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Universidad del País Vasco

Euskal Herriko Unibertsitatea

STUDY OF THE LONG-LASTING EFFECTS OF ETHANOL CONSUMPTION DURING ADOLESCENCE ON CANNABINOID TYPE 1 RECEPTOR-DEPENDENT SYNAPTIC TRANSMISSION AND PLASTICITY IN DENTATE GYRUS SYNAPSES

DOCTORAL THESIS

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Leioa, 2018

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Universidad del País Vasco
Euskal Herriko Unibertsitatea

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MEDIKUNTZA ETA ERIZAINTZA FAKULTATEA
FACULTAD DE MEDICINA Y ENFERMERÍA

Doctoral Thesis presented by Lda.

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Financed by Predoctoral (PhD) contract from Ministerio de Economía y Competitividad (MINECO) of Spanish Government

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Leioa, 2018

AGRADECIMIENTOS

Después de estos años de doctorado, echo la vista atrás y muchas son las personas que de una u otra manera han contribuido a que haya podido realizar este trabajo, que más que un trabajo es una pasión.

En primer lugar, quiero agradecer a mis directores; el Dr. Pedro Grandes y la Dra. Nagore Puente. Gracias a Pedro, por confiar en mí y darme la oportunidad de desarrollar mi vocación investigadora. Por poner a mi alcance todo lo necesario para que fuera posible, y darme todas las facilidades para compaginar mi vida en el laboratorio con mi vida personal. Gracias especialmente por todo el apoyo en Canadá durante mi estancia. Gracias a Nagore, por ser mí maestra, por ser más que una jefa una compañera.

Gracias a todos los que formáis parte del equipo; a los profes: Sonia, Inma, Leire, Juan, Almudena e Izaskun. Por enseñarme vuestra manera de trabajar, porque siempre habéis sacado tiempo para resolver mis dudas tanto para hacer experis como para realizar trámites. Especialmente agradecer a Izas, porque además de ser una investigadora estupenda es una magnífica persona y un ejemplo a seguir. A las post-doc: Ianire e Irantzu. Infinitas gracias por estar ahí en todo momento, por vuestro apoyo y confianza, por acogerme en vuestras casas. Ya sabéis que sois mis vascas favoritas. Gracias Iani, por verme siempre con tan buenos ojos y valorarme tanto, en definitiva... por quererme tanto. Gracias “marida”, por llegar en el mejor momento y darme la sensatez y alegría que derrochas, ha sido un placer trabajar codo con codo contigo. Voy a echarte mucho de menos. Gracias también a los futuros Doctores: Naiara, Jon, Itziar B, Aitor, Svein e Itziar T, por todos los favores dentro y fuera del labo, por las cañas brindadas y por todos los momentos de risas que siempre añoraré. Gracias a Elsa, por sus conocimientos y consejos tanto a nivel laboral como

personal. No puedo olvidar, a las personas que ya no están en el laboratorio; Ana, Paula, Elvi y Yolanda. Gracias por vuestro cariño y amistad.

En especial a mi “Euskosister”, gracias por ser tan buena y generosa conmigo, por darme tanto cariño, porque desde que nos conocimos me has acompañado en cada paso del camino. Muchos viajes compartidos, risas y preocupaciones, pero siempre a mi lado. No he podido tener mejor suerte contigo. Aprovecho para agradecer a Álex, por mostrarme siempre su punto de vista y ampliar el mío. Continuamos con los planes, que nos quedan muchos.

Gracias a los compis de departamento, en especial a Cata, Andrea, Mari Paz, Juan Carlos, Alazne y Raffaela, por los momentos de risas, confidencias y consejos. Os deseo la mejor de las suertes y no me cabe duda que la tendréis.

"I would like to continue saying thank all people I met during my stay at the University of Victoria; to Dr. Brian Christie, because he opened to me the doors of his laboratory; to Dr. Patrick Nahirney, for his meticulous way of transmitting me his knowledge. Thanks a lot to all members of the lab: Melissa, Katie, Scott, Christine, Juan, Cristina, Luis and Jenny, also Moha. Thanks Juan and Cristina for the support provided, for our delightful dinners and weekly beers. And, thanks a lot Luis, Jenny and Moha because all of you are my Canadian family. See you somewhere…"

Gracias a nuestros colaboradores; Dr. Fernándo Rodríguez de Fonseca, Dr. Juan Suárez, Dr. Joan Sallés, Dr. Sergio Barrondo y Dr. Gontzal García del Caño, por confiar en el proyecto y aportar unos magníficos resultados que nos han ayudado a comprender “lo que tenemos entre manos". Gracias al Dr. Giovanni Marsicano, Dr. Beat Lutz y Dra. Susana Mato, por proporcionarnos los animales transgénicos tan valiosos en este trabajo. A la Dra. Cristina Miguelez, por poner a nuestro alcance sus aparatos de comportamiento. Al servicio de

microscopía de la uni, Ricardo, Álex, Irene y Jon, por resolver siempre con una sonrisa todas mis dudas y problemas con el electrónico.

Gracias a mis amigos de Madrid, a "las de Siempre": Rocío, Cristina, Mar, Mamen y Cris, por comprenderme, en ocasiones casi sin entenderme, por vuestras visitas a Bilbo, por todos los planes organizados facilitándome la vida. A Carlos, por interesarte siempre en mis cosas, escucharme hasta el aburrimiento y tener siempre una opinión sincera para mí. A Mora, Pablo, Mónica, Víctor y Toni, por acompañarme en éste y en todos los proyectos de mi vida. Por vuestro infinito cariño y apoyo.

A mis amigas "a la distancia": a Virginia por enseñarme que kilómetros de separación no son barrera para la amistad. Gracias por tus consejos, por tu continuo apoyo y por estar siempre dispuesta a echarme una mano. A Elena por enseñarme que hay amigos que por mucho que pase el tiempo siempre están. Porque esta "aventura", aunque separadas, la hemos pasado juntas.

Infinitas gracias a María y a José, por todas las facilidades, comprensión y apoyo que siempre me han dado.

A mis padres, a los que quiero con locura. Gracias por respetar y apoyar todas mis decisiones, por los consejos, por hacer lo posible y lo imposible por mí, por las continuas llamadas, por la confianza que depositáis en mí. Gracias por tranquilizarme cuando la preocupación me desbordaba, porque sin vosotros nada de esto hubiera sido posible. A mi tío Eugenio, porque es el mejor tío. A mis hermanos, Javi, Puri y Felito, por ser un ejemplo a seguir y enseñarme que los obstáculos se saltan. Os quiero mucho. A mis cuñados, Sonia y Raúl, por valorarme y animarme siempre a continuar. A Eva, por su constante interés y comprensión. A Jose, porque sé que estarías muy orgulloso. Te echamos mucho de menos.

A mis sobrinos, Alba, Lucía, Julia, Tomás, Carmen y Jaime por regalarme momentos increíbles que hacen desconectar a cualquiera. Sois mis tesoritos y os quiero muchísimo.

Por último, agradecer a Álvaro, mi compañero de vida, mi todo, porque contigo sobran las palabras. Gracias por apoyarme en cada una de mis decisiones aunque eso conllevara irme lejos o aún más lejos. Por ser más fuerte que la situación, por ser más racional que yo. Te amo.

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1.SUMMARY

Binge drinking (BD), especially during adolescence, is a leading public health concern. Research within the past decade has made it clear that the ethanol (EtOH) consumption and the endocannabinoid (eCB) system reciprocally interact to modify neural activity and behavior. However, the long-lasting impact of adolescent EtOH intake on the localization and function of the Type I Cannabinoid (CB_1) receptor in adult brain and, ultimately, on neurobehavior, remains unknown.

In this doctoral study, adult male C57BL/6J mice were used to investigate the localization and function of the CB_1 receptor at the excitatory medial perforant path (MPP) synapses in the dentate molecular layer of the hippocampus. We focused on these synapses because: first, they integrate the hippocampal excitatory tri-synaptic circuit involved in learning and memory; second, MPP synapses show high efficiency in neuronal activation; and third, but not least, the persistent EtOH intake during the adolescence damages the entorhinal cortex and dentate gyrus and impairs synaptic transmission and plasticity. In particular, we studied the involvement of CB_1 receptors and the eCB system in long-term depression of the excitatory MPP-granule cell synapses (CB_1-eLTD), a form of synaptic plasticity. Then, the consequences of the adolescent EtOH intake on MPP synaptic transmission and plasticity were examined in adulthood after exposure of adolescent male C57BL/6J mice (postnatal day 32) to a 4 day binge drinking in the dark procedure over a period of 4 weeks (from postnatal day 32 to 56) followed by two weeks of EtOH-withdrawal.

To reach the proposed objectives, we conducted a multidisciplinary experimental approach based on electrophysiology, immunohistochemistry, behavior and molecular biology techniques. The main results of the Doctoral Thesis are:

First, low frequency stimulation (10 min, 10 Hz) of the medial perforant path triggers CB_1-eLTD at the MPP synapses. This eLTD is group I metabotropic glutamate receptor

(mGluR)-dependent, requires intracellular calcium influx and 2-arachydonoyl-glycerol (2-AG) synthesis.

Second, the CB_1-eLTD at MPP synapses is absent in adult mice after adolescent EtOH consumption. Furthermore, CB_1 receptor activation inhibits field excitatory postsynaptic potentials (fEPSPs) evoked after MPP stimulation in adult shams, but not in EtOH-exposed mice.

Third, adolescent EtOH intake significantly reduces the CB_1 receptor expression in excitatory synaptic terminals localized in the dentate MPP termination zone, decreases the [^{35}S]guanosine-5*-O-(3-thiotriphosphate) ([^{35}S] GTPγS) basal binding and Gαi2 subunit and significantly increases the monoacylglycerol lipase (MAGL) mRNA and protein in adult hippocampus.

Fourth, mice exposed to EtOH display a significant lower recognition memory, spatial memory and associative memory, as well as a significant reduction in motor coordination and balance after two weeks of the last session of EtOH. However, no significant permanent anxiety or depressive-like behaviors are detected.

Fifth, the increase of endogenous 2-AG by the MAGL inhibitor JZL184 rescues the CB_1-eLTD and reverses the significant loss of recognition memory observed in EtOH-treated mice.

In conclusion, adolescent binge drinking leads to deficits in CB_1 receptor-dependent excitatory transmission and plasticity at the MPP-granule cell synapses that correlate with memory loss and motor disturbance in adult mice. Furthermore, both the CB_1-eLTD and memory can be recovered in EtOH mice by increasing the endogenous levels of 2-AG.

2. INTRODUCTION

2.1 ETHANOL AS A DRUG OF ABUSE

Addictive drugs have short–reward effects but also long-lasting effects on brain circuitry leading the pattern of repetitive drug intake to tolerance, dependence, withdrawal and sensitization and, ultimately, addiction. There are two levels in drug addiction, the first is related to drug use seeking for hedonic feelings eliciting pleasure and reward, bringing individuals to benefit for repetition. This regular consume produces molecular changes in the brain that alters brain function and, as a consequence, behavior leading to drug seeking evolving to addiction in which drug seeking and compulsive consumption set as the center and only meaning in life. The behavioral changes are guided by dopamine release in the reward system, independently of how frequent the drug is taken. Furthermore, dopamine acting in the hippocampus and cerebral cortex potentiates associative memories linked to drug-related cues leading to drug seeking. Long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission are thought to be at the basis of the physiological mechanisms of different types of learning and memory. Thus, changes in synaptic plasticity by drug use are thought to rule addictive behaviors.

Ethyl alcohol or ethanol (EtOH) (CH3-CH2-OH) is probably the most commonly consumed addictive drug in the world (SAMHSA, 2011) and is an important health and social problem worldwide (WHO, 2014). EtOH is a weak drug; a quantity of grams is needed to produce a pharmacological effect. Unlike other substances of abuse, EtOH is able to modify the permeability of some ion channels, the functionality of several receptors particularly sensitive to the action of EtOH, the organization of aqueous molecules in the extracellular matrix, and the solubility of ligands or ions that interact with membrane receptors (Franks and Lieb, 1994; Peoples and Weight, 1995). Nevertheless, this type of interactions produces small effects and only occurs at high concentrations of EtOH (> 100 mM). In contrast, interactions at specific sites of proteins, which are critical for its function, seem to be much

more important (Yamakura *et al.*, 2001), since they occur at lower concentrations of EtOH (10-50 mM). These changes can be short- or long-lasting, but reversible, or permanent and associated with degenerative processes in specific brain areas (Fadda and Rossetti, 1998).

2.1.1 Adolescent ethanol consumption

EtOH has become the most widely used toxic substance during adolescence (Pautassi *et al.*, 2009). EtOH heavily impacts on the structure and function of the brain, particularly during adolescence (Pascual *et al.*, 2007; Clark *et al.*, 2012; Keshavan *et al.*, 2014; Liu and Crews, 2015; Montesinos *et al.*, 2015; Vetreno and Crews, 2015; Adermark and Bowers, 2016; Spear, 2016a). Because EtOH modifies brain maturation, adolescent EtOH drinking associates with deficits in attention, learning, memory, intellectual development or visual-spatial functions (Brown and Tapert, 2004; Nagel *et al.*, 2005; Zeigler *et al.*, 2005; Lacaille *et al.*, 2015) that correlate with a loss in hippocampal, prefrontal cortex and cerebellar volumes and a ventricular expansion in young people drinking at early age (Shear *et al.*, 1992; De Bellis *et al.*, 2000, 2005; Nagel *et al.*, 2005; Medina *et al.*, 2008; Lisdahl *et al.*, 2013).

Binge drinking (BD) is the typical pattern of alcohol consumption in adolescents and youth. It is characterized by an intermittent consumption of large amounts of EtOH in short periods of time (3 or more drinks in 1-2 hours) followed by a period of abstinence (Courtney and Polich, 2009). This intake pattern causes large and rapid spikes in blood EtOH concentration (BEC) that brings serious consequences in terms of acute toxicity but also leads to vulnerability for later EtOH abuse and dependence (Amodeo *et al.*, 2017). BD correlates with cognitive damage as abusive EtOH consumption has deleterious effects on the adolescent brain (Lacaille *et al.*, 2015). The neocortex, limbic system and cerebellum are brain regions particularly sensitive to the neurotoxic effects of EtOH during early life (Crews *et al.*, 2000; Squeglia *et al.*, 2009; Karanikas *et al.*, 2013). EtOH causes a significant

loss of hippocampal neurons, astrocytes and microglia (Oliveira *et al*., 2015), hippocampal shrinkage (De Bellis *et al*., 2000) and mitochondrial dysfunction that leads to brain inflammation, synaptic dysfunction and memory loss (Crews *et al*., 2000). Furthermore, EtOH intake during adolescence causes damage to the perirhinal cortex, entorhinal cortex and dentate gyrus (Crews *et al*., 2000) that play a key role in memory tasks and mood, as well as to cerebellar Purkinje cells (Sarna and Hawkes, 2003; Jaatinen and Rintala, 2008) which are essential in the cerebellar motor control (Lamont and Weber, 2012). Moreover, all these effects are long lasting (Coleman *et al*., 2011, 2014; Forbes *et al*., 2013). Actually, BD alters brain volume in animal models and mimics the alteration found in young drinkers (Crews *et al*., 2000; Coleman *et al*., 2011, 2014; Forbes *et al*., 2013; Vetreno *et al*., 2016) and EtOH-exposed adolescent animals are more sensitive and show memory and learning dysfunctions (Markwiese *et al*., 1998; White and Swartzwelder, 2005) which can extend into adulthood (Sircar and Sircar, 2005; Pascual *et al*., 2007).

Given the incidence of BD in adolescents and young adults and the lesion effects of EtOH in the central nervous system (CNS), it is critical to understand both the long-term consequences of this exposure and methods by which this damage can be overcome by therapeutic interventions. The persistent behavioral effects of EtOH in adolescence are accompanied by disturbance of synaptic plasticity and neurotransmission. Thus, numerous studies have shown that EtOH alters several neurotransmitter and neuromodulatory systems, in particular, the endocannabinoid (eCB) (Hungund *et al*., 2003; Basavarajappa, 2007; Mitrirattanakul *et al*., 2007; Adermark *et al*., 2011; Talani and Lovinger, 2015; Varodayan *et al*., 2017), glutamatergic (Hoffman and Tabakoff, 1996; Fadda and Rossetti, 1998; Alele and Devaud, 2005; Heinz *et al*., 2005; Larsson *et al*., 2005), Gamma-Aminobutyric acid (GABA) (Mehta and Ticku, 2005; Fleming *et al*., 2007, 2012, 2013; Centanni *et al*., 2014), or dopaminergic system (Coleman *et al*., 2011; Boutros *et al*., 2014; Shnitko *et al*., 2014; Vetreno *et al*., 2014; Spoelder *et al*., 2015) in many brain areas. Moreover, it is well

documented that the eCB system regulates the EtOH-induced changes in excitatory and inhibitory transmission and participates in EtOH addictive behaviors of consumption, motivation, reinforcing and dependence (Rimondini *et al.* 2002; Colombo *et al.*, 2005; Thanos *et al.*, 2005; Economidou *et al.*, 2006; Mitrirattanakul *et al.* 2007; Basavarajappa *et al.*, 2008; Kelm *et al.*, 2008; Vinod *et al.*, 2008, 2012; Roberto *et al.*, 2010; Pava *et al.*, 2012; Pava and Woodward 2012; Talani and Lovinger, 2015) and, reciprocally, EtOH modulates the behavioral and neural eCB-dependent effects (Pava *et al.*, 2012; Talani and Lovinger, 2015).

2.2 THE ENDOCANNABINOID SYSTEM

The eCB system is a complex neuromodulatory endogenous signalling system widely distributed throughout the mammalian organism that participates in multiple metabolic pathways regulating cell physiology. This system is composed of cannabinoid receptors, endogenous ligands (endocannabinoids) and their synthesizing and degrading enzymes, intracellular signalling pathways regulated by endocannabinoids as well as transport systems (Piomelli, 2003, 2014; De Petrocellis *et al.*, 2004; Marsicano and Lutz, 2006; Kano *et al.*, 2009; Katona and Freund, 2012; Pertwee, 2015; Lu and Mackie, 2016). The eCB system is widely distributed in the central and peripheral nervous system (Katona and Freund, 2012; Lu and Mackie, 2016), and also in many other organs (Piazza *et al.*, 2017), where it regulates brain functions by acting on different cell types and cellular compartments (Katona and Freund, 2012; Lu and Mackie, 2016; Gutiérrez-Rodríguez *et al.*, 2017; Busquets-Garcia *et al.*, 2018). The alteration of the eCB system participates in the pathogenesis of multiple neurological and neuropsychiatric disorders (Pertwee, 2009).

2.2.1 Cannabinoid receptors

Cannabinoid receptors are known to be present in many vertebrate species, including rodents, monkeys and humans (Elphick and Egertová, 2005). The first classical receptor characterized by radiometric methods was the type 1 cannabinoid receptor (CB_1 receptor; CB_1) (Devane *et al.*, 1988); its molecular structure was identified first in rat (Matsuda *et al.*, 1990), then in human (Gérard *et al.*, 1991) and later in mouse (Akinshola *et al.*, 1999). The second classical receptor characterized was the type 2 cannabinoid receptor (CB_2 receptor), which was characterized from rat spleen myeloid cells (Munro *et al.*, 1993). Both receptors are members of the G-protein-coupled receptors (GPCRs) superfamily. GPCRs are widely distributed in the CNS and immune system and are characterized by seven hydrophobic transmembrane segments connected by intracellular and extracellular loops, an N-terminal extracellular domain that possesses glycosylation sites and a C-terminal intracellular domain coupled to a $G_{i/o}$ protein (Howlett *et al.*, 2002).

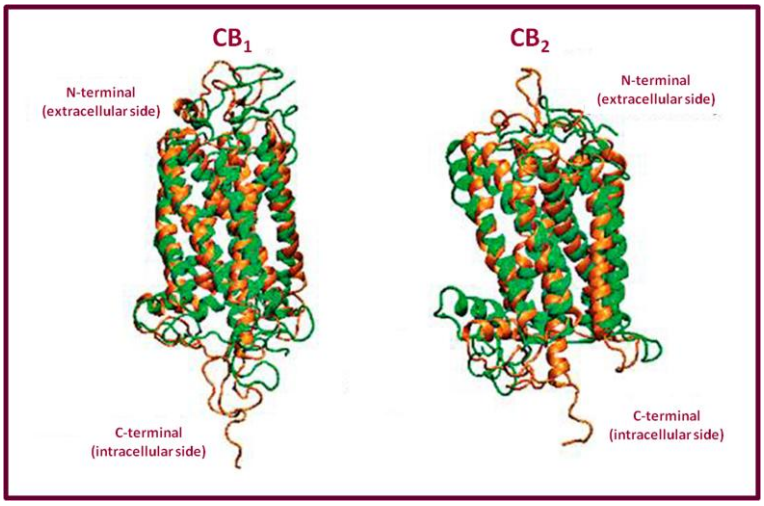

Figure 1. Structure of classical cannabinoid receptors
(Modified from Ramos *et al.*, 2011).

- **CB_1 Receptor**

The **CB_1 receptor** is one of the most abundant GPCR in the brain (Herkenham *et al.*, 1991; Tsou *et al.*, 1998; Moldrich and Wenger, 2000). Its expression is widespread, heterogeneous

and has crucial roles in the brain during prenatal and postnatal development and participates in many brain functions ranging from food intake to cognition through the modulation of synaptic transmission and plasticity (Marsicano *et al.*, 2002; Monory *et al.*, 2006; Marsicano and Kuner, 2008; Bellocchio *et al.*, 2010; Puente *et al.*, 2011; Castillo, 2012; Katona and Freund, 2012; Steindel *et al.*, 2013; Ruehle *et al.*, 2013; Soria-Gómez *et al.*, 2014, 2015; Hu and Mackie, 2015; Katona, 2015; Martín-García *et al.*, 2016; Bonilla-Del Río *et al.*, 2017; Gutiérrez-Rodríguez *et al.*, 2017, 2018).

The CB_1 receptor is the target of (−)-trans-Δ9-tetrahydrocannabinol (THC), the main psychoactive compound of Cannabis plants. Hence, the CB_1 receptor distribution in the brain closely fits into the deleterious effects of cannabinoids on locomotion, perception, learning, memory or the cannabinoid-positive effects as anti-convulsant or food intake enhancers, and its low amount in the brainstem correlates with the low toxicity and lethality of marijuana (Bellocchio *et al.*, 2010; Han *et al.*, 2012; Katona and Freund, 2012; Hebert-Chatelain *et al.*, 2014a,b, 2016; Soria-Gómez *et al.*, 2014; Martín-García *et al.*, 2016; Lu and Mackie, 2016; Mechoulam, 2016). CB_1 receptors are abundant in the basal ganglia (substantia nigra reticulata, globus pallidus, striatum, entopeduncular nucleus), cortex, nucleus accumbens, cerebellum, hippocampus (Howlett *et al.*, 1990; Tsou *et al.*, 1998; Hu and Mackie, 2015; Martín-García *et al.*, 2016), and poorly expressed in the hypothalamus, brainstem and spinal cord (Herkenham *et al.*, 1990; 1991; Mailleux and Vanderhaeghen, 1992; Tsou e*t al.*, 1998; Hu and Mackie, 2015).

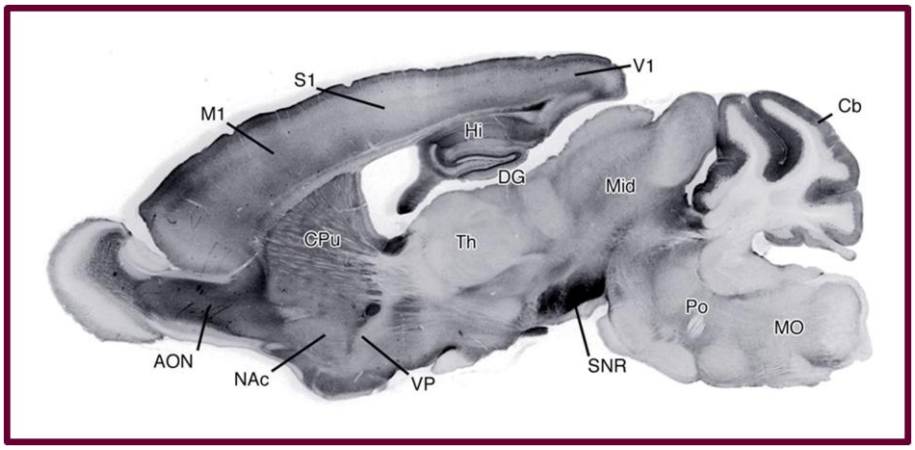

Figure 2. Distribution of CB1 receptors in a parasagittal section of the adult mouse brain. AON: anterior olfactory nucleus, Cb: cerebellar cortex, CPu: caudate putamen, DG: dentate gyrus, Hi: hippocampus, M1: primary motor cortex, Mid: midbrain, MO: medulla oblongata, NAc: nucleus accumbens, Po: pons, S1: primary somatosensory cortex, SNR: substantia nigra pars reticulata, Th: thalamus, V1: primary visual cortex, VP: ventral pallidum. (Modified from Kano *et al.*, 2009).

Mice with CB_1 receptor gene deletion (*CB*$_1$-KO) lack CB_1 receptor protein expression (Steiner *et al.*, 1999; Zimmer *et al.*, 1999; Marsicano *et al.*, 2002; for review: Zimmer, 2015) and, therefore, only unspecific CB_1 receptor immunolabelling is observed in mutant tissue. Conditional mutant mice lacking CB_1 receptor mainly from cortical glutamatergic neurons (Glu-*CB*$_1$-KO) and from GABAergic neurons (GABA-*CB*$_1$-KO) (Monory *et al.*, 2006, 2007) show a selective decrease in the brain pattern of CB_1 receptor staining but not in the same degree as in *CB*$_1$-KO; in particular, the CB_1 receptor immunoreactivity is greatly reduced in the GABA-*CB*$_1$-KO and less in the Glu-*CB*$_1$-KO compared with the wild type (Monory *et al.*, 2006, 2007; Marsicano and Kuner, 2008; Steindel *et al.*, 2013; Martín-García *et al.*, 2016) indicating that CB_1 receptors are more abundantly expressed in GABAergic neurons than in glutamatergic neurons. An exception would be the great reduction in CB_1 receptor staining observed in the granule cell layer of the Glu-*CB*$_1$-KO olfactory bulb (Soria-Gómez *et al.*, 2014). Substantia nigra pars reticulata lacks CB_1 receptor immunoreactivity in GABA-*CB*$_1$-KO, and a large decrease in CB_1 receptor staining is observed in the GABA-*CB*$_1$-KO hippocampus but not at the zone of the glutamatergic commissural/associational synapses in the inner 1/3 of the dentate ML (Monory *et al.*, 2006,

2007; Marsicano and Kuner, 2008; Martín-García *et al.*, 2016). Conversely, the weak pattern of CB_1 receptor immunostaining in genetic rescue mice expressing CB_1 receptors only in dorsal telencephalic glutamatergic neurons (Glu-*CB*$_1$-RS) (de Salas-Quiroga et al., 2015; Lange et al., 2017; Ruehle et al., 2013; Soria-Gómez et al., 2014; Gutiérrez-Rodríguez et al., 2017, 2018) relative to the rescue mice expressing CB_1 receptors only in GABAergic neurons (GABA-*CB*$_1$-RS) (de Salas-Quiroga *et al.*, 2015; Lange *et al.*, 2017; Remmers *et al.*, 2017; Gutiérrez-Rodríguez *et al.*, 2017, 2018) correlates with the low CB_1 receptor distribution in glutamatergic neurons and high in GABAergic cells, respectively. However, a conspicuous CB_1 receptor staining in Glu-*CB*$_1$-RS is observed in the striatum, cortex, olfactory tubercle, amygdala, hippocampus (strata oriens and radiatum of the hippocampal Ammon's horn) and, remarkably, in the inner 1/3 of the dentate ML of Glu-*CB*$_1$-RS (Monory *et al.*, 2006; Ruehle *et al.*, 2013; Gutiérrez-Rodríguez *et al.*, 2017). In GABA-*CB*$_1$-RS, strong CB_1 receptor immunoreactivity is seen in the cortex, anterior olfactory nucleus, piriform cortex, globus pallidus, entopeduncular nucleus, amygdala, and substantia nigra, and moderate to strong in the striatum (Gutiérrez-Rodríguez *et al.*, 2017). In the hippocampus, heavy CB_1 receptor immunoreaction is present throughout the hippocampus, particularly in the Ammon's horn pyramidal cell layer, at the limit between the strata radiatum and the lacunosum-moleculare and in the inner one-third of the dentate ML (Gutiérrez-Rodríguez *et al.*, 2017, Remmers *et al.*, 2017).

One critical aspect in the understanding and discovery of new cannabinoid-based drugs to treat addiction, and also other brain and organic diseases, is to elucidate where the main players of the eCB system, and particularly the CB_1 receptor, is localized subcellularly in the brain. Then, this knowledge will provide the anatomical substrate for the development of innovative strategies oriented towards the selective hit of specific CB_1 receptor populations at defined subcellular compartments and cell organelles by pharmacological or genetic tools. Brain CB_1 receptors are mostly localized in axon terminals and preterminals

away from the presynaptic active zones (Kawamura *et al.*, 2006; Uchigashima *et al.*, 2007; Katona and Freund, 2012).

Under normal conditions, CB_1 receptor expression is very high in inhibitory GABAergic synaptic terminals mostly in cortical and hippocampal cholecystokinin (CCK)-positive GABAergic interneurons (Kawamura *et al.*, 2006; Ludányi *et al.*, 2008; Marsicano and Kuner, 2008; Katona and Freund, 2012; De-May and Ali, 2013; Steindel *et al.*, 2013; Hu and Mackie, 2015; Lu and Mackie, 2016; Gutiérrez-Rodríguez *et al.*, 2017), low in excitatory glutamatergic synapses (Marsicano *et al.*, 2003; Domenici et al., 2006; Takahashi and Castillo, 2006; Katona *et al.*, 2006; Monory *et al.*, 2006; Kamprath *et al.*, 2009; Bellocchio *et al.*, 2010; Puente *et al.*, 2011; Reguero *et al.*, 2011; Ruehle *et al.*, 2013; Soria-Gómez *et al.*, 2014; Gutiérrez-Rodríguez *et al.*, 2017) and very low in brain astrocytes (Rodriguez *et al.*, 2001; Navarrete and Araque, 2008, 2010; Stella, 2010; Han *et al.*, 2012; Bosier *et al.*, 2013; Metna-Laurent and Marsicano, 2015; Viader *et al.*, 2015; Oliveira da Cruz *et al.*, 2016; Kovács *et al.*, 2017; Gutiérrez-Rodríguez *et al.*, 2018). The activation of the scarce CB_1 receptors expressed in astrocytes promotes astroglial differentiation and regulates synaptic transmission and plasticity through the modulation of neuron-astrocyte crosstalk. Furthermore, astroglial CB_1 receptors activation by acute cannabinoids impairs working memory (Han *et al.*, 2012); also, CB_1 receptors in astrocytes control the leptin receptor expression in cultured cortical and hypothalamic astrocytes needed for energy supply to the brain (Bosier *et al.*, 2013). CB_1 receptors are expressed in oligodendrocytes and neural precursors too (Molina-Holgado *et al.*, 2002; Aguado *et al.*, 2005; Benito *et al.*, 2007; Garcia-Ovejero *et al.*, 2009; Mato *et al.*, 2009; Gomez *et al.*, 2010) and intracellular CB_1 receptors have been unequivocally localized to neuronal mitochondria (Bénard *et al.*, 2012; Hebert-Chatelain *et al.*, 2014a; 2014b; Koch *et al.*, 2015) where they regulate memory through the modulation of energy metabolism (Hebert-Chatelain *et al.*, 2016) as well as to astroglial mitochondria (Gutiérrez-Rodríguez *et al.*, 2018).

We assessed the CB_1 receptor distribution in subcellular compartments of the CA1 of the Hi as the proportion of CB_1 receptor-dependent silver-intensified gold particles in GABAergic terminals (~56%), glutamatergic terminals (~12%), astrocytes (~6%) and mitochondria (~15%) (Bonilla-Del Río *et al.*, 2017; Gutiérrez-Rodríguez *et al.*, 2018). Noticeably, 11% of the immunoparticles were localized to other compartments, and, importantly, the labeling disappeared in the *CB_1*-KO (Bonilla-Del Río *et al.*, 2017; Gutiérrez-Rodríguez *et al.*, 2018). Other brain cells constitutively expressing CB_1 receptors are oligodendrocytes (Molina-Holgado *et al.*, 2002; Benito *et al.*, 2007; Garcia-Ovejero *et al.*, 2009; Mato *et al.*, 2009; Gomez *et al.*, 2010) and probably microglia (Bonilla-Del Río *et al.*, unpublished observations).

CB_1 receptors also localize in adipose tissue, muscle, liver, heart, gastrointestinal tract, pancreas, spleen, tonsils, prostate, testicle, uterus, ovary, skin, eye, or presynaptic sympathetic nerve terminals (Galiègue *et al.*, 1995; Ishac *et al.*, 1996; Pertwee, 2001; Maccarone *et al.*, 2016; Zou and Kumar, 2018). They are also present at mitochondria of skeletal (gastrocnemius and rectus abdominis) and myocardial muscles (Mendizabal-Zubiaga *et al.*, 2016) whose activation by THC reduces mitochondria coupled respiration (Mendizabal-Zubiaga *et al.*, 2016).

- **CB_2 Receptor**

The **CB_2 receptor** was first described in spleen (Munro *et al.*, 1993) and, in addition to this organ, it was believed to be only present in the immune system (tonsils, B and T lymphocytes, natural killer cells, macrophages and CD8 and CD4 T-lymphocytes) (Galiègue *et al.*, 1995; Ameri, 1999; Cabral *et al.*, 2015). However, CB_2 receptors are also expressed in heart, endothelium, bone, liver, pancreas, testicle (Zou and Kumar, 2018). The localization of CB_2 receptors in the CNS is a controversial issue as not specific CB_2 receptor antibodies are available so far (Atwood and Mackie, 2010; Lu and Mackie, 2016).

CB_2 receptors are expressed in reactive microglia and also astrocytes (Fernández-Ruiz *et al.*, 2007; López *et al.*, 2018).

- **Other Cannabinoid Receptors**

There are also other receptors that mediate the effects of endocannabinoids (Pertwee, 2015). For instance, the transient receptor potential vanilloid 1 (TRPV1) activated by anandamide and other molecules (Maccarrone *et al.*, 2008; De Petrocellis and Di Marzo, 2009; Tóth *et al.*, 2009; Alhouayek *et al.*, 2014; Rossi *et al.*, 2015); the transient receptor potential ankyrin 1 (TRPA1) receptors (De Petrocellis *et al.*, 2008), peroxisome proliferator-activated receptors, namely PPAR-α (Sun *et al.*, 2006; Alhouayek *et al.*, 2014) and non-CB_1/CB_2 GPCRs such as G protein-coupled receptor 55 (GPR55) (Ryberg *et al.*, 2007).

2.2.2 Signal transduction mechanism

The analysis of [^{35}S]guanosine-5*-O-(3-thiotriphosphate) ([^{35}S] GTPγS) binding demonstrated that CB_1 receptors at glutamatergic synapses are more efficiently coupled to G protein signaling than GABAergic CB_1 receptors (Steindel *et al.*, 2013). Signal transduction through CB_1 and CB_2 receptors occurs mainly by their interaction with G proteins of the $G_{i/o}$ subtype which leads, among other effects, to adenylyl cyclase inhibition with the consequent decrease of cyclic adenosine monophosphate (cAMP), and transient blockade of protein kinase type A (PKA)-mediated short-term effects. $G_{i/o}$ also stimulates the pathways of several intracellular kinases, such as mitogen-activated protein kinase (MAPK) or extracellular signal–regulated kinase (ERK) (Pertwee, 1997; Galve-Roperh *et al.*, 2002). In addition, CB_1 receptors (but not CB_2) are coupled, via $G_{i/o}$ proteins, to ion channels of different types, so that activation of CB_1 receptors leads to a negative regulation of -N, -L and -P / Q, calcium channels and positive currents of potassium rectifiers (Pertwee, 1997; de Fonseca *et al.*, 2005). Finally, the activation of CB_1 receptors (and not CB_2) stimulates other kinases, such as phosphatidyl-inositol 3 kinase and protein kinase B

(Galve-Roperh *et al.*, 2002). All these effects are related to the control of neuronal excitability and to the inhibitory influence of cannabinoid agonists on neurotransmitter release (Di Marzo *et al.*, 1998; Ohno-Shosaku *et al.*, 2002; De Petrocellis, *et al.*, 2004).

2.2.3 Endocannabinoids in the central nervous system

The endocannabinoids are lipid messengers considered as promiscuous molecules since they activate CB_1 and CB_2 receptors and other receptors (Piomelli, 2003; Kano *et al.*, 2009; Pertwee *et al.*, 2010; Katona and Freund, 2012; Lutz *et al.*, 2015; Lu and Mackie, 2016; Zou and Kumar, 2018). The physiology and pharmacology of the endocannabinoids are complex due to both the vast distribution of the numerous components and the features of the system. The endocannabinoids exert their influence in a paracrine and autocrine manner, and probably even in endocrine mode, because their lipid nature allows them to diffuse and cross membranes. They are cannabinoid receptor agonists that constitute a family of molecules that are not accumulated in secretory vesicles but rather synthesized on demand and released right after to the extracellular space following physiological and pathological stimuli (Piomelli, 2003; Kano *et al.*, 2009; Pertwee *et al.*, 2010; Katona and Freund, 2012; Lutz *et al.*, 2015; Lu and Mackie, 2016; Zou and Kumar, 2018).

The two main endocannabinoids are derivatives of polyunsatured fatty acids, N-arachidonoylethanolamine (anandamide, AEA) (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995). AEA produces the "tetrad" effects of cannabinoids (i.e., catalepsy, antinociception, hypolocomotion, and hypothermia) in rodents (Fride and Mechoulam, 1993) whereas 2-AG plays a key role in most of the CB_1 receptor-dependent modulation of synaptic transmission and plasticity (Kano *et al.*, 2009). 2-AG concentration in brain tissue is about 200-fold higher than AEA (Bisogno *et al.*, 1999) and correlates well with the cannabinoid receptor density in the brain (Sugiura *et al.*, 2006). However, this is not the case for AEA that accumulates in brain regions with high

cannabinoid receptor density (hippocampus, cortex, striatum) and also in regions with low receptor expression (thalamus, brainstem) (Felder and Glass, 1998). 2-AG is an agonist with high efficacy on both CB_1 and CB_2 receptors (Lynn and Herkenham, 1994; Slipetz *et al.*, 1995; Gonsiorek *et al.*, 2000; Sugiura *et al.*, 2000), while the AEA efficacy is low at CB_1 (partial agonist) and very low at CB_2 receptors (weak partial agonist/antagonist) (Showalter *et al.*, 1996; Gonsiorek *et al.*, 2000; Sugiura *et al.*, 2000; Luk *et al.*, 2004).

There is a great variety of biochemical pathways for the synthesis, transport, release and degradation of endocannabinoids. Thus, the biosynthetic enzymes phospholipase D selective N-acylphosphatidylethanolamine (NAPE-PLD) for AEA and diacylglycerol lipases (DAGL) α and β for 2-AG, as well as the hydrolytic enzymes fatty acid amide hydrolase (FAAH) for AEA inactivation and monoacylglycerol lipase (MAGL) for 2-AG, among others, are responsible for the distinctive physiological and pathophysiological roles of both endocannabinoids (Kano *et al.*, 2009; Fezza *et al.*, 2014; Piomelli, 2014; Lu and Mackie, 2016; Zou and Kumar, 2018).

The AEA precursor N-arachidonoyl phosphatidylethanolamine (NAPE) is generated by the transfer of arachidonic acid (AA) from phosphatidylcholine to phosphatidylethanolamine by the Ca^{2+} dependent N-acyltransferase (NAT) (Cadas *et al.*, 1996; Kano *et al.*, 2009; Fezza *et al.*, 2014). Then, AEA is synthesized by the N-acylphosphatidylethanolamine specific phospholipase D (NAPE-PLD) that hydrolyses NAPE localized in cell membranes (Okamoto *et al.*, 2004; Kano *et al.*, 2009). The AEA half-life is very short because of its quick uptake by a high affinity transporter (AMT, *anandamide membrane transporter*) distributed in neurons and glia (Di Marzo *et al.*, 2015). AEA is inactivated by FAAH present in many organs and also in the brain (Dinh *et al.*, 2002; Ueda, 2002; Kano *et al.*, 2009) where its postsynaptic localization meets with presynaptic CB_1 receptors (Egertová *et al.*, 2003; Kano *et al.*, 2009; Hu and Mackie, 2015). FAAH is serine-hydrolase bound to

intracellular membranes that catalyzes AEA into arachidonic acid and ethanolamine (Fezza *et al.*, 2014). There are two more hydrolases for AEA degradation: FAAH-2 and the lysosomal *N*-acylethanolamine cisteine-amidohydrolase (NAAA).

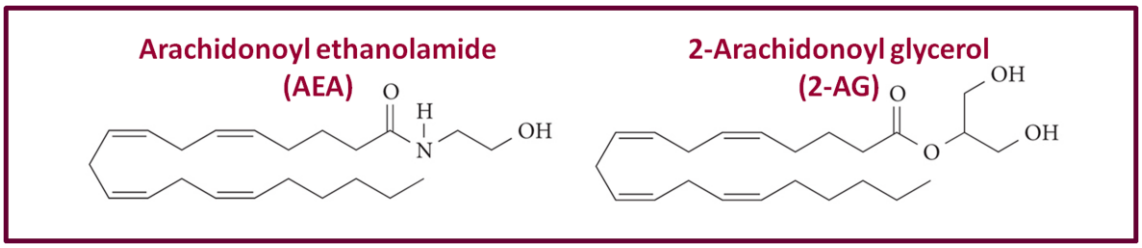

Figure 3. Chemical structures of the main endocannabinoids. Arachidonoyl ethanolamide (anandamide; AEA) and 2-arachidonoyl glycerol (2-AG) (Modified from Mechoulam *et al.*, 2014).

2-AG participates in the CB_1-dependent retrograde signalling and is an intermediate metabolite for lipid synthesis providing AA for prostaglandin synthesis (Kano *et al.*, 2009; Fezza *et al.*, 2014; Lu and Mackie, 2016). Neuronal membrane depolarization or the activation of Gq-coupled GPCRs triggers the synthesis of 2-AG (Kano *et al.*, 2009). The diacylglycerol (DAG) precursors come from the hydrolysis of membrane phosphatidylinositol by phospholipase C, β or δ. The degradation of these precursors by DAGL-α and DAGL-β drives 2-AG synthesis (Kano *et al.*, 2009; Gao *et al.*, 2010; Tanimura *et al.*, 2010; Lu and Mackie, 2016; Zou and Kumar, 2018). The DAGLα isoform synthesizes the greatest amount of 2-AG; DAGLβ synthesizes 2-AG under certain circumstances (Di Marzo *et al*, 2015). MAGL is a serine-hydrolase that catalyzes 2-AG into AA and glycerol (Dinh *et al.*, 2002; Ueda, 2002; Kano *et al.*, 2009); this enzyme is mainly found in presynaptic terminals (Kano *et al.*, 2009; Straiker *et al.*, 2009; Hu and Mackie, 2015; Lu and Mackie, 2016). Also, the α/β-hydrolase domain 6 (ABHD6) and domain 12 (ABHD12) degrade 2-AG (Blankman *et al.*, 2007; Kano *et al.*, 2009; Fezza *et al.*, 2014). AEA and 2-AG are also metabolized by lipooxygenases and cyclooxygenase-2 (COX-2) (Kano *et al.*, 2009; Lu and Mackie, 2016).

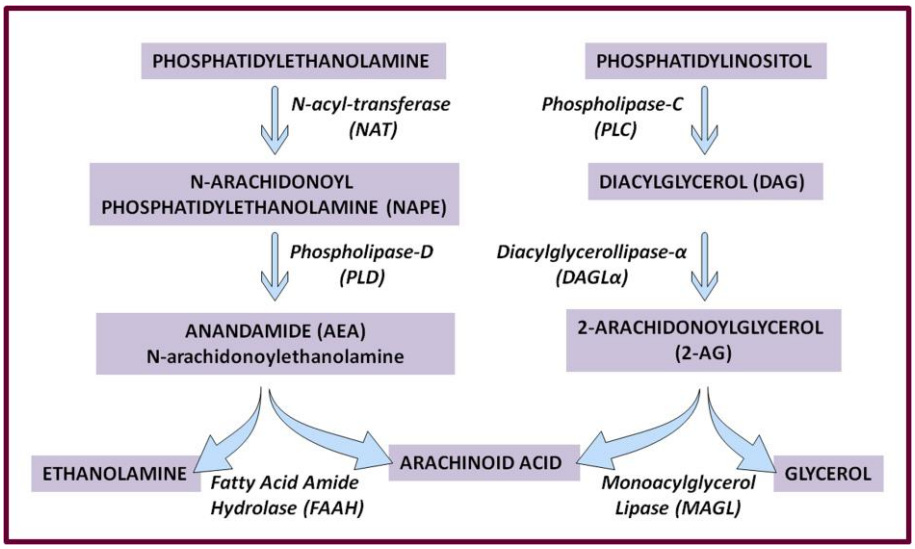

Figure 4. Major pathways for synthesis and degradation of anandamide and 2-AG (Modified from Lee *et al.*, 2015).

2.3 ETHANOL CONSUMPTION, ADOLESCENCE AND CANNABINOID SYSTEM

Several preclinical approaches have been developed in order to study the effects of EtOH consumption. The drinking in the dark (DID) procedure has emerged as a valuable tool in both mouse (Crabbe *et al.*, 2011) and rat (Holgate *et al.*, 2017) to investigate the effects of alcohol administration during adolescence. In rodents, adolescence runs between postnatal day (pnd) 28 and 42 but neurobehavioral signs can persist in male rats up to 55-60 pnd (Spear, 2000). In this model, the animals are given access to EtOH (or tap water) for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day. We have chosen the DID method because: (1) EtOH self-administration is closer to voluntary alcohol intake in humans; (2) intermittent access to EtOH provides a cycle of consumption-withdrawal that relates to escalating EtOH consumption patterns; (3) it has been adapted to the adolescent period (see Carnicella *et al.*, 2014; Crews *et al.*, 2016; Spear, 2016b).

The CB_1 receptor has a crucial role in the EtOH behaviors, as receptor agonists stimulate EtOH intake and antagonists reduce voluntary EtOH consumption, preference and craving (Colombo *et al.*, 2002; Economidou *et al.*, 2006). Likewise, preference and EtOH intake are reduced in mice lacking CB_1 receptors (Hungund *et al.*, 2003) and chronic EtOH exposure decreases CB_1 receptor mRNA expression, receptor density and functionality (Basavarajappa *et al.*, 1998; Ortiz *et al.*, 2004; Mitrirattanakul *et al.*, 2007; Vinod *et al.*, 2006; 2008; 2010) which associate with a long-lasting increase in endocannabinoids in the hippocampus after withdrawal (Mitrirattanakul *et al.*, 2007; Rubio *et al.* 2009). So, the interaction of the eCB system with EtOH was thought to be a good target candidate for treatment of EtOH addiction.

We have recently tested the effect of EtOH consumption during adolescence on the expression of the CB_1 receptor in the adult hippocampus. The model applied was the DID procedure in which adolescent male mice were subjected to a 4-day DID (Rhodes *et al.*, 2007) over a period of 4 weeks. Accordingly, we assessed the CB_1 receptor expression in the CA1 hippocampus as the proportion of silver-intensified 1.4 nm gold particles bound to Fab' fragments of rabbit anti-goat immunoglobulin G antibodies (1:100, Nanoprobes Inc.) directed to goat polyclonal anti-CB_1 receptor antibodies (2 µg/ml corresponding to a 1:100 dilution, #CB1-Go-Af450, Frontier Institute Co.; RRID: AB_257130). The pattern of CB_1 receptor distribution was altered under conditions of EtOH (Bonilla-Del Río *et al.*, 2017). Interestingly, there were not detected differences between the proportion of CB_1 receptor particles localized to inhibitory terminals, mitochondria and other membrane compartments. Furthermore, there was a striking decrease in CB_1 receptor labeling in astrocytes as well as in the CB_1 receptor immunopositive astrocytic processes and in the density of receptor labeling of the adult hippocampus after EtOH intake during adolescence (Bonilla-Del Río *et al.*, 2017). Furthermore, the astrocytes were swollen much like after exposure to acute EtOH (Adermark and Bowers, 2016; Allansson *et al.*, 2001; Othman *et al.*, 2002; Pava and

Woodward, 2012). Chronic EtOH exposure alters the glial fibrillary acidic protein and, consequently, the astrocyte morphology (Renau-Piqueras *et al.*, 1989). In the last years, astroglial CB_1 receptors have been shown to play a role in brain function, cognition and behavior (Navarrete and Araque, 2008, 2010; Han *et al.*, 2012; Min and Nevian, 2012; Araque *et al.*, 2014; Navarrete et al., 2014; Gómez-Gonzalo *et al.*, 2015; Metna-Laurent and Marsicano, 2015; Oliveira da Cruz, *et al.*, 2016). The reduced CB_1 receptor expression in astrocytes and their morphological changes observed after adolescent EtOH consumption should have consequences on the molecular architecture and synaptic plasticity mechanisms at the tripartite synapse (Dzyubenko *et al.*, 2016). Furthermore, altered astrocytes upon EtOH consumption associate with an increase in the glutamate transporter GLAST (EAAT1) (Flatscher-Bader *et al.*, 2006; Rimondini *et al.*, 2002); however, GLAST-null mice with functional CB_1 receptors synapses have less EtOH consumption, motivation and reward (Karlsson *et al.*, 2012). Thus, GLAST expression and consequently the regulation of the extracellular glutamate, seems to be a key piece in the EtOH addictive behaviors. Whether the drastic reduction of astroglial CB_1 receptors observed in the mature hippocampus after adolescent EtOH intake affects GLAST expression in astrocytes is still an unanswered question. If there were an interaction between CB_1 receptors and GLAST in astrocytes, it would have clinical implications as to selective astroglial CB_1 receptor modulation might impact on GLAST.

Another purview to be considered is the neuroinflammatory mechanisms turned on by BD in adolescence that entails impaired synaptic plasticity, long-term behavioral and cognitive deficits, and late alcohol abuse and addiction (Nestler, 2001; Montesinos *et al.*, 2016). Astrocytes are able to release pro-inflammatory molecules (Farina *et al.*, 2007) and astroglial CB_1 receptors are involved in anti-inflammatory responses in reactive astrocytes (Metna-Laurent and Marsicano, 2015; Ortega-Gutiérrez *et al.*, 2005; Sheng *et al.*, 2005). Hence, the drastic reduction in CB_1 receptors in astrocytes upon adolescent EtOH intake

might be accompanied by an impairment of the astrocyte-mediated anti-inflammatory reaction. Thus, depending on the pattern of EtOH intake, therapeutic strategies based on the use of anti-inflammatory drugs could be designed in order to treat EtOH addiction and the perturbed behavior and cognition associated. Furthermore, the drastic decrease in CB_1 receptors in astrocytes and their morphological changes observed in the adult brain after EtOH intake during adolescence, represent a novel pharmacological target to palliate the structural, functional and behavioral consequences of the adolescent BD in adulthood.

We also observed that the CB_1 receptor expression on glutamatergic synapses in the adult CA1 hippocampus was lower after EtOH exposure during adolescence (Bonilla-Del Río *et al.*, 2017) with no effect on the expression and localization of CB_1 receptors in GABAergic synapses (Bonilla-Del Río *et al.*, 2017; Gutiérrez-Rodríguez *et al.*, 2017). As already mentioned, CB_1 receptors have been recently shown to localize to mitochondria ($mtCB_1$ receptors) of neurons and astrocytes. The $mtCB_1$ receptors modulate mitochondrial respiration having important functional impact on synaptic transmission, behavior and memory. Thus, the decrease in cellular respiration yielded by the exposure to acute cannabinoids relates to $mtCB_1$ receptors activation that turns on intramitochondrial Gαi protein signaling with the consequent soluble-adenylyl cyclase inhibition and shutdown of the PKA-dependent phosphorylation of specific subunits of he mitochondrial electron transport system (Hebert-Chatelain *et al.*, 2016). This effect of cannabinoids on bioenergetic production through $mtCB_1$ receptors impacts on memory formation, as mutant mice lacking CB_1 receptors in hippocampal mitochondria do not exhibit amnesia after cannabinoid administration (Hebert-Chatelain *et al.*, 2016) in the NOR task (Puighermanal *et al.*, 2009). Furthermore, cannabinoids reduce mitochondrial mobility (Boesmans *et al.*, 2009) needed for energy support (Sheng and Cai, 2012). The potential role of $mtCB_1$ receptors in addictive behaviors remains to be elucidated. However, there are anatomical indications showing that the proportion of CB_1 receptor particles on mitochondria in sham

and EtOH hippocampus of adult brain in mice exposed to the model of adolescent BD (Bonilla-Del Río *et al.*, 2017) was similar to our previous findings (Bénard *et al.*, 2012; Hebert-Chatelain *et al.*, 2016). Hence, no changes in the CB_1 receptor expression on this organelle could be detected upon BD during adolescence. Furthermore, an increase in AEA was detected in EtOH animal models (Vinod *et al.*, 2006) and ventral striatum of postmortem human alcoholics (Vinod *et al*., 2010) together with a decrease in the AEA degrading enzyme fatty acid amide hydrolase (FAAH) and CB_1 receptor expression (Vinod *et al.*, 2010). A decrease in CB_1 receptor expression and a reduced G protein coupling of the receptor was also observed in the striatum, hippocampus, nucleus accumbens and amygdala of FAAH knockout mice (Vinod *et al.*, 2008).

Altogether, the investigations have firmly established a role for the eCB system in mediating the reinforcing properties of EtOH and EtOH dependence. So, the reciprocal interaction between the eCB system and EtOH has been thought as a good target candidate for treating EtOH addiction. Accordingly, how the manipulation of the eCB system interferes positively with the long-term changes induced by EtOH is one of the main goals of this Doctoral Thesis.

2.4 HIPPOCAMPAL FORMATION

The hippocampal formation (HF) is part of the limbic system. Its C-shaped structure contains 3 subregions: Dentate Gyrus, CA of the Hippocampus (which is subdivided into CA1, CA2, CA3 and CA4 areas) and the subiculum. The adjoining area is the parahippocampal region which is divided into 5 subregions: perirhinal, entorhinal, and postrhinal cortex, presubiculum and parasubiculum.

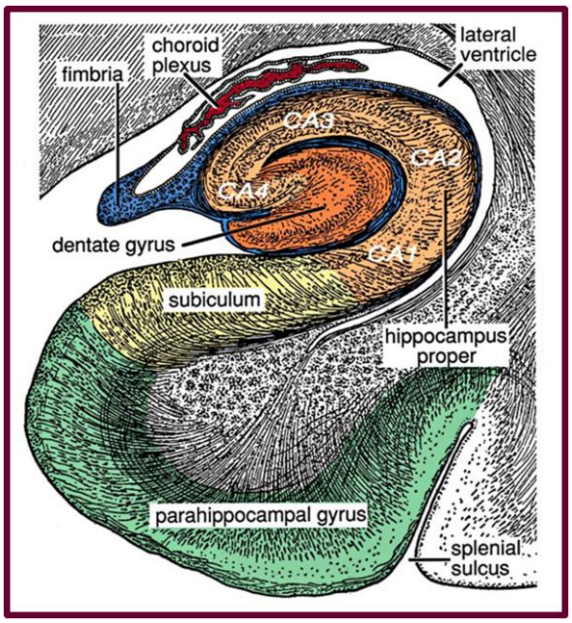

Figure 5. Schematic drawing of the hippocampal formation and the parahippocampal gyrus. A dorsal plane section from the right cerebral hemisphere (rostral toward the left and medial toward the bottom). The HF consists of the dentate gyrus (dark orange), hippocampus (pale orange), and subiculum (yellow). The latter is continuous with the entorhinal cortex covering the parahippocampal gyrus (green). The hippocampus (Ammon´s horn) is divided into four regions (CA1-CA4). Output axons (blue) from the HF run superficially in the alveus and then in the fimbria. (Modified from Ranson and Clark, 1959).

The HF plays an essential role in spatial and contextual memory, as well as in learning and mood regulation. In addition, disorders such as anxiety, depression, neurodegenerative diseases and addiction, including EtOH addiction, are related to alterations in regions of the HF.

2.4.1 Dentate gyrus

The DG is involved in the formation of episodic memory (Aimone *et al.*, 2011). Thus, behavioral studies have shown that animals with damaged DG are not able to distinguish between similar events or objects, without any other behavioral deficit (anxiety, depression, etc.) (Gilbert *et al.*, 2001).

The DG has three layers: the molecular layer (ML) which contains the perforant path fibers that connect the entorhinal cortex (EC) with the DG; the granule cell layer mainly constituted by glutamatergic excitatory granule cells; and the polymorphic layer or hilus

that contains the mossy cells and many other cell types (Amaral and Witter, 1989; Amaral *et al.*, 2007).

There is a high connectivity between the HF regions and other regions of the brain. The progression of synaptic activation is unidirectional in the HF (Amaral *et al.*, 1990): the entry point is the ML of DG that receives sensory information of the EC through the perforant path. An additional component of the perforant path originates in layer III and terminates in the CA1 field of the hippocampus and the subiculum. Then, the granule cells of the DG give rise to the mossy fibers that terminate both within the polymorphic layer of the DG and within stratum lucidum of the CA3 field of the hippocampus. They give rise to the ipsilateral Schaffer collaterals that terminate on the dendritic spines of the pyramidal cells in the CA1 stratum radiatum. These CA1 pyramidal cells project in turn into the subicular complex, which completes the local trisynaptic circuit and subsequently projects back into the EC. This trisynaptic circuit is the main route of activity flow through the hippocampus (Nicoll and Schmitz, 2005; Nakashiba *et al.*, 2008).

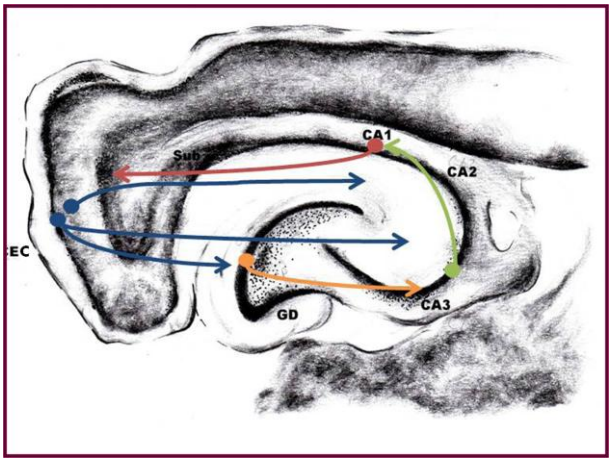

Figure 6. Diagram of the hippocampal trisynaptic circuit. Sensory information comes from the perforant pathway (blue arrows) to the granule cells. Their axons, the mossy fibers, project onto the CA3 pyramidal cells (orange arrow) which through Schaffer collaterals (green arrow) connect with the CA1 pyramidal cells which return projections to the neuronal layer of EC (red arrow) (Modified from Hernández *et al.*, 2015).

2.5 WORKING HYPOTHESIS

The adolescent brain is characterized by continuous maturation and structural development processes (Kyzar *et al.*, 2016). Alcohol abuse during this critical period causes long-term alterations in neurotransmitter synthesis and release, signaling cascades, neuronal morphology, gene expression, axonal outgrowth, dendritic pruning or synaptic transmission and plasticity (Keshavan *et al.*, 2014). EtOH intake profoundly impairs neural transmission in reward pathways, and the long-term structural changes and synaptic plasticity deficits in these circuits over time likely underlie the brain dysfunction observed after chronic EtOH consumption (Pava and Woodward, 2012; Lovinger and Roberto, 2013; Lovinger and Alvarez, 2017) that are thought to be at the basis of addictive behaviors (Vetreno and Crews, 2015). The eCB system is one of the main neuromodulatory systems of the brain that play important roles in the regulation of EtOH intake. Alterations of the eCB metabolism and signaling pathways during critical periods of brain development cause long-lasting behavioral abnormalities in adulthood (Subbanna *et al.*, 2013, 2015). Moreover, EtOH consumption alters eCB-dependent synaptic plasticity leading to long-term cognitive impairments (DePoy *et al.*, 2015; Crews *et al.*, 2016; Nimitvilai *et al.*, 2016; Lovinger, 2017; Bonilla del Río *et al.*, 2017; Marco *et al.*, 2017; Rico-Barrio *et al.*, 2018) and, reciprocally, the eCB system plays a pivotal role in the EtOH drinking behavior and the development of alcoholism (Basavarajappa and Hungund, 2002; Lovinger, 2017).

In spite of the ample information on the reciprocal interaction between EtOH and the eCB system, the long-lasting effects of EtOH exposure during adolescence on the eCB system and, ultimately, on behavior are only beginning to be uncovered (Bonilla-Del Río, *et al.*, 2017; Marco *et al.*, 2017; Rico-Barrio *et al.*, 2018). Based on this, we hypothesized that excessive EtOH consumption during the adolescence should elicit important molecular,

anatomical and physiological alterations of the eCB system disrupting brain functions in which this system plays key roles, such as synaptic plasticity and memory.

3. OBJECTIVES

The general goal of this Doctoral Thesis was to investigate the existence of eCB-dependent synaptic plasticity in the MPP of the adult mouse hippocampus in healthy conditions and after chronic EtOH intake during adolescence. In particular, we studied the molecular organization of the eCB system and the CB_1 receptor function at excitatory synapses of the dentate molecular layer. For this investigation, we developed an interdisciplinary strategy that combined molecular biology, biochemistry, anatomy, electrophysiology and behavior.

The specific objectives of the Doctoral Thesis were to:

1. Characterize the excitatory synaptic transmission after CB_1 receptor activation in the dentate medial perforant path (MPP) under normal conditions and in adult mice exposed to EtOH during adolescence.
2. Investigate the intrinsic mechanisms of the excitatory long-term depression mediated by activation of CB_1 receptors in the MPP in sham and adult mice chronically exposed to EtOH during adolescence.
3. Compare the CB_1 receptor expression and efficiency of the receptor in hippocampi from sham and EtOH mice.
4. Determine the anatomical distribution of the CB_1 receptor in the MPP of DG in sham and EtOH mice.
5. Quantify 2-AG and arachidonic acid in sham and EtOH mice.
6. Study the cognitive consequences in adult mice after adolescence EtOH exposure.

4. MATERIALS AND METHODS

4.1 ANIMALS

Experiments were performed on male C57BL/6J (Janvier Labs, Le Genest-Saint-Isle, France) and CB_1-KO mice and their wild-type (CB_1-WT) littermates (3 weeks old). They were housed in pairs of littermates in standard Plexiglas cages (17 cm x 14.3 cm x 36.3 cm) and allowed to habituate to the environment for at least 1 week before experimental procedures were initiated. All animals were maintained at approximately 22 °C with a 12:12 h light:dark cycle (red light on at 9:00 h). Mice had *ad libitum* access to food throughout all experiments and water except during EtOH access, as noted later. The protocols for animal care and use were approved by the Committee of Ethics for Animal Welfare of the University of the Basque Country (CEEA/M20/2016/073; CEIAB/2016/074) and were in accordance to the European Communities Council Directive of 22nd September 2010 (2010/63/EU) and Spanish regulations (Real Decreto 53/2013, BOE 08-02-2013). Great efforts were made in order to minimize the number and suffering of the animals used.

4.2 DRINKING IN THE DARK PROCEDURE

Adolescent male mice (pnd 32-56) were randomly assigned to either the water (sham) or EtOH experimental group. Mice were treated with a 4-day DID procedure (Bonilla-Del Río *et al.*, 2017; Marco *et al.*, 2017) for a total of 4 weeks. Each week, animals were weighed 1 h before lights out on days 1, 2, 3 and 4. On days 1-4, starting 3 h into the dark cycle, all animals were housed individually in standard Plexiglas cages (17 cm x 14.3 cm x 36.3 cm) and were exposed to a single bottle of EtOH [20% EtOH (v/v) prepared from EtOH 96% (Alcoholes Aroca S.L., Madrid, Spain)] or tap water for 2 h on days 1-3, and for additional 2 h on day 4. The EtOH exposure was followed by 3 days respite *(see Figure 7 for details)*. To ensure that the effects were the result of voluntary EtOH intake, the amount of EtOH

ingested by animals throughout the treatment was measured as TEI = [EtOH consumption x EtOH % (v/v) x EtOH density/Body Weight]/2 or 4 h, as required. TEI is the average of the quantity of total EtOH intake (in grams of EtOH, per kilogram of the animal, per hour) thorough adolescence of the EtOH exposed mice. EtOH consumption corresponds to the average of the quantity of the liquid in milliliters ingested for each animal in each session. EtOH % (v/v) is equivalent to graduation of EtOH used (20% (v/v)) and EtOH density to 0.78 grams of EtOH per milliliter, and finally it is divided by body weight of each animal in kilograms.

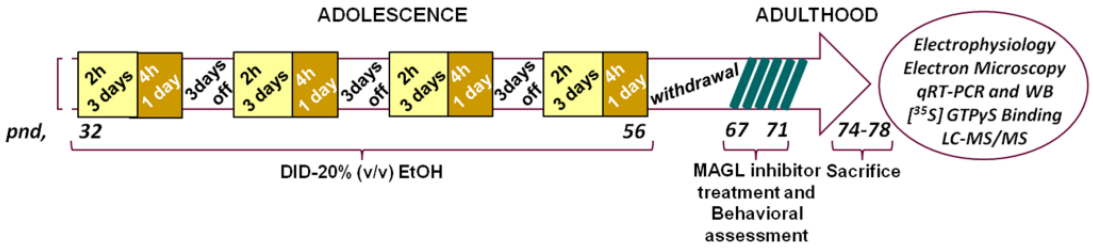

Figure 7. Experimental timeline. EtOH mice had free EtOH access (20% (v / v)) during 4 weeks in adolescence (pnd 32-56). Each week, the mice were exposed to 2 or 4 h of free EtOH access. In the remaining 3 days of the week, animals were kept resting in their respective cages. After two weeks of withdrawal (adulthood), mice (5-13 per experimental group) were treated with subchronic monoacilglicerol lipase (MAGL) inhibitor (JZL184) or vehicle during 5 consecutive days (pnd 67-71). The novel object recognition (NOR) test was run the last 3 days of JZL184 treatment (pnd 69-71). The remaining mice were subjected to spatial and associative recognition memory tests as well as rotarod, beam walking balance, tail suspension and light-dark box tests, during adulthood (pnd 69-71) and then sacrificed to process the brain tissue for different techniques in adulthood (pnd 74-78).

4.3 IN VITRO ELECTROPHYSIOLOGY

4.3.1 Slice preparation

Adult male C57BL/6J and *CB_1*-KO mice (pnd 74 – 78) were anesthetized by inhalation of isoflurane and the brains were rapidly removed and placed in a sucrose-based solution at 4 °C that contained: 87 mM NaCl, 75 mM sucrose, 25 mM glucose, 7 mM $MgCl_2$, 2.5 mM KCl, 0.5 mM $CaCl_2$ and 1.25 mM NaH_2PO_4.

Coronal sections (300 µm-thick) were obtained with a vibratome (Leica Microsistemas S.L.U.), then were recovered at 32-35 °C and superfused (2 mL/min) in the recording chamber with artificial cerebrospinal fluid (ACSF) containing: 130 mM NaCl, 11 mM glucose, 1.2 mM $MgCl_2$, 2.5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM NaH_2PO_4 and 23 mM $NaHCO_3$, equilibrated with 95% O_2/5% CO_2. All experiments were carried out at 32-35 °C. The superfusion medium contained picrotoxin (100 µM) to block type A Gamma-Aminobutyric acid ($GABA_A$) receptors. All drugs were added at the final concentration to the superfusion medium.

4.3.2 Extracellular field recordings

For extracellular field recordings, a glass recording pipette was filled with ACSF. The stimulation electrode was placed in the MPP and the recording pipette in the inner 1/3 of the ML of the DG (*see Figure 8 for details)*.

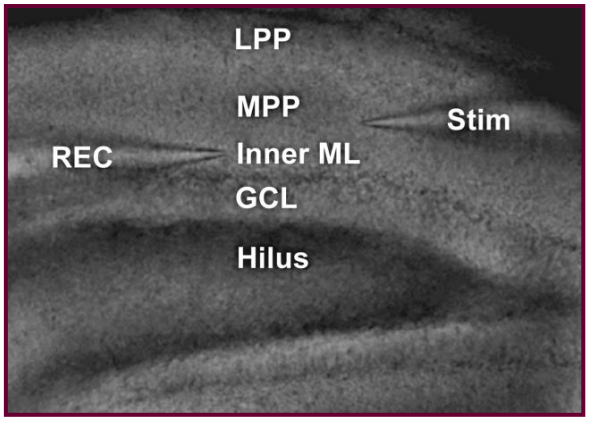

Figure 8. Image of sagittal section of the mouse dentate gyrus.
Stim: stimulation electrode.
REC: Recording electrode.
GCL: granule cell layer.
Inner ML: inner molecular layer.
MPP: medial perforant path.
LPP: lateral perforant path.

To evoke field excitatory postsynaptic potential responses (fEPSPs), repetitive control stimuli were delivered at 0.1 Hz (Stimulus isolater ISU 165, Cibertec, Spain; controlled by a Master-8, A.M.P.I.). An Axopatch-200B (Axon Instruments/Molecular Devices, Union City, CA, USA) was used to record the data, which were filtered at 1–2 kHz, digitized at 5 kHz on a DigiData 1440A interface (Axon Instruments/Molecular Devices, Union City, CA, USA) and collected on a computer using Clampex 10.0 (Axon Instruments/Molecular

Devices, Union City, CA, USA) and analyzed using Clampfit 10.2 (Axon Instruments/Molecular Devices, Union City, CA, USA). At the start of each experiment an input-output curve was constructed. Stimulation intensity was selected for baseline measurements that yielded between 40–60% of the maximal amplitude response. We used a stimulation protocol that Manzoni's group first introduced (Robbe *et al*., 2002) and that is based on naturally occurring frequencies. So, low frequency stimulation (LFS, 10 min, 10 Hz) protocol was used to induce LTD of glutamatergic inputs that can be reliably observed when recording extracellular fEPSPs (Puente *et al*., 2011).

4.3.3 Data analysis

Slope, area and amplitude of fEPSPs were measured (graphs depict area). The magnitude of the LTD after tetanic stimulation was calculated as the percentage change between baseline (averaged excitatory responses for 10 min before tetanus) and last 10 min of stable responses, normally at 30 min after the end of the tetanus. The slices used for each experimental condition (n) were obtained from at least 3 mice.

For the estimation of the paired-pulse ratio (PPR), 30 pairs of pulses were delivered with a 50 ms interval between individual pulses that composed the pair. The PPR of the evoked excitatory field recordings was calculated by dividing the mean of all 30 fEPSP2 (2nd evoked responses) slopes by the mean of all 30 corresponding fEPSP1 (1st evoked responses).

4.4 ELECTRON MICROSCOPY

4.4.1 Pre-embedding immunogold method

A pre-embedding silver-intensified immunogold method was used for the localization of the CB_1 receptor at the MPP termination zone in the middle 1/3 of the dentate ML (Gutierrez-

Rodriguez *et al.*, 2017). Adult C57BL/6J and *CB_1*-KO animals (n = 3, pnd 76) were deeply anesthetized with ketamine/xylazine (80/10 mg/kg body weight) and transcardially perfused at room temperature (RT, 20-25 ºC) with phosphate buffered saline (PBS, 0.1 M, pH 7.4) and fixed with 300 ml of 4% formaldehyde (freshly depolymerized from paraformaldehyde), 0.2% picric acid, and 0.1% glutaraldehyde in phosphate buffer (PB) (0.1 M, pH 7.4) prepared at 4 ºC. Coronal hippocampal vibrosections were cut at 50 µm and collected in a 0.1 M PB (pH 7.4) at RT. Sections were pre-incubated in a blocking solution of 10% bovine serum albumin (BSA), 0.1% sodium azide, and 0.02% saponin prepared in Tris-HCl buffered saline (TBS, pH 7.4) for 30 min at RT. Then hippocampal sections were incubated with the primary goat polyclonal anti-CB_1 receptor antibody (2 µg/ml, #CB_1-Go-Af450, Frontier Science Co.;RRID: AB_257130) in 10% BSA/TBS containing 0.1% sodium azide and 0.004% saponin on a shaker for 2 days at 4 ºC. After several washes in 1% BSA/TBS, tissue sections were incubated in a secondary 1.4 nm gold-labeled rabbit anti-goat Immunoglobulin-G (Fab' fragment, 1:100, Nanoprobes Inc., Yaphank, NY, USA) in 1% BSA/TBS with 0.004% saponine on a shaker for 4 h at RT. Thereafter, after washing hippocampal sections in 1% BSA/TBS overnight at 4 ºC, they were postfixed in 1% glutaraldehyde in TBS for 10 min and washed in double-distilled water. Following washes in double-distilled water, gold particles were silver-intensified with a HQ Silver kit (Nanoprobes Inc., Yaphank, NY, USA) for about 12 min in the dark and then washed in 0.1M PB. Stained sections were osmicated (1% OsO4 (v/v) in 0.1M PB, 20 min), dehydrated in graded alcohols to propylene oxide and plastic-embedded in Epon resin 812. Ultrathin sections of 50 nm were collected on mesh nickel grids, stained with 2.5% lead citrate for 20 min, and examined them in a Philips EM208S electron microscope. Tissue preparations were photographed by using a digital camera (Digital Morada Camera, Olympus) coupled to the electron microscope. Adjustments in contrast and brightness were made to the figures using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

4.4.2 Semi-quantification analysis

The pre-embedding immunogold method was applied simultaneously to the sections collected from all animals (n = 3 each condition). Immunogold-labeled hippocampal sections were visualized under a light microscope in order to select portions of the middle 1/3 of the dentate ML with good and reproducible CB_1 receptor immunolabeling. All electron micrographs were taken at 18,000x magnification and showed similar labeling intensity indicating that the selected areas were at the same depth. Furthermore, to avoid false negatives, only ultrathin sections within the first 1.5 μm from the surface of the tissue block were examined. Metal particles on presynaptic membranes were visualized and counted. Positive labeling was considered if at least one immunogold particle was on the presynaptic membrane or within approximately 30 nm of the membranes. Image-J (NIH, USA; RRID:SCR_003070) was used to measure the membrane length. Sampling was always carefully and accurately carried out in the same way for all the animals studied and experimenters were blinded to the condition of the subject during CB_1 receptor quantification.

328 excitatory synapses in sham and 313 in EtOH-treated mice were measured. Percentages of CB_1 receptor positive profiles, density (particles/μm membrane) of CB_1 receptor immunoparticles in terminals and proportion of CB_1 receptor immunoparticles in different compartments versus total CB_1 receptor expression in cellular membranes were determined and displayed as mean ± standard error mean (SEM) using a statistical software package (GraphPad Prism, GraphPad Software Inc, San Diego, USA; RRID:SCR_002798). The Kolmogorov-Smirnov normality test was applied before running statistical tests, and subsequently data were analyzed using the nonparametric Kruskal-Wallis test. Since there were no differences between them, all data within each line were pooled.

To study the molecular effects of EtOH intake during adolescence, the following techniques were performed in collaboration. The laboratory of Dr. Joan Sallés (Department of Pharmacology, Faculty of Pharmacy, University of the Basque Country UPV/EHU, Vitoria-Gasteiz, Spain, CIBERSAM, Spain) performed Western blotting of Gαi/o subunits, [^{35}S] GTPγS binding assays and measurements of 2-AG and arachidonic acid by liquid chromatography tandem mass spectrometry (LC-MS/MS). The laboratory of Dr. Fernando Rodríguez de Fonseca (Hospital Regional Universitario de Málaga, Instituto de Investigación Biomédica de Málaga, IBIMA, Málaga, Spain) carried out the quantitative real-time PCR (qRT-PCR) and Western blotting of components of the eCB and glutamatergic systems.

I describe these techniques and the results obtained with them as a part of this thesis with the permission and approval of Dr. Joan Sallés, Dr. Gontzal García del Caño, Dr. Sergio Barrondo, Dr. Xabier Aretxabala, Dr. Fernando Rodríguez de Fonseca and Dr. Juan Suárez.

4.5 RNA ISOLATION AND qRT-PCR ANALYSIS

Total RNA was extracted from the mouse hippocampus (~25-50 mg) from sham and EtOH adult mice (n = 16) by using the Trizol method, as previously described (Serrano *et al.*, 2012). Purified RNA (1 µg) and random hexamers were used to generate first strand cDNA using transcriptor reverse transcriptase. cDNA was used as a template for qRT-PCR. The relative quantification was normalized to the expression of the housekeeping gene Actb and calculated by using the ΔΔCt method. Primers used for the qRT-PCR reaction were obtained based on TaqMan® Gene Expression Assays (ThermoFisher) (Table 1).

Table 1. Primers used in qRT-PCR analyses (ThermoFisher).

Gene ID	GenBank accession numbers	ID	Product size (bp)
Actb	NM_007393.3	Mm00607939_s1	115
Cnr1	NM_007726.3	Mm01212171_s1	66
Dagla	Mm00813830_m1	NM_198114.2	69
Daglb	Mm00523381_m1	NM_144915.3	*72*
Mgll	NM_001166249.1	Mm00449274_m1	78
Napepld	NM_178728.5	Mm00724596_m1	85
Faah	NM_010173.4	Mm00515684_m1	62
Grm5	Mm00690332_m1	NM_001081414.2	97

Abbreviations: Actb, beta actin; Cnr1, cannabinoid receptor type 1, brain; Dagla, diacylglycerol lipase, alpha; Daglb, diacylglycerol lipase, beta; Mgll, monoacylglycerol lipase; Napepld, N-acyl phosphatidylethanolamine phospholipase D; Faah, fatty acid amide hydrolase; Grm5, glutamate receptor metabotropic 5.

4.6 HIPPOCAMPAL MEMBRANE PREPARATION

Western blots of Gαi/o subunits and [^{35}S] GTPγS binding assays were performed using mouse hippocampal membranes (P2 fraction) from sham and EtOH adult mice (n = 6-7). Hippocampal sections were thawed in ice-cold 20 mM TBS, pH 7.4, containing 1 mM EGTA (TBS/EGTA buffer) prior to homogenization, and then homogenized in 20 times the volume of the same hypotonic buffer using a glass homogenizer. First, cell debris was discarded by centrifugation at 1,000 g (10 min, 4 ºC) and then membranes were obtained by centrifugation at 40,000 g (30 min, 4 ºC). Finally, the pellet was re-suspended and re-centrifuged under the same conditions. Membranes were aliquoted in microcentrifuge tubes, centrifuged again (40,000 g, 30 min, 4 ºC), and the pellets were stored at -75 ºC prior to use. Protein content was determined using the Bio-Rad dye reagent with bovine γ-globulin as a standard.

4.7 PROTEIN DETERMINATION BY WESTERN BLOT ASSAYS

4.7.1 Gαi/o subunits

Western blot experiments of Gαi/o subunits were performed as previously described with minor modifications (Montaña *et al*., 2012). Briefly, hippocampal membranes (P2 fractions) from sham and EtOH adult mice (n = 2-3) were boiled in urea-denaturing buffer [20 mM TBS, pH 8.0, 12% glycerol, 12% Urea, 5% dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue] for 5 min. Increasing amounts of denatured proteins were resolved by electrophoresis on SDS–polyacrylamide (SDS–PAGE) gels (10%) using the Mini Protean II gel apparatus (Bio-Rad, Hercules, CA, USA). Proteins were transferred to polyvinylidene fluoride membranes (Amersham Bioscience, Buckinghamshire, UK) using the Mini TransBlot transfer unit (Bio-Rad, Hercules, CA, USA) at 90 V constant voltage for 1 h at 4 ºC. Blots were blocked in 5% non-fat dry milk/PBS containing 0.5% BSA and 0.2% Tween for 1 h, and incubated overnight at 4 ºC with antibodies against specific antibodies against different Gαi/o subunits subtypes, Gαo, Gαi1, Gαi2 and Gαi3 (Table 2). Blots were washed and incubated with specific horseradish peroxidase (HRP) conjugated secondary antibodies diluted to 1:10,000 in blocking buffer for 2 h at RT. Immunoreactive bands were incubated with the ECL system according to the manufacturer instructions (Amersham Bioscience, Buckinghamshire, UK).

4.7.2 Endocannabinoid and glutamatergic systems

Protein extracts (~15 µg) from the whole hippocampus of the sham and EtOH adult mice (n = 5-8) were separated in gradient SDS-PAGE gels and electroblotted onto nitrocellulose membranes (Crespillo *et al.*, 2011). Then, CB_1 receptor, MAGL and mGluR5 proteins were detected by overnight incubation in the corresponding primary antibodies (Table 2). Then,

HRP-conjugated anti-rabbit IgG (H+L) or anti-mouse secondary antibodies (Promega) diluted 1:10,000 was added for 1 h at RT. After the enhanced chemiluminiscence detection (Santa Cruz) in an Autochemi-UVP Bioimaging System, bands were quantified with ImageJ software (Rasband, W.S., ImageJ, U.S; RRID:SCR_003070).

Table 2. Primary antibodies used in Western blot analyses.

Protein ID	RRID	Molecular mass (KDa)	Source of antibody	Ref. nº.	Antibody dilution
β-actin	AB_47674	45	Sigma	A5316	1:1,000
CB_1 Receptor	AB_447623	52	Abcam	Ab23703	1:200
MAGL	AB_327809	35	Cayman	100035	1:100
mGluR5	AB_2571804	132	Frontier	GO47	1:200
Gαo	AB_2111641	40	Santa Cruz	sc-387	1:5,000
Gαi1	AB_2247692	41	Santa Cruz	sc-391	1:5,000
Gαi2	AB_2111472	41	Santa Cruz	sc-7276	1:1,000
Gαi3	AB_2279066	45	Santa Cruz	sc-262	1:50,000

Abbreviations: β-actin, beta actin; CB_1 Receptor, cannabinoid receptor type 1, brain; MAGL, monoacylglycerol lipase; mGluR5, glutamate receptor metabotropic 5; Gαo, Gαi-1, Gαi-2 and Gαi-3 are Gαi/o subunits subtypes.

4.8 [^{35}S] GTPγS BINDING ASSAYS

The [^{35}S] GTPγS binding assays were performed following the procedure described elsewhere (Barrondo and Sallés, 2009). Hippocampal membranes (P2 fraction; 25 µg protein) from sham and EtOH adult mice (n = 4) were thawed, and incubated at 30 ºC for 2 h in [^{35}S] GTPγS-incubation buffer (0.5 nM [^{35}S] GTPγS, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0,2 mM DTT, 50 µM GDP, and 50 mM TBS, pH 7.4). The CB_1 receptor agonist CP 55.940 (10^{-11} – 10^{-5} M, eight concentrations) was added to determine receptor-stimulated [^{35}S] GTPγS binding. Nonspecific binding was defined in the presence of 10 µM unlabelled GTPγS. Basal binding was assumed to be the specific [^{35}S] GTPγS binding in the

absence of agonist. The reactions were terminated by rapid vacuum and filtration through Whatman GF/C glass fibre filters and the remaining bound radioactivity was measured by liquid scintillation spectrophotometry.

For analysis of data from [^{35}S] GTPγS binding assays, individual CP 55.940 concentration-response curves were fitted by nonlinear regression to the four parameter Hill equation, which is the following: E = Basal + Emax-Basal/1 + 10 (LogEC50-Log [A])^{nH}. Where E denotes effect, log [A] the logarithm of the concentration of agonist, nH the midpoint slope, LogEC50 the logarithm of the midpoint location parameter, and Emax and basal the upper and lower asymptotes, respectively. When required, simultaneous model-fitting with parameter-sharing across datasets was performed using GraphPad Prism (GraphPad Prism 5, GraphPad Software Inc, San Diego, USA; RRID:SCR_002798).

4.9 MEASUREMENT OF ENDOGENOUS 2-AG AND ARACHIDONIC ACID BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

The determination of the endogenous 2-AG levels was carried out as described by Schulte *et al*. (2012) with minor modifications (García del Caño *et al*., 2015). Samples of hippocampus from sham and EtOH adult mice (n = 5) were stored at -80 ºC until extraction. Samples (25 mg wet weight) were weighed into borosilicate tubes containing 0.5 mL ice-cold 0.1 M formic acid and were homogenized with the aid of a 5 mm-steel ball using the Digital Sonifier (Model S250 Branson, USA) for 1 cycle of 10 seconds at 10% amplitude. Aliquots (50 µL) of the homogenate were placed into silanized microcentrifuge tubes containing ice-cold 0.1 M formic acid, and were spiked with 20 µL acetonitrile containing the internal standards [deuterated 2-AG-d5 (final concentration 100 nM), deuterated 1-AG-

d5 (final concentration 100 nM), and deuterated AA-d8 (final concentration 500 nM)] and with 10 µL of the appropriate concentration of 2-AG and AA in its natural form, to give a final volume of 500 µL. Ethylacetate/hexane (1,000 µL; 9:1, v/v) were added to extract the cortical homogenate, again with the aid of the Digital Sonifier for 1 cycle of 10 s at 10% amplitude. Then the tubes were centrifuged for 10 min at 10,000 g at 4 ºC, and the upper (organic) phase was removed, evaporated to dryness under a gentle stream of nitrogen at 37 ºC and re-dissolved in 500 µL acetonitrile.

Analysis was performed as previously described (Schulte *et al*., 2012; García del Caño *et al*., 2015) on a LC-MS/MS system based on Agilent technologies (Wilmington) consisting of a 6410 Triple Quad mass spectrometer equipped with an electrospray ionization source operating in positive ion mode, and a 1200-series binary pump system. 2-AG and AA were separated with a Phenomenex Luna 2.5 µm C18(2)-HST column, 100 x 2 mm, combined with a Security Guard pre-column (C18, 4x2 mm; Phenomenex) with solvents A (0.1% formic acid in 20:80 acetonitrile/water, v/v) and B (0.1% formic acid in acetonitrile), using the following gradient: 55-90% B (0-2 min), then held at 90% B (2-7.5 min) and re-equilibrated at 55% B (7.5-10 min). The column temperature was 25 ºC, the flow rate was 0.3 mL/min, the injection volume was 10 µL and the needle was rinsed for 60 s using a flushport with Water/Acetonitrile (80:20) as the eluent. The electrospray ionization interface was operated using nitrogen as a nebulizer and desolvation gas, and using the following settings: temperature 350 ºC, nebulizer pressure 40 psi, and capillary voltage + 4800 V. The following precursor-to-product ion transitions were used for multiple-reaction monitoring: 2-AG and 1-AG m/z 379.4→287; 2-AG-d5 and 1-AG-d5 m/z 384→287; AA-d8 and AA m/z 313→126 and 305→93, respectively. Dwell times were 20 milliseconds and the pause between multiple-reaction monitoring transitions was 5 ms. Data acquisition and

analysis were performed using Agilent Masshunter Quantitative Analysis software (Agilent, Santa Clara, CA, USA; RRID:SCR_015040).

4.10 BEHAVIORAL STUDIES

All behavioral experiments were performed in the last days of the withdrawal *(see Figure 7 for details)* period under the same light and temperature conditions. Adult male C57BL/6J mice were kept into a temperature-controlled (22 °C) behavioral room 1 h before each test and kept there under red light to acclimatize to this new environment before starting with each test. All behavioral tests were monitored by two blinded observers to the treatment who used at least one stopwatch. To remove olfactory cues, all apparatus and objects were cleaned with EtOH (70% v / v) and then rinsed with water between each animal tested.

4.10.1 Novel object recognition

Non-spatial recognition memory was assessed by novel object recognition (NOR) test (Rico-Barrio *et al.*, 2018) based on the spontaneous tendency of rodents to explore a novel object rather than a familiar one. This test was performed in a square-shape open field box made of non-transparent plexi-glass (dimensions: 40 cm length x 40 cm height x 40 cm width) under red 10 lux lighting conditions. On the first 2 days of the behavioral test (pnd 69-70) sham and EtOH adult mice (n = 13) were habituated to the apparatus and allowed to explore the empty arena for 10 min each day. On the third day, (pnd 71) an acquisition session was carried out. In this session two identical familiar objects were placed at an equal distance in two adjacent corners of the arena, at 7 cm from the walls. A mouse was placed in the middle of the square keeping the head opposite to both objects and allowed to investigate and explore them for 10 min. After 2 h, the mouse returned to the apparatus and test session was performed where one of two familiar well-known objects was replaced by a novel one *(see Figure 9A for more details)*. In this way, animals were allowed to freely

explore familiar and novel objects for 10 min. The time exploring each object (sniffed, whisked or looked at no more than 2 cm away) during acquisition and test sessions was manually recorded. Animals who did not reach in the acquisition phase a total exploration time of 20 s were excluded from the data analysis. Total exploration time and discrimination index (DI) during test session were calculated and represented. Discrimination index was calculated as DI = (TN − TF) / (TN + TF). Where TN indicates the time spent on novel object and TF the time spent on familiar object.

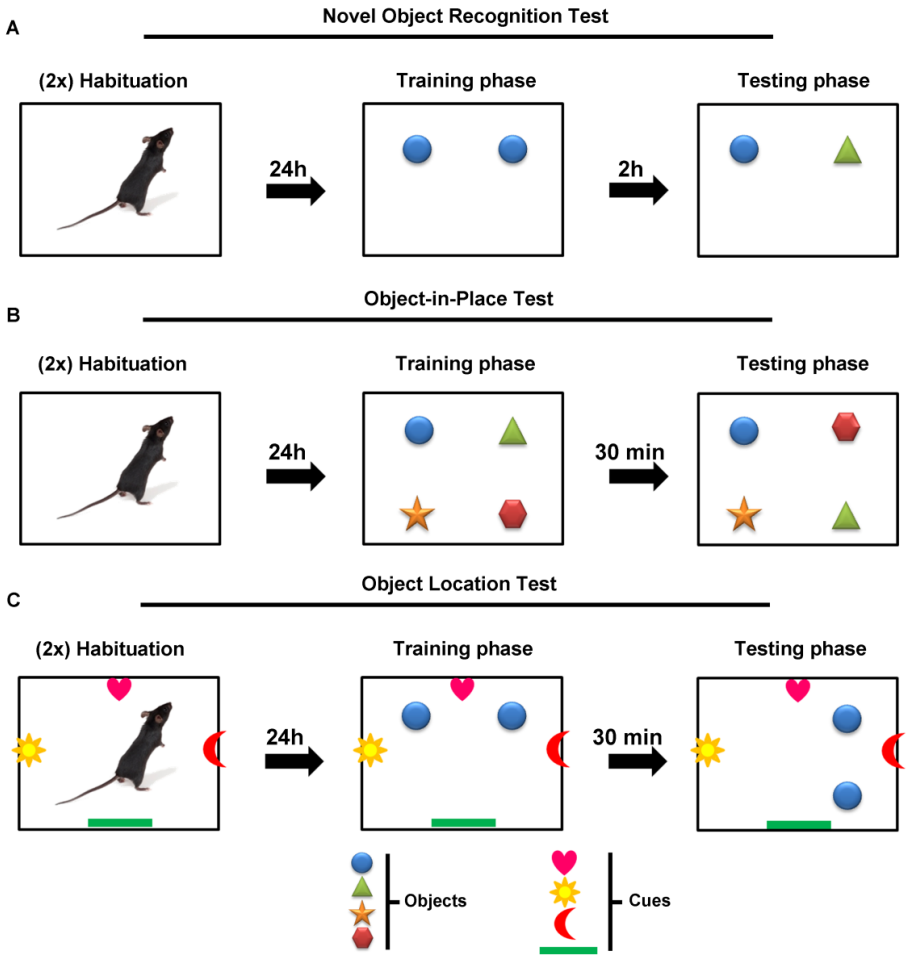

Figure 9. Schematic diagram illustrating the three memory tests assessed. **(A)** Novel object recognition test, **(B)** Object-in-place test and **(C)** Object location test. Objects and cues used in these behavioral tests.

- **MAGL inhibitor treatment combined with novel object-recognition test**

A total of 37 adult C57BL/6J mice (5-13 animals per experimental group) were treated subchronically with MAGL inhibitor (JZL184) or vehicle (8 mg/kg, intraperitoneally)

during 4 days before and the day of the test session (pnd 67-71) *(see Figure 9 for details)*. JZL184 or vehicle was injected 1 h before all sessions of the NOR test, time in which the animals kept resting in the behavior room. Then, short-term memory was tested with NOR 2 h after the training session *(see Figure 9A for more details)*. Animals who did not reach in the acquisition phase a total exploration time of 20 s were excluded from the data analysis. Total exploratory time and DI during testing phase were calculated and represented.

4.10.2 Object-in-place test

Associative recognition memory was analyzed by object-in-place (OiP) test in the same apparatus and conditions used for NOR test (Rico-Barrio *et al.*, 2018). On the first 2 days of the behavioral test (pnd 69-70) sham and EtOH adult mice (n = 12-10) had 2 days of habituation (10 min each day). In the acquisition phase (pnd 71), each mouse was placed in the center of the arena with one different object in each of the 4 corners at about 7 cm from the walls, and were allowed to explore them for 10 min. In the test phase 30 min later, two of the objects exchanged positions while the other two remained in the same location *(see Figure 9B for more details)*. Different combinations of the objects were considered in order to avoid place preferences. Animals not reaching a total exploration time of 20 s in the acquisition phase were excluded from the analysis. Total exploratory time and DI during test session were calculated and represented.

4.10.3 Object location test

Spatial recognition memory was assessed by the object location (OL) test in the same apparatus and conditions as for NOR and OiP tests (Rico-Barrio *et al.*, 2018). In this test, visual cues fixed on the walls were constantly visible from the arena to help spatial orientation of the mice *(see Figure 9C for more details)*. On the first 2 days of the behavioral test (pnd 69-70) sham and EtOH adult mice (n = 9-10) were habituated to the

apparatus and allowed to explore the empty arena with cues for 10 min each day. During the acquisition session on the third day (pnd 71), each animal was placed in the center of the quadrate and was allowed to explore for 10 min two identical parallel objects placed at 7 cm from the walls. The animals were then transferred to their home cages for 30 min. In the test session, one of the two identical objects was moved to a new location while the other object remained in the same position as in the acquisition phase. The mice were allowed for 10 min to freely investigate and explore the apparatus with one of the objects in a novel location *(see Figure 9C for more details)*. All combinations of the objects were considered to avoid preferences for a particular location. Mice that were not able to explore more than 20 s were excluded from the study. Total exploratory time and DI during test phase were calculated and represented.

4.10.4 Rotarod

Rotarod equipment (Panlab, Spain) is widely used to evaluate motor coordination of rodents. It consists on a horizontal rotating spindle *(see Figure 10)* with a padded surface under the apparatus. To achieve the necessary skills to perform the test properly, all animals were trained over 3 days before the test phase (Rico-Barrio *et al.*, 2018). The first 2 days (pnd 68-69) of the training phase, sham and EtOH adult mice (n = 11) were placed on the rotating rod (5 min at 30 min intervals, thrice a day) at a constant rotation speed of 4 rpm (pnd 68) and 20 rpm (pnd 69). They were put back on the rod each time a mouse fell off until the 5 min session was completed. On the third day (pnd 70), mice experienced a progressive speed increase from 4 rpm up to 40 rpm over a period of 5 min. They were trained for 3 sessions with 30 min-resting intervals. The final test was performed on the fourth day (pnd 71) with the same

Figure 10. Rotarod apparatus
(Picture c*ourtesy of German Mouse Clinic, Múnich)*

accelerating protocol as in the third day. The rotarod was controlled by an advanced microprocessor which provided precise timing control and accurate speed regulation. When an animal dropped onto the individual sensing platform underneath, the latency to fall and the falling speed were recorded digitally. To represent graphically, only two of the three measures recorded were taken into account.

4.10.5 Beam walking balance test

This test detects subtle balance deficits. The apparatus consists of a 120 cm-long beam supported by two pillars suspended 60 cm above foam pads and the home cage was placed at the end of the beam *(see Figure 11)* (Rico-Barrio *et al.*, 2018). It is based on the mouse´s ability to cross a graded series of beams. Two circular (2 cm- and 1 cm-diameter) wood beams were used. On the first day of training phase (pnd 69), sham and EtOH adult mice (n = 10) were trained to traverse the largest diameter beam for three consecutive times. On the second training day (pnd 70), they have to cross both the wide and the narrow beam each for three consecutive times. The test session was performed likewise the following day (pnd 71). The time taken to cross the wide and the narrow beam and the number of foot slips off was determined. Only two of the three measures obtained in each parameter were taken into account.

Figure 11. Beam Walking Balance apparatus.

4.10.6 Tail suspension test

The tail suspension test analyzes depressive-related behaviors in rodents by using a horizontally suspended solid metal bar *(See Figure 12)* (Rico-Barrio *et al.*, 2018). Sham and EtOH adult mice (n = 10-12 respectively) (pnd 71) were individually suspended (60 cm above a padded floor) by means of a tape wrapped around the tail (1 cm from the tail tip). Each mouse was tested for 6 min and the immobility time during the last 4 min was recorded. Immobility was considered when the animal was passively suspended in full motionless.

Figure 12. Tail suspension apparatus.

4.10.7 Light-dark box

The light-dark box test is one of the most useful tools to evaluate unconditioned anxiety in rodents. It is based on their spontaneous exploratory behavior in response to a novel environment and light. The light-dark box apparatus has an illuminated (40 Watios light lamp) open compartment and a dark cover compartment both connected by a restricted opening, so the mouse move freely between them *(See Figure 13)*. On the testing day (pnd 71), the mouse was placed in the dark box for 10 s and the gate remained open for 10 min. The percentage of time spent in the light compartment was manually recorded.

Figure 13. Light-Dark Box apparatus.
(Picture courtesy of Stoelting Co, USA)

4.10.8 Open field

Thigmotaxis refers to the tendency of rodents to avoid open areas remaining close to the walls (no more than 6 cm from them) during exploration. This parameter is used as a general measure of anxiety-related behavior (Rico-Barrio *et al.*, 2018). Sham and EtOH adult mice (pnd 69; n = 12) were individually taken from the home cage and placed for 5 min in the middle of a square (40 cm x 40 cm x 40 cm) opaque arena which was subdivided into a 30 cm-inner zone and a 10 cm-outer zone. Each animal was allowed to explore it freely and then was returned to the home cage. (i) The time spent exploring the outer zone (6 x 6 cm from the wall) and (ii) the time spent exploring the center of the apparatus (28 x 28 cm) was manually recorded.

4.11 STATISTICAL ANALYSIS

All values are given as mean ± S.E.M with *p* values and sample size (n). Shapiro-Wilk test and Kolmogorov-Smirnov was used to confirm normality of the data. Electrophysiological data was analyzed by using parametric or non-parametric two-tailed Student's t-test and two-way analysis of variance (ANOVA) to compare the effects of CB_1 agonist and LFS in sham and EtOH mice, comparing baseline and post-manipulation fEPSPs between the two groups. Subsequent post hoc analysis (Bonferroni post-test) was used when required. Electron microscopy data was analyzed by parametric or non-parametric two-tailed Student's t-test or one-way ANOVA with subsequent post hoc analysis (Bonferroni post-test) when compared the percentage of CB_1 receptor immunopositive excitatory terminals in sham, EtOH-treated and *CB_1*-KO mice. qRT-PCR, western blot, [^{35}S] GTPγS binding and LC-MS/MS assays were analyzed by parametric or non-parametric two-tailed Student's t-test, as required. Data obtained from NOR test was analyzed using two-way ANOVA with subsequent post hoc analysis (Bonferroni post-test) to evaluate the long term DID effect,

JZL184 treatment and the interaction between DID effect and JZL184 treatment. The Pearson correlation coefficient was used to analyze the relation between EtOH intake and BEC. The significance level was set at $p < .05$ for all comparisons. All statistical tests were performed with GraphPad Prism (GraphPad Prism 5, GraphPad Software Inc, San Diego, USA; RRID:SCR_002798).

4.12 DRUGS

All drugs used in the electrophysiological experiments were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and added at the final concentration to the superfusion medium (see Table 3 for drugs information).

JZL184 was administered intraperitoneally in a volume of 10 mL/Kg, dissolved in 15% DMSO (Sigma-Aldrich): 4.25% polyethylene glycol 400 (Sigma-Aldrich): 4.25% Tween-80 (Sigma-Aldrich): 76.5% saline.

2-AG and AA and their deuterated analogs 2-AG-d5 and AA-d8, used for LC/MS determinations, were obtained from Cayman Chemical Company.

Table 3. Drugs used in Electrophysiology recordings.

Drug*	Description	Concentration of use	Incubated Time	Supplier
Picrotoxin	$GABA_A$ receptor antagonist	[100 µM]	All recording	Tocris BioScience (Bristol, United Kingdom)
CP 55.940	Potent, non-selective cannabinoid receptor agonist	[10 µM]	All recording	
WIN 55.212-2 (Win-2)	Highly potent cannabinoid receptor agonist	[5 µM]	All recording	
AM251	Potent CB_1 antagonist; also GPR55 agonist	[4 µM]	All recording	
D-APV	Potent, selective NMDA antagonist; more active form of DL-AP5	[50 µM]	All recording	
3.5-DHPG	Selective group I mGluR agonist	[50 µM]	All recording	
MPEP	mGluR5 antagonist and positive allosteric modulator at mGluR4	[10 µM]	All recording	
CPCCoEt	Selective non-competitive mGluR1 receptor antagonist	[50 µM]	All recording	
U73122	Pospholipase C inhibitor	[5 µM]	1 h of additional pre-incubation	
URB 597	Potent and selective FAAH inhibitor	[2 µM]	20 min of additional pre-incubation	

JZL184	MAGL inhibitor	[50 µM]	1 h of additional pre-incubation	
AM404	AEA transport inhibitor	[30 µM]	All recording	
Nimodipine	Ca^{2+} channel blocker (L-type)	[1 µM]	All recording	
Thapsigargin	Potent inhibitor of SERCA ATPase	[2 µM]	1 h of additional pre-incubation	
RHC-80267	DAG inhibitor	[100 µM]	All recording	Santa Cruz Biotechnology Inc (Spain)
THL	Lipase Inhibitor	[10 µM]	All recording	

***** All drugs were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich).

5. RESULTS

5.1 CB_1 RECEPTOR-DEPENDENT EXCITATORY SYNAPTIC TRANSMISSION AND PLASTICITY AT MPP-GRANULE CELL SYNAPSES IN SHAM MICE

Exogenous CB_1 receptor activation by either CP 55.940 [10µM] or Win-2 [5µM] depressed excitatory synaptic transmission at MPP-granule cell synapses in sham mice as shown by Mann-Whitney test (*p < .05; ***p < .001 versus (*vs.*) baseline, respectively) (Figure 14*A, C*, CP 55.940: (n = 7) 16.97 ± 5.67% of inhibition; Win-2: (n = 6) 33.45 ± 7.53% of inhibition). This suppression was prevented by co-perfusion with the selective CB_1 receptor antagonist AM251 [4µM] (p > .05 *vs.* baseline) (Figure 14*B, C* (n = 4) 1.53 ± 12.15% of inhibition).

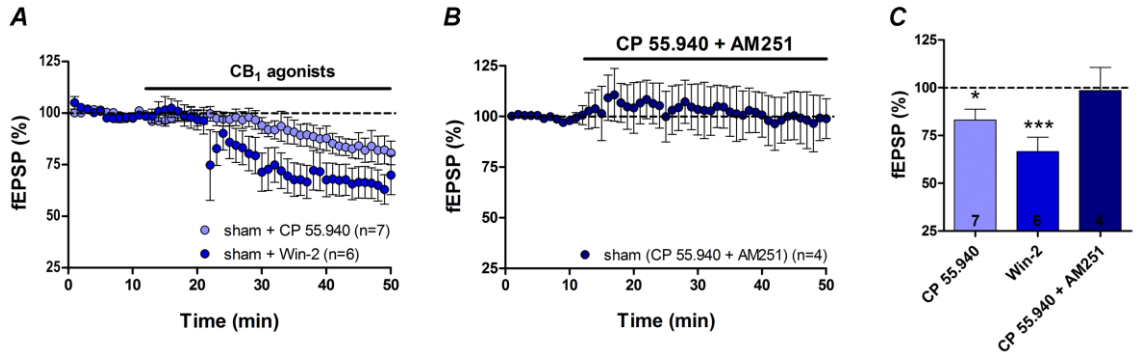

Figure 14. Endocannabinoid excitatory synaptic transmission at MPP synapses in sham mice. For representation, the experiments were normalized to its baseline. ***A,*** Time course plot of average fEPSP areas are represented. The CB_1 receptor agonist, CP 55.940 [10 µM] (light blue circles) and Win-2 [5 µM] (blue circles) reduces fEPSP. ***B,*** Simultaneous application of a selective CB_1 receptor antagonist (AM251) [4µM] and agonist (CP 55.940) [10 µM] (dark blue circles) blocks the synaptic depression observed in ***A***. Black horizontal bars on the top show the exposition time of the drugs. ***C,*** Summary bar histogram of the experiments performed: CP 55.940 [10 µM], Win-2 [5µM], CP 55.940 + AM251 cocktail [10 µM + 4 µM, respectively]. Baseline is represented by the dotted line. Numbers in the bars are individual experiments. Data are expressed as mean ± SEM. Mann Whitney test (*p < .05; ***p < .001; p > .05 versus (*vs.*) baseline, respectively).

On the other hand, LFS at 10 Hz for 10 min is known to induce CB_1-eLTD in other synapses (Chiu and Castillo, 2008). In our experiments, 10 Hz for 10 min triggered a novel CB_1-eLTD at MPP-granule cell synapses (**$p < .01$ *vs.* baseline), which was blocked by AM251 ($p > .05$ *vs.* baseline) (Figure 15*A*, *F* sham: (n = 20) 16.50 ± 5.75% of inhibition; AM251: (n = 8) -8.27 ± 6.26% of inhibition) but not by perfusion of the N-methyl-d-aspartate receptor (NMDA) antagonist D-APV [50µM] (*$p < .05$ *vs.* baseline) (Figure 15*A*, *F* (n = 9) 11.33 ± 4.19% of inhibition). The CB_1-eLTD was absent in global CB_1 receptor knockout (CB_1-KO) mice (Figure 15*B*, *F* CB_1-WT: (n = 5) 12.77 ± 5.75% of inhibition; CB_1-KO: (n = 8) -13.14 ± 4.81% of inhibition). In addition, the slight potentiation in the fEPSP (***$p < .001$ *vs.* baseline) was suppressed by D-APV ($p > .05$ *vs.* baseline) (Figure 15*B*, *F* CB_1-KO + D-APV: (n = 8) -1.74 ± 3.72% of inhibition). This novel CB_1-eLTD was accompanied by an increase in the paired pulse ratio (PPR) slope (*$p < .05$ *vs.* Pre-LFS) (Figure 15*C* (n = 10)), indicating the presynaptic locus of the CB_1-eLTD in agreement with the CB_1 receptor location in axon terminals. Noticeably, another low frequency stimulation protocol, 1Hz stimulation for 10 min also induced LTD in sham mice (***$p < .001$ *vs.* baseline) (Figure 15*D*, *F* (n = 5) 25.98 ± 4.08% of inhibition). Furthermore, the 10 Hz 10 min LFS did not induce CB_1-eLTD at mossy cell fiber (MCF) synapses (*$p < .05$ *vs.* baseline), as previously shown (Chiu and Castillo, 2008) (Figure 15*E*, *F* (n = 11) -11.8 ± 1.00% of inhibition) and D-APV blocked the small potentiation observed ($p > .05$ *vs.* baseline) (Figure 15*E*, *F* (n = 11) -3.1 ± 4.14% of inhibition). Altogether, these results demonstrate that LFS is able to induce a novel CB_1-eLTD at MPP granule cell synapses in untreated mice.

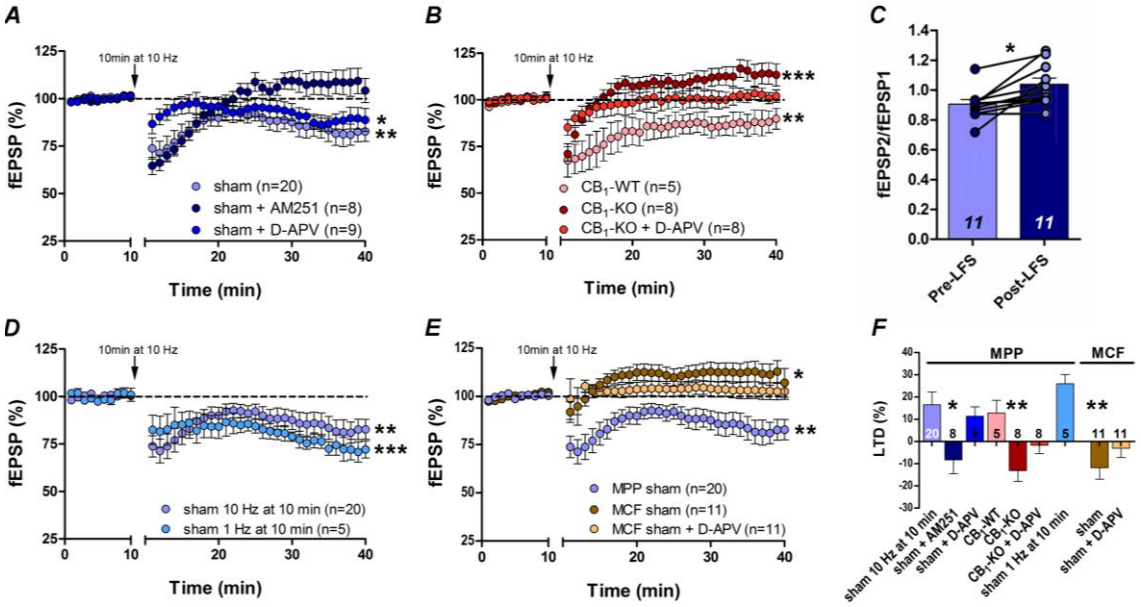

Figure 15. CB_1 receptor-dependent excitatory long-term depression (CB_1-eLTD) at medial perforant path (MPP) synapses in sham mice. For representation, each section of the experiment was normalized to its baseline before CB_1-eLTD induction at the time marked by the X-axis break. The average of the fEPSP areas is shown. ***A,*** Low frequency synaptic stimulation (LFS, 10 min, 10 Hz) triggers CB_1-eLTD at MPP in sham (light blue circles; Student's t test, two tailed, $t_{38} = 2.89$; $^{**}p < .01$ versus (*vs.)* baseline). AM251 [4µM] blocks CB_1-eLTD in sham (dark blue circles; Student's t test, two tailed, $t_{14} = 1.39$; $p > .05$ *vs.* baseline) and D-APV [50 µM] does not cause any change of CB_1-eLTD in sham (blue circles; Student's t test, two tailed, $t_{16} = 2.68$; $^{*}p < .05$ *vs.* baseline). ***B,*** CB_1-eLTD is induced in CB_1 receptor wild-type (*CB_1*-WT) littermate mice (light red circles; Mann Whitney test; $^{**}p < .01$ *vs.* baseline) but not in global CB_1 knock out (*CB_1*-KO*)* (dark red circles; Mann Whitney test; $^{***}p < .001$ *vs.* baseline). The slight but significant long-term potentiation (LTP) in *CB_1-KO* (dark red circles) was suppressed after application of the N-methyl-d-aspartate receptor (NMDA) antagonist D-APV (red circles; Mann Whitney test; $p > .05$ *vs.* baseline). ***C,*** Paired-pulse ratio (PPR) was calculated with slope of 30 sweeps i.e. 10 min before and 20 min after stimulation protocol. PPR augments after LFS. Student's t test, two tailed, $t_{20} = 2.63$; $^{*}p < .05$ *vs.* Pre-LFS. Numbers in the bars are individual experiments. ***D,*** LFS (10 min, 10 Hz) triggers CB_1-eLTD at MPP (light blue circles; Student's t test, two tailed, $t_{38} = 2.89$; $^{**}p < .01$ *vs.* baseline), and LFS (10 min, 1 Hz) also triggers CB_1-eLTD at MPP (blue circles; Student's t test, two tailed, $t_8 = 6.32$; $^{***}p < .001$ *vs.* baseline). ***E,*** Unlike the CB_1-eLTD observed in MPP of sham mice (light blue circles; Student's t test, two tailed, $t_{38} = 2.89$; $^{**}p < .01$ *vs.* baseline), LFS induces a slight LTP at Mossy Cell Fiber (MCF) (brown circles; Student's t test, two tailed, $t_{20} = 2.31$; $^{*}p < .05$ *vs.* baseline) which is absent under D-APV [50 µM] application (light brown circles; Student's t test, two tailed, $t_{20} = 0.73$; $p > .05$ *vs.* baseline). ***F,*** Summary bar histogram of the experiments performed: sham, sham + AM251 [4µM], sham + D-APV [50 µM], *CB_1*-WT, *CB_1*-KO and *CB_1*-KO + D-APV [50 µM] in MPP and, sham and sham + D-APV [50 µM] in MCF. Mann Whitney test ($p > .05$; $^{*}p < .05$; $^{**}p < .01$ *vs.* sham in MPP). Numbers in the bars are individual experiments. Data are expressed as mean ± SEM.

Finally, at more physiological conditions without picrotoxin (PTX), 10 min, 10 Hz LFS triggered long-term potentiation (LTP) (**p < .01 *vs.* baseline) (Figure 16*A*, *D* (n = 5) -49.79 ± 11.28% of inhibition) that was unaffected by D-APV [50 μM] (*p < .05 *vs.* baseline) (Figure 16*B*, *D* (n = 5) -34.41 ± 16.81% of inhibition) but blocked by AM251 [4μM] (p > .05 *vs.* baseline) (Figure 16*C*, *D* (n = 4) -2.26 ± 13.84% of inhibition) suggesting that CB_1 receptor-modulation of GABAergic transmission might be involved independently of NMDA receptors.

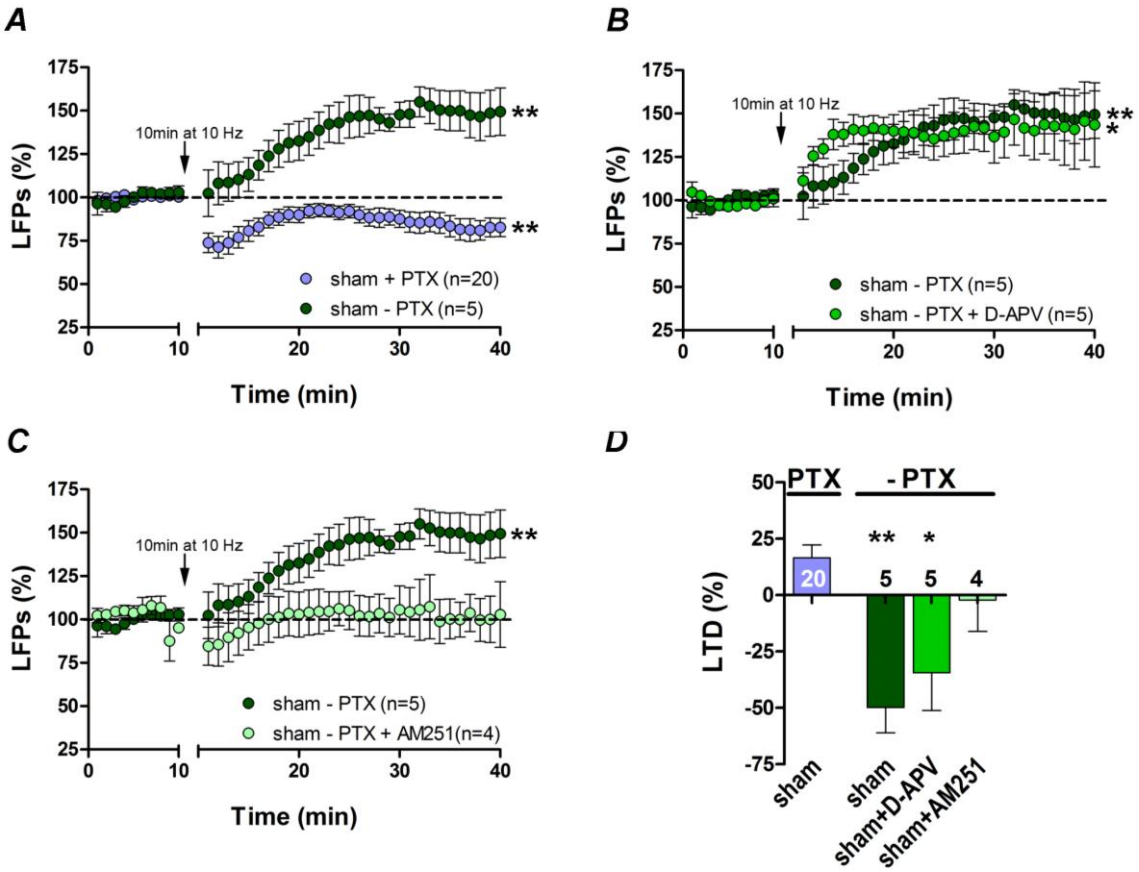

Figure 16. Endocannabinoid synaptic plasticity at physiological conditions in medial perforant path (MPP) synapses of sham mice. For representation, each section of the experiment was normalized to its baseline before LFS (10 min, 10 Hz) protocol at the time marked by the X-axis break. The average of the local field potentials (LFPs) areas is shown. ***A,*** As shown in figure 15, regular experiments with picrotoxin (PTX) [100 μM] trigger CB_1-eLTD in MPP after LFS (blue circles; Student's t test, two tailed, $t_{38} = 2.89$; ***p* < .01 versus (*vs.)* baseline); however, without PTX, LFS triggers a long-term potentiation (LTP) in the MPP (dark green circles; Mann Whitney test; ***p* < .01 *vs.* baseline). ***B,*** This LTP (dark green circles; Mann Whitney test; ***p* < .01 *vs.* baseline) is unaffected by D-APV [50 μM] application (green circles; Student's t test, two tailed, $t_8 = 2.08$; *p* > .05 *vs.* baseline). ***C,*** CB_1 receptor antagonist AM251 [4μM] blocks LTP (light green circles; Mann Whitney test; **p* < .05 *vs.* baseline). ***D,*** Summary bar histogram of the experiments performed with PTX [100 μM]: sham, and without PTX: sham, sham + D-APV [50 μM], sham + AM251 [4μM]. Mann Whitney test (*p* > .05; **p* < .05; ***p* < .01 *vs.* sham). Numbers in the bars are individual experiments. Data are expressed as mean ± SEM.

5.2 CB_1-eLTD MECHANISMS AT MPP-GRANULE CELL SYNAPSES IN SHAM MICE

5.2.1 Role of Group I mGluRs and intracellular Ca^{2+}

The group I metabotropic glutamate receptor (mGluR) agonist 3.5-DHPG [50 µM] significantly decreased fEPSP in sham mice (*p < .05 *vs.* baseline) (Figure 17*A* (n = 4) 26.68 ± 10.22% of inhibition). Conversely, 3.5-DHPG [50 µM] occluded subsequent CB_1-eLTD induced by LFS (p > .05 *vs.* baseline) (Figure 17*B, D* (n = 11) -4.8 ± 6.43% of inhibition). Indeed, the CB_1-eLTD was abolished by application of either the mGluR5 antagonist MPEP (p > .05 *vs.* baseline) (Figure 17*C, D* (n = 13) -4.8 ± 6.43% of inhibition) or the mGluR1 antagonist CPCCoEt (p > .05 *vs.* baseline) (Figure 17*C, D* (n = 10) -9.49 ± 6.70% of inhibition), indicating that group I mGluRs activation and CB_1-eLTD share common mechanisms. Furthermore, the L-type Ca^{2+} channel blocker, nimodipine [1 µM], was ineffective at blocking CB_1-eLTD of the fEPSP, suggesting that this calcium channel is not involved in the CB_1-eLTD induced by MPP stimulation (Figure 17*D*, (n = 8) 25.65 ± 10.20% of inhibition). However, thapsigargin [2 µM, >1 h], a sarco/endoplasmic reticulum Ca^{2+}-ATPase pump blocker, prevented CB_1-eLTD at the MPP synapses (Figure 17*D*, (n = 12) -17.88 ± 7.35% of inhibition). Altogether, these results indicate that activation of group I mGluRs, and release from intracellular Ca^{2+} stores are necessary for the induction of CB_1-eLTD at the MPP-granule cell synapses.

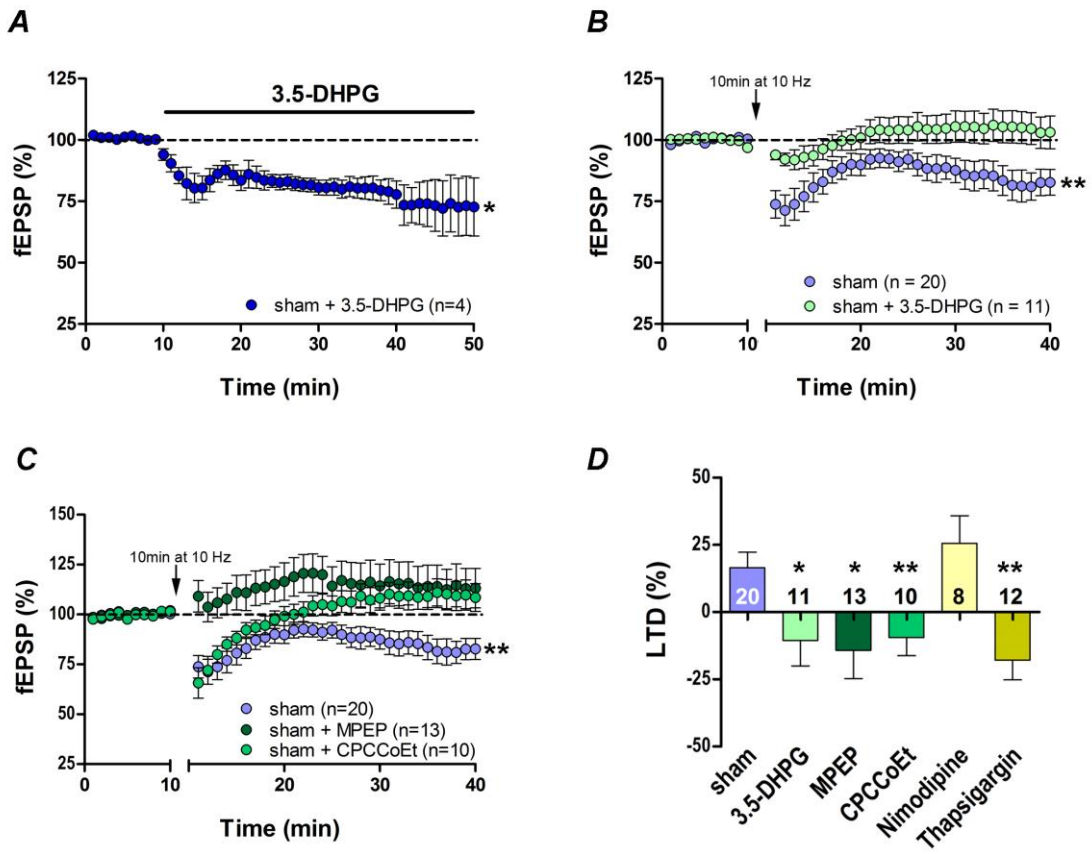

Figure 17. CB_1 receptor-dependent excitatory long-term depression (CB_1-eLTD) is mediated by group I mGluRs and rise of Ca^{2+} from intracellular stores in sham. For representation, the experiments were normalized to its baseline. The average of fEPSP areas is shown. ***A,*** The group I mGluR agonist, 3.5-DHPG [50 μM] reduces fEPSPs (dark blue circles; Mann Whitney test; **p* < .05 versus (*vs.*) baseline). Black horizontal bar on the top shows the exposition time of the drug. ***B,*** Co-application of 3.5-DHPG [50 μM] with LFS protocol (blue circles; Student's t test, two tailed, t_{20} = 0.74; *p* > .05 *vs.* baseline) prevents the CB_1-eLTD observed in MPP (light green circles; Student's t test, two tailed, t_{38} = 2.89; ***p* < .01 *vs.* baseline). ***C,*** MPEP [10 μm], the antagonist of mGluR5 (dark green circles; Mann Whitney test; *p* > .05 *vs.* baseline) and CPCCoEt [50 μM], the antagonist of mGluR1 (green circles; Mann Whitney test; *p* > .05 *vs.* baseline) block CB_1-eLTD (blue circles; Student's t test, two tailed, t_{38} = 2.89; ***p* < .01 *vs.* baseline). ***D,*** Summary bar histogram of the experiments performed: sham, MPEP [10 μM], CPCCoEt [50 μM], nimodipine [1 μM] and thapsigargin [2 μM, >1 h]. Numbers in the bars are individual experiments. Mann Whitney test; *p* > .05; **p* < .05; **p < .01 *vs.* sham. All data are expressed as mean ± SEM.

5.2.2 2-AG underlies the novel CB_1-LTD at MPP-synapses

The LFS stimulation was unable to elicit CB_1-eLTD at MPP synapses in the presence of the DAGL inhibitors THL [10 µM] or RHC-80267 [100 µM] ($p > .05$ *vs.* baseline) (Figure 18*A, C,* THL: (n = 7) -14.17 ± 7.31% of inhibition; RHC-80267: (n = 4) -11.12 ± 6.16% of inhibition). Also, LFS was unable to elicit CB_1-eLTD in the presence of the phospholipase C (PLC) inhibitor U73122 [5 µM, >1 h] (Figure 18*C,* (n = 6) - 18.56 ± 6.15% of inhibition). Thus, PLC activity is also required for the synthesis of 2-AG. Furthermore, the MAGL inhibitor, JZL184 [50 µM, >1 h], also blocked the CB_1-eLTD observed in sham mice after LFS (Figure 18*C,* (n = 12) -6.93 ± 3.54% of inhibition) suggesting that 2-AG degradation may be a limiting factor for CB_1-eLTD induction. By contrast, bath application of URB597 [2 µM, >20 min], a potent and selective inhibitor of FAAH, did not affect CB_1-eLTD (*$p <$ .05 *vs.* baseline) (Figure 18*B, C,* (n = 10) 18.14 ± 8.52% of inhibition) supporting the idea that AEA is not involved in the CB_1-eLTD at the MPP-granule cell synapses.

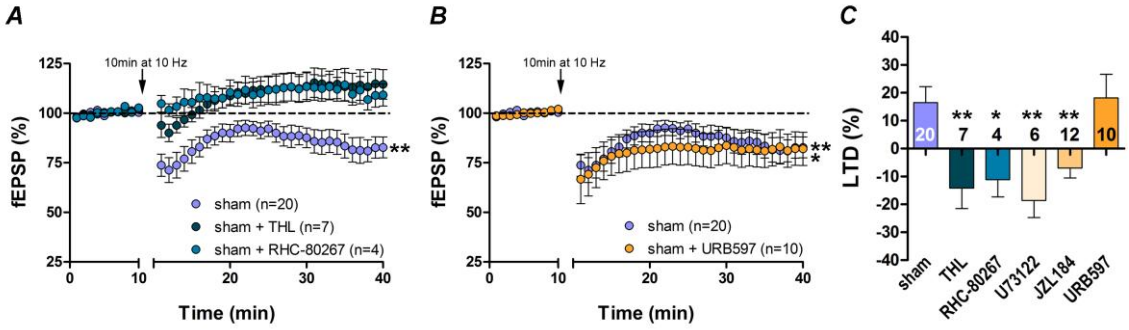

Figure 18. The 2-arachydonoyl-glycerol (2-AG) production is required to induce CB_1 receptor-dependent excitatory long-term depression (CB_1-eLTD) at MPP synapses in sham. ***A,*** DAGL inhibitors (THL [10 µM] and RHC-80267 [100 µM]) block CB_1-eLTD (dark blue circles; Student's t test, two tailed, $t_{12} = 1.93$; $p > .05$ versus (*vs.*) baseline and blue circles; Mann Whitney test; $p > .05$ *vs.* baseline, respectively) in sham mice (light blue circles; Student's t test, two tailed, $t_{38} = 2.89$; $^{**}p < .01$ *vs.* baseline). ***B,*** The fatty acid amide hydrolase (FAAH) inhibitor URB597 [2 µM, >20 min] does not affect CB_1-eLTD (orange circles; Student's t test, two tailed, $t_{18} = 2.12$; $^{*}p < .05$ *vs.* baseline) observed in sham (blue circles; Student's t test, two tailed, $t_{38} = 2.89$; $^{**}p < .01$ *vs.* baseline). ***C,*** Summary bar histogram of the experiments performed: sham, THL [10 µM], RHC-80267 [100 µM], U73122 [5 µM, >1 h], JZL184 [50 µM, >1 h] and URB597 [2 µM, >20 min]. Numbers in the bars are individual experiments. Mann Whitney test; $p > .05$; $p < .05^{*}$; $p < 0.01^{**}$ *vs.* sham. All data are expressed as mean ± SEM.

5.3 VOLUNTARY ORAL ETHANOL CONSUMPTION AND BLOOD ETHANOL CONCENTRATION

To ensure that the following effects were the result of voluntary alcohol intake, the amount of alcohol ingested by animals throughout the treatment was measured (Figure 19*A*, (n = 30) 2.19 ± 0.10 g/Kg/h). In addition, a blood sample at the end of the 4-h session of the last week of treatment was analyzed and yielded an average of 62.67 ± 2.67 mg/dl (Figure 19*B*, (n = 12)). Indeed, a significant correlation between EtOH intake and BEC was observed (Figure 19*C*, (n = 12)).

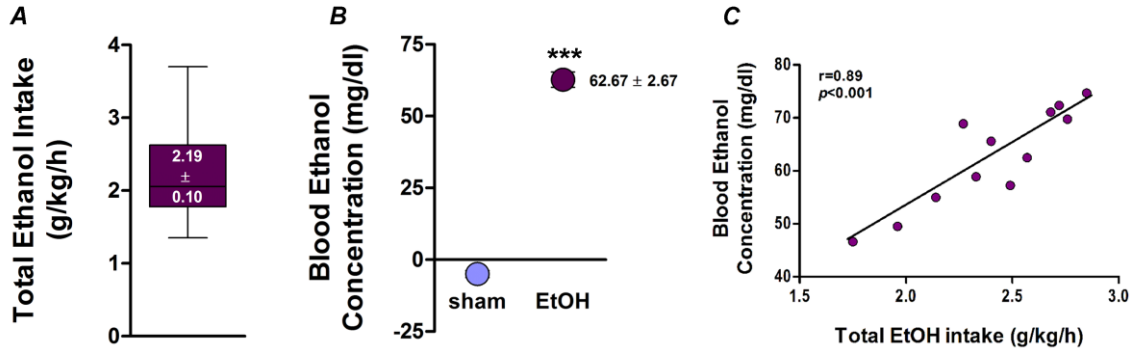

Figure 19. Voluntary oral ethanol (EtOH) consumption and Blood EtOH Concentration (BEC). ***A,*** Average of Total EtOH intake (g/kg/h) throughout adolescence period (Postnatal day, pnd 32 - 56). ***B,*** BEC (mg/dl) of C57BL/6J mice at the last day of EtOH treatment (pnd 56). Student's t test, two tailed, $t_{22} = 23.15$; ***$p < .0001$ *versus* sham. ***C,*** Correlation between Total EtOH Intake throughout adolescence period and BEC measured at the end of the EtOH access. ***$p < .001$. All data are expressed as mean ± SEM.

5.4 ADOLESCENT ETHANOL INTAKE IMPAIRS ADULT CB_1 RECEPTOR-MEDIATED EXCITATORY TRANSMISSION AND CB_1-eLTD AT MPP-GRANULE CELL SYNAPSES

The input–output relationships between fEPSPs slope relative to stimulus intensity in sham and EtOH-treated mice revealed significant differences (*p < .05 *vs.* sham) (Figure 20A) suggesting that adolescent EtOH consumption affects basal synaptic transmission in the adult. Besides, the CB_1 receptor-induced suppression of the fEPSP in sham was not observed in the EtOH group after withdrawal (p > .05 *vs.* baseline) (Figure 20*B, C* (n = 10) CP 55.940 [10 μM]: (n = 10) -0.34 ± 8.96% of inhibition; Win-2 [5 μM]: (n = 7) -4.67 ± 7.08% of inhibition). Furthermore, the CB_1-eLTD elicited by MPP stimulation (10 min, 10 Hz) was absent in EtOH-treated mice (p > .05 *vs.* baseline) (Figure 20*D, E*, (n = 16) -3.07 ± 2.77 of inhibition). These findings demonstrate that chronic exposure to EtOH during adolescence has long-term impacts on the CB_1-receptor-mediated excitatory synaptic transmission and CB_1-eLTD at the MPP-granule cell synapses in the mature brain.

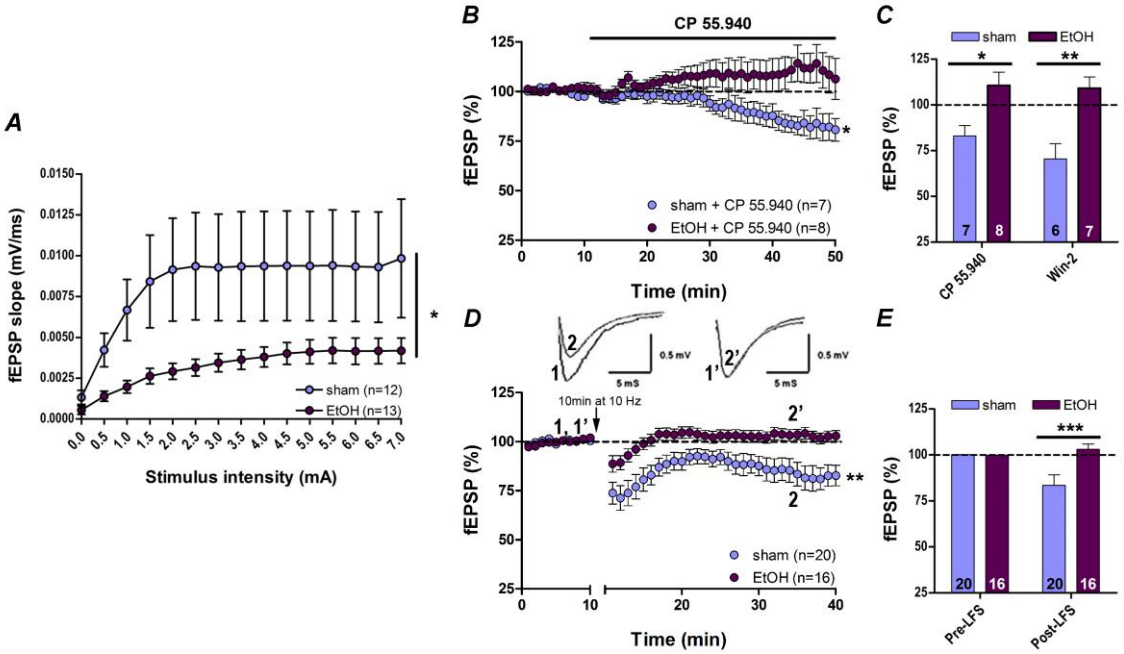

Figure 20. EtOH intake during adolescence impairs adult CB_1 receptor-mediated excitatory transmission and CB_1 receptor-dependent excitatory long-term depression (CB_1-eLTD) at medial perforant pathway (MPP) synapses. ***A,*** Input-output curves where mean fEPSP slopes (mv/ms) are plotted against the stimulation intensities in hippocampal slices of sham (blue circles) and EtOH (purple circles). To analyze these data the area under the curve of each condition was calculated. Mann Whitney test; $*p < 0.05$ versus (*vs.*) sham. ***B,*** Time course plot of average of fEPSP areas are represented. CP 55.940 [10 µM] reduces fEPSPs in sham (blue circles; Student's t test, two tailed, $t_{12} = 2.98$; $*p < .05$ *vs.* baseline) but not in EtOH (purple circles; Mann Whitney test; $p > 0.05$ *vs.* baseline). Black horizontal bar on the top shows the exposition time of the drug. ***C,*** Summary bar histogram of the transmission experiments: sham + CP 55.940 [10 µM], sham + Win-2 [5 µM], EtOH + CP 55.940 [10 µM], EtOH + Win-2 [5 µM]. Baseline is represented by the dotted line. Two-way ANOVA (overall EtOH-treatment effect: $F_{1,24} = 23.00$; $***p < .001$ and Bonferroni post-test $*p < .05$; $**p < .01$). Numbers in the bars are individual transmission experiments. ***D,*** Low frequency stimulation (LFS, 10 min, 10 Hz) triggers CB_1-eLTD in sham (blue circles; Student's t test, two tailed, $t_{38} = 2.89$; $**p < .01$ *vs.* baseline) but not in EtOH group (purple circles; Mann Whitney test; $p > 0.05$ *vs.* baseline). Above traces represent the average of 30 consecutive fEPSPs taken at the times indicated on the time-course graph. ***E,*** Summary bar histogram of CB_1-eLTD experiments performed: sham and EtOH. Mann Whitney test; $**p < 0.01$ *vs.* sham. Numbers in the bars are individual experiments. All data are expressed as mean ± SEM.

5.5 ADOLESCENT ETHANOL INTAKE INDUCES SIGNIFICANT CHANGES IN SOME ENDOCANNABINOID GENES AND PROTEINS IN THE MATURE HIPPOCAMPUS

The expression of both the CB_1 receptor gene, Cnr1 and its protein was significantly reduced after EtOH exposure during adolescence followed by 2 weeks of EtOH withdrawal (**p < .01; *p < .05 *vs.* sham, respectively) (Figure 21*A, B*). In contrast, a significant increase in the MAGL gene, Mgll and its protein relative to sham was detected (**p < .01; **p < .01 *vs.* sham, respectively) (Figure 21*C, D)*. In addition, mGluR5 mRNA was slightly but significantly decreased upon adolescent exposure to EtOH but no significant changes were observed in protein levels (*p < .05; p > .05 *vs.* sham, respectively) (Figure 21*E, F*). Furthermore, the Dagla and Daglb genes encoding for DAGL-α and DAGL-β enzymes, the 2-AG synthesizing enzymes, and Napepld and Faah genes encoding for the AEA synthesizing and degradation enzymes respectively, did not show any significant change as a result of the adolescent EtOH exposure (p > .05 *vs.* sham) (Figure 21*G-J*).

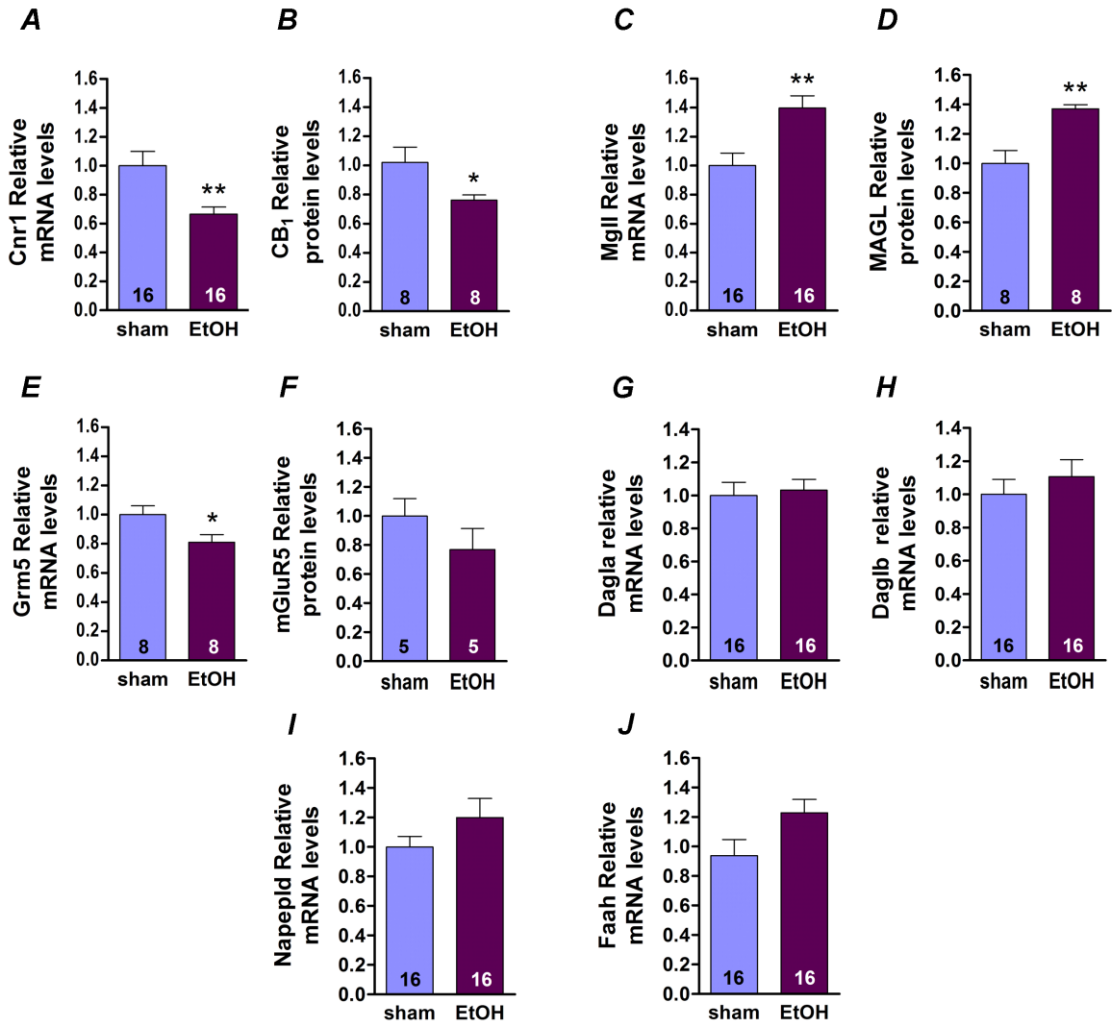

Figure 21. Molecular changes on endocannabinoid and glutamatergyc systems after EtOH intake during adolescence. ***A, B,*** Relative Cnr1 mRNA and CB_1 receptor protein levels in adult hippocampus (Hi) of sham and EtOH-treated mice during adolescence. Student's t test, two tailed, $t_{30} = 3.01$; $^{**}p < .01$ versus (*vs.*) sham and Student's t test, two tailed, $t_{14} = 2.34$; $^{*}p < .05$ *vs.* sham, respectively. ***C, D,*** Relative Mgll mRNA and MAGL protein levels in adult Hi of sham and EtOH-treated mice during adolescence. Student's t test, two tailed, $t_{30} = 3.30$; $^{**}p < .01$ *vs.* sham and Student's t test, two tailed, $t_{14} = 4.03$; $^{**}p < .01$ *vs.* sham, respectively. ***E, F,*** Relative Grm5 mRNA and mGluR5 protein levels in adult Hi of sham and EtOH-treated mice during adolescence. Student's t test, two tailed, $t_{14} = 2.35$; $^{*}p < .05$ *vs.* sham and Mann Whitney test; $p > .05$ *vs.* sham, respectively ***G, H,*** Relative mRNA levels of Dagla and Daglb in adult Hi of sham and EtOH-treated mice during adolescence. Student's t test, two tailed, $t_{29} = 0.31$; $p > .05$ *vs.* sham and Student's t test, two tailed, $t_{30} = 0.78$; $p > .05$ *vs.* sham, respectively. ***I, J,*** Relative Napepld and Faah mRNA levels in adult hippocampus of sham and EtOH-treated mice during adolescence. Student's t test, two tailed, $t_{29} = 1.32$; $p > .05$ *vs.* sham and Student's t test, two tailed, $t_{29} = 2.02$; $p > .05$ *vs.* sham, respectively. Numbers in the bars are the samples analyzed. All data are expressed as mean ± SEM.

5.6 ADOLESCENT EXPOSURE TO ETHANOL ALTERS ARACHIDONIC ACID BUT NOT 2-AG IN THE MATURE BRAIN

The endogenous 2-AG and AA were assessed by liquid chromatography and mass spectrometry. Basal 2-AG in sham (6.92 ± 0.42 nmol/g) and EtOH (6.65 ± 0.84 nmol/g) were not significantly different ($p > .05$ *vs.* sham) (Figure 22*A*). However, AA levels were significantly lower in sham (21.18 ± 1.79 nmol/g) than in EtOH-treated mice (76.30 ± 4.61 nmol/g) (**$p < .01$ *vs.* sham) (Figure 22*B*).

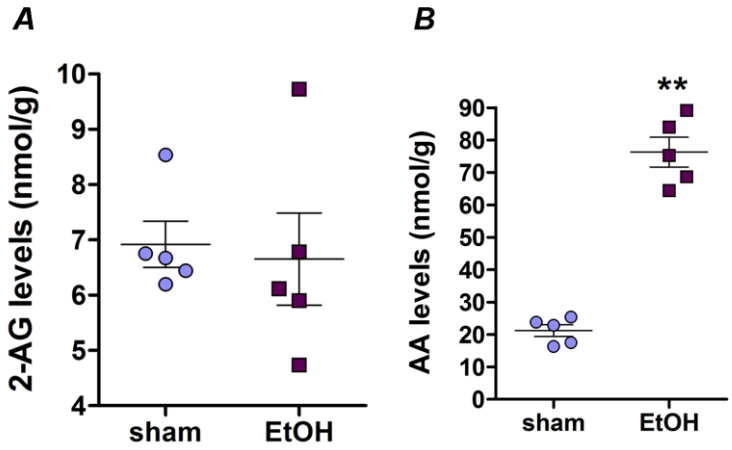

Figure 22. Measurement of 2-arachidonoyl-glycerol (2-AG) and arachidonic acid (AA) levels. ***A,*** 2-AG levels in individual P2 fractions from hippocampal brain samples of adult sham (n = 5, blue circles) and EtOH-treated mice (n = 5, purple squares) during adolescence. Mann Whitney test; $p > 0.05$ versus (*vs.)* sham. ***B,*** AA levels in individual P2 fractions from hippocampal brain samples of adult sham (n = 5, blue circles) and EtOH-treated mice (n = 5, purple squares) throughout adolescence. Mann Whitney test; **$p < .01$ *vs.* sham. All data are expressed as mean ± SEM.

5.7 SUBCELLULAR LOCALIZATION OF CB_1 RECEPTORS IN THE ADULT DENTATE MPP TERMINATION ZONE AFTER CHRONIC ETHANOL EXPOSURE DURING ADOLESCENCE

CB_1 receptor immunogold particles in the middle 1/3 of the dentate ML of sham and EtOH mice were mainly localized on inhibitory and excitatory axon terminals forming synapses with dendrites and dendritic spines, respectively (Figure 23*A-D*). The CB_1 receptor immunolabeling was absent in the global *CB_1*-KO mice (***$p < .001$ *vs.* sham) (Figure 23*E, G,* 2.83 ± 1.51%), demonstrating the specificity of the anti-CB_1 receptor antibody used.

To determine whether adolescent EtOH intake caused a global change in CB_1 receptor expression in the mature hippocampus the proportion of the total CB_1 receptor gold particle distribution was examined in excitatory terminals (14.68% ± 1.93% particles), inhibitory terminals (45.25% ± 3.97% particles), mitochondria (11.91% ± 1.13% particles), dendrites (11.84% ± 1.19% particles) and other membranes (16.32% ± 1.83% particles) of sham and EtOH-treated mice (excitatory terminals: 9.52% ± 0.93% particles (*$p < .05$ *vs.* sham); inhibitory terminals: 49.70% ± 5.08% particles ($p > .05$ *vs.* sham); mitochondria: 11.80% ± 1.38% particles ($p > .05$ *vs.* sham); dendrites: 12.84% ± 1.54% particles ($p > .05$ *vs.* sham); other membranes: 17.19% ± 2.08% particles ($p > .05$ *vs.* sham)) (Figure 23*F,* (n = 3)). In addition, the proportion of CB_1 receptor-labeled excitatory terminals dropped significantly after EtOH exposure (Figure 23*G,* (n = 3) 17.78% ± 1.95% in EtOH *vs.* 26.31% ± 2.93 in sham). Finally, no statistical differences were found in CB_1 receptor immunoparticle density (particles/µm) between excitatory boutons of sham (0.64 ± 0.03) and EtOH treated mice (0.58 ± 0.03) (Figure 23*H*).

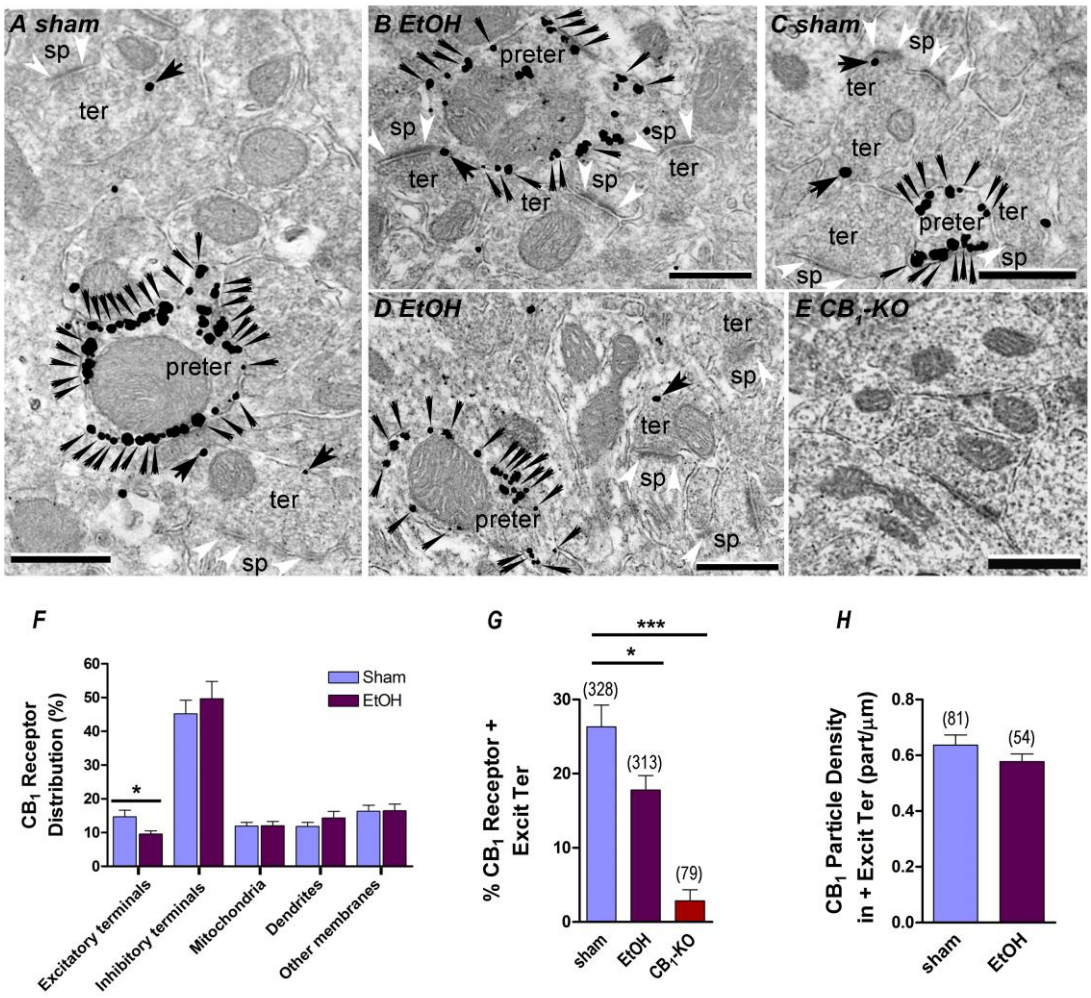

Figure 23. Ultrastructural location of CB_1 receptors in the middle 1/3 of the dentate molecular layer. ***A-D,*** CB_1 receptor immunogold labeling (black arrows) is observed on both excitatory terminals (ter) forming asymmetric synapses (white arrowheads) with dendritic spines (sp) and on inhibitory preterminals (preter) in sham and EtOH-exposed mice. Scale bars: 0.5µm. ***E,*** No CB_1 receptor immunolabeling is detected in global *CB_1*-KO mice. Scale bars: 0.5µm. ***F,*** Proportion of CB_1 receptor labeling in different compartments normalized to the total CB_1 receptor signal in sham and EtOH mice. Student's t test, two tailed, $t_{40} = 2.26$; $*p < .05$ for excitatory terminals and Student's t test, two tailed, $t_{40} = 0.70$, $t_{40} = 0.06$, $t_{40} = 0.52$ and $t_{40} = 0.32$ for the rest of compartments respectively. ***G,*** Percentage of CB_1 receptor-immunopositive excitatory synaptic terminals in sham, EtOH and CB_1-*KO* mice. One-way ANOVA ($F_{2,58} = 18.64$, $***p < .001$) and Bonferroni post hoc comparisons ($*p < .05$; $***p < .001$ *vs.* sham, respectively). The number of synaptic terminals analyzed is in parentheses on the top of each column. ***H,*** CB_1 receptor density (particles/µm) in CB_1 receptor positive excitatory terminals in sham and EtOH-treated mice. Mann Whitney test; $p > .05$. The number of synaptic terminals assessed is in parentheses on the top of each column. All data are expressed as mean ± SEM.

5.8 CP 55.940 STIMULATED [^{35}S] GTPγS BINDING ASSAYS

[^{35}S] GTPγS binding assays were performed with the CB_1 receptor agonist CP 55.940 in hippocampal membranes obtained from both sham and EtOH-treated mice. As shown in Figure 24*A*, CP 55.940 was able to stimulate [^{35}S] GTPγS binding in a concentration dependent manner in both cases without significant differences in efficacy (Emax) (Figure 24*A*, sham: (n = 4) 103.7 ± 4.2; EtOH: (n = 4) 95.3 ± 5.7). However, the potency of CP 55.940 stimulated [^{35}S] GTPγS binding was 3-4 fold higher in sham than in EtOH-treated mice (EC_{50}) (Figure 24*A*, sham: (n = 4) 45.7 ± 13.2 nM; EtOH: 148.5 ± 24.1 nM). Furthermore, a significant reduction (~18%) in [^{35}S] GTPγS basal binding was observed in hippocampal membranes of EtOH mice (inset of the figure 24*A*, sham: (n = 4) 99.9 ± 1.6; EtOH: 82.9 ± 2.1).

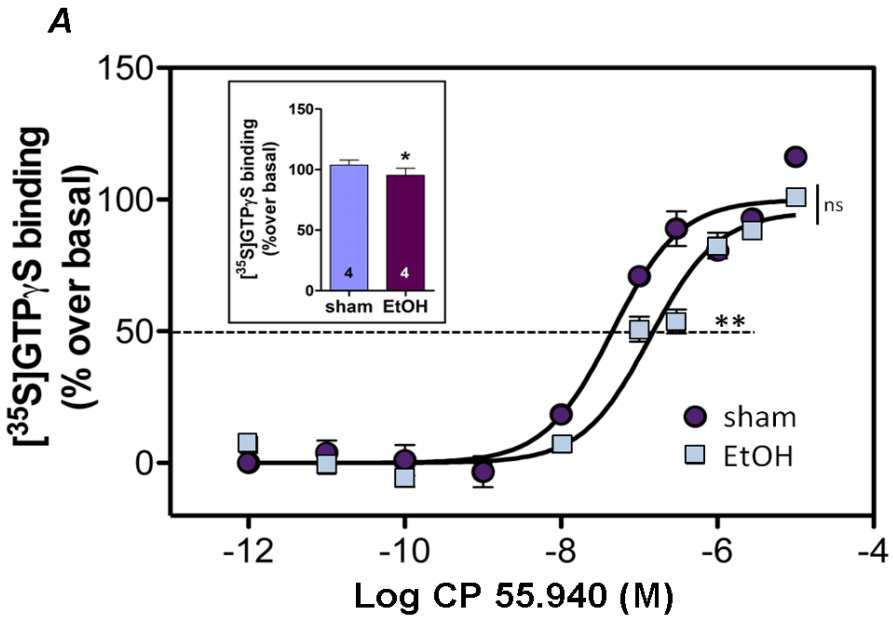

Figure 24. Effect of ethanol (EtOH) intake during adolescence on CB_1 receptor functionality. ***A,*** CP 55.940-stimulated [^{35}S]guanosine-5*-O-(3-thiotriphosphate) ([^{35}S] GTPγS) binding in hippocampal membranes from sham and EtOH-treated mice. Concentration curves were constructed using mean values ± SEM from four different experiments performed in duplicate. Mann Whitney test; $p > .05$, ns; $*p < .05$; $**p < .01$ versus (*vs.*) sham. Bar graphs in the inset depict the relative percentage of [^{35}S] GTPγS basal binding levels in sham and EtOH. Mann Whitney test; $*p < .05$ *vs.* sham. Numbers in the bars are the samples analyzed. Data in the inset are mean ± SEM.

5.9 EXPRESSION OF Gαi/o SUBUNIT IN HIPPOCAMPAL MEMBRANES FROM ADULT SHAM AND ETHANOL-TREATED MICE DURING ADOLESCENCE

In order to evaluate whether the changes observed in [^{35}S] GTPγS binding assays were related to any alteration in G-protein expression, the relative expression levels of different Gαi/o subunits were determined by western blotting. To this aim, increasing amounts of hippocampal membranes were loaded, and the linear relationship between the amount of protein and the relative optical density (OD) was established in the range of 2-16 μg for all the proteins evaluated. No differences in the Gαo, (n = 2), Gαi1 (n = 2) and Gαi3, (n = 3) subunits were found between sham and EtOH-treated mice (*p* > .05 *vs.* sham) (Figure 25*A-C)*. However, the Gαi2 subunit showed a small but significant (16%) decrease in hippocampal membranes of EtOH mice relative to sham (Figure 25*D,* (n = 3)).

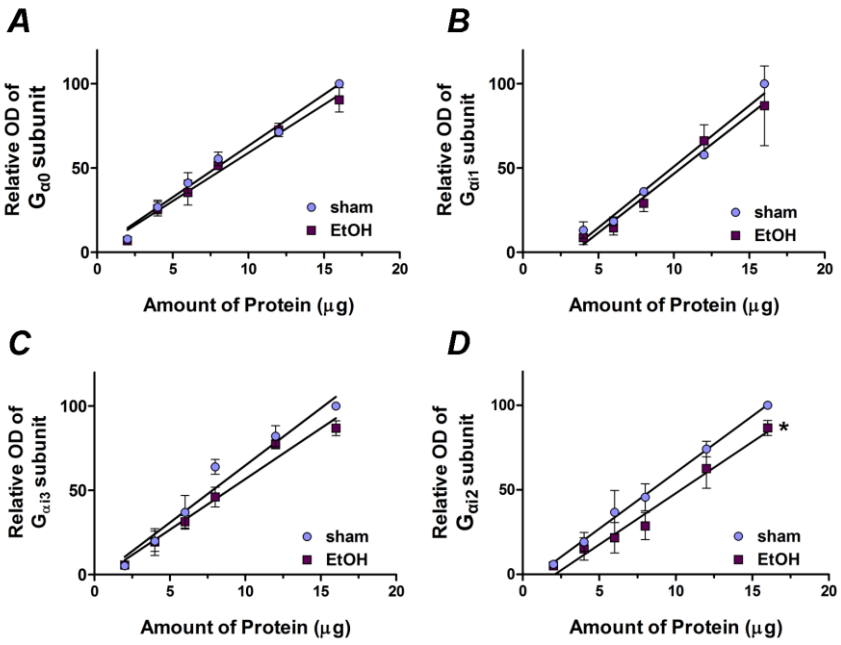

Figure 25. Regression analysis of *A,* Gαo (n = 2); ***B,*** Gαi-1 (n = 2); ***C,*** Gαi-2 (n = 3) and ***D,*** Gαi-3 (n = 3) G-protein subunits in hippocampal membrane samples from adult sham and EtOH-treated mice during adolescence. t test with Welch's correction; $p > .05$; *$p < .05$. All data are expressed as mean ± SEM.

5.10 2-AG ENHANCEMENT NORMALIZES CB_1-LTD IN ETHANOL-TREATED MICE

Bath application of JZL184 [50 µM, >1 h] rescued CB_1-eLTD in EtOH-treated mice (Figure 26*A, C,* (n = 14) 15.02 ± 4.61% of inhibition), indicating that the endogenous 2-AG tone is affecting CB_1-eLTD at MPP following EtOH exposure. Furthermore, the eLTD restored by JZL184 was CB_1 receptor dependent since AM251 [4 µM] blocked CB_1-eLTD (Figure 26*C,* (n = 8) -3.57 ± 6.37% of inhibition). However, URB597 [2 µM, 20 min] did not produce any change on the evoked fEPSP (Figure 26*B, C,* (n = 5) -2.86 ± 3.95% of inhibition). Also, the AEA transporter inhibitor, AM404 [30 µM] did not elicit CB_1-eLTD (Figure 26*C,* (n = 5) -14.75 ± 4.23%). These findings reveal that the pharmacological blockade of 2-AG degradation rescues CB_1-eLTD in adult MPP-granule cell synapses after adolescent EtOH exposure.

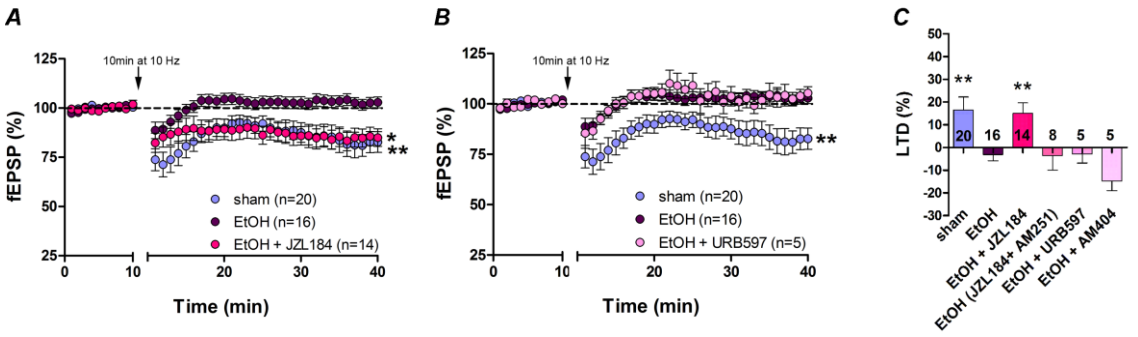

Figure 26. Enhancement of 2-AG signaling normalizes CB_1 receptor-dependent excitatory long-term depression (CB_1-eLTD) in ethanol (EtOH) mice. *A,* Time course plot of average fEPSP areas upon application of the low frequency stimulation (LFS, 10 min, 10 Hz) in sham (light blue circles: Student's t test, two tailed, $t_{38} = 2.89$; $^{**}p < .01$ versus (*vs.*) baseline), EtOH (dark purple circles; Mann Whitney test; $p > .05$ *vs.* baseline) and EtOH mice with Monoacylglicerol lipase (MAGL) inhibitor (JZL184, pink circles; Mann Whitney test; $^{*}p < .05$ *vs.* baseline). JZL184 recovers CB_1-eLTD in EtOH-treated mice. ***B,*** Time course plot of average fEPSP areas upon application of the LFS protocol in sham (light blue circles; Student's t test, two tailed, $t_{38} = 2.89$; $^{**}p < .01$ versus (*vs.*) baseline), EtOH (dark purple circles; Mann Whitney test; $p > .05$ *vs.* baseline) and EtOH mice with the fatty acid amide hydrolase (FAAH) inhibitor URB597 (light pink circles; Mann Whitney test; $p > 0.05$ *vs.* baseline). URB597 has no effect on the loss CB_1-eLTD after EtOH exposure. ***C,*** Summary bar histogram of the experiments performed: sham, EtOH, EtOH + JZL184 [50 µM, >1 h], EtOH + (JZL184 + AM251) cocktail [JZL184: 50 µM, >1 h; AM251: 4 µM, >30 min], EtOH + URB 597 [2 µM, >20 min] and EtOH + AM404 [30 µM]. Mann Whitney test; $^{**}p < .01$; $p > .05$ vs. EtOH. Numbers in the bars are individual experiments. All data are expressed as mean ± SEM.

5.11 BEHAVIORAL TESTING

5.11.1 Memory evaluation

Adult mice exposed to EtOH during adolescence showed a statistically lower short-term recognition, spatial and associative memory. In particular, a significant decrease in the DI in the NOR (***p < .001 *vs.* sham) (Figure 27*A*, sham: (n = 13) 0.45 ± 0.05; EtOH: (n = 13) 0.03 ± 0.03), OiP (***p < .001 *vs.* sham) (Figure 27*C*, sham: (n = 12) 0.30 ± 0.04; EtOH: (n = 10) 0.003 ± 0.035) and OL tests (**p < .01 *vs.* sham) (Figure 27*E*, sham: (n = 9) 0.17 ± 0.04; EtOH: (n = 10) -0.01 ± 0.04) was observed in EtOH compared to sham adult mice. However, their total exploration time between the familiar and the new object or location was similar between sham and EtOH groups in all tasks: NOR (Figure 27*B*, sham: (n = 13) 34.78 ± 4.01; EtOH: (n = 13) 31.14 ± 3.87), OiP (Figure 27*D*, sham: (n = 12) 75.71 ± 5.50 and EtOH: (n = 10) 64.90 ± 9.45) and OL test (Figure 27*F*, sham: (n = 9) 41.73 ± 4.16; EtOH: (n = 10) 31.10 ± 6.77). Taken together, all these results suggest that chronic consumption of EtOH during adolescence alters memory processes dependent, at least in part, on the hippocampal circuits.

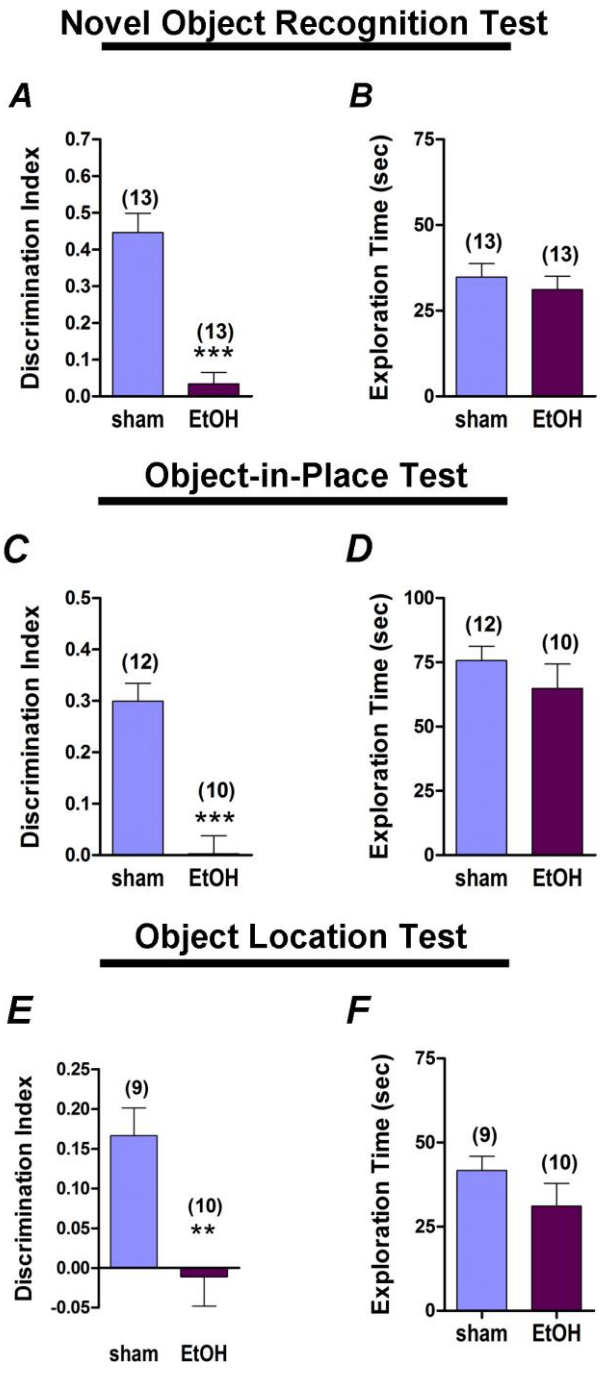

Figure 27. Ethanol (EtOH) intake during adolescence leads cognitive impairment on memory. ***A,*** Recognition memory was affected in adult mice after EtOH exposure during adolescence. Unpaired t test, ***$p < .001$ ***B,*** Object exploration time (sec) during test phase of NOR was unaffected between experimental groups. Student's t test, two tailed, $t_{25} = 2.36$; $p > .05$. ***C,*** Associative memory was altered in adult mice after EtOH exposure during adolescence. Unpaired t test, ***$p < .001$. ***D,*** Object exploration time (sec) during test phase of OiP test was unaffected between experimental groups. Student's t test, two tailed, $t_{20} = 1.03$; $p > .05$. ***E,*** Spatial memory was disrupted in adult mice after EtOH exposure during adolescence. Student's t test, two tailed, $t_{17} = 3.46$; **$p < .01$. ***F,*** Object exploration time (sec) during test phase of OL was unaffected between experimental groups. Student's t test, two tailed, $t_{17} = 1.30$; $p > .05$. The number of mice used in each test is in parentheses on the top of each column. All data are expressed as mean ± SEM.

- **JZL184 reverses cognitive impairment induced by EtOH treatment**

In the NOR test, adult mice treated with EtOH during adolescence showed a much lower short-term memory discrimination index than the sham, as we have shown above. However, systemic JZL184 administration (8 mg/kg ip) abolished the memory impairment associated with EtOH intake (Figure 28*A*, (n = 5-13)). Additionally, no differences in the total exploration time were observed among the experimental groups (Figure 28*B*, (n = 5-13)).

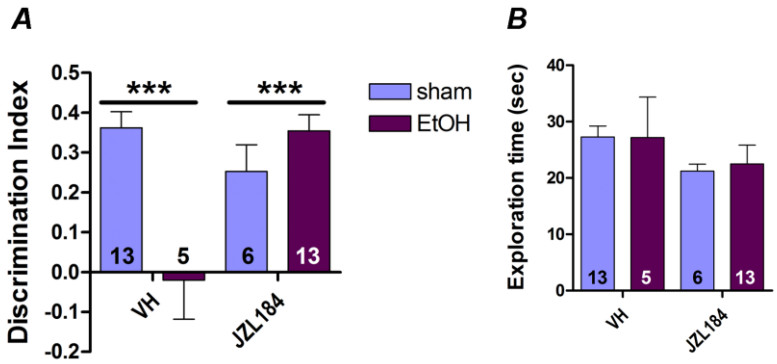

Figure 28. JZL184 reverses adult cognitive impairment after adolescent EtOH intake. ***A,*** Short-term memory was tested 2 h after the training session. Discrimination index of each experimental group in 10 min testing session of the novel object recognition test. Two-way ANOVA (EtOH treatment *versus* (*vs.*) JZL184 treatment interaction: $F_{1,33}$ = 16.75; ***p < .001 and Bonferroni post-test (***p < .001). ***B,*** Total exploration time (sec) of objects in the 10 min test session. Two-way ANOVA (EtOH treatment *vs.* JZL184 treatment interaction: $F_{1,33}$ = 0.03; p > .05. Numbers in the bars are individual experiments. Each bar represents the mean ± SEM.

5.11.2 Long-term effects on motor coordination and balance after chronic ethanol consumption during adolescence

Rotarod test showed a significant lower latency to fall off (*p < .05) (Figure 29*A*, sham: (n = 11) 127.4 ± 13.12; EtOH: (n = 11) 87.41 ± 12.65) and a lower rotating speed at falling (*p < .05) (Figure 29*B*, sham: (n = 11) 19.27 ± 1.61; EtOH: (n = 11) 14.27 ± 1.54) in mature mice after EtOH exposure during adolescence. On the other hand, mature EtOH mice spent more time to cross the narrow beam (*p < .05) (Figure 29*C*, sham: (n = 10) 22.77 ± 4.93; EtOH: (n = 10) 43.63 ± 5.71) and exhibited a higher number of foot slips (**p < .01) (Figure 29*D*, sham: (n = 10) 5.91 ± 0.32; EtOH: (n = 10) 8.61 ± 0.62) during walking balance test. However, no significant changes in time to cross the broad beam were detected (p > .05) (Figure 29*E*, sham: (n = 10) 12.14 ± 2.40; EtOH: (n = 10) 17.96 ± 2.37). These results show that adolescence BD leads to motor incoordination and imbalance both controlled by the cerebellum.

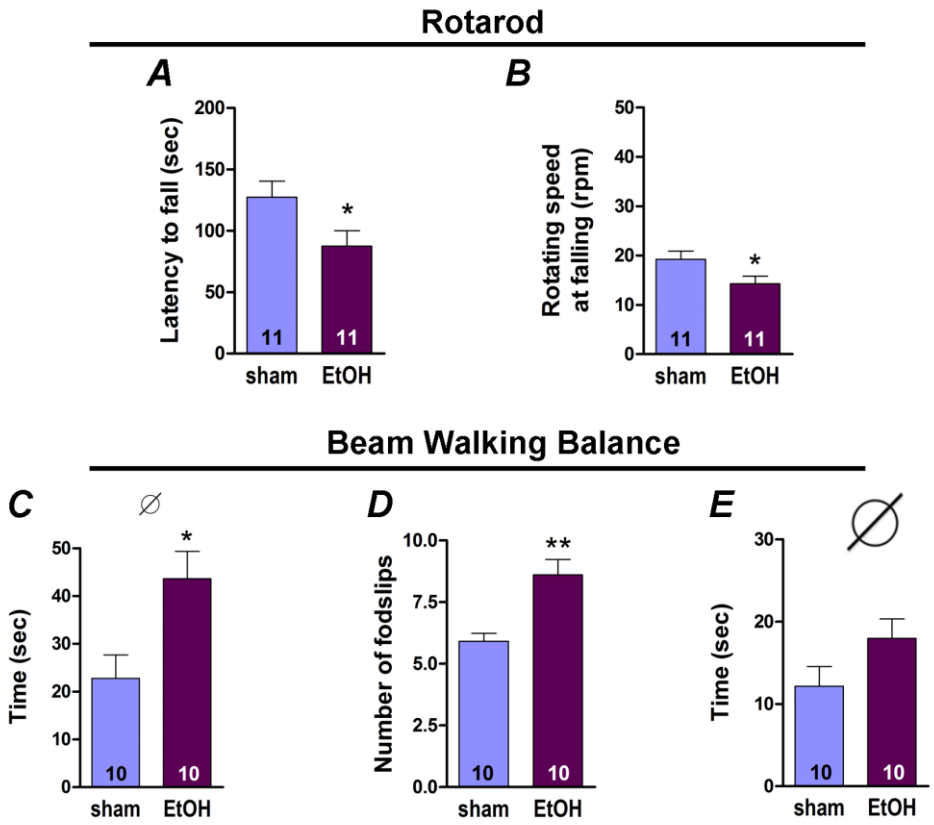

Figure 29. Ethanol (EtOH) intake during adolescence leads motor incoordination and imbalance in mature mice. ***A,*** Latency to fall (sec) in adult sham and EtOH mice during test phase of rotarod task. Student's t test, two tailed, $t_{20} = 2.19$; **p* < .05 versus *(vs.)* sham. ***B,*** Rotating speed at falling (rpm) during test phase of rotarod task in sham and EtOH mice. Student's t test, two tailed, $t_{20} = 2.24$; **p* < .05 *vs.* sham. ***C,*** Latency to traverse the narrow beam (sec) in sham and EtOH-treated mice during test phase of beam walking balance task. Mann Whitney test; **p* < .05 *vs.* sham. ***D,*** Number of foodslips in sham and EtOH mice during test phase of beam walking balance task. Mann Whitney test; ***p* <.01 *vs.* sham. ***E,*** Time spent to cross de broad beam in sham and EtOH-treated mice during training phase of beam walking balance task. Unpaired t test; *p* > .05 *vs.* sham. Numbers in the bars indicate the animals used in each behavioral test. All data are expressed as mean ± SEM.

5.11.3 Depressive and anxiety-like behaviors in adulthood after ethanol consumption throughout adolescence

Tail suspension test showed no significant changes in depressive-like behaviors in adult mice after chronic EtOH exposure during adolescence ($p > .05$) (Figure 30*A*, sham: (n = 10) 95.18 ± 12.90; EtOH: (n = 12) 119.7 ± 11.73). In addition, anxiety-like behavior was measured by open field and light dark box tests. The opend field task did not show any difference of the time spent in center ($p > .05$) (Figure 30*B*, sham: (n = 12) 105.2 ± 6.46; EtOH: (n = 12) 101.8 ± 11.53) and peripheral zone ($p > .05$) (Figure 30*B*, sham: (n = 12) 194.8 ± 6.46; EtOH: (n = 12) 198.2 ± 11.53) between experimental groups. Further, the light-dark box did not show statistically significant changes in any of the four parameters measured in adult mice after adolescence EtOH exposure ($p > .05$): time spent in light compartment (Figure 30*C*, sham: (n = 12) 222.9 ± 19.56; EtOH: (n = 12) 165.5 ± 22.59); time spent in dark compartment (Figure 30*C*, sham: (n = 12) 377.1 ± 19.56; EtOH: 434.5 ± 22.59); latency to enter the light compartment for the first time (Figure 30*D*, sham: (n = 12) 16.36 ± 3.34; EtOH: (n = 12) 14.45 ± 2.76) and number of transition events between compartments (Figure 30*E*, sham: (n = 12) 37 ± 2.42; EtOH: (n = 12) 30.36 ± 2.33).

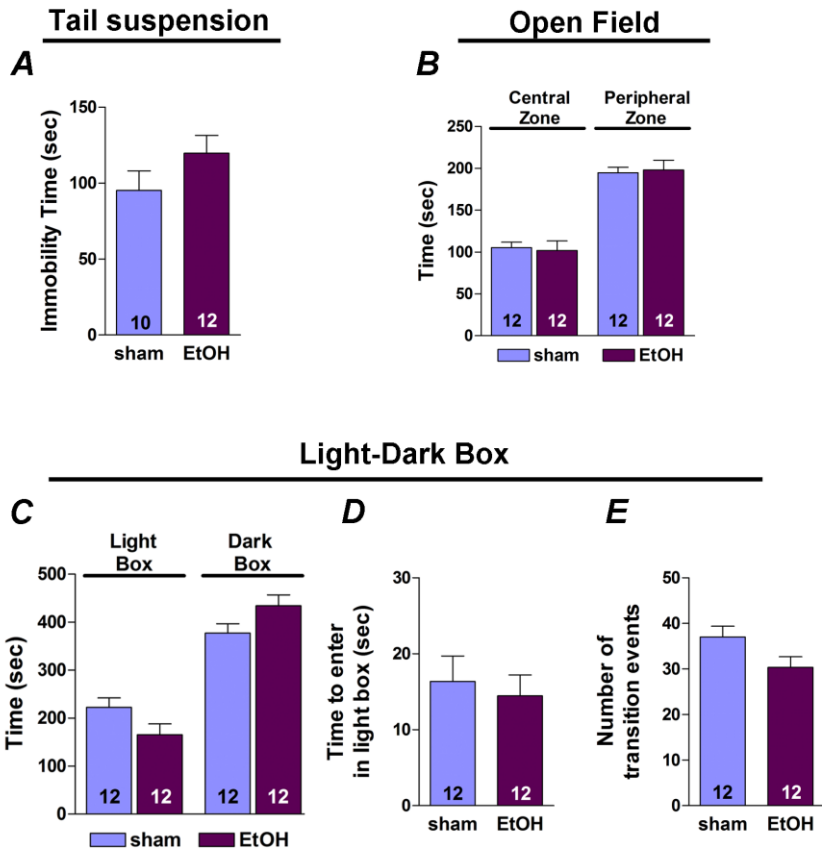

Figure 30. No persistent depressive- and anxiety-like behaviors are observed after chronic ethanol (EtOH) exposure during adolescence. ***A,*** Immobility time (sec) of adult sham and EtOH-treated mice during tail suspension trial. Student's t test, two tailed, $t_{20} = 1.41$; $p > .05$ versus (*vs.)* sham. ***B,*** Time spent in the center (sec) and peripheral (sec) zone of adult sham and EtOH mice during open field test. Mann Whitney test; $p > .05$ vs. sham and Student's t test, two tailed, $t_{22} = 0.26$; $p > .05$ *vs.* sham. ***C,*** Time spent in light box (sec) and dark box (sec) of sham and EtOH mice during light-dark box task. Student's t test, two tailed, $t_{22} = 1.92$; $p > .05$ *vs.* sham and Student's t test, two tailed, $t_{21} = 1.58$; $p > .05$ *vs.* sham, respectively. ***D,*** Time of both experimental groups to enter in light box (sec) during the light-dark box task. Student's t test, two tailed, $t_{22} = 0.44$; $p > .05$ *vs.* sham. ***E,*** Number of transitions events of sham and EtOH-treated mice during light-dark box task. Student's t test, two tailed, $t_{22} = 1.97$; $p > .05$ *vs.* sham. Numbers in the bars indicate the animals used in each behavioral test. All data are expressed as mean ± SEM.

6. DISCUSSION

The main findings of this Doctoral Thesis have shown that chronic EtOH intake during adolescence severely disrupts CB_1 receptor-mediated excitatory transmission and long-term depression of the excitatory synaptic transmission in adult MPP-granule cell synapses that results in recognition memory impairment. Moreover, the adolescent binge consumption also alters motor coordination and balance but not triggers depression or anxiety-like behaviors. Finally, both the loss of the CB_1-eLTD at MPP and the NOR memory impairment were reversed by the selective MAGL antagonist, JZL184.

The endocannabinoids play a crucial role in the induction of long-term synaptic plasticity in the brain (Chevaleyre and Castillo, 2004; Kreitzer and Malenka, 2005; Chiu and Castillo, 2008; Huang *et al.*, 2008; Yasuda *et al.*, 2008; Lafourcade and Alger, 2008; Carey *et al.*, 2011; Puente *et al.*, 2011; Cachope, 2012; Araque *et al.*, 2017). In addition, alterations of the eCB metabolism and signaling pathways during critical periods of brain development cause long-lasting behavioral abnormalities that can be observed into adulthood (Subbanna *et al.*, 2013, 2015). EtOH consumption alters eCB-dependent synaptic plasticity leading to long-term cognitive impairments (DePoy *et al.*, 2015; Crews *et al.*, 2016; Nimitvilai *et al.*, 2016; Lovinger, 2017; Bonilla-Del Río *et al.*, 2017; Marco *et al.*, 2017) and, reciprocally, the endocannabinoids play a pivotal role in the EtOH drinking behavior and in the development of alcoholism (Basavarajappa and Hungund, 2002; Lovinger, 2017).

6.1 MECHANISMS UNDERLYING A NOVEL CB_1-eLTD AT MPP-GRANULE CELL SYNAPSES

We found a CB_1 receptor-dependent inhibition of MPP-granule cell excitatory synaptic transmission, and a novel CB_1-eLTD induced by MPP LFS (10 min, 10 Hz) that has been previously used to consistently induce eCB-dependent LTD in other brain regions (Lafourcade *et al.*, 2007; Puente *et al.*, 2011). Other protocols of LFS (10 min, 1 Hz) known

to trigger LTD, also induced LTD in the MPP-granule cell synapses. We found that the magnitude of CB_1-eLTD was unaffected by the NMDA receptor antagonist D-APV suggesting that NMDA receptors were not involved in the CB_1-eLTD, despite the fact that CB_1-eLTD may require NMDA receptor activity at other synapses (Sjöström *et al*., 2003; Bender *et al*., 2006). However, the slight potentiation observed in *CB_1*-KO mice after LFS could be triggered by an increase in glutamate release and NMDA receptor activation (Errington *et al*., 1987) since the potentiation disappeared after bath perfusion of D-APV. Interestingly, the potentiation was not observed after EtOH consumption, suggesting that NMDA receptor signaling may also be impaired by this drinking pattern (Carpenter-Hyland *et al*., 2004, Carpenter-Hyland and Chandler, 2007). Consistent with a previous report (Chiu and Castillo, 2008), the 10 min 10 Hz protocol did not induce LTD at the excitatory mossy cell fiber synapses in the innermost 1/3 dentate ML highly expressing CB_1 receptors (Tsou *et al*., 1998; Katona *et al*., 2006; Kawamura *et al*., 2006; Monory *et al*., 2006), but rather a small D-APV-sensitive LTP.

Another relevant finding was that the LFS used to induce CB_1-LTD (Lafourcade *et al*., 2007; Puente *et al*., 2011) triggers a CB_1 receptor-dependent, but NMDA receptor-independent, LTP. These results indicate that the eCB synthesis elicited by LFS enhances excitability probably due to a predominant inhibition of GABA release over glutamate release. These results are in agreement with previous studies showing that both 2-AG and CB_1 receptor signaling are required for LTP at the lateral perforant path synapses (Wang *et al*., 2016). Also, CA1 LTP was facilitated by 2-AG and CB_1 receptor signaling (Silva-Cruz *et al*., 2017). Moreover, Chevaleyre and Castillo (2003; 2004) suggested that the eCB-mediated I-LTD (LTD at inhibitory synapses) underlie changes in CA1 pyramidal excitability and exert long-lasting modulatory effects on excitatory LTP. Altogether, these

previous studies and the present work indicate that neuronal excitability and long-term synaptic plasticity at excitatory synapses are critically dependent on the level of inhibition.

Mouse age (pnd 74-80), temperature of the in vitro experiments (32-35 ºC) and/or the stimulation paradigm could be critical factors for the novel CB_1-eLTD induction at the MPP-granule cell synapses in the DG. For instance, the eCB production by 3-sec postsynaptic depolarization of DG granule cells suppresses glutamatergic inputs in the innermost 1/3 dentate ML but not of the entorhinal-dentate pathway (Chiu and Castillo, 2008). Yet, postsynaptic transient receptor potential vanilloid 1 (TRPV1) activation at MPP-granule cell synapses suppresses excitatory transmission, and brief postsynaptic depolarizations (1 Hz) induce AEA-mediated TRPV1-LTD in a CB_1 receptor independent manner (Chávez *et al*., 2010). In fact, TRPV1 is highly concentrated in postsynaptic dendritic spines to asymmetric perforant path synapses in the outer 2/3 of the ML (Puente *et al*., 2015). Furthermore, TRPV1-LTD required mGluR5 activation, but not mGluR1, and involved postsynaptic α-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA) receptor internalization (Chávez *et al*., 2010). In our study, LFS of MPP inputs activated both mGluR1 and mGluR5 leading to an increase in intracellular Ca^{2+} released from the sarco/endoplasmic reticulum. The TRPV1-LTD induced by a similar LFS (10 min, 10 Hz) in the bed nucleus of the stria terminalis (BNST) was mediated by postsynaptic mGluR5-dependent release of AEA acting on postsynaptic TRPV1 receptors, and was strongly inhibited by depletion of intracellular Ca^{2+} stores (Puente *et al*., 2011).

We found that the 2-AG-dependent CB_1-eLTD at MPP synapses activates presynaptic CB_1 receptors distributed on excitatory synaptic terminals in the middle 1/3 of the dentate ML. In the BNST, however, dendritic L-type Ca^{2+} channels and the subsequent release of 2-AG acting on presynaptic CB_1 receptors triggered retrograde short-term depression (Puente *et al*., 2011). Hence, the eCB-LTD can be induced at either presynaptic or postsynaptic loci of

the BNST synapses depending on the stimulation paradigm, and that it recruits either presynaptic CB_1 receptors or postsynaptic TRPV1 activated by 2-AG or AEA, respectively (Puente *et al.*, 2011). Together, these findings further suggest that the precise subcelullar localization of the eCB components in specific cell types and synapses are key players for the induction of diverse forms of synaptic plasticity through distinct signaling mechanisms.

6.2 LONG-TERM EFFECTS OF ETHANOL INTAKE DURING ADOLESCENCE

The disruption of the adult CB_1 receptor-mediated excitatory transmission and CB_1-eLTD after adolescent EtOH intake is similar to previous findings (Guerri and Pascual, 2010; Adermark *et al.*, 2011; Renteria *et al.*, 2014, 2017). Furthermore, the absence of CB_1-eLTD was accompanied by a defect in recognition memory in adulthood. This could be explained by several mechanisms, such as reduction in neurogenesis (Anderson *et al.*, 2012; Vetreno and Crews, 2015), increase in neuroinflammation (Blanco and Guerri 2007; Pascual *et al.*, 2011) or increase in neurodegeneration (Obernier *et al.*, 2002). However, the impairments detected in the mature mouse after adolescent EtOH consumption seem to be correlated with the disturbance of cannabinoid signaling, as both the loss of excitatory synaptic plasticity and the NOR deficits were reversible by the selective MAGL antagonist. Moreover, the adolescent EtOH intake caused a significant decrease in the relative CB_1 receptor protein and mRNA, as previously shown (Basavarajappa *et al.*, 1998; Mitrirattanakul *et al.*, 2007; Rubio *et al.*, 2009).

We have recently demonstrated that the amount of CB_1 receptor immunoparticles in excitatory terminals in the hippocampal CA1 subregion was lower in EtOH-treated than in sham mice, in addition to a significant reduction in CB_1 receptor labeling in astrocytic processes (Bonilla-del Río *et al.*, 2017). In the present Doctoral Thesis, a ~35% decrease in

the CB_1 receptor particle distribution was found in excitatory terminals of the medial dentate ML and no changes in the CB_1 receptor distribution were detected in other cellular compartments. Furthermore, the CB_1 receptor immunopositive excitatory terminals decreased by 32% in EtOH-treated *vs.* sham. Hence, the reduction in CB_1 receptors in excitatory terminals could account for at least part of the deficits in the adult CB_1 receptor-dependent LTD after adolescent EtOH intake. However, whether there are also any glial cell-associated changes in CB_1 receptor expression in the medial dentate ML is unknown, as we have previously shown to occur in the CA1 hippocampus (Bonilla-Del Río *et al.*, 2017). Adolescent mice subjected to a 4-day model of BD had a 40% decrease in astroglial processes expressing CB_1 receptors and a 30% drop in receptor density in adult CA1 stratum radiatum astrocytes relative to sham (Bonilla-Del Río *et al.*, 2017). In addition, the proportion of total CB_1 receptor particles found on astrocytes in EtOH was much lower than in sham. Also, astrocytes were swollen in adult CA1 upon cessation of EtOH intake in adolescence (Bonilla-Del Río *et al.*, 2017). Because of the disrupted cell morphology, the astroglial CB_1 receptor expression was analyzed on a similar number of astroglial processes that were counted up in about 30% larger area in EtOH than in sham. Astrocytic swelling seems to be a phenomenon associated with EtOH consumption that leads to astroglial dysfunction (Adermark and Bowers, 2016) upon disruption of the glial fibrillary acidic protein found in the astrocyte intermediate filaments (Renau-Piqueras *et al.* 1989). Furthermore, long-term behavioral and cognitive impairments, synaptic plasticity disturbance, late alcohol abuse and addiction related to BD during the adolescence have been associated with neuroinflammatory mechanisms (Nestler 2001; Montesinos *et al.* 2016) as mentioned already (see below for further discussion).

Another possibility is that the function of CB_1 receptor signaling was affected during the adolescent EtOH intake. We have reported that EtOH treated mice did not show CB_1

receptor agonist-induced decrease in glutamate release as observed in sham mice, suggesting a reduced CB_1 receptor function in the MPP-granule cell synapses upon EtOH treatment, as previously shown in other brain regions (Pava and Woodward, 2012; Pava, 2014; Basavarajappa and Hungund, 1999; Mitrirattanakul et al., 2007; Vinod et al., 2006). CB_1 receptors signal in neurons through coupling to Gαi/o proteins (Kano *et al.* 2009) and mitochondrial CB_1 receptors have been shown to signal through Gαi proteins, as pertussis toxin blocks the decrease in mitochondrial cAMP, protein kinase A, complex I activity and respiration induced by cannabinoids (Hebert-Chatelain *et al.* 2016). Interestingly, no changes in the mitochondrial CB_1 receptors in adult upon adolescent intermittent EtOH intake were observed in our study. In astrocytes, there are pieces of evidence indicating that CB_1 receptors, in addition to Gαi/o proteins, also signal through Gαq proteins enabling astroglial CB_1 receptors to couple to different intracellular signaling pathways (Metna-Laurent and Marsicano, 2015). These biochemical differences might also have consequences on CB_1 receptor-binding proteins, like the G-protein-associated sorting protein 1 (GASP1) responsible for linking CB_1 receptors to degradation, or the cannabinoid receptor associated protein 1a (CRIP1a) involved in the CB_1 receptor function modulated by antagonists (Vinod *et al*. 2012).

CB_1 receptors located in glutamatergic synapses are more efficiently coupled to G protein signaling cascades (Steindel *et al.*, 2013); hence, the remaining CB_1 receptors at the MPP synapses could compensate for the CB_1 receptor reduction elicited by the adolescent EtOH consumption. We found a significant reduction in CP 55.940 potency for stimulating [^{35}S] GTPγS binding and [^{35}S] GTPγS basal binding that agrees with the decrease in CB_1 receptor binding (Basavarajappa *et al.*, 1998; Vinod *et al.*, 2006) and G-protein cycling after EtOH (Basavarajappa and Hungund, 1999). Furthermore, we also detected a specific reduction in Gαi2 subunit that may be responsible for the observed reduction in [^{35}S] GTPγS basal

binding and also for the impairment in CB_1 receptor signaling, which may be related to the absence of CB_1-eLTD and deficits in the NOR test in the EtOH-treated mice. Actually, a lack of Gαi2 subunit leads to abnormalities in learning efficiency, sociability and social recognition (Hamada *et al.*, 2017). As a compensatory mechanism, there was an increase in MAGL in our EtOH model as shown by others (Subbanna *et al.*, 2015), but no changes in the mRNA expression for the 2-AG biosynthetic enzymes were detected. Consequently, 2-AG levels would be expected to decrease in animals exposed to EtOH during adolescence. Curiously, there were no changes in 2-AG levels after withdrawal. However, a substantial increase in AA was found, suggesting a 2-AG increase during or after EtOH exposure (Basavarajappa *et al.*, 2000) that could eventually be normalized by further 2-AG degradation caused by the observed MAGL increase.

Adolescent EtOH impairs NOR memory after cessation of consumption, as previously shown (García-Moreno *et al.*, 2002; Farr *et al.*, 2005; García-Moreno and Cimadevilla, 2012) which may be due to its effects on hippocampal, parahippocampal and neocortical structures leading to a deficit in recognition memory formation (Tanimizu *et al.*, 2017), as discussed later. Interestingly, MAGL inhibition was able to overcome the functional and behavioral disturbances induced by EtOH, most likely due to the increase in 2-AG. Actually, pharmacological or genetic ablation of MAGL was shown to enhance long-term synaptic plasticity, improve cognitive performance through CB_1 receptor-mediated mechanisms, suppress neuroinflammation and prevent neurodegeneration after harmful insults (Long *et al.*, 2009; Chen *et al.*, 2012). Thus, upon agonist (2-AG)-induced stimulation of Gai/o subunits, inhibition of MAGL could overcome the loss of CB_1 receptors in glutamatergic terminals due to the high coupling efficiency of this CB_1 receptor population (Basavarajappa and Hungund, 1999), leading to functional (CB_1-eLTD) and behavioral (recognition memory) recovery in adult mice after EtOH treatment during

adolescence. As noted earlier, there is a growing body of literature demonstrating that adolescent EtOH exposure has more profound behavioral and neurobiological effects than similar treatments in adulthood (Beaudet *et al.*, 2016; Spear, 2016b; Wolstenholme *et al.*, 2017), however further research is needed to study whether the deficit in eCB plasticity and the NOR observed here are also observed if the EtOH intake occurs in adulthood.

Taken together, the increase in MAGL, the decrease in CB_1 receptors in excitatory terminals and their loss of efficacy could be underlying the loss of CB_1-eLTD at the MPP-granule cell synapses and the memory impairment observed in mature mice after EtOH exposure during adolescence (Figure 31). The present results can be taken into account for future investigations oriented to the search of new therapies to minimizing the potential consequences in adulthood of the irresponsible EtOH intake during early periods of life.

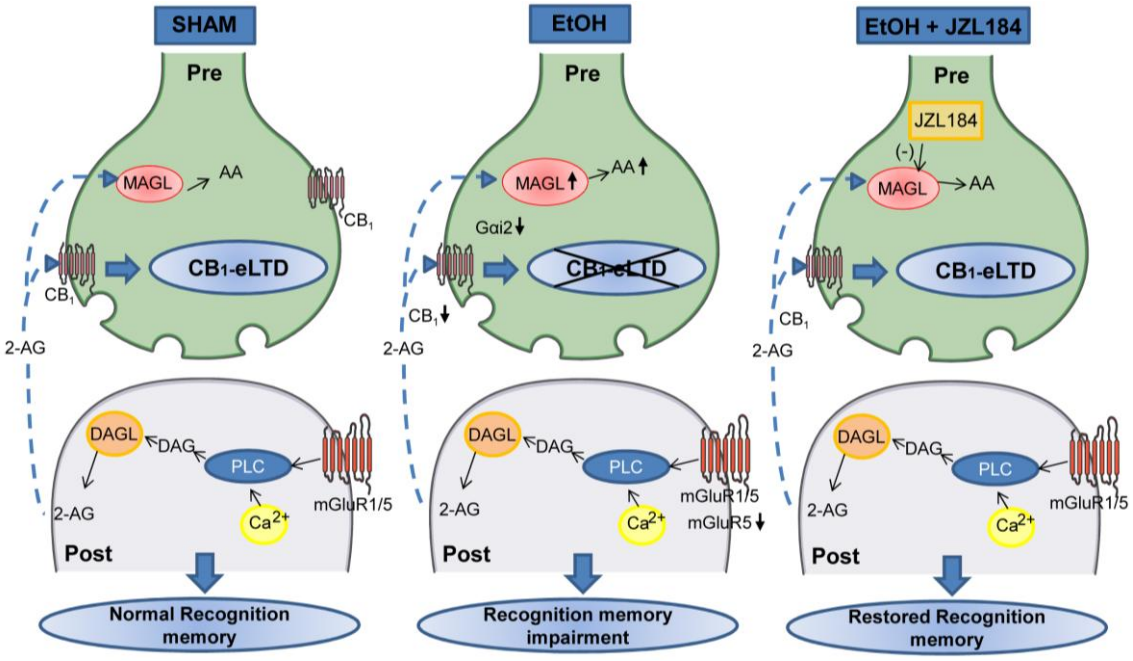

Figure 31. Schematic representation of the mechanisms involved in the novel CB_1-eLTD plasticity at MPP synapses, the long-lasting effects of EtOH exposure during adolescence on plasticity and behavior (recognition memory) and after treatment with a MAGL inhibitor. In sham mice, the activation of group I metabotropic glutamate receptors (mGluRs), phospolipase C and intracellular Ca^{2+} channels with the subsequent 2-AG production leads to the CB_1 receptor mediated LTD at excitatory synapses. Adolescent EtOH decreases in the adult hippocampus: CB_1 receptors in excitatory synaptic terminals and proportion of CB_1 receptor immunopositive

excitatory synaptic terminals in MPP termination zone (middle 1/3 of the dentate molecular layer); [^{35}S] GTPγS basal binding; Gαi2 subunit; mGluR5 mRNA. Adolescent EtOH increases in the adult hippocampus: MAGL mRNA and protein; arachidonic acid (AA). These changes abolish CB_1-eLTD and impair recognition memory. The MAGL inhibitor JZL184 recovers CB_1-eLTD and recognition memory. Figure design is adapted from Servier Medical Art.

6.3 LONG-TERM BEHAVIORAL EFFECTS OF ETHANOL INTAKE DURING ADOLESCENCE

The brain undergoes important structural and functional changes along the adolescent period that makes it more vulnerable to the deleterious effects of EtOH (Bonilla-Del Río *et al*., 2017) that can persist long after the end of EtOH consumption. The effects of adolescent EtOH intake on NOR memory have been widely studied (García-Moreno *et al*., 2002; Farr *et al*., 2005; García-Moreno and Cimadevilla, 2012; Swartzwelder *et al*., 2015; Beaudet *et al*., 2016; Sanchez-Marin *et al*., 2017). However, more studies were necessary to check the long-term impact of the adolescent pattern of EtOH intake on associative and spatial recognition memory.

The impairment in recognition, spatial and associative memory detected in early adulthood after chronic EtOH intake during adolescence in our study, is consistent with recent findings showing cognitive and behavioral deficits (Sanchez-Marin *et al*., 2017) as well as previous observations demonstrating that adolescent BD causes a decrease in hippocampal neurogenesis that persists into adulthood, altering brain plasticity and perturbing cognitive function (Pascual *et al*., 2007; Rodríguez-Arias *et al*., 2011; Vetreno and Crews, 2015). Newborn neuronal generation is directly related to hippocampal-dependent cognitive processes (Shors *et al*., 2001) and is highly sensitive to dysregulation by EtOH (Crews *et al*., 2006; Patten *et al*., 2016). In fact, adolescent rats subjected to intermittent exposure to alcohol exhibit a reduction in dentate neurogenesis lasting into adulthood (Vetreno and Crews, 2015). Also, white matter volume deficits, selective gray matter damage, changes in

neuroprogenitor proliferation (by Ki-67 immunopositivity) and caspase-3 expression in the dentate gyrus have been shown to be involved in the EtOH cognitive impairment (Crews *et al.*, 2016). All these alterations can culminate in reduced hippocampal volume and brain-derived neurotrophic factor (BDNF) in the adult hippocampus (De Bellis *et al.*, 2000; Sakharkar *et al.*, 2016). Interestingly, these evidences seem to be exclusively related to EtOH intake during adolescence because they were not observed after EtOH drinking in adulthood (Broadwater *et al.*, 2014).

In line with this, recent studies have demonstrated the importance of the immune system in the neuropathological consequences of adolescent EtOH. BD activates the inflammatory TLR4/NFκB signaling response in glial cells, which leads to the release of cytokines/chemokines and free radicals that correlates with neurophysiological, cognitive, and behavioral dysfunctions (Pascual *et al.*, 2018). Actually, the EtOH effect on hippocampal, parahippocampal and neocortical structures leading to a deficit in recognition memory formation (Tanimizu *et al.*, 2017) might be explained by an increase in neuroinflammation (Blanco and Guerri, 2007; Pascual *et al.*, 2011; see Crews and Vetreno, 2015). Astrocytes participate in the inflammatory response through their capacity to release pro-inflammatory molecules (Farina *et al*. 2007) that can be diminished by anti-inflammatory reactions mediated by endocannabinoids acting on astroglial CB_1 receptors (Metna-Laurent and Marsicano 2015). Hence, because of the drastic reduction in CB_1 receptors in adult astrocytes that we have recently demonstrated in the CA1 hippocampus (Bonilla-Del Río *et al.*, 2017), it is reasonable to expect an impairment of the astroglial anti-inflammatory reaction in response to adolescent EtOH intake. Furthermore, the altered astroglial morphology should affect the extracellular matrix components and the perineuronal nets sat between the astrocytes and the synapses, so impairing the homeostasis at the tripartite synapse. The supposedly resulting disturbance of neurotransmitter clearance

and gliotransmission may lead to deficits in synaptic plasticity (Dzyubenko *et al*. 2016) that ought to underlie the brain dysfunction observed after chronic EtOH consumption (Lovinger and Roberto 2013; Lovinger and Alvarez 2017; Pava and Woodward 2012). The astroglial glutamate aspartate transporter GLAST (EAAT1) appears to be up-regulated upon EtOH exposure (Rimondini *et al*. 2002) which should favor glutamate clearance from the synaptic cleft. However, this compensation seems not to be relevant for the EtOH effects, as mice lacking GLAST but equipped with functional presynaptic CB_1 receptors show less alcohol consumption, motivation, and reward (Karlsson *et al*. 2012).

Other possible mechanisms implicated in the recognition memory deficit by EtOH might be the increase in neurodegeneration (Obernier *et al.*, 2002; Broadwater *et al.*, 2014) or a reduction in neurogenesis (Anderson *et al.*, 2012; Broadwater *et al.*, 2014; Vetreno and Crews, 2015), that both persist into adulthood (Vetreno and Crews, 2015), leading to alterations in brain plasticity (Eisch and Harburg, 2006; Fontaine *et al.*, 2016) and cognitive functions (Nixon and Crews, 2002; Vetreno and Crews, 2015).

We have observed that chronic EtOH exposure in adolescence leads to long-term impairment of motor coordination and balance as shown in the rotarod and the beam walking balance test usually associated with cerebellar functions (Yamamoto *et al.*, 2003). These results are consistent with previous reports (Forbes *et al*., 2013) showing that early EtOH consumption alters cerebellar function (Lamont and Weber, 2012) indicating Purkinje cell vulnerability to EtOH (Sarna and Hawkes, 2003; Jaatinen and Rintala, 2008; Pierce *et al.*, 2011) that leads to loss of these cells (Forbes *et al*., 2013), cerebellar atrophy (Andersen, 2004; Jaatinen and Rintala, 2008) and motor deficits (Forbes *et al*., 2013). Also, a loss of prefrontal grey matter is correlated with motor, emotional and memory impairments in human alcoholics (West *et al*, 2018). Importantly, prefrontal development persists into adolescence and may be particularly vulnerable to EtOH-induced damage.

Significant differences in long-term anxiety and depressive-like behaviors between sham and EtOH groups were not found in our study probably due to the use of male mice in the experimental sampling. Evidences from human and animal studies suggest that the female brain is more affected by EtOH than the male brain (Marco *et al.*, 2017; West *et al*, 2018). Besides, females are at greater risk of EtOH-induced brain injury (Prendergast, 2004) and exhibited higher rates of anxiety and depression than males (Harris et al., 2017). Furthermore, longer EtOH withdrawal, like in our study, could lead to adaptations that may reduce the long-term anxiety and depression-like behaviors, since other investigations reported that EtOH-exposed mice have abnormal plasticity in amygdala and prefrontal cortex (Stephens and Duka, 2008; Kroener *et al*., 2012; Burgos-Robles *et al.*, 2013) as well as anxiety at shorter withdrawal periods (Sanchez-Marin *et al.*, 2017). Interestingly, exposure to an enriched environment yields a significant recovery of memory, motor coordination and balance impaired after adolescent EtOH drinking (Rico-Barrio *et al.*, 2018).

The long-lasting effects of the adolescent binge drinking on the CB_1 receptors localized in glutamatergic synapses, demonstrated in the present Doctoral Thesis, as well as on the astroglial CB_1 receptors and astroglial morphology shown in our previous study (Bonilla-Del Río *et al.*, 2017), suggest the existence of an architectural stumble of the neuron-astrocyte crosstalk at the tripartite synapse that has a severe impact on synaptic function and behavior in the adult brain. Lastly, the reciprocal interactions between the eCB system and the acute and chronic effects of EtOH have been taken as targets for treatment of alcohol addiction. Therefore, the changes in CB_1 receptors in glutamatergic neurons described in this Thesis and in astrocytes (Bonilla-Del Río *et al.*, 2017) together with the disturbance of the cannabinoid system in the hippocampus illustrated here,

might represent novel targets of interest to palliate the structural, functional and behavioral consequences of the adolescent bing drinking at later periods of life.

7. CONCLUSIONS

The conclusions of this Doctoral Thesis are the following:

1. Field excitatory postsynaptic potentials evoked by medial perforant path stimulation in the dentate molecular layer were inhibited upon CB_1 receptor activation in adult sham, but not in EtOH-exposed mice.
2. Low frequency stimulation (10 min, 10 Hz) of the medial perforant path triggered a novel CB_1 receptor-dependent long-term depression (CB_1-eLTD) at the excitatory medial perforant path-granule cell synapses that was absent in adult mice after adolescent EtOH consumption.
3. The CB_1-eLTD was group I metabotropic glutamate receptor (mGluR)-dependent, required intracellular calcium influx from the sarco/endoplasmic reticulum and 2-arachydonoyl-glycerol (2-AG) synthesis.
4. Adolescent EtOH intake significantly decreased CB_1 receptor mRNA and protein, reduced CB_1 receptor distribution and proportion of immunopositive excitatory synaptic terminals in the medial perforant path, decreased [^{35}S]guanosine-5*-O-(3-thiotriphosphate) ([^{35}S] GTPγS) basal binding and guanine nucleotide-binding (G) protein Gαi2 subunit, significantly increased monoacylglycerol lipase (MAGL) mRNA and protein and increased arachidonic acid, all in the adult hippocampus.
5. The absence of CB_1-eLTD in adulthood after adolescent EtOH consumption associated with impaired recognition, spatial and associative memory.
6. Adolescent EtOH intake caused persistent motor coordination and balance deficits, but not anxiety or depressive-like behaviors in adulthood.
7. Monoacylglycerol lipase inhibition recovered the CB_1 receptor-dependent eLTD and recognition memory in EtOH-treated mice.
8. Altogether, 2-AG recovers the long-term deficit in CB_1-eLTD and memory disturbance after repeated adolescent exposure to EtOH.

8. ABBREVIATIONS

- AA: Arachidonic acid.
- ABHD6: α/β-hydrolase domain containing 6.
- ABHD12: α/β-hydrolase domain containing 12.
- ACSF: Artificial cerebrospinal fluid.
- Actb: beta actin gene.
- AEA: Arachidonoyl-ethanolamine or anandamide.
- AMPA: α-amino-3-hydroxy-5-methyl-isoxazole propionic acid.
- AMT: Anandamide membrane transporter.
- ANOVA: Analysis of variance.
- AON: Anterior olfactory nucleus.

- BD: Binge drinking.
- BDNF: Brain-derived neurotrophic factor.
- BEC: Blood ethanol concentration.
- BNST: Bed nucleus of the stria terminalis.
- BSA: Bovine serum albumin.

- cAMP: Cyclic adenosine monophosphate.
- Cb: Cerebellar Cortex.
- CB_1-eLTD: CB_1 receptor-dependent excitatory long-term depression.
- CB_1: Type I Cannabinoid receptor.
- CB_2: Type II Cannabinoid receptor.
- CB_1-*KO*: Cannabinoid type-1 receptor knock-out mouse.
- CB_1-*WT*: Cannabinoid type-1 receptor wild type mouse.
- CCK: cholecystokinin.
- Cnr1: cannabinoid receptor type 1 gene.
- CNS: Central Nervous System.
- CPu: Caudate Putamen.
- CRIP1a: Cannabinoid receptor associated protein 1a.

- DAG: Diacylglycerol.
- DAGL: Diacylglycerol lipase.
- Dagla: Diacylglycerol lipase alpha gene.
- Daglb: Diacylglycerol lipase beta gene.
- DG: Dentate Gyrus.
- DI: Discrimination index.
- DID: Drinking in the dark.
- DMSO: Dimethyl sulfoxide.

- EC: Entorhinal Cortex.
- EC_{50}: Half maximal effective concentration
- eCB: endocannabinoid.
- Emax: Efficacy maximum.
- ERK: Extracellular signal–regulated kinase.
- EtOH: Ethyl alcohol or ethanol.

- FAAH: Fatty acid amide hydrolase.
- Faah: Fatty acid amide hydrolase gene.
- fEPSPs: Field excitatory postsynaptic potentials.

- GABA: Gamma-Aminobutyric acid.
- $GABA_A$: Type A Gamma-Aminobutyric acid.
- GABA-*CB_1*-KO: GABA-CB_1 knock-out mouse.
- GABA-*CB_1*-RS: GABA-CB_1 knock-out rescue mouse.
- GASP1: G-protein-associated sorting protein 1.
- GCL: Granule cell layer.
- GLAST: Glutamate aspartate transporter.
- Glu-*CB_1*-KO: Glutamatergic CB_1 knock-out mouse.
- Glu-*CB_1*-RS: Glutamatergic CB_1 knock-out rescue mouse.
- GPCRs: G-protein-coupled receptors.
- GPR55: G protein-coupled receptor 55.
- Grm5: glutamate receptor metabotropic 5.

- HF: Hippocampal Formation.
- Hi: Hippocampus.
- HRP: Horseradish peroxidase.

- I-LTD: Long term depression at inhibitory synapses.

- LC-MS/MS: Liquid chromatography tandem mass spectrometry.
- LFS: Low-frequency stimulation.
- LPP: Lateral perforant pathway.
- LTD: Long-Term Depression.
- LTP: Long-Term Potentiation.

- M1: Primary Motor Cortex.
- MAGL: Monoacylglicerol lipase.
- MAPK: Mitogen-activated protein kinase.
- MCF: Mossy Cell Fiber.
- Mgll: Monoacylglycerol lipase gene.
- mGluR: Group I metabotropic glutamate receptor.
- mGluR5: Metabotropic glutamate receptor 5.
- mGluR1: Metabotropic glutamate receptor 1.
- Mid: Midbrain.
- ML: Molecular layer.
- MO: Medulla Oblongata.
- MPP: Medial perforant pathway.

- NAAA: *N*-acylethanolamine cisteine-amidohydrolase.
- NAc: Nucleus Accumbens.
- NAPE: N-arachidonoyl phosphatidylethanolamine.
- NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D.
- Napepld: N-acyl phosphatidylethanolamine phospholipase D gene.
- NAT: N-acyltransferase.
- NMDA: N-methyl-d-aspartate receptor.
- NOR: Novel object recognition.

- OD: Optical density.
- OiP: Object-in-place.
- OL: Objct location.

- PB: Phospate buffer.
- PBS: Phosphate buffered saline.
- PKA: Protein kinase type A.
- PLC: Phospholipase C.
- Pnd: Postnatal day.
- Po: Pons.
- PPAR-α: Peroxisome Proliferator-Activated Receptors.
- PPR: Paired pulse ratio.
- Preter: Preterminal.
- PTX: Picrotoxin.

- REC: Recording electrode.
- RT: Room temperature.

- S1: Primary Somatosensory Cortex.
- SDS: Sodium dodecyl sulfate.
- SDS-PAGE: SDS–polyacrylamide.
- SEM: Standard error mean.
- SNR: Substantia Nigra pars Reticulate.
- Sp: Dendritic spine.
- Stim: stimulation electrode.

- TBS: Tris-HCl buffered saline.
- TEI: Total ethanol intake.
- Ter: Terminal.
- TF: Time spent in familiar object.
- Th: Thalamus.
- THC: (−)-trans-Δ9-tetrahydrocannabinol
- TN: Time spent in novel object.
- TRPA1: Transient receptor potential ankyrin 1.
- TRPV1: Transient potential receptors of vanilloid type 1
- TRPV1-LTD: Long term depression mediated by TRPV1 receptor.

- V1: Primary Visual Cortex.
- VP: Ventral Pallidum.

- 2-AG: 2-Arachidonoyl-Glycerol.
- [^{35}S] GTPγS: [^{35}S]guanosine-5*-O-(3-thiotriphosphate).
- β-actin: beta actin protein.

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