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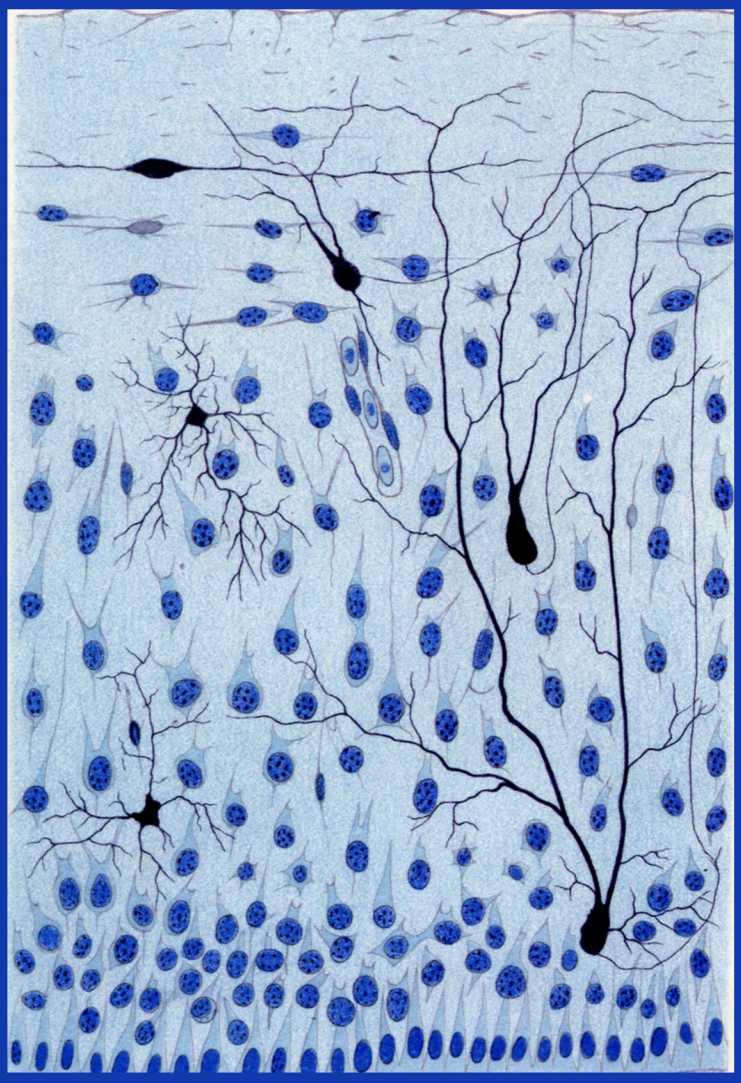

Tesis Doctoral

Jorge Montesinos Selfa

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Papel de los receptores TLR4 en los mecanismos moleculares neuroinflamatorios y patológicos de la exposición al alcohol durante la adolescencia

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CERTIFICO:

Que la memoria de Tesis Doctoral realizada por **D. Jorge Montesinos Selfa** que lleva por título "***Papel de los receptores TLR4 en los mecanismos moleculares neuroinflamatorios y patológicos de la exposición al alcohol durante la adolescencia***" ha sido llevada a cabo bajo mi dirección y la codirección de la Dra. María Pascual Mora y reúne todos los requisitos necesarios para su juicio y calificación.

Lo que suscribe en Valencia, a 23 de Octubre de 2016.

Fdo.: Dra. Consuelo Guerri Sirera

Fdo.: Dra. María Pascual Mora

AGRADECIMIENTOS

11024. Mi número de tarjeta, el vigésimo cuarto nuevo pase de acceso al CIPF del 2011. Aquel día me recibía una desconocida para mí, jamás presagié que cinco años más tarde yo lideraría el movimiento 'Por un Centro de Investigación Pilar Fortea'. La cosa empezaba bien, muy bien.

'Ya baja la Dra. Guerri a recogerte'- me dijo Pilar. '¿Una IP bajando a recoger al nuevo de prácticas?'- pensé. Tras diez minutos de entrevista, aunque Chelo no me dejase hablar mucho, yo ya estaba prendido. Quería ciencia y la quería en el I05. Por suerte, Chelo también me quería allí. Cualquiera que la conozca sabe que es enérgica, inagotable y muy curranta. Gracias por enseñarme que hay que luchar por lo que uno quiere, que la suerte no te encuentra si no estás trabajando y por apostar por mí. Sin duda, no hay mejor investigadora que tú para amadrinarme en esta aventura.

La compañía no ha sido poca estos años. Los vaivenes de la crisis político-económica han hecho que cada año una postdoc me acompañase: Sara, Anabel, Maya. No tengo palabras para agradeceros todo lo que me habéis enseñado, junto a vosotras he mejorado como científico y como persona. 'Hoy solo lisamos noventa muestras y cargamos ocho ELISAs', así arranca cualquier lunes con María. Al final, el tándem ha funcionado mejor en el lab que en la pista de pádel. Aunque nos hemos reído todo el rato, no será poca la faena que hemos sacado adelante (con la banda sonora de Fernando de fondo). El día a día se hace muy ameno al trabajar con compañer@s como vosotros. GRACIAS, tanto a las que seguís (Majo, Silvia) como los que ya han pasado (miedo me da la fiesta del enlace Morillo&Ureña). Mención especial a mis tetes Toni y Ful, da gusto coincidir con gente tan crack y llevarme dos buenos amigos. Las astrositadas no son lo mismo sin ustedes.

Si el prestigio científico de un Centro se midiese por la calidad humana de sus trabajadores, ¡¡me rio de Harvard!! Voy a echaros muchísimo de menos; almorzar y comer con vosotr@s, cruzarnos por los pasillos, coincidir revelando (menos en Agosto), grabar lipdubs, diseñar tarjetas y discutir por elegir regalo común de cumpleaños. En definitiva, gracias por haberme hecho ahorrarme el psicólogo estos años y reírnos sin parar.

GRACIAS A MI FAMILIA. A mis padres, mi hermana, mis tí@s, abuelas y abuelos, prim@s (cada vez más). Por apoyarme siempre, por enseñarme que nada se regala, pero siempre hay que luchar sonriendo. Dan igual 200 o 6000 kilómetros cuando lo que nos une es así de fuerte. Sois mi orgullo, lo mejor que me ha pasado.

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“Cuando huye la suerte,

¿sabes qué hay que hacer?

¡Sigue nadando, sigue nadando,

sigue nadando, nadando, nadando!”

Dory. *Buscando a Nemo* (2003)

ABREVIATURAS

ADN	Ácido desoxirribonucleico
AP-1	Activator protein-1
ARN	Ácido ribonucleico
cAMP	Cyclic adenosine monophosphate
CD11b	Cluster of differentiation 11b
CD14	Cluster of differentiation 14
Cdk5	Cyclin dependent kinase 5
CpG	Pares de citosina y guanina enlazados por fosfato
CSF	Colony stimulating factor
ERK	Extracellular regulated kinase
HMGB1	High mobility group box 1
IFN	Interferon
IκB	Inhibitor of κB
IKK	IkB kinase
IL	Interleukin
IP-10	IFN-γ -inducible protein 10
IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon regulatory factor 3
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MCP1	Monocyte chemoattract protein 1
MD2	Myeloid differentiation factor 2
MIP-1α	Macrophage inflammatory protein 1α
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor κB
Pág.	página
TIRAP	Toll-Interleukin 1 Receptor (TIR) Domain Containing Adaptor Protein
TNF-α	Tumor necrosis factor
TRAF6	TNF receptor associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRAM	TRIF-related adaptor molecule

ÍNDICE

1. INTRODUCCIÓN

1.1 El consumo de alcohol en la adolescencia

El consumo de alcohol en España ha registrado en las últimas décadas un importante crecimiento entre la población juvenil y adolescente, convirtiéndose en la droga más consumida por esta población y causando importantes problemas sociales, sanitarios y de salud pública (WHO, 2010). La última Encuesta Estatal sobre Uso de Drogas en Estudiantes de Enseñanzas Secundarias (ESTUDES, 2016) muestra que la prevalencia del consumo de alcohol entre los jóvenes de 14-18 años es de 76.8%, seguida por la de tabaco 31.4%, y la de cannabis 25.4%. Otros datos de interés en esta encuesta revelan que: 1) La edad media de inicio en el consumo de alcohol es de 13-14 años; 2) El 55.9% de los chicos y 59.3% de la chicas practican el consumo intensivo de alcohol, incluyendo el botellón y las borracheras; 3) Aumenta la presencia de las chicas, a las edades más tempranas (14, 15 y 16 años) y para patrones de consumo intensivo; 4) Los menores obtienen alcohol con mucha facilidad y su percepción de riesgo es escasa; 5) destaca el consumo durante los fines de semana y en forma de *binge drinking* o consumo en atracón, definido como el consumo de cinco o más unidades alcohólicas en una misma ocasión.

La alta prevalencia del consumo de alcohol por los jóvenes ocurre en un momento en el que el cerebro adolescente se encuentra en desarrollo, y dicha plasticidad, aunque favorable para adaptarse a entornos nuevos, le confiere una especial vulnerabilidad a los efectos adversos del alcohol u otras drogas (Spear y Swartzwelder, 2014).

1.2 La adolescencia: período crítico del neurodesarrollo

La adolescencia es el período crítico del desarrollo que abarca desde la pubertad hasta que se alcanza la madurez sexual y psicológica. Según la Organización Mundial de la Salud, la adolescencia comprende desde los 10 hasta los 19 años (WHO, 2010). Esta etapa se caracteriza por la aparición de notables cambios morfológicos y funcionales que, junto al incremento de niveles hormonales, interactúan con factores culturales, económicos y psicosociales dando forma a la identidad y conducta del adolescente (Spear, 2013). Desde una perspectiva evolutiva, en la adolescencia se adquieren habilidades de independencia que

favorecen el éxito tras la separación de la protección familiar. Así, aumentan los comportamientos relativos a la búsqueda de independencia, como la interacción social y la búsqueda de novedad y riesgo (Kelley *et al.*, 2004).

Durante la última década, el desarrollo de técnicas de resonancia magnética nuclear funcional ha permitido demostrar con claridad que el cerebro adolescente está en fase de desarrollo, y su maduración no culmina hasta los 21-25 años (Giedd, 2004; Toga *et al.*, 2006). En modelos animales, como ratón y rata, se estima que la equivalencia al desarrollo adolescente comprende entre los 25 y 65 días postnatales, pudiéndose hablar de una adolescencia temprana entre los 25-45 días, mientras de 45-65 días se consideran animales adolescentes tardíos o jóvenes adultos (Spear, 2015). Durante la adolescencia, ciertas áreas cerebrales sufren importantes reestructuraciones y fenómenos de plasticidad, causando cambios morfológicos, funcionales y conductuales (Giedd, 2008) que conducen a su completa maduración. La inmadurez de ciertas áreas cerebrales se asocia con la impulsividad, búsqueda de nuevas sensaciones ante cualquier estímulo, que junto con la baja percepción de los peligros, hace que la adolescencia sea una etapa que favorece conductas de alto riesgo (Casey *et al.*, 2008).

En el desarrollo cognitivo es clave la habilidad para suprimir acciones o pensamientos inapropiados en favor de las conductas dirigidas a objetivo, la flexibilidad cognitiva y la planificación (Casey *et al.*, 2000, 2002, 2005). El desarrollo de dichas conductas, conocidas como funciones ejecutivas, se inicia en la niñez alcanzando su eficiencia óptima al final de la adolescencia (Murty *et al.*, 2016). Para la consecución de una capacidad ejecutiva óptima se precisa: (i) el control inhibitorio de la impulsividad, (ii) la autorregulación, planificación y toma de decisiones y (iii) el procesamiento integrado y eficiente de la información para una respuesta coherente al entorno (Luna *et al.*, 2015). A nivel anatómico, diferentes estructuras del circuito mesolímbico (área tegmental ventral, núcleo accumbens, núcleo caudado y amígdala) participan en el proceso de recompensa y, por tanto, generan conductas impulsivas de aproximación a estímulos placenteros (comida, bebida, sexo) (Ernst *et al.*, 2005), mientras que la corteza prefrontal (CPF) es la encargada del control inhibitorio de la impulsividad y el

control ejecutivo en tareas de planificación, autorregulación/autocontrol y desarrollo cognitivo (Casey, 2015).

De este modo, la maduración de la CPF es crucial para controlar las conductas impulsivas generadas por las regiones mesolímbicas. Sin embargo, las zonas cerebrales más modernas desde el punto de vista filogenético son también las últimas en completar su desarrollo ontogenético. Así, las regiones mesolímbicas maduran antes que las corticales (Bava y Tapert, 2010). Además, la maduración cortical ocurre asincrónicamente, iniciándose en las regiones posteriores, que desarrollan funciones más básicas como las motoras y las visuales, y va avanzando hacia la zona frontal (Gogtay *et al.*, 2004). Por tanto, es este desequilibrio entre las áreas maduras, que motivan la búsqueda de recompensa, y las áreas inmaduras (CPF), que planifican e inhiben este impulso, lo que favorece que el adolescente adopte conductas exploratorias de alto riesgo hacia estímulos novedosos (Hittner y Swickert, 2006). Cabe señalar que la CPF puede dividirse en tres regiones atendiendo a su citoarquitectura: la CPF medial, lateral y orbitofrontal (Fuster y Bressler, 2015).

En el nacimiento, el cerebro dispone de muchas más neuronas y sinapsis de las que van a permanecer en la edad adulta (Oppenheim, 1991; Huttenlocher y Dabholkar, 1997). La sobreproducción de neuronas y sinapsis juega un papel importante a la hora de establecer una correcta conectividad, puesto que las neuronas y sinapsis que no se usan con frecuencia, serán eliminadas (Rakic *et al.*, 1994). Aunque la mayor pérdida neuronal ocurre en las primeras etapas postnatales **(Fig. 1)**, la eliminación de sinapsis o 'poda sináptica' es un proceso clave en la transformación del cerebro adolescente (Spear, 2013). La poda sináptica adolescente es altamente específica y puede llegar a suponer hasta una pérdida del 50% de las conexiones en ciertas regiones (Rakic *et al.*, 1994), las cuales adquieren una mayor funcionalidad, eficacia y especialización (Lenroot y Giedd, 2006).

Otro proceso determinante del neurodesarrollo adolescente es la mielinogénesis, por la cual los precursores de oligodendrocitos proliferan, maduran y recubren los axones con mielina (Butt y Berry, 2000) **(Fig. 1)**. Se sabe que la mielina actúa como sustancia aislante y permite aumentar la velocidad de la transmisión eléctrica en forma de potenciales de acción,

facilitando la especialización, funcionalidad (Paus *et al.*, 2001), y capacidad para comunicar información entre las diferentes áreas cerebrales (Salami *et al.*, 2003). Además de reducir el coste energético de la transmisión eléctrica, la mielinización modula la ritmicidad y sincronía del impulso nervioso, aumentando la complejidad de la comunicación neuronal (Fields y Stevens-Graham, 2002). El proceso de mielinización permite que áreas más alejadas dentro del cerebro tengan una comunicación rápida y efectiva, logrando establecer redes funcionales de mayor complejidad y alcance (Rubia *et al.*, 2007). De nuevo, la mielinización de los lóbulos frontales es la última en ocurrir (Lenroot y Giedd, 2006). De este modo, el cerebro adolescente sufre una pérdida de materia gris debido a la poda sináptica y un aumento de la materia blanca debido a la mielinización, ambos procesos en conjunto suponen el estrechamiento de la corteza cerebral ya que disminuye el volumen de materia gris en relación al volumen de materia blanca (Sowell *et al.*, 2010; Tau y Peterson, 2010).

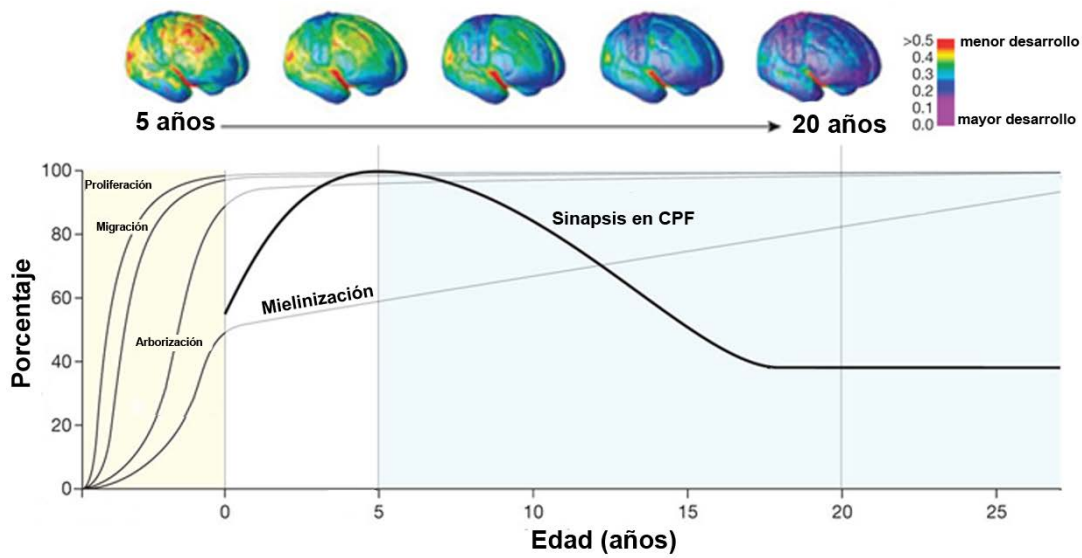

Figura 1. Neurodesarrollo durante los primeros años de vida. El cerebro sufre importantes cambios estructurales y celulares desde el nacimiento hasta la edad adulta, destacando la mielinización y la eliminación de sinapsis.

El neurodesarrollo adolescente no se basa exclusivamente en modificaciones morfológicas y celulares, sino que ocurren también cambios neuroquímicos que juegan un papel importante. Por ejemplo, en el circuito mesocorticolímbico, que comunica la corteza cerebral con el sistema límbico de recompensa, se genera un cambio en el patrón de producción y

utilización de la dopamina, un neurotransmisor clave en la comunicación neuronal (Wahlstrom *et al.*, 2010). De hecho en la CPF, la síntesis y recuperación de dopamina así como los niveles de receptores para dopamina y glutamato son mayores durante la adolescencia que en la fase adulta (Pascual *et al.*, 2009), mientras que en el sistema límbico ocurre lo opuesto (Andersen *et al.*, 1997; Teicher *et al.*, 1993). Por ello, la inmadurez de la arquitectura del cerebro, que lo dota de alta impulsividad y baja inhibición, se considera una de las principales causas que pueden conllevar al inicio del consumo de alcohol en la adolescencia (Silveri, 2012).

1.3 El cerebro adolescente es vulnerable a los efectos del alcohol

Numerosos estudios han demostrado que la inmadurez del cerebro adolescente hace que los efectos del etanol sean diferentes en el cerebro adolescente que en el adulto. De hecho, el adolescente necesita mayores cantidades de alcohol para desarrollar los síntomas negativos del consumo de alcohol, como las alteraciones de la función motora (White *et al.*, 2002), la sedación (Moy *et al.*, 1998) y la veisalgia o resaca (Acheson *et al.*, 1999). Sin embargo, el cerebro adolescente es más susceptible que el adulto a los efectos neurotóxicos del etanol (Pascual *et al.*, 2009; Guerri y Pascual, 2010).

Estudios de neuroimagen funcional en humanos ponen de manifiesto que el consumo abusivo de alcohol durante la adolescencia daña al cerebro y altera su maduración, y estos eventos se asocian con déficits cognitivos que pueden ser permanentes y mantenerse hasta la edad adulta. Entre los efectos que causa el alcohol se encuentran las alteraciones en la integridad de la materia blanca (McQueeny *et al.*, 2009) o la actividad funcional de la CPF (Crego *et al.*, 2010). Los estudios de neuroimagen demuestran además que el patrón de consumo en forma de 'atracón', botellón o *binge drinking* tan popular entre adolescentes, provoca una reducción en el tamaño del hipocampo (De Bellis *et al.*, 2000; Welch *et al.*, 2013) y de la CPF (Welch *et al.*, 2013, Medina *et al.*, 2009). Además también se ha demostrado un bajo rendimiento escolar (Hill y Mrug, 2015) y alteraciones en procesos de memoria (Squeglia *et al.*, 2012a; Tapert *et al.*, 2004), efectos que son más acusados cuanto antes comienza el

consumo/abuso del alcohol (Squeglia *et al.*, 2012a). Estudios neurofisiológicos en adolescentes han indicado que el consumo en 'atracón' o *binge drinking* disminuye la actividad perceptual, la atención y la toma de decisiones (Maurage *et al.*, 2012). Este tipo de consumo de forma intermitente, y caracterizado por alternar altas dosis de alcohol con períodos de abstinencia, evita la neuroadaptación del circuito corticolímbico que conecta la CPF con el núcleo accumbens (NAc) y la amígdala (George *et al.*, 2012). De este modo, los animales que muestran un patrón *binge drinking* presentan una desconexión funcional entre estas áreas cerebrales, favoreciendo la sensibilización de la CPF, y facilitando la transición a la dependencia alcohólica. De hecho, el consumo de alcohol siguiendo un patrón de *binge drinking* durante la adolescencia aumenta el riesgo de desarrollar dependencia alcohólica en la edad adulta (Grant, 1998).

Diversos estudios en modelos animales han mostrado evidencias de la vulnerabilidad del cerebro adolescente a los efectos neurotóxicos del alcohol (Pascual *et al.*, 2007; Carpenter-Hyland y Chandler, 2007). Por ejemplo, en un modelo experimental en ratas adolescentes con un tratamiento intermitente de alcohol, que simula el consumo intensivo o *binge drinking* durante los fines de semana, se observó que se producían cambios en los sistemas dopaminérgicos y glutamatérgicos en la CPF y en el NAc de animales adolescentes, mientras que el mismo tratamiento no ocasionaba cambios en los animales adultos (Pascual *et al.*, 2009). Además, el consumo en atracón durante la adolescencia en las ratas aumentaba la preferencia a consumir alcohol durante la fase adulta (O'Tousa *et al.*, 2013; Pascual *et al.*, 2009). Estos estudios coinciden con los trabajos de Grant (1998), que demostraban que la edad de inicio del consumo de alcohol se correlacionaba con la prevalencia al abuso y dependencia al alcohol en la etapa adulta. Cuanto antes se comienza a beber, más probabilidad tiene el individuo de desarrollar problemas relacionados con el alcohol o con otras drogas. En nuestro país, la edad media de inicio del consumo se ha estimado que es de 14 años, edad en la que se tendría una probabilidad 4-5 veces mayor de tener problemas de abuso de alcohol y alcoholismo que si se inicia a los 21 años. Así mismo, los antecedentes familiares de alcoholismo potencian dicho efecto (Grant, 1998).

Los estudios en modelos animales, principalmente ratas y ratones, también han demostrado que el consumo intensivo de alcohol durante la adolescencia tiene efectos a largo plazo, causando:

- Cambios en los niveles de neurotransmisores y receptores importantes en el neurocircuito de recompensa **(Fig. 2)**; como el glutamatérgico, dopaminérgico (Pascual *et al.*, 2009; Trantham-Davidson *et al.*, 2016), serotoninérgico (Vetreno *et al.*, 2016a), colinérgico (Coleman *et al.*, 2014) y gabaérgico (Centanni *et al.*, 2014).
- Alteraciones morfológicas (Coleman *et al.*, 2014), como anomalías en la estructura cortical (Vetreno *et al.*, 2016b) e hipocampal (Risher *et al.*, 2015a) y pérdida de la neurogénesis hipocampal (Vetreno y Crews, 2015).
- Cambios epigenéticos en genes relacionados con plasticidad neuronal (Kyzar *et al.*, 2016, Pascual *et al.*, 2012) y alteración en el número de dendritas sinápticas en neuronas piramidales de la CPF (Trantham-Davidson *et al.*, 2016) y neuronas hipocampales (Risher *et al.*, 2015b).
- Daños en las fibras de mielina (Pascual *et al.*, 2014; Vargas *et al.*, 2014).
- Deterioros electrofisiológicos (Ehlers y Criado, 2010; Avegno *et al.*, 2016).
- Aumento de la preferencia por consumir alcohol (Pascual *et al.*, 2009) y déficits conductuales en tareas tanto de memoria, aprendizaje y atención (Pascual *et al.*, 2012; Pascual *et al.*, 2009) como de flexibilidad cognitiva (Coleman *et al.*, 2014; Gass *et al.*, 2014).
- Aumento de mediadores inflamatorios en el cerebro (Pascual *et al.*, 2014; Pascual *et al.*, 2007).

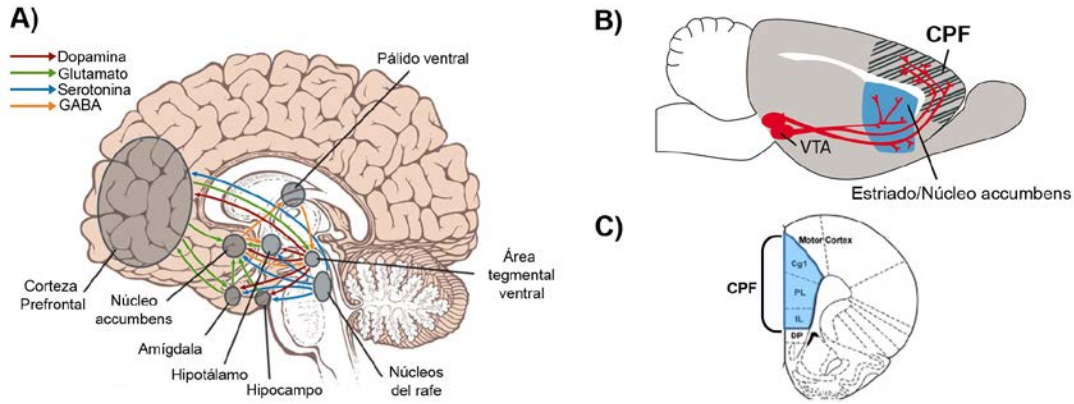

Figura 2. La corteza prefrontal es un componente clave del neurocircuito de recompensa. (A) Conexiones corticolímbicas participantes en el circuito de recompensa y su distinta naturaleza bioquímica. **(B)** Corte sagital que muestra la localización del área tegmental ventral (VTA) y la CPF en ratones. **(C)** Corte frontal (Bregma: 3.0 mm) que muestra la CPF murina, análoga de la CPF medial humana, y sus distintas subregiones: el giro del cíngulo anterior (Cg1), el área prelímbica (PrIL) y el área infralímbica (IL).

Estudios clínicos y preclínicos muestran que los adolescentes son más sensibles que los adultos a los efectos reforzantes positivos causados por la exposición aguda al alcohol, pero menos sensibles a los efectos negativos (Spear y Swartzwelder, 2014). Por ejemplo, los efectos sedantes, motores y anxiolíticos causados por la exposición al alcohol son menos acusados en animales adolescentes que adultos (Sakharkar *et al.*, 2012; Sakharkar *et al.*, 2014) y también muestran ser menos susceptibles a los síntomas de la abstinencia al alcohol (Chung *et al.*, 2008). Sin embargo, los ratones adolescentes muestran mayor sensibilidad a los efectos reforzantes positivos del consumo de alcohol (Spear y Varlinskaya, 2005; Spear, 2014), incluso llegando a autoadministrarse niveles de alcohol causantes de taquicardia (Ristuccia y Spear, 2008). Esto puede explicarse por la mayor capacidad metabólica que presentan los adolescentes (Morris *et al.*, 2010), aunque también se ha descrito una menor tasa de eliminación, siendo de 4.5 mg/dl/h para ratas neonatales y de 42 mg/dl/h para ratas adultas (Kelly *et al.*, 1987).

Finalmente, también cabe señalar que la vulnerabilidad a los efectos del alcohol presenta diferencias de género. Por ejemplo, las mujeres que inician el consumo de alcohol durante la

adolescencia presentan mayor riesgo de daño cerebral y problemas conductuales que los hombres (Squeglia *et al.*, 2009). Así mismo, el consumo de alcohol durante la adolescencia se asocia a un mayor daño frontocortical y peor capacidad visuo-espacial en mujeres que en hombres (Squeglia *et al.*, 2012b). También se han encontrado mayores déficits en las funciones ejecutivas en chicas adolescentes con historial de consumo de drogas (Giancola y Parker, 2001; Moss *et al.*, 1994).

1.4 Sistema inmunitario en el sistema nervioso central: microglía y astrocitos

El sistema inmunitario está integrado por distintas líneas de defensa principales, como son las barreras físico-mecánicas, químicas y biológicas que protegen al organismo intentando frenar la entrada y colonización de patógenos. La inmunidad innata (natural o inespecífica) nos permite hacer frente a la mayor parte de los agentes patógenos que llegan al organismo. Sin embargo, la inmunidad adquirida (adaptativa o específica) proporciona al organismo una respuesta específica frente a cada agente infeccioso y presenta memoria inmunológica específica. El sistema inmunitario innato es el sistema dominante de protección en la mayoría de los organismos (Litman *et al.*, 2005), siendo la inflamación uno de las primeras respuestas ante una infección (Kawai y Akira, 2006).

En el sistema nervioso central (SNC), la inflamación se asocia con la activación del sistema inmune innato, y se manifiesta en cerebro por una estimulación de las células gliales residentes y por la presencia de infiltrados (Griffiths *et al.*, 2007). Entre las células gliales, las principales células con función inmune son la microglía y la astroglía, jugando un papel clave en la respuesta inflamatoria.

La microglía, o macrófagos parenquimales del cerebro, se estimulan rápidamente en respuesta a cualquier tipo de lesión o infección, y adquieren funciones como la fagocitosis, la producción y secreción de mediadores inflamatorios o la presentación antigénica (Hanisch, 2002). En este estado activado, la microglía expresa proteínas como el complejo mayor de histocompatibilidad MHCII (Perlmutter *et al.*, 1992) o la β-integrina CD11b (Roy *et al.*, 2006).

Por otro lado, los astrocitos además de contribuir al establecimiento y mantenimiento de la barrera hematoencefálica (Prat *et al.*, 2001) y permitir la extravasación de infiltrados como monocitos y linfocitos (Weiss *et al.*, 1998), son importantes reguladores de la inflamación cerebral (Dietrich *et al.*, 2003). De hecho, en la mayoría de casos, el daño cerebral se presenta junto a una hipertrofia astrocítica, conocida como astrogliosis reactiva (Ridet *et al.*, 1997). La activación de los astrocitos juega un papel dual, conteniendo la inflamación de manera local permitiendo aislar el daño o infección, o liberando factores inhibitorios de crecimiento (Crews *et al.*, 2004; Sofroniew, 2009), aunque este último papel está actualmente en debate (Anderson *et al.*, 2016).

La sobre-activación del sistema neuroinmunitario se ha asociado a enfermedades neurodegenerativas (Glass *et al.*, 2010), a desórdenes neuropsiquiátricos (Hodes *et al.*, 2015; Rosenblat y McIntyre, 2016) y a la drogadicción (Jacobsen *et al.*, 2016).

1.5 Los TLR son receptores del sistema inmune innato

Tanto los astrocitos como la microglía expresan receptores característicos del sistema inmune innato que actúan como sensores de patógenos y de daño tisular. Estos receptores, llamados receptores de reconocimiento de patrones (PRR, *pattern recognition receptors*), inician la respuesta del sistema inmune innato al ser capaces de detectar la presencia de patrones moleculares asociados a patógenos (PAMP, *pathogen-associated molecular patterns*), característicos de un amplio abanico de microorganismos (Akira *et al.*, 2001). Además, también son capaces de reconocer patrones moleculares asociados a daño (DAMP, *damage-associated molecular patterns*), que forman parte del propio organismo, aunque se encuentran en una conformación aberrante o en una localización no habitual, generalmente como parte de una situación patológica (Bianchi, 2007).

Entre los PRR, cabe destacar los receptores *toll-like* (TLR, *toll-like receptor*), que fueron los primeros en ser identificados en *Drosophila melanogaster*, como una defensa contra infecciones microbianas (Medzhitov *et al.*, 1997). Aunque existe una alta conservación evolutiva entre humano y ratón, existen 10 miembros de receptores TLR en humanos (TLR1 a

TLR10), y 12 en ratones, pues disponen de TLR11, TLR12 y TLR13, pero el TLR10 murino es un pseudogen (Kumar *et al.*, 2011). Los TLR son proteína transmembrana de clase I que presentan un dominio citoplasmático conservado denominado TIR (Toll/IL-1R), crucial para la señalización, y un dominio extracelular con repeticiones ricas en leucina formando una estructura cóncava de gran importancia en el reconocimiento de los PAMP/DAMP (Kawai y Akira, 2010). TLR1, TLR2, TLR4, TLR5 y TLR6 se localizan en la membrana plasmática, mientras que TLR3, TLR7, TLR8 y TLR9 se localizan intracelularmente en el sistema endosómico-lisosomal (Nishiya y DeFranco, 2004). Sin embargo, la localización subcelular de algunos TLR depende del tipo celular (Matsumoto *et al.*, 2002).

El TLR2 forma heterodímeros con el TLR1 o el TLR6, lo que les permite reconocer una amplia variedad de componentes microbianos como el ácido lipoteicóico de las bacterias Gram positivas o el zimosán característico de los hongos. TLR5 detecta la flagelina, componente del flagelo bacteriano, mientras que el TLR3, el TLR7 y el TLR8 participan en el reconocimiento de virus a través del ARN producto de la replicación viral. TLR9 reconoce motivos CpG sin metilar, característicos del ADN bacteriano (Takeuchi y Akira, 2010).

1.6 El receptor TLR4: localización y señalización

Aunque la astroglía y la microglía expresan la mayoría de los TLR (Okun *et al.*, 2011), hay que destacar en particular la expresión del TLR4, cuyo ligando específico es el lipopolisacárido (LPS), que es el componente mayoritario de la membrana externa de las bacterias Gram negativas (Kawai y Akira, 2010), aunque también reconoce otros ligandos endógenos como el fibrinógeno, el ácido hialurónico, proteínas de choque térmico (HSP60/70) o la proteína HMGB1 (Rifkin *et al.*, 2005). Las neuronas también expresan TLR4 y responden al LPS, aunque las vías de señalización difieren en cierta medida de las gliales (Leow-Dyke *et al.*, 2012).

El reconocimiento del LPS se inicia cuando la proteína sérica LBP (*LPS binding protein*) se une al LPS y lo transfiere al co-receptor CD14, que a su vez lo transfiere a la proteína adaptadora MD-2. CD14 y MD-2 no contienen dominios citoplasmáticos, por lo que carecen de actividad de transducción de señales por sí solas. De este modo, se forma un complejo

LPS/MD-2/TLR4, que al dimerizar con otro complejo LPS/MD-2/TLR4, provoca un cambio conformacional en el dominio citoplasmático del TLR4, que permite la unión del dominio TIR con otras proteínas como TIRAP o TRAM (Kawai y Akira, 2010) **(Fig. 3)**.

Por un lado, la interacción con TIRAP permite la activación de la vía dependiente de MyD88. A nivel de membrana, la proteína MyD88 recluta y activa a IRAK-4 que hiperfosforila a IRAK-1, formando un complejo con TRAF6. El complejo IRAK-4/IRAK-1/TRAF6 transfiere TRAF6 al complejo TAB1/TAB2/TAK1, liberándolo al citosol. Tras su liberación, TAK1 provoca la activación por fosforilación de las MAPKs (p38, ERK, JNK), que conducen a la activación del factor de transcripción AP-1 (formado por c-Fos y c-Jun). TAK1 también activa a las IKKs, que fosforilan a las IkBs, provocando su degradación y la liberación de NF-κB. La subunidad p65 de NF-κB se activa por fosforilación y transloca al núcleo donde actúa como regulador transcripcional de genes pro-inflamatorios como las citoquinas TNF-α, IL-1β o IL17A, las quimioquinas MCP-1 o MIP-1α o las enzimas pro-inflamatorias como las oxidasas, la óxido nítrico sintasa inducible (iNOS) o la ciclooxigenasa-2 (COX-2), que catalizan la síntesis de superóxido, óxido nítrico y prostaglandinas, respectivamente (Akira y Sato, 2003; Morgan y Liu, 2011).

Por otro lado, la vía dependiente de TRIF está mediada por TRAM y TRIF que interactúan con TBK1, la cual induce la fosforilación y activación de IRF3, conduciendo a su dimerización y translocación al núcleo, regulando la transcripción de genes como IFN-β o IP-10. Además, TRIF también interacciona con TRAF6, lo que provoca una activación de NF-κB, similar a la que induce la ruta de MyD88, aunque más tardía (Yamamoto *et al.*, 2003).

Cabe señalar que la localización subcelular del TLR4 también juega un papel crucial en la regulación de su activación. De hecho, la dimerización del TLR4 ocurre en microdominios de membrana ricos en colesterol y esfingolípidos llamados *lipid rafts* o balsas lipídicas (Triantafilou *et al.*, 2002). Así, algunas moléculas que disgregan dichos microdominios, como la saponina o la filipina, son capaces de inhibir la respuesta del TLR4, a través de las rutas dependientes de MyD88 y TRIF (Kagan *et al.*, 2008). Tras su activación, el TLR4 es endocitado a endosomas y se favorece la interacción con la proteína TRAM, por lo que inhibidores de la

endocitosis como clorpromacina, bloquean la vía dependiente de TRIF, pero no la vía dependiente de MyD88 (Pascual-Lucas *et al.*, 2014; McGettrick y O'Neill, 2010).

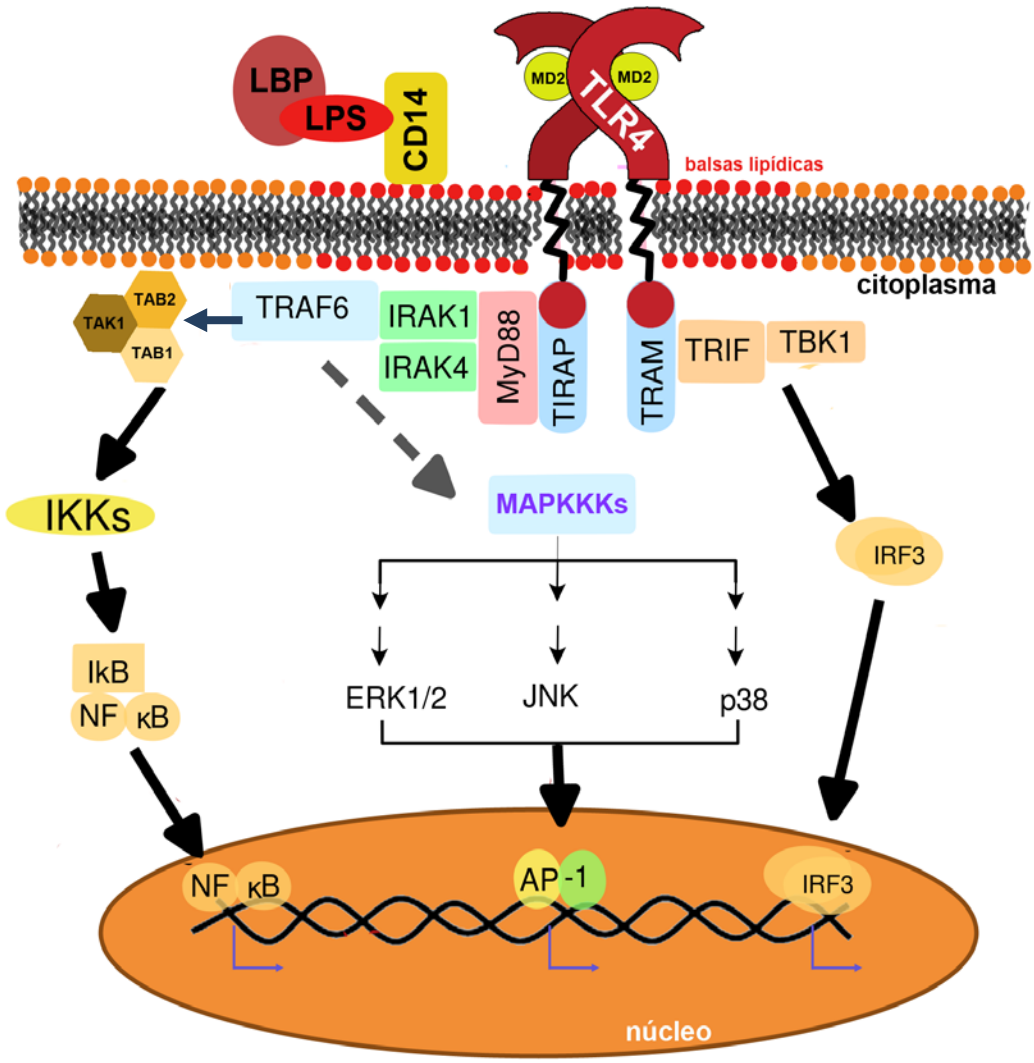

Figura 3. **Vía de señalización del receptor TLR4.** En la activación del TLR4 participan distintos co-receptores y proteínas adaptadoras, culminando en la transcripción de genes asociados a factores de transcripción como NF-κB, AP-1 o IRF-3.

1.7 El alcohol causa neuroinflamación a través de los receptores TLR4

Los primeros estudios que demostraban que el consumo de alcohol producía neuroinflamación y daño neural se publicaron en 2004 (Vallés *et al.*, 2004; Blanco *et al.*, 2004). Posteriormente, se confirmó que el alcohol potencia la respuesta inmune inducida por el LPS, al activar la actividad transcripcional de NF-κB, y la consecuente expresión de genes pro-inflamatorios en cultivos organotípicos de rata (Qin *et al.*, 2008).

Sin embargo, el efecto directo del etanol sobre el receptor TLR4 se demostró utilizando cultivos primarios de células microgliales y astrogliales. Estos trabajos demostraban que el etanol, en ausencia de endotoxinas, provoca la translocación del receptor TLR4 a las balsas lipídicas, activando su señalización (MAPK, NF-κB, AP-1, IRF3) y la posterior liberación de mediadores inflamatorios y citoquinas (Blanco *et al.*, 2008; Fernandez-Lizarbe *et al.*, 2009). Además, de manera similar a como lo hace el LPS, el etanol provoca la endocitosis del TLR4 en astrocitos (Pascual-Lucas *et al.*, 2014). De hecho, al bloquear la acción del TLR4 en astrocitos, mediante la tecnología del ARN de interferencia o usando cultivo primario de astrocitos o microglía de ratones deficientes en TLR4 (TLR4-KO) (Alfonso-Loeches *et al.*, 2010; Fernandez-Lizarbe *et al.*, 2009), se observa que el etanol no causa respuesta inflamatoria.

El papel crucial del TLR4 en los efectos inflamatorios causados por el consumo de alcohol se demostró *in vivo* claramente al usar ratones TLR4-KO con un tratamiento crónico de alcohol (10% v/v durante 5 meses), observando que la ausencia de TLR4 evitaba el aumento de mediadores inflamatorios en la corteza cerebral, así como la aparición de gliosis, daño mielínico y degeneración neural (Alfonso-Loeches *et al.*, 2012; Alfonso-Loeches *et al.*, 2010). Además, estudios tanto en cerebros post-mortem de individuos alcohólicos, como en modelos animales demuestran que el abuso de alcohol aumenta la expresión de genes del sistema neuroinmune, como el TLR4, TLR2 y HMGB1 (Crews y Vetreno, 2016).

1.8 Mielina: función y composición molecular

En el SNC, los oligodendrocitos generan una estructura multilaminar membranosa, llamada membrana mielínica o mielina, que envuelve a los axones neuronales para posibilitar la conducción saltatoria del impulso nervioso. La mielina, a diferencia de otros tipos de membranas biológicas, presenta un alto porcentaje de lípidos, en torno al 70% de su peso seco, siendo principalmente colesterol, galactosilceramida y esfingomielina (Baumann y Pham-Dinh, 2001). La estructura multilaminar de la mielina surge por el enrollamiento de la membrana del oligodendrocito, por lo que las partes externas de la membrana quedan yuxtapuestas creando la línea intraperiodo, mientras que el citoplasma constituye la línea densa mayor **(Fig. 4)**. Los segmentos mielinizados de los axones se conocen como internodos, mientras que hay segmentos no mielinizados llamados nódulos de Ranvier, donde se concentran los canales de sodio que generan los potenciales de acción que conducen el impulso saltatorio. Los internodos presentan una alta compactación de la mielina, sin embargo, en sus extremos, los paranodos, la mielina no se encuentra tan compactada y presenta una composición molecular ligeramente distinta (Baumann y Pham-Dinh, 2001).

En la mielina se encuentra un conjunto de proteínas exclusivas, resaltando: PLP, MBP, MOG, MAG, CNPasa y NG-2 **(Fig. 4)**. La **PLP** (*proteolipid protein*) es una proteína integral de membrana que posee la propiedad física de ser soluble en solventes orgánicos. Presenta un tamaño molecular aparente de 26 kDa. Su secuencia aminoacídica, fuertemente conservada en la evolución, contiene múltiples sitios de unión a membrana (Bizzozero y Good, 1991). También sufre modificaciones post-traduccionales de unión a ácidos grasos como palmitato, oleato y estearato (Weimbs y Stoffel, 1992). Además de otras funciones celulares como el transporte de colesterol, PLP actúa como estabilizador de la línea intraperiodo, al anclar las membranas externas yuxtapuestas de la mielina (Werner *et al.*, 2013).

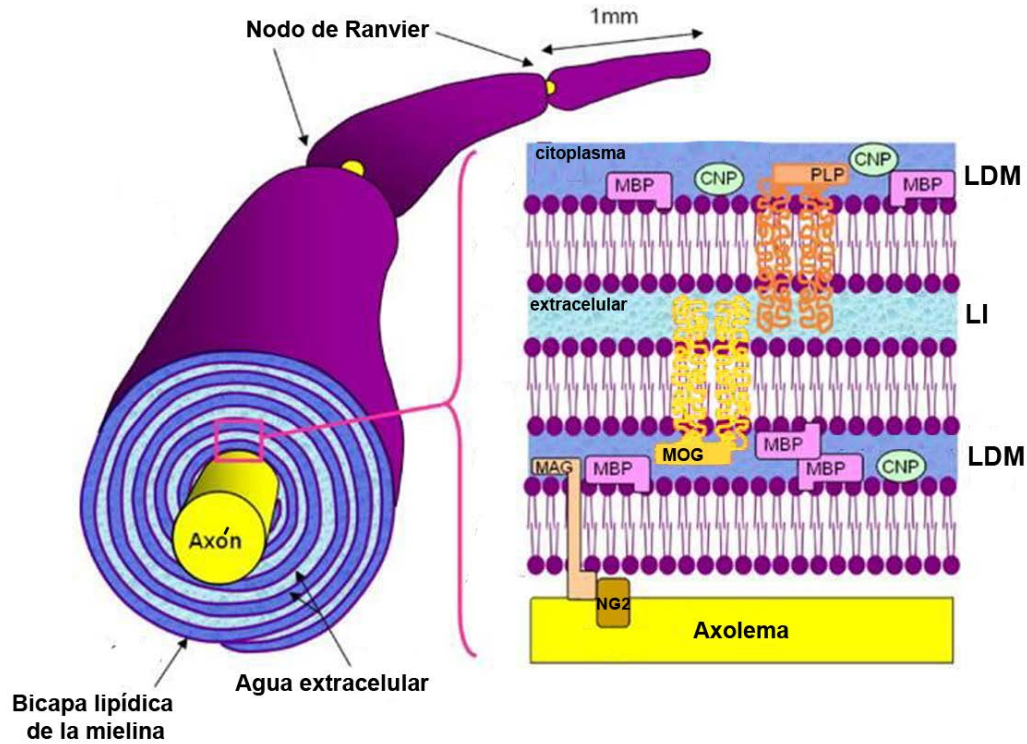

Figura 4. Estructura molecular de la mielina. Las proteínas mielínicas se organizan en la cara extracelular o la línea intraperiodo (LI) o en la cara citoplasmática o línea densa mayor (LDM), de la vaina de mielina que envuelve el axolema o axón.

La única proteína conocida como indispensable para la formación de la mielina es **MBP** (*myelin basic protein*) (Chernoff, 1981). Se encuentra en la cara citoplasmática de las membranas de mielina, es decir, en la línea densa mayor. Su rápida fosforilación y su carga positiva le permiten interactuar con fosfolípidos aniónicos como la fosfatidilserina y el fosfatidilinositol, con lo que juega un papel dinámico en la regulación estructural de la mielina (Boggs, 2006). Existen varias isoformas de esta proteína generadas por *splicing* alternativo que varían según la especie: cuatro en humano, y seis en el ratón siendo mayoritarias las isoformas de 21.5, 18.5 y 14 kDa (Boggs, 2006; Harauz y Boggs, 2013).

La enzima **CNPasa** (*2',3'-cyclic nucleotide-3'-phosphodiesterase*) se expresa mayoritariamente en oligodendrocitos y juega un papel importante en la mielinización(Gravel *et al.*, 1996), protegiendo a los axones del daño al transformar localmente la toxina mitocondrial 2',3'-cAMP en el neuroprotector adenosina (Verrier *et al.*, 2013).

MAG (*myelin-associated glycoprotein*) es una proteína con un único dominio transmembrana que se encuentra exclusivamente en las membranas oligodendrogliales periaxonales del SNC, donde desempeña funciones de interacción célula a célula como la adhesión mielina-axón, la señalización entre la neurona y el oligodendrocito (Quarles, 1997) y la inhibición del crecimiento axonal tras una lesión (McKerracher y Rosen, 2015). La glicoproteína transmembrana **MOG** (*myelin-oligodendrocyte glycoprotein*) contiene un dominio tipo inmunoglobulina y se localiza en la superficie de la lámina de mielina, permitiéndole actuar como transmisor de la información extracelular (Gardinier *et al.*, 1992).

Por último, **NG2** (*neuron-glia antigen 2*) es un proteoglicano condroitín sulfato, que se expresa en la superficie de las células precursoras de los oligodendrocitos, los cuales se distribuyen ampliamente en la materia gris y blanca del SNC (Somkuwar *et al.*, 2014). Estas células tienen capacidad auto-renovadora y pueden madurar en oligodendrocitos premielinizantes y mielinizantes. NG2, al interactuar con componentes de la matriz extracelular, participa en diferentes funciones como la migración, la regulación del citoesqueleto y la regulación génica (Sakry y Trotter, 2016).

1.9 La sinapsis como unidad funcional de la comunicación neuronal

Con el fin de transmitir el impulso nervioso, el potencial de acción recorre la longitud del axón hasta donde se comunica con la siguiente neurona. La conexión entre dos neuronas se conoce como sinapsis. Las sinapsis que recibe una neurona se localizan en su mayor parte a nivel de las dendritas, son las llamadas sinapsis axo-dendríticas. En menor medida también se producen sinapsis axo-somáticas y axo-axónicas, que ocurren a nivel del soma o del axón, respectivamente. Independientemente de donde se localicen, desde el punto de vista funcional existen dos mecanismos de transmisión sináptica: la transmisión eléctrica y la transmisión química.

En la sinapsis eléctrica, las membranas de las neuronas pre-sinápticas y post-sinápticas están unidas por una unión de tipo *gap*, o unión comunicante. Esta unión deja en su centro un canal de comunicación a través del cual fluye la corriente iónica de una neurona a otra de

forma directa. Sin embargo, en la sinapsis química no existe continuidad en las membranas. Generalmente, las sinapsis químicas están formadas por el elemento pre-sináptico en el terminal del axón, donde se liberará el neurotransmisor al espacio intersináptico hasta el elemento post-sináptico en la dendrita donde se encuentran los receptores de los neurotransmisores **(Fig. 5)**.

Los neurotransmisores se acumulan en vesículas sinápticas, que son estructuras de doble membrana lipídica, con un diámetro de 40 a 100 nm, localizadas en las zonas activas pre-sinápticas (Di Carlo, 1967). Estas vesículas median la rápida exocitosis de los neurotransmisores en el rango de milisegundos tras el influjo de calcio (< 0.5 ms), para ello, existen numerosas vesículas próximas a zonas de la membrana plasmática con grupos de canales de calcio dependientes de voltaje que responden a la depolarización de la membrana causada por el potencial de acción (Beaumont *et al.*, 2005).

La exocitosis de los neurotransmisores en la hendidura sináptica depende de la unión de las vesículas sinápticas a la membrana presináptica **(Fig. 5)**. Este proceso es llevado a cabo por un conjunto de proteínas llamadas SNARE (*soluble N-ethylmaleimide-sensitive factor attachment protein receptor*). Las proteínas SNARE que están unidas a las vesículas se denominan v-SNARE mientras que las asociadas a la membrana presináptica se llaman t-SNARE. La interacción de estos dos grupos de proteínas forma una estructura de cuatro hélices que favorece que ambas membranas se aproximen íntimamente iniciando la fusión (Jahn y Scheller, 2006). La fusión de membranas es dependiente de las proteínas SNARE en todos los tipos celulares, aunque en las neuronas, la maquinaria protéica es sensible a los niveles de calcio con el fin de ser modulable por la entrada de calcio generada por el potencial de acción (Han y Jackson, 2006).

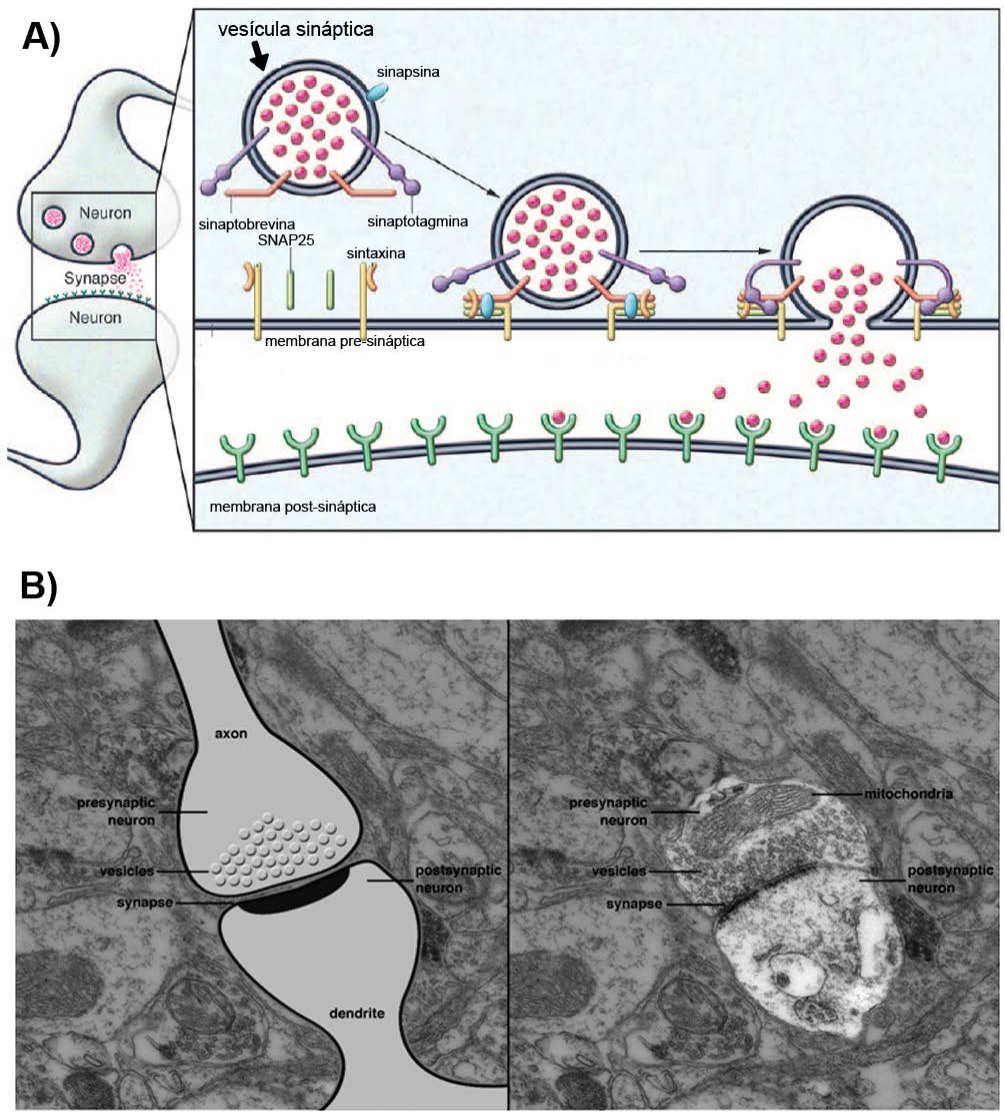

Figura 5. **Maquinaria molecular responsable de la neurotransmisión de una sinapsis química. (A)** Las proteínas SNARE orquestan la exocitosis de los neurotransmisores. **(B)** Imagen de microscopía electrónica que esquematiza los distintos componentes estructurales de una sinapsis química.

Aunque el conjunto de proteínas SNARE es complejo y variado (Ramakrishnan *et al.*, 2012) cabe destacar entre las v-SNARE, las **sinapsinas**, que regulan la transmisión sináptica al controlar las reservas de vesículas sinápticas. Se encargan de anclar las vesículas al citoesqueleto de actina, para que las vesículas se sitúen cerca de la zona activa y estén listas para la exocitosis. Destaca la sinapsina IIa como moduladora de la reserva de vesículas glutamatérgicas (Gitler *et al.*, 2008). Por otro lado, las **sinaptotagminas** actúan como los

sensores de calcio en neuronas que facilitan la nucleación del complejo (van den Bogaart *et al.*, 2011) y la fusión vesicular (Kuo *et al.*, 2011); y las **sinaptobrevinas**, que facilitan la formación del poro de fusión perturbando la membrana vesicular mediante su dominio C-terminal transmembrana (Ngatchou *et al.*, 2010)

Del mismo modo, entre las proteínas t-SNARE, cabe destacar a las proteínas citoplasmáticas **SNAP** (*synaptosomal-associated proteins*), que se asocian a la membrana pre-sináptica a través de la palmitoilación de ciertos residuos de cisteína, pues carecen de dominio transmembrana(Gonzalo *et al.*, 1999). Dentro de esta familia, destaca SNAP-25 por interactuar con ciertas sinaptotagminas y canales de calcio en la regulación de la exocitosis neuronal (Zhang *et al.*, 2002). También las **sintaxinas** interactúan con sinaptotagminas o con los canales de calcio participando en distintas etapas de la fusión de membranas y la exocitosis (Kee *et al.*, 1995, Wu *et al.*, 1999).

1.10 Papel de los mecanismos epigenéticos como reguladores de la actividad neuronal

La epigenética se define como un patrón de cambios estables en la organización y función de un cromosoma, que provoca un fenotipo específico, sin modificar la secuencia de ADN (Berger *et al.*, 2009). Estos fenotipos incluyen cambios químicos, como la metilación o la acetilación de las colas terminales de las histonas sobre las cuales se empaqueta y organiza el ADN, el cual también puede encontrarse metilado (Kouzarides, 2007). Estas modificaciones epigenéticas modulan la transcripción génica, actuando generalmente la metilación del DNA como represor de la transcripción, pues dificulta el acceso de la maquinaria molecular de transcripción (Boyes y Bird, 1991), mientras que la acetilación de las histonas lo favorece (Gräff y Tsai, 2013). La metilación de las histonas puede tener efecto activador o represor en función de la secuencia concreta de ADN y del residuo de la histona que es modificada (Kouzarides, 2007).

Estos cambios epigenéticos son llevados a cabo por distintas enzimas que catalizan las diferentes reacciones químicas. Por ejemplo, el nivel de acetilación de un determinado

residuo es controlado por el equilibrio entre las enzimas histona acetiltransferasa (HAT), que une grupos acetil al residuo de la histona, y la actividad histona deacetilasa (HDAC), que elimina dichos grupos (Peserico y Simone, 2011). Las marcas epigenéticas y los niveles de expresión y activación de las enzimas responsables fluctúan durante el desarrollo y en respuesta a ciertos estímulos, por lo que se postula que perturbaciones de este sistema en periodos críticos del desarrollo como la adolescencia pueden causar una multitud de alteraciones que lleguen a perdurar en el tiempo (Kyzar *et al.*, 2016).

Numerosos estudios ponen de manifiesto que el alcohol causa modificaciones epigenéticas que conducen a cambios en la expresión génica, en la plasticidad sináptica, en la morfología de las espinas dendríticas y en el comportamiento (Kyzar y Pandey, 2015). Concretamente, el tratamiento agudo con alcohol provoca la inhibición de las enzimas HDAC, aumentando la acetilación de histonas en genes reguladores de la plasticidad sináptica como *bdnf* (*brain-derived neurotrophic factor*) o *arc (activity-regulated cytoskeletal-associated protein)*, incrementando su expresión génica y, por tanto, la densidad de espinas sinápticas (Pandey *et al.*, 2008). Sin embargo, los efectos opuestos se observan durante la abstinencia (You *et al.*, 2014). En los promotores de los genes *bdnf* y *arc* se localizan unos elementos de respuesta CRE (*cyclic AMP response elements*), a los que se une el factor transcripcional CREB (*CRE binding protein*) cuando se encuentra fosforilado o activado (Nestler, 2005). Además, el tratamiento intermitente con alcohol durante la adolescencia se ha asociado a cambios epigenéticos en genes como *cfos*, *fosb* o *cdk5* (Pascual *et al.*, 2012), que actúan como reguladores transcripcionales participando en la regulación de la plasticidad neuronal y la conducta (Nestler *et al.*, 1999).

La convergencia de todas estas rutas de control de la plasticidad sináptica da lugar a la modificación de la estructura de las sinapsis. La plasticidad sináptica puede explicarse como la variación del número de espinas dendríticas, la modificación de la estructura dendrítica existente o la alteración de los componentes moleculares presentes en ella; todos estos mecanismos conducen a alterar la comunicación neuronal, y en última instancia la conducta, pudiendo ser factores determinantes que favorezcan la adicción (Nestler, 2001). Por ejemplo, la sensibilización de las sinapsis glutamatérgicas por remodelamiento molecular puede

ocurrir por la inserción de receptores de glutamato tipo AMPA (ácido α-amino-3-hidroxi-5-metilo-4-isoxazolpropiónico) en la membrana post-sináptica (Keifer y Zheng, 2010). Estos receptores reaccionan inmediatamente a la unión del glutamato y generan una transmisión excitatoria más rápida que los receptores de glutamato tipo NMDA (N-metil-D-aspartato), por tanto el ratio de receptores tipo AMPA (subunidad GluR1) frente a NMDA (subunidad NR1) presente en la membrana post-sináptica puede informar de la plasticidad neuronal, y se ha asociado a procesos de aprendizaje y memoria (Ménard *et al.*, 2015; Self *et al.*, 2004) y de conductas adictivas (van Huijstee y Mansvelder, 2014).

Además, la regulación puede ocurrir en sentido inverso, ya que los cambios en los sistemas dopaminérgicos y glutamatérgicos pueden inducir modificaciones epigenéticas asociadas con la sensibilización conductual relacionada con el uso de drogas de abuso (Li *et al.*, 2004; Schroeder *et al.*, 2008).

2. OBJETIVOS

Dada la vulnerabilidad del cerebro adolescente y la capacidad del etanol de causar una respuesta inflamatoria a través del receptor TLR4, la **hipótesis** que se examina en este trabajo es que "*la exposición intermitente a elevados niveles de alcohol durante la adolescencia activa la respuesta del sistema inmune innato y los receptores TLR4 en cerebro, causando neuroinflamación, daño neural y disfunciones cognitivas que se mantienen a largo plazo*".

Para ello, se han abordado distintos objetivos que intentan explorar distintos mecanismos moleculares que pueden verse afectados por la exposición intermitente al alcohol durante la adolescencia (EIAA). Para evaluar los mecanismos moleculares dependientes de la activación de TLR4 se han utilizado ratones normales de genotipo normal o silvestre (*wild type*; WT) y deficientes en TLR4 (TLR4 *knock-out* o TLR4-KO).

OBJETIVOS

Publicación I. Estudiar si la EIAA causa daño en CPF a través de la respuesta de los TLR4 induciendo:

- Neuroinflamación y activación de las vías de señalización asociadas a los TLR4.
- Alteraciones en la mielina y en proteínas sinápticas que pueden o no ser reversibles.
- Déficits cognitivos a largo plazo.

Publicación II. Investigar si la EIAA provoca cambios de plasticidad neuronal asociados con la adicción, y si éstos dependen del TLR4. Para ello se analizará el efecto de la EIAA sobre:

- Los niveles de los receptores glutamatérgicos (GluR1 y NR1) en sinaptosomas, a corto y largo plazo en la edad adulta.
- Posibles cambios epigenéticos en las vías de señalización, relacionadas con plasticidad neuronal en la CPF a corto y largo plazo.
- Los niveles de ansiedad y preferencia por alcohol en animales adultos.

Publicación III. Valorar posibles biomarcadores de neuroinflamación y estudiar sus diferencias de género, examinando:

- Las diferencias en el perfil de citoquinas y quimioquinas, en plasma de chicos y chicas adolescentes tras la intoxicación aguda por alcohol, así como los niveles de expresión del TLR4.
- La respuesta inflamatoria periférica (suero) y central (CPF) tras la exposición aguda y la EIAA en ratones adolescentes machos y hembras.
- Las diferencias de marcadores inflamatorios en suero y CPF entre ratones adolescentes WT y TLR4-KO.

3. METODOLOGÍA

Con el fin de evaluar los efectos neuroinflamatorios del alcohol en el cerebro adolescente, se usaron ratones hembra C57BL/6 de 30 días de edad tanto normales (WT) como deficientes en el TLR4 (TLR4-KO). Los ratones TLR4-KO fueron cedidos por el Dr. S. Akira (Universidad de Osaka, Suita, Japón). Estos animales fueron generados mediante reemplazamiento por recombinación homóloga en células embrionarias de ratón a E14. Así, las regiones transmembrana y citoplasmática de TLR4 (aminoácidos 86-835) se sustituyeron por el casete de resistencia a neomicina (Hoshino *et al.*, 1999).

Modelo de exposición intermitente al alcohol durante la adolescencia (EIAA)

A estos animales se les aplicó un modelo establecido de tratamiento *binge-like* o "atracón" de forma intermitente. Este patrón de consumo, de altas cantidades de alcohol en un corto periodo de tiempo, conduce a unos niveles de alcohol en sangre y en cerebro relativamente elevados. El patrón de intermitencia se desarrolló imitando al abuso de alcohol durante los fines de semana llevada a cabo por los adolescentes. Siguiendo el protocolo inicialmente descrito en ratas por nuestro grupo (Pascual *et al.*, 2007), se realizaron 8 administraciones intraperitoneales de etanol (3 g/kg, diluido al 25% en suero fisiológico) durante los días 30, 31, 34, 35, 38, 39, 42, 43. Los animales controles recibieron el mismo patrón de administración pero con suero fisiológico. Una dosis de alcohol causa un máximo de concentración a 30 minutos tras la inyección de 178 ± 17 mg/dL de alcohol en sangre, tanto para animales WT y TLR4-KO.

Las muestras de CPF (zona medial; Fig. 2C) o suero se recolectaron 24 horas (corto plazo/adolescencia) o 3 semanas (largo plazo/jóvenes adultos) tras la última administración de alcohol o suero salino y se conservaron según las necesidades específicas de cada técnica. Las pruebas conductuales se realizaron en jóvenes adultos (3 semanas tras el tratamiento).

Intoxicación etílica aguda en pacientes adolescentes

Se utilizó suero de pacientes que presentaban sintomatología de intoxicación etílica aguda, con alcoholemia superior a 1 g/L, así como un grupo control equivalente (pág. 66). Las características clínicas, epidemiológicas y analíticas se describen con detalle en la página 67.

4. RESULTADOS

4.1. Publicación I

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TLR4 elimination prevents synaptic and myelin alterations and long-term cognitive dysfunctions in adolescent mice with intermittent ethanol treatment

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ABSTRACT

The adolescent brain undergoes important dynamic and plastic cell changes, including overproduction of axons and synapses, followed by rapid pruning along with ongoing axon myelination. These developmental changes make the adolescent brain particularly vulnerable to neurotoxic and behavioral effects of alcohol. Although the mechanisms of these effects are largely unknown, we demonstrated that ethanol by activating innate immune receptors toll-like receptor 4 (TLR4), induces neuroinflammation and brain damage in adult mice. The present study aims to evaluate whether intermittent ethanol treatment in adolescence promotes TLR4-dependent pro-inflammatory processes, leading to myelin and synaptic dysfunctions, and long-term cognitive impairments. Using wild-type (WT) and TLR4-deficient (TLR4-KO) adolescent mice treated intermittently with ethanol (3.0 g/kg) for 2 weeks, we show that binge-like ethanol treatment activates TLR4 signaling pathways (MAPK, NFκB) leading to the up-regulation of cytokines and pro-inflammatory mediators (COX-2, iNOS, HMGB1), impairing synaptic and myelin protein levels and causing ultrastructural alterations. These changes were associated with long-lasting cognitive dysfunctions in young adult mice, as demonstrated with the object recognition, passive avoidance and olfactory behavior tests. Notably, elimination of TLR4 receptors prevented neuroinflammation along with synaptic and myelin derangements, as well as long-term cognitive alterations. These results support the role of the neuroimmune response and TLR4 signaling in the neurotoxic and behavioral effects of ethanol in adolescence.

1. Introduction

Alcohol binge drinking is prevalent in adolescence, and a high percentage of European and USA teenagers have reported heavy episodic drinking, defined as consuming five or more drinks on one occasion in the past 2 weeks (Danielsson et al., 2012; Johnston et al., 2013). These high rates of heavy alcohol use are concerning since many studies have indicated that heavy alcohol consumption can impair neurocognitive development which affects several neuropsychological domains, including memory, executive functioning, visuospatial skills, and sustained attention (Brown et al., 2000; Giancola et al., 2001). Longitudinal studies have also shown adverse effects of adolescent drinking on the development of visuospatial processing, attention and working memory (Hanson et al., 2011; Squeglia et al., 2009; Tapert et al., 2002).

Adolescence is an important brain maturation period during which some brain regions undergo remodeling and functional changes, which increase neuronal connectivity and change synaptic plasticity (Alfonso-Loeches and Guerri, 2011; Toga et al., 2006). The prefrontal cortex (PFC), which coordinates higher-order cognitive processes and executive functions, is the last brain region to mature (Mills et al., 2014). Gray matter reduction begins primarily in early adolescence (≈12–14 years) in posterior brain regions and progresses to more anterior regions (Gogtay et al., 2004), such as the PFC, to continue into early adulthood (mid-20s) (Sowell et al., 2001). Reduction in gray matter, which is particularly evident in the PFC, is associated with the pruning of excess neurons (Paus, 2005), and with synapse stabilization. Concomitantly, the white

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matter volume and myelination of fiber tracts increases in the PFC during adolescence, event associated with enhanced neuronal conduction and communication (Barnea-Goraly et al., 2005; Giedd et al., 1999; Pfefferbaum, 2004). All these processes are an integral component of neurocognitive development and generate efficient information processing and improved cognition (Squeglia et al., 2013). These extensive developing changes in brain maturation might explain the adolescent brain's vulnerability to the deleterious effects of ethanol (Alfonso-Loeches and Guerri, 2011; Jacobus and Tapert, 2013). PFC-dependent behaviors have been described to be altered in human alcoholics (Fortier et al., 2009; Kamarajan et al., 2010) and also in PFC-damaged rodents (Bissonette et al., 2008), or after an acute ethanol challenge (Brown et al., 2007). Neuroimaging studies have also reported changes in microstructural and functional myelin integrity in different brain areas in adolescents with alcohol abuse (Bava et al., 2009a; Schweinsburg et al., 2010). These myelin alterations may be related with attention and spatial working memory deficits in human adolescents who participate in heavy alcohol abuse (Tapert et al., 1999, 2004).

PFC is one of the brain regions more affected by ethanol drinking during adolescence and can cause PFC-mediated control deficits in adulthood (Gass et al., 2014). The underlying mechanisms of neurotoxic and behavioral effects of ethanol in adolescence are presently unknown. Our previous studies indicated that by activating the innate immune system, particularly the toll-like receptor 4 (TLR4) signaling response, ethanol triggers the activation of transcription factors NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) and AP-1 (activating protein-1), which induce the production of cytokines and inflammatory mediators, that cause brain damage. Indeed, we have shown that by activating TLR4 responses in glial cells, chronic alcohol consumption induces neuroinflammation, gliosis, demyelination and brain damage (Alfonso-Loeches et al., 2010, 2012; Blanco et al., 2005; Fernandez-Lizarbe et al., 2009), and these effects have been associated with behavioral impairments (Pascual et al., 2011).

Our previous studies have also provided evidence that binge-like ethanol treatment in adolescent rats induces some inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and ciclooxygenase-2 (COX-2), in the cerebral cortex and the hippocampus, causing neural death and cognitive alterations (Pascual et al., 2007). Neural death can, in turn, enhance neuroinflammation by the release of danger-associated molecular pattern (DAMP) molecules, such as high-mobility group box-1 (HMGB1), a nuclear protein which is passively released during stress, cell injury and necrosis, or is actively secreted as a cytokine (Andersson and Tracey, 2011). HMGB1 can bind and activate several receptors, including TLRs, receptors for advanced glycation end products (RAGE) and C-X-C chemokine receptor 4 (CXCR4) (Yang et al., 2013). Recent studies have demonstrated that ethanol treatment induces the release of HMGB1 in the brain and liver, which contributes to ethanol-induced pathology in both organs (Ge et al., 2014; Vetreno et al., 2013; Whitman et al., 2013).

Despite the involvement of TLR4 in adult neuroinflammation, whether TLR4 activation participates in the myelin and synaptic dysfunction, and long-term neuroinflammatory and cognitive behavioral impairments associated with binge drinking in adolescence is presently unknown. This study reveals, for the first time, the critical role of TLR4 receptors in neuroinflammation and brain damage induced by intermittent ethanol treatment in the PFC of adolescent wild-type (WT) mice since changes in neither the production of cytokines and inflammatory mediators nor in myelin and synaptic structures were observed in TLR4-deficient (TLR4-KO) mice treated with alcohol. Elimination of the TLR4 function also prevents ethanol-induced long-lasting cognitive alterations, which suggests the role of TLR4 signaling and neuroinflammation in ethanol-induced brain alterations in adolescence.

2. Materials and methods

2.1. Animals and treatments

Female C57BL/6 WT (Harlan Ibérica, Barcelona, Spain) and TLR4-KO knockout (KO) mice (C57BL/6 background, kindly provided by Dr. S. Akira, Osaka University, Suita, Japan) aged 30 days were used. All the animals were kept under controlled light and dark (12/12 h), temperature (23 °C), and humidity (60%) conditions. All the experimental procedures were carried out in accordance with the guidelines approved by the European Communities Council Directive (86/609/ECC) and by Spanish Royal Decree 1201/2005. The animal experiments were also approved by the *Ethical Committee of Animal Experimentation of the* Príncipe Felipe Research Center (Valencia, Spain).

For the binge ethanol treatment, WT and TLR4-KO mice were housed (4 animals/cage) and maintained with water and solid diet *ad libitum*. Morning doses (9–10 a.m.) of either saline or 25% (v/v) ethanol (3 g/kg) in isotonic saline were administered intraperitoneally to 30-day-old mice on 2 consecutive days with 2-day gaps without injections for 2 weeks (PND 30 to PND 43), as previously described (Pascual et al., 2007). Then, some mice were maintained without alcohol treatment until postnatal day (PND) 65. A single dose of ethanol to adolescent mice resulted in a peak of BECs of 178.23 ± 16.75 mg/dL at 30 min post-injection. Some animals were sacrificed by decapitation 24 h after the last (8th) ethanol or saline administration (PND 44, short-term ethanol effects) or after 3 weeks upon ethanol or saline administration (PND 65, long-term ethanol effects). Brains from adolescent (PND 44) and young adult mice were collected, and the PFC were dissected and stored at −80 °C until use. In addition, some animals were anesthetized, perfused with paraformaldehyde (PF)/glutaraldehyde and used for the electron microscopy analysis. Behavioral studies were conducted 3 weeks after the last dose of ethanol or saline treatment (long-term effects).

2.2. Western blot analysis

The Western blot technique was performed in the PFC tissue lysates, as described elsewhere (Fernandez-Lizarbe et al., 2009). To analyze the HMGB1 levels, nuclear and cytosolic fractions were isolated following the procedure of (Ishida et al., 2002). The primary antibodies used were: CNPase (2′,3′-cyclic-nucleotide 3′-phosphodiesterase), PLP (proteolipid protein), MBP (myelin basic protein), MAG (myelin-associated glycoprotein), MOG (myelin oligodendrocyte glycoprotein) and HMGB1 (Abcam, Cambridge, UK); NG2 (neuron/glial antigen 2), p-ERK (extracellular signal-regulated kinase), p-JNK (c-Jun N-terminal kinase), p-p38 and p-p65 (Cell Signaling Technology, Leiden, The Netherlands); iNOS (BD Transduction Laboratories, California, USA) and COX-2 (Cayman Chemical, Michigan, USA); synapsin IIa, syntaxin 4, SNAP-25 (synaptosomal-associated protein-25) and synaptotagmin (BD Transduction Laboratories). Membranes were washed, incubated with the corresponding HRP-conjugated secondary antibodies and developed using the ECL system (ECL Plus; Thermo Scientific, Illinois, USA). All the membranes were stripped and incubated with the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibodies (Chemicon, California, USA), Lamin A/C (BD Transduction Laboratories), NeuN (neuronal nuclei protein) (Chemicon, California, USA) or the corresponding total form of each phosphorylated protein (ERK, JNK, p38 and p65; Santa Cruz Biotechnology) as loading controls. Band intensity was quantified with the ImageJ 1.44p analysis software (National Institutes of Health, USA). The densitometry analysis is shown in arbitrary units normalized to the respective loading control.

2.3. Cytokine analysis

PFC-homogenized extracts were analyzed simultaneously to determine the levels of several cytokines and chemokines using the Milliplex MAP kit premixed mouse cytokine/chemokine magnetic bead panel (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The analyzed cytokines and chemokines included tumor necrosis factor (TNF)-α, IL-17A, monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α). Data were acquired in a Luminex 100 System and analyzed by the MasterPlex CT version 1.2.0.7 software (Hitachi MiraiBio, USA). The interleukin (IL)-1β levels were determined in the PFC lysates with an ELISA kit (eBioscience, Vienna, Austria) following the manufacturer's protocol.

2.4. Determination of nitric oxide and H_2O_2 levels

Nitric oxide (NO) levels in PFC lysates were assessed by the Griess reaction, as described previously (Fernandez-Lizarbe et al., 2009). The H_2O_2 concentration in the PFC-homogenized extracts was estimated by a fluorometric assay following the manufacturer's instructions (Oxiselect H_2O_2 assay kit, Cell Biolabs Inc., San Diego, USA).

2.5. Brain tissue preparation and electron microscopy

Mice were anesthetized by an intraperitoneal injection of sodium penthobarbital (60 mg/kg) and fentanyl (0.05 mg/kg) for analgesia. Animals were then perfused transcardially with 0.9% saline containing heparin, and followed immediately by 2% PF and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for tissue fixation. PF-fixed brains were removed, postfixed overnight at 4 °C with the same fixative solution, and were then stored at 4 °C in PBS. Coronal 200 μm sections from the PFC were cut on a vibratome Leica VT-1000 (Leica, Heidelberg, Germany). Sections were post-fixed with 2% osmium, rinsed, dehydrated and embedded in Durcupan resin (Fluka, Sigma–Aldrich, St. Louis, USA). Semithin sections (1.5 μm) were cut with an Ultracut UC-6 (Leica, Heidelberg, Germany) and stained lightly with 1% toluidine blue. Finally, ultra-thin sections (0.08 μm) were cut with a diamond knife, stained with lead citrate (Reynolds solution) and examined under a transmission FEI Tecnai G2 Spirit electron microscope (FEI Europe, Eindhoven, Netherlands) using a digital camera Morada (Olympus Soft Image Solutions GmbH, Münster, Germany). Then, images were analyzed by using the MetaMorph software analysis (version 7.0).

2.6. Behavioral testing

2.6.1. Object recognition test

The object recognition test was performed in an open box (24 × 24 × 15 cm) using two types of objects: two small river stones (A) and a small non toxic plastic toy (B). The task procedure has been previously described in (Pascual et al., 2011), and consists in three phases: habituation, training session (T1) followed by a test session (T2). In the training session, the mouse is placed in the open-field arena that contains two identical sample objects placed in the middle of the testing box for 3 min. After a retention interval (1 min), the animal is returned to the open-field arena with two objects during the 3-min test session: one is identical to the sample and the other is novel. Object exploration was defined as the orientation of the animal's snout toward the object within a range of 2 cm, or less, from the object. The basic measure in the object recognition test was the discrimination index, calculated as $[D.I. = (t_{novel} - t_{familiar})/(t_{novel} + t_{familiar}) \times 100\%]$.

2.6.2. Passive avoidance test

A step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy) was employed for the passive avoidance test. This cage is made of Perspex sheets and is divided into two compartments (15 × 9.5 × 16.5 cm each). The safe compartment is white and illuminated by a light fixture (10 W) fastened to the cage lid, whereas the "shock" compartment is dark and made of black Perspex panels. Passive-avoidance tests were carried out following the procedure described in (Aguilar et al., 2000). On the day of training, each mouse was placed in the illuminated compartment and after a 60-s period of habituation, the door leading to the dark compartment was opened. When the animal had placed all four paws in the dark compartment, a foot shock (0.5 mA, 3 s) was delivered, and the animal was immediately removed from the apparatus and returned to its home cage. The time taken to enter the dark compartment (step-through latency) was recorded. Retention was tested 24 h and 72 h later following the same procedure, but without shock. The maximum step-through latency was 300 s.

2.6.3. Olfactory behavior test

Mice were screened for olfactory deficits by an odor-habituation test. Odors ($n = 3$; pentanol, propyl butyrate and nonane; Sigma–Aldrich, Madrid, Spain) were diluted 1×10^{-3} in mineral oil and applied to a cotton applicator stick. The stick was then enclosed in a piece of odorless plastic tubing to prevent the liquid odor coming into contact with the testing chamber or animal, but still allowed volatile odor delivery. Following the procedure of (Wesson et al., 2010), odors were delivered for 4 successive 20-s trials (1 block), separated by 30-s inter-trial intervals by inserting the odor stick into a port on the side of the animal's home cage. The duration of time spent investigating, defined as snout-oriented sniffing within 1 cm of the odor presentation port, was recorded. To analyze the olfactory behavior data, odor investigation durations within the individual trials were integrated across all the odors. In addition to measuring odor habituation (discrimination), the habituation index (the investigatory values from the 1st trial odor presentation, minus those from the 4th trial odor presentations) was also determined.

2.7. Statistical analysis

The results are reported as mean ± SEM. Statistical significance for Western blots and inflammatory mediators was determined by an unpaired Student's *t*-test. Electron microscopy analysis was performed by three-way ANOVA with three variables: "Genetics" (WT/TLR4-KO); "Treatment" (saline/ethanol) and "Age" (short-term/long-term). Bonferroni *post hoc* tests or two-way ANOVA comparisons was used to test for differences among groups. The data from the object recognition test were analyzed by a mixed ANOVA with two "between" subject variables: "Genetics" (WT/TLR4-KO) and "Treatment" (saline/ethanol). The passive avoidance and the olfactory behavioral test data were analyzed using a mixed analysis of variance (MANOVA) with the same two "between" variables, and also with a mixed ANOVA with these two "between" variables and a "within" subject variable – "Days" – for passive avoidance, with three levels (training, 24-h test and 72-h test) or – "Trial" – for the olfactory behavioral test with four levels (1st, 2nd, 3rd and 4th trial).

3. Results

3.1. Intermittent ethanol treatment activates TLR4 signaling pathways and promotes inflammatory mediators in the PFC of adolescent mice

To demonstrate the involvement of TLR4 signaling in the inflammatory response induced by intermittent ethanol treatment

in adolescent animals, we evaluated the activation of different kinases, which are associated with the activation of the TLR4 signaling pathway (Akira and Takeda, 2004), including MAPK and NFκB. Fig. 1A shows that intermittent ethanol treatment promotes both the phosphorylation of ERK, p38 and JNK and the activation of transcription factor NFκB in the PFC of WT mice. Since the activation of TLRs leads to a transcriptional induction of several inflammatory mediators and cytokines, we measured the levels of COX-2, TNF-α, IL-1β, IL-17A and chemokines such as MCP-1 and MIP-1α. Our results demonstrated that the intermittent ethanol treat-

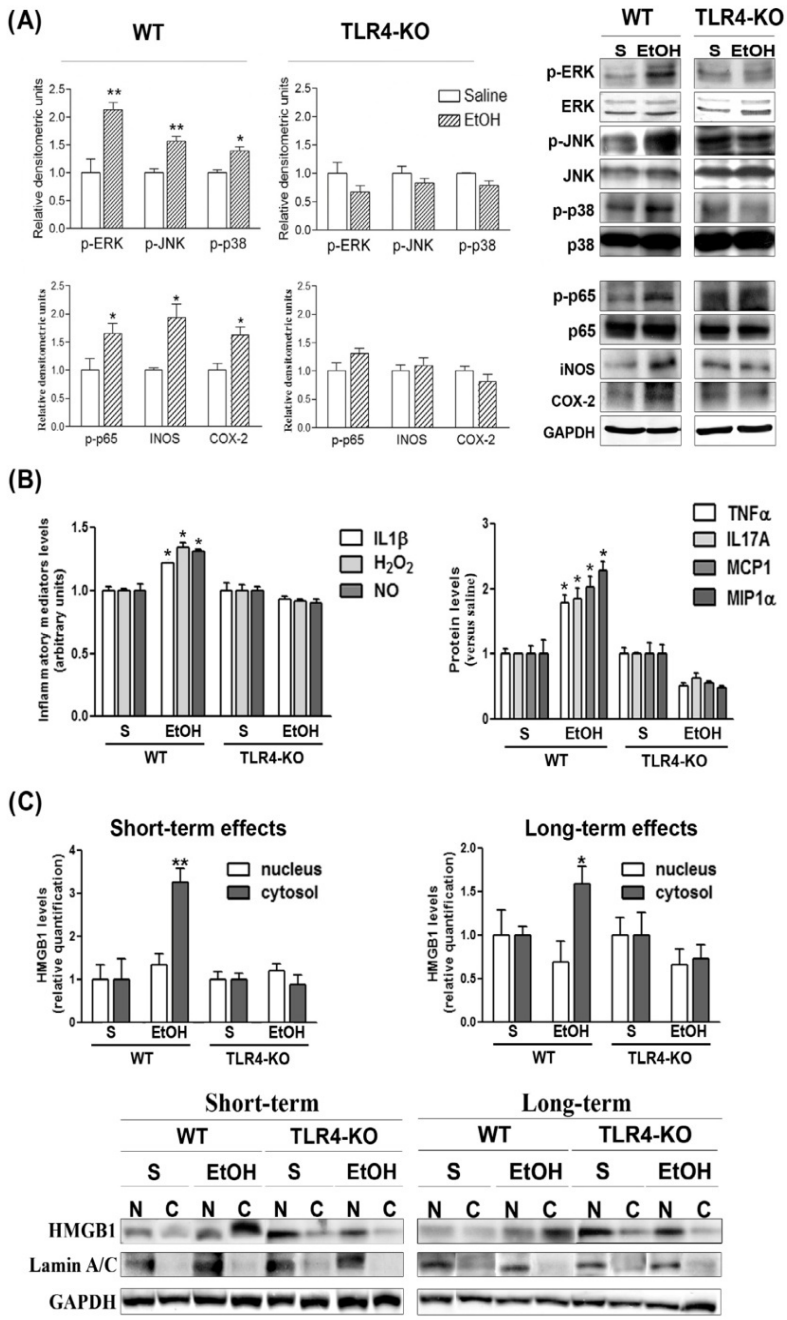

Fig. 1. Effects of intermittent ethanol treatment on TLR4 signaling in the PFC of WT and TLR4-KO mice treated in adolescence. (A) Immunoblot analysis and quantification of p-ERK, p-JNK, p-p38, p-p65, iNOS and COX-2 in the PFC of adolescent WT and TLR4-KO mice treated with ethanol or saline (S) in adolescence. A representative immunoblot of each protein is shown. (B) The levels of cytokines (TNF-α, IL-17A, IL-1β), chemokines (MCP-1, MIP-1α), NO and H_2O_2 in the PFC of adolescent WT and TLR4-KO mice treated with ethanol or saline (S) in adolescence. (C) The nuclear (N) and cytoplasmic (C) levels of HMGB1 in the PFC of adolescent (short-term) and young adult (long-term) WT and TLR4-KO mice treated with ethanol or saline (S) in adolescence. A representative immunoblot of each protein is shown. Lamin A/C levels represent the nuclear loading control. Data represent mean ± SEM, $n = 8$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, as compared to their respective saline groups.

ment in WT mice up-regulates the levels of COX-2, TNF-α, IL-1β, IL-17A, MCP-1 and MIP-1α. However, ethanol treatment neither induced the activation of the different kinases or NFκB nor the production of cytokines and inflammatory mediators in the PFC of TLR4-KO adolescent mice.

We further evaluated the potential oxidative stress induced by ethanol treatment since redox molecules, such as NO, H_2O_2 and iNOS, are key mediators of immunity and inflammation. Fig. 1A and B demonstrates that the levels of NO, H_2O_2 and iNOS in intermittent ethanol-treated adolescent WT mice significantly increased, whereas no differences were observed in TLR4-KO mice when compared with untreated mice.

We finally analyzed the possible activation of HMGB1. Considering that this nuclear protein can be released under stress or tissue damage, we assessed the expression levels and the translocation dynamics of HMGB1 in the PFC of not only adolescent mice at the end of ethanol treatment, but also of young adult mice. Fig. 1C shows that whereas the nuclear HMGB1 levels did not significantly change in the PFC of the WT mice treated with ethanol, the cytoplasmic levels of this protein were up-regulated in adolescents and remained elevated, but at lower levels, in the young adult mice exposed to ethanol in adolescence. However, no changes in either the nuclear or cytoplasmic HMGB1 levels were observed in the PFC of TLR4-KO mice.

3.2. Role of TLR4 in the myelin alterations induced by intermittent ethanol treatment in the PFC of adolescent animals

We next analyzed the potential role of TLR4 signaling in the reduction of myelin proteins and white matter dysfunctions observed in alcohol-drinking adolescent rats (Pascual et al., 2014) and humans (Bava et al., 2009b, 2013). To assess this aim, we determined the levels of different myelin proteins in ethanol-treated and untreated WT and TLR4-KO adolescent mice. We observed that ethanol treatment significantly down-regulated PLP, MBP, CNPase and NG2 in the PFC of WT mice (Fig. 2). We next determined whether ethanol-induced changes in myelin proteins were reversible or irreversible, and if they were maintained in adulthood. To answer this question, we measured the levels of different myelin proteins in the PFC of WT mice, which had been exposed to ethanol in adolescence and were then maintained without alcohol for 3 weeks (long-term effects). We noted that while the NG2 levels remained lower if compared with untreated adult mice, other proteins that decreased in the PFC of ethanol-treated adolescents, such as PLP, CNPase and MBP, recovered to the control values in ethanol-treated adult mice (Fig. 2). No significant changes in the levels of the different myelin proteins analyzed were observed in PFC of adolescent or young-adult TLR4-KO mice treated with alcohol in adolescence (Fig. 2).

An ultrastructural study (Fig. 3) provided further evidence that ethanol treatment disturbed the myelin structure in the PFC of adolescent WT mice. We noted that ethanol-treatment induced inter-laminar splitting of myelin sheaths (Fig. 3A, arrows) and irregular fiber shapes of myelin (Fig. 3A). Some myelin disarrangements were also observed in the PFC of adult mice exposed to ethanol in adolescence (long-term ethanol effects), which might be related with the changes in myelin proteins, such as NG2, MOG and MAG, noted in these mice (see Fig. 2). Nevertheless, ethanol-treated TLR4-KO mice showed no ultrastructural myelin alterations in either adolescence or adulthood (Fig. 3B).

3.3. TLR4 response is associated with changes in the synaptic elements in the PFC of intermittent ethanol-treated mice

Presynaptic terminals are enriched with multiple specialized proteins, which are important factors in the communication between neurons and axonal neurotransmission (Washbourne et al., 2002). We therefore evaluated the expression of several synaptic proteins in the PFC of both WT and TLR4-KO mice. These proteins included synapsin IIa, syntaxin 4, SNAP-25 and synaptotagmin, which are components of the SNARE complex

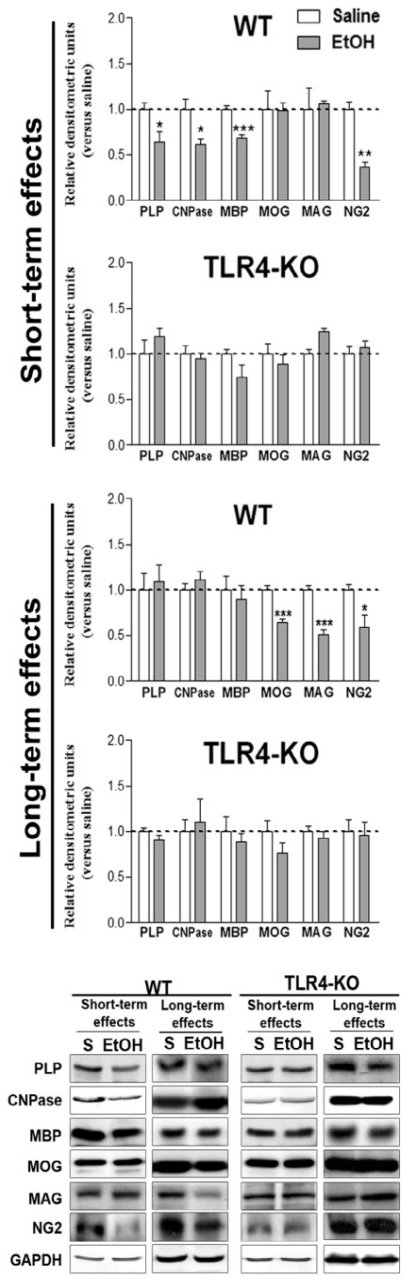

Fig. 2. TLR4 involvement in the down-regulation of the myelin protein levels induced by intermittent ethanol treatment in adolescence. The immunoblot analysis and quantification of PLP, CNPase, MBP, MOG, MAG, and NG2 in the PFC of adolescent (24 h after the last treatment administration, short-term) and adult (3 weeks after the last treatment administration, long-term) WT and TLR4-KO mice treated with ethanol or saline (S) in adolescence. A representative immunoblot of each protein is shown. Data represent mean ± SEM, $n = 8$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared to their respective saline groups.

(Giraudo et al., 2006). Fig. 4 shows that intermittent ethanol treatment in adolescence down-regulated the protein expression of synapsin IIa, syntaxin 4, SNAP-25 and synaptotagmin in WT mice, whereas no changes were observed in the saline control counterparts of ethanol-treated TLR4-KO mice. The changes noted in these proteins were not maintained in the PFC of the adult animals treated with alcohol in adolescence (long-term effects) (Fig. 4).

We also assessed the potential ultrastructural alterations in the synaptic elements in the WT and TLR4-KO mice treated with or without intermittent ethanol in adolescence (Fig. 5A). For this purpose, several morphological aspects of the synapses were analyzed by electron microscopy, such as vesicle number, thickness of postsynaptic density, as well as width of synaptic cleft and synaptic curvature. The three-way ANOVA analysis showed only a signifi-

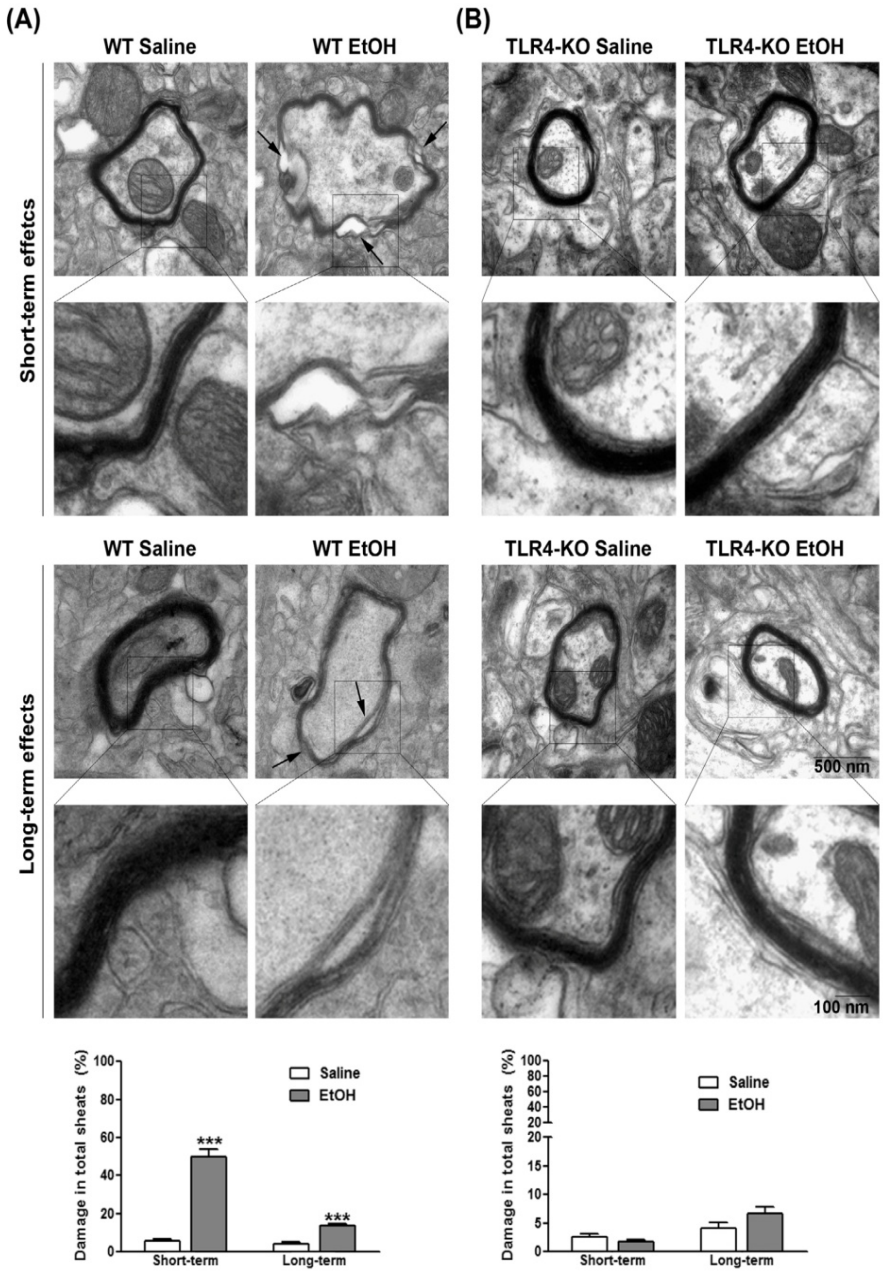

Fig. 3. The electron microscopy analysis shows the role of TLR4 in the myelin sheath disarrangements induced by intermittent ethanol treatment in adolescence. The representative transmission electron micrographs from the PFC of adolescent (24 h after the last treatment administration) and adult (3 weeks after the last treatment administration) WT (A) and TLR4-KO (B) mice treated with ethanol or saline (S) in adolescence are shown. Arrows indicate inter-laminar splitting of myelin sheaths. Bars represent the percentage of myelin sheath damaged. Data represent mean ± SEM, $n = 3–4$ mice/group. $^{***}p < 0.001$, as compared to their respective saline groups.

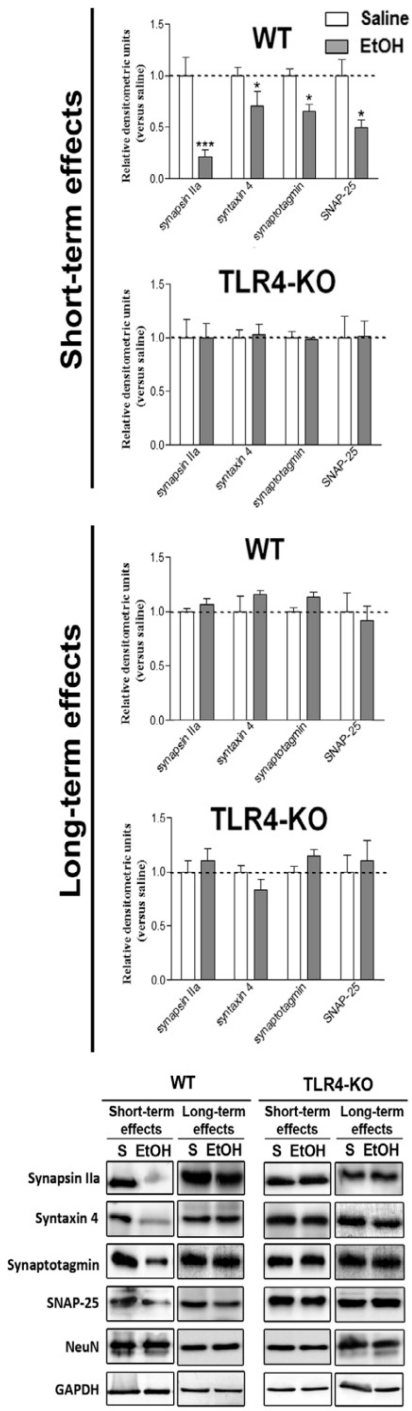

Fig. 4. Role of TLR4 in the synaptic protein expression alterations induced by intermittent ethanol treatment in adolescence. The immunoblot analysis and quantification of synaptic proteins, synapsin IIa, syntaxin 4, SNAP-25 and synaptotagmin in the PFC of WT and TLR4-KO mice treated with ethanol or saline (*S*) in adolescence were analyzed in the adolescent (PND 44, short-term) or adult (PND 65, long-term) stage 24 h or 3 weeks after the last treatment administration, respectively. A representative immunoblot of each protein is shown. Data represent mean ± SEM, $n = 8$ mice/group. $^{*}p < 0.05$, $^{***}p < 0.001$, as compared to their respective saline groups.

cant interaction between age × Treatment × Genetics for the variable 'vesicle number' [$F(1,158) = 7604$; $p < 0.01$]. The two-way ANOVA comparison between Treatment × Genetics was also significant [$F(1,158) = 20.595$; $p < 0.001$] for this variable, and the comparison between Treatment × Strain was significant for 'postsynaptic density thickness' [$F(1,242) = 9.518$; $p < 0.05$] and between Treatment × age for 'synaptic cleft width' [$F(1,232) = 17.038$; $p < 0.001$]. Intermittent ethanol treatment in adolescence reduced vesicle number (Fig. 5B) and postsynaptic density thickness (Fig. 5C), but increased synaptic cleft width (Fig. 5D) in WT mice. Some of these effects (vesicle number and postsynaptic density thickness) remained until the adult stage. Likewise, ethanol-treated WT mice showed no changes in the concave synapses (associated with strong synaptic activation), but an overall increase in the convex synapses was noted, an effect that is related with loss of synaptic efficacy (Ghaffari-Farazi et al., 1999) (Fig. 5E). This latter effect was observed in adolescent animals and remained in adult mice (long-term ethanol effects). Ethanol-treated TLR4-KO mice showed no short- and long-term ultrastructural changes in the synaptic elements if compared with their saline control counterparts (Fig. 5).

3.4. Intermittent ethanol treatment causes long-term cognitive dysfunctions associated with TLR4 response

In order to assess whether the neuroinflammation, myelin and synaptic alterations induced by binge ethanol treatment in the PFC of adolescent mice were able to induce long-term cognitive impairments in adult mice exposed to ethanol in adolescence, and whether these behavioral alterations are associated with the activation of the TLR4 pathway, we performed several cognitive behavioral tasks. We used object recognition, passive avoidance and the olfactory behavior test, which have been related with cortical cognitive function (Akirav and Maroun, 2006; Nobakht et al., 2011; Rolls, 2004).

3.4.1. Object recognition test

This task measures cognitive functions, such as learning and memory (Antunes and Biala, 2012). The two-way ANOVA analysis in the object recognition test (Fig. 6A) reveals a significant effect of the Genetics × Treatment interaction [$F(1,50) = 8.736$; $p < 0.01$]. Moreover, ethanol-treated WT mice failed to recognize the familiar object, and its discrimination index was significantly lower than in the rest of the groups ($p < 0.001$).

3.4.2. Passive avoidance test

A passive avoidance test was used to assess memory function based on the association formed between an aversive stimulus, such as mild foot shock, and a specific environmental context. This test measured short-term or long-term memory. The results of the passive avoidance test are presented in Fig. 6B. The ANOVA revealed a significant effect of the variable Days [$F(2,114) = 85.997$; $p < 0.001$] as all the groups presented longer step-through latencies during both test sessions in comparison to the training session ($p < 0.001$). A MANOVA showed an effect of the variable genetics [$F(3,55) = 4.118$; $p < 0.01$]. On the training day, TLR4-KO mice took significantly longer to enter the dark compartment than WT mice ($p < 0.002$). The variable treatment also showed a significant effect [$F(3,55) = 5.487$; $p < 0.002$]. All the ethanol-treated mice presented shorter latencies than their saline control counterparts during the 24-h test session ($p < 0.001$). However, only the ethanol-treated WT mice demonstrated lower latencies during the 72-h test session than their saline counterparts ($p < 0.05$).

3.4.3. Olfactory behavior test

The olfactory habituation test was used to assess a subject's ability to discriminate between novel and familiar odors. The ANOVA showed an effect of the Trial × Genetics × Treatment interactions [$F(3,171) = 2.641$; $p < 0.05$]. All the groups were eventually able to habituate to odors by the 2nd, 3rd or 4th trial, except those WT treated with ethanol (Fig. 6C). The MANOVA showed a significant effect of the Treatment × Genetics interaction [$F(4,54) = 3.906$; $p < 0.007$]. In the 1st, 2nd and 3rd trials, saline WT mice explored the odor for a significantly longer time than saline TLR4-KO mice ($p < 0.01$; $p < 0.001$ and $p < 0.05$, respectively). Likewise in these three trials, ethanol treatment shortened the exploration time only in WT mice ($p < 0.01$ in all cases). The habituation index (Fig. 6D) showed that the WT ethanol-treated mice presented a significantly lower index than saline WT ($p < 0.01$), which implies a less marked difference between the first and last trial ($p < 0.01$). Thus, our results show that only ethanol-treated WT mice were unable to habituate to odors in the fourth trial.

4. Discussion

Increasing evidence supports the role of the neuroimmune response in the pathophysiology of many neurological and psychiatric disorders, such as neurodegeneration, depression and epilepsy (Capuron and Miller, 2011; Jones and Thomsen, 2013), and in alcohol abuse disorders (Mayfield et al., 2013). Studies done in the last decade have also indicated the adverse consequences of heavy drinking in adolescence, which can induce myelin alterations and other structural and functional brain dysfunctions to affect memory, executive functioning, visuospatial skills and attention processes in human adolescents (Parada et al., 2012). The underlying molecular mechanisms of these cognitive effects induced by ethanol are largely unknown. The present findings provide evidence for the critical role of the innate immune response and TLR4 signaling in the ethanol-induced PFC damage and long-term cognitive dysfunctions induced by binge-like ethanol exposure in adolescence in mice. Here we show that by activating the TLR4 response, binge-like ethanol exposure in adolescence of mice induces the production of cytokines, inflammatory mediators and reactive oxygen species (ROS), such as hydrogen peroxide, to promote myelin sheath disarrangements and changes in the synaptic ultrastructure, along with changes in myelin and synaptic protein levels. Some of these changes remained until adulthood, and can participate in the long-lasting cognitive and memory impairments observed in mice exposed to ethanol in adolescence. Mice deficient in the TLR4 function are protected against ethanol-induced neuroinflammation, myelin and synaptic derangements and the long-term cognitive effects induced by ethanol in adolescence, which suggests the critical role of innate immune receptor TLR4 in these effects.

Adolescence is a critical brain maturation period throughout which certain brain areas, like the PFC, continue to develop and are very sensitive to the neurotoxic effects of ethanol in both humans and experimental animals (Pascual et al., 2007). Numerous studies provide evidence for the sensitivity of the developing PFC to neurotoxic effects of ethanol. In fact, neuroimaging studies have evidenced in human adolescents that heavy alcohol drinking reduces the PFC volume (De Bellis et al., 2005; Medina et al., 2008), alters myelin integrity in the superior longitudinal fasciculus (Elofson et al., 2013) and reduces myelin fiber tracts with frontal connections (Bava et al., 2013). Heavy alcohol consumption also accelerates the cortical thinning of the right middle frontal gyrus, and decreased white matter volume when compared to demographically matched non-using teens (Luciana et al., 2013). These structural alterations can be correlated with persistent neurobehavioral deficits and alterations in several neuropsychological domains, including memory, executive functioning, visuospatial skills, and attention (Brown et al., 2000; Hanson et al., 2011; Squeglia et al., 2009; Tapert et al., 2002).

According to human data, the present findings show that repeated ethanol administration in adolescence alters the myelin structure (irregular myelin fiber shapes, inter-laminar splitting of myelin sheaths) and reduces the expression of several myelin-associated proteins. In addition, we also provide evidence, for the first time, that ethanol in adolescence alters synaptic machinery, down-regulates synaptic proteins and causes ultrastructural alterations in synapses, such as a reduction in both postsynaptic thickness and synaptic vesicle number, and an increase in the convex synaptic curvature and synaptic cleft width, effects that are related with poor efficacy in synaptic transmission (Ghaffari-Farazi et al., 1999; Jing et al., 2004; Kovalenko et al., 2006). Indeed, synaptic proteins (e.g., SNAP-25, synaptophysin, syntaxin and others), which are all implicated in the regulation of synaptic transmission and synaptic vesicle cycling, have been reported to be altered in either their expression level or their posttranslational modifications after distinct insults including chronic ethanol exposure (Zahr et al., 2013) or ischemia (Kovalenko et al., 2006). These data suggest that binge-like ethanol treatment in adolescence can disrupt synaptic plasticity by altering the synaptic machinery and myelin structure which affects the normal PFC processes and impairs PFC-related functions; e.g., executive function, learning, memory (Tapert et al., 1999, 2004). Accordingly, we show herein that binge-like ethanol treatment in adolescence induces long-lasting dysfunctions in learning and memory processes in young-adults. Thus, by running three tests (object recognition, passive avoidance and olfactory behavior), which have been associated with cortical cognitive function (Akirav and Maroun, 2006; Nobakht et al., 2011; Rolls, 2004), we show that intermittent ethanol treatment in adolescence lowers the discrimination index in the object recognition task, the exploring time and the odor habituation index in the olfactory behavior test in young-adult mice. Similarly, these animals also showed less latency to leave the safe compartment (illuminated), which suggests an impairment in cognitive functions in young-adult mice exposed to ethanol in adolescence.

The present findings also support the critical role of the innate immune receptors TLR4 response in the myelin and synaptic dysfunctions and the long-term cognitive effects induced by intermittent ethanol exposure in adolescence since the mice lacking the TLR4 function (TLR4-KO) are protected against ethanol-induced PFC damage and cognitive dysfunctions. Indeed, our previous studies demonstrate that by interacting with membrane microdomains lipid rafts (Blanco et al., 2005, 2008), ethanol activates TLR4 signaling in glial cells to trigger the activation of signaling pathways (MAPK, NFκB), which promote the release of cytokines and inflammatory mediators to cause neural damage (Alfonso-Loeches et al., 2010; Fernandez-Lizarbe et al., 2009) in the adult brain. The present results further demonstrate that intermittent binge-like ethanol exposure induces in the PFC of adolescent WT, but not in TLR4-KO mice, MAPK (p-ERK, p-P38, p-JNK) and transcription factor NFκB-p65 activation to trigger the production of inflammatory cytokines (IL-1β, TNF-α, IL-17A), chemokines (MCP-1 and MIP-1α) and inflammatory mediators (iNOS, COX-2), which can cause neuroinflammation and brain damage. These results suggest that the proinflammatory environment induced by glial cells (Alfonso-Loeches et al., 2010; Fernandez-Lizarbe et al., 2009) in the PFC can participate in the synaptic and myelin dysfunctions observed in mice exposed to ethanol in adolescence. According to this hypothesis, proinflammatory agents and the associated cytotoxic products induced during neuroinflammation can damage neurons (Rao et al., 2012) and synaptic elements (Mandolesi et al., 2013).

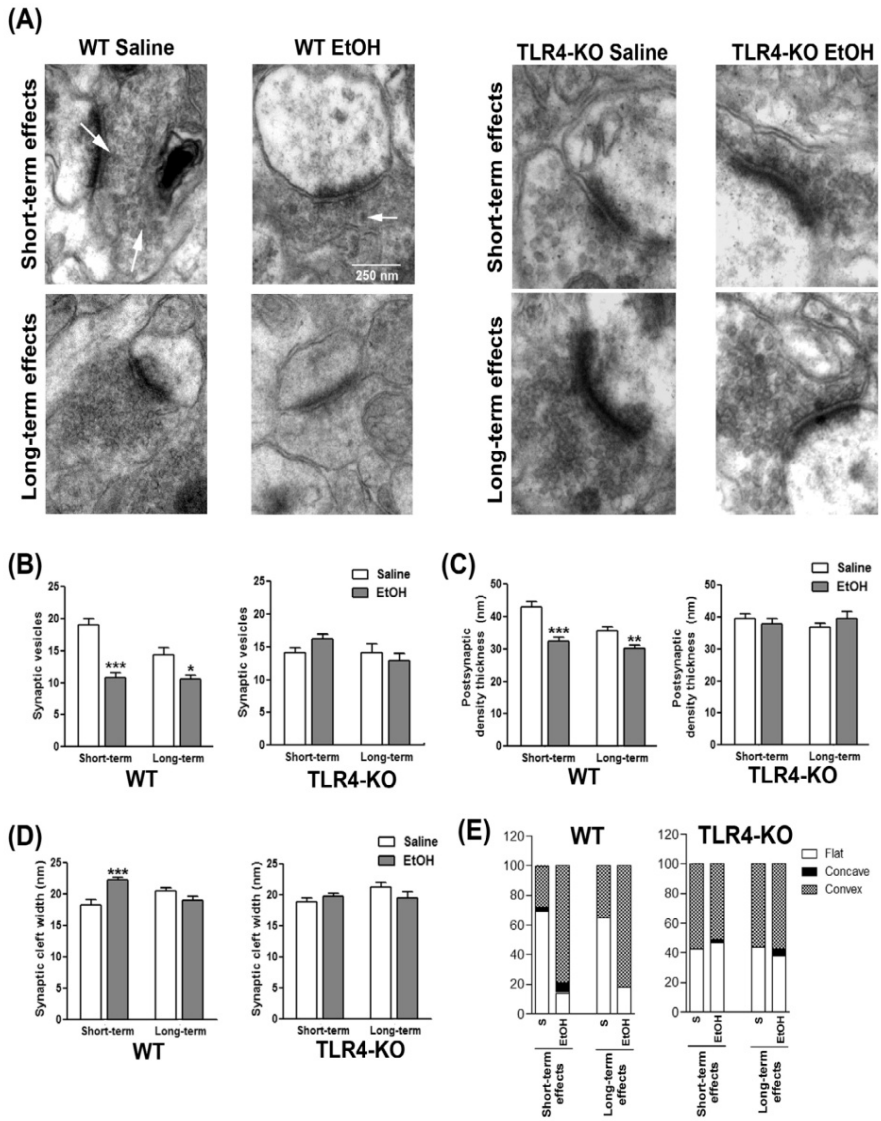

Fig. 5. The electron microscopy analysis shows the role of TLR4 in the alteration of the synaptic structure induced by intermittent ethanol treatment in adolescence. (A) The representative transmission electron micrographs from the PFC of adolescent (24 h after the last treatment administration) or adult (3 weeks after the last treatment administration) WT and TLR4-KO mice treated with ethanol or saline (S) in adolescence are shown. Arrows mark vesicles. Bars represent the vesicle number (B), the thickness of postsynaptic density (C), the synaptic cleft width (D) and the percentage of convex, concave and flat synapses (E). Data represent mean ± SEM, $n = 3$–4 mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared to their respective saline groups.

Other studies have also demonstrated that lipopolysaccharide (LPS), by activating TLR4 signaling, impairs long-term potentiation in CA1 of hippocampal slices (Costello et al., 2011), and COX-2 up-regulation has been associated with synaptic and memory alterations (Chen et al., 2013). Moreover, LPS not only induces the release of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), ROS and iNOS production, which have been shown to induce demyelination and axonal damage in cerebellar cultures (di Penta et al., 2013), but also promotes the down-regulation of several myelin genes in human oligodendrocytes (Jana and Pahan, 2013). It is noteworthy that TLR4 receptors can also be activated by different ligands, including endogenous molecules (saturated fatty acids, heat shock proteins, etc.) (Yu et al., 2010), ethanol metabolite ethyl-glucuronide (Lewis et al., 2013) and opioids (Coller and Hutchinson, 2012; Wang et al., 2012), which suggests its role in the regulation of non infectious inflammation (Yu et al., 2010). In addition, neuroimmune signaling and TLRs have also been implicated in drug dependence (Coller and Hutchinson, 2012).

Recent studies have shown that HMGB1, an endogenous cytokine that activates TLR4, is up-regulated in the cortex of adolescent rats exposed to ethanol and that HMGB1 remains elevated from late adolescence to young adulthood (Vetreno et al., 2013). HMGB1 is a non-histone chromatin protein that is passively released by dying cells or damaged tissue, which is associated with inflammatory response (Andersson and Tracey, 2011; Yang et al., 2010), but is also secreted by activated immune cells and functions as a late mediator of inflammation (Andersson and Tracey, 2011; Bonaldi

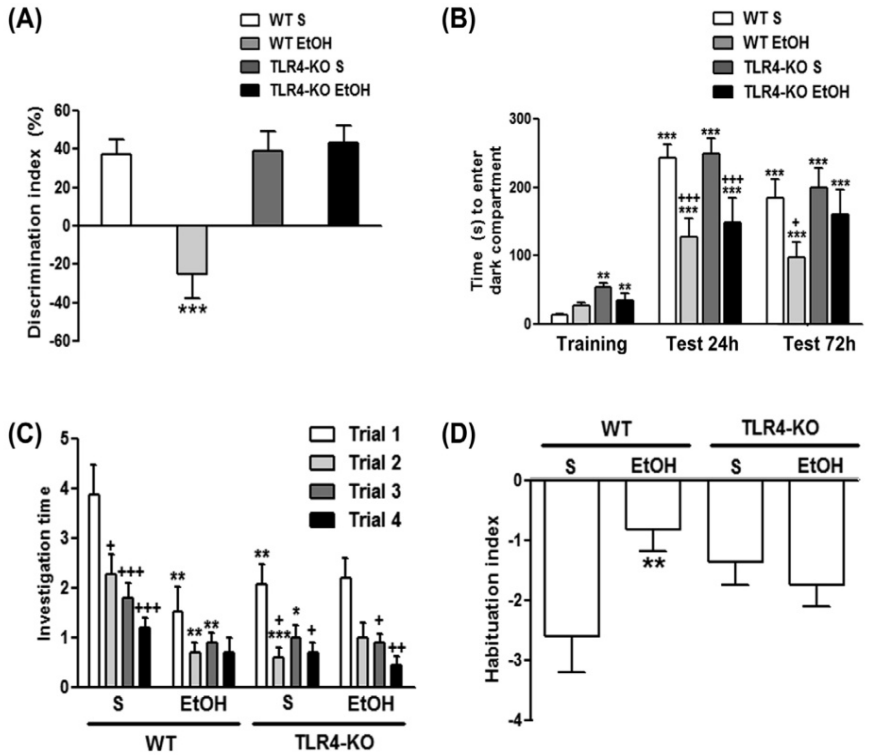

Fig. 6. Role of TLR4 in the object recognition task, passive avoidance test and olfactory behavior test in adult WT and TLR4-KO mice treated or untreated with ethanol in adolescence. (A) Bars represent the mean (±SEM, $n = 15$ mice/group) of the discrimination index in the object memory recognition task. $^{***}p < 0.001$ differences with the rest of the groups. (B) Bars represent the time taken to enter in the dark compartment of the passive avoidance test in the training and test sessions (24 h and 72 h after training). Data are presented as mean (±SEM), $n = 15$ mice/group. $^{***}p < 0.001$, as compared to the training session, $^{**}p < 0.01$ as compared to saline- or ethanol-treated WT mice, and $^{+}p < 0.05$, $^{+++}p < 0.001$, as compared to the saline-treated WT and TLR4-KO mice. (C) Bars represent the odor investigation time in the four trials. Data are presented as mean (±SEM), $n = 15$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared to WT saline group, and $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ as compared to the trial 1. (D) Bars represent the habituation index (discrimination). Data are presented as mean (±SEM), $n = 15$ mice/group. $^{**}p < 0.01$, as compared to the saline-treated WT group.

et al., 2003). The present study confirms that HMGB1 is up-regulated in the PFC of adolescent WT mice treated with ethanol, and that the levels of this protein remain somewhat high in young adult animals, suggesting the persistent up-regulation of a danger innate immune signal. These findings lead to the question as to what is (are) the mechanism(s) involved in maintaining the up-regulation of this protein in adult mice exposed to ethanol in adolescence. Using rat hippocampal-entorhinal cortex, a recent study suggested that by inhibiting neuronal histone deacetylases (HDACs), ethanol promotes the nucleocytoplasmic mobilization of HMGB1, which results in increased acetylated HMGB1 (Zou and Crews, 2014). Nevertheless, although epigenetic processes can contribute to the persistent up-regulation of HMGB1, further experiments are required to demonstrate its molecular mechanism, whether neuronal or/and glial cells are involved in this process and if the up-regulation of HMGB1 is responsible for the synaptic and myelin dysfunction induced by ethanol. Alternatively, ethanol-induced inflammatory and ROS production during brain maturation can cause irreversible structural and/or functional changes that induce long-term cognitive/behavioral effects, as observed in fetal alcohol spectrum disorders (for a review, see Alfonso-Loeches and Guerri, 2011).

To conclude, the results presented herein provide the critical role of the TLR4 response in brain damage and cognitive dysfunctions induced by ethanol exposure in adolescence. These findings support the role of the neuroimmune system as a target of alcohol effects and may provide a better therapeutic approach to not only improve the cognitive deficits induced by binge ethanol drinking, but to also restore neuroinflammation and neuronal communications, effects that can also participate in drug addiction.

Acknowledgments

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Involvement of TLR4 in the long-term epigenetic changes, rewarding and anxiety effects induced by intermittent ethanol treatment in adolescence

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ABSTRACT

Studies in humans and experimental animals have demonstrated the vulnerability of the adolescent brain to actions of ethanol and the long-term consequences of binge drinking, including the behavioral and cognitive deficits that result from alcohol neurotoxicity, and increased risk to alcohol abuse and dependence. Although the mechanisms that participate in these effects are largely unknown, we have shown that ethanol by activating innate immune receptors, toll-like receptor 4 (TLR4), induces neuroinflammation, impairs myelin proteins and causes cognitive dysfunctions in adolescent mice. Since neuroimmune signaling is also involved in alcohol abuse, the aim of this study was to assess whether ethanol treatment in adolescence promotes the long-term synaptic and molecular events associated with alcohol abuse and addiction. Using wild-type (WT) and TLR4-deficient (TLR4-KO) adolescent mice treated intermittently with ethanol (3 g/kg) for 2 weeks, we showed that binge-like ethanol treatment in adolescent mice promotes short- and long-term alterations in synaptic plasticity and epigenetic changes in the promoter region of bdnf and fosb, which increased their expression in the mPFC of young adult animals. These molecular events were associated with long-term rewarding and anxiogenic-related behavioral effects, along with increased alcohol preference. Our results further showed the participation of neuroimmune system activation and the TLR4 signaling response since deficient mice in TLR4 (TLR4-KO) are protected against molecular and behavioral alterations of ethanol in the adolescent brain. Our results highlight a new role of the neuroimmune function and open up new avenues to develop pharmacological treatments that can normalize the immune signaling responsible for long-term effects in adolescence, including alcohol abuse and related disorders.

1. Introduction

Adolescence is a developmental stage during which the brain undergoes remodeling and functional changes in synaptic plasticity, as well as neuronal connectivity in different regions, including the cortical and subcortical structures which undergo modifications in white- and gray-matter densities (Gogtay et al., 2004; Sowell et al., 2001). Adolescence is also a time when alcohol use and abuse are initiated, and adolescent binge drinking impacts the developing brain, particularly the prefrontal cortex (PFC), causing cognitive dysfunction and impairing attentional functioning (Koskinen et al., 2011), visuospatial ability (Giancola et al., 1998; Tapert et al., 2002) and executive control (White et al., 2011).

Adolescence binge drinking has also been linked to greater risk-taking and novelty-seeking behavior, and to higher prevalence of drug abuse and risk of relapse (Blakemore, 2008). Both prospective and retrospective human studies have revealed that alcohol use onset is a reliable predictor of later problematic use and dependence on alcohol and other drugs (DeWit et al., 2000; Grant and Dawson, 1997; Hawkins et al., 1997; Labouvie et al., 1997). Stress is also a consistent predictor of increased alcohol use and alcohol-related disorders, and stressors in adolescence may be particularly detrimental (Casement et al., 2015; Green et al., 2010; Lloyd and Turner, 2008; McLaughlin, 2010).

Although the molecular mechanisms of alcohol actions in the adolescent brain are not well understood, we have demonstrated that by activating the innate immune receptor TLR4 signaling in glial cells, ethanol triggers signaling pathways, which induce the release of inflammatory mediators and consequent brain damage (Alfonso-Loeches et al., 2010). Thus, using adolescent rats (Pascual et al., 2007) and mice (Montesinos et al., 2015), we

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demonstrated that intermittent binge-like alcohol treatment triggers pro-inflammatory cytokines and mediators (iNOS and COX-2) in the brain, which causes inflammatory damage in the PFC and impairs synaptic and myelin structures (Montesinos et al., 2015). Binge ethanol exposure during rodent adolescence also leads to the persistent loss of neurogenesis in the hippocampus (Vetreno and Crews, 2015). All these events have been associated with long-lasting cognitive dysfunctions (Montesinos et al., 2015; Pascual et al., 2007; Vetreno and Crews, 2015) and anxiety-like behavior (Vetreno and Crews, 2015; Vetreno et al., 2015) in young adult mice treated with alcohol in adolescence. The role of neuroinflammation and the TLR4 response in the actions of ethanol on the adolescent brain has been supported by data which show that anti-inflammatory compounds (Pascual et al., 2007) or the genetic elimination of TLR4 receptors prevents neuroinflammation, along with synaptic and myelin derangements, long-term cognitive alterations (Montesinos et al., 2015; Pascual et al., 2007), as well as motor impairment (Wu et al., 2012). However, whether inflammation and up-regulation of brain cytokines are also involved in some long-term behavioral effects as anxiety disorders, addiction and alcohol preference remains unknown.

TLRs are key regulators of immune activation in the CNS in response to pathogens and damage, and they initiate the innate and adaptive immunity during infection (Iwasaki and Medzhitov, 2004). Nevertheless, ethanol can activate the TLR4 response triggering cytokines, chemokines and pro-inflammatory signaling which modulate some behavioral effects of alcohol, such as the regulation of voluntary ethanol intake in rodent models (see rev. Blednov et al., 2011; Robinson et al., 2014). Ethanol intake also activates the immune system in humans since changes in the expression of immune-related genes have been reported in human alcoholic brains (Lewohl et al., 2000). Recent studies have also shown the involvement of neuroimmune signaling in the regulation of neuroplasticity, and in learning and memory processes (Williamson and Bilbo, 2013; Yirmiya and Goshen, 2011).

Increasing evidence suggests a role of epigenetic mechanisms in the long-lasting behavioral effects induced by alcohol consumption, including alcohol preference and addiction-related behavioral abnormalities as anxiety disorders (Nestler, 2014; Pandey et al., 2015; Sakharkar et al., 2014). Epigenetic mechanisms can affect the chromatin structure by regulating the expression of the genes involved in addiction in specific brain regions, including the ventral tegmental area and nucleus accumbens (Renthal and Nestler, 2008). For instance, changes in histone acetylation in the promoter region of cFos, Cdk5 and FosB have been associated with ethanol-induced place conditioning in adult rats treated with ethanol in adolescence (Pascual et al., 2012). One major encephalic structure involved in the expression of emotional states and anxiety-like behaviors is the prelimbic medial prefrontal cortex (mPFC) (Saitoh et al., 2014; Vialou et al., 2014). This brain area has bidirectional connections with a wide range of neuromodulatory systems (e.g. dorsal raphe, ventral tegmental area, locus coeruleus) and plays an important role in adaptive responses to rewarding and stressful events (Euston et al., 2012). Recent studies have suggested that repeated binge and withdrawal episodes in young adults may sensitize the mPFC to additional dysfunction thus facilitating the transition to alcohol dependence (George et al., 2012). However, the role of mPFC in the long-term synaptic remodeling and behavioral changes induced by binge-like ethanol treatment in adolescence is uncertain.

Here we report that intermittent binge-like ethanol treatment in adolescence induces long-term aberrant synaptic remodeling, increases histone acetylation at the promotor region of the bdnf and fosb genes and up-regulates their expression in the mPFC of young adult animals. These events were associated with long-term rewarding and anxiogenic-related behavior effects, along with alcohol preference, in young adult mice treated with alcohol in adolescence. Our findings also provide evidence for the participation of the neuroimmune system and TLR4 signaling response in the neurochemical and behavioral dysfunctions caused by alcohol abuse in adolescence since TLR4-deficient mice (TLR4-KO) are protected against the neurochemical and behavioral actions of ethanol on the adolescent brain.

2. Materials and methods

2.1. Animals and treatments

Female C57BL/6 WT (Harlan Ibérica, Barcelona, Spain) and TLR4 knockout (KO) mice (C57BL/6 background, kindly provided by Dr. S. Akira, Osaka University, Suita, Japan) aged 30 days were used. All the animals were kept under controlled light and dark (12/12 h), temperature (23 °C), and humidity (60%) conditions. All the experimental procedures were carried out in accordance with the guidelines approved by the European Communities Council Directive (86/609/ECC) and by Spanish Royal Decree 1201/2005. The animal experiments were also approved by the Ethical Committee of Animal Experimentation of the Príncipe Felipe Research Center (Valencia, Spain). Female mice were used because previous studies showed more ethanol-induced inflammatory damage in female than in male mice (Alfonso-Loeches et al., 2013), and also to correlate the present results with previous findings on adolescent female mice exposed to binge-like ethanol treatment (Montesinos et al., 2015).

For the intermittent ethanol treatment, WT and TLR4-KO mice were housed (4 animals/cage) and maintained with water and solid diet *ad libitum*. Morning doses of either saline or 25% (v/v) ethanol (3 g/kg) in isotonic saline were administered intraperitoneally to 30-day-old mice on 2 consecutive days, with 2-day gaps with no injections, for 2 weeks (PND30 to PND43), as previously described (Pascual et al., 2007). Some mice were maintained without alcohol treatment until postnatal day (PND) 65. A single dose of ethanol to adolescent mice resulted in a peak of BECs of 178.23 ± 16.75 mg/dL at 30 min post-injection. Some animals were sacrificed by decapitation 24 h after the last (8th) ethanol or saline administration (PND44, short-term ethanol effects), or after 3 weeks upon ethanol or saline administration (PND 65, long-term ethanol effects). Brains from adolescent (PND 44) and young adult mice were collected, and the mPFC were dissected and stored at −80 °C until use. Behavioral tests were performed with young adult mice (PND 65).

2.2. Western blot analysis

The Western blot technique was performed in the mPFC tissue lysates, as described elsewhere (Fernandez-Lizarbe et al., 2009). The primary antibodies used were: acetyl H3 lysine 9 (Ac-H3 K9) FosB, ΔFosB, p-CREB, NF-κB p-p65, tri-methyl H3 lysine 4 (3me-H3 K4) (Cell Signaling Technology, Leiden, The Netherlands); acetyl histone 4 (H4) lysine 5 (Ac-H4 K5), acetyl H4 lysine 12 (Ac-H4 K12), cyclin-dependent kinase-5 (Cdk5), major histocompatibility complex class II (MHCII), cluster of differentiation molecule 11b (CD11b) (Abcam, Cambridge, UK), brain-derived neurotrophic factor (BDNF) (Santa Cruz Biotechnology, Madrid, Spain), GluR1 and NR1 (Millipore Iberica, Madrid, Spain). Membranes were washed, incubated with the corresponding HRP-conjugated secondary antibodies and developed with the ECL system (ECL Plus; Thermo Fisher Scientific, Illinois, USA). All the membranes were stripped and incubated with GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (Chemicon, California, USA), postsynaptic density protein 95 (PSD95), total H3 (T-H3), total H4 (T-H4) (Abcam) or the

corresponding total form of NF-κB p65 (Santa Cruz Biotechnology) or cAMP response element-binding protein (CREB) (Cell Signaling Technology) as loading controls. Band intensity was quantified with the ImageJ 1.44p analysis software (National Institutes of Health, USA). The densitometry analysis is shown in arbitrary units normalized to the respective loading control. Table 1S (Supplementary information) shows the basal levels of the different proteins evaluated in WT and TLR4-KO mice. No significant differences were observed in the basal levels of the analyzed proteins when compared with the saline-treated adolescent (PND 44) or young adult (PND 65) WT and TLR4-KO mice.

2.3. RNA isolation and quantitative RT-PCR

mPFC tissue was lysed in 1 mL of Tri-Reagent solution (Sigma–Aldrich, Madrid, Spain) and RNA was isolated according to the manufacturer's instructions. The amount of purified RNA was estimated in a Nanodrop device (ND-1000, NanoDrop Technologies, Thermo Fisher Scientific). Next 2 μg of each sample underwent DNAase treatment (Life Technologies, Madrid, Spain) and were reverse-transcribed with the Transcriptor First Strand cDNA Synthesis kit (Roche, Madrid, Spain). cDNA was amplified in triplicate in a rapid thermal cycler (LightCycler Instrument; Roche) in 10 μL of LightCycler 480 SYBR Green I Master (Roche) using 0.5 μM of each oligonucleotide. Primers were designed by the Primer Blast program (NCBI), covering at least one intron, whenever possible, in order to avoid genomic DNA amplification. Samples underwent 40–50 RT-PCR cycles (2 min at 95 °C; 10 s at melting temperature and 18 s at 72 °C). For each primer pair, melting temperature was empirically optimized. The sequences of the primer pairs used in this study were: bdnf exon, 5′-TAC CTG GAT GCC GCA AAC AT-3′ (forward) and 5′-GCT GTG ACC CAC TCG CTA AT-3′ (reverse); bdnf intron, 5′-CGC CAA GGT GGA TGA GAG TT-3′ (forward) and 5′-TTC GGC TTT GCT CAG TGG AT-3′ (reverse); fosB, 5′-ACC TGA CGG CTT CTC TCT TTA C-3′ (forward) and 5′-GTG AGG ACA AAC GAG GAA GTG-3′ (reverse); fosB + ΔfosB, 5′-AGA GCC AGG CCT AGA AGA CC-3′ (forward) and 5′-ACG GTT CCT GCA CTT AGC TG-3′ (reverse); cyclophilin A, 5′-GTC TCC TTC GAG CTG TTT GC-3′ (forward) and 5′-GAT GCC AGG ACC TGT ATG CT-3′ (reverse). The mRNA levels of housekeeping gene cyclophilin A were used as an internal control for normalization. The relative quantification of the PCR products was done using the LightCycler 480 software (Roche). Bdnf mRNA levels were assessed by amplifying an exonic region (bdnf exon), which was common for the distinct bdnf isoforms, and by amplifying an intronic region (bdnf intron) to screen possible genomic DNA contamination. Δfosb mRNA levels were calculated as previously described (Jurado et al., 2007). Briefly, fosb and fosb + Δfosb total amounts of mRNA were calculated and the difference was assigned as the amount of the Δfosb transcript.

2.4. Synaptosome fractionation

Briefly, mPFC tissue was homogenized in sucrose buffer (0.32 M sucrose, 10 mM Tris pH 7) and clarified at 3,000 rpm for 15 min. Supernatants were centrifuged at 11,000 rpm for 20 min. The resulting pellet was resuspended in sucrose buffer and submitted to a Ficoll-400 (Sigma–Aldrich) separation gradient (4%, 6%, and 13%). After centrifuging at 25,500 rpm for 1 h, the synaptosome enriched fraction (interphase between the 6% and 13% phases) was collected and lysed by adding a hypo-osmotic solution for 1 h at room temperature (0.5 mM DTT, 20 mM Tris pH 8.5). To obtain the synaptosome preparation, this solution was further centrifuged at 24,000 rpm for 30 min and the obtained pellet was resuspended in hypo-osmotic solution and used for protein detection by Western blot.

2.5. Chromatin Immunoprecipitation assay and quantification by real-time PCR

The EpiQuik™ Tissue Acetyl-Histone H4 ChIP kit (Epigentek, NY, USA) was used to perform ChIP assays, following the manufacturer's instructions. The H4 acetylation levels at each gene promoter of interest were determined by measuring the amount of that gene in chromatin immunoprecipitates by real-time PCR. Specific primers were designed to amplify proximal promoter regions, <200 bp long. For fosb: 5′-GGT CCC GGA GGC ATA AAT TC-3′ (forward) and 5′-TCA CGC CTC CAA GAA GAA GAA-3′ (reverse); bdnf promoter IV: 5′-GCG CGG AAT TCT GAT TCT GG-3′ (forward) and 5′-AAA GTG GGT GGG AGT CCA-3′ (reverse). Finally, β-tubulin was used as a control: 5′-TAG AAC CTT CCT GCG GTC GT-3′ (forward) and 5′-TTT TCT TCT GGG CTG GTC TC-3′ (reverse). Input or total DNA (non-immunoprecipitated) and immunoprecipitated DNA were PCR-amplified in triplicate in the presence of LightCycler 480 SYBR Green I Master (Roche). Ct values from each sample were obtained with the Lightcycler 480 software (Roche). Relative quantification of amplified template was performed as described by (Tsankova et al., 2004).

2.6. Behavioral testing

2.6.1. Open field behavior

Spontaneous locomotor behavior was recorded in an open field (30 × 30 × 35 cm) for a 10-min period by an automated tracking control (EthoVision 3.1; Noldus Information Technology, VA, USA). The studied parameters are the following: distance (cm), time spent in the center (s), number of times visiting (frequency) the center and speed (cm/s).

2.6.2. Elevated plus maze

Elevated plus maze (EPM) tests were carried out by essentially following the procedure described by (Daza-Losada et al., 2009). Briefly, EPM consisted in two open arms (OA) (30 × 5 × 0.25 cm) and two closed arms (CA) (30 × 5 × 15 cm) with a central platform (5 × 5 cm). The entire apparatus was raised 45 cm above floor level. The measurements taken during the test period were frequency of entries, and time and percentage of the time spent in each section of the apparatus.

2.6.3. Conditioning place preference

Acquisition of conditioning place preference (CPP) consisted in three phases and took place during the dark cycle following an unbiased procedure in initial spontaneous preference terms (for a detailed explanation of the procedure, see (Daza-Losada et al., 2007). Briefly, during preconditioning (Pre-C), the time spent by the animal in each compartment over a 15-min period was recorded. Animals which showed strong unconditioned aversion or preference for any compartment were excluded from the study. In the second phase (conditioning), animals underwent two pairings per day. First, they received an injection of physiological saline before being confined to the vehicle-paired compartment for 30 min. After a 4-h interval, they received cocaine immediately before being confined to the drug-paired compartment for 30 min. In the third phase (post-conditioning; Post-C), the time spent by the untreated mice in each compartment was recorded over a 15-min period.

All the groups in which CPP was confirmed subsequently underwent an extinction procedure. Animals were subjected to an extinction session every 3 days, which consisted in placing them in the apparatus for 900 s until the time spent in the drug-paired compartment was similar to that of the Pre-C phase. CPP was considered extinguished when there was no significant difference between the time spent in the drug-paired compartment during

the extinction session and that spent in the same compartment during Pre-C (Student's *t*-test). The effects of a priming dose of cocaine (1.5, 0.75, 0.375, and 0.187 mg/kg) were evaluated 24 h after confirming extinction. Reinstatement tests were the same as for Post-C, except that animals were tested 15 min after administering the respective dose of drug employed in the conditioning phase.

2.6.4. Ethanol consumption assessment: two bottle choice paradigm

The two-bottle choice test was adapted from (Blednov et al., 2011). One week before starting the experiment (PND 58), mice were singly placed into cages where two drinking tubes that contained water were available continuously for habituation purposes. At PND 65, mice were offered a choice between 3% ethanol (v/v) and water for 2 days. Tube positions were changed daily to control for position preferences. The volume of consumed ethanol and water was measured every 24 h per mouse. Ethanol preference was calculated as the volume of ethanol consumed per total liquid intake and was averaged for each ethanol concentration. Immediately following 3% ethanol, a choice between 6% (v/v) ethanol and water was offered for 2 days and, finally, 10% (v/v) ethanol versus water for 2 days. Throughout the experiment, food was available *ad libitum*. Evaporation/spillage estimates were calculated by placing two bottles in an empty cage, one contained water and the other contained the appropriate ethanol solution.

2.6.5. Preference for non alcohol tastants: the two-bottle choice paradigm

The young adult WT and TLR4-KO mice treated with or without ethanol were tested for saccharin and quinine consumption. One tube always contained water, and the other contained the tastant solution. Mice were serially offered saccharin (0.033% and 0.066%) or quinine hemisulfate (0.05 and 0.1 mM). Preference was calculated. Each concentration was offered for 4 days, and bottle position was changed daily. For each tastant, the low concentration was always presented first, followed by the higher concentration.

2.7. Statistical analysis

All the statistical analyses were performed with GraphPad Prism v5.01 (GraphPad Software Inc., CA, USA). Data represent mean ± SEM. The statistical significance for the Western blots, qRT-PCR and ChIP was determined by a two-way ANOVA. For each genotype, the data from the open field, the EPM and the two-bottle choice test were analyzed by an ANOVA with one "between" subject variable, "Treatment", at two levels (S or EtOH). CPP was analyzed by a mixed ANOVA with the same one "between" variable, plus a "within" subject variable, "Days", at two levels (Pre- and Post-test). The differences between the extinction and reinstatement tests were analyzed by a Student's *t*-test. Bonferroni adjustment was employed to make post hoc comparisons. Values of $p < 0.05$; $p < 0.01$; $p < 0.001$ were considered statistically significant.

3. Results

3.1. Intermittent binge-like ethanol treatment causes long-term synaptic remodeling in the mPFC

We reported that intermittent binge-like ethanol drinking in adolescence alters synaptic terminals in the PFC and causes cognitive effects (Montesinos et al., 2015). However, whether this drinking pattern in adolescence can induce long-term aberrant synaptic remodeling in the mPFC, inducing anxiety-like behaviors and alcohol consumption (Saitoh et al., 2014; Vialou et al., 2014), is presently unknown. To answer this question, we measured the ratio of the GluR1 AMPA receptor subunit and the NR1 NMDA receptor subunit (GluR1/NR1 ratio) levels in the synaptosome-enriched fractions from the mPFC as a marker of synaptic remodeling in glutamatergic synapses (Menard et al., 2015; Self, 2014). We found that while the NR1 levels were significantly up-regulated in the mPFC of WT mice 24 h after the last ethanol treatment, the GluR1/NR1 ratio did not change (Fig. 1A). However, the GluR1 levels and the GluR1/NR1 ratio significantly increased in the synaptosome-enriched fractions from the mPFC of young adult mice exposed to ethanol in adolescence, which suggests that intermittent alcohol treatment can promote long-term glutamatergic synaptic plasticity. Notably, the GluR1 and NR1 levels in the synaptosome-enriched fractions from the TLR4-KO mPFC showed no differences between the saline and the ethanol treatment (Fig. 1A), which supports the contribution of TLR4 to the neuronal synaptic remodeling induced by ethanol treatment in adolescent mice. The two-way ANOVA analyses for short-term effects of NR1 revealed a significant effect of treatment [$F(1,12) = 28.39$, $p < 0.001$], genotype [$F(1,12) = 7.86$, $p < 0.05$] and interaction [$F(1,12) = 7.86$, $p < 0.05$]. Likewise, for long-term effects of GluR1, the two-way ANOVA analyses indicated a significant effect of treatment [$F(1,12) = 6.61$, $p < 0.05$], genotype [$F(1,12) = 4.80$, $p < 0.05$] and interaction [$F(1,12) = 4.80$, $p < 0.05$] and for GluR1/NR1 there is a significant effect of genotype [$F(1,12) = 11.52$, $p < 0.01$] and interaction [$F(1,12) = 11.52$, $p < 0.01$].

To evaluate the potential contribution of microglia activation and TLR4 signaling to the long-term synaptic remodeling observed in the young adult mice exposed to alcohol in adolescence, we assessed the levels of some microglial markers, MHCII and CD11b (Akiyama and McGeer, 1990; Benveniste et al., 2001), and the TLR4 signaling (e.g. NF-κB-p-p65) in the mPFC of adolescent (short-term) and young (long-term) adult mice. Fig. 1B shows that binge-like ethanol treatment increased the phosphorylated levels of NF-κB-p65 (p-p65/p65) in the short- and long-term in WT mice. Interestingly, the levels of the microglia markers, MHCII and CD11b, increased in the mPFC of both the adolescent and young adult WT mice treated with alcohol in adolescence (Fig. 1B), which indicates that alcohol triggers microglia activation and that this activation persisted in the adult brain. No significant changes were observed in the levels of MHCII, CD11b and NF-κB-p65 when compared to the ethanol-treated and untreated TLR4-KO mice on short- or long-term treatments (Fig. 1B). The two-way ANOVA analyses for short-term effects of p-p65 revealed a significant effect of treatment [$F(1,16) = 8.30$, $p < 0.05$], for MHCII there is a significant effect of treatment [$F(1,12) = 17.03$, $p < 0.01$], genotype [$F(1,12) = 24.08$, $p < 0.001$] and interaction [$F(1,12) = 24.08$, $p < 0.001$] and for CD11b there is a significant effect of treatment [$F(1,16) = 7.44$, $p < 0.05$]. For long-term effects of p-p65 the two-way ANOVA analyses revealed a significant effect of treatment [$F(1,16) = 6.93$, $p < 0.05$], for MHCII there is a significant effect of treatment [$F(1,12) = 11.24$, $p < 0.01$] and for CD11b there is a significant effect of genotype [$F(1,16) = 5.05$, $p < 0.05$] and interaction [$F(1,16) = 5.05$, $p < 0.05$].

3.2. Long-term alterations in mPFC synaptic remodeling are associated with changes in histone acetylation in the adolescent and young adult mice treated with ethanol in adolescence

The conformational state of chromatin and histone modifications, including histone acetylation-deacetylation, is regulated in adult neurons in association with learning and memory processes (Levenson et al., 2004), and in response to drugs (Kumar et al., 2005). Acetylation of histones H3 and H4 at gene promoters is

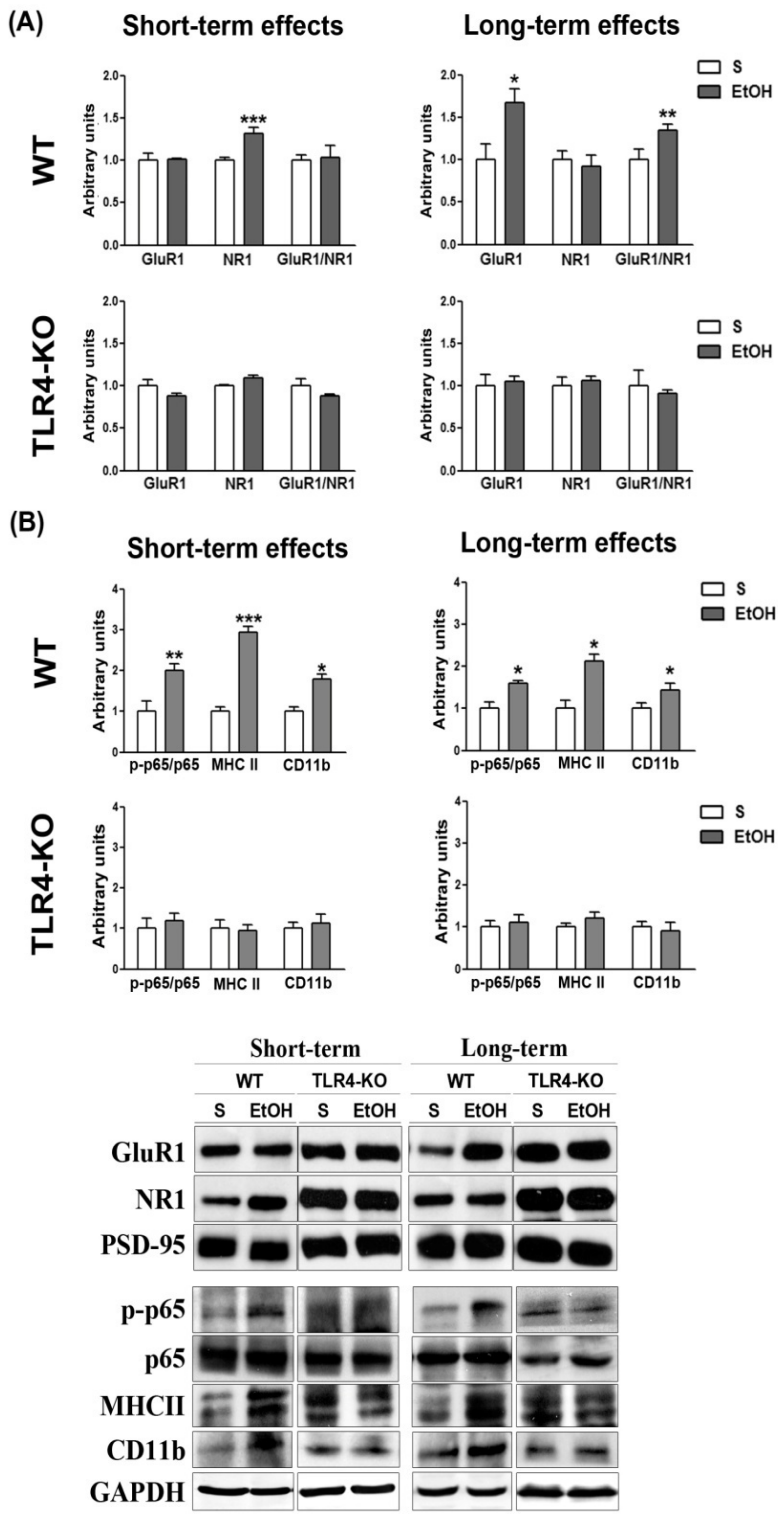

Fig. 1. Effects of intermittent ethanol treatment on synaptic remodeling. (A) Levels of GluR1 and NR1 were measured in synaptosome-enriched fractions from the mPFC of WT and TLR4-KO mice 24 h (short-term) or 3 weeks (long-term) after ethanol (EtOH) or saline (S) intermittent treatment. PSD95 represents the synaptosome loading control. (B) The immunoblot analysis and quantification of p-p65 (NF-κB), MHCII and CD11b in the mPFC of adolescent (short-term effects) and young adult (long-term effects) WT and TLR4-KO mice treated with ethanol or saline (S) in adolescence. GAPDH and p65 represent the loading control. A representative immunoblot of each protein is shown. Data represent mean ± SEM, $n = 5$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to their respective saline-treated group.

linked to increased gene activity, while deacetylation is linked with the suppression and silencing of gene activity.

Our previous studies have demonstrated that binge-like ethanol treatment in adolescent rats induces acetylation changes in the adolescent brain (Pascual et al., 2012). Therefore, to gain further insights into the mechanisms involved in the long-term synaptic changes observed in the mPFC of adolescent mice treated with binge-like ethanol treatment, we assessed the levels of the acetylation of the histones H3 (K9) and H4 (K5, K12) and the tri-methylation of H3 (K4) in the mPFC of WT and TLR4-KO adolescent mice. Fig. 2 illustrates that while the acetylation levels at H3 (K9) and H4 (K5, K12) were up-regulated, the tri-methylation of the H3 (K4) levels significantly decreased in the adolescent mPFC after ethanol treatment (short-term effects) compared to their saline counterparts. We then evaluated whether the changes in the histone acetylation or methylation observed in the mPFC of adolescent mice were maintained in the young adult mice treated with alcohol in adolescence (long-term effects). The results in Fig. 2 reveal that whereas changes in the acetylation of H3 (K9) and H4 (K12), along with the tri-methylation of H3 (K4), recovered in young adult mice, the levels of acetylated H4 (K5) were still elevated in the young adult mice treated with ethanol in the adolescence. Finally, we also assessed the potential role of the TLR4 response in the ethanol-induced epigenetic changes in the mPFC of adolescent mice. As noted in Fig. 2, no significant changes were observed in the mPFC of either the adolescent or young adult TLR4-KO mice treated with ethanol in adolescence compared to their saline counterparts. This finding supports the role of TLR4 signaling in

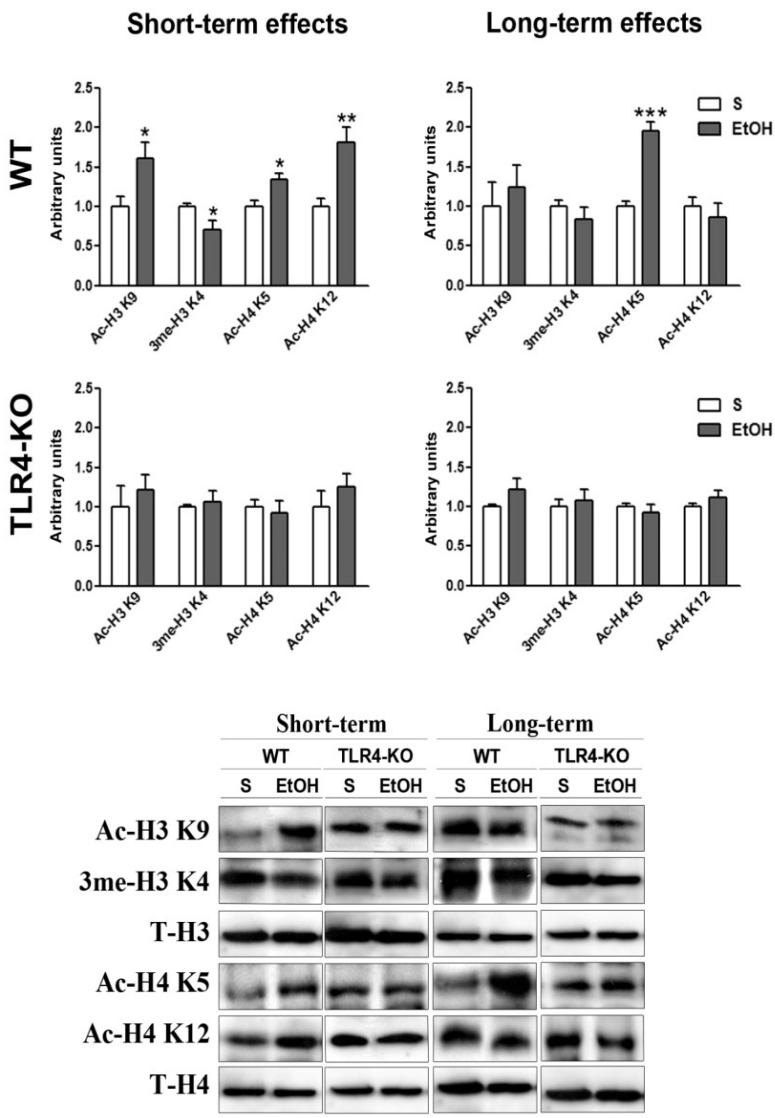

Fig. 2. Effects of intermittent ethanol treatment on post-translational modifications on H3 and H4 in the mPFC of adolescent (short-term, 24 h after the last treatment administration) and young adult (long-term, 3 weeks after the last treatment administration) WT and TLR4-KO mice treated with ethanol (EtOH) or saline (S) in adolescence. Analysis and quantification of the Ac-H3 K9, Ac-H4 K5, Ac-H4 K12, 3me-H3 K4 protein levels. Total H3 (T-H3) and total H4 (T-H4) represent the loading control. A representative immunoblot of each protein is shown. Data represent mean ± SEM, $n = 8$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to their respective saline-treated group.

the epigenetic alterations caused by intermittent ethanol administration. The two-way ANOVA analyses for short-term effects of acetylated H3 (K9) revealed a significant effect of treatment [$F(1,16) = 5.66$, $p < 0.05$], for the tri-methylated H3 (K4) there is a significant effect of genotype [$F(1,16) = 6.06$, $p < 0.05$] and interaction [$F(1,16) = 6.06$, $p < 0.05$], for the acetylated H4 (K5) there is a significant effect of genotype [$F(1,16) = 5.73$, $p < 0.05$] and interaction [$F(1,16) = 5.73$, $p < 0.05$] and for the acetylated H4 (K12) there is a significant effect of treatment [$F(1,16) = 9.84$, $p < 0.01$]. Likewise, the two-way ANOVA analyses for long-term effects of acetylated H4 (K5) revealed a significant effect of treatment [$F(1,16) = 30.19$, $p < 0.001$], genotype [$F(1,16) = 40.94$, $p < 0.001$] and interaction [$F(1,16) = 40.94$, $p < 0.001$].

3.3. Intermittent binge-like ethanol treatment in adolescence impairs the BDNF, FosB and ΔFosB-Cdk5 pathways in the mPFC and triggers epigenetic long-term alterations

Long-term changes in addiction have been associated with neural plasticity, and with the activation of both BDNF and the transcriptional factors, ΔFosB and CREB (cAMP responsive element-binding protein), which are involved in synaptic structure regulation (Hope et al., 1994; Nestler, 2001). Therefore, to further evaluate the molecular changes in the aberrant synaptic plasticity induced by binge-like ethanol treatment in adolescence, we assessed the gene and protein expression of neurotrophic factor BDNF and the transcription factors CREB and ΔFosB in the mPFC of the ethanol-treated adolescent WT mice. Our results showed that binge-like ethanol treatment up-regulated the BDNF levels (both the precursor and the mature form) and increased the phosphorylated levels of CREB (p-CREB/CREB), which indicates that alcohol treatment activates the CREB–BDNF pathway (Fig. 3A, short-term). Notably, we also observed that ethanol treatment up-regulated the protein expression of FosB, ΔFosB and Cdk5 (a downstream protein synthesized by ΔFosB activation) in the mPFC of the ethanol-treated WT mice (Fig. 3A).

We next evaluated the potential long-term changes in the transcriptional factors and proteins involved in synaptic plasticity in young adult mice after 3 weeks without ethanol (Fig. 3B). We found that the protein levels of FosB, ΔFosB, Cdk5 and BDNF, but not of p-CREB/CREB, remained high in the mPFC of the young adult WT mice treated with alcohol in adolescence (Fig. 3B, long-term), which implies permanent long-term alterations. In addition, the mRNA levels of FosB, ΔFosB and BDNF increased in the young adult WT mice treated with ethanol (Fig. 4A). Remarkably, neither the changes induced by binge-ethanol treatment in the proteins associated with synaptic plasticity in adolescence nor long-term alterations were observed in TLR4-KO mice (Figs. 3A, B and 4A). The two-way ANOVA analyses for the short-term effects of ΔFosB revealed a significant effect of treatment [$F(1,28) = 5.40$, $p < 0.05$], genotype [$F(1,28) = 13.06$, $p < 0.01$] and interaction [$F(1,28) = 13.06$, $p < 0.01$], for Cdk5 there is a significant effect of treatment [$F(1,22) = 10.48$, $p < 0.01$] and interaction [$F(1,22) = 10.48$, $p < 0.01$], and for FosB there is a significant effect of treatment [$F(1,23) = 10.04$, $p < 0.01$], genotype [$F(1,23) = 5.98$, $p < 0.05$] and interaction [$F(1,23) = 5.98$, $p < 0.05$]. The two-way ANOVA analyses of pre-BDNF revealed a significant effect of treatment [$F(1,16) = 6.42$, $p < 0.05$] and for mat-BDNF there is a significant effect of genotype [$F(1,16) = 7.80$, $p < 0.05$] and interaction [$F(1,16) = 7.80$, $p < 0.05$]. For the long-term effects of ΔFosB, the two-way ANOVA analyses revealed a significant effect of treatment [$F(1,28) = 16.04$, $p < 0.001$], genotype [$F(1,28) = 11.82$, $p < 0.01$] and interaction [$F(1,28) = 12.25$, $p < 0.01$], for Cdk5 there is a significant effect of treatment [$F(1,21) = 7.89$, $p < 0.05$], genotype [$F(1,21) = 5.52$, $p < 0.05$] and interaction [$F(1,21) = 5.52$, $p < 0.05$], and for FosB there is a significant effect of genotype [$F(1,24) = 5.56$, $p < 0.05$] and interaction [$F(1,24) = 5.56$, $p < 0.05$]. The two-way ANOVA analyses of p-CREB indicated a significant effect of genotype [$F(1,16) = 4.68$, $p < 0.05$] and interaction [$F(1,16) = 4.68$, $p < 0.05$] and for mat-BDNF there is a significant effect of treatment [$F(1,16) = 6.05$, $p < 0.05$], genotype [$F(1,16) = 7.82$, $p < 0.05$] and interaction [$F(1,16) = 7.82$, $p < 0.05$]. In addition, the two-way ANOVA analyses for the mRNA levels of FosB revealed a significant effects of genotype [$F(1,14) = 10.01$, $p < 0.01$] and interaction [$F(1,14) = 10.01$, $p < 0.01$], for ΔFosB there is a significant effect of treatment [$F(1,16) = 4.76$, $p < 0.05$], genotype [$F(1,16) = 5.93$, $p < 0.05$] and interaction [$F(1,16) = 5.93$, $p < 0.05$] and for BDNF there is a significant effect of treatment [$F(1,13) = 31.05$, $p < 0.001$], genotype [$F(1,13) = 22.81$, $p < 0.001$] and interaction [$F(1,13) = 22.81$, $p < 0.001$].

Finally, we wondered whether the histone acetylation levels in the promoter region of the *bdnf* and *fosb* genes could be responsible for their up-regulation at both the mRNA and protein levels. To answer this question, we performed ChIP assays with an antibody against acetylated H4 and we quantified the amount of DNA associated with the modified histone by real-time PCR. Our data showed a significant increase in H4 acetylation at the bdnf (∼1.2-fold) and fosb (∼1.45-fold) promoters in the mPFC of the young adult WT animals treated with ethanol in adolescence compared to their saline counterparts. These results indicate long-term effects induced by ethanol treatment during adolescence (Fig. 4B). However, no significant changes were detected between the ethanol-treated or the saline-treated TLR4-KO young adult mice. The two-way ANOVA analyses of fosb revealed a significant effect of treatment [$F(1,12) = 4.90$, $p < 0.05$] and for bdnf there is a significant effect of treatment [$F(1,12) = 4.88$, $p < 0.05$] and genotype [$F(1,12) = 4.79$, $p < 0.05$].

3.4. Intermittent ethanol treatment causes long-term behavioral alterations associated with the TLR4 response

Since bdnf and fosB are candidate genes associated with vulnerability to drug addiction (Nestler, 2001; Tsai, 2007), we evaluated whether ethanol treatment in the adolescence could induce increased vulnerability to develop addictive behavior, and whether TLR4 is also involved in these effects. For this objective, we performed the two-bottle choice paradigm to test alcohol drinking preference, as well as the place conditioning paradigm, which has been widely used to measure the conditioned rewarding effects of many drugs of abuse, including cocaine (Tzschentke, 2007).

To assess the alcohol preference of the WT or TLR4-KO mice treated, or not, with ethanol in adolescence, we performed the two-bottle choice paradigm. The ANOVA revealed a significant effect of the variable ethanol preference [$F(1,40) = 12.20$, $p < 0.01$] on WT mice. The intermittently ethanol-treated WT mice exhibited greater ethanol preference than their saline counterparts ($p < 0.05$ for ethanol 6%; $p < 0.01$ for ethanol 10%). No significant difference was detected between the saline or ethanol-treated TLR4-KO mice (Fig. 5A).

Since differences in alcohol consumption between mutant mice could be related to changes in taste (Blednov et al., 2008), we also studied the consumption of saccharin and quinine following a 24-h two-bottle choice test in the young adult WT and TLR4-KO mice treated or not with ethanol in adolescence in order to determine if taste could account for the reduced alcohol consumption in the ethanol-treated TLR4-KO mice. Fig. 5B shows that all the mice distinguished the two taste solutions at both concentrations from water drinking. Thus the ethanol-treated and the untreated WT mice preferred saccharin solution, but avoided quinine solution. Similar results were obtained between the untreated WT and TLR4-KO mice. These results suggest that both genotypes perform

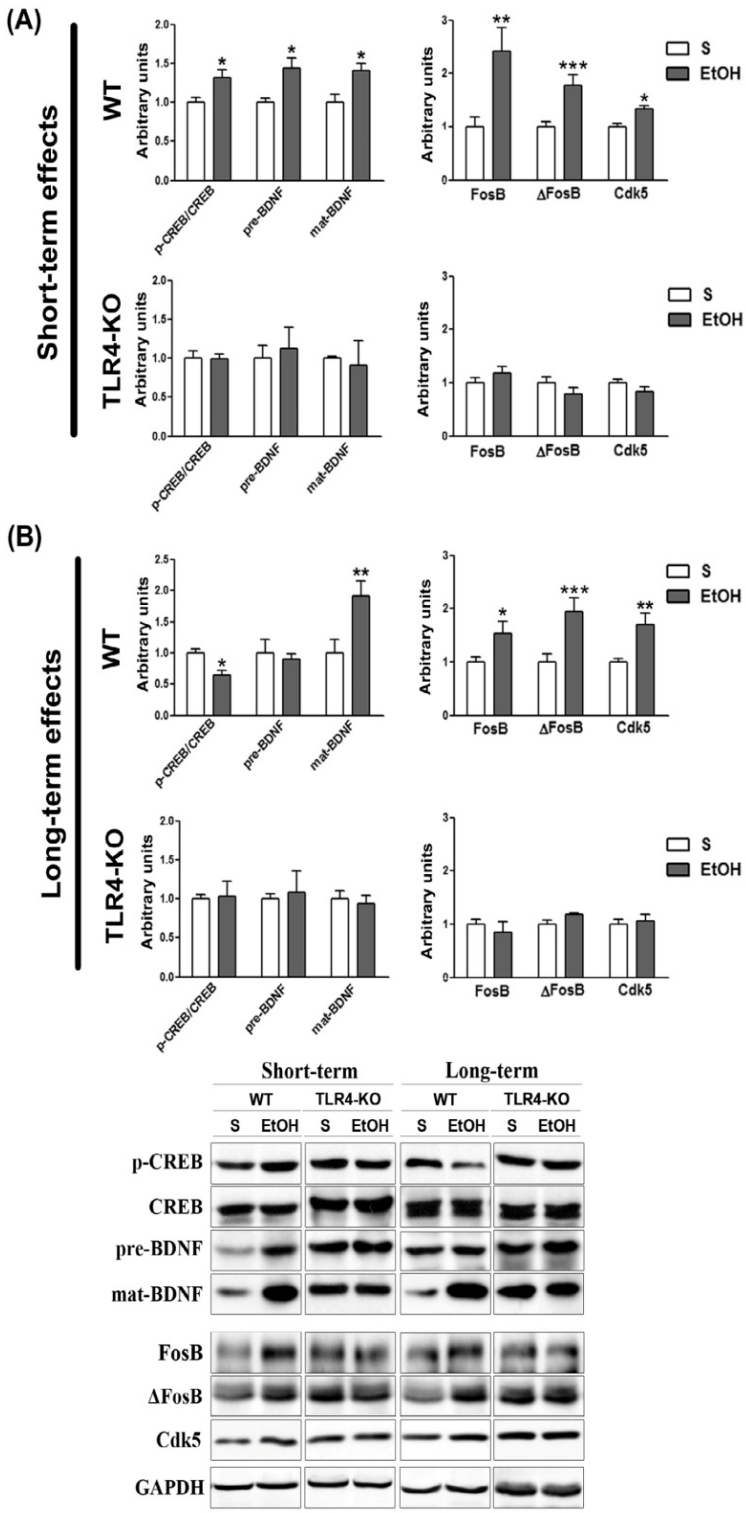

Fig. 3. TLR4 involvement in CREB–BDNF, FosB and ΔFosB-Cdk5 activation induced by intermittent ethanol treatment in adolescence. The immunoblot analysis and quantification of p-CREB, CREB, mature BDNF (mat-BDNF, 14 kDa), precursor BDNF (pre-BDNF, 32 kDa), FosB, ΔFosB, Cdk5 in the mPFC of adolescent (short-term effects) (A) and young adult (long-term effects) (B) WT and TLR4-KO mice treated with ethanol or saline (S) in adolescence. GAPDH and CREB represent the loading control. A representative immunoblot of each protein is shown. Data represent mean ± SEM, $n = 8$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to their respective saline-treated group.

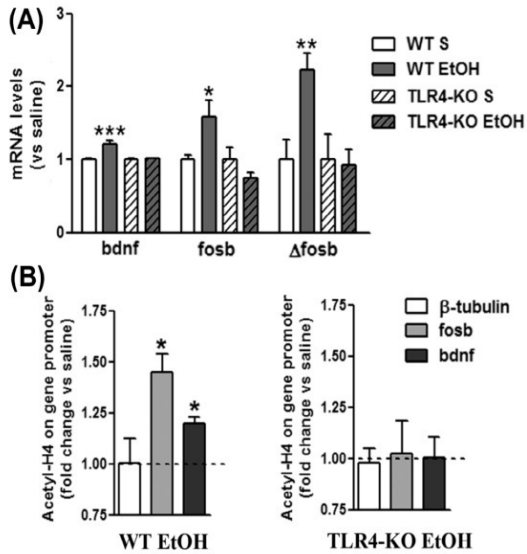

Fig. 4. TLR4 elimination prevents the up-regulation of the expression levels of bdnf, fosb and Δfosb in the mPFC of young adult mice associated with H4 acetylation after an intermittent ethanol-treatment in adolescence. (A) Quantification of the mRNA levels of bdnf, fosb and Δfosb in the mPFC of the young adult WT and TLR4-KO mice treated intermittently either with ethanol (EtOH) or saline (S) in adolescence. Data represent mean ± SEM, $n = 6$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to their respective saline counterparts. (B) Acetylation levels of H4 in the gene promoter of β-tubulin, bdnf and fosb in the mPFC of the young adult WT or TLR4-KO mice treated in adolescence with ethanol (EtOH) or saline (S). To assess antibody binding specificity, we immunoprecipitated chromatin samples with non immune IgG, which precipitated negligible levels of the genes under study (data not shown). As an internal control, we measured the H4 acetylation levels at the promoter of the β-tubulin gene, whose expression levels were not changed by ethanol treatment. As expected, H4 acetylation at this promoter did not differ between groups. Data represent mean ± SEM, $n = 4$–5 mice/group. $^{*}p < 0.05$, compared to their respective saline counterparts.

a comparable orosensory function, and the increase in the ethanol-treated WT mice's ethanol preference could be due to a specific alcohol reward effect.

We evaluated whether intermittent ethanol treatment in adolescence affects the rewarding effects of cocaine. For this purpose, we assessed the CPP induced by cocaine (3 mg/kg) in the young adult mice treated with ethanol in adolescence (Fig. 5C). In the WT animals, the ANOVA revealed a significant effect of the variable days [$F(1,28) = 16.65$, $p < 0.05$], and the interaction days × treatment [$F(1,28) = 9.51$, $p < 0.001$]. The WT ethanol-exposed group spent more time in the drug-paired compartment during the Post-C test than during the Pre-C test ($p < 0.001$). The WT mice exposed to ethanol in adolescence also spent more time in this compartment in Post-C than the WT saline-exposed mice ($p < 0.001$). As previously reported (Montagud-Romero et al., 2014), ethanol exposure in adolescence increases the conditioned rewarding effects of cocaine since the WT ethanol-exposed mice required 12 sessions for preference to be extinguished, and a priming dose of 1.5 mg/kg of cocaine reinstated preference ($p < 0.001$), which required 7 more sessions before being extinguished. Further priming doses (0.75 and 0.375 mg/kg of cocaine) reinstated preference after 4 and 10 extinction sessions, respectively. These results confirm that ethanol exposure in adolescence increases the rewarding effects of cocaine and also increases vulnerability to reinstate preference for the environment cues associated with cocaine effects. However, in TLR4-KO animals, the ANOVA did not reveal any significant effect between the ethanol- and saline-treated animals because neither group developed CPP. Taken together these data suggest that intermittent ethanol treatment promotes changes in motivation by altering the reinforcing effects of cocaine in young adult animals.

3.5. Changes in histone acetylation in the mPFC of the adolescent mice treated intermittently with alcohol in adolescence were associated with both long-lasting anxiety-related behavior and TLR4 function

Several studies have supported the functional connectivity of the mPFC with the amygdala and the role of the mPFC in stress regulation and anxiety-like behaviors (Vialou et al., 2014). Dysregulation of the mPFC impairs executive control over motivated behavior and appears to be an early index of neuroadaptation that leads to excessive binge drinking (George et al., 2012). Elimination of TLR4 has also been reported to prevent the anxiety-related behavior associated with chronic alcohol consumption (Pascual et al., 2015). We therefore set out to analyze the role of TLR4 in long-lasting anxiety-related behavior 3 weeks after an intermittent ethanol treatment in adolescence administered to WT and TLR4-KO mice, along with their saline counterparts. Two anxiety-related behavioral paradigms, the open field test and the EPM test, were assessed.

The open field test results are shown in Table 1. The ANOVA of the WT mice data showed an effect of treatment for total distance [$F(1,20) = 4.82$, $p < 0.04$], seconds spent in the center [$F(1,20) = 14.70$, $p < 0.001$], frequency to enter the center [$F(1,20) = 17.18$, $p < 0.001$] and speed [$F(1,20) = 4.75$, $p < 0.04$]. Intermittently ethanol-treated WT mice travelled fewer cm at less speed than the saline-treated mice, and their frequency to enter the center and the time spent in the center were lower. Conversely, intermittent ethanol treatment induced no changes in TLR4-KO mice.

The EPM test is based on the natural aversion of mice to open, elevated areas, and also on the natural spontaneous exploratory behavior that they display in novel environments. The number of open arm entries, time spent in open arms, and percentage of open arm entries are generally used to characterize the anxiolytic effects of drugs. The ANOVA of WT mice revealed an effect of the variable treatment on the time [$F(1,32) = 4.77$, $p < 0.04$], and the percentage of time [$F(1,32) = 5.12$, $p < 0.03$], spent in the open arms of the maze, the time spent in the closed arms [$F(1,32) = 5.75$, $p < 0.02$], and the number of open entries [$F(1,32) = 60.05$, $p < 0.04$]. The time and the percentage of the time that the intermittently ethanol-treated WT mice spent in the open arms decreased, but the time they spent in the closed arms and performing less open entries was longer than the saline-treated WT animals (see Table 2). No significant effects were observed in the ethanol-treated TLR4-KO mice compared to their saline counterparts.

Data from both these tests suggest that intermittent ethanol treatment in adolescence induces long-term anxiogenic-related behavior (less frequency to enter and less time spent in the center of the open field, less time and fewer number of entries in the open arms of the EPM, etc.), and that the elimination of TLR4 protects against these behavioral alterations.

4. Discussion

Different studies in humans and experimental animals have demonstrated the vulnerability of the adolescent brain to actions of ethanol and the long-term consequences of binge drinking. These include not only the behavioral and cognitive deficits that result from alcohol neurotoxicity in the highly vulnerable adolescent PFC (Liu and Crews, 2015; Montesinos et al., 2015; Pascual et al., 2007), but also increased susceptibility to alcohol disorders and dependence (DeWit et al., 2000; Grant and Dawson, 1997; Hawkins et al., 1997; Hingson et al., 2008; Labouvie et al., 1997).

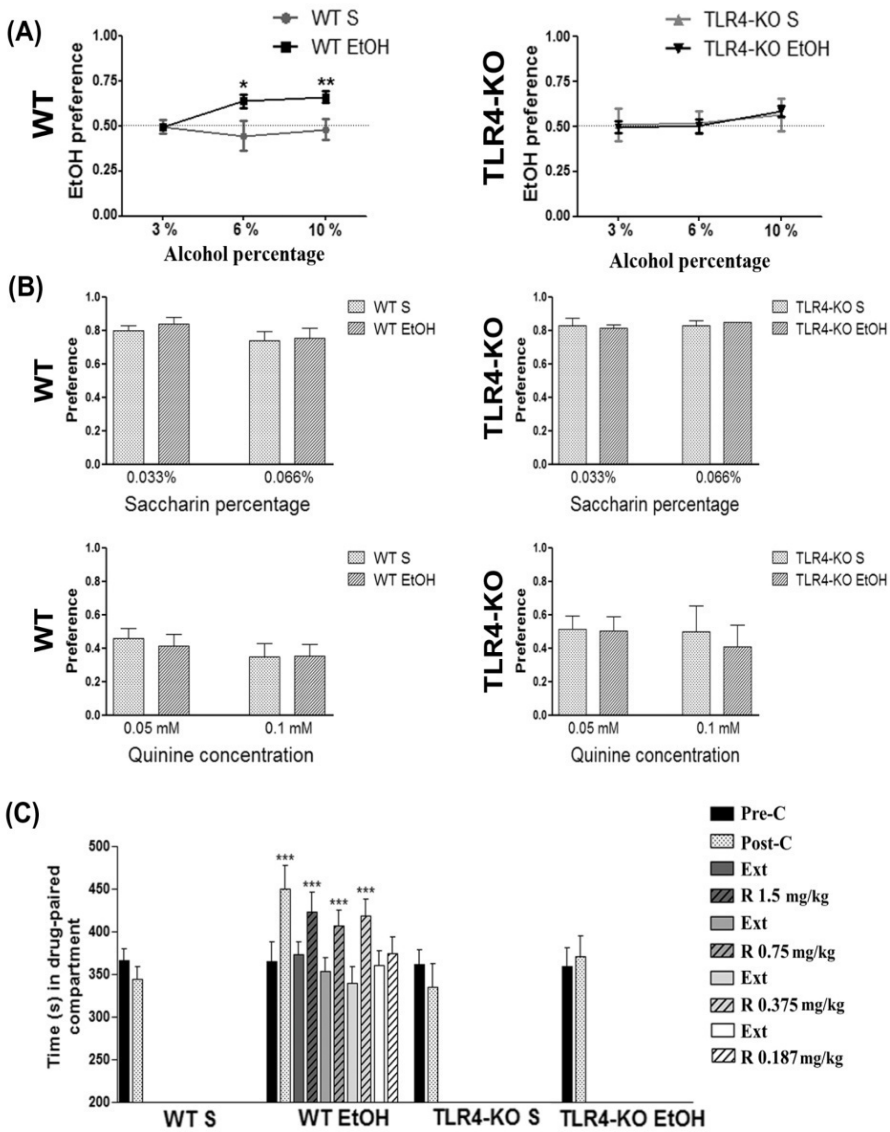

Fig. 5. Role of TLR4 in the two-bottle choice and conditioned place preference tests in the young adult WT and TLR4-KO mice intermittently treated with ethanol (EtOH) or saline (S) in adolescence. (A) Bars represent the mean ± SEM of ethanol preference in a 48-h two-bottle choice paradigm between ethanol and water at increasing ethanol concentrations: 3%, 6% and 10%. Data are presented as mean ± SEM, $n = 12$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the saline-treated WT mice. (B) Saccharin or quinine preference is represented as the average of all the daily measures at each tastant concentration. Data present mean ± SEM, $n = 10$ mice/group. (C) Bars represent the time in seconds spent in the drug-paired compartment before conditioning sessions during the pre-conditioning test (Pre-C), the post-conditioning test (Post-C), the extinction test (Ext) and the reinstatement test (R). Data are presented as mean ± SEM, $n = 14–17$ mice/group. $^{***}p > 0.001$ significant difference in the time spent in the drug-paired compartment during the pre-conditioning versus the post-conditioning tests, or during the reinstatement versus the extinction test.

However, the molecular mechanisms of the actions of ethanol in the adolescent brain are not well understood. The present study provides evidence that binge-like ethanol treatment in adolescence promotes long-term alterations in synaptic plasticity, along with epigenetic changes in the promoters of the genes associated with addiction in the mPFC of young adult animals. These molecular events were linked with long-term increases in the rewarding response for cocaine and preference for alcohol, together with an anxiogenic-related behavior. Our results also show the participation of neuroimmune system activation and the TLR4 signaling response in the neurotoxic, neurochemical and behavioral dysfunctions caused by alcohol abuse in the adolescence since TLR4-deficient mice (TLR4-KO) are protected against these ethanol effects on the adolescent brain. This suggests a role of the innate immune response in the long-term effects induced by alcohol abuse in adolescence.

Previous studies have demonstrated the susceptibility of the adolescent brain to toxic effects of ethanol (Liu and Crews, 2015; Montesinos et al., 2015; Pascual et al., 2007; Vetreno and Crews, 2015) since binge-like ethanol in the adolescence not only induces an innate immune response through TLR4 signaling by triggering an increase in pro-inflammatory mediators, but also causes neural damage, and myelin and synaptic dysfunctions, in the PFC, which lead to long-term cognitive impairment (Montesinos et al., 2015).

Table 1
Role of TLR4 in the open field in young adult WT and TLR4-KO mice intermittently treated with ethanol (EtOH) or saline during the adolescence. Data are presented as mean (± SEM), $n = 14–17$ mice/group. $^{*}p < 0.05$, $^{***}p < 0.001$, as compared to their respective saline-treated group.

	WT Saline	WT EtOH	TLR4-KO Saline	TLR4-KO EtOH
Total distance moved (cm)	3642 ± 261	3015 ± 170*	2524 ± 178	2842 ± 168
Time in center (s)	58 ± 10	21 ± 3***	32 ± 4	27 ± 7
Frequency to enter center	48 ± 6	22 ± 6***	21 ± 2	28 ± 3
Latency to center (s)	13 ± 4	21 ± 10	9 ± 3	14 ± 5
Speed (cm/s)	6 ± 0.5	5 ± 0.3*	4.2 ± 0.3	4.7 ± 0.3

Table 2
Role of TLR4 in the EPM in young adult WT and TLR4-KO mice intermittently treated with ethanol (EtOH) or saline during the adolescence. OA, open arms and CA, closed arms. Data are presented as mean (± SEM), $n = 15$ mice/group. $^{*}p < 0.05$, as compared to their respective saline-treated group.

	WT Saline	WT EtOH	TLR4-KO Saline	TLR4-KO EtOH
Time in OA (s)	113 ± 11	82 ± 8*	96 ± 14	95 ± 18
% time in OA	48 ± 4	35 ± 4*	42 ± 6	39 ± 6
Time in CA (s)	117 ± 10	148 ± 10*	133 ± 14	143 ± 16
Time in center (s)	70 ± 6	70 ± 4	68 ± 6	65 ± 9
OA entries	10 ± 1	7 ± 1*	7 ± 1	6 ± 1
% OA entries	48 ± 3	41 ± 3	51 ± 4	47 ± 5
Closed entries	11 ± 1	10 ± 1	7 ± 1	7 ± 1
Total entries	21 ± 2	18 ± 1	13 ± 1	13 ± 1

Notably when using the same animal model and alcohol treatment, ethanol can also promote long-term aberrant plasticity in certain brain regions, such as the mPFC, by inducing the activation of the CREB–BDNF pathway, and transcription factors and proteins, such as FosB, ΔFosB and Cdk5, which are implicated in addiction-related neural plasticity (Hope et al., 1994; Nestler, 2001). The up-regulation in the expression of some of these genes was associated with the histone acetylation in the promoter region of bdnf and fosB, which led to the up-regulation of the protein and gene expression of BDNF and ΔFosB in the mPFC of young adult mice. Remarkably, repeated exposures to drugs of abuse have been associated with increased ΔFosB levels (Nestler, 2001) and also with modifications in the chromatin structure in specific gene promoters (e.g. fosb, bdnf, etc.), which lead to long-term gene expression changes and contribute to neurobehavioral alterations or addictive-like behavior (Pandey et al., 2015; Walker et al., 2015). Chromatin modifications and epigenetic changes have been also described in alcoholic brains (Ponomarev et al., 2012), which are correlated with brain pathology and plasticity associated with alcohol abuse and dependence behavior (Ponomarev, 2013). Binge-like ethanol treatment in rat adolescence also alters dopaminergic and glutamatergic neurotransmission, effects that have been associated with changes in the acetylation of histones H3 and H4 in the rat frontal cortex, the nucleus accumbens and the striatum (Pascual et al., 2009) and also with acetylation of histones in the fosb promoter in adolescent rats (Pascual et al., 2012). According to these findings, we also showed that binge-like ethanol treatment causes modifications in histone acetylation in young adult mice, effects that are associated with long-term rewarding behavior and alcohol preference in young adult mice exposed to alcohol in adolescence.

It is interesting to note that, although some studies have demonstrated that ethanol exposure up-regulates NF-κB while it decreases CREB in hippocampal-entorhinal complex slice culture (Zou and Crews, 2006), other *in vivo* findings have shown that ethanol up-regulates p-CREB expression in different brain areas of ethanol-treated adolescent mice (Soares-Simi et al., 2013). Ethanol-induced CPP has also been associated with p-CREB up-regulation in the hippocampus, prefrontal cortex and striatum (Varela et al., 2014) and Groblewski et al. (2012) reported that while p-CREB increased during ethanol-induced CPP, the levels of p-CREB lowered following the extinction process. In line with these last findings, we found that p-CREB levels increased 24 h after the last ethanol treatment in the mPFC, but lowered after 3 weeks of ethanol removal in young adult mice.

The present study also shows that binge-like ethanol treatment in adolescence induces preference for ethanol, and increases the conditioned rewarding effects and vulnerability to drug-induced cocaine reinstatement. Thus the large number of extinction sessions required in ethanol-treated animals suggests that cocaine has greater motivational properties, as reflected by the persistence of drug-seeking behavior when it is absent. Likewise, using the reinstatement of extinguished preference through a priming injection of a drug, a reliable model used to assess the mechanisms of drug-craving and relapse (Aguilar et al., 2009), our results also indicated the greater vulnerability of the ethanol-treated WT mice. Similarly, the anxiogenic profile exhibited by ethanol-treated animals could increase the vulnerability of alcohol abuse since anxiety and substance use disorders are strongly associated, and are major contributors to higher rates of alcohol (Carota and Calabrese, 2013) and cocaine dependence (Prast et al., 2014).

The results of this study provide further evidence for the critical role of the innate immune system response and, in particular, the TLR4 signaling response in the short- and long-term effects on synaptic plasticity, alterations in histone acetylation, behavioral effects and alcohol preference and motivation since the elimination of the TLR4 function abolished all effects of ethanol. Indeed neuroimmune signaling has been reported to participate in neuroplasticity, learning and memory processes (Williamson and Bilbo, 2013; Yirmiya and Goshen, 2011), in alcohol dependence (Mayfield et al., 2013) and in the regulation of voluntary ethanol intake in rodent models (Blednov et al., 2005). Injecting TLR4 siRNA into the central amygdala of the alcohol-preferring rats reduced operant ethanol self-administration (Liu et al., 2011), which supports the role of TLR4 pathways and brain immune signaling in alcohol consumption. Activation of TLR4 signaling in mice with chronic alcohol consumption also promotes chromatin changes, effects that have been associated with short- and long-term behavioral effects (Pascual et al., 2011b). Furthermore, there is considerable evidence to indicate that changes in the neuroimmune gene expression contribute to the development of alcohol addiction in adolescents who binge drink (Crews and Vetreno, 2011), and the pharmacological blockade of TLR4 activation suppresses some drug reward/reinforcement (Bachtell et al., 2015). Accordingly, most long-term motivation rewarding behavioral effects and alcohol preference were not observed in the TLR4-KO young adult mice treated with alcohol in adolescence. These findings suggest that TLR4-KO animals are protected against the ethanol-induced TLR4-dependent neuroinflammation, PFC damage and behavioral dysfunctions that occur in adult mice exposed to alcohol in adolescence. It is important to note that, although TLR4-KO are protected against some effects of ethanol, alcohol intake and preference are similar in both naïve WT and TLR4-KO mice (Pascual et al., 2011a), and sedation and motor impairment induced by an acute ethanol administration are only attenuated in TLR4-KO when compared with WT mice (Wu et al., 2012).

One limitation of using knockout mice during development was that compensatory changes, as well as other factors (e.g., interaction with other genes), could influence the neurochemical and behavioral effects of ethanol in adolescence and their interpretation (Gingrich and Hen, 2000). Furthermore, when using TLR4-KO mice, from which TLR4 was globally deleted, it is difficult to

establish the role of specific brain area or circuit associated with a particular behavior, such as anxiety or alcohol intake. Finally, although ethanol can directly activate the TLR4 signaling response in glial cells by triggering neuroinflammation (Alfonso-Loeches et al., 2010; Fernandez-Lizarbe et al., 2009), ethanol can also induce a TLR4 response from immune peripheral cells and the release of proinflammatory cytokines, which can cross the blood brain barrier and contribute to neuroinflammation and behavioral dysfunctions.

Briefly, the results of the present study highlight the role of the TLR4 pathway in the epigenetic changes, along with the long-term anxiety and rewarding effects induced by intermittent ethanol administration in the adolescence. Our results provide a novel mechanism of the neuroimmune function and open up new avenues to develop pharmacological treatments that can normalize the immune signaling responsible for long-term effects in adolescence, including alcohol abuse and related disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2015.12.006.

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Supplementary Material Table 1S

Table 1S. Basal level of the proteins analyzed between untreated WT and TLR4-KO mice in the adolescence (PND 44) or young adulthood (PND 65).

		Short-term effects		Long-term effects	
		Mean ± SEM	p	Mean ± SEM	p
Ac-K9 H3	WT S	1.00 ± 0.22		1.00 ± 1.97	
	TLR4-KO S	0.83 ± 0.19	0.58	0.86 ± 0.12	0.55
3me-K4 H3	WT S	1.00 ± 0.28		1.00 ±0.07	
	TLR4-KO S	1.13 ± 0.23	0.74	1.18 ± 0.08	0.15
Ac-K5 H4	WT S	1.00 ± 0.09		1.00 ± 0.13	
	TLR4-KO S	0.88 ± 0.16	0.50	0.87 ± 0.11	0.52
Ac-K12 H4	WT S	1.00 ± 0.11		1.00 ± 0.09	
	TLR4-KO S	1.08 ± 0.17	0.71	0.94 ± 0.03	0.63
pre-BDNF	WT S	1.00 ± 0.11		1.00 ± 0.08	
	TLR4-KO S	0.77 ± 0.18	0.32	1.04 ± 0.17	0.84
mat-BDNF	WT S	1.00 ± 0.25		1.00 ± 0.07	
	TLR4-KO S	1.22 ± 0.07	0.49	1.08 ± 0.10	0.55
Cdk5	WT S	1.00 ± 0.08		1.00 ± 0.06	
	TLR4-KO S	1.06 ± 0.29	0.85	1.15 ± 0.11	0.27
p-CREB	WT S	1.00 ± 0.05		1.00 ± 0.04	
	TLR4-KO S	1.18 ± 0.15	0.25	1.02 ± 0.05	0.74
FosB	WT S	1.00 ± 0.11		1.00 ± 0.08	
	TLR4-KO S	0.88 ± 0.09	0.42	1.01 ± 0.05	0.92
ΔFosB	WT S	1.00 ± 0.17		1.00 ±0.07	
	TLR4-KO S	0.88 ± 0.25	0.69	1.04 ± 0.12	0.78
GluR1	WT S	1.00 ± 0.10		1.00 ± 0.22	
	TLR4-KO S	1.09 ± 0.18	0.06	1.13 ± 0.12	0.66
MHC-II	WT S	1.00 ± 0.27		1.00 ± 0.49	
	TLR4-KO S	1.13 ± 0.43	0.81	1.24 ± 0.40	0.72
NR1	WT S	1.00 ± .13		1.00 ± 0.22	
	TLR4-KO S	0.92 ± 0.11	0.67	0.96 ± 0.21	0.90
p-p65	WT S	1.00 ± 0.12		1.00 ± 0.17	
	TLR4-KO S	1.18 ± 0.09	0.24	0.97 ± 0.14	0.17

4.3. Publicación III

Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

ORIGINAL ARTICLE

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Gender differences in the inflammatory cytokine and chemokine profiles induced by binge ethanol drinking in adolescence

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ABSTRACT

Heavy binge drinking in adolescence can cause long-term cognitive and behavioral dysfunctions. Recent experimental evidence indicates the participation of immune system activation in the effects of ethanol in the adolescent brain and suggests gender differences. The present study aims to assess plasma cytokine and chemokine levels in male and female adolescents and young adults during acute alcohol intoxication and to correlate these results with the toll-like receptor 4 (TLR4) response. The potential role of the TLR4 signaling response was also assessed in plasma and prefrontal cortex (PFC) of adolescent wild-type and TLR4-knockout male and female mice with binge ethanol treatment. The results showed that alcohol intoxication increased the plasma levels of several cytokine and chemokine [interferon-γ, interleukin (IL)-10, IL-17A, IL-1β, IL-2, IL-4, IL-6, IL-8, fractalkine, monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α)] and the upregulation of TLR4 mRNA levels occurred in intoxicated females, while elevation of colony-stimulating factor was only observed in the plasma of males. In wild-type female adolescent mice, intermittent ethanol treatment increased the levels of several cytokines (IL-17A and IL-1β) and chemokines (MCP-1, MIP-1α and fractalkine) in PFC and in serum (IL-17A, MCP-1 and MIP-1α), but significant differences in the fractalkine levels in PFC were observed only in male mice. No changes in serum or prefrontal cortex cytokine and chemokine levels were noted in ethanol-treated male or female TLR4-knockout mice. Our findings revealed that females are more vulnerable than males to inflammatory effects of binge ethanol drinking and suggested that TLR4 is an important target of ethanol-induced inflammation and neuroinflammation in adolescence.

Keywords Adolescent humans, adolescent mice, gender difference, inflammation, TLR4.

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INTRODUCTION

Heavy alcohol binge drinking is increasing in adolescents from different European countries (Danielsson, Wennberg, Hibell, & Romelsjo 2012), including some Mediterranean ones where a shift from natural moderate wine alcohol consumption to heavy binge drinking has been noted (Peretti-Watel, Beck, & Legleye 2006; Tur, Puig, Pons, & Benito 2003), which can lead to many alcohol-related problems among adolescents (Danielsson *et al.* 2012). Evidence obtained from human and experimental animals has shown the harmful consequences of alcohol binge drinking during a developmental stage of adolescent brain maturation. Thus, alterations in cortical and white matter structure (Squeglia, Jacobus, & Tapert 2014), deficits in memory, poor academic performance (Hanson, Cummins, Tapert, & Brown 2011), as well as visual learning (Sanhueza, Garcia-Moreno, & Exposito 2011) and executive function impairments (Goudriaan, Grekin, & Sher 2007; Scaife & Duka 2009) have been reported in adolescents with alcohol abuse. Alcohol consumption in adolescence continues into adulthood and is also associated with later alcohol problems and alcohol dependence (Hermos, Winter, Heeren, & Hingson 2008).

Data on experimental animals have also revealed that binge-like ethanol treatment in adolescence impairs both prefrontal cortex (PFC) maturation (Montesinos *et al.* 2015) and hippocampal neurogenesis (Vetreno & Crews 2015), induces structural changes in the hippocampus and cerebellum (Vetreno, Yaxley, Paniagua, & Crews 2015), alters the myelin structure (Montesinos *et al.* 2015) and causes long-term cognitive and behavioral dysfunctions (Montesinos *et al.* 2015; Schulteis, Archer, Tapert, & Frank 2008; Vetreno *et al.* 2015).

Although the molecular mechanisms of alcohol actions in the adolescent brain remain elusive, our previous studies have shown that ethanol is capable of activating immune receptors toll-like receptor 4 (TLR4) by triggering signaling pathways, which induce the release of inflammatory mediators and consequent brain damage in the adult (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri 2010) and adolescent (Montesinos *et al.* 2015) brain. Using adolescent rats (Pascual, Blanco, Cauli, Minarro, & Guerri 2007) and mice (Montesinos *et al.* 2015), we have demonstrated that intermittent binge-like alcohol treatment triggers pro-inflammatory cytokines and mediators (iNOS and COX-2) in the brain, which cause inflammatory damage in the PFC, and impairs synaptic and myelin structures (Montesinos *et al.* 2015) and long-term cognitive dysfunctions (Montesinos *et al.* 2015; Pascual *et al.* 2007) in young adult mice treated with alcohol in adolescence. The role of neuroinflammation and the TLR4 response in ethanol actions on the adolescent brain has been supported by data that have indicated that anti-inflammatory compounds (Pascual *et al.* 2007) or the genetic elimination of TLR4 prevents neuroinflammation, synaptic and myelin disarrangements and long-term cognitive alterations (Montesinos *et al.* 2015; Pascual *et al.* 2007). Neuroimmune gene expression activation has also been suggested to contribute to the risk of alcoholism or to other brain diseases associated with neuroinflammation and linked to adolescent drinking (Vetreno, Qin, & Crews 2013). Some studies have demonstrated that binge drinking differentially affects adolescent male and female brain morphometry and greater deficits in visual memory have been shown in female than in male adolescents (Squeglia *et al.* 2012). Animal studies have also revealed that ethanol-induced neuroinflammatory damage is greater in females than in males (Alfonso-Loeches, Pascual, & Guerri 2013).

Neuroinflammation is associated with increased levels of brain cytokines and chemokines. We have recently shown that some cytokines/chemokines can be used as biomarkers of alcohol-induced brain inflammation and behavioral response in animals after chronic ethanol intake (Pascual, Balino, Aragon, & Guerri 2015). However, whether plasma cytokines and/or chemokines can also be used as markers of neuroinflammation in adolescents with binge drinking habits is presently unknown.

By using samples from adolescent and young individuals and mice adolescents, the present study was designed to assess if acute alcohol intoxication differently affects the plasma immune response (cytokines and chemokines) in males and females and whether the immune response is associated with the TLR4 response. We herein demonstrated that alcohol intoxication induced higher plasma levels of cytokines/chemokines in females than in males. We found an association between elevated cytokines/chemokines and the upregulation of blood TLR4 mRNA levels in females, but not in males. We also provide evidence that intermittent ethanol treatment increases the levels of several cytokines/chemokines in serum and in the PFC, along with the upregulation of the TLR4 expression of adolescent female mice. However, no significant differences in the cytokines and chemokines levels were observed in adolescent male mice, when compared with untreated counterparts. The present work further showed how female and male adolescent TLR4-knockout (KO) mice were protected against the ethanol-induced immune response in both plasma and the brain. These findings suggest that human female adolescents and young adults are more vulnerable than males to the inflammatory effects of binge ethanol drinking and confirm that TLR4 is an important target of ethanol-induced inflammation.

MATERIALS AND METHODS

Human study

Subjects

Our clinical sample included 27 adolescent and young adults (48.1 percent females) who were admitted to the Emergency Department of the University Hospital of Salamanca (Salamanca, Spain) with moderate to severe acute alcohol intoxication, as previously reported (Lopez-Moreno *et al.* 2015). Acute alcohol intoxication was defined by clinical signs and symptoms (e.g. motor incoordination, unsteady gait, impaired reasoning, slurred speech, confusion or disorientation), blood alcohol levels (BALs) >1 g/l and consumption of at least five (50 g, males) or four (40 g, females) standard drinks during the 6 hours before admission, although the total amount of drinking or the time from first and last ethanol intake was not exactly known for each individual. Patients were excluded if they had any other acute (e.g. trauma or infection) or chronic illness, took any medication or if their toxicological urinary analysis or clinical data demonstrated that they had used illegal drugs (apart from cannabis). Individuals' clinical, epidemiological and analytical characteristics are shown in Table 1.

Table 1 Characteristics of healthy control individuals and alcohol-intoxicated individuals.

	Healthy control individuals		*Alcohol-intoxicated individuals*	
	Male ($n = 8$)	*Female* ($n = 9$)	*Male* ($n = 14$)	*Female* ($n = 13$)
Age (years)	23.4 (1.1)	24.3 (1.1)	20.4 (1.2)	19.8 (1.1)
BALs (g/l)	0	0	2.5 (0.1)**	2.2 (0.2)**
AST levels (IU/l)	20.8 (2.0)	16.6 (1.3)	34.6 (4.5)*	18.5 (1.0)***
ALT levels (IU/l)	23.2 (5.5)	14.6 (1.7)	25.8 (5.8)	13.4 (0.9)
ALP levels (IU/l)	58.4 (5.2)	57.0 (9.6)	92.7 (11.8)	70.4 (8.3)
GGT levels (IU/l)	16.4 (3.0)	11.3 (1.2)	25.9 (6.1)	14.0 (1.4)
White blood cell count/μl	6830.0 (1190.1)	7302.5 (786.9)	9337.1 (606.3)	7870.8 (454.1)
Individuals who reported weekend drinking (%)[a]	0	0	6 (60.0%)	2 (28.6%)

Quantitative variables are presented as the mean (SEM) and qualitative variables are presented as absolute frequencies (percentage). ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BALs = blood alcohol levels; GGT = γ-glutamyl transpeptidase; IU = international units; SEM = standard error of the mean. *$P < 0.05$. **$P < 0.001$, as compared with the healthy control group. ***$P < 0.01$, as compared with the alcohol-intoxicated males. [a]Four male individuals and six female individuals refused to answer the questionnaire regarding drinking patterns.

Seventeen healthy controls (eight males and nine females) were also included in the study and were recruited among medical and nursing students. Controls did not consume alcohol apart from light sporadic drinking, did not report alcohol consumption during the 72 hours prior to blood extraction and reported no binge drinking episodes during the three previous months. These subjects showed normal hematological and plasma biochemical parameters (Table 1) and reported no chronic or acute illness. The study was approved by the Ethics Committee of the University Hospital of Salamanca, and written informed consent was obtained from each participant. Blood samples were obtained from the patients upon admission for use in standard care and also for research purposes. These blood samples were used to determine the BAL, blood count and liver function tests and for the mRNA and cytokine analyses. Samples were processed and analyzed for this study only after the patients were able to provide informed consent.

Quantitation of cytokines and chemokines in human plasma samples

Whole blood samples (2.5 ml) from each individual were drawn into lithium heparin tubes and PAXgene Blood RNA tubes (Qiagen, Valencia, CA, USA). Plasma samples were obtained by centrifuging the lithium heparin tubes immediately after collection and were stored at −80°C. Samples were analyzed simultaneously to determine the levels of several cytokines and chemokines using the Milliplex MAP kit premixed human cytokine/chemokine magnetic bead panel (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The analyzed cytokines and chemokines included colony-stimulating factor (CSF), interferon (IFN)-γ, interleukin (IL)-10, IL-17A, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, tumor necrosis factor (TNF)-α, fractalkine, monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α). Data were acquired in a Luminex 100 System and analyzed by the MASTERPLEX CT software, version 1.2.0.7 (Hitachi MiraiBio, USA).

Blood RNA isolation and quantitative reverse transcription polymerase chain reaction

The PAXgene Blood RNA tubes with each subject's blood were kept at room temperature for at least 2 hours and were stored at −20°C. Total RNA was isolated using the PAXgene Blood miRNA Kit (Qiagen) following the manufacturer's instructions and was stored at −80°C. RNA concentration and purity were examined in a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For mRNA expression analysis, complementary DNA was synthesized by reverse transcription using a commercial High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's manual. Relative quantitative real-time polymerase chain reaction was performed using SYBR Green PCR master mix (Applied Biosystems) and gene-specific primer sets: TLR4, 5′-CGA GGA AGA GAA GAC ACC AGT-3′ (forward) and 5′-CAT CAT CCT CAC TGC TTC TGT-3′ (reverse); actin, 5′-CCA ACC GCG AGA AGA TGA-3′ (forward) and 5′-CCA GAG GCG TAC AGG GAT AG-3′ (reverse). PCR experiments were performed in duplicate on a StepOnePlus™ Real-Time PCR System (Applied Biosystems), and primer specificity was verified by melt curve analysis. Threshold cycle (C_T; number of cycles to reach threshold of detection) was determined for each

reaction, and gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

Animal study

Animals and treatments

Male and female C57BL/6 wild-type (WT) (Harlan Ibérica, Barcelona, Spain) and TLR4-KO mice (C57BL/6 background, kindly provided by Dr. S. Akira, Osaka University, Suita, Japan), aged 30 days, were used. All the animals were kept under controlled light and dark (12/12 hours), temperature (23°C) and humidity (60 percent) conditions. All the experimental procedures were approved by the Ethical Committee of Animal Experimentation of the Príncipe Felipe Research Center (Valencia, Spain) and were carried out in accordance with the guidelines approved by the European Communities Council Directive (2010/63/EU) and by Spanish Royal Decree 1201/2005.

For the binge ethanol treatment, both the male and female WT and TLR4-KO mice were housed (four animals per cage) and maintained with water and solid diet ad libitum. Morning doses of either saline or 25 percent (v/v) ethanol (3 g/kg) in isotonic saline (15 µl/g) were administered intraperitoneally to the 30-day-old mice on two consecutive days with 2-day gaps without injections for 2 weeks (PND30 to PND43), as previously described (Pascual *et al.* 2007). For the biochemical studies, animals were anesthetized with sodium pentobarbital (0.06 mg/kg) 24 hours after the eight ethanol administration and whole blood was collected from the hepatic portal vein. After centrifugation, the separated serum was stored at −80°C until further analysis. Although the reproducibility of the biochemical value was similar in both the plasma and serum samples, we collected serum samples in mice because serum contains higher metabolite concentrations than plasma (Yu *et al.* 2011), which make it possible to provide more sensitive results in biomarker detections. Brains were removed, and the PFC was dissected and stored at −80°C until used.

Quantitation of cytokines and chemokines in mouse serum and PFC

The serum and the homogenized extracts from the PFC were used to determine the levels of IL-1β (eBioscience, Vienna, Austria), fractalkine (R&D Systems, Abingdon, UK), IL-17A, MCP-1 and MIP-1α (Peprotech, Barcelona, Spain) with an enzyme-linked immunosorbent assay kit, following the manufacturer's instructions.

Western blot analysis

The Western blot technique was performed in the PFC tissue lysates, as described elsewhere (Fernandez-Lizarbe, Pascual, & Guerri 2009). The primary antibodies used were NFκB p-p65 (1:500, Cell Signaling Technology, Leiden, The Netherlands), TLR4 (1:1000, Santa Cruz Biotechnology, Madrid, Spain), NFκB p65 (1:500) (Santa Cruz Biotechnology) and GAPDH (1:2500, glyceraldehyde 3-phosphate dehydrogenase, Chemicon, CA, USA). Samples were incubated overnight with the corresponding antibodies.

Statistical analysis

The results are reported as mean ± standard error of the mean. The biochemical data were analyzed by a two-way ANOVA (gender × alcohol intoxication or treatment), followed by Tukey's multiple comparison test (GRAPHPAD PRISM v7.00, GraphPad Software Inc., CA, USA). The correlations of the data in Fig. 2 and Supporting Information Table S1 were determined by Pearson's coefficient (SPSS program v17.0, IBM, Madrid, Spain). A linear regression model was also generated with the BALs as a score predictor of the cytokine or chemokine levels (SPSS program, v17.0).

RESULTS

Gender differences in the plasma cytokine and chemokine levels after alcohol intoxication in human adolescents and young adults

The median age of females and males was 18.0 years [interquartile range (IQR) 18.0–22.5] and 19.0 years (IQR 16.8–24.5), respectively. These ages have been considered as late adolescence (ages 16–20 years) or young adulthood (ages 21–25 years) (Brown *et al.* 2008; NIAAA 2009). In addition, the biochemical analysis of plasma during the intoxication period demonstrated a median BAL of 2.10 g/l (IQR 1.80–2.50) for females and one of 2.40 g/l (IQR 2.28–2.73) for males. No other drugs of abuse were found. The data showed a greater dispersion of the BALs in females than in males. Control subjects were used, with median ages of 23.0 years (IQR 21.2–26.2) for females and 23.0 years (IQR 21.5–25.7) for males.

Two-way ANOVA with Tukey's *post hoc* test revealed that alcohol intoxication in the female subjects caused a significant upregulation in the levels of plasma cytokine, e.g. IL-1β, IL-2, IL-6, IL-10, IL-8, IFN-γ, IL-17A, IL-4 and chemokine, e.g. MIP-1α, fractalkine and MCP-1, compared with the control counterparts group (Fig. 1a). When the plasma cytokines levels were assessed in the ethanol-intoxicated males, only significant elevations were noted for CSF compared with the plasma samples of the control individuals. No differences were observed between healthy male and female subjects in the analyzed cytokines and chemokines. Two-way ANOVA

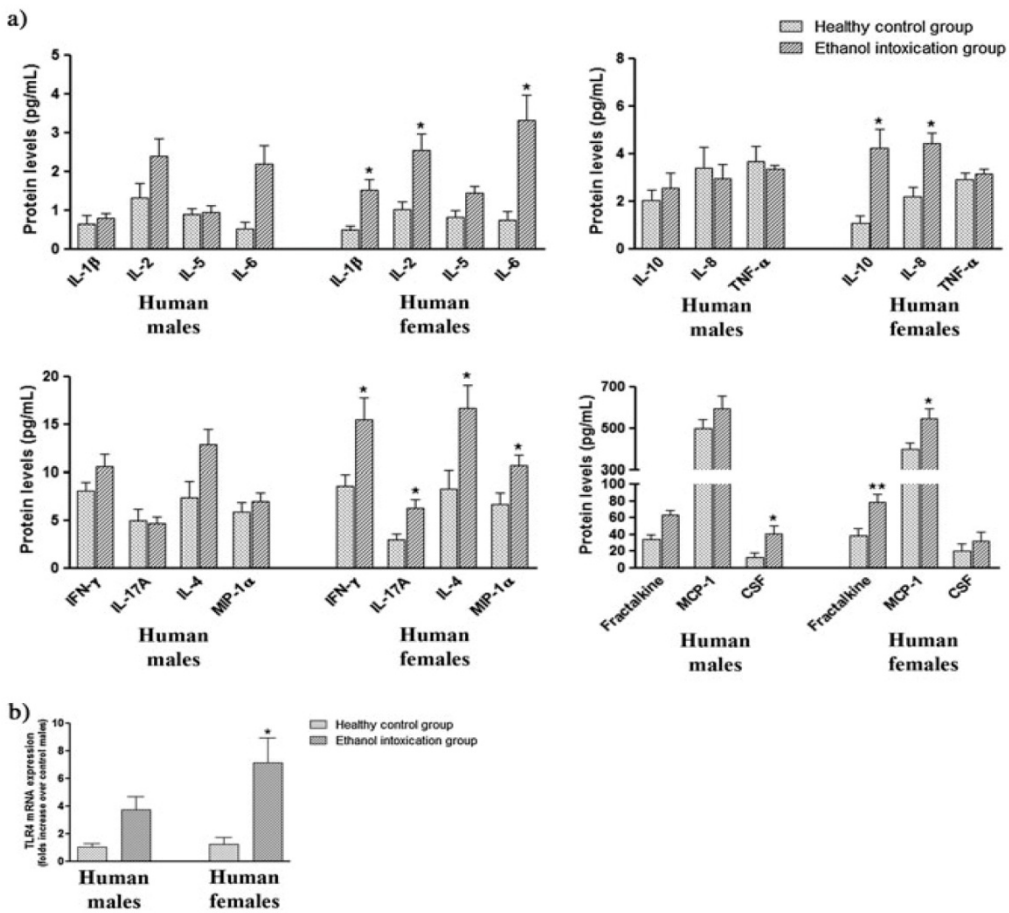

Figure 1 Human plasma levels of interleukin (IL)-1β, IL-2, IL-5, IL-6, IL-10, IL-8, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-17A, IL-4, macrophage inflammatory protein (MIP)-1α, fractalkine, monocyte chemoattractant protein (MCP)-1 and colony-stimulating factor (CSF) of the males and females. (a) Graphs represent the plasma data of females and males after acute ethanol intoxication and the data of the corresponding control abstainers. Data represent mean ± standard error of the mean (SEM), $n = 8–14$ individuals/group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with their respective control abstainers. (b) Graphs represent the levels of the toll-like receptor 4 (TLR4) mRNA expression from the blood of female and male individuals after acute ethanol intoxication and also of the corresponding control abstainers. Results are expressed relatively to values of the control group (AU, arbitrary units; mean ± SEM), $n = 8–14$ individuals/group. $^{*}P < 0.05$ compared with their respective control abstainers

analyses were conducted to compare whether the effects of alcohol intoxication on the plasma cytokine and chemokine levels differed between the female and male subjects. These analyses revealed a significant interaction effect on IL-17A [$F(1, 39) = 4.24$, $P < 0.05$] and a main effect of alcohol intoxication on CSF [$F(1, 37) = 7.44$, $P < 0.01$], IFN-γ [$F(1, 39) = 7.15$, $P < 0.05$], IL-10 [$F(1, 34) = 5.63$, $P < 0.05$], IL-1β [$F(1, 35) = 6.42$, $P < 0.05$], IL-2 [$F(1, 38) = 10.95$, $P < 0.01$], IL-4 [$F(1, 36) = 14.30$, $P < 0.001$], IL-6 [$F(1, 33) = 14.62$, $P < 0.001$], fractalkine [$F(1, 39) = 16.97$, $P < 0.001$], MIP-1α [$F(1, 37) = 7.08$, $P < 0.05$], IL-8 [$F(1, 38) = 6.00$, $P < 0.05$] and MCP-1 [$F(1, 33) = 6.37$, $P < 0.05$]. No significant interactions were found on CSF [$F(1, 37) = 2.29$, $P > 0.05$], IFN-γ [$F(1, 39) = 1.53$, $P > 0.05$], IL-10 [$F(1, 34) = 2.91$, $P > 0.05$], IL-1β [$F(1, 35) = 3.65$, $P > 0.05$], IL-2 [$F(1, 38) = 0.35$, $P > 0.05$], IL-4 [$F(1, 36) = 0.07$, $P > 0.05$], IL-5 [$F(1, 38) = 2.37$, $P > 0.05$], IL-6 [$F(1, 33) = 0.27$, $P > 0.05$], TNF-α [$F(1, 39) = 0.88$, $P > 0.05$], fractalkine [$F(1, 39) = 0.45$, $P > 0.05$], MIP-1α [$F(1, 37) = 1.27$, $P > 0.05$], IL-8 [$F(1, 38) = 2.03$, $P > 0.05$] and MCP-1 [$F(1, 33) = 2.33$, $P > 0.05$]. Similarly, no main effect of ethanol intoxication was observed on IL-17A [$F(1, 39) = 2.92$, $P > 0.05$], IL-5 [$F(1, 38) = 3.40$, $P > 0.05$], TNF-α [$F(1, 39) = 0.03$, $P > 0.05$] and gender on CSF [$F(1, 37) = 0.72$, $P > 0.05$], IFN-γ [$F(1, 39) = 2.26$, $P > 0.05$], IL-10 [$F(1, 34) = 0.21$, $P > 0.05$], IL-17A [$F(1, 39) = 0.04$,

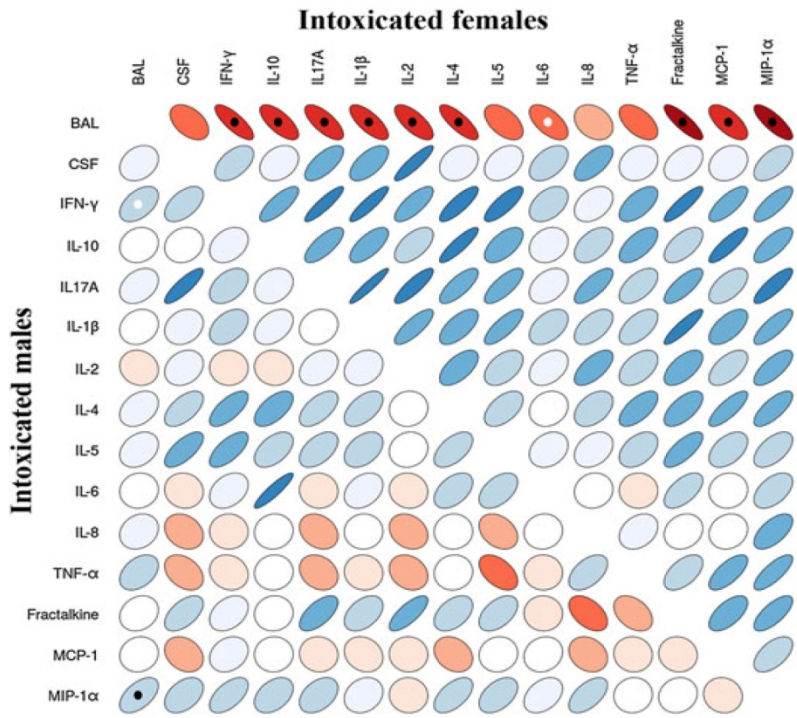

Figure 2 Pearson's coefficient between the plasma levels of cytokines or chemokines and the blood alcohol levels (BALs) in females and males with alcohol intoxication. Plot represents the correlation coefficient and the significant *P* value between the plasma BALs and the levels of cytokines or chemokines in females and males intoxicated with alcohol. The width of the ellipses and the color gradient indicate the strength of the correlation. Blue ellipses represent positive correlations, and negative ones are represented by red ellipses. Black points represent statistically significance correlations, and white ones denote a tendency to be significant

$P > 0.05$], IL-1β [$F(1, 35) = 1.65$, $P > 0.05$], IL-2 [$F(1, 38) = 0.03$, $P > 0.05$], IL-4 [$F(1, 36) = 0.45$, $P > 0.05$], IL-5 [$F(1, 38) = 0.45$, $P > 0.05$], IL-6 [$F(1, 33) = 0.79$, $P > 0.05$], TNF-α [$F(1, 39) = 2.54$, $P > 0.05$], fractalkine [$F(1, 39) = 1.47$, $P > 0.05$], MIP-1α [$F(1, 37) = 3.65$, $P > 0.05$], IL-8 [$F(1, 38) =$, $P > 0.05$] and MCP-1 [$F(1, 33) = 0.29$, $P > 0.05$]. These results indicated that females were more vulnerable than males to alcohol effects.

To study whether there was a correlation between the high plasma levels of cytokines and chemokines and the BALs during the intoxicated period, we used a Pearson's correlations analysis. Figure 2 and Supporting Information Table S1 show a significant negative correlation in the intoxicated females for cytokines and chemokines, IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-17A, fractalkine, MCP-1 and MIP-1α and a significant tendency for IL-6, which became evident at high alcohol levels. Among the intoxicated male subjects, we observed a correlation only between the BALs and MIP-1α, although a significant tendency was noted for IFN-γ (Fig. 2 and Supporting Information Table S1). We also performed a linear regression model in the BALs of both alcohol-intoxicated females and males with the different cytokines and chemokines to estimate possible linearity between both variables. This analysis improved the evaluation of the relationship between the levels of each cytokine/chemokine with BALs. The linear regression analysis gave similar significant results to Pearson's correlation (Table 2 and Supporting Information Fig. S1).

Acute ethanol intoxication increases the TLR4 mRNA levels in the blood of adolescent and young females, but not of males

To gain further insights into the actions of ethanol on the innate immune response and as we have previously demonstrated the role of TLR4 in the ethanol-induced immune response in mice (Alfonso-Loeches *et al.* 2010; Fernandez-Lizarbe *et al.* 2009; Montesinos *et al.* 2015), we assessed the mRNA expression of TLR4 in the whole blood samples of the females and males after acute alcohol intoxication. Two-way ANOVA with Tukey's *post hoc* test indicated that alcohol intoxication significantly increased the blood mRNA levels of TLR4 in females compared with the control group's values (Fig. 1b).

Table 2 Linear regression analysis between the plasma levels of cytokines or chemokines and the BALs in females and males with alcohol intoxication.

	Intoxicated females			*Intoxicated males*		
	Slope	*Constant*	*P*	*Slope*	*Constant*	*P*
CSF	−28.58	106.14	0.180	31.88	−37.35	0.388
IFN-γ	−8.54	34.61	0.006	7.45	−7.57	0.091
IL-10	−2.59	10.06	0.024	1.01	0.09	0.658
IL-17A	−3.39	14.13	0.021	2.84	−2.26	0.264
IL-1β	−1.03	3.91	0.016	0.26	0.13	0.572
IL-2	−1.41	5.57	0.022	−0.62	3.89	0.701
IL-4	−6.77	30.16	0.007	6.11	−2.01	0.255
IL-5	−0.51	2.60	0.121	0.63	−0.58	0.311
IL-6	−2.27	8.68	0.070	0.38	1.28	0.823
IL-8	−0.75	5.91	0.412	3.56	−3.94	0.512
TNF-α	−0.51	4.28	0.103	0.81	1.35	0.175
Fractalkine	−37.94	162.81	0.002	14.04	28.65	0.517
MCP-1	−158.20	849.56	0.039	83.85	359.32	0.758
MIP-1α	−4.44	20.83	0.005	5.71	−7.41	0.050

Data marked in dark grey are statistically significant, while data in light grey denote a tendency to be significant. CSF = colony-stimulating factor; IFN = interferon; IL = interleukin; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; TNF = tumor necrosis factor.

However, no significant differences in the gene expression levels of this receptor was noted in the blood of either intoxicated males versus healthy males or healthy control males versus female subjects. The two-way ANOVA analyses revealed a main effect of alcohol intoxication [$F(1, 28) = 10.73$, $P < 0.01$], but no significant effects in either gender [$F(1, 28) = 1.84$, $P > 0.05$] or interaction [$F(1, 28) = 1.42$, $P > 0.05$].

Gender differences in the cytokine and chemokine levels in the serum and PFC of the WT mice after intermittent ethanol exposure

To further investigate gender differences in the ethanol response in adolescent and young individuals, we used an experimental model of intermittent ethanol treatment in adolescence. Adolescent female and male WT mice, intermittently treated with ethanol or saline, were used to mimic the drinking pattern of adolescents. We determined BALs after a single ethanol dose to evaluate possible gender differences. After this single ethanol dose, BALs peaked at 164.5 ± 2.5 mg⁄dl in the WT female mice and at 161.6 ± 1.5 mg⁄dl in the WT male mice at 30 minutes post-injection with similar ethanol clearance at 5 hours post-injection (data not shown). We also evaluated the BAL levels in both male and female mice after eight intermittent ethanol doses. No statistical variations in the BAL levels in either the female and male mice were observed after the eighth ethanol injection (at 30 minutes post-injection, 165.4 ± 1.1 mg⁄dl in the WT female mice and 170.1 ± 0.8 mg⁄dl in the WT male mice).

The levels of cytokines and chemokines were also assessed in the serum and PFC of the saline-treated or ethanol-treated WT mice. Two-way ANOVA with Tukey's *post hoc* test revealed that intermittent ethanol treatment significantly increased the serum levels of IL-17A, MCP-1 and MIP-1α of the WT adolescent female mice compared with the saline WT female mice (Fig. 3a). However, the same ethanol treatment did not significantly alter the serum cytokine/chemokine levels in the adolescent male mice compared with the saline control group. In addition, significant differences in the serum MIP-1α levels were noted between the male and female saline-treated WT mice (Fig. 3a). To determine the differences between the WT male and female mice in the serum cytokine and chemokine levels, two-way ANOVAs were assessed. These analyses revealed a significant effect of interaction on IL-17A [$F(1, 28) = 10.22$, $P < 0.01$] and a main effect of gender [$F(1, 28) = 6.83$, $P < 0.05$] and interaction [$F(1, 28) = 4.30$, $P < 0.05$] on MIP-1α. No significant effects were found on IL-1β {gender [$F(1, 28) = 2.34$, $P > 0.05$], treatment [$F(1, 28) = 0.06$, $P > 0.05$], gender and treatment [$F(1, 28) = 0.01$, $P > 0.05$]}, IL-17A {treatment [$F(1, 28) = 2.14$, $P > 0.05$], gender [$F(1, 28) = 0.03$, $P > 0.05$]}, MCP-1 {gender [$F(1, 28) = 0.45$, $P > 0.05$], treatment [$F(1, 28) = 3.91$, $P > 0.05$], gender and treatment [$F(1, 28) = 1.78$, $P > 0.05$]}, MIP-1α {treatment [$F(1, 28) = 0.70$, $P > 0.05$]} and fractalkine {gender [$F(1, 28) = 2.45$, $P > 0.05$], treatment [$F(1, 28) = 0.01$, $P > 0.05$], gender and treatment [$F(1, 28) = 0.58$, $P > 0.05$]}.

We next assessed whether the differences in the serum levels of cytokines and chemokines observed

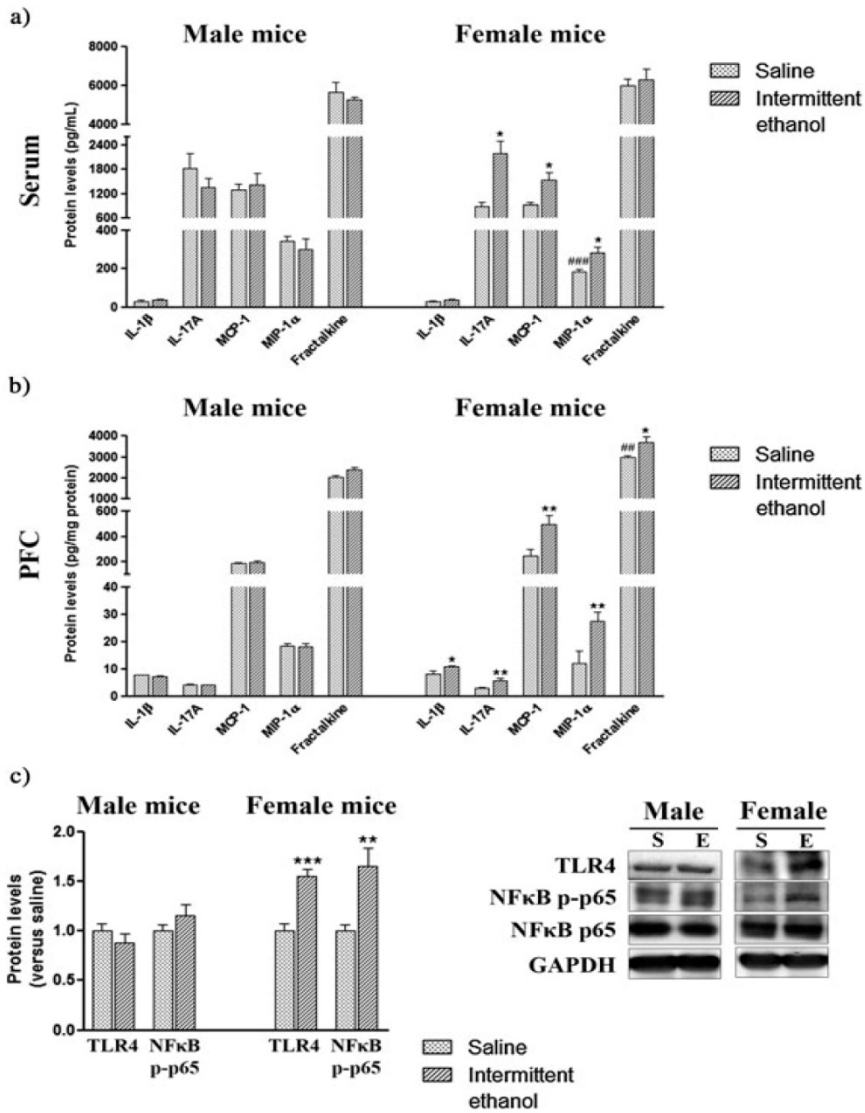

Figure 3 The prefrontal cortex (PFC) levels of interleukin (IL)-17A, IL-1β, fractalkine, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α of the male and female WT adolescent mice. Mice at PND 30 were treated intraperitoneally with eight intermittent ethanol doses (3 g/kg) or physiological saline. Graphs represent data from the (a) serum and (b) PFC of the female and male adolescent WT mice, treated or not with ethanol. Data represent mean ± standard error of the mean (SEM), $n = 8$ mice/group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with their respective saline-treated group, ## $P < 0.01$, ### $P < 0.001$ compared with the saline-treated male group. (c) Immunoblot analysis and quantification of TLR4 and NFκB p-p65 from the PFC of the female and male adolescent WT mice treated intermittently, or not, with ethanol. A representative immunoblot of each protein is shown. Data represent mean ± SEM, $n = 6$ mice/group. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with their respective saline-treated group

between the ethanol-treated WT adolescent male and female mice were associated with the changes in these pro-inflammatory molecules in the PFC. Figure 3b shows how intermittent ethanol treatment upregulated the levels of IL-1β, IL-17A, MCP-1, MIP-1α and the fractalkine levels in the PFC of the WT adolescent female mice compared with the untreated adolescent female WT mice, as revealed by using two-way ANOVA with Tukey's *post hoc* test. Significant differences were also noted in the fractalkine levels of PFC between the male and female saline-treated WT mice (Fig. 3b). Two-way ANOVAs were assessed to determine the differences between the adolescent WT male and female mice in the cytokine and chemokine levels of the PFC. The overall

analyses revealed a significant effect of variables gender, treatment and the gender–treatment interaction on IL-1β {gender [$F(1, 28) = 8.26$, $P < 0.01$], gender and treatment [$F(1, 28) = 5.87$, $P < 0.05$]}, IL-17A {treatment [$F(1, 28) = 7.07$, $P < 0.05$], gender and treatment [$F(1, 28) = 7.71$, $P < 0.01$]}, MCP-1 {gender [$F(1, 28) = 16.04$, $P < 0.001$], treatment [$F(1, 28) = 8.08$, $P < 0.01$], gender and treatment [$F(1, 28) = 7.22$, $P < 0.05$]}, MIP-1α {treatment [$F(1, 28) = 6.79$, $P < 0.05$], gender and treatment [$F(1, 28) = 7.08$, $P < 0.05$]} and fractalkine {gender [$F(1, 28) = 49.76$, $P < 0.001$] and treatment [$F(1, 28) = 11.63$, $P < 0.01$]}, with no significant effects on IL-1β {treatment [$F(1, 28) = 3.23$, $P > 0.05$]}, IL-17A {gender [$F(1, 28) = 0.42$, $P > 0.05$]}, MIP-1α {gender [$F(1, 28) = 0.24$, $P > 0.05$]} and fractalkine {gender and treatment [$F(1, 28) = 1.35$, $P > 0.05$]}.

We further assessed the protein expression of TLR4 and the phosphorylation of the transcription factor NFκB p65 subunit involved in the TLR4 signaling pathway, in the PFC of the WT male and female mice treated with saline or intermittent ethanol treatment. Two-way ANOVA with Tukey's *post hoc* test revealed that the levels of TLR4 and NFκB p65 phosphorylation significantly increased in the WT female mice, but not in males, treated intermittently with ethanol (Fig. 3c). For both proteins, the two-way ANOVAs revealed a significant effect of treatment {TLR4: [$F(1, 20) = 8.30$, $P < 0.01$], NFκB p-p65: [$F(1, 20) = 12.43$, $P < 0.01$]}, gender {TLR4: [$F(1, 20) = 20.10$, $P < 0.001$], NFκB p-p65: [$F(1, 20) = 4.83$, $P < 0.05$]} and interactions {TLR4: [$F(1, 20) = 20.10$, $P < 0.001$], NFκB p-p65: [$F(1, 20) = 4.83$, $P < 0.05$]}.

We also analyzed the cytokine and chemokine levels after an acute ethanol dose (1.5 hours post-injection). Unpaired Student's *t*-test analyses indicated that while an upregulation of IL-17A, IL-1β and MCP-1 took place in the serum of the WT female mice after one ethanol dose, no changes were observed in the cytokine/chemokine levels of the PFC, although both the TLR4 expression and NFκB p65 phosphorylation increased (Supporting Information Fig. S2).

Finally, we assessed the cytokine levels in the TLR4-KO adolescent female and male mice treated with either saline or ethanol. As shown in Fig. 4, ethanol treatment did not significantly change the cytokine/chemokine levels in either the serum (Fig. 4a) or PFC (Fig. 4b) in the TLR4-KO female and male mice.

These results indicated that the WT female mice were more vulnerable than the WT male mice to ethanol-induced immune activation, and also suggested the involvement of the innate immune receptors TLR4 response in ethanol effects.

DISCUSSION

The present findings indicated major gender differences in the immune response of ethanol. Thus, at the equivalent BALs, both the human and mice adolescent females had higher levels of plasma cytokines and chemokines than the adolescent males after acute alcohol intoxication. These results also provide evidence for the role of the TLR4 response in these effects because the TLR4 mRNA levels increased in the blood of the human adolescent females, but not in the males. Accordingly, the TLR4-KO adolescent female and male mice were protected against the inflammatory/neuroinflammatory response of ethanol.

The susceptibility of young females versus males to ethanol actions in adolescence has been pointed out in several studies. For instance, females who begin drinking in adolescence and who continue to drink heavily may be at a higher risk of brain damage and of behavioral problems later in life compared with male adolescents (Squeglia, Spadoni, Infante, Myers, & Tapert 2009). Accordingly, drinking in adolescence is associated with greater frontal cortical thinning in females than in males, effects that are linked to worse visuospatial and inhibition of attention performance (Squeglia *et al.* 2012; Squeglia *et al.* 2009). Executive functioning deficits have also been found in female substance-using teens (Giancola, Shoal, & Mezzich 2001; Moss, Kirisci, Gordon, & Tarter 1994). These results suggested that females could be much more susceptible than males to the neurodevelopmental dysfunction associated with heavy alcohol use in adolescence. In line with these results, a recent study performed in adult males and females has also indicated that after an acute ethanol dose, females had higher levels of BALs and circulating endotoxin levels than males (Bala, Marcos, Gattu, Catalano, & Szabo 2014). Although these gender differences in the immune response effects of ethanol are uncertain, differences in alcohol metabolism, hormonal changes and different alcohol distribution due to the body/fat ratio between females and males (Frezza *et al.* 1990; Wechsler, Dowdall, Davenport, & Rimm 1995) might explain some of the observed gender differences.

According to the human data, our results also showed how the cytokine and chemokine levels were higher in the serum and PFC of the adolescent female mice than in the male mice. These effects were associated with the TLR4 response because the TLR4 levels increased in the PFC of the WT female mice after different intermittent doses of ethanol in adolescence. These gender differences were not related to the differences found in BALs after administering ethanol because similar BALs levels were noted in the male and female mice. However, some studies (Middaugh, Frackelton, Boggan, Onofrio, & Shepherd

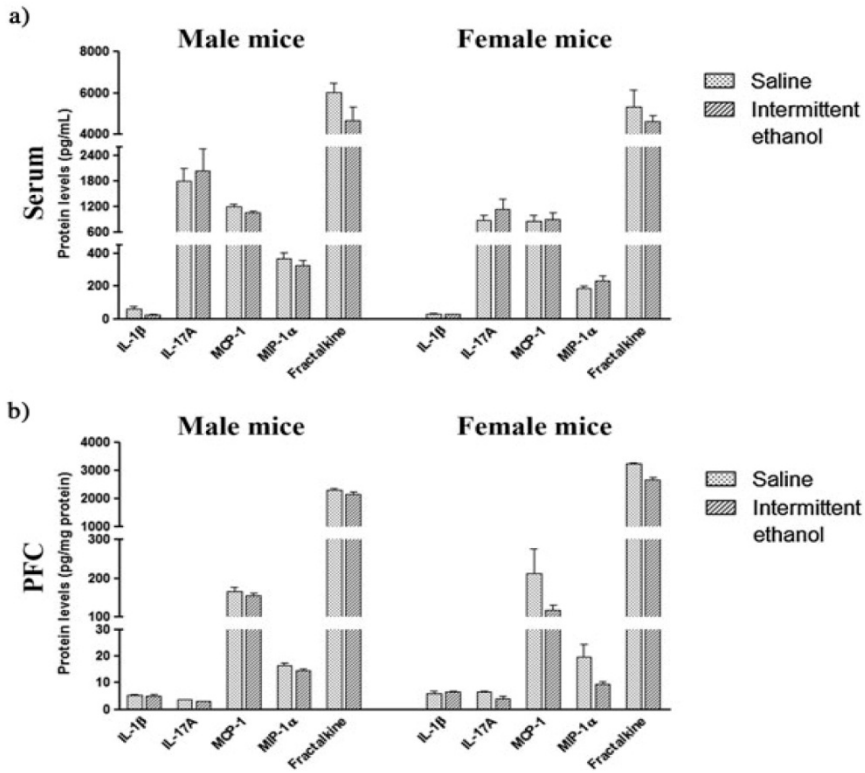

Figure 4 Serum levels of interleukin (IL)-17A, IL-1β, fractalkine, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α of the male and female toll-like receptor 4-knockout (TLR4-KO) adolescent mice. Mice at PND 30 were treated intraperitoneally with eight intermittent ethanol doses (3 g/kg) or physiological saline. Graphs represent data from the (a) serum and (b) prefrontal cortex (PFC) of the female and male adolescent TLR4-KO mice treated, or not, with ethanol. Data represent mean ± standard error of the mean (SEM), $n = 8$ mice/group

1992) report higher BALs in 2-month-old male mice than in female mice. We further showed that eliminating the TLR4, by using TLR4-KO mice, abolished the immune response in the serum and PFC of both male and female adolescent mice, which supports the role of these receptors in the ethanol-induced immune response. According to our results, the upregulation of both TLR4 immunoreactivity and pro-inflammatory cytokines in the PFC of adolescent rats treated intermittently with ethanol has previously been demonstrated (Vetreno *et al.* 2013), which supports the involvement of the immune response in ethanol effects.

In line with the results obtained in adolescent mice, we previously demonstrated some gender differences in adult mice because the neuroinflammation and astrogliosis associated with chronic alcohol intake were higher in female mice than in male mice and the TLR4-KO female mice were protected against neuroinflammatory ethanol actions (Alfonso-Loeches *et al.* 2013). The potential role of TLR4 in ethanol-induced immune activation was further supported by the human data that showed an upregulation of TLR4 gene expression in the blood of females, but not of males. This suggests the participation of the TLR4 response in the gender differences of the ethanol-induced cytokine and chemokine immune response after acute ethanol intoxication. According with these findings, a recent study shows that women exhibited a more profound pro-inflammatory response (e.g. IL-6 and TNF-α) than men after an intravenous LPS (TLR4 ligand) injection (Engler *et al.* 2016). In fact, alveolar macrophages of the female rats treated with LPS spontaneously released significantly more nitric oxide than the male rats (Spitzer 1997). Conversely, other studies have shown that estradiol attenuates the LPS-induced expression of CXCL8 in human peripheral blood monocytes (Pioli *et al.* 2007). Spitzer & Zhang (1996) demonstrated that the LPS challenge elicits a milder phagocytic response in the ethanol-treated liver polymorphonuclear leukocytes and Kupffer cells of female rats than in male rats (Spitzer & Zhang 1996). Differences in species and immune cells response might account for these divergences.

Our results also provide evidence that both human and mice adolescent females had higher levels of peripheral cytokines and chemokines than the adolescent males during or after acute alcohol intoxication. Changes and upregulation in plasma cytokine levels have also been reported in adolescent students with heavy binge drinking (Ward, Lallemand, & de Witte 2014), although no gender differences were assessed. Using the data of the human adolescents, we also examined the association between the BALs and the cytokine and chemokine circulating levels in both female and male adolescents with acute alcohol intoxication. When the linear regression analyses were used, we observed a potent linearity between the BALs and several cytokines and chemokines for both genders. Our results also showed that at the same BALs, e.g. 2 g/l, females displayed a stronger immune response than males. We also noticed that while 70 percent of the females with BALs of ~2 g/l (~45 mM) had higher levels of cytokines and chemokines than males, 30 percent of the females obtained very high BALs (3.2 g/l and ~75 mM) and displayed lower levels of cytokines and chemokines than at 2 g/l. This paradoxical effect could be explained by the biphasic effects of ethanol on the TLR4 response. Unfortunately, the negative correlation was not found in the males because they did not reach BALs over 75 mM. We previously showed that ethanol exerts biphasic action on TLR4; at low/moderate concentrations (10–50 mM), it interacts with membrane lipid rafts by activating the TLR4 response, while at a high concentration (75–100 mM), it disrupts lipid rafts and inhibits the receptor response and the release of cytokine (Blanco & Guerri 2007; Fernandez-Lizarbe, Pascual, Gascon, Blanco, & Guerri 2008). Likewise, corticoids have pro-inflammatory or anti-inflammatory effects in humans (Yeager, Pioli, & Guyre 2011). A single alcohol dose that resulted in a BAL of 100-mg percent activates the hypothalamo–pituitary–adrenal axis and leads to elevated cortisol levels (Richards, Fernandez, Caulfield, & Hawrylowicz 2000; Spencer & Hutchison 1999). It is therefore possible that high BALs could decrease the levels of cytokines/chemokines in ethanol-intoxicated females by inhibiting the TLR4 response or promoting the release of glucocorticoids and their anti-inflammatory response.

Activation of the immune system and the TLR4 might also occur through the release of gut bacteria components, as previously demonstrated in healthy adults after acute binge drinking (Bala *et al.* 2014). Interestingly, this study also shows that the levels of blood alcohol and circulating endotoxin were higher in women than in men, which corroborates the importance of gender differences in alcohol effects. To date, it remains unknown whether an ethanol-induced immune response is due to the release of endotoxin (Bala *et al.* 2014) and/or to direct ethanol action on the TLR4 response. However, if we consider that alcohol in the absence of LPS can trigger the TLR4 response in microglia and in RAW 264.7 macrophage cells (Fernandez-Lizarbe *et al.* 2008; Fernandez-Lizarbe *et al.* 2009), it is conceivable to suggest that both ethanol and LPS could be involved in the TLR4-dependent peripheral immune response. Another question is whether the cytokines and chemokines induced by an acute ethanol dose in the PFC could originate in the brain and/or is the result of a peripheral response to ethanol. Because alcohol can reach the brain and can trigger the TLR4 signaling response in glial cells within minutes (Alfonso-Loeches *et al.* 2010; Fernandez-Lizarbe *et al.* 2009), a direct effect of alcohol on the TLR4 response could be considered before peripheral inflammation could reach the brain. Nonetheless, we cannot rule out the coexistence of other mechanisms.

Finally, the present results also suggested that cytokines and/or chemokines can be used as biomarkers of ethanol-induced neuroinflammation in female adolescents because alcohol intoxication and/or binge-drinking increases the peripheral levels of IL-17A, MCP-1 and MIP-1α in both human and mice adolescent females and also because the levels of these cytokines/chemokines were also significantly high in the PFC of the adolescent female mice. In line with these results, we recently demonstrated that chronic ethanol consumption increased the levels of cytokines (IL-1β, IL-17A and TNF-α) and chemokines (MCP-1, MIP-1α and fractalkine) in the striatum and serum (MCP-1, MIP-1α and fractalkine) of WT male mice (Pascual *et al.* 2015). These variations in cytokine and chemokine levels between both models could be related with the different ages of the animals (adolescents versus adults), ethanol treatment (binge drinking versus chronic treatment) or brain area analyzed.

It is important to point out the limitations of our results when using human samples, particularly the small sample size and the unknown drinking pattern of several participants. In fact, the poor statistical power associated with a small sample size could have prevented us from finding significant differences in the plasma cytokine/chemokine levels between the control and ethanol-intoxicated males. Available results from our sample, however, are in agreement with previous data showing that twice as many adolescent males reported heavy binge drinking (≥5 standard drinks on at least 1 day in the recorded diary week) compared with females (Olsson *et al.* 2016). If we take this into account, it is most likely that adolescent and young adult males in our study have had more episodes of binge drinking than females, although it is uncertain to which extent this could explain our results. Another limitation of this study is that the human ethanol samples were collected during the intoxication period, while the murine samples were collected 24 hours after the last ethanol dose. Human

studies indicate that a binge drinking episode causes a transient pro-inflammatory state, which is followed by an anti-inflammatory state (e.g. Afshar *et al.* 2015).

Taken together, these results indicate that female adolescents are more vulnerable than male adolescents to the inflammatory effects of binge alcohol drinking. The findings also suggest that circulating cytokines and chemokines might serve as clinical biomarkers of neuroinflammation and that the TLR4 response is an important target of the ethanol-induced immune and neuroimmune response in adolescence.

Acknowledgements

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Authors Contribution

CG, MP, JM, FJL and MM were responsible for the study concept and design. PCA and JLT contributed to the collection of human samples and clinical data and to the acquisition of the human TLR4 mRNA data. JM and MP contributed to the acquisition of animal samples and to carry on the enzyme-linked immunosorbent assay and Western blot experiments and to analyze all data. FGG contributed to the correlation and linear regression analyses. MP, JM and CG drafted the manuscript. MM, JLT, PCA and FJL provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table 1S: Pearson's correlation between the plasma levels of cytokines or chemokines and the BALs in females and males with alcohol intoxication. Data marked in dark grey are statistically significant, while data in light grey denote a tendency to be significant.

Figure 1S: Examples of dispersion data between BALs and fractalkine, IFN-γ and IL-4 in females and males with alcohol intoxication. It is also shown the linear regression between BALs and fractalkine, IFN-γ and IL-4 in intoxicated females, which their slope and constant are shown in Table 2.

Figure 2S: The PFC and serum levels of IL-17A, IL-1β, MCP-1 and MIP-1α of the female WT and TLR4-KO adolescent mice. Female mice at PND 30 were treated intraperitoneally with one ethanol dose (3 g/kg) or physiological saline. After 1.5 h of the injection, serum was collected and the PFC was removed. Graphs represent data from the **(A)** PFC and **(B)** serum of the female WT and TLR4-KO adolescent mice. Data represent mean ± SEM, $n = 8$ mice/group. * $p < 0.05$ compared to their respective saline-treated group, and according to an unpaired Student's *t*-test. **(C)** Immunoblot analysis and quantification of TLR4 and NFκB p-p65 from the PFC of the female WT mice treated with an acute ethanol dose (1.5 h). A representative immunoblot of each protein is shown. Data represent mean ± SEM, $n = 6$ mice/group. * $p < 0.05$, *** $p < 0.001$ compared to their respective saline-treated group, and according to an unpaired Student's *t*-test.

Table 1S. Spearman's correlation between the plasma levels of cytokines or chemokines y the BALs in females y males with alcohol intoxication. Data marked in dark grey are statistically significant, while data in light grey denote a tendency to be significant.

	Intoxicated females		Intoxicated males	
	Correlation coefficient	p	Correlation coefficient	p
CSF	-0.609	0.061	0.092	0.765
IFN-γ	-0.720	0.019	0.573	0.041
IL-10	-0.511	0.131	0.277	0.359
IL-17A	-0.505	0.137	0.277	0.360
IL-1β	-0.640	0.046	0.131	0.669
IL-2	-0.671	0.034	0.021	0.946
IL-4	-0.571	0.085	0.470	0.105
IL-5	-0.455	0.186	0.240	0.429
IL-6	-0.765	0.010	0.095	0.758
IL-8	-0.111	0.761	0.439	0.134
TNF-α	-0.419	0.225	0.419	0.154
Fractalkine	-0.926	0.001	0.155	0.613
MCP-1	-0.689	0.027	-0.159	0.603
MIP-1α	-0.535	0.060	0.506	0.077

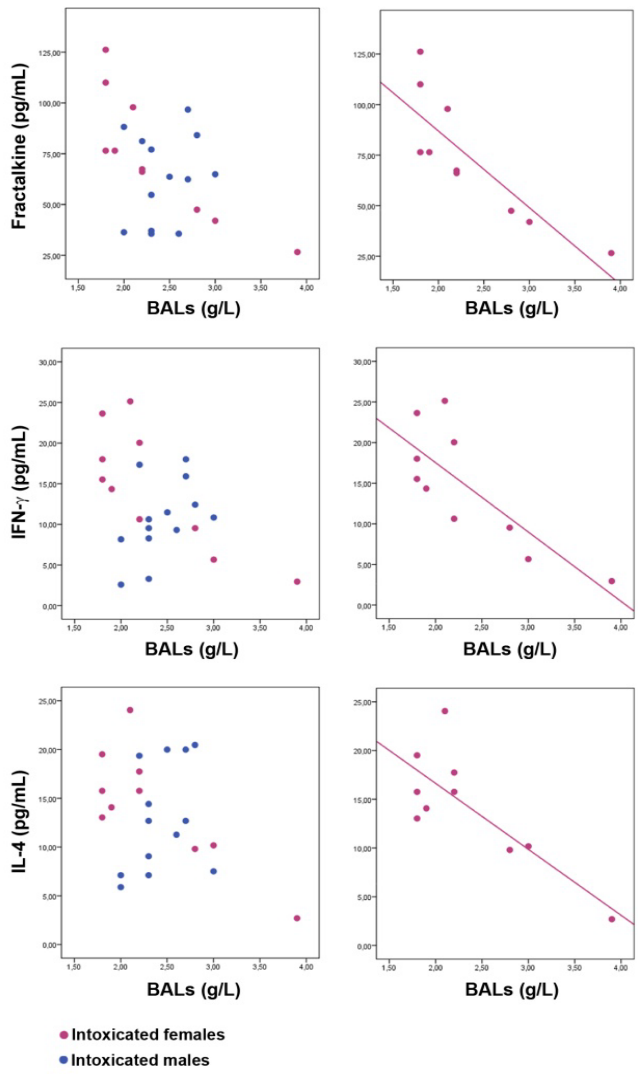
Fractalkine (pg/mL)
IFN-γ (pg/mL)
IL-4 (pg/mL)
BALs (g/L)
Intoxicated females
Intoxicated males

Figure 1S

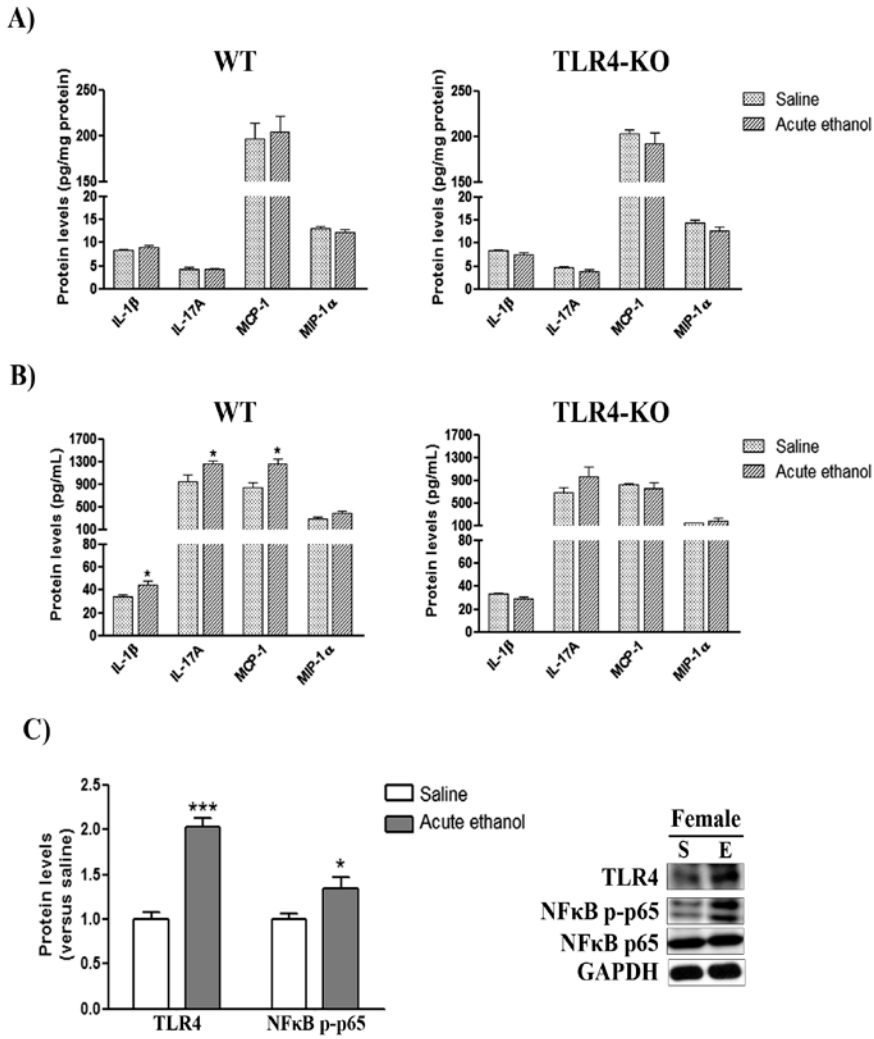
A)
WT
TLR4-KO
Saline
Acute ethanol
Protein levels (pg/mg protein)
IL-1β
IL-17A
MCP-1
MIP-1α
B)
WT
TLR4-KO
Saline
Acute ethanol
Protein levels (pg/mL)
IL-1β
IL-17A
MCP-1
MIP-1α
C)
Saline
Acute ethanol
Protein levels (versus saline)
TLR4
NFκB p-p65
Female
S
E
TLR4
NFκB p-p65
NFκB p65
GAPDH

Figure 2S

4.4. Publicación IV

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Critical Review

Impact of the Innate Immune Response in the Actions of Ethanol on the Central Nervous System

Jorge Montesinos*, Silvia Alfonso-Loeches*, and Consuelo Guerri

The innate immune response in the central nervous system (CNS) participates in both synaptic plasticity and neural damage. Emerging evidence from human and animal studies supports the role of the neuroimmune system response in many actions of ethanol (EtOH) on the CNS. Research studies have shown that alcohol stimulates brain immune cells, microglia, and astrocytes, by activating innate immune receptors Toll-like receptors (TLRs) and NOD-like receptors (inflammasome NLRs) triggering signaling pathways, which culminate in the production of pro-inflammatory cytokines and chemokines that lead to neuroinflammation. This review focuses on evidence that indicates the participation of TLRs and the inflammasome NLRs signaling response in many effects of EtOH on the CNS, such as neuroinflammation associated with brain damage, cognitive and behavioral dysfunction, and adolescent brain development alterations. It also reviews findings that indicate the role of TLR4-dependent signaling immune molecules in alcohol consumption, reward, and addiction. The research data suggest that overactivation of TLR4 or NLRs increases pro-inflammatory cytokines and mediators to cause neural damage in the cerebral cortex and hippocampus, while modest TLR4 activation, along with the generation of certain cytokines and chemokines in specific brain areas (e.g., amygdala, ventral tegmental area), modulate neurotransmission, alcohol drinking, and alcohol rewards. Elimination of TLR4 and NLRP3 abolishes many neuroimmune effects of EtOH. Despite much progress being made in this area, there are some research gaps and unanswered questions that this review discusses. Finally, potential therapies that target neuroimmune pathways to treat neuropathological and behavioral consequences of alcohol abuse are also evaluated.

Key Words: Alcohol, TLR4 and NLRP3, Neuroimmune Signaling, Neuroinflammation, Brain Damage, Behavior.

INTRODUCTION TO THE NEUROIMMUNE SYSTEM

THE ROLE OF the immune system and its relevance in the pathogenesis of alcoholic liver disease, as well as susceptibility of alcoholics to infections, has been well established (Szabo and Saha, 2015). Nevertheless in the last decade, new insights into mechanisms of the immune system response have helped us advance in our knowledge about the effects of ethanol (EtOH) on immune system dysregulation and its contribution in a wide range of alcohol-associated disorders (Szabo and Saha, 2015), including neuroinflammation and central nervous system (CNS) dysfunctions (Crews et al., 2015).

For many years, the CNS was considered an immune-privileged site, until it was well established that immune surveillance occurs in the normal CNS (Shrestha et al., 2013) and that inflammatory responses can mediate either physiological or pathological processes. Under normal conditions, immune mechanisms are activated by environmental and psychological stimuli, which modulates the communication pathways from the peripheral immune system to the brain, and also by the signals produced by immune-like processes that involve neuro-glial communication. These signals are mediated by the secretion of low levels of inflammatory cytokines (e.g., interleukin [IL]-1, IL-6, and tumor necrosis factor [TNF]-α; Boulanger, 2009), which modulate the release of neurotransmitters and hormones (e.g., glucocorticoids; Yirmiya and Goshen, 2011), and promote neuronal plasticity and neurogenesis. However, sustained inflammation or a persistent stimulus that results from either environmental factors or the formation of endogenous factors (e.g., pathogens, protein aggregates, toxins, psychological stress) impairs memory, neural plasticity, and neurogenesis. Overactivation of the neuroimmune system is involved in neurodegenerative diseases (Glass et al., 2010), neuropsychiatric disorders (Hodes et al., 2015; Rosenblat and McIntyre, 2016), and drug addiction (Jacobsen et al., 2016).

Evidence in human alcoholic brain and animal models has demonstrated that alcohol abuse up-regulates the

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expression of neuroimmune genes and microglial transcript, which supports the role of the innate immune system response in many EtOH actions on the CNS (Crews et al., 2015; Mayfield et al., 2013; Robinson et al., 2014). This article reviews the cellular and molecular mechanisms by which EtOH is capable of activating different signaling pathways associated with the 2 important targets of the neuroimmune response, the innate immune receptors Toll-like receptor 4 (TLR4) and NLRP3. Activation of these receptors allows the induction of cytokines and chemokines, which promotes neuroinflammation, brain damage, behavioral and cognitive dysfunction, and addiction. The neuroimmune mechanism of alcohol offers new approaches to develop more effective pharmacotherapies to treat alcohol-related neuropathology and alcohol abuse.

INNATE IMMUNE RECEPTORS, TLRS, AND NOD-LIKE RECEPTORS, IN THE CNS

The CNS is immunologically active and is able to recognize and respond to pathogens, toxins, and danger molecules by activating the innate immune response (Lampron et al., 2013). Inflammatory responses to infectious agents are initiated by pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs, stranger signals), molecular "signatures" of whole classes of microorganism (Akira et al., 2001). This immune response does not provide long-lasting immunity or memory, unlike what happens in the adaptive immune system.

Toll-Like Receptors

Among PRRs, TLRs are the first identified and best characterized evolutionarily conserved family of receptors, discovered in the *Drosophila melanogaster* as a major defense against microbial infection (Medzhitov et al., 1997). The TLR family comprises 10 members (TLR1 to TLR10) in humans, but the most widely studied is TLR4, which are receptors that recognize the endotoxin lipopolysaccharide (LPS), a part of the external membrane of *gram*-negative bacteria. TLRs are class I transmembrane proteins, the extracellular domains of which contain leucine-rich repeats involved in protein–protein interactions and ligand recognition. TLR1, TLR2, TLR4, TLR5, and TLR6 are all receptors located on the cell surface, while TLR3, TLR7, TLR8, and TLR9 are located within the endosome/lysosome membrane (Nishiya and DeFranco, 2004). However, the subcellular localization of some nucleic acid-sensing TLRs, such as TLR3, depends on cell type (Matsumoto et al., 2002, 2003).

Stimulation of TLRs by PAMPs evokes the activation of different signaling pathways, including nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), which results in the production of cytokines, chemokines, and reactive oxygen species (ROS) that serve to destroy pathogens (Fig. 1).

Among glial cells, astroglia and microglia are the immune cells in the CNS and express most of the TLRs, particularly TLR4 (Okun et al., 2011). Neurons also express TLR4 and can respond to LPS by releasing cytokines and chemokines, although TLR4 signaling pathways differ between neurons and glial cells (Leow-Dyke et al., 2012; Okun et al., 2011).

Brain endothelial cells, an important component of the blood–brain barrier (BBB), also express TLR4 (Nagyoszi et al., 2010). These cells can receive immune signals from either the brain or peripheral blood by releasing cytokines within the brain which, in turn, can activate glial cells or neurons. However, it is uncertain whether CNS inflammation results from the neural or peripheral TLR4 response, although some evidence suggests a cross-talk between both systems as peripheral TLR4 signaling can mediate the CNS immune response (Roach et al., 2013).

NOD-Like Receptors

Another type of important innate immune PRRs is NOD-like receptors (NLRs) (nucleotide-binding domain leucine-rich repeat containing); these being cytoplasmic receptors that sense and detect invading pathogens and can initiate an innate immune response. NLR family members NLRP1, NLRP3, NLRC4, and AIM2 can also be activated by PAMPs or by nonmicrobial danger signals or damaged-associated molecular patterns (DAMPs).

The link between TLRs and NLRs occurs by the activation of NF-κB by TLRs, which induces pro-IL-1β until a second stimulus (e.g., ions or membrane perturbations, ROS, or extracellular ATP) promotes the recruitment of the large multiprotein complex, called inflammasome, by activating caspase-1. This, in turn, cleaves pro-ILβ into its bioactive molecule IL-1β, along with other cytokines of this family (IL-18, IL-33) (Guo et al., 2015). The discovery of inflammasome (Martinon, 2012) has advanced our knowledge of the role of the innate immune system in several immune pathologies, including neurodegeneration (Guo et al., 2015) (Fig. 1), as IL-1β is a potent pro-inflammatory cytokine that can promote sickness and depression (Konsman et al., 2008; Raison et al., 2006). Among the different NLRs inflammasomes, NLRP3 (or cryopyrin) is the best characterized, whose activation is evidenced in cryopyrin-associated periodic syndromes (Willingham et al., 2007). NLRP3 is expressed in microglia (Hanamsagar et al., 2012), astrocytes (Lu et al., 2014; Tezel et al., 2012), and neurons (Zou and Crews, 2012). Indeed, EtOH can activate different inflammasomes, including NLRP3, in neural cells by triggering the production of IL-1β, IL-18, and IL-33 (Alfonso-Loeches et al., 2014, 2016) (Fig. 1).

Both membrane TLRs and cytoplasmic NLRs are not only activated by pathogens or PAMPs, but are also highly effective at sensing and responding to the released components of DAMPs or "alarmins" (Bianchi, 2007). DAMP-triggered "sterile inflammation" is most important in the

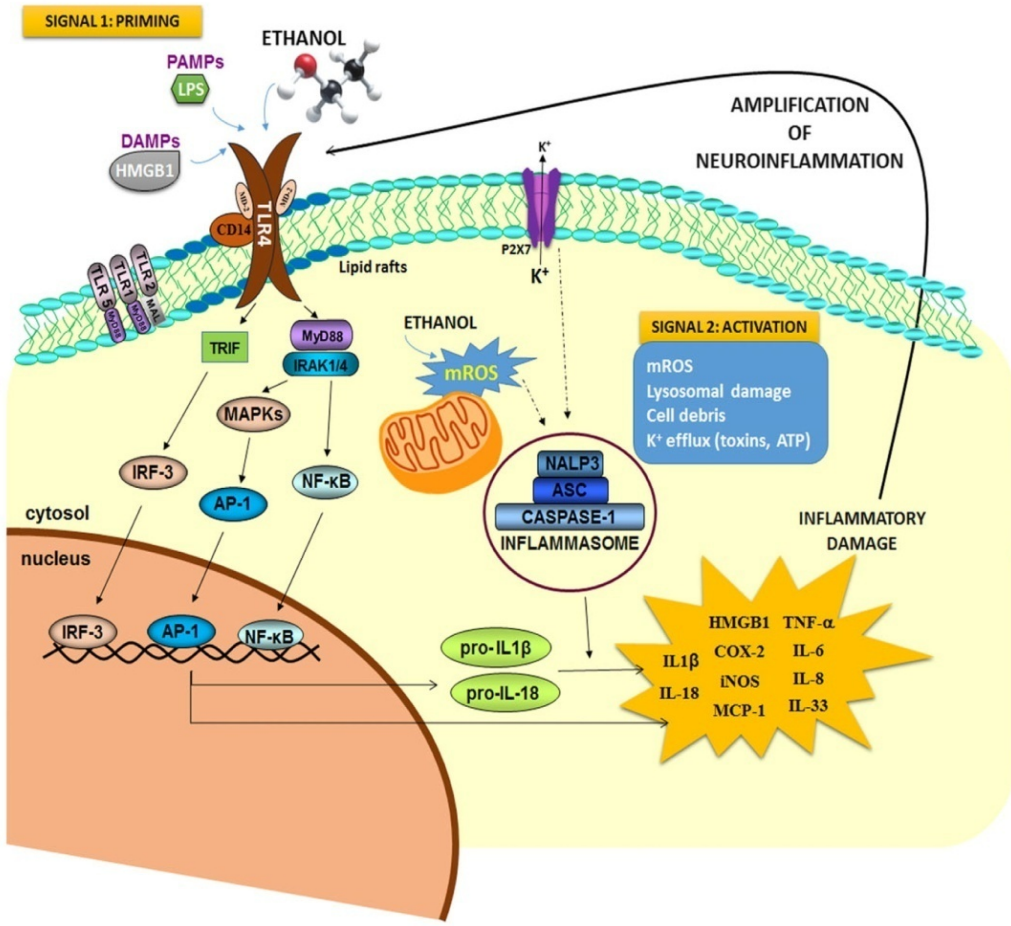

Fig. 1. Diagram of TLR4 and NLRP3 inflammasome receptors activation and alcohol-induced neuroinflammation. Activation of TLR4 by PAMPs (LPS) or ethanol or DAMPs (HMGB1) is initiated by the recruitment of TLR4 with adaptor proteins CD14 and MD2 into lipid rafts. This activates and recruits several proteins and kinases (MyD88, IRAK 1-4, TRIF) and triggers fast downstream signaling pathways (i.e., MAPKs) and transcription nuclear factors (NF-κB, AP-1, IRF3), which culminate in the generation of cytokines (i.e., pro-IL-1β, IL-6, IL-8, and TNF-α), chemokines (i.e., MCP-1), and inflammatory mediators (iNOS and COX-2), leading to neuroinflammation. Stimulation of TLR4 also promotes the production of pro-IL1β (priming signal) which, along with the presence of other stimuli (second signal, i.e., mitochondrial (m)ROS generation, lysosomal damage, K^+ efflux, some toxins or ATP), triggers NLRP3 inflammasome multiprotein complex recruitment by activating cysteine-protease caspase-1, which cleaves pro-IL1β into active IL-1β, as well as other cytokines such as IL-18 and alarmins (IL-33, HMGB1). Alcohol activates NLRP3 inflammasome in glial cells by stimulating the generation of pro-IL1β via TLR4 along with mROS formation (second signal) to generate IL-1β, IL-18, and IL-33 which, in turn, can amplify the neuroinflammatory response. MyD88: myeloid differentiation primary response gene 88; IRAK: interleukin-1 receptor-associated kinase-1; ROS: reactive oxygen species; TRIF: TIR-domain-containing adaptor-inducing IFNβ; MAPK: mitogen-activated protein kinase; IRF3: Interferon regulatory factor 3; ASC: speck-like protein containing a CARD; AP-1: activator protein 1.

neuroinflammatory disorders that occur in the absence of any foreign pathogens, as in neurodegenerative diseases (Glass et al., 2010). One relevant alarmin is the high-mobility group box 1 (HMGB1) protein, a nuclear protein which, given a certain stimulus, is hyperacetylated on lysine residues and translocates into the cytosol. HMGB1 is an endogenous ligand for different TLRs (TLR2, TLR4, TLR9), RAGE (receptor for advanced glycation products), and TREM-1 (triggering receptor expressed in myeloid cells 1) receptors and leads to the activation of NF-κB, and also to cytokine and chemokine production (El Mezayen et al., 2007; Ibrahim et al., 2013).

Advanced knowledge in TLRs and NLRs response by endogenous compounds, as alarmins or DAMPs, has highlighted their role in many neurological and neurodegenerative disorders (Amor et al., 2010; Hanamsagar et al., 2012). Several studies have also demonstrated that alcohol directly or indirectly activates TLRs and NLRs response by contributing to not only EtOH-induced neuroinflammation, behavior, and neural damage (Alfonso-Loeches et al., 2010, 2012, 2014; Montesinos et al., 2016), but also to alcoholic liver injury (Szabo and Lippai, 2014). This scenario demonstrates the contribution of both innate immune receptors in EtOH-induced inflammation and even organ damage.

TLRS AND NLRS AS MOLECULAR TARGETS IN ETOH-INDUCED NEUROINFLAMMATION

The first experimental studies to show that alcohol intake can activate brain signaling pathways by promoting pro-inflammatory cytokine production and neural death were published in 2004 (Valles et al., 2004). More recently, it has been confirmed that EtOH potentiates the LPS response by promoting the up-regulation of NF-κB DNA binding activity and pro-inflammatory gene expression in rat brain slices (Qin et al., 2008). However, the direct effect of EtOH on TLR4 was demonstrated in primary cultured astroglial and microglial cells. These studies proved that EtOH, in the absence of any toxin, can recruit TLR4 and IL-1RI receptors into membrane *lipid rafts* by activating their signaling response (MAPK, NF-κB, AP-1) and the release of cytokines and inflammatory mediators in medium (Blanco et al., 2005, 2008; Fernandez-Lizarbe et al., 2009). EtOH, as LPS (TLR4 ligand) does, promotes TLR4 receptor endocytosis that is dependent on clathrin and rafts/caveolae, and EtOH-induced TLR4 signaling is associated with receptor internalization and trafficking (Pascual-Lucas et al., 2014). Blocking TLR4 in glial cells using either siRNA or cells from TLR4-deficient mice (TLR4-knockout [KO]) abolishes EtOH effects on the inflammatory response in cultured astrocytes and microglia (Alfonso-Loeches et al., 2010; Fernandez-Lizarbe et al., 2009).

However, the critical role of TLR4 in the EtOH-induced inflammatory response and glial activation was clearly demonstrated in vivo using mice with chronic EtOH intake. These studies showed that EtOH treatment causes gliosis, induces MAPK and NF-κB signaling activation, neuroinflammation (up-regulation of inflammatory cytokines and mediators), and promotes myelin disruptions and apoptosis in the cerebral cortex of WT mice. TLR4-KO mice are protected against neuroinflammation and the neuropathological alterations induced by chronic EtOH abuse (Alfonso-Loeches et al., 2010, 2012). Chronic EtOH intake also impairs both ubiquitin–proteasome and autophagy–lysosome pathways by inducing brain accumulation of ubiquitinated proteins and reducing autophagy (events dependent on TLR4 activation), which might participate in EtOH-induced brain damage and neurodegeneration (Pla et al., 2014).

It is noteworthy that the neuroimmune effects of alcohol remain for long periods after alcohol exposure, as demonstrated by Qin and colleagues (2008). This study showed that EtOH potentiates the LPS-induced increase of TNF-α, monocyte chemoattractant protein-1 (MCP-1 or CCL2), and IL-1β in the liver, brain, and serum. However, the up-regulation of cytokines observed in the mouse brain remained high, whereas the peripheral levels returned to normal (Qin et al., 2008). This suggests that immune signaling in the brain can be much more persistent compared to peripheral immune activation. Long-term effects of EtOH on neuroimmune system activation have also been observed in adult animals exposed to alcohol in adolescence (Montesinos et al., 2016; Vetreno et al., 2013).

According to experimental data, changes in the expression of neuroimmune genes, such as microglial transcripts MCP-1 (He and Crews, 2008), and specific microRNAs involved in immune function regulation (Lewohl et al., 2011) have been found in postmortem alcoholic brains, which thus supports the role of the neuroimmune system in alcoholism.

EtOH also activates other immune receptors, such as NLRP3 inflammasome, in astroglia and microglia from the cerebral cortex of chronic EtOH-treated mice, as well as in primary cultured astroglial and microglial cells (Alfonso-Loeches et al., 2014, 2016), by triggering the production of IL-1β, IL-18, and IL-33, and lead to cell death by pyroptosis, a type of necrotic cell death. Activation of NLRP3 inflammasome is TLR4-dependent as it is initiated by the generation of pro-IL-1β by TLR4 signaling (priming signal) which, along with EtOH-induced mitochondria (m)ROS production (second signal), triggers the recruitment of the inflammasome complex, caspase-1 activation, and pro-inflammatory cytokine IL-1β release (Alfonso-Loeches et al., 2014) (Fig. 1). Notably, activation of TLR4/NLRP3-inflammasome signaling in microglial cells promotes leukocyte infiltration, which compromises BBB permeability (Alfonso-Loeches et al., 2016). Activation of NLRP1 and NLRP3 inflammasomes also contributes to inhibitory effects of EtOH on hippocampal neurogenesis (Zou and Crews, 2012). Studies in vivo further demonstrated that alcohol up-regulates and activates NLRP3/ASC inflammasome, which leads to caspase-1 activation and IL-1β production in the cerebellum. IL-1β amplifies neuroinflammation, while the disruption of IL-1/IL-1R signaling prevents alcohol-induced inflammasome activation and neuroinflammation (Lippai et al., 2013). These results support the key role of NLRP3/ASC and IL-1β production in EtOH-induced neuroinflammation.

The question raised from the above findings is whether alcohol-induced activation of TLR4 and NLRs response is caused by direct EtOH interactions with these receptors or by DAMPs such as HGMB1. Despite EtOH rapidly crossing the BBB and being able to initiate inflammatory events by activating TLR4/NLRs in glial cells, neuroinflammation amplification by these processes could occur via a mechanism in which endogenous TLR4 ligands, such as HGMB1, activate these receptors, and thus contribute to brain damage (Crews and Vetreno, 2014). Additionally, EtOH induces the release of LPS from the gut into the bloodstream (Mandrekar and Szabo, 2009), which activates TLR4 in liver Kupffer cells and peripheral monocytes, which can trigger the production of cytokines that can cross the BBB via diffusion or active transport, or even by BBB alterations (see Fig. 2). Disruption of BBB integrity has been recently reported in postmortem alcoholic brain (Rubio-Araiz et al., 2016), but TLR4-KO mice were protected against EtOH-induced BBB disruption (Alfonso-Loeches et al., 2016; Rubio-Araiz et al., 2016). At present, it remains uncertain

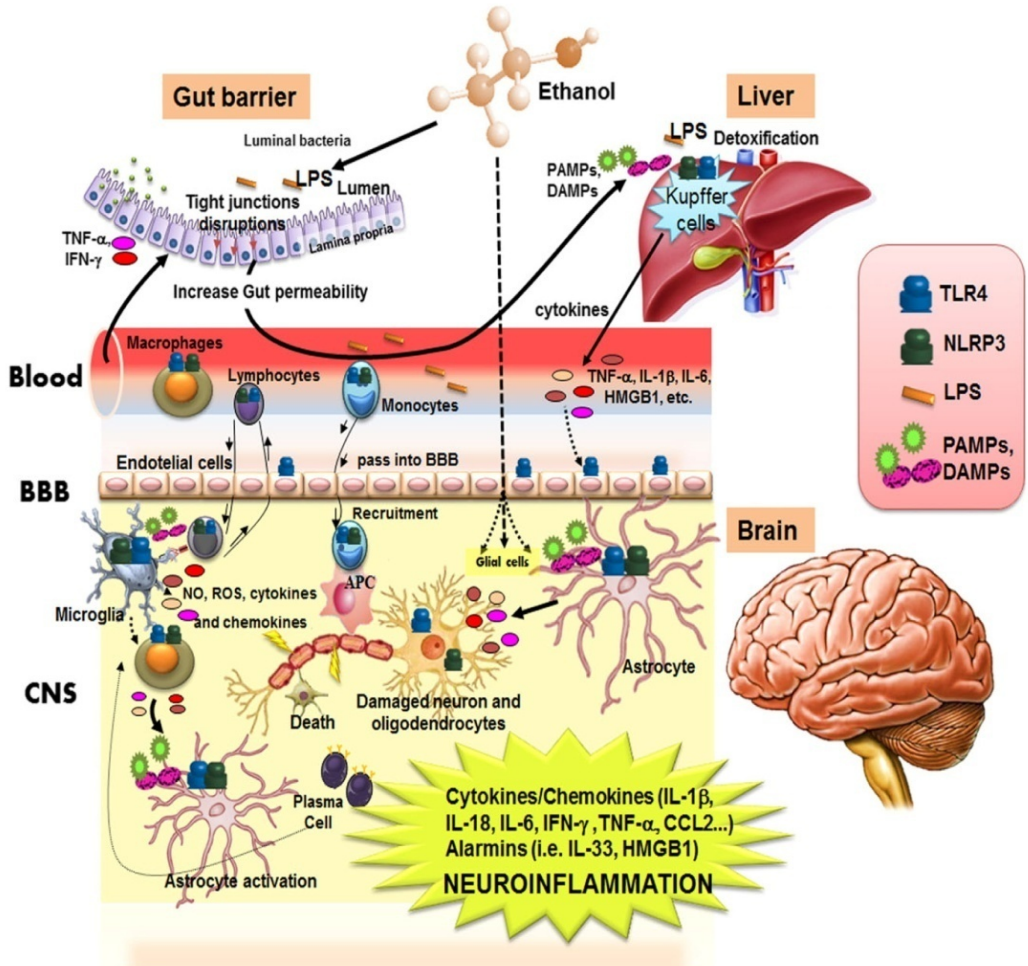

Fig. 2. Brain and periphery immune system interactions. Alcohol, by inducing tight junctions' disruptions in the epithelium of intestinal mucosa, increases its permeability to LPS (Mandrekar and Szabo, 2009) and allows LPS to enter systemic circulation. Then, LPS in the bloodstream reaches the liver and stimulates TLR4 in liver Kupffer cells to result in an increase in pro-inflammatory cytokines and chemokines which can cross the blood–brain barrier (BBB) and activate brain endothelial cells, microglia, and astrocytes. Peripheral cytokines and/or the direct effect of ethanol on the TLR4/NLRP3 signaling pathway can trigger cytokines/chemokines/alarmins (e.g., IL-1β, IL-18, IL-33, HMGB1, CCL2) release, causing astrogliosis, microgliosis, and leukocyte infiltration. High pro-inflammatory environment can induce neuroinflammation, myelin disruptions, and neural damage. Once the BBB is injured, plasma cells and antigen-presenting cells (APCs), including macrophages, enter the CNS and amplify the neural damage.

whether 1 mechanism or more can be involved in EtOH-induced brain cytokine production and if it depends or not on the alcohol drinking pattern (acute vs. chronic). A recent study has found increased levels of peripheral pro-inflammatory cytokines in noncirrhotic alcohol-dependent subjects compared with control subjects, which correlated with alcohol craving (Leclercq et al., 2012). This suggests a role for the gut–brain axis in alcohol dependence. Further research is needed to understand the contribution of not only the peripheral versus central immune response in EtOH-induced neuroimmune activation, but also of BBB integrity.

Finally, although chronic EtOH consumption or binge EtOH drinking can cause neuroinflammation and brain damage, transient exposure to low/moderate levels of EtOH increases the levels of anti-inflammatory cytokines, such as IL-10 and TGFβ in monocytes (Mandrekar et al., 2006) and peritoneal macrophages (Pascual et al., 2011b), which suggests that low/moderate EtOH intake may offer some beneficial actions on the immune system response. Recent studies (Doremus-Fitzwater et al., 2014; Gano et al., 2016) also support the anti-inflammatory role of acute EtOH intoxication as the gene expression of IL-1β and TNF-α was suppressed in the hippocampus, amygdala, and bed nucleus of the stria terminalis after 3 hours, while the IL-1β mRNA levels increased during withdrawal (18 hours postinjection). However, it is important to note that gene expression not always mimics protein levels as cytokine and chemokine expression is finely regulated in multiple posttranscriptional (Kovarik et al., 2015) and posttranslational (Tang et al., 2007) steps.

ROLE OF TLRS/NLRS SIGNALING IN ALCOHOL DRINKING BEHAVIOR

EtOH-induced changes in neuroimmune signaling also appear to have a significant impact on behavior and alcohol drinking. Several animal studies have demonstrated that blocking TLR4-MyD88 signaling changes the EtOH response. Null mutations in either TLR4 or MyD88 cut the motor impairment recovery time induced by a single EtOH injection to BALB/c wild-type (WT) mice (Wu et al., 2012). C3H/HeJ mice, a strain that is naturally TLR4 deficient, displayed reduced operant EtOH self-administration compared to controls (Harris and Blednov, 2013). However, naïve C57BL/6-TLR4-KO mice had a similar alcohol intake and preference to WT counterparts (Pascual et al., 2011a), although TLR4-KO mice were protected against the escalation of alcohol preference caused by intermittent EtOH treatment (Montesinos et al., 2016). C57BL/6-TLR4-KO mice are also protected against the EtOH-induced up-regulation of brain cytokines and chemokines, long-term cognitive dysfunctions, and anxiety-related behavioral impairments observed in WT mice during a withdrawal period after chronic EtOH treatment (Pascual et al., 2011a, 2015).

Activation of TLR4 signaling triggers cytokine and chemokine production, which can also influence alcohol drinking and behavioral patterns. For instance, mice that lack chemokine MCP-1 or CCL2, or its receptor CCR2, display a less marked preference for alcohol (Blednov et al., 2005). Interestingly, EtOH-triggered MCP-1 release is TLR4 dependent as using either mice that lack TLR4 (Montesinos et al., 2015) or TLR4 silencing in glial cells (June et al., 2015) eliminates MCP-1 release. The targeted elimination of MCP-1/CCL2 in central amygdala (CeA) or the ventral tegmental area (VTA) blunts binge drinking (June et al., 2015), while a chronic intracerebroventricular infusion of MCP-1/CCL2 increases alcohol operant self-administration (Valenta and Gonzales, 2016).

Inflammasome NLRs activation results in IL-1β production which, in turn, activates IL-1R1, and both of which influence alcohol response. Blednov and colleagues (2015) showed that deletion of the il1r1 gene (that encodes IL-1R1, a receptor activated by EtOH) reduces sensitivity to sedative effects of EtOH and enhances acute EtOH withdrawal severity, but EtOH consumption remains unaltered. Deletion of the IL-1R1 antagonist gene, il1rn (IL-1ra), strongly reduces alcohol intake in mice (Blednov et al., 2015), while a bilateral infusion of IL-1Ra into the basolateral amygdala reduces EtOH consumption without affecting sucrose consumption or open-field locomotor activity (Marshall et al., 2016). Similar results have been obtained using KO mice with deleted immune-related genes, such as B2 m (beta-2 microglobulin), IL-6, Ctss (cathepsin S), and Ctsf (cathepsin F) (Blednov et al., 2012). These genes code for the proteins involved in the inflammatory and immunological functions of microglia and astrocytes.

Finally, more work is needed to search for the potential role of different TLRs and their interactions with TLR4 in the actions of EtOH on the brain. Indeed, some studies demonstrate that EtOH-induced TLR2 and TLR4 interactions (Fernandez-Lizarbe et al., 2013) trigger higher neuroinflammation and neuronal death than TLR2 or TLR4. Mice that lack TLR4 or TLR2 are largely protected against EtOH-induced cytokine and chemokine release, and behavioral-associated effects during alcohol abstinence (Pascual et al., 2015).

In summary, experimental evidence mainly from KO mice has indicated that TLR4, as well as some cytokines and chemokines, participate in alcohol intake modulation. A critical limitation of using KO mice during development is potential compensatory changes, as well as other factors (e.g., interaction with other genes that may depend on the genetic background of the mice strain) that could influence neurochemical and behavioral effects of EtOH. Despite this limitation, the data obtained using different mice strains support the role of immune molecules in alcohol drinking. An alternative approach is to target TLR4 in adult brain glial cells (knock-in or conditional transgenic mice) or to block the expression of a target gene in a specific brain area. A recent study, which employed siRNA vectors to target TLR4/MCP-1 infused into CeA and VTA, demonstrated that blocking the activity of TLR4/MCP-1 blunted binge drinking (June et al., 2015). The authors indicated that the signal was sustained during alcohol drinking by an increased expression of corticotropin-releasing factor and its feedback regulation of TLR4 expression, effects that could contribute to the transition to alcohol dependence.

NEUROIMMUNE SIGNALING AND NEUROTRANSMISSION

An important new area of interest is the relationship between neuroimmune signaling (e.g., cytokines and chemokines) and modulation of the synaptic function. It is well established that glutamatergic and GABAergic neurotransmissions are critical in the actions of EtOH in the CNS. Several studies have demonstrated that EtOH promotes the production and release of several cytokines and chemokines, such as IL-1β, TNF-α, CCL2, IL-6, CX_3CL_1, and that most of these molecules are involved in synaptic transmission and plasticity from different neuronal populations (Donzis and Tronson, 2014). For instance, CXCL12 (stromal cell-derived factor 1 alpha) and its receptor CXCR4 regulate the synaptic release of glutamate and GABA. This cytokine can also act postsynaptically by activating G protein-coupled inwardly rectifying potassium channels (a voltage-gated K channel Kv2.1) and modulating several neurotransmitter systems in the brain (e.g., GABA, glutamate, opioids, and cannabinoids) (Guyon, 2014). IL-1β and IL-6 are also involved in the modulation and maintenance of long-term potentiation (LTP) in vivo (del Rey et al., 2013). Recent studies have suggested that TLR4/HMGB1 signaling may impact neuronal

excitability by interacting with NMDA receptors (Balosso et al., 2014; Maroso et al., 2010), and LPS facilitates epileptiform activity in vitro by enhancing excitatory synaptic transmission and neuronal excitability (Gao et al., 2014). EtOH-induced HMGB1 release is also associated with NOX2/NLRP1 inflammasome signaling, which represents a new mechanism of EtOH-associated neuron injury (Wang et al., 2015). During brain injury however, TLR4 activates glutamatergic currents by non-NMDA receptors, which suggests a new mechanism by which immune activation influences neuronal excitability in neurological disorders (Li et al., 2015). These results indicate that many cytokines/chemokines can modulate neurotransmission and can promote synaptic plasticity. Nonetheless, overproduction of pro-inflammatory cytokines or chemokines can also induce neural damage, which contributes to neuroinflammation (Donzis and Tronson, 2014; Gao et al., 2014).

Regarding EtOH effects, depending on the concentration and/or treatment used, EtOH can promote or impair LTP in critical brain regions such as the VTA or hippocampus. For example, hippocampal–entorhinal cortex brain slice cultures exposed to EtOH (100 mM) for 72 hours showed increased levels of several cytokines, including MCP-1, which leads to glutamate excitotoxicity and neuronal death (Zou and Crews, 2010). However, hippocampal brain slices with astrocyte-targeted MCP-1 treated with lower alcohol concentrations (20 to 60 mM for 20 minutes) displayed a protective effect against the depressing effects of EtOH on hippocampal LTP and synaptic plasticity (Bray et al., 2013).

As previously mentioned, the interaction between of GABA(A) α2 and TLR4 expression in the CeA has also been shown as an important contributor to excessive alcohol consumption (Liu et al., 2011). Interestingly, a single injection of LPS also produces a long-lasting increase in alcohol consumption, as well as changes in alcohol-conditioned taste aversion and the firing rate of dopamine neurons in the VTA, which suggest that immune signaling activation promotes alcohol consumption and alters certain aspects of alcohol reward/aversion (Blednov et al., 2011). It is noteworthy that the pharmacological blockade of TLR4/MD2 activity with (+)-naloxone potently reduced morphine-induced elevations of extracellular dopamine in rat nucleus accumbens (NAc) (Hutchinson et al., 2012), which supports a role of TLR4/central pro-inflammatory immune signaling in drug reward. As naloxone and derivatives (e.g., naltrexone, nalmefene) are effective drugs for reducing alcohol consumption, the question of whether TLR4 signaling is involved in alcohol reward and addiction is raised (Jacobsen et al., 2016).

In summary, considerable research is needed to understand the effects of EtOH on the neuroimmune system, and how the different cross-talk between immune components and neural cells modulates synaptic plasticity and neurotransmission under moderate, acute, or chronic alcohol consumption, or by binge drinking. However, experimental data suggest that modest TLR4 activation, along with the production of some cytokines or chemokines in specific brain regions (e.g., CeA, VTA, NAc), might modulate alcohol drinking and reward. Another unanswered question revolves around potential gender differences in alcohol-induced neuroinflammation as human (Pascual et al., in press) and animal studies have suggested greater neuroinflammation in females than in males (Alfonso-Loeches et al., 2013). The development of new tools, such as the delivery of inhibitors or silencing RNA vectors, to target specific immune components within a particular brain region will allow us to advance in our knowledge of the role of the neuroimmune system in the actions of alcohol on the CNS.

NEUROIMMUNE SYSTEM, ALCOHOL, AND THE ADOLESCENT BRAIN

Evidence during the last decades indicates that the brain continues to develop throughout adolescence and undergoes important structural and functional changes in synaptic plasticity and neural connectivity (Spear, 2013). These changes in the functional maturation of the neural pathways that connect individual brain regions are essential for successful cognitive, motor, and sensory functions from infancy into adulthood (Luna et al., 2015). Changes in the prefrontal cortices and key subcortical structures within the medial temporal lobe, which include the amygdala, hippocampus, and NAc, are the main remodeled brain regions (Selemon and Friedman, 2013; Spear, 2000). Many human and experimental studies have demonstrated that the adolescent brain is prone to deleterious effects of EtOH (Spear, 2015). Adolescent intermittent binge drinking, which is the most prevalent pattern of alcohol consumption among teenagers, causes numerous structural and functional changes in the brain. For instance, abnormalities in the cortical (Vetreno et al., 2016) and hippocampal (Risher et al., 2015) structure, loss of hippocampal neurogenesis (Vetreno and Crews, 2015), and long-lasting cortical synaptic and myelin disarrangements have been observed in animal models (Montesinos et al., 2015). These structural changes are associated with long-term cognitive dysfunctions (Montesinos et al., 2015, 2016) and loss of behavioral flexibility (Gass et al., 2014). EtOH drinking in adolescence also sensitizes EtOH activation in NAc, while blunting prefrontal cortex responses in adult rats (Liu and Crews, 2015), effects which might predispose to alcohol-related problems in adults.

Along with these changes, neuroinflammation occurs in the adolescent brain after alcohol exposure (Guerri and Pascual, 2010). Microglia and astrocytes are activated and promote the release of pro-inflammatory cytokines, chemokines, and reactive nitrogen and oxygen species which, in turn, promote neural death (Broadwater et al., 2014; Pascual et al., 2007; Qin et al., 2008; Ward et al., 2009). Binge-like EtOH treatment increased the expression of TLR4, TLR3, TLR2, RAGE, HMGB1, TNF-α, and IL-1β mRNA levels, in the prefrontal cortex of adolescent rats (Pascual et al., 2014; Vetreno and Crews, 2012), and some immune molecules,

such as TLR4, RAGE, and HMGB1, along with microglia activation, remained up-regulated for long periods after alcohol consumption, such as early adulthood (Montesinos et al., 2016; Vetreno et al., 2013). According to these experimental results, overexpression of HMGB1 and TLRs has also been observed in the human orbital frontal cortex, and the expression of these molecules correlated highly with lifetime alcohol consumption and age of drinking onset (Crews and Vetreno, 2016). Interestingly, the genetic elimination of TLR4 prevented binge-like EtOH-induced inflammatory damage in mice, along with myelin and synaptic disarrangements (Montesinos et al., 2015, 2016).

As previously mentioned, HMGB1 is an alarmin that activates TLR4 and RAGE to promote signaling pathways that generate cytokine/chemokine production. Some studies suggest that EtOH promotes HMGB1 release by damaged neurons (Zou and Crews, 2014) by eliciting brain neuroimmune signaling through microglial TLR4 response activation. Nevertheless, other findings indicate that EtOH-induced mitochondrial ROS can trigger TLR4/NLRP3 activation in astroglia and microglial cells (Alfonso-Loeches et al., 2014, 2016) which, in turn, might promote HMGB1 release (Lippai et al., 2013).

The importance of this HMGB1 lies in it being maintained 3 weeks after EtOH administration (Montesinos et al., 2015), which might be a long-lasting activator of inflammation to mediate persistence of EtOH inflammatory damage. Further studies are needed to understand the cellular and molecular mechanism by which HMGB1 is released, and how it is maintained for long periods after alcohol intake. Epigenetic changes induced by EtOH or posttranslational modification differences in oxidation of highly conserved redox-sensitive cysteine can promote the stability of HMGB1 and potentiate the inflammatory responses of EtOH (e.g., Magna and Pisetsky, 2014).

POTENTIAL TREATMENTS AND BIOMARKERS OF ALCOHOL ABUSE

As neuroimmune signaling is involved in many actions of EtOH on the brain, including neural damage, behavior, and addiction, the drugs used to reduce microglia activation or anti-inflammatory compounds will be good candidates to treat neuroinflammation associated with alcohol abuse. In fact, a recent work shows that T5342126, a selective TLR4 inhibitor, decreased EtOH drinking in both EtOH-dependent and nondependent mice, but also showed dose-dependent nonspecific effects represented by reduced animal locomotor activity, saccharine intake, and lower body core temperature (Bajo et al., 2016). Moreover, minocycline, a pharmacological inhibitor of microglia activation, has been shown to reduce EtOH intake in male and female C57BL/6J mice by a 2-bottle choice voluntary drinking model (Agrawal et al., 2011). The IL-1 receptor antagonist, anakinra, blocks the effects of NLRP3 inflammasome activation on the brain (Lippai et al., 2013) and reduces alcohol-induced sedation in mice (Wu et al., 2011). Ibudilast, a nonselective PDE inhibitor that crosses the BBB, suppresses TNF-α release, as well as astrocyte and microglial activation (Ledeboer et al., 2007), and reduces alcohol consumption (Bell et al., 2015). Some nonsteroidal anti-inflammatory compounds such as indomethacin, which inhibits cyclooxygenase enzyme (COX-2), reduce neuroinflammation and block behavioral deficits in rats exposed to EtOH in adolescence (Pascual et al., 2007). Blocking HMGB1, by means of antagonists such as glycyrrhizin and ethyl pyruvate, prevents the brain damage caused by chronic EtOH consumption (Whitman et al., 2013). Peroxisome proliferator-activated-type gamma receptors (PPAR) reduce innate immune signaling activation, while pioglitazone, a PPAR agonist, suppresses alcohol drinking and relapse to alcohol seeking in rats (Stopponi et al., 2011). A recent study also demonstrated that oleoylethanolamide, which has anti-inflammatory/neuroprotective properties, prevents neuroimmune HMGB1/TLR4/NF-κB danger signaling in the rat frontal cortex and depressive-like behavior induced by EtOH binge administration (Antón et al., 2016). Given the aforementioned important role of MCP-1 in alcohol dependence, inhibitors of its synthesis, such as bindarit (Mora et al., 2012; Severini et al., 2014), open up new avenues to treat alcoholism by modulating the immune system.

Regarding biomarkers for neuroinflammation, recent studies have shown that during chronic alcohol intake, some cytokines (IL-1β, IL-17, TNF-α) and chemokines (MCP-1, MIP-1α, CX_3CL_1) were up-regulated in mice striatum and serum (Pascual et al., 2015). However, after 24 hours of alcohol deprivation, IFN-γ levels were induced and associated with enhanced EtOH-induced anxiogenic-related behavior. Notably, these neuroimmune responses were associated with TLR4 and TLR2 responses (Pascual et al., 2015).

Emerging evidence also supports the role of small noncoding RNA, specifically miRNAs, as regulators of neuroinflammation, and as potential biomarkers in the pathophysiology of addiction and other neurodegenerative diseases and/or neurological disorders (Kocerha et al., 2009). Alcohol abuse alters the expression of miRNAs by regulating alcohol-induced neural damage (Most et al., 2016; Natarajan et al., 2015). Recent studies also indicate that chronic alcohol intake induces miR-155 in the cerebellum in a TLR4-dependent manner by up-regulating the TNF-α and MCP-1 expression levels (Lippai et al., 2013). Likewise, miR339-5p has been shown to inhibit EtOH-induced pro-inflammatory mediators and neuroinflammation by regulating NF-κB pathway (Zhang et al., 2014).

In general terms, there is significant evidence to support the role of neuroimmune signaling in many actions of EtOH on the CNS, which offers new insights into researching for biomarkers of neuroinflammation, and to develop effective pharmacotherapies for alcohol abuse and dependence.

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5. DISCUSIÓN GENERAL

5.1. La exposición intermitente al alcohol en la adolescencia (EIAA) altera el desarrollo cognitivo causando daño neuroinflamatorio a través del receptor TLR4

Diversos estudios ponen de manifiesto que el cerebro continúa su desarrollo durante la adolescencia, y sufre un conjunto de cambios estructurales y funcionales (Spear, 2013), que permiten la conexión de distintas áreas cerebrales de una manera eficaz y efectiva, permitiendo la correcta maduración cognitiva de la infancia a la edad adulta (Luna *et al.*, 2015). Este neurodesarrollo y maduración de las diferentes estructuras cerebrales hace que el cerebro adolescente sea especialmente vulnerable a los efectos perjudiciales del alcohol (Spear, 2016).

Como ya se ha comentado (pág. 29), el patrón de consumo de alcohol más extendido en la adolescencia es el consumo intensivo de alcohol en pocas horas (también conocido como botellón, 'en atracón' o *binge*), y es especialmente neurotóxico ya que en un periodo corto de tiempo se alcanzan altas concentraciones de alcohol en sangre y en cerebro, provocando numerosas alteraciones estructurales y funcionales en el cerebro.

Aunque los mecanismos por los que el consumo intermitente de alcohol durante la adolescencia causa alteraciones estructurales y funcionales, no están totalmente esclarecidos, datos de la última década indican que dicho patrón de consumo activa la astroglía y la microglía liberando mediadores inflamatorios como citoquinas, quimioquinas o especies reactivas del oxígeno (Pascual *et al.*, 2007; Qin *et al.*, 2008; Ward *et al.*, 2009). Datos nuestros y de otros laboratorios demuestran que la neuroinflamación que causa el consumo de alcohol está asociada a una activación de los receptores TLR del sistema neuroinmune (TLR2, TLR3, TLR4) (Pascual *et al.*, 2014; Vetreno y Crews, 2012). La activación de estos receptores, junto a un aumento de su expresión, se mantendría a largo plazo tras el cese de la exposición a etanol (Vetreno *et al.*, 2013). Estudios de nuestro laboratorio han demostrado que cuando se bloquea o elimina la función del receptor TLR4 (mediante el uso de anticuerpos bloqueantes, ARN de interferencia o ratones deficientes en TLR4) se evita la neuroinflamación causada tanto por la exposición aguda (*in vitro*) como por el consumo crónico (*in vivo*) de alcohol (Fernandez-Lizarbe *et al.*, 2009, Alfonso-Loeches *et al.*, 2010).

Usando el modelo de EIAA, nuestros resultados muestran un aumento significativo de los mediadores pro-inflamatorios en la CPF de ratones adolescentes (citoquinas, quimioquinas, especies reactivas del oxígeno, como el óxido nítrico o el peróxido de hidrógeno), así como la activación de rutas de señalización (MAPKs, NF-κB) asociadas al TLR4 (pág. 38). Sin embargo, los ratones deficiente en TLR4 no presentan este aumento de componentes inflamatorios, ni de las rutas de señalización asociadas, sugiriendo que la activación del TLR4 por la EIAA desencadena la neuroinflamación **(Fig. 6)**. Estos resultados demuestran, por primera vez, que la ausencia del TLR4 protege del daño neuroinflamatorio en la CPF causado por la EIAA.

La deficiencia en TLR4 también protege de los daños sinápticos y en la mielina asociados con la EIAA tanto a nivel molecular como estructural. A largo plazo, la alteración de los niveles de proteínas sinápticas y de ciertas proteínas de la mielina (PLP, CNPasa, MBP) se normalizaron, junto con una importante recuperación de la estructura de la vaina de mielina (en 3 semanas tras la EIAA, el daño en la mielina pasó del 54±4 % al 15±2 %). Sin embargo, la estructura sináptica no mostró una recuperación completa, ya que se observaron cambios importantes en las sinapsis de animales adultos tras la EIAA (pág. 43). De hecho, estos cambios estructurales en las sinapsis (reducción del número de vesículas presinápticas y de la longitud de la densidad postsináptica) ocurrían junto a las alteraciones en el cociente GluR1/NR1 en sinaptosomas (pág. 53), sugiriendo que la EIAA altera el sistema glutamatérgico en la CPF de animales adultos. Algunos trabajos indican que la EIAA altera a largo plazo otros sistemas como el dopaminérgico (Pascual *et al.*, 2009; Trantham-Davidson *et al.*, 2016), el serotoninérgico (Vetreno *et al.*, 2016a), el colinérgico (Coleman *et al.*, 2014) y el gabaérgico (Centanni *et al.*, 2014). Un dato interesante de nuestros resultados es que los cambios glutamatérgicos en la CPF de los animales adolescentes tras la EIAA no se observan en ausencia del receptor TLR4, sugiriendo su implicación en los efectos del alcohol sobre la transmisión glutamatérgica. A este respecto, existe una extensa literatura abordando la participación del TLR4 y de moléculas pro-inflamatorias como IL-1β y TNF-α en la neurotransmisión (pág. 88-89).

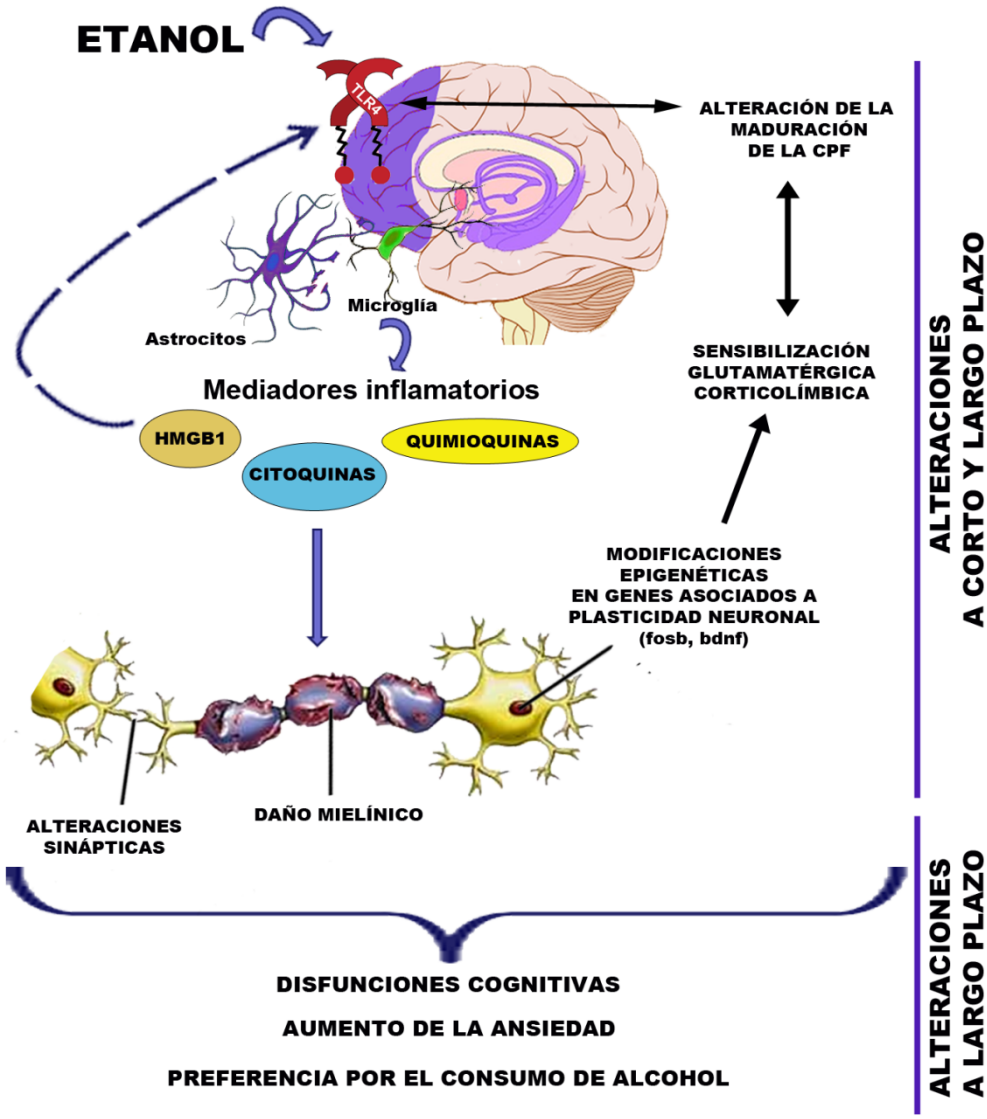

Figura 6. La exposición intermitente al alcohol durante la adolescencia activa el receptor TLR4 causando neuroinflamación, daños en la CPF y alteraciones conductuales. La activación del TLR4 en la CPF provoca la liberación de mediadores inflamatorios que conlleva a daños sinápticos y mielínicos, la mayoría de los cuales se mantiene hasta la edad adulta a través de la señalización inflamatoria del TLR4/HMGB1. Los cambios epigenéticos causados por el alcohol en genes involucrados en la neuroplasticidad se asocian a la sensibilización glutamatérgica corticolímbica, conduciendo, junto con los déficits sinápticos y mielínicos, a alteraciones conductuales en la edad adulta.

Estas alteraciones a nivel molecular y estructural de las sinapsis y de la mielina en la CPF de animales adolescentes tras la EIAA, pueden conllevar a déficits cognitivos en la edad adulta. De hecho, ratones adultos tras la EIAA ejecutan peor distintas pruebas de aprendizaje y memoria, como la prueba de reconocimiento de objeto y la de evitación pasiva (pág. 44). Sin embargo, no se encontraron diferencias en los ratones deficientes en TLR4 tras la EIAA. De nuevo, estos resultados apuntan a que la EIAA causa neuroinflamación a través de la activación del TLR4, provocando daños sinápticos y en la mielina que pueden afectar al correcto neurodesarrollo de las funciones cognitivas **(Fig. 6)**.

Resultados de este trabajo también demuestran que la EIAA induce una liberación de la alarmina HMGB1 en la CPF (pág. 38). HMGB1 es una proteína asociada a la cromatina, que ante ciertos estímulos o estrés celular, se hiper-acetila en determinados residuos de lisina y sufre una translocación desde el núcleo hacia el citosol, desde donde se libera al medio extracelular actuando como mediador inflamatorio (Klune *et al.*, 2008). La EIAA induce la translocación de la proteína HMGB1 del núcleo al citoplasma, manteniendo elevados sus niveles en el citosol durante un largo periodo de tiempo tras la interrupción del tratamiento con alcohol, llegando incluso hasta la edad adulta (pág. 38). Algunos trabajos han demostrado que la HMGB1 activa los receptores TLR4 (Klune *et al.*, 2008). De hecho, la EIAA induce un aumento de la HMGB1 (pág. 38) que se asocia con el incremento de los niveles de TLR4 (Pascual *et al.*, 2014), y de marcadores de la activación microglial (pág. 53) en la CPF de animales adultos. Nuestros resultados sugieren que la EIAA induce una liberación de la HMGB1, que activa al TLR4 y a la microglía **(Fig. 6)**, e inicia un ciclo de retroalimentación positiva que mantiene la inflamación a largo plazo, incluso 3 semanas tras el tratamiento con etanol.

Actualmente se desconoce la fuente celular de la liberación de HMGB1 tras la EIAA, pero trabajos recientes sugieren que esta proteína puede ser liberada por neuronas (Zou y Crews, 2014), microglía (Frank *et al.*, 2016) o astrocitos (Zhang *et al.*, 2015). Incluso se ha descrito que la liberación neuronal de HMGB1 es dependiente del TLR4 en cultivos organotípicos (Zou y Crews, 2014). Por tanto, estudios futuros sobre el mecanismo celular y molecular de

activación del TLR4/HMGB1 tras la EIAA, pueden ofrecer dianas terapéuticas prometedoras para el tratamiento del daño neuroinflamatorio y conductual asociado al consumo de alcohol.

5.2. Los mecanismos epigenéticos participan en la vulnerabilidad a la conducta adictiva tras la EIAA: papel del TLR4

Los resultados del presente trabajo muestran que los ratones adultos tratados con etanol durante la adolescencia presentan mayores niveles de ansiedad, parámetro medido mediante pruebas conductuales como la exploración en campo abierto y el laberinto elevado en cruz (pág. 59). Además, estos animales muestran una mayor preferencia por el consumo de alcohol y mayor vulnerabilidad en la prueba de condicionamiento de lugar con cocaína (pág. 58). Sin embargo, los animales TLR4-KO no presentaron estos cambios conductuales.

El papel regulador del sistema neuroinmunitario en el consumo de alcohol ha sido sugerido en numerosos trabajos (pág. 88). Sin embargo, el papel de los TLR4 en el consumo de alcohol es un tema reciente, tal y como demuestran nuestros resultados. Nuevos hallazgos apoyan nuestros datos, y demuestran que cuando se bloquea el TLR4 en la amígdala o el área tegmental ventral (mediante la administración de ARN de interferencia específico contra TLR4), evita el abuso del alcohol en ratas con alta preferencia por el alcohol (June *et al.*, 2015).

Nuestros resultados corroboran otros estudios que demuestran que los animales adultos expuestos al etanol durante la adolescencia presentan un aumento de la ansiedad (Sakharkar *et al.*, 2016; Pandey *et al.*, 2015), y una mayor preferencia por el consumo de alcohol (Pandey *et al.*, 2015; Alaux-Cantin *et al.*, 2013; Broadwater *et al.*, 2013). Aunque se desconocen los mecanismos moleculares del mantenimiento a largo plazo de estos efectos conductuales, algunos autores han sugerido el papel de los mecanismos epigenéticos sobre genes asociados a neuroplasticidad, como *bdnf*, en diferentes componentes del sistema mesolímbico, como la amígdala o el NAc. Por ejemplo, la EIAA disminuye en amígdala el nivel de expresión de *bdnf* y la acetilación de histonas en su promotor (Pandey *et al.*, 2015). Nuestros resultados muestran que en la CPF, la EIAA causa un aumento en la acetilación a largo plazo de la histona 4, tanto

en los niveles totales (pág. 54) como en el promotor del gen *bdnf* (pág. 57), así como su expresión a nivel del ARN mensajero y de la proteína (pág. 56-57). Por tanto, podemos sugerir que los cambios epigenéticos inducidos por la EIAA son específicos de la región cerebral tratada, y su inter-regulación puede conducir a cambios conductuales **(Fig. 6)**.

Así mismo, nuestros resultados indican que la EIAA afecta a largo plazo al gen *fosb*, que codifica a las proteínas FosB y ΔFosB. En la CPF de ratones adultos tras EIAA observamos un aumento en la acetilación de la histona 4 en el promotor del gen *fosb* (pág. 57), así como un aumento del nivel de expresión de FosB y ΔFosB (pág. 56-57). La acumulación de ΔFosB se ha descrito como un mecanismo molecular mediador de la ansiedad (Vialou *et al.*, 2014) y del mantenimiento de la conducta adictiva a largo plazo (Nestler, 2001). Nuestros datos apoyan esta hipótesis, ya que los ratones tras EIAA son más vulnerables al tratamiento con cocaína (pág. 58).

Otro dato de interés es que los cambios epigenéticos que se observan en la CPF de animales tratados con etanol dependen de la activación del TLR4, ya que no ocurren en ratones TLR4-KO. A partir de estos resultados, se podría sugerir que la implicación del TLR4 sobre los cambios epigenéticos puede estar mediada por su función reguladora sobre NF-κB, que a su vez actúa como regulador de CREB y BDNF (Kaltschmidt *et al.*, 2006). Además, la respuesta del TLR4 (Raby *et al.*, 2011; Pope *et al.*, 2010), de FosB y de ΔFosB (Nomaru *et al.*, 2014) modulan el sistema del complemento, componente importante del sistema inmune innato, y que participa en el proceso de eliminación de sinapsis llevado a cabo por la microglía durante la adolescencia (Stephan *et al.*, 2012, Stevens *et al.*, 2007).

5.3. La exposición al alcohol en la adolescencia causa mayor inflamación en mujeres

Estudios en humanos (Hommer *et al.*, 2001; Mann *et al.*, 2005) y en animales (Alfonso-Loeches *et al.*, 2013; Wilhelm *et al.*, 2014) han observado mayor vulnerabilidad de las hembras a los efectos neurotóxicos del alcohol. Nuestros datos muestran que las chicas adolescentes con intoxicación etílica aguda presentan mayores niveles plasmáticos de

expresión del ARN mensajero del TLR4 (pág. 69), así como un aumento de proteínas pro-inflamatorias (IFN-γ, IL-10, IL-17A, IL-1β, IL-2, IL-4, IL-6, IL-8, fractalquina, MCP-1 y MIP-1α), mientras que la intoxicación etílica no causa estos efectos en chicos adolescentes, con niveles de alcoholemia similares al de las chicas. Utilizando nuestro modelo murino de EIAA, corroboramos el aumento de los niveles del TLR4 en la CPF de ratones hembra, así como el aumento de los niveles en el suero y en la CPF (pág. 72) de algunas de estas proteínas pro-inflamatorias (IL-17A, MCP-1 y MIP-1α). Por tanto, estas citoquinas y quimioquinas pueden servir como posibles biomarcadores del daño neuroinflamatorio. Cabe destacar que la EIAA no causó el aumento de los marcadores de inflamación ni en el suero ni en la CPF de los ratones macho WT (pág. 72) y TLR4-KO (pág. 74), y de los ratones hembra TLR4-KO (pág. 74).

Debido a que nuestros resultados no muestran diferencias significativas entre mujeres y hombres en los niveles basales de las distintas moléculas inflamatorias analizadas, nos preguntamos si existe un mecanismo de respuesta inflamatoria al alcohol que difiere entre mujeres y hombres adolescentes, que sustente la mayor vulnerabilidad observada en chicas. Las diferencias de género observadas en la inflamación pueden ser debidas a diferencias en el metabolismo del etanol o en su distribución debido al mayor porcentaje de grasa corporal de las mujeres.

Además, estudios recientes comparan la respuesta inflamatoria de células gliales provenientes de ratones y ratas hembra y macho. Dichos trabajos *in vitro* demuestran que la respuesta inflamatoria al etanol en astrocitos procedentes de hembras es mayor que en astrocitos procedentes de machos (Wilhelm *et al.*, 2016). Así mismo, en presencia de la hormona sexual femenina estradiol, la respuesta inflamatoria al LPS se atenúa en microglía *in vitro* de machos pero se potencia en microglía de hembras (Loram *et al.*, 2012). La regulación de la inflamación por el estradiol, cuyos niveles aumentan significativamente en hembras durante la adolescencia, podría explicar nuestros resultados que muestran mayor vulnerabilidad de las chicas adolescentes a los efectos inflamatorios de la intoxicación aguda al alcohol. De hecho, el estradiol actúa sobre los receptores de los estrógenos aumentando los niveles de expresión del TLR4 (Rettew *et al.*, 2009), y potenciando sus vías de señalización (Calippe *et al.*, 2010).

La EIAA también tiene efectos a largo plazo sobre la respuesta del eje hipotalámico-hipofisario-adrenal, que varían en función del sexo (Logrip *et al.*, 2013). Este eje controla la síntesis y liberación de glucocorticoides como el cortisol, que por un lado se asocia a la potenciación de la neuroinflamación y neurotoxicidad en hembras, pero por otro lado induce inmunosupresión en machos (Wilhelm *et al.*, 2015). Estudios que usan cultivos organotípicos hipocampales corroboran estos datos, ya que demuestran que el tratamiento con etanol causa mayor sensibilidad a los efectos tóxicos del cortisol en hembras que en machos (Walls *et al.*, 2013). Además, se ha descrito que la pre-exposición a glucocorticoides potencia la respuesta inflamatoria causada por la estimulación del receptor TLR4 usando LPS, tanto en el SNC como en el hígado (Frank *et al.*, 2010).

Se ha demostrado que el consumo de alcohol puede desencadenar un aumento de la permeabilidad intestinal, permitiendo la extravasación de componentes bacterianos, como el LPS, al torrente sanguíneo (Crews *et al.*, 2015). De hecho, los niveles de LPS séricos tras el consumo de alcohol son mayores en mujeres que en hombres (Bala *et al.*, 2014). Tanto el LPS sérico como el alcohol pueden alcanzar la barrera hematoencefálica, causando su disrupción y provocando una respuesta neuroinflamatoria (Crews *et al.*, 2015). Cabe destacar que tanto la permeabilidad intestinal (Guo *et al.*, 2015) como la disrupción de la barrera hematoencefálica (Rubio-Araiz *et al.*, 2016) causadas por el consumo de alcohol están mediadas por la acción del TLR4. Estos estudios utilizan modelos de consumo voluntario o administración intragástrica de alcohol en animales adultos, mientras que nuestro modelo de EIAA se basa en inyecciones intraperitoneales con cierto efecto en la inflamación intestinal (Lee *et al.*, 2014). El uso de protocolos de descontaminación bacteriana intestinal (Gárate *et al.*, 2011) puede ayudar a delimitar la contribución de la translocación del LPS intestinal en los efectos neuroinflamatorios observados.

Finalmente, nuestros resultados resaltan el papel del sistema neuroinmune, y concretamente, del receptor TLR4 en el daño neurotóxico y conductual causado por la EIAA. Por tanto, terapias dirigidas al SNC con anti-inflamatorios (Pascual *et al.*, 2007), o inhibidores que puedan eliminar la sobre-activación del receptor TLR4 (pág. 90) podrían ser efectivas para paliar o tratar el daño neuroinflamatorio asociado al consumo del alcohol.

6. CONCLUSIONES

La exposición intermitente al alcohol durante la adolescencia:

- Induce neuroinflamación a través de la activación del receptor TLR4, conduciendo a daños sinápticos y en la estructura de la mielina de la CPF de ratones adolescentes WT, que conllevan a disfunciones cognitivas a largo plazo.
- Causa la remodelación de las sinapsis glutamatérgicas en la CPF adolescente, que se mantiene hasta la edad adulta.
- Induce cambios epigenéticos a corto y largo plazo en la CPF que se asocian con la activación de mecanismos moleculares de plasticidad neuronal y específicamente en los genes *bdnf* y *fosb*.
- Provoca mayores niveles de ansiedad y conductas asociadas a la adicción (preferencia por alcohol y condicionamiento por preferencia de lugar a cocaína) en animales adultos.
- No ocasiona dichas alteraciones en animales deficientes en TLR4 (TLR4-KO), sugiriendo el papel de este receptor en las alteraciones observadas que induce el alcohol.
- En ratones adolescentes hembra, induce un aumento en los niveles de varias citoquinas y quimioquinas en CPF y en suero, pero solo incrementó los de fractalquina en CPF de los ratones macho. El mismo tratamiento no causó cambios significativos en los niveles de citoquinas/quimioquinas de plasma o CPF de ratones TLR4-KO.
- Los resultados en chicos y chicas adolescentes con intoxicación por alcohol demuestran, que las chicas presentan un aumento de los niveles plasmáticos de citoquinas y quimioquinas, así como de la expresión del TLR4. Sin embargo, para niveles de alcoholemia similares, los chicos solo presentan elevaciones plasmáticas de CSF.

Finalmente, los resultados demuestran que el abuso de alcohol durante la adolescencia induce cambios neuroquímicos y conductuales importantes que se mantienen hasta la edad adulta, y que la activación de los receptores TLR4 desempeña un papel relevante en la neuropatología del abuso de alcohol durante la adolescencia.

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ANEXOS

Ilustración de la portada

Corte horizontal de la corteza de la pared medial de *Salamandra Maculata*. Tomada de 'Haller B. Arch. Mikrosk. Anat. Entwiklungsgesch. 1908. 71. 350-466 (Lám. 29, Fig. 6)'

Figura 1

Modificada de 'Rethinking schizophrenia. Insel TR. Nature. 2010. 468(7321):187-93'.

Figura 2

(A) Modificada de 'Medscape'. (www.medscape.org/viewarticle/838083)

(**B)** Modificada de 'Dopamine modulation of learning and memory in the prefrontal cortex: insights from studies in primates, rodents, and birds. Puig MV, Rose J, Schmidt R, Freund N. Front Neural Circuits. 2014. 8:93'.

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Figura 3

Modificada de 'Wikipedia'. (commons.wikimedia.org/wiki/File:Toll-like_receptor_pathways.svg)

Figura 4

Modificada de 'Magnetic resonance imaging of myelin. Laule C, Vavasour IM, Kolind SH, Li DK, Traboulsee TL, Moore GR, MacKay AL. Neurotherapeutics. 2007. 4(3):460-84'.

Figura 5

(A) Modificada de 'Richard Scheller and Thomas Südhof receive the 2013 Albert Lasker Basic Medical Research Award. Hurst JH. J Clin Invest. 2013. 123(10):4095–4101'.

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Figura 6

Modificada de: http://connersclinic.com/lyme-and-multiple-sclerosis. De http:// www.clker.com/cliparts/W/T/n/U/v/c/astrocyte-md.png. De http:// biohumana35. blogspot. com.es /2013/03/sistema-nervioso-neuroglia-neurona.html

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