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NEUROBIOLOGICAL CONSEQUENCES OF ADOLESCENT EXPOSURE TO SYNTHETIC CANNABINOIDS PRESENT IN SPICE/K2 PREPARATIONS

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A mi florecilla favorita del jardín, Amelia

ABSTRACT

Synthetic cannabinoids are new psychoactive substances commonly found in herbal mixtures known as "Spice" in Europe or "K2" in the United States. Their consumption, primarily through smoking, has increased exponentially in recent years. Marketed as "alegal" alternatives to marijuana, synthetic cannabinoids are often perceived as safer options. However, clinical and preclinical reports indicate that synthetic cannabinoids are significantly much more potent and toxic than natural cannabis. Thus, acute intoxication has been associated with tachycardia, hypertension, hallucinations, anxiety, seizures, and most alarming, death. Growing evidence suggests that adolescence represents a period of heightened vulnerability to the central effects of drugs. Specifically, early cannabinoid consumption is a known risk factor for the development of psychiatric and cognitive disorders later in life. Notably, adolescents and young adults are reported to be the primary consumers of these new substances. However, little is known about the long-lasting consequences of chronic synthetic cannabinoid consumption during adolescence. Using behavioral and biochemical approaches, this thesis aims to investigate the potential neurobiological effects of adolescent exposure to synthetic cannabinoids in male and female mice. Concretely, two specific synthetic cannabinoids were selected: JWH-018, the earliest synthetic cannabinoid found in Spice/K2 preparations, and AB-FUBINACA, a more recent compound. Our findings reveal significant sex-dependent behavioral and neurobiological alterations. Adolescent exposure to JWH-018 primarily induced psychotic-like alterations in males. In addition, this behavioral effect was associated with disruptions in perineuronal nets and increased microglial reactivity in the prefrontal cortex, a brain area closely related to psychiatric disorders. On the other hand, AB-FUBINACA adolescent exposure induced psychotic-like symptoms and cognitive impairments in female mice, along with reduced dendritic spine density and altered neuronal arborization in the prefrontal cortex. In addition, both synthetic cannabinoids induced anxiety-like alterations in a sex-dependent manner. Overall, the findings of the present thesis highlight the strong potential of synthetic cannabinoids to induce psychotic-like effects, among others, while also confirming adolescence as a period of particular vulnerability to cannabinoid effects.

RESUMEN

Los cannabinoides sintéticos son nuevas sustancias psicoactivas comúnmente encontradas en mezclas herbales conocidas como "Spice" en Europa o "K2" en Estados Unidos. Su consumo, principalmente fumado, ha aumentado exponencialmente en los últimos años. Se comercializan como alternativas "alegales" a la marihuana y a menudo se perciben como opciones más seguras. Sin embargo, informes clínicos y preclínicos indican que los cannabinoides sintéticos son significativamente más potentes y tóxicos que el cannabis natural. De hecho, la intoxicación aguda se ha asociado con taquicardia, hipertensión, alucinaciones, ansiedad, convulsiones y, lo más alarmante, muertes. Es importante destacar que la evidencia creciente sugiere que la adolescencia representa un período de especial vulnerabilidad a los efectos de las drogas. En particular, el consumo temprano de cannabinoides es un factor de riesgo conocido para el desarrollo de trastornos psiquiátricos y cognitivos en etapas posteriores de la vida. Los informes más recientes indican que los adolescentes y adultos jóvenes son los principales consumidores de estas nuevas sustancias. Sin embargo, se sabe poco sobre las consecuencias a largo plazo del consumo crónico de cannabinoides sintéticos durante la adolescencia. Por ello, el objetivo de esta tesis fue evaluar los posibles efectos neurobiológicos derivados de la exposición a cannabinoides sintéticos durante la adolescencia en ratones macho y hembra. Para alcanzar este objetivo, se realizaron diferentes estudios de conducta y análisis bioquímicos. Se seleccionaron dos cannabinoides sintéticos: JWH-018, el primer cannabinoide sintético identificado en las preparaciones de Spice/K2, y AB-FUBINACA, un compuesto hallado más recientemente. Nuestros resultados revelan alteraciones neurobiológicas y conductuales dependientes del sexo. La exposición a JWH-018 durante la adolescencia produjo principalmente alteraciones de tipo psicótico en machos. Además, estos efectos psicóticos se asociaron con alteraciones en redes perineuronales y con un aumento de la reactividad microglial en la corteza prefrontal, una región cerebral estrechamente relacionada con los trastornos psiquiátricos. Por otro lado, la exposición a AB-FUBINACA durante la adolescencia indujo síntomas de tipo psicótico y alteraciones en memoria en hembras, junto con una reducción en la densidad de espinas dendríticas y alteraciones en la arborización neuronal en la corteza prefrontal. Adicionalmente, ambos cannabinoides sintéticos indujeron alteraciones de tipo ansioso de forma dependiente del sexo. En conjunto, los hallazgos de esta tesis destacan el alto potencial de los cannabinoides sintéticos para inducir efectos de tipo psicóticos, entre otros, al tiempo que refuerzan que la adolescencia es un período particularmente vulnerable a las consecuencias negativas de los cannabinoides.

Abbreviations

2-AG	2-arachidonoylglycerol
AEA	Anandamide
AUC	Area under the curve
BDNF	Brain derived neurotrophic factor
CB1R	Cannabinoid receptor type 1
CB2R	Cannabinoid receptor type 2
CNS	Central nervous system
CS	Conditional stimulus
CUD	Cannabis use disorder
DAGL	Diacylglycerol lipase
DSM-5-TR	Diagnostic and statistical manual of mental disorder 5, text revised
EMCDDA	European monitoring center for drugs and drug addiction
EPM	Elevated plus maze
ESPAD	European school survey project on alcohol and other drugs
FAAH	Fatty acid amide hydrolase
GAD67	Glutamic acid decarboxylase 67
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GnRH	Gonadotropin-releasing hormone
IBA-1	Ionized calcium-binding adapter molecule 1
i.p.	Intraperitoneal
INE	Instituto nacional de estadística
IL	Infralimbic area
ITI	Inter trial interval
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
NAPE-PLD	N-acyl-phosphatidylethanolamine specific phospholipase D
NOP	Novel object place
NOR	Novel object recognition
OBF	Orbitofrontal area
OEDA	Observatorio Español de las Drogas y Adicciones
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PFC	Prefrontal cortex
PL	Prelimbic area
Pleckhg2	Pleckstrin Homology And RhoGEF Domain Containing G2
PnC	Caudal pontine reticular nucleus
PND	Post-natal day
PNN	Perineuronal nets

PPI	Prepulse inhibition of startle reflex test
PPT	Pedunculopontine nucleus
PSD95	Postsynaptic density protein 95
PV	Parvalbumin
RhoGEF	Rho guanine nucleotide exchange factor
RNA-seq	RNA sequencing
ROI	Region of interest
SCBs	Synthetic cannabinoids
Sh3tc1	SH3 Domain And Tetratricopeptide Repeat-Containing Protein 1
SYP	Synaptophysin
THC	Δ^9-tetrahydrocannabinol
US	Unconditional stimulus
WHO	World Health Organization

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Introduction

ENDOCANNABINOID SYSTEM AND CANNABINOIDS I

1. Endocannabinoid system

The use of Cannabis sativa plant dates back to 12.000 years ago, with early reports documenting its cultivation in Central Asia (Li, 1973). Initially, cannabis was cultivated for its fibers, used in ropes and nets, however, its social consumption gradually became widespread (Crocq, 2020; Li, 1973). The migration of nomadic people contributed and facilitated its global distribution around the world. Later, during the 1960s the recreational use of marijuana increases exponentially. Even then, there were widespread speculations about the possible harmful or therapeutic effects of using preparations derived from the Cannabis sativa plant. Consequently, efforts were made to identify the different components, named cannabinoids, responsible for the diverse effects of cannabis (Crocq, 2020). In 1965 efforts culminated when Δ^9-tetrahydrocannabinol (THC) was isolated from the plant (Mechoulam and Gaoni, 1965) and later on considered as the main responsible for the psychoactive properties of marijuana (Beardsley et al., 1987; Little et al., 1988). It was not until 1988 when the cannabinoid receptor type 1 (CB1R) was discovered (Devane et al., 1988), revealing that cannabinoid compounds exert their biological effects through the activation of specific endogenous receptors. By homology cloning, Matsuda and collaborators discovered a similar receptor, the cannabinoid receptor type 2 (CB2R) (Matsuda et al., 1990). This discovery was followed by the identification of the endogenous lipidic ligands of cannabinoid receptors, which were referred to as endocannabinoids: anandamide (AEA) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). Subsequently, enzymes involved in endocannabinoid biosynthesis and degradation were identified (Cravatt et al., 1996; Dinh et al., 2002a; Bisogno et al., 2003; Okamoto et al., 2004), being these, the last members of what became known as the endocannabinoid system (Figure 1).

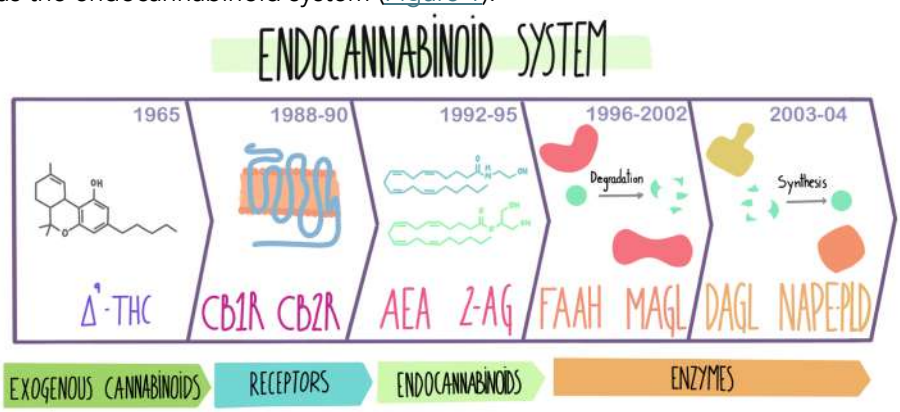


Figure 1. Major events in the history of the endocannabinoid system. *THC, Δ9-tetrahydrocannabinol; CB1R and CB2R, cannabinoid receptor 1 and 2; AEA, anandamide; 2-AG, 2-arachidonoylglyclerol; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE-PLD, N-acyl transferase with a selective phospholipase D.*

A significant feature of this system is that it acts as a retrograde modulator of numerous neurotransmitters (Ohno-Shosaku et al., 2001), being widely distributed throughout the brain. Consistently, the endocannabinoid system is involved in the modulation of numerous physiological functions such as brain development, motivation, memory, pain perception, stress, among others (Chen, 2015, Cristino et al., 2020). Exogenous cannabinoid ligands can act through hijacking the endocannabinoid system, and the resulting biological impairments are directly linked to the neuroanatomical distribution and physiological role of this endogenous system. This theme will be discussed throughout this thesis.

1.A. Cannabinoid Receptors

Classical cannabinoid receptors, CB1R and CB2R, belong to the G protein-coupled receptor family and are mainly coupled to Gi/o proteins (Howlett and Abood, 2017). Diverse studies also point to the existence of other receptors that bind cannabinoid ligands, such as G protein-coupled receptor 55 (GPR55) (Pertwee, 2007), the peroxisome proliferator- activated receptor (PPAR) (O'Sullivan, 2007), or the transient receptor potential cation channel subfamily V member 1 (TRPV1) (Marzo and Petrocellis, 2010). **CB1R** is characterized by 7 transmembrane domains being the most abundant G-coupled receptor in the central nervous system (CNS) (Cristino et al., 2020). It is the major cannabinoid receptor involved in the psychoactive effects of THC and other cannabinoid ligands (Stella, 2023). The brain distribution of CB1R in both rodents (Herkenham et al., 1991; Tsou et al., 1998) and humans (Westlake et al., 1994; Burns et al., 2007) has been well characterized. Basal ganglia, cerebellum and hippocampus are the brain areas where the greatest abundance of CB1R has been observed (Herkenham et al., 1991; Tsou et al., 1998). This receptor is also expressed in peripheral tissues including heart, lung, adrenal glands, retina, liver, gonads, adipocytes and immune and vascular systems (Pertwee et al., 2010). Functional CB1R is mainly expressed in presynaptic terminals of both excitatory and inhibitory neurons (Mátyás et al., 2008), but it has also been observed in astrocytes (Sánchez et al., 2001; Robin et al., 2018). **CB2R** shares with its homolog CB1R, the 7 transmembrane domain structure. However, they exhibit only 44% sequence homology at the protein level (Zou and Kumar, 2018). CB2R is mainly found in the peripheral tissues, predominantly in immune cells (Munro et al., 1993; Liu et al., 2009; Simard et al., 2022) since its main function is the modulation of the immune system (Cristino et al., 2020). Differing from CB1R, CB2R expression is residual in the CNS under physiological conditions. In contrast, it is a dynamic inducible receptor upregulated in pathological conditions, including schizophrenia, depression, addiction, and others (Banaszkiewicz et al., 2020; Morcuende et al., 2022). At a cellular level, a broad consensus is emerging on the important role of CB2R in microglial cells (Komorowska-Müller and Schmöle, 2021; Reusch et al., 2022) and, to a lesser extent, in astrocytes (Jia et al., 2020) and neurons (Liu et al., 2017). Interestingly, recent studies suggest that CB2R is involved in several neuropsychiatric disorders, and it is proposed as a potential biomarker or target for the diagnosis or treatment of different mental illnesses (Kibret et al., 2022; Kumar, 2024; Onaivi, 2023).

· Cannabinoid cellular signaling

Although CB1R and CB2R are mainly coupled to Gi/o protein (Howlett and Abood, 2017), it has been reported that CB1R can also activate Gs and Gq in certain circumstances in a cell type- and ligand-dependent manner (Demuth and Molleman, 2006). Through coupling to Gαi/o, the activation of both CB1R and CB2R induces the inhibition of adenylate cyclase and, in turn, decreases cAMP levels which reduces the activity of protein kinase A (Howlett, 2005; Bosier et al., 2010). In addition, by paring Gβγi/o, CB1R and CB2R can also activate some members of the MAPK family by inducing phosphorylation (Bouaboula et al., 1995; Howlett, 2005) (Bosier et al., 2010). Both Gαi/o and Gβγi/o couplings, ultimately lead to the modulation of gene expression (Howlett, 2005; Bosier et al., 2010). Other kinase signaling cascades such as the phosphoinositide 3-kinase pathway, glycogen synthase kinase 3 and protein kinase C can as well be activated by CB1R (Bouaboula et al., 1995; Gómez Del Pulgar et al., 2000; Ozaita et al., 2007). Moreover, CB1R agonism regulates the activity of diverse ion channels, including K^+ and Ca^{2+} channels, inhibiting the release of neurotransmitters by triggering the repolarization of the plasmatic membrane (Figure 2) (Deadwyler et al., 1995; Vásquez et al., 2003).

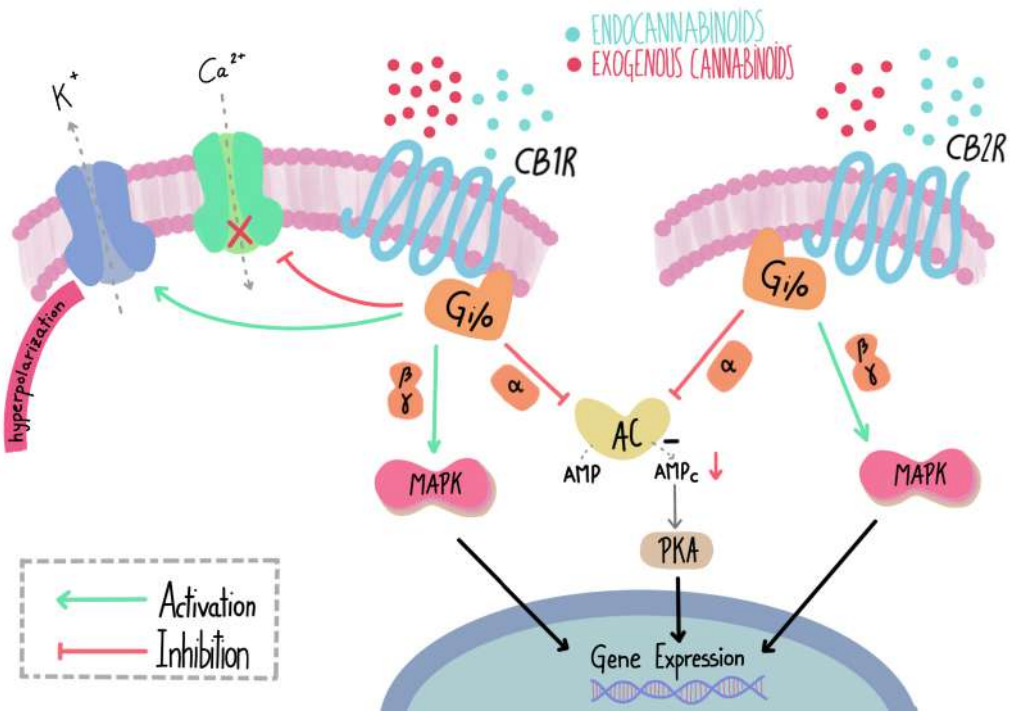


Figure 2. Main cannabinoid receptor signaling pathways. *Through coupling Gi/o both CB1R and CB2R modify gene expression by inhibiting AC activity and activating MAPK cascades. CB1R controls neurotransmitter release by a negative regulation of Ca^{2+} channels and positive rectifications through K^+ channels. AC, adenylyl cyclase; MAPK, members of the mitogen-activated protein kinase cascade; PKA, protein kinase A. Adapted from (Bosier et al., 2010).*

1.B. Endocannabinoids and enzymes

The successful identification and cloning of the CB1R and CB2R prompted the characterization of their endogenous agonists. AEA was the first endocannabinoid isolated (Figure 3A) (Devane et al., 1992) and it behaves as a partial agonist of both cannabinoid receptors, although presents lower affinity for CB2R than CB1R (Reggio, 2010). 2-AG, the second endogenous cannabinoid described (Figure 3B) (Mechoulam et al., 1995; Sugiura et al., 1995), is full agonist at both CB1R and CB2R, with higher affinity an efficacy than AEA (Reggio, 2010). Additionally, 2-AG has been found to be 170 times more concentrated than AEA in the brain (Stella et al., 1997). Beside these molecules, other putative endocannabinoids have also been identified, such as 2-arachidonylglycerolether (Hanus et al., 2001) and O-arachidonoylethanolamine (Porter et al., 2002).

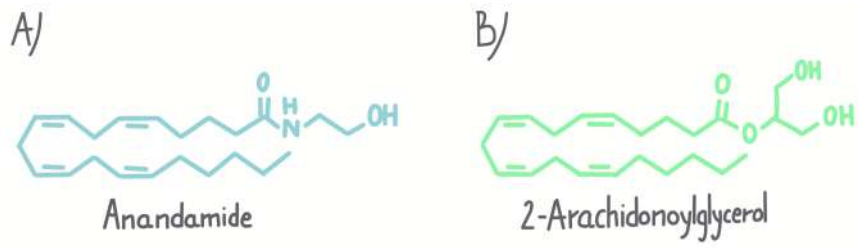


Figure 3. Structures of the most well-known endocannabinoids.

A feature that hallmark endocannabinoids from other neurotransmitters is that are not stored in presynaptic vesicles. Conversely, AEA and 2-AG are produced and released on demand in response to increased intracellular Ca^{2+} concentration (Castillo et al., 2012). Once released from the postsynaptic neurons, endocannabinoids travel backward across synapses and activate CB1R on presynaptic terminals, acting as rapid retrograde synaptic messengers (Figure 4) (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2002). In order to give a quick response to the requirement of these endocannabinoids in the synaptic cleft, there must be an efficient regulation of the biosynthesis and degradation of these lipidic ligands. In brief, AEA is catalyzed from N-acyl-phosphatidylethanolamine by NAPE-specific phospholipase D (NAPE-PLD) (Figure 4) (Di Marzo et al., 2004, 2005; Okamoto et al., 2004) and 2-AG results from the hydrolysis of diacylglycerol by a diacylglycerol lipase (DAGL) (Figure 4) (Bisogno et al., 2003; Di Marzo et al., 2005). Once their molecular targets have been reached, endocannabinoids are removed from the synaptic cleft to be subsequently degraded by their specific enzymes. Fatty acid amide hydrolase (FAAH) is the enzyme involved in the hydrolysis of AEA to arachidonic acid and ethanolamine (Figure 4) (Cravatt et al., 1996), while 2-AG is hydrolyzed mainly by the monoacylglycerol lipase (MAGL) into arachidonic acid and glycerol (Figure 4) (Dinh et al., 2002a, 2002b). Both are intracellular enzymes, but FAAH is primarily expressed in the soma and dendrites of postsynaptic neurons (Egertová et al., 2003), whereas MAGL is mainly in presynaptic terminals (Gulyas et al., 2004).

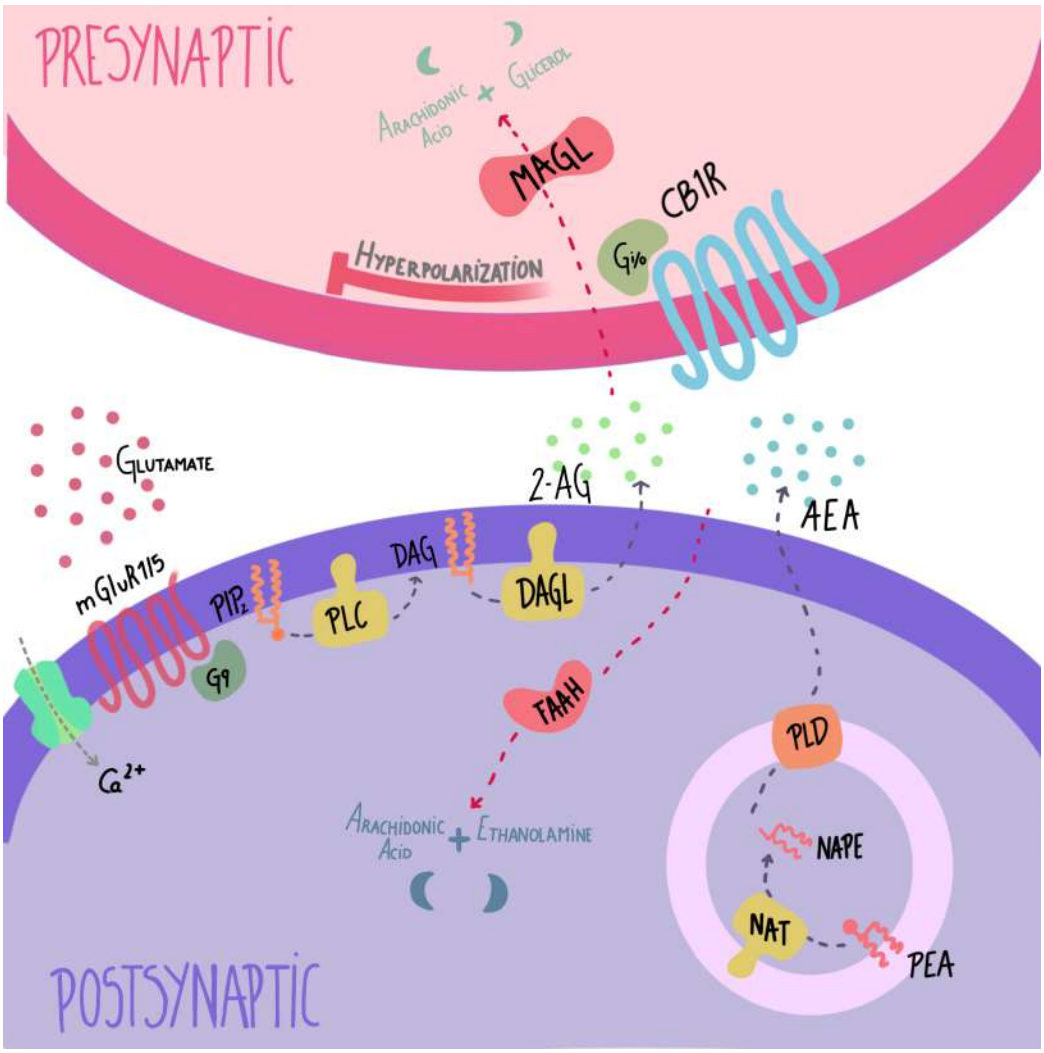


Figure 4. Endocannabinoid biosynthesis, retrograde release and degradation in response to increased intracellular levels of Ca^{2+}. *After Ca^{2+} entry into the postsynaptic terminal, the biosynthetic pathways of AEA and 2-AG get activated. Respectively, the –N-acyl transferase (NAT) and a selective phospholipase D (PLD)– located in intracellular membranes and the diacylglycerol lipase (DAGL) in the plasma membrane synthetized AEA and 2-AG. On demand, both endocannabinoids are released retrogradely and bind CB1R located mainly in presynaptic neurons. Subsequently, the endocannabinoids are removed from the synaptic cleft by rapid diffusion through the cell membrane. The monoacylglycerol lipase (MAGL) located in presynaptic terminal is the most classic route for the degradation of 2-AG, although alternative routes have also been described through α/β-hydrolase domain containing 6 (ABHD6) (Dinh, Freund, et al., 2002). On the other hand, the FAAH located greatly in postsynaptic terminals is responsible for the degradation of AEA. 2-AG; 2- arachidonoylglycerol; AA, arachidonic acid; AE, ethanolamine AEA, anandamide; DAG, diacylglycerol; mGluR1/5, metabotropic glutamate receptor type 1 or 5; NAPE, N-arachidonoyl-phosphatidyl-ethanolamine; PEA, phosphatidylethanolamine; PIP2, phosphatidylinositol bisphosphate. Adapted from (Di Marzo et al., 2004).*

1.C. Anatomical distribution and physiological functions of the endocannabinoid system

Studies focus on the distribution of the different components of the endocannabinoid system are motivated by the idea of obtaining a better understanding of the multiple physiological functions in which this system is involved. However, its distribution and density

is known to be age-dependent. Consistently, CB1R at embryonic and perinatal period, showed an atypical pattern of distribution and expression in comparison to an adult brain. CB1R labeling is notably increased in white-matter regions, particularly in fiber tracts, as well as in the midbrain and brainstem, areas where it almost disappears in adulthood (Fernández-Ruiz et al., 2000). Furthermore, at this early stage, high CB1R concentrations have also been found in cerebral cortex, hippocampus and cerebellum (Figure 5A) (Fernández-Ruiz et al., 2000). Concerning endocannabinoids, 2-AG and AEA have been identified in rodents brain during embryonic development, although in different amounts. Moreover, an abrupt increase of 2-AG levels in the first day of birth has also been described (Figure 5D) (Berrendero et al., 1999). Therefore, the endocannabinoid system plays a fundamental role in the proper development of the CNS during embryonic and perinatal periods. Endocannabinoids primarily contribute to axonal growth, synaptogenesis, as well as neuronal proliferation and migration, among others (de Fonseca et al., 1993; Fernández-Ruiz et al., 2000). During adolescence, a transient increase in CB1R has been observed in prefrontal cortex (PFC), limbic, striatal and midbrain areas of rodents and humans (Figure 5B) (de Fonseca et al., 1993; Bukiya, 2019). In addition, different studies demonstrate an eventual decrease in 2-AG levels in the PFC and nucleus accumbens during adolescence (Figure 5D) (Ellgren et al., 2008; Peters and Naneix, 2022). These transient changes occurring during this developmental stage are crucial for ensuring the proper functioning of the CNS. In fact, one of the main objectives of the present thesis is to assess the impact of exogenous modulation of the endocannabinoid system during this critical period, considered of particular susceptibility. Notably, in adulthood, CB1R is the most abundant G protein-coupled receptor in the CNS, which elucidates its contribution in numerous physiological functions (Figure 5C). As mentioned, the highest density of CB1R has been observed in the basal ganglia, cerebellum and hippocampus. However, this receptor has also been found in PFC, amygdala, thalamus, hypothalamus, among others (Herkenham et al., 1991). The abundant expression in the cerebellum and basal ganglia has been related to the control of motor coordination (Fernández-Ruiz and González, 2005; Kishimoto and Kano, 2006). Learning and memory are important physiological functions influenced by this system, due to its expression in the hippocampus (Kano et al., 2009; Puighermanal et al., 2009). In addition, the abundance of CB1R in amygdala and PFC has been associated with emotional processing, such as regulation of stress and anxiety (Lutz et al., 2015). Further, acting at peripheral level, the endocannabinoid system modulates the immune and cardiovascular systems and controls gastrointestinal motility and metabolism, among others (Grotenhermen and Müller-Vahl, 2003; Mehrpouya-Bahrami et al., 2017; Wang et al., 2020). In conclusion, the endocannabinoid system regulates numerous physiological functions throughout life, making it particularly susceptible to external disruptions due to drug use, especially during early developmental stages.

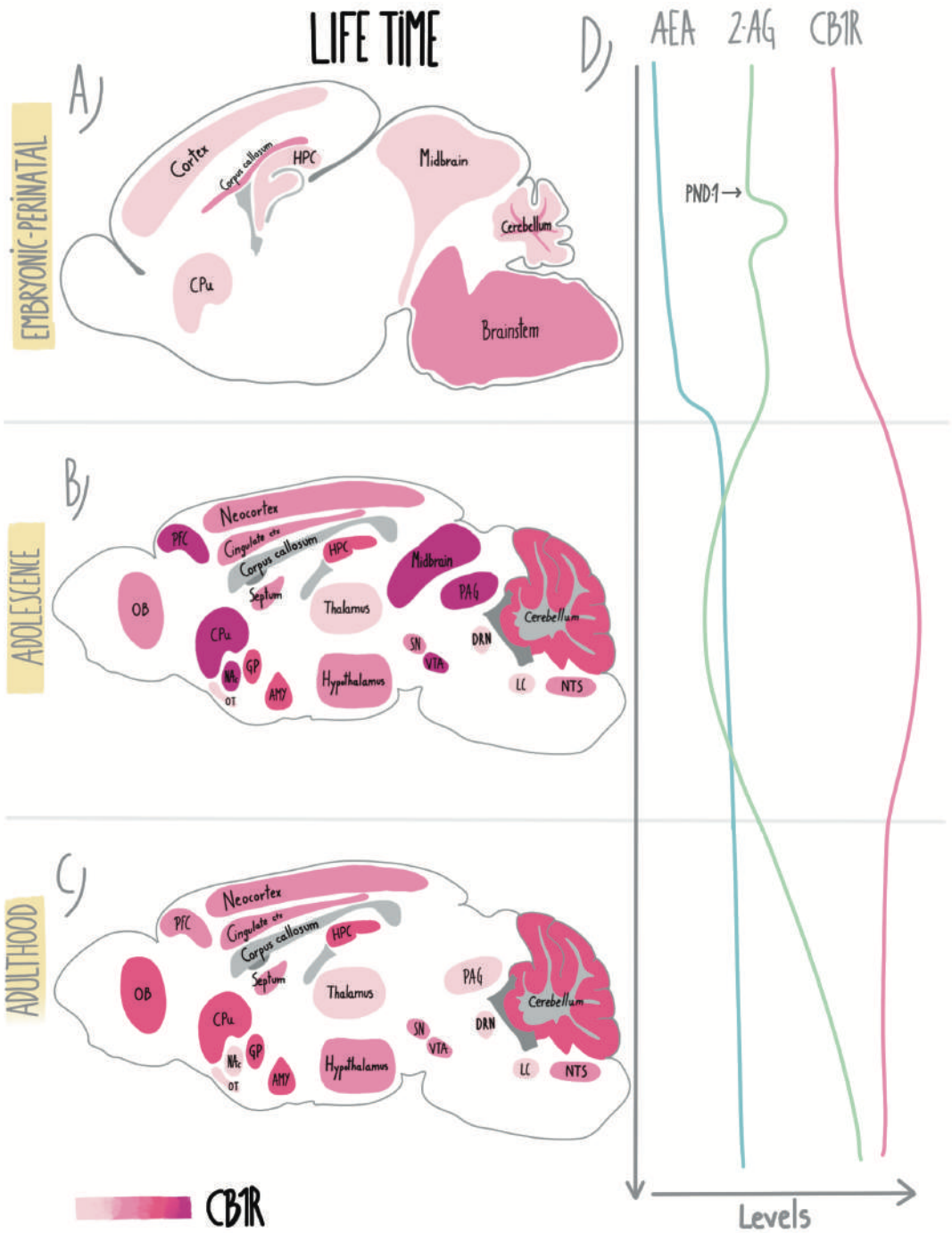


Figure 5. Distribution of CB1R and fluctuations of the endocannabinoid system throughout life. *Sagittal sections of mouse brain showing different CB1R location and density in embryonic/perinatal (A), adolescent (B) and adulthood (C) stages. The intensity of pink reflects CB1R concentration. Developmental trajectories of the components of the endocannabinoid system (D). 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AMY, amygdala; ctx, cortex; CPu, caudate-putamen; DRN, dorsal raphe; GP, globus pallidus; HPC, hippocampus; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal gray; PFC, prefrontal cortex; PND, post-natal day; SN, substantia nigra; VTA, ventral tegmental area. Adapted from (Herkenham et al., 1991; de Fonseca et al., 1993; Tsou et al., 1998; Fernández-Ruiz et al., 1999)*

2. Natural and Synthetic Cannabinoids

According to the literature, the first harvests of Cannabis sativa are documented more than 12.000 years ago in Asia (Li, 1973). Although it was initially used to generate textile fibre, cannabis consumption was already accepted and common (Crocq, 2020). It is well documented that later civilizations took advantage of its therapeutic benefits, however, there are also evidences of its use for recreational purposes due to its psychoactive effects (Zuardi, 2006; Crocq, 2020). According to the World Health Organization (WHO), psychoactive substances are compounds that, once consumed or administered, can influence mental processes (perception, consciousness, cognition or mood and emotions). Interestingly, the manifestation of hallucinations, dysphoria and even addictive-like states upon cannabis consumption are mentioned in diverse antique texts, although most transcripts collect detailed information about its curative benefits (Zuardi, 2006; Cook et al., 2015). This dichotomy between "therapeutic benefit" and "psychoactive effect" aroused interest in investigating which compounds within the plant offer therapeutic benefits in the absence of psychoactive effects.

2.A. Phytocannabinoids

The *Cannabis sativa L.* plant belongs to the *Cannabaceae* family and only has one genus (*Cannabis*) with one specie (*sativa*), whose properties vary significantly depending on the environment (ElSohly and Slade, 2005). The number of natural compounds identified in the plant amounts to more than 500. However, the most specific of the hemp plant are the C_{21} terpenophenolic cannabinoids (ElSohly and Slade, 2005), commonly named phytocannabinoids, which sum up to 125 (Radwan et al., 2021). The remaining constituents include phenols, flavonoids, terpenes, alkaloids and others (Radwan et al., 2021). The first phytocannabinoid isolated was cannabinol (Wood et al., 1899), which is part of the main active compounds extracted from the plant along with THC, cannabidiol and ^{8}-tetrahydrocannabinol (Pertwee, 2005). Among them, THC is the main psychoactive component in cannabis extracts (Gaoni and Mechoulam, 1964) (Figure 6A), and is considered a partial agonist of both CB1R and CB2R (Pertwee et al., 2010). However, it exerts its psychoactive effects mainly through CB1R (Schurman et al., 2020). The alterations produced by THC on the CNS are diverse, including catalepsy, hypothermia, analgesia and hypolocomotion, well-known as tetrad effects of cannabinoids (Moore and Weerts, 2022). THC is one of the two phytocannabinoids licensed for medical use, in combination with cannabidiol (proportion 1:1), for reducing muscle spasticity resulting from multiple sclerosis (Sativex®) (Table A1). On the contrary, cannabidiol is a non-psychoactive phytocannabinoid which has recently gained attention (Figure 6B) due to its potential as neuroprotective, anti-inflammatory, anticonvulsant, among others (Van den Elsen et al., 2014; Singh et al., 2023). In fact, some regulatory agencies have approved the use of Epidiolex® for the treatment of seizures in Lennox-Gastaut and Dravet syndromes (for more information see Table A1). In addition, in 2024, Spain introduced new regulations for the preparation and dispensing of

standardized magistral cannabis formulas for therapeutic use. These formulations will be available to patients with multiple sclerosis-related spasticity, severe refractory epilepsy, chemotherapy-induced nausea and vomiting, and refractory chronic pain (Sanidad, Gabinete de Prensa, 2024).

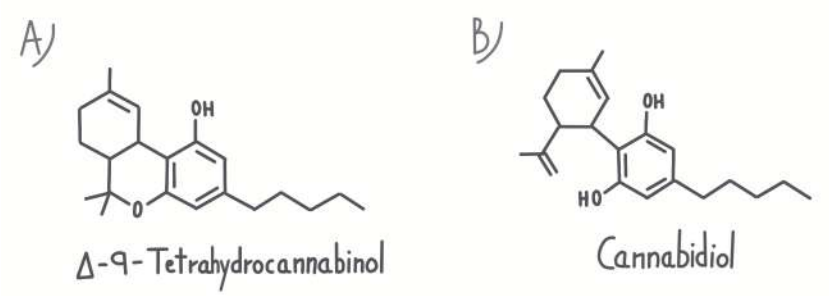


Figure 6. Chemical structure of the most relevant phytocannabinoids.

Therefore, legalization of cannabis and derivates for medical or recreational use is a current hot topic with controversial opinions among the scientific community and society. Marijuana consumption is becoming very popular and represents a public health concern since cannabis is increasingly perceived as safe by the public (Cook et al., 2015). On the other hand, there is evidence documenting that THC levels in hemp plants have been rising over the years to enhance its psychoactive effects (Figure 7) (Lafaye et al., 2017). Considering all these factors, the study of the behavioral alterations induced by marijuana consumption is a crucial issue. Mainly during adolescence, as is known as a vulnerable period for the harmful consequences of cannabis exposure.

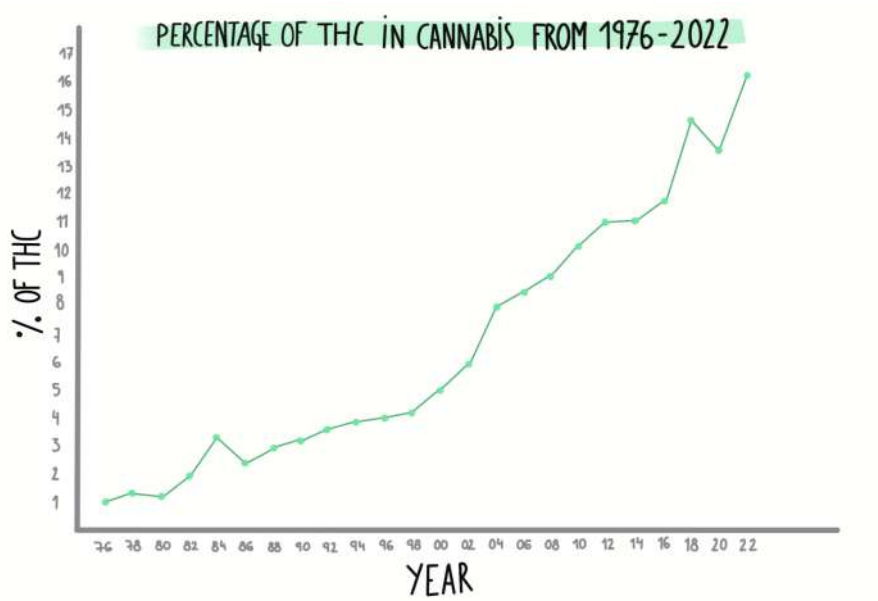


Figure 7. Percentage of THC in cannabis from 1976 to 2022. *Contemporaneous cannabis contains 10 times more THC than it did 50 years before. Adapted from (EMCDDA, 2022)*

· Cannabis use epidemiology

Cannabis preparations are the most consumed illegal drugs worldwide. The latest European Drug Report (2024) (EMCDDA) estimates that the 8% of population (15-64 years old) have consumed cannabis during their life. However, regarding young adults (15-24 years old), the group most likely to experience problems with these drugs, the consumption increased to 18.6% in the last year. In addition, around three quarters of adult users are men, and the majority (54%) are under 35 (EMCDDA, 2024). Regarding the most recent national data collected in ESTUDES, 2023, the prevalence of cannabis use in the last year among young people (14-18 years old) was 22.5% in men and 21% in women. Furthermore, the most concerning data from the INE (Instituto Nacional de Estadística) reveal that 93.7% of minors (under 18) admitted to detoxification programs in 2021 were due to cannabis use (Figure 8) (OEDA, 2021). Moreover, the 44% of drug-related hospital emergencies were due to intoxications with cannabis (EMCDDA, 2024).

All these data highlight that cannabis consumption constitutes a substantial public health concern given its association with low academic achievement, unemployment, violence and risk for developing psychiatric disorders (Rubino and Parolaro, 2016; Connor et al., 2021; Barry et al., 2022).

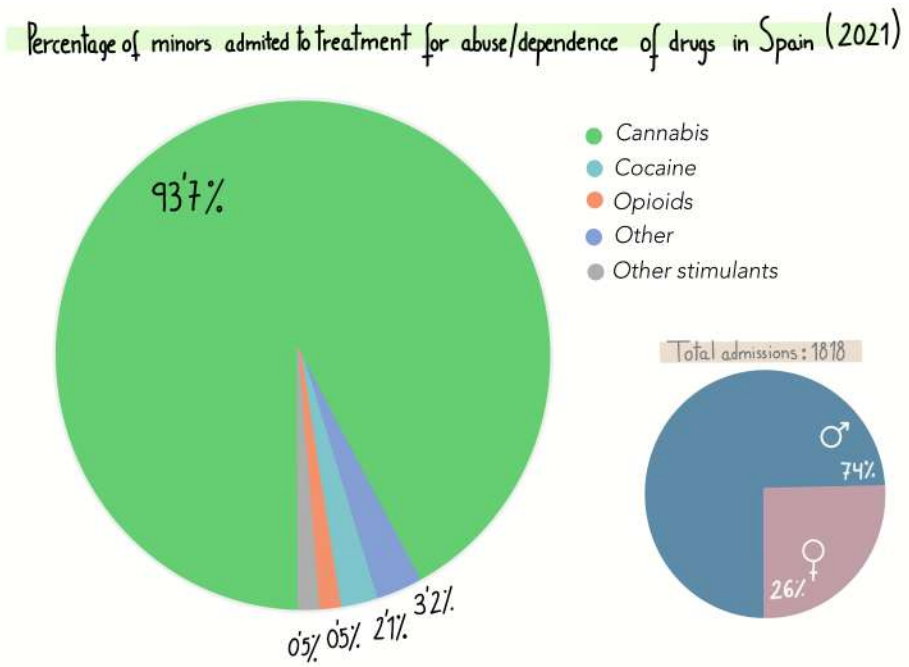


Figure 8. Percentage of minors (under 18 years old) admitted to treatment for abuse or dependence of drugs in Spain in 2021. *Of the 1,818 adolescents who entered to detoxification programs, 93% was due to problems with cannabis use. In addition, 74% of them were men (OEDA, 2021).*

· Cannabis use, abuse and dependence

The most common way to consume cannabis is by smoking it in co-use with tobacco. Traditionally, the female flowers from Cannabis sativa (marijuana) are the organs employed for smoking. However, the consumption of the resinous part (hashish) is increasing in the last years, possibly due to its higher THC content. Recently, new products containing THC are appearing, such as edibles (sweets, chocolate, …) or e-cigarette liquids (Holt et al., 2022). Acute effects of cannabis products last for approximately two to three hours (depending on the administration route) and are often described as a pleasant and relaxing experience, characterized by euphoria, sedation, and "floating" feelings (Stella, 2023). In addition, beside these emotions, users show physical alterations including tachycardia, increased appetite and bronchodilatation, among others (Karila et al., 2014). Moreover, it is also common the occurrence of negative effects such as dysphoria, anxiety, panic reactions, and sometimes positive psychotic symptoms (e.g., hallucinations, delusions and paranoia) (Karila et al., 2014). These last side effects described are the main responsible for hospital emergencies in Spain (Figure 9) (OEDA, 2023).

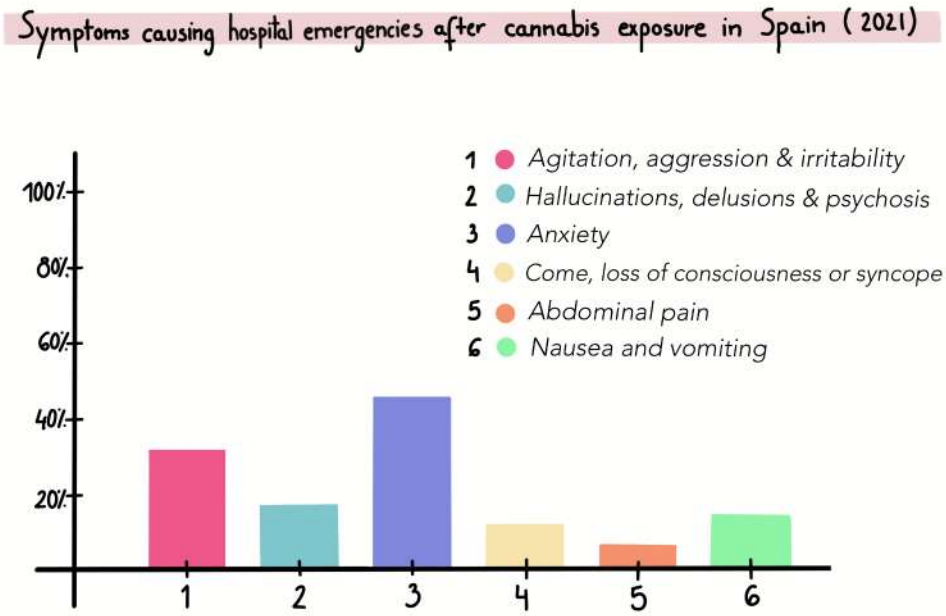


Figure 9. Symptoms causing hospital emergencies due to cannabis use in 2021 in Spain. *In most cases (44%) anxiety is the cause of admission. Agitation and aggression are the second reason (32%), followed by hallucinations and psychosis (16%). Adapted from (OEDA, 2023).*

Chronic cannabis use is known to produce cognitive deficits as well as impairments in memory and attention, which may persist even after cessation. These effects worsen with increasing years of use and with the initiation during adolescence (Broyd et al., 2016). Daily use of cannabis, can also give rise to dysregulation of emotional processes and psychiatric afflictions (Rubino and Parolaro, 2016). Other long-term consequences include impaired respiratory function, cardiovascular disease, and others (Tashkin and Roth, 2019; Richards et al., 2020).

It is quite clear that cannabis produces severe consequences both in acute and chronic consumption, even more severe when exposure occurs during the adolescent development stage. This is one of the main topics that will be discussed during this thesis.

Compared to other drugs, the addictive potential of cannabis has been questionable during the last years. However, the Diagnostic and statistical manual of mental disorders-5 (DSM-5-TR) now recognizes cannabis-related disorders including cannabis use disorder (CUD), cannabis intoxication, and cannabis withdrawal. CUD is broadly defined as the inability to stop consuming cannabis even when it is causing physical or psychological harm (Connor et al., 2021). CUD occurs in approximately 1 in 10 regular users and the risk of progression from cannabis use to CUD increases with frequency of use (Connor et al., 2021). Additionally, the market around cannabis is changing enormously with the emergence of new synthetic drugs derived from cannabis that seem to be more addictive and produce more severe consequences than classic cannabis (Ford et al., 2017). This could lead to the emergence of new CUD-like disorders due to the consumption of synthetic derivatives, although more research is needed to understand the health implications this would entail.

2.B. Synthetic Cannabinoids

Since the characterization of THC (Mechoulam and Gaoni, 1965), researchers and industry started to synthesize cannabinoid analogues to provide better understanding of the endocannabinoid system and in turn with the idea of finding new therapeutic options for different pathologies. In 1974, Pfizer developed the first synthetic cannabinoid (SCB), for antinociceptive purposes, included in the group of cyclohexylphenols (CP 55,940) (De Luca and Fattore, 2018). Over the following years, scientists continue to develop more potent synthetic variants of THC as research tools, and as these compounds were discovered, the information was made publicly available (Alves et al., 2020). Unfortunately, around the 2000s, clandestine laboratories misused this research for selling these compounds as an alternative to marijuana. Moreover, the rejected substances from pharmaceutical industry ended up on the drug market as unregulated highs as well (Papaseit et al., 2018; Alves et al., 2020). However, it was not until 2008 when the EMCDDA first detected the SCB JWH-018 in an herbal product (Ford et al., 2017; Alves et al., 2020). Seized of these herbal mixtures began around 2010 and were confiscated mainly under the names of Spice (in Europe) and K2 (in United States) (EMCDDA, 2016). The main characteristics of Spice/K2 will be described in the next section. During that year (2010), the "List I" of illegal SCBs possession was published, however, new chemically distinct SCBs emerged on the market simultaneously (Ford et al., 2017). Since then and until today, the lists of prohibited SCBs have increased enormously, although clandestine laboratories continue to produce ingenious alternative molecules (EMCDDA, 2024).

The exponential growth of this synthetic market is a major public health trouble given the high number of new SCBs available compared to the little knowledge we have about their negative effects. Therefore, this need of information has motivated the realization of this doctoral thesis.

· SCBs classification

Classical SCBs were synthetized based on the initial hypothesis that THC exerted its effect through three structural motifs: the C9 methyl group, the phenolic alcohol, and the pentyl chain extending from C3 (Worob and Wenthur, 2020). This group includes dronabinol and nabilone, which are THC analogs approved in some countries as capsules to reduce nausea and vomiting caused by chemotherapy (Syndros®, Cesamet®). Classic SCBs also include HU-derivatives, such as HU-210 (Alves et al., 2020). Cannabinoids defined as "nonclassical" include bicyclic and tricyclic structures, among these CP 55,940 and its analogs (Alves et al., 2020; Worob and Wenthur, 2020). However, later molecules synthesized with cannabimimetic effects were structurally very dissimilar to THC. Based on the aminoalkylindole scaffold, chemists began to generate libraries of cannabinoid receptor ligands with higher affinity to CB1R and CB2R than classical SCBs. Aminoalkylindoles, the biggest group, is further divided into 7 subgroups, of which naphthoylindoles (e.g. JWH-018, JWH-015) were the first to be found and appeared in the highest concentrations in Spice/K2 first preparations (Alves et al., 2020; Tettey et al., 2021). The complete classification is represented in Table 1 (Tettey et al., 2021). Moreover, aminoalkylindazoles have emerged more recent and are subdivided into: naphthoylindazoles (e.g. THJ-018, THJ-2201) and indazole carboxamides (e.g. AB- FUBINACA, AB-CHMINACA) which are the most abundant in contemporary herbal preparations (Tettey et al., 2021). Prototypical aminoalkylindole and aminoalkylindazole structures can be demarcated into four regions: a core, a head, a linker, and a tail (Figure 10), all subject to modifications (Alves et al., 2020). Hybrid SCBs with combinations of classical and non-classical cannabinoids have also been described (e.g. AM-403) (Alves et al., 2020; Tettey et al., 2021). Semi-synthetic cannabinoids (e.g. HHC) have emerged enormously in the last two years and are now one of the groups that most concerns to regulatory entities (EMCDDA, 2024). In addition, synthetic analogues of endocannabinoids have also been developed and are classified as eicosanoids (e.g. methanandamide) (Alves et al., 2020; Tettey et al., 2021). Although most SCBs have psychoactive effects, some of the groups mentioned lacks them.

In brief, the enormous number of Spice/K2 seizures during the last years has contribute to the characterization and classification of the different SCBs, that today sum up to 254 forbidden (EMCDDA, 2024).

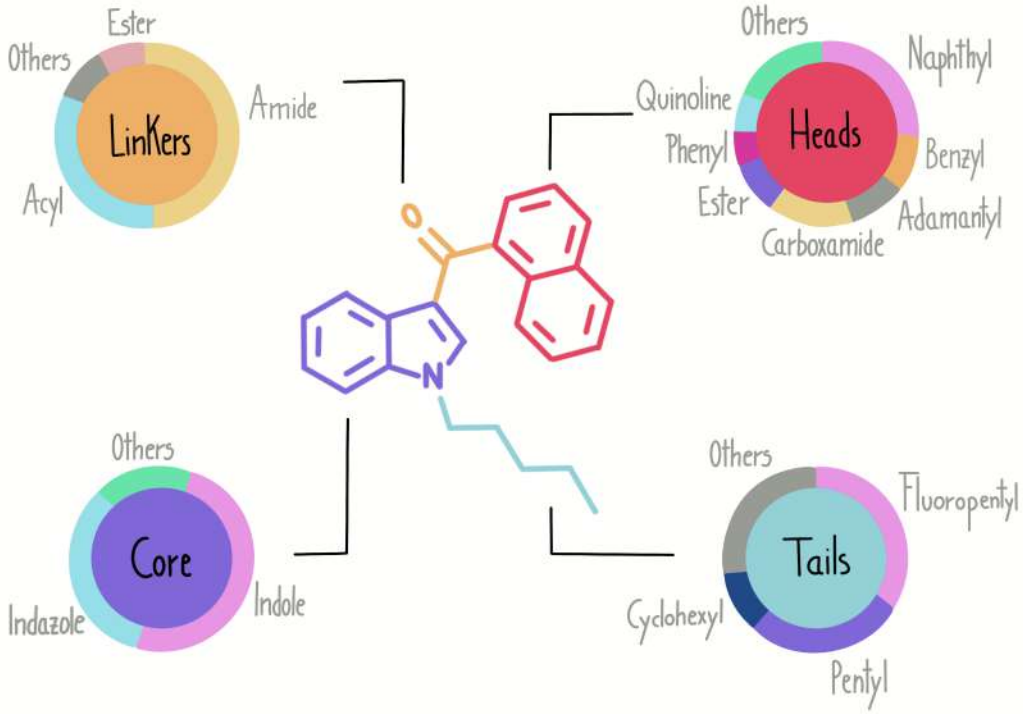


Figure 10. Most prevalent chemical substitutions in SCBs identified in seized material. *Regarding the "core", indole and indazole are the most frequent alternatives employed. Common "head" groups consist of large aryl or valine derivatives. Both regions are linked often by an amide, acyl or ester bond. In addition, fluoropentyl, pentyl and cyclohexyl are the widespread groups described as tails (Worob and Wenthur, 2020).*

Table 1. Main classification of SCBs

A) Classical cannabinoids e.g. HU-210, HU-308, AM-906
B) Nonclassical cannabinoids e.g. CP 47,497, CP 55,940, CP 55,244
C) Aminoalkylindoles i. Naphtoylindoles (e.g. JWH.018, JWH-105, WIN 55,212-2) ii. Phenylacetylindoles (e.g. JWH-250, JWH-251) iii. Benzoylindoles (e.g. AM-694, RSC-4) iv. Naphthylmethylindoles (e.g. JWH-184, JWH-192, JWH-196) v. Cyclopropoylindoles (e.g. UR-144, XLR-11) vi. Adamantoylindoles (e.g. AD-001, AM1248) vii. Indoles Carboxamides (e.g. APICA, 5F-APICA)
D) Aminoalkylindazoles i. Naphthoylindazoles (e.g. THJ-018, THJ-2201) ii. Indazole carboxamides (e.g. AB- FUBINACA, AB-CHMINACA)
E) Hybrid cannabinoids e.g. AM-403, PB-22
F) Semi-synthetic cannabinoids e.g. HHC
G) Eicosanoids e.g. Methanandamide
H) Others e.g. CRA-13, JWH-307

· What is Spice/K2?

Spice/K2 is sold in brightly colored bags which contains between 0.5 and 3 grams of dried green/brown plant material. These herbs, mainly Melissa, Mentha or Thymus (EMCDDA, 2024), do not have any psychoactive effect per ser, and are used to create the false impression that the product is of natural origin. Different type and amount of SCBs are soaked or sprayed onto the herbs. Once the solvent (acetone or ethanol) evaporates and the plant material is dried with the SCBs attached to them, the product can be crushed and packaged, often in very different concentrations between the different bags with the same packaging (Spaderna et al., 2013). Then, the product is ready to be sold down the internet as "legal high", typically displaying on the packaging a disclaimer with the message "not for human use" (Figure 11) (Papaseit et al., 2018; Alves et al., 2020). In addition, some herbal mixtures have been shown to contain numerous other compounds such as sympathomimetic agents (e.g. clenbuterol), -opioid receptor agonists (e. g. mitragynine) and benzodiazepines (e.g. phenazepam), among others (Manseau, 2016). The huge number of SCBs, the fact that each bag has a different concentration and composition, together with the detail that non-cannabimimetic drugs are added to these preparations, make Spice/K2 a dangerous cocktail for consumers, mainly since little is known about the acute and chronic effects of these drugs.

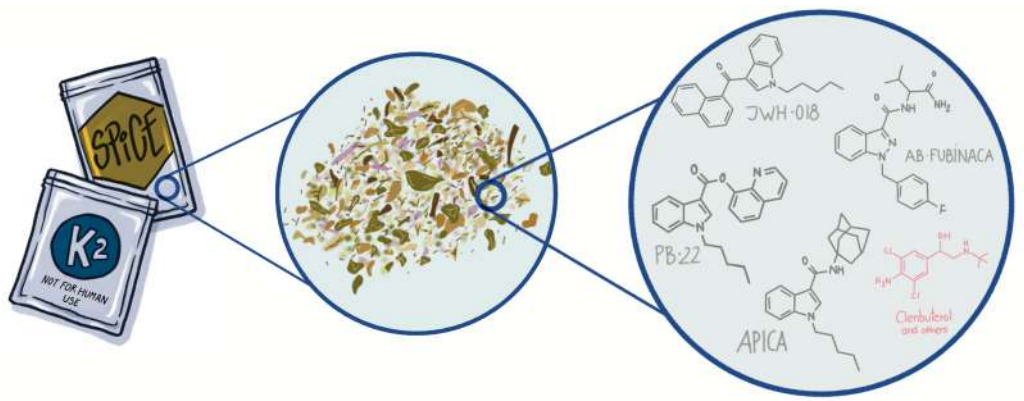


Figure 11. Graphical representation of marketed Spice/K2 preparations. *Herbal mixtures packaged in metallic colored bags are sprinkled with different type and amount of SCBs, although the presence of contaminants such as clenbuterol, benzodiazepines and others has also been described.*

· SCBs epidemiology

SCBs constitute the largest group of new psychoactive substances and compared with other new drugs, the increase in consumption of SCBs has been particularly remarkable (Figure 12) (EMCDDA, 2024). The main incentives for SCBs use could be curiosity, low price, positive psychoactive effects, the belief that the drug is safe, and the potential for passing drug testing (Alves et al., 2020). According to the most recent data from the EMCDDA, of the 950 new psychoactive substances monitored, 26 were detected in 2023 for the first time, of which 9 were new synthetic cannabinoids (Figure 12). Unfortunately, the different countries still collect little information on the prevalence of consumption of these new drugs. In some

cases, data are on classified together with cannabinoids and in others are reported as new synthetic substances in general, so giving real data on global consumption poses a challenge. However, it seems that the European School Survey Project on Alcohol and Other Drugs confirms that the prevalence of consumption of SCBs in young people (15 - 16 years old) is 1.1 - 5.2 % (Espad, 2019). Additionally, seizure data also shed light this lack of information and in 2022, 24 countries reported a total seizure of 281 kilograms of SCBs as herbal material (EMCDDA, 2024).

Therefore, it is clear that business around SCBs is growing by leaps and bounds, posing a risk to the health of consumers, especially the youngest ones, since they are the most vulnerable group and have one of the highest prevalences of consumption.

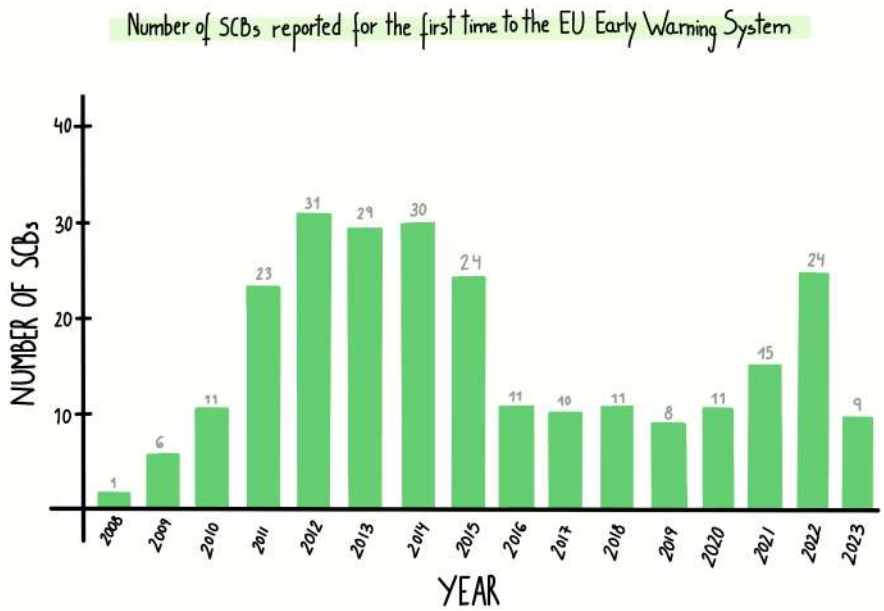


Figure 12. Number of SCBs reported from the first time to the EU Early Warning System. *The SCBs market has grown significantly since 2008, bringing a total of 254 cannabinoids persecuted by Europe legislation. Adapted from (EMCCDDA, 2024).*

· SCBs use, abuse and dependence

Similar to marijuana, smoking the herbal extracts is also the most common way of consuming SCBs, although new alternatives have recently emerged. E-cigarette consumption is gaining popularity among young people and is sold under the names of "Buda-blue", "C-liquid" or "Herbal e-liquid" (Figure 13). Further, oral administration is also possible, but less usual because of late onset effects (Figure 13). In addition, some fewer common cases of rectal administration of SCBs have been reported (Ford et al., 2017; Papaseit et al., 2018; Alves et al., 2020). Depending on the SCBs consumed and on the route of administration, the onset of action is within minutes of smoking, and intoxication lasts 2–5 hours. Therefore, as there are plenty SCBs, the lasting profile of effects compared to THC (2-3h) varies greatly, with some having been described with shorter durability

(e.g. JWH-018; 1-2h) and others more prolonged (CP 47,497; 5-6h) (Vandrey et al., 2012; Fattore, 2016).

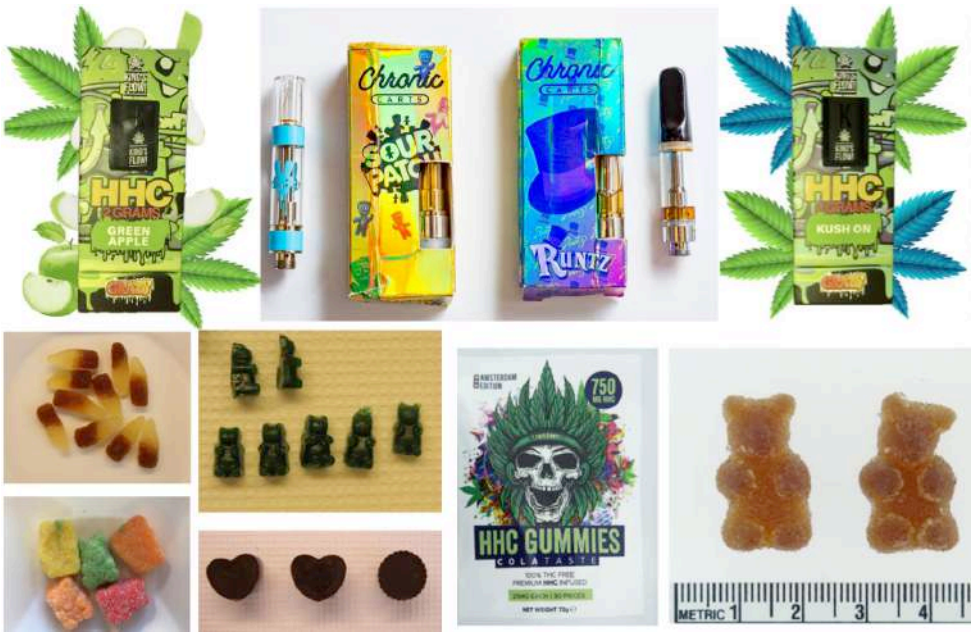


Figure 13. Representative images of alternative products to Spice/K2 containing SCBs which mainly include edibles and e-liquids.

While often advertised as "safe" and/or "legal" alternatives to marijuana on the internet, SCBs effects have proved to be dangerous, and markedly more toxic than, those produced by marijuana (Ford et al., 2017). Although some of the acute symptoms characteristics of marijuana are common with SCBs (e.g. relaxing feelings, sedation, …), side effects (e.g. seizures, tachycardia, depressed breathing, chest pain and psychotic symptoms) are more potent and frequent, as well as responsible for numerous hospital emergencies (Fattore, 2016). More severe consequences such as kidney damage, rhabdomyolysis and even death have also been described following acute SCB intoxication (Trecki et al., 2015; Fattore, 2016).

Chronic consumption of SCBs frequently induces tolerance, thus discontinuation has been reported to induced withdrawal symptoms similar to those reported with marijuana cessation (Nacca et al., 2013; Macfarlane and Christie, 2015; DSM-5-TR, 2017). In addition, there are increasing number of studies associating chronic exposure to SCBs with memory, psychiatric and dependence disorders (Li et al., 2019; Bilel et al., 2020; Trexler et al., 2020; Pintori et al., 2021), among others, a topic that will be discussed deeper in later sections of this introduction. It is also worth noting that most data related to the effects of SCBs are collected from hospital reports, with a lack of results from basic experimentation. Specifically, this absence of information has motivated the completion of this thesis, with the aim of exploring potential consequences of SCBs chronic exposure.

JWH-018

The aminoalkylindole JWH-018 was synthesized in the 1990s along with other naphthoylindole derivatives (Figure 14) (Huffman et al., 1994). As mentioned above, it was the first SCB reported in Spice/K2 preparations and was also quite frequent in these herbal products during many years (EMCDDA, 2024). Although it constitutes part of the so-called "first generation of SCBs", is still common to find JWH-018 in some recent preparations (Alam and Keating, 2020). In addition, current hybrid-SCBs contains JWH-018 as part of its molecule (e.g. PB-22) (Brandt et al., 2021). JWH-018 is a potent agonist at CB1R and CB2R, showing approximately a four-fold increased activity at the CB1R and about a ten-fold affinity at the CB2R compared with THC (Vigolo et al., 2015). In animal models, JWH-018 reproduces the typical "tetrad" symptoms of THC, although its effects are much more potent at lower doses (Marshell et al., 2014; Pintori et al., 2021). A characteristic that JWH-018 shares with other SCBs is that after phase I degradation, the numerous metabolites generated (13 for JWH-018), remain highly active on CB1R (Brents et al., 2011; Alves et al., 2020). Moreover, there are current studies that relate acute exposure to JWH-018 with psychiatric alterations (e.g. anxiety, psychosis, hallucinations) (Vigolo et al., 2015; Bilel et al., 2020; Theunissen et al., 2022), as well as cognitive impairments (Barbieri et al., 2016; Li et al., 2019), seizures (Malyshevskaya et al., 2017), and dependence (Pintori et al., 2021). However, the possible long-lasting effects induced by chronic JWH-018 exposure have been poorly studied.

Considering its prevalence in Spice/K2 preparations, JWH-018 was chosen as a representative to explore the effects of chronic SCBs use, with a focus on adolescescence.

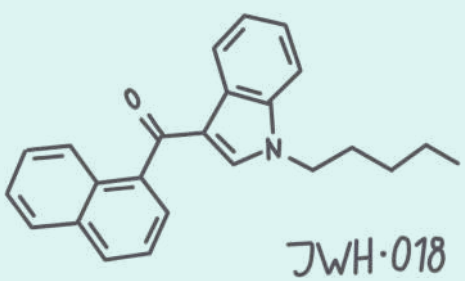


Figure 14. Chemical structure of JWH-018.

AB-FUBINACA

Unlike JWH-018, this indazole derivative has been identified in more recent Spice/K2 preparations, specifically AB-FUBINACA was first reported in Japan in 2013 (Figure 15) (Uchiyama et al., 2012). Interestingly, Pfizer was the first to synthesize and patent this and other aminoalkylindazole derivatives for their potential analgesic properties in 2009 (Buchler I, 2009). AB-FUBINACA binds with similar affinity to CB1R and CB2R, showing more efficient Ki values than THC (Banister et al., 2015). Notably, up to 11 active metabolites have been described after the consumption of AB-FUBINACA, which prolongs its toxicity (Castaneto et al., 2015; Minakata et al., 2021). One of the main differences with respect to JWH-018 is the pharmacokinetic and pharmacodynamic profile, as AB-FUBINACA clearance is known to be slower (Toennes et al., 2017, 2018; Brandon et al., 2021). Thus, twelve hours after consumption, JWH-018 is difficult to detect in urine (Toennes et al., 2017, 2018), whereas AB-FUBINACA can be detected even after 24 hours (Hsin-Hung Chen et al., 2016; Brandon et al., 2021). Not surprising, alterations in the "tetrad test battery" were observed in animal models after administration of AB-FUBINACA (Trexler et al., 2020). Furthermore, intake of AB-FUBINACA has been related with acute euphoric state, marked hallucinogenic and hypnotic actions (Canazza et al., 2017). Several hospitalizations following Spice/K2 consumption containing AB-FUBINACA in high doses were due to seizures, tachycardia, myocardial infarction, pneumonia, rhabdomyolysis, anxiety, delirium, psychosis, and aggressive behaviors (Trecki et al., 2015; Canazza et al., 2017). Although later sections of this document delve deeper into the effects reported after JWH-018 or AB-FUBINACA administration, chronic exposure to AB-FUBINACA in animal models has been mainly related to memory impairments (Kevin et al., 2017; Alzu'bi et al., 2024). Therefore, due to its similarities with other derivatives in its group and its high presence in recent Spice/K2 preparations, AB-FUBINACA was also considered as a representative compound to study the long-term consequences of chronic adolescent exposure.

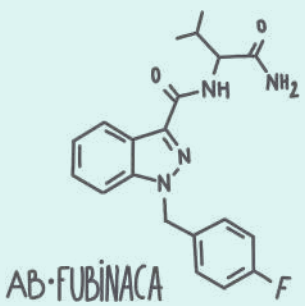


Figure 15. Chemical structure of AB-FUBINACA.

II BEHAVIORAL MODELS FOR STUDYING CANNABINOID EFFECTS

The long-lasting effects of recreational cannabis consumption have been a concern for many years. Therefore, numerous investigations have been carried out, in both humans and animal models, and a close relationship between chronic cannabis use and the appearance of multiple disorders, mainly psychiatric have been found. However, the importance of the developmental moment of exposure is becoming more and more established, since early exposure is associated with an increase of deleterious consequences (Rubino et al., 2015; Rubino and Parolaro, 2016). Psychiatric disorders are behavioral, emotional, or cognitive dysfunctions which are commonly associated with impairments in important areas of functioning (social, occupational and interpersonal) (WHO, 2022). Interestingly, dysregulation of emotional processes, alterations in memory or cognition, and psychotic behaviors are the most prevalent dysfunctions described followed chronic cannabis use (Broyd et al., 2016; Rubino and Parolaro, 2016; Abela et al., 2019; Moore and Weerts, 2022). Therefore, the increasing prevalence of SCBs use suggests a growing risk of associated psychiatric and cognitive dysfunctions. In fact, there are previous data supporting memory impairments (Compton et al., 2012; Li et al., 2019; Alves et al., 2020; Alzu'bi et al., 2024) psychotic-like states (Spaderna et al., 2013; Fattore, 2016; Rubino and Parolaro, 2016; Alves et al., 2020; Theunissen et al., 2022), and anxiety-like alterations (Frontera et al., 2018; Pushkin et al., 2019; Pintori et al., 2021; Margiani et al., 2022) after SCBs administration in rodents. Despite this significant progress, much remains unknown regarding the long-term consequences of SCBs exposure, particularly during adolescence. In this context, animal models represent a valuable tool for assessing the impact of these substances on the CNS, as well as for investigating potential sex differences, one of the main objectives of this thesis.

1. Behavioral models to study anxiety and fear

1.A. Definition of anxiety and fear

Anxiety and fear are adaptative defensive responses to protect animals from dangerous stimuli (Tovote et al., 2015). The distinction between these two emotions is not always clear, but the critical difference between both is that fear is a rapid response to a real imminent threat that can active defensive responses, while anxiety is a long-lasting mood that occurs before a potential possible threat (McNaughton and Corr, 2004; McNaughton and Zangrossi, 2008). Anxiety often results in an apprehensive mood accompanied by increased arousal and vigilance in preparation for future danger. Therefore, anxiety and fear are adaptive responses that have a pivotal role in survival (Tovote et al., 2015). However, if the response is extreme in intensity or duration, or occurs due to inappropriate stimuli, it can become pathological, leading to the development of anxiety or trauma- and stressor-related disorders (Kindt, 2014; DSM-5-TR, 2017). Some anxiety disorders also include alterations with abnormal fear processing, whereas others are defined by more general pathological anxiety states (Table 2).

Table 2. Anxiety disorders and trauma and stressor- related disorders according to DSM-5-TR
ANXIETY DISORDERS
1.Separation Anxiety Disorder
2. Selective Mutism
3. Specific Phobia a. Animal b. Natural environment c. Blood-injection-injury d. Situational e. Other
4. Social Anxiety Disorder (Social Phobia)
5. Panic Disorder
6. Panic Attack Specifier
7. Agoraphobia
8. Generalized Anxiety Disorder
9. Substance/Medication-Induced Anxiety Disorder
TRAUMA AND STRESSOR-RELATED DISORDERS
1. Reactive Attachment Disorder
2. Disinhibited Social Engagement Disorder
3. Posttraumatic Stress Disorder
4. Acute Stress Disorder
5. Adjustment Disorder

The innate defense circuit against threats is composed by the amygdala, hypothalamus, hippocampus, periaqueductal grey and PFC (LeDoux, 2000; Maren et al., 2013), areas in which the endocannabinoid system is highly expressed (Herkenham et al., 1991; Tsou et al., 1998). Therefore, exogenous cannabimimetic activation of this system in these specific areas can lead to the appearance of several impairments. Accordingly, a study conducted in Spain found that approximately 60% of patients admitted for cannabis dependence treatment also presented comorbid psychiatric disorders, including anxiety and fear-related disorders (Table 3) (Pascual et al., 2016). Thus, in order to study the effects of cannabinoids in anxiety and fear, two categories of tests have been used over the years: (1) innate anxiety-related tests and (2) conditioned or learned fear- related tests.

Table 3. Comorbidity of mental disorders in current cannabis abusers (Pascual et al., 2016)

PSYCHIATRIC DISORDER	PERCENTAGE	DSM-5 GROUP DISORDER
Major Depressive Episode	19,5 %	Depressive Disorders
Dysthymia	14,3 %	Depressive Disorders
Suicide attempt	30,1 %	*Common to several disorders*
Bipolar Disorder	29,3 %	Bipolar and Related Disorders
Agoraphobia	15,8 %	Anxiety Disorders
Social Phobia	10,5 %	Anxiety Disorders
Obsessive Compulsive Disorder	8,3 %	Obsessive-Compulsive Disorders
Posttraumatic Stress Disorder	4,5 %	Trauma and Stress-related Disorder
Psychosis	12 %	*Common to several disorders*
Bulimia	1,5 %	Feeding and Eating Disorders
Generalized Anxiety Disorder	24,1 %	Anxiety Disorders

1.B. Innate anxiety-related tests

The current tests for studying anxiety in rodents include the elevated plus-maze (EPM), the zero-maze, the light/dark box and the open-field tests (File et al., 2004; Ennaceur and Chazot, 2016), among others. Most are based on the inherent conflict between exploration of a novel area and avoidance of its aversive features specific to each test (File et al., 2004; Cryan and Holmes, 2005).

· Elevated plus maze

EPM is one of the most widely used tests for assessing anxiety-like behaviors in rodents (Ennaceur and Chazot, 2016). The apparatus consists of a cross-shaped maze with two enclosed and two open arms elevated above the ground (File et al., 2004). Rodents naturally prefer enclosed arms but may explore open arms due to its curious character (La-Vu et al., 2020). Therefore, the percentage of time spent in open arms is the most common index to measure anxiety (File et al., 2004; Chen et al., 2024). In agreement, anxiolytic treatments (e.g. benzodiazepines) increase open-arm exploration, whereas anxiogenic compounds reduce it (Figure 16) (Chen et al., 2024). Specific details of the protocol employed in this work will be provided later in the "Materials and Methods" section. Further, additional test to evaluate anxiety are summarized in Table A2.

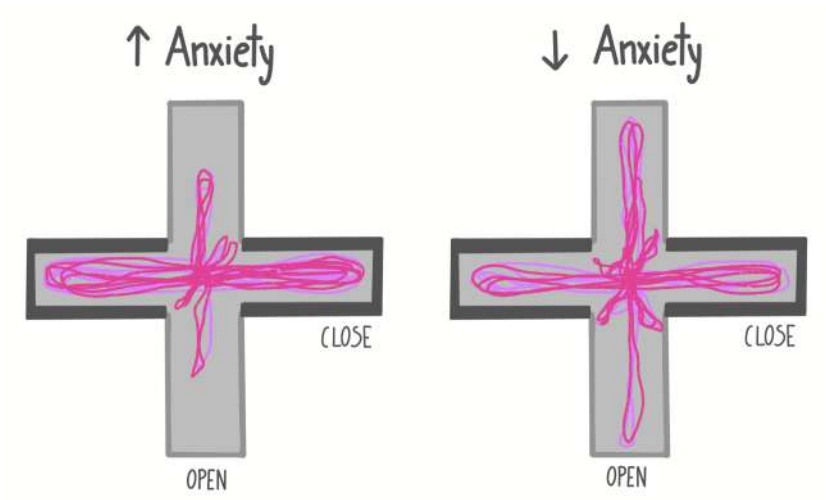


Figure 16. Illustrative description of EPM performance. *Possible movement patterns of a mice in the EPM test. Increased anxiety (left) is reflected by a preference for the closed arms, while decreased anxiety (right) is indicated by greater exploration of the open arms. The pink lines represent the mice's trajectories.*

1.C. Conditioning fear-related tests

Although an important part of fear responses are innately programmed, the process of learning is crucial to avoid similar dangerous situations in the future (Kindt, 2014). The classical fear-conditioning model is the most commonly used to study acquisition and extinction of aversive memories.

· Fear conditioning

Pavlovian fear conditioning involves an associative learning process (acquisition) in which a neutral conditional stimulus (CS; e.g. light, context or tone) is paired with an aversive unconditional stimulus (US; e.g. electric foot-shock). According to the nature of the CS, fear conditioning can be referred to as "cue" (elemental cue as tone or light) or "contextual" (environmental representation) (Figure 17) (Riebe et al., 2012). After repeated pairings (CS + US), the presentation of the CS alone elicits fear responses (expression). The main index to evaluate fear is the freezing behavior, characterized by the absence of movements except for those necessary for breathing (LeDoux, 2000). Interestingly, repeated exposure to CS in the absence of US leads to the dissociation of both stimuli (CS - US) and in a reduction in freezing behavior (extinction) (Figure 17) (Tovote et al., 2015). The ability to correctly acquire or extinguish fear memories is essential for an adaptative control of fear response. Therefore, dysregulations in both process, enhanced acquisition or resistance to extinction of fear memories, are related to the aforementioned fear-related disorders (Table 2).

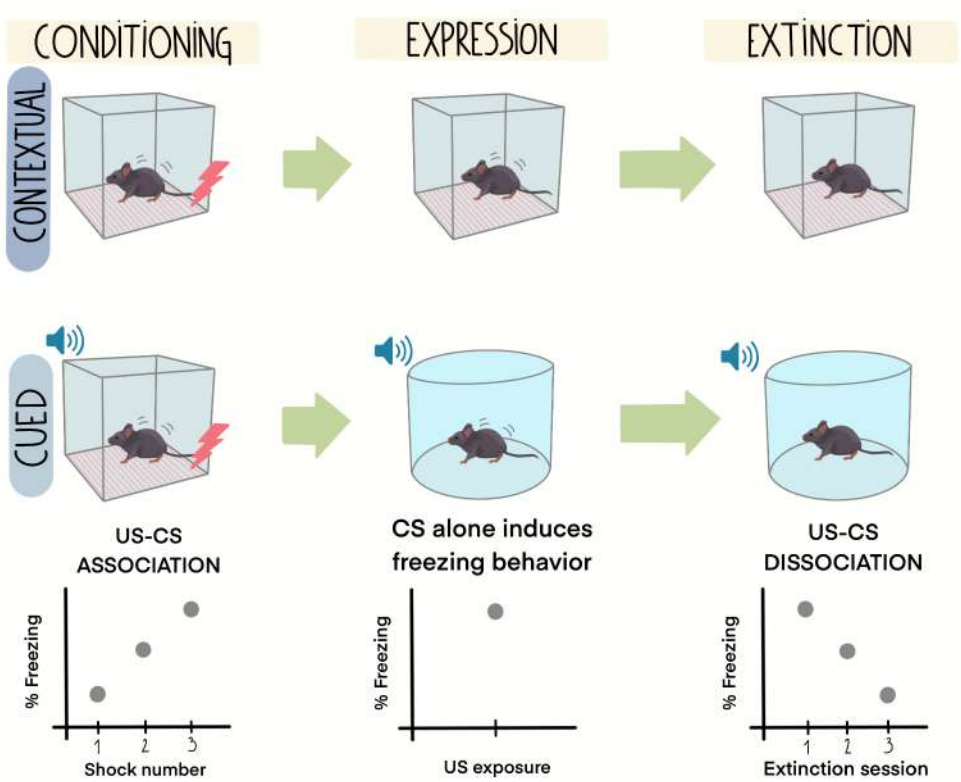


***Figure 17. Contextual and cued fear conditioning and extinction paradigms**. During the conditioning phase, a neutral conditioned stimulus (**CS**; chamber or cue tone) is paired with an aversive unconditioned stimulus (**US;** footshock). As a consequence of an associative process, a new exposure to the CS in absence of US, induces freezing behavior, which is an index of fear (expression). Interestingly, repeated exposure to CS without US progressively leads to fear extinction, expressed as a reduction in the freezing behavior. Adapted from (Flores and Berrendero, 2019).*

The endocannabinoid system has been described to play a crucial role in the correct extinction of aversive memories (Gunduz-Cinar, 2021; Gunduz-Cinar et al., 2023; Lutz et al., 2015; Mizuno et al., 2022; Mizuno and Matsuda, 2021; Ten-Blanco et al., 2022). Disrupting this system through exogenous modulation may, therefore, contribute to the comorbidity described between cannabinoid abuse and fear-related disorders (Pascual et al., 2016).

2. Behavioral models to study memory

2.A. Definition and classification of memory

Memory is a cognitive brain function by which the acquired knowledge of the world is encode, stored and later retrieved (Kandel, 2001). There are different types of memory that can be classified according to their durability or content. With respect to the time the information is available for the subject, memory can be categorized into several groups; however, in a simplified approach, it is generally divided into short-term and long-term memories. Further, long-term memories can be subdivided based on its content into declarative or non-declarative memories (Squire and Dede, 2015). Declarative memory requires a conscious process of integration of facts or events and can be defined as semantic (knowledge of general facts and concepts) or episodic memories (related to personal experiences) (Tulving and Markowitsch, 1998). During this thesis we have focused mainly on evaluating declarative memory in rodents. Conversely, non-declarative memory is the unconscious recollection of learning capacities (habits or skills) (Figure 18) (Squire and Dede, 2015).

Memory impairment is one of the most consistently reported cognitive effects of cannabis consumption (Broyd et al., 2016). However, little is known about SCBs exposure and memory deficits. Thus, animal models provide a valuable tool to further explore the long-term consequences of SCBs on memory function.

2.B. Behavioral models to assess memory deficits in rodents

Numerous behavioral tests have been developed for studying the neurobiological mechanisms of memory and learning in rodents. There are three categories of behavioral tests based on the type of memory or process evaluated: (1) recognition tests, (2) spatial memory tasks and (3) conditional learning paradigms (Lang et al., 2023). A description of the most popular test used are summarized in Table 3A.

Recognition tests are manly based on the instinct exploratory behavior, since rodents tend to explore new environments, objects and subjects acquiring novel information (Chao et al., 2022; Cohen and Stackman, 2015). Within this group we can find the Novel Object Recognition (NOR), the Novel Object Place (NOP), and the Spontaneous alternation tasks (Table A4). Concretely, in this work, the NOR was employed to explore potential memory consequences of chronic exposure to SCBs during adolescence.

· Novel Object Recognition

This recognition test is probably the most popular test for evaluating declarative memories in rodents (Cohen and Stackman, 2015). NOR is based on the natural tend that rodents have

to explore novel objects rather than familiar ones (Antunes and Biala, 2012). Commonly, in this task animals are exposed to two identical objects (training session). Then, in a delayed trial, one of the familiar object is replaced by a new different item (test session). Thus, an animal with correct memory will remember the familiar object and spend more time exploring the novel one (Figure 18) (Antunes and Biala, 2012; Cohen and Stackman, 2015). Specific details of the protocol employed along this work will be provided later in the "Materials and Methods" section. Modifications in the duration period between training and test sessions (inter trial interval; ITI) are commonly used to evaluate different memories. For studying short-term memories, both sessions should be separated for minutes to 3-4 hours. However, 24-48 hours of delay are required for long-term memory studies (Antunes and Biala, 2012; Moore et al., 2013). Although NOR is mainly performed in an open field arena, some studies have used other Y- or V-shaped mazes for reducing contextual information and increase the time of exploration (Figure 18) (Busquets-Garcia et al., 2013; da Cruz et al., 2020).

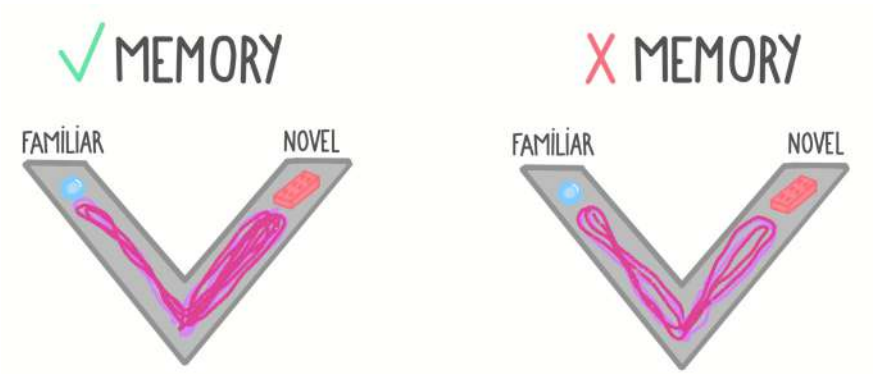


Figure 18. Illustrative representation of the NOR test performed in a V-maze. *The figure depicts the possible movement patterns of rodents during the NOR test. Successful memory (left) is indicated by a preference for exploring the novel object, while impaired memory (right) is reflected by similar exploration of both objects. The pink lines represent the trajectories of the animals.*

3. Behavioral models to evaluate psychotic-like effects

3.A. Definition of psychosis

Psychosis is a mental state characterized by a loss of contact with the reality and is composed of several symptoms (Gaebel and Zielasek, 2015). Classical texts defined psychosis as a clinical syndrome coursing with hallucinations and delusions. However, novel clinical guidelines also indicate the presence of thoughts disorders, behavioral disorganization and catatonia (DSM-5-TR, 2022; Freudenreich, 2020). Given the theme of the present thesis, we will describe this section in more detail.

Delusions are false beliefs held with conviction even in face of evidence of the contrary (Freudenreich, 2020). It is also defined as misinterpretations of reality (distorted perception of what is seen) that normally have an impossible content. In contrast, **hallucinations** are

characterized by perceptions without an objectively identifiable stimuli (Waters and Fernyhough, 2017; Corlett et al., 2019). Auditory, visual, tactile and less common, olfactory hallucinations have been described in different conditions such as schizophrenia, dementia, drug use, epilepsy, among many others (Nakamura and Koo, 2016; Corlett et al., 2019; Freudenreich, 2020). **Thought disorder**, is known as an impairment in the speech or a problem in how a patient expresses something. These symptoms sometimes are accompanied by **psychomotor abnormalities** which can be manifest as a lack of movement or the opposite, exaggerated and uncontrollable movements (Freudenreich, 2020).

Psychosis is a set of symptoms common to different disorders or medical conditions, considered primary if it is symptomatic of a psychiatric disorder or secondary if it is caused by other medical organic afflictions (Figure 19) (Griswold et al., 2015; Freudenreich, 2020). The duration of the psychosis together with the presence or absence of other symptoms are key points for a correct diagnosis (DSM-5-TR, 2022; Freudenreich, 2020).

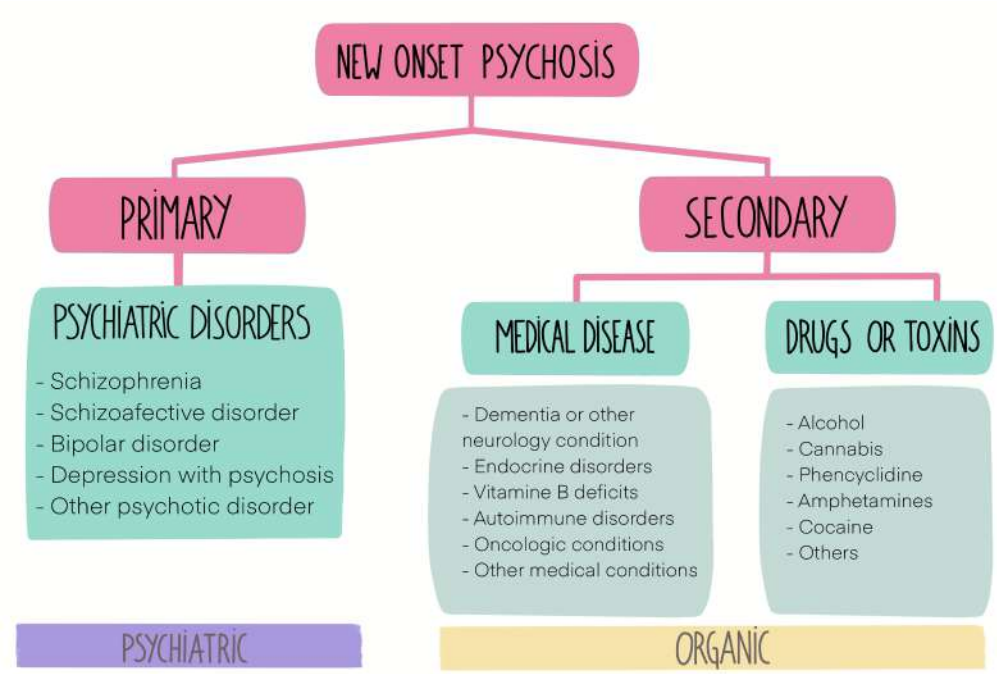


***Figure 19. Differential diagnosis of new onset psychosis**. Adapted from (Gaebel and Zielasek, 2015; Griswold et al., 2015)*

3.B. Psychotic disorders: focus on Schizophrenia

Among psychiatric disfunctions, psychotic disorders are characterized by the presence of the defined psychotic symptoms alone or in combination with others (Table 4) (Murlanova and Pletnikov, 2023). The main afflictions included as psychotic disorders are schizophrenia, schizoaffective disorder, schizophreniform disorder, brief psychotic disorder and substance/ medication-induced psychotic disorder (Table 4) (DSM-5-TR, 2022). However, other medical

non-psychotic conditions also include transitional symptoms of psychosis such as mood disorders, obsessive-compulsive disorder, posttraumatic stress disorders, neurological conditions, autoimmune afflictions, among others (DSM-5-TR, 2022; Freudenreich, 2020).

Table 4. Psychotic disorders, symptoms and duration of symptoms (DSM-5-TR, 2022)

	SUBSTANCE/ MEDICATION INDUCED PSYCHOTIC DISORDER	BRIEF PSYCHOTIC DISORDER	SCHIZOPHRENIFORM DISORDER	SCHIZOPHRENIA	SCHIZOAFFECTIVE DISORDER
Symptoms duration	During consumption or withdrawal	1 day – 1 month	1 month – 6 months	> 6 months + at least 1 month of positive symptoms	at least 2 weeks of positive symptoms
Symptoms	· Delusions · Hallucinations (**one** or both) **ICD-11-CM include:** · Alcohol · **Cannabis** · Phencyclidine · Hallucinogens · Inhalants · Sedative · Amphetamine · Cocaine	· Delusions · Hallucinations · Disorganized speech · Grossly disorganized or catatonic behavior (**one** or more)	· Delusions · Hallucinations · Disorganized speech · Grossly disorganized or catatonic behavior (**two** or more) · Negative Symptoms	· Delusions · Hallucinations · Disorganized speech · Grossly disorganized or catatonic behavior (**two** or more) · Negative Symptoms · Cognitive and personal dysfunction	· Delusions · Hallucinations · Disorganized speech (**two** or more) · Mood symptoms
Sexual dimorphism				• **Men** more prevalent. • **Women** more positive symptoms, less negative or cognitive symptoms.	• **Women** more prevalent.

- **Positive symptoms**
- **Negative symptoms**
- **Cognitive symptoms**
- **Mood symptoms**

SEVERITY AND DURATION

· Schizophrenia: prevalence and description

Schizophrenia is the most prevalent psychotic disorder affecting 1 in 100 people worldwide (DSM-5-TR, 2022; McCutcheon et al., 2020; Richetto and Meyer, 2021). Nevertheless, national data revealed higher prevalences (4.5% in men and 2.9% in women), with a greater incidence in men compared to women (BDCAP, 2020), consistent with previous literature (DSM-5-TR, 2022; Sommer et al., 2020). The schizophrenia is a disease characterized by the presence of positive (delusions, hallucinations, catatonia, etc), negative (social withdrawal, apathy, deficits in motivation and reward-related functions) and functional symptoms (interpersonal relations, or self-care) (DSM-5-TR, 2022; Freudenreich, 2020; Richetto and Meyer, 2021). In addition, cognitive impairments may also appear in these patients (Freudenreich, 2020; McCutcheon et al., 2020; Richetto and Meyer, 2021). Although the psychotic features of schizophrenia typically emerge in the late teens or early adulthood (mid-20 in men and late-20s in women), the majority of individuals manifests previous slow

and gradual signs and symptoms (prodromal phase) (Figure 20) (Selemon and Zecevic, 2015; DSM-5-TR, 2022; Freudenreich, 2020; Richetto and Meyer, 2021). Earlier first episode of psychosis is associated with a worse prognosis and consistently, as the age of onset is sex-dependent, men show lower educational achievement, more prominent negative symptoms, cognitive impairments and in general a worse outcome (DSM-5-TR, 2022).

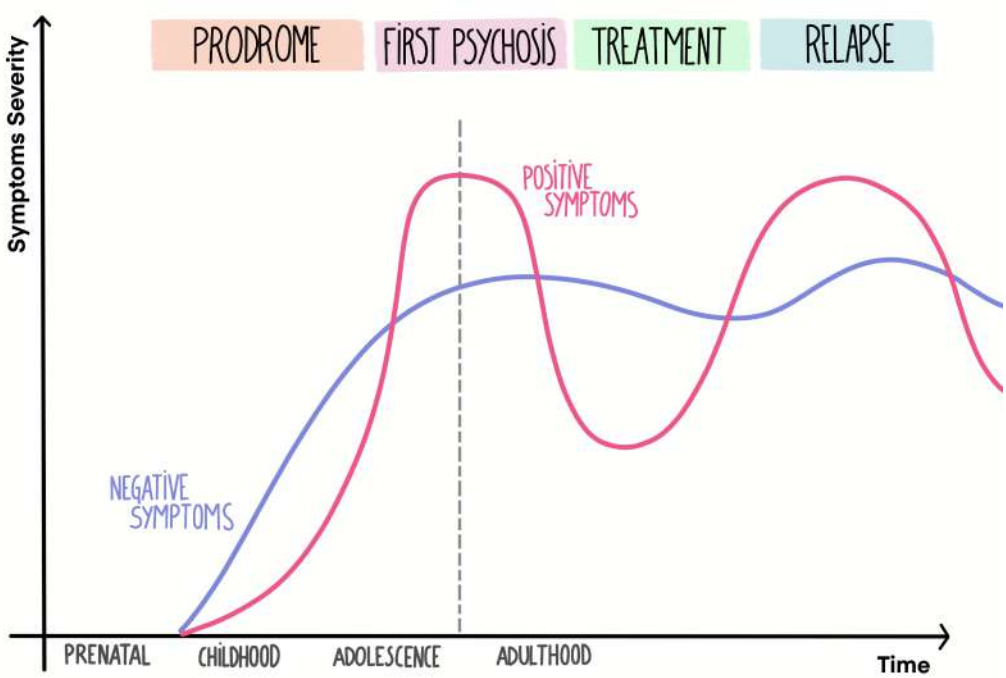


Figure 20. Typical course of schizophrenia. *The course of the disease usually begins with mild negative symptoms (prodrome phase). Once the first psychotic episode appears, pharmacological measures are usually taken to improve positive symptoms. However, relapses are very common due to the abandonment of the treatment. Adapted from (Freudenreich, 2020).*

· Etiology of schizophrenia

Like most psychiatric disorders, the etiology of schizophrenia appears to be multifactorial (DSM-5-TR, 2022; Freudenreich, 2020; McCutcheon et al., 2020; Richetto and Meyer, 2021). Studies have demonstrated that is a highly heritable disorder, with more than 100 loci significantly related to schizophrenia development (Ripke et al., 2014; Prata et al., 2019). However, the importance of environmental factors and their interactions with genetics are known to exponentially increase the risk of suffering from this psychotic disease (Figure 21) (McCutcheon et al., 2020; Richetto and Meyer, 2021).

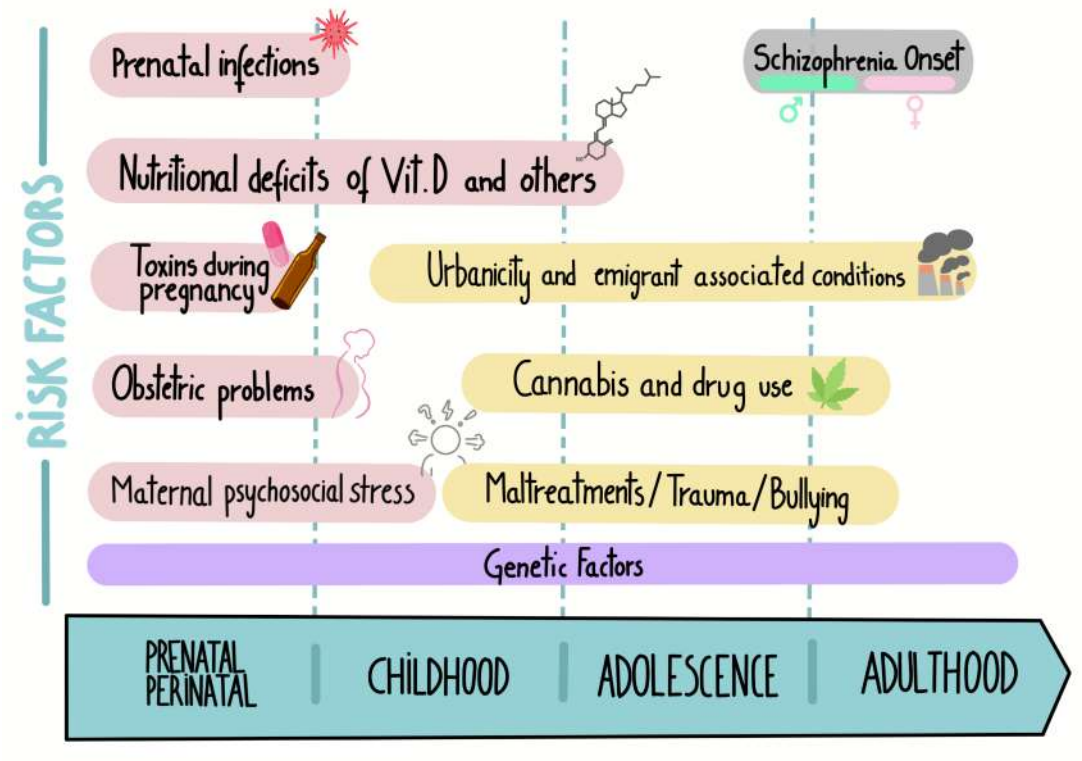


Figure 21. Environmental risk factors for the development of schizophrenia during lifetime.

Cannabis abuse has been closely associated with the development of schizophrenia and other psychotic illnesses, showing an associative ODDs ratio of 5.17 (Table 5) (Radua et al., 2018; McCutcheon et al., 2020). The use of cannabis with high THC content increases the risk to the onset of a first episode of psychosis and subsequent schizophrenia (Hasan et al., 2020; Johnson et al., 2021). Thus, this effects could be exacerbated in the case of SCBs abuse. Consistently, acute intoxication with SCBs has been shown to induced psychotic-like symptoms, and chronic exposure, psychotic-like alterations in humans (Papanti et al., 2013; Van Amsterdam et al., 2015; Fattore, 2016; Ford et al., 2017).

Although the interaction environment-genes is a key point in the course of this disease, there are increasing data supporting the importance of the stage of development in which this interaction occurs, pointing early-life stages as periods of special vulnerability (Figure 21) (Radua et al., 2018; Nettis et al., 2020; Patel et al., 2021; Stark et al., 2021; Krantz et al., 2023). This fact is of particular importance since our work focuses on adolescence as a period of heightened susceptibility to the development of psychotic-like alterations.

Table 5. Association of environmental factors with the risk of schizophrenia (Radua et al., 2018; McCutcheon et al., 2020).

RISK FACTOR	ODDS RATIO (95% CI)
Obstetric complications	1.84 (1.25-2.70)
Winter birth in the northern hemisphere	1.04 (1.02-1.06)
Childhood trauma	2.87 (2.07-3.98)
Urban living	2.19 (1.55-3.09)
Migration (first generation)	2.10 (1.72-2.56)
Cannabis use	**5.17** (3.64-7.36)

· Physiopathology of schizophrenia

Regarding the physiopathology of the disease, multiple theories are being proposed focusing on disturbances found in different neurotransmitter systems (Stępnicki et al., 2018; McCutcheon et al., 2020). The discovery of chlorpromazine as the first antipsychotic drug turned the attention on the involvement of the dopaminergic system in schizophrenia (Lau et al., 2013a; Stępnicki et al., 2018). The dopaminergic hypothesis is based on imbalance levels of dopamine, combining prefrontal hypodopaminergia responsible for cognitive and negative symptoms and striatal hyperdopaminergia associated to positive symptoms (Lau et al., 2013a; Stępnicki et al., 2018; McCutcheon et al., 2020). However, alterations in glutamatergic, serotoninergic, GABAergic and other systems have also been observed, opening enormously the field of study of this disease. (Meador-Woodruff and Healy, 2000; Goff and Coyle, 2001; Abi-Dargham, 2007; Fernandez-Espejo et al., 2009; Eggers, 2013; Zamberletti et al., 2014; Shetty and Bates, 2016). In this thesis, the contribution of the GABAergic system takes special importance since GABAergic interneurons are known to be crucial regulators in the CNS. Thus, GABA disturbances can cause imbalance between excitatory and inhibitory signaling in the PFC, a characteristic issue described in schizophrenic patients (Hashimoto et al., 2003; Shetty and Bates, 2016). Among other GABAergic alterations found in this psychotic illness, impairments in parvalbumin- (PV) containing interneurons have been widely reported (Gonzalez-Burgos et al., 2015; Lodge et al., 2009). PV is a calcium binding protein expressed in specific GABAergic interneurons that play a key role controlling gamma oscillations, simultaneously altered in schizophrenia (Gonzalez-Burgos et al., 2015). During development, the connectivity and maturation of these interneurons are regulated by the presence of perineuronal nets (PNNs), specialized regions of the extracellular matrix, which are frequently located surrounding this PV-interneurons (Alcaide et al., 2019). Interestingly, reductions in the density of PNNs in the PFC have been reported in patients with schizophrenia and other psychotic disorders (Mauney et al., 2013; Enwright et al., 2016; Alcaide et al., 2019). These and other disturbances in the PFC are strongly linked to impaired somatosensory filtering (Ellenbroek et al., 1996; Rajakumar et al., 2004; Tapias-Espinosa et al., 2023; Suzuki et al., 2024). This impairments are considered a hallmark of schizophrenia, as patients struggle to differentiate between relevant and irrelevant stimuli (Braff et al., 1978; Mcghie and Chapam, 1961; Mena et al., 2016). Prepulse inhibition of startle reflex (PPI) is a well-established method for investigating sensory gating deficits in both humans (Swerdlow et al., 2018; San-Martin et al., 2020; Sato,

2020; Mao et al., 2023) and rodents (Buccafusco, 2009; Powell et al., 2009, 2012), and has contributed enormously to better understand sensorial processing dysfunctions found in psychotic disorders (Swerdlow et al., 2016).

3.C. Prepulse inhibition

Sensorimotor gating, measured by the PPI test, is a function of the CNS that filters out irrelevant sensory information during early processing, allowing attention to focus on more salient elements of the environment (Braff et al., 1978; Shoji and Miyakawa, 2018). Specifically, PPI test measures how a weak sensorial stimuli (prepulse) can inhibit the startle response induced by a subsequent stronger stimulus (pulse) (Figure 22) (Swerdlow, 2009). Muscular responses are the most frequently measured to assess sensorimotor gating, evaluated through eye-blink reflex in humans and whole-body flinch in rodents, following an acoustic startle-eliciting stimulus (Shoji and Miyakawa, 2018; San-Martin et al., 2020, 2022). Reduced PPI is reported in schizophrenia (Braff et al., 1978; Swerdlow et al., 2018; San-Martin et al., 2020, 2022), but also in patients with other psychiatric disorders such as bipolar disorder, post-traumatic stress disorder, obsessive compulsive disorder, and others (Kohl et al., 2013; Swerdlow et al., 2016; San-Martin et al., 2022; Schulz et al., 2023). PPI has been classically used to validate animal models of schizophrenia and other psychotic disorders, as well as to investigate and develop new antipsychotic treatments. This is based on the idea that PPI deficits can be partially or totally restored through pharmacological interventions (Swerdlow, 2009; Powell et al., 2012; Shoji and Miyakawa, 2018). In addition, in this particular disease in which exposure to certain environmental factors increases the risk of developing schizophrenia, PPI serves as a crucial tool for identifying and assessing potential risk factors, including SCBs exposure.

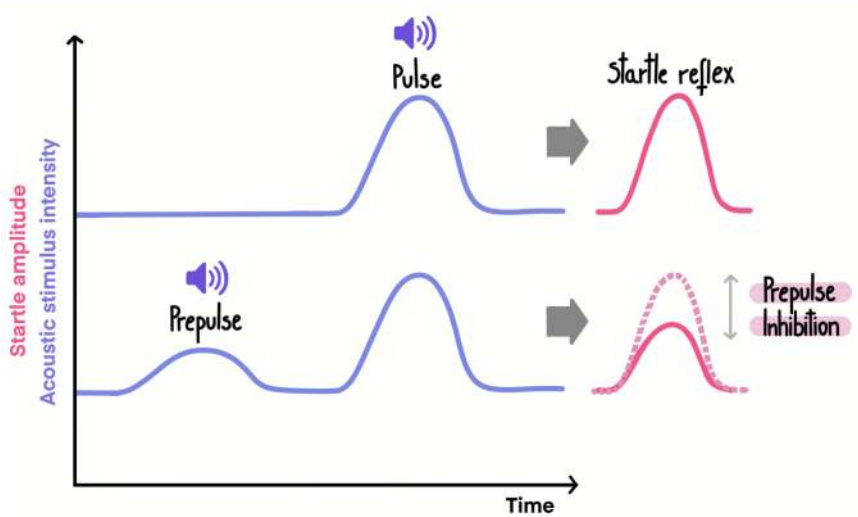


Figure 22. Prepulse Inhibition paradigm. *Innately, when a high-intensity sound pulse is presented, the organism generates a startle response. This reflex is inhibited when the pulse is preceded by a lower intensity prepulse. This reduction is known as prepulse inhibition, and greater inhibition correlates with better somatosensory filtering.*

· Neural circuit of prepulse inhibition

The brain mechanisms underlying the modulation of PPI are not completely known, although numerous studies have identified several brain areas implicated in this complex process. To delve deeper seems important to understand the basis of startle reflex. The acoustic startle reflex is a survival mechanism that triggers rapid muscular and autonomic physiological responses, alerting individuals to sudden and loud auditory stimuli (Davis et al., 1941). The primary startle reflex circuitry has been described in rats (Osen et al., 1991; Lee et al., 1996), but its modulation is not well stablished in humans (Gómez-Nieto et al., 2020). In brief, after an auditory input, cochlear root neurons innervating the caudal pontine reticular nucleus (PnC) get activated. Then, the PnC projects to different motoneurons (facial, cranial, spinal) that rapidly induce muscle contractions (Figure 23) (Lee et al., 1996; Gómez-Nieto et al., 2014). This startle reflex can be reduced when a weak non-startling stimulus (prepulse) precedes a strong acoustic startling stimulus (pulse) (Swerdlow, 2009). Since alterations in the PPI are associated with neuropsychiatric disorders, efforts have been made to map the neurobiological substrates of such motor response inhibition (Koch and Schnitzler, 1997; Fulcher et al., 2020; Gómez-Nieto et al., 2020). Although not all processes are clear, numerous brain areas are known to be involved in its modulation such as PFC, medial septum, hippocampus, nucleus accumbens, striatum, amygdala and ventral pallidum (Figure 23) (Koch and Schnitzler, 1997; Swerdlow et al., 2016; Gómez-Nieto et al., 2020).

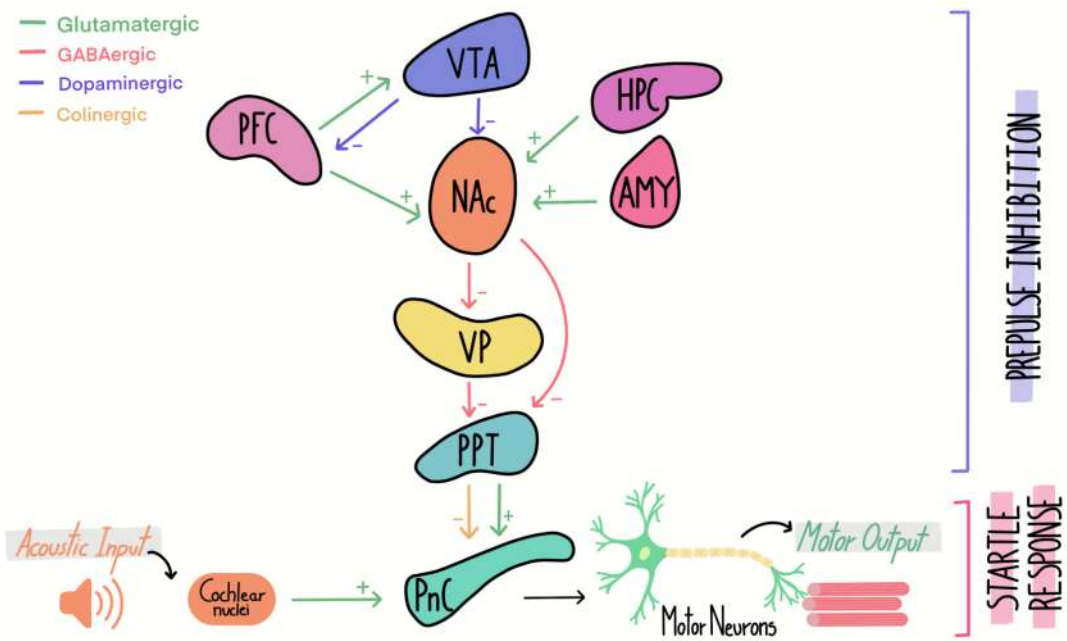


Figure 23. Acoustic startle response and prepulse inhibition reflex circuitry proposed in rodents. *PFC, prefrontal cortex; VTA; ventral tegmental area; HPC, hippocampus; AMY, amygdala; NAc, nucleus accumbens; VP, ventral pallidum; PPT, pedunculopontine nucleus; PnC, caudal pontine reticular nucleus. Adapted from (Koch and Schnitzler, 1997; Fulcher et al., 2020)*

However, all these areas converge on a common center, the pedunculopontine nucleus (PPT), which is the final responsible for the correct inhibition of the startle response when a prepulse is presented. Dopaminergic, cholinergic, GABAergic and glutamatergic systems have been found to be involved in the PPI, intervening in different areas (Figure 23) (Koch and Schnitzler, 1997; Swerdlow et al., 2016; Fulcher et al., 2020). Therefore, homeostatic balance of these systems seems essential for appropriate PPI. The biological significance of PPI impairments remains unclear, so further research is needed to fully understand the physiology and pathophysiology underlying PPI deficits.

· Performing prepulse inhibition in rodents

Evaluating somatosensory filtering in rodents has been very useful since there is a high degree of homology in PPI measures compared with humans (Swerdlow, 2009). Therefore, to evaluate the animals ability to inhibit a startle response when a prepulse is presented, the rodent is placed in chamber equipped with a sensor that detects animals movements (Valsamis and Schmid, 2011). For assessing PPI, white noise is used as auditory stimulus, with different intensities selected for each event. All the events are randomly presented given that PPI is not a form of conditioning (Swerdlow, 2009). Rodents are exposed to pulse alone events (110-120 dB) which evoked startle responses; prepulse alone events (60-85 dB) that should not induce any muscular reaction and also to prepulse + pulse events in which an inhibition of the startle reflex may occur. This inhibition, represented as a reduction in the amplitude of the muscular reflex (Figure 22), is used to evaluate sensorimotor gating. Variations on the basic PPI protocol are common, so specific details of the procedure employed in this thesis are well described in the methodology section. The time interval between the prepulse and the pulse emission can vary (20-300 ms), although this variable holds more significance in human studies than in rodents. In humans, automatic inhibition occurs during the initial 30-60 ms, whereas inhibition in longer intervals (120-140 ms) appears to be sensitive to attentional mechanisms and conscious manipulation (Swerdlow, 2009). Interestingly, PPI responses can vary in age- and sex-dependent manner. In both humans and rodents, PPI ability is higher in middle adulthood than in adolescence or old age, reaching maximal levels by the end of the second decade in humans (Swerdlow, 2009; Shoji and Miyakawa, 2019; de Oliveira et al., 2023). On the other hand, sexual differences are also found in PPI, with higher inhibition percentages described in men or male compared with woman or female rodents (Aasen et al., 2005; Naysmith et al., 2022). The influence of the female hormonal cycle on PPI could explain part of these differences, as high estrogen levels have been shown to impair inhibitory capacity in both humans and rodents (Koch, 1998; Jovanovic et al., 2004). All of these data underscore the importance of assessing sexual variability in response to potential PPI alterations, such as those induced by SCBs exposure, a topic explored throughout this thesis.

3.D. Other behavioral models for evaluating schizophrenia

Alterations in somatosensory filtering, measured by the PPI, are a common characteristic of patients with schizophrenia. However, this alteration is primarily associated with the positive symptoms of the disease. However, negative or cognitive symptoms which are the main barrier to proper functioning in schizophrenic patients (Freudenreich, 2020), can be evaluated in animal models by using different combinations of behavioral assays (see Table A4) (Ellenbroek and Cools, 2000; Ang et al., 2020). Negative symptoms include anhedonia (inability to experience pleasure from positive stimuli), avolition (decreased goal-directed motivational behavior), asociality (social withdrawal), and blunted affect (diminished facial and emotional expression), all close related to core symptoms of depression (Ellenbroek and Cools, 2000; Ang et al., 2020; Freudenreich, 2020). Therefore, an approach to evaluate negative symptoms is to use behavioral models of depression (Table A4). In addition, cognitive symptoms of the disease can be measure by using different task in which the PFC is involved (Lesh et al., 2011), such as working memory, attention and visual memory and other learning tests (Table A4) (Ang et al., 2020).

III RISK FACTORS INVOLVED IN THE HARMFUL EFFECTS ASSOCIATED WITH CANNABINOID USE

As previously mentioned, exogenous modulation of the endocannabinoid system can lead to the appearance of various long-term health problems, including psychiatric and cognitive disorders. There are risk factors that have been shown over the years to increase and potentiate the risk of suffering the harmful consequences of cannabinoid consumption. The frequency and amount of consumption, early-life exposure, high THC content and biological sex are the main risk factors linked to impairments after cannabinoid use (Rubino and Parolaro, 2015, 2016). Specifically, adolescence and sex-vulnerability are the predisposing factors that will be described in detail next due to their relevance in this thesis.

1. Adolescence as a period of vulnerability

Adolescence, derived from the Latin *adolescere* (meaning 'to grow up'), is a crucial developmental period between childhood and adulthood, marked by significant behavioral and cognitive changes (Spear, 2000). During this time, individuals acquire adult behavioral abilities, experience increased social interactions and show heightened novelty-seeking and risk-taking (Schneider, 2013; Peters and Naneix, 2022). Although adolescence is observed in both humans and rodents, the exact boundaries between periods remain difficult to define (Spear, 2000; Schneider, 2013). Considering the interindividual variability, also influenced by sex, adolescence is defined as spanning from age 10 to the mid-20s in humans (Figure 24) (Blakemore, 2012; Peters and Naneix, 2022). In rodents, adolescence starts around post-natal day (PND) 20-30 and lasts until PND 60-70 (Figure 24) (Schneider, 2013; Peters and Naneix, 2022), although there are some differences in the boundaries between rat and mice and also among males and females (Schneider, 2013).

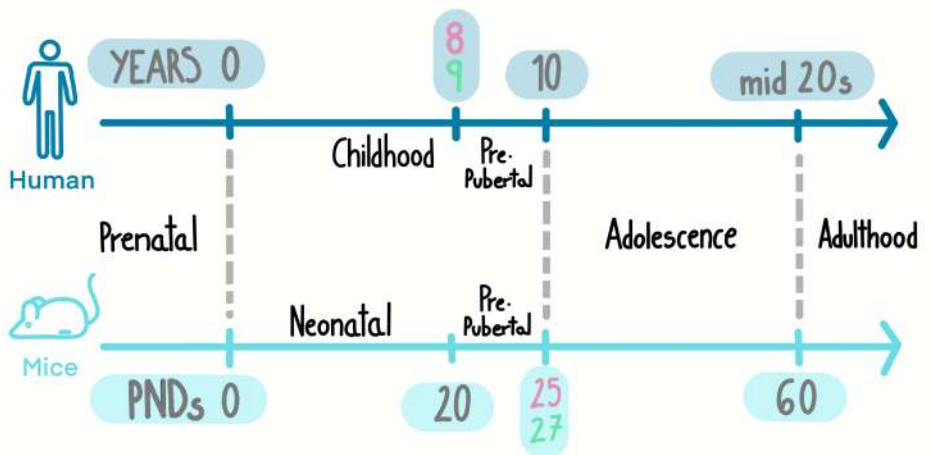


Figure 24. Approximate developmental boundaries for humans and mice. PND, post-natal day. *The ages colored in green correspond to males and those in pink to females. Adapted from (Schneider, 2013).*

At a neurobiological level, the brain of adolescents undergoes dramatic changes, including a l**oss of gray matter** and, conversely, an **increase in white matter** (Figure 25) (Giedd et al., 1999; Rubino and Parolaro, 2016; Peters and Naneix, 2022). This pattern of change is area- and time-dependent, with earlier changes occurring in more primitive areas, while frontal and temporal reorganization take place at later stages (Sowell et al., 1999; Gogtay et al., 2004; Blakemore, 2012). In early adolescence, there is usually an overproduction of axons and synapses, followed by a rapid pruning phase in later adolescence (Schneider, 2013; Rubino and Parolaro, 2016; Peters and Naneix, 2022). Therefore, connections and communication between cortical and subcortical areas are in a state of transition during this period, contributing to the formation of more efficient neural networks. Interestingly, this maturation and refinement of the circuitry connectivity has been reported to occur mainly in the PFC (Drzewiecki and Juraska, 2020; Kolk and Rakic, 2022), but also in the amygdala, striatum and thalamus (Rubino and Parolaro, 2016). In addition, the excitatory-inhibitory balance is greatly different in adolescence compared with adulthood (Kilb, 2012; Rubino and Parolaro, 2016). Specifically, prefrontal **GABAergic system matures** across adolescence in both humans and rodents, contributing enormously to enhance the inhibitory function (Figure 25), particularly important in this area (Kilb, 2012; Drzewiecki and Juraska, 2020). Thus, maturation of fast-spiking inhibitory interneurons expressing PV occurs during this period in the PFC (Juraska and Drzewiecki, 2020; Gibel-Russo et al., 2022). As already mentioned, the contribution of the PNNs is essential for the correct maturation of this inhibitory system (Figure 25) (Kilb, 2012; Paylor et al., 2016; Reichelt et al., 2019), an extracellular matrix that simultaneously increases in density from adolescence to adulthood in the PFC of both male and female rodents (Figure 25). (Baker et al., 2017; Drzewiecki et al., 2020; Delevich et al., 2021).

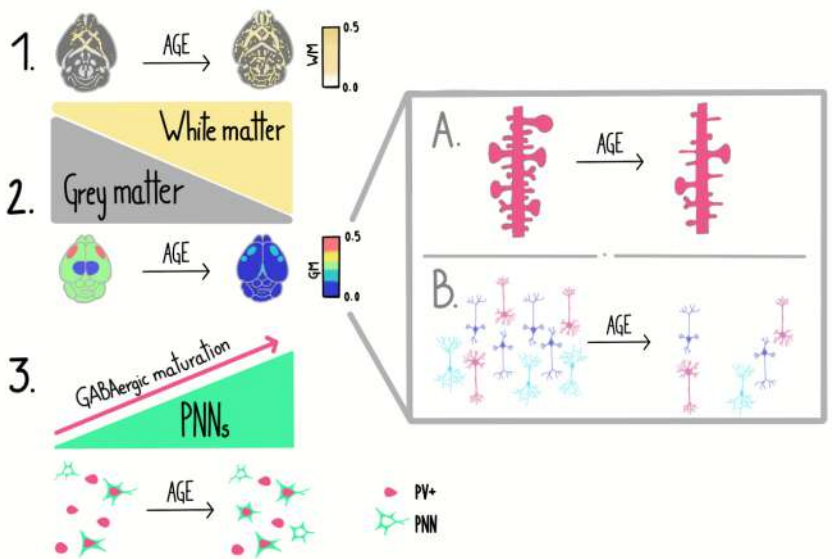


Figure 25. Summary of the main brain changes occurring during adolescence. *Throughout adolescence, brain experiences several changes including: 1. increase in white matter (WM), 2. reduction of grey matter (GM) by synaptic (A) and neuron pruning (B) and 3. increase in perineuronal nets (PNNs) contributing to the maturation of the GABAergic system.*

On the other hand, it is important to emphasize the involvement of the endocannabinoid system in the maturation of the CNS during adolescence, which entails transitory changes in both the distribution and quantity of the different components of the endocannabinoid system as explained previously (see Figure 5) (de Fonseca et al., 1993; Fernández-Ruiz et al., 1999, 2000).

The harmonious sequence of these dynamic changes is essential for achieving a well-formed and functional adult brain. Therefore, any external interference in these critical developmental systems may serve as a risk factor for mental health issues. Thus, in addition to the vulnerability that adolescence brings, disruptions in the endocannabinoid system through external modulation by SCBs, will further increase the risk of developing psychiatric and cognitive disorders later in life.

2. Sex-dependent vulnerability

2.A. Puberty: a key point in neuronal development

Although puberty is part of the adolescence, is defined as the acquisition of reproductive competence, and represents a process of vital importance for various species (Schneider, 2013). An increase in pulsatile gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus initiates puberty, leading to gonadal maturation, steroid hormone release, and ultimately sexual maturity (Schneider, 2013). Notably, similar to humans, female rodents complete sexual maturity earlier than males (Figure 26). In female mice, puberty is described to occur between PND 22-35, whereas in males take place from PND 26 to PND 40 (Schneider, 2013).

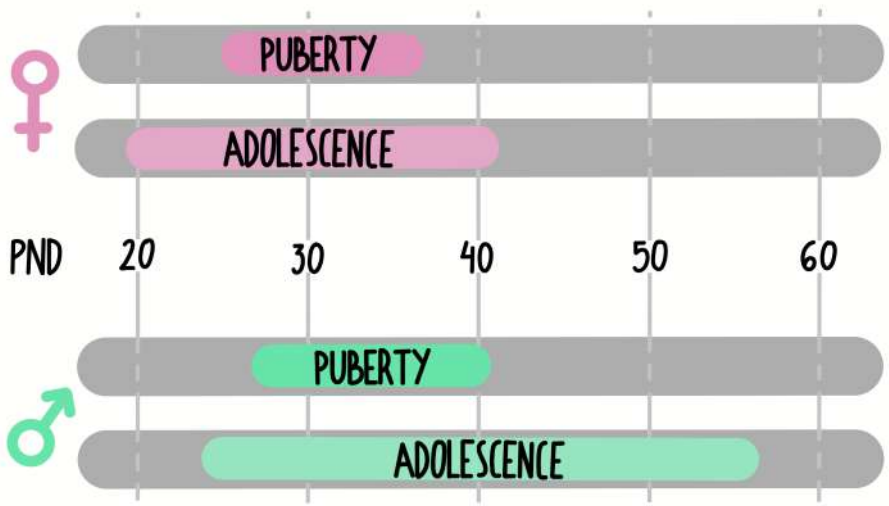


Figure 26. Schematic representation of time boundaries of puberty and adolescence between male and female mice. *PND, post-natal day.*

The process differs completely between sexes. In females, it is marked by the opening of the vagina and the first ovulation, while in males, a rise in testosterone levels results in preputial separation (Schneider, 2013). Numerous neurodevelopmental changes occur during puberty, directly linked to the presence of steroid hormones (Figure 27) (Schneider, 2013; Delevich et al., 2021). For example, testosterone levels have been related to increased axonal caliber contributing to the augmentation in white matter volume described during adolescence (Perrin et al., 2008). Synaptic pruning has been found to coincide with puberty onset in rats, occurring earlier in females (PND 35) than in males (PND 40) (Figure 27) (Drzewiecki et al., 2016; Delevich et al., 2021). Moreover, gonadal manipulation can block and alter spine pruning during adolescence (Delevich et al., 2021), suggesting that PFC pruning is influenced by sexual hormones. In addition, loss of entire neurons (neuronal pruning) also contributes to gray matter thinning in the PFC during adolescence (Markham et al., 2007) and is described to occur in a sex-dependent manner (Delevich et al., 2021). Female rats, but not males, have been reported to experience dramatic neuron loss in the PFC between PND 35 and PND 45 (Figure 27) (Willing and Juraska, 2015). Interestingly, prepubertal gonadectomy reverses this neuronal pruning in females, while in males no effect was observed (Koss et al., 2015), suggesting that pubertal hormones drives physiological neuron loss in female rats (Delevich et al., 2021). Regarding the maturation of inhibitory neurotransmission in the PFC, it has been described that ovarian hormones enhance this process specifically in layer II/III of the PFC, whereas ovariectomy procedure blocked this process in female mice (Piekarski et al., 2017). Further, PV interneurons in the PFC express estrogen receptor beta in both male and female rats, being therefore susceptible to hormonal modulation (Blurton-Jones and Tuszynski, 2002). In both sexes, PNNs coincidentally increase after the onset of puberty (Figure 27), suggesting the involvement of gonadal hormones in this developmental progression (Baker et al., 2017; Drzewiecki et al., 2020). However, female rats have been described to show a special pattern of expression, since a transient increase of PNN in prelimbic area of the PFC is observed in PND 35 (Figure 27) (Drzewiecki et al., 2020). Moreover, PNNs are differentially altered in prelimbic and infralimbic regions of the PFC following sex-specific patterns in rats, suggesting that prelimbic and infralimbic areas respond differentially to gonadal hormones at pubertal onset (Gildawie et al., 2020). Therefore, this sex-dependent neurobiochemical differences during development could make males and females differentially susceptible to external perturbations, producing unique psychiatric and cognitive alterations later in life. Moreover, sexual differences have also been described in the endocannabinoid system, as well as in the behavioral effects of exogenous cannabinoids modulation such as SCBs, a topic discussed below.

Figure 27. Schematic representation of biochemical changes occurring in the prefrontal cortex linked to puberty onset in mice. *OVX, ovariectomy; PND, post-natal day; PNNs, perineuronal nets. Adapted from (Juraska and Drzewiecki, 2020; Delevich et al., 2021)*

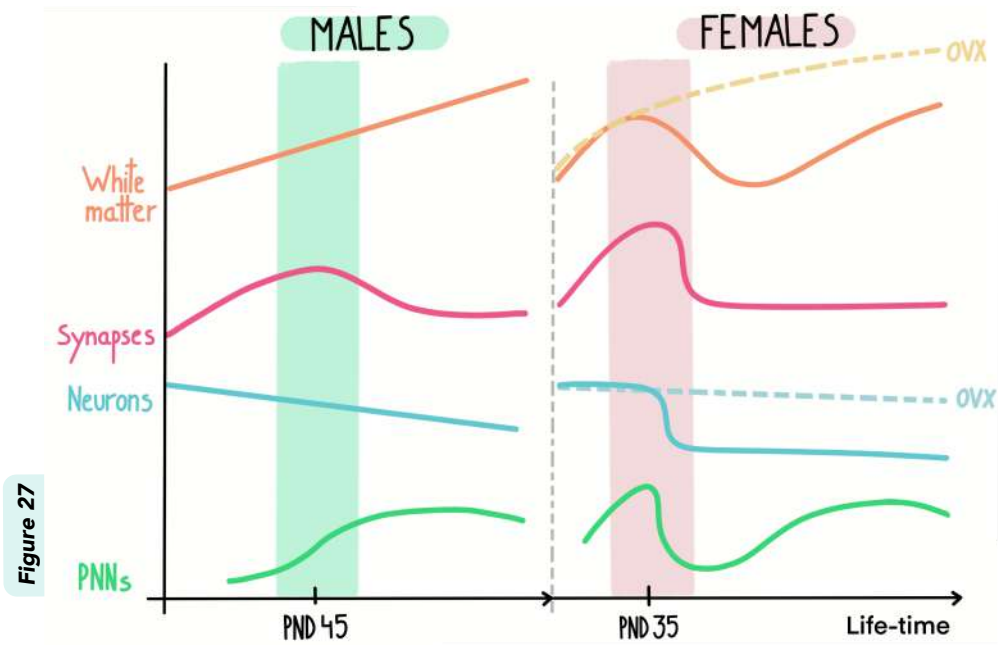


Figure 27

2.B. *Sexual dimorphisms in the endocannabinoid system*

The endocannabinoid system plays a significant role in adolescent neurodevelopment, and, interestingly, differences in the expression and activity of this system have been observed between males and females (Ginder et al., 2022; Bernabeu et al., 2023). *Marco et al 2014* described increased mRNA levels of *Cnr1*, *Trpv1*, *Faah*, *Magl*, *Dagl* (*α* and *β*) and *Nape-pld* in the PFC of adolescent female rats, despite, *Magl*, *Dagl*α and *Nape-pld* in amygdala were found to decrease in comparison to male rats (Marco et al., 2014). It is also widely reported that females show lower levels of CB1R in most brain areas, although its functionality and activity have been described to be greater than in males (Mateos et al., 2011; Llorente-Berzal et al., 2013a). Further, CB1R expression and functionality are reported to fluctuate during the estrus cycle of female rats, but also after gonadectomy and steroid replacement (de Fonseca et al., 1994; Mize and Alper, 2000), which could contribute to the different behavioral long-term effects found between males and females after cannabinoid exposure (Fattore and Fratta, 2010; Dow-Edwards et al., 2016; Ginder et al., 2022). Moreover, gonadal hormones also regulate CB1R density and transcription in male rats (González et al., 2000; Busch et al., 2006), supporting a CB1R sexual dimorphic functionality. Preclinical studies also showed hormone-dependent neurobiochemical responses to the administration of exogenous cannabinoids, suggesting the importance of gonadal hormones in the appearance of harmful consequences (Gorzalka et al., 2010; Winsauer et al., 2011; Craft et al., 2013; Wagner, 2016). However, most of the studies evaluating the impact of cannabinoid exposure are conducted exclusively in one sex (male or female), without directly comparing both sexes. This fact makes challenging to determine sex-specific vulnerabilities and makes it difficult to draw resounding conclusions.

BEHAVIORAL IMPAIRMENTS AFTER CANNABINOID EXPOSURE IN ANIMAL MODELS IV

Considering the different risk factors mentioned above, the main alterations observed in preclinical studies in rodents after acute and chronic exposure to cannabinoids will be now described. Given the complexity of this topic, studies carried out with THC have been included, although the main focus will be on SCBs effects. In agreement with the epidemiological data, preclinical studies suggest that the main alterations observed after cannabinoid exposure are psychiatric and cognitive impairments (Rubino and Parolaro, 2015, 2016; Fattore, 2016; Lafaye et al., 2017). In addition, both clinical and preclinical data emphasize the importance of the developmental period of exposure as a key factor for the onset of harmful and long-lasting effects (Schneider, 2013; Rubino and Parolaro, 2015, 2016). However, sexual dimorphisms in cannabinoid-induced alterations should be also take into account (Rubino and Parolaro, 2015; Cooper and Craft, 2018). To further explore these behavioral impairments, data from various studies involving acute and chronic cannabinoid administration have been collected in Tables A6, A7 and A8 and the main conclusions will be described below. Tables are located in annex I.

1. Anxiety-like effects

Conflicting data related to anxiety effects induced by cannabinoids have been observed over the years (Table A6). These contradictory results could be due to various factors, including experimental conditions and protocols, treatments, dosage, animal strain and others. In this section, studies assessing different anxiety test have been included, such as EPM, open field, light/dark box and marble burying.

1.A. Acute exposure to cannabinoids on anxiety behavior

Notably, acute THC exposure is known to influence anxiety in a biphasic dose-dependent manner. Thus, low doses induced anxiolytic effects, whereas high doses seems to be anxiogenic (Petrie et al., 2021a). On the other hand, SCBs acute administration has yielded contradictory results, with some studies reporting anxiolytic effects (Patel and Hillard, 2006; Li et al., 2019; Pineda Garcia et al., 2024), while others observed anxiogenic responses at equivalent doses (Arévalo et al., 2001; Pineda Garcia et al., 2024). Therefore, drawing a definitive conclusion about the acute effects of SCBs on anxiety remains challenging, however the evidence suggests that SCBs could modulate anxiety responses.

1.B. Chronic exposure to cannabinoids on anxiety behavior

Despite the important involvement of the endocannabinoid system in the modulation of emotional processing, long-term effects of chronic THC exposure during adolescence on anxiety are not entirely consistent. Most studies showed no alterations on anxiety-like behaviors in adult rodents after chronic adolescent exposure to THC (Iemolo et al., 2021;

Kasten et al., 2017; Petrie et al., 2021b; Rubino, Vigano', et al., 2008). However, the administration of SCBs offer mixed results. As with THC, some studies have found no long-term alterations following adolescent exposure to SCBs (Higuera-Matas et al., 2009; Llorente-Berzal et al., 2011a; Mateos et al., 2011; Aguilar et al., 2017; Farinha-Ferreira et al., 2022; Gonçalves et al., 2023). However, chronic administration of indole-derived compounds (JWH-018 or WIN 55,212-2) during adolescence has been shown to induced anxiety-like alterations in adulthood (Margiani et al., 2022; Schneider et al., 2005). These findings could suggest that adolescent SCBs exposure elicit a more potent long-term impact on anxiety than THC. Moreover, chronic SCBs exposure during adulthood have been shown to induced anxiety-like responses in male rodents (Hill and Gorzalka, 2006; MacRì et al., 2013; Pintori et al., 2021). These results indicate that adolescence is not necessarily a period of vulnerability for anxiety-like alterations induced by chronic SCBs exposure.

Regarding sex, most reports evaluating both sexes show equivalent behavior responses on anxiety tests between males and females after adolescent exposure to THC or SCBs. However, the majority of them did not find alterations on anxiety after cannabinoid exposure (Biscaia et al., 2003; Higuera-Matas et al., 2009; Iemolo et al., 2021; Llorente-Berzal et al., 2011a; Rubino, Vigano', et al., 2008). On the other hand, most studies reporting anxiety alterations after cannabinoid adolescent exposure have been conducted exclusively in males (Schneider et al., 2005; Frontera et al., 2018; Margiani et al., 2022). This makes it difficult to determine whether one sex is more vulnerable than the other to anxiety-related consequences following cannabinoid exposure. Therefore, further research is needed to draw more definitive conclusions in this regard.

2. Memory impairments

One of the clearest associations described in animal models after acute or chronic exposure to cannabinoids is the appearance of memory deficits (Table A7). This effect is observed in most of the studies regardless of the test employed, the ITI, the specie and strain. Since this thesis focused solely on recognition memory, only studies assessing this specific type of memory were considered, including NOR, NOP, and Y-maze.

2.A. Acute exposure to cannabinoids on memory

Consistently, acute administration of both THC and SCBs have been shown to impair short- and long-term recognition memory (Barbieri et al., 2016; Ito et al., 2019b; Schreiber et al., 2019; Corli et al., 2023a, 2023b). However, while defining an exact dose equivalence between THC and different SCBs is difficult, synthetic compounds have been shown to induce cognitive deficits at significantly lower doses than THC. In addition, it is worth noting that acute administration of JWH-018 and AB-FUBINACA has been found to disrupt recognition memory, a key aspect for this thesis (Li et al., 2019; Schreiber et al., 2019; Corli et al., 2023b). These findings suggest that acute cannabinoid administration disrupts memory, with SCBs exerting these effects at lower doses.

2.B. *Chronic exposure to cannabinoids on memory*

Long-lasting cognitive impairments after chronic adolescent exposure to THC are highly consistent in both humans and rodents. In agreement, recognition memory deficits have been observed in adult rodents after THC adolescent exposure (Llorente-Berzal et al., 2013b; Zamberletti et al., 2015; Renard et al., 2017b; Iemolo et al., 2021; Poulia et al., 2021; De Felice et al., 2023). With respect to SCBs, most reports have demonstrated memory alterations in adulthood after chronic adolescent treatment, regardless of strain, drug, memory paradigm, administration protocol and washout period (Mateos et al., 2011; Abush and Akirav, 2012; Renard et al., 2013; Aguilar et al., 2017; Kevin et al., 2017). Specifically, AB-FUBINACA chronic exposure during adolescence has been reported to alter recognition memory in adult male rats, an issue examined in this thesis (Kevin et al., 2017). On the other hand, adult chronic treatment with SCBs have been reported to preserve recognition memory, suggesting that adolescence is a period of vulnerability to long-term cognitive deficits induced by cannabinoids (O'Shea et al., 2004; Renard et al., 2013; Schneider et al., 2008).

Regarding potential sex differences, numerous studies have observed memory impairments in both male (Schneider et al., 2008; Mateos et al., 2011; Abush and Akirav, 2012; Renard et al., 2013, 2017b; Aguilar et al., 2017; Kevin et al., 2017; Iemolo et al., 2021; Poulia et al., 2021; De Felice et al., 2023) and female rodents after adolescent cannabinoid exposure (O'Shea et al., 2004; Mateos et al., 2011; Llorente-Berzal et al., 2013b; Zamberletti et al., 2015; Iemolo et al., 2021). Therefore, based on the available data, both sexes appear to be susceptible to the negative consequences of cannabinoids on memory.

3. Psychotic-like effects

The association between acute or chronic exposure to cannabinoids and the development of psychotic alterations is widely reported at epidemiological and preclinical level. Adolescence and high cannabinoid use seem to be crucial factors in the onset of new psychosis, which could potentially lead to schizophrenia later in life. In this section, we will discuss the potential pro-psychotic effect of cannabinoids based on different studies assessing the PPI model (Table A8).

3.A. *Acute exposure to cannabinoids on psychotic-like symptoms*

High doses of THC have been shown to impair sensorimotor filter, but only when the acute dose is given to adolescent or young adult rodents (Nagai et al., 2006; Busquets-Garcia et al., 2017). In contrast, SCBs acute exposure impairs PPI in adult rodents even at low doses (Bilel et al., 2020; Corli, et al., 2023a; Martin et al., 2003; Schneider and Koch, 2002). These data suggest that acute SCBs have a greater pro-psychotic potential than THC, regardless of the dose and age of exposure.

3.B. Chronic exposure to cannabinoids on psychotic-like symptoms

With respect to the long-lasting effects of chronic exposure to THC during adolescence on PPI, most of the studies revealed no effect (Long et al., 2010; Silva et al., 2016; Renard et al., 2017a; Ibarra-Lecue et al., 2018; Poulia et al., 2021; Moreno-Fernández et al., 2024). However, there are also recent reports that have found PPI impairments in adult rodents after adolescent THC treatment (Abela et al., 2019; Iemolo et al., 2021; Lamanna-Rama et al., 2024). Interestingly, these studies observing PPI deficits used high doses of THC, indicating a potential dose-dependent harmful effect (Abela et al., 2019; Iemolo et al., 2021; Lamanna-Rama et al., 2024). On the contrary, SCBs adolescent exposure frequently alters PPI in adulthood regardless of the drug, dose and species (Abboussi et al., 2020; Aguilar et al., 2017; Llorente-Berzal et al., 2013a; Schneider et al., 2005; Schneider and Koch, 2003; Wegener and Koch, 2009). In addition, adult chronic exposure to different SCBs did not induce long-term PPI alterations (Gleason et al., 2012; Klug and van den Buuse, 2012; Pintori et al., 2021; Schneider and Koch, 2003), suggesting an age-dependent effect of SCBs on PPI disruptions. In contrast, long-lasting PPI impairments were observed following high-dose JWH-018 exposure in adulthood (Bilel et al., 2023), whereas chronic low-dose did not produce PPI deficits in adult rats (Pintori et al., 2021), highlighting a possible dose-dependent effect of JWH-018 in adult rodents.

Concerning sexual dimorphisms, although PPI impairments in both males (Schneider and Koch, 2003; Schneider et al., 2005; Wegener and Koch, 2009; Aguilar et al., 2017; Abela et al., 2019; Abboussi et al., 2020; Lamanna-Rama et al., 2024) and females (Llorente-Berzal et al., 2011b; Iemolo et al., 2021) have been reported after cannabinoid exposure (both THC and SCBs) during adolescence, there are few studies that make direct comparison between sexes. Therefore, it can be concluded that both sexes may be susceptible for the psychotic-like effects induced by cannabinoids. However, when males and females are directly compared within the same study, females exhibit greater susceptibility to the negative consequences of cannabinoids in the PPI test (Llorente-Berzal et al., 2011b; Iemolo et al., 2021). However, further research will be necessary to better define potential sexual vulnerabilities.

As a whole, acute SCBs exposure alters anxiety, memory and PPI in the different studies reviewed. In addition, studies evaluating the long-lasting effects of chronic SCBs exposure during adolescence primarily report memory deficits and psychotic-like alterations. Additionally, anxiety-like alterations have also been documented, albeit less extensively. Moreover, memory and PPI impairments appear to be exposure-age dependent, occurring only when administration takes place during adolescence and not in adulthood. Finally, studies evaluating sexual vulnerability to cannabinoid effects are inconclusive, so further research is required to reach definitive conclusions. Regarding THC studies, the findings suggest that SCBs elicit more frequent and pronounced harmful effects compared to THC, representing a major public health problem.

2

Objective

General objective

It is well established that cannabis use during adolescence can lead to psychiatric and cognitive disorders later in life. The emergence of new SCBs represents a significant risk, particularly for adolescents who show the highest rate of SCBs use, potentially impacting in several neurodevelopmental processes and behaviors. Therefore, the main objective of the present thesis is to evaluate the neurobiological consequences of chronic SCBs use during adolescence, as well as to assess potential sex differences in the behavioral and biochemical alterations found.

Specific objectives

1. Objective 1. To study potential effects of exposure to JWH-018, the first SCB identified in Spice/K2 preparations.

a. To evaluate short- and long-term consequences of adolescent exposure to JWH-018 on anxiety-, fear- and psychotic-related disorders considering sexual dimorphisms.
b. To explore the potential neurobiological mechanisms related to the behavioral impairments previously observed after adolescent JWH-018 exposure.

This objective was successfully achieved and published in Translational Psychiatry:
"Adolescent exposure to the Spice/K2 cannabinoid JWH-018 impairs sensorimotor gating and alters cortical perineuronal nets in a sex-dependent manner" (Izquierdo-Luengo et al., Translational Psychiatry, 2023, 13 (1): 176).

2. Objective 2. To elucidate potential alterations after AB-FUBINACA exposure, as a representative of the recent SCBs found in Spice/K2 preparations.

a. To evaluate long-term consequences of adolescent exposure to AB-FUBINACA on anxiety-, fear-, cognitive-, social-, depressive- and psychotic-related disorders considering sexual differences.
b. To study potential neurobiological mechanisms related to the behavioral alterations observed after adolescent AB-FUBINACA exposure.

This objective was successfully achieved and published in iScience:
"Long-term consequences of adolescent exposure to the synthetic cannabinoid AB-FUBINACA in male and female mice" (Izquierdo-Luengo et al., iScience, 2025, 28 (2): 111857).

3

Materials & Methods

EXPERIMENTAL DESIGNS I

1. SCBs treatment in adolescent mice

1.A. JWH-018 treatment - Objective 1

To evaluate the short- and long-term effects of the exposure to JWH-018 during adolescence on locomotion, anxiety-like behavior, cued fear conditioning and extinction and prepulse inhibition of the startle reflex in both male and female mice 4 batches of each sex were used as shown in the Figure 28A. Although the temporal boundaries of adolescence are not exactly defined neither in humans nor in rodents (Brust et al., 2015), based on previous studies (Aguilar et al., 2017; Biscaia et al., 2003; Saravia et al., 2019; Zamberletti et al., 2015), mice were treated intraperitoneally (i.p) with increasing doses of JWH-018 (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) or vehicle in order to avoid drug tolerance for 15 days. Short- and long- term effects were analyzed 5 (PND 54) and 20 (PND 69) days respectively after the end of the treatment, as described in Figure 28A. The interval of time between adolescent treatment and the different behavioral assays was based on previous reports (Ibarra-Lecue et al., 2018; Saravia et al., 2019). Different cohorts of animals were used for the experiments of locomotion, anxiety and fear extinction (males, n = 15, short-term, n = 14–16, long-term; females, n=10–11, short-term, n=13–15, long-term), and for the experiments of PPI (males, n=10–16, short-term, n=11–17, long-term; females, n = 11–12, short-term, n = 17–18, long-term). Tissues were obtained 24 h after the PPI test to carry out biochemical experiments in male and female mice. For short-term, an additional experimental batch was used to complete the number of mice required. For immunofluorescence experiments, the number of mice was 5–7 (males, short-term), 5–7 (males, long-term) and 6-7 (females, long-term). In RT-qPCR experiments, the number of mice employed was 8–9 (males, short-term) and 6–10 (males, long- term).

1.B. AB-FUBINACA treatment - Objective 2

To archive objective 2, we evaluated long-term effects due to adolescent exposure to AB-FUBINACA on anxiety-like behavior, cued fear conditioning and extinction, novel object memory, sociability, depressive-like behavior and PPI of the startle reflex in both male and female mice in different cohorts (Figure 28C). As with JWH-018, animals were i.p. treated with increasing doses of AB-FUBINACA (PND 35-39: 1 mg/kg, PND 40-44: 1.5 mg/kg, and PND 45-49: 2 mg/kg) or vehicle for 15 days. Long-term effects were analyzed 20 days after the end of the treatment (PND 69). Behavioral studies were carried out in 6 different batches (3 per sex) as described in Figure 28C. The first was used for locomotion, anxiety and fear extinction experiments (males, n = 12; females, n =12), the second, for performing object recognition, sociability and forced swimming test (males, n = 14-15; females, n = 11-13) and the third for the experiments of PPI (males, n = 13-15; females, n = 13–15). Tissues for

biochemical experiments were extracted 24 h after the PPI test. For RNAseq experiments, the number of mice used was 4 females per group. Protein extraction for G-LISA assay was conducted using 11 female mice per group. An additional experiment was performed for Golgi staining (n = 4 female mice per group).

2. SCBs treatment in adult mice

To elucidate whether adolescence could be a period of vulnerability to the effects previously observed with JWH-018 and AB-FUBINACA, a similar protocol was performed in adult mice.

2.A. JWH-018 treatment - Objective 1

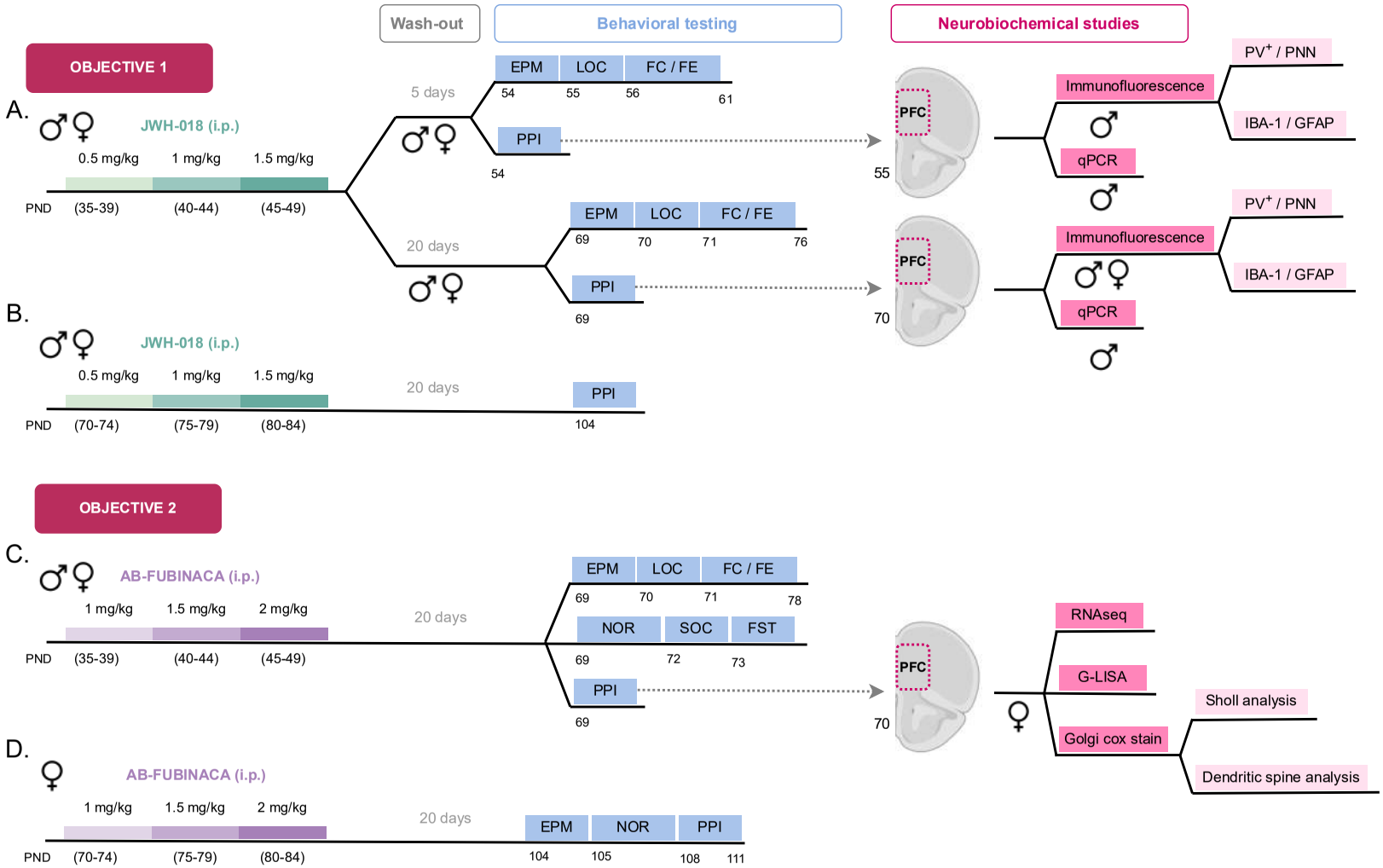
Starting at PND 70, male and female mice were i.p administered with increasing doses of JWH-018 (PND 70–74: 0.5 mg/kg, PND 75–79: 1 mg/kg, and PND 80–84: 1.5 mg/kg) or vehicle (Figure 28B). Behavioral testing was performed at PND 104 (Figure 28B), 20 days after the end of the treatment. The number of mice used was 11–13 for males, and 11–15 for females.

2.B. AB-FUBINACA treatment - Objective 2

Female mice were i.p. injected with increasing doses of AB-FUBINACA (PND 70-74: 1 mg/kg, PND 75-79: 1.5 mg/kg, and PND 80-84: 2 mg/kg) or vehicle as shown in Figure 28D. Behavioral evaluation started 20 days after the end of the treatment (PND 104), as described in Figure 28D. The number of female mice used was 12–17.

Figure 28. Schematic representation of the experimental designs carried out to achieve the main objectives of the thesis. *(A) Adolescent treatment with JWH-018 and subsequent behavioral and biochemical assays. (B) Adult treatment of JWH-018 and behavioral studies. (C) Adolescent treatment with AB-FUBINACA and subsequent behavioral and biochemical assays. (D) Adult treatment with AB-FUBINACA and behavioral protocol. i.p. intraperitoneal, EPM elevated plus maze, FC fear conditioning, FE fear extinction, GFAP glial fibrillary acidic protein, IBA-1 ionized calcium-binding adapter molecule 1, LOC locomotion, PFC prefrontal cortex, PND post-natal day, PNN perineuronal nets, PPI prepulse inhibition test, PV+ positive parvalbumin interneurons*

Figure 28


Wash-out
Behavioral testing
Neurobiochemical studies
OBJECTIVE 1
A.
JWH-018 (i.p.)
0.5 mg/kg
1 mg/kg
1.5 mg/kg
PND
(35-39)
(40-44)
(45-49)
5 days
EPM
LOC
FC / FE
54
55
56
61
PPI
54
20 days
EPM
LOC
FC / FE
69
70
71
76
PPI
69
PFC
55
PFC
70
Immunofluorescence
qPCR
PV+ / PNN
IBA-1 / GFAP
B.
JWH-018 (i.p.)
0.5 mg/kg
1 mg/kg
1.5 mg/kg
PND
(70-74)
(75-79)
(80-84)
20 days
PPI
104
OBJECTIVE 2
C.
AB-FUBINACA (i.p.)
1 mg/kg
1.5 mg/kg
2 mg/kg
PND
(35-39)
(40-44)
(45-49)
20 days
EPM
LOC
FC / FE
69
70
71
78
NOR
SOC
FST
69
72
73
PPI
69
PFC
70
RNAseq
G-LISA
Sholl analysis
Golgi cox stain
Dendritic spine analysis
D.
AB-FUBINACA (i.p.)
1 mg/kg
1.5 mg/kg
2 mg/kg
PND
(70-74)
(75-79)
(80-84)
20 days
EPM
NOR
PPI
104
105
108
111

II ANIMALS

Adolescent and adult C57BL/6J male and female mice (Charles River) were used in these experiments. Mice were housed 3-5 per cage in a temperature (21±1°C) and humidity (55± 10%)-controlled room under a 12 h light/dark cycle. All behavioral studies were performed during light period. Food and water were available *ad libitum*. Experimental procedures were conducted in accordance with the guidelines of the European Communities Directive 2010/63/EU and Spanish Regulations RD 1201/2005 and 53/2013 regulating animal research and approved by the local ethical committee (CEEA-UFV). Experiments in males and females were conducted in alternating weeks.

III DRUGS

JWH-018 (Tocris) and AB-FUBINACA (Cayman Chemical) were prepared in a 5% ethanol, 5% Tween-80 and 90% saline solution and were intraperitoneally administered at increasing doses for 15 consecutive days at 10 ml/kg of body weight. For JWH-018 experiments, doses of 0.5, 1 and 1.5 mg/kg were selected based on previous studies (Ossato et al., 2015; Barbieri et al., 2016). AB-FUBINACA doses of 1, 1.5 and 2 mg/kg were administered every five days, as with JWH-018, according to earlier reports (Banister et al., 2015; Trexler et al., 2020).

IV BEHAVIORAL STUDIES

1. Elevated plus maze

Anxiety-like behavior was assessed by using an elevated plus maze, which consisted in four arms (25 x 5 cm) set in cross from a central square (5 x 5 cm) and raised 30 cm from the ground. Two opposite arms were delimited by vertical walls (closed arms), although the two other arms had unprotected edges (open arms) (Figure 29). The apparatus was indirectly illuminated with 40-50 lux. The 5 mins test was recorded through a videocamara located on top of the maze. Results are expressed as total entries to the open and closed arms, and the percentage of time spent in the open arms with respect to the total amount of time spent in both closed and open arms.

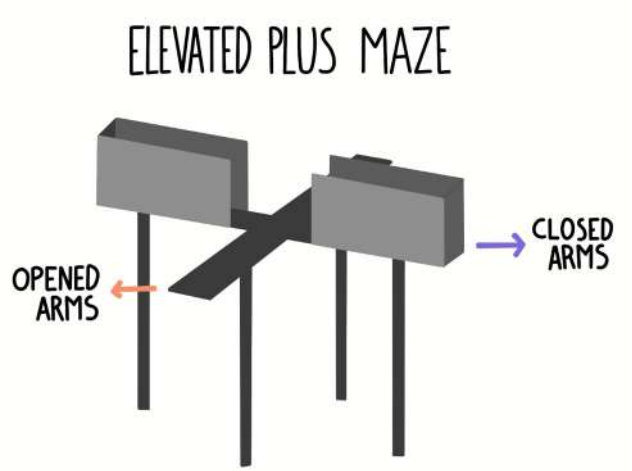


Figure 29. Schematic representation of the elevated plus maze.

2. Locomotion

Changes in locomotor activity were assessed by using activity boxes (27 x 27 x 21 cm, Cibertec). Mice were individually placed in locomotor cages with low luminosity (<50 lux). Activity was measured as the total number of times the animal crossed an infrared beam during 15 mins.

3. Cued fear conditioning and extinction

Training and testing were performed as in preceding experiments with slight modifications (Flores et al., 2014; Saravia et al., 2019). Mice were individually placed in the chamber (LE116, Panlab, Harvard Instruments) made of black walls with a transparent front door. The box (25 x 25 x 25 cm) was located inside a soundproof module to provide background noise and to reduce outside sound. The chamber floor was formed by parallel metal bars (2 mm of diameter and 6 mm spaced) connected to a shock generator (LE100- 26 module, Panlab, Harvard Instruments). A high-sensitivity weight transducer (load cell unit) was used to record the signal generated by the animal movement intensity. The software PACKWIN V2.0 automatically quantified the percentage of immobility for each experimental phase. Between animal trials, the chamber was cleaned with 70% ethanol and water to avoid olfactory cues. The conditioning session consisted of a 180 s habituation followed by three cue tones (3 Hz, 80 dB) of 30 s long. Each cue (CS) co-terminates with a 0.7 mA foot-shock of 1 s duration (US) (Figure 30). The interval between cues lasted 10 s. Fear extinction sessions (E1-E5) were performed 24, 48, 72, 96 and 120 h after the conditioning day in a novel environment (white walls, transparent cylinder, and smooth floor). During E1, mice were habituated to the new context for 180 s, whereas in E2-E5 this acclimatation period was reduced to 60 s. Then, mice were re-exposed to the CS (4 cue tones, 30 s long, 10 s between tones) (Figure 30). Fear memory was assessed as the mean percentage of time that mice spent freezing during the 4 cue tones of each extinction session. Freezing behavior, a

rodent's natural response to fear, was automatically recorded and defined as complete lack of movement, except for breathing for more than 800 ms. Data from fear extinction were expressed as percentage of freezing behavior.

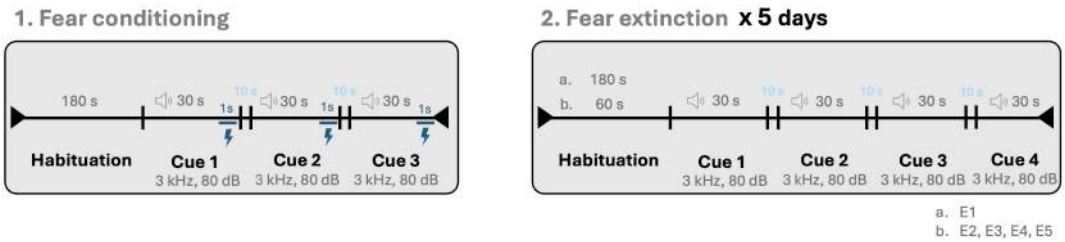


Figure 30. Fear conditioning and extinction protocol employed. *dB decibels, E1-E5 extinction sessions.*

4. Prepulse inhibition

PPI of startle reflex was conducted in two automated StartFear combined chambers (LE116, Panlab, Harvard Instruments) which were calibrated to ensure equivalent sensitivity and sound. Mice were daily habituated to a non-restrictive Plexiglas cylinder anchored to a high sensitivity transducer for 5 min with background white noise (65 dB) 4 days prior to test. The test started with an acclimatation period of 5 min followed by 5 pulse trials (120 dB, 40 ms, white noise) to establish baseline acoustic startle response. The experimental protocol consisted of 10 blocks with 3 or 12 trials each, randomly presented with an inter-trial interval of 10-30 s: no stimulus (12x) (65dB, white noise), pulse alone (12x) (120dB, 40 ms, white noise), pulse precede by 4 prepulse intensities (12x each) (4, 8, 12 and 16 dB above background noise, 20 ms duration, 100 ms before pulse, white noise) and prepulse alone (3x each) (Figure 31). Finally, 5 pulse trials were delivered (Figure 31). The first and last five trial pulses were excluded from the final analysis. Startle amplitude was automatically detected by PACKWIN V2.0 software. PPI was calculated as: 100 x (mean startle response – mean prepulse inhibited startle response) / (mean startle response).

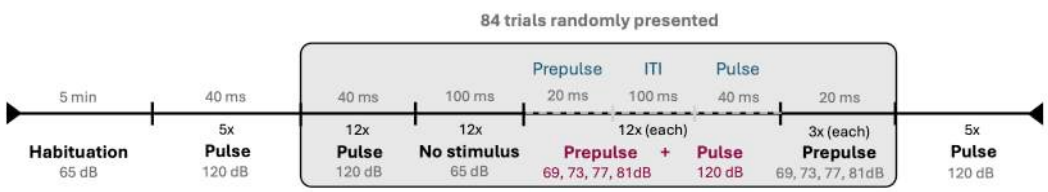


Figure 31. Schematic representation of the prepulse inhibition protocol. *dB decibels, ITI inter trial interval.*

5. Novel object recognition

Object-recognition memory was performed by using a V-shaped maze made of matte black methacrylate with two corridors (30 x 4.5 cm and 15 cm high) joined at a 90º angle. Mice were first habituated for 9 min to the maze. The day after, animals were trained and exposed to two identical objects (blue marbles) located at both limits of the maze and were allowed to explore for 9 min. On the test day, 24 h later, mice were again placed in the maze for 9 min, but one of the familiar objects was replaced with a novel one (Figure 32). Object exploration was defined as the orientation of the nose to the object at less than 2 cm. The total time the animal spent exploring each object was computed and the discrimination index was calculated as the difference between the time spent exploring novel vs familiar object divided by the total time exploring the two objects.

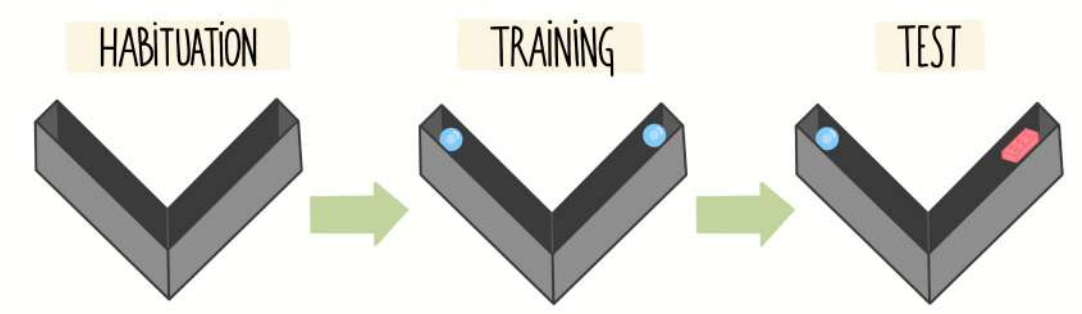


Figure 32. Schematic representation of the novel object recognition (NOR) test. *This test is composed of three phases, a first habituation to the contextual maze, a training session in which the animal is exposed to two identical objects and the test in which one familiar object is replace for a new one.*

6. Three-chamber social interaction test

Sociability testing occurred in a three-chamber maze made of transparent methacrylate with three exact compartments (20 x 20 x 40 cm) separated by sliding doors (5 x 8 cm) (Figure 33). After a 5 min habituation in the central chamber, the session to evaluate social affiliation/ motivation started. A same-sex conspecific stranger was placed in a cylindrical cage that allows interaction in one of the side compartments, while the other compartment remained empty. The doors opened, and the mouse was allowed to explore the different compartments freely for 10 minutes. Typically, mice exhibit a preference for spending more time with other mice than alone, demonstrating sociability (Figure 33) (Kaidanovich-Beilin et al., 2010). Interaction times, measured as the time that the animal´s head was inside of a zone surrounding cylindrical cages enclosures at less than 5 cm distance, were recorded and analyzed.

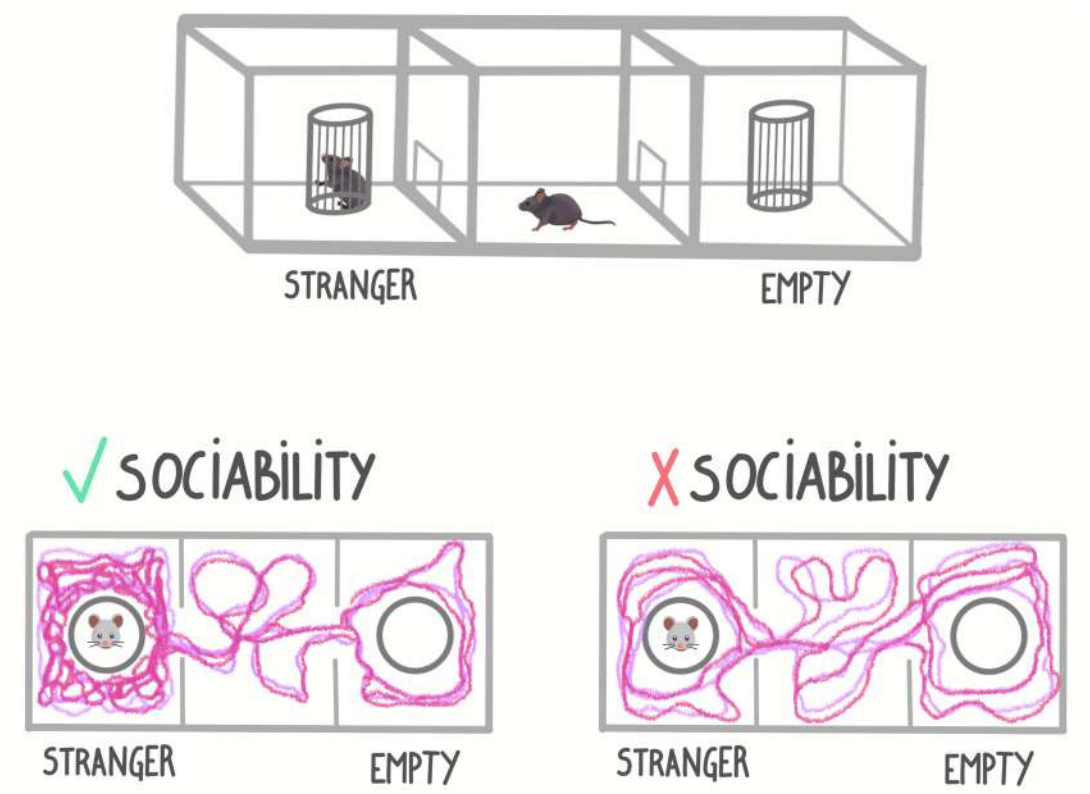


Figure 33. Illustrative representation of the sociability test performed in the three-chamber test. *The illustration at the top is a schematic representation of the three-chamber maze. The two images below illustrate the potential outcomes performing this test. Social behavior (left) is demonstrated when the mouse of study spends more time exploring the cylinder containing another mouse compared to the empty cylinder. Impaired social behavior (right) is indicated when the mouse explores both compartments equally.*

7. Forced swimming test

To evaluate depressive-like behaviors, animals were placed in a transparent methacrylate cylinder (20 cm of diameter) filled with water (22-24°C) up to 15 cm to prevent mice from touching the bottom. They were allowed to swim freely for 6 min under normal light conditions. The cumulative duration of immobility, an index of depressive-like state, during the last 4 min was calculated. Immobility was defined as the absence of movements except for those slights to maintain balance in the water.

BIOCHEMICAL STUDIES V

1. Immunofluorescence

1.A. Tissue preparation

Twenty-four hours after PPI test, mice were deeply anesthetized by i.p. injection of ketamine, xylazine and saline solution prior to intracardiac perfusion. Mice were perfused with 1X phosphate buffer saline (PBS) followed by 4% paraformaldehyde. Afterward, brains were post-fixed in 4% paraformaldehyde 24 h and then dehydrated by sequential transfer to 15% and 30% of sucrose in PBS 1X (4° C). Coronal frozen sections of 20 m thickness were obtained in a cryostat from 1.98 to 1.54 mm relative to bregma for PFC. Brain slices were stored in a cryoprotective solution (20% Glycerol, 30% ethylenglycol in PBS 1X) at -20°C until use.

1.B. Immunofluorescence

· Parvalbumin and perineuronal nets

Floating slices were 3 times rinsed in PBS 1X and then treated with blocked solution (4% normal goat serum, 0.1% Triton X-100, 0.1% bovine serum albumin in PBS 1X) for 1.5 h at room temperature. Slices were incubated overnight at 4°C with the primary antibodies prepared in blocked solution. Rabbit anti-PV (1:2000, ab11427, Abcam) and Wisteria floribunda agglutinin combined with fluorescein (1:1000, FL-1351, Vector Laboratories) to label PNNs were used. Next day, after three rinses with PBS 1X (10min), sections were incubated with the secondary antibody AlexaFluor-594 (1:500, A-11012, Invitrogen) for PV labeling at room temperature for 1 h in blocked solution. Slices were washed 3 times in PBS 1X and mounted with Fluoromount-DAPI (Invitrogen).

· Iba-1 and GFAP immunofluorescence

The same protocol previously described was used, applying the specific antibodies. Primary antibodies used were rabbit anti- Iba-1 (1:1000, 019-19741, Wako) and guinea pig anti-GFAP (1:1000, 173 004, Synaptic system) to label microglial cells and astrocytes, respectively. The secondary antibodies employed were AlexaFluor-594 (1:500, A-11012, Invitrogen) for Iba-1 and AlexaFluor-488 (1:500, A-11073, Invitrogen) for GFAP labeling.

1.C. Image and analysis

· Parvalbumin and perineuronal nets

Immunostained sections were observed under a Zeiss LSM 900 confocal microscope, using a 20x/0.5 dry objective (Zeiss, CLSM, Germany). Images were acquired through a z-plane (1µm/stack, 8 stacks, 16-bit, 1024 × 1024) and the z-stack was obtained through a maximum

projection. A 500 µm squared region of interest (ROI) was delimited for quantification in each infralimbic (IL), prelimbic (PL) and orbitofrontal (OBF) subregions of the prefrontal cortex. The number of positive PV, PNNs and % of PV surrounded by PNNs was semiautomatically detected by using the Pipsqueak tool ® (Slaker et al., 2016) for FIJI (FIJI is just ImageJ) software. For all areas, 5–7 images per animal were quantified (n = 5-7 animals per group).

· IBA-1 and GFAP

The stained sections were analyzed at 40x/0.5 objective using a Zeiss LSM 900 confocal microscope (Zeiss, CLSM, Germany). Images were taken through a z-plane (0.5 µm/stack, 10 stacks, 16-bit, 1024 × 1024) and the quantification was carried out through a sum slides projection (32- bit). A quantification ROI of 320 × 320 µm located in the intermediate region between the IL and PL subareas of the prefrontal cortex was chosen. FIJI (FIJI is just Image J) software was used to calculate fluorescence intensity of GFAP stain. The "freehand selection" tool was used to quantify soma area and perimeter of Iba-1-stained cells. Five to seven images per animal were analyzed (n = 6 mice per group).

2. Quantitative RT-PCR analysis

Prefrontal cortex tissues were extracted 24 h after the PPI test and immediately stored at −80 °C (n = 6-10 mice per group). The RNA was purified with the RiboPureTM KIT (Invitrogen), and the reverse transcription was performed with 1µg of total RNA and the SuperScriptTM II Reverse Transcriptase (Invitrogen). PCR reactions were conducted using PrimePCRTM Probe Assay (Bio- Rad) to quantify mRNA levels of glutamic acid decarboxylase, 67 kDa isoform (GAD67) (ID: qMmuCEP0060617), brain derived neurotrophic factor (BDNF) (ID: qMmuCEP0058759), synaptophysin (SYP) (ID: qMmuCIP0035577), CB1R (ID: qMmuCEP0038879) and CB2R (ID: qMmuCEP0039299). To evaluate postsynaptic density protein 95 (PSD95) (ID: 4453320), TaqManTM Gene Expression Assay (Applied BiosystemsTM) was used. GAPDH (ID: qMmuCEP0039581) expression was used as endogenous control gene for normalization. PCR assays were carried out with the CFX Connect Real-Time PCR Detection System (Bio-Rad). The fold changes in gene expression of JWH-018 treated animals in comparison with controls were calculated using the $2^{-\Delta\Delta Ct}$ method.

3. RNA sequencing

Total RNA was purified from PFC tissues of vehicle (n = 4) and AB-FUBINACA treated female (n = 4) mice 24 h after PPI test, with the RiboPureTM Kit (Invitrogen). RNA integrity > 7 was confirmed by TapeStation (Aligent). Sequencing libraries were prepared using TruSeq Stranded mRNA Sample Prep Kit (Illumina) following manufacturer's instructions. Libraries were validated by using KAPA Library Quantification Kit for Illumina according to the qPCR Quantification Protocol Guide (KAPA Biosystems) and quantified by TapeStation (Aligent). Libraries were submitted to an Illumina NovaSeq and sequencing was performed using a 2

x 150 bp paired end configuration. Pseudo-alignment and quantification were then made with Salmon algorithm (reference genome GRCh38) (PMID: 28263959). Correlation analysis, principal component study and differential expression analysis were performed with DESeq2 package (PMID: 25516281). Differential expression gene (DEG) analyses were done using the parametric Wald test, with Benjamini-Hochberg adjustment method (padj). Genes with padj < 0.05 and a cutoff of 2-fold change were considered significantly DEGs. Raw data corresponding to RNA sequencing analyses were deposited at the NCBI SRA, ID number: PRJNA 1167322.

4. G-LISA Cdc42 Activation Assay

Activity of Cdc42 GTPases in PFC tissues extracted 24 h after PPI, was measured by G-LISA Activation Assay (Cytoskeleton Inc.; BK127) according to the manufacturers protocol (n= 11 mice per group). Tissues were lysed with an appropriate lysis buffer and centrifugated (10,000 x g, 1min, 4°C). Supernatants were immediately frozen and kept at −20°C till the G-LISA Activity Assay. Protein concentration was measured by Precision Red™ Advanced Protein Assay (Cytoskeleton Inc.). Most articles that use the G-LISA kit worked with cells instead of tissue, so a fine-tuning had to be carried. An amount of 3 mg/ml of sample was needed in this case, data that differ from the original protocol. The GTP-bound Cdc42 levels were performed according to the manufacturer's protocol (Cytoskeleton Inc.) and measured with a spectrophotometer at 490 nm.

5. Golgi-Cox procedure

5.A. Staining protocol

Twenty days after adolescent exposure to AB-FUBINACA (n = 4) or vehicle (n = 4), female mice were sacrificed and the whole brain was quickly and carefully removed from the skull. The Golgi staining procedure was conducted in accordance with manufacturer's instructions, FD Rapid GolgiStain kit (FD NeuroTechnologies, Inc.; PK401A Cell Systems Biology). In summary, brains were immersed in solution A/B for 10 days in dark (with a change of the solution A/B after the first 24 h). Subsequently, they were transferred to solution C for 4–5 days prior to being sliced (with a change of the solution C after the first 24 h). Coronal sections of 160 µm thickness, spanning from 1.98 to 1.54 mm with respect to bregma for the PFC, were obtained by using a cryostat following the protocol described by Zhong et al (Zhong et al., 2019). After the sections dried completely on gelatin-coated slides, they were incubated in staining solution D/E for 10 min. Subsequently, stained sections were rinsed with destilled water and underwent for dehydration by a series of consecutive immersion in ethanol solutions with increasing concentrations (50, 75, 95 and 100%). Following this, samples were subjected to clearing using xylene and then mounted with DPX (DP00500500, Scharlau).

5.B. Image and analysis

· Sholl analysis

Stained sections were photographed at a 10x dry objective using a Zeiss LSM 900 confocal microscope (Zeiss, CLSM, Germany). A Z projection was employed to ensure capturing the entire neuron (1 µm/stack, 16-bit, 1024 x 1024). For sholl analysis, only neurons of layer II/III of the PFC completely impregnated within Golgi stain and that could be traced along their entire length were selected. Six independent neurons from each animal were randomly selected. To assess neuron remodeling and analysis, we used the Neuroanatomy plugin (Simple Neurite Tracer, semi-automatic tool) in FIJI (FIJI is just Image J). Finally, a principal component analysis (PCA) was performed to identify possible different groupings of the neuronal populations, by using an unbiased approach. For that, we identified 13 markers of neuron complexity: number of intersections (every 20 µm), total length (µm), number of terminal ends, total bifurcations, convex hull volume (μm^3), average branch order, number of late-order branches, number of first, second, and third-order branches, average length of first and second-order branches, and the ratio total length –late order branches. PCA was performed using R package version 2.11 of FactoMineR.

· Dendritic spine analysis

Section images were captured under a Zeiss LSM 900 confocal microscope, using a 60x/2x oil objective (Zeiss, CLSM, Germany) with 1.4 NA. Images were acquired through a z-plane (0.3µm/stack, 16-bit, 2048 x 2048). Secondary and tertiary dendrites of individual pyramidal neurons from layers II/III of the PFC were selected. In addition, we chose 4-5 apical and 4-5 basal dendrites per animal for the analysis. To calculate spine density, a minimum dendrite length of 20 µm long was required. Reconstruction of dendrites and spine classification was performed by using the "FilamentTracer" tool of IMARIS software (Bitplane). Projections from dendrites were classified into 4 types based on their morphological characteristics: "stubby" were less than 0.7 µm in length and did not appear to have a neck; "thin spines", larger than 0.7 µm and had elongated spine necks with small heads; "mushroom-like" were also more than 0.7 µm of length, but were characterized by a short neck and large spine head; and "branched" spines that had elongated spine necks with 2 or more spine heads (Figure 34). Interestingly, spine morphology is indicative of functional capacity and maturity (Hering and Sheng, 2001). Thus, although a strict classification of dendritic spines is increasingly debated, traditionally long-thin spines have been considered the most immature, while mushroom-shaped spines are regarded as the most mature ones (Risher et al., 2014; Pchitskaya and Bezprozvanny, 2020).

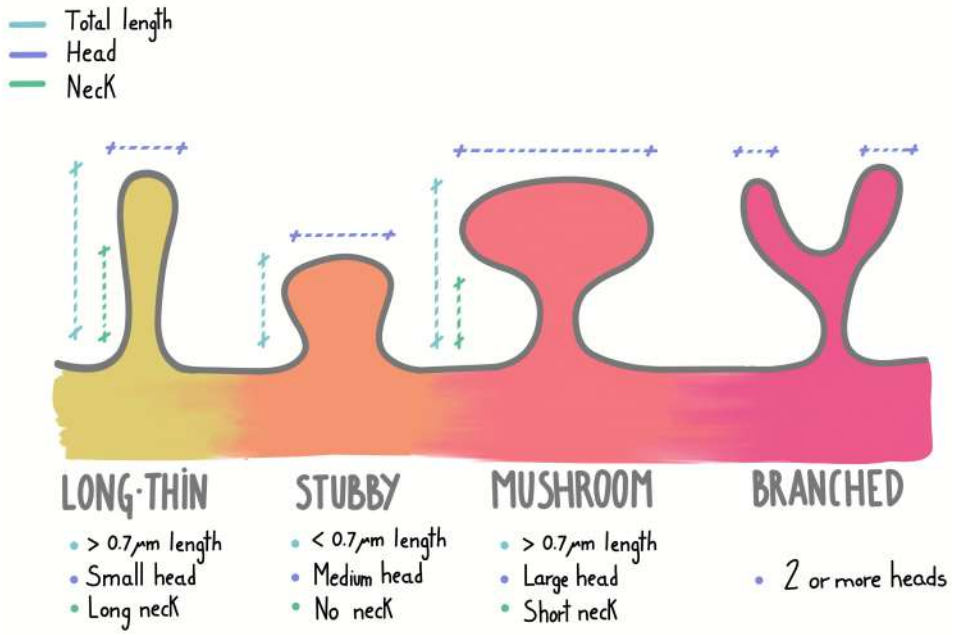


Figure 34. Dendritic spine classification based on morphological characteristics.

VI STATISTICAL ANALYSIS

Normality and homoscedasticity were evaluated before the final analysis (Kolmogorov-Smirnov test and Bartlett's test, respectively). Statistical analysis was carried out using unpaired Student t-test (with Welch's correction when heteroscedasticity), two-way ANOVA, and two-way ANOVA of repeated measures followed by Bonferroni or Newman-keuls post hoc comparisons after significant interactions between factors. In case of missing values, a mixed-model ANOVA was performed. Nonparametric Mann-Whitney test was used when data did not fit a normal distribution. To study correlations between two variables, the Pearson´s coefficient was employed. Outliers were excluded if they were >2 standard deviations from the mean. All data are expressed as mean ± SEM. A p value <0.05 was used to determine statistical significance. The statistical analysis was performed using STATISTICA (StatSoft) software and GraphPad Prism 9. A summary table of the statistics employed can be found in annex I (Tables A9 and A10).

4

Results

OBJECTIVE 1: JWH-018 EFFECTS I

JWH-018 was the first SCB ever reported in Spice/K2 preparations around 2008 (EMCDDA, 2024). Psychotic symptoms, hallucinations, anxiety and panic attacks have been reported, among many other effects described following acute consumption of JWH-018 (Fattore, 2016; Ford et al., 2017). In addition, animal studies reveal both acute and chronic effects related to anxiety and psychotic-like symptoms (MacRì et al., 2013; Li et al., 2019; Bilel et al., 2020; Pintori et al., 2021; Margiani et al., 2022; Corli et al., 2023b). On the other hand, it is worth noting that adolescence is described as a period of vulnerability to the central effects of cannabis (Rubino and Parolaro, 2015, 2016) and teenagers and young adults have been reported to show the highest ratio of SCBs consumption (EMCDDA, 2024). Therefore, the main objective of this chapter is to analyze the short- and long-term behavioral and molecular alterations induced by chronic treatment with JWH-018 in adolescent male and female mice.

1. Impact of adolescent JWH-018 treatment on body weight

In order to evaluate the short- and long-term consequences of chronic consumption during adolescence, increasing doses of JWH-018 were given for 15 days (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg). Body weight was monitored daily during JWH-018 treatment, revealing reduced weight gain in JWH-018-treated mice compared to controls in both sexes (Figure 35) (treatment effect: $F_{1,112} = 8,987$, $p < 0.005$ and $F_{1,85} = 4,410$, $p < 0.05$, for male and female mice, respectively). The effect on weight becomes increasingly pronounced between the control and JWH-018-treated group as the dose increases, represented by the area under the curve in both males (AUC day 6-10 $p < 0.01$; 11-15 $p < 0.001$) (Figure 35A) and females (AUC day 6-10 $p < 0.05$; 11-15 $p < 0.01$) (Figure 35B). These results are consistent with previous reports that evaluate effects of chronic THC adolescent exposure (Scherma et al., 2016; Saravia et al., 2019).

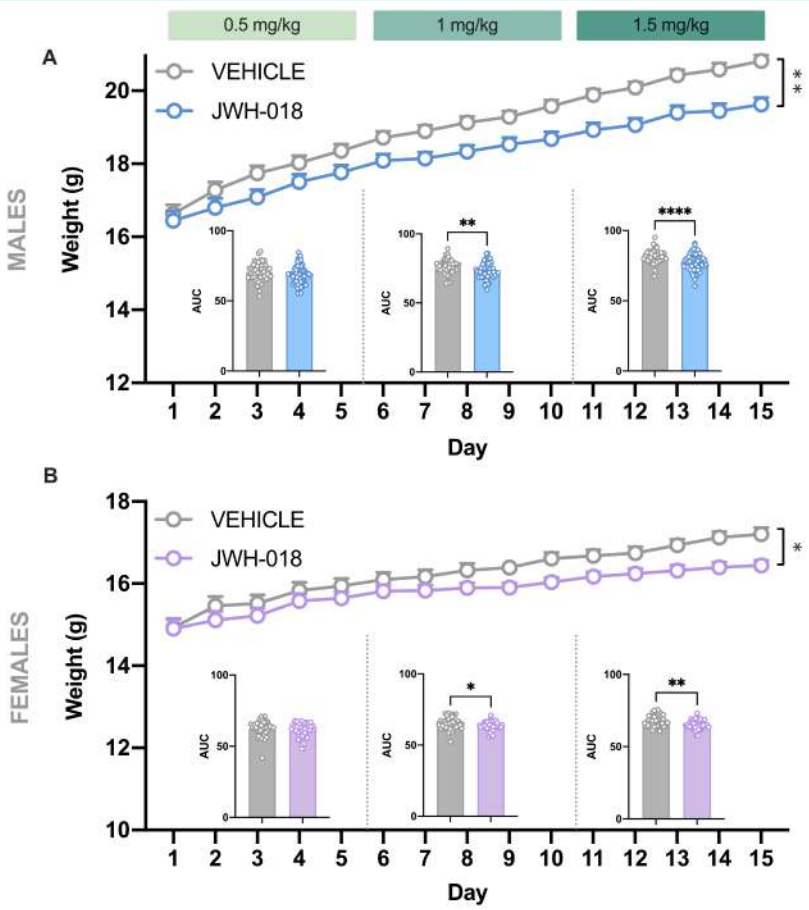


Figure 35. Adolescent exposure to JWH-018 alters body weight in male and female mice. *Effects of treatment with JWH-018 during adolescence in body weight of adolescent male (A) and female (B) mice (n= 43-58 mice per group). Daily weight in grams during the 15 days of treatment and AUC values every five days are shown. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (comparison between JWH-018 and vehicle; two-way ANOVA of repeated measures (A, B), Student's t-test (AUC A, AUC B). AUC, area under the curve.*

2. Short- and long-term effects on anxiety and fear conditioning and extinction in adolescent mice exposed to JWH-018

It is well stablished that adolescent exposure to cannabinoids leads to dysregulation of emotional processes later in adulthood (Rubino and Parolaro, 2011, 2016). The exponential growth in SCBs consumption among young people explains the importance of delving into the possible harmful consequences associated with emotional processing. To achieve this objective, anxiety-like behavior, fear memory processing and locomotor activity were evaluated 5 (short-term) or 20 (long-term) days after the end of the treatment with JWH-018 (Figure 36).

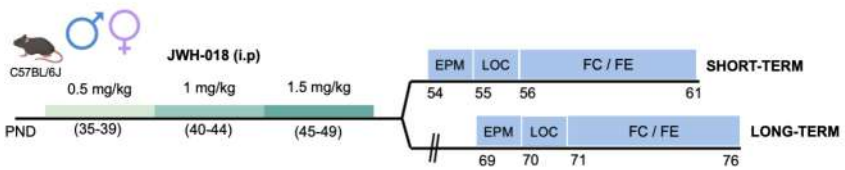


Figure 36. Schematic representation of the experimental design. *EPM elevated plus maze, FC fear conditioning, FE fear extinction, LOC locomotion, PND post-natal day*

After assessing the EPM for testing anxiety-like responses, males at short-term exposed to JWH-018 revealed an anxiogenic-like effect compared to the control group ($p < 0.05$) (Figure 37A). However, this effect was specific to that period as during adulthood males showed an anxiolytic-like tendency ($p = 0.053$) (Figure 37B). On the contrary, female mice treated with JWH-018 exhibited no differences in anxiety at short-term (Figure 37C), although there was a clear anxiogenic-like trend at long-term ($p = 0.054$) (Figure 37D). No differences in the total number of entries were observed in either males or females (Figure 37A, B, C, D).

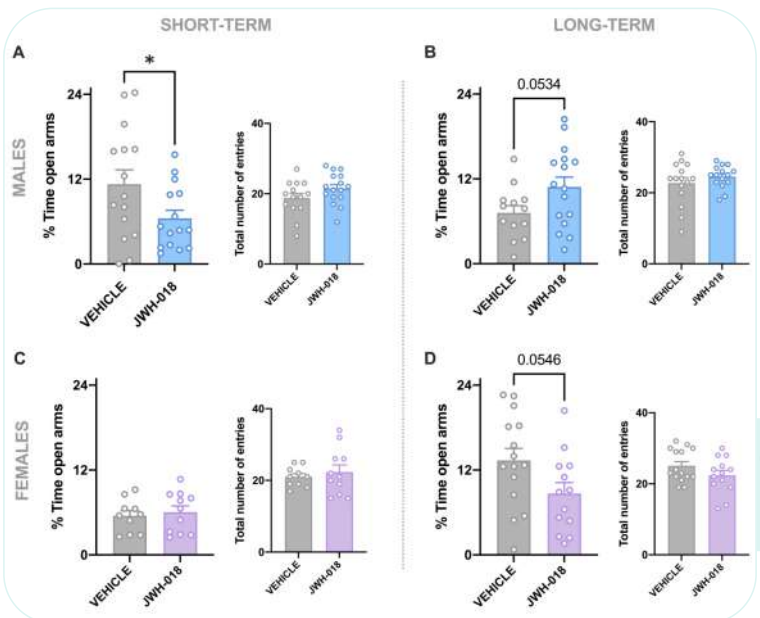


Figure 37

Figure 37. JWH-018 exposure during adolescence alters anxiety-like behavior depending on the sex and the time interval between treatment and behavioral analysis*. (A–D) Effects of adolescent exposure to JWH-018 (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) or vehicle in anxiety-like behavior in the EPM in male mice at short- (A) and long-term (B) and female mice at short- (C) and long- term (D) (n = 10–16 mice per group). Percentage of time spent in the open arm and total number of entries are shown. Data are expressed as mean ± SEM. *p < 0.05 (comparison between JWH-018 and vehicle; Student's t-test).*

To evaluate potential alterations in the processing of aversive memories, a fear conditioning paradigm was employed. Chronic JWH-018 exposure during adolescent did not modify fear extinction in males (Figure 38A, B) or females (Figure 38C, D) at any time-point. However, fear conditioning was higher in females at long-term (interaction cue x treatment: $F_{2,52}$ = 3.937, p < 0.05) treated with JWH-018 during adolescence in comparison with controls (Figure 38D). This effect was not observed in males at short- and long-term (Figure 38A, B) or females at short-term (Figure 38C).

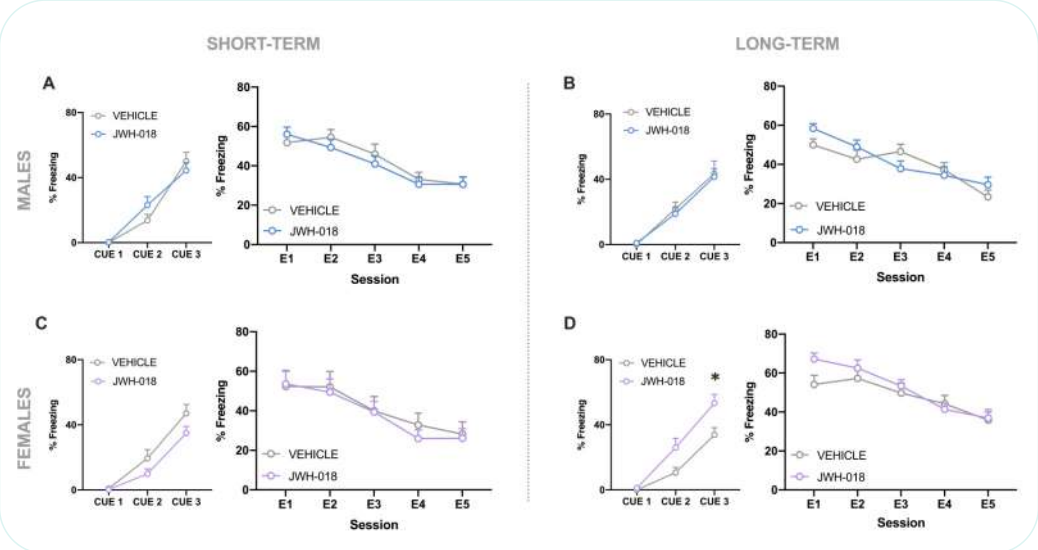


Figure 38. JWH-018 exposure during adolescence impairs fear conditioning, but not fear extinction in a sex- and time-dependent manner*. (A-D) Effects of adolescent exposure to JWH-018 (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) or vehicle in fear conditioning and extinction in male mice at short- (A) and long-term (B) and female mice at short- (C) and long- term (D) (n = 10–16 mice per group). Time courses of the freezing levels scored during cued fear conditioning and cue fear extinction trials are shown for fear memory processing. Data are expressed as mean ± SEM. *p < 0.05 (comparison between JWH-018 and vehicle; two-way ANOVA of repeated measures). E1-E5 extinction trials.*

Interestingly, the mean percentage of freezing along the 3 cues during the fear conditioning session in adult females negatively correlates with the percentage of time the animals spent in the open arms (p < 0.05) (Figure 39). This suggests that the anxiogenic-like tendency previously observed in the EPM in females at long-term could be associated with the observed conditioning impairment to an aversive stimuli.

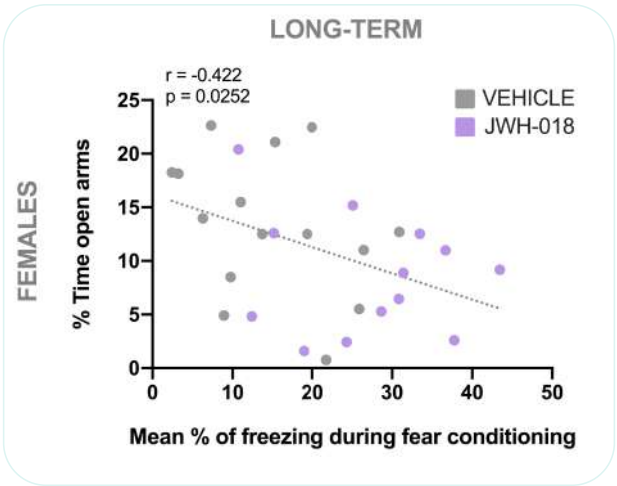


Figure 39. Negative correlation between the percentage of time in open arms and the mean percentage of freezing during fear conditioning in female mice at long-term. *(n = 10–16 mice per group) (Pearson's correlation coefficient)*

Additionally, alterations in locomotion were examined by using an actimetry system. No changes in locomotion were observed in either males (Figure 40A, C) or females (Figure 40B, D) at any time-point.

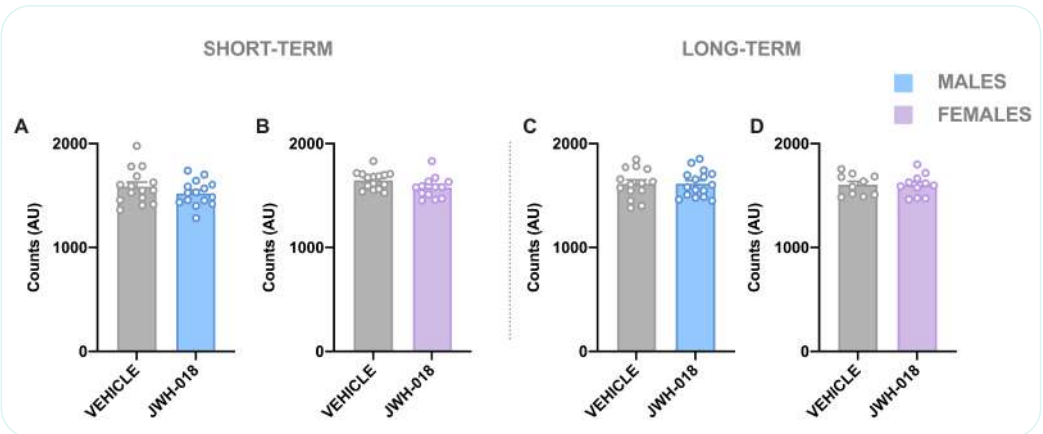


Figure 40. Adolescent exposure to JWH-018 does not modify locomotion in male and female mice. *(A-D) Effects of JWH-018 exposure during adolescence on locomotor activity in male mice at short- (A) and long-term (C), and female mice at short- (B) and long- term (D) (n = 10-16 mice per group). Data are expressed as mean ± SEM. AU: arbitrary units.*

Altogether, these results suggest sex-specific effects on unconditioned anxiety resulting from adolescent exposure to JWH-018.

3. Short- and long-term consequences on sensorimotor gating in adolescent mice exposed to JWH-018

Cannabis consumption is known to increase the risk of developing psychotic disorders by 2-4 fold (Moore et al., 2007; Marconi et al., 2016). The higher frequency and potency of the cannabimimetic, the greater risk (Rubino and Parolaro, 2015; Di Forti et al., 2019). Impairments in PPI of startle reflex are observed in patients with schizophrenia (Mena et al., 2016) and other psychotic disorders (Kohl et al., 2013; San-Martin et al., 2022), and are considered a marker of psychotic-like alterations (Carceles-Cordon et al., 2020). Therefore, to further investigate the role of SCBs in the appearance of psychotic-like effects, a second group of batches treated with JWH-018 during adolescence were employed. PPI test was also performed 5 (short-term) or 20 (long-term) days after the end of treatment in both males and females (Figure 41).

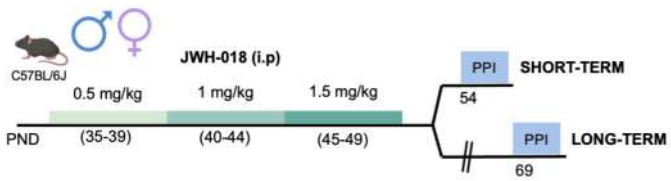
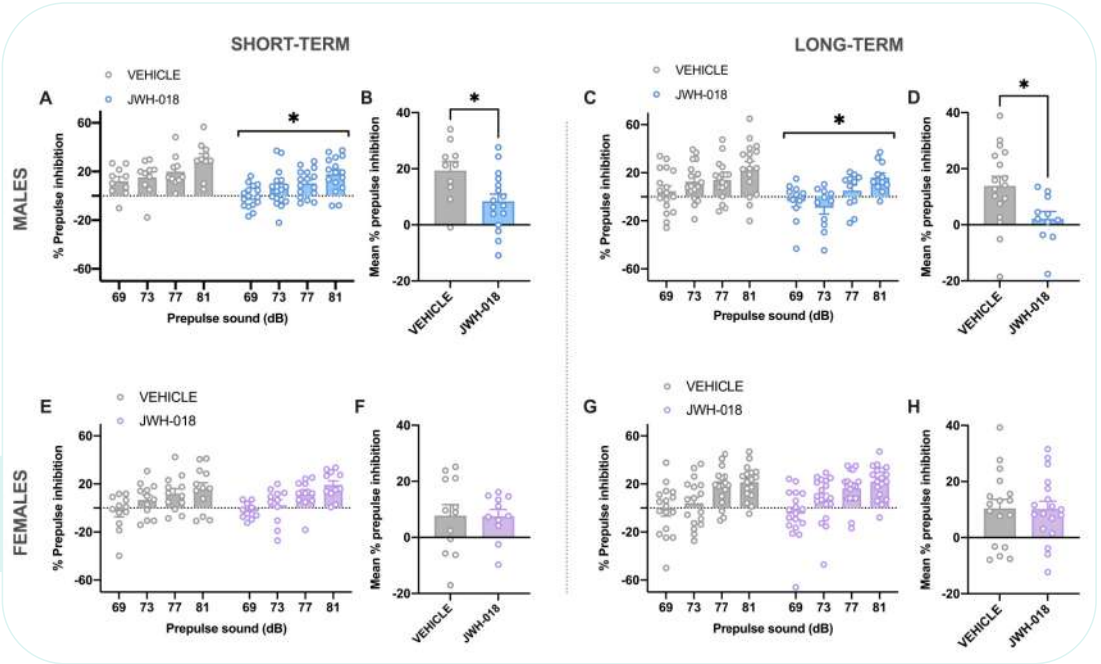


Figure 41. Schematic representation of the protocol employed. *PNS post-natal day, PPI prepulse inhibition test.*

Interestingly, male mice treated with JWH-018 showed a significant decrease in the percentage of PPI compared to the control group in both short- (treatment effect: $F_{1,24} = 6.79$, $p < 0.05$) (Figure 42A) and long-term (treatment effect: $F_{1,26} = 6.06$, $p < 0.05$) (Figure 42C). A general reduction due to JWH-018 exposure was also observed when representing the mean PPI score at both time periods in male mice ($p < 0.05$) (Figure 42B, D). However, females treated with JWH-018 during adolescence did not exhibit alterations in the PPI of startle reflex either at short- or at long-term (Figure 42E, F, G, H). Notably, JWH-018 exposure did not induce any baseline changes in the startle response amplitude in males (Figure 43A, C) or females (Figure 43B, D), eliminating any possible influence of the startle reflex on the PPI changes observed in male mice.

Figure 42. JWH-018 exposure during adolescence impairs sensorimotor gating in male, but not female, mice at short- and long-term*. (A-G) Effects of adolescent exposure to JWH-018 (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) or vehicle in sensorimotor gating in male mice at short- (A, B) and long-term (C, D), and female mice at short- (E, F) and long-term (G, H) (n = 10–18 mice per group). Percentage of prepulse inhibition and mean of the percentage of prepulse inhibition are shown. Data are expressed as mean ± SEM. *p < 0.05 (comparison between JWH-018 and vehicle group; two-way ANOVA with repeated measures, treatment A, C; Student's t-test B, D). dB decibels.*

Figure 42



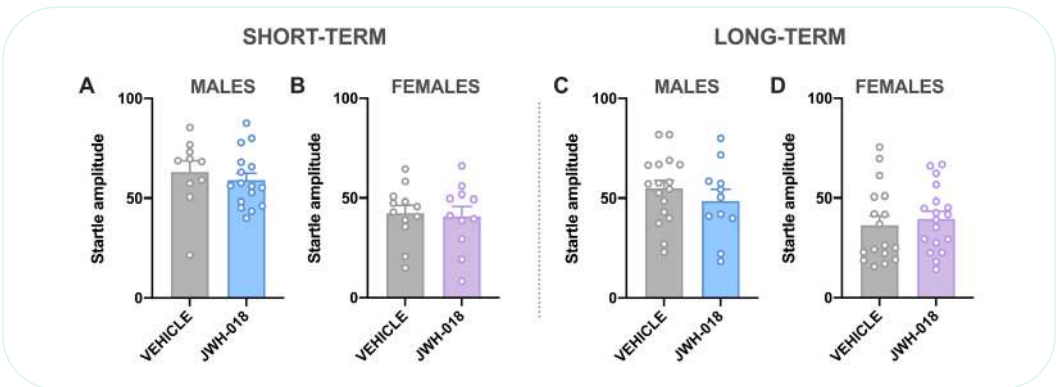


Figure 43. JWH-018 treatment does not alter startle reflex amplitude in any experimental group. *(A-D) Effects of adolescent exposure to JWH-018 (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) or vehicle in startle response amplitude in male mice at short- (A) and long-term (C), and female mice at short- (B) and long-term (D) (n = 10–18 mice per group). Startle response amplitude for pulse is shown. Data are expressed as mean ± SEM.*

These results suggest that JWH-018 exposure could be a risk factor for the development of psychotic-like alterations at short- and long-term in a sex dependent-manner. Accordingly, several reports found long-term PPI impairments after chronic administration of other aminoalkylindole derivatives during adolescence in male rodents (Schneider and Koch, 2003; Schneider et al., 2005; Wegener and Koch, 2009; Aguilar et al., 2017; Abboussi et al., 2020).

4. Adult chronic exposure to JWH-018 on sensorimotor gating

To further investigate if adolescence represents a period of vulnerability to the alterations in PPI previously described, the aforementioned protocol was replicated, but JWH-018 was administered for 15 days directly on adulthood. The PPI test was conducted 20 days following the last day of treatment in both male and female mice.

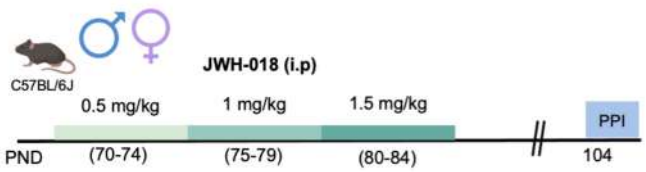


Figure 44. Schematic representation of experimental design through adult male and female mice. PND postnatal day, *PPI prepulse inhibition test.*

Remarkably, no changes in the sensorimotor gating were observed between vehicle and JWH-018 group in male mice (Figure 45A, B, C), highlighting that early life exposure to the SCB is a risk factor for the development of psychotic symptoms.

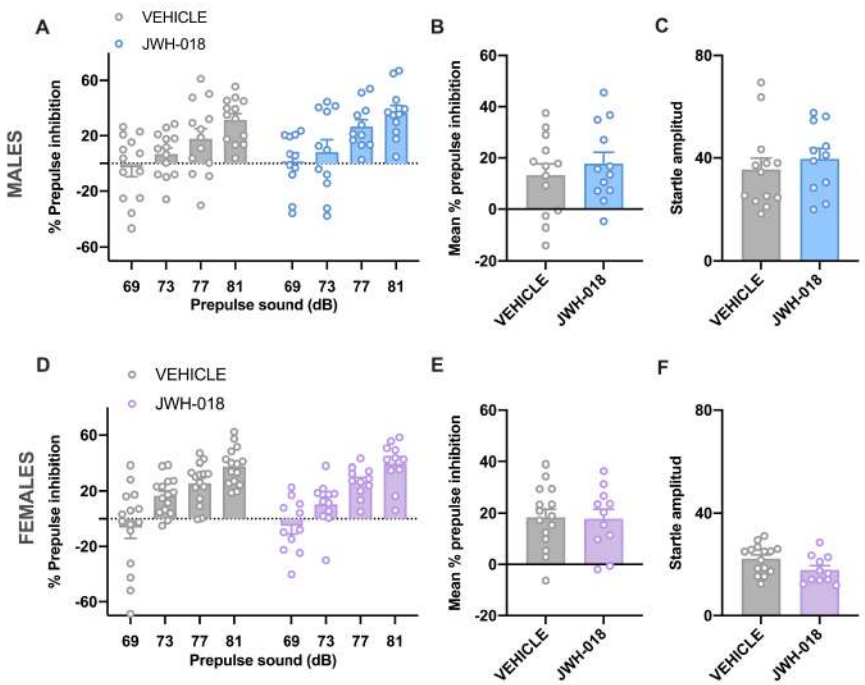


Figure 45. JWH-018 exposure during adulthood does not modify sensorimotor gating in male and female mice*. (A-F) Effects of exposure to JWH-018 during adulthood (PND 70–74: 0.5 mg/kg, PND 75–79: 1 mg/kg, and PND 80–84: 1.5 mg/kg) or vehicle in male (A-C) and female (D-F) mice (n = 11–15 mice per group). Percentage of prepulse inhibition, mean of the percentage of prepulse inhibition, and startle response amplitude are shown. Data are expressed as mean ± SEM. dB decibels.*

Supporting these findings, previous reports described the presence of somatosensory filtering alterations following SCBs exposure during adolescence, but not when administered in adulthood (Schneider and Koch, 2003; Gleason et al., 2012). In addition, adult females did not modify PPI responses after chronic treatment with JWH-018, in agreement with absence of effect observed during adolescence (Figure 45D, E, F).

5. Short- and long-term consequences on the density of cortical parvalbumin-expressing interneurons and perineuronal nets in adolescent mice exposed to JWH-018

To explore potential neurobiochemical alterations underlying the observed behavioral changes, the brains of animals treated during adolescence and subjected to PPI test were perfused or extracted 24 h later. We have primarily focused on the PFC, given its close association with the correct modulation of somatosensory filtering (Tóth et al., 2017). In addition, as described in the introduction section, it has been reported that individuals with schizophrenia exhibit alterations in the GABAergic system in the PFC, particularly within PV+ interneurons (Lodge et al., 2009; Gonzalez-Burgos et al., 2015). During development, PNNs play a critical role in the maturation of the GABAergic system and are mainly found surrounding PV-expressing neurons. Further, alterations in the density of these PNNs have also been found in patients with psychiatric disorders (Mauney et al., 2013; Enwright et al., 2016; Alcaide et al., 2019). Therefore, based on the previous data, potential alterations in the density of PV+ neurons and PNNs in the PFC were further investigate given the deficits observed in PPI in male mice treated with JWH-018 during adolescence.

Remarkably, JWH-018 treatment induced a significant decreased in PNNs density in males at short-term within the infralimbic (IL) and prelimbic (PL) areas of the PFC ($p < 0.05$), but not in the orbitofrontal cortex (OBF) (Figure 46A, G). However, these alterations were either fully reversed in the PL or partially reversed in the IL ($p = 0.07$) at long-term (Figure 46B). In accordance, there were no significant differences in PNNs density at long-term in females exposed to JWH-018 during adolescence (Figure 47A). Regarding PV-expressing neurons, no significant differences were observed following JWH-018 exposure in both male (Figure 46C, D, G) and female mice (Figure 47B) across the different PFC subregions analyzed. In male mice, but not in females (Figure 47C), a near-significant reduction in the percentage of PV+ neurons surrounded by PNNs was detected in the IL region of the PFC at both short- ($p = 0.060$) and long-term ($p = 0.059$) (Figure 46E, F).

Figure 46. JWH-018 exposure during adolescence alters perineuronal nets density in cortical subregions in male mice. *(A–F) Number of PV+, PNNs and PV+ surrounded by PNNs in the IL, PL and OBF in male mice exposed to JWH-018 during adolescence at short- (A, C, E) and long-term (B, D, F). Tissues were obtained 24 h after the prepulse inhibition test (n = 5–7 mice per group). G Representative images of each cortical subregion obtained by fluorescence microscopy labelling PV+ (red) and PNNs (green) of short-term experiments. Scale bar represents 100 μm. Data are expressed as mean ± SEM. *p < 0.05 (comparison between JWH-018 and vehicle; Student's t-test). IL infralimbic prefrontal cortex, PL prelimbic prefrontal cortex, OBF orbitofrontal cortex, PV+ positive parvalbumin neuron, PNNs perineuronal nets.*

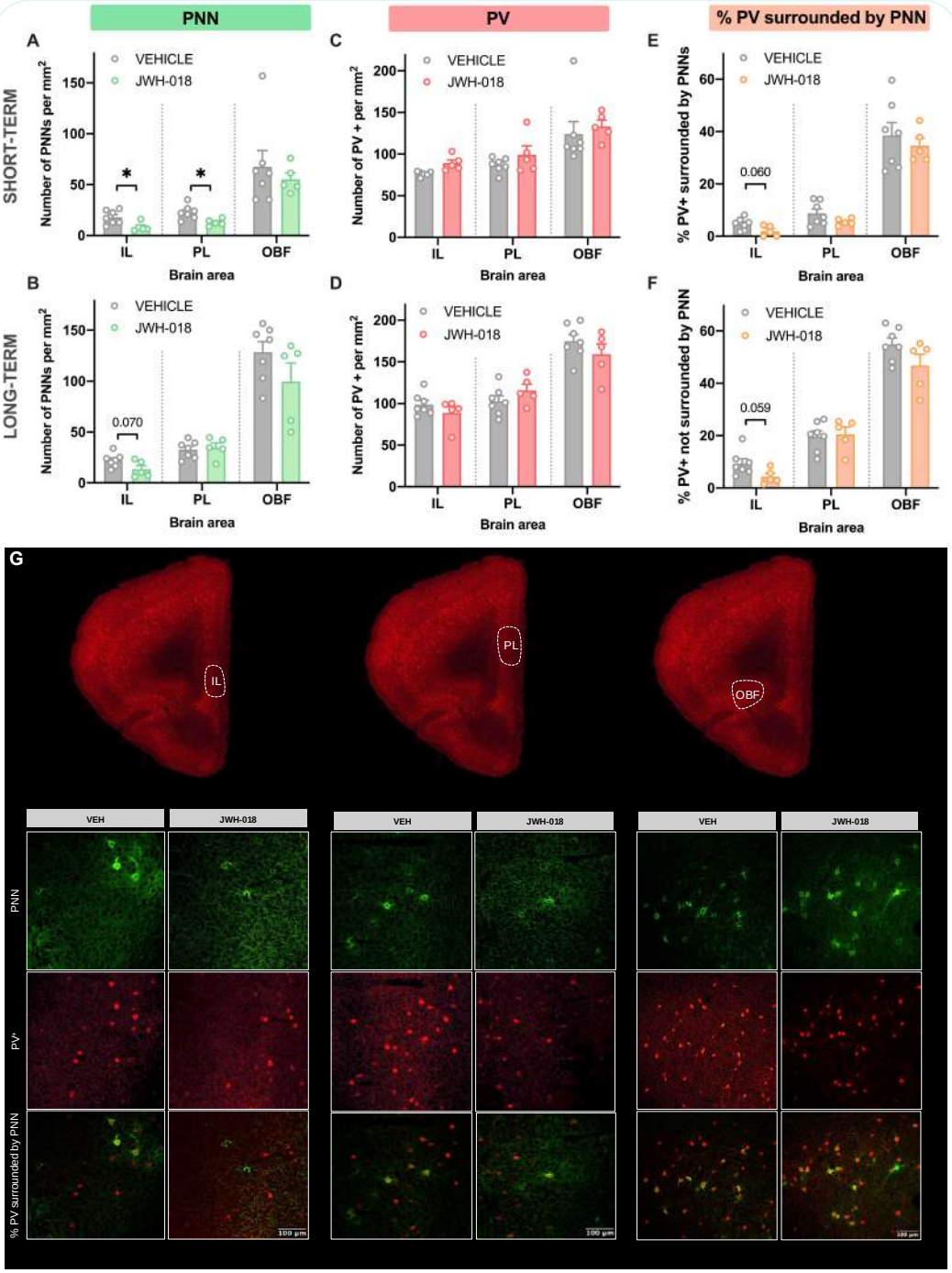

PNN
PV
% PV surrounded by PNN
SHORT-TERM
LONG-TERM
A
B
C
D
E
F
G
VEHICLE
JWH-018
Number of PNNs per mm²
Number of PV + per mm²
% PV+ surrounded by PNNs
% PV+ not surrounded by PNN
IL
PL
OBF
Brain area
0.060
0.070
0.059
VEH
JWH-018
PNN
PV+
% PV surrounded by PNN

Figure 46

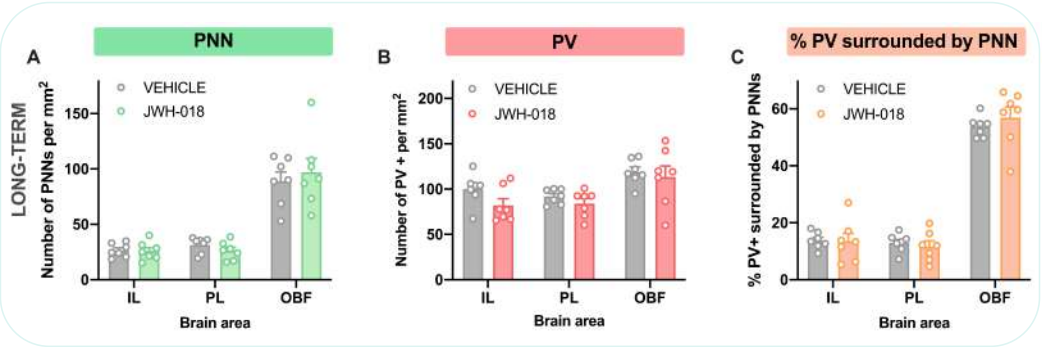


Figure 47. Adolescent exposure to JWH-018 does not induce alterations in cortical perineuronal nets and PV+ interneurons in female mice. *(A-C) Effects of JWH-018 exposure during adolescence in the number of PV+, PNNs and PV+ surrounded by PNNs in the IL, PL, and OBF of female mice at long-term (n = 7 mice per group). Tissue was obtained 24 h after the prepulse inhibition test. Data are expressed as mean ± SEM. IL: infralimbic prefrontal cortex; PL: prelimbic prefrontal cortex; OBF: orbitofrontal cortex; PV+: positive parvalbumin neuron; PNNs: perineuronal nets.*

6. Short- and long-term consequences on the microglia morphology and GFAP immunoreactivity in astrocytes in adolescent mice exposed to JWH-018

Microglial cells have been traditionally associated with immune functions in the CNS (Salter and Beggs, 2014; Li and Barres, 2018). However, recent studies have highlighted their involvement in other essential roles critical for maintaining brain homeostasis (Schafer and Stevens, 2015; Kierdorf and Prinz, 2017). PNNs act as scaffolds, stabilizing synapses during critical periods of neuroplasticity. As such, the condensation and removal of PNNs are tightly regulated at both genetic and enzymatic levels. While astrocytes' role in extracellular matrix remodeling has been well-documented over the past years (Silver and Miller, 2004; Yiu and He, 2006; Lau et al., 2013b; Raposo and Schwartz, 2014), the involvement of microglia in this process has only recently been explored (Crapser et al., 2021; Tewari et al., 2022). Further, pathological activation of both cell types has been shown to damage PNNs via the release of proteases and proteolytic enzymes that compromise their structural integrity (see Figure 48) (Crapser et al., 2020; Tewari et al., 2022). Therefore, we evaluated microglial and astrocytic activation through immunofluorescence in the PFC to delve into the alterations described in PNNs.

Figure 48. Representative image of microglial regulation of PNN integrity. *Microglia play a crucial role in regulating PNNs under both physiological and pathological conditions. In a healthy brain, microglia contribute to the maintenance of baseline PNNs and the perisynaptic extracellular matrix by continuously releasing proteases, protease inhibitors, or activators, as well as engaging in phagocytic processes. In addition, experimental depletion of local microglia has been shown to increase PNN deposition and density. Under pathological conditions or injury, microglial activation or dysfunction result in heightened phagocytosis and/or protease secretion. This can lead to the degradation of PNNs and excessive synaptic loss.*

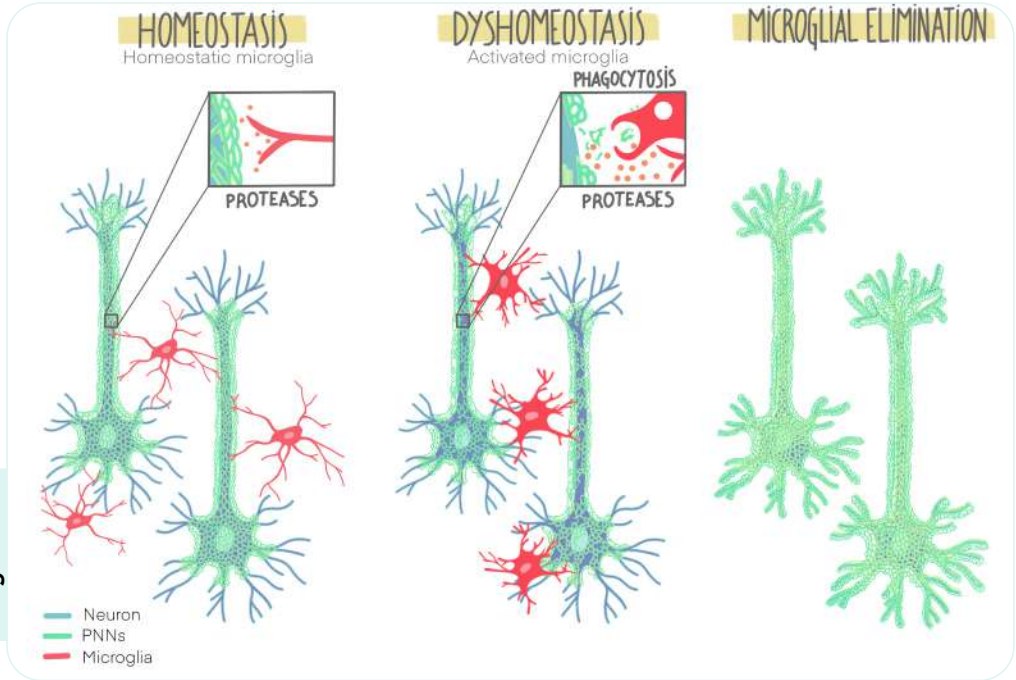


Figure 48

Notably, although no changes were observed in the total number of IBA-1 positive cells after JWH-018 exposure (Figure 49A, B and Figure 50A), an enhancement in the area of the microglia soma was observed in the PFC of males, but not females (Figure 50B), at short- ($p < 0.01$) and long-term ($p < 0.05$) (Figure 49C, D, G). Consistent with these findings, a marked trend toward an increase in the microglial soma perimeter was observed in the short term ($p = 0.06$) (Figure 49E, G), with this increase becoming significant in the long term in males ($p < 0.01$) (Figure 49F). This effect was neither observed in females at long-term (Figure 50C). Therefore, these results suggest that chronic JWH-018 treatment during adolescence in males induces a shift in microglial state, potentially toward a more reactive phenotype, as evidenced by the overall increase in soma size (Kohman and Rhodes, 2013).

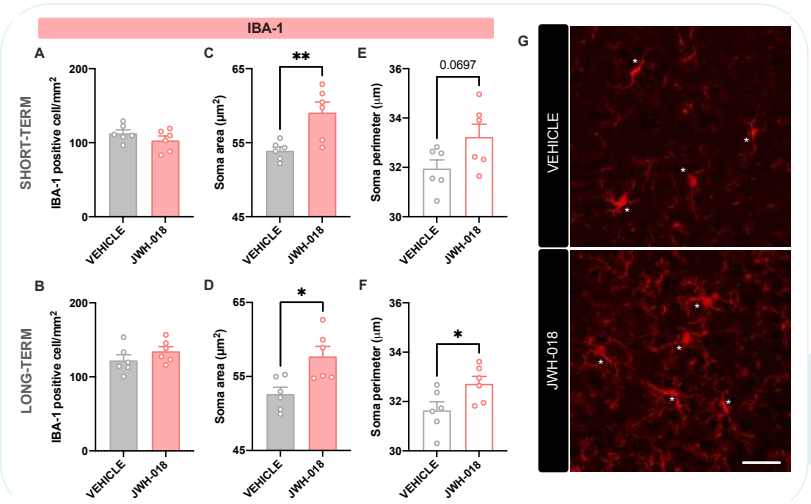


Figure 49

***Figure 49. JWH-018 exposure during adolescence induces microglia activation in the PFC in male mice**. (A-F) IBA-1 positive cells, area and perimeter of soma of Iba-1-stained cells in the PFC in male mice exposed to JWH-018 during adolescence at short- (A, C, E) and long-term (B, D, F). Tissue was obtained 24 h after the prepulse inhibition test (n = 6 mice per group). Representative images of males at long-term obtained by fluorescence microscopy labelling Iba-1 in red (G). Scale bar represents 50 µm. Asterisks indicate the microglia found in each field. Data are expressed as mean±SEM. *p < 0.05; **p < 0.01 (comparison between JWH-018 and vehicle; Student's t-test). Iba-1 ionized calcium-binding adapter molecule 1.*

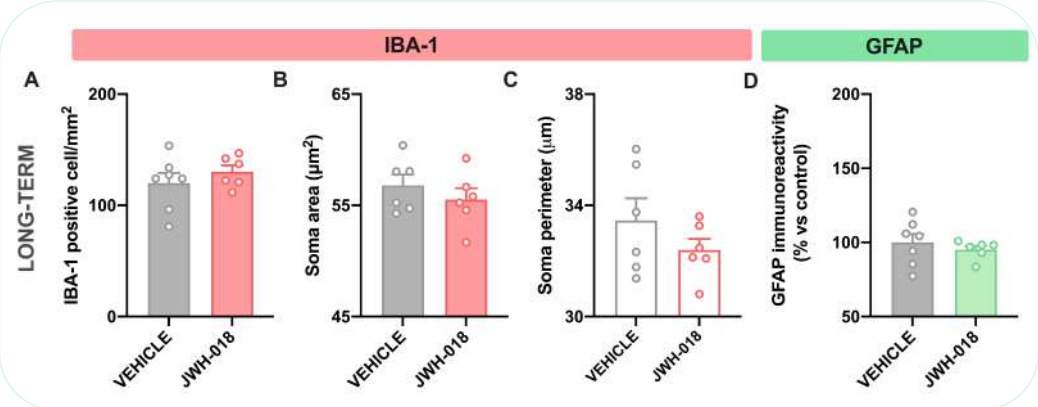


***Figure 50. Adolescent exposure to JWH-018 does not induce cortical alterations in microglia or astrocytes in female mice.** (A-C) Effects JWH-018 exposure during adolescence in the number of Iba-1 positive cells, area and perimeter of the soma of Iba-1- stained cells and GFAP immunoreactivity (D) in the PFC of female mice at long-term (n = 6-7 mice per group). Tissue was obtained 24 h after the prepulse inhibition test. Data are expressed as mean ± SEM. Iba-1; Ionized calcium-binding adapter molecule 1; GFAP: glial fibrillary acidic protein.*

In addition, the microglial reactivity measured by the soma area of male mice at short-term negatively correlates with the density of PNNs in both the IL ($p < 0.05$) (Figure 51A) and PL ($p < 0.05$) (Figure 51B). These results indicate that JWH-018 adolescent exposure induces changes in microglial reactivity in the PFC associated with a significant reduction of the density of PNNs in male mice. These structural changes might play a role in the PPI deficits observed in male mice.

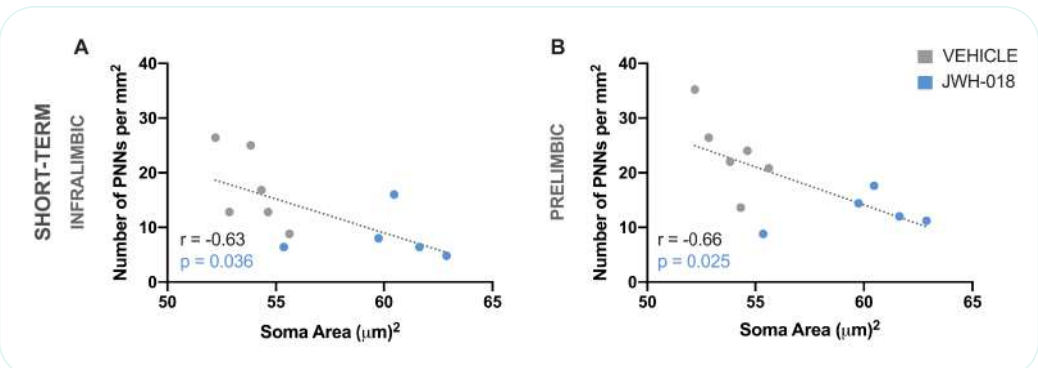


***Figure 51. Negative correlation between the number of PNNs and microglial reactivity in male mice.** (A-B) Significant correlation was observed between the number of PNNs in the IL (A) and PL of male mice at short-term (Pearson´s Correlation). PPNs; perineuronal nets.*

In support of this, a significant correlation was observed between the percentage of inhibition, when representing the prepulses of 69 and 73 dB, and the density of PNNs in the IL of males at short- ($p < 0.05$) (Figure 52A) and long-term ($p < 0.05$) (Figure 52B).

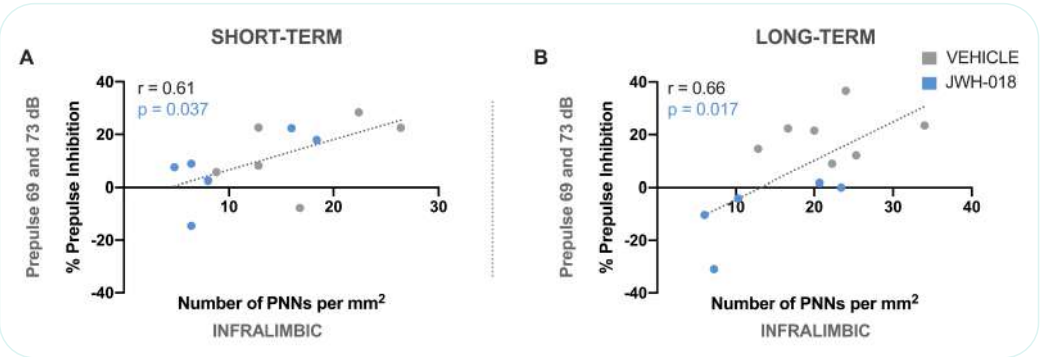


Figure 52. Significant correlations between the percentage of prepulse inhibition and the density of PNNs in the IL in male mice. *Adolescent exposure to JWH-018 leads to positive correlations between the percentage of prepulse inhibition (when representing the prepulses of 69 and 73 dB) and the density of PNNs in the IL in male mice at short- (A) and long-term (B). PNNs: perineuronal nets; dB: decibels.*

Regarding astrocytes, an increase of GFAP immunoreactivity following JWH-018 treatment was described in males at long-term in the PFC ($p < 0.05$) (Figure 53B). However, this change was not observed in males at short-term (Figure 53A) or in females at long-term (Figure 50D).

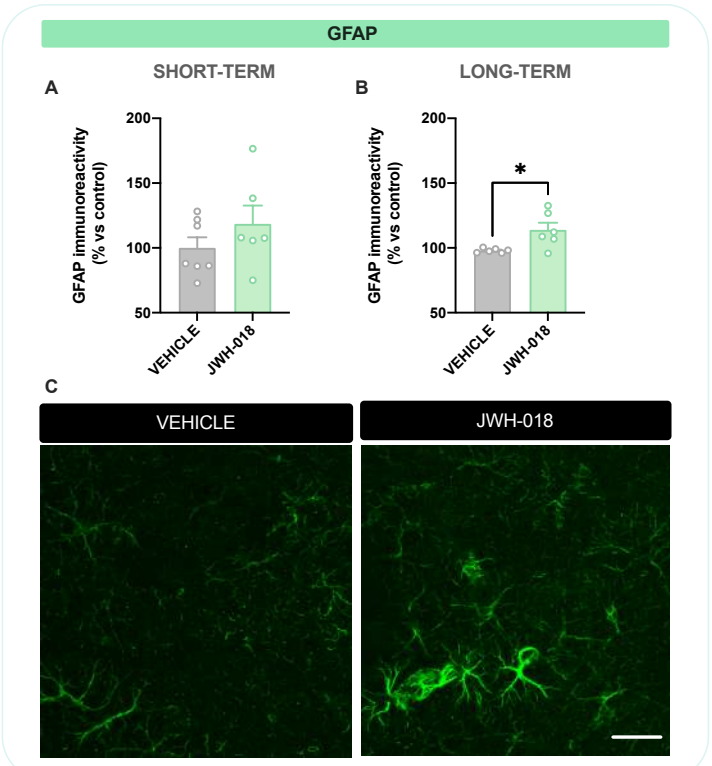


Figure 53. Adolescent exposure to JWH-018 increases GFAP immunoreactivity in male mice. *Astrocytes immunoreactivity in the PFC of male mice exposed to JWH-018 or vehicle during adolescence at short- (A) and long-term (B). Tissue was obtained 24 h after the prepulse inhibition test (n = 6 mice per group). Representative images of adult males obtained by fluorescence microscopy labelling GFAP in green (C). Scale bar represents 50 µm. Data are expressed as mean±SEM. *p < 0.05 (comparison between JWH-018 and vehicle; Student's t-test). GFAP glial fibrillary acidic protein.*

7. Short- and long-term consequences on the expression of GAD67, SYN, PSD95, BDNF, CB1R and CB2R in mice exposed to JWH-018 during adolescence

Finally, we explored whether JWH-018 exposure in male mice during adolescence could alter the expression pattern of genes linked to inhibitory neurotransmission, synaptic plasticity, and also CB1R and CB2R. In the short-term, mRNA levels of GAD67 ($p < 0.01$) and CB2R ($p < 0.05$) were significantly reduced in the PFC (Figure 54A), suggesting that these alterations were restricted to the early post-treatment period (Figure 54B). No significant differences were detected in SYN, PSD95, BDNF, or CB1R expression at either time point (Figure 54A, B).

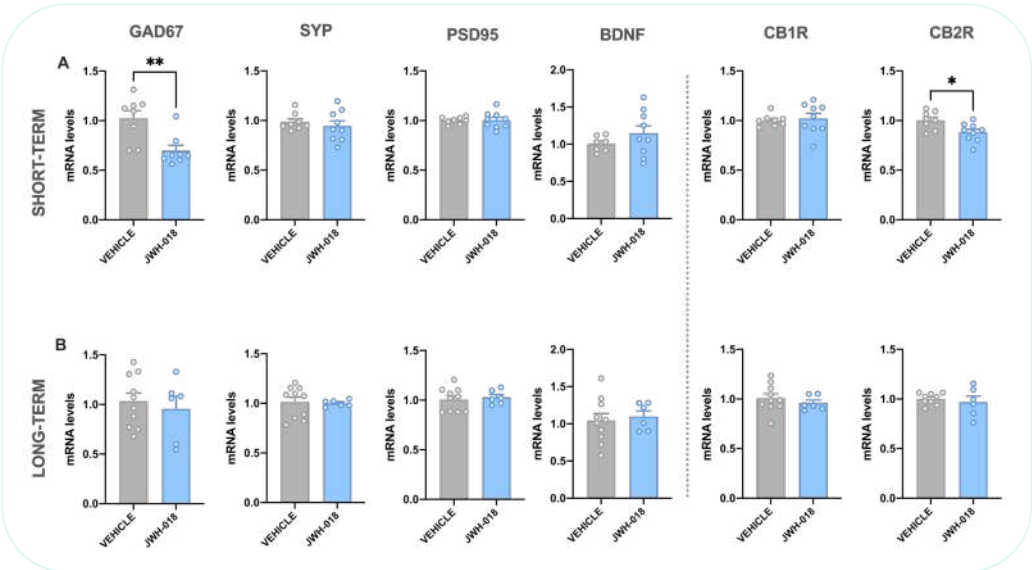


Figure 54. JWH-018 exposure during adolescence induces a decrease in the expression of GAD67 and CB2R in the PFC in male mice. *(A-B) mRNA levels of GAD67, SYP, PSD95, BDNF, CB1R and CB2R in the PFC in male mice exposed to JWH-018 during adolescence at short- (A) and long-term (B). Tissue was obtained 24 h after the prepulse inhibition test (n = 6–10 mice per group). Data are expressed as mean ± SEM. *p < 0.05; **p < 0.01 (comparison between JWH-018 and vehicle; Mann-Whitney test for GAD67 expression, and Student's t-test for CB2R expression). GAD67 glutamic acid decarboxylase 67, SYP synaptophysin, PSD95 postsynaptic density protein 95, BDNF brain derived neurotrophic factor, CB1R cannabinoid receptor type 1, CB2R cannabinoid receptor type 2.*

Consistently, reductions in cortical GAD67 expression have been previously described in schizophrenic patients (Hashimoto et al., 2003; Curley et al., 2011), and is also associated with psychotic-like symptoms in rats exposed to THC (Zamberletti et al., 2014). Further, previous reports have associated PPI impairments with reduction in both GAD67 and CB2R levels in the PFC (Toriumi et al., 2016; Ten-Blanco et al., 2022b).

As a whole, our findings suggest that the consumption of substances containing JWH-018 during adolescence could significantly impact on health, representing a major public health concern.

OBJECTIVE 2: AB-FUBINACA EFFECTS II

AB-FUBINACA is a SCB classified within the aminoalkylindazole derivatives, mainly found in most recent preparations of Spice/K2. As the case of JWH-018, acute consumption of AB-FUBINACA and analogues has been associated with hallucinations, deliriums, anxiety, psychosis and aggressive behaviors (Trecki et al., 2015; Canazza et al., 2017), but also with deaths (Trecki et al., 2015). Consistently, behavioral studies in rodents treated with aminoalkylindazoles have identified alterations related to anxiety, psychosis, and memory in both acute and chronic protocols (Kevin et al., 2017; Bilel et al., 2019; Schreiber et al., 2019; Pineda Garcia et al., 2024). However, research into the effects of SCBs has mostly focused on indole derivatives such as JWH-018, while impact on behavior of other SCBs classes, such as indazole derivatives, have been poorly studied. Moreover, preclinical research has rarely explored the consequences of adolescent exposure to SCBs, a critical developmental period susceptible to central effects of drugs. Therefore, the aim of this section was to evaluate behavioral and biochemical alterations induced after adolescent exposure to AB-FUBINACA in adult male and female mice.

1. Effects of adolescent AB-FUBINACA exposure on body weight

Similar to JWH-018, male and female mice were treated for 15 days with escalating doses of AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg), and body weight was registered daily. A significant reduction in weight gain between AB-FUBINACA and vehicle-treated groups was revealed in both males (Figure 55A) and females (Figure 55B) (interaction day x treatment: $F_{14,1162} = 10.79$, $p < 0.0001$ and $F_{14,1372} = 4.99$, $p < 0.0001$, for male and female mice, respectively). These differences in weight became significant once the higher dose was reached, as indicated by the area under the curve in male (AUC day 11-15 $p < 0.05$) and female (AUC day 11-15 $p < 0.001$) mice (Figure 55A, B).

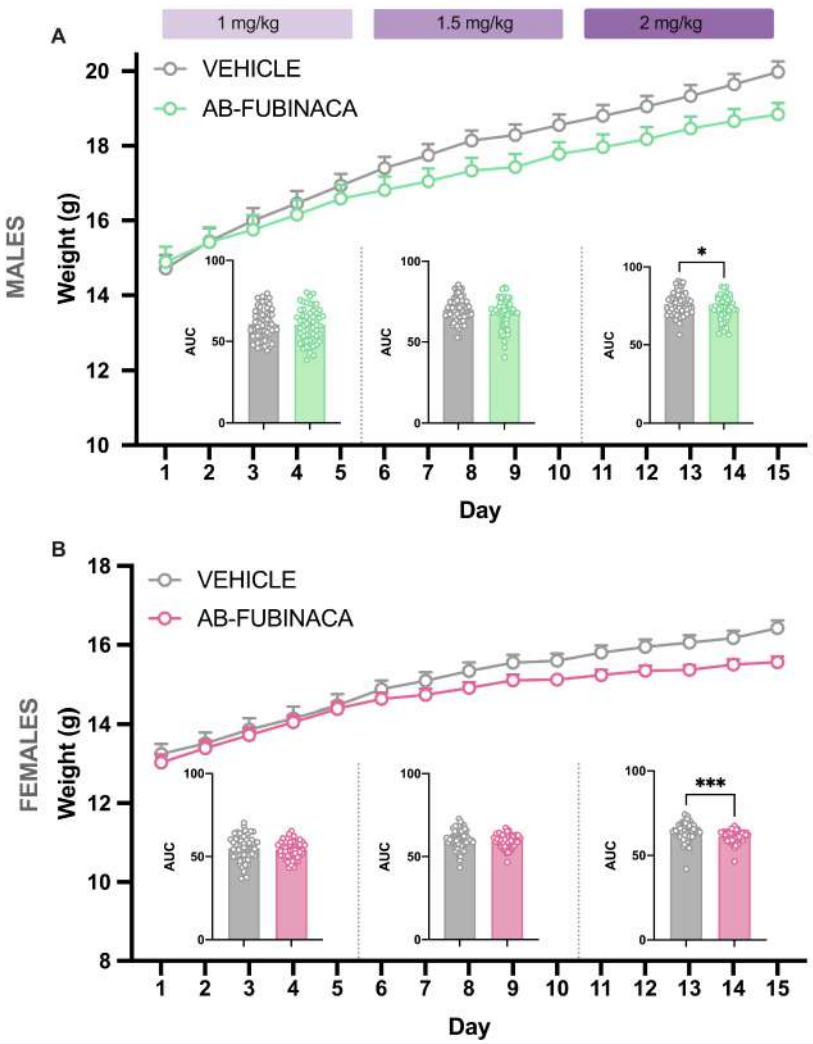


Figure 55. Adolescent exposure to AB-FUBINACA alters body weight in male and female mice. *Effects of treatment with AB-FUBINACA during adolescence in body weight of adolescent male (A) and female (B) mice (n= 54-61 mice per group). Daily weight in grams during the 15 days of treatment and AUC values every five days are shown. Data are expressed as mean ± SEM. *p < 0.05, ***p < 0.001 (comparison between AB-FUBINACA and vehicle; Student's t-test (A); Mann-Whitney test (B)). AUC, area under the curve.*

2. Long-term consequences on anxiety and conditioning and extinction of fear in mice exposed to AB-FUBINACA during adolescence

Unlike the previous protocol employed to study JWH-018 alterations, AB-FUBINACA effects were exclusively explored at long-term, 20 days after the finishing of the treatment. Potential changes in anxiety, fear conditioning and extinction and locomotion were examined as described in Figure 56.

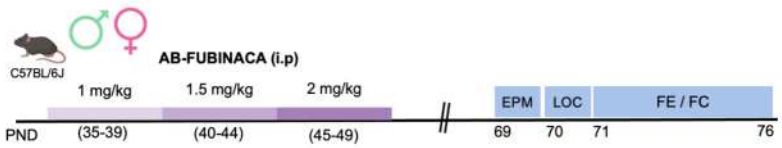


Figure 56. Schematic representation of the experimental design. *EPM elevated plus maze, FC fear conditioning, FE fear extinction, LOC locomotion, PND post-natal day*

AB-FUBINACA treatment during adolescence induced an opposite effect on anxiety between sexes when performing the EPM. While males spent more time in the open arms indicating an anxiolytic-like effect ($p < 0.05$) (Figure 57A), females exhibited an anxiogenic-like response compared to the control group ($p < 0.05$) (Figure 57B). No changes were observed in the total number of entries in either males or females (Figures 57A, B).

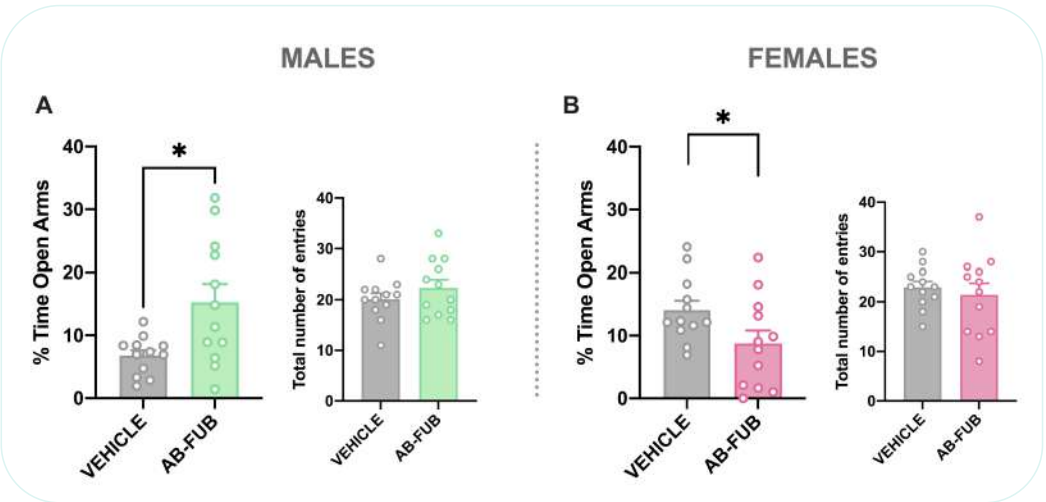


Figure 57. AB-FUBINACA treatment during adolescence alters anxiety-like behavior in a sex-dependent manner. *(A-B) Effects of adolescent exposure to AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) or vehicle in anxiety-like behavior in male (A) and female (B) mice (n = 12 mice per group). Percentage of time spent in the open arm and total number of entries are shown for the EPM. Data are expressed as mean ± SEM. *p < 0.05 (comparison between AB-FUBINACA and vehicle; Student's t-test).*

Regarding fear-related behavior, female, but not male mice, exposed to AB-FUBINACA during adolescence exhibited higher fear acquisition compared to the control group (interaction cue x treatment: $F_{2,40} = 4.91$, $p < 0.05$) (Figure 58A, C). However, neither males nor females displayed changes in the extinction of aversive memories (Figure 58B, D).

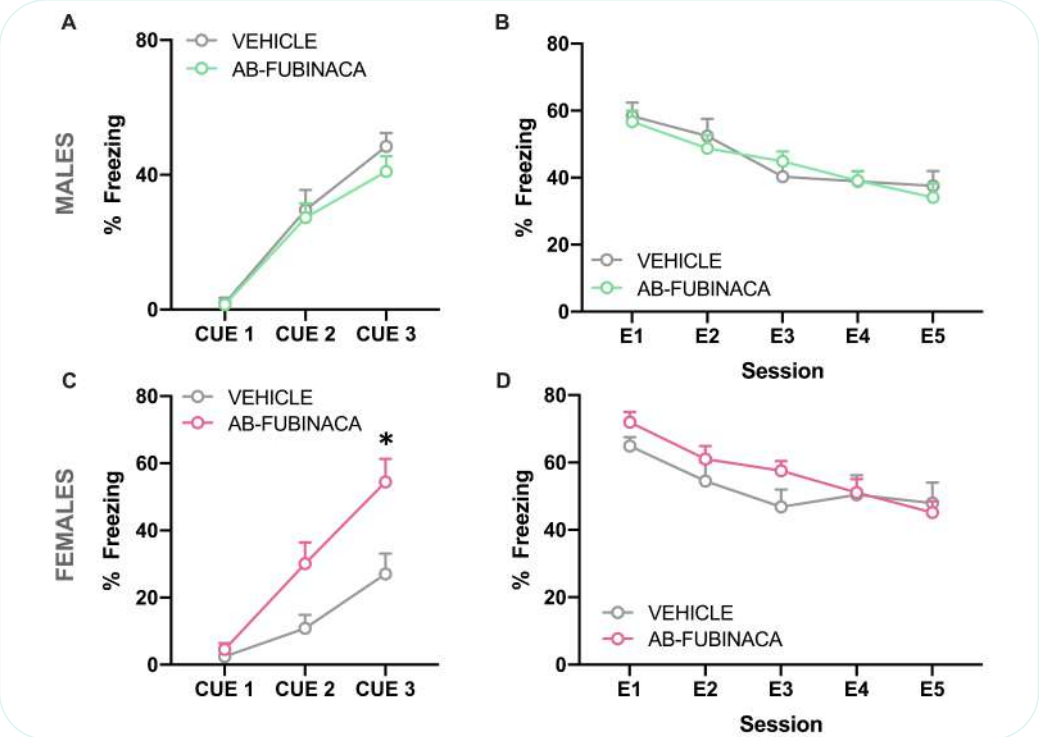


Figure 58. Adolescent exposure to AB-FUBINACA alters fear conditioning in female mice. *(A-D) Effects of treatment with AB-FUBINACA during adolescence in fear conditioning (A,C) and extinction (B, D) in adult male (A, B) and female (C,D) mice (n = 12 mice per group). Time course of the freezing levels scored during each cue and extinction session are shown. Data are expressed as mean ± SEM. *p < 0.05 (comparison between AB-FUBINACA and vehicle group; two-way ANOVA with repeated measures (C)).*

Interestingly, a negative correlation between the anxiety score, measured by the EPM, and the percentage of freezing during the conditioning session was found in female mice ($p < 0.05$) (Figure 59). This suggests that an anxiety-like state induced by AB-FUBINACA treatment could be related to a higher conditioning to an aversive stimulus.

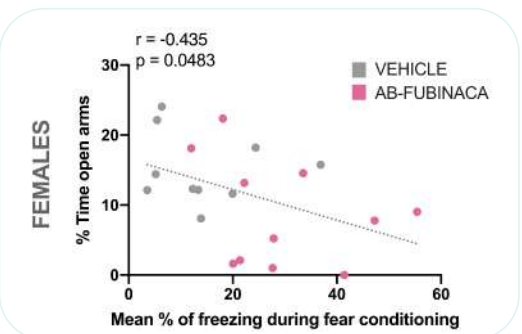


Figure 59. Negative correlation between the percentage of time in open arms and the mean percentage of freezing during fear conditioning in female mice*. (n = 10–11 mice per group) (Pearson's correlation coefficient)*

Finally, no differences where observed in locomotion between AB-FUBINACA- and vehicle-treated groups, either in males (Figure 60A) or females (Figure 60B).

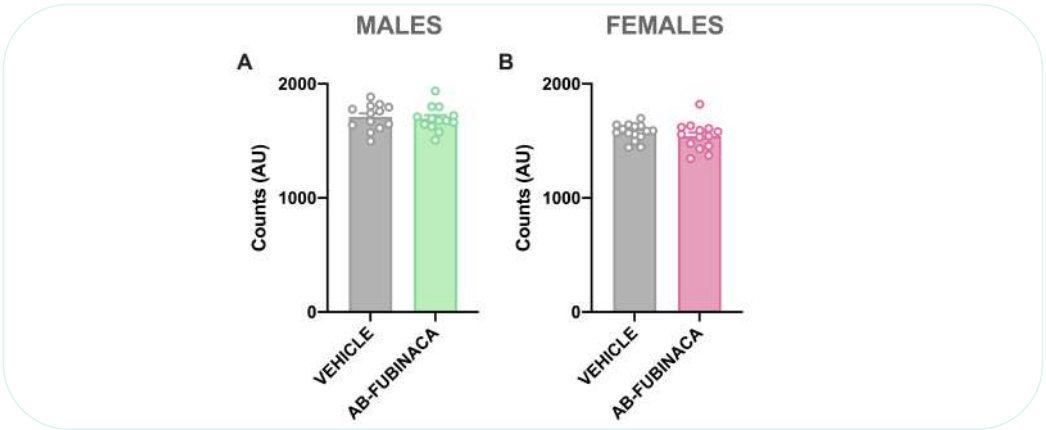


Figure 60. Adolescent exposure to AB-FUBINACA does not modify locomotion in male and female mice. *(A-B) Effects of treatment with AB-FUBINACA during adolescence on locomotor activity in adult male (A) and female (B) mice (n = 13-15 mice per group). Data are expressed as mean ± SEM. AU, arbitrary units.*

These findings suggest that AB-FUBINACA exposure during adolescence induces sex-specific effects on unconditioned anxiety which could be associated with higher fear conditioning in female mice.

3. Long-term consequences on non-emotional memory, sociability and depression in mice exposed to AB-FUBINACA during adolescence

Adolescent cannabinoid use significantly affects different functional domains (Rubino and Parolaro, 2008, 2016), including non-emotional memory deficits and emotional disturbances, such as depression and impaired social behavior (Gruber, 2002; Hayatbakhsh et al., 2007). Based on these findings, this study aimed to examine the potential effects of adolescent exposure to AB-FUBINACA on memory, sociability, and depressive-like behaviors (Figure 61).

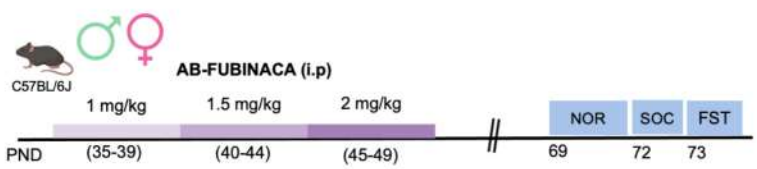


Figure 61. Schematic representation of the experimental design. *FST forced swimming test, NOR novel object recognition, PND post-natal day, SOC sociability test.*

Interestingly, memory was not affected in male mice exposed to AB-FUBINACA by using the NOR test (Figure 62A). However, the discrimination index of females treated with this SCB was significantly lower than the vehicle-treated group ($p < 0.05$) (Figure 62C), suggesting sex-dependent memory deficits. *Llorente-Berzal* and colleagues reported a similar sexual dimorphism in memory following THC treatment during adolescence, pointing to females as more vulnerable to alterations in recognition memory (Llorente-Berzal et al., 2013b).Total time of exploration was not affected in any experimental group (Figure 62B, D).

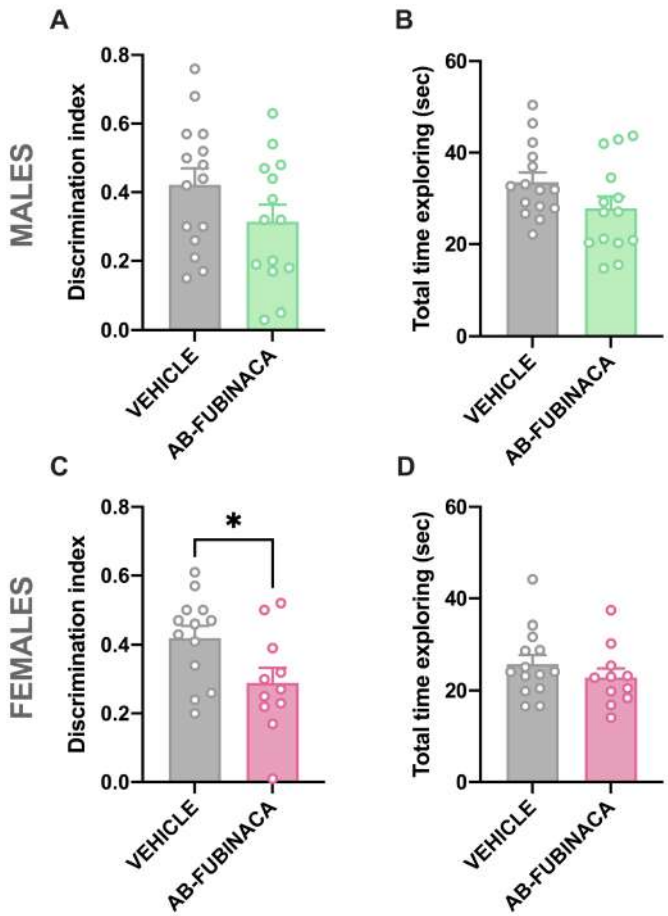


Figure 62. AB-FUBINACA treatment during adolescence alters novel object recognition memory in a sex dependent manner. *(A-D) Effects of adolescent exposure to AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) or vehicle in memory in the NOR in male (A, B) and female (C, D) mice (n = 11-15 mice per group). The discrimination index (A, C) and the total time of exploration (B, D) are shown. Data are expressed as mean ± SEM. *p < 0.05 (comparison between AB-FUBINACA and vehicle group; Student's t-test (C).*

By using the three-chamber test, we evaluated potential alterations in social behavior. No differences were found between AB-FUBINACA- or vehicle-treated groups in either male or female mice (Figure 63A, B).

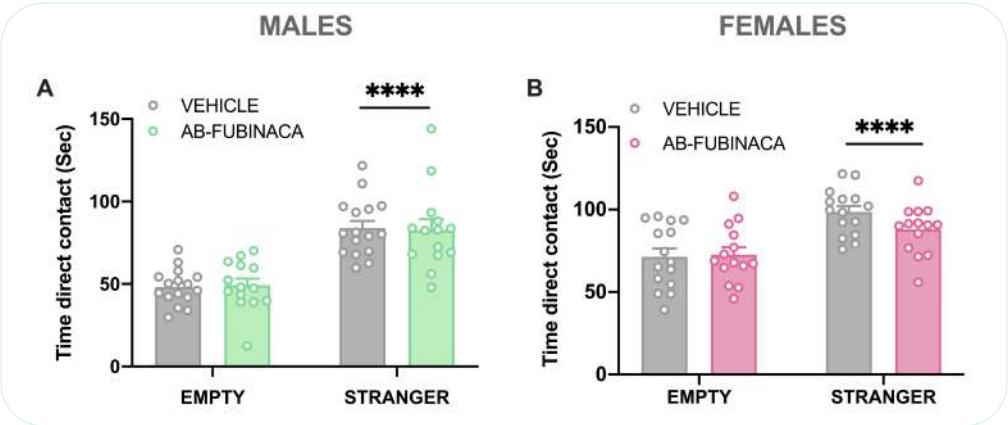


Figure 63. Adolescent exposure to AB-FUBINACA did not alter social behavior. *(A-B) Effects of adolescent exposure to AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) or vehicle in social behavior in the three-chamber test in male (A) and female (B) mice (n = 14-16 mice per group). The total time in direct contact with each compartment is shown. Data are expressed as mean ± SEM. ****p < 0.001 (comparison between empty and stranger compartments; two-way ANOVA, compartment effect (A, B).*

Finally, chronic treatment during adolescence with AB-FUBINACA showed a significant decreased in the immobility time in male mice ($p < 0.01$), but not females, in the forced swimming test compared to the control group (Figure 64A, B). This reduction in immobility behavior suggests a protective phenotype against depressive-like behaviors in male mice treated with AB-FUBINACA.

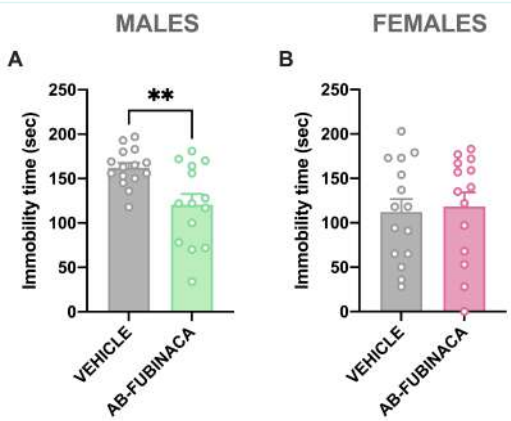


Figure 64. AB-FUBINACA treatment during adolescence modulates depressive-like behavior in a sex dependent manner. *(A-B) Effects of adolescent exposure to AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) or vehicle in depressive-like behavior in the FST in male (A) and female (B) mice (n = 14-15 mice per group). The immobility time is shown. Data are expressed as mean ± SEM. **p < 0.01, (comparison between AB-FUBINACA and vehicle group; Student's t-test (A).*

4. Long-term consequences on sensorimotor gating in mice exposed to AB-FUBINACA during adolescence

Next, we aimed to assess potential long-term psychotic-like effects by using the PPI test. Considering that Spice/K2 consumption has been associated to several acute psychotic episodes (Ford et al., 2017), we explored possible similar long-term consequences. Thus, potential alterations in the somatosensory filter were evaluated 20 days after the end of AB-FUBINACA or vehicle treatment in both male and female mice (Figure 65).

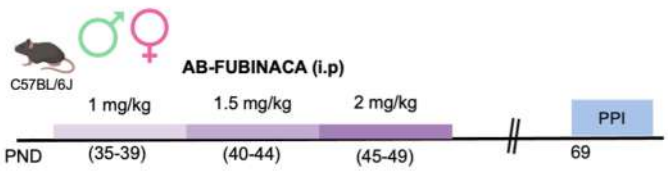


Figure 65. Schematic representation of the experimental procedure. *PPI prepulse inhibition test, PND post-natal day*

Interestingly, in contrast to the previous observations with JWH-018, treatment during adolescence with AB-FUBINACA did not alter sensorimotor gating in adult male mice (Figure 66A, B). Furthermore, no modifications were observed in the startle response in these animals (Figure 66C). However, females exhibited significantly lower inhibition levels at the different prepulse intensities following chronic AB-FUBINACA exposure during adolescence (treatment effect: $F_{1,26} = 6.04$, $p < 0.05$) (Figure 66D). In addition, an overall reduction of PPI due to AB-FUBINACA exposure was also observed when representing mean PPI score ($p < 0.05$) (Figure 66E). This effect was independent of baseline changes in startle amplitude (Figure 66F), ruling out an impact of startle reaction in the modifications of PPI observed. Consistently, *Iemolo* and collaborators described PPI impairments after chronic exposure to THC during adolescences in adult female mice (Iemolo et al., 2021). Taken as a whole, these results support that adolescent exposure to AB-FUBINACA elicits sex-dependent disruptions in sensorimotor gating, an index related to psychotic disorders.

Figure 66. AB-FUBINACA treatment during adolescence alters sensorimotor gating in a sex-dependent manner. *(A-F) Effects of adolescent exposure to AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) or vehicle in sensorimotor gating in male (A, B, C) and female (D, E, F) mice (n = 13-15 mice per group). Percentage of prepulse inhibition (A, D), mean of the percentage of prepulse inhibition (B, E), and startle response amplitude (C, F) are shown. Data are expressed as mean ± SEM. *p < 0.05 (comparison between AB-FUBINACA and vehicle group; two-way ANOVA with repeated measures, treatment (D); Student's t-test (E). dB decibels.*

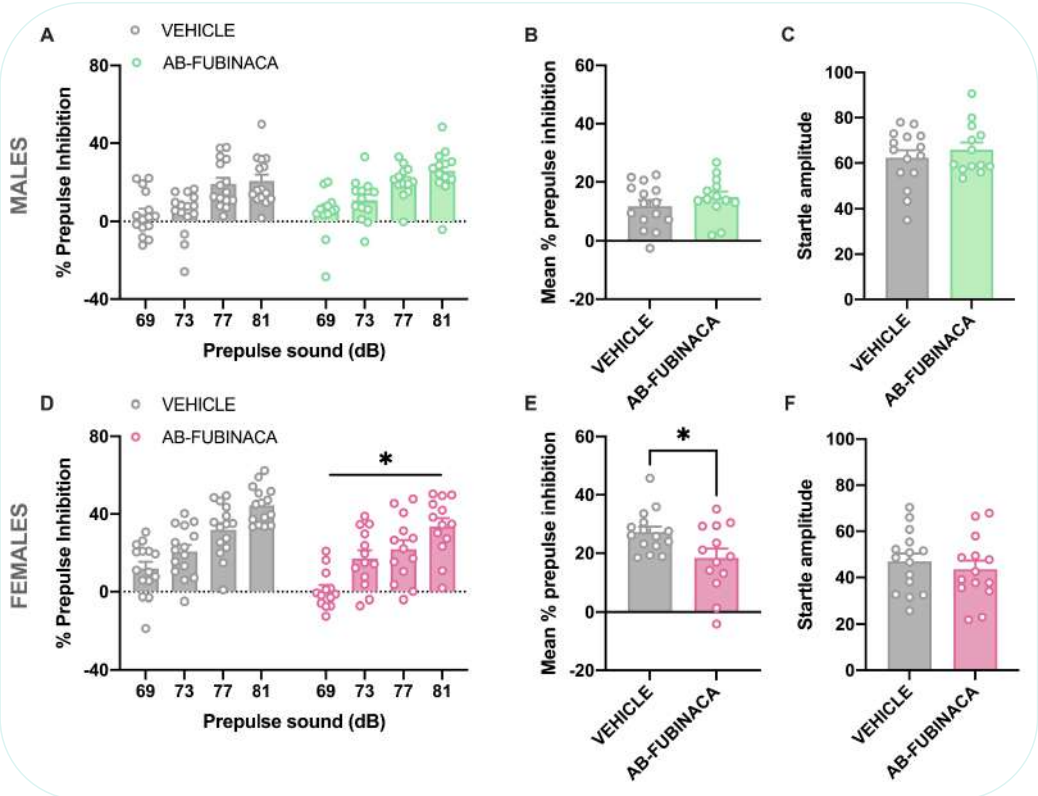


Figure 66

5. Long-term consequences of AB-FUBINACA exposure during adulthood in female mice

Numerous studies support that adolescence represents a critical window of vulnerability. The immaturity of the brain, together with the numerous biochemical changes occurring during this period, makes it particularly susceptible. Considering that most harmful consequences observed following AB-FUBINACA exposure during adolescence were described in females, this experiment was conducted only in female mice. Therefore, to assess whether the period of exposure is a key factor in the alterations previously observed, the SCB was administered during adulthood (PND 70-84). Twenty days after the treatments ended, EPM, NOR and PPI tests were performed to assess potential alterations on anxiety, non-emotional memory and psychosis, respectively (Figure 67).

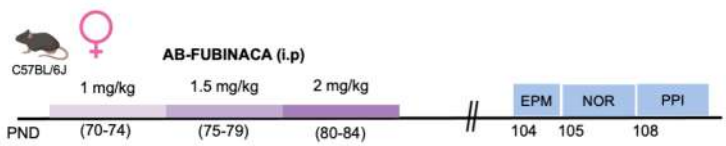


Figure 67. Schematic representation of the experimental design. *EPM elevated plus maze, NOR novel object recognition, PND post-natal day, PPI prepulse inhibition test.*

AB-FUBINACA adult treatment did not induced alterations in the EPM (Figure 68A, B) or NOR in female mice (Figure 68C, D). These results highlight that adolescence is a vulnerable developmental period for the detrimental impact of AB-FUBINACA exposure on anxiety and memory.

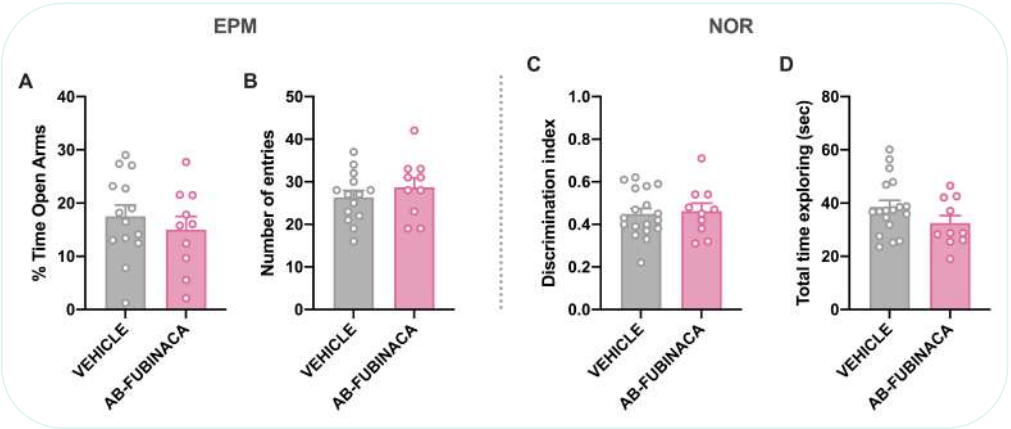


Figure 68. AB-FUBINACA treatment during adulthood did not alter anxiety-like behavior or memory in female mice. *(A) Schematic representation of the experimental design. (A-D) Effects of adult exposure to AB-FUBINACA (PND 69–73: 1 mg/kg, PND 74–78: 1.5 mg/kg, and PND 79–83: 2 mg/kg) or vehicle in anxiety-like behavior in the EPM (A, B) and non-emotional memory in the NOR (C, D) in female mice (n = 10-18 mice per group). Percentage of time spent in the open arm and total number of entries are shown for the EPM and the discrimination index and total time of exploration are represented for the NOR. Data are expressed as mean ± SEM. EPM elevated plus maze, NOR novel object recognition.*

Conversely, chronic AB-FUBINACA treatment during adulthood induced a reduction in PPI response (Figure 69A, B), despite showing a similar startle reflex amplitude to the control group (Figure 69C). A similar impairment was observed when females were treated during adolescence, suggesting that the effect of AB-FUBINACA on sensorimotor gating in females is independent of the period of exposure. In addition, these findings highlight the potent pro-psychotic-like consequences associated to AB-FUBINACA exposure.

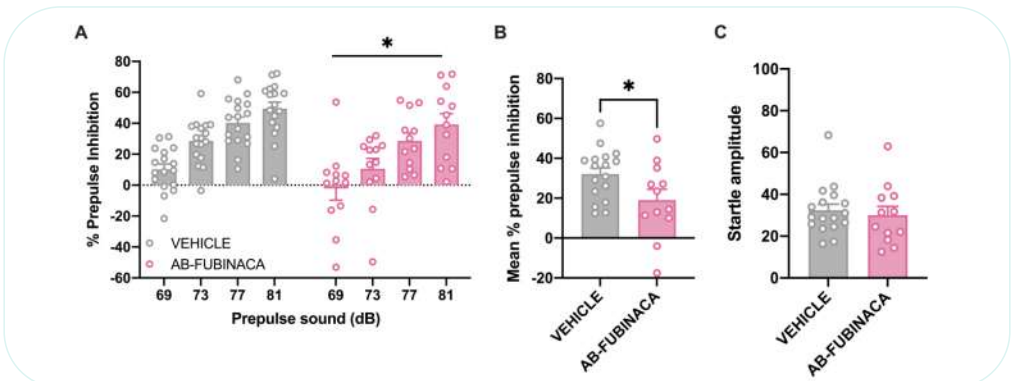


Figure 69. AB-FUBINACA treatment during adulthood alters sensorimotor gating in female mice. *(B-C) Effects of adult exposure to AB-FUBINACA (PND 69–73: 1 mg/kg, PND 74–78: 1.5 mg/kg, and PND 79–83: 2 mg/kg) or vehicle in sensorimotor gating in female mice (n = 12-17 mice per group). Percentage of prepulse inhibition (A), mean of the percentage of prepulse inhibition (B), and startle response amplitude (C) are also shown. Data are expressed as mean ± SEM. *p < 0.05 (comparison between AB-FUBINACA and vehicle group; two-way ANOVA with repeated measures, treatment (A); Student's t-test (B). dB decibels.*

6. Transcriptome analysis in adult female mice exposed to AB-FUBINACA during adolescence

As previously mentioned, the PFC is a key brain region involved in several functions central to this thesis, including sensorimotor gating regulation (Tóth et al., 2017), emotional response processing (LeDoux, 2000), and memory formation (Funahashi, 2017; Chao et al., 2022). Based on the above, to explore potential neurobiochemical mechanisms underlying the previous observed behavioral alterations, primarily psychosis, RNA sequencing (RNA-seq) of the PFC was conducted (Figure 70A). Brains were collected from female mice treated with AB-FUBINACA during adolescence, 24 h after completing the PPI test. Principal component analysis is shown in Figure 70B. The RNA-seq revealed that AB-FUBINACA treatment induced an upregulation of *Plekhg2* and *Sh3tc1* (adjusted $p < 0.05$ and cutoff of 2-fold change) 20 days after the finishing of the treatment (Figure 70C,D).

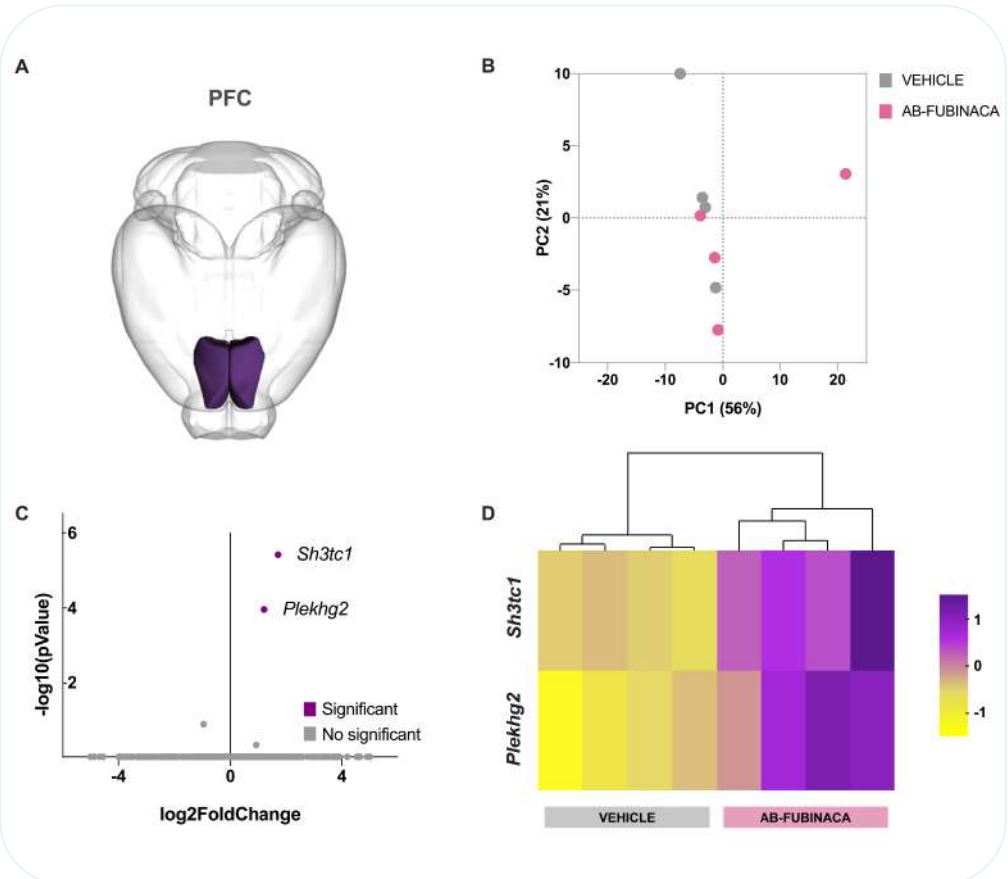


Figure 70. AB-FUBINACA treatment during adolescence induces an up-regulation of PLEKHG2 and SH3TC1 genes in the PFC of adult females. *(A) Schematic representation of the extracted area. (B) Principal component analysis. (C) Volcano plot summarizing the differentially expressed genes (DEGs) of AB-FUBINACA vs vehicle treated mice (n = 4 mice per group). (D) DEGs in each AB-FUBINACA and vehicle treated mouse clustered with a heat map. PFC prefrontal cortex, DEGs differentially expressed genes.*

Notably, significant negative correlations between the percentage of PPI and the relative expression of both *Plekhg2* ($p < 0.001$) (Figure 71A) and *Sh3tc1* genes ($p < 0.05$) (Figure 71B) in the PFC of female mice were found.

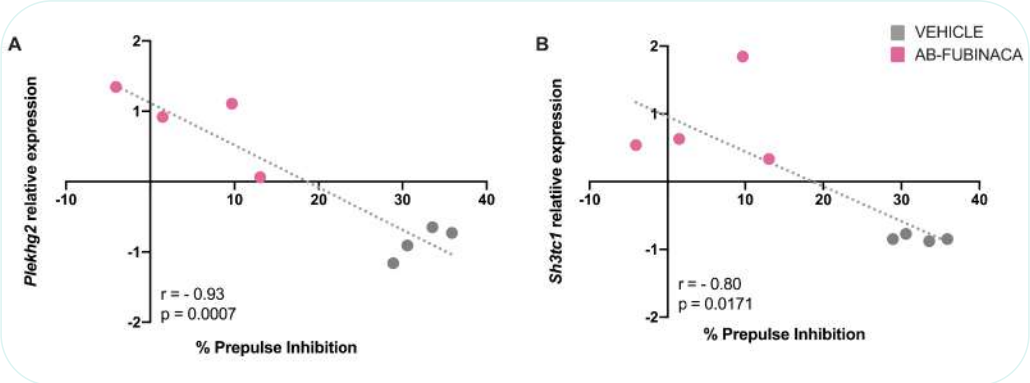


Figure 71. Significant negative correlation between the percentage of PPI and the expression of both DEGs. *A-B) Correlation between percentage of prepulse inhibition and Plekhg2 (A) and Sh3tc1 (B) relative expression (n = 4 mice per group) (Pearson correlation coefficient).*

Plekhg2 gene, encoding a Rho family-specific guanine nucleotide exchange factor (RhoGEF) (Figure 72A), is known to act as a positive regulator of Cdc42 and Rac (Runne and Chen, 2013; Sugiyama et al., 2017). This RhoGEF activation regulates various cellular process in the CNS, including cortical neuron migration during corticogenesis and spine formation (Govek et al., 2011; Ito et al., 2019a). In addition, modulation of *Plekhg2* gene has been shown to impaired dendritic arborization and caused defects in dendritic spine morphology formation in mice (Nishikawa et al., 2022). Further, GTP-dependent activation of Cdc42 has been reported to be in some cases modulated by an SH3-binding domain (Barfod et al., 1993; Hildebrand et al., 1996; Wheatley and Rittinger, 2005). As *Sh3tc1* gene encodes a specific SH3-binding domain (Figure 72B), we investigated the activation profile of Cdc42 through *Plekhg2* by preforming a G-LISA assay. No significant differences between treated and control groups were found in Cdc42 activation in the PFC (Figure 72C). These results suggest that *Plekhg2* might be regulating other RhoGEFs, or alternatively, that Cdc42 activation may need to be measured at a time point closer to the end of AB-FUBINACA treatment.

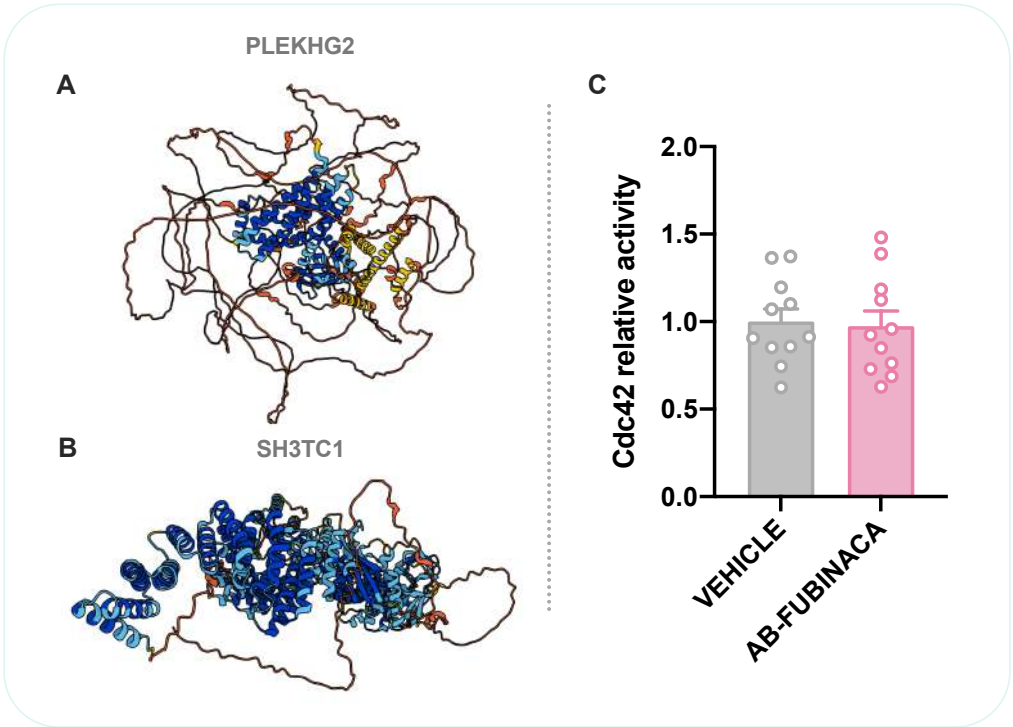


Figure 72. Adolescent exposure to AB-FUBINACA does not modify Cdc42 activity in PFC of adult female mice. *(A-B) Schematic representation of predicted PLEKHG2(A) and SH3TC1 (B) proteins obtained in AlphaFold. (C) Cdc42 relative activity measured by a G-LISA assay (n = 11 mice per group). Data are expressed as mean ± SEM.*

7. Structural plasticity analysis in adult female mice exposed to AB-FUBINACA during adolescence

We then examined whether structural plasticity in the pyramidal neurons of the PFC was altered in female mice 20 days after chronic AB-FUBINACA treatment during adolescence. By performing a sholl analysis, females exposed to AB-FUBINACA showed lower dendritic arborization than the control group (treatment effect: $F_{1,46} = 6.01$, $p < 0.05$; interaction treatment x radius: $F_{14,370} = 4.68$, $p < 0.0001$) (Figure 73A, B). PCA of different factors measured in the sholl analysis revealed distinct neuronal populations between AB-FUBINACA- and vehicle- treated groups (Figure 73C).

Figure 73. AB-FUBINACA treatment during adolescence induces alterations in dendritic arborization in the PFC of adult females. *Effects of treatment with AB-FUBINCA or vehicle during adolescence in dendritic arborization (A-C) in the prefrontal cortex of adult female mice. (A) Representative traces of reconstructed neurons in AB-FUBINACA or vehicle-treated female mice. Scale bar = 50 µm. (B) Sholl analysis represented by the number of intersections every 20 µm. (C) Plot comparing the total number of reconstructed neurons (n = 6 neurons/mouse and n = 4 mice per group) across principal components PC1 and PC2. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 (comparison between AB-FUBINACA and vehicle group; mixed-model ANOVA, interaction treatment x radius (B); PC principal components 1 and 2.*

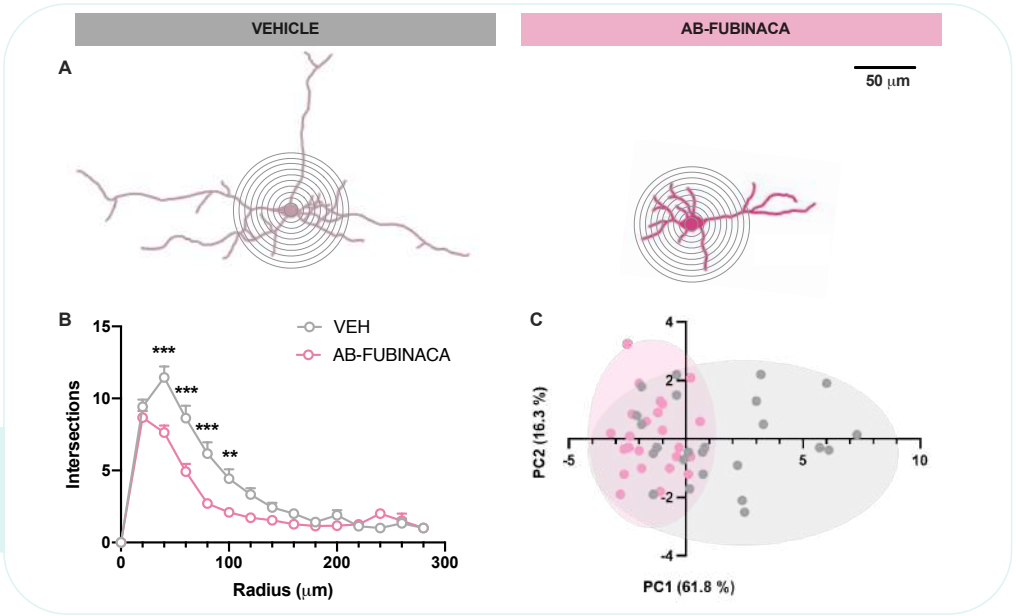


Figure 73

In addition, SCBs exposure during adolescence significantly reduced the total length of neurons in adult females ($p < 0.05$) (Figure 74A, B), primarily due to a decreased of the length of basal ($p< 0.001$) (Figure 74C), but not apical dendrites (Figure 74D). These differences in dendritic arborization were revealed as the branching order increased (secondary $p < 0.05$; tertiary $p < 0.01$) (Figure 74E, F, G).

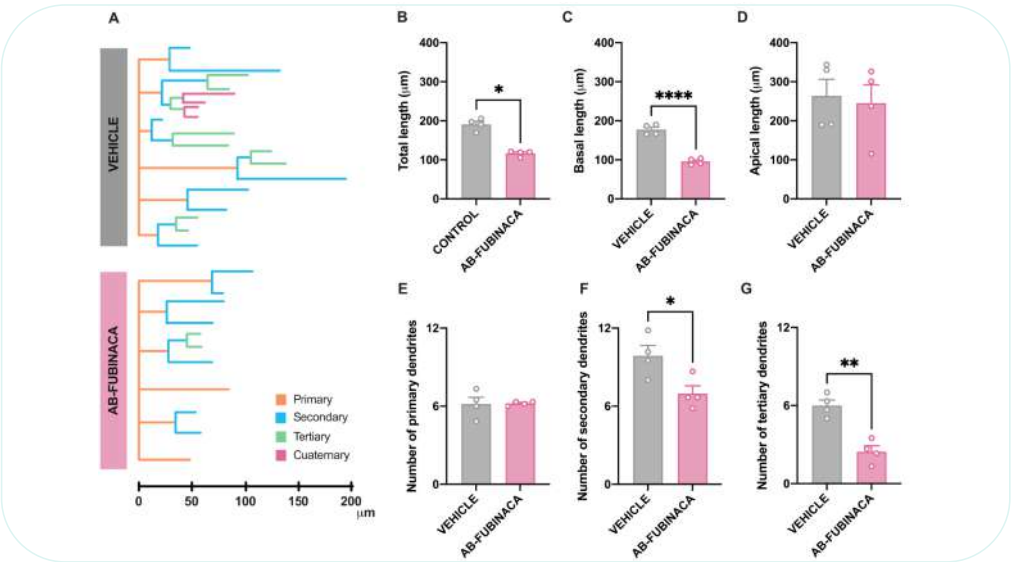


Figure 74. Adolescent AB-FUBINACA treatment alters neuron length and dendritic arborization in the PFC of females 20 days after. *(A) Representative dendrogram of cortical neurons in AB-FUBINACA or vehicle treated female mice. Dendrogram is colored by branch order. (B-D) Dendritic length expressed as total (B), basal (C) and apical (D). (E-G) Number of dendrites classified by orders in adult females treated with AB-FUBINACA or vehicle during adolescence. Primary (E), secondary (F) and tertiary dendrite orders (G) are shown (n = 6 neurons/animal, n = 4 mice per group). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001 (comparison between AB-FUBINACA and vehicle group; Student's t-test (C, F, G); Mann-Whitney test (B)).*

Interestingly, total apical (Figure 75A,B,C), but not basal (Figure 75D,E), dendritic spine density decreased in female mice exposed to AB-FUNIACA ($p < 0.01$). Specifically, mushroom-like spines, which are considered mature, showed a lower density after SCB exposure in females ($p < 0.05$) (Figure 75C). However, no changes were observed in long-thin, stubby and branched spines between groups (Figure 75C). Together, these findings suggest that adolescent exposure to AB-FUBINACA in female mice leads to alterations in dendritic arborization and the density of mature spines within the PFC of adult animals. In addition, these structural changes could be linked to psychotic-like symptoms, as evidenced by deficits observed in the PPI test. Notably, reduced dendritic spine density has been frequently reported in postmortem brain tissues of patients with schizophrenia, particularly in cortical regions like the PFC and visual cortex (Hill et al., 2006; Moyer et al., 2015; Reyes-Lizaola et al., 2024). Additionally, evidence suggests that a reduction in cortical pyramidal neuron size, rather than neuron loss, may primarily contribute to the grey matter decline observed in schizophrenia (Glausier and Lewis, 2013).

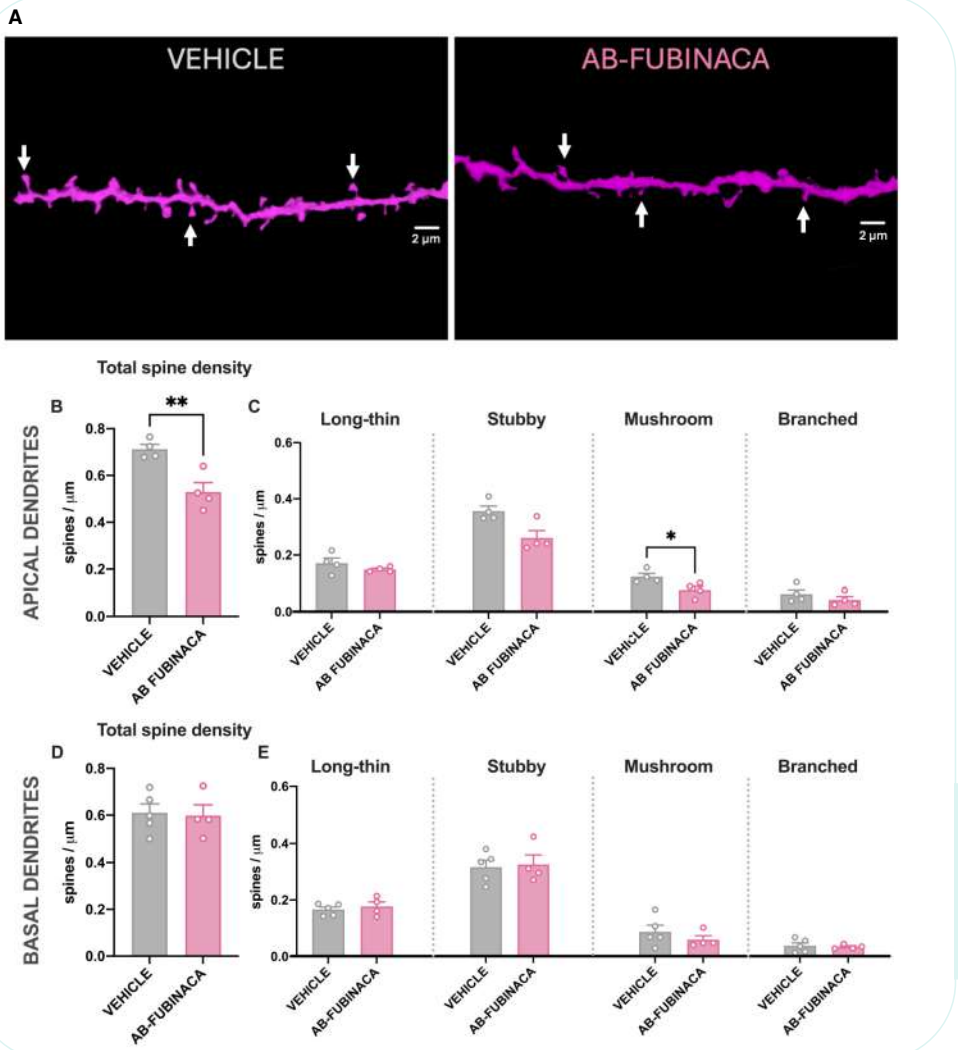


Figure 75

Figure 75. AB-FUBINACA treatment during adolescence induces alterations in dendritic spine density in the PFC of adult females. *Effects of treatment with AB-FUBINCA or vehicle during adolescence in dendritic spine density (A-E) in the PFC of adult female mice. (A) Representative images of apical dendritic spines from AB-FUBINACA and vehicle treated female mice. Scale bar = 2 µm. (B,D) Total spine density of apical (B) and basal (D) dendrites in AB-FUBINACA and vehicle treated female mice (n = 4-5 neurons/mouse and n = 4 mice per group). (C,E) Spine density grouped according to their morphological characteristics in apical (C) and basal (E) dendrites are shown. Arrows point different spines. Data are expressed as mean ± SEM. **p < 0.01 (comparison between AB-FUBINACA and vehicle group; Student's t-test (B,C).*

In conclusion, these results suggest that consumption of mixtures containing indazole derivatives during adolescence could lead to the appearance of several harmful effects. This might contribute to the development of public health policies focused on preventing the use of these substances, particularly among adolescents.

5

Discussion

SCBs contained in Spice/K2 preparations are new psychoactive substances whose growth and accessibility in the market have increased exponentially. Sold as "alegal" alternatives to marijuana use, SCBs are often perceived as safer options. However, data from hospital emergencies indicate that SCBs are significantly more toxic and potent than cannabis (Ford et al., 2017). Epidemiological studies highlight that adolescents are the main consumers of Spice/K2 preparations (EMCDDA, 2024), and early cannabinoid exposure is a known risk factor for developing psychiatric and cognitive disorders later in life. Although clinical and preclinical studies have already described similar alterations after acute SCBs use, few reports have explored the consequences of chronic SCBs exposure during adolescence. Therefore, the main objective of this thesis was to further investigate potential neurobiological consequences of adolescent exposure to SCBs in both male and female mice. Specifically, we aimed to evaluate the effects induced by two different SCBs. First, JWH-018, frequently found in high concentrations in the earliest preparations of these herbal mixtures (EMCDDA, 2024). Second, AB-FUBINACA, a representative of a more recent group of SCBs, commonly associated with hospital emergencies due to severe adverse effects (Adams et al., 2017; EMCDDA, 2024; Lam et al., 2017). In particular, this work has primarily focused on studying psychiatric and cognitive behavioral disruptions and associated neurobiochemical changes, while also exploring potential sex differences.

BEHAVIORAL EFFECTS OF CHRONIC EXPOSURE TO SCBs DURING ADOLESCENCE I

During adolescence, the brain undergoes numerous morphological, expression, and signaling changes aimed at achieving the organization and efficiency characteristic of an adult brain (Giedd et al., 1999; Sturman and Moghaddam, 2011; Rubino and Parolaro, 2016; Juraska and Drzewiecki, 2020). These transformations are essential for proper development, making this period particularly vulnerable to external disturbances (Rubino and Parolaro, 2008, 2016). Additionally, the endocannabinoid system plays a crucial role in CNS maturation, exhibiting a dynamic, time- and region-dependent nature during adolescence (Fernández-Ruiz et al., 1999, 2000; Ellgren et al., 2008; Rubino and Parolaro, 2016). Consequently, overstimulation of the endocannabinoid system during adolescence through drug use has been identified as a significant risk factor for developing alterations in memory and emotional processing, among others (Rubino and Parolaro, 2008, 2015, 2016). Based on this, to further evaluate potential effects of SCBs exposure during adolescence in cognition and emotional processing, two specific SCBs (JWH-018 or AB-FUBINACA) were administered to adolescent male and female mice. Although defining the exact boundaries of adolescence in rodents remains challenging, drug administration began at PND 35, following established literature and prior experimental protocols (Schneider, 2013; Saravia et al., 2019; Peters and Naneix, 2022). In addition, to minimize potential tolerance effects, SCBs treatments were administered over 15 days using an escalating dose regimen (0.5 - 1 - 1.5 mg/kg for JWH-018 and 1 - 1.5 - 2 mg/kg for AB-FUBINACA, 5 days each dose). Regarding JWH-018, behavioral tests were assessed both in the short- and long-term (5- or 20-days post-treatment respectively). However, for AB-FUBINACA, the behavioral evaluation was conducted exclusively in the long term (20 days post-treatment). In the present thesis, we reported that both JWH-018 and AB-FUBINACA treatment during adolescence modified anxiety-like behavior in a sex-specific manner (Figure 76). Male, but not female mice, exposed to JWH-018 showed an anxiogenic phenotype 5 days after the treatment ended. However, anxiolytic-like effect were observed in adult males treated with either JWH-018 or AB-FUBINACA. In contrast, an anxiogenic-like behavior was described in adult female mice following JWH-018 or AB-FUBINACA exposure during adolescence. These findings suggest that JWH-018 and AB-FUBINACA exposure induce similar anxiety-related alterations at long-term, but in an opposite manner between sexes, emphasizing a pronounced sexual dimorphism. Inconsistent findings have been reported regarding the effects of adolescent cannabinoid exposure on anxiety. In general, most studies using the EPM have not shown differences in anxiety in adult male or female rodent due to THC (Rubino et al., 2008a; Saravia et al., 2019; Zuo et al., 2022) or SCBs adolescent exposure (Higuera-Matas et al., 2009; Llorente-Berzal et al., 2011a; Mateos et al., 2011). However, chronic adolescent treatment with CP55,940 or WIN 55,212-2 has shown to induce an anxiolytic-like effect in adult male (Biscaia et al., 2003; Pushkin et al., 2019) and female

(Biscaia et al., 2003) rodents in the EPM. In contrast, a recent study showed that JWH-018 self-administration during adolescence increases compulsive behaviors in male mice, as evidenced by elevated nestlet shredding and marble burying (Margiani et al., 2022). While marble burying can sometimes reflect anxiety-like behavior, in this case its increase appears linked to compulsivity rather than anxiety, given that another study reported heightened burying scores without changes in EPM performance after JWH-018 exposure in adult male rats (Pintori et al., 2021). Regarding AB-FUBINACA, no effects on anxiety were observed in adult male rats by performing an emergence test after adolescent exposure (Kevin et al., 2017). On the other hand, anxiety disorders characterized by pathological fear, such as post-traumatic stress disorder and phobias, have been related to impairments in fear memory retention and extinction. Although under our experimental conditions JWH-018 or AB-FUBINACA adolescent exposure did not impact cued fear extinction in either male or female mice, both SCBs induced heightened fear conditioning in adult females compared with the controls. This result correlates with the anxiogenic-like effect observed in adult females following adolescent exposure to both SCBs, suggesting a close relation between anxiety-like states and heightened conditioning to aversive stimulus. Consistent with these findings, THC exposure during adolescence did not alter fear extinction in male and female adult mice (Saravia et al., 2019; Stollenwerk and Hillard, 2021). Nevertheless, a recent report has described that chronic THC vaporizations potentiate fear responses predominantly in females (Lightfoot et al., 2025). In agreement, adult rats treated with WIN 55,212-2 during adolescence exhibited enhanced fear conditioning, although in this study the sex was not specified (Gleason et al., 2012).

Memory impairments are among the most consistently associated consequences of early cannabinoid exposure. In fact, numerous rodent studies have reported long-term deficits in the NOR test following adolescent exposure to cannabinoids (Rubino and Parolaro, 2016). Interestingly, these impairments do not commonly appear when cannabinoid treatment is administered during adulthood, suggesting adolescence vulnerability for this detrimental effect (O'Shea et al., 2004; Schneider et al., 2008; Renard et al., 2013). Based on this, we aimed to assess potential memory deficits, as well as sociability and depression-related behaviors, following adolescent exposure to AB-FUBINACA. These behaviors were not evaluated in the case of JWH-018 treatment due to experimental and logistical constraints. Memory impairments were observed in adult female, but not male, mice treated with AB-FUBINACA during adolescence (Figure 76). In agreement, Llorente-Berzal and collaborators, 2013b described that adult female, but not male rats, treated with escalated doses of THC during adolescence manifested recognition impairments in the NOR test. On the contrary, other article reported memory impairments in adult male rats treated with AB-FUBINACA from PND 31 to 55 (Kevin et al., 2017). This result, which contrasts with our findings in males is not surprising, as the previous literature indicates that adolescent exposure to cannabinoids could induced memory and learning impairments in both sexes (Rubino and Parolaro, 2015). Adolescence vulnerability to memory deficits is suggested under our experimental conditions, as adult females treated with AB-FUBINACA did not

exhibit impairments in the NOR test. By contrast, a recent study found memory alterations in adult mice treated chronically with AB-FUBINACA, however, the test was conducted 24 h after discontinuation (Alzu'bi et al., 2024). A timing difference between the two studies that could account for the discrepancies observed. In addition, the sex of the rodents used in the previous report (Alzu'bi et al., 2024) was not specified, making difficult direct comparison with our results.

Under our experimental conditions, neither male nor female mice treated with AB-FUBINACA during adolescence exhibited social deficits at long-term. In agreement, AB-FUBINACA chronic administration during adolescence did not alter social interaction in adult male rats (Kevin et al., 2017). However, several previous data showed that chronic THC administration during adolescence significantly reduced sociability in both sexes in adulthood (Quinn et al., 2008; Realini et al., 2011; Kevin et al., 2017; Renard et al., 2017a). On the other hand, the FST revealed that adolescent exposure to AB-FUBINACA significantly reduces the immobility time in adult males, but not females (Figure 76). This reduction is classically associated with an antidepressive-like response. In agreement, chronic administration of HU-210, a classic SCB found in some Spice/K2 mixtures (UNODC), has been reported to reduced immobility time in adult male rats in the FST (Jiang et al., 2005; Morrish et al., 2009), Moreover, high doses of AB-FUBINACA has been shown to exert antidepressive-like effects in the FST in male mice, though this study focused solely on acute effects (Schreiber et al., 2019). In general, studies on the effects of cannabinoid exposure during adolescence in both humans and rodents suggest that adolescent females appear to be more sensitive than males to the long-lasting emotional effects induced by chronic cannabinoid consumption (Rubino and Parolaro, 2011; Craft et al., 2013). Specifically, males exhibit pronounced effects of exogenous cannabinoids on food intake and energy homeostasis, whereas females show greater sensitivity in terms of depression and anxiety, but also in the tetrad effects of cannabinoids (Dow-Edwards et al., 2016). In agreement with our findings, although females did not exhibit alterations in the FST, they appear to be more vulnerable to JWH-018 and AB-FUBINACA across the different behaviors evaluated that were associated with emotional processing.

PSYCHOTIC-LIKE EFFECTS OF CHRONIC EXPOSURE TO SCBs DURING ADOLESCENCE II

Pro-psychotic alterations are frequently reported after acute SCBs intoxication. Thus, hallucinations, delusions, paranoia and panic attacks are some of the symptoms causing hospital admissions due to Spice/K2 consumption (Ford et al., 2017). In addition, cannabinoid adolescent exposure is a known risk factor for the development of psychotic disorders later in life (Gleason et al., 2012; Marconi et al., 2016; Richetto and Meyer, 2021). Therefore, using the PPI as preclinical model, we assessed potential psychotic-like effects following adolescent exposure to JWH-018 or AB-FUBINACA in male and female mice. Impairments in sensorimotor gating, a hallmark of psychotic disorders such as schizophrenia (Mena et al., 2016; Sato, 2020), were evaluated by using the PPI test. A critical finding of our study was that adolescent JWH-018 exposure significantly reduced PPI of startle reflex at short- and long-term in male, but not female, mice (Figure 76). On the contrary, AB-FUBINACA adolescent treatment induced a reduction of PPI in adult female, but not male mice (Figure 76). In addition, the effect of JWH-018 on psychotic-like symptoms was age-dependent, as evidenced by the absence of alterations when administration was performed directly in adulthood. In contrast, AB-FUBINACA impaired PPI in females regardless of the age of exposure, indicating that AB-FUBINACA is a potent pro-psychotic agent. Although studies on the effects of THC exposure during adolescence on PPI yield mixed findings, early exposure to SCBs appears to modulate PPI in a more consistent manner (Zhikun et al., 2024). Nonetheless, according to some reports, adolescent administration of THC significantly reduced PPI in both male (Abela et al., 2019; Lamanna-Rama et al., 2024; Renard, et al., 2017a) and female (Iemolo et al., 2021) rodents. However, an increase of the sensorimotor filter has also been described in other study (Garcia-Mompo et al., 2020). Interestingly, adolescent exposure to the aminoalkylindole derivative WIN 55,212-2 has been reported to induce long-term PPI alterations in male rodents (Schneider and Koch, 2003; Schneider et al., 2005; Wegener and Koch, 2009; Gleason et al., 2012; Aguilar et al., 2017; Abboussi et al., 2020). In agreement with our results with JWH-018, this psychotic effect appears to be age-dependent, as adult aminoalkylindole administration did not elicit long-term disruptions in somatosensory filtering either with WIN 55,212-2 (Schneider and Koch, 2003; Bortolato et al., 2005; Gleason et al., 2012) or JWH-018 (Pintori et al., 2021). However, sub-chronic administration of high doses of JWH-018 to adult male rats has been shown to disrupt PPI (Bilel et al., 2023). Although there could be a significant bias, given that the startle response was also modified (Bilel et al., 2023). The consequences of adolescent or adult exposure to AB-FUBINACA or other aminoalkylindazoles on PPI were not previously assessed, making it difficult to discuss and compare with the previous results observed with JWH-018. Clinical studies suggest that males are more vulnerable than females to

developing psychiatric disorders, such as schizophrenia, following cannabinoid exposure (Hjorthoj et al., 2023). In agreement, epidemiological data show that men consume cannabis at higher rates and earlier ages than women (EMCDDA, 2022; ESTUDES, 2023; Rubino and Parolaro, 2015). This suggests that the observed vulnerability in men could be influenced by greater cannabis use, rather than an actual sex susceptibility *per se*. On the other hand, it is worth noting that positive symptoms appear much earlier in men than in women (mid-20 and late-20s respectively) (DSM-5-TR, 2022). Therefore, different effects could have been observed if PPI had been conducted at a different time point in female mice. In this context, hypothetically, females treated with JWH-018 could have shown PPI impairments if a longer wash-out period had been explored. In agreement, Abela et al., 2019 suggest that the wash-out period is crucial for observing sensorimotor gating deficits, as THC treatment during adolescence impaired PPI only after an intermediate testing period.

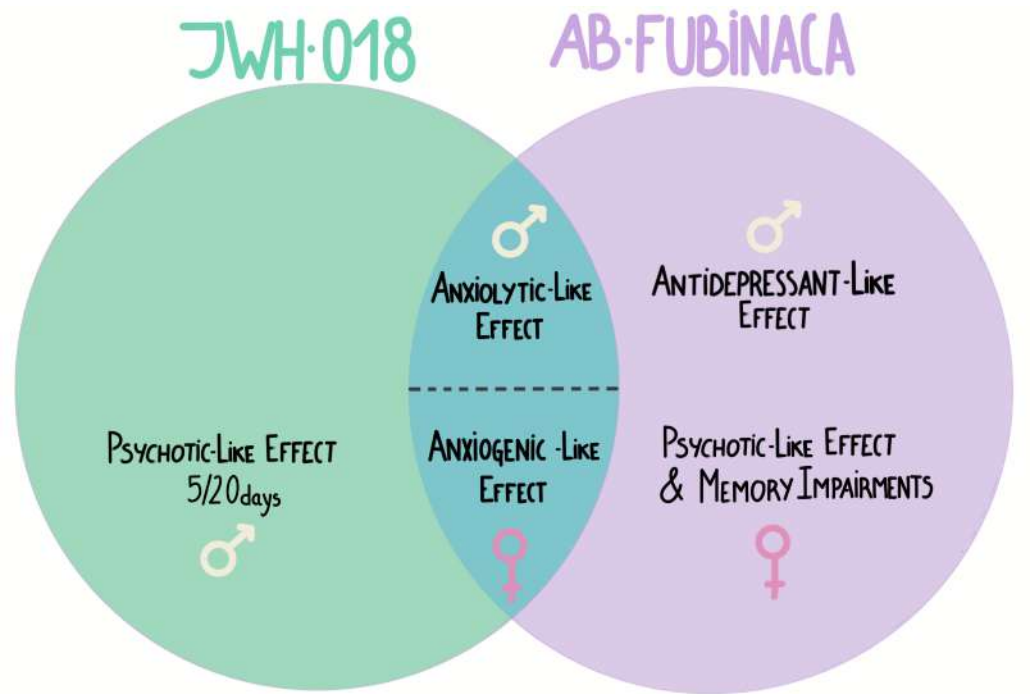


Figure 76. Schematic representation of the different behavioral alterations observed after JWH-018 and AB-FUBINACA exposure during adolescence.

An explanation for the sexual differences observed following JWH-018 or AB-FUBINACA treatment is challenging. However, an attempt to highlight certain factors relevant to understanding some of these differences, both in terms of sex-dependent effects intra or inter-drug, will be discussed. Sexual differences in the endocannabinoid system but also in behavioral and biochemical effects of exogenous cannabinoids have been described for years. However, many of these studies are outdated and predominantly focus on adulthood,

neglecting critical periods such as adolescence. Therefore, definitive answers regarding sexual dimorphisms in the effects of cannabinoid exposure during adolescence remain elusive. Actually, an important limitation could be due to the small number of studies that directly compare the effects between both sexes, as well as the scarce cannabinoid bibliography including females in the different experimental procedures. Regarding the endocannabinoid system, differences in CB1R density and functionality have been reported between males and females. Males generally exhibit higher CB1R levels, whereas females show greater receptor functionality and activity (Mateos et al., 2011; Rubino and Parolaro, 2015; Dow-Edwards et al., 2016). Additionally, CB1R expression and activation are closely linked to sex hormones, leading to fluctuations during puberty and throughout different life stages, contributing to sex-dependent cannabinoid alterations (Gorzalka et al., 2010; Craft et al., 2013). It is also known that CB1R in females is more prone to desensitization following chronic cannabinoid exposure, potentially contributing to sexual differences in the harmful consequences induced by cannabinoids (Rubino and Parolaro, 2011, 2015; Bassir Nia et al., 2018). However, although both JWH-018 and AB-FUBINACA, along with their metabolites, are potent CB1R agonists, they have also been shown to interact with other receptors outside the endocannabinoid system, such as serotonin receptors (Elmore and Baumann, 2018; Yano et al., 2020; Canoura et al., 2024; Corli et al., 2024). This aspect may contribute to some of the drug-specific differences observed. Additionally, it has been reported that although both cannabinoids strongly bind CB1R, their coupling to Gi/o or Gs subunits differs considering *in vitro* studies. Specifically, although Gi/o is the primary pathway activated by SCBs, AB-FUBINACA showed enhanced efficacy for the Gs pathway compared to other SCBs, including JWH-018 (Sachdev et al., 2020). This result suggests different receptor/effector coupling balance in the presence of the different SCBs. While the biological significance of differential coupling between Gi/o or Gs is not yet fully understood, it is possible that the signaling pathways triggered by each SCBs differ, ultimately leading to distinct effects and different susceptibility between male and females for each drug.

With respect to metabolism, it is well established that females produce higher levels of 11-OH-THC, the active metabolite of THC, which could make them more vulnerable to the negative consequences of cannabis (Rubino and Parolaro, 2015; Bassir Nia et al., 2018; Sallam et al., 2023). Although potential sex differences in the generation of active metabolites of JWH-018 and AB-FUBINACA have not been extensively studied, it can be hypothesized that such differences could exist. Interestingly, the metabolism of JWH-018 is primarily mediated by CYP1A2 and CYP2C9 enzymes, leading to the formation of -COOH and -OH metabolites, which are inactive and active, respectively (Chimalakonda et al., 2012). *In vitro* studies indicate that polymorphisms in CYP2C9 can modulate the rate of metabolism, potentiating susceptibility to the toxic effects of JWH-018 (Patton et al., 2018). Although the homology between human and murine CYP2C enzymes is not exact, the murine CYP2C family exhibits notable functional and distributional similarities to its human counterpart (Renaud et al., 2011). Notably, female C57BL/6N mice have been reported to

express significantly higher levels of CYP2C enzymes than males (up to 800% more) in liver and kidney (Renaud et al., 2011). If CYP2C family also regulates JWH-018 metabolism in mice, a more rapid metabolic activity in females could explain the absence of psychotic-like alterations after chronic exposure to JWH-018 during adolescence. On the other hand, considering pharmacodynamics, a common characteristic of cannabinoids is their lipophilic nature. It has been reported that SCBs containing an indole group display greater lipophilicity than those with an indazole core (Kakehashi et al., 2020), suggesting that JWH-018 is more hydrophobic than AB-FUBINACA. Unlike humans, male rodents exhibit a higher proportion of body fat compared to females (Fattore and Fratta, 2010), and this characteristic could contribute to the greater susceptibility of males to the psychotic-like effects induced by JWH-018.

As already mentioned, providing an explanation to the different psychotic-like effects observed between males and females with JWH-018 and AB-FUBINACA is difficult at present and further investigation is needed to explore the different hypothesis discussed above.

NEUROBIOCHEMICAL ALTERATIONS AFTER CHRONIC EXPOSURE TO SCBs DURING ADOLESCENCE III

The PFC brain area that plays a key role in cognitive processing, emotionally guided behaviors and proper somatosensory filtering, continues to mature until the end of adolescence (Ginder et al., 2022; Bernabeu et al., 2023; Tapias-Espinosa et al., 2023; Suzuki et al., 2024). This immaturity of the PFC significantly contributes to adolescence being a period of heightened malleability, making it particularly sensitive to external stimuli (Ginder et al., 2022; Bernabeu et al., 2023). The endocannabinoid system plays a crucial role in the maturation of corticolimbic areas, particularly the PFC (Meyer et al., 2018; Bernabeu et al., 2023). Therefore, the hijacking of the endocannabinoid system through SCBs could disrupt the proper maturation of the PFC, thus impairing its proper functionality. Notably, this cortical development has been shown to be influenced by sex (Delevich et al., 2021; Ginder et al., 2022; Bernabeu et al., 2023), which could make males and females differentially susceptible to external disturbances, such as SCBs exposure. The different behavioral alterations observed following adolescent treatment with JWH-018 or AB-FUBINACA suggest a potential involvement of the PFC. In particular, the alterations observed in PPI with both SCBs not only corroborate the potent pro-psychotic effects of these new synthetic drugs, but also highlight the potential risk of developing psychotic disorders, such as schizophrenia, due to chronic SCBs exposure. Dysfunction of the PFC is a dominant aspect of schizophrenia and other psychiatric conditions (Dienel et al., 2022; Gao et al., 2022; Bilecki and Maćkowiak, 2023), but also contributes to PPI impairments (Ellenbroek et al., 1996; Rajakumar et al., 2004; Tapias-Espinosa et al., 2023; Suzuki et al., 2024). Therefore, brains of mice that underwent through the PPI test were collected to investigate potential neurobiochemical alterations in the PFC. The study took different biochemical approaches between JWH-018 and AB-FUBINACA.

Regarding **JWH-018**, potential alterations in PNNs and positive-PV expressing neurons were evaluated in the PFC of males at short- and long-term. Briefly, PNNs are condensed aggregates of extracellular matrix that play an important role in the maturation of fast-spiking GABAergic interneurons expressing PV (Wingert and Sorg, 2021). Remarkably, alterations in the density of PNNs in the PFC have been observed in patients with schizophrenia (Mauney et al., 2013; Enwright et al., 2016), but also in animal models that mimic this disorder (Paylor et al., 2016; Matuszko et al., 2017). In addition, disruptions of GABAergic neurotransmission have also been associated with psychotic disorders, primarily related to alterations in PV-containing interneurons (Gonzalez-Burgos et al., 2015; Enwright et al., 2016; Wingert and Sorg, 2021; Tapias-Espinosa et al., 2023). Our results revealed a reduction in the density of PNNs in the IL and PL areas at short-term in male mice exposed to JWH-018 during adolescence. Furthermore, the percentage of PV+ neurons surrounded by PNN in the IL tended to decrease even in the long-term. These disturbances could be

impacting in the correct inhibition in the PPI test, since a positive correlation between PNNs density and the percentage of PPI was found in the IL area. Unlike JWH-018, THC adolescent exposure did not alter PNNs density in the PFC of adult male mice (Garcia-Mompo et al., 2020). However, the interval between cannabinoid treatment and PNNs density evaluation was shorter in our study, and JWH-018 is also much more potent that THC, facts that could explain the differences observed between these two studies. In fact, a protocol assessing the effects of early life adversity observed a transient reduction in PNNs surrounding PV+ neurons in the PFC of adolescent male mice, which returned to control levels in adulthood, suggesting an age-dependent vulnerability in this PNNs alteration (Page and Coutellier, 2018). On the other hand, female mice treated with JWH-018 showed no changes in PNNs or PV markers, which aligns with their unaltered PPI response. Therefore, the susceptibility observed in males could be due to an earlier maturation process of the PNNs in females than in males (Drzewiecki and Juraska, 2020). Thus, JWH-018 treatment might be more harmful to the immature PNNs of males than to the mature PNNs of females, probably contributing to the presence of PPI disruptions only in males.

PNNs change their composition and organization constantly, providing specific medium to neurons during development, aging and specific pathological conditions (Reichelt et al., 2019; Drzewiecki et al., 2020). By facilitating PNNs remodeling, glial cells are considered essential regulators of their functions both in health and disease (Crapser et al., 2020, 2021; Tewari et al., 2022). In fact, microglial depletion in healthy mice dramatically increases PNNs density, which produces important disbalance of excitatory and inhibitory signaling (Liu et al., 2021). Moreover, PNNs deficits observed across diverse disorders have been associated with microglial activation (Crapser et al., 2021). While microglia play a crucial role in the removal of PNNs due to their inherent phagocytic activity, astrocytes are primarily responsible for the synthesis and release of extracellular matrix components and their proteolytic enzymes (Tewari et al., 2022). Thus, reactive astrocytes also play a role in PNNs disruption, primarily after brain injury and trauma (Tewari et al., 2022). Our results showed that adolescent JWH-018 exposure changes microglial and astrocytic reactivity in the PFC of adult male mice. In agreement, Margiani et al., 2022 found microglial changes in several brain areas such as nucleus accumbens and caudate-putamen of adult male mice 20 days after JWH-018 self-administration during adolescence. Moreover, alterations in microglial reactivity in the PFC have been linked to PPI disruptions in different preclinical models of schizophrenia (Ayilara and Owoyele, 2023; Fujikawa et al., 2024; Lopez-Rodriguez et al., 2014et al., 2024; Lopez-Rodriguez et al., 2014), but are also found in patients with this disease (Nguyen et al., 2023). Under our experimental conditions, no changes were observed in the microglia or astrocytes of female mice treated with JWH-018. While this lack of changes in microglia aligns with the absence of PPI alterations observed in females, it contrasts with some of the existing cannabinoid literature. Recent investigations indicate that adolescent THC exposure increases the proportion of reactive microglia in the PFC of adult females, although these studies were conducted in rats (Zamberletti et al., 2015; Gabaglio et al., 2021; Freels et al., 2024).

Microglial cells are known to exhibit significant sex differences, including in morphology, gene expression and reactivity, among others (Villa et al., 2019). Specifically, a recent transcriptomic study identified up to 500 DEGs between male and female microglia, supporting that sex influences microglial reactivity (Villa et al., 2018). Remarkably, these sex-specific microglial characteristics persisted even after transplantation into a brain of the opposite sex (Villa et al., 2018). The maturation of these cells follows distinct trajectories between males and females, with females reaching maturity earlier (Villa et al., 2019). Additionally, male microglia have been reported to be more sensitive to immunomodulatory agents such as LPS (Hanamsagar et al., 2017). These factors may contribute to the susceptibility observed in males, but not in females, to developing psychotic-like alterations following adolescent exposure to JWH-018. Specifically, this SCB could act as an immunoactive agent, triggering microglial and astrocytic activation, which in turn reduces PNNs density in the PFC, ultimately leading to PPI impairments in male mice (Figure 77).

GAD67, the enzyme responsible for most GABA synthesis, has been reported to be downregulated in the PFC of individuals with schizophrenia (Hashimoto et al., 2003; Curley et al., 2011). Consistently, GABAergic unbalance in the cerebral cortex is a key factor of schizophrenia physiopathology (Stępnicki et al., 2018), and contributes to PPI disruptions (Toriumi et al., 2016). Interestingly, JWH-018 exposure during adolescence reduced the expression of GAD67 in the PFC of males in the short-term, although this change was transitory and return to control levels in adult male mice (Figure 77). In agreement, THC treatment during adolescence reduced GAD67 levels in the PFC of adult rats that showed alterations related to psychotic-like phenotype (Gabaglio et al., 2021; Renard, Szkudlarek, et al., 2017; Zamberletti et al., 2014). However, GAD67 reduction did not lead to differences in PV protein levels, as observed in our study (Zamberletti et al., 2014; Renard et al., 2017b).

Moreover, a transitory decrease in the expression of CB2R was observed after adolescent JWH-018 treatment. The contribution of the CB2R in somatosensory filtering process has been increasingly explored in recent years. Notably, our group reported that the 129S1/SvImJ mouse strain, which exhibits elevated CB2R levels in the PFC, displays an enhanced ability to inhibit the startle response in the PPI test (Ten-Blanco et al., 2022b). Furthermore, pharmacological activation of CB2R through different agonists has been shown to reverse PPI impairments induced by MK-801, a classic NMDA receptor antagonist (Khella et al., 2014; Cortez et al., 2022). CB2R knockout mice exhibit psychotic-like effects and show PPI disruptions (Ortega-Alvaro et al., 2011). The selective deletion of CB2R in midbrain dopaminergic neurons was sufficient to induce these psychotic-related behaviors (Canseco-Alba et al., 2024). This finding highlights CB2R as a potential regulator of dopaminergic pathways, which are disrupted in schizophrenia, supporting its role as a promising target for modulating schizophrenia-related alterations (Ferranti and Foster, 2022). The previous results could suggest a potential association between the downregulation of CB2R and the psychotic-like alterations observed after adolescent JWH-018 exposure (Figure 77).

To summarize, our data demonstrate significant and sex-dependent lasting behavioral and neurobiological alterations resulting from JWH-018 administration during adolescence.

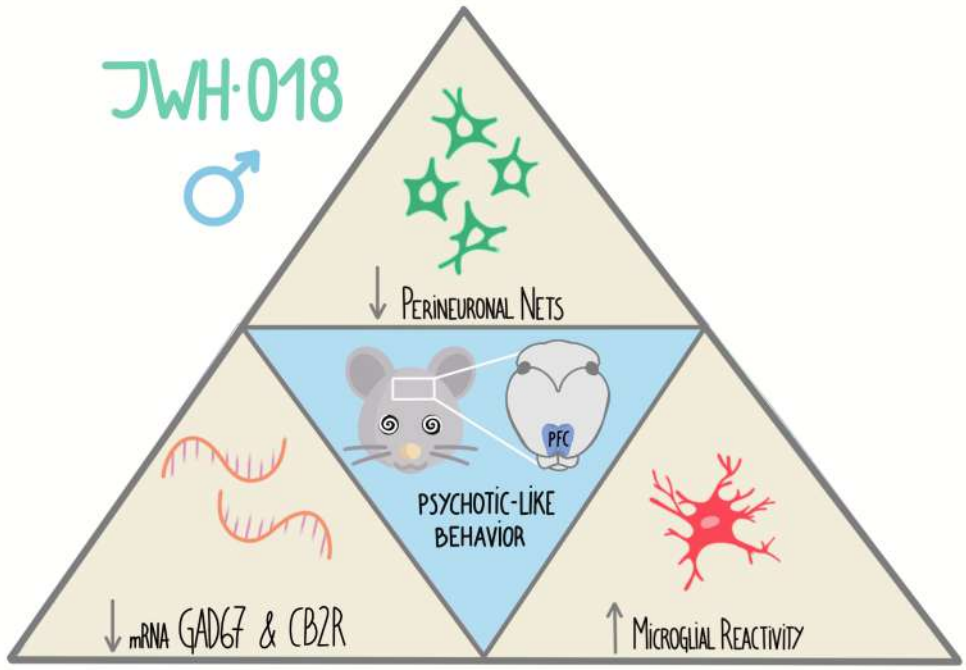


Figure 77. Schematic representation of the main neurobiological alterations found in the PFC of male mice that showed PPI impairments following adolescent exposure to JWH-018. *Chronic treatment with JWH-018 during adolescence induced microglial reactivity, reduced PNNs density, and decreased GAD67 and CB2R mRNA levels. These neurobiological changes could underlie the psychotic-like behavior found in male mice at short- and long-term after adolescent SCB exposure.*

Regarding **AB-FUBINACA**, by performing an RNAseq, we identified that *Sh3tc1* and *Plekhg2* genes were up-regulated in the PFC of females 20 days after AB-FUBINACA adolescent exposure (Figure 78). As described before, the tissues were collected 24 hours after the PPI test, in which female mice showed impairments followed AB-FUBINACA discontinuation. *Plekhg2* encodes a guanine nucleotide exchange factor involved in activating Rho GTPases, including Rac and Cdc42, by promoting GDP/GTP exchange (Runne and Chen, 2013; Sugiyama et al., 2017; Nishikawa et al., 2021). Abnormalities in this gene have been associated with postnatal microcephaly and intellectual disability (Edvardson et al., 2016). Interestingly, a recent *in vivo* study revealed that *Plekhg2* plays a critical role in axon, dendrite, and synapse development in cortical neurons of mice (Nishikawa et al., 2022). In agreement, Rho GTPases, specifically Rac1, Cdc42, and RhoA, have been closely associated with proper dendrite and dendritic spine formation (Negishi and Katoh, 2002; Konietzny et al., 2017; Basu and Lamprecht, 2018). Although we did not explore whether alterations in the *Plekhg2* gene affect specific Rho GTPases, evidence links

CB1R activation to their modulation (Bromberg et al., 2008). Previous studies suggest that Gi/o signaling converges on Rac1 and Cdc42, promoting actin cytoskeletal reorganization (Bromberg et al., 2008). Gi/o-CB1R coupling during development has been shown to facilitate neurite outgrowth through Rac1 (He et al., 2005). Moreover, CB1R activation via WIN 55,212-2 rapidly remodels neuronal cytoskeleton by inducing Rho-GTPase-dependent actomyosin contractility (Roland et al., 2014). If this activation persists due to chronic WIN 55,212-2 exposure, it potentially results in lasting neuronal and brain morphology changes (Roland et al., 2014). Taken together, these data suggest that exogenous modulation of the Plekhg2-RhoGTPase complex by AB-FUBINACA exposure during adolescence could disrupt actine reorganization, thus altering dendritic spine formation. Congruent with this, AB-FUBINACA administration to adolescent female mice induced lower dendritic arborization and a reduction of total length of dendrites of pyramidal neurons located in the PFC in adulthood. Furthermore, reductions in the density of total and mushroom-shaped (mature) dendritic spines were also observed in the same brain region. A strong relationship between PFC dysmorphology and PPI alterations is suggested in this work, which could, in turn, contribute to the development of psychotic-like symptoms following adolescent exposure to AB-FUBINACA (Figure 78). In agreement, reduced complexity of pyramidal neurons in the PFC has also been described in adult male rats following chronic adolescent exposure to both CP 55,940 (Renard et al., 2016) and THC (Miller et al., 2019). Moreover, females treated during adolescence with THC showed a decreased number of dendritic spines in layers II/III of the PFC at different time-points (Rubino et al., 2015). Although a limitation of this study is the lack of neurobiochemical experiments in male mice that do not show PPI alterations, there is previous data reporting no reduction in spine density in the PFC of adult male rats that were exposed to THC during adolescence (Miller et al., 2019). However, changes in spine density in other brain areas such as the hippocampus or amygdala have been described after THC adolescent treatment in males (Rubino et al., 2009; Saravia et al., 2019). In addition, reductions in dendritic spine density have been commonly observed in the PFC of patients with schizophrenia (Hill et al., 2006; Moyer et al., 2015), as well as smaller size of cortical pyramidal neurons, which contribute to the characteristic grey matter reduction associated with this disorder (Glausier and Lewis, 2013). As a whole, these results indicate that AB-FUBINACA exposure during adolescence could lead to the appearance of biochemical and behavioral alterations related to psychotic-like disorders in a sex-dependent manner.

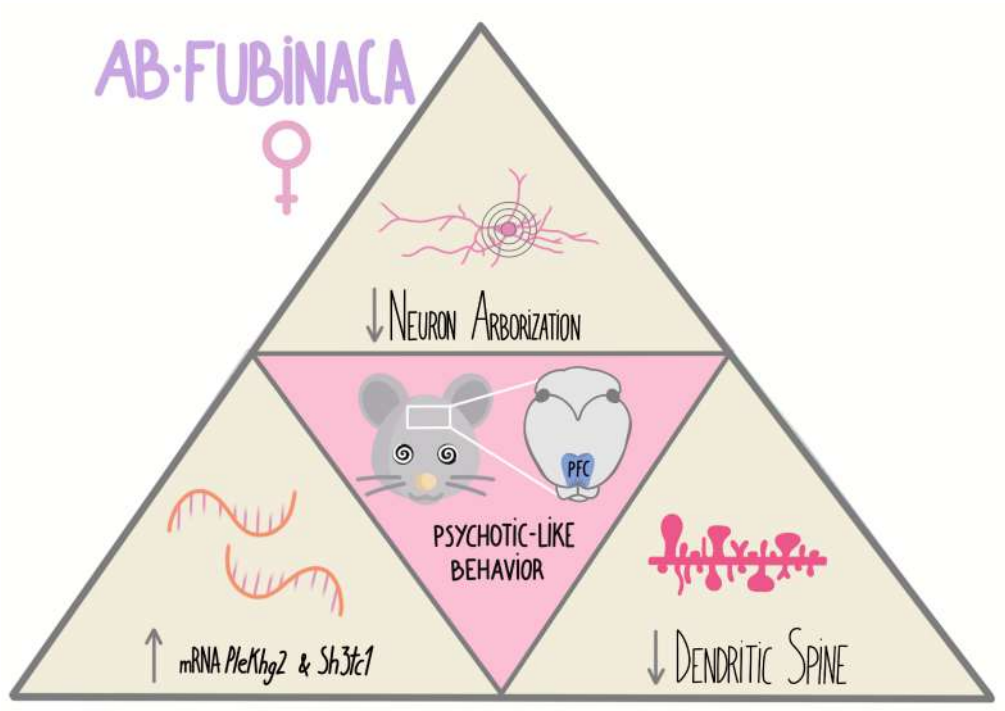


Figure 78. Schematic representation of the main neurobiological alterations found in the PFC of female mice that showed PPI alterations following adolescent exposure to AB-FUBINACA*. RNAseq analysis revealed an increase expression of PleKhg3 and Sh3tc1genes 20 days after AB-FUBINACA exposure during adolescence. Plekhg2 gene has been associated with proper dendritic arborization and dendritic spine formation, both of which were disrupted following AB-FUBINACA administration during adolescence. Specifically, adult females exhibited reduced neuronal arborization and a decrease in total and mushroom-shaped dendritic spines. These brain changes could be related to the psychotic-like alterations described in females after AB-FUBINACA chronic exposure during adolescence.*

In summary, despite discrepancies in some behavioral alterations observed following JWH-018 or AB-FUBINACA adolescent exposure, both SCBs induce significant impairments in emotional and cognitive processes. Moreover, both drugs exhibit a strong pro-psychotic profile closely associated with PFC dysfunctions characteristic of schizophrenia and other psychiatric disorders. It is also worth noting that these new psychoactive substances are primarily consumed by smoking Spice/K2 herbal preparations, which typically contain a mixture of different type and concentrations of SCBs. This likely results in the simultaneous presence of indole- and indazole-derived SCBs, potentially exacerbating their detrimental effects and increasing the vulnerability of both sexes to their harmful consequences. Therefore, this study has important clinical and public health policy implications in terms of restricting adolescent access to SCBs, given their heightened susceptibility to the neurotoxic effects of these drugs.

6

Conclusion

The findings reported in the current thesis allow to draw the following conclusions:

1 Adolescent treatment with JWH-018, a SCB found in Spice/K2 preparations, induces behavioral long-term consequences in a sex-dependent manner.

2 Chronic JWH-018 treatment during adolescence, but not adulthood, results in both short- and long-term psychotic-like effects in male mice. These findings suggest that adolescence constitutes a period of special vulnerability to the central effects of JWH-018.

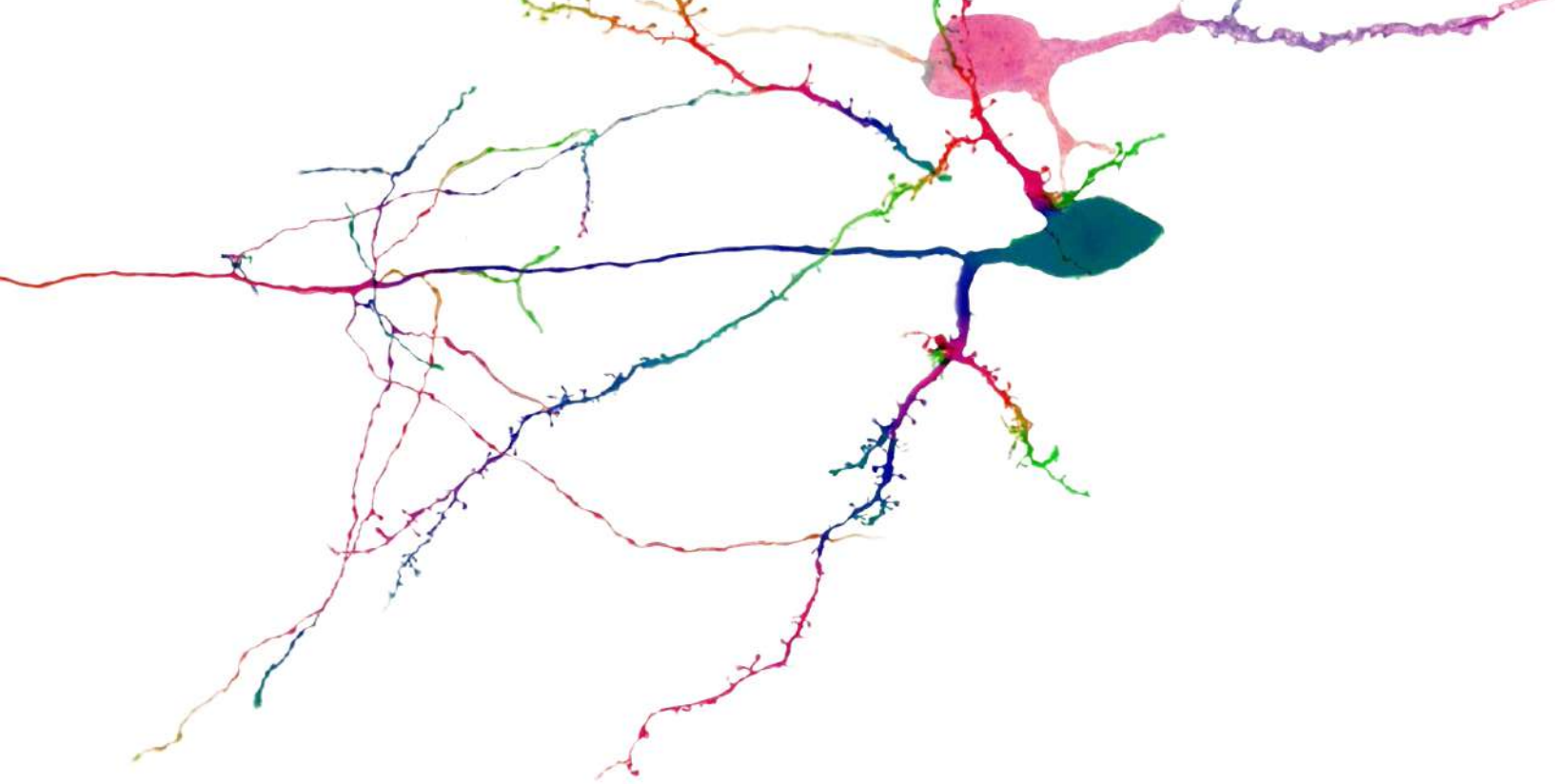
3 Psychotic-like alterations found with JWH-018 adolescent exposure could be associated with neurobiological changes in the PFC, including modifications in PNNs and microglia morphology. Both biochemical alterations have been previously described in patients with psychotic-like disorders.

4 Adolescent exposure to AB-FUBINACA, a SCB present in recent Spice/K2 herbal mixtures, leads to long-term behavioral impairments in females, including memory deficits and anxiety-like behavior.

5 Chronic AB-FUBINACA treatment during both adolescence and adulthood induces persistent psychotic-like alterations in female mice, highlighting the potent pro-psychotic effects of this compound.

6 Psychotic-like effects observed after AB-FUBINACA exposure may be associated with structural alterations in the PFC, including a reduction in mature dendritic spine density and altered dendritic arborization. These morphological changes have been previously observed in patients with psychotic-like disorders.

7



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ANNEX I

Table A1. Medicinal products cannabinoid-based approved			
Name	Composition	Indication	Regulators
Cesamet® **Canemes®**	Nabilone	Nausea and vomiting associated with chemotherapy	FDA approved AEMPS for exceptional cases (imported)
Marinol® **Syndros®**	Dronabinol	1. Anorexia in AIDS patients 2. Nausea and vomiting associated with chemotherapy	FDA approved AEMPS for exceptional cases (imported)
Sativex®	CBD/THC	Muscle spasticity resulting from multiple sclerosis	FDA approved AEMPS approved
Epidyolex®	CBD	Seizures associated with Lennox-Gastaut syndrome or Dravet syndrome	FDA approved AEMPS approved

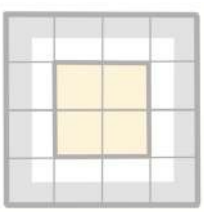
Table A2. Summary of some behavioral test to study anxiety in rodents.		
NAME OF THE TEST	**DESCRIPTION**	**SCHEME**
Zero Maze	This test is similar to EPM but is performed in a round maze which has two segments protected by high walls and two other portions unprotected. This test is used to eliminate the decision making EPM entails. The percentage of time the animal spends in the open sections is calculated as an index of anxiety.	
Light/Dark or Black & White box	The light/dark box consists of two compartments connected by a small entrance. One small dark compartment and another bigger and highly illuminated chamber. Large, lighted areas are perceived as potentially dangerous places for rodents, while shaded areas imply safety. The percentage of time the animal spends in the white compartment is the main value calculated in this test.	
Open Field	This tests is performed in a squared big area protected with high walls. The maze is divided in two main areas, the central are highly illuminated and the border area close to the walls. The animal is placed in the maze and the more anxious it is, the more time it will spend exploring the outer edge.	

Table A3. Summary of some behavioral test to study memory and learning in rodents.

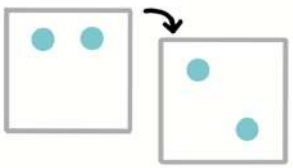
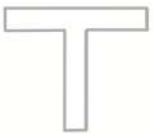
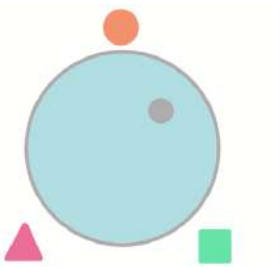
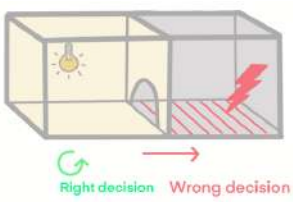
TYPE OF TEST	NAME OF THE TEST	DESCRIPTION	SCHEME
Recognition	Novel Place Recognition	The test is based on the animal's ability to recognize the new location of a previous explored object. After being exposed to two exact objects, under normal conditions, a rodent should spend more time exploring the object with a new location, rather than the one located at the same place.	
	Spontaneous alternation tasks	This test, classically used to evaluate working memory, is performed in a Y- or V-shaped maze. Rodents naturally prefer to explore a new arm than a previously visited, so it is possible to assess alternation ability as an index of learning.	
Spatial Memory	Morris Water Maze	This test is performed in a small pool filled with water, which is a stressor for rodents. An escape platform is submerged, and its location must be learned through visual cues after repeated sessions. This test lasts several days, and the time the animal spends finding the platform is an indicator of learning.	
	Barnes Maze	This test is assessed on a circular surface that is brightly lit (as a stressor) and contains 20 holes, of which 1 leads to an escape place. Similar to the previous test, the idea is to evaluate the animal's ability to learn the location of the escape hole after several consecutive sessions by finding the correct location using spatial cues.	
Conditioned Learning	Radial Arm Maze	In this test, there is a reinforcing element at the end of some of the arms. Therefore, the animal must remember the arm previously visited in order to find more reinforcers in the other new arms. This test is usually assessed to food-deprived animals. The number of incorrect re-entries are evaluated as an index of memory.	
	Active Avoidance	This test is performed in a box with two connected compartments. One contains a light, and the other compartment release an electric shock after this light turns on. The animal's ability to associate both stimuli and stay in the illuminated chamber to avoid the foot shock is evaluated as an index of correct learning process.	

Table A4: Neurobehavioral correlations between human clinical symptoms of schizophrenia and animal models		
	CLINICAL SYMPTOM	**BEHAVIORAL PARADIGM**
Positive Symptoms	Stereotypic movements and hyperactivity	Hyperlocomotion · OFT · Actimetry box
	Impairment in sensorimotor filtering	· PPI
Negative Symptoms	Anhedonia	· Sucrose preference · Forced swimming test · Tail suspension test
	Avolition	· Forced swimming test · Tail suspension test.
	Asociality	· Three-chamber test
	Blunted affect	· Sucrose preference · EPM
Cognitive Symptoms	Impairment of spatial working memory	· Radial arm maze · Y-maze · Morris water maze · Barnes maze
	Impairment of non-spatial working memory	· NOR · Odor span task.
	Attention and executive function impairment	· Attentional set-shifting task · 5-choice serial reaction time task

Table A5: Keys, abbreviations and symbols to understand the cannabinoid effects summary tables:				
	Common to the 3 tables	**Anxiety table**	**Memory table**	**PPI table**
Symbols	**=** "no effect"	↑ " anxiogenic" ↓ " anxiolytic"	↓ "memory impairment"	↓ % of Prepulse inhibition
Abbreviations	**i.p.** intraperitoneal **h,** hours **n.s.** not specified **PND,** post-natal day **TT,** treatment **WO,** Washout period	**EPM**, elevated plus maze **LDB**, light/dark box **MB**, marble burying **NS**, neste shredding **OFT**, open field test	**ITI**, inter trial interval **NOP**, novel object place **NOR**, novel object recognition	**PPI**, prepulse inhibition test
Colors	· Purple: SCBs · Dark green: THC · Orange: no WO period · Light green: Male · Pink: Female			

Table A6: Acute and chronic effects of cannabinoid exposure on anxiety-like behavior (For abbreviations and symbols see table A5 above)

Age start	Cannabinoid/s	Dose	Protocol	Specie and strain	Sex	Behavioral test	Result	Summary	Reference
				ACUTE EFFECTS					
PND 49	a.JWH-018 b.THC	**Acute** a.1 mg/kg b.3 mg/kg	Immediately prior to test	Mice (C57BL/6J)	Male	OFT	↑ % Center JWH-018 and THC	↓	(Li et al., 2019)
PND (42-56)	AF-AMB	Acute 0.003-0.3 mg/kg	15 mins prior to test	Mice (C57BL/6J)	Male	OFT	↑ % Center	↓	(Ito et al., 2019b)
PND 49	a.MDMB-CHMINACA b.APICA c.5F-ADB-PINACA	**Acute** 1 mg/kg	60-, 180- and 300- prior to test	Mice (C57BL/6J)	Male	OFT	60 mins ↑ % Center MDMB and 5F-ADB 180 mins ↓ % Center APICA and 5F-ADB 300 mins ↓ % Center all drugs	↑↓ Time- and drug-dependent	(Pineda Garcia et al., 2024)
PND 70	HU-210	**Acute** 10-50 mg/kg	30 mins prior to test	Rats (LE)	Male	EPM	10 mg/kg ↑ % OA 50 mg/kg ↓ % OA	↑↓ dose-dependent	(M. N. Hill and Gorzalka, 2004)
Adults (n.s.)	THC	**Acute** 0.5-2.5 mg/kg	30 mins prior to test	Rats (CD)	Male	OFT EPM	OFT ↓ % Center at 5 mg/kg EPM ↓ % OA at 2 and 5 mg/kg	↑ dose-dependent	(Manwell et al., 2019)
				Rats (CD)	Female	OFT EPM	OFT ↓ % Center at 2 and 5 mg/kg EPM ↓ % OA at 2 and 5 mg/kg	↑ dose-dependent	(Manwell et al., 2019)
Adults (n.s.)	a.CP55,940 b. WIN 55,212–2 c.THC	**Acute** a.0.001-0.3mg/kg mg/kg b.0.3-10 mg/kg c.0.25-10 mg/kg	30 mins prior to test	Mice (CD1)	Male	EPM	a. ↑ % OA 0.01;0.03 and 0.3 mg/kg b. ↑ % OA 1 and 3 mg/kg c. ↓ % OA 1-10 mg/kg	↓ SCBs ↑ THC	(Patel and Hillard, 2006)
Adults (n.s.)	CP55,940	**Acute** 0.075-1.25 mg/kg	30 mins prior to test	Rats (Wistar)	Male	EPM	↓ % OA	↑	(Arévalo et al., 2001)

CHRONIC EFFECTS									
PND 28	THC	**Chronic** 10 mg/kg/day	TT: PND 28-48 WO: 25 days Test: PND 63	Mice (C57BL/6J)	Male	OFT LDB	OFT = % Center LDB = % Light	=	(Iemolo et al., 2021)
					Female	OFT LDB	OFT = % Center LDB = % Light	=	(Iemolo et al., 2021)
a. PND 27 **b. PND 68**	THC	**Chronic** 10 mg/kg/day	TT: PND 28-48 WO: 28 days Test: PND 63	Mice (C57BL/6J) Mice (DBA/2J)	Male	OFT EPM	PND27 = % Center = % OA PND 68 ↑ % Center = % OA	=	(Kasten et al., 2017)
PND 35	THC	**Chronic** 2.5-10 mg/kg/2x day	TT: PND 35-45 WO: 30 days Test: PND 75	Rats (SD)	Male	OFT EPM	= % Center = % OA	=	(Rubino, Vigano', et al., 2008)
					Female	OFT EPM	= % Center = % OA	=	(Rubino, Vigano', et al., 2008)
PND 15	WIN 55,212–2	**Chronic** 1.2 mg/kg/day	TT: PND 15-40 WO: 45 days Test: PND 85	Rats (Wistar)	Male	OPF	↓ % Center	↑	(Schneider et al., 2005)
PND 28	CP55,940	**Chronic** 0.4 mg/kg/day	TT: PND 28-38 WO: 62 days Test: PND 100	Rats (Wistar)	Male	OFT EPM	= % Center = % OA	=	(Higuera-Matas et al., 2009)
					Female	OFT EPM	= % Center = % OA	=	(Higuera-Matas et al., 2009)
PND 28	CP55,940	**Chronic** 0.4 mg/kg/day	TT: PND 28-42 WO: 1 days Test: PND 43	Rats (Wistar)	Male	EPM	= % OA	=	(Llorente-Berzal et al., 2011a)
					Female	EPM	= % OA	=	(Llorente-Berzal et al., 2011a)
PND 28	CP55,940	**Chronic** 0.4 mg/kg/day	TT: PND 28-43 WO: 27 days Test: PND 70	Rats (Wistar)	Male	EPM	= % OA	=	(Mateos et al., 2011)
					Female	EPM	= % OA	=	(Mateos et al., 2011)
a. PND 28	WIN 55,212–2	**Chronic** 2 mg/kg/day	TT: PND 28-47 and WO: 1 or 23 days Test: PND 48 and 70	Mice (Swiss)	Male	OFT	PND 48 = % Center PND 70 = % Center	=	(Gonçalves et al., 2023)

PND 30	WIN 55,212–2	**Chronic** 3 mg/kg/day	TT: PND 30-35 WO: 0 days Test: PND 35	Mice (CD1)	Male	OFT EPM	↓ % Center =	↑ test-dependent	(Frontera et al., 2018)
PND 31	JWH-018	**Chronic** (Autoadmin) 2.5–15 µg/kg/25 µl infusion	TT: PND 31-55 WO: 22 days Test: PND 77	Mice (CD1)	Male	MB NS	MB ↑ NS ↑	↑	(Margiani et al., 2022)
PND 34	WIN 55,212–2	**Chronic** 5 mg/kg/day	TT: PND 34-48 WO: 21 days Test: PND 70	Mice (OF1)	Male	EPM	= % OA	=	(Aguilar et al., 2017)
PND 35	HU-210	**Chronic** ↑ doses: 0.025-0.1 mg/kg x2/day	TT: PND 35-45 WO: 30 days Test: PND 75	Rats (SD)	Female	OFT EPM	= % Center = % OA	=	(Farinha-Ferreira et al., 2022)
PND 35	CP55,940	**Chronic** 0.4 mg/kg/day	TT: PND 35-45 WO: 30 days Test: PND 75	Rats (Wistar)	Male	EPM	↑ % OA	↓	(Biscaia et al., 2003)
					Female	EPM	↑ % OA	↓	(Biscaia et al., 2003)
PND 38	WIN 55,212–2	**Chronic** 0.2 or 2 mg/kg/day	TT: PND 38-49 WO: 21 days Test: PND 70	Mice (C57BL/6J)	Male	OFT EPM	0.2 mg/kg OFT = % center EPM = % OA 2 mg/kg OFT = % center EPM ↑ % OA	↑ test- and dose-dependent	(Pushkin et al., 2019)
					Female	OFT EPM	0.2 and 2 mg/kg OFT = % center EPM = % OA	=	(Pushkin et al., 2019)
PND 45	WIN 55,212–2	**Chronic** 1.2 mg/kg/day	TT: PND 45-60 WO: 1, 10, 20 and 75 days Test: PND 61, 70, 80 and 135.	Rats (SD)	Male	OFT	↑ % Center 1 day = % Center rest of the time points	= *Except day 1	(Abush and Akirav, 2012)
PND 70	HU-210	**Chronic** 0.005-0.1 mg/kg/day	TT: PND 70-82 WO: 1 day Test: PND 83	Rats (LE)	Male	OFT	0.005 mg/kg = % Center 0.1 mg/kg ↓ % Center	↑ dose-dependent	(M. N. Hill and Gorzalka, 2006)
PND 70	WIN 55,212–2	**Chronic** 2 mg/kg/day	TT: PND 70-89 WO: 1 day Test: PND 90	Mice (Swiss)	Male	OFT	= % Center	=	(Gonçalves et al., 2023)
Adults (n.s.)	JWH-018	**Chronic** 0.25 mg/kg/day	TT: 14 days WO: 1 hour, 1 and 7 days	Rats (SD)	Male	EPM MB	1h and 1 day EPM ↓ % OA MB ↑	↑ time-dependent	(Pintori et al., 2021)

							7 days EPM = % OA MB ↑		
PND 180	a.JWH-018 b.WIN 55212-2	**Chronic** a.0.03-0.3 mg/kg b. 2 mg/kg	TT: PND 180-188 WO: 0 days Test: PND 180 and 188	Mice (CD1)	Male	OFT EPM	PND 188 OFT a. ↓ % Center b. ↓ % Center EPM a. = % OA b. ↓ % OA PND 180 OFT and EPM a. and b. ↓ % Center ↓ % OA	↑	(MacRì et al., 2013)

Table A7: Acute and chronic effects of cannabinoid exposure on recognition memory (For abbreviations and symbols see table A5 above)

Age start	Cannabinoid/s	Dose	Protocol	Specie and strain	Sex	Behavioral test	Result	Summary	Reference
ACUTE EFFECTS									
PND 35	a.JWH-018 b.JWH-018-Cl c.JWH-018-Br d.THC	**Acute** a.0.01-1 mg/kg b.0.01-1 mg/kg c.0.01-1 mg/kg d.0.1-3 mg/kg	15 mins after training phase	Mice (CD1)	Male	NOR · ITI 2 hours · ITI 24 hours	**ITI 2 hours** ↓ DI JWH´s 0.1, 0.3 and 1 mg/ kg ↓ DI THC 3 mg/kg **ITI 24 hours** ↓ DI JWH´s 0.1, 0.3 and 1 mg/ kg = DI THC	↓	(Barbieri et al., 2016)
PND 45	WIN 55,212–2	**Acute** 1.2 mg/kg	24 hours prior to test	Rats (SD)	Male	NOR NOP	= DI = DI	=	(Abush and Akirav, 2012)
PND 50	a.JWH-018 b.THC	**Acute** 1 mg/kg 3 mg/kg	Immediately prior to test	Mice (C57BL/6J)	Male	NOR	↓ DI JWH-018 = DI THC	↓ SCBs = THC	(Li et al., 2019)
PND 50	a.AB-FUBINACA b.AB-CHMINACA c.PB-22 d.THC	**Acute** a.0.05-0.4 mg/kg b.0.125-1 mg/kg c.0.05-0.4 mg/kg d.1.25-25 mg/kg	60 min prior to test	Mice (CD1)	Male	Y-Test	a. ↓ DI 4 mg/kg b. ↓ DI 0.125, 0.5 & 1 mg/kg c. ↓ DI 0.05, 0.1 & 0.4 mg/kg d. ↓ DI 2.5 & 12.5 mg/kg	↓	(Schreiber et al., 2019)
PND 50	a.MDMB-CHMINACA b.APICA c.5F-ADB-PINACA	**Acute** a, b and c 1 mg/kg	60-, 180- and 300-mins prior to test	Mice (C57BL/6J)	Male	NOR	**60 mins** ↓ DI MDMB and 5F-ADB **180 mins** ↓ DI MDMB **300 min** =	↓ time-dependent	(Pineda Garcia et al., 2024)
PND 50	AF-AMB	**Acute** 0.003-0.3 mg/kg	15 mins prior to test	Mice (C57BL/6J)	Male	NOR	↓ DI	↓	(Ito et al., 2019)
PND (100-120)	JWH-018	**Acute** 0.01-0.1 mg/kg	120 mins or 24 hours prior to test	Mice (CD1)	Male	NOR NOP	**120 mins** ↓ DI 0.1 mg/kg **24 hours** ↓ DI 0.1 mg/kg	↓	(Corli et al., 2023b)
PND (100-120)	MAM-2201	**Acute** 0.01-1 mg/kg	15 mins after training phase	Mice (CD1)	Male	NOR · ITI 2 hours · ITI 24 hours	**ITI 2 hours** ↓ DI 0.1 and 1 mg/kg **ITI 24 hours** ↓ DI 0.1 and 1 mg/kg	↓	(Corli et al., 2023a)

Adults (n.s.)	JWH-081	**Acute** 1.25 mg/kg	30 mins prior to test	Mice (C57BL/6J)	Male	NOR · ITI 1hours · ITI 4 hours · ITI 24 hours	**ITI 1hours** ↓ DI **ITI 4 hours** ↓ DI **ITI 24 hours** = DI	↓ ITI dependent	(Basavarajappa and Subbanna, 2014)
				CHRONIC EFFECTS					
PND 28	THC	**Chronic** 10 mg/kg/day	TT: PND 28-48 WO: 15 days Test: PND 63	Mice (C57BL/6J)	Male	NOR modified	↓ DI	↓	(Iemolo et al., 2021)
					Female	NOR modified	↓ DI	↓	(Iemolo et al., 2021)
PND 28	THC	**Chronic** ↑ doses: 2.5, 5 and 10 mg/kg	TT: PND 28-45 WO: 30 days Test: PND 75	Rats (Wistar)	Male	NOR	= DI	=	(Llorente-Berzal et al., 2013b)
					Female	NOR	↓ DI	↓	(Llorente-Berzal et al., 2013b)
PND 35	THC	**Chronic** ↑ doses: 2.5, 5 and 10 mg/kg	TT: PND 35-45 WO: 30 days Test: PND 75	Rats (SD)	Male	NOR · ITI 1 hours · ITI 5 hours NOP	**ITI 1 hours** = DI **ITI 5 hours** ↓ DI NOP ↓ DI	↓	(De Felice et al., 2023)
PND 35	THC	**Chronic** ↑ doses: 0.3, 1 and 3 mg/kg	TT: PND 35-45 WO: 30 days Test: PND 75	Rats (SD)	Male	NOR	↓ DI	↓	(Poulia et al., 2021)
PND 35	THC	**Chronic** ↑ doses: 2.5-10 mg/kg **x2**/day	TT: PND 35-45 WO: 20 days Test: PND 65	Rats (SD)	Female	NOR NOP	↓ DI ↓ DI	↓	(Zamberletti et al., 2015)
PND 35	THC	**Chronic** ↑ doses: 2.5-10 mg/kg **x2**/day	TT: PND 35-46 WO: 20 days Test: PND 66	Rats (SD)	Male	NOR	↓ DI	↓	(Renard et al., 2017b)
a.PND 28 b.PND 70	WIN 55,212–2	**Chronic** 2 mg/kg/day	TT: PND 28-47 and PND 70-89 WO: 1 and 23 days Test: PND 48 & 70 / PND 90	Mice (Swiss)	Male	NOR	**a.PND 48** = DI **b.PND 70** = DI	=	(Gonçalves et al., 2023)
PND 28	CP 55,940	**Chronic** 0.4 mg/kg/day	TT: PND 28-43 WO: 27 days Test: PND 70	Rats (Wistar)	Male	NOR NOP	↓ DI =	↓	(Mateos et al., 2011)
					Female	NOR NOP	= ↓ DI	↓	(Mateos et al., 2011)
a.PND 29 b.PND 70	CP 55,940	**Chronic**	TT: PND 29-50 & PND 70-91	Rats (Wistar)	Male	NOR NOP	**a.PND 29** ↓ DI	↓ adolescence	(Renard et al., 2013)

		↑ doses: 0.15-0.3 mg/kg	WO: 27 days Test: PND 77 & PND 118	Rats (Lister)			↓ DI **b. PND 70** = DI = DI	-dependent	
a.PND 30 **b.PND 56**	CP 55,940	**Chronic** ↑ doses: 0.15-0.3 mg/kg	TT: PND 30-51 & PND 56-77 WO: n.s. days Test: n.s.	Rats (Wistar)	Female	NOR	**a.PND 30** ↓ DI **b. PND 56** = DI	↓ adolescence -dependent	(O'Shea et al., 2004)
PND 31	a.AB-PINACA b.AB-FUBINACA c.THC	**Chronic** ↑ doses a.0.2-1 mg/kg b.0.2-1 mg/kg c.1-5 mg/kg	TT: PND 31-55 WO: 15 days Test: PND 70	Rats (Wistar)	Male	NOR · ITI: 2 min · ITI: 60 min	**ITI 2 min** ↓ DI AB-FUB **ITI 60 min** ↓ DI all drugs	↓	(Kevin et al., 2017)
PND 34	WIN 55,212–2	**Chronic** 0.5 mg/kg/day	TT: PND 34-48 WO: 21 days Test: PND 69	Mice (OF1)	Male	NOR	↓ DI	↓	(Aguilar et al., 2017)
a.PND 40 **b.PND 80**	WIN 55,212–2	**Chronic** 1.2 mg/kg/day	TT: PND 40-65 & PND 80-105 WO: 1 and 15 days Test: PND 66-80 & PND 106-120	Rats (Wistar)	Male	NOR	**a.PND 40** · WO 1 day ↓ DI · WO 15 days ↓ DI **b.PND 80** · WO 1 day = DI · WO 15 days = DI	↓ adolescence -dependent	(Schneider et al., 2008)
PND 45	WIN 55,212–2	**Chronic** 1.2 mg/kg/day	TT: PND 45-60 WO: 1, 10, 20 and 75 days Test: PND 61, 70, 80 and 135	Rats (SD)	Male	NOR NOP	↓ DI 1 and 10 days ↓ DI 1, 10, 20 and 75 days	↓ time-dependent	(Abush and Akirav, 2012)
Adults (n.s.)	JWH-018	**Chronic** 6 mg/kg	TT: PND 7 days WO: 14 days	Mice (CD1)	Male	NOR NOP	↓ DI ↓ DI	↓	(Bilel et al., 2023)

Table A8: Acute and chronic effects of cannabinoid exposure on somatosensory filtering (For abbreviations and symbols see table A5 above)

Age start	Cannabinoid/s	Dose	Protocol	Specie and strain	Sex	Behavioral test	Result	Summary	Reference
ACUTE EFFECTS									
PND 70	WIN 55,212–2	**Acute** 0.5 - 5 mg/kg	45 mins prior to test	Mice (Swiss)	Male	PPI	= PPI	=	(Marques et al., 2020)
PND (100-120)	JWH-018 JWH-018-Cl JWH-018-Cl	**Acute** 0.1 mg/kg	15 mins prior to test	Mice (CD1)	Male	PPI	↓ % PPI all drugs	↓	(Bilel et al., 2020)
PND (100-120)	MAM-2201	**Acute** 0.01 - 1 mg/kg	15 or 120 mins prior to test	Mice (CD1)	Male	PPI	**15 mins** ↓ % PPI 0.1 y 1 mg/kg **120 mins** ↓ % PPI 0.1 y 1 mg/kg	↓	(Corli et al., 2023a)
Adults (n.s.)	APINACA	**Acute** 0.1 - 3 mg/kg	15 mins prior to test	Rats (SD)	Male	PPI	↓ % PPI 3 mg/kg ↓ Startle 3 mg/kg	↓ dose-dependent	(Bilel et al., 2019)
Adults (n.s.)	WIN 55,212–2	**Acute** 1.2 mg/kg	10 mins prior to test	Rats (Wistar)	Male	PPI	↓ % PPI	↓	(Schneider and Koch, 2002)
Adults (n.s.)	WIN 55,212–2	**Acute** 0.5 - 2mg/kg	40 mins prior to test	Rats (SD)	Male	PPI	= PPI	=	(Bortolato et al., 2005)
Adults (n.s.)	CP 55,940	**Acute** 0.01-0.1mg/kg	10 mins prior to test	Rats (SD)	Male	PPI	↓ % PPI 0.1 mg/kg ↓ Startle 0.1 mg/kg	↓	(Martin et al., 2003)
CHRONIC EFFECTS									
PND 21	THC	**Chronic** 10 mg/kg/day	TT: PND 21-50 WO: 5 days Test: PND 55	Mice (CD1)	Male	PPI	= PPI	=	(Ibarra-Lecue et al., 2018)
PND 28	THC	**Chronic** 3 mg/kg/day	TT: PND 28-44 WO: 59 days Test: PND 103	Rats (SD)	Male	PPI	= PPI	=	(Moreno-Fernández et al., 2024)
					Female	PPI	= PPI	=	(Moreno-Fernández et al., 2024)
PND 28	THC	**Chronic** 10 mg/kg/day	TT: PND 28-48 WO: 15 days Test: PND 63	Mice (C57BL/6J)	Male	PPI	= PPI	=	(Iemolo et al., 2021)
					Female	PPI	↓ % PPI	↓	(Iemolo et al., 2021)
PND 28	THC	**Chronic** 10 mg/kg/day	TT: PND 28-38 WO: 32 days Test: PND 70	Rats (Wistar)	Male	PPI	↓ % PPI	↓	(Lamanna-Rama et al., 2024)
PND 29	THC	**Chronic** 3 mg/kg/day	TT: PND 28-38 WO: 10 days Test: PND 48	Rats (SD)	Male	PPI	= PPI	=	(Silva et al., 2016)
					Female	PPI	= PPI ↑ Startle	=	(Silva et al., 2016)
PND 30 PND 60	THC	**Chronic**	TT: PND 30-41 PND 60-71	Rats (SD)	Male	PPI	= PPI	=	(Renard et al., 2017a)

		↑ doses: 2.5, 5 and 10 mg/kg	WO: 30 days Test: PND 71or 101						
PND 31	THC	**Chronic** 10 mg/kg/day	TT: PND 31-52 WO: 0 or 1 day Test: PND 51 and 53	Mice (C57BL/6J)	Male	PPI	**0 days** = PPI **1 day** = PPI	=	(Long et al., 2013)
PND 35	THC	**Chronic** ↑ doses: 2.5, 5 and 10 mg/kg	TT: PND 35-45 WO: 5 days, 4 or 6 months Test: PND 50, 161 and 241	Rats (LE)	Male	PPI	**5 days** = PPI **4 months** ↓ % PPI **6 months** = PPI	↓ time-dependent	(Abela et al., 2019)
PND 35	THC	**Chronic** ↑ doses: 0.3, 1 and 3 mg/kg	TT: PND 35-45 WO: 30 days Test: PND 75	Rats (SD)	Male	PPI	= PPI	=	(Poulia et al., 2021)
PND 15	WIN 55,212–2	**Chronic** 1.2 mg/kg/day	TT: PND 14-40 WO: 45 days Test: PND 85	Rats (Wistar)	Male	PPI	↓ % PPI	↓	(Schneider et al., 2005)
PND 28	WIN 55,212–2	**Chronic** 2 mg/kg/day	TT: PND 28-47 WO: 0 and 23 days Test: PND 48 and 70	Mice (Swiss)	Male	PPI	= PPI both time points	=	(Gonçalves et al., 2023)
PND 28	CP 55,940	**Chronic** 0.4 mg/kg/day	TT: PND 28-42 WO: 1 days Test: PND 43	Rats (Wistar)	Male	PPI	= PPI	=	(Llorente-Berzal et al., 2011a)
					Female	PPI	↓ % PPI	↓	(Llorente-Berzal et al., 2011a)
PND 30	WIN 55,212–2	**Chronic** 1.2 mg/kg/day	TT: PND 30-50 WO: 20 days Test: PND 70	Rats (Wistar)	Male	PPI	↓ % PPI ↑ Prepulse perception	↓	(Abboussi et al., 2020)
a.PND 30 **b.PND 63**	WIN 55,212–2	**Chronic** 2 mg/kg/day	TT: PND 30-40 PND 63-73 WO: a.80 days b.47 days Test: PND 120	Mice (C57BL/6J)	**(n.s)**	PPI	a. PND 30 ↓ % PPI b. PND 63 = PPI	↓ adolescence-dependent	(Gleason et al., 2012)
PND 32	WIN 55,212–2	**Chronic** 2,5 mg/kg/day	TT: PND 32-55 WO: 21 days Test: PND 76	Mice (C56BL/6J)	Male	PPI	= PPI	=	(O'Tuathaigh et al., 2012)
PND 34	WIN 55,212–2	**Chronic** 0.5 mg/kg/day	TT: PND 34-48 WO: 21 days Test: PND 70	Mice (OF1)	Male	PPI	↓ % PPI	↓	(Aguilar et al., 2017)

PND 40	WIN 55,212–2	**Chronic** 1,2 mg/kg/day	TT: PND 40-65 WO: 15 or 40 days Test: PND 80 and 105	Rats (Wistar)	Male	PPI	↓ % PPI at both time-points	↓	(Wegener and Koch, 2009)
PND 42	CP 55,940	**Chronic** 0.4 mg/kg/day **+ Acute** dose test day	TT: PND 42-63 WO: 14 days Test: PND 77	Mice (n.s.)	Male	PPI	= PPI	=	(Klug and van den Buuse, 2013)
					Female	PPI	= PPI * ↑ PPI with acute dose	=	(Klug and van den Buuse, 2013)
a.PND 40 **b.PND 70**	WIN 55,212–2	**Chronic** 1,2 mg/kg/day	TT: PND 40-65 WO: 20, 55 and 85 days Test: PND 85, 120 and 150	Rats (Wistar)	Male	PPI	**PND 40** ↓ % PPI at all time-points **PND 70** = PPI	↓ adolescence-dependent	(Schneider and Koch, 2003)
PND 56	CP 55,940	**Chronic** 0.2 mg/kg/day	TT: PND 56-70 WO: 14 days Test: PND 84	Rats (Wistar)	Male	PPI	= PPI	=	(Klug and van den Buuse, 2012)
					Female	PPI	= PPI	=	(Klug and van den Buuse, 2012)
PND 70	WIN 55,212–2	**Chronic** 2 mg/kg/day	TT: PND 70-89 WO: 1 day Test: PND 90	Mice (Swiss)	Male	PPI	= PPI	=	(Gonçalves et al., 2023)
Adults (n.s.)	JWH-018	**Chronic** 0.25 mg/kg/day	TT: 14 days WO: 1 hour, 1 or 7 days Test: after 1 hour, 1 or 7 days	Rats (SD)	Male	PPI	**1 hour** ↓ % PPI **24 hour** = PPI **7 days** = PPI	↓ time-dependent	(Pintori et al., 2021)
Adults (n.s.)	JWH-018	**Chronic** 6 mg/kg/day	TT: 6 days WO: 14 days Test: after 14 days	Mice (CD1)	Male	PPI	↓ % PPI ↓ Startle amplitude	↓	(Bilel et al., 2023)
Adults (n.s.)	WIN 55,212–2	**Chronic** 0.5 & 1.2 mg/kg/day	TT: 7 or 21 days WO: 40 mins	Rats (SD)	Male	PPI	= PPI	=	(Bortolato et al., 2005)

Table A9: statistical summary of all experiments shown for Objective 1

Assay	Parameters	Sex	Treatment effect	Interaction	N of animals	Normality	Equal variances	Figure	Statistical test
WEIGHT	Grams/day	Males	p = 0.0034 $F_{(1, 112)}$=8.987	p <0.0001 $F_{(14, 1568)}$=10.68	56-58 per group	Yes	Yes	35	ANOVA of RM
	AUC 1-5		p = 0.0928	-		Yes	Yes		T test
	AUC 6-10		p = 0.0018	-		Yes	Yes		T test
	AUC 11-15		p = <0.0001	-		Yes	Yes		T test
	Grams/day	Females	p = 0.0387 $F_{(1, 85)}$=4.410	p <0.0001 $F_{(14, 1372)}$=4.992	43-44 per group	Yes	Yes		ANOVA of RM
	AUC 1-5		p = 0.315	-		No	-		Mann-Whitney test
	AUC 6-10		p = 0.0369	-		Yes	Yes		T test
	AUC 11-15		p = 0.0003	-		Yes	Yes		T test
EPM SHORT-TERM	%Time OA	Males	p = 0.048	-	15 per group	Yes	No	37	T test Welch´s correction
	Total entries		p = 0.317	-		Yes	Yes		T test
	%Time OA	Females	p = 0.650	-	10-11 per group	Yes	Yes		T test
	Total entries		p = 0.547	-		Yes	Yes		T test
EPM LONG-TERM	%Time OA	Males	p = 0.0534	-	13-16 per group	Yes	Yes		T test
	Total entries		p = 0.123	-		Yes	Yes		T test
	%Time OA	Females	p = 0.0546	-	13-15 per group	Yes	Yes		T test
	Total entries		p = 0.153	-		Yes	No		T test Welch´s correction
FC SHORT-TERM	% Freezing/cue	Males	p = 0.752	p = 0.865	15 per group	Yes	Yes	38	ANOVA of RM
	% Freezing/cue	Females	p = 0.0769	p = 0.184	10-11 per group	Yes	Yes		ANOVA of RM
FE SHORT-TERM	% Freezing/session	Males	p = 0.676	p = 0.490	15 per group	Yes	Yes		ANOVA of RM
	% Freezing/session	Females	p = 0.780	p = 0.831	10-11 per group	Yes	Yes		ANOVA of RM
FC LONG-TERM	% Freezing/cue	Males	p = 0.740	p = 0.0987	13-16 per group	Yes	Yes		ANOVA of RM
	% Freezing/cue	Females	p = 0.0026 $F_{(1, 26)}$=11.10	p = 0.0256 $F_{(2, 52)}$=3.93	13-15 per group	Yes	Yes		ANOVA of RM
FE LONG-TERM	% Freezing/session	Males	p = 0.684	p = 0.490	13-16 per group	Yes	Yes		ANOVA of RM
	% Freezing/session	Females	p = 0.402	p = 0.0885	13-15 per group	Yes	Yes		ANOVA of RM
CORR EPM-FC LONG-TERM	%Time OA-Mean % of freezing	Females	p = 0.0252 r = -0.422	-	13-15 per group	-	-	39	Pearson´s Correlation
LOC SHORT-TERM	AU	Males	p = 0.236	-	15 per group	Yes	Yes	40	T test
	AU	Females	p = 0.915	-	10-11 per group	Yes	Yes		T test
LOC	AU	Males	p = 0.977	-	13-16 per group	Yes	Yes		T test

LONG-TERM	AU	Females	p = 0.0668	-	13-15 per group	Yes	Yes		T test
PPI SHORT-TERM	% PPI	Males	p = **0.0154** $F_{(1, 24)}$=6.79	p = 0.890	10-16 per group	Yes	Yes	42,43	ANOVA of RM
	Mean % PPI		p = **0.0155**	-		Yes	Yes		T test
	Startle response		p = 0.517	-		Yes	Yes		T test
	% PPI	Females	p = 0.967	-	11-12 per group	Yes	Yes		ANOVA of RM
	Mean % PPI		p = 0.967	-		Yes	Yes		T test
	Startle response		p = 0.809	-		Yes	Yes		T test
PPI LONG-TERM	% PPI	Males	p = **0.0209** $F_{(1, 26)}$=6.05	p = 0.192	11-17 per group	Yes	Yes		ANOVA of RM
	Mean % PPI		p = **0.0209**	-		Yes	Yes		T test
	Startle response		p = 0.378	-		Yes	Yes		T test
	% PPI	Females	p = 0.967	-	17-18 per group	Yes	Yes		ANOVA of RM
	Mean % PPI		p = 0.983	-		Yes	Yes		T test
	Startle response		p = 0.5070	-		No	-		Mann-Whitney test
PPI ADULTS	% PPI	Males	p = 0.455	-	11-13 per group	Yes	Yes	45	ANOVA of RM
	Mean % PPI		p = 0.472	-		Yes	Yes		T test
	Startle response		p = 0.496	-		Yes	Yes		T test
	% PPI	Females	p = 0.913	-	11-15 per group	Yes	Yes		ANOVA of RM
	Mean % PPI		p = 0.913	-		Yes	Yes		T test
	Startle response		p = 0.058	-		Yes	Yes		T test
PNN SHORT-TERM	PNN - IL	Males	p = **0.0214**	-	5-7 images per animal and 5-7 animals per group	Yes	Yes	46	T test
	PNN - PL		p = **0.0139**	-		Yes	Yes		T test
	PNN - OBF		p = 0.781	-		No	-		Mann-Whitney test
PNN LONG-TERM	PNN - IL		p = **0.0707**	-		Yes	Yes		T test
	PNN - PL		p = 0.715	-		Yes	Yes		ANOVA of RM
	PNN - OBF		p = 0.169	-		Yes	Yes		T test
PV+ SHORT-TERM	PV+ - IL		p = 0.075	-		No	-		Mann-Whitney test
	PV+ - PL		p = 0.313	-		Yes	No		T test Welch´s correction
	PV+ - OBF		p = 0.106	-		Yes	Yes		Mann-Whitney test
PV+ LONG-TERM	PV+ - IL		p = 0.755	-		No	-		Mann-Whitney test
	PV+ - PL		p = 0.214	-		Yes	Yes		T test
	PV+ - OBF		p = 0.285	-		Yes	Yes		T test
% PV+ / PNN SHORT-TERM	%PV+/PNN - IL		p = **0.0602**	-		Yes	Yes		T test
	%PV+/PNN - PL		p = 0.109	-		Yes	No		T test Welch´s correction

	%PV+/PNN - PL		p = 0.557	-		Yes	Yes		T test
% PV+ / PNN LONG-TERM	%PV+/PNN - IL		p = **0.059**	-		Yes	Yes		T test
	%PV+/PNN - PL		p = 0.812	-		Yes	Yes		T test
	%PV+/PNN - PL		p = 0.107	-		Yes	Yes		T test
PNN LONG-TERM	PNN - IL	Females	p = 0.906	-	5-7 images per animal and 6-7 animals per group	Yes	Yes	47	T test
	PNN - PL		p = 0.228	-		Yes	Yes		T test
	PNN - OBF		p = 0.597	-		Yes	Yes		T test
PV+ LONG-TERM	PV+ - IL		p = 0.244	-		No	-		Mann-Whitney test
	PV+ - PL		p = 0.236	-		Yes	Yes		T test
	PV+ - OBF		p = 0.682	-		Yes	Yes		T test
% PV+ / PNN LONG-TERM	%PV+/PNN - IL		p = 0.924	-		Yes	Yes		T test
	%PV+/PNN - PL		p = 0.581	-		Yes	Yes		T test
	%PV+/PNN - PL		p = 0.444	-		Yes	Yes		T test
IBA-1 SHORT-TERM	IBA-1+ cells	Males	p = 0.231	-	5-7 images per animal and 6 animals per group	Yes	Yes	49	T test
	IBA-1 soma area		p = **0.012**	-		Yes	No		T test Welch´s correction
	IBA-1 soma perim.		p = 0.069	-		Yes	Yes		T test
IBA-1 LONG-TERM	IBA-1+ cells	Males	p = 0.237	-		Yes	Yes		T test
	IBA-1 soma area		p = **0.0106**	-		Yes	Yes		T test
	IBA-1 soma perim.		p = **0.0407**	-		Yes	Yes		T test
	IBA-1+ cells	Females	p = 0.382	-		Yes	Yes	50	T test
	IBA-1 soma area		p = 0.390	-		Yes	Yes		T test
	IBA-1 soma perim.		p = 0.263	-		Yes	Yes		T test
GFAP SHORT-TERM	GFAP immunoreactivity	Males	p = 0.266	-		Yes	Yes	53	T test
GFAP LONG-TERM	GFAP immunoreactivity	Males	p = 0.266	-		Yes	No		T test Welch´s correction
	GFAP immunoreactivity	Females	p = 0.485	-		Yes	Yes	50	T test
CORR PNN-IBA-1 SHORT-TERM	PNNs-soma area-IL	Males	p = **0.036** r = -0.63	-	5-6 per group	-	-	51	Pearson´s Correlation
	PNNs-soma area-PL	Males	p = **0.025** r = -0.66	-	5-6 per group	-	-		Pearson´s Correlation
CORR PNN-PPI SHORT-TERM	PNNs-%PPI-IL	Males	p = **0.037** r = 0.61	-	5-6 per group	-	-	52	Pearson´s Correlation
CORR PNN-PPI LONG-TERM	PNNs-%PPI-IL	Males	p = **0.017** r = 0.66	-	5-6 per group	-	-		Pearson´s Correlation

qPCR SHORT-TERM	GAD67 mRNA levels	Males	p = 0.003	-	7-9 per group	No	-	54	Mann-Whitney test
	SYP mRNA levels		p = 0.517	-		Yes	Yes		T test
	PSD95 mRNA levels		p = 0.911	-		Yes	No		T test Welch´s correction
	BDNF mRNA levels		p = 0.227	-		Yes	No		T test Welch´s correction
	CB1R mRNA levels		p = 0.736	-		Yes	No		T test Welch´s correction
	CB2R mRNA levels		p = 0.015	-		Yes	Yes		T test
qPCR LONG-TERM	GAD67 mRNA levels	Males	p = 0.615	-	6-10 per group	Yes	Yes		T test
	SYP mRNA levels		p = 0.870	-		Yes	Yes		T test
	PSD95 mRNA levels		p = 0.692	-		Yes	Yes		T test
	BDNF mRNA levels		p = 0.687	-		Yes	Yes		T test
	CB1R mRNA levels		p = 0.432	-		Yes	Yes		T test
	CB2R mRNA levels		p = 0.610	-		Yes	No		T test Welch´s correction

Table A10: statistical summary of all experiments shown for Objective 2

Assay	Parameters	Sex	Treatment effect	Interaction	N of animals	Normality	Equal variances	Figure	Statistical test
WEIGHT	Grams/day	Males	p = 0.1782	p <0.0001 $F_{(14, 1162)}$=10.79	54 per group	Yes	Yes	55	ANOVA of RM
	AUC 1-5		p = 0.8472	-		Yes	Yes		T test
	AUC 6-10		p = 0.0848	-		Yes	Yes		T test
	AUC 11-15		p = 0.0114	-		Yes	Yes		T test
	Grams/day	Females	p = 0.1304	p <0.0001 $F_{(14, 1372)}$=4.992	61 per group	Yes	Yes		ANOVA of RM
	AUC 1-5		p = 0.6559	-		Yes	No		T test Welch´s correction
	AUC 6-10		p = 0.1061	-		Yes	No		T test Welch´s correction
	AUC 11-15		p = 0.0003	-		No	-		Mann-Whitney test
EPM	%Time OA	Males	p = 0.0142	-	12 per group	Yes	No	57	T test Welch´s correction
	Total entries		p = 0.2708	-		Yes	Yes		T test
	%Time OA	Females	p = 0.0489	-		Yes	Yes		T test
	Total entries		p = 0.5767	-		Yes	No		T test Welch´s correction
FC / FE	% Freezing/cue	Males	p = 0.3794	p = 0.5869	12 per group	Yes	Yes	58	ANOVA of RM
	% Freezing/session	Males	p = 0.8378	p = 0.6544		Yes	Yes		ANOVA of RM
	% Freezing/cue	Females	p = 0.0045 $F_{(1, 20)}$ = 10.26	p = 0.0123 $F_{(2, 40)}$ = 4.914	12 per group	Yes	Yes		ANOVA of RM
	% Freezing/session	Females	p = 0.3262	p = 0.2794	12 per group	Yes	Yes		ANOVA of RM
CORR EPM/FC	%Time OA-Mean % of freezing	Females	p = 0.0483 r = -0.435	-	10-11 per group	-	-	59	Pearson´s Correlation
LOC	AU	Males	p = 0.7450	-	13 per group	Yes	Yes	60	T test
	AU	Females	p = 0.3411	-	14-15 per group	Yes	Yes		T test
NOR	Discrimination index	Males	p = 0.1286	-	14-15 per group	Yes	Yes	62	T test
	Total time exploring		p = 0.0927	-		Yes	Yes		T test
	Discrimination index	Females	p = 0.0274	-	11-13 per group	Yes	Yes		T test
	Total time exploring		p = 0.3134	-		Yes	Yes		T test
SOC	Time direct contact/compartment	Males	p = 0.9942 *Compartment effect: <0.0001	p = 0.8034	14-16 per group	Yes	Yes	63	ANOVA
	Time direct contact/compartment	Females	p = 0.2777 *Compartment effect: <0.0001	p = 0.1826	14-15 per group	Yes	Yes		ANOVA
FST	Immobility time	Males	p = 0.0056	-	14-15 per group	Yes	No	64	T test Welch´s correction

	Immobility time	Females	p = 0.7770	-		Yes	Yes		T test
PPI	% PPI	Males	p = 0.2515	p = 0.6083	13-15 per group	Yes	Yes	66	ANOVA of RM
	Mean % PPI		p = 0.2515	-		Yes	Yes		T test
	Startle response		p = 0.4442	-		Yes	Yes		T test
	% PPI	Females	p = 0.0210 $F_{(1, 26)}$ = 6.040	p = 0.5177	13-15 per group	Yes	Yes		ANOVA of RM
	Mean % PPI		p = 0.0210	-		Yes	Yes		T test
	Startle response		p = 0.5070	-		Yes	Yes		T test
EPM Adults	%Time OA	Females	p = 0.4515	-	10-14 per group	Yes	Yes	68	T test
	Total entries		p = 0.3836	-		Yes	Yes		T test
NOR Adults	Discrimination index	Females	p = 0.4483	-	13-14 per group	Yes	Yes		T test
	Total time exploring		p = 0.1385	-		Yes	Yes		T test
PPI Adults	% PPI	Females	p = 0.0329 $F_{(1, 27)}$ = 5.056	p = 0.6999	12-17 per group	Yes	Yes	69	ANOVA of RM
	Mean % PPI		p = 0.0329	-		Yes	Yes		T test
	Startle response		p = 0.7730	-		Yes	Yes		T test
Correlation PPI-RNAseq	%PPI-Plekhg2	Females	p = 0.0007 r = -0.9336	-	4 per group	-	-	71	Pearson´s Correlation
	%PPI-Sh3tc1		p = 0.0171 r = -0.8003	-		-	-		Pearson´s Correlation
Sholl Analysis	Sholl	Females	p = 0.0181 $F_{(1,46)}$ = 6.01	p <0.0001 $F_{(14, 370)}$= 4.680	6 neurons per mice and 4 mice per group	Yes	Yes	73	Mixed-ANOVA
	Length		p = 0.0286	-		No	-	74	Mann-Whitney test
	Basal length		p <0.0001			Yes	Yes		T test
	Apical length		p = 0.778	-		Yes	Yes		T test
	N of primary dendrites		p = 0.9408	-		Yes	No		T test Welch´s correction
	N of secondary dendrites		p = 0.0265	-		Yes	Yes		T test
	N of tertiary dendrites		p = 0.0013	-		Yes	Yes		T test
Dendritic spines analysis	Total spine density (APICAL)	Females	p = 0.0064	-	4-5 neurons per mice and 4 mice per group	Yes	Yes	75	T test
	Long thin (APICAL)		p = 0.3143	-		Yes	No		T test Welch´s correction
	Stubby (APICAL)		p = 0.1143	-		No	-		Mann-Whitney test
	Mushroom (APICAL)		p = 0.0361	-		Yes	Yes		T test
	Branched (APICAL)		p = 0.3298	-		Yes	Yes		T test
	Total spine density (BASAL)		p = 0.8403	-		Yes	Yes		T test

	Long thin (BASAL)		p = 0.5636	-		Yes	Yes		T test
	Stubby (BASAL)		p = 0.8307	-		Yes	Yes		T test
	Mushroom (BASAL)		p = 0.3810	-		No	-		Mann-Whitney test
	Branched (BASAL)		p = 0.7749	-		Yes	Yes		T test
CDC42 G-LISA	Relative activity	Females	p = 0.8208	-	11 per group	Yes	Yes	72	T test

ANNEX II

ARTICLE OPEN

Check for updates

Adolescent exposure to the Spice/K2 cannabinoid JWH-018 impairs sensorimotor gating and alters cortical perineuronal nets in a sex-dependent manner

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The consumption of synthetic cannabinoids during adolescence is reported to be a risk factor for the appearance of psychiatric disorders later in life. JWH-018 was identified as one of the primary psychoactive components present in Spice/K2 preparations. This study evaluated the short- and long-term consequences of exposure to JWH-018 during the adolescence in anxiety-like behavior, fear extinction, and sensorimotor gating in male and female mice. Alterations in anxiety varied depending on the time interval between treatment and behavioral analysis along with sex, while no changes were observed in the extinction of fear memory. A decrease in prepulse inhibition of the startle reflex was revealed in male, but not female, mice at short- and long-term. This behavioral disturbance was associated with a reduction in the number of perineuronal nets in the prelimbic and infralimbic regions of the prefrontal cortex in the short-term. Furthermore, adolescent exposure to JWH-018 induced an activation of microglia and astrocytes in the prefrontal cortex of male mice at both time intervals. A transitory decrease in the expression of GAD67 and CB2 cannabinoid receptors in the prefrontal cortex was also found in male mice exposed to JWH-018. These data reveal that the treatment with JWH-018 during the adolescence leads to long-lasting neurobiological changes related to psychotic-like symptoms, which were sex-dependent.

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INTRODUCTION

Recreational use of synthetic cannabinoids (SCBs), a novel range of psychoactive substances which have similar effects like Δ^9-tetrahydrocannabinol (Δ^9-THC), is an increasing public health problem mainly in Western societies [1]. SCBs were promoted by internet retailers and European 'head shops' as meditation potpourris and tropical incense products under names such as K2 and Spice [2]. Wrapped in foil packages, these herbal mixtures typically contain a combination of several structural classes of SCBs which have been linked to more adverse health effects than natural cannabinoids [3]. Thus, acute intoxication with SCBs has been related to tachycardia, hypertension, visual and auditory hallucinations, mydriasis, agitation and anxiety, seizures, tachypnea, nausea and vomiting [1]. Most alarming, SCBs abuse in some individuals can result in death [4]. Adolescents [5] and military personnel [6] are the most frequent users probably due to easy accessibility and limited availability of selective and sensitive rapid analytical methods for screening these compounds [7].

The naphthoylindole JWH-018 was identified as one of the primary psychoactive components present in Spice/K2 preparations [8]. Initially developed for therapeutic purposes, JWH-018 is considered the prototypical compound of the so-called "first-generation" class of synthetic cannabinoids. JWH-018 is a potent agonist at cannabinoid type-1 (CB1R) and cannabinoid type-2 (CB2R) receptors, showing approximately a four-fold increased activity at the CB1R and about a ten-fold affinity at the CB2R compared with Δ^9-THC [9]. In animal models, JWH-018 reproduces the typical "tetrad" effects of THC which are hypothermia, analgesia, hypolocomotion and catalepsy [10], impairs memory retention [11, 12] as well as sensorimotor responses [13, 14], and triggers electrographic seizures [15]. Recently, repeated JWH-018 administration was found to induce an anxiety-like phenotype, transitory reductions of sensorimotor gating, and an aversive state upon withdrawal [16]. However, the possible long-lasting behavioral and biochemical changes induced by adolescent JWH-018 exposure are poorly understood.

As previously mentioned, adolescents and young adults show the highest rate of SCBs use [5], which is of particular concern because this period is crucial to generate efficient neuronal pathways by constant neuroplastic shaping, synaptic reorganization and neurochemical changes [17]. Indeed, preclinical studies indicate that cannabinoid exposure during adolescence impacts neurodevelopmental processes and behavior [18], including those normally mediated by the endocannabinoid system [19, 20].

In this study, we investigated the short- and long-term consequences of adolescent exposure to JWH-018 on key neurobehavioral responses associated with SCBs toxicity in humans. Anxiety, fear extinction, and sensorimotor gating were

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evaluated in male and female mice after treatment with JWH-018 during the adolescence. Possible neurochemical alterations related to these behavioral responses were also studied.

MATERIALS AND METHODS

Animals

Adolescent and adult C57BL/6J male and female mice (Charles River) were used in these experiments. Mice were housed 3–4 per cage in a temperature (21 ± 1 °C)—and humidity (55 ± 10%)-controlled room under a 12 h light/dark cycle. All behavioral studies were performed during the light period. Tests were conducted in alternate weeks in male and female mice. Mice were randomly assigned in the different experimental groups. Food and water were available ad libitum. All behavioral data were obtained by experimental observers blinded to the experimental conditions. Experimental procedures were conducted in accordance with the guidelines of the European Communities Directive 2010/63/EU and Spanish Regulations RD 1201/2005 and 53/2013 regulating animal research and approved by the local ethical committee (CEEA-UFV).

Drugs

JWH-018 (Tocris) was prepared in a 5% ethanol, 5% Tween-80 and 90% saline solution, and was intraperitoneally (i.p.) administered at doses of 0.5, 1 and 1.5 mg/kg (10 ml/kg of body weight). Doses used were based on previous studies [11, 13] in mice.

Experimental designs

JWH-018 treatment during adolescence. The short- and long-term effects of the exposure to JWH-018 during the adolescence on locomotion, anxiety-like behavior, cued fear conditioning and extinction, and prepulse inhibition (PPI) of the startle reflex were evaluated in both male and female mice. The temporal boundaries of adolescence, considered a vulnerable period to the central effects of drugs [21, 22], are not exactly defined neither in humans nor in rodents [23]. Therefore, based on previous studies [24], mice were treated with increasing doses of JWH-018 (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) or vehicle in order to avoid drug tolerance for 15 days. Short- and long-term effects were analyzed 5 (PND 54) and 20 (PND 69) days respectively after the end of the treatment, as described in Figs. 1A and 2A. The interval of time between adolescent treatment and the different behavioral assays is based on previous reports [24, 25]. Different cohorts of animals were used for the experiments of locomotion, anxiety and fear extinction (males, $n = 15$, short-term, $n = 14$–16, long-term; females, $n = 10$–11, short-term, $n = 13$–15, long-term), and for the experiments of PPI (males, $n = 10$–16, short-term, $n = 11$–17, long-term; females, $n = 11$–12, short-term, $n = 17$–18, long-term). Tissues were obtained 24 h after the PPI test to carry out biochemical experiments in male mice. For short-term, an additional experimental batch was performed to complete the number of mice required. For immunofluorescence experiments, the number of mice was 6–7 (short-term) and 5–7 (long-term). For RT-PCR experiments, the number of mice was 8–9 (short-term) and 6–10 (long-term). The number of animals used in this study is in the usual range of similar experiments previously published. Each experimental sequence was performed once.

JWH-018 treatment during adulthood. To elucidate whether adolescence is a period of susceptibility to the effects of JWH-018, a similar protocol was performed in adult male and female mice (Fig. 3A). Starting at PND 70, mice were administered with increasing doses of JWH-018 (PND 70–74: 0.5 mg/kg, PND 75–79: 1 mg/kg, and PND 80–84: 1.5 mg/kg). Behavioral evaluation was performed at PND 104 (Fig. 3A), 20 days after the end of the treatment. The number of mice used was 11–13 for males, and 11–15 for females.

Behavioral experiments

Elevated plus maze. Anxiety-like behavior was evaluated using an elevated plus maze (EPM), which consisted in four arms (25 × 5 cm) set in cross from a central square (5 × 5 cm) and raised 30 cm from the ground. Two opposite arms were delimited by vertical walls (closed arms), although the two other arms had unprotected edges (open arms). The apparatus was indirectly illuminated with 40–50 lux. The 5 min performance was recorded through a videocamara located above the maze. Results are expressed as total entries to the open and closed arms, and the percentage of time spent in the open arms with respect to the total amount of time spent in both closed and open arms.

Cued fear conditioning and extinction. Experiments were performed as previously reported with slight modifications [24, 26]. Mice were individually placed in the test chamber (LE116, Panlab, Harvard Instruments) made of black methacrylate walls with a transparent front door. The box (25 × 25 x 25 cm) was located inside a soundproof module with a ventilation fan to provide a background noise and attenuate nearby sounds. The chamber floor was formed by parallel stainless-steel bars (2 mm of diameter and 6 mm spaced) connected to a shock generator (LE100-26 module, Panlab, Harvard Instruments). A high-sensitivity weight transducer (load cell unit) was used to record the signal generated by the animal movement intensity. Experimental software PACKWIN V2.0 automatically calculated the percentage of immobility time for each experimental phase. Between each animal trial, the chamber was cleaned with 70% ethanol and then water to avoid olfactory cues. Mice were individually conditioned after a 180 s habituation with 3 cue tones (3 kHz, 80 dB) of 30 s long (10 s interval). Each cue tone (conditioned stimulus, CS) co-terminated with a 0.7 mA foot-shock of 1 s duration (unconditioned stimulus, US). Fear extinction sessions (E1-E5) took place 24, 48, 72, 96 and 120 h after the conditioning day in a novel environment (white walls, transparent cylinder, and smooth floor), and after an acclimatation period, 4 cue tones (CS) were presented with an interval period of 10 s. Freezing behavior, a rodent's natural response to fear, was automatically evaluated and defined as complete lack of movement, except for breathing for more than 800 ms. Data were expressed as percentage of freezing behavior during the time the sound was active.

Prepulse Inhibition of the startle reflex. Prepulse inhibition (PPI) of the startle reflex was tested in two automated StartFear combined system chambers (LE116, Panlab, Harvard Instruments) which were calibrated to ensure equivalent stabilimeter sensitivity. Mice were daily habituated to a non-restrictive Plexiglas cylinder anchored to a high sensitivity transducer for 5 min with background white noise (65 dB) 4 days prior to test. The test started with an acclimatation period of 5 min followed by 5 pulse trials (120 dB, 40 ms) for startle accommodation. The experimental protocol consisted of 10 blocks with 3 or 12 trials each, randomly presented with an inter-trial interval of 10–30 s: no stimulus (12x) (background white noise), pulse alone (12x) (120 dB, 40 ms), pulse preceded by 4 prepulse intensities (12x each) (4, 8, 12 and 16 dB above background noise, 20 ms duration, 100 ms before pulse) and prepulse alone (3x each). Finally, 5 pulse trials were delivered. Initial and final pulses were not included in the final analysis. A background white noise was generated throughout the whole experiment. Startle amplitude was automatically detected by PACKWIN V2.0 software. PPI was calculated as: 100 x (mean startle response – mean prepulse inhibited startle response) / (mean startle response).

Locomotion. Changes in locomotor activity were assessed by using locomotor activity boxes (27 × 27 x 21 cm, Cibertec). Mice were individually placed in locomotor cages with low luminosity. Activity was measured as the total number of times the animal crossed an infrared beam during 15 min.

Tissue preparation for immunofluorescence

Twenty-four h after the PPI test, mice were deeply anesthetized by i.p. injection of ketamine, xylazine and saline solution prior to intracardiac perfusion. Mice were perfused with 1X phosphate buffer saline (PBS) followed by 4% paraformaldehyde. Afterward, brains were post-fixed in 4% paraformaldehyde 24 h and then dehydrated by sequential transfer to 15 and 30% of sucrose in PBS 1X (4 °C). Coronal frozen sections of 20 µm thickness were obtained in a cryostat from 2.10 to 1.54 mm relative to bregma for prefrontal cortex. Brain slices were stored in a cryoprotective solution (20% glycerol, 30% ethylenglycol in PBS 1X) at −20 °C until use. The number of male mice was 6–7 (short-term) and 5–7 (long-term).

Immunofluorescence

Parvalbumin and perineuronal nets. Floating slices were 3 times rinsed in PBS 1X and then treated with blocked solution (4% normal goat serum, 0.1% Triton X-100, 0.1% bovine serum albumin in PBS 1X) for 1.5 h at room temperature. Slices were incubated overnight at 4 °C with the primary antibodies prepared in blocked solution. Rabbit anti-parvalbumin (PV) (1:2000, ab11427, Abcam) and Wisteria floribunda agglutinin combined with fluorescein (1:1000, FL-1351, Vector Laboratories) to label

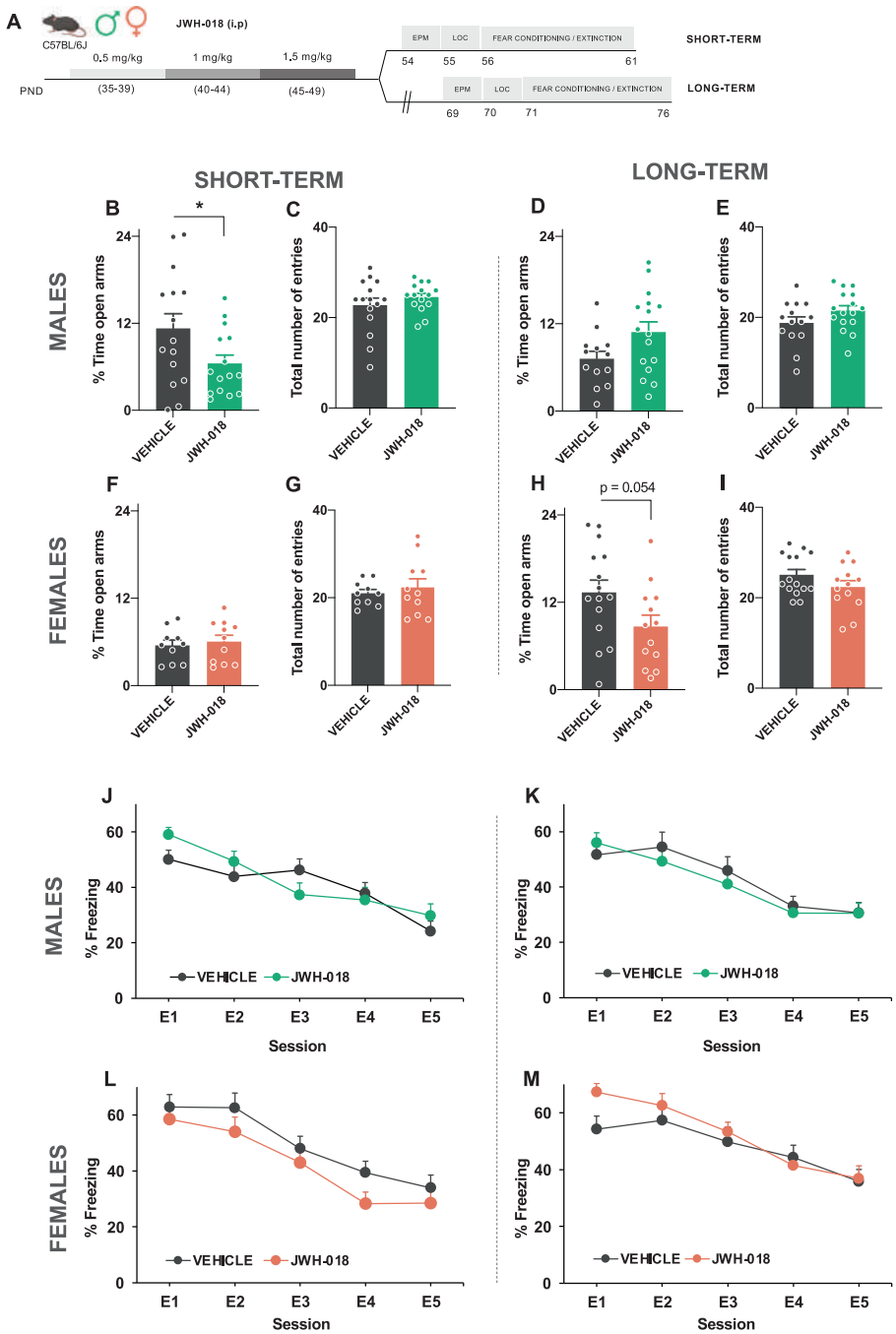

Fig. 1 JWH-018 exposure during adolescence alters anxiety-like behavior, but not fear extinction, depending on the sex and the time interval between treatment and behavioral analysis. A Schematic representation of the experimental design. **B–M** Effects of adolescent exposure to JWH-018 (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) or vehicle in anxiety-like behavior in the EPM **B–I** and fear conditioning and extinction **J–M** in male mice at short- **B**, **C**, **J** and long-term **D**, **E**, **K**, and female mice at short- **F**, **G**, **L** and long-term **H**, **I**, **M** ($n = 10–16$ mice per group). Percentage of time spent in the open arm and total number of entries are shown for the EPM. Time course of the freezing levels scored during cued fear extinction trials is shown for fear memory processing. Data are expressed as mean ± SEM. *$p < 0.05$ (comparison between JWH-018 and vehicle; Student's t-test). PND postnatal day, EPM elevated plus maze, E1-E5 extinction trials.

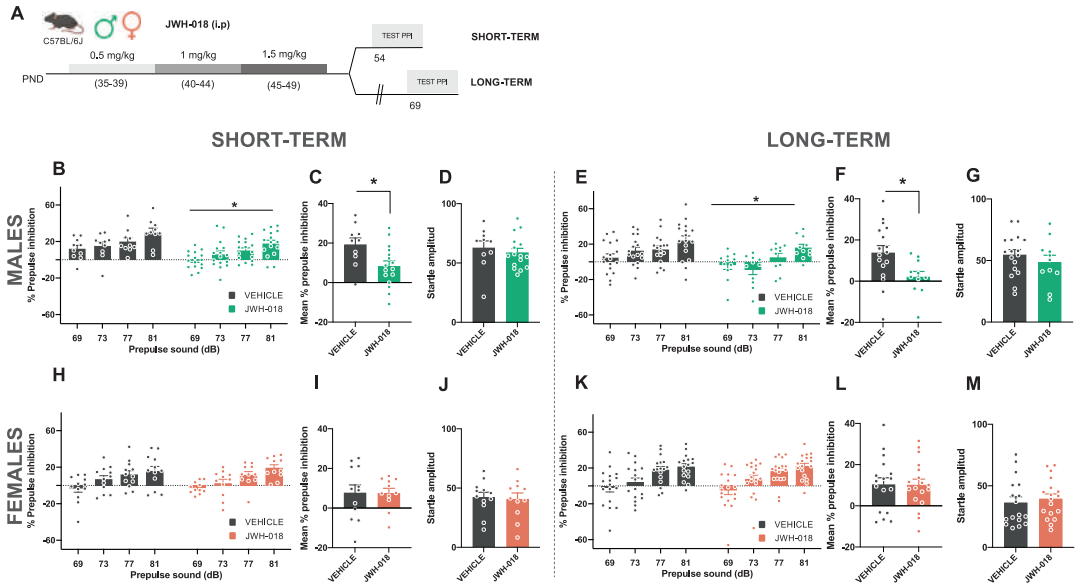

Fig. 2 JWH-018 exposure during adolescence impairs sensorimotor gating in male, but not female, mice at short- and long-term. **A** Schematic representation of the experimental design. **B**–**M** Effects of adolescent exposure to JWH-018 (PND 35–39: 0.5 mg/kg, PND 40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) or vehicle in sensorimotor gating in male mice at short- **B**–**D** and long-term **E**–**G**, and female mice at short- **H**–**J** and long-term **K**–**M** ($n = 10$–18 mice per group). Percentage of prepulse inhibition, mean of the percentage of prepulse inhibition, and startle response amplitude are shown. Data are expressed as mean ± SEM. *$p < 0.05$ (comparison between JWH-018 and vehicle group; two-way ANOVA with repeated measures, treatment **B**, **E**; Student's t-test **C**, **F**). PND postnatal day, dB decibels.

perineuronal nets (PNNs) were used. Next day, after three rinses with PBS 1X (10 min), sections were incubated with the secondary antibody AlexaFluor-594 (1:500, A-11012, Invitrogen) for PV labeling at room temperature for 1 h in blocked solution. Slices were washed 3 times in PBS 1X and mounted with Fluoromount-DAPI (Invitrogen).

Iba-1 and GFAP. The same protocol previously described was used, applying the specific antibodies. Primary antibodies used were rabbit anti-Iba-1 (1:1000, 019-19741, Wako) and guinea pig anti-GFAP (1:1000, 173 004, Synaptic system) to label microglial cells and astrocytes, respectively. The secondary antibodies employed were AlexaFluor-594 (1:500, A-11012, Invitrogen) for Iba-1 and AlexaFluor-488 (1:500, A-11073, Invitrogen) for GFAP labeling.

Immunofluorescence image analysis

Parvalbumin and perineuronal nets. Immunostained sections were observed under a Zeiss LSM 900 confocal microscope, using a 20x/0.5 dry objective (Zeiss, CLSM, Germany). Images were acquired through a z-plane (1µm/stack, 8 stacks, 16-bit, 1024 × 1024) and the z-stack was obtained through a maximum projection. A 500 µm squared region of interest (ROI) was delimited for quantification in each infralimbic (IL), prelimbic (PL) and orbitofrontal (OBF) subregions of the prefrontal cortex. The number of positive PV, PNNs and % of PV surrounded by PNNs was semiautomatically detected by using the Pipsqueak tool ® [27] for FIJI (FIJI is just ImageJ) software. For all areas, 5–7 images per animal were quantified.

Iba-1 and GFAP. The stained sections were analyzed at 40 × /0.5 objective using a Zeiss LSM 900 confocal microscope (Zeiss, CLSM, Germany). Images were taken through a z-plane (0.5 µm/stack, 10 stacks, 16-bit, 1024 × 1024) and the quantification was carried out through a sum slides projection (32-bit). A quantification ROI of 320 × 320 µm located in the intermediate region between the IL and PL subareas of the prefrontal cortex was chosen. FIJI (FIJI is just Image J) software was used to calculate fluorescence intensity of GFAP stain. The "freehand selection" tool was used to quantify soma area and perimeter of Iba-1-stained cells. Five to seven images per animal were analyzed.

Quantitative RT-PCR analysis

Prefrontal cortex tissues were extracted 24 h after the PPI test and immediately stored at −80 °C (n = 8–9 (short-term) and n = 6–10 (long-term)). The RNA was purified with the RiboPure™ KIT (Invitrogen) and the reverse transcription was performed with 1µg of total RNA and the SuperScript™ II Reverse Transcriptase (Invitrogen). PCR reactions were conducted using PrimePCR™ Probe Assay (Bio-Rad) to quantify mRNA levels of glutamic acid decarboxylase, 67 kDa isoform (GAD67) (ID: qMmuCEP0060617), brain derived neurotrophic factor (BDNF) (ID: qMmuCEP0058759), synaptophysin (SYP) (ID: qMmuCIP0035577), CB1R (ID: qMmuCEP0038879) and CB2R (ID: qMmuCEP0039299). To evaluate postsynaptic density protein 95 (PSD95) (ID: 4453320), TaqMan™ Gene Expression Assay (Applied Biosystems™) was used. GAPDH (ID: qMmuCEP0039581) expression was used as endogenous control gene for normalization. PCR assays were carried out with the CFX Connect Real-Time PCR Detection System (Bio-Rad). The fold changes in gene expression of JWH-018 treated animals in comparison with controls were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Before the analysis, all data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Bartlett's test). Statistical analysis was carried out using unpaired Student t-test (with Welch's correction when appropriate), two-way ANOVA of repeated measures followed by Newman-Keuls post hoc comparisons after significant interactions between factors. When parametric normality test was violated, a Mann-Whitney nonparametric test was used. Pearson's correlation coefficient was used to analyze the relationship between two variables. Outliers were excluded if they were >2 standard deviations from the mean. All data are expressed as mean ± SEM. A p value < 0.05 was used to determine statistical significance. The statistical analysis was performed using STATISTICA (StatSoft) software and GraphPad Prism 9.

RESULTS

Short- and long-term consequences on anxiety and fear extinction in adolescent mice exposed to JWH-018

Adolescent male and female mice were treated with increasing doses of JWH-018 during 15 days (PND 35–39: 0.5 mg/kg, PND

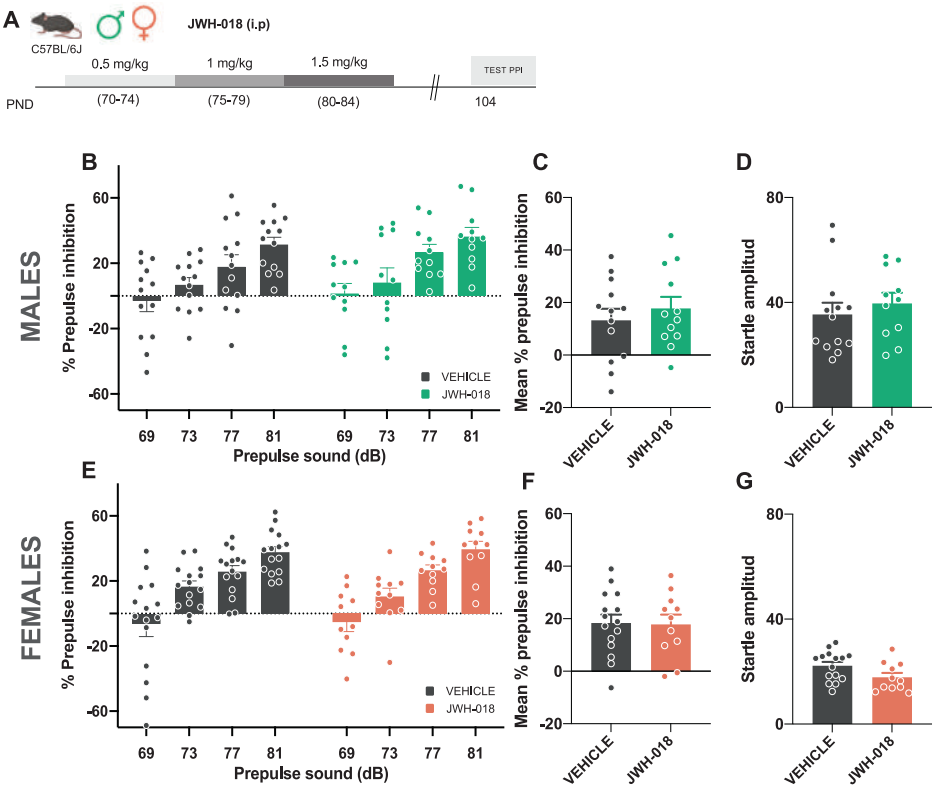

Fig. 3 JWH-018 exposure during adulthood does not modify sensorimotor gating in male and female mice. A Schematic representation of the experimental design. **B**–**G** Effects of exposure to JWH-018 during adulthood (PND 70–74: 0.5 mg/kg, PND 75–79: 1 mg/kg, and PND 80–84: 1.5 mg/kg) or vehicle in male (**B**–**D**) and female (**E**–**G**) mice ($n = 11$–15 mice per group). Percentage of prepulse inhibition, mean of the percentage of prepulse inhibition, and startle response amplitude are shown. Data are expressed as mean ± SEM. PND postnatal day, dB decibels.

40–44: 1 mg/kg, and PND 45–49: 1.5 mg/kg) (Fig. 1A). Body weight was daily evaluated along JWH-018 treatment. The weight gain of mice treated with JWH-018 was lower than those exposed to vehicle in both sexes (Supplementary Fig. 1) (treatment effect: $F_{1,27} = 4.88$, $p < 0.05$ and $F_{1,22} = 5.12$, $p < 0.05$, for male and female mice, respectively), in agreement with previous reports evaluating effects of adolescent THC exposure [24, 28]. Locomotor activity, anxiety-like behavior and fear memory processing were analyzed 5 (short-term) or 20 (long-term) days after the finishing of JWH-018 treatment (Fig. 1A). No changes in locomotion were observed in either males or females (Supplementary Fig. 2). By using the EPM, JWH-018 induced an anxiogenic-like effect in males in the short-term ($p < 0.05$) (Fig. 1B). This effect was specific to the early period as they recovered when they reached the adulthood (Fig. 1D). In contrast, no differences in anxiety were observed in female mice in the short-term (Fig. 1F), while there was a clear long-term anxiogenic trend ($p = 0.054$) (Fig. 1H). Total number of entries were not modified in either males or females (Fig. 1C, E, G, I). Aversive memory processing was evaluated by a cued fear conditioning paradigm. The administration of JWH-018 did not alter cued fear extinction in both males (Fig. 1J, K) and females (Fig. 1L, M) in the short- and the long-term. These results suggest the existence of sex-specific effects in unconditioned anxiety due to JWH-018 exposure during the adolescence.

Short- and long-term consequences on sensorimotor gating in adolescent mice exposed to JWH-018

Impairments of PPI of the startle reflex, a sensorimotor gating process, are observed in patients with schizophrenia [29] and is considered a marker of psychotic-like behavior [30]. By using the same experimental protocol of JWH-018 administration previously described (Fig. 2A), we studied possible PPI alterations in both male and female mice. Interestingly, a significant decrease of PPI of the startle reflex was revealed in male mice in both short- (treatment effect: $F_{1,24} = 6.79$, $p < 0.05$) (Fig. 2B) and long-term (treatment effect: $F_{1,26} = 6.06$, $p < 0.05$) (Fig. 2E). An overall reduction of PPI due to JWH-018 exposure was observed when representing mean PPI score at both time periods ($p < 0.05$) (Fig. 2C, F). This effect was independent of baseline changes in startle amplitude (Fig. 2D, G), ruling out an impact of startle reaction in the PPI modifications observed. In contrast to male mice, adolescent exposure to JWH-018 did not modify PPI of the startle reflex in females (Fig. 2H, I, K, L). The magnitude of startle reflex was also not altered by JWH-018 injection (Fig. 2J, M) in these mice. These results suggest a sex-dependent alteration on sensorimotor gating due to the treatment with the synthetic cannabinoid JWH-018.

To elucidate whether immature brain represents a period of development more susceptible to the effects of JWH-018, we evaluated the consequences of the synthetic cannabinoid

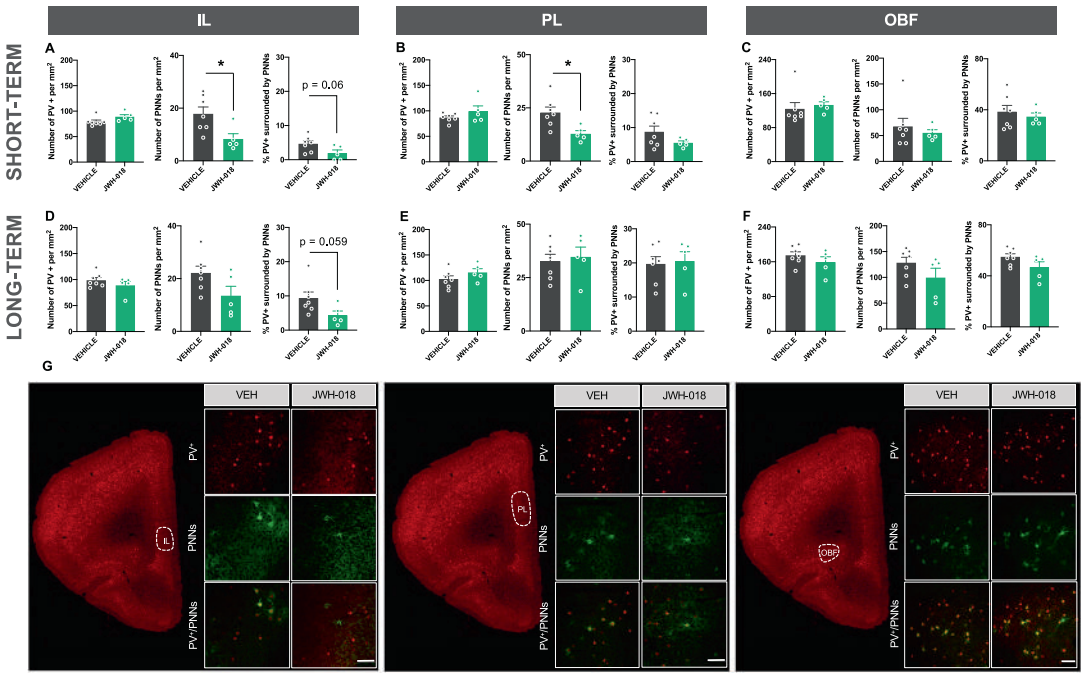

Fig. 4 JWH-018 exposure during adolescence alters perineuronal nets density in cortical subregions in male mice. A–F Number of PV+, PNNs and PV+ surrounded by PNNs in the IL (**A**, **D**), PL (**B**, **E**) and OBF (**C**, **F**) in male mice exposed to JWH-018 during adolescence at short- (**A**–**C**) and long-term (**D**–**F**). Tissue was obtained 24 h after the prepulse inhibition test ($n = 5$–7 mice per group). **G** Representative images of each cortical subregion obtained by fluorescence microscopy labelling PV+ (red) and PNNs (green) of short-term experiments. Scale bar represents 100 µm. Data are expressed as mean ± SEM. *$p < 0.05$ (comparison between JWH-018 and vehicle; Student's t-test). IL infralimbic prefrontal cortex, PL prelimbic prefrontal cortex, OBF orbitofrontal cortex, PV+ positive parvalbumin neuron, PNNs perineuronal nets.

exposure directly on adult animals (Fig. 3A). Possible changes in sensorimotor gating were assessed 20 days following the last day of JWH-018 administration (Fig. 3A), as previously studied after treatment during the adolescent period. Notably, no differences in PPI were observed between vehicle and JWH-018 groups in male mice (Fig. 3B–D), indicating that adolescence represents a sensitive window for the harmful consequences of JWH-018 exposure. Moreover, JWH-018 administration in adult females did not modify PPI (Fig. 3E–G), in agreement with the lack of effect previously observed in adolescent female mice treated with this synthetic cannabinoid.

Short- and long-term consequences on the density of cortical parvalbumin-expressing interneurons and perineuronal nets in adolescent mice exposed to JWH-018

The prefrontal cortex is a brain area directly related to the modulation of sensorimotor gating [31]. Moreover, studies on both patients and animal models of schizophrenia have found alterations in the subpopulation of cortical GABAergic interneurons expressing PV [32, 33]. During development the connectivity and maturation of these interneurons are regulated by the presence of PNNs, specialized regions of the extracellular matrix, which are frequently surrounding PV-expressing neurons [34]. Interestingly, some studies have revealed that patients with schizophrenia show a reduced density of PNNs in the prefrontal cortex [35, 36]. We next studied possible alterations in the density of cortical interneurons expressing PV and PNNs in adolescent male mice exposed to JWH-018, given the deficits previously observed in PPI of the startle reflex in these animals. Notably, the density of PNNs significantly decreased in the IL (Fig. 4A, G) and PL (Fig. 4B, G) ($p < 0.05$), but not in the OBF (Fig. 4C, G), in male mice 5 days after the end of the treatment with JWH-018. These changes were reversible as no long-term differences were found between groups (Fig. 4D–F). In agreement, the density of PNNs was similar in adult females exposed to JWH-018 during adolescence (Supplementary Fig. 3). The number of neurons expressing PV was not altered by JWH-018 exposure in both males (Fig. 4A–G) and females (Supplementary Fig. 3) in any of the brain regions of the prefrontal cortex analyzed. We also found an almost significant decrease in the percentage of PV neurons surrounded by PNNs in the IL of male, but not in female (Supplementary Fig. 3), mice in both the short- ($p = 0.06$) and long-term ($p = 0.059$) (Fig. 4A, D, G).

Short- and long-term consequences on the microglia morphology and GFAP immunoreactivity in astrocytes in adolescent mice exposed to JWH-018

Microglia are a key defense mechanism within the brain, but their activation can result in damage to PNNs through either the release of proteolytic enzymes such as matrix metalloproteinases [37] or directly by stripping PNNs from the neuronal surface [38]. In addition, astrocytes release an array of diverse matrix-remodeling proteases and their inhibitors to tightly control the structural integrity of PNNs [37]. Interestingly, an enhancement of the area of the microglia soma was observed in the prefrontal cortex of male mice exposed to JWH-018 during the adolescent period in both short ($p < 0.01$) (Fig. 5A, G) and long-term ($p < 0.05$) (Fig. 5D, G). Accordingly, the perimeter of microglia soma showed a strong trend to increase in the short term ($p = 0.06$) (Fig. 5B, G) while this enhancement was significant in the long-term

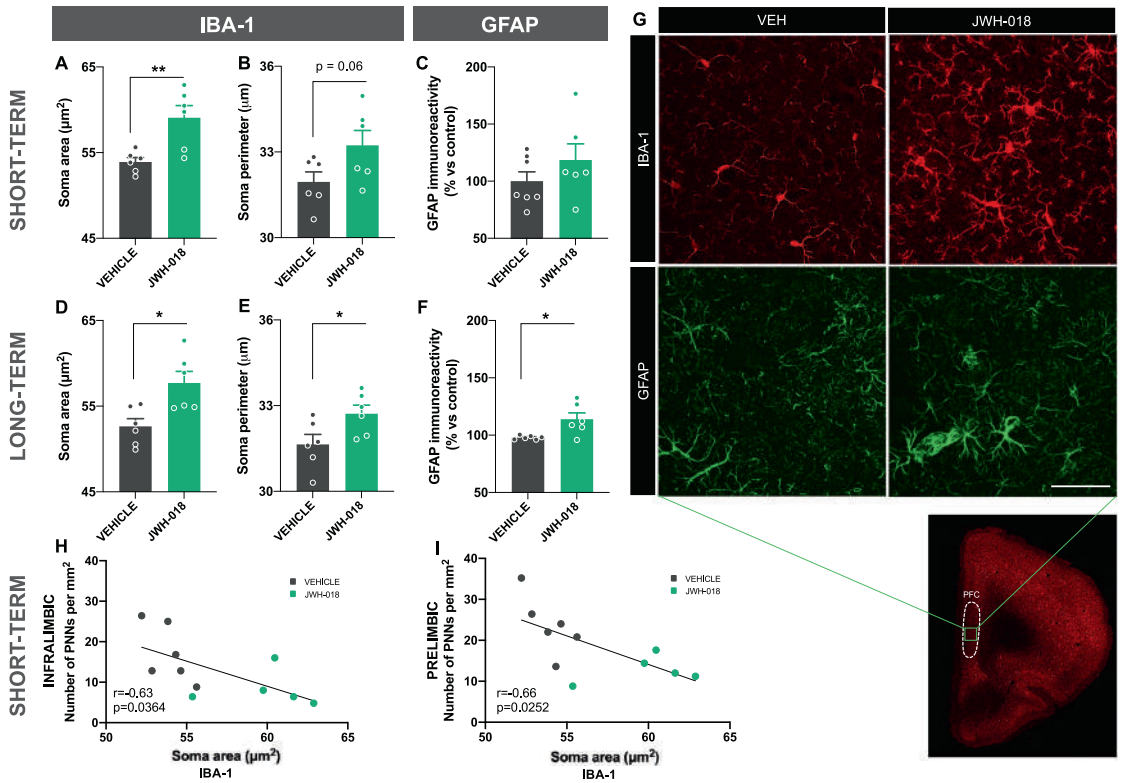

Fig. 5 JWH-018 exposure during adolescence induces microglia activation in the prefrontal cortex in male mice. A, **B**, **D**, **E** Area and perimeter of microglia soma of Iba-1-stained cells, and **C**, **F** GFAP immunoreactivity in the prefrontal cortex in male mice exposed to JWH-018 during adolescence at short- (**A**–**C**) and long-term (**D**–**F**). Tissue was obtained 24 h after the prepulse inhibition test ($n = 6$ mice per group). **G** Representative images of adult males obtained by fluorescence microscopy labelling Iba-1 (red) and GFAP (green). Scale bar represents 50 µm. **H**, **I** Correlations between the soma area of Iba-1-stained cells and the number of PNNs in the IL (**H**) and PL (**I**) 24 h after the prepulse inhibition test in male mice at short-term (Pearson's correlation coefficient). Data are expressed as mean ± SEM. $*p < 0.05$; $**p < 0.01$ (comparison between JWH-018 and vehicle; Student's t-test). Iba-1 ionized calcium-binding adapter molecule 1, GFAP glial fibrillary acidic protein, IL infralimbic prefrontal cortex, PL prelimbic prefrontal cortex, PNNs perineuronal nets.

($p < 0.05$) (Fig. 5E, G) in these animals. No changes were observed in the total number of Iba-1 positive cells (Supplementary Fig. 4). These results indicate that JWH-018 treatment induces a microglia morphology shift to a reactive state characterized by larger amoeboid soma [39]. However, no alterations were revealed in both area and perimeter of the microglia soma in females (Supplementary Fig. 3). A significant negative correlation between microglial activation (soma area) and PNNs density in the IL ($p < 0.05$) (Fig. 5H) and PL ($p < 0.05$) (Fig. 5I) was found in the short-term in male mice exposed to JWH-018. All together, these results suggest that exposure to this synthetic cannabinoid in adolescent male mice involves changes in microglial reactivity in the prefrontal cortex which are associated with a decrease of the density of PNNs. These histopathological alterations could contribute to the PPI deficits present in these mice. Indeed, a significant correlation between the percentage of PPI, when representing the prepulses of 69 and 73 dB, and the density of PNNs was found in the IL in the short- and the long-term ($p < 0.05$) (Supplementary Fig. 5) in male mice.

JWH-018 exposure in male, but not in female (Supplementary Fig. 3), mice enhanced GFAP immunoreactivity in the prefrontal cortex in the long-term ($p < 0.05$) (Fig. 5F, G). No modifications were observed in the short-term in these mice (Fig. 5C).

Short- and long-term consequences on the expression of GAD67, SYN, PSD95, BDNF, CB1R and CB2R in adolescent mice exposed to JWH-018

We finally explored whether the expression of molecules related to inhibitory neurotransmission and plasticity could be altered in the short- and long-term in male mice exposed to the synthetic cannabinoid JWH-018 during the adolescence. Possible changes in the expression of CB1R and CB2R were also investigated. A significant decrease was found in the mRNA levels of GAD67 ($p < 0.01$) and CB2R ($p < 0.05$) in the prefrontal cortex in the short-term (Fig. 6A). These alterations were specific to the early period after JWH-018 treatment (Fig. 6B). No differences in the expression of SYN, PSD95, BDNF and CB1R were observed in the short- and the long-term (Fig. 6A, B) in these mice.

DISCUSSION

Our data show that exposure to the Spice/K2 cannabinoid JWH-018 in adolescent mice induces long-term behavioral consequences which were sex-dependent. Notably, JWH-018 treatment during adolescence, but not in adulthood, triggered impairments of PPI of the startle reflex in young and adult male mice. These alterations were associated with changes in the

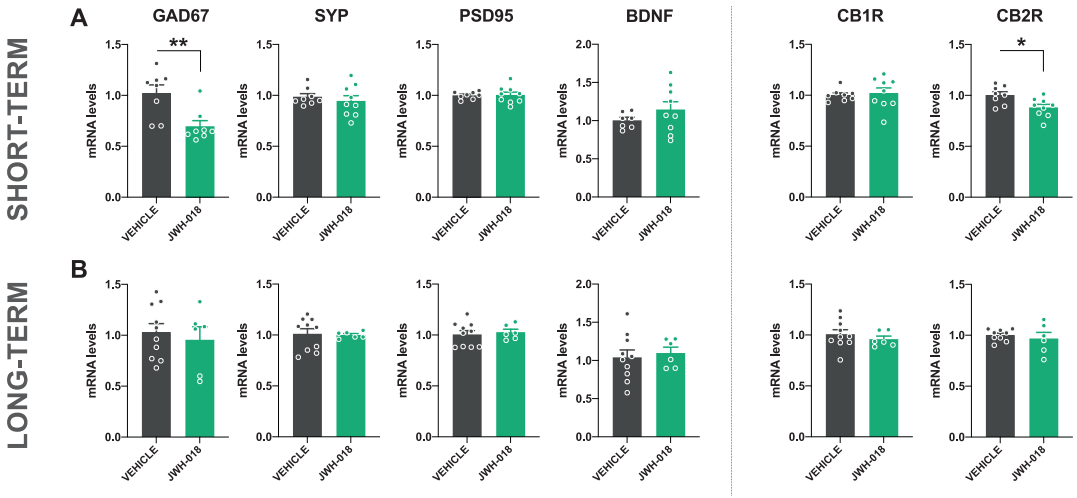

Fig. 6 JWH-018 exposure during adolescence induces a decrease in the expression of GAD67 and CB2R in the prefrontal cortex in male mice. A, **B** mRNA levels of GAD67, SYP, PSD95, BDNF, CB1R and CB2R in the prefrontal cortex in male mice exposed to JWH-018 during adolescence at short- **A** and long-term **B**. Tissue was obtained 24 h after the prepulse inhibition test ($n = 6$–10 mice per group). Data are expressed as mean ± SEM. $*p < 0.05$; $**p < 0.01$ (comparison between JWH-018 and vehicle; Mann-Whitney test for GAD67 expression, and Student's t-test for CB2R expression). GAD67 glutamic acid decarboxylase 67, SYP synaptophysin, PSD95 postsynaptic density protein 95, BDNF brain derived neurotrophic factor, CB1R cannabinoid receptor type 1, CB2R cannabinoid receptor type 2.

density of PNNs and microglia morphology in the prefrontal cortex.

Adverse outcomes associated with the consumption of SCBs are distinct from, and markedly more toxic than, those produced by marijuana [3]. Anxiety, panic attacks, and psychotic symptoms are among the most common psychiatric detrimental effects reported from several clinical case studies [3]. However, there is little information about the behavioral and neurochemical consequences of adolescent exposure to these drugs. In the present study, we reported that administration of JWH-018 during adolescence modified anxiety-like behavior in a sex-specific manner. An anxiogenic phenotype was revealed in male, but not in female, mice five days after the JWH-018 administration. In contrast, an anxiogenic trend were observed in females, but not in males, when the animals reached the adulthood. By using the EPM, most of the studies have not shown differences in anxiety in adult male [24, 40, 41] or female [40, 41] rodents due to adolescent Δ^9-THC exposure, although an anxiogenic-like effect was observed in adult male mice in another report [42]. A recent study revealed an increase of repetitive/compulsive behaviors at adulthood in adolescent male mice exposed to JWH-018, as shown in the nestlet shredding and marble burying tests [43]. Although marble burying test can be used to assess anxiety-like behavior, the heightened marble burying activity of JWH-018 treated male mice could reflect compulsive-like rather than anxiety-like states considering that in a previous study adult male rats showed increased burying scores, but not anxiety alterations in the EPM test, 7 days after JWH-018 discontinuation [16]. Moreover, adolescent exposure to the recently detected synthetic cannabinoid 5F-MDMB-PICA displayed an anxiety-like and compulsive-like state in adult male mice [44]. On the other hand, anxiety disorders characterized by pathological fear, such as post-traumatic stress disorder and phobias, are associated with extinction deficits of aversive memories. Under our experimental conditions, cued fear extinction was not affected in either male or female mice by adolescent exposure to JWH-018. Consistent with this result, the administration of Δ^9-THC during adolescence did not alter fear extinction in male and female adult mice [24], although impairment of this response was revealed when THC was combined with stress exposure [24].

A critical finding of our study was that adolescent JWH-018 exposure induces a reduction of PPI of the startle reflex at short- and long-term in male, but not in female, mice. Notably, the effect of JWH-018 was age-dependent as revealed by the lack of alterations in the PPI test when the same drug administration regimen was performed directly in adulthood. PPI is a classic preclinical model of sensorimotor gating, with high translational validity between humans and rodents [45], that measures the ability to filter out insignificant sensory information, a cognitive abnormality also seen in schizophrenia [46]. Previous studies evaluating the effects of Δ^9-THC during adolescence on PPI in rodents have yielded mixed results. Thus, Δ^9-THC administration induced persistent PPI deficits in adult male rats [47, 48] while an increase in PPI was observed in a recent report [49] in male mice. The consequences of adolescent exposure to JWH-018 on PPI in adulthood were not previously assessed although, in agreement with our study, chronic administration of this synthetic cannabinoid in adult male rats did not induce changes in sensorimotor gating 1 or 7 days after the end of the pharmacological treatment [16]. Interestingly, we found that the effects of adolescent exposure to JWH-018 on PPI responses were modulated by sex, being males more vulnerable than females. Although sex differences are a known and important part of mental illness, they are often overlooked in animal models. Preclinical and clinical studies show differences between sexes in the therapeutic potential and abuse liability of cannabis and cannabinoids [50]. Moreover, sex has been described as a major factor modulating pharmacokinetic, behavioral, and brain activity effects of Δ^9-THC in adolescent rats [51]. On the other hand, sex differences in CB1R expression have been found between male and female rodents throughout the brain. In this sense, CB1R protein or mRNA was found to be greater in male versus female rats in the prefrontal cortex [52]. In any case, future experiments will be necessary to determine the factors related to this sex-dependent effect including puberty timing, different

biochemical responses, tolerance from repeated injections, and hormonal status (i.e., estrous cycle phase) [51].

PNNs, condensed aggregates of the extracellular matrix, are important players in the regulation of the maturation and plasticity of interneurons, especially fast-spiking GABAergic interneurons expressing PV [53]. Although the functions of PNNs are not clear yet, they have been involved in the modulation of different cognitive processes and could be considered as a future potential therapeutic target for the treatment of diseases characterized by maladaptive memories [54]. Interestingly, PNNs in the prefrontal cortex are dysregulated in patients with schizophrenia [34, 35], and in animal models that mimic this disorder [55, 56]. Indeed, a link between these specialized regions of the extracellular matrix and the presence of psychosis has been suggested [34]. We observed a reduction in the density of PNNs in the IL and the PL at short-term due to the adolescent exposure to JWH-018 in male mice. Moreover, the percentage of PV neurons surrounded by PNNs in the IL tended to decrease even in the long-term. These alterations could be related to the PPI disruptions present in these animals since a correlation between the density of PNNs and the percentage of PPI was found in the IL. In contrast to the effects of JWH-018, Δ^9-THC administration during the adolescence did not modify the density of PNNs in the prefrontal cortex in adult male mice [49]. This discrepancy could be due to the different time interval between treatment and testing (shorter in our study), and the higher potency of JWH-018 compared to THC.

Glial cells are among the major extrinsic factors that facilitate the remodeling of PNNs, thereby acting as key regulators of their diverse functions in health and disease [37, 57]. PNNs abundance is dramatically upregulated throughout the healthy adult brain following microglial depletion in mice [58]. Interestingly, PNNs deficits/decreases have been observed across diverse disorders, generally associated with microglial activation [57]. In addition, reactive astrocytes also play a role in PNN disruption after brain injury and trauma [37]. Notably, adolescent JWH-018 exposure induced an activation of microglia and astrocytes in the prefrontal cortex of adult male mice. In agreement, a recent study showed a long-term microglia activation in several brain areas such as the nucleus accumbens and caudate-putamen following adolescent self-administration of JWH-018 [43]. Taken together, it is tempting to hypothesize that exposure to JWH-018 to adolescent mice induces changes in glial cells reactivity and PNNs density in the prefrontal cortex, leading to the appearance of sensorimotor gating deficits in adulthood.

Several studies have shown reductions in the expression of GAD67 in the prefrontal cortex of schizophrenia patients [59, 60], which contribute to dysfunction of this brain region. Simultaneous reductions in the expression of GABAergic markers in the prefrontal cortex and in PPI have been also reported [61]. Notably, adolescent exposure to JWH-018 reduced the expression of GAD67 in the prefrontal cortex in the short term, although normal levels were found in adult male mice. In this sense, lower levels of GAD67 in the prefrontal cortex were shown after Δ^9-THC administration during adolescence, which resulted in a psychotic-like phenotype in adult female rats [62]. Furthermore, a transitory decrease in the expression of CB2R, but not CB1R, was observed due to adolescent JWH-018 treatment. Several studies present evidence of the contribution of CB2R to the modulation of sensorimotor gating. Interestingly, an increased expression of CB2R in the prefrontal cortex has been recently related to enhanced PPI of the startle reflex in male 129S1/SvImJ mice [63]. Accordingly, CB2R knockout mice showed disrupted PPI at different prepulse intensities [64], while MK801-induced decrease in PPI was attenuated by the CB2R agonists JWH015 [65] and HU-910 [66]. On the other hand, 5-HT_{2c} receptors could also be involved in the PPI deficits caused by adolescent JWH-018 exposure. Indeed, the activation of this serotonin receptor has been previously shown to reverse PPI deficits induced by apomorphine in rats [67], and by the NMDA antagonist MK801 in mice [68]. Moreover, we observed a decrease in the mRNA levels of 5-HT_{2c} receptor in the prefrontal cortex of adolescent male mice exposed to JWH-018 in the short-term (data not shown), a mechanism that will be worth exploring in the future.

In summary, our data show important lasting behavioral and neurobiological changes associated with JWH-018 treatment during adolescence. This study has profound clinical and public health policy implications in terms of limiting adolescents to cannabinoid synthetic compounds exposure that may be particularly neurotoxic during certain neurodevelopmental windows.

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AUTHOR CONTRIBUTIONS

CI-L, MT-B, MP-R and IP-P performed behavioral and molecular experiments. RP analyzed images. FB and IP-P conceived the idea. FB funded this project. CI-L and FB wrote the manuscript. All co-authors edited and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests or personal relationships with other people or organizations that could inappropriately influence their work in this paper.

ADDITIONAL INFORMATION

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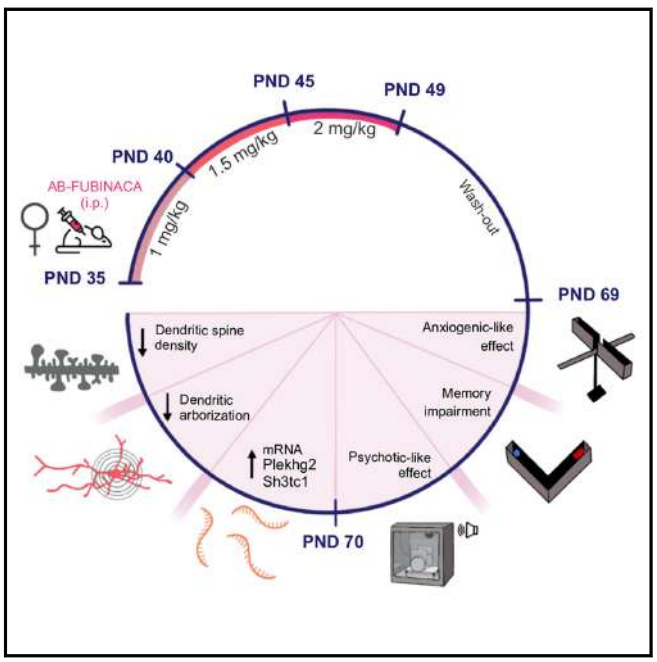
Article

Long-term consequences of adolescent exposure to the synthetic cannabinoid AB-FUBINACA in male and female mice

Graphical abstract



Highlights

- Adolescents are more vulnerable to developing psychiatric disorders after SCB use
- AB-FUBINACA adolescent exposure alters memory and causes anxiety in adult female mice
- AB-FUBINACA adolescent exposure induces psychotic-like effects in adult female mice
- AB-FUBINACA adolescent exposure causes dendritic spine loss in female mice's PFC

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In brief

Natural sciences; Biological sciences; Neuroscience; Behavioral neuroscience

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Long-term consequences of adolescent exposure to the synthetic cannabinoid AB-FUBINACA in male and female mice

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SUMMARY

The consumption of synthetic cannabinoids during adolescence is reported to be a risk factor for the appearance of psychiatric disorders later in life. AB-FUBINACA is a member of the indazole carboxamide family of synthetic cannabinoids present in Spice/K2 preparations. The present study sought to investigate the long-term effects of AB-FUBINACA consumption during adolescence in both male and female mice. AB-FUBINACA revealed several sex-dependent behavioral alterations. In this sense, the administration of this synthetic cannabinoid in female, but not male, mice induced psychotic-like symptoms which were associated with changes in dendritic arborization and density of mature dendritic spines in pyramidal neurons of the prefrontal cortex, as well as with an up-regulation of differentially expressed genes in this brain area. This study helps to clarify the potential late detrimental effects of this potent synthetic cannabinoid that may derive from its use during adolescence.

INTRODUCTION

Synthetic cannabinoids (SCBs) are emerging drugs of abuse detected in herbal incense products and sold under names such as K2 (in North America) or Spice (in Europe), primarily through the internet.[1] Compared to Δ^9-tetrahydrocannabinol (Δ^9-THC), the principal psychoactive ingredient of cannabis, SCBs are potent full agonists of brain cannabinoid receptors, leading to more potent effects than those produced by Δ^9-THC.[2] Indeed, the consumption of SCBs has been increasingly associated with severe intoxications and even deaths, thus representing a global public health problem.[3] Adolescents and young adults show the highest rate of SCB use[4] which is of particular concern because adolescence is a critical period in brain development and maturation.[5] Despite the dramatic increase of SCB use among young adults[6,7] their impact on brain function and behavior in adulthood represents an important unanswered health question.

SCBs can be categorized into distinct groups and subgroups depending on their chemical structure. In 2010, naphthoylindoles, such as JWH-018 and JWH-073, and cyclohexylphenols, such as CP-47,497, were the primary SCBs found in seized Spice/K2 products.[8] Research into the mechanisms of SCBs-related toxicity has mostly focused on these naphthoylindoles (e.g., JWH series) while the toxicological profiles and impact on the behavior of other SCBs classes (e.g., indole- and indazole-based SCBs) have been hardly studied.[2]

AB-FUBINACA is a member of the indazole carboxamide family of SCBs which includes other compounds that have been linked to deaths in the United States such as AB-CHMINACA or AB-PINACA.[9] AB-FUBINACA presents potent affinity for the CB1 cannabinoid receptor (CB1R) (Ki = 0.9 nM).[10] In animal models, AB-FUBINACA reproduced the typical "tetrad" effects of Δ^9-THC which are hypothermia, analgesia, hypolocomotion, and catalepsy.[11] Regarding anxiety, the acute administration of AB-FUBINACA in adult mice induced anxiolytic- or anxiogenic-like effects depending on the dose.[12] This SCB has also been shown to produce physical dependence[13] and impair recognition memory[14] after chronic administration in mice. However, lasting neurobiological consequences induced by adolescent exposure to AB-FUBINACA remain poorly understood.

In this study, we investigated the long-term consequences of adolescent exposure to AB-FUBINACA on key neurobehavioral responses associated with SCB toxicity in humans. Anxiety, fear extinction, recognition memory, sociability, depression, and sensorimotor gating were evaluated in male and female mice after treatment with AB-FUBINACA during adolescence. Given the behavioral results observed, possible structural plasticity alterations and RNA-Seq profile were analyzed in the prefrontal cortex of female mice exposed to this SCB.

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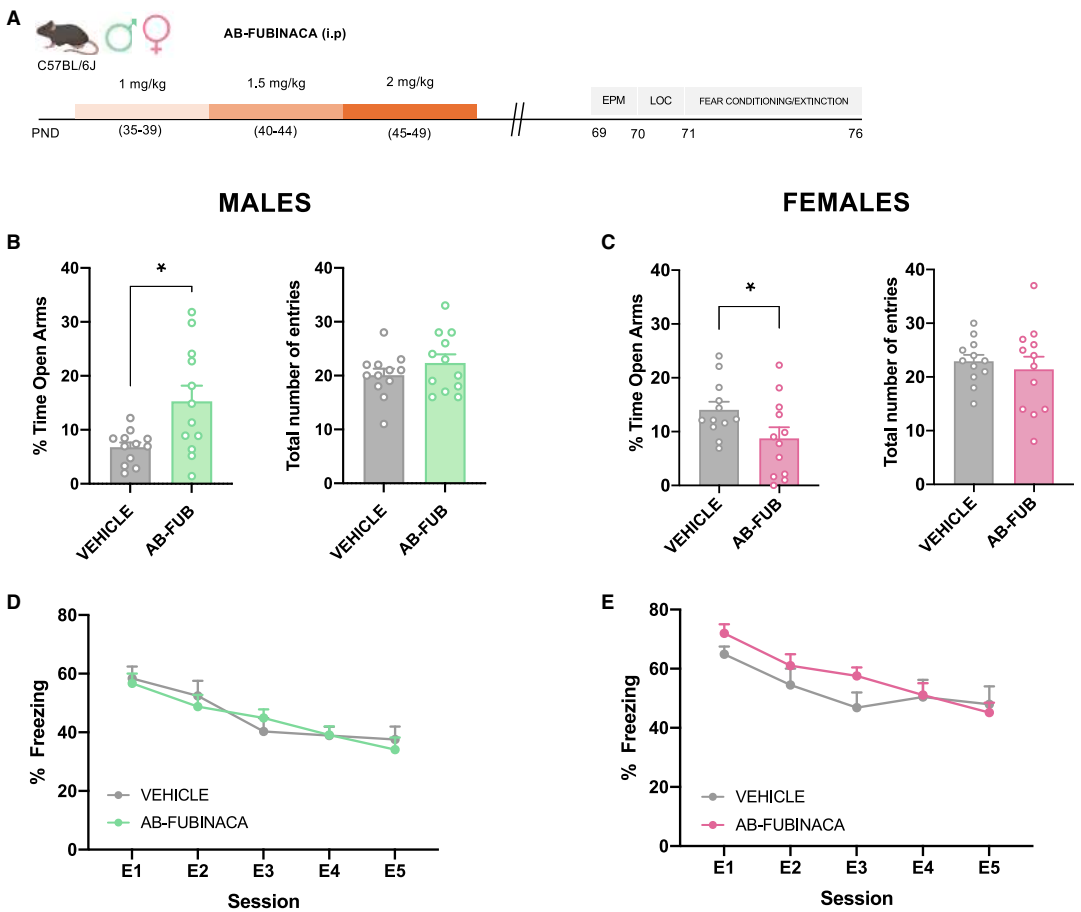

Figure 1. AB-FUBINACA treatment during adolescence alters anxiety-like behavior in a sex-dependent manner

(A) Schematic representation of experimental design.

(B–E) Effects of adolescent exposure to AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) or vehicle in anxiety-like behavior in the EPM (B, C) and fear extinction (D, E) in male (B, D) and female (C, E) mice ($n = 12$ mice per group). Percentage of time spent in the open arm and total number of entries are shown for the EPM. Time course of the freezing levels scored during cued fear extinction trials is shown for fear memory processing. Data are expressed as mean ± SEM. *$p < 0.05$ (comparison between AB-FUBINACA and vehicle; Student's t test). PND postnatal day, EPM elevated plus maze, LOC locomotion, E1-E5 extinction trials.

RESULTS

Long-term consequences in adolescent mice exposed to AB-FUBINACA on the extinction of fear and anxiety

Adolescent male and female mice were treated with increasing doses of AB-FUBINACA for 15 days (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) (Figure 1A). Body weight was daily evaluated along with AB-FUBINACA treatment. The weight gain of mice treated with AB-FUBINACA was lower than those exposed to the vehicle in both sexes (Figure S1) (interaction day x treatment: $F_{14,1162} = 10.79$, $p < 0.0001$ and $F_{14,1372} = 4.99$, $p < 0.0001$, for male and female mice, respectively), in agreement with previous reports evaluating effects of adolescent cannabinoid exposure. Locomotor activity, anxiety-like behavior, and fear memory processing were analyzed 20 days after the finishing of the AB-FUBINACA treatment (Figure 1A). No changes in locomotion were observed in either males or females (Figure S2). By using the elevated plus maze (EPM), AB-FUBINACA induced an opposite effect on anxiety depending on the sex. Anxiolytic- or anxiogenic-like effects ($p < 0.05$) were observed, respectively, in male and female mice, without changes in the total number of entries (Figures 1B and 1C). Aversive memory processing was evaluated by a cued fear conditioning and extinction paradigm. The administration

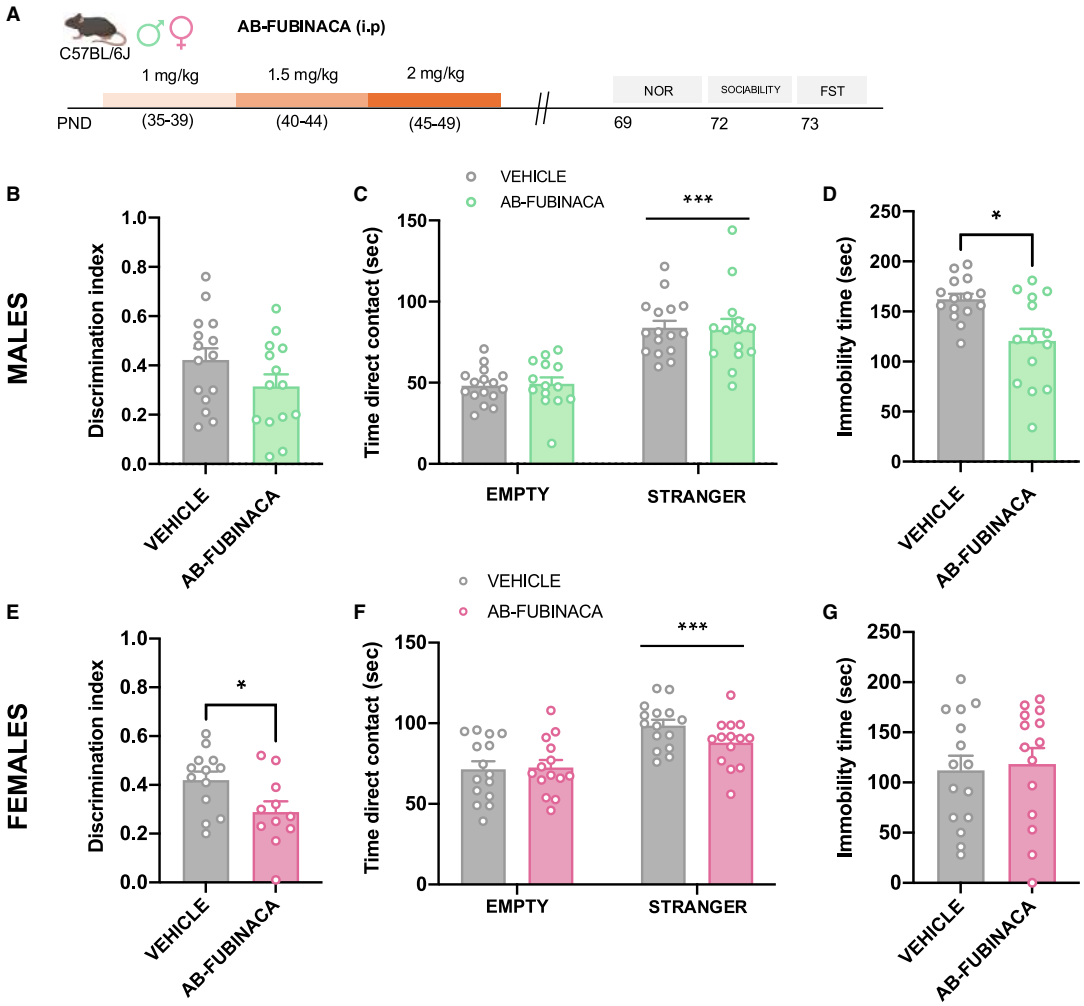

Figure 2. AB-FUBINACA treatment during adolescence alters novel object recognition memory and depressive-like behavior in a sex dependent manner

(A) Schematic representation of experimental design.

(B–G) Effects of adolescent exposure to AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) or vehicle in memory in the NOR (B, E), sociability in the three chamber test (C, F), and depressive-like behavior in the FST (D, G) in male (B, C, D) and female (E, F, G) mice (n = 11–15 mice per group). The discrimination index is shown for the NOR, total time in direct contact with each compartment is shown for sociability test, and immobility time is shown in the FST. Data are expressed as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ (comparison between AB-FUBINACA and vehicle group; two-way ANOVA, compartment (C, F); Student's t test (D, E). PND postnatal day, NOR novel object recognition, FST forced swimming test.

of AB-FUBINACA did not alter fear extinction in both males (Figure 1D) and females (Figure 1E). However, fear conditioning was higher in females (interaction cue x treatment: $F_{2,40} = 4.91$, $p < 0.05$) treated with AB-FUBINACA in comparison with controls, while no differences were observed in the case of male mice (Figure S3). These results suggest the existence of sex-specific effects in anxiety due to AB-FUBINACA exposure during adolescence.

Long-term consequences in adolescent mice exposed to AB-FUBINACA on non-emotional memory, sociability, and depression

To study possible durable alterations in non-emotional memory, sociability, and depression, mice were exposed to a similar protocol of AB-FUBINACA administration (Figure 2A). By using the novel object recognition test (NOR), memory was not affected in male mice as showed the lack of changes

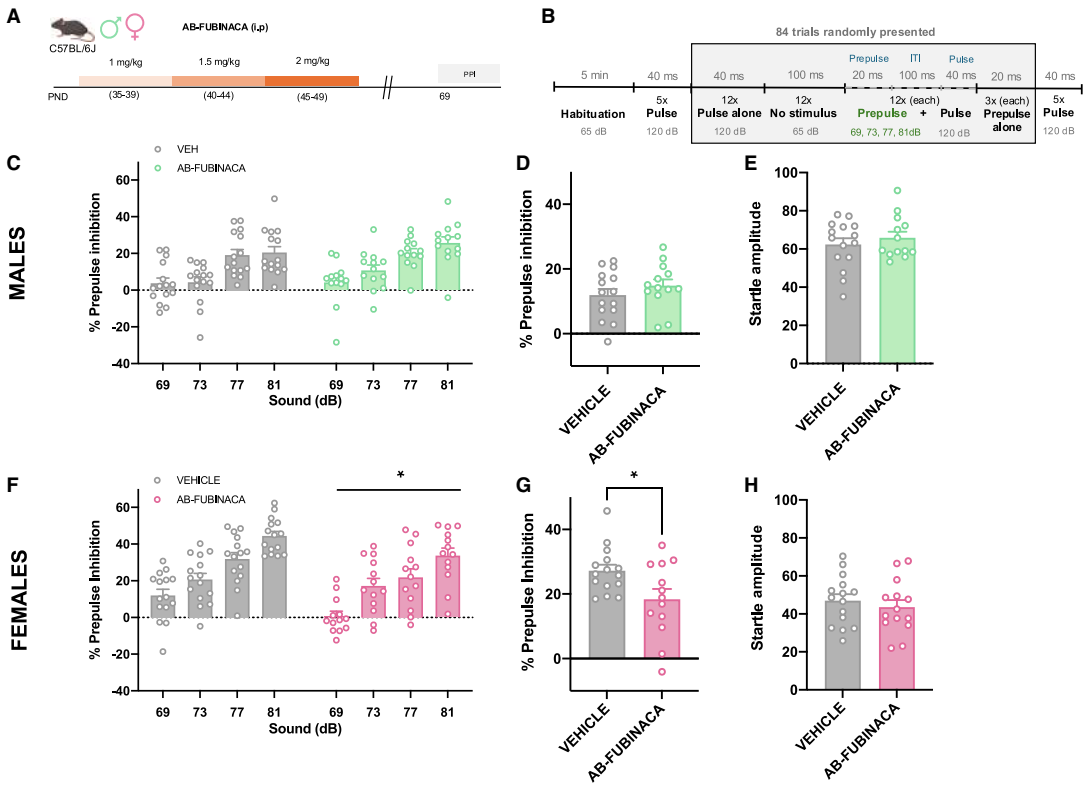

Figure 3. AB-FUBINACA treatment during adolescence alters sensorimotor gating in a sex-dependent manner

(A) Schematic representation of experimental design.

(B) Graphic diagram of the PPI protocol.

(C–H) Effects of adolescent exposure to AB-FUBINACA (PND 35–39: 1 mg/kg, PND 40–44: 1.5 mg/kg, and PND 45–49: 2 mg/kg) or vehicle in sensorimotor gating in male (C, D, E) and female (F, G, H) mice (n = 13–15 mice per group). Percentage of prepulse inhibition (C, F), mean of the percentage of prepulse inhibition (D, G), and startle response amplitude (E, H) are shown. Data are expressed as mean ± SEM. *$p < 0.05$ (comparison between AB-FUBINACA and vehicle group; two-way ANOVA with repeated measures, treatment (F); Student's t test (G). PND postnatal day, PPI prepulse inhibition test, dB decibels, ITI inter-trial interval.

in the discrimination index between control and treated animals (Figure 2B). Interestingly, the discrimination index was lower in females exposed to AB-FUBINACA during the adolescent period in comparison with controls ($p < 0.05$) (Figure 2E), indicating the existence of memory deficits in these animals. Total exploration time was not altered in any of the different experimental groups (Figure S4). On the other hand, social behavior was not affected in either males or females as revealed by similar contact times between controls and mice treated with AB-FUBINACA (Figures 2C and 2F). Finally, a decrease in immobility time was observed in adult male mice treated with AB-FUBINACA during adolescence ($p < 0.05$) (Figure 2D) in the forced swimming test (FST), while this difference was not present in the case of female mice (Figure 2G). Therefore, adolescent exposure to AB-FUBINCA induced different effects on memory and depressive-like behavior in adult male and female mice.

Long-term consequences in adolescent mice exposed to AB-FUBINACA on sensorimotor gating

Impairments of prepulse inhibition (PPI) of the startle reflex, a sensorimotor gating process, are observed in patients with schizophrenia and are considered a marker of psychotic-like behavior.[15] We studied possible PPI alterations in both adult male and female mice treated with AB-FUBINACA during the adolescent period (Figure 3A). Details of the experimental protocol of PPI used are presented in Figure 3B. AB-FUBINACA exposure did not modify the PPI of the startle reflex in males (Figures 3C and 3D). The magnitude of the startle reflex was also not altered by AB-FUBINACA injection (Figure 3E) in these mice. Notably, a significant decrease in PPI of the startle reflex was revealed in female mice (treatment effect: $F_{1,26} = 6.04$, $p < 0.05$) (Figure 3F). An overall reduction of PPI due to AB-FUBINACA exposure was also observed when representing the mean PPI score ($p < 0.05$) (Figure 3G). This effect was

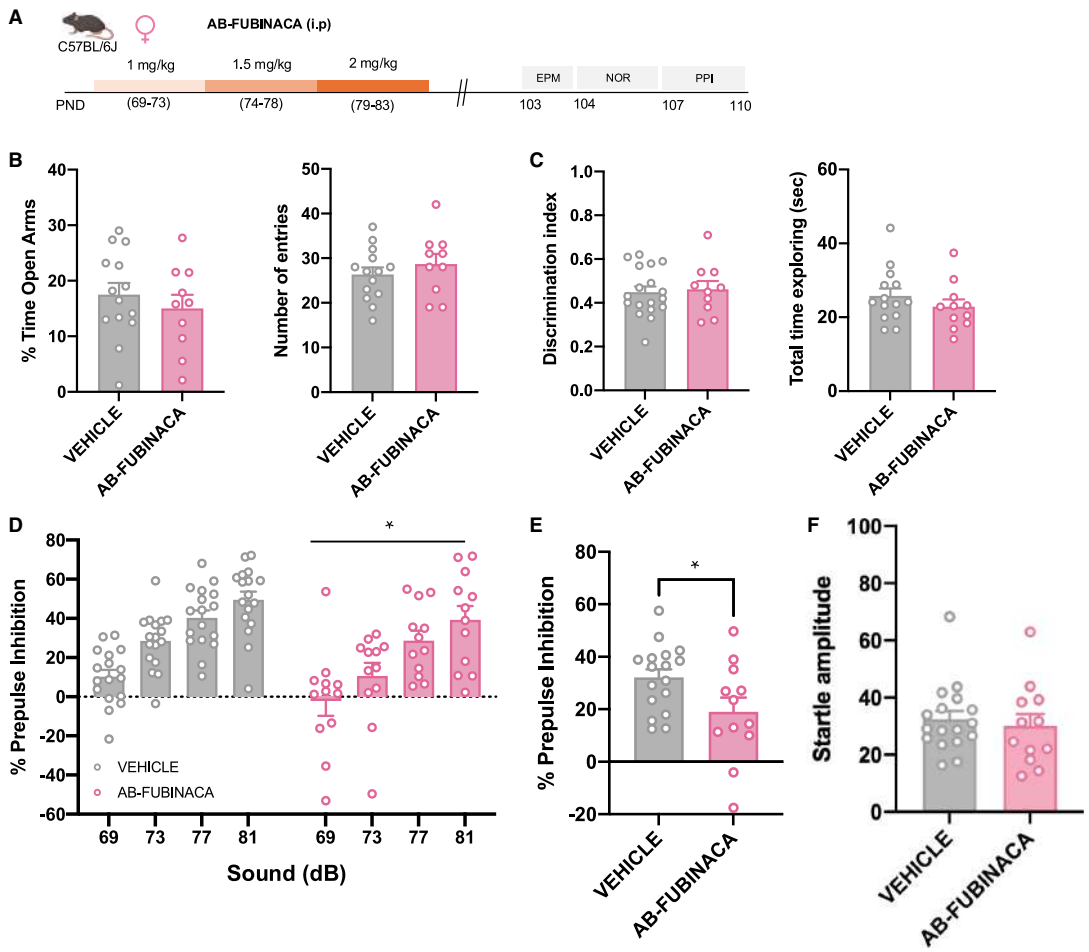

Figure 4. AB-FUBINACA treatment during adulthood alters sensorimotor gating, but neither anxiety-like behavior nor memory, in female mice

(A) Schematic representation of experimental design.

(B–F) Effects of adult exposure to AB-FUBINACA (PND 69–73: 1 mg/kg, PND 74–78: 1.5 mg/kg, and PND 79–83: 2 mg/kg) or vehicle in anxiety-like behavior in the EPM (B), memory in the NOR (C), and sensorimotor gating (D, E, F) in female mice (n = 10–17 mice per group). Percentage of time spent in the open arm and total number of entries are shown for the EPM, the discrimination index and total time of exploration are represented for the NOR. Percentage of prepulse inhibition (D), mean of the percentage of prepulse inhibition (E), and startle response amplitude (F) are also shown. Data are expressed as mean ± SEM. *$p < 0.05$ (comparison between AB-FUBINACA and vehicle group; two-way ANOVA with repeated measures, treatment (D); Student's t test (E). PND postnatal day, EPM elevated plus maze, NOR novel object recognition, PPI prepulse inhibition test, dB decibels.

independent of baseline changes in startle amplitude (Figure 3H), ruling out an impact of startle reaction in the modifications of PPI observed. To evaluate whether the deficits observed in PPI would be preserved in the long term, we chose a time point quite far from the initial measurement (80 days after ending treatment with AB-FUBINACA). However, the deficits in PPI were not maintained at this point (Figure S5). Taken together, these results suggest a sex-dependent alteration in sensorimotor gating due to the adolescent exposure to the SCB AB-FUBINACA.

To elucidate whether an immature brain represents a period of development more susceptible to the effects of AB-FUBINACA, we evaluated the consequences of the treatment with this SCB directly in adult female animals (Figure 4A). Possible changes in anxiety, non-emotional memory, and sensorimotor gating were assessed 20 days following the last injection of AB-FUBINACA (Figure 4A), as previously studied during the adolescent exposure to this drug. Notably, no differences in the EPM (Figure 4B) and NOR (Figure 4C) tests were observed between

vehicle and AB-FUBINACA groups. These results indicate that adolescence represents a sensitive window for the harmful consequences of AB-FUBINACA exposure on anxiety and memory. In contrast, AB-FUBINACA administration in adult females induced a decrease in PPI (Figures 4D and 4E), without changes in the magnitude of startle reflex (Figure 4F), as previously observed in adolescent female mice treated with this SCB. Therefore, the alterations induced by AB-FUBINACA in sensorimotor gating in females were independent of the temporal window of administration and emphasized the detrimental consequences of AB-FUBINACA exposure in the appearance of psychotic-like symptoms.

Transcriptome analysis in adult female mice exposed to AB-FUBINACA during adolescence

The prefrontal cortex is a brain area directly related to the modulation of sensorimotor gating.[16] We next used RNA-Seq to examine the molecular profile of this brain region in female mice exposed to AB-FUBINACA during adolescence (Figure 5A), given the deficits previously observed in PPI of the startle reflex in these animals. Prefrontal cortex samples used for the transcriptome analysis were obtained from the set of animals previously subjected to PPI. RNA-Seq identified two differentially expressed genes (DEGs) (adjusted $p < 0.05$ and cutoff of 2-fold change) which were upregulated in mice injected with AB-FUBINACA (Figure 5B). These two DEGs (*Plekhg2* and *Sh3tc1*) in each vehicle- and AB-FUBINACA-treated mouse were clustered with a heatmap (Figure 5C). Principal component analysis (PCA) is shown in Figure S6. Significant negative correlations between the percentage of PPI and the relative expression of *Plekhg2* ($p < 0.001$) (Figure 5D) and *Sh3tc1* ($p < 0.05$) (Figure 5E) were found in the prefrontal cortex of female mice treated with vehicle or AB-FUBINACA.

Structural plasticity analysis in adult female mice exposed to AB-FUBINACA during adolescence

The plekhg2 gene, which encodes a Rho family-specific guanine nucleotide exchange factor, has been recently associated with dendritic spine morphology formation and corticogenesis.[17] Moreover, Rho family guanosine triphosphatases (GTPases), including Cdc42, are known to regulate various cellular processes, such as morphology, gene transcription, proliferation, and migration through actin cytoskeletal rearrangement. Adolescent AB-FUBINACA exposure did not modify Cdc42 activity in the prefrontal cortex of female adults (Figure S7), suggesting that other Rho GTPases could be regulated by *Plekhg2*. We next analyzed the existence of possible structural plasticity alterations in pyramidal neurons of the prefrontal cortex of adult female mice treated with AB-FUBINACA during adolescence. Sholl analysis showed lower dendritic arborization in AB-FUBINACA-treated mice compared to controls as revealed by mixed-model ANOVA (treatment effect: $F_{1,46} = 6.01$, $p < 0.05$; interaction treatment x radius: $F_{14,370} = 4.68$, $p < 0.0001$) (Figures 6A and 6B). Total length dendrites were shorter in mice exposed to the SCB than in controls ($p < 0.05$) (Figures 6A, 6C, and S8A). This effect was revealed as the branching order increased (Figures S8B–S8D). Moreover, convex hull volume was also lower ($p < 0.01$) in female mice treated with AB-FUBINACA (Figure 6D). PCA identified different groupings of the neuronal populations, by using an unbiased approach (Figure 6E). Total apical (Figures 6F and 6G), but not basal (Figures S8E and S8F), dendritic spine density decreased in female mice exposed to AB-FUBINACA ($p < 0.01$). Moreover, the density of mushroom (mature) spines ($p < 0.05$) was lower in mice treated with the synthetic cannabinoid in comparison with controls (Figure 6H). No changes were observed in long thin, stubby, and branched spines (Figure 6H). As a whole, these results indicate that adolescent AB-FUBINACA exposure in female mice involves changes in dendritic arborization and in the density of mature spines in the prefrontal cortex of adult animals. These alterations were associated with the presence of psychotic-like symptoms, as revealed by the deficits in the PPI test.

DISCUSSION

Our results show important sex-dependent long-term behavioral effects due to adolescent exposure to the SCB AB-FUBINACA, present in Spice/K2 preparations. Notably, treatment with AB-FUBINCA during adolescence induced impairments of PPI of the startle reflex in adult female mice, but not in males. These deficits were also manifested when the cannabinoid was directly administered in adult female animals, suggesting a potent effect of this compound to produce psychotic-like symptoms. The presence of PPI alterations was associated with a decrease in the density of mature dendritic spines and a lower dendritic arborization in the prefrontal cortex of cannabinoid-exposed mice.

The weight gain of mice treated with AB-FUBINACA was lower than controls, as previously reported in the case of other cannabinoids such as Δ^9-THC[18,19] or JWH-018.[20] An anxiogenic-like effect induced by AB-FUBINACA could explain the changes in body weight. Indeed, one week after the finishing of the treatment, a higher level of anxiety-like behavior in adolescent rodents exposed to Δ^9-THC compared to controls was found in the EPM test in previous studies.[18] Nonspecific inhibition of ingestion, secondary to the potential sedative effects of AB-FUBINACA could also be involved in this effect on body weight.

The administration of AB-FUBINACA during adolescence affected anxiety in a sex-dependent manner. Thus, anxiolytic-like effects were observed in adult males, while anxiogenic-like responses were revealed in female adult mice. Consistent with this, fear conditioning was higher in females treated with AB-FUBINACA compared to controls, although no differences in fear memory acquisition were observed in adult female mice exposed to Δ^9-THC during adolescence in a previous study.[21] Controversial data have been shown regarding the effects of adolescent cannabinoid exposure on anxiety. In general, by using the EPM, most of the studies have not shown differences in anxiety in adult male[21–23] or female[22,23] rodents due to adolescent Δ^9-THC exposure, although an anxiogenic-like effect was observed in adult male mice in another study.[24] Regarding the effects of SCBs, male adolescent rats treated with AB-FUBINACA did not show changes in anxiety when reaching adulthood by using the emergence test.[25] However, an anxiogenic trend was observed in adult female, but not male, animals exposed to the SCB JWH-018 during adolescence.[20] On the

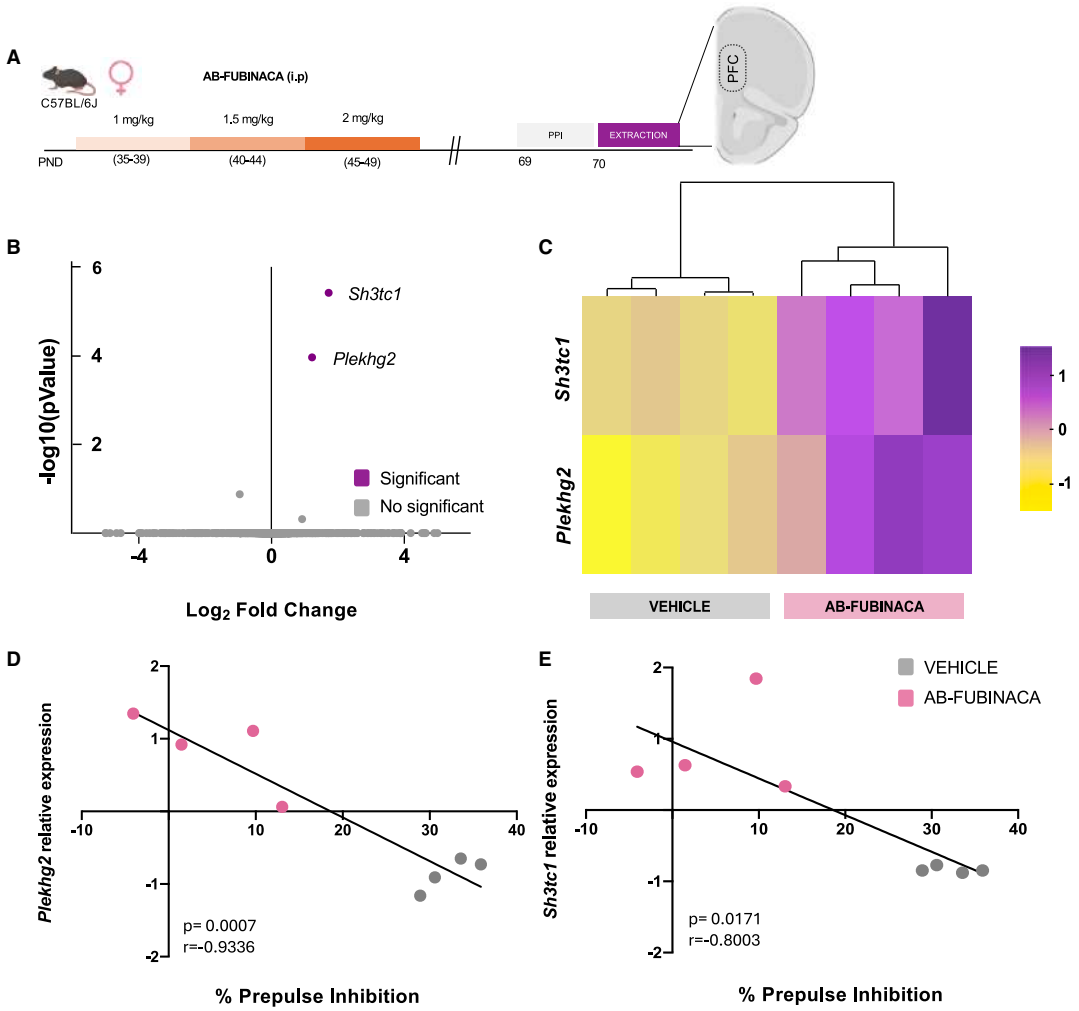

Figure 5. AB-FUBINACA treatment during adolescence induces an up-regulation of PLEKHG2 and SH3TC1 genes in the prefrontal cortex of adult females

(A) Schematic representation of experimental design.

(B) Volcano plot summarizing DEGs of AB-FUBINACA vs. vehicle treated mice (n = 4 mice per group).

(C) DEGs in each AB-FUBINACA and vehicle treated mouse clustered with a heatmap.

(D and E) Correlation between percentage of prepulse inhibition and *Plekhg2* (D) and *Sh3tc1* (E) relative expression (Pearson correlation coefficient). PND postnatal day, PPI prepulse inhibition test, PFC prefrontal cortex, DEGs differentially expressed genes.

other hand, anxiety disorders characterized by pathological fear, such as post-traumatic stress disorder and phobias, are associated with extinction deficits of aversive memories. Under our experimental conditions, cued fear extinction was not affected in either male or female mice by adolescent exposure to AB-FUBINACA. In agreement, the administration of Δ^9-THC or JWH-018 during adolescence did not alter fear extinction in male and female adult mice.[20,21]

The detrimental effects of adolescent exposure to synthetic or natural cannabinoids on novel object recognition are well-known.[26] Interestingly, similar treatments during adulthood did not produce such long-term deleterious effects.[27,28] Accordingly, we observed a memory impairment in adult female, but not male, mice treated with AB-FUBINACA during adolescence, while this effect was not preserved when a similar experimental procedure was carried out in adult animals. However, a recent

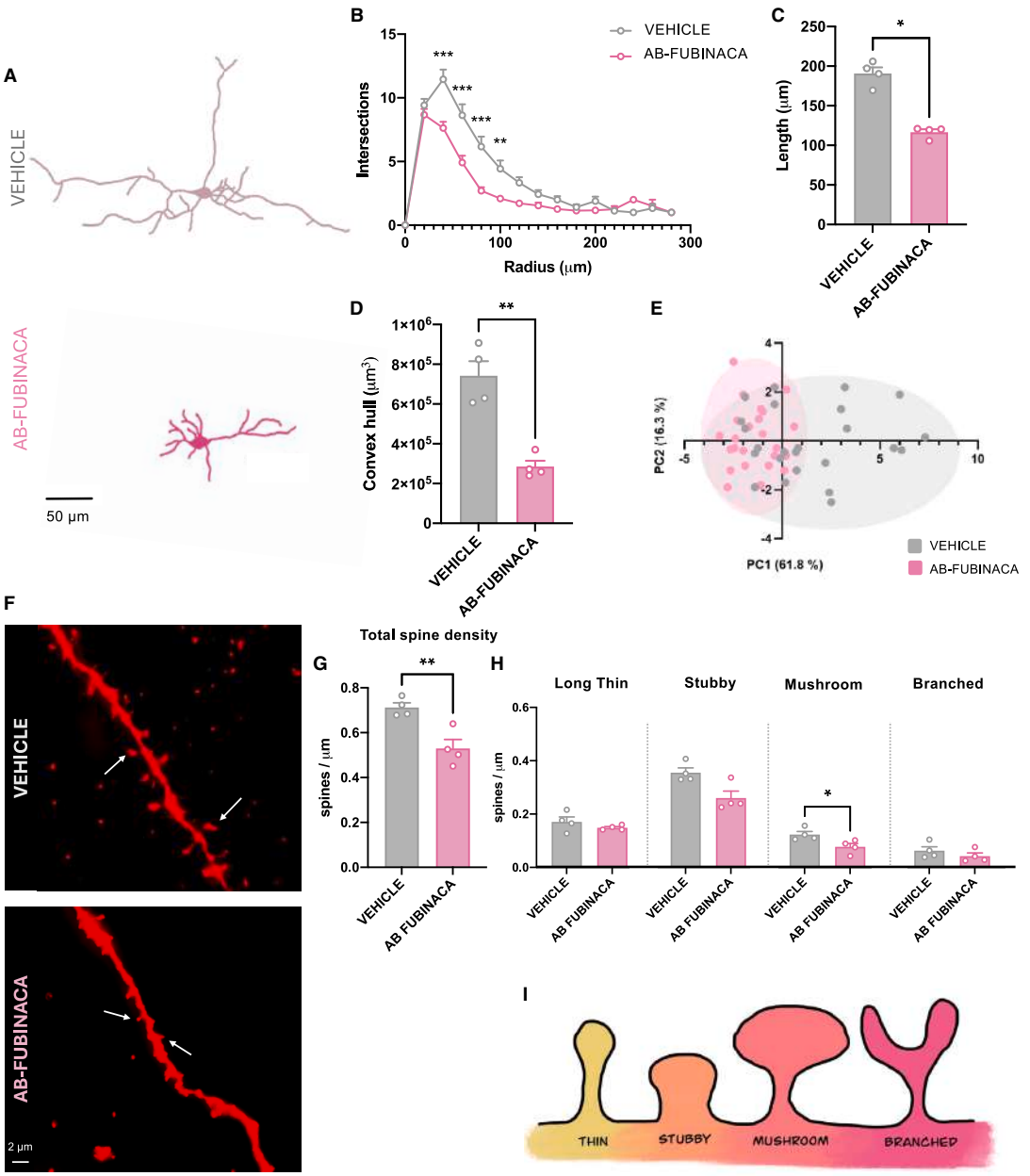

Figure 6. AB-FUBINACA treatment during adolescence induces alterations in dendritic arborization and dendritic spine density in the prefrontal cortex of adult females

Effects of treatment with AB-FUBINCA or vehicle during adolescence in dendritic arborization (A-E) and dendritic spine density (F-I) in the prefrontal cortex of adult female mice.

(A) Representative traces of reconstructed neurons in AB-FUBINACA or vehicle-treated female mice. Scale bar = 50 µm.

(B) Sholl analysis represented by the number of intersections every 20 µm.

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study has shown an impairment of the recognition memory in adult mice exposed to chronic AB-FUBINACA administration,[14] although this effect was observed in the short-term, 24 h after the last drug injection. On the other hand, an antidepressant-like effect was found in male mice exposed to AB-FUBINACA during adolescence, as revealed by the decrease in the immobility time in the FST test. Notably, both preclinical and epidemiological data suggest that females are more vulnerable than males to the deleterious effects of adolescent cannabinoid exposure on mood,[29] although no alteration in depressive-like behavior was observed in female mice in our study.

PPI of the startle reflex is a classic preclinical model of sensorimotor gating assessment which involves a sensory filtering mechanism to prevent sensory information overload.[30] Deficits in PPI have been observed in several psychiatric disorders, particularly in schizophrenia.[31] Adolescent AB-FUBINACA exposure induced a reduction of PPI of the startle reflex in female, but not male, adult mice. Notably, this detrimental effect was conserved when the same drug administration regimen was performed directly in adulthood, suggesting that this SCB has an important potential to produce psychotic-like symptoms. Different results have been described regarding the effects of cannabinoid exposure during adolescence on PPI in rodents. Thus, Δ^9-THC or JWH-018 administration induced persistent PPI deficits in adult male rats[32,33] and mice,[20] respectively. However, an increase in PPI has been also observed in male mice treated with Δ^9-THC during adolescence.[34] On the other hand, the chronic administration of JWH-018 in adult rodents did not induce changes in PPI in male rats[35] while causing sensorimotor gating deficits in male mice.[36] Interestingly, we found that the behavioral alterations induced by adolescent exposure to AB-FUBINACA were modulated by sex, being females more vulnerable than males. Thus, anxiogenic-like responses, memory impairment, and PPI deficits were observed in females, but not in male mice. Despite the well-accepted observation that several neuropsychiatric disorders are sex-related, few articles have dealt with sex vulnerability to adolescent cannabinoid exposure, both at the preclinical and clinical levels. In this sense, animal models seem to suggest that females are more sensitive to the effects of cannabinoids than males in the emotional sphere.[37] Sex has been described as a major factor modulating the pharmacokinetic and brain activity effects of Δ^9-THC in adolescent rats.[38] Future experiments will be required to study the influences of these and other factors (i.e.,:., tolerance from repeated injections, hormonal status), in the sex-dependent effects induced by AB-FUBINACA.

The prefrontal cortex is a brain area directly related to the modulation of sensorimotor gating.[16] By using RNA-Seq, we found that *Sh3tc1* and *Plekhg2* genes were up-regulated in the prefrontal cortex of female adults exposed to AB-FUBINACA during adolescence. *The plekhg2* gene encodes a guanine nucleotide exchange factor promoting GDP/GTP exchange to activate Rho GTPases, including Rac and Cdc42.[39] Abnormalities of the *Plekhg2* gene are involved in postnatal microcephaly and intellectual disability.[40] Specifically, all these patients suffered with profound mental retardation, dystonia, postnatal microcephaly, and a suggestive neuroimaging pattern which consisted of paucity of white matter and dorsal tegmental tracts, and particularly pons, involvement.[40] Interestingly, the *Plekhg2* gene has been recently shown to be essential for axon, dendrite, and synapse development in mouse cortical neurons *in vivo*.[17] The physiological and balanced expression of this gene, but not a defect or an excess of this expression, probably will be important for a correct biological function. Congruent with this, we observed a lower dendritic arborization and a reduction of total length dendrites in prefrontal cortex pyramidal neurons of adult female mice exposed to AB-FUBINACA during the adolescent period. Furthermore, a decrease in the density of total and mushroom-shaped (mature) dendritic spines was also revealed in the same brain region in this group of animals. These observations provide novel evidence of a potential link between prefrontal cortex dysmorphology and PPI alterations, which in turn could contribute to the presence of psychotic-like symptoms due to AB-FUBINACA adolescent exposure. In this sense, adolescent exposure to the SCB CP55,940 in male rats leads to reduced basal dendrite arborization in pyramidal neurons in the prefrontal cortex of adults,[31] suggesting that cannabinoids may impede the structural maturation of neuronal circuits in this brain region, thus inducing impaired cognitive function in adulthood. Reduced dendritic spine density in the hippocampal dentate gyrus was also associated with the impairment of spatial working memory in adult male rats treated with Δ^9-THC during adolescence.[41]

In summary, our findings reveal long-term behavioral alterations associated with chronic adolescent exposure to AB-FUBINACA, a member of the indazole carboxamide family of SCBs. Understanding the detrimental consequences of SCB abuse in the young population is crucial to developing drug-specific treatments for intoxication and effective education and prevention programs.

Limitations of the study

We show important long-term sex-dependent behavioral effects induced by adolescent exposure to AB-FUBINACA. SCBs

(C) Total dendritic length.
(D) Volumetric data of neuron dendrites in μm3.
(E) Plot comparing the total number of reconstructed neurons (n = 6 neurons/mouse and n = 4 mice per group) across principal components PC1 and PC2.
(F) Representative images of apical dendritic spines from AB-FUBINACA and vehicle treated female mice. Scale bar = 2 μm.
(G) Total spine density of apical dendrites in AB-FUBINACA and vehicle treated female mice (n = 4–5 neurons/mouse and n = 4 mice per group).
(H) Spine density grouped according to their morphological characteristics in apical dendrites.
(I) Schematic representation of the morphological classification of the dendritic spines. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (comparison between AB-FUBINACA and vehicle group; mixed-model ANOVA, interaction treatment x radius (B); Mann-Whitney U test (C); Student's t test (D, G, H). PC principal components 1 and 2.

present in Spice/K2 preparations seem to produce more detrimental effects than those produced by Δ^9-THC, although no direct comparison between AB-FUBINACA and Δ^9-THC was carried out in this study.

We used similar doses of AB-FUBINACA in male and female mice. Testing different doses in male and female animals would be of interest to evaluate whether the behavioral differences between sexes observed are qualitative or could be affected by a particular dose range.

We identify AB-FUBINACA as a drug of abuse with a high potential to produce psychotic-like symptoms. AB-FUBINCA is frequently detected in Spice/K2 preparations, but these herbal smoking mixtures can contain diverse bioactive compounds contributing to the detrimental effects associated with their consumption in humans.

We show PPI alterations in adult female mice, but not in males, exposed to AB-FUBINCA during adolescence. Given these results, we studied potential changes in structural plasticity in the prefrontal cortex of female mice. Additional studies are necessary to evaluate possible modifications in dendrite morphology in the prefrontal cortex of male mice treated with this SCB.

RESOURCE AVAILABILITY

Lead contact

Further information should be directed to and will be fulfilled by the lead contact, Fernando Berrendero (fernando.berrendero@ufv.es).

Materials availability

This study did not generate unique reagents.

Data and code availability

- The RNA-seq data have been deposited at the NCBI SRA database (ID number: PRJNA 1167322) and are publicly available as of the date of publication. Accession number is listed in the key resources table.
- This article does not report the original code.
- Any additional information required to reanalyze the data reported in this article is available from the lead contact upon request.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

CI-L, MP-R, MT-B, RM-T, and IP-P performed behavioral and biochemical experiments. MA-A analyzed images. FB and IP-P conceived the idea. FB funded this project. CI-L and FB wrote the article. All co-authors edited and approved the final version of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHOD DETAILS
  - Drugs
  - Experimental designs
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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
AB-FUBINACA	Cayman	CAY-14039; CAS: 1185282-01-2
Critical commercial assays		
RiboPureTM Kit	Invitrogen	Cat#10107824
G-LISA Activation Assay	Cytoskeleton Inc.	Cat#BK127
FD Rapid GolgiStain kit	FD NeuroTechnologies, Inc	Cat#PK401A
Deposited data		
RNAseq	NCBI SRA	PRJNA 1167322
Experimental models: Organisms/strains		
Male and female C57BL/6J mice	Charles Rivers	632C57BL/6J
Software and algorithms		
GraphPad Prism 9.2.0	Graphpad	https://www.graphpad.com/
STATISTICA ®	StatSoft	https://www.statsoft.de
Sholl Analysis – Neuroanatomy plugin – FIJI	FIJI free software	https://fiji.sc
Dendritic spine– Filament tracer tool - IMARIS	Oxford Instruments	BPA-IM-Tracer97
PCA – FactoMineR – R package version 2.11	r- project free software	https://doi.org/10.32614/CRAN.package.FactMineR
Other		
Chamber for fear conditioning and PPI test	PanLab	Cat#LE116
Actimetry	Cibertec	Not specified
Confocal microscope	Zeiss, CLSM	LSM 900

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

Adolescent male and female, and adult female C57BL/6J mice (Charles River, France) were used in these experiments. Animals were housed 4-5 per cage in a temperature (21 ± 1°C)-and humidity (55 ± 10%)-controlled room under a 12 h light/dark cycle. Food and water were available *ad libitum*. All behavioral studies were performed during light period. Experimental procedures were conducted in accordance with the guidelines of the European Communities Directive 2010/63/EU and Spanish Regulations RD 1201/2005 and 53/2013 regulating animal research and approved by the local ethical committee (CEEA-UFV) (283.2/21).

METHOD DETAILS

Drugs

AB-FUBINACA (Cayman Chemical) was prepared in a 5% ethanol, 5% Tween-80 and 90% saline solution and was intraperitoneally (i.p.) administered at doses of 1, 1.5 and 2 mg/kg (10 ml/kg of body weight). Dosage was based on previous studies.[10,13,14]

Experimental designs

AB-FUBINACA treatment in adolescent mice

We evaluated long-term effects due to adolescent exposure to AB-FUBINACA on anxiety-like behavior, cued fear conditioning and extinction, object memory, sociability, depressive-like behavior and prepulse inhibition (PPI) of the startle reflex in both male and female mice. The temporal boundaries of adolescence, a vulnerable period to the central effects of drugs[5,26] are not precisely defined in either humans or rodents.[26] Thus, based on previous studies,[20,21] the treatment started at PND 35. Mice were i.p. treated with increasing doses of AB-FUBINACA (PND 35-39: 1 mg/kg, PND 40-44: 1.5 mg/kg, and PND 45-49: 2 mg/kg) or vehicle for 15 days. Long-term effects were analyzed 20 (PND 69) days after the end of the treatment. The interval of time between adolescent treatment and the different behavioral assays were based on previous reports.[20,21] Behavioral studies were carried out in 6 different batches (3 per sex) as described in Figures 1A, 2A, and 3A. The first was used for locomotion, anxiety and fear extinction experiments (males, n = 12; females, n =12), the second, for performing object recognition, sociability and forced swimming tests (males, n = 14-15;

females, n = 11-13) and the third for the experiments of PPI (males, n = 13-15; females, n = 13–15). Tissues were obtained 24 h after the PPI test to carry out biochemical experiments. For RNAseq experiments, the number of mice used was 4 per group. An additional experiment was performed for Golgi staining (n = 4 mice per group). The number of animals used in this study is in the usual range of similar experiments previously published.[42,43]

AB-FUBINACA treatment in adult mice

To determine if adolescence is a period of vulnerability to the effects observed in AB-FUBINACA treated mice, a similar protocol was conducted in adult mice (Figure 4A).

Aligning with the prior experimental framework where PND 69 marked adulthood, intraperitoneal injections of AB-FUBINACA or vehicle started at this point, increasing the doses as previously described (PND 69-73: 1 mg/kg, PND 74-78: 1.5 mg/kg, and PND 79-83: 2 mg/kg). Behavioral evaluation started 20 days after the end of the treatment (PND 103), as described in Figure 4A.

Behavioral experiments

Elevated plus maze

Anxiety-like behavior was assessed by using an elevated plus maze (EPM), which consisted in four arms (25 × 5 cm) set in cross from a central square (5 × 5 cm) and raised 30 cm from the ground. Two opposite arms were delimited by vertical walls (closed arms), although the two other arms had unprotected edges (open arms). The apparatus was indirectly illuminated with 40-50 lux. The 5 min test was recorded through a videocamera located on top of the maze. Results are expressed as total entries to the open and closed arms, and the percentage of time spent in the open arms with respect to the total amount of time spent in both closed and open arms.

Cued fear conditioning and extinction

Training and testing were performed as in preceding experiments with slight modifications.[20,21] Mice were individually placed in the chamber (LE116, Panlab, Harvard Instruments) made of black walls with a transparent front door. The box (25 × 25 × 25 cm) was located inside a soundproof module to provide background noise and to reduce outside sound. The chamber floor was formed by parallel metal bars (2 mm of diameter and 6 mm spaced) connected to a shock generator (LE100- 26 module, Panlab, Harvard Instruments). A high-sensitivity weight transducer (load cell unit) was used to record the signal generated by the animal movement intensity. The software PACKWIN V2.0 automatically quantified the percentage of immobility for each experimental phase. Between animal trials, the chamber was cleaned with 70% ethanol and water to avoid olfactory cues. The conditioning session consisted of a 180 sec habituation followed by three cue tones (3 Hz, 80 dB) of 30 sec long. Each cue (conditioned stimulus, CS) co-terminates with a 0.7 mA foot-shock of 1 sec duration (unconditioned stimulus, US). The interval between cues lasted 10 sec. Fear extinction sessions (E1-E5) were performed 24, 48, 72, 96 and 120 h after the conditioning day in a novel environment (white walls, transparent cylinder, and smooth floor). During E1, mice were habituated to the new context for 180 sec, whereas in E2-E5 this acclimatation period was reduced to 60 sec. Then, mice were re-exposed to the CS (4 cue tones, 30 sec long, 10 sec between tones). Fear memory was assessed as the mean percentage of time that mice spent freezing during the 4 cue tones of each extinction session. Freezing behavior, a rodent's natural response to fear, was automatically recorded and defined as complete lack of movement, except for breathing for more than 800 ms. Data from fear extinction were expressed as percentage of freezing behavior.

Locomotion

Changes in locomotor activity were assessed by using activity boxes (27 × 27 × 21 cm, Cibertec). Mice were individually placed in locomotor cages with low luminosity. Activity was measured as the total number of times the animal crossed an infrared beam during 15 min.

Novel object recognition test

Object-recognition memory was performed by using a V-shaped maze made of matte black methacrylate with two corridors (30 × 4.5 cm and 15 cm high) joined at a 90° angle. Mice were first habituated for 9 min to the maze. The day after, animals were trained and exposed to two identical objects located at both limits of the maze and were allowed to explore for 9 min. On the test day, 24 h later, mice were again placed in the maze for 9 min, but one of the familiar objects was replaced with a novel one. Object exploration was defined as the orientation of the nose to the object at less than 2 cm. The total time the animal spent exploring each object was computed and the discrimination index was calculated as the difference between the time spent exploring novel vs familiar object divided by the total time exploring the two objects.

Three-chamber social interaction test

Sociability testing occurred in a three-chamber maze made of transparent methacrylate with three exact compartments (20 × 20 × 40 cm) separated by sliding doors (5 × 8 cm). After a 5 min habituation in the central chamber, the session to evaluate social affiliation/motivation started. A same-sex conspecific stranger was placed in a cylindrical cage that allows interaction in one of the side compartments, while the other compartment remained empty. The doors opened, and the mouse was allowed to explore the different compartments freely for 10 minutes. Typically, mice exhibit a preference for spending more time with other mice than alone, demonstrating sociability. Interaction times, measured as the time that the animal head was inside of a zone surrounding cylindrical cages enclosures at less than 5 cm distance, were recorded.

Forced swimming test

To evaluate depression-like behaviors, animals were placed in a transparent methacrylate cylinder (20 cm of diameter) filled with water (22-24°C) up to 15 cm to prevent mice from touching the bottom. They were allowed to swim freely for 6 min under normal light

conditions. The cumulative duration of immobility during the last 4 min was calculated. Immobility was defined as the absence of movements except for those slights to maintain balance in the water.

Prepulse inhibition of startle reflex

PPI of startle reflex, a measure of sensorimotor gating, was conducted in two automated StartFear combined chambers (LE116, Panlab, Harvard Instruments) which were calibrated to ensure equivalent sensitivity and sound. Mice were daily habituated to a non-restrictive Plexiglas cylinder anchored to a high sensitivity transducer for 5 min with background white noise (65 dB) 4 days prior to test. The test started with an acclimatation period of 5 min followed by 5 pulse trials (120 dB, 40 ms) to establish baseline acoustic startle response. The experimental protocol consisted of 10 blocks with 3 or 12 trials each, randomly presented with an inter-trial interval of 10-30 s: no stimulus (12×) (65dB), pulse alone (12×) (120 dB, 40 ms), pulse precede by 4 prepulse intensities (12× each) (4, 8, 12 and 16 dB above background noise, 20 ms duration, 100 ms before pulse) and prepulse alone (3× each) (Figure 3B). Finally, 5 pulse trials were delivered. The first and last five trial pulses were excluded from the final analysis. Startle amplitude was automatically detected by PACKWIN V2.0 software. PPI was calculated as: 100 × (mean startle response – mean prepulse inhibited startle response) / (mean startle response).

RNA sequencing

Total RNA was purified from prefrontal cortex tissues of vehicle (n = 4) and AB-FUBINACA treated female (n = 4) mice 24 h after PPI test, with the RiboPureTM Kit (Invitrogen). RNA integrity > 7 was confirmed by TapeStation (Aligent). Sequencing libraries were prepared using TruSeq Stranded mRNA Sample Prep Kit (Illumina) following manufacturer's instructions. Libraries were validated by using KAPA Library Quantification Kit for Illumina according to the qPCR Quantification Protocol Guide (KAPA Biosystems) and quantified by TapeStation (Aligent). Libraries were submitted to an Illumina NovaSeq and sequencing was performed using a 2 × 150 bp paired end configuration. Pseudo-alignment and quantification were then made with Salmon algorithm (reference genome GRCh38) (Patro et al.[44]). Correlation analysis, principal component study and differential expression analysis were performed with DESeq2 package (Love et al.[45]). Differential expression gene (DEG) analyses were done using the parametric Wald test, with Benjamini-Hochberg adjustment method (padj). Genes with padj < 0.05 and a cutoff of 2-fold change were considered significantly DEGs. Raw data corresponding to RNA sequencing analyses were deposited at the NCBI SRA, ID number: PRJNA 1167322.

G-LISA Cdc42 Activation Assay

Activity of CDC42 GTPases in prefrontal cortex tissue extract was measured by G-LISA Activation Assay (Cytoskeleton Inc.; BK127) according to the manufacturers protocol. Tissues were lysed with an appropriate lysis buffer and centrifugated (10,000 × g, 1min, 4°C). Supernatants were immediately frozen and kept at −20°C till the G-LISA Activity Assay. Protein concentration was measured by Precision Red^{TM} Advanced Protein Assay (Cytoskeleton Inc.). Most articles that use the G-LISA kit work with cells instead of tissue, so a fine-tuning had to be carried. An amount of 3 mg/ml of sample was needed in these case, data that differs from the original protocol. The GTP-bound Cdc42 levels were performed according to the manufacturer's protocol (Cytoskeleton Inc.) and measured with a spectrophotometer at 490 nm.

Golgi staining procedure

Twenty days after adolescent exposure to AB-FUBINACA (n = 4) or vehicle (n = 4), female mice were sacrificed and the whole brain was quickly and carefully removed from the skull. The Golgi staining procedure was conducted in accordance with manufacturer's instructions, FD Rapid GolgiStain kit (FD NeuroTechnologies, Inc.; PK401A Cell Systems Biology). In summary, brains were immersed in solution A/B for 10 days in dark (with a change of the solution A/B after the first 24 h). Subsequently, they were transferred to solution C for 4–5 days prior to being sliced (with a change of the solution C after the first 24 h). Coronal sections of 160 μm thickness, spanning from 1.98 to 1.54 mm with respect to bregma for the prefrontal cortex, were obtained by using a cryostat following the protocol described by Zhong et al.[46] After the sections dried completely on gelatin-coated slides, they were incubated in staining solution D/E for 10 min. Subsequently, stained sections were rinsed with distilled water and underwent for dehydration by a series of consecutive immersion in ethanol solutions with increasing concentrations (50, 75, 95 and 100%). Following this, samples were subjected to clearing using xylene and then mounted.

Image acquisition and analysis for sholl analysis

Stained sections were photographed at a 10× dry objective using a Zeiss LSM 900 confocal microscope (Zeiss, CLSM, Germany). A Z projection was made to ensure capturing the entire neuron (1 μm/stack, 16-bit, 1024 × 1024). For sholl analysis, only neurons of layer II/III of the prefrontal cortex completely impregnated within Golgi stain and that could be traced along its entire length were selected. Six independent neurons from each animal were randomly selected. To assess neuron remodeling and analysis, we used the Neuroanatomy plugin (Simple Neurite Tracer, semi-automatic tool) in FIJI (FIJI is just Image J). Finally, a principal component analysis (PCA) was performed to identify possible different groupings of the neuronal populations, by using an unbiased approach. For that, we identified 13 markers of neuron complexity: number of intersections (every 20 μm), total length (μm), number of terminal ends, total bifurcations, convex hull volume (μm^3), average branch order, number of late-order branches, number of first, second, and third-order branches, average length of first and second-order branches, and the ratio total length –late order branches. PCA was performed using R package version 2.11 of FactoMineR.

Image acquisition and analysis for the morphology of dendritic spines

Section images were captured under a Zeiss LSM 900 confocal microscope, using a 60×/2× oil objective (Zeiss, CLSM, Germany) with 1.4 NA. Images were acquired through a z-plane (0.3μm/stack, 16-bit, 2048 × 2048). Secondary and tertiary dendrites of individual pyramidal neurons from layers II/III of the prefrontal cortex were selected. In addition, we chose 4-5 apical and 4-5 basal dendrites per animal for the analysis. To calculate spine density, a minimum dendrite length of 20 μm long was required. Reconstruction of dendrites and spine classification was performed by using the ''FilamentTracer'' tool of IMARIS software (Bitplane). Projections from dendrites were classified into 4 types based on their morphological characteristics: ''stubby'' were less than 0.7 μm in length, lacked a large spine head, and did not appear to have a neck; ''thin spines,'' larger than 0.7 μm and had elongated spine necks with small heads; ''mushroom-like'' were also more than 0.7 μm of length, but were characterized by a short neck and large spine head; and ''branched'' spines that had elongated spine necks with 2 or more spine heads.

QUANTIFICATION AND STATISTICAL ANALYSIS

Normality and homoscedasticity were evaluated before the final analysis (Kolmogorov-Smirnov test and Bartlett's test, respectively). Statistical analysis was carried out using unpaired Student t-test (with Welch's correction when heteroscedasticity), two-way ANOVA, and two-way ANOVA of repeated measures followed by Bonferroni post hoc comparisons after significant interactions between factors. In case of missing values, a mixed-model ANOVA was performed. Nonparametric Mann-Whitney test was used when data did not fit a normal distribution. To study correlations between two variables, the Pearson's coefficient was employed. Outliers were excluded if they were >2 standard deviations from the mean. All data are expressed as mean ± SEM. A p value <0.05 was used to determine statistical significance. The statistical analyses performed for each behavioral and molecular experiment are described in Table S1. The statistical analysis was performed using STATISTICA (StatSoft) software and GraphPad Prism 9.

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congresos a los que he ido. Tu forma de demostrar amor es diferente a la del resto de personas, sin embargo, nunca me has hecho dudar del amor que nos tenemos. Te quiero mucho.

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Mami y Papi, gracias por estar delante y detrás de cada logro que he conseguido. Por cada aperitivo dedicado a un premio de un congreso, a un paper publicado o, ahora, a una tesis ya depositada. Papi, siempre me has hecho sentir especial, cuidada, querida y escuchada. Me has apoyado en cada paso que he dado en este difícil mundo de la ciencia y, literalmente, no habría podido ir a ningún congreso sin tu ayuda, jajaja. Has celebrado conmigo cada pequeño avance y te has alegrado por mí con cada historia que te he contado. Gracias por ser el mejor padre del mundo; no quiero que me faltes nunca. Mami, llegar hasta aquí sin ti habría sido imposible. He crecido viendo tu tenacidad, tu deseo de superarte, de ir más allá, de crecer, de prosperar y de ser mejor profesional, persona y madre. Cada uno de mis logros lleva un trocito de ti, de tu forma de enseñarme a ver la vida. Gracias por cuidarme tanto, incluso cuando quizá no me lo merecía. Gracias por las conversaciones difíciles y por darme un lugar seguro cuando la vida me ponía una piedrecita en el camino. Gracias por escucharme, por enseñarme, por hacerme una tabla de multiplicar de bolsillo, por ayudarme con los esquemas de conocimiento del medio, por repasar la lección conmigo por las mañanas, por explicarme los números reales, por imprimirme todos los ejercicios de sintaxis que existían en internet, por leerte los libros de historia cristiana por mí y por todas las cosas que sé que seguirás haciendo por mí en la vida. Te quiero mucho.

Y, por último, quiero agradecer a **Jose**, mi culin. Convertir estas líneas en un simple agradecimiento y no en una carta de amor me resulta complicado, pero voy a intentarlo. Cuando nos conocimos allá por 2012, ninguno de los dos habría imaginado que un día estaría aquí, en el sillón de nuestra casa, escribiendo los agradecimientos de una tesis que jamás estuvo en mis planes. Prácticamente hemos crecido y madurado juntos, y por eso me cuesta pensar en este logro sin reconocerte como una parte fundamental de él. Gracias por ayudarme a crecer como persona, por enseñarme a valorarme, por mostrarme cómo relativizar los problemas y por ponerme siempre los pies en la tierra. Estos últimos años también me has enseñado que el crecimiento profesional solo tiene valor cuando la vida personal lo supera. Y si hay un buen motivo para madrugar y hacer un experimento más de la tesis, es acabar el día compartiendo tiempo contigo. Siempre te estaré agradecida por cada detalle que has tenido estos años, pero en especial por todo lo que has hecho durante esta última etapa de escritura de tesis: por cada merienda, comida o cena preparada, por cada beso o abrazo, por teletrabajar mientras yo leía en alto la misma frase ocho veces seguidas y por ayudarme a elegir hasta el color más insignificante. Los logros personales siempre son motivo de alegría, pero compartirlos con alguien especial es lo que realmente nos llena el corazón. Y compartiendo contigo, cariño, mi corazón siempre se siente pleno. Te quiero.

Y para finalizar ya de verdad, quiero dejarle un mensaje a mi yo del futuro. Cris, sigue disfrutando tanto con cada cosa que haces y sigue intentado ser la mejor versión de ti cada día. Esto es solo el principio de todas las cosas que puedes hacer y recuerda… *"Donde otros ven límites, la pasión ve desafíos; donde otros se detienen, la pasión inventa caminos"*- Cris feat. Deepseek.