Relevance of cannabinoids in preclinical models of psychiatric disorders

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Por orden de aparición, para mamá, para papá, para Nuni, para Oli, para Jaia

Para Rebeca

Vení, volá, sentí

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'*What does your date look like? – She's sparkly, looks like a holiday'. – Rain Man –*

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- M.L. Soto-Montenegro, V. García-Vázquez, **N. Lamanna-Rama**, G. López-Montoya, M. Desco, E. Ambrosio, 'Neuroimaging reveals distinct brain glucose metabolism patterns associated with morphine consumption in Lewis and Fischer 344 rat strains', *Sci. Rep*., 12(1):4643, Mar 17, 2022 doi: 10.1038/s41598-022-08698-9. PMID: 35301397; PMCID: PMC8931060.
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adolescence. Young Spanish Molecular Imaging Network (ySMIN) – Meeting, Madrid, Spain. Poster. January 2017.

· ABBREVIATIONS ·

2-AG	2-arachidonoylglycerol
CBD	Cannabidiol
CB1R	Cannabinoid receptor type 1
CB2R	Cannabinoid receptor type 2
CC	Corpus callosum
CNS	Central nervous system
COX2	Cyclooxygenase 2
CP	CP-55,940
DAGL	Diacylglycerol lipase
ECS	Endocannabinoid system
F344	Fischer
FA	Fractional anisotropy
FAAH	Fatty acid amide hydrolase
FC	Frontal cortex
[^{18}F]-FDG	[^{18}F]-Fluorodeoxyglucose
fMRI	Functional magnetic resonance imaging
^{1}H-MRS	Magnetic resonance spectroscopy
HO1	Heme oxygenase-1
HPA	Hypothalamic-pituitary-adrenal
Iba1	Ionized calcium binding adaptor molecule 1
iNOS	Inducible nitric oxide synthase
IOS	Inflammation and oxidative stress
IRF3	Interferon Regulatory Factor
Keap1	Kelch-like ECH-associated protein 1
LEW	Lewis
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase

MAM	Methylazoxymethanol acetate
MD	Mean diffusivity
MIS	Maternal immune stimulation
MRI	Magnetic resonance imaging
MSA	Morphine self-administration
NAcc	Nucleus accumbens
NAPE	N-acyl phosphatidylethanolamine-specific phospholipase D
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NQO1	NAD(P)H dehydrogenase [quinone] 1
NRF2	Nuclear factor erythroid 2-related factor 2
PAG	Periaqueductal grey
PET	Positron Emission Tomography
PFC	Prefrontal cortex
Poly I:C	Polyinosinic:polycytidylic acid
ROI	Region of interest
ROS	Reactive oxygen species
SPECT	Single-photon emission computed tomography
THC	delta-9-tetrahydrocannabinol
TL	Telomere length
TLR3	Toll-like 3 receptors
TRPV1	Transient receptor potential cation channel subfamily V member 1
UNODC	United Nations Office on Drugs and Crime
VP	Ventral pallidum
VTA	Ventral tegmental área

· Table of contents ·

· Introduction ·

· INTRODUCTION ·

"**Drugs can kill**". Those are the first three words of the World Dug Report 2023 from the United Nations Office on Drugs and Crime (UNODC) [1]. Nowadays, we have a broad spectrum of knowledge about the harmful effects of different natural and synthetic drugs available on the market, but this has not always been the case. This thesis will explore the potential effects of cannabinoid derivates in different animal models of psychiatric disorders, finding both harmful and (surprising) beneficial effects, thus providing us a palette of greys which, let's be honest, could reasonably be expected.

Throughout this thesis, we are going to navigate through the history of cannabis, from its first use thousands of years ago, to its exponential growth in use and knowledge during the last third of the last century. The endocannabinoid system was a cutting-edge discovery, which initiated interest in cannabinoids both in their effects on the human body, and in their interaction with other neural systems, such as the dopaminergic reward system. Not much later, the implications of cannabis in adolescence were postulated, opening a wide range of possibilities in the study of cannabinoids. Consequently, researchers began to wonder whether cannabinoids might influence the consumption of other drugs and, the possible interactions with other endogenous systems, such as the opioid system, and their association with the onset of psychiatric disorders, such as schizophrenia. In this sense, preclinical models have provided a great impulse for the understanding of such processes and effects of cannabinoids. Nevertheless, it is important to note that the field of cannabinoid research is rapidly evolving, and although substantial progress has been made, many questions remain to be answered.

Cannabis

History of cannabis

Like many other (now legally regulated) drugs, cannabis has not always been treated as harmful or negative among society. The history of cannabis is rich and varied, spanning thousands of years, and diverse cultures and civilizations around the world. Cannabis, also known as marijuana, has played a significant role in human societies for a variety of purposes, including medicinal, spiritual, and recreational use [2]. The origins of cannabis can be traced back to Central Asia, the region where the Cannabis plant, in particular *Cannabis sativa*, is believed to have originated. The cultivation and use of the plant by humans date back thousands of years. Ancient archaeological evidence, including the remains of cannabis seeds and pollen, suggests that it was cultivated as early as 4000 BCE in regions such as China and Taiwan [3]. In ancient civilizations, cannabis was valued for its **versatile properties**. Its fibres

were used to produce textiles, rope, and paper, while its seeds served as a valuable source of nutrition. Cannabis was also recognized for its **medicinal properties** [2], with early documentation of its use in traditional Chinese medicine, where it was employed to treat various ailments, including pain, inflammation, and digestive disorders. As human civilizations developed, cannabis gained cultural and religious significance in several ancient societies. In ancient Egypt, cannabis had a profound presence in religious rituals. The **Ebers Papyrus**, an ancient Egyptian medical text dating back to 1550 BCE, includes references to cannabis as a treatment for various disorders. Additionally, cannabis was used in the embalming process, indicating its association with death and the afterlife. Similarly, in India, cannabis holds a prominent place in religious practices and rituals. The ancient sacred texts of the Hindu religion, known as the Vedas, mention cannabis as one of the five sacred plants. Cannabis, known as "ganja" or "bhang," was associated with the worship of the deity Shiva and was used in spiritual ceremonies and meditation practices.

Global exploration and trade routes from the 15th to 18th centuries played a crucial role in the spread of cannabis to different parts of the world. European explorers and traders became acquainted with cannabis during their travels to Africa, Asia, and the Americas. They observed its diverse uses and recognized its **economic potential**. Cannabis cultivation for industrial purposes, particularly the production of hemp fibres, expanded significantly during this period. Hemp fibres were valued for their strength and versatility, used in the production of sails, rope, and clothing. European powers, such as Spain, France, and England, encouraged the cultivation of cannabis for economic reasons, resulting in the establishment of hemp plantations in their colonies. The first works reporting the **clinical effects** of cannabis date back to the early 1840s. In London, a physician at the St. Marylebone Infirmary, John Clendinning M.D., F.R.S., exposed more than fifteen case reports on different types of ailments, including headaches, fever, or even side effects of opiates [4]. Few years later, in 1878, Russel Reynolds, a very influential British physician, compiled more than 30 years of experience in the therapeutic use of cannabis in The Lancet, finding the effects that medical cannabis has today according to medical research [5].

The perception of cannabis use began to change during the 19th and early 20th centuries, particularly in the United States, mainly due to the rise of the temperance movement and the association of cannabis with immigrant communities, which contributed to a growth in negative perceptions. Racial and cultural prejudices played an important role in the stigmatization of cannabis use, leading to discriminatory policies and legislation. Thus, in the early 20th century, the first wave of cannabis prohibition emerged, starting with the **TAX Act** in the United States, which effectively criminalized the possession and sale of cannabis, and

hindered cannabis research [3]. In addition, a 15-year follow-up study in a large Swedish cohort found that cannabis was a risk factor for schizophrenia [6].

In recent decades, there has been a resurgence of interest in the **medicinal properties** of cannabis. The discovery of the endocannabinoid system in the 1990s shed light on the physiological mechanisms through which cannabinoids interact with the body [7]. This scientific knowledge, coupled with anecdotal evidence and patient advocacy, led to the medical cannabis movement. Several countries and states have stablished laws allowing the medical use of cannabis to alleviate the symptomatology in various pathologies, such as chronic pain, nausea, and epilepsy. Furthermore, the recreational use of cannabis has been legalized in some regions, recognizing its cultural significance, and addressing the social and economic implications of prohibition.

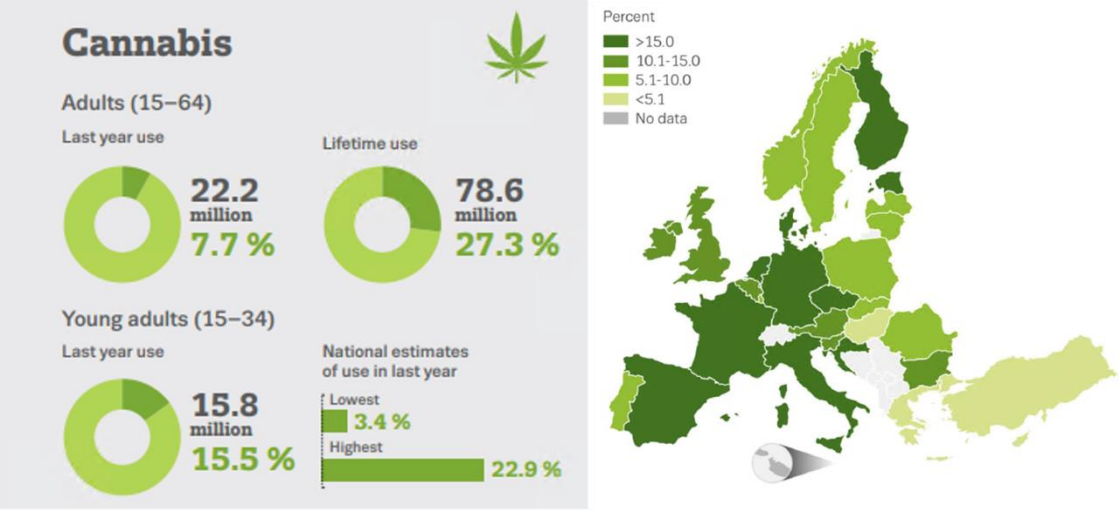


Figure 1.1. Estimation of adult cannabis consumers in Europe. On the left, adult and young adult estimation of cannabis consumers in Europe (year 2022). On the right, representation of the percentage of adult cannabis consumers among European countries (year 2022) [8].

The endocannabinoid system

The endocannabinoid system (ECS) is a complex signalling system found in humans and other mammals. It plays a crucial role in maintaining homeostasis, or balance, within the body [9]. The discovery of the ECS can be attributed to the pioneering research of Dr. Raphael Mechoulam and his team in the 1960s, who characterised some phytocannabinoids for the first time, including delta-9-tetrahydrocannabinol (THC) [10] and cannabidiol (CBD) [11]. This led to the intriguing exploration of the ECS, reaching the identification of **cannabinoid receptors** (CBRs) in the early 1990s and, subsequently, of their endogenous ligands, endocannabinoids. The ECS consists of three main components: receptors, endocannabinoids, and enzymes.

The two primary receptors identified within the ECS are the cannabinoid receptor type 1 (**CB1Rs**) and the cannabinoid receptor type 2 (**CB2Rs**). Both are G protein-coupled receptors, but vary on their abundance, distribution, and functions [9]. CB1Rs, the most abundant, are primarily found in the central nervous system (CNS), including the brain and spinal cord, while CB2Rs are predominantly located in peripheral tissues, particularly within the immune system. CB1Rs are densely distributed in brain regions associated with cognition, memory, coordination, and emotion, such as cerebellum, nucleus accumbens (NAcc), amygdala, cortex, and hippocampus [12]. Their activation by endocannabinoids or exogenous cannabinoids leads to various effects on the CNS, mainly focused on inhibition of neurotransmitter release, and therefore highly localized in GABAergic neurons. This type 1 receptors are also present in astrocytes and microglia, where they have an important role in neuroplasticity [9]. CB2Rs, on the other hand, are predominantly found in immune cells and contribute to immune regulation. However, CB2Rs are generating interest regarding their presence in microglia, where they are thought to have an anti-inflammatory effect [9,13]. Thus, of late, CB2Rs are being linked to psychiatric disorders, given the inflammatory basis present in most of them [13].

TABLE 1.1. Main characteristics of CB1 and CB2 cannabinoid receptors

	 **CB1Rs**	 **CB2Rs**
Location	CNS: cerebellum, nucleus accumbens, amygdala, cortex, hippocampus	Peripheral tissues: immune system
Function of the location	Cognition, memory, coordination, emotion	Immune regulation
Effects of their activation	Inhibits neurotransmiter release (GABAergic neurons)	Anti-inflammatory
Function on microglia	Neuroplasticity	Phychiatric disorders

Table 1.1. Table represents the main location and functions of the two main cannabinoid receptors.

The first isolated endocannabinoid, **anandamide**, named after the Sanskrit word for "bliss," is involved in mood regulation, pain perception, and appetite, while 2-arachidonoylglycerol (**2-AG**) plays a role in immune function and neuroprotection [9]. Despite being the most studied endocannabinoid to date, anandamide is only a partial agonist of CBRs, and can therefore interact with several other receptors, including the transient receptor potential cation channel subfamily V member 1 (TRPV1) [14], which some authors describe as CB3R [7]. One of the reasons why anandamide is vastly studied is its modulatory effect in the reward system. Anandamide can regulate the reinforcing and addictive effects of drugs of abuse, both cannabinoids and non-cannabinoids. However, as many drugs of abuse, depending on the conditions, anandamide could act in opposite directions, increasing side effects, or decreasing behavioural reinforcement activity [14]. On the other hand, 2-AG is the most abundant endocannabinoid, a total agonist of CBRs, and plays a central role in neuroinflammation. It is known that a higher concentration of 2-AG (or a decrease of its degradation enzyme) can lead to anti-inflammatory effects. The anandamide effect on the brain reward system and 2-AG modulation of neuroinflammation, make endocannabinoids an important component when studying psychiatric disorders [15].

Finally, enzymes constitute the third significant component of the ECS. These enzymes are responsible for the synthesis and degradation of endocannabinoids, tightly regulating their levels within the body. While the two main synthesis enzymes are N-acyl phosphatidylethanolamine-specific phospholipase D (**NAPE**) and diacylglycerol lipase

(**DAGL**), which are responsible for the synthesis of anandamide and 2-AG, respectively, research is mainly focused on fatty acid amide hydrolase (**FAAH**) and monoacylglycerol lipase (**MAGL**), which breaks down anandamide and 2-AG, respectively [9]. The main relevance of these enzymes relies on their potential as targets for various treatments aimed at decreasing their activity in the degradation of endocannabinoids, thereby increasing the concentration of either anandamide or 2-AG.

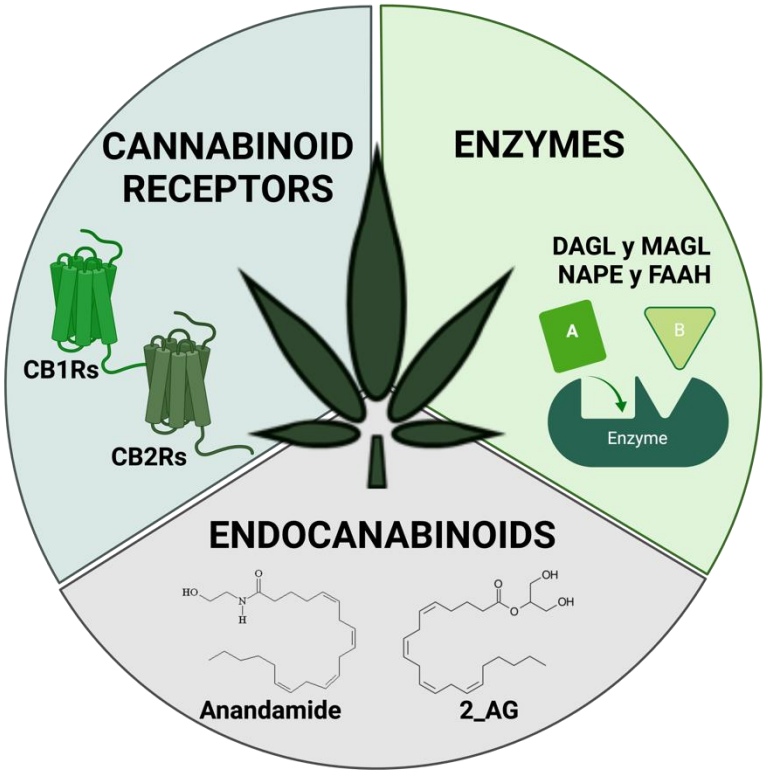


Figure 1.2. Scheme of the endocannabinoid system components. Schematic representation of the three main components of the endocannabinoid system: receptors (CB1 and CB2), enzymes (DAGL, MAGL, NAPE and FAAH), and endocannabinoids (anandamide and 2-AG).

The ECS is involved in a wide range of physiological processes, contributing to the **maintenance of homeostasis** within the body. It helps regulate different functions, such as pain perception, mood, appetite, sleep, immune response, and inflammation [7,9,12,14,15]. One of the primary roles of the ECS is modulating pain sensation. Endocannabinoids, particularly anandamide, interact with CB1Rs in the brain and spinal cord, reducing the transmission of pain signals. This mechanism contributes to the potential analgesic effects of cannabis and the therapeutic use of cannabinoids in pain management [14,15]. The ECS also plays a vital role in mood regulation and mental well-being. Endocannabinoids, acting on CB1Rs, influence neurotransmitter release and contribute to the regulation of emotions, stress response, and reward mechanisms. Dysregulation of the ECS has been implicated in mood disorders, such as depression and anxiety [7]. Furthermore, the ECS has immunomodulatory

functions, with CB2Rs playing a significant role in immune regulation and inflammation. Endocannabinoids and CB2Rs are found in immune cells, and their activation can influence immune response and inflammatory processes. This has implications for various inflammatory conditions, autoimmune disorders, and neurodegenerative diseases [9].

Cannabis-derived products and synthetic cannabinoids

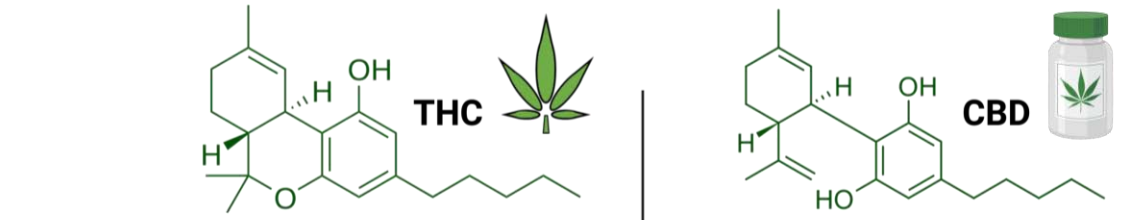
Despite both **THC** and **CBD** being described in the 1960s, deeper investigation was not carried out until much later. Some of the effects of THC prompted the interest in this compound, particularly in relation to its analgesic effects, but the non-psychotropic characteristics of CBD did not attract interest until very recent years. Nowadays, CBD is considered a potential treatment for several disorders, mainly due to its neuroprotective, anti-inflammatory, and antioxidant effects [16].

Regarding their mechanism of action, THC is the main psychoactive compound of *Cannabis sativa* and yet, it is only a partial agonist of CBRs, with a very similar affinity as the endocannabinoid anandamide [17]. In this regard, other synthetic cannabinoid agonists have higher affinity to CBRs, such as WIN55,212-2 and CP-55,940 [18]. It so happens that the objective of synthetising these compounds is to enhance the potency of cannabinoid effects, improving their affinity and binding efficacy to cannabinoid receptors, or the modulation of FAAH and MAGL, degradative enzymes of the endocannabinoid system. This would amplify their specific therapeutic effects while eliminating undesirable psychoactive properties, and, overall, provide new tools to deepen our understanding of the endocannabinoid system [19]. Within the broad spectrum of synthetic cannabinoids, aminoalkyindoles, like WIN55,212, form the major and most powerful family of synthetic cannabinoids, being a full agonist, but producing similar effects to those of other cannabinoids [18]. However, one of the most widely used synthetic cannabinoids is CP-55,940, which is a partial agonist for acetylcholine inhibition, and can also act as a very potent agonist of CB1/2Rs. Again, its physiological effects are similar to those of other cannabinoids [18].

Conversely, CBD has very low affinity to CBRs, and therefore has no psychoactive activity. Instead, CBD can interact with other receptors, such as serotoninergic, TRPV1, or μ-opioid receptors [20,21]. Therefore, unlike THC, CBD has a good safety profile and does not produce euphoric or cognitive effects. In fact, it can counteract some of the negative effects of THC, such as anxiety, psychotic symptoms, and cognitive deficits [16,17]. Neuroimaging studies revealed opposite effects on the brain between CBD and THC, with CBD reducing anxiety and exhibiting distinct patterns of brain activity [22]. CBD is well-tolerated and has demonstrated benefits in reducing depressive and psychotic symptoms, improving attention

and memory, and has been linked to the recovery of hippocampal volume in cannabis users [23].

TABLE 1.2. MAIN EFFECTS OF THC AND CBD



	Psicoactive	Non-psicoactive
Main effects	- Intoxicating - Euphoria - Stimulates appetite - Muscle relaxation - Anti-inflammatory - Alliviates pain	- Non-intoxicating - Anti-inflammatory - Reduces nausea - Reduces the effects of THC - Anti-convulgant - Anti-oxidant - Anxyolitic
Side effects	- Anxiety - Paranoia - Memory impairment - Muscle relaxation - Anti-inflammatory - Alliviates pain	- No side effeects
Medical applications	- Chemotherapy-induced nausea & loss of appetite - Multiple sclerosis - Glaucoma - Pain - Stress - Insomnia	- Migranes - Epilepsy - Arthritis - Inflammatory based disorders - Inflammation - Stress - Anxiety - Depression

Table 1.2. Table compiles the main effects and medical applications of both THC and CBD.

Given that the main mechanism of action of THC is due to its interaction with CBRs, the location of these receptors in the brain will determine the extent and nature of THC effects [17]. CB1Rs are mainly located in regions involved in emotion and cognition, such as the amygdala, NAcc, hippocampus, striatum, and prefrontal cortex. Thus, activation of these receptors by THC can lead to various effects, including euphoria, anxiety, and alterations in perception, thought content, reward, and psychotic symptoms. Studies examining the acute administration of THC to healthy individuals consistently reported increased feelings of "linked," "intoxicated," and "high," accompanied by impaired cognition, anxiety, and psychotic symptoms [22,23]. Functional magnetic resonance imaging (fMRI) studies suggested that THC affects fronto-striatal and limbic/paralimbic brain regions, contributing to its effects on psychotic symptoms, learning, and emotion processing [22]. Other neuroimaging studies have shown that acute THC administration increased striatal dopamine neurotransmission [24]. Regular cannabis use is also associated with reduced availability of dopamine transporters and dopamine synthesis in the striatum, potentially impacting reward processing, memory, and

executive function [24], despite other authors associating these effects with other factors, such as withdrawal symptoms [23].

However, the intricate functioning of the ECS and its involvement in various physiological processes have led to growing interest in its potential therapeutic applications. Modulating the ECS with exogenous cannabinoids, such as THC and CBD, has shown promises in the treatment of several conditions [19–21,23]. Cannabinoids have been widely investigated for their potential analgesic properties, via interactions with TRPV receptors, mainly produced with CBD [16,20]. Cannabinoids have also demonstrated anti-inflammatory effects, making them potential candidates for managing inflammatory conditions like rheumatoid arthritis or inflammatory bowel disease. As for the realm of mental health, cannabinoids have shown their potential to alleviate anxiety and depression symptoms. By modulating the release of neurotransmitters and influencing mood-regulating systems, cannabinoids may provide relief for those individuals with these conditions [20]. Additionally, the ECS has been implicated in neuroprotective mechanisms. Activating CB1Rs may help protect brain cells from oxidative stress, inflammation, and excitotoxicity, which are involved in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [21]. Finally, through its impact in the dopaminergic reward system, endocannabinoids can influence in drug reward and behavioural implications of drug abuse [25].

Addictions

All sentient organisms possess an inherent drive to seek out positive stimuli and avoid negative ones, a fundamental behaviour that increases the odds of their survival. The nervous system plays a pivotal role in coordinating this process, employing a range of physiological and cognitive processes such as homeostasis, perception, learning, emotions, and decision-making to shape an organism's response to stimuli [26]. Despite variations across species and individuals, there are underlying similarities in how different organisms respond to positive and negative stimuli, emphasizing their importance for survival. In the case of humans, our cognitive abilities have evolved to facilitate the identification and refinement of highly rewarding stimuli, **notably including drugs**, which have the potential to override the effects of natural rewards. The interaction between the availability of such drugs, **environmental influences**, and individual vulnerabilities significantly affects the likelihood of drug consumption and the subsequent development of substance use disorders. The detrimental consequences of addiction are starkly evident in the current opioid epidemic and the alarming rates of death associated with alcohol and tobacco use, highlighting the profound societal impact of addictive disorders [27].

Significant advances in neuroscientific methodologies have greatly enhanced our understanding of the underlying neurobiology of drug reinforcement and addiction. Addiction is now recognized as a chronic and relapsing disorder characterized by an intense compulsion to consume drugs and a loss of control over drug-seeking behaviour. Its development arises from intricate interactions between the pharmacological effects of drugs, individual vulnerabilities, social determinants, and genetic factors [26]. MRI studies have observed reductions in cerebral blood flow in the PFC and increased cerebellar activation in patients with ethanol addiction [28]. Moreover, MR spectroscopy (^{1}H-MRS) studies have demonstrated elevated glutamate levels in different brain regions both in clinical and preclinical studies, which recover at the end of the withdrawal period [29]. In fact, both clinical and preclinical investigations have provided compelling evidence that genetic variability plays a substantial role in addictions. Genetic factors have been found to contribute significantly, accounting for 40% to 60% of the variation in susceptibility to drug dependence [30]. In this context, inbred rat strains, such as the Lewis, Fischer 344, and Wistar strains, have emerged as valuable tools for the study of variations in susceptibility to drug addiction. These rat strains exhibit different vulnerability to drug exposure, displaying notable differences in the expression of dopaminergic, opioid, or NMDA receptors [31]. Their usage in research has shed light on the intricate mechanisms underlying addiction vulnerability. In-depth research into these complex mechanisms, particularly those related to the dopaminergic reward system, is imperative for comprehending the factors that contribute to addiction susceptibility and for developing effective strategies for prevention and recovery.

Dopaminergic reward system

In the dopaminergic reward system, dopamine plays a pivotal role in mediating the rewarding effects of drugs. Addictive substances exert their influence by modulating dopamine neurons located in the **ventral tegmental area** (VTA). Within the VTA and substantia nigra, dopamine neurons are crucial for motivated behaviours and the formation of habits. These neurons project to the **NAcc** and dorsal striatum, which respectively encode the salience of rewards and actions driven by habit [26]. The NAcc acts as a crucial information processing centre within this system, receiving signals from the VTA and transmitting them to various regions. This intricate network of connectivity extends its influence on areas including the **prefrontal cortex** (PFC), ventral pallidum (VP), **hippocampus**, and lateral hypothalamic area, thereby exerting a significant impact on neuroendocrine secretion [26]. Notably, the PFC also engages in interactions with dopamine neurons, exerting influence over action control [27]. The functional heterogeneity of VTA dopamine neurons arises from their diversity in terms of connectivity, co-release of neurotransmitters, and expression of receptors [26]. These neurons exhibit a firing pattern characterized by tonic firing that establishes baseline dopamine levels,

and phasic firing that encodes responses to salient stimuli [26]. Furthermore, recreational drugs, such as opioids and cannabinoids, engage other neurotransmitter systems, which contribute to the reinforcement of behaviours and modulation of emotional states [25]. The significance of non-dopaminergic factors in reward processing has been relatively understudied, despite their crucial role. Administration of drugs elicits neuroadaptations in various neurotransmitter systems, including glutamate, GABA, opioid, endocannabinoid, acetylcholine, serotonin, and noradrenaline, collectively regulating distinct aspects of reward, aversion, and motivation [25].

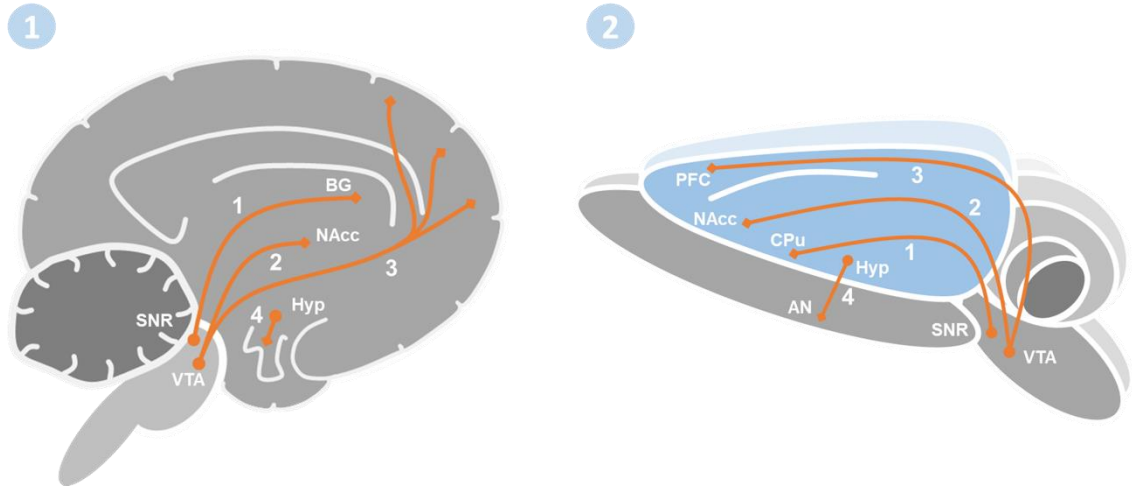


Figure 1.3. Different pathways of the reward system in human and rat brain. Dopamine pathways in the 1) human and 2) rat brain: (1) Nigrostriatal pathway, (2) Mesolimbic pathway, (3) Mesocortical pathway, and (4) Tuberoinfundibular pathway. Abb.: PFC: prefrontal cortex; BG: basal ganglia; NAcc: nucleus accumbens; Hyp: hypothalamus; CPu: caudate-putamen; AN: arcuate nucleus; SNR: subtantia nigra, VTA: ventral tegmental area.

The reward system, as part of the nervous system, undergoes significant changes during **adolescence**, which amplify the risks associated with drug consumption [26]. Many of these risks can be directly attributed to the ongoing development of the PFC. This cortical region is only partially developed during adolescence and reaches its maturation in early adulthood [32]. Additionally, some white matter structures undergo volume and functionality changes and cell reorganization during this period [33]. These developmental changes in the reward system can lead to different responses when facing a disturbance, such as those produced by drug consumption [26]. Furthermore, cannabis use among adolescents has been associated with an increased risk of engaging in other substance use, including tobacco, alcohol, and other drugs of abuse [34]. Interestingly, the '**Gateway hypothesis**', proposed by Kandel [35], suggests that cannabis consumption during adolescence may act as a "gateway" that predisposes individuals to subsequent use of more potent or addictive drugs in adulthood. According to this hypothesis, initial exposure to a relatively mild drug creates neurobiological and behavioural conditions that increase the likelihood of progressing to the use of harder

drugs at adulthood. In this sense, opioids are one of the most consumed drugs, together with some stimulants, such as cocaine, after the early exposure to cannabis [34]. Moreover, opioids could be a very dangerous group of drugs when facing addictions [36].

Opioids and opioid system

Opioids are the second most widely consumed group of recreational drugs, following cannabinoids [1]. These substances pose significant health risk, mainly attributed to the potential for overdose and the associated diseases resulting from their administration method. Globally, over **60 million individuals** consume opioids, and some countries, including the United States, have experienced a severe and ongoing crisis related to these drugs. In this sense, the non-medical use of opioids in the U.S. was originated in 1997 and coincided with an increase in opioid prescriptions for pain-management. By 2020, the prevalence of non-medical opioid use among individuals aged 15-64 had reached 3.4%, with over 9.5 million people in the United States having engaged in such use. Tragically, between 2010 and 2020, over 70,000 lives were lost due to opioid overdose [1].

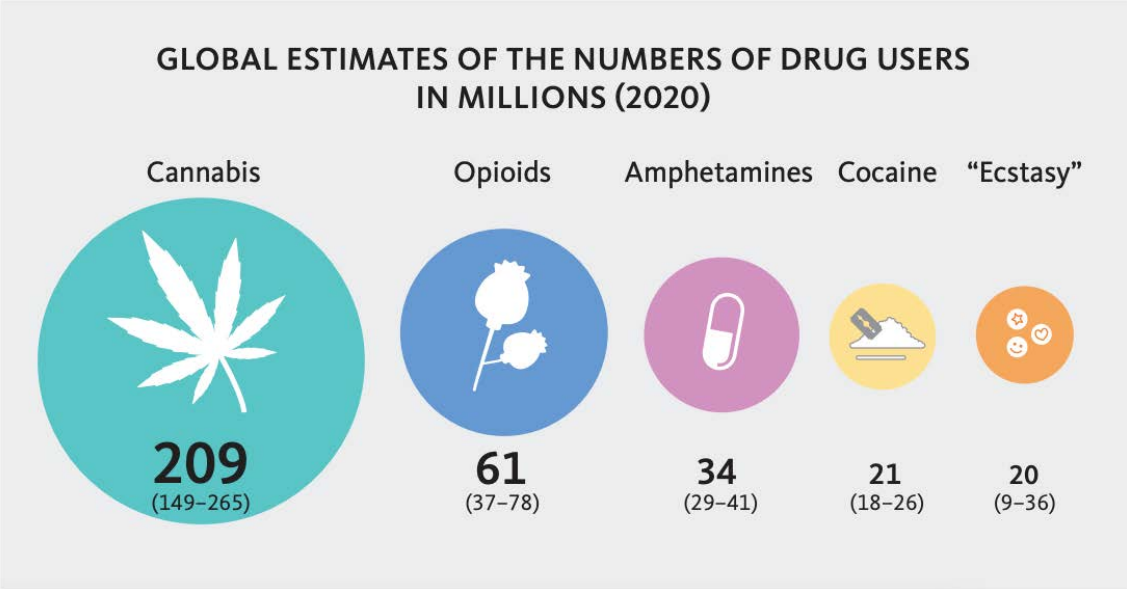


Figure 1.4. Global number of drug users. Estimation of consumers of the different groups of drugs available worldwide (year 2020) [1].

Opioids, such as **heroin and morphine**, among others, exhibit potent pharmacological effects. Consequently, their consumption can be detrimental not only during the acute exposure but also during periods of abstinence, characterized by potentially severe withdrawal symptoms, and high risk of experiencing craving and relapse even after successful withdrawal [37]. Despite the recognized risks of opioids regarding withdrawal and craving, preclinical studies have primarily focused on abstinence periods lasting 25-28 days or shorter, which is

equivalent to no more than 3 years in humans [38]. Among opioids, morphine remains as one of the most widely consumed worldwide. Morphine is an opium derivate, extracted from ***Papaver somniferum***, and is commonly employed in hospitals for the palliative treatment of post-operative or chronic pain. Its extensive utilization stems from its potent analgesic properties [36].

TABLE 1.3. Main characteristics of μ-, δ- and κ-opioid receptors

	 μ - receptor	 δ - receptor	 κ - receptor
Location	Cortex, nucleus accumbens, amygdala, periaqueductal grey and thalamus	Basal ganglia	Hypothalamus, periaqueductal grey,
Effects	Analgesia, euphoria, constipation, respiratory depression	Analgesia, convulsions, anxiolysis	Analgesia, diuresis, dysphoria

Table 1.3. Table represents the main location and functions of the three main opioid receptors. Adapted from [39].

Opioids bind to specific binding sites in the brain known as opioid receptors, which form an endogenous system that controls opioid functions. This endogenous opioid system plays a crucial role in various physiological functions, including pain regulation, gastrointestinal processes, endocrine functions, autonomic control, learning, and memory [36,37]. The main opioid receptors are μ-, δ- and κ-opioid receptors, predominantly distributed in the CNS. Activation of these opioid receptors can produce analgesic effects, but μ-opioid receptors are particularly important in mediating the effects of opioids and drug reward. This may be partially explained by their distribution in key brain regions such as the cerebral cortex, thalamus, periaqueductal grey, hippocampus, amygdala, and NAcc, which are closely associated with the reward system [26]. In the context of opioid dependence, preclinical studies using imaging techniques have highlighted the implication of regions such as the VP, corpus callosum, and retrosplenial cortex [40]. Additionally, PET studies have shown morphine administration to decrease brain metabolism in regions related to motivation and emotion [41].

Another important issue regarding opioids is their combination with other drugs of abuse, especially cannabis. There is evidence that patients with prescribed opioid treatment for non-cancer pain tend to increase opioid use when combined with cannabis exposure [42].

Thus, there appears to be a bidirectional relationship between these drugs, which could have potential detrimental effects if maintained over time [25].

Neurodevelopment

Neurodevelopment is a complex and dynamic process that occurs throughout **different stages of life** [43]. Drug consumption is not the only factor that can cause abnormalities in proper neurodevelopment, nor is adolescence the only critical period in this complex process. Significant events during gestation also contribute greatly to the proper formation of the adult brain and nervous system, making the gestational period a **decisive time window for neurodevelopment** [44]. Any disruption or alteration of these processes, whether during gestation or adolescence, could imply a wide range of difficulties in adulthood.

During gestation, which encompasses the prenatal and perinatal periods, remarkable transformations take place. In the first trimester of pregnancy, the foetus initiates the development of its nervous system from scratch. This period involves **neurulation**, a process that gives rise to the formation of the neural tube and neural crest [45]. Subsequently, these structures undergo regionalization, leading to the emergence of distinct and differentiated parts of the brain and the rest of the nervous system. During the progression of pregnancy into the second and third trimesters, a vital process known as **neurogenesis** initiates, playing a fundamental role in neurodevelopment [46]. Newly formed brain structures differentiate into specific populations of neurons, which migrate to distinct regions of the brain. Additionally, the neurotransmitter synthesis also begins during this time, further contributing to the intricate development of the nervous system. Neurogenesis starts in the final weeks of the first trimester and reaches its peak in the early weeks of the third trimester. It is important to note that certain neural cell populations retain the capacity to induce neuronal differentiation even after birth, showcasing the ongoing complexity of neurodevelopment [45].

During the third trimester of gestation, **synaptogenesis** begins, marking an important stage in neurodevelopment. Neurons, already differentiated and in their proper locations within the brain, undergo changes that enable the formation of new synapses and the establishment of neural networks. This process reaches its peak at approximately two years of age. Simultaneously, **myelination**, another crucial aspect of neurodevelopment, initiates during late gestation but predominantly occurs postnatally, extending into adolescence, and almost reaching adulthood. Disruptions during the second and/or third trimester, such as infections, malnutrition, and stress, have the potential to interfere with proper differentiation, migration, and synapse formation of neurons. These alterations have been associated with an increased risk of developing psychiatric disorders [47]. Although new-born brains bear a resemblance to

those of adults, ongoing neurodevelopmental events are necessary for the correct formation of the brain and nervous system. Synaptogenesis continues throughout the first two years after birth, while myelination continues gradually until approximately 25 years of age. Additionally, synaptic pruning, occurring predominantly during childhood and adolescence, eliminates less frequently used synapses, strengthening the remaining ones. This process peaks before puberty and lasts into adulthood [45].

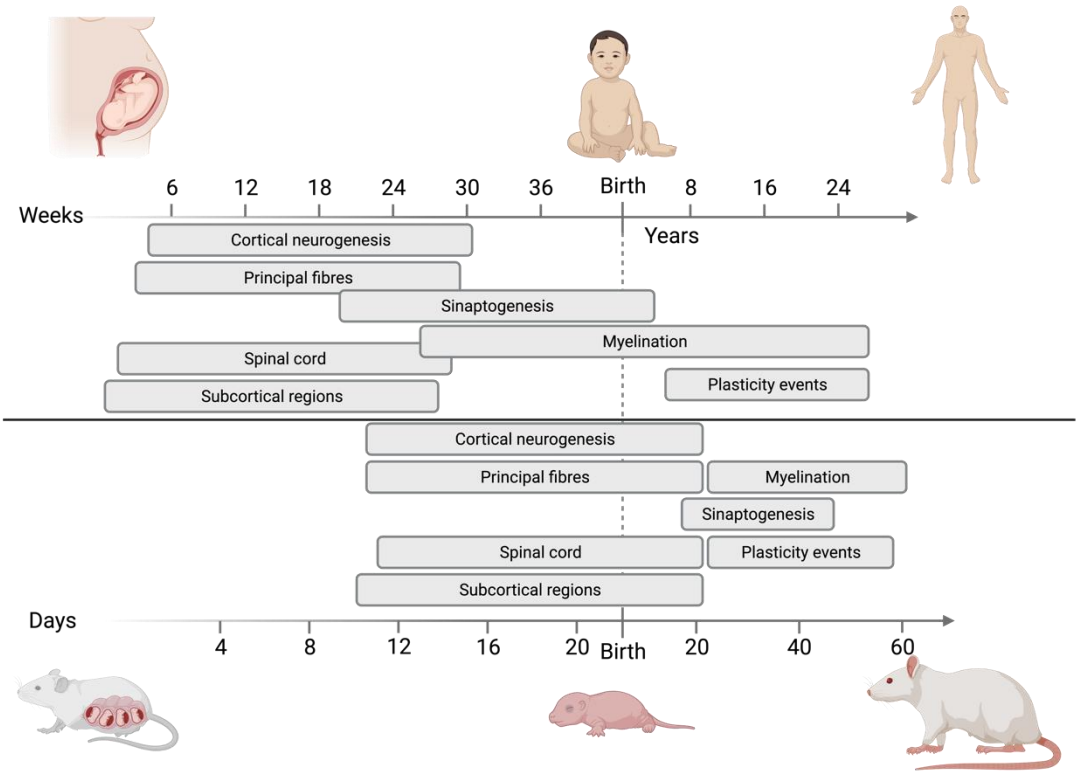


Figure 1.5. Neurodevelopment in humans and rats. Scheme shows the different neurodevelopmental processes that occur both in humans and rats, with their corresponding time windows. Adapted from [48].

During childhood and adolescence, **environmental factors** can significantly impact brain development and contribute to the emergence of mental disorders. Particularly, prenatal infections can increase vulnerability to disruptive factors later in life, potentially leading to the development psychiatric disorders in adulthood, such as schizophrenia or autism [44,48]. Understanding the intricacies of these neurodevelopmental processes and the potential effects of perturbations is crucial for promoting optimal brain development and implementing appropriate interventions to support mental health and well-being.

Cannabis and adolescence

Adolescence is a critical developmental stage characterized by significant physical, cognitive, and emotional changes. During this period, individuals strive to establish their identities and navigate through a variety of social, academic, and personal challenges. It is

also a time when experimentation and **risk-taking behaviours** tend to increase. Consequently, adolescents are particularly vulnerable to the influence of peer pressure and societal factors, including the use of substances like cannabis [33]. Brain maturation is a dynamic process that extends from conception into adulthood, characterized by the interplay of neuronal plasticity in response to environmental stimuli and physiological changes. Different brain regions undergo distinct periods of sensitive development, while synaptic pruning and myelination intensify in the frontal, parietal, and temporal regions during neurocognitive maturation [49]. The PFC, responsible for executive functions, undergoes pronounced synapse overproduction followed by gradual elimination. This period of profound neurodevelopment coincides with **heightened vulnerability to substance use**, although individual factors and socioenvironmental influences exert pivotal roles in shaping patterns of substance use behaviour [50].

The prevalence of cannabis use among adolescents has raised concerns among parents, educators, and healthcare professionals. Numerous studies indicate that cannabis remains one of the most commonly used illicit drugs among adolescents worldwide [1]. The prevalence of cannabis use is higher among adolescents compared to adults, with evaluation starting as early as 10 years old, according to the World Drug Report [1]. The reasons behind **adolescent cannabis consumption** are multifaceted, including curiosity, peer influence, and the desire for novel experiences. Additionally, some adolescents may also turn to cannabis as a form of self-medication for stress, anxiety, or other emotional difficulties they may encounter [51]. Moreover, the increasing acceptance and legalization of cannabis in various regions may contribute to the perception that it is a relatively harmless substance.

However, it is essential to acknowledge that cannabis consumption during adolescence can have profound consequences on physical, cognitive, and mental health. The adolescent brain is going through critical developmental processes, with neural connections and structures still maturing [45]. The presence of cannabinoids can disrupt these processes and potentially alter **brain development**, leading to long-term cognitive impairments. Adolescent exposure to THC can have detrimental effects on various cognitive functions, including memory, attention, and decision-making [52]. In this sense, neuroimaging studies have demonstrated a reduction in the volume of the hippocampus, which is a region closely associated with memory processes, among cannabis users aged 14 to 19 [53]. Moreover, PET studies have revealed the impact of THC consumption during adolescence on brain glucose metabolism, particularly affecting limbic regions that play a significant role in emotions and motivation [54]. Thus, regular cannabis use during adolescence has been associated with impaired attention, decreased IQ, and a higher likelihood of developing mental health

disorders, such as anxiety and depression [55]. In addition, this use during adolescence has been linked to the development of schizophrenia, acting as a stressor, and triggering this psychiatric disorder [56]. In this context, PET studies have provided evidence of increased dopamine levels in cannabis consumers, patients with psychotic disorders and their first-degree relatives, unveiling the connection between cannabis use and psychiatric disorders [57]. This relation has been studied in both clinical and preclinical scenarios, leading to the assumption that the dysregulation of the ECS may play a significant role in the onset of schizophrenia [50].

Schizophrenia, a neurodevelopmental disorder

Schizophrenia is one of the most abundant psychiatric disorders, with a prevalence of almost 1% worldwide with a higher incidence in men compared to women. Symptoms usually appear in early-adulthood, or even late-adolescence, and the exact classification of symptoms is still a subject of debate [43]. Most authors categorize them in two main groups, positive and negative, while others also include cognitive and affective symptoms [44]. **Positive symptoms** mainly include perceptive alterations, such as visual or auditory hallucinations, or delusions, that can significantly impact an individual's perception of reality and contribute to the disruption of their thought processes. On the other hand, **negative symptoms** encompass a loss of motivation, diminished emotional expression (anhedonia), and anxiety. These symptoms often lead to a reduced capacity for functioning and engaging in daily activities. In addition to positive and negative symptoms, cognitive symptoms have gained recognition as an important aspect of schizophrenia. These symptoms encompass deficits in memory, attention, executive functions, and difficulties in processing new information [58]. **Cognitive impairments** can significantly impact an individual's ability to perform tasks, maintain relationships, and lead a fulfilling life. It is worth noting that schizophrenia also has an important impact on individual's health, with a reduction in life expectancy compared to the general population, a higher prevalence of comorbidities, and a greater susceptibility to develop addictions [59].

Schizophrenia is a complex disorder with a **multifactorial aetiology**, making it challenging to prevent. While genetic factors were initially believed to be the sole contributor, it is now understood that multiple factors can influence the aetiology of schizophrenia. The heritability of schizophrenia is estimated to be around 60-80%, with most of the implicated genes associated with protein coding [60]. In addition to genetic factors, various environmental factors have been identified as potential contributors to the development of schizophrenia and other neurodevelopmental disorders, such as depression or anxiety [48]. As mentioned earlier, **cannabis consumption during adolescence** has emerged as a significant environmental factor that may increase the risk of developing psychiatric disorders, including schizophrenia,

in adulthood. However, prenatal infections have also been found to play an important role [43], [44]. In this regard, the use of preclinical models is a valuable tool to further investigate the mechanism underlying this disorder.

Preclinical neurodevelopmental models and schizophrenia

Preclinical models play a central role in advancing research, although they have inherent limitations that prevent them from fully replicating the complexity of human conditions and processes. Psychiatric disorders have a high social component, that cannot be reproduced in preclinical models for obvious reasons [61]. In addition, certain symptoms experienced by individuals with schizophrenia, such as hallucinations or other positive symptoms, are impossible to measure in animals. In the case of schizophrenia, no preclinical model can fully mimic the entire symptomatology of the disorder, but they can capture specific aspects of it [62].

The validity of preclinical models is based on three main foundations [63]. The first foundation is **face validity**, which refers to the extent to which a model exhibits characteristics or symptoms that resemble those observed in humans. The second foundation is **predictive validity**, which assesses the ability of a preclinical model to accurately predict the effects of therapeutic interventions or experimental manipulations. This involves testing weather the responses observed in the preclinical model agree with the expected responses seen in humans. The third foundation is **construct validity**, which focuses on the level of similarity in the biological dysfunctions obtained in the animal model and those observed in humans, which is based on how well the aetiology of the disease is understood. By meeting the three validity foundations, preclinical models provide researchers the opportunity to investigate the underlying mechanisms, test potential therapeutic interventions, and gain a deeper understanding of the disorder [63].

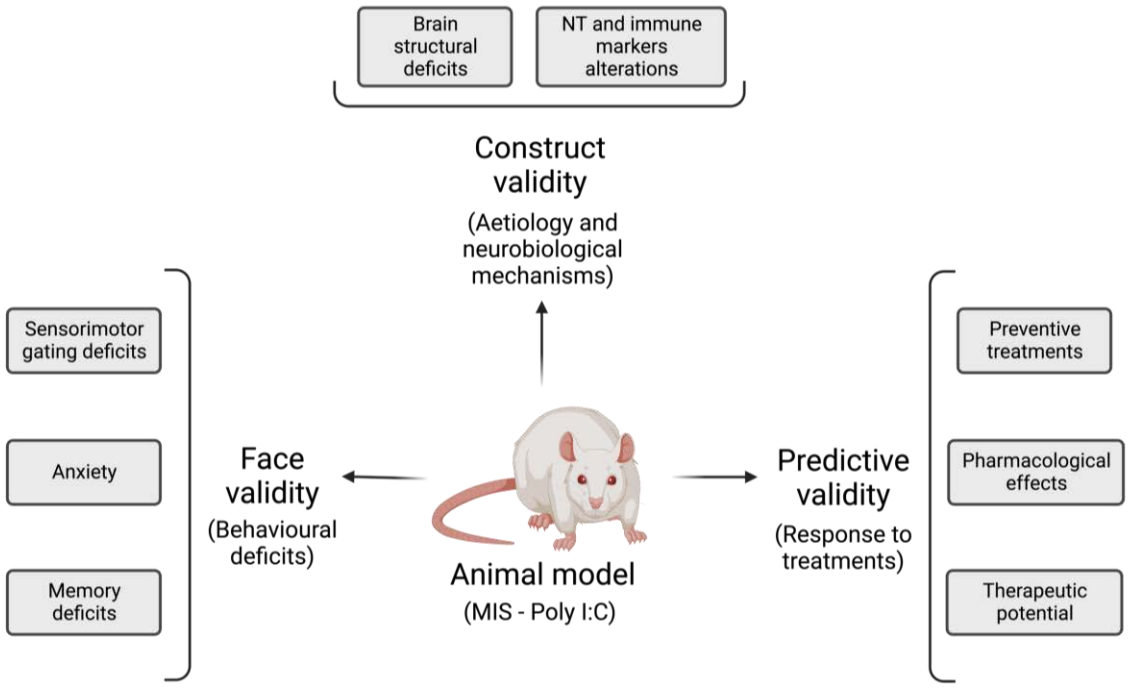


Figure 1.6. MIS model validity foundations. Representation of the three foundations for the validity of preclinical models exemplified in the maternal immune stimulation (MIS) model.

In this context, several preclinical models have been developed for the study of schizophrenia, including **genetic, pharmacological, and neurodevelopmental models**. Genetic models are generally based on the manipulation or deletion of a gene associated with schizophrenia. One of the most commonly used genetic models involves the deletion of the DISC-1 gene [62], which is involved in the expression of several synaptic proteins. Other relevant genes that could be used for modelling schizophrenia are 2q11.2 [64] and neuregulin-1 [65], which are associated with different behavioural and neuroanatomical alterations related to schizophrenia. Pharmacological models, on the other hand, use different drugs that can induce specific functions or symptoms relevant to schizophrenia. Ketamine [66] and MK-801 [67] are commonly used drugs in pharmacological models of schizophrenia due to their ability to produce psychotic-like effects. Neurodevelopmental models are based on environmental factors that can disrupt the proper neurodevelopment in specific time windows of vulnerability for the brain [68]. Some environmental factors used in these models include stressful situations, inflammatory processes, or drug exposure. As mentioned before, there are several stages of neurodevelopment whose interruption could have long-term implications. In this sense, stressors can be produced during gestation, childhood, or adolescence.

The importance of inflammation and oxidative stress

While there is not an absolute consensus on the exact mechanisms underlying the disruption of neurodevelopmental processes, most authors suggest that both **inflammation and oxidative stress (IOS)** play a crucial role in this phenomenon [69]. Inflammation is a biological defensive mechanism that aids in the initial repair of damage. However, persistent and intense inflammation can have adverse effects. It is closely interconnected with other mechanisms, including oxidative stress and apoptosis [70]. Dysregulation of these processes over time, can lead to detrimental responses and contribute to the schizophrenia-associated symptomatology.

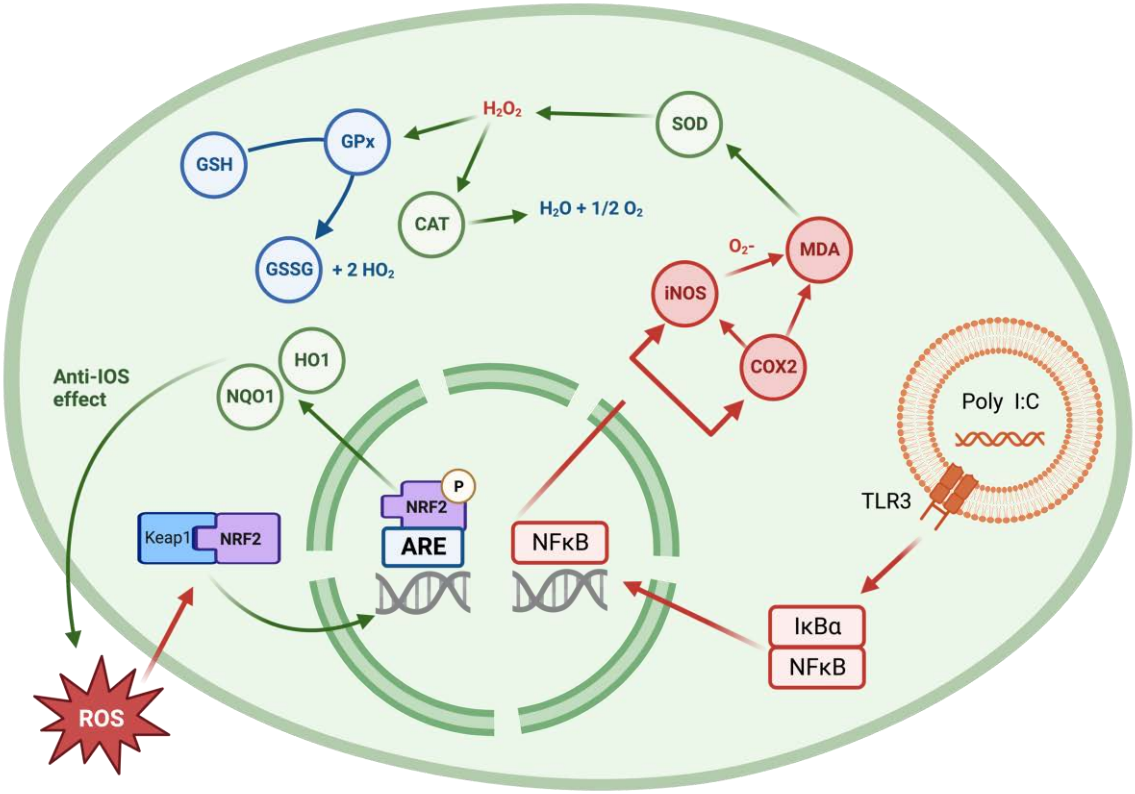


Figure 1.7. Inflammatory and oxidative stress markers after Poly I:C administration. Schematic representation of the anti-IOS and pro-IOS cellular pathways after PolyI:C viral signalling. PolyI:C is recognised by endosomal TLR3, and targets IκBα for degradation thus releasing NF-κB dimers that translocate to the nucleus, activating the transcription of inflammatory and oxidonitrosative mediators, including iNOS and COX2. This leads to an accumulation of ROS. The resultant oxidative damage is evidenced by the increase in lipid peroxidation products, such as MDA. Under oxidative stress, Keap1 oxidates and suffers a conformational change, preventing its binding to NRF2. NRF2 is phosphorylated and translocated to the nucleus, where it binds to ARE, inducing the synthesis of the enzymes NQO1, HO1, SOD, CAT and GPx, among others. These antioxidant enzymes transform free radicals into harmless molecular products, hence scavenging the amount of ROS and helping to maintain the oxidative stress balance. Abb.: ARE: antioxidant response elements; iNOS: inducible nitric oxide synthase; CAT: catalase; COX2: cyclooxygenase 2; Keap1: Kelch-like ECH-associated protein 1; GPx: glutathione peroxidase;

GSH: ; glutathione GSSG: glutathione disulfide; HO1: heme oxygenase 1; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; MDA: malondialdehyde; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NQO1: NAD(P)H dehydrogenase [quinone] 1; NRF2: nuclear factor erythroid 2-related factor 2; ROS: reactive oxygen species; SOD: superoxide dismutase; TLR3: toll-like receptor 3. Adapted from [71].

The involvement of uncontrolled inflammation and oxidative stress in the pathogenesis of this disorder is supported by the dysregulation of various markers, both inflammatory and oxidative, observed in patients at different stages of the disorder. While the precise mechanisms through which these processes contribute to the manifestation of schizophrenia symptoms are not fully understood, emerging evidence suggests a significant role of microglia [72]. These cells respond to harmful stimuli by producing inflammatory cytokines, and then anti-inflammatory compounds to restore the proper homeostasis when the stressful situation ends. However, under prolonged stress, microglia may continue to generate reactive oxygen species (ROS), inflammatory molecules, including inducible nitric oxide synthase (iNOS) or cyclooxygenase 2 (COX2), and oxidative stress mediators, such as the ionized calcium binding adaptor molecule 1 (Iba1) or Kelch-like ECH-associated protein 1 (Keap1), among others [69,73,74]. In this sense, increased levels of IOS markers have been observed in post-mortem brain samples of patients with schizophrenia [75]. Additionally, different preclinical schizophrenia-like models have shown similar alterations in these markers [76].

Maternal immune stimulation (MIS) model

The MIS model is based on the association between infections during the prenatal period and the long-term development of symptoms associated with neurodevelopmental disorders, including schizophrenia. Gestational infection may be mimicked by immunological stimulation of the dam with immune-stimulating agents, such as **polyinosinic:polycytidylic acid (Poly I:C)** or lipopolysaccharide (LPS) [44]. Both immunostimulants induce a significant rise in body temperature for a few days after the treatment and increase anxiety levels in treated animals [77]. Administration of Poly I:C or LPS during gestation leads to the release of pro-inflammatory cytokines, that can cross the placental barrier and affect the developing foetuses, thereby increasing the risk for the future development of psychiatric symptoms. The timing when the insult is performed during gestation has been used to induce different immune responses in the dam, which have been associated with **various neurodevelopmental disorders**, such as autism [78] or schizophrenia [44]. In mice, the most commonly employed window for inducing schizophrenia-like symptoms is early gestation, typically between 8.5 and 9.5 days of gestation. In rats, the preferred timing is mid-gestation, typically between 14.5 and 15.5 days [48].

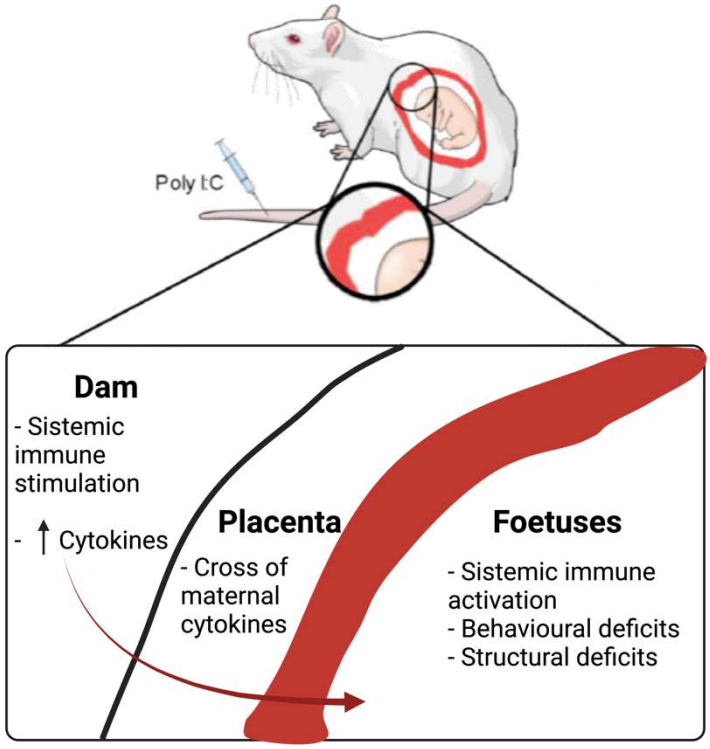


Figure 1.8. The maternal immune stimulation model. Effects of the Poly I:C administration on the dam and its foetuses. The stimulation of the immune system by the administration of Poly I:C release cytokines and interleukins that cross the placenta and stimulate the foetus immune system. Adapted from [79].

Poly I:C is a double-stranded RNA that is used experimentally to model viral infections *in vivo*. Poly I:C interacts specifically with Toll-like 3 receptors (TLR3), triggering a robust immune response with a high involvement of the interferon pathway. TLR3 receptors are involved in innate immune responses in mammals, triggering a **pro-inflammatory response** that involves the activation of signalling pathways mediated by Interferon Regulatory Factor (IRF3) and NF-κB [80]. This signalling cascade ultimately results in the production and release of pro-inflammatory cytokines that can reach different cellular targets, including neurons and glia cells. The immune response induced by Poly I:C, mediated through TLR3, resembles the immune response triggered by certain viruses, such as influenza or herpes simplex virus in humans. It has been observed that infection during gestation with these viruses is associated with the increased risk of neurodevelopmental disorders like schizophrenia [43,44,68]. If the peak of the immune response to this infection occurs during gestation, as is the case in the Poly I:C-induced model, there is a potential for excessive pro-inflammatory cytokines to cross the placental barrier and interact with the foetuses. During this critical period of development, the foetuses' immune system is still maturing and may not be adequately equipped to handle an acute pro-inflammatory environment. This disruption can upset the delicate balance of cytokines in the developing brain at an early stage.

The administration of Poly I:C can elicit a broad spectrum of behavioural, neuroanatomical, and molecular deficits in the offspring [68]. At the behavioural level, MIS animals exhibit impairments in prepulse inhibition (a measure of sensorimotor gating), social behaviours, and memory [71]. Additionally, increased anxiety-like behaviours, hyperactivity, and symptoms associated with depression have been observed. Regarding brain morphology, MIS offspring showed reductions in cortical and hippocampal volume, along with enlarged ventricles [81]. Alterations in white matter integrity and neurogenesis have also been observed [82]. Other studies have reported increased microglial activity, consistent with the model's genesis and the corresponding human disorder, as well as alterations in dopaminergic, glutamatergic, and GABAergic neurotransmission [83]. Lastly, at the molecular level, notable changes involve upregulation of pro-inflammatory cytokines in distinct brain regions [44].

However, given the inherent risk nature of this neurodevelopmental model, researchers have sought to explore additional environmental factors that could further increase this risk of developing mental disorders, thereby strengthening this animal model [62]. This has led to the use of combinations of multiple environmental risk factors to enhance the deficits observed in the MIS model, which can sometimes be subtle. Some of these additional risk factors include stress induction through maternal deprivation or THC exposure during adolescence [62].

Motivation

Given this background, it seems obvious that there’s still a large gap regarding the effects of the different cannabinoids, either harmful, like THC, or beneficial, like CBD. This gap is particularly important when the consumption happens during adolescence, as the harmful effects can be even more detrimental in this phase. However, there are some evident difficulties when studying this subject in clinical research. In this sense, preclinical studies could play a significant role, as long as they meet the validation requirements. Additionally, in vivo functional neuroimaging studies, as the ones used in this Thesis, are becoming an essential tool for the evaluation of different models, are becoming an invaluable tool for the examination of brain functionality alterations resulting from pharmacological manipulations. By employing PET, researchers can investigate, depending on the tracer used, the brain metabolism, or the dynamic changes in neurotransmitter systems associated with drug abuse and withdrawal states. This technique enables the non-invasive assessment of neurochemical and functional alterations in key brain regions, shedding light on the underlying neurobiology of addictive behaviours.

As far as we know, the studies presented in this Thesis were the first to evaluate different preclinical models of psychiatric disorders using these useful techniques and combining diverse methodologies in order to perform a wide analysis of the field of study.

· Hypothesis and objectives ·

· Hypothesis and objectives ·

Thus, this thesis aims to investigate the effects of different cannabinoids in two preclinical models of psychiatric disorders, specifically focusing on addiction and schizophrenia. Therefore, our hypotheses are: 1. Drug addiction vulnerability may be associated with different pattern of brain glucose metabolism. 2. Administration of a cannabinoid agonist during adolescence will impact on the endocannabinoid system and, consequently will induce different sensitivity to other drugs at adulthood. In addition, these changes may differ between males and females. 3. Administration of THC during adolescence will exacerbate the schizophrenia-like abnormalities induced by the MIS challenge, while CBD will prevent some of these abnormalities.

The specific objectives are linked with the three chapters of this thesis:

Chapter I.

1. To evaluate the effects of morphine self-administration on behaviour in two different rat strains with different vulnerability to drugs.

2. To assess the effects of morphine self-administration on brain glucose metabolism in two different rat strains with different vulnerability to drugs.

Chapter II.

1. To evaluate the behavioural effects of morphine self-administration in male and female Wistar rats that were pre-exposed to a cannabinoid agonist during adolescence.

2. To study the short- and long-term effects of morphine self-administration on brain glucose metabolism in male and female rats pre-exposed to a cannabinoid agonist during adolescence.

3. To examine the long-term effects of morphine self-administration on the endocannabinoid system in male and female rats pre-exposed to a cannabinoid agonist during adolescence.

Chapter III.

1. To evaluate the association between THC consumption during adolescence and the exacerbation of schizophrenia-like abnormalities in the MIS model

2. To assess the potential preventive effects of CBD on schizophrenia-like abnormalities in the MIS model.

Each one of these objectives has been thoroughly addressed in the three articles presented within this thesis.

· Chapter I ·

Neuroimaging reveals distinct brain glucose metabolism patterns associated with morphine consumption in Lewis and Fischer 344 rat strains

This chapter includes the following journal publication:

M.L. Soto-Montenegro, V. García-Vázquez, N. Lamanna-Rama, G. López-Montoya, M. Desco, E. Ambrosio, 'Neuroimaging reveals distinct brain glucose metabolism patterns associated with morphine consumption in Lewis and Fischer 344 rat strains', *Sci. Rep*., 12(1):4643, Mar 17, 2022 doi: 10.1038/s41598-022-08698-9. PMID: 35301397; PMCID: PMC8931060.

· Neuroimaging reveals distinct brain glucose metabolism patterns associated with morphine consumption in Lewis and Fischer 344 rat strains ·

Abstract

Vulnerability to addiction may be given by the individual's risk of developing an addiction during their lifetime. A challenge in the neurobiology of drug addiction is understanding why some people become addicted to drugs. Here, we used positron emission tomography (PET) and statistical parametric mapping (SPM) to evaluate changes in brain glucose metabolism in response to chronic morphine self-administration (MSA) in two rat strains with different vulnerability to drug abuse, Lewis (LEW) and Fischer 344 (F344). Four groups of animals were trained to self-administer morphine or saline for 15 days. 2-deoxy-2-[18F]-fluoro-d-glucose (FDG)-PET studies were performed on the last day of MSA (acquisition phase) and after 15 days of withdrawal. PET data were analyzed using SPM12. LEW-animals self-administered more morphine injections per session than F344-animals. We found significant brain metabolic differences between LEW and F344 strains in the cortex, hypothalamus, brainstem, and cerebellum. In addition, the different brain metabolic patterns observed after the MSA study between these rat strains indicate differences in the efficiency of neural substrates to translate the drug effects, which could explain the differences in predisposition to morphine abuse between one individual and another. These findings have important implications for the use of these rat strains in translational morphine and opiate research.

Introduction

Addiction vulnerability is the individual's risk of developing an addiction during their lifetime. Clinical and preclinical studies have shown that among the biological factors involved in this disorder, genetic variability plays an important role in humans [1–4], accounting for at least 40–60% of the variation in vulnerability to drug dependence [5]. In this regard, the use of inbred rats has proven to be a valuable tool for identifying differences in vulnerability to drug addiction. The Lewis (LEW) and Fischer 344 (F344) inbred rat strains have been the most widely used in modeling genetic vulnerability to drugs. LEW and F344 rats differ with respect to drug self-administration. Consequently, LEW rats more readily self-administer drugs, such as alcohol, opiates, and cocaine, than F344 rats [6–13]. At the neurochemical level, F344 rats have high basal levels of tyrosine hydroxylase (TH) protein, proenkephalin, dopamine transporter, glutamate, and μ-opioid receptors in the nucleus accumbens (NAcc) [14,15]. F344 rats also have high levels of μ-opioid receptors in the striatum, lateral globus pallidus, basolateral and lateral amygdaloid nucleus, periaqueductal gray matter (PAG), substantia nigra, and locus coeruleus measured by autoradiography [11]. LEW rats have lower active dopaminergic neurons in the ventral tegmental area (VTA) [16] but greater increases in

extracellular dopamine in ventral striatum and lower levels of dopamine metabolites than F344 rats [17]. In addition, LEW rats have high basal levels of TH protein and dopamine D1 and NMDA receptors in the VTA [12,18–20], as well as greater activity of the μ-opioid receptor in the nucleus accumbens, septal nuclei, thalamus, VTA, raphe nuclei, and locus coeruleus [11]; but lower basal levels of glutamate and GABA in the NAcc [21].

Furthermore, both strains show differences in the reactivity of the hypothalamic-pituitary-adrenocortical axis, such as reduced corticosterone responses to stress and morphine in LEW-animals among others [22,23]. Recently, genetic factors have been involved in the control of the vulnerability to drugs of abuse, with differences in the transcription of NGFI-B and Nor1 in the caudate-putamen, involved in the control of behaviors [24], or signalling pathway of mTOR (Raptor and Eif4ebp2 expression) that has an important role in the long-lasting neuroadaptations that occur on the progression of addictive behaviour in the amygdala [13].

In line with these observations, functional neuroimaging techniques, such as positron emission tomography (PET), single-photon emission computed tomography, functional magnetic resonance imaging (fMRI), and electro-encephalography, have been widely used to demonstrate the emotional and cognitive-behavioral components of addiction [25,26], as well as to study the neurotoxic effects of drugs in the brain [27–34]. In this respect, PET with 2-deoxy-2-[18F]-fluoro-d-glucose (FDG) has traditionally been the most commonly used technique for direct quantification of regional brain glucose metabolism in clinical and preclinical studies [35–40]. It constitutes an invaluable tool for investigating the in vivo changes in brain metabolism as a result of pharmacological manipulation. Given that the LEW and F344 strains show differential behaviours that model addiction and therefore different vulnerability to drugs, our working hypothesis is that the metabolism of morphine in reward areas of the brain would be higher in the LEW strain than in the F344 strain. As far as we know, this is the first study to examine the effects of morphine self-administration on glucose metabolism using in vivo FDG-PET and statistical parametric mapping (SPM) in two rat strains with different vulnerability to drug abuse.

Methods

Animals

Thirteen male F344 and fourteen LEW inbred rats (250–300 g) were obtained from the animal facility of Universidad Nacional de Educación a Distancia (UNED). Animals were housed individually when they reached post-natal day 75 (PND75) to leave sufficient time between individual housing and brain metabolic studies and thus avoid any non-specific effects of isolation stress on the metabolic measurements. All animal procedures were conducted in

conformity with Directive 2010/63/EU of the European Parliament and of the Council, the ARRIVE guidelines, and approved by the Ethics Committee for Animal Experimentation of UNED and Hospital Gregorio Marañón (number ES280790000087).

Drug administration and experimental protocol

Figure 1A shows the drug treatment and the design of the study. Twelve operant chambers (Coulbourn Instruments, Allentown, PA, USA) were used for the operant food-reinforced behaviour and morphine self-administration studies. A lever designed to register a response to 3.0 g of force was placed on the front wall of the chamber [11]. Food and morphine operant data were acquired and stored on IBM computers (Med Associates, PA, USA).

Saline or morphine sulphate self-administration (1 mg/kg/injection in 12 h daily sessions) was studied with a fixed-ratio 1 (FR1) schedule of reinforcement for 15 days [13].

Animals were divided into four groups based on the factors studied: rat strain (LEW and F344) and drug treatment (morphine and saline). Sample size of the groups were: 1) LEW-saline (n = 6); 2) LEW-morphine (n = 8); 3) F344-saline (n = 6); and 4) F344-morphine (n = 7).

Fixed-ratio1 food-reinforced behavior. This experimental procedure was performed at PND75. The FR1 schedule of food reinforcement is a commonly used behavioral procedure to determine whether a drug is reinforcing or not [41]. To do this, animals are trained and learn the association between behavior (pressing the lever) and response (pellet reward). Before training begins, animals were food deprived to 95%-90% of their free-feeding weight and submitted to an FR1 schedule of food reinforcement in which a single press of the lever turned on a light stimulus above the lever that signalled pellet delivery and reward availability (45 mg; Noyes Pellets, USA). Each pellet delivery was followed by a 30-s timeout period in which responses had no scheduled consequence (FR1: TO 30 s). This food-reinforced behavior was acquired over 5 days (30 min each day). After this phase, the animals had ad libitum access to food and were able to recover their free-feeding weight.

Morphine self-administration (MSA). When the response rate was sufficient (more than 50 responses per session) and stable (less than 10% variation across three consecutive sessions), animals were operated with an intravenous catheter in the right jugular vein. Polyvinylchloride tubing (0.064 i.d.) was implanted approximately at the level of the atrium under anesthesia with ketamine (40 mg/kg, i.p.) and diazepam (10 mg/kg, i.p.). The catheter was tunnelled and located in the midscapular region; it then passed through a spring tether system (Alice King, Chatham, CA, USA) that was mounted on the skull of the rat with dental cement. Animals were given at least five days to recover from the surgery, and the catheters were flushed daily with 0.5 ml of an antibiotic solution (gentamicin 40 mg/ml) dissolved in heparinized saline to prevent infection and to maintain catheter patency.

The drug-reinforced behaviour study was performed during the dark cycle since rodents are nocturnal animals, coinciding with their period of greatest activity. Animals did not have free access to food during MSA protocol. It involved two phases: 1) The acquisition phase, which started at PND100 and consisted of sessions lasting 12 h per day (starting at 8:00 pm) with morphine sulphate (1 mg/kg, dissolved in saline [0.9% NaCl] solution) or saline alone under an FR1 schedule of reinforcement for 15 consecutive days; and 2) The withdrawal phase, where the drugs were discontinued for 15 consecutive days. During acquisition sessions, one active lever press resulted in morphine/saline infusion delivered over 10 s and followed by a 10-s time-out. A light cue located above the active lever indicated the availability of the drug; this was turned off only during drug delivery, time out, and at the end of each session. A limit of 50 infusions per session was set in order to avoid overdosing.

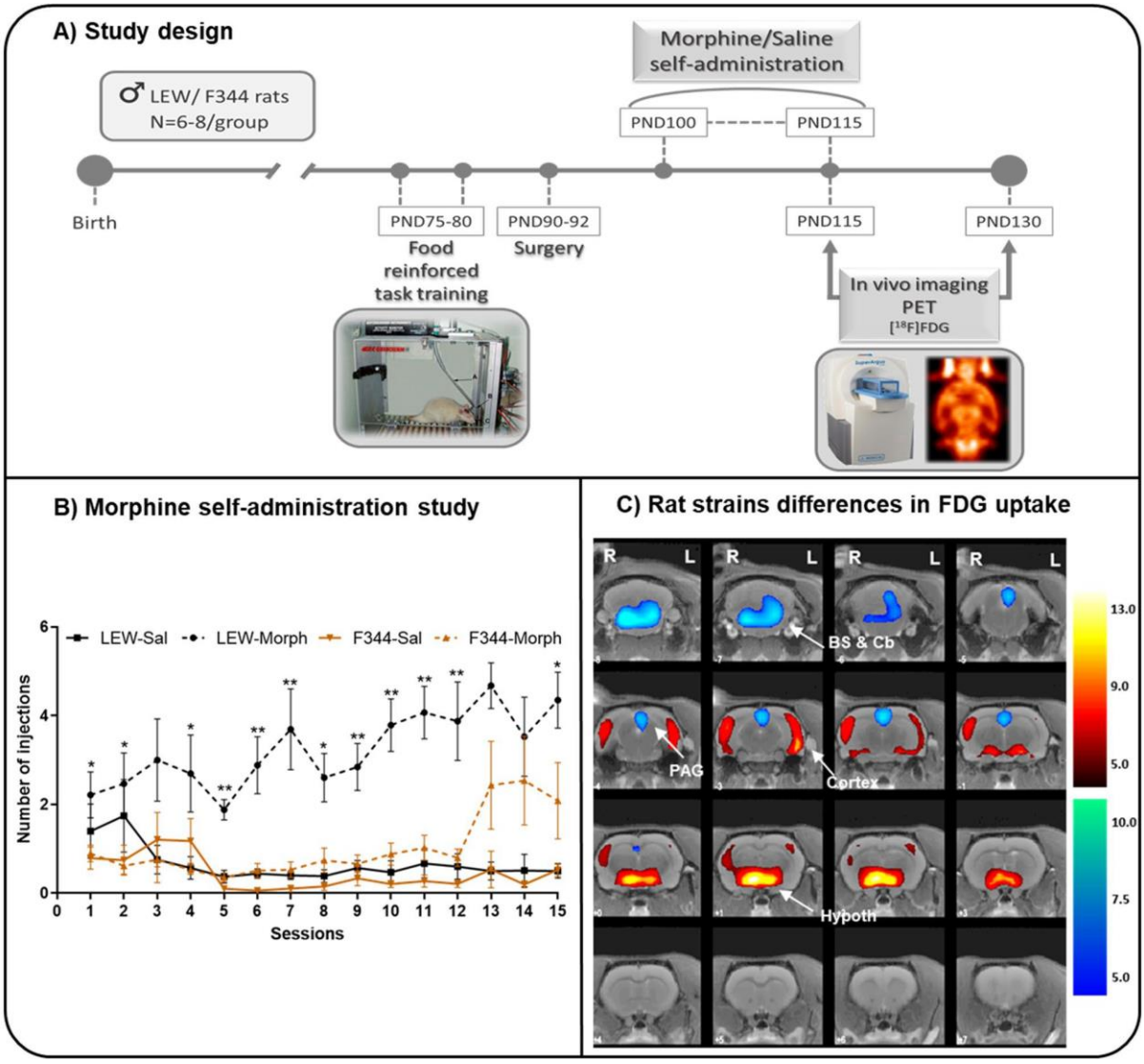


Figure 1. Study design, behavioral study and differences in brain glucose metabolism between rat strains. (A) Representative diagram of the chronology of the experimental procedures performed during the study according to the age of the animals. Abbrev.: 18FDG, [18F]-Fluorodeoxyglucose; PET, positron emission tomography. (B) MSA study in LEW and F344 rats. Morphine (1 mg/kg) or saline self-administration in adult

LEW and F344 rats under an FR1 schedule of reinforcement. The values are expressed as the mean ± SEM: LEW-saline (n = 6), LEW-morphine (n = 8), F344-saline (n = 6), and F344-morphine (n = 7). The number of morphine injections per session was greater for LEW animals than for F344 animals. 3-way ANOVA followed by Bonferroni's multiple comparisons test [*p < 0.05 and **p < 0.01 vs saline animals]. (C) PET results in T-maps overlaid on aT2-MRI reference showing increased FDG uptake (hot colors) or decreased FDG uptake (cold colors) in Saline-LEW animals compared with Saline-F344 animals in the first scan (PND 115). Similar results were obtained for the second scan (PND 130) (data not shown). Saline-LEW animals showed higher FDG uptake in the hypothalamus and the cerebral cortex and lower FDG uptake in the brainstem, cerebellum and PAG than Saline-F344 animals. Statistics corrected for multiple comparisons (FWE, p < 0.05). Region of interest (BS: brainstem, Cb: Cerebellum, Hypoth: hypothalamus, PAG: periaqueductal gray matter). Side: left (L) and right (R). k: cluster size, T: Student t. FDG uptake: increase (↑) and decrease (↓). p: p value (unc: uncorrected, FWE: family-wise error).

FDG-PET imaging studies

PET images were acquired on two separate days: the first session was at the end of the MSA phase (−acquisition phase−) and the second session was at the end of the withdrawal phase.

Imaging was performed using a dedicated small animal PET scanner (rPET, SUINSA Medical Systems, Madrid). 2-deoxy-2-[18F]fluoro-d-glucose (FDG: 73.63 ± 8.88 Mbq) was administered as a regular injection through the catheter inside the jugular vein, and, after an uptake period of 35 min, the animals were imaged for 60 min under isoflurane anesthesia (5% for induction and 1–1.5% for maintenance in 100% O2).

Tomographic images were reconstructed with a 3D filtered back projection (3D-FBP) algorithm [42] using a 12th-order Butterworth filter at a 35% Nyquist frequency cut-off. The FOV of our system was 68 mm transaxial and 47 mm axial. The trans-axial and axial resolutions of the PET scanner were 1.65 mm and 1.9 mm full width at half-maximum (FWHM), respectively. The voxel size of the reconstructed images was 0.81 × 0.81 × 0.81 mm3, the energy window was 400–700 keV, and decay, alignment, normalization, and deadtime corrections were applied. The decay correction was based on the theoretical half-life of the radiosotope used. The normalization correction was based on the acquisition of an uniform and homogeneous phantom consisting of a cylinder of 68Ge of the appropriate size for the FOV system And finally, the alignment and deadtime corrections were based on periodic calibrations of the equipment according to the manufacturer's instructions [43].

Magnetic resonance study

An MRI study of one animal at PND100 was acquired with a 7-Tesla Biospec 70/20 scanner (Bruker, Ettlingen, Germany). A 1H linear volume coil from Bruker was used for an homogeneous excitation of the sample and a 1H receive-only 2 × 2 rat brain array surface coil

from Bruker was used for the reception of the signal. The animal was anesthetized with sevoflurane (4.5% for induction and 2.5% for maintenance in 100% O2) and placed on a stereotactic device to prevent movement during the acquisition. A T2-weighted spin echo sequence was acquired, with TR = 4062 ms, TE = 33 ms, flip angle = 90°, RARE factor = 8. FOV = 3.7 × 3.7 cm, matrix size = 256 × 256, slice thickness = 0.8 mm (37 slices). The inhomogeneity of magnetic field caused by the surface antenna was corrected.

This MRI study was only used as an anatomical template in order to display the results of the statistical analysis, since PET imaging has a lower anatomical resolution. This MRI template corresponds to a male Wistar rat with the same age and weight as the F344 and LEW animals, with no anatomical differences between then since they are inbred rats of the Wistar strain.

Data analysis

Morphine self-administration. The statistical analysis of behavioral data consisted in a repeated-measures three-way ANOVA including the strain (LEW vs F344), treatment (morphine vs saline), and repetition time as factors. The average number of self-administered injections was also determined and analyzed using two-way ANOVA followed by a post hoc Bonferroni correction.

PET: statistical parametric mapping analysis. PET image post-processing and intensity normalization were performed following protocols previously described by our group [44,45]. Briefly, the reconstructed images were spatially registered using rigid transformations with an automatic algorithm based on mutual information [46]. All PET data were smoothed with a 2-mm FWHM isotropic Gaussian kernel. A brain mask was manually segmented onto the MRI template and applied to the PET studies to remove extracerebral voxels and to ensure that only voxels mapping brain tissue were included in the analysis. Image intensity was normalized to the brain average value (100%).

The statistical analysis was performed using SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/) and consisted in the analysis of variance (ANOVA) of three fixed factors with repeated measures on one of them: strain (inter-subject factor; levels: LEW, F344), condition (inter-subject factor; levels: saline, morphine), and time (within-subject factor, levels: at the end of the MSA phase, at the end of the withdrawal phase). Results were considered significant at a threshold of $p < 0.01$, uncorrected at the voxel level, but cluster-based–corrected by Family-Wise Error (FWE) in order to avoid a type II error. Only strain differences were significant for multiple comparisons using the family-wise error (FWE) rate with a significance level of $p < 0.05$. A 10-voxel clustering (spatial-extent) threshold was

also applied to reduce the possibility of a type I error; therefore, significant regions smaller than ten adjacent activated voxels were not admitted.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Results

Morphine self-administration

The three-way ANOVA revealed a statistically significant effect of the strain ($p < 0.01$), treatment ($p < 0.001$), and time ($p < 0.001$). We also found an interaction for strain × treatment ($p < 0.001$) and time × treatment ($p < 0.001$). Thus, the number of morphine injections per session was greater for LEW animals than for F344 animals ($p < 0.01$) (Fig. 1B). In addition, the average number of self-administered injections over the 15 sessions was greater in the LEW-morphine (48.58 ± 6.71) group than in the LEW-saline (9.90 ± 3.32) and F344 rats (morphine: 15.36 ± 4.40; saline: 6.66 ± 1.79). From almost the beginning, LEW-morphine animals showed an increasing learning curve that was maintained asymptotically the rest of sessions. In contrast, F344 animals started an increasing learning curve in the last 3 sessions.

PET: Statistical Parametric Mapping analysis

Table 1 shows the differences in brain glucose metabolism between rat strains, and the brain metabolic changes after the MSA study.

SPM analysis revealed a significant effect of the rat strain with higher metabolism in the hypothalamus and the cerebral cortex and lower FDG uptake in the brainstem (locus coeruleus) and PAG in the Saline-LEW animals than in the Saline-F344 animals (Fig. 1C). Similar results were obtained for the first (PND 115) and second scan (PND 130) (data not shown).

TABLE 1. Brain metabolic changes in F344 and LEW animals after MSA study and between rat strains

		1. Brain metabolic changes after the MSA study														
		(A) ACQUISITION PHASE							(B) WITHDRAWAL PHASE							
		ROI	Side	t-value	p_{unc} peak	p_{FWE} peak	P_{FWE} cluster	K	ROI	Side	t-value	p_{unc} peak	p_{FDR} peak	P_{FDR} cluster	K	
F344	↑	Cortex	R	4.42	<0.001	0.128	0.053	129	Cortex	L	4.28	<0.001	0.160	0.197	67	
			L	13.08		0.145	0.248	57	Cb	–	4.05		0.230	0.254	56	
	↓	MC	–	4.66	<0.001	0.085	0.216	63	RSC-MC	–	5.00	<0.001	0.046	0.055	127	
		Pir C	R	4.03		0.237	0.700	13								
			L	3.62	0.001	0.421	0.314	47								
LEW	↑	Cb	–	3.57	0.001	0.452	0.272	53	Cb		3.54	0.001	0.467	0.079	109	
									Pir C	R	5.32	<0.001	0.026	0.361	41	
										L	3.70	0.001	0.380	0.505	27	
	↓	SSC-Th-CC	L	4.37	<0.001	0.137	0.076	111	C-Th-Hipp-CP-CC	R	4.63	<0.001	0.089	<0.001	<0.001	
										L	5.09		0.009			
		2. Differences in brain glucose metabolism between rat strains														
		ROI	Side	t-value	p_{unc} peak	p_{FWE} peak	P_{FWE} cluster	K		ROI	Side	t-value	p_{unc} peak	p_{FWE} peak	P_{FWE} cluster	K
	↑	Hypoth	–	13.08	<0.001	<0.001	<0.001	420	↓	BS & Cb	–	9.72	<0.001	<0.001	<0.001	257
		Cortex	R													
			L	9.72	<0.001	<0.001	<0.001			PAG	–	7.49	<0.001	<0.001	<0.001	

Table 1. (1) Brain metabolic changes in F344 and LEW animals after MSA study. The comparison shows differences in glucose brain metabolism between morphine-treated animals and saline-treated animals, in the acquisition phase (1.A) and the withdrawal phase (1.B). (1.A) F344-morphine animals showed higher FDG uptake in the cortical area and lower FDG uptake in the motor and piriform cortex than the F344- saline animals. LEW-morphine animals showed lower FDG uptake in the somatosensorial and cingulate cortex and the thalamus and higher FDG uptake in the cerebellum than the LEW-saline animals. (B) F344- morphine animals showed higher FDG uptake in the left cortex and cerebellum and lower FDG uptake in the restrosplenial and motor cortices than the F344-saline animals. LEW-morphine animals showed higher FDG uptake in the cerebellum and piriform cortex and lower FDG uptake in the cortex, thalamus, hippocampus, and caudate putamen than the LEW-saline animals. (2) Differences in brain glucose metabolism between LEW-Sal animals and F344-Sal animals. Saline-LEW animals showed higher FDG uptake in the hypothalamus and the cerebral cortex and lower FDG uptake in the brainstem, cerebellum and PAG than Saline-F344 animals. ROI: Region of interest. Side: left (L) and right (R). k: cluster size, T: Student t. FDG uptake: increase (↑) and decrease (↓). p: p value (unc: uncorrected, FWE: family-wise error). Abbrev.: BS: brainstem; C: cortex; Cb: cerebellum; CC: cingulate cortex; CP: caudate-putamen; Ent C: entorhinal cortex; Hipp: hippocampus; Hypoth: hypothalamus; PAG: periaqueductal gray matter; MC: motor cortex; Pir C: piriform cortex; RSC: retrosplenial cortex; SSC: somatosensorial cortex; Th: thalamus].

During the drug acquisition phase, F344-morphine animals showed higher FDG uptake in the cortical area and lower FDG uptake in the motor and piriform cortex than the F344-saline animals (Fig. 2A). LEW-morphine animals showed lower metabolism in the somatosensorial and cingulate cortex and the thalamus and higher FDG uptake in the cerebellum than the LEW-saline animals (Fig. 2A).

During drug withdrawal, F344-morphine animals showed higher metabolism in the left cortex and lower FDG uptake in the restrosplenial and motor cortices than the F344-saline animals (Fig. 2B). LEW-morphine animals showed higher metabolism in the cerebellum and piriform cortex and lower FDG uptake in the cortex, thalamus, hippocampus, and caudate putamen than the LEW-saline animals (Fig. 2B).

Discussion

To our knowledge, this is the first report to show that brain glucose metabolism differs from strain to strain, and that this finding may account, among others, for the differences in morphine self-administration (MSA) between LEW and F344 animals.

One of the core traits shown by rat strains with differential vulnerability to drug addiction is the capacity to self-administer more drug than other rat strains [1,4,47,48]. According to this, drug intake is very high in some vulnerable animals and very low in other resistant ones [48]. In this regard, LEW rats are more likely to transition to addiction and are more likely to relapse than F344 rats [9].

In this study, we found that LEW rats self-administered more morphine injections per session than F344 rats, as previously reported by others and also by our group [9–11]. Furthermore, the average number of self-administered injections over the 15 sessions was higher in LEW rats than in F344 rats, as previously shown [9,10]. Our data reinforce the fact that the LEW strain is a morphine-vulnerable phenotype predisposed to higher doses of morphine intake than the F344 strain.

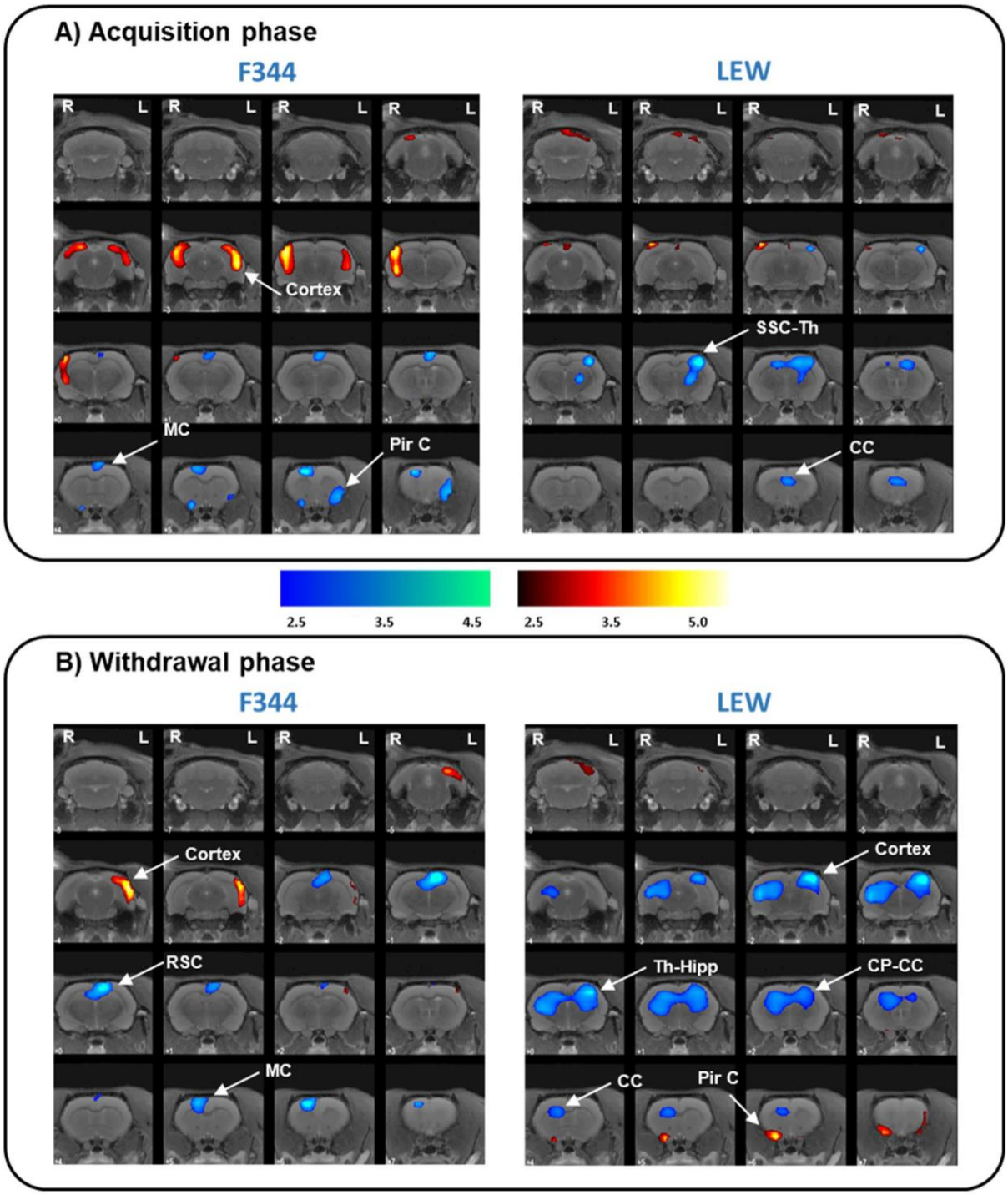


Figure 2. Brain glucose metabolism patterns associated with morphine consumption in F344 and LEW rat strains. PET results in T-maps overlaid on a T2-MRI reference showing increased FDG uptake (hot colors) or decreased FDG uptake (cold colors). PET results in the F344-morphine (left) and LEW-morphine (right) animals compared to the Sal-F344 and Sal-LEW animals respectively, in the acquisition phase (A) and the withdrawal phase (B). (A) F344-morphine animals showed higher FDG uptake in the cortical area and lower FDG uptake in the motor and piriform cortex than the F344- saline animals. LEW-morphine animals showed lower FDG uptake in the somatosensorial and cingulate cortex and thalamus and higher FDG uptake in the cerebellum than the LEW-saline animals. (B) F344-morphine animals showed higher FDG uptake in the left cortex and lower FDG uptake in the restrosplenial and motor cortices than the F344-saline animals. LEW-morphine animals showed higher FDG

uptake in the cerebellum and piriform cortex and lower FDG uptake in the cortex, thalamus, hippocampus, and caudate putamen than the LEW-saline animals. LEW-saline (n = 5), LEW- morphine (n = 6), F344-saline (n = 7), and F344-morphine (n = 6). Threshold for statistical significance of $p< 0.01$. Region of interest (C: cortex, CC: cingulate cortex, Cb: Cerebellum, CP: caudate putamen, Hipp: hippocampus, MC: motor cortex, PirC: piriform cortex, RSC: retrosplenial cortex, Sept: septum, SSC: somatosensorial cortex; Th: thalamus). Side: left (L) and right (R). k: cluster size, T: Student t. FDG uptake: increase (↑) and decrease (↓). p: p value (unc: uncorrected, FWE: family-wise error).

The role of individual differences in the responses to drugs of abuse, and thus predisposition to addiction is not new, and it has been widely demonstrated in humans and laboratory animals [4]. An important issue in drug addiction is to understand whether a person is predisposed to addiction. Thus, differences in vulnerability to drug dependence may be associated with differences in the efficiency of neural substrates to translate the drug effects and, in turn, to differences in brain metabolism. In this respect, the development of PET scanners for laboratory animals enabling the in vivo study of the neurotoxic effects of drugs in a non-invasive follow-up provides an alternative way to explore the underlying mechanisms of morphine-induced neurotoxicity. In the present study, [18F]-FDG was used as a marker of cerebral glucose consumption, which indicates neuronal activity [49]. Our [18F]-FDG data showed that the LEW animals exhibited increased glucose metabolism in cortical areas, including the somatosensory and the entorhinal cortices and the hypothalamus compared with the F344 animals. These areas are of great importance in brain reward circuits [50], and may account for the differences between the studied strains. Specifically, the orexigenic neurons located in the lateral hypothalamus [51] plays an important role in morphine-induced reward, withdrawal, and synaptic plasticity [50]. Therefore, the number of LH orexin neurons has been proposed as a potent predictor of addiction vulnerability [52]. The entorhinal cortex is the main input and output structure of the hippocampus, and the hippocampus is critical for the memory processes of drug-seeking and drug-conditioned stimuli53. Thus, in studies with natural reinforces such as food, there are differences in learning between LEW and F344 rats, with LEW rats learning faster than F344 rats [54]. Sensory system information plays an important role in responses related to drug addiction, and, specifically, the somatosensory system is required for the positive reward property of drugs [55]. In this sense, the higher level of glucose metabolism in this area in the LEW rats might indicate differences in sensory information between the strains.

In general, addiction involves pathological learning in the neural processes related to the reward system and, therefore, underlies long-term associative memory deficits [56]. Here, we demonstrated differences in glucose metabolism in the entorhinal cortex between LEW and F344 rats. This area is closely related to learning and memory and connects the hippocampal neocortex and the hypothalamus, which is the main source of afferents to the hippocampus

[57]. Research has shown genetic differences between both strains in synaptic plasticity in the hippocampus [58,59], which are translated into spatial learning and memory deficits. Thus, F344 animals are less effective at performing some behavioral tasks, such as the radial arm maze test and the Morris maze test60, in that they are more liable to errors and take longer to learn the task [61]. The increased metabolism in this area in the LEW strain, together with the fact that more morphine was self-administered than the F344 strain, might be related to the higher learning capacity shown in the LEW strain.

We also found higher metabolism levels in the hypothalamus (including the mammillary bodies, the VTA, and the medial forebrain bundle) in the LEW animals than in F344 animals. Of note, the mammillary bodies are also involved (with anterior and dorsomedial nuclei of the thalamus) in recognition memory [62]. Together with the increased metabolism in the cortical area, these results represents a plausible explanation for the greater learning capacity of the LEW strain. In addition, the hypothalamus is part of the hypothalamic–pituitary–adrenal (HPA) axis, and differences in HPA axis reactivity between LEW and F344 strains have been reported47. Therefore, the increased glucose metabolism in the hypothalamus in the LEW strain would support this alteration in the HPA axis, which is consistent with the alteration of the reward and motivational processes reported for these strains [63]. In contrast, glucose metabolism was lower in the brainstem and PAG in the LEW strain than in the F344 strain. The brainstem controls basic vital functions, such as heart rate, breathing, and sleeping, but it is also involved in emotional responses and episodes of distress. In this respect, an increased state of anxiety in LEW rats has been associated with alteration of the HPA axis [63]. Furthermore, while the PAG is a key area in acute and chronic pain processing, it is also involved in mediating fear-evoked behavior, which is in turn related to anxiety and depression [64]. Taken together, these results could also account for the differences in HPA axis reactivity between the strains.

Regarding the morphine self-administration study, during acquisition, morphine-F344 animals showed increased glucose metabolism in cortical areas, with more changes in the left hemisphere than in the right, and decreased FDG uptake in the motor and piriform cortices than saline-F344 animals. These changes were mainly maintained during drug withdrawal. However, the metabolism pattern of the morphine-LEW animals was completely different when compared to saline-LEW animals, with decreases in the somatosensorial cortex, thalamus, and cingulate cortex that were maintained and extended during withdrawal. The different brain metabolic patterns observed after the MSA study between these rat strains indicate differences in the efficiency of neural substrates to translate the drug effects and, in turn, possible differences in vulnerability to morphine abuse. Few in vivo imaging studies have evaluated the effect of morphine or other opioids on glucose metabolism using PET or single photon

emission computed tomography (SPECT) in humans, probably because of the radioactive nature of these techniques, being most of them from the 1990s and beginning of this century. London and coworkers showed that acute administration of morphine in humans reduces glucose uptake in the brain by 10% on average [32], with more changes in the left hemisphere than in the right hemisphere. In our study, repeated exposure to morphine induced different patterns of brain changes depending on the rat strain. During acquisition phase, morphine resulted in reduced glucose metabolism in cortical areas in both strains. This pattern of cortical reduction increased after withdrawal in the LEW animals but not in the F344 animals. Repeated exposure to morphine is usually accompanied by the development of tolerance and dependence. In addition, although the exact mechanisms underlying these phenomena are not yet fully understood, they are known to be associated with drug-induced neuroadaptations [56]. In this respect, nuclear magnetic resonance spectroscopy studies demonstrated that acute administration of morphine produces a significant decrease in glycine and glutamate levels that were dramatically increased or overcompensated, when naloxone was used to precipitate withdrawal [65].

Thus, in our F344 animals, chronic morphine may have triggered some compensatory mechanisms in order to normalize glucose metabolism, which were not triggered in the LEW animals.

During drug withdrawal in the LEW strain, glucose metabolism decreased dramatically in brain regions associated with reward and drug dependence, such as the hippocampus, thalamus, caudate-putamen, and cingulate cortex; while glucose metabolism changes in the F344 strain were modest compared to the LEW strain. In humans, neurobiological abnormalities in the regional cerebral metabolic rate for glucose were found in chronic opiate users several years after methadone detoxification [66]. This widespread pattern of abnormal cortical activity involved the anterior cingulate cortex, left mid-cingulate cortex, left insula, and right superior frontal cortex [66], similar to some of our cortical changes. In addition, SPECT studies have shown perfusion deficits during heroin withdrawal in several brain areas, including the temporal lobe [67] and the frontal, parietal, and temporal areas in a chronic opioid user after one week of interrupted administration [68]. In our study, 15 days of morphine abstinence induced a pattern of abnormal brain activity in the LEW animals similar to that found in humans. However, this abnormal metabolic pattern was not found in the F344 animals, suggesting that the genetic background (and other factors) makes one individual more susceptible to developing morphine addiction than another. Furthermore, the LEW strain showed increased metabolism in the piriform cortex. This structure is an olfactory region, and its involvement in the behavioral effects of drugs is limited. Thus, the increased expression of the activity marker c-Fos in the piriform cortex has been associated with cocaine-induced conditioned place

preference [69] and has also been associated with relapse in opioid seeking after food choice–induced voluntary abstinence [70]. Therefore, the increased metabolism in the piriform cortex in the LEW strain could respond to morphine seeking after the withdrawal period and could explain in part the implication of the piriform cortex in morphine addiction and dependence.

Nonetheless, our study had several limitations. First, we only evaluated males. The effect of morphine on females could be different, so further studies would be advisable to determine how the gender may influence on the effect of morphine in brain metabolism. Second, we have not corrected for multiple comparisons since individual analysis methods (SPM) provide some correction, being a common practice in exploratory works [71]. In addition, Bonferroni correction assumes independence of the voxels, which is not true in brain imaging studies and would underestimate the real effects.

In conclusion, we found significant brain metabolic differences between LEW and F344 strains in brain regions associated with reward and drug dependence. In addition, the different brain metabolic patterns observed after the MSA study between these rat strains indicate differences in the efficiency of neural substrates to translate the drug effects, which could explain the differences in predisposition to morphine abuse between one individual and another. These findings have important implications for the use of these rat strains in translational morphine and opiate research.

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Author contributions

M.L.S. Acquisition and analysis of imaging studies, writing-original draft. V.G. Analysis of imaging studies. N.L.R. Analysis of behavioral studies, writing-original draft. G.L.M. Animal handle, review and editing. M.D. Review and editing. E.A. Conception of the study, animal handle, review and editing. All authors reviewed and approved the final version of the manuscript to be published.

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· Chapter II ·

Neuroimaging revealed long-lasting glucose metabolism changes to morphine withdrawal in rats pretreated with the cannabinoid agonist CP-55,940 during periadolescence

This chapter includes the following journal publication:

N. Lamanna-Rama, K S. MacDowell, G. López, J.C. Leza, M. Desco, E. Ambrosio, M.L. Soto-Montenegro, 'Neuroimaging revealed long-lasting glucose metabolism changes to morphine withdrawal in rats pretreated with the cannabinoid agonist CP-55,940 during periadolescence', *Eur Neuropsychopharmacol*. 69:60-76, Apr, 2023. doi: 10.1016/j.euroneuro.2023.01.005. Epub 2023 Feb 11. PMID: 36780817.

· Chapter III ·

THC improves behavioural schizophrenia-like deficits that CBD fails to overcome: a comprehensive multilevel approach using the Poly I:C maternal immune stimulation

This chapter includes the following journal publication (under review):

N. Lamanna-Rama, R. Romero-Miguel, M. Casquero-Veiga, K S. MacDowell, C. Santa-Marta, S. Torres-Sánchez, E. Berrocoso, J.C. Leza, M. Desco, M.L. Soto-Montenegro, 'THC improves behavioural schizophrenia-like deficits that CBD fails to overcome: a comprehensive multilevel approach using the Poly I:C maternal immune stimulation, *Psychiatry Res.* (Under review)

· THC improves behavioural schizophrenia-like deficits that CBD fails to overcome: a comprehensive multilevel approach using the Poly I:C maternal immune stimulation ·

Abstract

Prenatal infections and cannabis use during adolescence are well-recognized risk factors for schizophrenia. As inflammation and oxidative stress (IOS) contribute to this disorder, anti-IOS drugs have been proposed as potential therapies. This study aimed to evaluate the association between delta-9-tetrahydrocannabinol (THC) use and schizophrenia-like abnormalities in a maternal immune stimulation (MIS) model. Additionally, we assessed the preventive effect of cannabidiol (CBD), a non-psychotropic/anti-IOS cannabinoid. THC and/or CBD were administered to Saline- and MIS-offspring during periadolescence. THC-exposed MIS-offspring showed significant improvements in sensorimotor gating deficits. Structural and metabolic brain changes were evaluated by magnetic resonance imaging, revealing cortical shrinkage in Saline- and enlargement in MIS-offspring after THC-exposure. Additionally, MIS-offspring displayed enlarged ventricles and decreased hippocampus, which were partially prevented by both cannabinoids. CBD prevented THC-induced reduction in the corpus callosum, despite affecting white matter structure. Post-mortem studies revealed detrimental effects of THC, including telomere length shortening and increased IOS. CBD partially prevented these pro-inflammatory alterations and modulated THC's effects on the endocannabinoid system. In conclusion, contrary to expectations, THC exhibited greater behavioural and morphometric benefits, despite promoting a pro-IOS state that CBD partially prevented. Further research is needed to elucidate the underlying mechanisms involved in the observed benefits of THC.

Keywords: cannabidiol; delta-9-tetrahidrocannabinol; endocannabinoid system; inflammation; magnetic resonance imaging; oxidative stress

Introduction

Prenatal infections and cannabis use during adolescence have been linked to the future development of psychiatric disorders, including schizophrenia, depression, and anxiety (Jenkins and Khokhar, 2021). However, understanding the precise role of cannabis in these disorders among vulnerable populations remains challenging. Although cannabis has been linked to the onset of psychosis, limited data are available on the clinical implications and long-term consequences of its use (Ahmed et al., 2021). The increasing use of cannabis among adolescents raises concerns about the early onset of psychosis (Dragioti et al., 2022), highlighting the need to investigate cannabis as a potential catalyst for schizophrenia in vulnerable individuals. There is a common belief that cannabis can alleviate symptoms of anxiety, depression, and sleep disturbances. Thus, there is a growing concern with the increasing accessibility and cannabis use, particularly due to recent legalization efforts in Canada and parts of the U.S. for recreational and medical purposes. Consequently, it becomes crucial to thoroughly investigate the effects of THC and CBD, especially among susceptible individuals.

Several hypotheses have been postulated to explain the association between cannabis and psychosis risk. A prominent hypothesis suggests that cannabis acts as a stressor, triggering psychotic relapses and worsening schizophrenia symptoms. Notably, delta-9-tetrahydrocannabinol (THC), the primary psychoactive component in Cannabis sativa, can induce neuropsychological impairments resembling schizophrenia in the general population. This suggests that dysfunction within the endocannabinoid system (ECS) contributes to the development of psychosis (D'Souza et al., 2004). Epidemiological studies have further demonstrated that cannabis significantly contributes to the onset of schizophrenia in vulnerable individuals (D'Souza et al., 2016b; Gage et al., 2016; Gage et al., 2017; Sherif et al., 2016). During adolescence, a critical period in neurodevelopment, alterations in the ECS influence synaptogenesis, synaptic pruning and myelination, ultimately leading to neurochemical and structural changes. These changes may affect cognition, emotions, and the risk of mental disorders (Realini et al., 2009; Rubino and Parolaro, 2008). Hence, it is crucial to identify the factors involved in psychosis onset and mental disorders and explore preventive measures.

Considering the involvement of inflammation and oxidative stress (IOS) in psychiatric disorders, including schizophrenia, anti-inflammatory and antioxidant (anti-IOS) drugs have been proposed as therapeutic options. Cannabidiol (CBD), a cannabinoid compound derived from Cannabis sativa, has gained attention due to its interesting antipsychotic,

neuroprotective, and anti-IOS properties (Osborne et al., 2017a; Osborne et al., 2017b). CBD can modulate the ECS, mitigating THC-induced symptoms and impairments when administered before THC (Bhattacharyya et al., 2010), and reducing psychotic experiences in patients with schizophrenia using cannabis with high CBD content (Schubart et al., 2011). This makes CBD an appealing strategy for preventing brain and behavioural deficits in psychiatric disorders.

In this context, animal models offer valuable insights into the effects of THC and CBD. The maternal immune stimulation (MIS) rat model, induced by PolyI:C, is a well-stablished neurodevelopmental model for schizophrenia. This model aids in investigating the association between maternal infection during pregnancy and the increased risk of schizophrenia onset in offspring (Zuckerman et al., 2003). Furthermore, the pathophysiological delay observed in this model makes it a valuable tool for evaluating preventive approaches (Casquero-Veiga et al., 2019; Casquero-Veiga et al., 2021b; Romero-Miguel et al., 2021). Therefore, using the MIS model, this study aimed to 1) evaluate the association between THC consumption during adolescence and the onset of schizophrenia-like abnormalities, and 2) assess the preventive effect of CBD treatment during periadolescence on schizophrenia-related abnormalities in adulthood. To our knowledge, this is the first study to investigate this topic using different magnetic resonance imaging (MRI) techniques, including structural (T2W), diffusion tensor imaging (DTI), and spectroscopy (1H-MRS). This multilevel approach allows for the evaluation of in vivo structural and metabolic changes following THC and CBD treatment in an animal model of schizophrenia. Additionally, this study includes behavioural tests and molecular assays in brain tissue to provide a multilevel exploratory assessment.

Methods

Animals and drug treatment

The study involved 96 male Wistar rats kept at a temperature of 24±0.5ºC under a 12 h dark/light cycle, with free access to food and water. Experimental procedures were conducted in conformity with the European Communities Council Directive 2010/63/EU, following the ARRIVE guidelines (Percie du Sert et al., 2020) and approved by the Ethics Committee for Animal Experimentation of Hospital General Universitario Gregorio Marañón (ES280790000087).

PolyI:C dissolved in saline (4 mg/kg, polyinosinic-polycytidylic acid, P0913, Sigma-Aldrich, Germany) or saline were intravenously injected to pregnant Wistar dams on

gestational-day 15. Two or three males per litter were randomly included in the study. THC (10 mg/kg/day) (Moore et al., 2021; Moore and Weerts, 2022) and/or CBD (10 mg/kg/day) (Osborne et al., 2017a) or their vehicle (Ethanol, Cremophor, Saline; 1:1:18) (Lamanna-Rama et al., 2023) were administered intraperitoneally between post-natal days (PND) 28–38 and PND35-49, respectively. Thus, animals were divided in eight groups (N=12/group) according to the study factors: MIS-model (Saline, MIS), THC treatment (VH, THC), and CBD treatment (VH, CBD) (Figure 1A).

Behavioural study

At PND70, sensorimotor gating deficits were measured by the prepulse inhibition (PPI) of the acoustic startle response, starting with 10 minutes of acclimatization (70-dB background noise) to the startle chamber (Cibertec, Spain), followed by five trials of startle stimulus (pulse, 120 dB). Subsequently, the animals underwent 10 trials of pseudo-randomly presented stimuli [pulse (120 dB), prepulse (74, 80, or 86 dB) + pulse (120 dB), or no stimulus (background noise)] followed by five trials of pulse (120 dB). Pulse and prepulse duration were 40 ms, whereas the prepulse-pulse interval was 100 ms, and the intertrial interval ranged from 10 to 20 seconds. PPI percentage was calculated as follows: 100 – ([startle response to prepulse + pulse)/ startle response to pulse) × 100]) (Garcia-Partida et al., 2022).

In vivo magnetic resonance (MR) studies

At adulthood (PND120), animals were scanned using a 7-Tesla Biospec 70/20 scanner (Bruker, Germany) while under sevoflurane anaesthesia (4.5% induction, 2.5% maintenance in 100% O2). The structural changes in grey (GM) and white matter (WM) (T2W), fibre integrity (DTI), and metabolic composition (1H-MRS) were examined.

MR structural imaging

Coronal T2-weighted (T2W) spin-echo sequence with TE=33 ms, TR=3,732 ms, averages 2 and slice thickness 0.4 mm were acquired. Matrix size was 256×256 pixels at a FOV of 3.5×3.5 cm2 (Casquero-Veiga et al., 2021a; Casquero-Veiga et al., 2021b; Romero-Miguel et al., 2021). A voxel-based morphometry (VBM) analysis of GM tissue was performed as previously described (Casquero-Veiga et al., 2019), processing the T2W acquisitions.

Additionally, eight regions of interest (ROIs) were manually segmented using MMWKS software (Pascau et al., 2009) by one blinded, experienced operator. ROIs were segmented according to a rat brain atlas (Paxinos and Watson, 2008) (coordinates from Bregma): brain

(5.20 to -9.30 mm), hippocampus (-1.80 to -6.30 mm), ventricles (2.70 to -8.30 mm), frontal cortex (FC) (5.20 to 0.20 mm), cortex (5.20 to -9.30 mm), corpus callosum (3.70 to -8.00 mm), caudate-putamen (2.20 to -3.80 mm) and cerebellum (-9.68 to -14.30 mm).

Diffusion tensor imaging (DTI)

A SE-EPI sequence was acquired, with TE=26.55 ms, TR=2,000 ms, and 3 repetitions. Other scan parameters were as follows: diffusion gradients applied in 27 directions (b-value = 1,000 s/mm2), 35 slices, 0.4 mm thickness, matrix size of 128 × 128 voxels and FOV of 35 × 35 mm. The corpus callosum (CC) (3.70 to -8.00 mm) was manually segmented using ImageJ (FIJI, NIH, USA) by one blinded, experienced operator. Fractional anisotropy (FA) and mean diffusivity (MD) were obtained from this brain area.

MR spectroscopy (1H-MRS)

Spectroscopic images were acquired with a PRESS mono-voxel sequence in the FC and hippocampus, applying the following parameters: TR=2,500 ms, TE=16.5 ms, acquisition points=2,048, 128–256 averages, and band width=3,301.06 Hz. The size of the voxel was adjusted to the anatomical structure (FC: 1.5×1.5×1.5 to 1.5×1.7×1.7 and hippocampus: 1.5×1.5×1.5 to 2.0×1.1×1.7). An unsuppressed water signal measured from the FC and hippocampus was used as an internal reference for quantification. In vivo 1H-MRS spectra were analysed using LCModel software (Version 6.2-0U)(Provencher, 2001). The LCModel basis set included spectra of 20 brain metabolites, but only those metabolites with a Cramer-Rao Lower Bound < 20%, corresponding to the concentration estimated error (0.2 mmol/g), were included in the quantitative analysis. Metabolites with overlapping resonances were represented as the sum of both species.

Post-mortem studies

All post-mortem studies were performed in brain tissue samples.

qPCR assay

DNA was extracted (eight animals/group) using a phenol-chloroform protocol (77617, Merck, Spain)(Green and Sambrook, 2017), previous incubation with lysis buffer and proteinase K (P2308, Merck, Spain) at 55ºC overnight. Then, mean telomere length (TL) was measured in duplicate from each sample by Absolute Rat TL Quantification qPCR Assay kit

(R8918, ScienCell Research Laboratories, USA), following the manufacturer's instructions. TL was calculated according to manufacturer's protocol.

Western blot (WB)

The cannabinoid receptors CB1R and CB2R and critical enzymes of the endocannabinoid metabolism, including N-acyl phosphatidylethanol-amine-specific phospholipase D (NAPE), diacylglycerol lipase (DAGL), fatty acid amidohydrolase (FAAH), and monoacylglycerol lipase (MAGL), were determined (Lamanna-Rama et al., 2023). Moreover, DAGL/MAGL and NAPE/FAAH ratios were calculated as indirect indices of 2-arachidonoylglycerol (2-AG) and anandamide levels, respectively (Notarstefano et al., 2020). The inflammatory and oxidonitrosative inducible enzymes iNOS and COX2, and the pan-microglial marker Iba1 were measured. Furthermore, as antioxidant elements of the Nrf2 pathway, KEAP1, HO1 and NQO1 were also measured (Romero-Miguel et al., 2021). These determinations were evaluated in cytosolic extracts from caudate-putamen, FC, hippocampus, and amygdala (eight animals/group). Protein levels were quantified using the Bradford method.

Proteins were loaded into an electrophoresis gel and then blotted onto a membrane with a semi-dry transfer system. Blots were blocked with 5% BSA (Sigma, Spain) and incubated overnight at 4°C with the following antibodies: rabbit anti-CB1 (ab23703, 1:1000 BSA 0.5%; Abcam, UK), rabbit anti-CB2 (101,550, 1:1000 BSA 0.5%; Cayman, Estonia), goat anti-DAGL (ab81984, 1:1000 BSA 0.5%; Abcam, UK), rabbit anti-FAAH (101,600, 1:1000 BSA 0.5%; Cayman, Estonia), rabbit anti-MAGL (100,035, 1:1000 BSA 0.5%; Cayman, Estonia), rabbit anti-NAPE (10,306, 1:1000 BSA 0.5%; Cayman, Estonia), rabbit anti-iNOS (sc-650, 1:750 BSA 2%; SCBT, USA), goat anti-COX2 (sc-1747, 1:750 BSA 2.5%; SCBT, USA), mouse anti-KEAP1 (MAB3024, 1:1000; R&D, USA), rabbit anti-HO1 (ab68477, 1:1000; Abcam, UK), goat anti-NQO1 (sc16464, 1:750 BSA 1%, SCBT, USA), rabbit anti-Iba/AIF-1 (17198, 1:1000 BSA 5%, CST, Madrid, Spain) and mouse anti-β-actin (A5441, 1:10000; Sigma, Spain). Horseradish peroxidase-linked secondary antibodies were used to detect each protein. Antibody binding was detected using a ChemiDoc Imaging System (BioRad, Germany). All blots were performed at least three times in separate assays. Digital images from WB were analysed using densitometry (ImageJ, NIH, USA). Values were normalized to the loading control (β-actin) and expressed as a percentage of variation compared to the control group.

Enzyme-linked immunosorbent assay (ELISA)

BDNF was quantified using a commercial ELISA-based kit (CSB- E04504r, Cusabio, USA) in cytosolic extracts. Nrf2 activity was determined by a commercial ELISA-based kit (600590,

Cayman Chemical, Estonia), using nuclear extracts. Both markers were measured in caudate-putamen, FC, hippocampus, and amygdala (eight animals/group), following the manufacturer's instructions.

Statistical analysis

VBM

GM analyses were conducted using SPM12 software (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/). Groups were compared using three-way ANOVA, setting a significance threshold of $p<0.01$ uncorrected (voxel-level significance), but cluster-based corrected by false discovery rate to avoid type II errors. Moreover, a 1000-voxel clustering threshold was applied to reduce type I error.

Behavioural, volumetric, DTI, 1H-MRS, and post-mortem data

Normality and homoscedasticity were tested by Shapiro–Wilk and Levenne's tests, respectively. If the data did not meet these assumptions, a two-step transformation approach was employed(Templeton, 2011). Normal and homoscedastic variables were evaluated using a three-way ANOVA followed by LSD post-hoc test; except for the PPI, which underwent repeated measures (RM) ANOVA followed by LSD post-hoc test. Non-normal and/or heteroscedastic variables were analysed using Kruskal–Wallis test, followed by Dunn's post-hoc test. A p-value<0.05 was considered statistically significant. Statistical analyses were performed using SPSS (IBM SPSS Statistics 20, Spain), while graphics were performed in GraphPad Prism (v8, GraphPad Software, San Diego, CA, USA).

Results

Behavioural study

PPI test (Figure 1B; Supplementary tables S1, S3).

RM-ANOVA showed a significant prepulse effect ($p<0.001$) and a 3-way interaction (MIS×THC×CBD) ($p<0.01$). Post-hoc tests revealed a significant PPI reduction at 80dB and 86dB in the MIS offspring (vs Saline-VH-VH group). THC significantly reversed this PPI reduction in MIS-offspring (vs MIS-VH-VH group) ($p<0.05$) but worsened in Saline-offspring (vs Saline-VH-VH group) ($p<0.05$). CBD did not significantly modify PPI in Saline- or MIS-offspring.

MR studies

VBM analysis (Figure 2A; Table 1).

In the Saline-offspring, THC enlarged ventral hippocampus and shrinked cortical areas (piriform, motor, cingulate and somatosensory -SSC- cortices); whereas CBD induced enlargement in the cerebellum, substantia nigra, dorsal hippocampus, and thalamus. In the MIS-offspring, THC led to enlargements in the cerebellum, superior colliculus, ventral hippocampus, motor and cingulate cortices and thalamus, whereas CBD induced enlargements in the cerebellum, periaqueductal grey (PAG), SSC and thalamus, and shrinkage of the caudate-putamen and auditory cortex. Furthermore, CBD induced greater morphometric changes in THC-treated MIS-offspring than in THC-treated Saline-offspring, with shrinkages in cortical areas (SSC, visual and auditory cortex) and medial amygdaloid nucleus and enlargements in the cerebellum, PAG, motor cortex, and ventral pallidum.

ROI analyses (Figure 2B, Supplementary Figure S1A, Supplementary tables S1, S3).

MIS-offspring showed reduced hippocampus and ventricular enlargement, the latter being prevented by THC and CBD. Lastly, THC increased caudate-putamen volume in both Saline- and MIS-offspring.

DTI analysis (Figure 2C, Supplementary Figure S1B, Supplementary tables S1, S3).

ANOVA showed a significant effect of CBD in the FA, showing reduced FA in both Saline (Saline-VH-CBD: $p<0.001$; Saline-THC-CBD: $p<0.001$) and MIS-offspring (MIS-VH-CBD: $p<0.01$; MIS-THC-CBD: $p<0.001$).

1H-MRS analysis (Figure 2D, Supplementary Figure S1C, Supplementary tables S1, S3).

Kruskal–Wallis test showed between-group differences in N-Acetylaspartate + N-Acetylaspartate glutamate (NAA) levels ($p<0.01$) in the FC, showing higher NAA levels in the MIS-THC-CBD group ($p<0.05$).

Post-mortem studies

TL analysis (Figure 3A, Supplementary tables S2, S4).

ANOVA showed a significant THC×CBD interaction ($p<0.01$). THC decreased TL in Saline-offspring. A similar trend was found in MIS-offspring.

ECS markers analysis (Figure 3B, Supplementary tables S2, S4).

Caudate-putamen: Kruskal–Wallis test showed between-group differences in CB2R ($p<0.01$). MIS-offspring showed increased CB1R. In addition, THC and CBD increased CB1R in Saline-offspring. Also, increased CB1R was found in THC-CBD-treated MIS-offspring. Finally, THC decreased NAPE/FAAH in Saline-offspring, while CBD increased NAPE/FAAH in Saline and MIS THC-treated animals.

FC: MIS-offspring showed increased DAGL/MAGL that was reverted by THC, and increased NAPE/FAAH that was prevented by CBD. THC also increased CB2R in MIS-offspring.

Hippocampus: Kruskal–Wallis test showed between-group differences in CB1R ($p<0.001$), CB2R ($p<0.001$) and NAPE/FAAH ($p<0.001$). ANOVA analysis showed a CBD effect ($p<0.05$) in DAGL/MAGL.

Amygdala: ANOVA results showed MIS ($p<0.01$) and THC ($p<0.001$) factors' effects in DAGL/MAGL; and a THC×CBD interaction ($p<0.001$) in CB2R. THC reduced NAPE/FAAH and CB1R in Saline- and MIS-VH animals, respectively, while increased CB2R and DAGL/MAGL in Saline-offspring. In addition, CBD increased CB2R in Saline-animals and DAGL/MAGL in Saline-THC-offspring.

IOS markers analysis (Figure 4, Supplementary Figure S1D, Supplementary tables S2, S4).

Caudate-putamen: Kruskal–Wallis test showed between-group differences in iNOS ($p<0.001$), COX2 ($p<0.01$), iba1 ($p<0.001$), Keap1 ($p<0.05$), and NQO1 ($p<0.01$). ANOVA showed a THC effect ($p<0.001$) in HO1. THC increased iNOS, and HO1 in Saline-offspring. CBD increased COX2 and HO1 in MIS THC-treated animals, while decreased iba1 in MIS THC-treated animals and HO1 in MIS-offspring.

FC: Kruskal–Wallis test showed between-group differences in iNOS ($p<0.001$) and COX2 ($p<0.01$). MIS-offspring showed increased COX2 and iNOS, being the latter prevented by CBD. THC increased iba1 and HO1 in MIS-offspring. CBD decreased Iba1 in THC-treated animals.

Hippocampus: Kruskal–Wallis test showed between-group differences in COX2 ($p<0.01$), Keap1 ($p<0.001$) and HO1 ($p<0.01$). MIS-offspring showed increased iNOS, iba1,

Keap1 and NQO1, all of them prevented by CBD. THC increased iNOS and Iba1 in both Saline- and MIS-offspring.

Amygdala: Kruskal–Wallis test showed between-group differences in COX2 ($p<0.001$), iba1 ($p<0.05$) and NQO1 ($p<0.001$). ANOVA showed a THC effect ($p<0.001$) in HO1, and a CBD effect in iNOS ($p<0.001$), Keap1 ($p<0.01$) and HO1 ($p<0.001$). MIS-offspring showed increased iNOS, prevented by CBD. THC decreased Keap1 in MIS-VH animals, while increased NQO1 in Saline-offspring. CBD increased HO1 in both Saline- and MIS-offspring, whereas reduced HO1 and COX2 in THC-treated MIS-offspring.

Discussion

This study aimed to evaluate the association between THC consumption during adolescence and schizophrenia-related abnormalities in vulnerable individuals, as well as to investigate the potential preventive effect of CBD on the occurrence of such abnormalities. Surprisingly, although THC exacerbated certain abnormalities in the MIS model, acting as a double-hit along with the MIS challenge, it also prevented the PPI deficit and some brain volumetric abnormalities. Conversely, CBD failed to improve this behavioural deficit but prevented some of the structural and biochemical alterations.

THC prevented PPI deficits in MIS-offspring

As previously reported (Casquero-Veiga et al., 2021b), MIS-animals exhibited sensorimotor gating deficits with loss of PPI. Surprisingly, THC prevented these deficits in MIS-offspring, whereas it induced them in Saline-offspring, as anticipated. This aligns with studies on chronic exposure to cannabinoids during periadolescence, which have shown similar PPI deficits to that observed in cannabis users (Abboussi et al., 2020; Roberts et al., 2021; Francis et al., 2022). However, there is limited research on PPI deficits in MIS-offspring, and none have demonstrated improvements as seen with THC. It has been suggested that THC may have different effects depending on the individual's inflammatory background (Roberts et al., 2021), explaining the contrasting results. Conversely, CBD did not prevent PPI deficits in MIS-offspring, although some improvement was observed. These findings are consistent with the ongoing debate on the beneficial effect of CBD on PPI deficits, as other studies have found no improvement or no effect on PPI in different schizophrenia-like rat models (Hoffman, 2021).

THC and CBD prevented some brain volumetric abnormalities in MIS-offspring

Our study replicated the volumetric anomalies observed in patients with schizophrenia and preclinical models, namely reduced hippocampus and enlarged ventricles (van Erp et al., 2016; Bouet et al., 2021; Casquero-Veiga et al., 2021b). CBD and, surprisingly, THC prevented these abnormalities. While hippocampal volume reductions have been widely reported in regular cannabis users (Chye et al., 2019), along with other GM shrinkages in adolescent cannabis users with schizophrenia (James et al., 2013), limited studies have explored the potential benefits of CBD in these individuals. Recent research has demonstrated CBD's ability to restore hippocampal volume loss in cannabis users, particularly those with extensive cannabis use history, highlighting its usefulness as an adjuvant in cannabis dependence treatments (Beale et al., 2018). In contrast, previous animal studies have yielded inconsistent findings regarding ventricular changes following THC or CBD treatment in different models (Drazanova et al., 2019; Stark et al., 2020). Nevertheless, our results suggest a beneficial effect of both THC and CBD on the ventricular system, although caution is advised due to potential adverse effects of THC on other domains.

Additionally, we observed cerebellum and PAG enlargement in CBD-treated MIS-offspring. CB1 receptors are widely distributed throughout these brain structures, which belong to a neural network involved in processing anxiety-like behaviour (Chin and Augustine, 2023; Ruehle et al., 2012) and psychotic-like experiences (Lorenzetti et al., 2016), and are implicated in schizophrenia neuropathology (Solowij et al., 2011). Hence, the cerebellum has been found to be reduced in schizophrenia and cannabis users. However, total cerebellar reductions in healthy users were associated with the duration and early age of onset of cannabis use (Solowij et al., 2011). Instead, we revealed that CBD could protect from such detrimental effects in MIS-offspring, similar to that reported in humans (Lorenzetti et al., 2016). The mechanism underlying these enlargements remains unclear. One possibility is that CBD, by reducing the pathology-related IOS and mitigating the effects of THC on brain function, prevents the activation of molecular mechanisms that could trigger neurotoxicity and result in neuroanatomical abnormalities (Henshaw et al., 2021; Kopustinskiene et al., 2022).

CBD prevented CC deficits in MIS-offspring

Our study showed a reduction in CC volume, the largest WM fibre structure in the brain, in MIS-offspring, replicating findings reported in patients with schizophrenia (Keshavan et al., 2020), cannabis users (Manza et al., 2020), and preclinical models (Kaneko et al., 2017; Xiu et al., 2014). Indeed, early-life exposure to PolyI:C is associated with demyelination of the CC and decrease of its calibre (Singh et al., 2021). As expected, THC exacerbated this deficit in Saline- and MIS-offspring. Notably, based on rodent data, the CC exhibits particularly high

expression of cannabinoid receptors during development, which makes it especially vulnerable to cannabis exposure during adolescence (Manza et al., 2020). In contrast, CBD treatment during adolescence prevented this WM deficit, a result that, to our knowledge, has never been reported before. Therefore, CBD may contribute to enhancing WM integrity, particularly in the CC, whose loss of integrity has been linked to the development of psychiatric disorders (Singh et al., 2021). We hypothesize that CBD may be protecting WM fibres from demyelination, although further studies are needed to corroborate this hypothesis.

Additionally, we found reduced FA in the CC in all CBD-treated groups. Clinical investigations in schizophrenia have identified reduced FA and increased MD in the CC in never-treated schizophrenia patients, whereas increased FA has been observed in antipsychotic-medicated schizophrenia patients (Clark et al., 2011; Tao et al., 2021). However, the effects of cannabinoids on WM integrity in animal models remain unclear. Our study found no differences in FA between non-treated MIS-offspring and control animals, similar to the findings in the lipopolysaccharide-induced MIS model (Capellan et al., 2023). Nevertheless, other authors have reported regional FA variations in the CC in the MAM model, including increased FA in the posterior area, and lower FA in the anterior part (Kaneko et al., 2017). It is known that cannabis use in adolescents is associated with lower FA in several WM tracts (Epstein and Kumra, 2015), which could partially explain the FA reduction in the THC-CBD groups but not in the VH-CBD groups. Further post-mortem microstructural imaging studies are required to understand these alterations in WM architecture.

THC modulated the ECS and telomere length

The exposure to synthetic or natural cannabinoids during vulnerable periods of adolescence can imbalance the ECS, dysregulating cannabinoid receptors and endocannabinoid components, such as anandamide and 2-AG (Renard et al., 2016). In this sense, post-mortem evaluation of the ECS revealed increased CB1R expression in the caudate-putamen in MIS-offspring. CB1R is known to be upregulated in an IOS environment (Morris et al., 2022), hence in patients with schizophrenia (Newell et al., 2006) and schizophrenia-like preclinical models (Almeida et al., 2019), which is consistent with our results. Conversely, THC decreased CB1R expression in the amygdala in MIS-offspring, as previously reported in cannabis-dependent subjects (D'Souza et al., 2016a) and animal models exposed to THC or cannabinoid agonists during adolescence (Lamanna-Rama et al., 2023; Sim-Selley et al., 2006), not being prevented by CBD. Notably, a remarkable increase in CB1R expression was observed in the caudate-putamen and FC when animals were

exposed to both CBD and THC, suggesting a synergistic effect of both cannabinoids in modulating CB1R in these areas.

Regarding CB2R, recent studies have associated its overexpression with a neuroinflammatory state (Alexandre et al., 2020). In this regard, we found that THC increased CB2R in the FC of MIS-offspring and in the amygdala of Saline-offspring, suggesting its potential role in promoting a pro-inflammatory environment. Interestingly, CBD prevented these effects in the amygdala only when THC was present, consistent with its previously described anti-IOS properties (Osborne et al., 2017a; Osborne et al., 2017b). Additionally, we observed increased ratios of the main ECS synthesis and degradation enzymes in the FC, providing insights into the levels of anandamide and 2-AG. Schizophrenia has been associated with increased circulating levels of anandamide (Potvin et al., 2020; Ibarra-Lecue et al., 2022); however, a decrease has also been described in post-mortem brains of these patients (Muguruza et al., 2013). Cannabis use has been linked to reduced anandamide levels and increased psychotic symptoms in vulnerable individuals, while CBD has been shown to alleviate these psychotic symptoms and increase serum anandamide levels (Leweke et al., 2018), possibly through the inhibition of peripheral anandamide reuptake or moderate inhibition of FAAH (Leweke et al., 2018). Our results are consistent with these previous findings, as we found that THC reduced anandamide levels in the FC, and CBD did not prevent this reduction. On the other hand, CBD increased 2-AG levels in the caudate-putamen and FC of THC-treated animals, potentially due to the higher IOS environment in the brains of these animals. It has been proposed that 2-AG acts as an endogenous suppressor of neuroinflammation in response to harmful insults (Chen, 2023), which supports our findings. Overall, our findings and previous literature (Freeman et al., 2019) indicate that CBD can modulate the effects of THC on the ECS system. The way CBD interacts with THC requires further research, considering the growing interest in THC-CBD formulations for recreational and medical purposes (Legare et al., 2022).

Regarding the pro-inflammatory state, THC shortened TL in Saline-offspring, which was not prevented by CBD, and a similar trend was found in MIS-offspring. Telomere shortening has been associated with certain chronic pathologies involving IOS (Price et al., 2013). However, despite the greater IOS impairments shown in patients with schizophrenia (Fraguas et al., 2019), its effects in TL remain controversial, showing TL shortening (Galletly et al., 2017), TL maintenance (Li et al., 2015), or even TL lengthening (Nieratschker et al., 2013). In our study, we would have expected TL shortening in MIS-offspring due to the pro-inflammatory state induced by the MIS challenge, but we only observed TL shortening in the THC-treated group. This result is consistent with the described TL shortening associated with

drug abuse, likely due to the oxidative stress induced by the drug itself (Lin et al., 2021; Yang et al., 2013). Few studies have addressed the association between cannabis consumption and TL, but most of them reported TL shortening compared to controls (Navarro-Mateu et al., 2021). It is noteworthy that CBD, despite preventing the pro-inflammatory state induced by MIS and THC, did not alter TL in either Saline- or MIS-offspring.

CBD partially prevented the pro-IOS state

Our MIS model showed a generalized activation of the proinflammatory pathway, mainly represented by increased expression of iNOS in most brain areas and COX2 in the FC, consistent with previous reports in the MIS model (Casquero-Veiga et al., 2019; Casquero-Veiga et al., 2021b; Chamera et al., 2020; Romero-Miguel et al., 2021) and the pro-inflammatory dysregulation observed in patients with schizophrenia in peripheral mononuclear blood cells (Garcia-Bueno et al., 2014; Leza et al., 2015). This proinflammatory disbalance, mediated by the activation of the NF-κB pathway, seems to persist through the microglia-neuron connection (Chamera et al., 2021), as indicated by the increase of reactive microglia cells (Iba-1) in the hippocampus and amygdala. Notably, the hippocampus was the most affected region by the MIS challenge in the Keap1-Nrf2-ARE network, with increased levels of iNOS, Iba1, Keap1, and NQO1. As expected, THC treatment exacerbated the pro-inflammatory state in all animals, as evidenced by increased Iba-1 in all brain areas, confirming this microglial marker as one of the most consistent markers of cannabis-induced inflammation (Lopez-Rodriguez et al., 2014). In general, cannabis use or THC treatment has been associated with the increase of pro-inflammatory markers in these subjects (Bayazit et al., 2017; Henshaw et al., 2021), although some studies have reported its anti-inflammatory properties (Kopustinskiene et al., 2022). These inconsistencies suggest that there are still missing pieces in the study of THC exposure during adolescence, a critical period for neurodevelopment (Renard et al., 2016), in order to fully understand the scope of THC effects in this vulnerable population.

Finally, CBD prevented some IOS alterations induced by the MIS challenge and THC. Specifically, CBD reduced iNOS and Iba1 in most brain areas, as well as Keap1 and NQO1 in the hippocampus. In this regard, considering the anti-IOS and antipsychotic properties of CBD (Osborne et al., 2017b), there is evidence of CBD reducing IOS markers in SCZ-like preclinical models (Loss et al., 2020), consistent with our findings. However, while CBD prevented most of the inflammatory response, changes at oxidative stress markers levels were less pronounced. Specifically, CBD treatment, independently of the animal's phenotype, increased HO1 expression in the amygdala, but its combination with THC decreased its expression. This

region is associated with fear processing, emotional states, and it is involved in the reward system (Koob and Volkow, 2016), and its disruption caused by THC treatment during adolescence could be a critical factor in this amygdalar imbalance, as previously observed in rats exposed to a cannabinoid agonist during adolescence (Lamanna-Rama et al., 2023).

Limitations

This study has some limitations. First, it was focused exclusively on male-offspring. Previous research showed that female MIS-offspring exhibited different patterns of brain metabolism, morphometry, plasticity and disease temporal course compared to males of the same age (Casquero-Veiga et al., 2022). Therefore, future studies should include females, adapting the time points to their pathology, to broaden our understanding of these findings. Second, no adjustments for multiple comparisons were applied in the voxel-level statistical analyses. These adjustments would be too restrictive for an exploratory approach, as in our study, since we aim to describe all possible influence without detriment to false negatives. Nevertheless, we applied cluster-level corrections to prevent type I errors.

Conclusions

Taken together, these results provide a multilevel approach to understand the effects of THC exposure during adolescence in vulnerable individuals, and its influence on schizophrenia-related signs and symptoms, as well as the potential preventive effect of CBD on the onset of these anomalies. Surprisingly, THC treatment did not exacerbate the MIS-related abnormalities at the behavioural and brain anatomical levels, despite promoting a pro-inflammatory/pro-oxidative state. Conversely, CBD prevented some of the structural deficits and reduced inflammatory markers expression through iNOS and Nrf2-ARE pathways but failed to improve sensorimotor gating deficits. Finally, the improvement of behavioural deficits by THC exposure during adolescence highlights the importance of further research aimed to uncover the underlying pathophysiological mechanisms involved.

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Declarations of interest

None

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Table 1. VBM analysis: Statistical data of cannabinoids-related effects during adolescence for Saline- and MIS-offspring.

Region	Side	T	K	↑/↓	P_{FDR} Cluster
Saline VH-VH vs Saline THC-VH					
Pir	R	3.69	4031	↓	<.001
Cg/SSC	L & R	3.21	5710		
vHipp	R	3.61	1727	↑	.028
MIS VH-VH vs MIS THC-VH					
vHipp	R	4.09	3730		<.001
Cg/MC	L	3.69	3007		.001
	R	2.97	1694		.009
SCol	L & R	3.52	2080	↑	.004
Th	L	3.45	1487		.014
Cb	R	3.45	2447		.003
	L & R	3.42	1442		.014

Region	Side	T	K	↑/↓	P_{FDR} Cluster
Saline VH-VH vs Saline VH-CBD					
Cb/S	R	4.13	2951		.001
	L	3.62	1667		.011
SNR	R	3.92	1376		
S	L	3.62	1224	↑	.019
Th	L	3.48	1186		
dHipp	R	3.33	3592		<.001
	L	2.96	1003		.031
MIS VH-VH vs MIS VH-CBD					
AC	R	3.21	1822	↓	.012
CPu	L	3.14	1146		.044
Cb	L & R	5.35	53885		<.001
	R	3.87	1302		.028
SSC	R	3.98	3071	↑	<.001
Th	L & R	3.64	1938		.006
PAG	L & R	3.21	1208		.028

Region	Side	T	K	↑/↓	P_{FDR} Cluster
Saline VH-VH vs Saline THC-CBD					
Cb	L & R	3.25	1557	↑	.028
MIS VH-VH vs MIS THC-CBD					
SSC	R	3.90	3663		
	L	3.08	2389		<.001
VC/AC	R	3.86	5498	↓	
mAN	L	3.85	1061		.030
VC	L	3.07	1355		.015
CB/PAG	L & R	4.47	23156		<.001
	R	4.46	1468		.012
VP	L	4.27	2201	↑	.002
	R	4.3.31	1024		.027
MC	R & R	4.16	1285		.016

Table shows the THC- and CBD-related effects on grey matter morphometric changes in the voxel-based analysis in Saline- and MIS-offspring. [AC: auditory cortex; A.U.: arbitrary units; CBD: cannabidiol; Cb: cerebellum; CPu: caudate-putamen; Hipp: hippocampus; K: cluster size; KW: Kruskal–Wallis; L: left hemisphere; mAN: medial amigdaloid-nucleus; MIS: maternal immune stimulation; NAA: N-acetylaspartate + N-Acetylaspartylglutamate; pFDR: p-value false discovery rate; PAG: periaqueductal grey; Pir: piriform cortex; R: right hemisphere; RSA: retrosplenial cortex; S: subiculum; SSC: somatosensory cortex; SCol: superior colliculus; SNR: substantia nigra; T: t-value; Th: thalamus; THC: 9-Tetrahidrocannabinol; VH: vehicle; VC: visual cortex].

Figure 1.

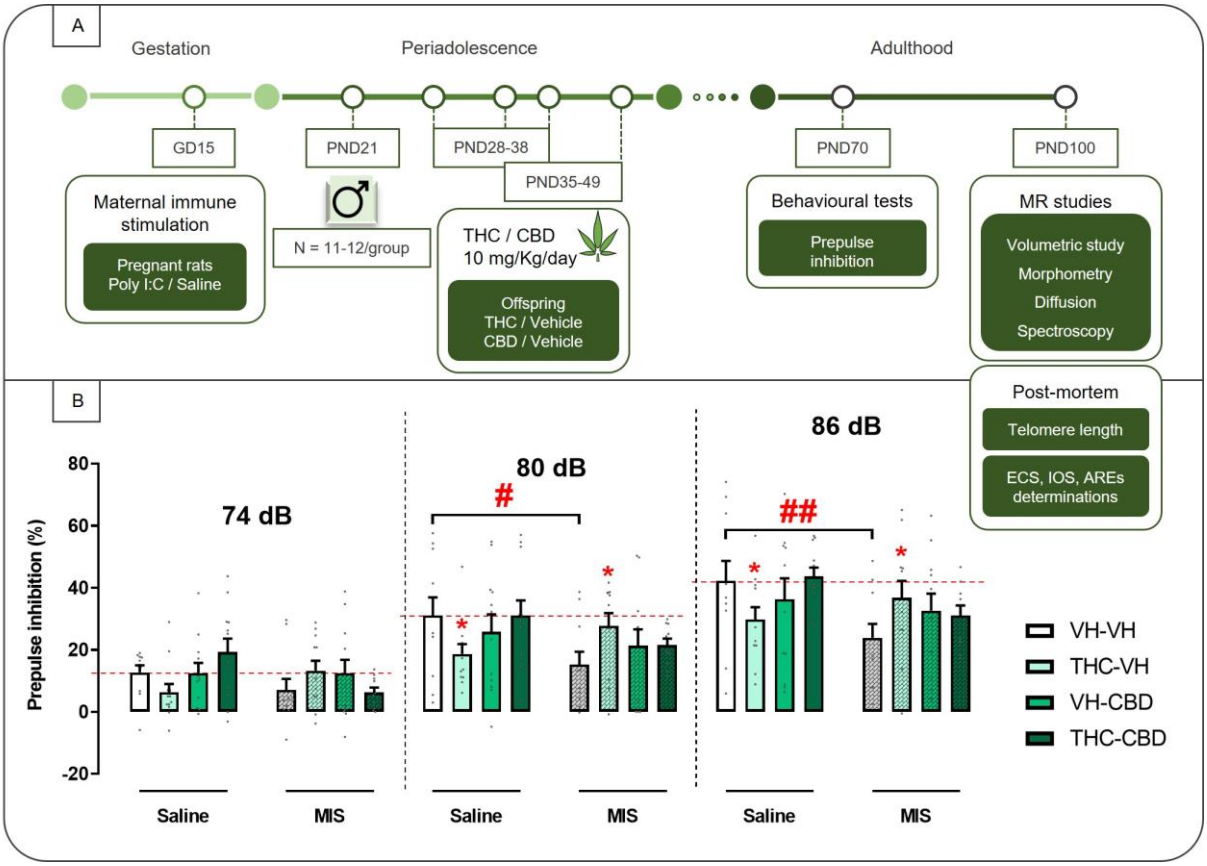

A
Gestation
Periadolescence
Adulthood
GD15
PND21
PND28-38
PND35-49
PND70
PND100
Maternal immune stimulation
Pregnant rats
Poly I:C / Saline
N = 11-12/group
THC / CBD
10 mg/Kg/day
Offspring
THC / Vehicle
CBD / Vehicle
Behavioural tests
Prepulse inhibition
MR studies
Volumetric study
Morphometry
Diffusion
Spectroscopy
Post-mortem
Telomere length
ECS, IOS, AREs determinations
B
74 dB
80 dB
86 dB
Prepulse inhibition (%)
Saline
MIS
VH-VH
THC-VH
VH-CBD
THC-CBD

Figure 2.

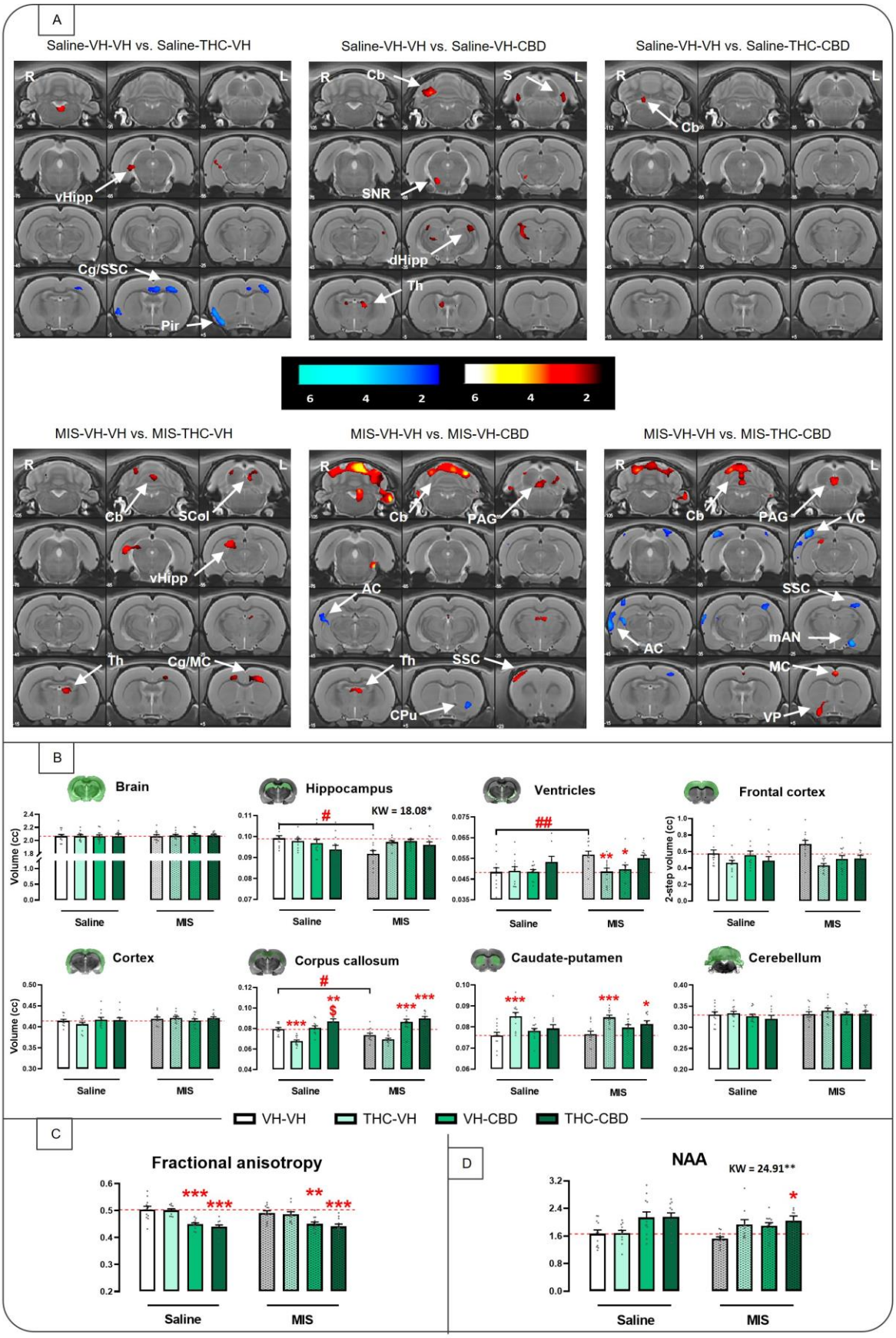

A
Saline-VH-VH vs. Saline-THC-VH
Saline-VH-VH vs. Saline-VH-CBD
Saline-VH-VH vs. Saline-THC-CBD
vHipp
Cg/SSC
Pir
SNR
dHipp
Th
Cb
6 4 2 6 4 2
MIS-VH-VH vs. MIS-THC-VH
MIS-VH-VH vs. MIS-VH-CBD
MIS-VH-VH vs. MIS-THC-CBD
Cb
SCol
vHipp
Th
Cg/MC
AC
PAG
SSC
CPu
VC
mAN
MC
VP
B
Brain
Hippocampus
KW = 18.08*
Ventricles
Frontal cortex
Volume (cc)
2-step volume (cc)
Saline
MIS
Cortex
Corpus callosum
Caudate-putamen
Cerebellum
VH-VH
THC-VH
VH-CBD
THC-CBD
C
Fractional anisotropy
D
NAA
KW = 24.91**

Figure 3.

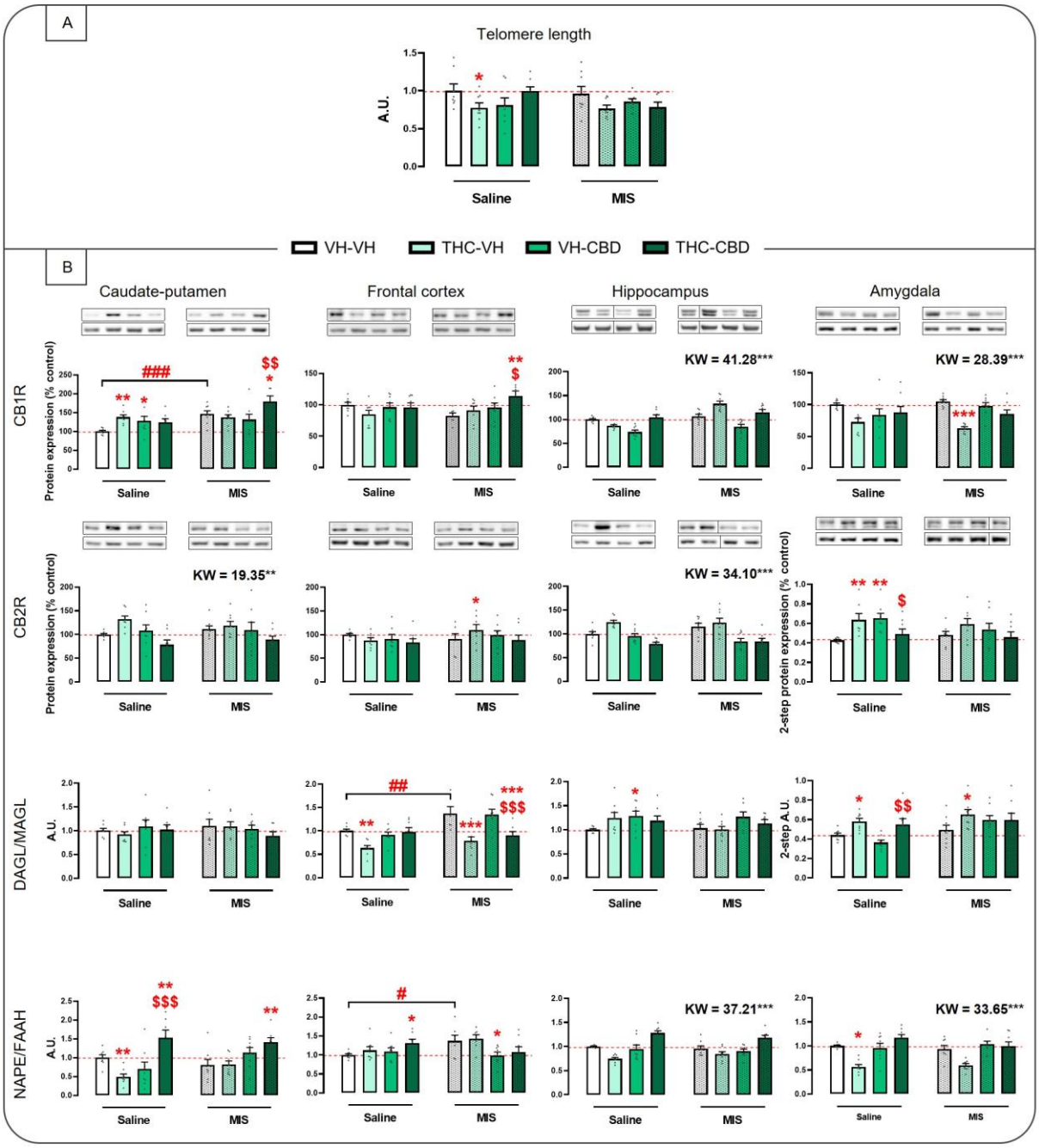
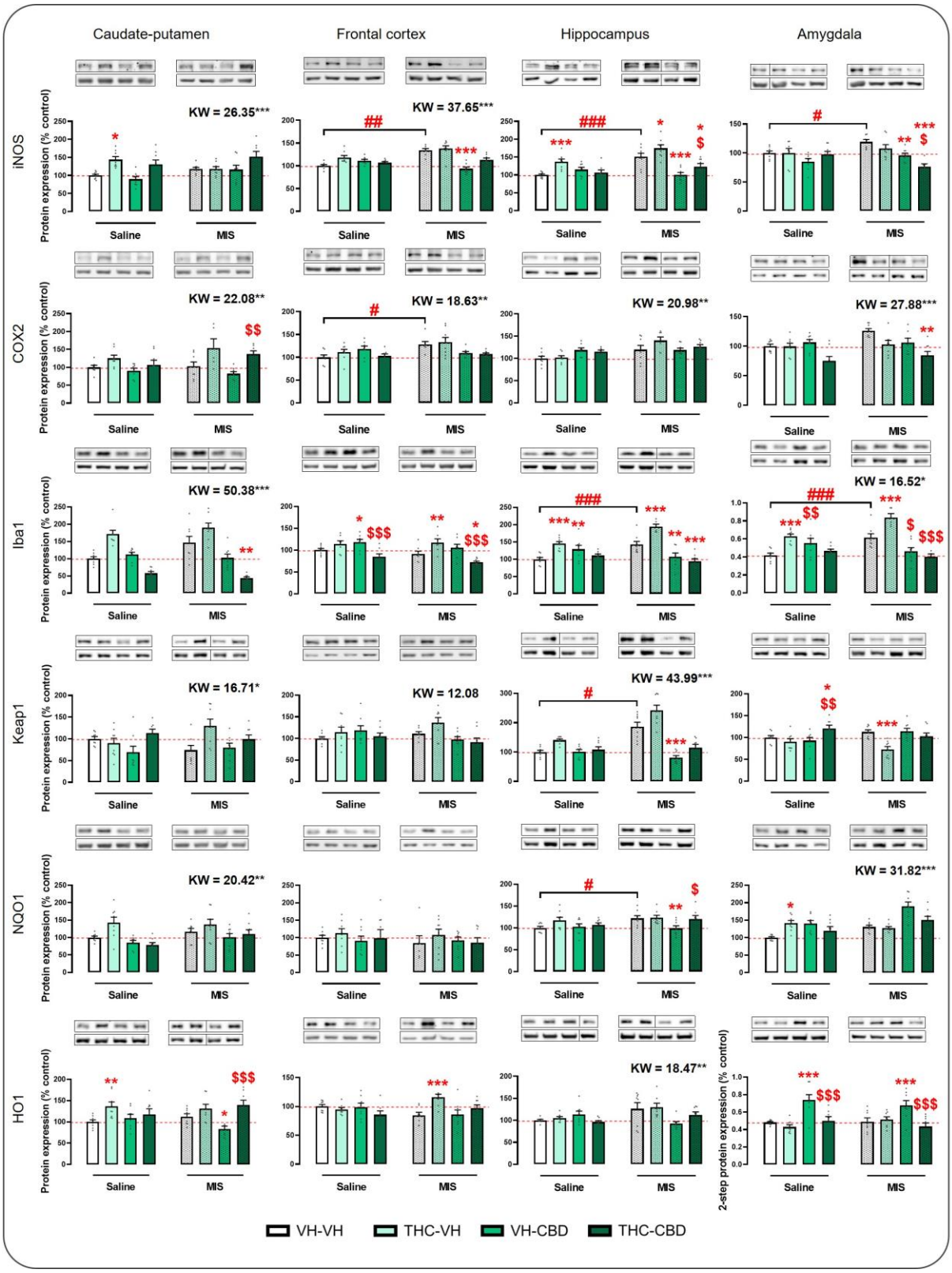
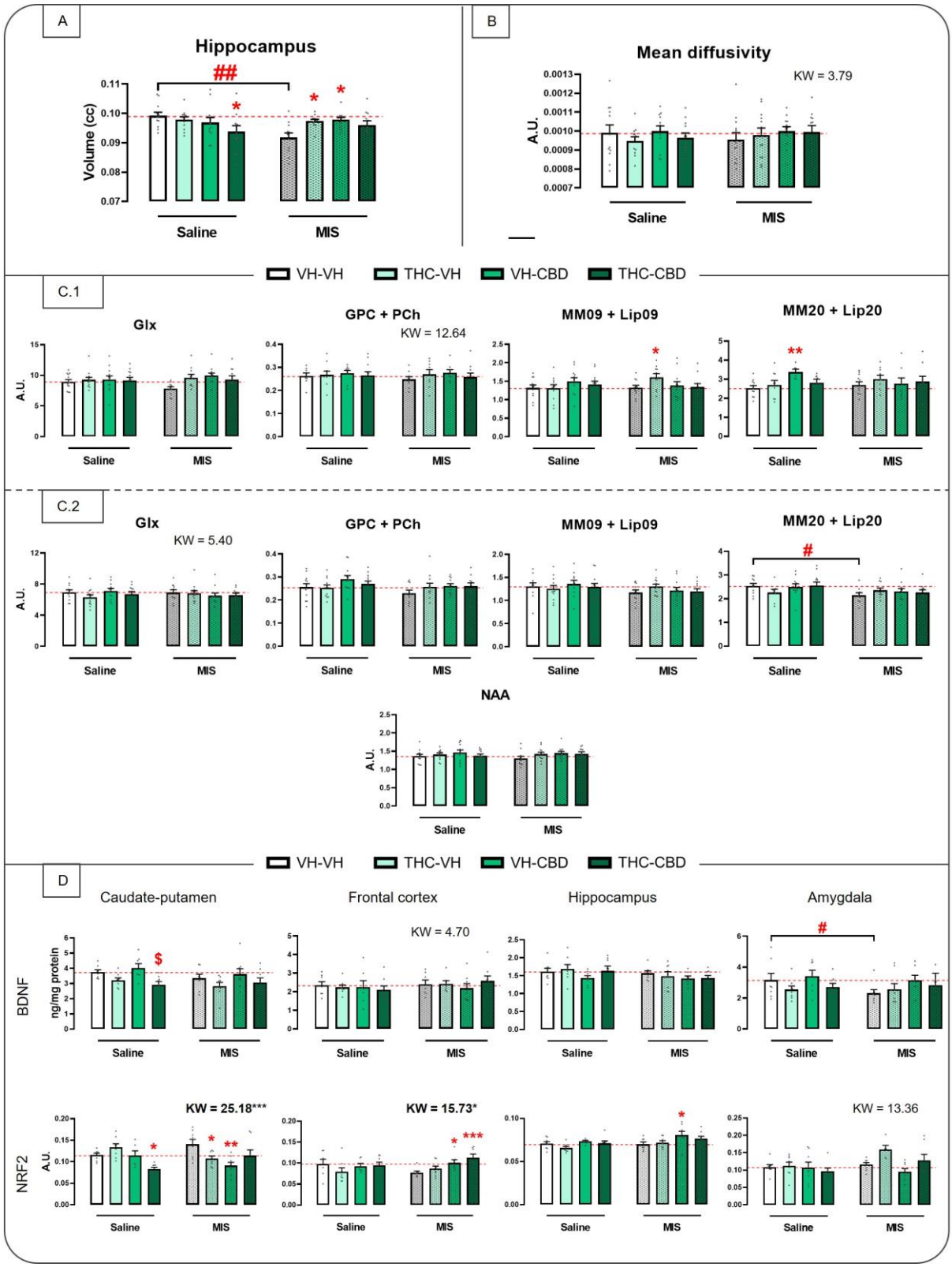

A
Telomere length
A.U.
Saline
MIS
VH-VH
THC-VH
VH-CBD
THC-CBD
B
Caudate-putamen
Frontal cortex
Hippocampus
Amygdala
CB1R
CB2R
DAGL/MAGL
NAPE/FAAH
Protein expression (% control)
2-step protein expression (% control)
2-step A.U.
KW = 41.28***
KW = 28.39***
KW = 19.35**
KW = 34.10***
KW = 37.21***
KW = 33.65***

Figure 4.


Caudate-putamen
Frontal cortex
Hippocampus
Amygdala
iNOS
COX2
Iba1
Keap1
NQO1
HO1
Protein expression (% control)
2-step protein expression (% control)
KW = 26.35***
KW = 37.65***
KW = 22.08**
KW = 18.63**
KW = 20.98**
KW = 27.88***
KW = 50.38***
KW = 16.52*
KW = 16.71*
KW = 12.08
KW = 43.99***
KW = 20.42**
KW = 31.82***
KW = 18.47**
Saline
MIS
VH-VH
THC-VH
VH-CBD
THC-CBD

Supplementary figure S1.



		MIS	THC	CBD	MIS.THC	MIS.CBD	THC.CBD	MIS.THC.CBD	Prepulse
Behavioural study									
Prepulse inhibition	**RM ANOVA**	$F_{(1,104)}$= 3.22	$F_{(1,104)}$= .129	$F_{(1,104)}$= .751	$F_{(1,104)}$= 1.13	$F_{(1,104)}$= .630	$F_{(1,104)}$= .116	**$F_{(1,104)}$= 7.19****	**$F_{(2,104)}$= 276.76*****
		MIS	**THC**	**CBD**	**MIS.THC**	**MIS.CBD**	**THC.CBD**	**MIS.THC.CBD**	**Kruskal–Wallis**
Magnetic resonance studies									
Manual ROI analysis	**Brain**	$F_{(1,87)}$ = .186	$F_{(1,87)}$ = <.001	$F_{(1,87)}$ = .010	$F_{(1,87)}$ = <.001	$F_{(1,87)}$ = .123	$F_{(1,87)}$ = .153	$F_{(1,87)}$ = .080	-
	Hippocampus	-	-	-	-	-	-	-	**KW = 18.08***
	Ventricles	**$F_{(1,87)}$ = 4.33***	$F_{(1,87)}$ = .185	$F_{(1,87)}$ = .508	$F_{(1,87)}$ = 2.25	$F_{(1,87)}$ = .856	**$F_{(1,87)}$ = 10.73****	$F_{(1,87)}$ = 2.98	-
	Frontal cortex	$F_{(1,87)}$ = .330	**$F_{(1,87)}$ = 14.09*****	$F_{(1,87)}$ = .593	$F_{(1,87)}$ = .357	$F_{(1,87)}$ = .742	**$F_{(1,87)}$ = 7.09****	$F_{(1,87)}$ = 3.58	-
	Cortex	$F_{(1,87)}$ = 2.96	$F_{(1,87)}$ = .022	$F_{(1,87)}$ = .396	$F_{(1,87)}$ = 1.88	$F_{(1,87)}$ = 1.81	$F_{(1,87)}$ = .804	$F_{(1,87)}$ = .049	-
	Corpus callosum	$F_{(1,87)}$ = .718	$F_{(1,87)}$ = 1.46	**$F_{(1,87)}$ = 103.03*****	$F_{(1,87)}$ = .788	**$F_{(1,87)}$ = 5.45***	**$F_{(1,87)}$ = 23.81*****	$F_{(1,87)}$ = 3.86	-
	Caudate-putamen	$F_{(1,87)}$ = .762	**$F_{(1,87)}$ = 22.64*****	$F_{(1,87)}$ = .824	$F_{(1,87)}$ = .020	$F_{(1,87)}$ = .765	**$F_{(1,87)}$ = 11.61*****	$F_{(1,87)}$ = .166	-
	Cerebellum	$F_{(1,87)}$ = 2.25	$F_{(1,87)}$ = .113	$F_{(1,87)}$ = 1.93	$F_{(1,87)}$ = .477	$F_{(1,87)}$ = .364	$F_{(1,87)}$ = .926	$F_{(1,87)}$ = .011	-
DTI analysis	**MD**	-	**-**	**-**	-	-	-	-	KW = 3.79
	FA	$F_{(1,85)}$ = 1.29	$F_{(1,85)}$ = 1.41	**$F_{(1,85)}$ = 81.27*****	$F_{(1,85)}$ = .003	$F_{(1,85)}$ = 1.81	$F_{(1,85)}$ = .279	$F_{(1,85)}$ = .002	-

	Glx	-	-	-	-	-	-	-	KW = 12.64
	GPC+PCh	$F_{(1,66)}$ = .123	$F_{(1,66)}$ = .107	$F_{(1,66)}$ = .325	$F_{(1,66)}$ = .041	$F_{(1,66)}$ = .029	$F_{(1,66)}$ = 1.44	$F_{(1,66)}$ = .225	-
^{1}H-MRS analysis - FC	**NAA**	-	-	-	-	-	-	-	**KW = 24.91****
	MM09+Lip09	$F_{(1,84)}$ = .178	$F_{(1,84)}$ = .349	$F_{(1,84)}$ = .099	$F_{(1,84)}$ = 1.67	$F_{(1,84)}$ = 3.33	$F_{(1,84)}$ = 2.43	$F_{(1,84)}$ = .837	-
	MM20+Lip20	$F_{(1,62)}$ = .013	$F_{(1,62)}$ = <.001	$F_{(1,62)}$ = 2.11	$F_{(1,62)}$ = 1.76	$F_{(1,62)}$ = 2.85	$F_{(1,62)}$ = 2.38	$F_{(1,62)}$ = .774	-
	Glx	-	-	-	-	-	-	-	KW = 5.40
	GPC+PCh	$F_{(1,83)}$ = 2.49	$F_{(1,83)}$ = .013	**$F_{(1,83)}$ = 4.69***	$F_{(1,83)}$ = 1.74	$F_{(1,83)}$ = .197	$F_{(1,83)}$ = 1.44	$F_{(1,83)}$ = .066	-
^{1}H-MRS analysis - Hipp	**NAA**	$F_{(1,86)}$ = .010	$F_{(1,86)}$ = .209	$F_{(1,86)}$ = 2.17	$F_{(1,86)}$ = .813	$F_{(1,86)}$ = .300	$F_{(1,86)}$ = 2.99	$F_{(1,86)}$ = .007	-
	MM09+Lip09	$F_{(1,84)}$ = 2.77	$F_{(1,84)}$ = .002	$F_{(1,84)}$ = .021	$F_{(1,84)}$ = 1.33	$F_{(1,84)}$ = .623	$F_{(1,84)}$ = .753	$F_{(1,84)}$ = .544	-
	MM20+Lip20	**$F_{(1,75)}$ = 5.04***	$F_{(1,75)}$ = .013	$F_{(1,75)}$ = .955	$F_{(1,75)}$ = 1.13	$F_{(1,75)}$ = .273	$F_{(1,75)}$ = .052	$F_{(1,75)}$ = 2.88	-

Table shows repeated measures (RM) ANOVA, three-way ANOVA F-test or Kruskal–Wallis (KW) test on the behavioural study and magnetic resonance studies and. [*p<0.05, **p<0.01, ***p<0.001]. [^{1}H-MRS: magnetic resonance spectroscopy; AA: amygdala; CBD: cannabidiol; CPu: caudate-putamen; DTI: diffusion tensor imaging; FA: fractional anisotropy; FC: frontal cortex; Glx: glutamine + glutamate; GPC+PCh: glycerophosphocholine + phosphocholine; Hipp: hippocampus; Lip: lipid; MD: mean diffusivity; MM: macromolecule; MIS: maternal immune stimulation; NAA: N-acetylaspartate + N-Acetylaspartylglutamate; ROI: region of interest; THC: 9-Tetrahidrocannabinol; VH: vehicle].

Supplementary table S2. Statistical data of cannabinoids-related effects during adolescence II.

		MIS	THC	CBD	MIS.THC	MIS.CBD	THC.CBD	MIS.THC.CBD	Kruskal–Wallis
Post-mortem studies									
Telomere length analysis	-	$F_{(1,56)}$= 1.25	$F_{(1,56)}$= 2.43	$F_{(1,56)}$= .062	$F_{(1,56)}$= 1.35	$F_{(1,56)}$= .305	**$F_{(1,56)}$= 7.09****	$F_{(1,56)}$= 2.16	-
BDNF analysis	**CPu**	$F_{(1,56)}$= 2.12	**$F_{(1,56)}$= 14.33*****	$F_{(1,56)}$= .412	$F_{(1,56)}$= .590	$F_{(1,56)}$= .544	$F_{(1,56)}$= .654	$F_{(1,56)}$= .510	-
	FC	-	-	-	-	-	-	-	KW = 4.70
	Hipp	$F_{(1,56)}$= 2.64	$F_{(1,56)}$= .527	$F_{(1,56)}$= 2.38	$F_{(1,56)}$= 1.45	$F_{(1,56)}$= .011	$F_{(1,56)}$= .576	$F_{(1,56)}$= .011	-
	AA	$F_{(1,54)}$= 2.35	$F_{(1,54)}$= 3.38	$F_{(1,54)}$= 3.46	$F_{(1,54)}$= 1.95	$F_{(1,54)}$= 1.18	$F_{(1,54)}$= .389	$F_{(1,54)}$= .337	-
ECS analysis - CPu	**CB1R**	**$F_{(1,53)}$= 13.93*****	**$F_{(1,53)}$= 6.84***	$F_{(1,53)}$= 2.33	$F_{(1,53)}$= .032	$F_{(1,53)}$= .233	$F_{(1,53)}$= .290	**$F_{(1,53)}$= 12.61*****	-
	CB2R	-	-	-	-	-	-	-	**KW = 19.35****
	DAGL/MAGL	$F_{(1,54)}$= .115	$F_{(1,54)}$= 1.22	$F_{(1,54)}$= .074	$F_{(1,54)}$= <.001	$F_{(1,54)}$= 2.66	$F_{(1,54)}$= .176	$F_{(1,54)}$= .306	-
	NAPE/FAAH	$F_{(1,55)}$= 1.33	$F_{(1,55)}$= 2.57	**$F_{(1,55)}$= 18.47*****	$F_{(1,55)}$= .005	$F_{(1,55)}$= .236	**$F_{(1,55)}$= 17.21*****	**$F_{(1,55)}$= 7.91****	-
ECS analysis - FC	**CB1R**	$F_{(1,55)}$= .150	$F_{(1,55)}$= .410	**$F_{(1,55)}$= 5.52***	**$F_{(1,55)}$= 5.38***	$F_{(1,55)}$= 2.40	$F_{(1,55)}$= 1.82	$F_{(1,55)}$= .081	-
	CB2R	$F_{(1,53)}$= 1.41	$F_{(1,53)}$= .200	$F_{(1,53)}$= 1.16	$F_{(1,53)}$= 1.28	$F_{(1,53)}$= <.001	$F_{(1,53)}$= .977	$F_{(1,53)}$= 1.83	-
	DAGL/MAGL	**$F_{(1,54)}$= 13.41*****	**$F_{(1,54)}$= 30.32*****	$F_{(1,54)}$= 1.99	**$F_{(1,54)}$= 8.70****	$F_{(1,54)}$= .435	**$F_{(1,54)}$= 5.64***	$F_{(1,54)}$= 1.48	-
	NAPE/FAAH	$F_{(1,54)}$= 1.36	$F_{(1,54)}$= 2.91	$F_{(1,54)}$= 2.33	$F_{(1,54)}$= .443	**$F_{(1,54)}$= 12.14*****	$F_{(1,54)}$= .202	$F_{(1,54)}$= .047	-
ECS analysis - Hipp	**CB1R**	-	-	-	-	-	-	-	**KW = 41.28*****

	CB2R	-	-	-	-	**-**	-	-	**KW = 34.10*** **
	DAGL/MAGL	$F_{(1,55)}$= 1.30	$F_{(1,55)}$= .010	$F_{(1,55)}$= 5.54*	$F_{(1,55)}$= 1.65	$F_{(1,55)}$= .293	$F_{(1,55)}$= 3.34	$F_{(1,55)}$= .843	-
	NAPE/FAAH	-	-	-	-	**-**	-	-	**KW = 37.21*** **
ECS analysis - AA	**CB1R**	-	-	-	-	**-**	-	-	**KW = 28.39*** **
	CB2R	$F_{(1,55)}$= .907	$F_{(1,55)}$= .313	$F_{(1,55)}$= <.001	$F_{(1,55)}$= .010	$F_{(1,55)}$= 1.27	**$F_{(1,55)}$= 15.45*** **	$F_{(1,55)}$= 1.61	-
	DAGL/MAGL	**$F_{(1,56)}$= 8.74** **	**$F_{(1,56)}$= 13.15*** **	$F_{(1,56)}$= .194	$F_{(1,56)}$= 1.53	$F_{(1,56)}$= 1.29	$F_{(1,56)}$= .657	$F_{(1,56)}$= 2.33	-
	NAPE/FAAH	-	-	-	-	**-**	-	-	**KW = 33.65*** **
IOS analysis - CPu	**iNOS**	-	-	-	-	-	-	-	**KW = 26.35*** **
	COX2	-	-	-	-	-	-	-	**KW = 22.08** **
	Iba1	-	-	-	-	-	-	-	**KW = 50.38*** **
	Keap1	-	-	-	-	-	-	-	**KW = 16.71***
	NQO1	-	-	-	-	-	-	-	**KW = 20.42** **
	HO1	$F_{(1,56)}$= .026	**$F_{(1,56)}$= 19.27*** **	$F_{(1,56)}$= 1.36	$F_{(1,56)}$= 1.32	$F_{(1,56)}$= .151	$F_{(1,56)}$= .126	**$F_{(1,56)}$= 5.84***	-
	NRF2	-	**-**	-	-	-	-	**-**	**KW = 25.18*** **
IOS analysis - FC	**iNOS**	-	-	-	-	-	-	-	**KW = 37.65*** **
	COX2	-	-	-	-	-	-	-	**KW = 18.63** **
	Iba1	$F_{(1,53)}$= 3.02	$F_{(1,53)}$= 2.18	$F_{(1,53)}$= 5.14	$F_{(1,53)}$= .491	$F_{(1,53)}$= 1.27	**$F_{(1,53)}$= 35.36*** **	$F_{(1,53)}$= .519	**-**
	Keap1	-	-	-	-	-	-	-	KW = 12.08

	NQO1	$F_{(1,54)}$= .536	$F_{(1,54)}$= .716	$F_{(1,54)}$= .696	$F_{(1,54)}$= .007	$F_{(1,54)}$= .051	$F_{(1,54)}$= .629	$F_{(1,54)}$= .315	-
	HO1	$F_{(1,54)}$= .039	$F_{(1,54)}$= 2.17	$F_{(1,54)}$= 2.83	**$F_{(1,54)}$= 13.46*** **	$F_{(1,54)}$= .174	$F_{(1,54)}$= 3.02	$F_{(1,54)}$= .575	-
	NRF2	-	-	-	-	-	-	-	**KW = 15.73***
	iNOS	**$F_{(1,56)}$= 17.86*** **	**$F_{(1,56)}$= 12.68*** **	**$F_{(1,56)}$= 29.58*** **	$F_{(1,56)}$= .652	**$F_{(1,56)}$= 16.12*** **	**$F_{(1,56)}$= 4.58***	**$F_{(1,56)}$= 4.26***	-
	COX2	-	-	-	-	-	-	-	**KW = 20.98****
	Iba1	**$F_{(1,53)}$= 5.55***	**$F_{(1,53)}$= 7.21****	**$F_{(1,53)}$= 34.44*** **	$F_{(1,53)}$= .144	**$F_{(1,53)}$= 28.57*** **	**$F_{(1,53)}$= 30.59*** **	$F_{(1,53)}$= .026	-
IOS analysis - Hipp	**Keap1**	-	-	-	-	-	-	-	**KW = 43.99*** **
	NQO1	**$F_{(1,56)}$= 5.04***	**$F_{(1,56)}$= 6.77***	$F_{(1,56)}$= 3.68	$F_{(1,56)}$= .011	$F_{(1,56)}$= 1.20	$F_{(1,56)}$= .218	$F_{(1,56)}$= 3.65	-
	HO1	-	-	-	-	-	-	-	**KW = 18.47****
	NRF2	**$F_{(1,54)}$= 5.90***	$F_{(1,54)}$= 1.70	**$F_{(1,54)}$= 9.49****	$F_{(1,54)}$= .400	$F_{(1,54)}$= .972	$F_{(1,54)}$= .231	$F_{(1,54)}$= 1.43	-
	iNOS	$F_{(1,53)}$= 1.26	$F_{(1,53)}$= 1.57	**$F_{(1,53)}$= 21.95*** **	**$F_{(1,53)}$= 7.74****	**$F_{(1,53)}$= 6.07***	$F_{(1,53)}$= .078	$F_{(1,53)}$= 1.75	-
	COX2	-	-	-	-	-	-	-	**KW = 27.88*** **
	Iba1	-	-	-	-	-	-	-	**KW = 16.52***
IOS analysis - AA	**Keap1**	$F_{(1,56)}$= .006	$F_{(1,56)}$= 3.24	**$F_{(1,56)}$= 8.45****	**$F_{(1,56)}$= 13.94*** **	$F_{(1,56)}$= .194	**$F_{(1,56)}$= 12.10*** **	$F_{(1,56)}$= .219	-
	NQO1	-	-	-	-	-	-	-	**KW = 31.82*** **
	HO1	$F_{(1,56)}$= .070	**$F_{(1,56)}$= 18.19*** **	**$F_{(1,56)}$= 13.32*** **	$F_{(1,56)}$= .383	$F_{(1,56)}$= 3.52	**$F_{(1,56)}$= 14.52*** **	$F_{(1,56)}$= .432	-
	NRF2	-	-	-	-	-	-	-	KW = 13.36

Table shows three-way ANOVA F-test or Kruskal–Wallis (KW) test on post-mortem studies. [*p<0.05, **p<0.01, ***p<0.001]. [AA: amygdala; AREs: anti-oxidant response elements; BDNF: brain-derived neurotrophic factor; CBD: cannabidiol; COX2: cyclooxygenase 2; CPu: caudate-putamen; DAGL: diacylglycerol lipase; ECS: endocannabinoid system; FAAH: fatty acid amidohydrolase; FC: frontal cortex; Hipp: hippocampus; HO1: heme oxygenase 1; Iba1: ionized calcium-binding adapter molecule 1; iNOS: inducible nitric oxide synthase; IOS: inflammation and oxidative stress; Keap1: Kelch-like ECH-associated protein 1; MAGL: monoacylglycerol lipase; MIS: maternal immune stimulation; NAPE: N-acyl phosphatidylethanolamine-specific phospholipase D; NQO1: NAD(P)H quinone oxidoreductase 1; NRF2: nuclear factor erythroid factor 2; THC: 9-Tetrahidrocannabinol; VH: vehicle].

Supplementary table S3. Size effect of the data of cannabinoids-related effects during adolescence I.

		MIS	THC	CBD	MIS.THC	MIS.CBD	THC.CBD	MIS.THC.CBD	Prepulse
Behavioural study									
Prepulse inhibition	**RM ANOVA**	η^2_p = .036 (3.6%)	η^2_p = .001 (.1%)	η^2_p = .008 (.8%)	η^2_p = .013 (1.3%)	η^2_p = .007 (.7%)	η^2_p = .001 (.1%)	η^2_p = .078 (7.8%)	η^2_p = .76 (76%)
		MIS	**THC**	**CBD**	**MIS.THC**	**MIS.CBD**	**THC.CBD**	**MIS.THC.CBD**	**Kruskal–Wallis**
Magnetic resonance studies									
Manual ROI analysis	**Brain**	η^2_p = .002 (.2%)	η^2_p = <.001 (<.1%)	η^2_p = <.001 (<.1%)	η^2_p = <.001 (<.1%)	η^2_p = .001 (.1%)	η^2_p = .001 (.1%)	η^2_p = .001 (.1%)	-
	Hippocampus	-	-	-	-	-	-	-	η^2_p = .206 (20.6%)
	Ventricles	η^2_p = .047 (4.7%)	η^2_p = .002 (.2%)	η^2_p = .006 (.6%)	η^2_p = .025 (2.5%)	η^2_p = .010 (1%)	η^2_p = .110 (11%)	η^2_p = .033 (3.3%)	-
	Frontal cortex	η^2_p = .004 (.4%)	η^2_p = .139 (13.9%)	η^2_p = .007 (.7%)	η^2_p = .004 (.4%)	η^2_p = .008 (.8%)	η^2_p = .075 (7.5%)	η^2_p = .040 (4%)	-
	Cortex	η^2_p = .033 (3.3%)	η^2_p = <.001 (<.1%)	η^2_p = .005 (.5%)	η^2_p = .021 (2.1%)	η^2_p = .020 (2%)	η^2_p = .009 (.9%)	η^2_p = .001 (.1%)	-
	Corpus callosum	η^2_p = .008 (.8%)	η^2_p = .017 (1.7%)	η^2_p = .542 (54.2%)	η^2_p = .009 (.9%)	η^2_p = .059 (5.9%)	η^2_p = .215 (21.5%)	η^2_p = .042 (4.2%)	-
	Caudate-putamen	η^2_p = .009 (.9%)	η^2_p = .206 (20.6%)	η^2_p = .009 (.9%)	η^2_p = <.001 (<.1%)	η^2_p = .009 (.9%)	η^2_p = .118 (11.8%)	η^2_p = .002 (.2%)	-
	Cerebellum	η^2_p = .025 (2.5%)	η^2_p = .001 (.1%)	η^2_p = .022 (2.2%)	η^2_p = .005 (.5%)	η^2_p = .004 (.4%)	η^2_p = .011 (1.1%)	η^2_p = <.001 (<.1%)	-

DTI analysis	**MD**	-	-	-	-	-	-	-	η^2_p = .033 (3.3%)
	FA	η^2_p = .015 (1.5%)	η^2_p = .016 (1.6%)	η^2_p = .489 (48.9%)	η^2_p = <.001 (<.1%)	η^2_p = .021 (2.1%)	η^2_p = .003 (.3%)	η^2_p = <.001 (<.1%)	-
^{1}H-MRS analysis - FC	**Glu+Gln**	-	-	-	-	-	-	-	η^2_p = .118 (11.8%)
	GPC+PCh	η^2_p = .002 (.2%)	η^2_p = .002 (.2%)	η^2_p = .005 (.5%)	η^2_p = .001 (.1%)	η^2_p = <.001 (<.1%)	η^2_p = .021 (2.1%)	η^2_p = .636 (63.6%)	-
	NAA+NAAG	-	-	-	-	-	-	-	η^2_p = .271 (27.1%)
	MM09+Lip09	η^2_p = .002 (.2%)	η^2_p = .004 (.4%)	η^2_p = .001 (.1%)	η^2_p = .019 (1.9%)	η^2_p = .038 (3.8%)	η^2_p = .028 (2.8%)	η^2_p = .010 (.1%)	-
	MM20+Lip20	η^2_p = <.001 (<.1%)	η^2_p = <.001 (<.1%)	η^2_p = .033 (3.3%)	η^2_p = .028 (2.8%)	η^2_p = .044 (4.4%)	η^2_p = .037 (3.7%)	η^2_p = .012 (1.2%)	-
^{1}H-MRS analysis - Hipp	**Glu+Gln**	-	-	-	-	-	-	-	η^2_p = .056 (5.6%)
	GPC+PCh	η^2_p = .029 (2.9%)	η^2_p = <.001 (<.1%)	η^2_p = .053 (5.3%)	η^2_p = .020 (2%)	η^2_p = .002 (.2%)	η^2_p = .017 (1.7%)	η^2_p = .001 (.1%)	-
	NAA+NAAG	η^2_p = <.001 (<.1%)	η^2_p = .002 (.2%)	η^2_p = .025 (2.5%)	η^2_p = .009 (.9%)	η^2_p = .003 (.3%)	η^2_p = .034 (3.4%)	η^2_p = <.001 (<.1%)	-
	MM09+Lip09	η^2_p = .032 (3.2%)	η^2_p = <.001 (<.1%)	η^2_p = <.001 (<.1%)	η^2_p = .016 (1.6%)	η^2_p = .007 (.7%)	η^2_p = .009 (.9%)	η^2_p = .006 (.6%)	-
	MM20+Lip20	η^2_p = .063 (6.3%)	η^2_p = <.001 (<.1%)	η^2_p = .013 (1.3%)	η^2_p = .015 (1.5%)	η^2_p = .004 (.4%)	η^2_p = .001 (.1%)	η^2_p = .037 (3.7%)	-

Table shows partial eta square (η^2_p) of every variable on the behavioural study and magnetic resonance studies. [^{1}H-MRS: magnetic resonance spectroscopy; AA: amygdala; CBD: cannabidiol; CPu: caudate-putamen; DTI: diffusion tensor imaging; FA: fractional anisotropy; FC: frontal cortex; Glx: glutamine + glutamate;

GPC+PCh: glycerophosphocholine + phosphocholine; Hipp: hippocampus; Lip: lipid; MD: mean diffusivity; MM: macromolecule; MIS: maternal immune stimulation; NAA: N-acetylaspartate + N-Acetylaspartylglutamate; ROI: region of interest; THC: 9-Tetrahidrocannabinol; VH: vehicle].

Supplementary table S4. Size effect of the data of cannabinoids-related effects during adolescence II.

		MIS	THC	CBD	MIS.THC	MIS.CBD	THC.CBD	MIS.THC.CBD	Prepulse
Post-mortem studies									
Telomere length analysis	-	η^2_p = .022 (2.2%)	η^2_p = .042 (4.2%)	η^2_p = .001 (.1%)	η^2_p = .023 (2.3%)	η^2_p = .005 (.5%)	η^2_p = .112 (11.2%)	η^2_p = .037 (3.7%)	-
BDNF analysis	**CPu**	η^2_p = .037 (3.7%)	η^2_p = .207 (20.7%)	η^2_p = .007 (.7%)	η^2_p = .011 (1.1%)	η^2_p = .010 (1%)	η^2_p = .012 (1.2%)	η^2_p = .009 (.9%)	-
	FC	-	-	-	-	-	-	-	η^2_p = .057 (5.7%)
	Hipp	η^2_p = .046 (4.6%)	η^2_p = .009 (.9%)	η^2_p = .041 (4.1%)	η^2_p = .026 (2.6%)	η^2_p = <.001 (<.1%)	η^2_p = .010 (1%)	η^2_p = <.001 (<.1%)	-
	AA	η^2_p = .027 (2.7%)	η^2_p = .008 (.8%)	η^2_p = .008 (.8%)	η^2_p = .022 (2.2%)	η^2_p = .015 (1.5%)	η^2_p = .002 (.2%)	η^2_p = .005 (.5%)	-
ECS analysis - CPu	**CB1R**	η^2_p = .208 (20.8%)	η^2_p = .114 (11.4%)	η^2_p = .042 (4.2%)	η^2_p = .001 (.1%)	η^2_p = .004 (.4%)	η^2_p = .005 (.5%)	η^2_p = .192 (19.2%)	-
	CB2R	-	-	-	-	-	-	-	η^2_p = .269 (26.9%)
	DAGL/MAGL	η^2_p = .002 (.2%)	η^2_p = .022 (2.2%)	η^2_p = .001 (.1%)	η^2_p = <.001 (<.1%)	η^2_p = .047 (4.7%)	η^2_p = .003 (.3%)	η^2_p = .006 (.6%)	-
	NAPE/FAAH	η^2_p = .024 (2.4%)	η^2_p = .045 (4.5%)	η^2_p = .251 (25.1%)	η^2_p = <.001 (<.1%)	η^2_p = .004 (.4%)	η^2_p = .238 (23.8%)	η^2_p = .126 (12.6%)	-
ECS analysis - FC	**CB1R**	η^2_p = .003 (.3%)	η^2_p = .007 (.7%)	η^2_p = .091 (9.1%)	η^2_p = .089 (8.9%)	η^2_p = .042 (4.2%)	η^2_p = .032 (3.2%)	η^2_p = .001 (.1%)	-
	CB2R	η^2_p = .021 (2,1%)	η^2_p = .004 (.4%)	η^2_p = .021 (2.1%)	η^2_p = .024 (2.4%)	η^2_p = <.001 (<.1%)	η^2_p = .018 (1.8%)	η^2_p = .033 (3.3%)	-

	DAGL/MAGL	η^2_p = .199 (19.9%)	η^2_p = .360 (36%)	η^2_p = .036 (3.6%)	η^2_p = .139 (13.9%)	η^2_p = .008 (.8%)	η^2_p = .095 (9.5%)	η^2_p = .027 (2.7%)	-
	NAPE/FAAH	η^2_p = .025 (2.5%)	η^2_p = .051 (5.1%)	η^2_p = .041 (4.1%)	η^2_p = .008 (.8%)	η^2_p = .184 (18.4%)	η^2_p = .004 (.4%)	η^2_p = .001 (.1%)	-
ECS analysis - Hipp	**CB1R**	-	-	-	-	-	-	-	η^2_p = .668 (66.8%)
	CB2R	-	-	-	-	-	-	-	η^2_p = .523 (52.3%)
	DAGL/MAGL	η^2_p = .023 (2.3%)	η^2_p = <.001 (<.1%)	η^2_p = .092 (9.2%)	η^2_p = .029 (2.9%)	η^2_p = .005 (.5%)	η^2_p = .057 (5.7%)	η^2_p = .015 (1.5%)	-
	NAPE/FAAH	-	-	-	-	-	-	-	η^2_p = .602 (60.2%)
ECS analysis - AA	**CB1R**	-	-	-	-	-	-	-	η^2_p = .403 (40.3%)
	CB2R	η^2_p = .016 (1.6%)	η^2_p = .006 (.6%)	η^2_p = <.001 (<.1%)	η^2_p = <.001 (<.1%)	η^2_p = .022 (2.2%)	η^2_p = .219 (21.9%)	η^2_p = .029 (2.9%)	-
	DAGL/MAGL	η^2_p = .140 (14%)	η^2_p = .182 (18.2%)	η^2_p = .002 (.2%)	η^2_p = .030 (3%)	η^2_p = .035 (2.5%)	η^2_p = .014 (1.4%)	η^2_p = .044 (4.4%)	-
	NAPE/FAAH	-	-	-	-	-	-	-	η^2_p = .334 (33.4%)
IOS analysis - CPu	**iNOS**	-	-	-	-	-	-	-	η^2_p = .394 (39.4%)
	COX2	-	-	-	-	-	-	-	η^2_p = .383 (38.3%)
	Iba1	-	-	-	-	-	-	-	η^2_p = .753 (75.3%)

	Keap1	-	-	-	-	-	-	-	η^2_p = .296 (29.6%)
	NQO1	-	-	-	-	-	-	-	η^2_p = .356 (35.6%)
	HO1	η^2_p = <.001 (<.1%)	η^2_p = .256 (25.6%)	η^2_p = .024 (2.4%)	η^2_p = .023 (2.3%)	η^2_p = .003 (.3%)	η^2_p = .002 (.2%)	η^2_p = .094 (9.4%)	-
	NRF2	-	-	-	-	-	-	-	η^2_p = .393 (39.3%)
	iNOS	-	-	-	-	-	-	-	η^2_p = .649 (64.9%)
	COX2	-	-	-	-	-	-	-	η^2_p = .328 (32.8%)
	Iba1	η^2_p = .054 (5.4%)	η^2_p = .040 (4%)	η^2_p = .088 (8.8%)	η^2_p = .009 (.9%)	η^2_p = .023 (2.3%)	η^2_p = .400 (40%)	η^2_p = .010 (1%)	-
IOS analysis - FC	**Keap1**	-	-	-	-	-	-	-	η^2_p = .243 (24.3%)
	NQO1	η^2_p = .010 (1%)	η^2_p = .013 (1.3%)	η^2_p = .013 (1.3%)	η^2_p = <.001 (<.1%)	η^2_p = .001 (.1%)	η^2_p = .012 (1.2%)	η^2_p = .006 (.6%)	-
	HO1	η^2_p = .001 (.1%)	η^2_p = .039 (3.9%)	η^2_p = .050 (5%)	η^2_p = .200 (20%)	η^2_p = .003 (.3%)	η^2_p = .053 (5.3%)	η^2_p = .011 (1.1%)	-
	NRF2	-	-	-	-	-	-	-	η^2_p = .236 (23.6%)
IOS analysis - Hipp	**iNOS**	η^2_p = .242 (24.2%)	η^2_p = .185 (18.5%)	η^2_p = .346 (34.6%)	η^2_p = .012 (1.2%)	η^2_p = .224 (22.4%)	η^2_p = .076 (7.6%)	η^2_p = .071 (7.1%)	-
	COX2	-	-	-	-	-	-	-	η^2_p = .372 (37.2%)

	Iba1	η^2_p = .004 (.4%)	η^2_p = .001 (.1%)	η^2_p = .049 (4.9%)	η^2_p = .132 (13.2%)	η^2_p = .013 (1.3%)	η^2_p = .001 (.1%)	η^2_p = .034 (3.4%)	-
	Keap1	-	-	-	-	-	-	-	η^2_p = .750 (75%)
	NQO1	η^2_p = .083 (8.3%)	η^2_p = .108 (10.8%)	η^2_p = .062 (6.2%)	η^2_p = <.001 (<.1%)	η^2_p = .021 (2.1%)	η^2_p = .004 (.4%)	η^2_p = .061 (6.1%)	-
	HO1	-	-	-	-	-	-	-	η^2_p = .298 (29.8%)
	NRF2	η^2_p = .098 (9.8%)	η^2_p = .030 (3%)	η^2_p = .150 (15%)	η^2_p = .007 (.7%)	η^2_p = .018 (1.8%)	η^2_p = .004 (.4%)	η^2_p = .026 (2.6%)	-
	iNOS	η^2_p = .023 (2.3%)	η^2_p = .029 (2.9%)	η^2_p = .293 (29.3%)	η^2_p = .127 (12.7%)	η^2_p = .103 (10.3%)	η^2_p = .001 (.1%)	η^2_p = .032 (3.2%)	-
	COX2	-	-	-	-	-	-	-	η^2_p = .463 (46.3%)
	Iba1	-	-	-	-	-	-	-	η^2_p = .261 (26.1%)
IOS analysis - AA	**Keap1**	η^2_p = <.001 (<.1%)	η^2_p = .055 (5.5%)	η^2_p = .131 (13.1%)	η^2_p = .199 (19.9%)	η^2_p = .003 (.3%)	η^2_p = .178 (17.8%)	η^2_p = .004 (.4%)	-
	NQO1	-	-	-	-	-	-	-	η^2_p = .540 (54%)
	HO1	η^2_p = .001 (.1%)	η^2_p = .245 (24.5%)	η^2_p = .192 (19.2%)	η^2_p = .007 (.7%)	η^2_p = .059 (5.9%)	η^2_p = .206 (20.6%)	η^2_p = .008 (.8%)	-
	NRF2	-	-	-	-	-	-	-	η^2_p = .259 (25.9%)

Table shows partial eta square (η^2_p) of every variable on post-mortem studies. [*p<0.05, **p<0.01, ***p<0.001]. [AA: amygdala; AREs: anti-oxidant response elements; BDNF: brain-derived neurotrophic factor; CBD: cannabidiol; COX2: cyclooxygenase 2; CPu: caudate-putamen; DAGL: diacylglycerol lipase; ECS: endocannabinoid system; FAAH: fatty acid amidohydrolase; FC: frontal cortex; Hipp: hippocampus; HO1: heme oxygenase 1; Iba1: ionized calcium-binding

adapter molecule 1; iNOS: inducible nitric oxide synthase; IOS: inflammation and oxidative stress; Keap1: Kelch-like ECH-associated protein 1; MAGL: monoacylglycerol lipase; MIS: maternal immune stimulation; NAPE: N-acyl phosphatidylethanolamine-specific phospholipase D; NQO1: NAD(P)H quinone oxidoreductase 1; NRF2: nuclear factor erythroid factor 2; THC: 9-Tetrahidrocannabinol; VH: vehicle].

· Discussion ·

· DISCUSSION ·

The goal of this thesis is to provide a general overview of the effects of different cannabinoids on preclinical models of psychiatric disorders, rather than delving into the intricacies of every underlying mechanism that may account for the results obtained in the different chapters. Thus, this thesis incorporates a diverse range of methodologies, including behavioural tests, advanced imaging techniques such as positron emission tomography (PET) and magnetic resonance imaging (MRI), and multiple post-mortem evaluations encompassing inflammation and oxidative stress (IOS) markers, endocannabinoid system (ECS) markers, and telomere length (TL). The incorporation of these diverse techniques makes this thesis a distinctive contribution, providing a broad perspective on the effects of cannabinoids in psychiatric disorders. Furthermore, by considering this thesis as an ongoing endeavour, its complexity has progressively evolved as we have progressed, commencing with a smaller set of tests, and expanding to a more comprehensive battery of assessments by the study's conclusion. This section intends to discuss and contextualize the obtained results, while integrating them within the existing scientific landscape.

Genetic and individual predisposition to develop diverse psychiatric disorders has long been a subject in this field. The first chapter of this thesis focused on elucidating a novel aspect, (brain glucose metabolism), of differential effects observed in an addiction model of morphine self-administration (MSA) between two distinct rat strains. In this sense, different rat strains have been used for this study, with the consequent differences in the potential interpretation of the results. Traditionally, the Lewis (LEW) strain has been regarded as more susceptible to addiction, while both LEW and Fischer (F344) rats have exhibited poorer responses to stressful situations and heightened sensitivity in the dopaminergic system, in comparison to Wistar rats. Consequently, Wistar rats have emerged as a widely employed model in addiction and other psychiatric disorder preclinical studies. They have demonstrated similar drug intake effects as the LEW strain, yet they possess a more stable profile in different behavioural tests, which is crucial for comprehending the diverse range of preclinical models for psychiatric disorders.

Therefore, prior to examining the effects of cannabinoids, the initial chapter of this thesis focused on comparing two rat strains in terms of MSA during adulthood. To accomplish this assessment, we employed a behavioural self-administration study in conjunction with PET imaging to investigate differences in brain glucose metabolism. At this juncture, we deemed it pertinent to explore these behavioural and metabolic changes in rats previously exposed to a cannabinoid agonist during adolescence. Consequently, in the subsequent chapter, we thoroughly examined these differences using the same methodologies employed in the first chapter, complemented by post-mortem evaluations of various ECS markers, while also conducting a long-term assessment that takes into account potential sex-dependent effects.

In the third chapter, we introduced a schizophrenia-like model (maternal immune stimulation, MIS) into the equation. As previously mentioned, cannabinoid usage during adolescence can yield diverse adverse effects and long-term consequences, not only limited to drug dependence, but also encompassing the development of other psychiatric disorders. Thus, this chapter examines the effects of two contrasting cannabinoids: delta-9-tetrahydrocannabinol (THC), the primary psychoactive component of cannabis, and cannabidiol (CBD), which lacks psychoactive effects but possesses antioxidant and anti-inflammatory properties. In this chapter, we expanded the range of experiments conducted, commencing with a behavioural test commonly regarded as the gold standard in schizophrenia preclinical research. Additionally, we performed various assessments utilizing MRI techniques, including structural studies, evaluations of fibre integrity, and metabolic analyses. Furthermore, we performed different post-mortem evaluations, adding the evaluation of IOS markers and TL measurements to the ECS marker determinations.

Addictions

In both Chapter I and Chapter II, we conducted the identical procedure for MSA, employing the same drug, during the same timeframe, and following the exact same protocol. The sole difference between the two chapters was the utilization of different rat strains, which allowed us to gather insights into the contrasting effects between LEW and F344 rats (Chapter I), as well as between male and female Wistar rats (Chapter II).

Morphine self-administration behavioural study

Regarding the **LEW and F344 strains**, it has been observed that LEW rats exhibit a higher susceptibility for addiction development and relapse compared to F344 rats [31]. In our results, we observed a higher number of self-administered morphine injections by LEW compared to F344 and controls. Our findings are consistent with previous research conducted by our group, which demonstrated an increased number of self-administered cocaine injections in LEW rats compared to F344 rats [84]. Similarly, other studies have reported similar results in relation to other addictive substances, such as alcohol or opiates [31,85]. When investigating addiction, it is crucial to ascertain an individual's susceptibility to addiction, as drug dependence susceptibility may be associated with differences in the efficacy of neural substrates in decoding drug effects, including differences in brain metabolism.

In relation to the **male and female Wistar rats**, our observations indicate that female rats displayed a significant increase in the average number of morphine injections, particularly following preexposure to CP-55,940 (CP), whereas males did not exhibit significant changes in self-administration. These findings are consistent with previous studies that have reported

an elevated level of MSA in females exposed to THC during the perinatal period, while no significant alterations were observed in males [86]. On the contrary, contrasting effects have been observed in other studies examining morphine or heroin self-administration, where males displayed an increase in their injection rates, as opposed to females that did not exhibit similar changes [87]. These divergent responses may be attributed to the utilisation of different rat strains, which could account for vulnerability differences [31]. Overall, these findings contribute to understanding sex differences in opioid self-administration, strengthening the notion of females finding opiates more reinforcing than males.

Short-term effects of morphine on brain glucose metabolism

Again, Chapter I and Chapter II employed the same methodology for studying brain glucose metabolism following MSA. For the PET imaging study, we utilized [^{18}F]-FDG as a marker to measure cerebral glucose consumption, serving as an indicator of neuronal activity. This approach revealed significant differences between the LEW and F344 strains, as well as male and female Wistar rats. The primary distinction in the PET studies conducted in these two chapters lies on the duration of the evaluation. While the LEW vs F344 comparison involved a fifteen-day withdrawal period, the Wistar rats were assessed after eight and fourteen weeks of withdrawal. It is worth noting that most studies investigating the effects of drug withdrawal typically focus on time periods no longer than 25–28 days. An important strength of our study is that we evaluated the effects over a period of up to fourteen weeks of withdrawal, which is equivalent to approximately 10 human years [38].

Specifically, the [^{18}F]-FDG-PET study revealed increased glucose metabolism in cortical areas, including the somatosensory and entorhinal cortices, as well as the hypothalamus, in **LEW rats compared to F344 rats**. These areas play a significant role in brain reward circuits [88] and may contribute to the observed differences between the two strains. Importantly, the increased glucose metabolism in the somatosensory system of LEW rats suggests potential differences in sensory information processing, as this region contributes to the positive rewarding properties of the drug [89]. Another affected cortical area, the entorhinal cortex, is critical for learning and memory and serves as a key link between the hippocampal neocortex and the hypothalamus, providing primary afferents to the hippocampus [90]. Genetic differences in synaptic plasticity in the hippocampus have been observed between the two strains, which may result in spatial learning and memory deficits in F344 animals [91]. Conversely, LEW rats exhibit enhanced learning capacity [92], which may be associated with the increased metabolism observed in the entorhinal cortex of this strain. Moreover, the lateral hypothalamus is known as a potent predictor of addiction vulnerability

[93], and therefore the effects we observed in this region suggest a greater vulnerability of LEW over F344 rats. In addition, the hypothalamus is a part of the hypothalamic-pituitary-adrenal (HPA) axis, which has been reported to present differences between the LEW and F344 animals [94]. The increased glucose metabolism in the hypothalamus of LEW rats supports the alteration of the HPA axis and aligns with the reported changes in reward and motivational processes associated with this strain [31].

When comparing LEW and F344 animals to their respective control groups, we observed distinct metabolic patterns between the two strains. Morphine self-administered F344 animals exhibited increased glucose metabolism in cortical areas, particularly in the left hemisphere, just after the MSA. These findings are consistent with observations of left hemisphere alterations in humans following acute morphine administration [95]. Additionally, F344 displayed decreased [^{18}F]-FDG uptake in the motor and piriform cortices compared to saline-treated F344 animals. These changes persisted throughout morphine withdrawal, being relatively small. Conversely, the metabolic pattern of morphine-exposed LEW animals differed significantly from that of saline-treated LEW animals. We observed reductions in glucose metabolism in the somatosensory cortex, thalamus, and cingulate cortex. These changes were sustained and extended during withdrawal. These distinct brain metabolic patterns following the MSA study indicate variations in the efficiency of neural substrates in translating the effects of drugs and may contribute to differences in vulnerability to morphine abuse. Chronic morphine exposure is typically accompanied by tolerance and dependence, which involve drug-induced neuroadaptations [96]. Previous studies have demonstrated significant decreases in glycine and glutamate levels following acute morphine administration, with subsequent dramatic increases or overcompensation during withdrawal [97]. In our F344 animals, chronic morphine exposure may have triggered compensatory mechanisms to normalize glucose metabolism, whereas the same mechanisms were not activated in LEW animals.

During morphine withdrawal in the LEW strain, glucose metabolism decreased dramatically in brain regions associated with reward and drug dependence, including the hippocampus, thalamus, caudate-putamen, and cingulate cortex. In humans, abnormal regional cerebral glucose metabolism has been observed in chronic opiate users several years after methadone detoxification [98]. These abnormalities primarily affect the anterior cingulate cortex, left mid-cingulate cortex, left insula, and right superior frontal cortex, which is consistent with some of the cortical changes observed in our study. Additionally, Single-photon emission computed tomography (SPECT) studies have demonstrated perfusion deficits in various brain areas, including the temporal lobe, frontal, parietal, and temporal areas, during heroin withdrawal in chronic opioid users [99]. In our study, we found abnormal brain activity patterns

in LEW animals after 15 days of morphine abstinence, resembling the findings observed in humans. However, these abnormal metabolic patterns were not evident in F344 animals, indicating that genetic background and other factors contribute to an individual's susceptibility to morphine addiction. Moreover, the increased metabolism in the piriform cortex of LEW rats, an olfactory region not typically associated with drug effects, may be linked to morphine seeking during the withdrawal period and partially explain the involvement of the piriform cortex in morphine addiction and dependence [100].

Regarding **morphine brain metabolic effects in Wistar rats**, males exhibited increased metabolism in the cortex and cerebellum, whereas females showed subcortical changes in the hippocampus and brainstem. These sex differences may play a role in the higher prevalence of compulsive drug-seeking behaviour observed in females. Dopaminergic cells in the midbrain play a crucial role in incentive salience and reward processes [101], which could explain the reinforcement of learned associations with repeated morphine exposure and the increased metabolism observed in the hippocampus. MSA resulted in long-lasting changes in brain glucose metabolism in females, particularly in areas related to the limbic system, such as the amygdala, piriform cortex, and entorhinal cortex. In this regard, the observed metabolic reductions in the amygdalo-piriform area during withdrawal can be related to functional decreases in the extended amygdala neurocircuitry and the dopaminergic component of the reward system [101]. Additionally, dysregulation of afferent projections from the prefrontal cortex (PFC) to the amygdala may contribute to these metabolic changes [101]. The piriform cortex, which is an integral part of the olfactory cortex, also plays a significant role in memory processing and encoding [102]. It forms connections with various limbic regions, including the amygdala, hippocampus, and rhinal cortex. Our findings are consistent with an fMRI study conducted on an animal model of precipitate morphine withdrawal [103], which demonstrated positive changes in BOLD contrast in regions such as the dentate gyrus, visual, auditory, insular, cingulate, and piriform cortices. These results highlight the significance of these structures in withdrawal processes and their contribution to impairments in attentional performance following prolonged opioid abstinence.

MSA induced sex-dependent differences in the mesolimbic reward system, specifically showing a decrease in hippocampal metabolism in females after a fourteen-week withdrawal period. The hippocampus plays a crucial role in drug-context memories and drug-cue associations [104], and it is known to undergo long-term structural and functional changes following drug withdrawal [105]. Importantly, most of our understanding regarding morphine and opioid withdrawal primarily stems from male subjects, despite women often facing greater challenges in sustaining abstinence and tend to progress more rapidly towards addiction [106]. One potential explanation for these differences lies in the influence of ovarian hormones,

particularly oestrogen and oestradiol. Ovarian hormones have been implicated in sensitizing dopaminergic neurons by augmenting their responsiveness to opioids [107], and have been associated with the redistribution of δ-μ-opioid receptors within hippocampal circuits [108]. These characteristics may contribute to the facilitation of opioid-related associative learning processes and heighten the vulnerability of females to opioid addiction.

Long-term effects of CP and morphine on brain glucose metabolism

As previously mentioned, the gateway hypothesis suggests that cannabis use during adolescence can increase the likelihood of developing an addiction to other drugs in adulthood. In this context, only Chapter II, but not Chapter I, used a preexposure to a cannabinoid agonist (CP) during periadolescence to assess its potential influence on future morphine addiction.

The effects of CP differed between male and female rats. Male rats displayed elevated glucose metabolism in limbic system areas such as the caudate-putamen, hippocampal-subiculum area, and visual cortex during adulthood, in line with previous findings from shorter withdrawal periods. On the other hand, females showed non-significant metabolic reductions in cortical areas throughout the study duration. However, it is important to note that the metabolic changes observed in males in limbic system areas did not persist over time, suggesting that males may recover from the adverse effects of CP-55,940 exposure during periadolescence. This finding is further supported by the region of interest (ROI) analysis, as males consistently exhibited normal global brain metabolic levels throughout the study until the fourteen-week withdrawal period.

Regarding the combination of CP and morphine, our findings revealed that the metabolic pattern induced by morphine was exacerbated in females when CP was administered, both after the MSA and fourteen weeks of withdrawal. These results suggest that CP may cause permanent alterations in female brain metabolism. Females exhibited enhanced metabolism in reward-related regions, including the brainstem, hippocampus, thalamus, periaqueductal grey (PAG), ventral tegmental area (VTA), habenula, and insular and somatosensory cortices. The presence of cannabinoid receptors (CB1R) and μ-opioid receptors in these brain areas suggests potential interactions between the endocannabinoid and opioid systems in reward and withdrawal processes [109]. The bidirectional relationship between CB1R and μ-opioid receptors contributes to the rewarding properties of drug abuse [110]. The observed metabolic changes at the end of MSA in females, but not in males, may be attributed to the higher number of morphine injections in females. Additionally, the PAG and habenula are particularly relevant in opioid addiction and withdrawal, as they modulate anxiety, fear, nociception, negative reinforcement, and impulsive behaviour [111–113]. The VTA-PAG

circuitry, along with the basolateral amygdala and nucleus accumbens (NAcc), forms a circuit involved in the stress response to chronic opioid use [114]. Gradual normalization of glucose metabolism in these areas occurs as the stressful situation of drug use diminishes.

Long-term effects of CP and morphine on endocannabinoid system

Exposure to cannabinoids during prenatal or adolescent stages has been linked to a wide range of behavioural changes and disruptions in the maturation of neurotransmitter systems, including the opioidergic system. Additionally, it can result in modifications in the density of cannabinoid receptors over time and other related effects. In our study, we observed reduced levels of CB1/2R and endocannabinoid enzymes (NAPE, MAGL, FAAH) in the NAcc of males exposed to CP-55,940 during periadolescence, whereas no such differences were observed in females. The downregulation of CB1R, particularly in cortical areas, is a known consequence of cannabinoid exposure [56,115]. However, the timeline of CB1R changes following abstinence or withdrawal remains a topic of debate. Some studies propose that CB1R number and function normalize within a few days or weeks after abstinence, as evidenced by in vivo PET imaging using the reversible [^{11}C]OMAR ligand. Similarly, in male mice, a 15-day THC administration led to a temporary downregulation of CB1R, which returned to normal levels after two weeks of withdrawal. Our findings contrast with previous literature, as our male animals exposed to CP-55,940 exhibited reduced CB1R levels in the NAcc even after 14 weeks of withdrawal [56]. Several factors may contribute to these discrepancies, including the specific cannabinoid agonist used, dosage and duration of exposure, timing of evaluation, and the specific brain region studied. Moreover, the interaction between cannabis and testosterone could also explain the observed sex differences in NAPE/FAAH and DAGL/MAGL ratios in males [116].

Historically, CB2R has been recognized as a peripheral cannabinoid receptor involved in immune regulation [117]. However, recent research has identified CB2R in glial cells [118] and neurons [119], particularly in brain areas associated with the reward system, indicating its relevance in the field of addiction [120]. CB2R activation has been linked to anti-inflammatory and neuroprotective properties, mainly by suppressing reactive microglia [121]. This makes CB2R a potential target for treating psychiatric disorders like schizophrenia and depression, as well as chronic neurodegenerative conditions [122]. In our study, we observed a reduction in CB2R expression in the NAcc of males exposed to CP-55,940 during periadolescence, similar to that found for CB1R expression. Given the potential neuroprotective role of CB2R in blocking microglial activation [121], the decrease in its expression could negatively impact the progression of withdrawal syndrome associated with cannabis abuse. Moreover, we observed

a decrease in NAPE, the primary enzyme responsible for anandamide synthesis, as well as reductions in degradative enzymes (FAAH and MAGL) in males pre-exposed to CP. Additionally, the NAPE/FAAH ratio showed a decline, while the DAGL/MAGL ratio exhibited an increase in CP-exposed males, indicating a decrease in anandamide and an elevation in 2-arachidonoylglycerol (2-AG) levels. Notably, these changes were reversed by morphine, suggesting an interesting interaction between the endocannabinoid and opioid systems, as previously reported [25]. In relation to these results, compensatory alterations in receptor expression may be expected. In line with this, our findings regarding cannabinoid receptors also revealed an interesting interaction between CP and morphine. Specifically, VH-animals displayed a decrease in CB1R expression and an increase in male CP-animals, while females exhibited the opposite pattern for CB2R expression.

Neurodevelopment

Chapter III introduced the well-stablished schizophrenia-like MIS model. In this extensively validated model, Poly I:C administration induces several deficits at different levels. Thus, we aimed to investigate the impact of adolescent THC exposure on the emergence of schizophrenia-like abnormalities, as well as to evaluate the potential preventive effects of CBD as an anti-IOS compound. Interestingly, our findings yielded some unexpected results, especially regarding the effects of THC on behaviour and brain structure.

THC prevented sensorimotor gating deficits in MIS-offspring

MIS model is known to show a marked sensorimotor gating deficit in the prepulse inhibition test [71]. We observed this deficit in our MIS animals, in accordance with previous studies. Surprisingly, **THC** prevented these deficits in MIS-offspring but induced them in Saline-offspring, in line with prior research on cannabis users [123] and animal models [124]. Limited investigation has focused on this issue in MIS-offspring, without prior evidence of a preventive effect of THC in sensorimotor gating deficits [125]. Hence, THC may not always effectively induce schizophrenia-like behavioural deficits [126], suggesting varied effects based on the individual's inflammatory background. In contrast, **CBD** did not prevent PPI deficits in MIS-offspring, albeit a slight tendency towards improvement was observed. The controversy surrounding the beneficial impact of CBD on PPI deficits aligns with other studies conducted on diverse schizophrenia-like rat models [127]. The observed enlargement of the prefrontal area in the volumetric study may contribute to the enhancement of PPI in MIS-offspring, as rodents' PPI is regulated by medial prefrontal cortices [128].

THC and CBD prevented some brain volumetric abnormalities in MIS-offspring

Reduced hippocampus volume and enlarged ventricles are well-established characteristics associated with schizophrenia, documented in both clinical [129] and preclinical research [71]. Our study successfully replicated these abnormalities and found that both CBD and, surprisingly, **THC** had a preventive effect on them. Previous studies have consistently reported reductions in hippocampal volume among regular cannabis users [130], including those with schizophrenia who initiated cannabis use during adolescence [131]. However, limited research has explored the potential therapeutic benefits of **CBD** specifically in this population. A recent study showcased the restorative properties of CBD in mitigating hippocampal volume loss in cannabis users, particularly those with prolonged cannabis use histories, suggesting its potential efficacy in the treatment of cannabis dependence [132]. Other rodent studies did not observe ventricular enlargement after perinatal THC administration in rats [133] or ventricular reduction following CBD treatment in the Methylazoxymethanol acetate (MAM) schizophrenia-like rat model [134]. Conversely, our findings indicate that both THC and CBD exhibit beneficial effects on the ventricular system. Nevertheless, it is crucial to exercise caution, as THC may have adverse effects in other fields of study.

Interestingly, we observed a cerebellar and PAG enlargement in MIS-offspring treated with CBD. These brain regions, known for their abundant distribution of CB1R receptors, have been implicated in anxiety-like behaviour [135], psychotic-like experiences [136], and the neuropathology of schizophrenia [137]. It is worth noting that the cerebellum is typically reduced in individuals with schizophrenia and cannabis users. However, the extent of cerebellar reductions in healthy cannabis users is dependent on the duration of use and the age at which cannabis consumption commenced [137]. In contrast, our findings indicate that CBD administration can protect against such detrimental effects in MIS-offspring, in line with similar observations in human studies [136]. The precise mechanisms underlying the enlargement of these brain areas in CBD-treated MIS-offspring remain unclear. One possibility is that CBD exerts anti-IOS effects, which could counteract the pathology-associated IOS pattern, thereby mitigating the effects of THC on brain function. This, in turn, may prevent the activation of molecular mechanisms that could trigger neurotoxicity and ultimately lead to neuroanatomical abnormalities [138].

CBD prevented white matter deficits in the corpus callosum in MIS-offspring

Our study revealed a reduction in the volume of the corpus callosum (CC), which is the brain's largest white matter fibre structure, in MIS-offspring. This finding is consistent with previous research in patients with schizophrenia [139], cannabis users [140], and preclinical

models of schizophrenia [141,142]. Early-life exposure to Poly I:C has been associated with CC demyelination and calibre reduction [143]. **THC** exacerbated this deficit in both Saline- and MIS-offspring, which aligns with rodent data showing high expression of cannabinoid receptors in the developing CC, making it vulnerable to cannabis exposure during adolescence [140]. In contrast, the administration of **CBD** during adolescence exhibited a novel and significant outcome by preventing the volumetric deficit in white matter. CBD may enhance the integrity of white matter, particularly in the CC, which serves as the primary communication pathway between the brain hemispheres and has implications for psychiatric disorders [143]. Our hypothesis would be that CBD may be protecting white matter fibres from demyelination, although further studies are needed to confirm this.

Moreover, we observed reduced fractional anisotropy (FA) in the CC across all CBD-treated groups. Clinical studies in schizophrenia have found decreased FA and increased mean diffusivity (MD) in the CC of untreated patients, but increased FA in medicated patients [144]. Yet, the effects of cannabinoids on white matter integrity in animal models remain unclear. Our study did not find FA differences between untreated MIS-offspring and control animals, which is similar to the outcomes of the lipopolysaccharide-induced MIS model [145]. However, other researchers have reported regional FA differences in the MAM model, including increased FA in the posterior area and lower FA in the anterior region of the CC [141]. Adolescent cannabis use has been associated with lower FA in various white matter tracts [146], which may partially explain the FA reduction observed in the THC-CBD groups, but not in the VH-CBD groups. Further post-mortem microstructural imaging studies are necessary to gain a better understanding of the changes in white matter architecture.

THC modulated the ECS and Telomere length

Exposure to cannabinoids during adolescence disrupts the ECS by affecting cannabinoid receptors and endocannabinoid components like anandamide and 2-AG [147]. Our post-mortem evaluation showed increased CB1R expression in the caudate-putamen of MIS-offspring, consistent with findings in individuals with schizophrenia [148] and preclinical models [149]. Conversely, **THC** reduced CB1R expression in the amygdala of MIS-offspring, as seen in cannabis-dependent subjects [56] and animal models exposed to cannabinoids during adolescence [115]. Interestingly, we observed a significant increase in CB1R expression in the caudate-putamen and frontal cortex (FC) in **THC-CBD** exposed animals, suggesting a synergic effect of these cannabinoids on this receptor in cortical and striatal areas.

Recent studies have linked the overexpression of CB2R to neuroinflammation [13]. In line with this, **THC** increased CB2R expression in the FC of MIS-offspring and in the amygdala

of Saline-offspring, indicating its role in enhancing neuroinflammation. Notably, CBD exhibited a preventive effect on these changes in the amygdala [150], only when THC was present, exerting its anti-inflammatory properties. Furthermore, we observed elevated ratios of the key enzymes involved in the synthesis and degradation of the endocannabinoids in the FC, shedding light on the levels of anandamide and 2-AG levels. Schizophrenia has been associated with both increased circulating levels of anandamide [151] and decreased levels in post-mortem brains [152]. Cannabis use has been linked to reduced anandamide levels and increased psychotic symptoms in susceptible individuals, while CBD has shown symptom relief and increased serum levels of anandamide, possibly by inhibiting its reuptake or modulating the activity of FAAH [153]. Our findings partially support these previous studies, as THC reduced anandamide levels in the FC, but CBD did not prevent this effect.

Conversely, **CBD** increased 2-AG levels in the caudate-putamen and FC of THC-treated animals, potentially due to the increased inflammatory environment in their brains. It has been suggested that 2-AG acts as an endogenous regulator of neuroinflammation when facing detrimental insults [154], providing further support for our findings. Overall, our findings, along with those of other researchers [155], indicate that CBD has the ability to modulate the effects of THC on the ECS system. Nevertheless, further research is needed to fully understand the interactions between CBD and THC, particularly given the growing interest in THC-CBD formulations for recreational and medical purposes.

In relation to the pro-inflammatory state, **THC** shortened TL in Saline-offspring, a trend that was also observed in MIS-offspring. TL shortening has been associated with chronic inflammatory conditions [156]. However, there is an ongoing debate regarding the impact of inflammation on TL in schizophrenia, with studies reporting varying results including TL shortening [157], TL maintenance [158], or even TL lengthening [159]. In our study, we initially anticipated TL shortening in MIS-offspring due to the pro-inflammatory state induced by the MIS challenge. However, we only observed TL shortening in the group treated with THC, deviating from our initial hypothesis. This observation is consistent with previous findings of TL shortening associated with substance abuse, likely attributed to the oxidative stress induced by the drug [160]. Although the association between cannabis use and TL has been investigated to a limited extent, most studies have reported TL shortening compared to control groups [161]. Importantly, despite its ability to mitigate the pro-inflammatory effects induced by the MIS challenge and THC, **CBD** did not exert any influence on TL in either the Saline- or MIS-offspring groups.

CBD partially prevented the pro-inflammatory and oxidative state

Our MIS model showed widespread activation of the proinflammatory pathway, with increased expression of iNOS in multiple brain areas and COX2 in the FC. These findings are consistent with previous observations in the MIS model [71,81] and the pro-inflammatory dysregulation seen in schizophrenia patients' peripheral mononuclear blood cells [73,74]. This persistent proinflammatory dysregulation, mediated by the NF-κB pathway, appears to involve the microglia-neuron interaction [162], as indicated by the increased presence of iba-1 in the hippocampus and amygdala. Remarkably, the MIS challenge had the greatest impact on the hippocampus within the Keap1-Nrf2-ARE network, resulting in elevated levels of iNOS, Iba1, Keap1, and NQO1 expression. Similarly, **THC** treatment exacerbated the pro-inflammatory state in all animals, as evidenced by increased Iba1 levels in all brain areas, confirming Iba1 as a reliable marker of cannabinoids-induced inflammation [163]. Generally, cannabinoids have been linked to increased pro-inflammatory markers [138], although it has also demonstrated anti-inflammatory properties [164]. These discrepancies indicate that there are still gaps in our understanding of the effects of THC exposure during adolescence, a critical period for neurodevelopment, in order to fully grasp the extent of THC's impact on this vulnerable population.

Finally, it is noteworthy that **CBD** mitigated some of the alterations induced by the MIS challenge and THC. Specifically, CBD demonstrated a reduction in the expression of iNOS and iba1 in most brain areas, as well as Keap1 and NQO1 in the hippocampus. These findings are consistent with existing evidence of CBD's anti-inflammatory and antipsychotic properties [165], as shown in preclinical schizophrenia-like models, further supporting our results. However, while most of the inflammatory response was effectively prevented by CBD, its influence on oxidative stress markers was less significant. Notably, CBD treatment increased the expression of HO1 in the amygdala, regardless of the animal's phenotype, although this effect was intensified when combined with THC. The amygdala, known for its involvement in fear processing, emotional states, and the reward system, could be a crucial factor in the amygdalar imbalance induced by THC treatment during adolescence, as observed in previous studies using a cannabinoid agonist on rats during this developmental stage [101].

Limitations and future lines

The studies presented in this thesis have certain limitations that lay a solid foundation for potential future directions that this investigation could pursue in the short or medium term.

First, it is important to consider the impact of anaesthesia on our research findings. It has been reported that anaesthesia can reduce brain glucose metabolism, neural activity, and cerebral blood flow. This could particularly affect our PET studies discussed in Chapters I and

II, which involved anaesthesia during image acquisition. However, it is worth noting that we used inhaled anaesthesia in all cases, which has less impact on neurological activity, and the entire period of [^{18}F]-FDG uptake was performed while awake.

Second, the influence of sex is an important but often overlooked factor in preclinical research. This is evident in Chapter II of this thesis, which included male and female rats and revealed sex-dependent differences. Both opioids and cannabinoids have been found to have differential effects based on sex, emphasizing the need to consider this factor in future research. Additionally, controlling for the oestrus cycle in female rats would be beneficial, which was not done in the second chapter. This could have impacted the post-mortem determinations of ECS markers depending on the timing of the sample extraction during the cycle. It is important to note that in the context of the MIS model, females were not included due to previous findings demonstrating that they exhibit distinct patterns of brain metabolism, morphometry and neuroplasticity during the time window covered in this study compared to males [166]. Given that the onset of schizophrenia occurs later in women than in men, further studies in female MIS-offspring at later PND are warranted to fully understand these processes.

Third, it is important to address the statistical analyses employed in SPM methods. The voxel-level statistical analyses in both PET and MRI did not include adjustments for multiple comparisons, although individual analysis methods in SPM did incorporate some correction. Implementing such adjustments would have been overly restrictive for our exploratory approach, as our aim was to capture all potential influences without compromising the risk of false negatives. Nonetheless, we did apply cluster-level corrections to mitigate type I errors. However, it is important to acknowledge that dedicated future studies are necessary to confirm our results.

· Conclusions ·

· CONCLUSIONS ·

Chapter I. Short-term effects of morphine self-administration on brain glucose metabolism in two different rat strains:

1. LEW rats self-administered more morphine than F344 rats.
2. LEW and F344 rats showed significant brain metabolism differences in regions associated with reward and drug dependence.
3. The different brain metabolic patterns observed after the morphine self-administration study between these rat strains indicate differences in the efficiency of neural substrates to translate the drug effects, which could explain the differences in predisposition to morphine abuse between one individual and another.

Chapter II. Long-term effects of morphine self-administration on brain glucose metabolism in male and female rats pre-exposed to CP-55,940 during periadolescence:

1. Females, but not males, showed increased morphine self-administration, especially when pre-exposed to CP during adolescence.
2. The brain metabolic pattern induced by morphine in females was exacerbated when CP was present, mainly at the end of morphine self- administration but also to a lesser extent during withdrawal, suggesting that CP may cause long-lasting metabolic changes in females.
3. Preexposure to CP during periadolescence modulated the reward and endocannabinoid systems, inducing long-lasting metabolic changes after a 14- week morphine withdrawal. This was more notable in females than in males, which could explain sex differences in vulnerability to the use of drugs of abuse such as opiates.

Chapter III. Impact of different cannabinoids in the Poly I:C maternal immune stimulation model of schizophrenia:

1. The MIS model showed sensorimotor gating deficits, structural abnormalities and increased IOS markers.
2. Surprisingly, THC treatment did not exacerbate the MIS-related abnormalities at the behavioural and brain anatomical levels, despite promoting a pro-inflammatory/pro-oxidative state and shortening telomere length.
3. CBD prevented some of the structural deficits and reduced inflammatory markers expression through iNOS and Nrf2-ARE pathways in MIS-offspring but failed to improve sensorimotor gating deficits.

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