation of neuronal energy deficiency by mTOR inhibition as a treatment for mitochondria-related neurodegeneration**. *eLIFE* 2016, **5**.
The protective effects of inhibition of mTOR signaling with rapamycin against neurodegeneration have been largely attributed to autophagy. However, in this work, the authors demonstrate that inhibition of mTOR with rapamycin significantly preserves neuronal ATP levels via a decrease in protein synthesis, a major energy-consuming process, when oxidative phosphorylation is impaired.

68. Tain LS, Mortiboys H, Tao RN, Ziviani E, Bandmann O, Whitworth AJ: **Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss**. *Nat Neurosci* 2009, **12**:1129–1135.

69. Imai Y, Gehrke S, Wang HQ, Takahashi R, Hasegawa K, Oota E, Lu B: **Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in drosophila**. *EMBO J* 2008, **27**:2432–2443.

70. Ng CH, Guan MS, Koh C, Ouyang X, Yu F, Tan EK, O'Neill SP, Zhang X, Chung J, Lim KL: **AMP kinase activation mitigates dopaminergic dysfunction and mitochondrial abnormalities in Drosophila models of Parkinson's disease**. *J Neurosci* 2012, **32**:14311–14317.

71. Choi JS, Park C, Jeong JW: **AMP-activated protein kinase is activated in Parkinson's disease models mediated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine**. *Biochem Biophys Res Commun* 2010, **391**:147–151.

72. Dulovic M, Jovanovic M, Xilouri M, Stefanis L, Harhaji-Trajkovic L, Kravic-Stevovic T, Paunovic V, Ardah MT, El-Agnaf OM, Kostic V, *et al.*: **The protective role of AMP-activated protein kinase in alpha-synuclein neurotoxicity in vitro**. *Neurobiol Dis* 2014, **63**: 1–11.

73. Xu Y, Liu C, Chen S, Ye Y, Guo M, Ren Q, Liu L, Zhang H, Xu C, Zhou Q, *et al.*: **Activation of AMPK and inactivation of Akt result in suppression of mTOR-mediated S6K1 and 4E-BP1 pathways leading to neuronal cell death in in vitro models of Parkinson's disease**. *Cell Signal* 2014, **26**:1680–1689.

74. Kim TW, Cho HM, Choi SY, Suguira Y, Hayasaka T, Setou M, Koh HC, Hwang EM, Park JY, Kang SJ, *et al.*: **(ADP-ribose) polymerase 1 and AMP-activated protein kinase mediate progressive dopaminergic neuronal degeneration in a mouse model of Parkinson's disease**. *Cell Death Dis* 2013, **4**:e919.

75. Jiang P, Gan M, Ebrahim AS, Castanedes-Casey M, Dickson DW, Yen SH: **Adenosine monophosphate-activated protein kinase overactivation leads to accumulation of alpha-synuclein oligomers and decrease of neurites**. *Neurobiol Aging* 2013, **34**:1504–1515.

76. ** Anandhan A, Lei S, Levytskyy R, Pappa A, Panayiotidis MI, Cerny RL, Khalimonchuk O, Powers R, Franco R: **Glucose metabolism and AMPK signaling regulate dopaminergic cell death induced by gene (alpha-Synuclein)-Environment (paraquat) interactions**. *Mol Neurobiol* 2017, **54**: 3825–3842.

In this work, our research group demonstrated that while autophagy is indeed a protective mechanism against the toxicity of gene-environment interactions linked to Parkinson's disease, the protective role of AMPK is associated with the regulation of cell metabolism (glucose) but not autophagy.

77. Garcia-Garcia A, Anandhan A, Burns M, Chen H, Zhou Y, Franco R: **Impairment of Atg5-dependent autophagic flux promotes paraquat- and MPP(+)-induced apoptosis but not rotenone or 6-hydroxydopamine toxicity**. *Toxicol Sci* 2013, **136**:166–182.

78. Domise M, Didier S, Marinangeli C, Zhao H, Chandakkar P, Buee L, Viollet B, Davies P, Marambaud P, Vingtdeux V: **AMP-activated protein kinase modulates tau phosphorylation and tau pathology in vivo**. *Sci Rep* 2016, **6**:26758.

79. Mairet-Coello G, Courchet J, Pieraut S, Courchet V, Maximov A, Polleux F: **The CAMKK2-AMPK kinase pathway mediates the synaptotoxic effects of Abeta oligomers through Tau phosphorylation**. *Neuron* 2013, **78**:94–108.

80. ** Seixas da Silva GS, Melo HM, Lourenco MV, Lyra ESNM, de Carvalho MB, Alves-Leon SV, de Souza JM, Klein WL, da-Silva WS, Ferreira ST, *et al.*: **Amyloid-beta oligomers transiently inhibit AMP-activated kinase and cause metabolic defects in hippocampal neurons**. *J Biol Chem* 2017, **292**: 7395–7406.

This study links an impairment in AMPK function induced by Aβ oligomers with alterations in neuronal metabolism and survival. Down-regulation of AMPK activity led to a decrease in glucose transporters (GLUTs) in dendritic processes, suggesting an impairment in glucose metabolism.

81. Ju TC, Chen HM, Lin JT, Chang CP, Chang WC, Kang JJ, Sun CP, Tao MH, Tu PH, Chang C, *et al.*: **Nuclear translocation of AMPK-alpha 1 potentiates striatal neurodegeneration in Huntington's disease**. *J Cell Biol* 2011, **194**:209–227.

82. Vazquez-Manrique RP, Farina F, Cambon K, Dolores Sequedo M, Parker AJ, Millan JM, Weiss A, Deglon N, Neri C: **AMPK activation protects from neuronal dysfunction and vulnerability across nematode, cellular and mouse models of Huntington's disease**. *Hum Mol Genet* 2016, **25**: 1043–1058.

83. Wang X, Meng D, Chang Q, Pan J, Zhang Z, Chen G, Ke Z, Luo J, Shi X: **Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway**. *Environ Health Perspect* 2010, **118**:627–634.

84. Chen L, Xu B, Liu L, Luo Y, Zhou H, Chen W, Shen T, Han X, Kontos CD, Huang S: **Cadmium induction of reactive oxygen species activates the mTOR pathway, leading to neuronal cell death**. *Free Radic Biol Med* 2011, **50**:624–632.

85. Agarwal S, Tiwari SK, Seth B, Yadav A, Singh A, Mudawal A, Chauhan LK, Gupta SK, Choubey V, Tripathi A, *et al.*: **Activation of autophagic flux against xenoestrogen bisphenol-a-induced hippocampal neurodegeneration via AMP kinase (AMPK)/Mammalian target of rapamycin (mTOR) pathways**. *J Biol Chem* 2015, **290**:21163–21184.

**mTOR/AMPK signaling in the brain: Cell metabolism, proteostasis and survival**

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**Supplementary Table 1. Effect of phosphorylation (depicted in Figure 1) on mTORC1-, mTORC2- and AMPK-dependent signaling pathways.** Autophosphorylation sites are not included in this table

| Target Protein (Alphabetical Order) | Kinase Involved and Phosphorylated Residues | Effect on the Target protein | References (Suppl) |
|---|---|---|---|
| **ACC**<br>Acetyl-CoA carboxylase | • **AMPK** → Ser80, and Ser1201.<br>Ser1216 does not play a regulatory role<br><br>By PKA as well but effect is unclear | **Inhibition -** Conformational changes leading to a decrease in the catalytic activity of ACC. ACC carboxylates acetyl-CoA to produce malonyl-CoA, a substrate of fatty acid biosynthesis. | [1,2] |
| **AKT/PKB**<br>Protein kinase B | • **PDK1** → Thr308 | **Activation -** Phosphorylation of the T-loop kinase domain in AKT. | [3] |
| | • **CAMKK2/β** → Thr308 | | [4,5] |
| | • **mTORC2** → Ser473 | **Activation -** Phosphorylation of the hydrophobic motif of AKT facilitates its interaction with PDK1. Might be dispensable for Akt activation under certain conditions. | [6-8] |
| | • **mTORC2** → Thr450 | *Folding* - Thr450 is constitutively phosphorylated and is required for proper folding of AKT | [9,10] |
| | • **GSK3** → Thr312 | **Inhibition -** Phosphorylation at the substrate binding site inhibits AKT activation | [11] |
| | • CDK2, TBK1, IKKε → Ser129, Ser137, Thr308, Ser 378, Ser473, Ser477, SerT479, Tyr? | Activation **-** Different mechanisms, see references for details. *Not relevant for this review.* | [12] |
| **AMPK**<br>AMP-activated protein kinase<br>Catalytic (α) and Regulatory (β or γ subunits) | • **LKB1** → αThr172 (Constitutive) | **Activation -** Phosphorylation of the T-loop kinase domain in AMPK. AMPK activation switches on catabolic pathways that generate ATP while switching off biosynthetic pathways that consume it. | [13-16] |
| | • **CaMKK2/β** → αThr172 (Independent of nucleotide levels) | | [17-19] |
| | • **ULK1** → β1Ser108 | **Activation -** Sensitizes AMPK to drugs/metabolites acting at the ADaM (allosteric drug and metabolism) site, | [20] |

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| | | independently of Thr172 phosphorylation. | |
| | • **ULK1** → αβγ? | **Inhibition -** *S*uppresses Thr172 phosphorylation and AMPK sensitivity to AMP via a negative feedback. | [21] |
| | • **AKT** → α1Ser487 | **Inhibition -** Inhibits subsequent phosphorylation of AMPK at Thr 172, and hence its activation. | [22,23] |
| | • **GSK3** → αT479 | **Inhibition -** Enhances the accessibility of the activation loop of the AMPKα subunit to phosphatases. | [24] |
| | • **S6K** → αSer491 | **Inhibition -** Reduces AMPK activity | [25] |
| **ATG14L**<br>Autophagy-Related Protein 14-Like Protein | • **ULK1** → Ser9 | **Activation -** Regulates ATG14L-Vps34 lipid kinase | [26] |
| **Beclin 1**<br>Coiled-coil, moesin-like BCL2-interacting protein | • **AMPK** → Ser93 and Ser96 | **Activation -** Promotes ATG14-dependent Beclin 1 activation. | [27] |
| | • **AMPK** → Thr 388 | **Activation -** Autophagy induction via the regulation of Vps34-Beclin 1-ATG14 complex formation.<br>**Inhibition -** Caspase 8-dependent cleavage. | [28]<br>[29] |
| | • **ULK1** → Ser14, Ser30 and Ser337 | **Activation -** As a complex with VPS34, Beclin 1 increases VPS34 lipid kinase activity and promotes autophagy initiation. | [30,31] |
| | • **AKT** → Ser234 and Ser295 | **Inhibition -** Sequesters Beclin 1 in a 14-3-3 / vimentin intermediate filament complex. | [32] |
| | • **EGFR** → Y229, Y233 and Y352 | **Inhibition -** Decreased Beclin 1-associated VPS34 lipid kinase activity. | [33] |
| | • DAPK3, CAMKII and MAPKAPK2/3 (MK2/3) → Ser90 | Activation - Different mechanisms, see references for details. *Not relevant for this review.* | [34-36] |
| **CAMKK2/β**<br>$Ca^{2+}$ / CaM-dependent protein kinase kinase 2/β | • **AMPK** → Thr144 | **Inhibition -** Reduces autonomous, but not $Ca^{2+}$ / CaM-activated CaMKK2/β activity. | [37] |
| | • CDK5 → Ser137 | | |
| | • **GSK3** → Ser129 and Ser133 (preceded by Ser137 phosphorylation by CDK5 | | [38] |
| **4E-BP**<br>Eukaryotic translation initiation factor binding protein | • **mTORC1** → Thr37, Thr46, Ser65 and Thr70 | **Inhibition -** Phosphorylation of Thr37 and Thr46 in 4E-BP serves as a priming event, which is followed by Thr 70 and Ser 65 phosphorylation. Phosphorylation of 4E-BP causes its release from eIF4E to allow cap-dependent protein translation. | [39,40] |
| | • **GSK3** → Thr37 and Thr46 | | [41] |
| | • p38, ERK, PIM2, ATM, CDK1 and LRRK2 → Thr37, Thr46, Ser65, Thr70 and Ser 112 | Inhibition - See references for details. *Not relevant for this review.* | [42] |

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| **EEF2K**<br>Eukaryotic elongation factor 2 kinase | • **AMPK** → Ser398, Ser491<br>A similar mechanism might be involved in activation by PKA → Ser500 [43] | **Activation -** Mechanism unknown but might be independent from changes in the sensitivity of EEF2K to $Ca^{2+}$ / CaM. | [44,45] |
| | • **mTORC1** → Ser70, Ser78, Ser392 and Ser396<br>**GSK** → Ser392 requires confirmation | **Inhibition -** Phosphorylation of Ser 392 and Ser396 facilitates phosphorylation of Ser70 and Ser78 controlling the binding of $Ca^{2+}$ / CaM to EEF2K. | [46] |
| | • **S6K** and RSK1 → Ser366 | **Inhibition -** Decreases the sensitivity of EEF2K to activation by $Ca^{2+}$ / CaM. Decreases eEF2 phosphorylation and inactivation by EEF2K. eEF2 mediates the translocation the mRNA–tRNA moiety on the ribosome at the elongation step. | [47,48] |
| | • p38δ and ERK → Ser359 | Inhibition (p38δ and ERK) and Activation (PKA) - See references for details. *Not relevant for this review.* | [46,49] |
| **FOXO3a**<br>The Forkhead Box O 3a | • **AMPK** → Thr179, Ser399, Ser413, Ser555, Ser588 and Ser626 | **Activation -** Increases FOXO3a DNA-binding and transcriptional activity. | [50] |
| | • **AKT** and **SGK**→ Thr 32, Ser253, Ser315 | **Inhibition -** Ser253 and Ser315 phosphorylation at the nuclear localization sequence (NLS) disrupt nuclear translocation and reveal the nuclear export signal (NES) of FOXO3a, respectively. | [51,52] |
| | • ERK, p38, JNK, MST1, IKK, CK1, DYRK1A/B and LMTK3 → Ser7, Ser12, Ser209, Ser215, Ser231, Ser232, Ser284, Ser294, Ser318, Ser321, Ser325, Ser344, Ser425, Ser487, Ser574, Ser644 | Inhibition (ERK, p38, CK1 and IKK) and Activation (JNK, MST1 and LMTK3) - See references for details. *Not relevant for this review.* | [53] |
| **Grb10**<br>Growth factor receptor-bound protein 10 | • **mTORC1** → Ser 501/503 | **Activation -** Promotes Grb10 stabilization leading to feedback inhibition of PI3K and ERK-MAPK pathways.<br>**Inhibition -** Promotes Grb10 dissociation from the receptor to interact with raptor, and thus suppresses mTORC1 signaling. | [54,55]<br>[56] |
| **GS**<br>Glycogen synthase | • **GSK3** → Ser641, Ser645, Ser649 and Ser653 | **Inhibition -** Decrease in glycogenesis | [57] |
| | • **AMPK** → Ser7 | | [58,59] |
| | • PKA, PhK, CaMKII, CK2, DYRK, PASK, p38 → Ser10, Ser641, Ser645, Ser653, Thr713, Thr718 and Ser724 | See references for details. *Not relevant for this review.* | [60] |
| **GSK3**<br>Glycogen Synthase Kinase 3<br>α or β | • **AKT**, **S6K**, **SGK** → αSer21 and βSer9<br>PKC and PKA as well | **Inhibition -** Phosphorylation of the N-terminal tail of GSK3 makes it to act as an inhibitory pseudosubstrate. | [61-64] |
| | • PKCζ, ERK and Dyrk1A → Thr23, Ser147 and Thr356 | Activation (PKCζ) and Inhibition (ERK and Dyrk1A) - See references for details. *Not relevant for this review.* | [65-67] |

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| **HIF1α** Hypoxia-inducible transcription factor 1α | | • **GSK3** → Ser551, Thr555 and Ser589; or Thr498, Ser502, Ser505, Thr506, and Ser510 | **Inhibition -** Promotes HIF-1α degradation via the SCF complex | [68,69] |
| | | • **AMPK** → Ser419 | **Inhibition -** Decreases HIF-1α levels | [70] |
| | | • **S6K** → Ser125 of PHD2 | **Inhibition -** Phosphorylation of PHD2 at Ser125 by S6K increases its ability to degrade HIF1α | [71] |
| | | • CDK1, Plk3, ERK and ATM → Ser576, Ser641, Ser643, Ser657, Ser668, Ser696 | Activation (CDK1, ERK, and ATM) or Inhibition (Plk3) **-** See references for details. *Not relevant for this review.* | [72] |
| **mTORC** Mechanistic (mammalian) target of rapamycin complex | **mTOR** | • **S6K** → Ser2448 | *Unknown* | [73,74] |
| | | • ? → Ser1261, Ser2159 and Thr2164 | **Activation -** Promote mTOR S2481 autophosphorylation and phosphorylation of downstream substrates | [75,76] |
| | **Deptor** | • **mTOR** → Ser293, Thr295, and/or Ser299 mTOR primes Deptor for phosphorylation at Ser286 and Ser287 by CK1. | **Activation -** Promotes the ubiquitination and degradation of DEPTOR, an inhibitor of mTORC, by phosphorylation of its degron. | [77] |
| | | • **S6K** and RSK1 → Ser286, Ser287 and Ser291. S6K primes the phosphorylation by RSK1 | | [78] |
| | | • p38γ and p38δ → Ser293 or Thr321 | | [79] |
| | C1 **PRAS40** | • **AKT** → Thr246 of PRAS | **Activation -** PRAS40 phosphorylation relieves its inhibitory action on mTORC1. However, knockout of PRAS40 can also exert a similar effect. | [80-84] |
| | | • **mTOR** → Ser183 and Ser221. Primed by Thr246 phosphorylation by AKT | | |
| | | • PKM2, DYRK3 and PIM1 → Ser202, Ser203 and Thr246 | | [85-87] |
| | C1 **Raptor** | • **mTOR** → Ser283 | **Activation -** An important step for Rheb-dependent activation of mTORC1 | [88,89] |
| | | • **GSK3** → Ser859 | **Activation -** Promotes mTOR and raptor interaction | [90] |
| | | • **AMPK** → Ser722 and Ser792 | **Inhibition -** Raptor phosphorylation inhibits mTORC1 | [91] |
| | | • **ULK1** → Ser855 and Ser859 | **Inhibition -** Phosphorylation of Raptor by ULK1 inhibits its ability to recognize substrates | [92] |
| | | • p38β, CDK1 and NLK → Ser283, Ser696, Thr706, Ser771 and Ser863 | Activation (p38β) and Inhibition (NLK) - See references for details. *Not relevant for this review.* | [93-95] |
| | C2 **mSIN1** | • **AKT** or **S6K** → Thr86 and Thr398 | **Activation -** A positive feedback loop that enhances mTORC2 kinase activity, which leads to phosphorylation of Akt Ser473.<br>**Inhibition -** Suppresses mTOR kinase activity by dissociating Sin1 from mTORC2 | [96,97]<br>[98] |
| | | • **mTOR** → ? | **Activation** - Regulates stability of mSIN1 and mTORC2 | [99] |

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| | Rictor | • **S6K** → Thr1135 | **Inhibition** - A negative-feedback mechanism affecting Akt phosphorylation by mTORC2. It might also be involved in complex interactions and in the TORC2-independent role of Rictor | [100-103] |
| | | • Plk1 → Ser1162 | *Unknown* - See references for details. *Not relevant for this review.* | [104] |
| **Nrf2**<br>Nuclear factor erythroid 2 - related factor 2 | | • **AMPK** → Ser558 | **Activation -** Phosphorylation occurs at the canonical export signal promoting nuclear accumulation of Nrf2. | [105] |
| | | • PKC and Fyn → Ser40 and Tyr568 | Activation (PKC) or Inhibition (Fyn) **-** See references for details. *Not relevant for this review.* | [106] |
| **p62/SQSTM1**<br>Sequestosome-1 | | • **AMPK** → Ser294 | **Activation -** p62 translocates to mitochondria and induces mitophagy. | [107] |
| | | • **ULK1** → Ser407 | **Activation -** Enhances the affinity of p62 to ubiquitin. | [108] |
| | | • **mTORC1** → Ser349 | **Activation -** Disrupts Keap1-mediated Nrf2 ubiquitination | [109] |
| | | • PKA, LRRK2, CDK1, p38δ, PKCδ, CK2, and TBK1 → Ser24, Thr138, Thr269, Ser272, Ser349, S403 | Activation **-** See references for details. *Not relevant for this review.* | [110-116] |
| **PFKFB**<br>(PFK-2/FBPase-2) 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase | | • **Akt** → Ser466 and Ser483 of PFKFB2 | **Activation -** Increases glycolysis, by controlling the levels of fructose 2,6-bisphosphate, an allosteric activator of phosphofructokinase 1 (PFK-1) | [117] |
| | | • **AMPK** → Ser466 of PFKFB2 and Ser461 of PFKFB3 | | [118] |
| | | • PKA, PKC and MAPKAPK2 (MK2) → Ser32 of PFKFB1; Ser84, Ser466, Thr475 and Ser483 PFKFB2; and Ser461 of PFKFB3 | Inactivation (Ser32 of PFKFB1) and Activation (Ser461 of PFKFB3) - See references for details. *Not relevant for this review.* | [119,120] |
| **PGC-1α**<br>Peroxisome-proliferator-activated receptor γ coactivator 1α | | • **AMPK** → Thr177 and Ser538 | **Activation -** Enhances the co-transcriptional activity of PGC-1α | [121] |
| | | • **AKT** → Ser570 | **Inhibition -** Inhibition of the recruitment of PGC-1α to cognate promoters | [122] |
| | | • **GSK3** → Thr295 (to be confirmed) | **Inhibition -** Enhances the proteasomal degradation of PGC-1α in the nucleus | [123,124] |
| | | • Clk2, PKA and p38 → Ser194, Ser241, Ser256, Thr262, Ser265 and Thr298 | Inhibition (Clk2) and Activation (PKA and p38) - See references for details. *Not relevant for this review.* | [124] |
| **PI3K**<br>Class I Phosphoinositide 3-kinase (p85 regulatory subunit) | | • **Src** family kinases → Tyr688 | **Activation -** Relieves p85's inhibitory activity on p110 | [125] |
| | | • **PKA** → Ser83 | **Activation -** Enhances Ras binding to p110 | [126] |
| | | • **Growth factor/cytokines** → Tyr508 | *Unknown* | [127] |
| | | • **p110** (catalytic) → Ser608 | **Inhibition -** Decreases p85–p110 activity | [128] |
| | | • PKC (PKD/PKCμ) and IKK → Ser361, | Inhibition - Inhibits binding of p85 to phospho-Tyr. See | [129,130] |

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| | Ser652 and Ser690 | references for details. *Not relevant for this review.* | |
| **PINK1**<br>PTEN-induced putative kinase 1 | • **AMPK** → Ser495 | **Activation -** Promotes the recruitment of Parkin to depolarized mitochondria to trigger mitophagy | [131] |
| **Rheb**<br>Ras homolog enriched in brain | • PRAK → Ser130 | **Inhibition -** Phosphorylation of Rheb by PRAK impairs inhibits Rheb-mediated mTORC1 activation. See references for details. *Not relevant for this review.* | [132] |
| **(p70)S6K**<br>Ribosomal protein S6 kinase | • **mTORC1** → Thr389 | **Activation -** Thr389 phosphorylation promotes docking of PDK1, which phosphorylates S6K at Thr229. | [133] |
| | • **PDK1** → Thr229 | **Activation -** Phosphorylation by PDK1 a critical component of the activation of S6K. | [134] |
| | • **GSK3** → Ser371 | **Activation -** Cooperates in the phosphorylation of Thr389 by mTORC1 | [135] |
| | • JNK1, ERK, p38, CDK1, CDK5 → Ser411, Thr421 and Ser424 | Activation (CDK5, JNK1) and Inhibition (CDK1) - See references for details. *Not relevant for this review.* | [136-139] |
| **SGK**<br>Serum and glucocorticoid-regulated kinase | • **mTORC2** → Ser 422 | **Activation -** Phosphorylation of the hydrophobic motif of SGK facilitates its interaction with PDK1. | [140] |
| | • **PDK1** → Thr256 | **Activation -** Phosphorylation of the T-loop kinase domain of SGK | [141,142] |
| **TBC1D1/4**<br>Rab GTPase-activating protein TBC (Tre-2/ BUB2/cdc) 1 domain. Family members 1 and 4 (AS160) | • **Akt** → Thr596 TBC1D1 and Thr642 TBC1D4 | **Activation -** Promotes traffic of glucose transporters to the plasma membrane. | [143-145] |
| | • **AMPK** → Ser237 TBC1D1; Ser588 and Ser711 TBC1D4 | | [144-147] |
| **TFEB**<br>Transcription factor EB | • **mTORC1** → Ser211 | **Inhibition -** Prevents TFEB nuclear translocation. | [148] |
| | • **AKT** → Ser467 | | [149] |
| | • MAP4K3 → Ser3 | | [150] |
| **TSC1/2**<br>Tuberous sclerosis complex TSC1-TSC2 | • **AMPK** → Ser 1227 and Thr1345 on TSC2 | **Activation -** Through its GAP (GTPase-activating protein) activity towards the small G-protein Rheb negatively regulates mTORC1 signaling. | [151,152] |
| | • **GSK3** → Ser1371, Ser1375, Ser1379, and Ser1383 on TSC2 | | [153] |
| | • **AKT** → Ser939, Ser981, Ser1130, Ser1132 and Thr1462 on TSC2 | **Inhibition –** Inhibition of TSC2's GAP action on Rheb (activity, localization, complex formation or degradation) | [154-156] |
| | • **SGK** → Ser 939, Ser981, Ser1130, Ser1132, Thr1462, and Ser1798 on TSC2 | **Inhibition -** Activation of mTORC1. | [152,157] |
| | • ERK, RSK, MAPKAP2 (MK2), IKK and CDK1 → Ser540, Ser664, Ser939, | Inhibition (ERK, RSK, IKK and CDK1) - See references for details. *Not relevant for this review.* | [152] |

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|---|---|---|---|
| | Ser1462, Ser1254 and Ser1798 on TSC2; Thr417, Ser487, Ser511, Ser584 and Thr1047 on TSC1 | | |
| **Ub** Ubiquitin | • **PINK1** → Ser65 | **Activation -** Accelerates the discharge of the thioester conjugate from the E2 conjugating enzyme and unlocks the autoinhibition of the catalytic cysteine in Parkin. Regulates Ub structure, chain assembly and hydrolysis | [158-161] |
| **ULK1** Unc-51 like autophagy activating kinase | • **AMPK** → Ser317 and Ser777 | **Activation -** Activates ULK1 and promotes autophagy | [162] |
| | • **AMPK** → Ser467 and Ser555 | | [163,164] |
| | • **AMPK** → Ser555 | **Activation -** Regulates mitophagy via ULK1 translocation to the mitochondria | [165,166] |
| | • **mTORC1** → Ser757 | **Inhibition -** Disrupts the interaction with AMPK. | [162,163] |
| | • **AKT** → Ser774 | *Unknown* | [166] |
| **Vps34/PI3KC3** Class III PI3K | • **AMPK** → Thr163 and Ser 165 | **Inhibition** - Vps34 phosphorylation suppresses PI(3)P production. | [27] |
| | • **ULK1** → Ser249 | *Unknown* | [31] |
| | • CDK1 → Thr159 | Inhibition - See references for details. *Not relevant for this review.* | [167] |

## REFERENCES


1. Davies SP, Sim AT, Hardie DG: **Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase**. *Eur J Biochem* 1990, **187**:183-190.
2. Ha J, Daniel S, Broyles SS, Kim KH: **Critical phosphorylation sites for acetyl-CoA carboxylase activity**. *J Biol Chem* 1994, **269**:22162-22168.
3. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA: **Mechanism of activation of protein kinase B by insulin and IGF-1**. *EMBO J* 1996, **15**:6541-6551.
4. Gocher AM, Azabdaftari G, Euscher LM, Dai S, Karacosta LG, Franke TF, Edelman AM: **Akt activation by Ca(2+)/calmodulin-dependent protein kinase kinase 2 (CaMKK2) in ovarian cancer cells**. *J Biol Chem* 2017, **292**:14188-14204.
5. Yano S, Tokumitsu H, Soderling TR: **Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway**. *Nature* 1998, **396**:584-587.
6. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM: **Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex**. *Science* 2005, **307**:1098-1101.
7. Hill MM, Andjelkovic M, Brazil DP, Ferrari S, Fabbro D, Hemmings BA: **Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase**. *J Biol Chem* 2001, **276**:25643-25646.
8. Moore SF, Hunter RW, Hers I: **mTORC2 protein complex-mediated Akt (Protein Kinase B) Serine 473 Phosphorylation is not required for Akt1 activity in human platelets [corrected]**. *J Biol Chem* 2011, **286**:24553-24560.
9. Facchinetti V, Ouyang W, Wei H, Soto N, Lazorchak A, Gould C, Lowry C, Newton AC, Mao Y, Miao RQ, et al.: **The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C**. *EMBO J* 2008, **27**:1932-1943.
10. Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL: **Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling**. *EMBO J* 2008, **27**:1919-1931.
11. Gulen MF, Bulek K, Xiao H, Yu M, Gao J, Sun L, Beurel E, Kaidanovich-Beilin O, Fox PL, DiCorleto PE, et al.: **Inactivation of the enzyme GSK3alpha by the kinase IKKi promotes AKT-mTOR signaling pathway that mediates interleukin-1-induced Th17 cell maintenance**. *Immunity* 2012, **37**:800-812.
12. Manning BD, Toker A: **AKT/PKB Signaling: Navigating the Network**. *Cell* 2017, **169**:381-405.
13. Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG: **Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase**. *J Biol Chem* 1996, **271**:27879-27887.
14. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D: **LKB1 is the upstream kinase in the AMP-activated protein kinase cascade**. *Curr Biol* 2003, **13**:2004-2008.
15. Lizcano JM, Goransson O, Toth R, Deak M, Morrice NA, Boudeau J, Hawley SA, Udd L, Makela TP, Hardie DG, et al.: **LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1**. *EMBO J* 2004, **23**:833-843.
16. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC: **The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress**. *Proc Natl Acad Sci U S A* 2004, **101**:3329-3335.
17. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG: **Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase**. *Cell Metab* 2005, **2**:9-19.
18. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D: **Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells**. *Cell Metab* 2005, **2**:21-33.

19. Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witters LA: **The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases**. *J Biol Chem* 2005, **280**:29060-29066.
20. Dite TA, Ling NXY, Scott JW, Hoque A, Galic S, Parker BL, Ngoei KRW, Langendorf CG, O'Brien MT, Kundu M, et al.: **The autophagy initiator ULK1 sensitizes AMPK to allosteric drugs**. *Nat Commun* 2017, **8**:571.
21. Loffler AS, Alers S, Dieterle AM, Keppeler H, Franz-Wachtel M, Kundu M, Campbell DG, Wesselborg S, Alessi DR, Stork B: **Ulk1-mediated phosphorylation of AMPK constitutes a negative regulatory feedback loop**. *Autophagy* 2011, **7**:696-706.
22. Horman S, Vertommen D, Heath R, Neumann D, Mouton V, Woods A, Schlattner U, Wallimann T, Carling D, Hue L, et al.: **Insulin antagonizes ischemia-induced Thr172 phosphorylation of AMP-activated protein kinase alpha-subunits in heart via hierarchical phosphorylation of Ser485/491**. *J Biol Chem* 2006, **281**:5335-5340.
23. Hawley SA, Ross FA, Gowans GJ, Tibarewal P, Leslie NR, Hardie DG: **Phosphorylation by Akt within the ST loop of AMPK-alpha1 down-regulates its activation in tumour cells**. *Biochem J* 2014, **459**:275-287.
24. Suzuki T, Bridges D, Nakada D, Skiniotis G, Morrison SJ, Lin JD, Saltiel AR, Inoki K: **Inhibition of AMPK catabolic action by GSK3**. *Mol Cell* 2013, **50**:407-419.
25. Dagon Y, Hur E, Zheng B, Wellenstein K, Cantley LC, Kahn BB: **p70S6 kinase phosphorylates AMPK on serine 491 to mediate leptin's effect on food intake**. *Cell Metab* 2012, **16**:104-112.
26. Wold MS, Lim J, Lachance V, Deng Z, Yue Z: **ULK1-mediated phosphorylation of ATG14 promotes autophagy and is impaired in Huntington's disease models**. *Mol Neurodegener* 2016, **11**:76.
27. Kim J, Kim YC, Fang C, Russell RC, Kim JH, Fan W, Liu R, Zhong Q, Guan KL: **Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy**. *Cell* 2013, **152**:290-303.
28. Zhang D, Wang W, Sun X, Xu D, Wang C, Zhang Q, Wang H, Luo W, Chen Y, Chen H, et al.: **AMPK regulates autophagy by phosphorylating BECN1 at threonine 388**. *Autophagy* 2016, **12**:1447-1459.
29. Song X, Kim SY, Zhang L, Tang D, Bartlett DL, Kwon YT, Lee YJ: **Role of AMP-activated protein kinase in cross-talk between apoptosis and autophagy in human colon cancer**. *Cell Death Dis* 2014, **5**:e1504.
30. Russell RC, Tian Y, Yuan H, Park HW, Chang YY, Kim J, Kim H, Neufeld TP, Dillin A, Guan KL: **ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase**. *Nat Cell Biol* 2013, **15**:741-750.
31. Egan DF, Chun MG, Vamos M, Zou H, Rong J, Miller CJ, Lou HJ, Raveendra-Panickar D, Yang CC, Sheffler DJ, et al.: **Small Molecule Inhibition of the Autophagy Kinase ULK1 and Identification of ULK1 Substrates**. *Mol Cell* 2015, **59**:285-297.
32. Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, White M, Reichelt J, Levine B: **Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation**. *Science* 2012, **338**:956-959.
33. Wei Y, Zou Z, Becker N, Anderson M, Sumpter R, Xiao G, Kinch L, Koduru P, Christudass CS, Veltri RW, et al.: **EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance**. *Cell* 2013, **154**:1269-1284.
34. Li X, Wu XQ, Deng R, Li DD, Tang J, Chen WD, Chen JH, Ji J, Jiao L, Jiang S, et al.: **CaMKII-mediated Beclin 1 phosphorylation regulates autophagy that promotes degradation of Id and neuroblastoma cell differentiation**. *Nat Commun* 2017, **8**:1159.
35. Wei Y, An Z, Zou Z, Sumpter R, Su M, Zang X, Sinha S, Gaestel M, Levine B: **The stress-responsive kinases MAPKAPK2/MAPKAPK3 activate starvation-induced autophagy through Beclin 1 phosphorylation**. *Elife* 2015, **4**.
36. Fujiwara N, Usui T, Ohama T, Sato K: **Regulation of Beclin 1 Protein Phosphorylation and Autophagy by Protein Phosphatase 2A (PP2A) and Death-associated Protein Kinase 3 (DAPK3)**. *J Biol Chem* 2016, **291**:10858-10866.
37. Nakanishi A, Hatano N, Fujiwara Y, Sha'ri A, Takabatake S, Akano H, Kanayama N, Magari M, Nozaki N, Tokumitsu H: **AMP-activated protein kinase-mediated feedback phosphorylation controls the Ca(2+)/calmodulin (CaM) dependence of Ca(2+)/CaM-dependent protein kinase kinase beta**. *J Biol Chem* 2017, **292**:19804-19813.

38. Green MF, Scott JW, Steel R, Oakhill JS, Kemp BE, Means AR: **Ca2+/Calmodulin-dependent protein kinase kinase beta is regulated by multisite phosphorylation**. *J Biol Chem* 2011, **286**:28066-28079.
39. Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N: **4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway**. *Genes Dev* 1998, **12**:502-513.
40. Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N: **Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism**. *Genes Dev* 1999, **13**:1422-1437.
41. Shin S, Wolgamott L, Tcherkezian J, Vallabhapurapu S, Yu Y, Roux PP, Yoon SO: **Glycogen synthase kinase-3beta positively regulates protein synthesis and cell proliferation through the regulation of translation initiation factor 4E-binding protein 1**. *Oncogene* 2014, **33**:1690-1699.
42. Qin X, Jiang B, Zhang Y: **4E-BP1, a multifactor regulated multifunctional protein**. *Cell Cycle* 2016, **15**:781-786.
43. Diggle TA, Subkhankulova T, Lilley KS, Shikotra N, Willis AE, Redpath NT: **Phosphorylation of elongation factor-2 kinase on serine 499 by cAMP-dependent protein kinase induces Ca2+/calmodulin-independent activity**. *Biochem J* 2001, **353**:621-626.
44. Browne GJ, Finn SG, Proud CG: **Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398**. *J Biol Chem* 2004, **279**:12220-12231.
45. Johanns M, Pyr Dit Ruys S, Houddane A, Vertommen D, Herinckx G, Hue L, Proud CG, Rider MH: **Direct and indirect activation of eukaryotic elongation factor 2 kinase by AMP-activated protein kinase**. *Cell Signal* 2017, **36**:212-221.
46. Wang X, Regufe da Mota S, Liu R, Moore CE, Xie J, Lanucara F, Agarwala U, Pyr Dit Ruys S, Vertommen D, Rider MH, et al.: **Eukaryotic elongation factor 2 kinase activity is controlled by multiple inputs from oncogenic signaling**. *Mol Cell Biol* 2014, **34**:4088-4103.
47. Wang X, Li W, Williams M, Terada N, Alessi DR, Proud CG: **Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase**. *EMBO J* 2001, **20**:4370-4379.
48. Browne GJ, Proud CG: **A novel mTOR-regulated phosphorylation site in elongation factor 2 kinase modulates the activity of the kinase and its binding to calmodulin**. *Mol Cell Biol* 2004, **24**:2986-2997.
49. Knebel A, Morrice N, Cohen P: **A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38delta**. *EMBO J* 2001, **20**:4360-4369.
50. Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP, Brunet A: **The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor**. *J Biol Chem* 2007, **282**:30107-30119.
51. Brunet A, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME: **Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a)**. *Mol Cell Biol* 2001, **21**:952-965.
52. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME: **Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor**. *Cell* 1999, **96**:857-868.
53. Wang X, Hu S, Liu L: **Phosphorylation and acetylation modifications of FOXO3a: Independently or synergistically?** *Oncol Lett* 2017, **13**:2867-2872.
54. Yu Y, Yoon SO, Poulogiannis G, Yang Q, Ma XM, Villen J, Kubica N, Hoffman GR, Cantley LC, Gygi SP, et al.: **Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling**. *Science* 2011, **332**:1322-1326.
55. Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, Peterson TR, Choi Y, Gray NS, Yaffe MB, et al.: **The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling**. *Science* 2011, **332**:1317-1322.
56. Liu M, Bai J, He S, Villarreal R, Hu D, Zhang C, Yang X, Liang H, Slaga TJ, Yu Y, et al.: **Grb10 promotes lipolysis and thermogenesis by phosphorylation-dependent feedback inhibition of mTORC1**. *Cell Metab* 2014, **19**:967-980.

57. Rylatt DB, Aitken A, Bilham T, Condon GD, Embi N, Cohen P: **Glycogen synthase from rabbit skeletal muscle. Amino acid sequence at the sites phosphorylated by glycogen synthase kinase-3, and extension of the N-terminal sequence containing the site phosphorylated by phosphorylase kinase**. *Eur J Biochem* 1980, **107**:529-537.
58. Embi N, Parker PJ, Cohen P: **A reinvestigation of the phosphorylation of rabbit skeletal-muscle glycogen synthase by cyclic-AMP-dependent protein kinase. Identification of the third site of phosphorylation as serine-7**. *Eur J Biochem* 1981, **115**:405-413.
59. Jorgensen SB, Nielsen JN, Birk JB, Olsen GS, Viollet B, Andreelli F, Schjerling P, Vaulont S, Hardie DG, Hansen BF, et al.: **The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading**. *Diabetes* 2004, **53**:3074-3081.
60. Jensen J, Lai YC: **Regulation of muscle glycogen synthase phosphorylation and kinetic properties by insulin, exercise, adrenaline and role in insulin resistance**. *Arch Physiol Biochem* 2009, **115**:13-21.
61. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA: **Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B**. *Nature* 1995, **378**:785-789.
62. Sakoda H, Gotoh Y, Katagiri H, Kurokawa M, Ono H, Onishi Y, Anai M, Ogihara T, Fujishiro M, Fukushima Y, et al.: **Differing roles of Akt and serum- and glucocorticoid-regulated kinase in glucose metabolism, DNA synthesis, and oncogenic activity**. *J Biol Chem* 2003, **278**:25802-25807.
63. Zhang HH, Lipovsky AI, Dibble CC, Sahin M, Manning BD: **S6K1 regulates GSK3 under conditions of mTOR-dependent feedback inhibition of Akt**. *Mol Cell* 2006, **24**:185-197.
64. Frame S, Cohen P, Biondi RM: **A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation**. *Mol Cell* 2001, **7**:1321-1327.
65. Song WJ, Song EA, Jung MS, Choi SH, Baik HH, Jin BK, Kim JH, Chung SH: **Phosphorylation and inactivation of glycogen synthase kinase 3beta (GSK3beta) by dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A)**. *J Biol Chem* 2015, **290**:2321-2333.
66. Tejeda-Munoz N, Gonzalez-Aguilar H, Santoyo-Ramos P, Castaneda-Patlan MC, Robles-Flores M: **Glycogen Synthase Kinase 3beta Is Positively Regulated by Protein Kinase Czeta-Mediated Phosphorylation Induced by Wnt Agonists**. *Mol Cell Biol* 2015, **36**:731-741.
67. Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, Bartholomeusz G, Li Y, Pan Y, Li Z, et al.: **Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin**. *Mol Cell* 2005, **19**:159-170.
68. Flugel D, Gorlach A, Michiels C, Kietzmann T: **Glycogen synthase kinase 3 phosphorylates hypoxia-inducible factor 1alpha and mediates its destabilization in a VHL-independent manner**. *Mol Cell Biol* 2007, **27**:3253-3265.
69. Cassavaugh JM, Hale SA, Wellman TL, Howe AK, Wong C, Lounsbury KM: **Negative regulation of HIF-1alpha by an FBW7-mediated degradation pathway during hypoxia**. *J Cell Biochem* 2011, **112**:3882-3890.
70. Hwang AB, Ryu EA, Artan M, Chang HW, Kabir MH, Nam HJ, Lee D, Yang JS, Kim S, Mair WB, et al.: **Feedback regulation via AMPK and HIF-1 mediates ROS-dependent longevity in Caenorhabditis elegans**. *Proc Natl Acad Sci U S A* 2014, **111**:E4458-4467.
71. Di Conza G, Trusso Cafarello S, Loroch S, Mennerich D, Deschoemaeker S, Di Matteo M, Ehling M, Gevaert K, Prenen H, Zahedi RP, et al.: **The mTOR and PP2A Pathways Regulate PHD2 Phosphorylation to Fine-Tune HIF1alpha Levels and Colorectal Cancer Cell Survival under Hypoxia**. *Cell Rep* 2017, **18**:1699-1712.
72. Kietzmann T, Mennerich D, Dimova EY: **Hypoxia-Inducible Factors (HIFs) and Phosphorylation: Impact on Stability, Localization, and Transactivity**. *Front Cell Dev Biol* 2016, **4**:11.
73. Chiang GG, Abraham RT: **Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase**. *J Biol Chem* 2005, **280**:25485-25490.

74. Holz MK, Blenis J: **Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase**. *J Biol Chem* 2005, **280**:26089-26093.
75. Acosta-Jaquez HA, Keller JA, Foster KG, Ekim B, Soliman GA, Feener EP, Ballif BA, Fingar DC: **Site-specific mTOR phosphorylation promotes mTORC1-mediated signaling and cell growth**. *Mol Cell Biol* 2009, **29**:4308-4324.
76. Ekim B, Magnuson B, Acosta-Jaquez HA, Keller JA, Feener EP, Fingar DC: **mTOR kinase domain phosphorylation promotes mTORC1 signaling, cell growth, and cell cycle progression**. *Mol Cell Biol* 2011, **31**:2787-2801.
77. Gao D, Inuzuka H, Tan MK, Fukushima H, Locasale JW, Liu P, Wan L, Zhai B, Chin YR, Shaik S, et al.: **mTOR drives its own activation via SCF(betaTrCP)-dependent degradation of the mTOR inhibitor DEPTOR**. *Mol Cell* 2011, **44**:290-303.
78. Zhao Y, Xiong X, Sun Y: **DEPTOR, an mTOR inhibitor, is a physiological substrate of SCF(betaTrCP) E3 ubiquitin ligase and regulates survival and autophagy**. *Mol Cell* 2011, **44**:304-316.
79. Gonzalez-Teran B, Lopez JA, Rodriguez E, Leiva L, Martinez-Martinez S, Bernal JA, Jimenez-Borreguero LJ, Redondo JM, Vazquez J, Sabio G: **p38gamma and delta promote heart hypertrophy by targeting the mTOR-inhibitory protein DEPTOR for degradation**. *Nat Commun* 2016, **7**:10477.
80. Kovacina KS, Park GY, Bae SS, Guzzetta AW, Schaefer E, Birnbaum MJ, Roth RA: **Identification of a proline-rich Akt substrate as a 14-3-3 binding partner**. *J Biol Chem* 2003, **278**:10189-10194.
81. Nascimento EB, Snel M, Guigas B, van der Zon GC, Kriek J, Maassen JA, Jazet IM, Diamant M, Ouwens DM: **Phosphorylation of PRAS40 on Thr246 by PKB/AKT facilitates efficient phosphorylation of Ser183 by mTORC1**. *Cell Signal* 2010, **22**:961-967.
82. Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH: **Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40**. *Nat Cell Biol* 2007, **9**:316-323.
83. Fonseca BD, Smith EM, Lee VH, MacKintosh C, Proud CG: **PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signaling downstream of this complex**. *J Biol Chem* 2007, **282**:24514-24524.
84. Wang L, Harris TE, Lawrence JC, Jr.: **Regulation of proline-rich Akt substrate of 40 kDa (PRAS40) function by mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation**. *J Biol Chem* 2008, **283**:15619-15627.
85. He CL, Bian YY, Xue Y, Liu ZX, Zhou KQ, Yao CF, Lin Y, Zou HF, Luo FX, Qu YY, et al.: **Pyruvate Kinase M2 Activates mTORC1 by Phosphorylating AKT1S1**. *Sci Rep* 2016, **6**:21524.
86. Zhang F, Beharry ZM, Harris TE, Lilly MB, Smith CD, Mahajan S, Kraft AS: **PIM1 protein kinase regulates PRAS40 phosphorylation and mTOR activity in FDCP1 cells**. *Cancer Biol Ther* 2009, **8**:846-853.
87. Wippich F, Bodenmiller B, Trajkovska MG, Wanka S, Aebersold R, Pelkmans L: **Dual specificity kinase DYRK3 couples stress granule condensation/dissolution to mTORC1 signaling**. *Cell* 2013, **152**:791-805.
88. Foster KG, Acosta-Jaquez HA, Romeo Y, Ekim B, Soliman GA, Carriere A, Roux PP, Ballif BA, Fingar DC: **Regulation of mTOR complex 1 (mTORC1) by raptor Ser863 and multisite phosphorylation**. *J Biol Chem* 2010, **285**:80-94.
89. Wang L, Lawrence JC, Jr., Sturgill TW, Harris TE: **Mammalian target of rapamycin complex 1 (mTORC1) activity is associated with phosphorylation of raptor by mTOR**. *J Biol Chem* 2009, **284**:14693-14697.
90. Stretton C, Hoffmann TM, Munson MJ, Prescott A, Taylor PM, Ganley IG, Hundal HS: **GSK3-mediated raptor phosphorylation supports amino-acid-dependent mTORC1-directed signalling**. *Biochem J* 2015, **470**:207-221.
91. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ: **AMPK phosphorylation of raptor mediates a metabolic checkpoint**. *Mol Cell* 2008, **30**:214-226.
92. Dunlop EA, Hunt DK, Acosta-Jaquez HA, Fingar DC, Tee AR: **ULK1 inhibits mTORC1 signaling, promotes multisite Raptor phosphorylation and hinders substrate binding**. *Autophagy* 2011, **7**:737-747.

93. Wu XN, Wang XK, Wu SQ, Lu J, Zheng M, Wang YH, Zhou H, Zhang H, Han J: **Phosphorylation of Raptor by p38beta participates in arsenite-induced mammalian target of rapamycin complex 1 (mTORC1) activation**. *J Biol Chem* 2011, **286**:31501-31511.
94. Yuan HX, Wang Z, Yu FX, Li F, Russell RC, Jewell JL, Guan KL: **NLK phosphorylates Raptor to mediate stress-induced mTORC1 inhibition**. *Genes Dev* 2015, **29**:2362-2376.
95. Gwinn DM, Asara JM, Shaw RJ: **Raptor is phosphorylated by cdc2 during mitosis**. *PLoS One* 2010, **5**:e9197.
96. Yang G, Murashige DS, Humphrey SJ, James DE: **A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation**. *Cell Rep* 2015, **12**:937-943.
97. Humphrey SJ, Yang G, Yang P, Fazakerley DJ, Stockli J, Yang JY, James DE: **Dynamic adipocyte phosphoproteome reveals that Akt directly regulates mTORC2**. *Cell Metab* 2013, **17**:1009-1020.
98. Liu P, Gan W, Inuzuka H, Lazorchak AS, Gao D, Arojo O, Liu D, Wan L, Zhai B, Yu Y, et al.: **Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis**. *Nat Cell Biol* 2013, **15**:1340-1350.
99. Aimbetov R, Chen CH, Bulgakova O, Abetov D, Bissenbaev AK, Bersimbaev RI, Sarbassov DD: **Integrity of mTORC2 is dependent on the rictor Gly-934 site**. *Oncogene* 2012, **31**:2115-2120.
100. Dibble CC, Asara JM, Manning BD: **Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1**. *Mol Cell Biol* 2009, **29**:5657-5670.
101. Treins C, Warne PH, Magnuson MA, Pende M, Downward J: **Rictor is a novel target of p70 S6 kinase-1**. *Oncogene* 2010, **29**:1003-1016.
102. Boulbes D, Chen CH, Shaikenov T, Agarwal NK, Peterson TR, Addona TA, Keshishian H, Carr SA, Magnuson MA, Sabatini DM, et al.: **Rictor phosphorylation on the Thr-1135 site does not require mammalian target of rapamycin complex 2**. *Mol Cancer Res* 2010, **8**:896-906.
103. Julien LA, Carriere A, Moreau J, Roux PP: **mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling**. *Mol Cell Biol* 2010, **30**:908-921.
104. Shao T, Liu X: **Identification of rictor as a novel substrate of Polo-like kinase 1**. *Cell Cycle* 2015, **14**:755-760.
105. Joo MS, Kim WD, Lee KY, Kim JH, Koo JH, Kim SG: **AMPK Facilitates Nuclear Accumulation of Nrf2 by Phosphorylating at Serine 550**. *Mol Cell Biol* 2016, **36**:1931-1942.
106. Niture SK, Khatri R, Jaiswal AK: **Regulation of Nrf2-an update**. *Free Radic Biol Med* 2014, **66**:36-44.
107. Ha S, Jeong SH, Yi K, Chung KM, Hong CJ, Kim SW, Kim EK, Yu SW: **Phosphorylation of p62 by AMP-activated protein kinase mediates autophagic cell death in adult hippocampal neural stem cells**. *J Biol Chem* 2017, **292**:13795-13808.
108. Lim J, Lachenmayer ML, Wu S, Liu W, Kundu M, Wang R, Komatsu M, Oh YJ, Zhao Y, Yue Z: **Proteotoxic stress induces phosphorylation of p62/SQSTM1 by ULK1 to regulate selective autophagic clearance of protein aggregates**. *PLoS Genet* 2015, **11**:e1004987.
109. Ichimura Y, Waguri S, Sou YS, Kageyama S, Hasegawa J, Ishimura R, Saito T, Yang Y, Kouno T, Fukutomi T, et al.: **Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy**. *Mol Cell* 2013, **51**:618-631.
110. Jiang X, Bao Y, Liu H, Kou X, Zhang Z, Sun F, Qian Z, Lin Z, Li X, Liu X, et al.: **VPS34 stimulation of p62 phosphorylation for cancer progression**. *Oncogene* 2017, **36**:6850-6862.
111. Linares JF, Amanchy R, Greis K, Diaz-Meco MT, Moscat J: **Phosphorylation of p62 by cdk1 controls the timely transit of cells through mitosis and tumor cell proliferation**. *Mol Cell Biol* 2011, **31**:105-117.
112. Kalogeropulou AF, Zhao J, Bolliger MF, Memou A, Narasimha S, Molitor TP, Wilson WH, Rideout HJ, Nichols RJ: **p62/SQSTM1 is a novel Leucine Rich Repeat Kinase 2(LRRK2) substrate that enhances neuronal toxicity**. *Biochem J* 2018.
113. Matsumoto G, Shimogori T, Hattori N, Nukina N: **TBK1 controls autophagosomal engulfment of polyubiquitinated mitochondria through p62/SQSTM1 phosphorylation**. *Hum Mol Genet* 2015, **24**:4429-4442.
114. Christian F, Krause E, Houslay MD, Baillie GS: **PKA phosphorylation of p62/SQSTM1 regulates PB1 domain interaction partner binding**. *Biochim Biophys Acta* 2014, **1843**:2765-2774.

115. Matsumoto G, Wada K, Okuno M, Kurosawa M, Nukina N: **Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins**. *Mol Cell* 2011, **44**:279-289.
116. Linares JF, Duran A, Reina-Campos M, Aza-Blanc P, Campos A, Moscat J, Diaz-Meco MT: **Amino Acid Activation of mTORC1 by a PB1-Domain-Driven Kinase Complex Cascade**. *Cell Rep* 2015, **12**:1339-1352.
117. Deprez J, Vertommen D, Alessi DR, Hue L, Rider MH: **Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades**. *J Biol Chem* 1997, **272**:17269-17275.
118. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L: **Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia**. *Curr Biol* 2000, **10**:1247-1255.
119. Ros S, Schulze A: **Balancing glycolytic flux: the role of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatases in cancer metabolism**. *Cancer Metab* 2013, **1**:8.
120. Novellasdemunt L, Bultot L, Manzano A, Ventura F, Rosa JL, Vertommen D, Rider MH, Navarro-Sabate A, Bartrons R: **PFKFB3 activation in cancer cells by the p38/MK2 pathway in response to stress stimuli**. *Biochem J* 2013, **452**:531-543.
121. Jager S, Handschin C, St-Pierre J, Spiegelman BM: **AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha**. *Proc Natl Acad Sci U S A* 2007, **104**:12017-12022.
122. Li X, Monks B, Ge Q, Birnbaum MJ: **Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator**. *Nature* 2007, **447**:1012-1016.
123. Anderson RM, Barger JL, Edwards MG, Braun KH, O'Connor CE, Prolla TA, Weindruch R: **Dynamic regulation of PGC-1alpha localization and turnover implicates mitochondrial adaptation in calorie restriction and the stress response**. *Aging Cell* 2008, **7**:101-111.
124. Fernandez-Marcos PJ, Auwerx J: **Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis**. *Am J Clin Nutr* 2011, **93**:884S-890.
125. Cuevas BD, Lu Y, Mao M, Zhang J, LaPushin R, Siminovitch K, Mills GB: **Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase**. *J Biol Chem* 2001, **276**:27455-27461.
126. Cosentino C, Di Domenico M, Porcellini A, Cuozzo C, De Gregorio G, Santillo MR, Agnese S, Di Stasio R, Feliciello A, Migliaccio A, et al.: **p85 regulatory subunit of PI3K mediates cAMP-PKA and estrogens biological effects on growth and survival**. *Oncogene* 2007, **26**:2095-2103.
127. Kavanaugh WM, Turck CW, Klippel A, Williams LT: **Tyrosine 508 of the 85-kilodalton subunit of phosphatidylinositol 3-kinase is phosphorylated by the platelet-derived growth factor receptor**. *Biochemistry* 1994, **33**:11046-11050.
128. Dhand R, Hiles I, Panayotou G, Roche S, Fry MJ, Gout I, Totty NF, Truong O, Vicendo P, Yonezawa K, et al.: **PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity**. *EMBO J* 1994, **13**:522-533.
129. Lee JY, Chiu YH, Asara J, Cantley LC: **Inhibition of PI3K binding to activators by serine phosphorylation of PI3K regulatory subunit p85alpha Src homology-2 domains**. *Proc Natl Acad Sci U S A* 2011, **108**:14157-14162.
130. Comb WC, Hutti JE, Cogswell P, Cantley LC, Baldwin AS: **p85alpha SH2 domain phosphorylation by IKK promotes feedback inhibition of PI3K and Akt in response to cellular starvation**. *Mol Cell* 2012, **45**:719-730.
131. Wang B, Nie J, Wu L, Hu Y, Wen Z, Dong L, Zou MH, Chen C, Wang DW: **AMPKalpha2 Protects Against the Development of Heart Failure by Enhancing Mitophagy via PINK1 Phosphorylation**. *Circ Res* 2018, **122**:712-729.
132. Zheng M, Wang YH, Wu XN, Wu SQ, Lu BJ, Dong MQ, Zhang H, Sun P, Lin SC, Guan KL, et al.: **Inactivation of Rheb by PRAK-mediated phosphorylation is essential for energy-depletion-induced suppression of mTORC1**. *Nat Cell Biol* 2011, **13**:263-272.
133. Pearson RB, Dennis PB, Han JW, Williamson NA, Kozma SC, Wettenhall RE, Thomas G: **The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain**. *EMBO J* 1995, **14**:5279-5287.

134. Alessi DR, Kozlowski MT, Weng QP, Morrice N, Avruch J: **3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro**. *Curr Biol* 1998, **8**:69-81.
135. Shin S, Wolgamott L, Yu Y, Blenis J, Yoon SO: **Glycogen synthase kinase (GSK)-3 promotes p70 ribosomal protein S6 kinase (p70S6K) activity and cell proliferation**. *Proc Natl Acad Sci U S A* 2011, **108**:E1204-1213.
136. Hou Z, He L, Qi RZ: **Regulation of s6 kinase 1 activation by phosphorylation at ser-411**. *J Biol Chem* 2007, **282**:6922-6928.
137. Papst PJ, Sugiyama H, Nagasawa M, Lucas JJ, Maller JL, Terada N: **Cdc2-cyclin B phosphorylates p70 S6 kinase on Ser411 at mitosis**. *J Biol Chem* 1998, **273**:15077-15084.
138. Lehman JA, Calvo V, Gomez-Cambronero J: **Mechanism of ribosomal p70S6 kinase activation by granulocyte macrophage colony-stimulating factor in neutrophils: cooperation of a MEK-related, THR421/SER424 kinase and a rapamycin-sensitive, m-TOR-related THR389 kinase**. *J Biol Chem* 2003, **278**:28130-28138.
139. Zhang J, Gao Z, Ye J: **Phosphorylation and degradation of S6K1 (p70S6K1) in response to persistent JNK1 Activation**. *Biochim Biophys Acta* 2013, **1832**:1980-1988.
140. Garcia-Martinez JM, Alessi DR: **mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1)**. *Biochem J* 2008, **416**:375-385.
141. Park J, Leong ML, Buse P, Maiyar AC, Firestone GL, Hemmings BA: **Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway**. *EMBO J* 1999, **18**:3024-3033.
142. Kobayashi T, Cohen P: **Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2**. *Biochem J* 1999, **339 ( Pt 2)**:319-328.
143. Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, Lienhard GE: **A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain**. *J Biol Chem* 2002, **277**:22115-22118.
144. Geraghty KM, Chen S, Harthill JE, Ibrahim AF, Toth R, Morrice NA, Vandermoere F, Moorhead GB, Hardie DG, MacKintosh C: **Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to IGF-1, EGF, PMA and AICAR**. *Biochem J* 2007, **407**:231-241.
145. Cartee GD: **Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle**. *Diabetologia* 2015, **58**:19-30.
146. Chen S, Murphy J, Toth R, Campbell DG, Morrice NA, Mackintosh C: **Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators**. *Biochem J* 2008, **409**:449-459.
147. Treebak JT, Taylor EB, Witczak CA, An D, Toyoda T, Koh HJ, Xie J, Feener EP, Wojtaszewski JF, Hirshman MF, et al.: **Identification of a novel phosphorylation site on TBC1D4 regulated by AMP-activated protein kinase in skeletal muscle**. *Am J Physiol Cell Physiol* 2010, **298**:C377-385.
148. Roczniak-Ferguson A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, Walther TC, Ferguson SM: **The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis**. *Sci Signal* 2012, **5**:ra42.
149. Palmieri M, Pal R, Nelvagal HR, Lotfi P, Stinnett GR, Seymour ML, Chaudhury A, Bajaj L, Bondar VV, Bremner L, et al.: **mTORC1-independent TFEB activation via Akt inhibition promotes cellular clearance in neurodegenerative storage diseases**. *Nat Commun* 2017, **8**:14338.
150. Hsu CL, Lee EX, Gordon KL, Paz EA, Shen WC, Ohnishi K, Meisenhelder J, Hunter T, La Spada AR: **MAP4K3 mediates amino acid-dependent regulation of autophagy via phosphorylation of TFEB**. *Nat Commun* 2018, **9**:942.
151. Inoki K, Zhu T, Guan KL: **TSC2 mediates cellular energy response to control cell growth and survival**. *Cell* 2003, **115**:577-590.
152. Huang J, Manning BD: **The TSC1-TSC2 complex: a molecular switchboard controlling cell growth**. *Biochem J* 2008, **412**:179-190.

153. Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, Yang Q, Bennett C, Harada Y, Stankunas K, et al.: **TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth**. *Cell* 2006, **126**:955-968.
154. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC: **Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway**. *Mol Cell* 2002, **10**:151-162.
155. Inoki K, Li Y, Zhu T, Wu J, Guan KL: **TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling**. *Nat Cell Biol* 2002, **4**:648-657.
156. Ballif BA, Roux PP, Gerber SA, MacKeigan JP, Blenis J, Gygi SP: **Quantitative phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling cassette and its targets, the tuberous sclerosis tumor suppressors**. *Proc Natl Acad Sci U S A* 2005, **102**:667-672.
157. Castel P, Ellis H, Bago R, Toska E, Razavi P, Carmona FJ, Kannan S, Verma CS, Dickler M, Chandarlapaty S, et al.: **PDK1-SGK1 Signaling Sustains AKT-Independent mTORC1 Activation and Confers Resistance to PI3Kalpha Inhibition**. *Cancer Cell* 2016, **30**:229-242.
158. Koyano F, Okatsu K, Kosako H, Tamura Y, Go E, Kimura M, Kimura Y, Tsuchiya H, Yoshihara H, Hirokawa T, et al.: **Ubiquitin is phosphorylated by PINK1 to activate parkin**. *Nature* 2014, **510**:162-166.
159. Wauer T, Swatek KN, Wagstaff JL, Gladkova C, Pruneda JN, Michel MA, Gersch M, Johnson CM, Freund SM, Komander D: **Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis**. *EMBO J* 2015, **34**:307-325.
160. Kazlauskaite A, Kondapalli C, Gourlay R, Campbell DG, Ritorto MS, Hofmann K, Alessi DR, Knebel A, Trost M, Muqit MM: **Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65**. *Biochem J* 2014, **460**:127-139.
161. Swaney DL, Rodriguez-Mias RA, Villen J: **Phosphorylation of ubiquitin at Ser65 affects its polymerization, targets, and proteome-wide turnover**. *EMBO Rep* 2015, **16**:1131-1144.
162. Kim J, Kundu M, Viollet B, Guan KL: **AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1**. *Nat Cell Biol* 2011, **13**:132-141.
163. Shang L, Chen S, Du F, Li S, Zhao L, Wang X: **Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK**. *Proc Natl Acad Sci U S A* 2011, **108**:4788-4793.
164. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, et al.: **Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy**. *Science* 2011, **331**:456-461.
165. Tian W, Li W, Chen Y, Yan Z, Huang X, Zhuang H, Zhong W, Wu W, Lin C, Chen H, et al.: **Phosphorylation of ULK1 by AMPK regulates translocation of ULK1 to mitochondria and mitophagy**. *FEBS Lett* 2015, **589**:1847-1854.
166. Bach M, Larance M, James DE, Ramm G: **The serine/threonine kinase ULK1 is a target of multiple phosphorylation events**. *Biochem J* 2011, **440**:283-291.
167. Furuya T, Kim M, Lipinski M, Li J, Kim D, Lu T, Shen Y, Rameh L, Yankner B, Tsai LH, et al.: **Negative regulation of Vps34 by Cdk mediated phosphorylation**. *Mol Cell* 2010, **38**:500-511.