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UNIVERSITÀ
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Ph.D. DEGREE IN

Neuroscience

Cycle XXXVII

TITLE OF THE Ph.D. THESIS

Cyclodextrins as a suitable carrier for the induction of feeding and analgesia by Delta-9 THC: a preclinical study in the female rat

Scientific Disciplinary Sector(s)

M-PSI/02 Psychobiology and Physiological Psychology

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Final exam. Academic Year 2023/2024
Thesis defense session: April 2025

Contents:

1. Introduction

Despite abundant preclinical evidence suggesting the therapeutic benefits of cannabinoid-based drugs, regulatory challenges continue to slow down the translation of these findings into clinical trials. Nonetheless, medicinal cannabinoids are being investigated for a variety of applications, including the treatment of nausea, anorexia, neurodegeneration, inflammation, excitotoxicity, and pain. The appetite-stimulating and antiemetic properties of cannabinoids have led to their approval for use in patients undergoing chemotherapy and those with AIDS. Moreover, cannabinoids are also being considered for neuroprotective purposes due to their antioxidative properties.

1.1. Endocannabinoid System: Overview

The endocannabinoid system (ECS) is a lipid signalling network that is essential for the regulation of numerous physiological processes across the body. This system is comprised of endocannabinoids, receptors, enzymes, and transport proteins, all of which function to maintain a range of physiological domains, including mood, appetite, nociception, and immune function (Lu and Mackie, 2021). The main component of ECS is the primary endogenous cannabinoids which act on cannabinoid receptors distributed extensively throughout both central and peripheral tissues (Joshi and Onaivi, 2019). Here I will delve into the synthesis and catabolism of endocannabinoids, the pharmacological distribution of cannabinoid receptors, and the interplay between phytocannabinoids and the ECS. Additionally, emerging findings regarding sex-specific variations as well as pharmaceutical cannabinoids will be discussed.

1.1.1. Synthesis and Function of Endocannabinoids:

Primary ligands in this system are N-arachidonoylethanolamine or anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Wang and Ueda, 2009). Endocannabinoids function as retrograde neurotransmitters and are synthesized "on demand" in response to elevated intracellular calcium levels (Alger and Kim, 2011). This mode of synthesis allows for a rapid and localized response to neuronal activity. AEA and 2-AG, the two most extensively characterized endocannabinoids, are synthesized through distinct metabolic pathways. AEA is produced

from N-arachidonoyl phosphatidylethanolamine (NAPE) via the action of N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (Tsuboi et al., 2018). In contrast, 2-AG is synthesized predominantly through the activity of diacylglycerol lipase (DAGL) on diacylglycerol (DAG) (Tsuboi et al., 2018). Specifically, DAGLα and DAGLβ are responsible for the biosynthesis of 2-AG, which is notably more abundant in the brain compared to anandamide and plays a more significant role in modulating synaptic activity (Tsuboi et al., 2018).

AEA and 2-AG are fundamental in regulating emotional reactivity, motivated behaviors, and energy homeostasis, largely through their interactions with the cannabinoid type 1 receptor (CB1r) in the brain (Yao and Mackie, 2009). These receptors, which are among the most prevalent G protein-coupled receptors (GPCRs) in the mammalian brain, are involved in physiological processes such as learning, memory, food intake, pain modulation, and mood regulation (Yao and Mackie, 2009)(Fig 1.).

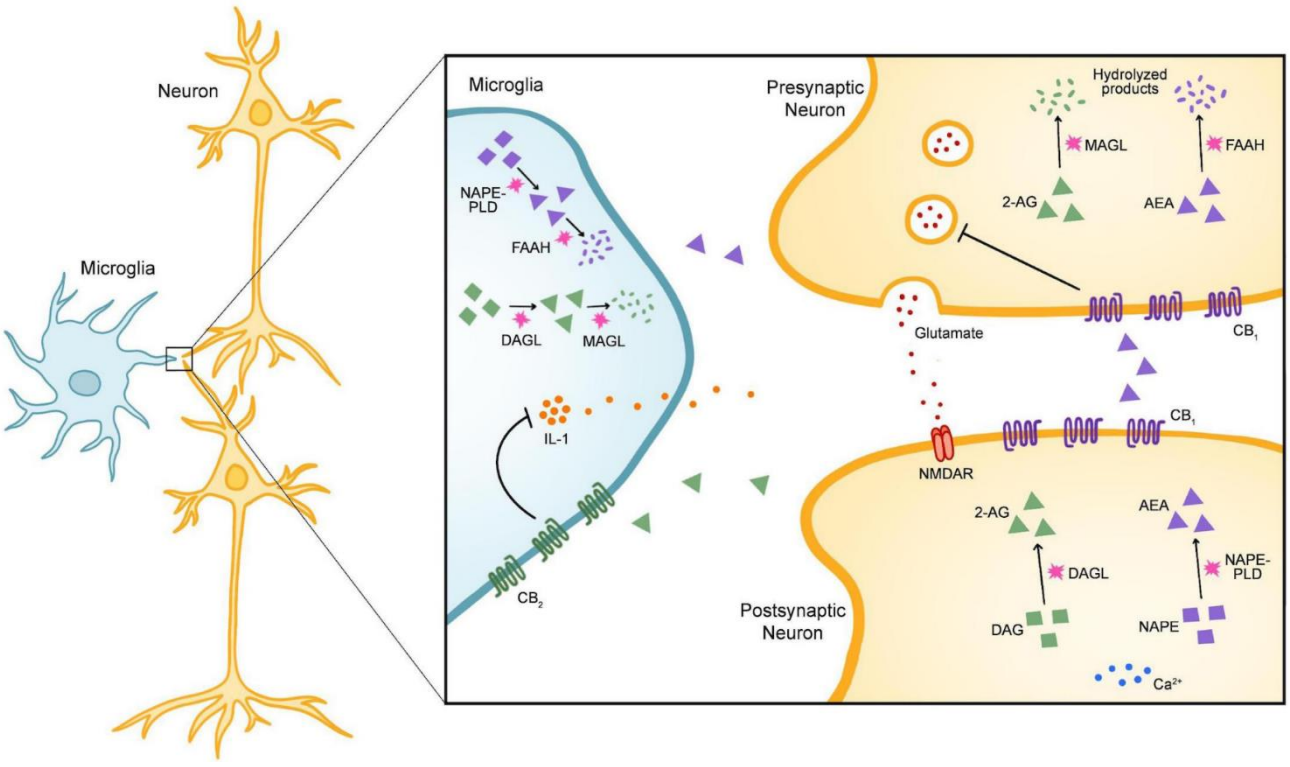


Figure 1. Overview of endocannabinoid system (Araujo et al., 2019)

1.2. Cannabinoid Receptors: CB1 and CB2

ECS exert its functions primarily through two main receptor types: cannabinoid receptors 1 (CB1) and 2 (CB2) (Yao and Mackie, 2009). In addition to CB1 and CB2, eCBs (endocannabinoids) and plant-derived cannabinoids also influence other GPCRs (G-protein-coupled receptors), such as GPR55, GPR119, and the serotonin receptor 5-HT1A (5-hydroxytryptamine receptor 1A) (Hillard, 2015). G-protein Gαi/o, a critical regulator of these receptors, is highly expressed in the developing central nervous system particularly in growing neurons and axons, highlighting the significance of GPCR signaling as fundamental in neurodevelopment (Bromberg et al., 2008). Furthermore, eCBs can directly interact with ionotropic receptors like transient receptor potential vanilloid (TRPV) channels, glycine receptors, and GABAA receptors (Hillard, 2015). These receptors are widely present in developing neurons and axons, playing a role in modulating neuronal excitability and synaptic transmission during early development (Gomes et al., 2020).

CB1 receptors are widely distributed throughout the central nervous system (CNS), particularly in areas such as the hippocampus, cerebellum, basal ganglia (including the striatum, globus pallidus, and substantia nigra), amygdala, hypothalamus, and cortical regions like the prefrontal cortex and cingulate gyrus. These regions are involved in essential functions including learning and memory (hippocampus), motor coordination and reward processing (basal ganglia), emotional regulation (amygdala), and executive decision-making (prefrontal cortex). The broad presence of CB1 receptors in these key regions highlights their significant

roles in coordinating cognitive, emotional, and motor processes, with important implications for various neuropsychiatric and neurodegenerative conditions (Fig. 2)(Hu and Mackie, 2015).

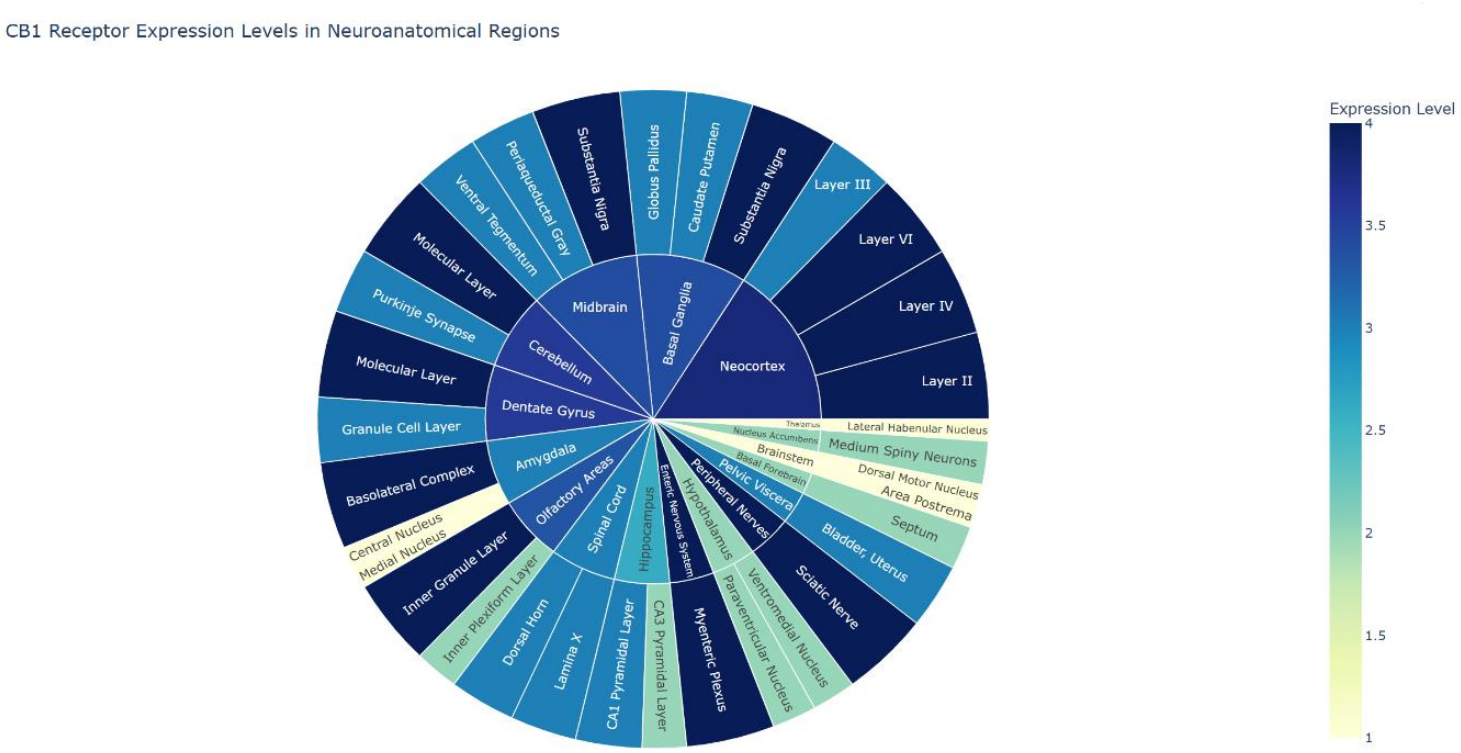


Figure 2. The sunburst chart illustrates the anatomical distribution of CB1 receptor expression levels across various neuroanatomical regions, based on the review by Hu and Mackie (2015). Regions are color-coded to reflect relative expression levels, ranging from low (light yellow, ~1) to high (dark blue, ~4). High CB1 receptor expression is observed in areas such as the neocortex, basal ganglia, hippocampal CA1 pyramidal layer, and cerebellar molecular layer, whereas regions like the central nucleus of the amygdala, medial nucleus, and peripheral structures exhibit lower expression. The figure highlights the critical role of CB1 receptors in brain regions associated with motor control, cognition, and sensory processing, as well as in peripheral regions like the spinal cord and myenteric plexus. (created in Plotpy library)

CB1 is the most prevalent cannabinoid receptor in the adult CNS, and it is predominantly expressed in inhibitory GABAergic neurons, while also being present at lower levels in excitatory glutamatergic neurons (Hu and Mackie, 2015). In mature neurons, the ECS displays a distinct compartmentalization: CB1 receptors are mainly found in axons and presynaptic terminals, diacylglycerol lipase-alpha (DAGLα) in somatodendritic postsynaptic regions, and monoacylglycerol lipase (MAGL) at presynaptic sites (Rivera et al., 2014). Consequently,

synaptic plasticity modulated by the ECS is largely driven by retrograde 2-AG signaling acting through presynaptic CB1 receptors, with specific compartmentalization leading the signaling direction (Kreitzer and Regehr, 2001, Ohno-Shosaku et al., 2001, Wilson and Nicoll, 2001). Additionally, in a different mode of paracrine ECS signaling, presynaptically synthesized AEA can function as an anterograde signal, inducing synaptic depression via postsynaptic TRPV1 receptors (Wilson and Nicoll, 2001, Grueter et al., 2010).

CB2 receptors, on the other hand, are primarily found in peripheral tissues, especially in immune cells, where they are involved in immune regulation and exhibit anti-inflammatory effects (Turcotte et al., 2016). They have also been identified in microglial cells within the CNS, which indicates a potential role in modulating neuroinflammation, with implications for neurodegenerative diseases (Vuic et al., 2022). Dysfunctions in the ECS, particularly involving CB1 and CB2 receptors, have been associated with neuropsychiatric conditions linked to dopamine and basal ganglia dysfunctions, such as drug addiction, psychosis, Parkinson's disease, and Huntington's disease (Behl et al., 2020, Kibret et al., 2022). This suggests a role for cannabinoid-based ligands in modulating these disorders through their impact on dopamine transmission (Fernández-Ruiz et al., 2010, Kibret et al., 2023). CB1 receptors are also found in peripheral tissues, including adipose tissue, liver, and skeletal muscle, suggesting the ECS's involvement in metabolic regulation, energy storage, and glucose homeostasis (Engeli, 2008).

1.2.1. Physiological Effects

CB1 receptor activation by endocannabinoids results in a variety of effects, including analgesia and modulation of mood and appetite (Zou and Kumar, 2018). These effects are mediated through multiple pathways, including inhibition of adenylate cyclase, activation of mitogen-activated protein kinases (MAPKs), and regulation of ion channels, which collectively reduce the release of excitatory and inhibitory neurotransmitters (Basavarajappa, 2007). This signaling cascade is evidence of the complexity of CB1 receptor physiology and its broad influence on neuronal function and behavior.

CB2 receptor activation, in contrast, is primarily associated with anti-inflammatory and immunosuppressive effects, pointing to the therapeutic potential for conditions involving inflammation, autoimmune dysfunction, and even neurodegeneration (Vuic et al., 2022). The broad expression of ECS components in the CNS underscores their involvement in numerous physiological functions (Herkenham et al., 1991, Mackie, 2008). Additionally, the ECS has been shown to influence neurogenesis, particularly in the hippocampus, where cannabinoid receptor activation may contribute to enhanced synaptic plasticity, stress resilience, and the mitigation of anxiety-like behavior (Rodrigues et al., 2024).

Most of the physiological effects of ECBs strictly depend on the brain distribution of CB receptors. What follows is an overview of some brain areas particularly rich in CBR expression, responsible for the effects of ECBs in feeding, pain perception and other relevant physiological functions.

Ventral tegmental area (VTA): The VTA is a major source of dopaminergic neurons that project to multiple areas, including the NAc and PFC. It's central to reward-related and motivational processes. In the context of food intake, VTA dopamine cell firing encodes the rewarding and motivational value of food, driving goal-directed feeding behavior. In the context of pain, VTA activity is linked to the affective and motivational dimensions of pain: pain can suppress VTA dopamine neuron firing (via increased GABAergic inhibition from the rostromedial tegmentum), leading to anhedonia and reduced motivation for natural rewards (Markovic et al. 2022). Notably, activating VTA dopamine neurons produces analgesic effects and even reduces the amount of opioid needed for pain relief (Taylor et al. 2020), indicating that VTA dopamine contributes to pain modulation, and the relief (reward) associated with analgesia (Taylor NE et al .2019). Thus, the VTA is a logical target for studying pain and feeding because it links the reward of eating with the relief of pain through common dopaminergic mechanisms.

The VTA is richly modulated by the endocannabinoid system despite dopamine neurons themselves having little to no CB1 receptor expression. CB1 cannabinoid receptors are present on presynaptic terminals in the VTA – notably on GABAergic interneurons and afferents

(including inhibitory inputs from NAc and excitatory glutamatergic inputs) (Sherry Shu-Jung Hu and Ken Mackie 2015).

Activation of CB1 in the VTA inhibits GABA release onto dopamine neurons, disinhibiting those dopamine cells and thereby increasing dopamine output to downstream targets (Melis et al. 2012). Through this mechanism, endocannabinoids (or exogenous cannabinoids like THC) in the VTA enhance the mesolimbic dopamine pathway, contributing to the rewarding and appetite-stimulating effects of cannabinoids (Koch 2017). Indeed, CB1 signaling in the VTA–NAc circuit has been shown to modulate hedonic feeding; enhancing CB1 activity increases appetite and food reward, whereas blocking CB1 suppresses feeding (Koch 2017).

Nucleus Accumbens (NAc): Neuroanatomically, the NAc is composed mainly of GABAergic medium spiny neurons that receive dopamine input from the VTA and glutamate inputs from regions like the PFC, amygdala, and hippocampus. The NAc is classically known for its role in reward, pleasure, and feeding: for example, endogenous opioid and endocannabinoid release in the NAc shell enhances the palatability of sweet foods and drives eating beyond metabolic need (Mitchell et al. 2018). Stimulating this region (e.g. via μ-opioid or endocannabinoid agonists) increases feeding and the enjoyment of food, whereas NAc dysfunction can lead to lack of motivation to eat. Importantly, the NAc is also involved in pain processing and analgesia. Although it has received less focus historically in pain research, the NAc has extensive connections with pain-related regions (prefrontal cortex, anterior cingulate cortex, periaqueductal gray, etc.) and plays a significant role in modulating pain signals (Harris and Peng. 2020). Activation of NAc circuits can produce analgesic effects; indeed, the NAc contains a high density of μ-opioid receptors and other neuromodulators (dopamine, GABA, glutamate, substance P, etc.) that when engaged, suppress pain signaling (Harris and Peng. 2020). Both preclinical and clinical studies have found that stimulating the NAc (for instance, via deep brain stimulation or pharmacological methods) can alleviate chronic pain (Harris and Peng. 2020). The NAc's dual role; promoting positive motivation (e.g. eating) and mitigating negative experiences (pain, stress), makes it a compelling region for investigating the interactions between pain perception and food intake.

The NAc is modulated by endocannabinoids in ways that influence both its reward and pain functions. The NAc contains abundant CB1 receptors, primarily on presynaptic terminals of glutamatergic and GABAergic inputs that regulate the output of NAc neurons. Endocannabinoid signaling within the NAc shell interacts with the endogenous opioid system to amplify feeding reward (Mitchell et al. 2018).

Cannabinoids and opioids often co-localize in the NAc highlighting a tightly connected mechanism for modulating motivation and pain (Mitchell et al. 2018). Neurochemically, the NAc's high μ-opioid receptor density and dopamine innervation work in concert with endocannabinoids: activation of CB1 in NAc can suppress inhibitory inputs and enhance dopaminergic and opioidergic effects, thus promoting reward and potentially producing an analgesic effect by activating accumbens output pathways that inhibit pain (for example, via projections to the brainstem pain modulatory centers) (Harris and Peng. 2020). In chronic pain states, dysregulation of endocannabinoid signaling in the NAc has been linked to pain-induced emotional disturbances (like depression), and normalizing this signaling can restore the NAc's modulatory balance (Fitzgibbon et al. 2016).

Prefrontal Cortex (PFC): The PFC plays a role in pain perception as a regulator of sensory components of pain. This region has dense connections to subcortical pain pathways and can exert descending control over pain processing. Projections from PFC to the NAc and other midbrain regions help inhibit pain responses as part of an endogenous analgesia system (Zhou et al. 2018). Experiments in naive rats show that optogenetically inhibiting PFC neurons (or their projection to the NAc) heightens both the sensory intensity of pain and the negative emotional reaction to it (Zhou et al. 2018).

Female rats prone to binge eating show less activation of excitatory PFC neurons by palatable food, and temporarily inactivating the mPFC causes a surge in uncontrolled consumption of treats (Sinclair et al. 2019).

CB1 receptors are widely expressed in the PFC, especially on presynaptic axon terminals of both excitatory (glutamatergic) and inhibitory (GABAergic) neurons. By activating these receptors, endocannabinoids fine-tune PFC output: generally, CB1 activation reduces neurotransmitter release, which can dampen excessive excitatory drive or inhibit interneurons

to alter network activity. In stress and pain contexts, endocannabinoid signaling in the medial PFC has been shown to gate emotional responses. For example, chronic neuropathic pain can cause imbalances in PFC endocannabinoid levels that lead to depressive-like states, and restoring normal endocannabinoid signaling in the mPFC can reverse these pain-induced emotional deficits (Mecca et al. 2021 Wang et al. 2023)

Periaqueductal gray(PAG): The PAG's classical role in pain perception is in activating the descending analgesia system: when stimulated (electrically or by opioids), the PAG drives inhibition of pain signals at the spinal cord level via relays like the rostral ventromedial medulla (RVM) (Plazzo et al. 2010). It is a primary site where endogenous opioids act to produce pain relief (the PAG is rich in opioid receptors), and it is critical for phenomena such as stress-induced analgesia. In addition to pain modulation, emerging evidence indicates the PAG also participates in food intake and reward-related behaviors. Anatomical tracing shows the PAG is interconnected with appetite and reward circuits (reciprocally connected with the PFC, hypothalamus, amygdala, parabrachial nucleus, and VTA) (Tyron et al. 2018). In their study, they conclude that PAG neurons process reward-related information, perhaps to mediate consummatory behaviors related to food consumption.

Recent studies demonstrate a direct role for PAG in feeding: temporarily inactivating the PAG in rats significantly reduces food consumption and slows feeding, implying that an active PAG is normally facilitating or permissive of eating behavior (Tyron et al. 2018). During a reward-seeking task, subsets of PAG neurons were excited by food reward and their activity scaled with reward magnitude further supporting the idea that the PAG processes reward-related information to promote consummatory behavior.

Functionally, cannabinoids in the PAG mimic some effects of opioids by suppressing GABA release and thereby disinhibiting the PAG output neurons that induce analgesia (Palazzo et al. 2010). Microinjections of CB1 agonists into the PAG produce measurable antinociception, activating the descending pain inhibition pathway, although the analgesia is typically somewhat less potent than that produced by morphine (Palazzo et al. 2010). This cannabinoid-induced analgesia in the PAG depends on downstream glutamate signaling, studies find it can be blocked by glutamate receptor antagonists, suggesting that endocannabinoids free PAG

output cells from GABA inhibition, allowing glutamatergic excitation of the RVM and spinal cord inhibitory interneurons to proceed (Palazzo et al. 2010). While the PAG's endocannabinoid contributions to feeding are not as extensively documented as in hypothalamic or reward areas, the anatomical links suggest that cannabinoid activity in the PAG can indirectly affect appetite through its integration of pain, stress, and hunger signals (Tyron et al. 2018).

1.2.2. Enzymatic Regulation of Endocannabinoid Signaling

Endocannabinoid signaling is regulated by the enzymatic degradation of endocannabinoids. AEA is primarily degraded by fatty acid amide hydrolase (FAAH), while 2-AG is hydrolyzed by MAGL (Bari et al., 2006). These enzymes play a crucial role in controlling the levels and activity of endocannabinoids, ensuring that their effects are temporally and spatially regulated to maintain physiological balance. FAAH and MAGL act as key checkpoints in the regulation of endocannabinoid signaling, with their activity directly influencing the duration and intensity of endocannabinoid-mediated effects (Bari et al., 2006, Martinez Ramirez et al., 2023).

Dysregulation of these enzymes is implicated in various pathological conditions, including anxiety, chronic pain, and neurodegenerative disorders, indicating that they could be targets for therapeutic intervention. For example, FAAH inhibition leads to elevated AEA levels, which can have anxiolytic and analgesic effects, offering potential therapeutic benefits for managing anxiety disorders and chronic pain (Patel and Hillard, 2006). Additionally, acute stress has been shown to increase the synthesis of endocannabinoids in limbic forebrain areas, while endocannabinoid release mediates opioid-independent stress-induced analgesia in the periaqueductal gray, emphasizing their role in the body's adaptive response to stress (Patel and Hillard, 2006, Hohmann et al., 2005). Chronic stress, however, has been found to downregulate ECS signaling, potentially contributing to the development of stress-related psychiatric disorders, further emphasizing the need for targeted modulation of ECS components to restore homeostasis in such conditions (Navarro et al., 2022) (Fig 3.).

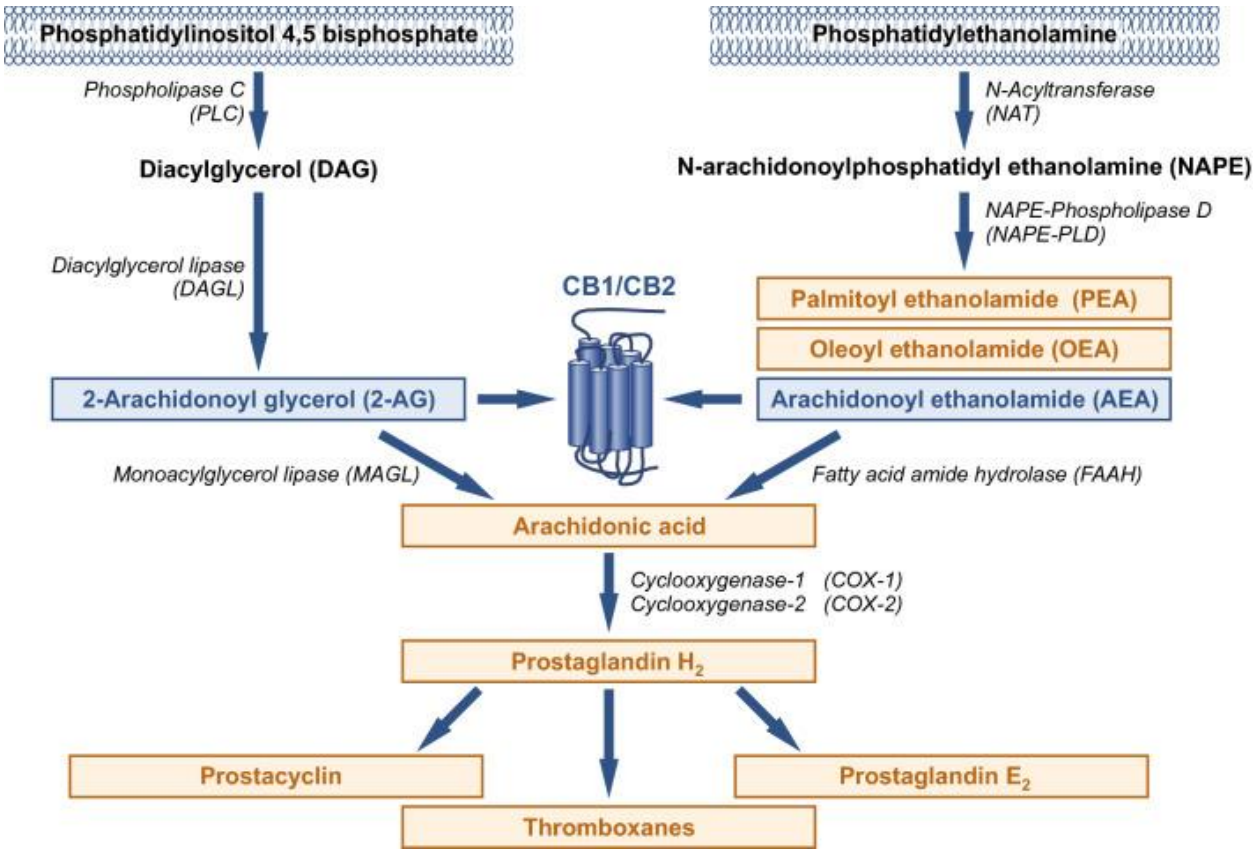


Figure 3 Pathways of endocannabinoid synthesis and degradation: Endocannabinoids (blue) are synthesized from membrane phospholipids via NAPE-PLD (AEA) or PLC/DAGL (2-AG). Degradation occurs through FAAH (AEA) or MAGL (2-AG), producing arachidonic acid (AA), which is converted into eicosanoids (orange) like prostaglandins via cyclooxygenase (Ruiz de Azua and Lutz, 2019).

1.3. Sex Differences in the Endocannabinoid System

Variations in receptor density, endocannabinoid levels, and enzymatic activity between males and females contribute to differential responses to both endogenous and exogenous cannabinoids. For example, female rats exhibit heightened sensitivity to the behavioral effects of cannabinoid drugs, which is partially attributable to lower body fat composition relative to males (Amissah et al., 2024, Ruiz et al., 2021). This results in higher concentrations of bioavailable cannabinoids in females, as these lipophilic compounds are less likely to be sequestered in body fat (Thomas et al., 1990).

Sex differences in the ECS are also mediated through bidirectional interactions with gonadal hormones (González et al., 2000). Fluctuating levels of CB1 receptor expression and endocannabinoid concentrations have been observed across different brain regions and

estrous cycle phases in female rats, suggesting that ECS activity is modulated by hormonal dynamics (Hillman et al., 2014, De Fonseca et al., 1994, Struik et al., 2018, Paola Castelli et al., 2014). Notably, González et al. observed that ovariectomized female rats exhibit reduced CB1 receptor density, whereas estradiol replacement restores receptor expression, indicating that estrogen levels are positively correlated with CB1 receptor expression (González et al., 2000). Moreover, estrogens appear to inhibit FAAH, the enzyme responsible for degrading AEA, thereby increasing the concentrations of this fatty acid neurotransmitter and enhancing cannabinoid receptor activation (Hill et al., 2007).

In males, evidence suggests that orchiectomy leads to a reduction in CB1 receptor mRNA expression in the anterior pituitary. However, this effect is not reversed by testosterone replacement (González et al., 2000). Additionally, activation of CB1 receptors has been linked to reductions in circulating testosterone and estradiol levels, mediated through the inhibition of luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH) release (Gorzalka and Dang, 2012). Thus, sexual dimorphism in the ECS appears to be shaped by both distinct patterns of cannabinoid metabolism and distribution, as well as reciprocal regulatory interactions between endocannabinoid signaling and gonadal hormones (Gorzalka and Dang, 2012).

These sex-specific differences emphasize the importance of incorporating sex as a critical biological variable in the development and clinical application of cannabinoid-based therapeutics, as these variations may significantly influence drug efficacy and safety across different populations. Furthermore, the ECS plays a major role in basal ganglia function—particularly in relation to reward processing, psychomotor control, and motivational behavior—indicating that sex differences could substantially modulate the effects of cannabinoid interventions on motor and reward-related systems (Kibret et al., 2023).

1.4. Phytocannabinoids and Their Therapeutic Potential

Phytocannabinoids (e.g., compounds derived from the Cannabis sativa plant) also modulate the ECS (Gertsch et al., 2010). Key phytocannabinoids include Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Caprioglio et al., 2022). THC acts as a partial agonist at both CB1 and CB2 receptors, with its psychoactive effects primarily resulting

from CB1 activation in brain regions involved in reward and pleasure, such as the nucleus accumbens (Spiller et al., 2019). This interaction contributes to THC's potential for abuse (Cooper and Haney, 2009). In contrast, CBD has a more complex interaction with the ECS, including indirect modulation of cannabinoid receptors, FAAH inhibition, and interaction with transient receptor potential (TRP) channels, thereby influencing multiple physiological pathways (Dávila et al., 2022, de Almeida and Devi, 2020).

Unlike THC, CBD lacks psychoactive effects and is noted for its anti-inflammatory, anticonvulsant, and anxiolytic properties (de Almeida and Devi, 2020). There is also growing evidence that cannabinoids have therapeutic effects on inflammatory and excitotoxic processes, which are implicated in disorders such as epilepsy, Parkinson's disease, amyotrophic lateral sclerosis, spasticity, and CNS injuries. Additionally, CBD has been shown to interact with serotonergic receptors, such as 5-HT1A, contributing to its anxiolytic and antidepressant effects (Miao et al., 2024).

1.5. Cannabis plant

Cannabis sativa and Cannabis indica are two primary subspecies within the Cannabis genus, with their morphological features, geographical origins, and phytochemical profiles. Botanically, Cannabis sativa and Cannabis indica have been treated as distinct species since the 18th century, based on the work of Carl Linnaeus and Jean-Baptiste Lamarck. Linnaeus identified Cannabis sativa in 1753 as a European hemp species cultivated for fibre (Clarke and Merlin, 2016b). Later, Lamarck classified Cannabis indica in 1785 as a species found in India, distinguished by its psychoactive properties and differing morphology (Clarke and Merlin, 2016b). C. sativa is known for being tall with narrow leaves, while C. indica tends to be shorter with wider leaves, morphological traits that reflect their respective adaptations to different environments: C. sativa is suited to warmer, temperate regions, while C. indica is adapted to harsher, mountainous climates (Piomelli and Russo, 2016, McPartland, 2017) (Fig. 4).

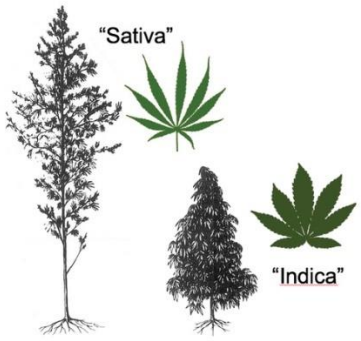


Figure 4. Cannabis Sativa and Cannabis Indica

That being said, there is an ongoing debate regarding dividing the species; some scientists propose that these two "species" are better considered subspecies of a single species due to their ability to interbreed and the extensive hybridization seen in modern cannabis (Brennan et al., 2015). Ernest Small's work has been influential in supporting the view that Cannabis sativa should be recognized as a single species with multiple subspecies, namely sativa (fibre-producing) and indica (psychoactive-producing) (Small, 2017). This perspective highlights the shared genetic foundation between the two subspecies, while also acknowledging their divergent applications (Clarke and Merlin, 2016a).

Despite these formal botanical distinctions, the terms “Sativa” and “Indica” have been adopted into the culture with different meanings. Modern cannabis users and breeders distinguish “Sativa” as strains producing an energetic and uplifting psychoactive effect, while “Indica” is associated with sedative, calming effects (McPartland, 2017). It should be noted that this classification is largely based on subjective reports of psychoactive experience rather than scientific evidence. In this regard, the usage of these terms does not align neatly with the botanical classification. Breeding has significantly hybridized cannabis strains, blurring the lines between the effects attributed to "Sativa" and "Indica." Many modern strains labeled as "Sativa-dominant" or "Indica-dominant" are hybrids with mixed genetic backgrounds and characteristics (Jin et al., 2021). This disconnect between common usage and scientific classification adds confusion to the marketplace and challenges researchers and consumers trying to understand cannabis based on its effects alone.

Research on the phytochemistry of cannabis shows that the chemical profiles, specifically cannabinoids and terpenes, are more useful for distinguishing strains than their morphology

or vernacular labels (Smith et al., 2022). Terpenoids also play a critical role in the effects attributed to different strains. Terpenoid profiling studies by Hood and Barry (1978) and Hillig (2004b) confirmed statistically significant differences in terpenoid content between the biotypes, notably in the presence of sesquiterpenoid alcohols like guaiol, eudesmol, and myrcene (Hood and Barry, 1978, Hillig, 2004). Several terpenoids, including limonene, pinene, caryophyllene, and linalool, have been identified in cannabis (Hanuš and Hod, 2020). Russo, in his review of the history of cannabis, suggests them having significant therapeutic activity, often acting synergistically with cannabinoids to enhance their effects (Russo, 2011). Moreover, the terpene β-myrcene, prevalent in Indica-dominant strains, is known for its sedative properties (Take).

The comparison of THC and CBD content between Cannabis sativa and Cannabis indica shows that there is no consistent or significant difference between these biotypes based solely on these two cannabinoids. A study by Hazekamp et al. (2016) found that THC and CBD levels did not distinguish between “Sativa” and “Indica” in their sample sizes. Specifically, the average THC/CBD ratios were 12.74/0.38 for Sativa strains and 13.71/0.30 for Indica strains, showing a small difference in the ratio but no significant distinction in absolute terms (Hazekamp et al., 2016). Elzinga et al. (2015) tested cannabis strains from U.S. medicinal dispensaries and found that Indica strains contained significantly higher THC levels (averaging 17.30%) compared to Sativa strains (13.84%). . For CBD, the levels across both types were generally low, with a mean of 0.6% and a median of 0.3%, indicating that most of the strains tested were THC-dominant hybrids rather than distinct Indica or Sativa plants (Elzinga et al., 2015).

1.6. Delta-9-tetrahydrocannabinol

1.6.1. Chemical composition:

More than 421 distinct chemical constituents, with over 60 different cannabinoids have been identified in cannabis (Huestis, 2007). In total, cannabis contains 18 distinct classes of compounds, including nitrogenous compounds, amino acids, hydrocarbons, sugars, terpenes, and fatty acids (Sharma et al., 2012, Kaur et al., 2023). This complex composition contributes to its recognized pharmacological and toxicological effects (Patel and Hillard, 2006). The

chemical profile of cannabinoids in the cannabis plant is obviously more complicated than just THC molecule alone, suggesting that the effects of cannabis may depend on the presence of numerous chemical compounds. ECS has been identified following the isolation of Δ9-tetrahydrocannabinol (Δ9-THC) and the endogenous receptors that it activates (Patel and Hillard, 2006). This molecule is the plant-derived cannabinoid and principal psychoactive compound found in the Cannabis plant.

The molecular formula of Δ9-THC is $C_{21}H_{30}O_2$, indicating that it consists of 21 carbon atoms, 30 hydrogen atoms, and 2 oxygen atoms (Triamchaisri and Lawtrakul, 2023). Structurally, Δ9-THC is classified as a terpenophenolic compound, with both aromatic (phenolic) and terpenoid moieties (Odieka et al., 2022). This chemical composition features a tricyclic core structure, along with a pentyl substituent on the aromatic ring, which is fundamental for its high-affinity binding to cannabinoid receptors in the human body (Fig. 5) (Bhattacharyya et al., 2014, Colizzi and Bhattacharyya, 2017).

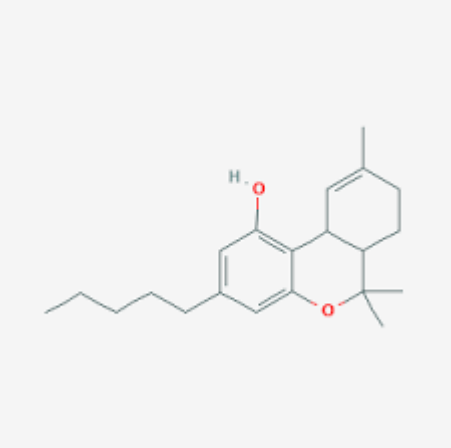


Figure 5. structural formula of THC.

The molecular arrangement of Δ9-THC, particularly the tricyclic ring system, provides the necessary three-dimensional conformation that enables effective interaction with the CB1 and CB2 receptors (Colizzi and Bhattacharyya, 2017). The molecule features a complex arrangement that includes a phenolic hydroxyl group, a cyclohexene ring, and a long aliphatic chain (Chiaramonte and Rosa, 2023). The presence of a double bond between the ninth and tenth carbon atoms in the cyclohexene ring is a defining feature of Δ9-THC, differentiating it from its isomer, Δ8-THC, which has a double bond in a different position and this structural

distinction significantly influences the pharmacological properties of the compound, including its potency and efficacy at cannabinoid receptors (Chiaramonte and Rosa, 2023, Goodman et al., 2023). This structural specificity not only dictates the potency of Δ^9-THC but also influences its bioavailability, metabolic stability, and lipophilicity.

The stereochemistry of Δ9-THC is also critical to its function. The molecule has specific stereocenters that result in different spatial arrangements of its atoms, which can affect how it interacts with biological targets. The (-)-trans configuration of Δ9-THC is responsible for its psychoactive effects, as it allows for optimal binding to the CB1 receptor and it has been shown that modifications to the molecular structure can significantly alter the binding affinity and activity of cannabinoids at the CB1 and CB2 receptors (Fichera et al., 2000, Colizzi and Bhattacharyya, 2017, Bhattacharyya et al., 2015). For instance, the introduction of different functional groups or alterations in the carbon chain length can enhance or diminish the psychoactive properties of the compound. This has led to the exploration of various synthetic analogues and derivatives of Δ9-THC for potential therapeutic applications (Fichera et al., 2000, Bhattacharyya et al., 2014).

In addition to its psychoactive effects, Δ9-THC has been studied for its potential therapeutic benefits, including analgesic, antiemetic, and appetite-stimulating properties (Huang et al., 2021). The chemical structure of Δ9-THC allows the compound to modulate various signaling pathways within the body; the ability of Δ9-THC to cross the blood-brain barrier is also attributed to its lipophilic nature, which is a consequence of its carbon-rich structure (Colizzi and Bhattacharyya, 2017, Vassall et al., 2023).

In 1990, researchers identified the first cannabinoid receptor, CB1, when they discovered that an orphan G protein-coupled receptor (SKR6) from a rat's cerebral cortex was responsible for mediating the pharmacological effects of THC. THC was first isolated in 1964 by Raphael Mechoulam, who subsequently elucidated its structure in 1967 (Russo, 2011, Mechoulam et al., 2014). The pharmacological profile of THC is characterized by its action as a partial agonist at the CB1 receptor which will be discussed later (Straiker, 2005). The ability of this compound to modulate dopaminergic activity in the brain's reward pathways further explains its role in the rewarding aspects of cannabis consumption (Norris et al., 2019, Fitoussi et al., 2018). In

addition to its psychoactive properties, THC has been recognized for its therapeutic potential in various medical conditions. Its analgesic, antiemetic, and orexigenic effects are under investigation in the treatment of chronic pain, nausea, and appetite loss associated with conditions such as cancer and HIV/AIDS (Dumbraveanu et al., 2023, Russo, 2011). However, the psychoactive effects of THC can pose challenges for patients seeking therapeutic benefits without cognitive impairment (Pepito, 2023, Chetia and Borah, 2020). This has led to increased interest in cannabinoids such as cannabidiol (CBD), which exhibits anxiolytic and antipsychotic properties and may mitigate some of the adverse effects of THC (Chetia and Borah, 2020, Zuardi et al., 2006).

The molecular characterization of CB1 has allowed researchers to develop techniques to increase the selectivity, metabolic stability, and efficacy of synthetic agents with cannabinoid and non-cannabinoid structures. In this regard, synthetic THC and related cannabinoids have shown therapeutic potential in various medical applications. Sativex, a standardized cannabis extract containing THC and cannabidiol, has been approved for multiple sclerosis-associated spasticity and chronic pain in some countries (Constantinescu and Tanasescu, 2012). Dronabinol, a synthetic THC, and Nabilone, a structurally similar agent have been approved for treating chemotherapy-induced nausea and as an appetite stimulant for cachexic AIDS patients (Constantinescu and Tanasescu, 2012, Galal et al., 2009). Research has explored the use of THC and other cannabinoids for appetite stimulation, with oral and rectal suppository forms showing efficacy (Voth and Schwartz, 1997). Finally, the discovery of cannabinoid receptors has led to the development of more selective and efficacious synthetic cannabinoids, targeting specific therapeutic effects while minimizing adverse reactions (Voth and Schwartz, 1997, Marzo and Petrocellis, 2006). Other novel research approaches focus on peripherally-restricted CB1R agonists and selective CB2R agonists, to optimize therapeutic benefits and reduce potential side effects (Marzo and Petrocellis, 2006).

1.6.2. General Pharmacokinetic and metabolism

The pharmacokinetics and metabolism of THC are complex and influenced by various factors including the method of administration, individual physiology, and the presence of

other cannabinoids. This includes absorption of THC by various routes of administration and from different formulations, the distribution throughout the body, its metabolism by different tissues and organs, and finally the elimination from the body in the feces, urine, sweat, oral fluid, and hair, as well as varied factors affecting these processes (Grotenhermen, 2003).

Understanding the pharmacokinetics of THC has posed significant challenges for research, largely due to the inherently low concentrations of cannabinoids in biological systems, their rapid and extensive metabolic processes, and their complex chemical properties (Huestis, 1999, McGilveray, 2005). These factors create difficulties in efficiently separating the compound of interest from biological tissues and from one another, and they also result in reduced recovery rates due to the tendency of cannabinoids to adsorb onto various organs (Chayasirisobhon, 2020). Early research often relied on radio-labeled cannabinoids, which provided highly sensitive but less specific quantification of individual cannabinoids (Herkenham et al., 1991). However, advances in mass spectrometry are enabling us to have a more precise and sensitive analysis of cannabinoids across a wide range of biological samples (Fig 6).

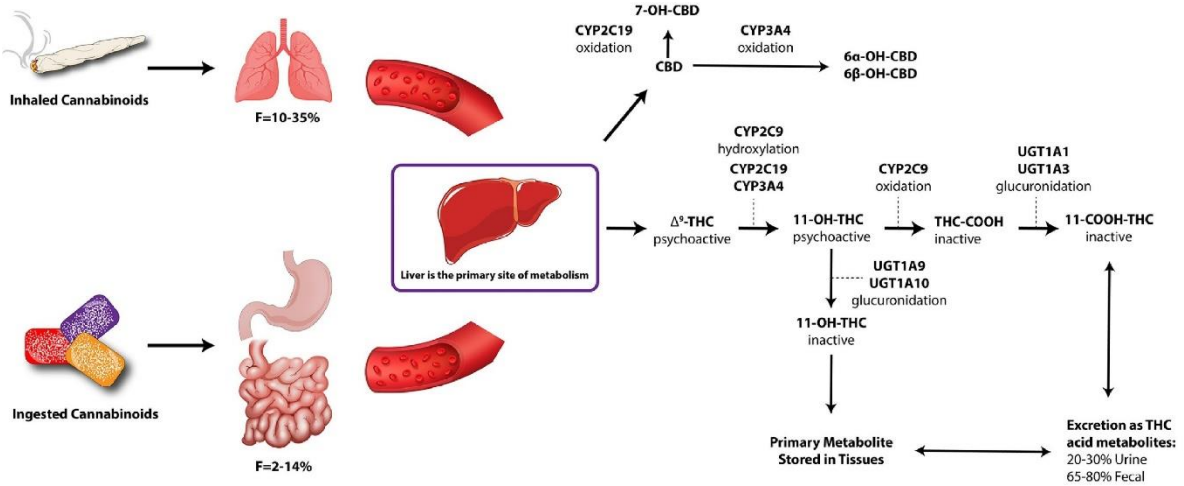


Figure 6 represents the metabolism of THC (Olt et al., 2021)

1.6.3. Administration:

The pharmacokinetics of cannabinoids absorption are profoundly affected by the route of administration and the formulation of the drug. Smoking is still the most prevalent method

of cannabis consumption, primarily due to the rapid and efficient absorption of THC through the membranes in the lungs into the bloodstream (Liyanage et al., 2023). Another common route of cannabis administration is oral ingestion which has distinct pharmacokinetic properties. This results in near-immediate psychoactive effects on the central nervous system, increasing its potential for abuse (McGilveray, 2005). Bioavailability following inhalation via smoking is highly variable, ranging from 2% to 56%. This variability is influenced by factors such as inhalation frequency, duration, volume, and interindividual differences. These factors are collectively referred to as "smoking topography" (McClure et al., 2012). THC appears in plasma almost immediately after the first inhalation, with peak concentrations typically observed approximately 9 minutes post-initiation, followed by a rapid decline to levels below 5 ng/ml within 2 hours (Huestis et al., 1992b).

Even under controlled conditions employing smoking protocols, substantial interindividual variability persists, largely due to differences in inhalation depth and self-titration behaviors, which involve adjusting the frequency and depth of inhalations to regulate THC intake to achieve the desired effect (Huestis et al., 1992b). Additionally, subjective expectations regarding the drug's rewarding effects further exacerbate variability in smoking patterns, as individuals may modulate their smoking behaviors to achieve elevated plasma THC levels when anticipating an active drug effect. For instance, Cami et al. demonstrated that subjects adjusted their smoking technique to achieve higher plasma THC concentrations when they anticipated receiving an active drug, compared to when they received placebo cigarettes (Camí et al., 1991). Although THC bioavailability through smoking is generally lower compared to intravenous administration, it remains sufficient to elicit the desired psychoactive effects. For example, peak plasma concentrations of THC have been reported at 84.3 ng/ml for a low-dose cigarette (1.75% THC) and 162.2 ng/ml for a high-dose cigarette (3.55% THC), highlighting the dose-dependent nature of THC delivery through smoking (Huestis et al., 1992a).

As already stated, smoking as a route of delivery is favored by users for its rapid onset of effects and the ability to self-titrate intake (Trigo et al., 2016). This variability in pharmacokinetics complicates the establishment of a dose-response relationship, resulting in challenges for clinical applications. In this regard, in clinical settings, this variability makes it

difficult to determine appropriate dosing regimens for therapeutic use (Russo, 2016). The study of cannabinoid, and in particular THC, pharmacokinetics following oral administration is not researched enough compared to the inhaled route. Smoking results in a rapid onset of effects and comes with adverse health effects of smoke inhalation. This major downside makes this route unsuitable for therapeutic use and medical application (Russo, 2016). The oral route of delivery will be discussed in detail later in upcoming chapters.

Rectal, sublingual, and dermal administrations of THC have been explored as alternative routes to the oral route, primarily to enhance bioavailability and reduce the impact of first-pass hepatic metabolism. Rectal administration has been studied through the use of THC suppositories, with research showing a bioavailability of 13.5% (Elsohly et al., 1991). Brenneisen et al. (1996) found that rectal administration in patients with spasticity resulted in peak plasma THC concentrations between 1.1 and 4.1 ng/ml within 2 to 8 hours (Jarho et al., 1998). Comparatively, oral THC administration led to higher variability in plasma concentrations, ranging from 2.1 to 16.9 ng/ml after 10 to 15 mg daily doses, but with slower and less efficient absorption (Brenneisen et al., 1996). Sublingual administration provides another alternative to avoid first-pass metabolism offering a method for rapid absorption directly into the bloodstream. Cannabis extracts, which contain both THC and CBD, have been evaluated in clinical trials, including studies on analgesia and spasticity (Brenneisen et al., 1996).

Dermal administration is another route under investigation. Stinchcomb et al. (2004) have investigated the potential of delta 8-THC transdermal patches or topical applications, which could allow for sustained release of the compound while avoiding gastrointestinal degradation and first-pass metabolism with fewer fluctuations in plasma concentration (Stinchcomb et al., 2004).

1.6.4. Distribution:

Once THC enters the bloodstream, it quickly distributes throughout the body, with a particular affinity for fatty tissues, where it can accumulate due to its lipophilicity (Sperry et al., 2021). This accumulation contributes to THC's prolonged effects and extended elimination half-life, which can vary widely depending on usage frequency and individual metabolism

(Sperry et al., 2021, Hansen et al., 2023). For instance, the elimination half-life can be as short as 2.75 hours after oral administration but may be significantly longer in chronic users because THC is stored in adipose tissues (Lucas et al., 2018). This rapid distribution, especially to highly perfused organs like the brain, lungs, liver, and heart, leads to a fast onset of effects after smoking, but concentrations decrease quickly as THC is metabolized and redistributed (Lucas et al., 2018, Lemberger, 1972). Comparatively, the pharmacokinetics of THC's major metabolite, 11-OH-THC, reveal important differences. Perez-Reyes et al. (1972) demonstrated that while equal doses of THC and 11-OH-THC produced similar psychoactive effects, the onset of these effects was quicker with 11-OH-THC (Perez-Reyes et al., 1973). This metabolite also left the bloodstream faster than THC, likely due to its lower protein binding and faster diffusion into the brain, which was further supported by animal studies showing more rapid brain penetration of 11-OH-THC compared to THC (Perez-Reyes et al., 1973). The distribution volume of THC is notably large, approximately 10 l/kg, despite THC being highly bound to plasma proteins (Perez-Reyes et al., 1973). Other studies estimate a steady-state of 3.4 l/kg (Grotenhermen, 2003). In chronic use, the slow accumulation of THC in less perfused tissues such as fat becomes a significant factor, as THC redistributes from the bloodstream into these stores over time. In prolonged exposure, THC can remain in fat tissues, potentially being released slowly back into the bloodstream, which can extend the duration of its effects (Johansson et al., 1989). Moreover, studies on tolerance have shown that repeated THC exposure does not necessarily lead to decreased uptake into the brain. For instance, Dewey et al. (1976) found no difference in THC distribution between tolerant and non-tolerant subjects, and tolerance to THC's behavioral effects was unrelated to decreased brain uptake (Dewey et al., 1976). Similarly, human tolerance studies revealed that while pharmacokinetic parameters like metabolic clearance and volume of distribution increased with chronic use, these changes did not fully explain the behavioral tolerance observed, pointing instead to pharmacodynamic adaptations (Dewey et al., 1976, Hunt and Jones, 1980).

1.6.5. Metabolism:

A key step in THC metabolism is its biotransformation in the liver, where enzymes like cytochrome P450 (CYP2C9, CYP3A4) convert THC into its primary metabolites: 11-hydroxy-THC

(11-OH-THC), which is active, and 11-nor-9-carboxy-THC (THC-COOH), which is inactive (Grotenhermen, 2003, Brenneisen et al., 2010, Leghissa et al., 2018). The presence of these metabolites in blood and urine provides useful biomarkers for drug testing, yet their roles and impacts are distinct, with THC-COOH being particularly valuable for long-term detection (Leghissa et al., 2018).

A comparative look at the two primary routes of THC administration—oral and inhalation (smoking or vaporizing)—reveals significant differences in metabolic outcomes. Oral THC is subject to first-pass metabolism, resulting in higher concentrations of 11-OH-THC in the bloodstream compared to inhalation (Wall and PEREZ-REYES, 1981). This metabolite, being more potent than THC itself, can contribute to stronger and longer-lasting psychoactive effects, which may explain the differential subjective experience between edible and smoked cannabis (Stinchcomb et al., 2004). In contrast, smoking or vaping THC delivers higher amounts of unmetabolized THC directly into systemic circulation, leading to a faster onset of effects but with lower production of 11-OH-THC (Schwilke et al., 2009, Torrens et al., 2023). Thus, while both methods of administration deliver THC's psychoactive properties, their pharmacological profiles diverge due to variations in how much of the active metabolite is produced and distributed.

Furthermore, the individual variability in THC metabolism introduces another layer of complexity. Factors like sex, age, body mass, and genetic polymorphisms in metabolic enzymes such as CYP2C9 can influence the rate and extent of THC metabolism (Grotenhermen, 2003). For example, some studies suggest that women may metabolize THC into 11-OH-THC more efficiently than men, potentially leading to prolonged or intensified effects (Grotenhermen, 2003, Narimatsu et al., 1991) However, clinical human studies have not consistently demonstrated significant sex-based differences in the plasma half-life of THC, indicating that other variables, such as hormone levels or body composition, may play a role. This lack of consensus highlights the need for more nuanced research into how demographic factors impact THC metabolism and its effects (Fattore et al., 2008).

Another important comparison lies in the detection and persistence of THC and its metabolites. THC, due to its lipophilicity, tends to accumulate in adipose tissues and is slowly

released back into the bloodstream over time, resulting in prolonged detection windows for THC-COOH in urine, especially in chronic users (Fattore et al., 2008, Schwilke et al., 2009). In contrast, acute users may clear THC and its metabolites much faster (Kelly and Jones, 1992). This variability is important in contexts such as drug testing, where the extended presence of THC-COOH may not necessarily correlate with recent cannabis use, but rather reflect residual excretion from fat stores (Kelly and Jones, 1992). In terms of THC transfer and accumulation in specific physiological contexts, both placental transfer and breast milk accumulation raise significant concerns (Bindesri et al., 2020). Research in rhesus monkeys indicates that while THC crosses the placenta, its levels in fetal tissues are significantly lower than in maternal blood (Monfort et al., 2022). This is partly due to the limited placental transfer of THC metabolites such as THC-COOH, which do not readily cross into fetal circulation (Bailey et al., 1987). This reduced fetal exposure to active metabolites may mitigate some of the potential developmental risks, although the presence of THC itself remains a concern. Comparatively, breast milk presents a more direct route for THC transfer to neonates, as the lipophilic nature of THC allows for its concentration in breast milk (Atkinson et al., 1988). This poses potential risks for infants exposed to THC through breastfeeding, particularly regarding neurological development, although the long-term effects remain under study. Animal studies provide additional insights into the interspecies variability of THC metabolism, offering a comparative understanding that can be cautiously extrapolated to humans. For example, rodents and guinea pigs exhibit species-specific differences in their production of THC metabolites. In rodents, 11-OH-THC is the primary metabolite, while guinea pigs show a preference for 8β-OH-THC (Huestis, 2007). Such differences suggest that while the general pathways of THC metabolism may be conserved across species, the relative production of certain metabolites can vary.

1.7. Pharmacological properties:

The molecular formula of THC is C21H30O2, and it has a molecular weight of approximately 314.46 g/mol (Hama and Sagen, 2009). This structure allows THC to interact effectively with the endocannabinoid system, primarily through its binding to the cannabinoid receptors CB1 and CB2 (Hama and Sagen, 2009, DeLong et al., 2010). As already mentioned,

one of the notable chemical properties of THC is its lipophilicity, which significantly influences its pharmacokinetics and distribution in the body. THC is highly lipophilic, with a pKa of approximately 10.6, which facilitates its rapid accumulation in fatty tissues and the central nervous system (Brunet et al., 2006). This lipophilic nature contributes to its prolonged half-life and the potential for accumulation with repeated use, leading to both therapeutic effects and the risk of adverse effects. The receptor binding affinity of THC is another critical aspect of its chemical properties. THC has been reported to have a Ki value of approximately 41 nM for the CB1 receptor and 36 nM for the CB2 receptor (Hama and Sagen, 2009). Recent research has focused on modifying the chemical structure of THC to enhance its therapeutic properties while minimizing psychoactive effects. Structural modifications, such as the elimination of the phenolic hydroxyl group, have been shown to alter THC's receptor affinity and activity (Gloriam et al., 2024). Additionally, the development of water-soluble THC analogues aims to improve bioavailability and therapeutic efficacy, particularly in formulations for oral administration (Breit et al., 2019). These modifications could lead to cannabinoids with improved pharmacokinetic profiles, potentially expanding their clinical applications.

1.8. Significance of THC as a medicinal agent:

The initial isolation of THC in the 1960s coincided with a growing interest in the potential therapeutic applications of cannabis, leading to a surge in research to understand the pharmacological properties of cannabinoids. As societal views toward cannabis are evolving, it is affecting the regulatory landscape which increases the acceptance of its medicinal use in various authorities. The use of cannabis goes back to ancient writings as far as 2900 B.C.E. The Shennong Ben Cao Jing, an ancient Chinese Pharmacopoeia holds the earliest documented mention of cannabis as a medicinal remedy. It suggests using cannabis to treat constipation, rheumatic pain, disorders of the female reproductive system, and malaria (Brand and Zhao, 2017). Modern research is in agreement with this encyclopedia, and it has been shown that THC produces pharmacological effects on many levels (Brand and Zhao, 2017, Stasiłowicz et al., 2021). These effects range from euphoria, muscle relaxation, and perceptual changes, to analgesic, orexigenic, anti-inflammatory, antipruritic, bronchodilatory, and anti-spasmodic properties (Mulia et al., 2021). However, this molecule is not without down sides and there

are documented adverse effects including anxiety, dysphoria, impaired memory, psychotic symptoms and immunosuppression (Mulia et al., 2021, Arboleda and Prosk, 2021). THC is also being used clinically for chemotherapy-induced nausea, as an appetite stimulant, and for chronic pain management under the names nabilone and dronabinol which are the only synthetic THC formulations approved in the United States (Boland et al., 2020, Anand et al., 2021). Despite the therapeutic promise of THC, concerns regarding its psychoactive effects and potential for abuse remain prevalent. The development of synthetic cannabinoids and formulations that minimize psychoactive effects while retaining therapeutic benefits is an area of active research (Joshi, 2021). Additionally, the exploration of cannabinoid combinations, such as THC and CBD, has shown potential for enhancing therapeutic effects while reducing adverse outcomes. This approach aligns with the concept of the "entourage effect," where the combined action of multiple cannabinoids and terpenes may produce synergistic benefits (Russo, 2011). One of the most well-documented medical applications of THC is in the management of pain, particularly cancer-related pain. A systematic review and meta-analysis (reference) indicated that cannabinoids, including THC, are effective in alleviating pain in patients with cancer, providing a viable alternative to traditional analgesics. The analgesic properties of THC are believed to stem from its ability to modulate pain pathways in the central nervous system, thereby reducing the perception of pain. In the context of neurological disorders, THC has demonstrated efficacy in treating symptoms associated with multiple sclerosis and can reduce muscle spasticity and improve mobility in patients suffering from MS (Novotná et al., 2011). It should be noted that the efficacy of THC could be dependent on the specific context. Various studies demonstrate that THC effectively reduces pain in both acute (e.g. traumatic injury) and chronic pain models, particularly in neuropathic pain, where it decreases hyperalgesia and allodynia through interactions primarily with CB1R and CB2R (Henderson-Redmond et al., 2021, Linher-Melville et al., 2023). However, notable sex differences exist in this regard, with females developing tolerance to THC's analgesic effects faster than males, although both sexes benefit from short-term use (Linher-Melville et al., 2023, Britch et al., 2020). Combining THC with CBD also shows enhanced pain relief, particularly in male subjects, with a 1:1 THC:CBD ratio sustaining anti-hypersensitive effects.

While CBD alone has minimal analgesic impact, it modulates immune responses, complementing THC's effects. This combination allows for better pain management, especially in cases where minimizing THC's psychoactive effects is desired (Linher-Melville et al., 2020). Furthermore, THC has shown promise in reducing opioid consumption, particularly when used in conjunction with opioids like morphine. This synergy offers potential relief for acute pain without requiring high opioid doses, thus mitigating risks associated with opioid use (Linher-Melville et al., 2020, Swartwood et al., 2020). Additionally, THC is particularly effective in managing cancer pain, though the high doses required for this analgesia often result in side effects such as sedation and mental clouding, which can limit its clinical use (Swartwood et al., 2020). In contrast, THC shows little efficacy in managing postoperative pain, with results comparable to placebo in clinical trials (Noyes Jr et al., 1975). This highlights the variability in THC's analgesic effectiveness across different types of pain and therapeutic contexts, suggesting it may not be suitable for all situations. Mechanistically, THC's analgesic effects seem to be primarily receptor-mediated, with minimal impact on serum cytokine levels, indicating that its pain relief is not primarily through immune modulation but rather through interactions with the ECS (Britch et al., 2020). In their study involving male and female rats, Stevie C. Britch et al. (2020) reported that THC had significant anti-edema and pain-relieving properties. The results suggest that THC could be more effective than CBD for managing inflammatory pain, as it retains its therapeutic effects with short-term use in both sexes and does not lead to immune system activation (Britch et al., 2020). The antiemetic properties of THC are also noteworthy, especially for patients undergoing chemotherapy. THC has been found to significantly reduce nausea and vomiting, side effects that are often debilitating for cancer patients receiving treatment (Kramer, 2015). Studies have indicated that CBD can counteract THC-induced anxiety and paranoia, making the combination more tolerable for patients (Gertsch et al., 2010). This synergistic relationship between THC and CBD is particularly relevant in the development of cannabis-based medicines, as it allows for a more balanced therapeutic profile that maximizes benefits while minimizing risks. Despite the wide range of therapeutic effects of THC, including its analgesic and antiemetic properties, there are

several challenges that limit its optimal clinical use. These challenges include issues related to dosing, psychoactive side effects, potential for abuse, and variability in patient response.

1.9. Current Challenges in THC Administration

One of the most prominent challenges in THC administration is finding the optimal therapeutic dose that balances efficacy with minimizing adverse effects. THC's psychoactive properties as well as the unpredictability of the therapeutic route (oral intake) is often a limiting factor. While lower doses of THC may provide therapeutic benefits without significant intoxication, higher doses—required for effective analgesia or appetite stimulation—can lead to anxiety, cognitive impairment, dizziness, hypotension, and dysphoria or depression (Stasiłowicz et al., 2021, Badowski, 2017). This psychoactive threshold not only affects patient comfort and quality of life but also presents a risk for potential abuse, particularly in long-term treatments for chronic conditions.

Oral administration of THC, whether through capsules, edibles, or tinctures, presents its own set of challenges. One of the primary limitations is the low bioavailability and high variability of THC when taken orally, typically ranging from 6% to 10% due to first-pass metabolism in the liver (Badowski, 2017, ElSohly et al., 2018). This means that a significant portion of the THC is metabolized before it reaches the systemic circulation, resulting in delayed onset of effects that can take 30 to 90 minutes to manifest (ElSohly et al., 2018). This delayed onset can lead to users consuming additional doses in an attempt to achieve the desired effects, increasing the risk of overdose and adverse reactions. Furthermore, the effects of orally administered THC can be unpredictable, as they depend on various factors, including the individual's metabolism, the presence of food in the stomach, and the specific formulation of the product (Colizzi and Bhattacharyya, 2017). For example, high-fat meals can enhance THC absorption, while high-fiber meals may impede it (Hingorani et al., 2013). This variability complicates dosing regimens and can lead to inconsistent therapeutic outcomes. Some available formulations to overcome these challenges (e.g., dronabinol oral solution) reduce pharmacokinetic and individual variability compared to capsules (Badowski, 2017).

Early research on oral delivery focused on simple extraction and purification techniques for oral administration of known THC amounts from cannabis plant material (Badowski, 2017).

In the same year, Perez-Reyes et al. (1973) studied the impact of different vehicles (including glycocholate and sesame oil) in administering oral THC in gelatin capsules (Perez-Reyes, 1990). Some of these vehicles were found to improve bioavailability, but there was significant variability in peak THC concentrations and absorption rates, even when the same vehicle was used. In 1980 Lucas and Laszlo pointed to the intra- and interindividual variability: "Δ9-tetrahydrocannabinol is erratically absorbed from the gastrointestinal tract, and dosage individualization may be necessary to control these patients" (Lucas and Laszlo, 1980).

Wall et al. (1983) reported oral THC bioavailability to range from 10% to 20% when participants were given either 15 mg (women) or 20 mg (men) of THC dissolved in sesame oil. Peak plasma concentrations were observed approximately 4 to 6 hours post-ingestion. However, in this study, they did not have the technology to distinguish between THC and metabolites (Lucas and Laszlo, 1980).

A more accurate estimate of oral THC bioavailability was presented by Ohlsson et al. (1980), who used mass spectrometry to measure the molecule of study in plasma. They found peak concentrations ranging from 4.4 to 11 ng/ml, occurring 1 to 5 hours after ingesting 20 mg of THC in a chocolate cookie. This study estimated THC bioavailability to be around 6%. These early studies concluded that oral THC absorption is generally slow, leading to lower plasma concentrations. Several factors contribute to its low oral bioavailability (ranging from 4% to 20%), including variable absorption, degradation in the stomach, and extensive first-pass metabolism in the liver, which converts THC to active 11-OH-THC and inactive metabolites. These challenges are still relevant to this day (Ohlsson et al., 1980).

Further investigation into oral THC's pharmacokinetics was driven by its therapeutic potential. In a study by Kim and Yoon (1996), THC, 11-OH-THC, and THC-COOH concentrations were measured in 17 volunteers following the ingestion of a single 10 mg Marinol capsule. The mean peak plasma concentrations were 3.8 ng/ml for THC, 3.4 ng/ml for 11-OH-THC, and 26 ng/ml for THC-COOH, with peaks occurring 1 to 2 hours post-ingestion. Notably, two THC peaks were frequently observed, likely due to enterohepatic recirculation. Compared to smoking, oral THC administration generally results in delayed effects, lower intensity, and a prolonged duration before returning to baseline (Heustis, 2005, Meier and Vonesch, 1997).

In another study (Nebro et al., 2004), researchers examined the pharmacokinetics and pharmacodynamics of oral THC administered via hemp oil and dronabinol. Six volunteers were given up to 14.8 mg of THC daily, divided into three doses taken with meals for five consecutive days. Plasma THC levels were measured using solid-phase extraction followed by GC/MS. After the highest doses (7.5 and 14.8 mg/day), peak plasma THC concentrations were less than 6.5 ng/ml, 11-OH-THC less than 5.6 ng/ml, and THC-COOH less than 43 ng/ml. Interestingly, the THC-COOH concentrations after a 7.5 mg/day dronabinol dose were similar to those observed with the 14.8 mg/day hemp oil dose. This higher bioavailability of THC in dronabinol was attributed to its encapsulation, which protects against degradation in the acidic stomach environment, and improved absorption from the sesame oil formulation. THC and 11-OH-THC levels dropped below detectable limits 25 hours after the last dose, while THC-COOH remained detectable for over 50 hours (Goodwin et al., 2006, Gustafson et al., 2003).

Recent cannabinoid research has focused on modifying the chemical structure of THC to enhance its therapeutic properties while minimizing psychoactive effects. Structural modifications, such as the elimination of the phenolic hydroxyl group, have been shown to alter THC's receptor affinity and activity. Additionally, the development of water-soluble THC analogues aims to improve bioavailability and therapeutic efficacy, particularly in formulations for oral administration (Breit et al., 2019) (Breit et al., 2019). These modifications could lead to cannabinoids with improved pharmacokinetic profiles, potentially expanding their clinical applications.

The chemical properties of THC, including its lipophilicity, receptor binding affinity, metabolic stability, and structural characteristics, play a crucial role in its pharmacological effects and therapeutic applications. However, the challenges in THC administration are not dependent on only the molecule itself.

1.9.1. Tolerance and Dependence:

Another significant limitation of THC administration is the development of tolerance and dependence. Chronic use of THC can lead to the desensitization of cannabinoid receptors, resulting in diminished therapeutic effects over time (Li et al., 2012). This necessitates higher doses to achieve the same level of symptom relief, which can increase the risk of adverse

effects and complicate treatment plans. Additionally, withdrawal symptoms may occur upon cessation of THC use, including irritability, insomnia, and anxiety, which can deter patients from adhering to the prescribed regimens (Kesner and Lovinger, 2021).

1.9.2. Individual Variability:

Individual variability in response to THC is a critical consideration in its administration. Factors such as genetics, sex, age, and pre-existing medical conditions can influence how patients metabolize and respond to THC (Kandasamy et al., 2018). For example, genetic polymorphisms in cannabinoid receptors can lead to differences in receptor sensitivity and downstream signaling pathways, resulting in varied therapeutic responses among individuals (Kandasamy et al., 2018, de la Ossa et al., 2013). This variability complicates the establishment of standardized dosing guidelines and highlights the need for personalized approaches to THC administration.

1.10. Rodent studies

In vivo animal studies have demonstrated that THC exhibits high-affinity binding to neuronal CB1 receptors. In animal models, activation of CB1 receptors by THC induces the classical "tetrad" effects: (1) hypolocomotion, (2) hypothermia, (3) catalepsy as assessed by the ring test, and (4) analgesia as measured in the tail-flick or hot-plate assays (Moore and Weerts, 2022, Wang et al., 2020). Furthermore, THC elevates dopamine release in the nucleus accumbens of rats and mice, implicating its involvement in reward circuitry and neural reward mechanisms (Wang et al., 2020, Cheer et al., 2004). In these studies, THC exhibits dual roles, functioning as both a proconvulsant and anticonvulsant, and can act as either an anxiolytic or anxiogenic agent, depending on the context (Devinsky et al., 2014). In addition to interacting with CB1 and CB2 receptors, THC also targets other receptors, including GPR55 and various transient receptor potential (TRP) channels, such as TRPV1, TRPV2, and TRPA1, as well as serotonin receptors like 5-HT2 (Pertwee, 2010). TRPV1 plays a key role in detecting temperature, heat, and pain, while TRPV2 is implicated in pathological conditions such as cancer and inflammation (Bujak et al., 2019, Shuba, 2021).

Similarly to humans, in rodent models, sex, strain, and age significantly influence THC's potency and effects. Female rats exhibit heightened sensitivity to 11-OH-THC, indicating sex-specific metabolic differences in THC processing (Wiley et al., 2021, Torrens et al., 2022). Furthermore, genetic variability across strains, such as Lewis and Fischer 344 rats, reveals significant differences in the rewarding properties of THC. Lewis rats, for example, show enhanced sensitivity to THC's rewarding effects compared to Fischer 344 and Sprague-Dawley rats, suggesting that genetic background is an important factor in mediating THC's psychoactive properties (Mokler et al., 1987). Similarly, results from a microdialysis study show that delta 9-THC produces a dose-dependent, strain-specific enhancement of basal DA efflux in Lewis strain rats when compared with Sprague Dawley rats (Mokler et al., 1987). Another study observed that inhalation of THC results in different serum concentrations and behavioral effects compared to intraperitoneal injection in female rats (Hume et al., 2024). Age-related differences also play a critical role in THC metabolism and distribution. Adolescent rats demonstrate higher plasma and brain concentrations of THC and its metabolites compared to adults, pointing to age-dependent variability in pharmacokinetics (Torrens et al., 2022). This suggests that younger individuals may be more sensitive to THC, which has significant implications for therapeutic use and potential side effects in younger populations. Moreover, THC produces both rewarding and aversive behavioral responses in rodents, mediated by different opioid receptors. The absence of mu-opioid receptors, for instance, abolishes THC-induced place preference, while the absence of kappa-opioid receptors eliminates THC-induced aversion, suggesting that opioid signaling is intricately linked to THC's behavioral effects (Corchero et al., 1998).

Activation of ECB is thought to affect appetite regulation as well as energy and tissue metabolism (Drori et al., 2018). THC’s orexigenic effects in rats are mediated by its engagement of hypothalamic homeostatic circuits and mesocorticolimbic reward pathways, with plasticity in CB1 receptor efficacy shaping behavioral outcomes (Zehra et al., 2018, O’Sullivan et al., 2021a). Food intake outcome shows variety based on dose, sex, treatment duration administration route, and diet type.

The biphasic effect of the utilized dose is evident in various studies. Low to moderate doses of THC generally increase food consumption, particularly of palatable foods, in the short term (Koch, 2001, Koch and Matthews, 2001, Williams et al., 1998b). This effect is observed with both peripheral and central administration, though central administration may produce longer-lasting effects (Koch and Matthews, 2001). However, high doses can decrease food intake (Koch and Matthews, 2001). The orexigenic effect is also more pronounced with high-fat diets compared to standard chow (Koch, 2001).

Interestingly, while acute THC administration can cause hyperphagia, rats typically compensate for overconsumption, resulting in similar 24-hour intakes (Williams et al., 1998b). Chronic THC treatment, however, may suppress food intake, with obese rats showing slower recovery than lean rats (Williams et al., 1998b). Acute THC administration (0.5–5 mg/kg, i.p.) reliably induces hyperphagia in rats, particularly under conditions of ad libitum access to palatable diets (Mattes et al., 1994, Ogden et al., 2019). This orexigenic effect peaks within 1–2 hours post-administration and correlates with increased meal frequency rather than meal duration, suggesting enhanced appetitive motivation.

The hypothalamus is a privileged target of cannabinoid actions on food intake and feeding modulation. Local injections of the CB1R antagonist AM251 into PVN potentiates both fasting and ghrelin-induced hyperphagia (Busquets-García et al., 2015).

In addition to the PVN, nuclei such as the arcuate nucleus (ARC), lateral hypothalamus (LH), and ventromedial hypothalamus (VMH) are involved in hunger and satiety. The ARC integrates peripheral signals like leptin and ghrelin via its distinct populations of orexigenic and anorexigenic neurons, thereby setting the initial drive for feeding (Junewoo Na et al. 2022). In other words, the ARC processes leptin and ghrelin signals to regulate feeding behavior. Leptin activates POMC neurons while inhibiting AgRP/NPY neurons, reducing appetite. In contrast, ghrelin stimulates AgRP/NPY neurons and suppresses POMC neurons, promoting food intake. This balance between orexigenic and anorexigenic neurons initiates feeding drive (Alexander Jais and Jens C Brüning 2021). Moreover, the LH, drives hunger and motivation to eat, whereas the VMH is implicated in satiety and the cessation of feeding (Daniel C Castro 2015).

Mechanistically, THC amplifies ghrelin signaling in the hypothalamus while suppressing activity in anorexigenic pro-opiomelanocortin (POMC) neurons, a process dependent on CB1 receptor agonism in the arcuate nucleus (Charalambous, 2022, Corchero et al., 1997). Conversely, chronic THC exposure induces tolerance, attenuating hyperphagic responses (McMahon, 2011). This tolerance aligns with CB1 receptor downregulation in main feeding-related brain regions, such as the hypothalamus and NAc, and parallels clinical observations of reduced appetite in habitual cannabis users (Barré et al., 2023, O'Sullivan et al., 2021a).

THC's orexigenic effects are not uniform across dietary contexts. Rats exhibit preferential consumption of high-fat or high-sucrose foods following THC administration, implicating cannabinoid modulation of reward circuitry (Cota D. et al 2003). Neurochemically, THC enhances dopamine release in the NAc and the VTA, synergistically driving the hedonic valuation of palatable foods (Roura-Martínez et al., 2019). Operant paradigms further demonstrate that CB1 agonists increase effortful food-seeking behaviors (e.g., lever-pressing), even under progressive ratio schedules, suggesting its role in enhancing motivation rather than solely satiety disruption (Williams and Kirkham, 2002). In contrast, antagonism of CB1 receptors by SR141716A (rimonabant) was shown to decrease motivation for, and intake of, palatable foods by inhibiting dopamine release into the NAc (Melis et al., 2007; Maccioni et al., 2008).

Sex-specific responses to THC in feeding behavior are also evident. Male rats typically exhibit more pronounced hyperphagia following acute THC compared to females, a disparity linked to estrogen's moderating effects on CB1 receptor signaling (Simone et al., 2015). Additionally, females show higher THC concentrations and stronger high-fat food preferences compared to males (Hume et al., 2022, Moore et al., 2021). Both vaped and injected THC produce comparable behavioral effects, including changes in food-motivated behavior, antinociception, and hypothermia (Moore et al., 2021). Prolonged THC administration in rats correlates with leptin reduction in plasma suggesting a dissociation between short-term appetite stimulation and long-term metabolic dysregulation (Eitan et al., 2023).

Research conducted in rodent models also indicates that significant tolerance to various effects, including antinociception, hypothermia, catalepsy, and reduced locomotor activity, emerges within days (3-6) to two weeks of chronic exposure (Maguma, 2010, Compton et al., 2013). This tolerance is not solely a learned response but has a strong physiological basis, involving mechanisms such as CB1 receptor desensitization and activation of the JNK pathway (Ibsen et al., 2017). Interestingly, tolerance development shows regional specificity in the brain, with differential responses observed between midbrain dopamine neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc) (Ibsen et al., 2017, Wu and French, 2000). Cross-tolerance with other cannabinoids (e.g. CP 55,940 and WIN 55,212) suggests shared mechanisms of action (Pertwee et al., 1993). Moreover, factors such as hormonal status and age influence the development and expression of tolerance. In this regard, female rats tolerance to chronic THC's behavioral effects depends on ovarian hormones and the age of initiation. Adolescents developed weaker tolerance than adults, and early THC use reduced sensitivity in intact females but increased sensitivity in ovariectomized (OVX) females—effects not observed with adult initiation or after a drug-free period post-adolescence (Winsauer et al., 2010). The phenomenon of tolerance is further evidenced by the precipitation of withdrawal symptoms upon administration of cannabinoid receptor antagonists in tolerant subjects. For instance, withdrawal symptoms were seen to be precipitated in THC tolerant rats using a cannabinoid receptor antagonist (SR141716A, rimonabant), characterized by disorganized patterns of constantly changing brief sequences of motor behavior without autonomic signs (Tsou et al., 1995).

1.11. The Role of Carriers in Drug Delivery

As extensively reported above, the limitations of existing THC administration methods encompass a range of challenges, including variability in dosing, bioavailability issues, health risks associated with inhalation, and the potential for tolerance and dependence. Alternative routes of THC administration have been explored to circumvent the challenges associated with oral delivery. Inhalation methods, while providing rapid onset of action, pose risks related to pulmonary health and are unsuitable for certain patient populations (Wilkinson et al., 2016).

Sublingual and buccal delivery systems offer improved bioavailability over oral administration but are limited by formulation challenges and patient compliance issues due to taste and mucosal irritation (Millar et al., 2018). Transdermal patches and nasal sprays are other avenues that have shown promise; however, they often require penetration enhancers to facilitate drug absorption, which can cause local irritation and have limited capacity for delivering therapeutic doses of THC (Paudel et al., 2010). These limitations highlight the need for an effective oral delivery system that can enhance the bioavailability of THC, provide consistent therapeutic effects, and improve patient compliance, especially for chronic conditions requiring long-term management.

In this regard, the role of carriers such as ethanol, Cremophor, and dimethyl sulfoxide (DMSO) in the drug delivery of THC has been a focal point in various animal studies aimed at enhancing the bioavailability and therapeutic efficacy of this cannabinoid. These carriers serve as solvents or emulsifiers that facilitate the solubility, stability, and absorption of THC, which is inherently lipophilic and poorly soluble in aqueous environments. This section will briefly explore the significance of these carriers in THC delivery systems, their mechanisms of action, and the implications for therapeutic applications.

Ethanol: Ethanol is frequently used as a solvent in the formulation of THC for animal studies due to its ability to dissolve lipophilic compounds effectively. The use of ethanol can enhance the solubility of THC, allowing for higher concentrations to be administered without the need for large volumes of liquid (Fuentes-Verdugo et al., 2021). This is particularly beneficial in studies where precise dosing is critical, as it enables researchers to achieve effective plasma concentrations of THC more efficiently. Moreover, ethanol can facilitate the absorption of THC through biological membranes. Its small molecular size and ability to disrupt lipid bilayers can enhance the permeability of THC across the gastrointestinal tract or through the skin when used in transdermal formulations (Lipson et al., 2020). However, the use of ethanol as a carrier must be carefully managed, as excessive amounts can lead to toxicity and adverse effects, particularly in sensitive populations or when used in high doses (Fuentes-Verdugo et al., 2021).

Cremophor: Cremophor, a polyethoxylated castor oil derivative, is another carrier commonly employed in THC formulations. It acts as an emulsifier, stabilizing oil-in-water emulsions and enhancing the solubility of hydrophobic drugs like THC (Elmes et al., 2019). The incorporation of Cremophor in THC formulations can improve the drug's bioavailability by facilitating its dispersion in aqueous environments, thereby increasing the surface area available for absorption (Khazaeli et al., 2023).

Cremophor has been shown to enhance the pharmacokinetics of THC in animal studies, leading to improved therapeutic outcomes. For instance, formulations containing Cremophor have demonstrated increased plasma concentrations of THC compared to those without this carrier, suggesting that Cremophor can significantly enhance the systemic availability of THC (Elmes et al., 2019a, de la Ossa et al., 2013). However, it is important to note that Cremophor can also induce hypersensitivity reactions in some individuals, which may limit its use in clinical applications (Elmes et al., 2019a).

DMSO: Dimethyl sulfoxide (DMSO) is a polar aprotic solvent known for its ability to penetrate biological membranes and enhance the absorption of various drugs. In the context of THC delivery, DMSO has been utilized to improve the solubility and bioavailability of THC formulations (Punyamurthula et al., 2016). Its unique properties allow it to facilitate the transdermal delivery of THC, making it a valuable carrier in studies focused on non-invasive administration routes (Taskar et al., 2019).

DMSO can also enhance the stability of THC in solution, reducing the degradation of the compound during storage and administration (Balguri et al., 2016). This stability is crucial for maintaining the therapeutic efficacy of THC, particularly in formulations intended for chronic use. However, the use of DMSO must be approached with caution, as it can alter the pharmacokinetics of other co-administered drugs and may cause skin irritation or other adverse effects when used in high concentrations (Agabio et al., 2017a).

1.11.1. Limitations and Implications for Therapeutic Applications

The use of carriers such as ethanol, Cremophor, and DMSO in THC formulations has significant implications for therapeutic applications. By enhancing the solubility and bioavailability of THC, these carriers can improve the pharmacological effects of THC in various

medical conditions, including chronic pain, nausea, and neurodegenerative disorders (Soliman, 2024, Gaur, 2024). The ability to achieve effective plasma concentrations with lower doses can also reduce the risk of adverse effects associated with higher THC doses.

Moreover, the choice of the carrier can influence the pharmacokinetic profile of THC, affecting its onset, duration of action, and overall therapeutic efficacy. For example, formulations that utilize DMSO may provide a more rapid onset of action due to enhanced absorption, which can be beneficial in acute pain management scenarios (Yamazoe et al., 2022). Conversely, formulations with Cremophor may offer sustained release characteristics, allowing for prolonged therapeutic effects (Elmes et al., 2019a, de la Ossa et al., 2013). On the other hand, the research on DMSO toxicity highlights the fact that this agent could cause neuronal apoptosis and significant developmental effects at doses as low as 0.3 ml/kg (Galvao et al., 2014, Hanslick et al., 2009). Combined with other chemicals, DMSO's toxicity could be synergistic and increase mortality and developmental delays in zebrafish embryos (Kim and Lee, 2021). DMSO could also disrupt gene expression and human cellular processes (Kang et al., 2020, Verheijen et al., 2019). Moreover, a study by Huang et al. showed significant behavioral abnormalities such as hypoactivity and hyperactivity syndromes in aquatic models with sublethal doses of DMSO (Huang et al., 2018). Detrimental effects of this carrier on spontaneous exploratory activity and impaired acquisition of conditioned behavior have been reported (Fossom et al., 1985). Although some studies report no significant neurotoxicity even with controlled infusion, the compound's broad impact on cellular integrity, behavior, and development raises an issue (Bakar et al., 2012, Vogin et al., 1970). Moreover, Cremophor (another widely used solubilizer) exhibits dose-dependent antinociceptive effects, compromising its utility in pain-related studies (Tabarelli et al., 2003, Liu et al., 2016). A study by Liu et al. (2016) further demonstrated that this carrier alters the inherent pharmacokinetic properties of co-administered compounds, reducing plasma volume of distribution and clearance in mice and rats. (Liu et al., 2016). The low-concentration effects of ethanol on locomotor activity and behavioral sensitization have also been reported previously (Liu et al., 2016, Chen et al., 2011).

Future research should focus on optimizing the formulation of THC with these carriers to maximize therapeutic benefits while minimizing potential risks. Investigating alternative carriers or combinations of carriers may also yield formulations with improved safety profiles and enhanced efficacy (Jongjitphisut et al., 2023, Bergeria et al., 2023). Additionally, studies exploring the interactions between THC and these carriers at the molecular level could provide insights into the mechanisms underlying their effects on drug delivery and absorption (Bergeria et al., 2023, Yang et al., 2019).

1.12. Cyclodextrins in Drug Delivery

As discussed earlier, the delivery of THC, particularly through the oral route, presents challenges due to its hydrophobic nature, leading to reduced bioavailability among other limitations. Moreover, oral drug delivery is a preferred method for its ease and patient compliance; yet hydrophobic compounds like THC require specific modifications to improve their solubility and stability. This limitation has led to the exploration of various drug carriers (as reported above) that can improve the bioavailability of poorly water-soluble drugs such as THC (Bragança et al., 2020, Hippalgaonkar et al., 2011). Regarding the mentioned studies on the safety profile of common carriers used in THC delivery, cyclodextrins (CDs) have emerged as promising excipients in this context, capable of tackling the common challenges in food and drug delivery.

Originally derived from starch by enzymatic treatment, CDs are cyclic oligosaccharides with a distinctive structure: a hydrophobic cavity and a hydrophilic exterior (Hippalgaonkar et al., 2011). These molecules were discovered in 1891 by Villiers but were not present in scientific literature until the beginning of the twentieth century with the discovery of alpha and beta CDs by Schardinger (Crini, 2014). It was not until 1930 that Freudenberg described the gamma form of CD and established their cyclic oligosaccharide structure. By the 1950s, cyclodextrins' abilities to form inclusion complexes, solubilize, and stabilize drugs were recognized, and the first patent was issued in 1953. By the late 1970s and early 1980s, they start to gain prominent scientific interest. Pure cyclodextrins became available for pharmaceuticals about 25 years later, with Japan marketing the first product, followed by Europe and the US (Crini, 2014, Loftsson and Duchene, 2007). Over time, CDs have been

extensively researched for their potential to act as carriers for drugs with limited aqueous solubility, improving this aspect as well as active ingredient stability and bioavailability. This has led to their application in a variety of formulations, including immediate and controlled-release forms; dosage forms such as solid, viscous, or liquid; and administration routes: oral, buccal, nasal, ophthalmic, dermal, and parenteral (Duchene and Bochot, 2016).

The effect of CDs on oral drug absorption can be explained in the context of The Biopharmaceutical Classification System (BCS) which was introduced by Amidon et al. and categorizes drugs based on their solubility and permeability profiles (Vikaas and Arun, 2012). This system identifies THC as a BCS Class II drug due to its low solubility but high permeability. Unlike class III and IV the increased bioavailability of inclusion complex of class II with CDs by oral route has been shown in multiple studies (Messner et al., 2011). Interestingly, CDs have the greatest effect on relatively small lipophilic molecules in this class. In addition, CDs can form both nano- and microparticles, which have shown their ability to penetrate human mucus more rapidly than can individually do drug molecules (Lai et al., 2007, Brewster et al., 2008). CDs are also utilized in enhancing the delivery of neuroactive steroids such as allopregnanolone for postpartum depression (PPD) treatment. This is demonstrated in brexanolone (ZULRESSO™), an intravenous therapy combining allopregnanolone (a $GABA_a$ receptor modulator critical for its antidepressant effects) with sulfobutylether-beta-cyclodextrin, which improves the compound's solubility and bioavailability. Supported by phase 3 clinical trials, brexanolone demonstrated significant efficacy in reducing depressive symptoms in women with moderate-to-severe PPD, evidenced by marked decreases in HAM-D scores compared to placebo across two dosage regimens (60 and 90 µg/kg/hour). These findings, led to brexanolone's landmark FDA approval in 2019 as the first dedicated PPD treatment (Scott, 2019, Meltzer-Brody et al., 2019).

Here we will dive into the chemical properties of different types of CDs, their safety profile, as well as medical applications.

The enzymatic decomposition of starch via cyclodextrin glucosyl transferase and by specific bacteria could produce highly selective oligosaccharide rings. Depending on the size of the ring (and the number of d-(+)-glucopyranose units accordingly), a distinction is made

between alpha, beta, and gamma CDs. They consist of six, seven, and eight glucose units, in order. The utilization of CDs with bigger rings is limited due to their structures and properties (Brewster et al., 2008, Ishida and Ho, 2021).

CDs have a toroidal structure, and their diameter varies with the number of glucopyranose units. The cavity is hydrophobic due to the hydroxyl group arrangement, while the outer surface is hydrophilic (Fig. 7). Primary hydroxyl groups, located on the smaller rim, can rotate and alter rim size, whereas secondary hydroxyls on the larger rim contribute to CDs' structural flexibility and solubility through intramolecular hydrogen bonding. Among CDs, β-CD is the most rigid due to a complete hydrogen bond belt, α-CD is more flexible with fewer bonds, and γ-CD is the most flexible and soluble (Hedges, 2009, Shieh and Hedges, 1996). The reversibly encapsulated lipophilic molecules within their hydrophobic cavities, form complexes stabilized by van der Waals forces (Ryzhakov et al., 2016).

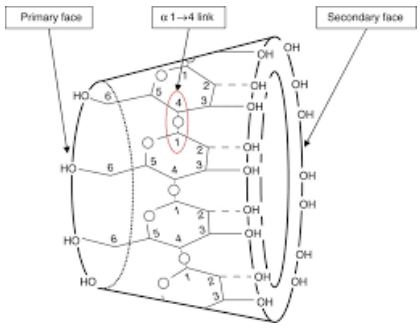


Figure 7. Cyclodextrin structure

Regarding their physical and chemical properties, they have lower water solubility than glucose, primarily due to intramolecular hydrogen bonding that limits hydration. Each CD type differs in solubility based on the strength of this bonding: β-CD, with the strongest hydrogen bond belt, is least soluble (1.85 g/100 mL at 25 °C), while γ-CD, with the weakest, is most soluble (23.2 g/100 mL at 25 °C), and α-CD falls in between (14.5 g/100 mL at 25 °C) (Astray et al., 2009). Solubility increases with temperature (Astray et al., 2009). CDs also have high acidity in their hydroxyl groups (pKa ~12) and become ionized and more soluble at pH >12, reaching 75.0 g/100 mL for β-CD at pH 12.5 (Astray et al., 2009, Hedges, 2009). In their solid state, α-, β-, and γ-CDs are thermally stable up to 300 °C, after which simultaneous melting and thermal decomposition occur (Hedges, 2009, Shieh and Hedges, 1996). CDs' cyclic α-1,4 glycosidic

bonds are more acid-resistant than those in starch due to structural stabilization. However, under strong acidic conditions (pH < 2) with agents like hydrochloric acid, CDs undergo hydrolysis into glucose and oligosaccharides. The reaction rate increases with stronger acidity and heat (Shieh and Hedges, 1996, Szejtli and Budai, 1976, Szejtli, 1988). CDs are highly stable in basic solutions and resist hydrolysis even at elevated temperatures. After ingestion, α-CD and β-CD pass largely undigested through the upper digestive tract, with approximately 99% reaching the large intestine, where intestinal bacteria ferment them into beneficial short-chain fatty acids (Szejtli, 1988, Nihei et al., 2018). γ-CD, unlike α- and β-CD, is nearly fully digested by salivary and pancreatic α-amylase, behaving similarly to starch as a slow-release carbohydrate (Saokham and Loftsson, 2017).

These molecules are widely regarded as safe, with the FAO/WHO JECFA confirming their suitability for use, although with different tolerances depending on CD type. Toxicity assessments for α-CD indicate a high tolerance across species; acute studies in rodents show LD50 values between 500–1000 mg/kg body weight, while extended exposure studies in rats, beagle dogs, and humans reveal no significant toxic effects, aside from minor gastrointestinal discomfort in humans consuming over 20 g in a single meal (Saokham and Loftsson, 2017, Antlsperger and Schmid, 1996). β-CD, on the other hand, has shown mild renal responses in long-term canine studies, such as elevated urinary protein and potassium levels, although no significant clinical symptoms have been noted, resulting in a JECFA-recommended ADI of 5 mg/kg body weight (bw) (Additives et al., 2016). γ-CD demonstrates an even higher tolerance, with both animal and human studies showing no significant toxicity at high doses; gastrointestinal tolerance studies in humans further corroborate its safety profile, revealing no adverse effects even at elevated intake levels (Additives et al., 2016).

Chemically modified CDs are commonly employed in pharmaceutical formulations because they offer enhanced solubilizing capacities relative to native counterparts (Szejtli, 1983). These include hydroxypropylated, randomly methylated, and sulfobutylated CDs. Among these formulations, 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) in particular is being used as active pharmaceutical ingredient for medical purposes (Stella and He, 2008). HPBCD is formally recognized in both the European and United States Pharmacopoeias and has

received orphan drug status for Niemann-Pick type C disease, a severe genetic disorder affecting cholesterol metabolism (Kovacs et al., 2022). This molecule functions by reducing cholesterol buildup in neurons, and potentially slowing disease progression (Chang et al., 2006). Currently, ongoing research is focused on investigating HP-β-CD's potential therapeutic application for other related disorders, such as atherosclerosis and neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (Becktel et al., 2022, Dohárszky et al., 2024). In this context, prior research has demonstrated the superior efficacy of HP-β-CD in enhancing the solubility of THC. Notably, Jarho et al. confirmed a thousand-fold increase in THC solubility using HP-β-CD; Mannila et al. investigated its application in sublingual delivery systems; and more importantly, our own earlier work validated its effectiveness in pain management through intrathecal administration (Agabio et al., 2017b, Jarho et al., 1998, Mannila et al., 2005). However, the impacts of administering THC orally using HP-β-CD on analgesia and other potential therapeutic purposes remain uninvestigated.

1.13. Research gap and aim of the study

Although there is a substantial body of research elucidating the pharmacological effects, metabolism, and therapeutic potential of Δ^9-THC, the exploration of optimized oral delivery modalities that enhance its bioavailability, stability, and therapeutic index remains relatively limited. A number of studies have explored the encapsulation of hydrophobic drugs, including cannabinoids, into cyclodextrin-based carriers to improve their solubility and delivery profiles; however, a critical gap persists specifically with regard to Δ^9-THC/HP-β-CD complexation for oral administration in rodent models. Most extant investigations into cyclodextrin inclusion complexes for lipophilic drugs emphasize alternative administration routes—such as intrathecal or sublingual—or focus on different cyclodextrin derivatives, frequently overlooking the oral route.

A previous study (Agabio et al., 2017) conducted in our Laboratories and aimed at investigating the potential use of HPbCD as a suitable carrier for central administration of delta-9-THC showed that animals who received ICV administration of THC complexed with HPbCD dose-dependently, exhibited significantly increased tail flick latency (by about 30%), indicating effective analgesic effects by the complex. Two ICV doses (30 or 135 µg per rat) were tested and antinociceptive outcomes alongside locomotor activity and body temperature monitored. In particular, rats that received the dose of 135 µg displayed a robust and sustained analgesic effect in the tail-flick test, reduced locomotor activity, and showed significant changes in body temperature (an interesting biphasic effect on body temperature: initial hypothermia followed by hyperthermia) compared with animals in the control group. At variance, animals who received ICV administration of 30 µg of THC complexed with HPbCD did not show significant differences in tail-flick latency or locomotor activity compared with animals in the control group. The antinociceptive effect produced by ICV administration of 135 µg of THC using HPbCD as a carrier was similar to that described in other preclinical studies in which analogous doses of THC were administered centrally using DMSO or cremophor as the solvent [Lichtman and Martin, 1991; Lichtman et al., 1996; Wakley and Craft, 2011]. Moreover, the results of this study also demonstrated that HPbCD is a safe carrier for the administration

of THC in animals; in fact, no adverse or toxic effects were observed under the experimental conditions used.

ICV administration in rats models to some extent intrathecal (IT) administration in humans and, although these preliminary results are of high relevance for the potential use of the complex for pain relief in severe human pathologies through invasive routes of administration, it raises the possibility that the complex could be also used in less invasive ways (e.g., through oral route) for the treatment of pain relief, and other symptoms such as inappetence, that characterize several pathological conditions.

As already discussed extensively in previous chapters, considering the inherent pharmacokinetic and pharmacodynamic complexities of Δ^9-THC, the oral delivery pathway poses notable challenges, including substantial first-pass metabolism, unpredictable absorption, and variable onset of action, which have thus far been addressed only superficially by inclusion complex strategies.

While some studies have demonstrated that HP-β-CD can markedly enhance the aqueous solubility of Δ^9-THC—thereby facilitating potential improvements in oral absorption—these findings remain largely confined to preliminary proofs-of-concept, often performed in vitro or using administration routes that are not physiologically reflective of typical therapeutic use patterns. For instance, formulations employing HP-β-CD for sublingual or parenteral delivery in rodents have provided useful insights into the potential of cyclodextrin complexes to improve the pharmacokinetics of Δ^9-THC. Yet these alternate methods do not replicate the challenges encountered when cannabis-derived compounds traverse the gastrointestinal tract, undergo extensive hepatic metabolism, or encounter diverse enzymatic and transport pathways along the alimentary canal. As a result, their translational value for oral therapeutics remains constrained. Moreover, recent literature, while abundant on the topic of cyclodextrin-mediated solubilization, tends to focus on other active pharmaceutical ingredients rather than Δ^9-THC, whose lipophilicity and sensitivity to oxidative degradation demand tailored complexation strategies. The few investigations that have considered oral HP-β-CD complexes involving cannabinoid compounds often lack the rigorous pharmacokinetic evaluations and comparative controls necessary to explain the mechanistic underpinnings of enhanced

bioavailability or to determine optimal dosing regimens. Comprehensive rodent studies that incorporate proper dose-ranging, multiple time-point sampling, and robust analytical quantifications of both parent drugs and metabolites remain evidently absent.

2. Aims:

2.1. General aims:

Based on these preliminary considerations, the general aim of this study was to bridge this gap. To achieve this, a study in which rats received different doses of the THC-HPbCD complex through the oral route and under an acute or chronic regimen of administration was performed. A multidisciplinary approach, allowing the investigation at different levels of analysis (behavioral, neurochemical, molecular and pharmacokinetics) was used to obtain a clear and possible complete picture of the potential clinical applications and mechanisms of action of the complex when given to stimulate food intake and induce analgesia. Possible adverse events were also evaluated.

2.2. Operative aims:

1. Assessment of pharmacological efficacy and selectivity of the THC-HPbCD complex:

To examine the behavioral effects THC–HPβCD complex on food intake, analgesia, locomotor activity and exploration in female Sprague Dawley rats under acute or chronic treatment regimens with different doses of THC.

2. Assessment of the neurochemical effects of the THC-HPbCD complex:

To evaluate the impact of the THC–HPβCD complex on dopaminergic neurotransmission in brain regions implicated in pain modulation and reward, including the periaqueductal gray (PAG), ventral tegmental area (VTA), nucleus accumbens (Acb), and prefrontal cortex (PFC), through analytical neurochemical techniques.

3. Assessment of the metabolic effects of the THC-HPbCD complex:

To evaluate the impact of the THC–HPβCD complex on peripheral fatty acid (FA) metabolism in the liver, through mass spectrometry techniques.

4. Assessment of the pharmacokinetics properties of the THC-HPbCD complex:

To determine the liver tissue concentrations of THC and its major metabolites (11-OH-THC and 11-COOH-THC) after chronic treatment through mass spectrometry techniques.

3. Materials and Methods

3.1. Chemicals and Reagents

Synthetic Δ9-tetrahydrocannabinol (THC, dronabinol) was acquired from THC Pharm GmbH (Frankfurt, Germany). Hydroxypropyl-β-cyclodextrin (HPβCD) was obtained from Sigma–Aldrich (St. Louis, MO). All other reagents, unless otherwise specified, were of the highest available purity.

3.2. Preparation of Formulations

A concentrated stock solution of THC (200 mg/mL) was made by dissolving 1 g of THC in 5 mL of ethanol at approximately pH 6.8. Subsequent complexation of THC with HPβCD was implemented using a previously described methodology (Agabio et al., 2017a).

By subsequent dilution steps, three final THC/HPβCD complex solutions were prepared at nominal doses of 0.3, 1 and 3 mg/kg. The vehicle solution was an aqueous 22% (w/v) HPβCD solution. All of them were kept equilibrated at 37°C with constant 100 rpm shaking for 24 hours prior to use. Doses used were chosen based on previous literature (see Agabio et al., 2017, and included references; Koch and Matthews, 2001; Kumar et al., 1986; Harris, 1971; Hlozek et al., 2017; Jarbe and DiPatrizio, 2005; Lazzari et al., 2010; Leighty, 1973; Smirnov and Kiyatkin, 2008; Sofia and Barry, 1974; Whitlow et al., 2002; Williams and Kirkham, 2002; Verty et al., 2009; Williams et al. 1998; Moore and Weerts, 2022).

The formulations of THC in aqueous HPβCD were analyzed using Fourier transform infrared (FT-IR) spectroscopy to study intermolecular interactions and molecular integrity. The comprehensive method has been already reported (Agabio et al., 2017a).

3.3. Animals

Female adult Sprague Dawley rats, weighing between 250–300 gr., were used in all the experiments. Upon arrival and for a period of 7 days, rats were acclimated to the environmental conditions of the animal facility before starting the experimental procedures. To minimize stress from handling during the experiments, each rat was daily handled and familiarized with the experimental procedures as specified below. The rats were housed in groups of four per cage with access to food and water ad libitum maintained at 22 ± 2°C, with

60% humidity and a 12-hour light/dark cycle (lights on from 08:00 to 20:00). Additional details about the experimental procedures are reported in the dedicated sections. All experimental procedures were performed in strict accordance with European Directive 2010/63/EU and Italian legislation (D.L. March 4, 2014, no. 26).

3.4. Experimental groups and drug administration

Before starting the treatments, rats were randomly divided into four treatment groups for the experiments on food intake: a control group (aqueous 22% (w/v) HPβCD solution), and three treatment groups (0.3 mg/kg, 1 mg/kg and 3 mg/kg THC in 22% HPβCD). Based on the results obtained in the studies on food intake, the experiments on motor activity and analgesia were instead performed by employing only two THC doses (0.3 mg/kg and 3 mg/kg THC in 22% HPβCD). Rats were treated for consecutive 15 days once a day between 9.00 and 11.00 am and food intake and behavioral assessments were performed at specific timepoints as specified below.

For oral gavage, rats were restrained gently to minimize stress, and a flexible gavage propylene needle (18 gauge) (Instech Laboratories, Inc.) was used to administer the solution directly into the gastric cavity. The rats were acclimated to handling and the gavage procedure over a period of prior to the start of the experiments to reduce stress and ensure consistent administration. The gavage needle was carefully inserted into the esophagus and guided into the stomach. The THC formulations in aqueous HPβCD or vehicle alone (3 ml/kg) were administered slowly to avoid any discomfort or injury. The gavage was performed with precision to ensure that the entire dose was delivered accurately into the gastric cavity without causing regurgitation or aspiration. Each administration session was monitored closely to confirm that the procedure was well-tolerated by the rats.

3.5. Determination of the phase of the estrus cycle

The phase of the estrus cycle was determined by morphological inspection of the vaginal smears collected by lavage, e.g., by inserting a plastic 200 µl pipette with a smooth silicone tip filled with saline solution (NaCl 0.9%) into the rat's vagina to a depth of approximately 2.0 mm. Briefly, the vaginal smear of each female rat was spread over a glass slide, let dry overnight, stained with May- Grunwald-Giemsa colouration, and observed under a phase contrast

microscope, with 10x and 40x objective lenses. The phase of the cycle was identified by the morphological features of the vaginal smear and the presence of the following 3 types of cells: a) round epithelial and nucleated cells, b) irregularly shaped and cornified cells, c) small and dark stained leukocytes. The different ratio among these cells was used to detect the following phases of the estrus cycle: Diestrus: prevalence of leukocytes; Proestrus: prevalence of round, large and nucleated epithelial cells which can be grouped in form of layers; Estrus: prevalence of large, pink stained, and irregular shaped cornified cells; Metestrus: leucocytes, epithelial and cornified cells in equal ratio as previously described (Contini et al., 2018, Robert et al., 2021).

3.6. Food Intake Assessment

Food intake was assessed every day starting after the first gavage administration (acute condition) and for the subsequent 15 days of treatment in order to monitor food intake during the chronic administration regimen. Animals were maintained under ad libitum feeding conditions, with no fasting period prior to the experiments, though food was removed in the 30 min before treatment to avoid differences in gastric filling due to food intake just before the treatment. In order to perform a precise assessment of food intake, all animals were individually housed in cages equipped with standard food and water dispensers. A precision scale with a sensitivity of 0.01 g was used to measure food consumption accurately. Food was weighed immediately before and at designated time points after drug or vehicle administration. For the acute assessment, food intake was measured at 60 minutes and 120 minutes post-treatment. Chronic food intake was evaluated daily over a 15-day treatment period, with measurements conducted at the same time points and also considering the total daily 24-hour intake. Body weights were also monitored daily before and throughout the 15-day chronic treatment period and expressed as a percentage of the baseline weight recorded on the last three days prior to the initiation of treatment (set as 100%).

3.7. Locomotor Activity

Locomotor activity was measured as already described (Angioni et al., 2016). Before the beginning of the experiments, rats were daily handled for at least one week to avoid stress due to manipulation during the experimental sessions. At the end of this period, each rat

underwent one habituation session that lasted for 1 hour in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions. On the day of the experiment, rats were transported from their home cages to the experimental room for a 20-minute habituation and thereafter treatments were performed. Rats were individually tested for motor activity under standardized environmental conditions (in a soundproof room with a light level of 30 lux) with a Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, Ohio). Each cage (42 cm x 42 cm x 63 cm) had two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor and a further set of 16 horizontal beams whose height was adapted to the size of the animals (20 cm). Horizontal and vertical activities were measured as total number of sequential infrared beam breaks (counts) in the horizontal or vertical sensors, recorded every 5 minutes, beginning immediately after placing the animals into the cage, over a test period of 15 minutes (Fig 8), while centre time indicated the time in seconds the rat passed in the central part of the cage, recorded at the same time frames. Locomotor activity was assessed 60 and 120 min after the first and the fifteenth (i.e., last one) drug administration.

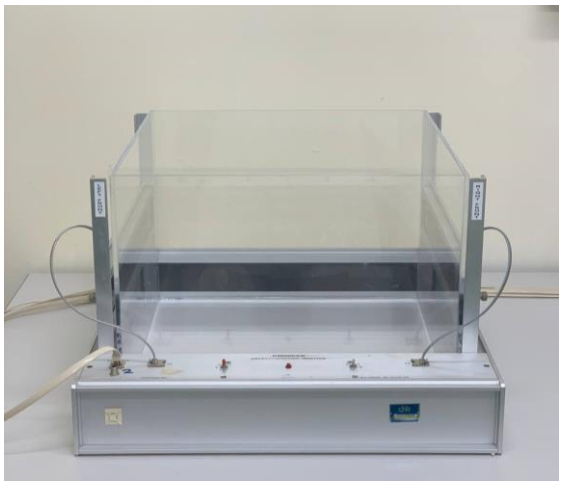


Figure 8. Locomotor Activity chamber. Image depicts the locomotor activity monitoring system used to measure spontaneous movement in rats. The apparatus includes a transparent chamber equipped with infrared beam sensors on two axes (horizontal and vertical) to record animal movement patterns. Rats are placed individually in the chamber during the testing phase, and locomotor activity is quantified based on the number of beam breaks detected by the sensors over a designated time period.

3.8. Tail Flick Test

The tail-flick latency was measured to assess the antinociceptive effects of the administered THC formulations (see Agabio et al., 2017). The day of the experiment rats were transported from their home cages to the experimental room for a 20-minute habituation period before treatment. Thereafter tail flick response was measured. At 30, 60, and 120 minutes after treatment. Tail flick response was assessed after the first treatment (acute response) as well as at the end of the fifteen days of treatment (chronic response).

The tail flick test is a method employed to evaluate antinociception in rats. During this procedure, the rat is placed on a specialized apparatus (TSE Systems, Bad Homburg, Germany). A focused beam of light, heated to 56°C, is projected onto the rat's tail, specifically targeting a point approximately 6 cm from the tip. This exposure to radiant heat prompts a tail flick response, which is the rapid withdrawal of the tail from the heat source. The duration between the onset of heat exposure and the tail-flick response is measured and recorded as the "tail flick latency". To ensure precision and to minimize the risk of tissue damage, the exposure is capped at a cut-off time of 20 seconds. The latency to tail withdrawal is carefully recorded for each rat, with the final reported value being the average of two consecutive measurements taken two minutes apart. This protocol ensures that the assessment of pain response is both accurate and humane.

Baseline latency was determined for each rat before treatment to account for individual variability. Following treatment, tail flick responses were recorded at multiple time points post-administration (30, 60, and 120 minutes). The percentage of maximum possible effect (%MPE) was calculated using the formula: %MPE = (experimental latency - mean baseline latency)/(cutoff latency -mean baseline latency)*100. All %MPE values were statistically analyzed to compare treatment effects over time, as detailed in the Statistics section (Fig. 9).

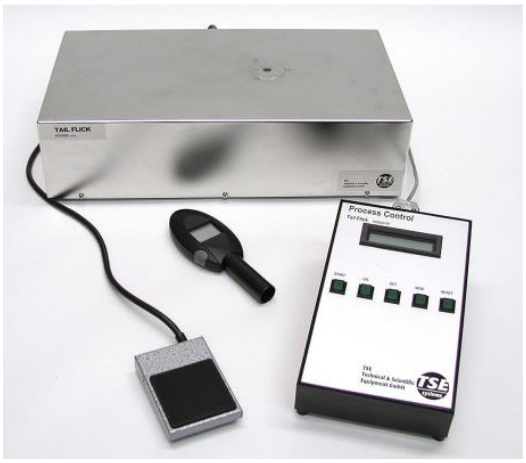


Figure 9. The tail flick test apparatus shown here consists of a heat source, a process control unit, and a foot pedal for precision measurement. The heat source delivers a focused beam of radiant heat to a specific point on the rat's tail, and latency to tail withdrawal (flick) is recorded as a measure of antinociception. The control unit allows precise timing of heat application, while the foot pedal ensures consistent operation. This setup ensures an accurate and humane assessment of pain sensitivity.

3.9. Tissue Collection and processing

Twenty-four hours after the last drug administration rats were sacrificed by decapitation. Immediately after decapitation, the brains were quickly extracted, rinsed and positioned in a rat brain matrix (Fig. 10).

Coronal brain slices of 2 mm as per Paxinos and Watson (Paxinos and Franklin, 2019), were made using a lancet. Subsequently, the regions containing the nucleus accumbens (Acb), prefrontal cortex (PFC), ventral tegmental area (VTA), and periaqueductal grey (PAG), were extracted through the micropunching technique, as previously described in Bharatiya et al. (Bharatiya et al., 2020)(Fig. 10). The extracted tissues were placed in 1.5 ml Eppendorf tubes, weighed, and stored at -80 °C for future analysis. For the homogenization process, tissue punches were sonicated in 0.1 M perchloric acid (1 mg of wet tissue per 20 µl of 0.1 M $HClO_4$), and then centrifuged at 23,000 g for 30 minutes using an Eppendorf 5424R centrifuge (Fisher Scientific, Illkirch, France). Consequently, the supernatant was filtered through microspin centrifuge tubes equipped with a 0.22-µm nylon filter at 10,000 g for 10 minutes and stored at -80 °C until the day of chromatographic analysis.

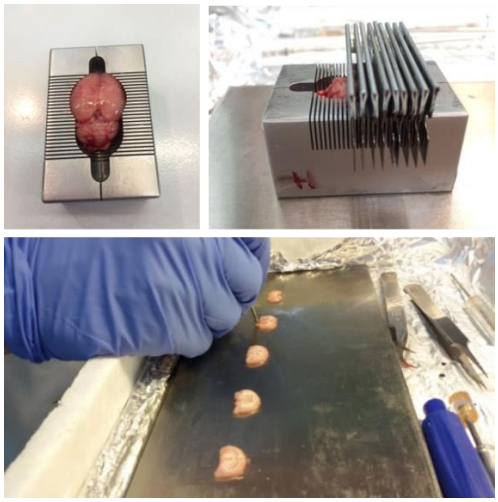


Figure 10. Brain Slicing and Punching Procedure. This series of images illustrates the method for extracting specific brain regions from rat brain tissue for biochemical analysis: Top Left: A freshly extracted rat brain is carefully positioned within a rat brain matrix, a precision stainless steel tool designed to facilitate uniform slicing of brain tissue. The brain matrix features parallel grooves spaced at regular 2-mm intervals, ensuring standardized coronal sectioning. Proper alignment of the brain within the matrix is critical to achieve accurate and reproducible brain slices. Top Right: The brain matrix with integrated blades shows the slicing process in action. A set of evenly spaced blades is inserted into the grooves, enabling the simultaneous sectioning of the brain into multiple coronal slices. This method ensures high consistency in slice thickness (2 mm), which is essential for downstream region-specific dissections. Care is taken to avoid tissue deformation or excessive handling during the slicing process. Bottom: Following sectioning, the coronal slices are laid out on a flat, chilled metal surface to maintain tissue integrity. A fine-tipped instrument (2.5-mm diameter tissue punch) is then used to extract the nucleus accumbens (Acb), prefrontal cortex (PFC), ventral tegmental area (VTA) and periaqueductal gray (PAG) region. Dissections are performed with precision to avoid contamination from adjacent brain regions. The punched tissue samples are immediately collected into labeled tubes, weighed, and stored at -80°C to preserve their biochemical composition for subsequent analysis (e.g., HPLC).

3.10. Chromatographic analyses on brain tissues

Dopamine was measured by injecting a 20 µL aliquot of the supernatant obtained from homogenates by using HPLC coupled to electrochemical detection using a 4011- dual cell (Coulochem II, ESA, Cambridge, MA, USA). Detection was performed in reduction mode at +350 and −180 mV. The HPLC was equipped with a Supelcosil C18 column (7.5 cm ×3.0 mm i.d., 3 µm particle size; Supelco, Supelchem, Milan, Italy), eluted with 0.06 M citrate/acetate pH 4.2, containing methanol 20 % v/v, 0.1 mM EDTA (ethylenediaminetetraacetic acid), 1 µM triethylamine, and 0.03 mM sodium dodecyl sulfate as a mobile phase, at a flow rate of 0.6 mL/min and room temperature. The sensitivity of the assay was 0.125 pg.

3.11. Fatty acid metabolism analyses in the liver

Lipids were extracted from liver samples using a modified Folch method [Folch, J.; Lees, M.; Sloane Stanley, G.H. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 1957, 226, 497–509].

Total lipid quantification was performed by the method of Chiang. Aliquots of the lipid fraction were mildly saponified in order to obtain free FAs (FFA) for High-Performance Liquid Chromatograph (HPLC) and Gas Chromatograph (GC) analysis. The separation and identification of UFA was carried out using an Agilent 1100 HPLC System (Agilent, Palo Alto, CA, USA) equipped with a diode array detector (DAD). SFAs were measured as FA methyl esters (FAME) by a GC (Agilent, Model 6890, Palo Alto) equipped with a flame ionization detector (FID).

Beyond simply metabolizing THC, the liver is a major site for the synthesis and breakdown of fatty acids, including those that serve as precursors for endocannabinoids (e.g., anandamide and 2-AG). By assessing fatty acid composition or metabolic pathways in the liver, we can detect changes that might influence or reflect endocannabinoid tone systemically.

Many endocannabinoids derive from arachidonic acid, an omega-6 fatty acid. Any significant alterations in hepatic fatty acid profiles—whether due to chronic THC exposure or dietary changes—could ultimately shift the balance of endocannabinoid production and breakdown. This, in turn, can impact signaling in the brain and peripheral tissues. Because feeding behavior, energy balance, and analgesia can all be influenced by endocannabinoid signaling, measuring changes in the liver's lipid or fatty acid metabolism offers a peripheral window into how chronic THC treatment might be reconfiguring the overall homeostatic environment. For instance, if chronic THC shifts the balance of hepatic lipids, it may feed back into the ECS (through newly synthesized or degraded endocannabinoids), potentially shaping long-term adaptations in behavior and physiology.

By examining both brain-specific neurotransmitter changes and liver metabolic profiles, we were aiming for a more holistic view of THC's systemic impact. The endocannabinoid system links many peripheral signals (e.g., energy stores, and immune responses) with central circuits (e.g., feeding and pain pathways). Thus, picking apart how THC alters fatty acid

metabolism in the liver may help explain or predict certain neural or behavioral adaptations observed in the brain data.

3.12. THC and metabolite analyses in the liver

An aliquot of lipid fraction was used to analyze delta9-THC and its metabolites (11-hydroxy-delta9-THC and 11-NOR-9-Carboxy-delta9-THC) and their quantification was carried out using an Agilent 1260 UHPLC system (Agilent, Palo Alto, CA, USA) equipped with a mass spectrometry Agilent Technologies QQQ triple quadrupole 6420 with ESI source, using positive mode (ESI+). A Poroshell 120 EC-C-18 column (Agilent, Palo Alto, CA, USA) with 2.7 µm particle size and 3 × 100 mm was used with a mobile phase of CH3OH/H2O/CHOOH (80/20/0.1, v/v/v) at a flow rate of 0.5 mL/min. N2 was used as a nebulizing gas with a pressure of 50 psig, a drying gas temperature of 300 ◦C, a flow of 11 L/min, and 4000 V capillary voltage.

The internal deuterated standard for quantification of THC and its metabolites by isotope dilution ([2H]3-11-OH-THC) was purchased from Merck Life Science S.r.l.

For each standard, the precursor ion [M+H]+ was determined during a full scan (SCAN) in MS, and subsequently, the obtained product ion (PI) was monitored for each transition in MRM mode in MS/MS. Source parameters, such as cone voltage or fragmentor (CV) and collision energy (CE), were optimized for each MRM transition.

Data acquisition was performed using the MassHunter workstation acquisition software (version B.08.02) and data were analyzed with the MassHunter software for qualitative analysis (version B.08.00 SP1) and quantitative analysis (version B.09.00).

3.13. Statistics

Data are presented as mean values ± SEM of absolute values (e.g., in the case of food intake) or percents (e.g., in the case of the tail flick reflex or body weight) and were analyzed by means of one- or two-way ANOVAs with the treatment as between subjects' factor and the time (i.e., day test or test fraction or timepoint) as within subject's factor. Before performing ANOVA, data sets of each of the different experimental variables were inspected for homogeneity of variances among the experimental groups with the Bartlett's or Levine's test depending on the case. Normality of data distribution was assessed by Shapiro-Wilks test. When ANOVAs revealed statistically significant main effects and/or interactions, pairwise

comparisons were performed by using the Tukey's multicomparison test. In all the other cases, Bonferroni's corrected multiple t tests were performed.

Statistical analyses were all carried out with PRISM, Graph Pad 8 Software (San Diego, USA) with the significance level set at $P < 0.05$.

4. Results

4.1. Influence of the estrus cycle on behavioral, neurochemical and molecular parameters across the treatment groups

Since the literature reports a significant influence of the cyclicity of sexual hormones during the estrus cycle not only in the regulation of sexual behavior but also in several other physiological and behavioral aspects, such as food intake(Bautista et al., 2012) and pain perception (Blanton et al., 2021), as well as a different pharmacological activity of cannabis derivatives in the different estrous phases (Struik et al., 2018), the estrous cycle was monitored in order to determine the exact phase of the cycle at the time of behavioral tests, sampling for pharmacokinetic studies and sampling for ex-vivo analyses of brain areas. This allowed us to perform a posteriori evaluation of any effects of the phase of the estrous cycle on the variables under examination. The results obtained (data not shown) indicated that the different phases of the estrous cycle were randomly distributed in the females across the treatment groups and timepoints for behavioral, neurochemical, molecular and pharmacokinetic assessments, reducing in this way the possibility that hormonal factors may have had biased effects on specific experimental groups or conditions.

4.2. Effect of acute treatment with the complex THC–HPβCD on food intake

As shown in Figure 11 acute treatment with the complex THC–HPβCD dose-dependently stimulated food intake in female rats. The effect was observed within the first hour after treatment, while no significant difference between groups was detected when considering the first two hours after treatment.

Accordingly, two-way ANOVA detected a significant treatment x time interaction [$F(3,37) = 3.46$, $p = 0.026$] and a significant effect of time [$F(1,37) = 68.23$, $p = 0.0001$]. Moreover, Tukey's multicomparison post-hoc test detected a significant difference between vehicle and the doses of 0.3 mg/kg ($p < 0.05$) and 1 mg/kg ($p < 0.01$) in the amount of food eaten within the first hour after the treatment. In contrast, no significant differences were observed between treatment groups when considering the first two hours after treatment, indicating a transient time-dependent effect of THC on acute feeding behavior (Fig. 11).

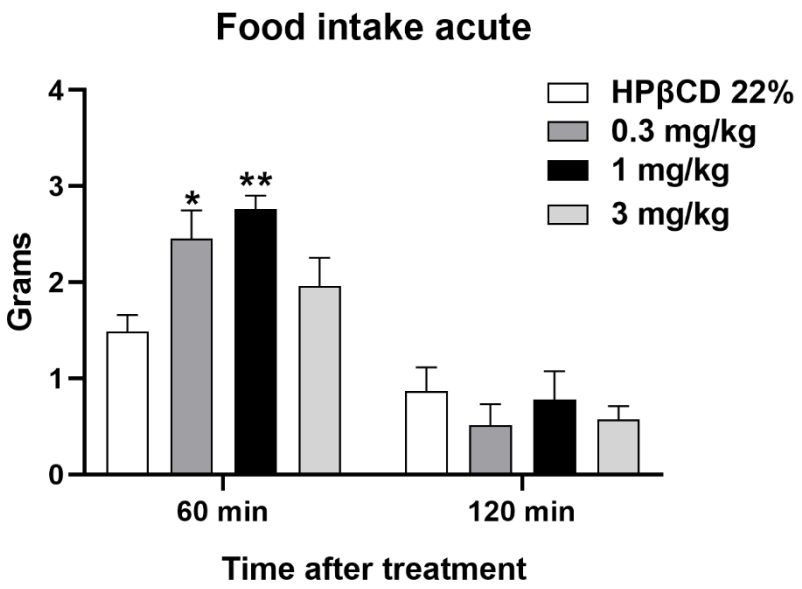


*Figure 11. The graph represents the acute effects of the THC–HPβCD complex administration on food intake at 1 hour and 2 hours post-treatment. *:P < 0.05 and **: P < 0.01 vs vehicle-treated rats, respectively. Two-way ANOVA followed by Tukey's post hoc test. Data are presented as means ± SEM of 6-12 rats/group.*

4.3. Effect of chronic treatment with the complex THC–HPβCD on food intake and body weight

The daily averaged food intake over the 15-day treatment period is shown in Figure 12. Food consumption was analyzed across three intervals: the first hour (0–1 h) (Fig. 12A), the second hour (1–2 h) (Fig. 12B), and the entire 24-hour period (0–24 h) (Fig. 12C).

As regards the 0–1 h interval, one-way ANOVA detected a significant effect of treatment [$F(3, 30.20) = 21.66$, $p < 0.0001$], and post-hoc analyses evidenced highly significant differences between vehicle-treated females and all the THC-treated groups ($P < 0.001$ for 0.3 mg/kg; $P < 0.0001$ for 1 and 3 mg/kg). Similarly to what was seen after acute treatment, the highest intake was observed at the 1 mg/kg dose, suggesting a dose-dependent modulation of feeding behavior in the acute phase.

As regards the 1-2 h interval, no significant differences were observed, indicating that the stimulatory effect of THC on feeding behavior is limited to the first hour.

Finally, the analysis of total food intake over the 24-hour period revealed a trend to decrease in food intake in THC-treated rats, regardless of the dose [$F(3, 52) = 5.21$, $p < 0.0032$]; in particular, post hoc analyses detected a significant reduction in food consumption in animals receiving the 3 mg/kg dose compared to the vehicle group ($P < 0.01$). No significant differences were observed for the 0.3 or 1 mg/kg groups, suggesting a biphasic dose-dependent effect of THC on overall food intake.

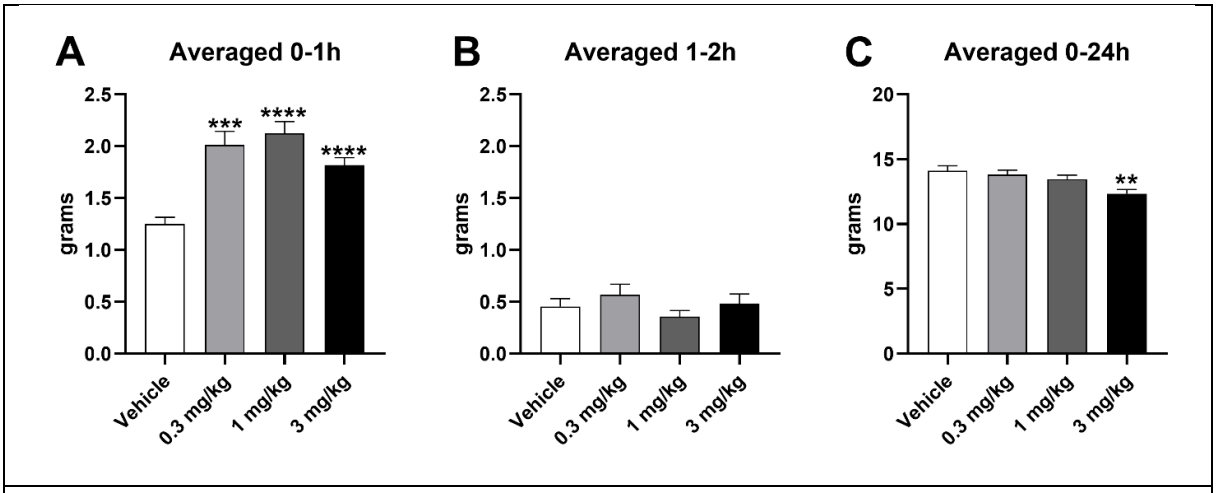


*Figure 12. The panels display the averaged food intake during the 15 days of chronic THC treatment, separated into three intervals: 0–1 hour (A), 1–2 hours (B), and 0–24 hours (C). **: P < 0.01, ***:P < 0.001 and ****: P < 0.0001 compared to vehicle -treated rats. One-way ANOVA followed by Bonferroni's post-hoc test. Data are expressed as means ± SEM of 6 rats/group.*

Over the 15-day of the chronic administration period, the effect of THC–HPβCD treatment on body weight was monitored daily. The results related to this parameter are reported in figure 13 and show that while at the lower dose of 0.3 mg/kg there is a trend towards increasing body weight, at the higher dose of 3 mg/kg rats tend to decrease their body weight. In particular, rats receiving vehicle maintained relatively stable body weight throughout the 15 days, with only minor fluctuations around baseline levels. Rats treated with the 0.3 mg/kg dose exhibited minimal changes in body weight, comparable to the vehicle group, with values remaining close to baseline throughout the study period, though a trend to increase was observed. In the 1 mg/kg treatment group, a slight downward trend in body weight was observed from Day 6 onward, with values stabilizing approximately 3–4% below baseline by Day 15. Rats receiving the 3 mg/kg dose exhibited a progressive and significant

reduction in body weight. By Day 15, body weight dropped to approximately 90% of baseline, with a significant difference compared to the vehicle group.

Accordingly, two-way ANOVA detected a significant treatment x time interaction [F(45, 270) = 1.91, p = 0.0009] and of time [F(15, 270) = 4.07, p = 0.0001]. Moreover, Tukey's multicomparison test evidenced a significant difference between vehicle- and 3 mg/kg treated rats at day 15 ($p < 0.05$) and a significant difference between 0.3 and 3 mg/kg treated rats at days 14 and 15 (both $p < 0.05$).

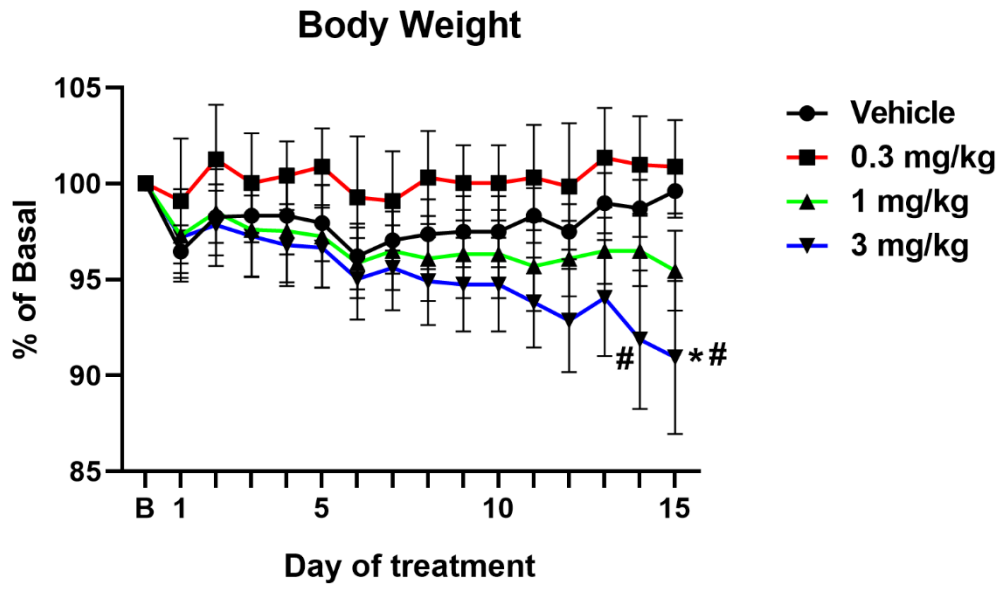


*Figure 13. The graph shows the effects of chronic THC–HPβCD treatment on body weight percentage relative to baseline (Day 0, 100%) over a 15-day period. Rats were treated with vehicle (●), 0.3 mg/kg (■), 1 mg/kg (▲), or 3 mg/kg (▼) doses of THC–HPβCD. *: P < 0.05 vs vehicle-treated rats; #: P < 0.05 0.3 vs 3 mg/kg treated-rats. Two-way ANOVA followed by Tukey's post hoc test . Data are expressed as mean ± SEM of 6 rats/group.*

4.4. Effect of acute and chronic treatment with the complex THC–HPβCD on analgesia

The analgesic effects of the complex were assessed by the Tail flick test (for details, see Material and Methods Section) both after the first (acute) and the fifteen (chronic) days of treatment.

As shown in Figure 14, results from acute treatment showed that while acute administration of the lower dose of 0.3 mg/kg was ineffective, that of 3 mg/kg THC induced

an increase in pain latency at both 30 and 60 minutes, as indicated by the Maximum Possible Effect (%MPE) (Fig. 14). Accordingly, two-way ANOVA revealed a significant effect of treatment ($F(2, 26) = 11.90$, $P < 0.002$; time, $F(2, 52) = 19.45$, $P < 0.0001$; and treatment × time interaction, $F(4, 52) = 11.21$, $P < 0.0001$). Moreover, Tukey's post hoc comparison revealed highly significant differences between 3 mg/kg and vehicle-treated rats both at 30 and 60 minutes after the treatment, although in a time-dependent manner, with a tendency of the analgesic effect to decrease and disappear over time (at 120 min).

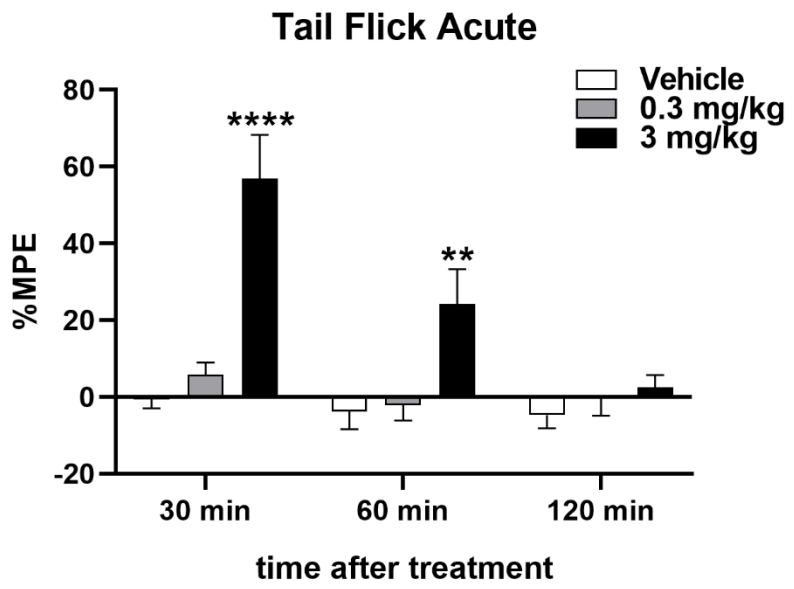


*Figure 14. Graph representing the analgesic effects of acute treatment with the THC-HPβCD complex. Rats were administered either a vehicle (22% w/v HPβCD) or formulations containing 0.3 mg/kg or 3 mg/kg of THC complexed with HPβCD via intragastric route. The tail flick response was measured at 30, 60, and 120 minutes post-treatment. The results, expressed as means ± SEM (n=9–10 rats per group), were statistically analyzed using two-way ANOVA followed by Tukey's post hoc tests. **P < 0.01, and ****P < 0.0001 compared to the vehicle-treated rats. %MPE (percentage of maximum possible effect, calculated as ([experimental latency – mean baseline latency]/[cut-off latency – mean baseline latency]) × 100).*

Furthermore, as shown in figure 15, a significant decrease in pain perception was also detected after the 15-day treatment period with the dose of 3 mg/kg, although with some differences in comparison with the acute effects; in particular, a lower analgesic power, as indicated by the lower MPE values and a longer duration of the effect, as indicated by the significance of the effects observed at 120 min after treatment, were observed in the chronic condition. Accordingly, two-way ANOVA revealed a significant effect of treatment ($F(2, 26) =$

9.25, P < 0.0009 and time, F(2, 52) = 3.41, P < 0.04, and Tukey's post hoc comparisons revealed significant differences of the dose of 3 mg/kg when compared to vehicle at 30, 60 and 120 min (P < 0.01-0.001).

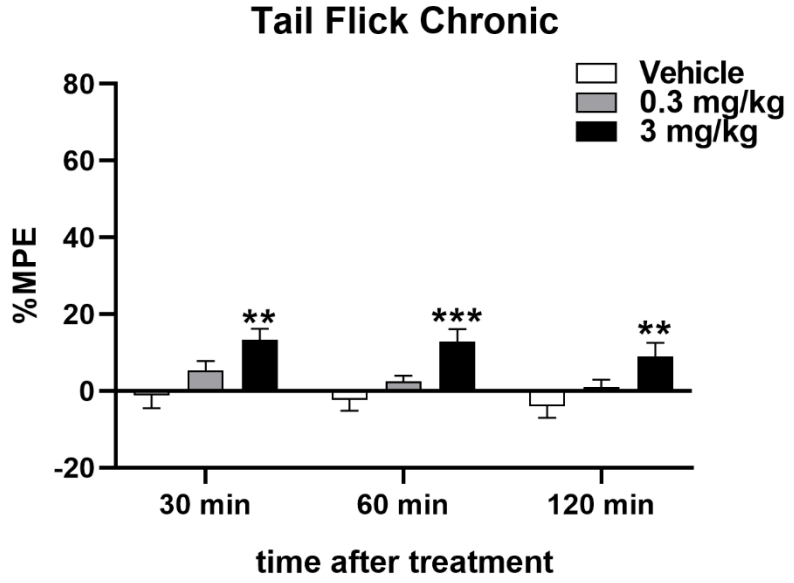


*Figure 15. Graph representing the analgesic effects of chronic treatment with the THC-HPβCD complex. Rats were administered either a vehicle (22% w/v HPβCD) or formulations containing 0.3 mg/kg or 3 mg/kg of THC complexed with HPβCD via intragastric route. The tail flick response was measured at 30, 60, and 120 minutes post-treatment. The results, expressed as means ± SEM (n=9–10 rats per group), were statistically analyzed using two-way ANOVA followed by Tukey's post hoc tests. **P < 0.01, and ***P < 0.001 compared to the vehicle-treated rats. %MPE (percentage of maximum possible effect, calculated as ([experimental latency – mean baseline latency]/[cut-off latency – mean baseline latency]) × 100).*

4.5. Effect of acute and chronic treatment with the complex THC–HPβCD on locomotor activity

As shown in Fig. 16, acute treatment with the THC-HPβCD complex resulted in a dose-dependent decrease in both horizontal and vertical locomotor activities at 60- and 120-minutes post-treatment that became particularly evident with the dose of 3 mg/kg. Accordingly, in horizontal activity two-way ANOVA revealed significant effects of time [F(1,15) = 4.561, p < 0.05] and treatment [F(2,15) = 23.20, p < 0.0001], with a significant time × treatment interaction [F(2,15) = 4.008, p < 0.05] and Tukey's multicomparison post hoc test displayed significant reductions of locomotor activity at 60 and 120 min after treatment (p < 0.0001 and p < 0.01, respectively). Similar results were also obtained for vertical activity, with

a significant effect of treatment [F (2, 15) = 11.66, p < 0.0009] and time × treatment interaction [F (2, 15) = 8.814, 0.0029] in two-way ANOVA. Post hoc Tukey's multicomparison also indicated significant reductions at 60 min after treatment (p < 0.0001). Finally, as regards the time spent in the centre of the arena, no significant differences were observed between treatment groups, although a pronounced trend to decrease was observed in rats treated with the THC dose of 3 mg/kg at both 60 and 120 min after the treatment.

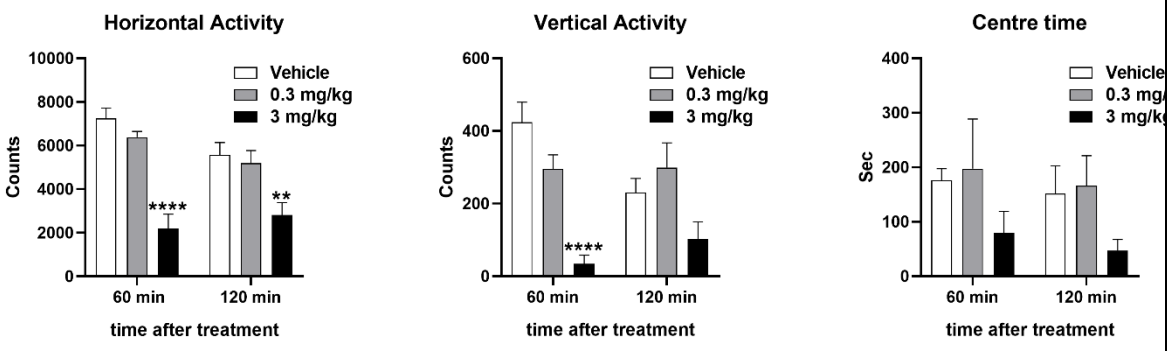


*Figure 16. Graphs depicting dose-dependent reductions in horizontal and vertical locomotor activities following acute THC treatment. Measurements were taken at 60- and 120-minutes post-treatment. ****: P < 0.0001 and **: P < 0.01 compared to vehicle-treated rats. Two-way ANOVA followed by Tukey's or Bonferroni's post hoc test Data are presented as means ± SEM of 9-10 rats/group.*

Similarly, as depicted in figure 17, the 15-day chronic treatment, led to a significant reduction in horizontal and vertical activity and in the time spent in the center of the arena at the dose of 3 mg/kg, being the lower dose of 0.3 mg/kg ineffective. However, in this case, the effects observed were of lower intensity, suggesting that some degree of tolerance developed to the suppressing effects on locomotor activity as consequence of the chronic treatment.

Accordingly, two-way ANOVA detected the following significances: horizontal activity [time: F (1, 26) = 112.8, p < 0.0001]; vertical activity [time: F (1, 26) = 101.0, p < 0.0001; treatment F (2, 26) = 6.41, p < 0.0054]; center time [time: F (1, 26) = 36.55, p < 0.0001; treatment F (2, 26) = 6.52, p < 0.0051].

Moreover, Bonferroni's post hoc comparisons detected a significant difference between vehicle and 3 mg/kg treated rats in vertical activity at both 60 and 120 min (both p < 0.05) and

a significant decrease in the time spent in the centre of the arena in rats treated with 3 mg/kg THC compared to vehicles in the first 60 min after treatment ($p < 0.05$).

In general, these findings indicate that both acute and chronic administrations of 3 mg/kg result in decreased locomotor activity, suggesting a consistent suppressing effect on movement and exploratory behavior across different treatment durations and depending on the specific parameter considered.

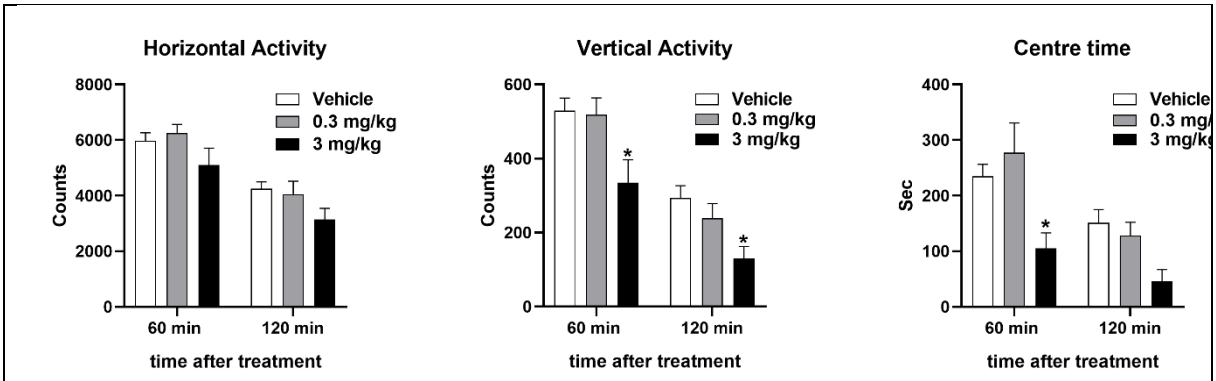


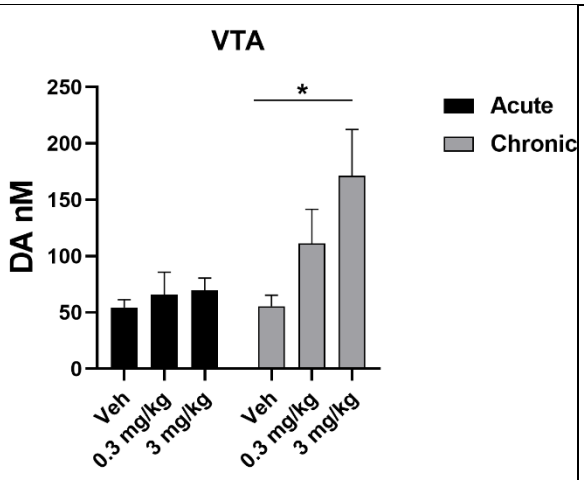
*Figure 17 Graphs depicting dose-dependent reductions in horizontal and vertical locomotor activities following chronic THC treatment. Measurements were taken at 60- and 120-minutes post-treatment. ****: P < 0.0001 and **: P < 0.01 compared to vehicle-treated rats. Two-way ANOVA followed by Tukey's or Bonferroni's post hoc test Data are presented as means ± SEM of 9-10 rats/group.*

4.6. Effect of acute and chronic treatment with the complex THC–HPβCD on tissue dopamine in the VTA, nucleus accumbens, prefrontal cortex and periaqueductal gray

The VTA, a key structure in the mesolimbic reward pathway, exhibited distinct dopamine (DA) tissue concentrations after acute and chronic THC treatment (Fig. 18).

As shown in figure 18, acute administration of THC complexed with HPβCD (0.3 mg/kg and 3 mg/kg) did not significantly alter dopamine (DA) levels in the VTA compared to the vehicle-treated group suggesting that acute THC administration, at the given doses, does not substantially affect dopaminergic activity in the VTA. In contrast, chronic administration of the THC complex dose-dependently elevated DA tissue levels in the VTA compared to the vehicle-treated group. Accordingly, two-way ANOVA showed significant effect of treatment [$F(1, 32) =$

5.66, P = 0.023] and a trend towards significance for the dose [F(1, 32) = 3.23, P = 0.053]. Moreover, Bonferroni's multiple comparisons indicated that DA tissue levels were significantly higher in the chronic 3 mg/kg group compared to the vehicle-treated group (P < 0.05).



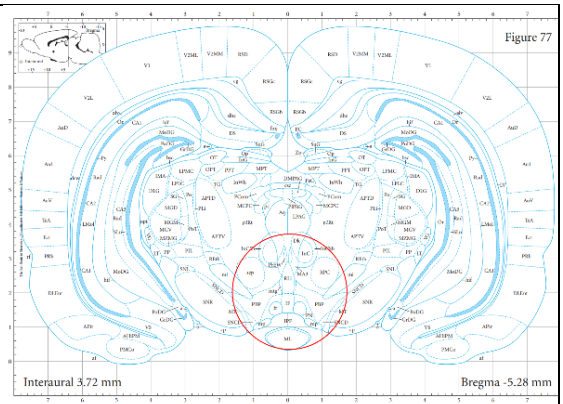
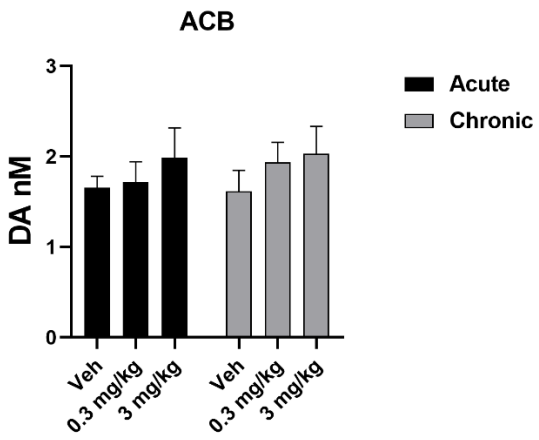


*Figure 18. Graph illustrating dopamine (DA) concentrations in the ventral tegmental area (VTA) tissue following acute and chronic THC treatments (left) and the tissue area containing the VTA collected by micropunching technique (right). *: P < 0.05 compared to the corresponding vehicle-treated group. Data were analyzed using two-way ANOVA with Bonferroni's post hoc comparisons. Data are presented as means ± SEM of 6 rats/group.*

The nucleus accumbens (Acb) represents a primary target of VTA dopamine projections and plays a key role in reward and reinforcement. Surprisingly, as shown in figure 19, THC administration both 0.3 mg/kg and 3 mg/kg doses did not significantly alter DA levels in the Acb under either acute or chronic conditions. Two-way ANOVA indicated no significant main effects or interactions. However, a trend towards higher concentrations in THC-treated rats can be observed both after acute and chronic treatment.



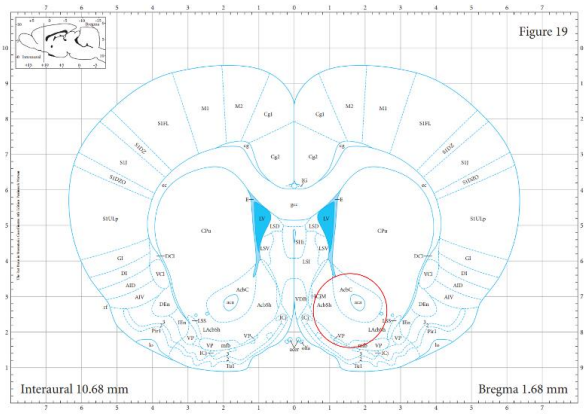
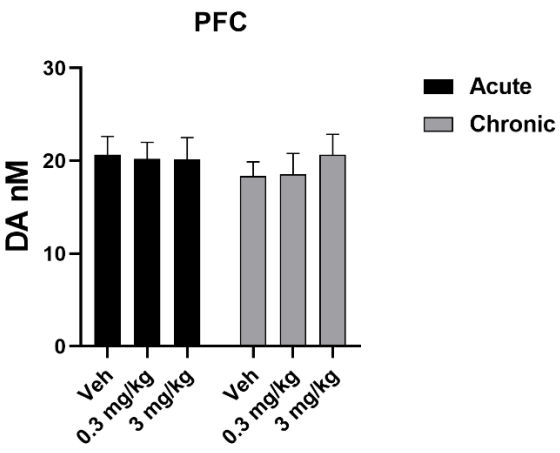


Figure 19. Graph illustrating dopamine (DA) concentrations in the nucleus accumbens (Acb) tissue following acute and chronic THC treatments (left) and the tissue area containing the VTA collected by micropunching technique (right). Data were analysed using two-way ANOVA (not significant). Data are presented as means ± SEM of 6 rats/group.

The prefrontal cortex (PFC) is implicated in executive function, decision-making, and mood regulation. It also receives extensive dopamine projections from the VTA; thus, it was assessed for dopaminergic changes following THC administration. As shown in figure 20, similarly to what observed in the Acb, neither 0.3 mg/kg nor 3 mg/kg doses of THC produced significant changes in DA tissue concentrations in the PFC after acute or chronic treatment.



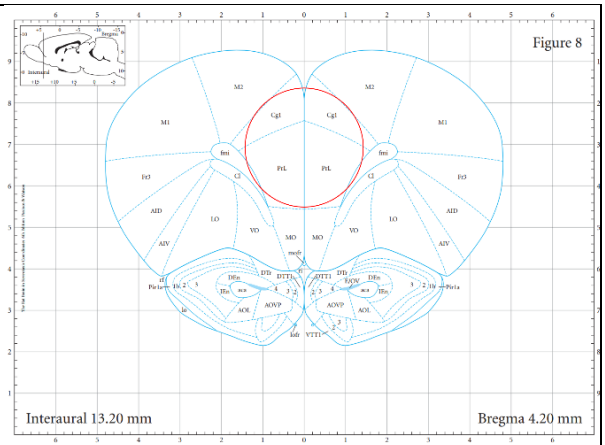
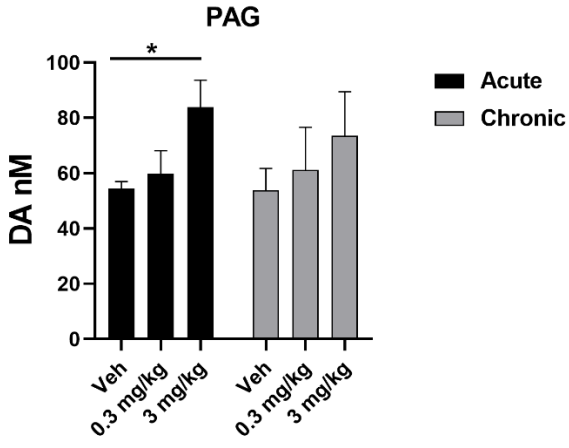


Figure 20 Graph illustrating dopamine (DA) concentrations in prefrontal cortex (PFC) tissue following acute and chronic THC treatments (left) and the tissue area containing the VTA collected by micropunching technique (right). Data were analysed using two-way ANOVA (not significant). Data are presented as means ± SEM of 6 rats/group.

As reported in figure 21, the periaqueductal grey (PAG), a critical brain region for pain modulation and analgesic processing, showed significant changes in dopamine tissue concentrations following acute but not chronic THC treatment (One-way ANOVA [$F(2, 15) = 4.36$, $P < 0.05$]). Accordingly**,** acute administration of THC at 3 mg/kg significantly elevated DA concentrations in the PAG tissue compared to the vehicle group ($P < 0.05$). This effect was dose-dependent, as the lower dose (0.3 mg/kg) did not produce a similar increase.



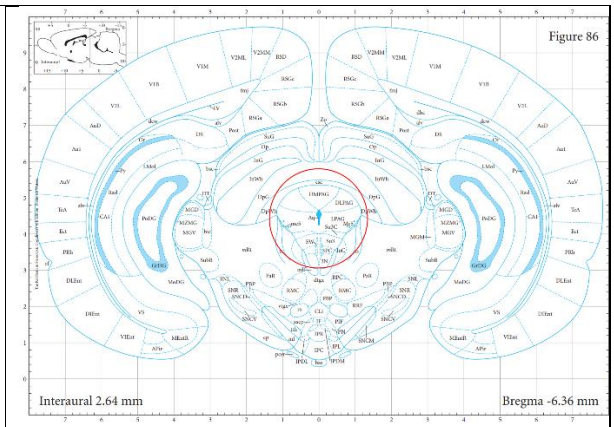


Figure 21. Graph illustrating dopamine (DA) concentrations in the periaqueductal grey (PAG) tissue following acute and chronic THC treatments (left) and the tissue area containing the VTA collected by micropunching technique (right). *: $P < 0.05$ compared to the corresponding vehicle-treated group. Data were analysed using two-way ANOVA with Bonferroni's post hoc comparisons. Data are presented as means ± SEM of 6 rats/group.

4.7. Levels of THC and its primary and secondary metabolites (11-OH-THC and THC-COOH) in liver tissue after chronic treatment

Levels of THC and its primary and secondary metabolites (11-OH-THC and THC-COOH) were quantified using LC-MS/MS. The results indicated that the levels of THC and its metabolites were significantly elevated in rats treated with 3 mg/kg of THC (see Fig. 22A, B, and C).

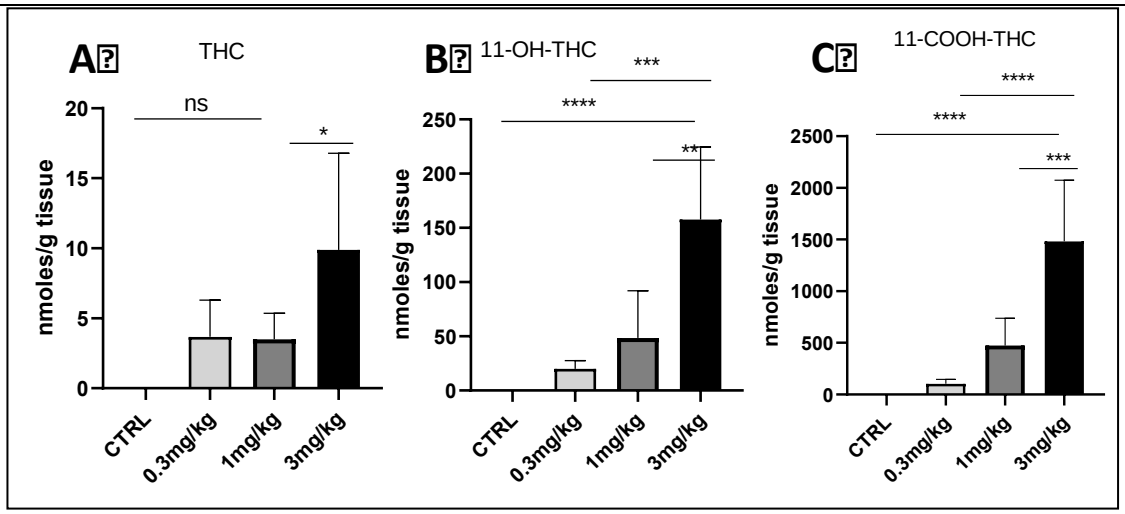


Figure 22*: A) THC B) 11-OH-THC and C) 11-COOH-THC, expressed as nmoles/g tissues, in liver of dams treated with different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg). Values are mean ± S.E.M. of n = 6. *p ≤ 0.05; **p ≤ 0.01.*

Consistent with these findings, a comparison of THC-COOH/THC ratios demonstrated a dose-dependent conversion pattern (Fig. 23A and B). This pattern was also observed in the OH-THC/THC ratio. However, when considering the COOH-THC/OH-THC ratio, a decrease was observed in rats treated with 3 mg/kg of THC, suggesting a reduced biotransformation of OH-THC to COOH-THC (Fig. 23C).

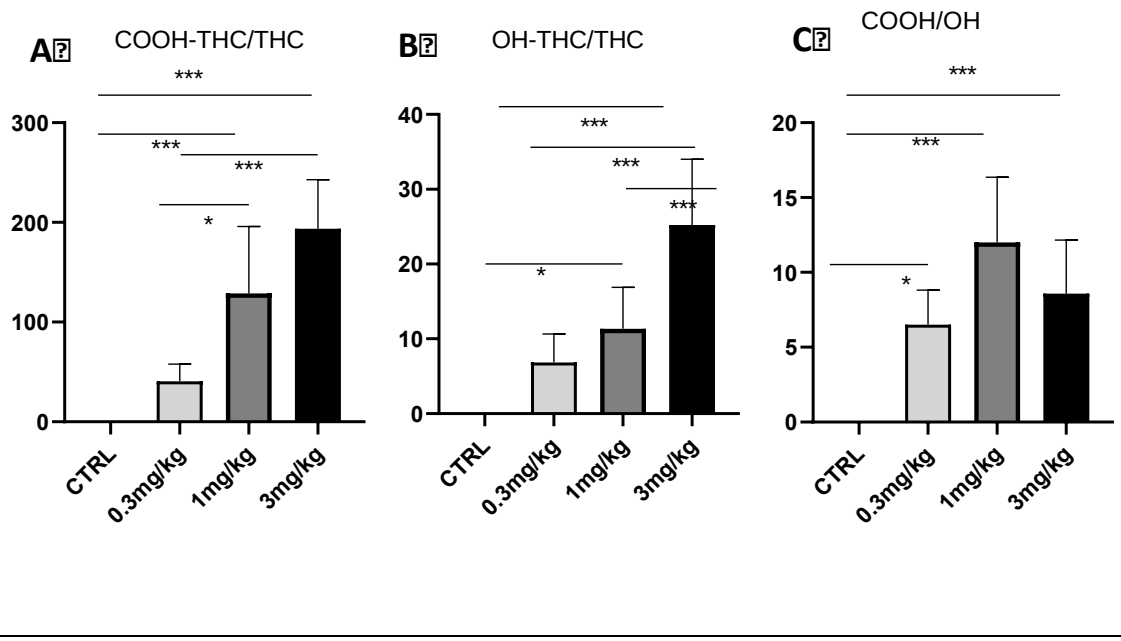


Figure 23: A) COOH-THC/THC ratio B) 11-OH-THC/THC ratio and C) 11-COOH-THC/OH-THC ratio in liver of dams treated with different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg). Values are mean ± S.E.M. of n = 6. *p ≤ 0.05; **p ≤ 0.01.

4.8. Analysis of Fatty Acids and Bioactive Metabolites in Liver

To evaluate the systemic metabolic effects of THC, we analyzed the fatty acid (FA) profile and FA-derived bioactive metabolites belonging to the endocannabinoidome (eCBome) in the liver.

A significant increase in total lipid content (expressed as mg/g tissue) was observed in the liver of rats treated with 1 mg/kg of THC, although this effect was not sustained at the higher dose of 3 mg/kg (Fig. 24A). This increase in total lipids was primarily due to elevated levels of monounsaturated fatty acids (MUFA) (Fig. 24B), which likely resulted from enhanced de novo lipogenesis (DNL) as shown by an increase of a metabolic DNL marker, the ratio between palmitoleic acid (POA), endogenously produced through DNL, and linoleic acid (18:2, LA), derived from the diet (Fig. 24C).

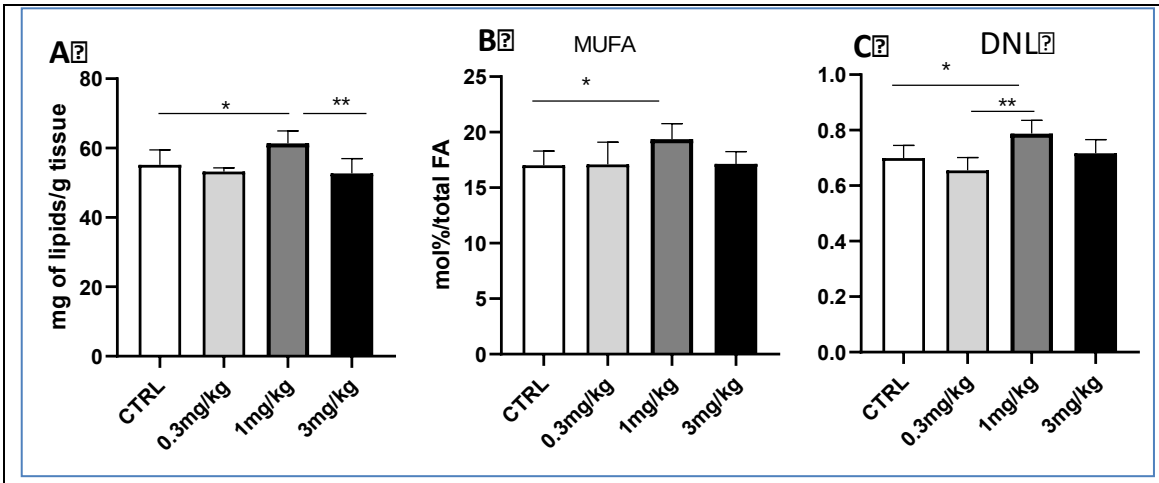


Figure 24: A) total lipids, expressed as mg/g tissue and B) Monounsaturated Fatty Acids (MUFA), expressed as mol% of total FA, and C) metabolic de novo lipogenesis (DNL) marker, calculated as the ratio of MUFA to linoleic acid (18:2, LA) derived from the diet, in liver of dams treated with different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg). Values are mean ± S.E.M. of n = 6. * = $p \leq 0.05$; ** = $p \leq 0.01$.

No significant changes in total hepatic polyunsaturated fatty acids (PUFA) levels were detected between the different treatment groups. However, a more detailed analysis of individual fatty acids showed a reduction in eicosapentaenoic acid (20:5n3, EPA) levels in dams treated with both 1 mg/kg and 3 mg/kg of THC compared to the control group (Figure 24A). EPA is synthesized through enzymatic desaturation and elongation steps from α-linolenic acid (ALA), the precursor present in the diet. The final step in this enzymatic process involves peroxisomal β-oxidation, for the formation of docosahexaenoic acid (22:6n3, DHA) (Figure 25).

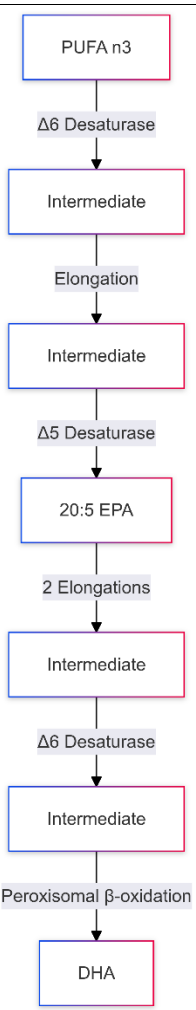


Figure 25 . Schematic representation of n-3PUFA metabolism.

In this study, DHA levels remained unchanged across treatment groups (Figure 26B). However, the DHA/EPA ratio increased in dams treated with 3 mg/kg of THC (Figure 26C), suggesting an enhanced peroxisomal β-oxidation activity (Shang et al. 2017).

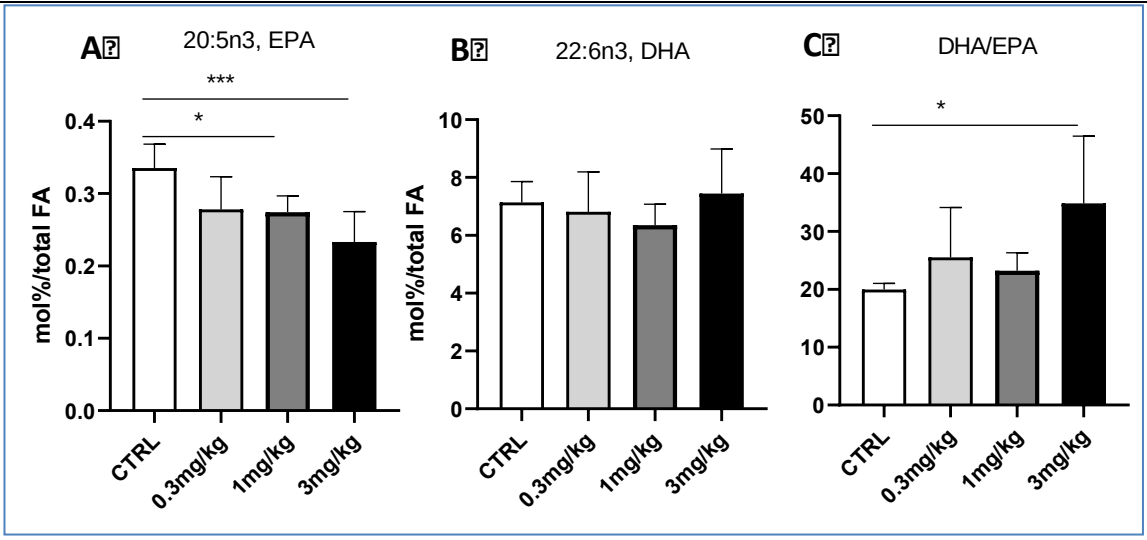


Figure 26: A) eicosapentaenoic acid (20:5n3, EPA), expressed as mol %/total FA and B) docosahexaenoic acid (22:6n3, DHA), and C) DHA/EPA ratio, in the liver of dams treated with different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg). Values are mean ± S.E.M. of n = 6. * = $p \leq 0.05$; ** = $p \leq 0.01$.

Since the observed changes in the liver FA profile could influence the biosynthesis of eCB and related compounds, such as N-acylethanolamine (NAEs) (Murru et al. 2021) we analysed these molecules. Specifically, we observed an increase of NAEs derived from FAs produced through DNL, including N-palmitoylethanolamine (PEA) derived from palmitic acid (16:0) and N-oleoylethanolamine (OEA) derived from oleic acid (18:1) in dams treated with 1 mg/kg of THC (Fig. 27B and C). Additionally, levels of anandamide or N-arachidonoilethanolamine (AEA) derived from arachidonic acid (Fig. 27A) and N-docosaexanoilethanolamine (DHEA) derived from DHA (Fig. 27D) were increased following 1 mg/kg THC treatment.

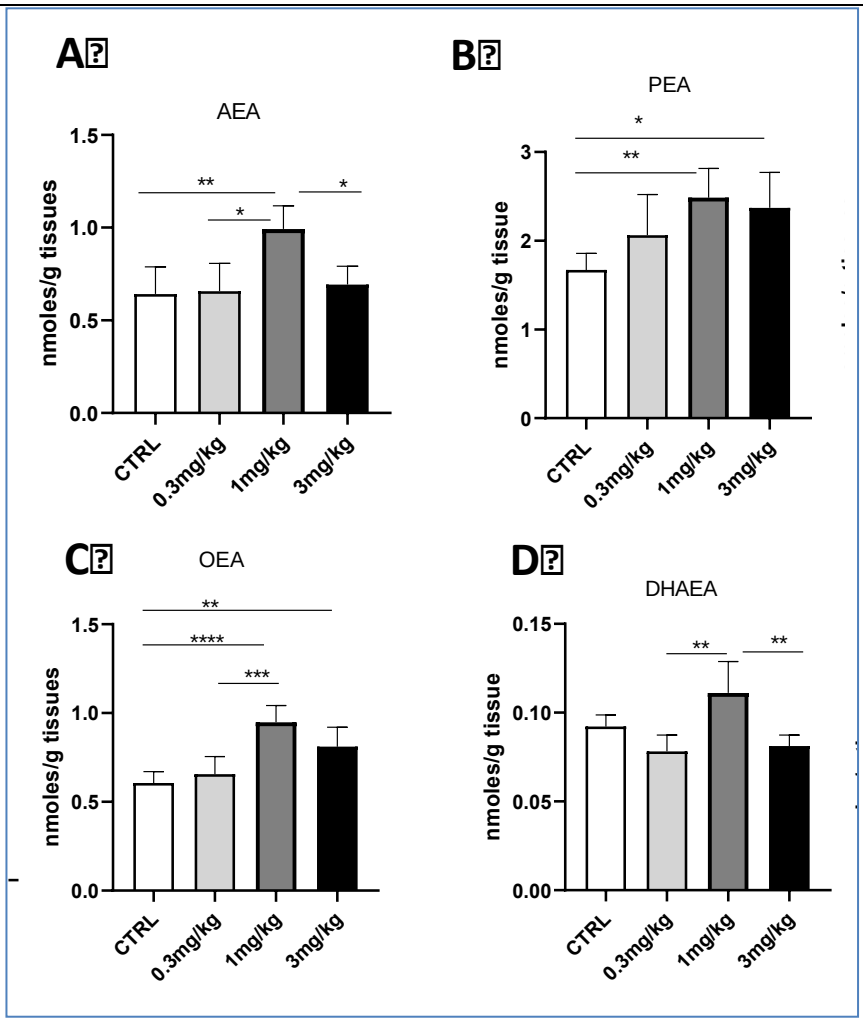


Figure 27: A) N-arachidonoilthanolamine or anandamide (AEA), B) N-palmitoylethanolamine (PEA), C) N-oleoylethanolamine (OEA), and D) N-docosaexanoilethanolamine (DHEA), as nmoles/g tissues, in liver of dams treated with different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg). Values are mean ± S.E.M. of n = 6. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 and ****p ≤ 0.0001.

In contrast, 2-arachidonoilglycerol (2-AG) levels increased with 3 mg/kg of THC treatment (Fig. 28A). These changes resulted in a significantly higher OEA/2-AG ratio, considered a potential index of the balance between endogenous PPARα and endocannabinoid system (ECS) activation, in the group treated with 1mg/kg of THC (Fig. 28B).

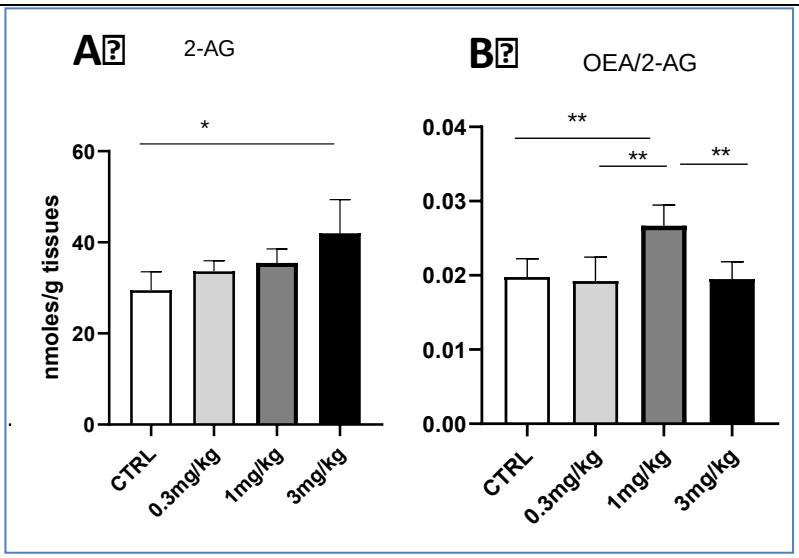


Figure 28*: A) 2-arachidonoilglycerol (2-AG) expressed as nmoles/g tissues, and B) OEA/2-AG ratio in liver of dams treated with different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg). Values are mean ± S.E.M. of n = 6. *p ≤ 0.05; **p ≤ 0.01.*

Considering that PEA and OEA are potent endogenous ligands for PPAR-α, and the observed increase in peroxisomal β-oxidation, as indicated by the elevated DHA/EPA ratio, may be regulated by the peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor that functions as a transcription factor, modulating the expression of numerous target genes involved in lipid, glucose, and aminoacidic metabolism ((Bharatiya et al., 2020)), we further examined whether THC treatment influenced Ppar-α gene expression in the liver. Although no significant differences in PPARα gene expression were observed between the treated groups and the control group (Fig. 29A), a significant positive correlation was detected between PPARα expression levels and the administered THC doses (Fig. 29B).

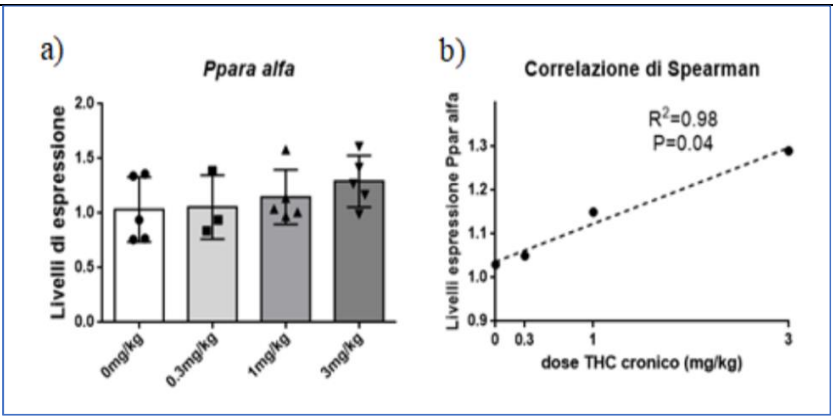


Figure 29: A) mRNA expression levels of peroxisome proliferator-activated receptor α (Pparα), a key regulator in energy and lipid metabolism in liver of dams treated with different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg), and B) Spearman correlation coefficients among the PPARα mRNA expression levels and different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg). Statistical significance $p \leq 0.05$.

Additionally, we found a significant increase of non-esterified fatty acids (FFA) in dams treated with 3mg/kg of THC (Fig. 30).

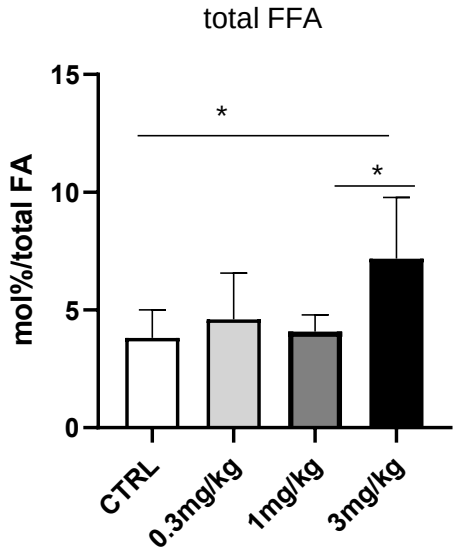


Figure 30: *total non-esterified fatty acids (FFA) expressed mol%/total FA in liver of dams treated with different concentration of THC (0.3mg/kg, 1mg/kg and 3mg/kg). Values are mean ± S.E.M. of n = 6. *p ≤ 0.05.*

5. Discussion:

The central problem addressed in this study is the challenge of achieving effective and consistent delivery of Δ9-THC via oral administration. THC, a highly lipophilic compound, exhibits limited aqueous solubility, which impedes its bioavailability and therapeutic efficacy when delivered through conventional methods. Previous studies have explored various carriers and routes of administration; however, these approaches often fall short due to issues such as poor absorption, rapid metabolism, or suboptimal pharmacokinetics. These limitations highlight the need for innovative delivery systems capable of enhancing the solubility, stability, and bioavailability of THC while ensuring consistent pharmacodynamic effects. Not only the role of HP-β-CD is to enhance the solubility of poorly water-soluble drugs like THC, thereby improving bioavailability and ensuring more effective and consistent dosing, but there are additional benefits that further justify its choice. HP-β-CD is known for its relatively non-toxic profile and is widely used in pharmaceutical formulations, making it a safe option for this research (Ren et al., 2016). It also enhances the stability of drugs, protecting them from degradation and potentially improving their pharmacokinetic profiles (Gould and Scott, 2005). In the context of this study, HP-β-CD likely facilitates the effective delivery of THC to target sites, such as CB1 receptors in the brain, which are crucial for regulating feeding behavior. Additionally, by controlling the release profile of THC, HP-β-CD may help reduce potential side effects associated with peak plasma levels. Overall, the combination of improved solubility, enhanced bioavailability, and controlled drug release makes HP-β-CD an optimal choice for this experimental setup, ensuring consistency and predictability in the pharmacological response

Here, we provide evidence that the oral administration of THC complex with HP-β-CD significantly enhances its pharmacological profile, achieving pronounced analgesic effects and modulating feeding behavior in female Sprague Dawley rats. Acute administration of THC-HP-β-CD led to a marked increase in tail-flick latency, confirming the effectiveness of this complex in producing rapid and robust antinociception. This analgesic effect, while slightly diminished, persisted over a 15-day chronic administration period, suggesting sustained efficacy and the potential for longer-term therapeutic use with minimal tolerance development. In parallel,

both acute and chronic treatment with THC-HP-β-CD consistently decreased horizontal and vertical locomotor activities, reflecting its significant influence on movement and exploratory behavior. Furthermore, the THC–HPβCD complex exhibited notable effects on feeding behavior. Acute administration at lower doses stimulated food intake, indicating a transient orexigenic effect, while chronic administration of higher doses reduced overall food consumption and led to significant weight loss. Neurochemical analyses revealed region-specific dopaminergic responses, with chronic THC–HPβCD administration increasing dopamine levels in the VTA, a key region implicated in reward and motivational pathways. Acute treatment also elevated dopamine concentrations in the PAG, aligning with the observed antinociceptive effects, as the PAG is integral to pain modulation and descending inhibitory pathways. These neurochemical outcomes suggest that the THC–HPβCD complex not only enhances analgesic efficacy but also engages central dopaminergic mechanisms.

The increase in DA levels following chronic administration of THC suggests a cumulative effect of repeated exposure, potentially through modulation of presynaptic CB1 receptors. These receptors are known to inhibit GABAergic inhibition of dopaminergic neurons, leading to enhanced dopaminergic output over time. This sustained increase in DA may underlie the gradual sensitization or neuroadaptations within the mesolimbic reward system often observed with chronic cannabinoid exposure.

As regards the Acb, our findings suggest that THC does not directly modulate dopaminergic neurotransmission in the NAc under the tested conditions, despite its psychoactive properties. This lack of effect might reflect region-specific CB1 receptor densities or downstream regulatory mechanisms that buffer acute and chronic alterations in DA release within the NAc.

The lack of significant changes in PFC DA levels under both acute and chronic THC treatments highlights the region's potential resistance to direct cannabinoid modulation. This could be attributed to the relatively lower CB1 receptor expression in the PFC compared to other brain regions like the VTA or basal ganglia, limiting THC's ability to influence dopaminergic activity in this region.

The observed increase in PAG DA levels aligns with THC's well-established antinociceptive effects. The PAG is a central hub for descending pain modulation pathways, and dopaminergic activity within this region is known to interact with opioid and cannabinoid systems to produce analgesia. THC's activation of CB1 receptors in the PAG likely disinhibits dopaminergic neurons, leading to enhanced DA release and subsequent modulation of pain perception. The absence of chronic effects may suggest a desensitization or downregulation of CB1 receptors within the PAG following repeated THC exposure, a phenomenon commonly observed with chronic cannabinoid administration. This could explain the reduced analgesic efficacy seen during the chronic phase in the tail flick test.

Metabolic processing analyses further supported the advantages of the novel formulation. A dose-dependent increase in liver concentrations of THC and its metabolites, including 11-OH-THC and 11-COOH-THC, was observed, indicating improved absorption and biotransformation efficiency. The elevated metabolite/THC ratios suggest that HP-β-CD facilitates enhanced solubility and bioavailability, optimizing THC's pharmacokinetics for oral administration. Collectively, these findings highlight the THC–HPβCD complex as a superior delivery platform, capable of achieving sustained pharmacological effects, minimizing variability associated with traditional formulations, and providing a more effective and targeted alternative for common oral cannabinoid carriers.

The findings from feeding behavior experiments demonstrate a dose- and time-dependent effect of the THC–HPβCD complex. Consistent with existing literature, our results align with studies indicating that cannabinoids can exert opposing effects on feeding behavior depending on dose and duration of exposure (Farrimond et al., 2012, Tarragon and Moreno, 2019).

In the acute phase, the low-dose group (0.3 mg/kg) demonstrated a significant increase in food intake at the 1-hour mark compared to the vehicle-treated rats, indicating a transient orexigenic effect. Interestingly, this effect was not replicated in the higher dose group (3 mg/kg), which showed food intake levels similar to the control. This suggests a biphasic dose-response relationship, where a low THC dose stimulates feeding behavior, while higher doses may lack this effect, potentially due to aversive or sedative properties that counteract its

orexigenic action. By the 2-hour mark, food intake across all groups converged, reflecting the short-lived nature of THC's acute impact on feeding which could be affected due to cataleptic effect as seen in locomotor activity. Similarly, Nelson et al. observed increased food intake at 3h and reduced weight gain with oral THC and sesame oil as carrier. This was only observed at low doses of 3mg/kg and not >5mg/kg doses (Nelson et al., 2019). These results have been repeated in previous research studies with different carriers at doses ranging from 0.5 to 2mg/kg and various administration routes (Koch, 2001, Glick and Milloy, 1972, Williams et al., 1998a).

In the chronic phase, the biphasic trend became more pronounced. During the 0–1 hour interval, all THC doses (0.3, 1, and 3 mg/kg) significantly increased food consumption compared to the vehicle group, with the 1 mg/kg dose eliciting the most pronounced effect. This supports the hypothesis that moderate doses of THC maximize the orexigenic effect. However, during the 1–2 hour interval, the stimulatory effect diminished across all doses, further emphasizing the transient nature of THC-induced feeding responses. Similarly, Koch et al. did not observe differences in food intake after 6 or 24hr period at low doses (Koch, 2001). Over the 24-hour monitoring period, a clear reduction in cumulative food intake was observed in the 3 mg/kg group compared to the vehicle group, suggesting that prolonged exposure to higher THC doses may suppress appetite over time. The long-lasting effects of oral THC is also evident in cannabinoid tetrad as shown by Moore et al (Moore and Weerts, 2022).

The biphasic effect observed during chronic treatment—increased feeding at 0.3 and 1 mg/kg but reduced cumulative intake at 3 mg/kg—aligns with existing literature on cannabinoid pharmacodynamics. Previous studies have shown that low to moderate doses of THC stimulate feeding, while higher doses can suppress appetite, likely due to CB1 receptor desensitization or the sedative effects of THC (Anderson-Baker et al., 1979, Koch and Matthews, 2001). The ability of the HPβCD formulation to achieve similar dose-dependent effects emphasizes its viability as a career and its potential to optimize oral THC delivery. The weight loss observed in the 3 mg/kg group suggests that prolonged high-dose THC administration may interfere with energy homeostasis, potentially through altered metabolic processes or suppression of feeding motivation (Cota et al., 2003). Body weight changes over

the 15-day chronic treatment period mirrored the trends in food intake. The 3 mg/kg dose caused a significant and progressive reduction in body weight, reaching approximately 90% of baseline levels by Day 15. Previous studies show the same effect on weight in rodents (Rahminiwati and Nishimura, 1999, Elsmore and Manning, 1974, Le Foll et al., 2013). This suggests that the chronic administration of high-dose THC not only reduces overall food intake but also impacts energy homeostasis, possibly through alterations in metabolic pathways or reduced feeding motivation. In contrast, the 0.3 mg/kg group maintained stable body weights comparable to the vehicle group, while the 1 mg/kg group exhibited a mild but non-significant decline in body weight. These results indicate that the effects of THC on body weight are dose-dependent, with higher doses exerting catabolic effects that may counteract its short-term orexigenic properties. This response could be attributed to the differential modulation of CB1 receptors by THC–HPβCD. At low doses, THC likely enhances CB1 signaling, promoting feeding. In contrast, high doses may lead to receptor desensitization or activation of downstream inhibitory pathways, resulting in reduced food intake and weight loss. Additionally, the enhanced bioavailability of THC due to HPβCD might amplify these effects, potentially explaining the pronounced weight loss in the high-dose group.

The orexigenic effect observed at low doses is reminiscent of findings showing CB1 receptor-mediated stimulation of feeding (Tarragon and Moreno, 2019, Kirkham, 2005)). However, the suppression of appetite at high doses suggests a mechanism involving receptor desensitization or alternative pathways, as previously hypothesized (Lau et al., 2017). This effect is likely facilitated through CB1 cannabinoid receptors located in the paraventricular nucleus (PVN) of the hypothalamus, as administration of THC directly into the PVN enhances feeding behavior—a response that can be mitigated by the use of a CB1 receptor antagonist (Verty et al., 2005). The THC induced eating behavior retains the normal species-typical sequence, suggesting that cannabinoids enhance the incentive value of food and support the role for endocannabinoids in the regulation of the appetitive aspects of feeding motivation (Williams and Kirkham, 2002). Additionally, dopamine D1 receptor signaling appears to be necessary for THC-induced feeding, as the dopamine D1-like receptor antagonist SCH 23390 suppresses THC-induced hyperphagia (Verty et al., 2004). This could explain the observed

increase in DA levels in VTA after chronic treatment. Previous studies have reported that THC stimulates appetite through CB1 receptor activation in hypothalamic and mesolimbic pathways (Koch, 2017). Kruse et al. (2019) argue that long-term changes in CBR1 expression within the VTA following oral THC administration exhibit sex-dependent differences, with male rats displaying greater impairments.(Kruse et al., 2019). Similarly, Chen et al. (1993) observed that microinjection of Δ9-THC into the VTA elevated somatodendritic dopamine levels within the VTA but did not alter dopamine concentrations in the NAc, aligning with findings from our study.(Chen et al., 1993). Regarding PFC, studies show both an increase and decrease of DA in this region. These differences could be both due to sex-specific effects, or differences in experimental methods (Pistis et al., 2002, Verrico et al., 2003). The ability of the THC–HPβCD complex to replicate this effect at low doses suggests that HPβCD improves THC absorption, enabling rapid and effective engagement of CB1 receptors. This finding contrasts with conventional oral THC formulations, which typically exhibit delayed and inconsistent bioavailability due to extensive first-pass metabolism (Grotenhermen, 2003).

An unexpected finding was the significant reduction in food intake in the high-dose (3 mg/kg) group during chronic treatment, this reduction was also evident in body weight. While previous studies have noted that high-dose THC can suppress appetite (Verty et al., 2005), the progressive weight loss observed in this study was more pronounced than anticipated. One possible explanation is that the enhanced solubility and bioavailability conferred by HPβCD intensified the effects of THC at higher doses, leading to cumulative metabolic changes or adverse effects such as sedation. This suggests that the HPβCD formulation may amplify both the therapeutic and side effects of THC, warranting careful dose optimization.

The results from tail-flick apparatus demonstrate that acute administration of 3 mg/kg THC-HP-β-CD increases tail flick latency, indicating an effective response of the animals to this complex in respect to pain. This effect was consistent with chronic administration, over the 15-day period. These findings align with previous research demonstrating the analgesic properties of THC with other carriers and routes of administration. Lichtman and Martin (1991) demonstrated this effect when tested in tail flick apparatus, in both intravenous and intrathecal route of THC compound dissolved in ethanol and emulphor (Lichtman and Martin,

1991). The same dissolution method was also deemed effective in another study by Smith et al (1999) when administrated subcutaneously and per os, 30 minutes after administration (Smith et al., 1998). However, the subcutaneous method requires a high dose of THC (100mg/kg) as reported by Bloom et al. where they observed only a 17% MPE (Bloom et al., 1977). A mixture of Cremophor/ethanol/water was utilized for the dissolution of THC in a study by Reche et al. (1996), where intravenous injection of doses of 1-2-4-8 mg/kg increased tail flick latency after 20 minutes (Reche et al., 1996). Another study by Cichewicz and McCarthy (2003) examined the antinociceptive properties of oral THC dissolved in ethanol (Cichewicz and McCarthy, 2003) where they also observed an increased latency in tail flick response 30 min post-administration.

Zeidenberg et al. (1973) reported that oral administration of capsules containing 15 mg of THC dissolved in sesame oil in humans was effective in blocking a painful thermal stimulus (Zeidenberg et al., 1973). A recent study by Moore et al. (2021) with oral administration of THC dissolved in sesame oil found an increased tail flick latency in both female and male rats (Moore and Weerts, 2022). This increased latency at 3mg/kg however was much less than what we have demonstrated with THC-HP-β-CD complex indicating the higher efficacy of this complex in delivery of investigated compound. Moreover, similar to the results from our study, they also observed that particularly in female rats this efficacy was declined after 315 min compared to 75 min time point indicating effects returning to baseline. The same group have proven the greater sensitivity of female rats in intraperitoneal THC, pointing to sex differences in response to various routes of THC administration (Moore et al., 2021). Interestingly, this sensitivity with i.p. route and same carrier (ethanol/Cremophor/water) was reduced in female mice which could be due to the species-specific effects of this compound, however, this also questions the reproducibility of results with this carrier in different studies (Henderson-Redmond et al., 2022). Moreover, as mentioned before, the nociceptive properties of Cremophor alone has been shown in a study by Taberelli et al. which questions the suitability of this carrier in analgesia studies (Tabarelli et al., 2003). A study of the analgesic efficacy of chronic oral THC in oil form (medium-chain triglyceride) aimed at reducing hypersensitivity of rats showed only this effect in male rats but not females (Linher-Melville et al., 2020). This is

in contrast to what we have seen in the current study, suggesting a possible superior ability of HP-β-CD in pain reduction. Also, another study with volitional consumption of THC dissolved in gelatin (2mg/kg) showed equivalent antinociception in both sexes pointing to the volatility of chronic oral effect based on carrier among other factors (Kruse et al., 2019). The reduction in the efficacy of chronic treatment in our study could be explained by the development of tolerance by oral route of THC administration shown in previous studies with different carriers (Moore and Weerts, 2022). This tolerance was not observed in the i.p. or vaporized method (Javadi-Paydar et al., 2018, Tai et al., 2015). Moreover, clinical results suggest a faster buildup of tolerance in females (Lunn et al., 2019).

Neurochemical analyses revealed a selective increase in dopamine concentrations in the PAG following acute treatment, consistent with PAG's role in descending inhibitory pain modulation pathways (Tobaldini et al., 2019). Chronic treatment also elevated dopamine levels in the VTA, supporting the hypothesis that enhanced bioavailability enables THC to engage key neural circuits involved in analgesia more effectively. Moreover, cannabinoid administration in the ventrolateral PAG can enhance antinociception, suggesting the potential for improved pain management (Wilson-Poe et al., 2013). The posterior ventrolateral PAG plays a critical role in mediating cannabinoid-induced antinociception and catalepsy through G protein-coupled mechanisms, as demonstrated by Lichtman et al. (1996) (Lichtman et al., 1996). Comparative analyses of cannabinoid effects reveal that while Δ9-THC and CP-55,940 elicit robust antinociceptive responses following intracerebroventricular administration, the endogenous ligand anandamide appears to lack similar efficacy under these conditions (Lichtman et al., 1996). This distinction underscores potential differences in receptor affinity, metabolism, or signaling pathways between synthetic and endogenous cannabinoids. In parallel, the work of Retz and Holaday (1986) highlights that the GABAergic agonist THIP produces dose-dependent analgesic and motor effects specifically within the ventrolateral PAG (Retz and Holaday, 1986). Taken together, these findings suggest that cannabinoid and GABAergic systems, though distinct, converge within the vlPAG to modulate pain and motor control, emphasizing the region’s functional versatility and its potential as a target for analgesic interventions.

Here we also found that both acute and chronic administration of THC-HP-β-CD resulted in significant reductions in horizontal and vertical locomotor activities. This effect on locomotor activity was dose-dependent and consistent across different treatment durations. Previous studies have shown that THC can affect locomotor activity, as evidenced by studies investigating the motoric effects of this compound. This was shown in a study by Tseng and Craft (2001) with a decrease in the number of photobeam breaks in motility apparatus in both male and female rats at 1mg/kg of THC dissolved in emulphor/ethanol/saline after 240 minutes (Tseng and Craft, 2001). This decrease in locomotion after i.p. THC administration in pluronic acid was observed to be more pronounced in female rats compared to males after 60 minutes (Harte and Dow-Edwards, 2010). The repeated dosing of subcutaneous THC dissolved in emulphor/ethanol/saline in higher doses (10, 30, 100, and 300mg/kg) for 9.5 days also decreased locomotor activity (Wiley and Burston, 2014). Interestingly, in the same study, the comparison of blood and brain levels of THC and its metabolite 11-OH-THC in both sexes did not show a significant difference attesting to the sexual dimorphism in metabolism and questioning the stability of the utilized carrier. Similar to the result from our study, delivery of THC in sesame oil via oral route also showed a decrease in locomotion when studied in male and female rats after 5 hours (Moore and Weerts, 2022). However, in our study, the effect observed with the same dose (3mg/kg) was evident after 2hrs, demonstrating the ability of HP-β-CD in oral delivery of THC. In separate studies, reduced hypolocomotion after oral THC in sesame oil administration was observed after 40 and 120 minutes, but this was achieved with a higher dose (5 and 10mg/kg, respectively) (Hložek et al., 2017, Dow-Edwards and Zhao, 2008). This effect was not evident in THC dissolved in ethanol by the same method of administration (Rock et al., 2016). The results from these studies showing a variety of carriers with varying effects suggest that the commonly used carriers might not be optimal for sustained THC delivery due to potential issues with drug release or stability. Moreover, contrasting results from other studies using sesame oil as an oral delivery vehicle underscore the complexities involved in THC carrier dynamics. While sesame oil successfully decreased locomotion at higher doses, the lack of effect using ethanol as a carrier suggests that lipid-based carriers might offer advantages in solubilizing and stabilizing THC for effective delivery.

In our study, utilizing HP-β-CD as a carrier for THC, demonstrated a faster onset and more pronounced effect at a lower dose (3 mg/kg) compared to THC dissolved in the aforementioned results. HP-β-CD's ability to enhance solubility and potentially improve the bioavailability of lipophilic molecules like THC is evident here, offering a notable improvement over other carriers in terms of efficiency and rapidity of drug delivery effects.

The enhanced solubility and bioavailability of THC when complexed with HP-β-CD are likely key factors in the observed effects. Cyclodextrins, such as HP-β-CD, have been shown to improve drug transport across biological barriers by altering membrane fluidity, which can enhance the absorption and efficacy of lipophilic drugs like THC (Loftsson et al., 2005, Kearse and Green, 2000). The study by Jarho et al. (1998) demonstrated that HP-β-CD could significantly increase the aqueous solubility of THC, confirming the findings of this study regarding the improved delivery and efficacy of THC-HP-β-CD complexes (Jarho et al., 1998). This is in line with previous studies using cyclodextrins for transcorneal, sublingual, and intracerebroventricular delivery of THC (Kearse and Green, 2000, Agabio et al., 2017a, Mannila et al., 2006), delta-8-tetrahydrocannabinol (Hippalgaonkar et al., 2011), and THC ester prodrug (Upadhye et al., 2010).

The enhanced analgesic and locomotor effects observed following oral administration of THC complex with HP-β-CD highlight the importance of carrier systems in modulating the efficacy of lipophilic compounds. Traditional carriers for THC, such as sesame oil, ethanol, and Cremophor, have been widely utilized and studied (Cichewicz and McCarthy, 2003, Lichtman and Martin, 1991, Moore and Weerts, 2022), yet they often require higher doses, display slower onsets of action, or potentially introduce confounding pharmacological effects. For instance, ethanol-based vehicles can offer rapid absorption but may limit THC’s stability or cause irritation (Cichewicz and McCarthy, 2003), whereas sesame oil formulations necessitate higher doses (e.g., >10 mg/kg) to achieve comparable analgesia or alterations in locomotor behavior (Dow-Edwards & Zhao, 2008; Hložek et al., 2017; Moore & Weerts, 2022)(Dow-Edwards and Zhao, 2008, Hložek et al., 2017, Moore and Weerts, 2022). Cremophor, while improving solubility, can introduce its own nociceptive properties, thus confounding analgesia studies (Tabarelli et al., 2003). In contrast, our findings demonstrate that HP-β-CD significantly

improves the efficiency of oral THC delivery, achieving robust analgesic and hypolocomotor effects at lower doses (3 mg/kg) and faster onset than commonly reported in the literature for other carriers. This superiority aligns with previous investigations showing that cyclodextrins can markedly enhance the aqueous solubility and membrane permeability of lipophilic drugs (Hippalgaonkar et al., 2011, Jarho et al., 1998, Loftsson et al., 2005). The molecular structure of cyclodextrins, especially HP-β-CD, forms inclusion complexes by hosting hydrophobic molecules like THC in their nonpolar cavity while maintaining a hydrophilic external surface, thus increasing drug solubility and potentially improving bioavailability (Loftsson et al., 2005, Kearse and Green, 2000).

This unique delivery profile might also stabilize THC's chemical structure against oxidative degradation or enzymatic hydrolysis in the gastrointestinal tract, resulting in more consistent plasma levels. Such stabilization could explain why we observed analgesic responses and reduced locomotor activity at lower doses and within a shorter time frame compared to THC dissolved in ethanol or oils. By enhancing the fraction of THC absorbed, HP-β-CD may enable more efficient interaction with CB1 receptors located in key pain modulatory areas (e.g., PAG) and motor control regions (Tseng and Craft, 2001).

Our results indicate that even with a more efficient carrier like HP-β-CD, tolerance emerges over time, although the initially higher efficacy at lower doses may offer a therapeutic advantage during the early phases of treatment. This advantage could be clinically significant: patients might benefit from effective analgesia with smaller doses initially, potentially delaying the onset of tolerance-related challenges seen with traditional carriers requiring larger or more frequent dosing (Lunn et al., 2019).

Previous research has underscored sex-dependent differences in THC response, with females often displaying greater sensitivity to THC's antinociceptive effects and sometimes more rapid tolerance development (Harte and Dow-Edwards, 2010, Henderson-Redmond et al., 2021). Studies utilizing various carriers have yielded inconsistent results, likely influenced by differences in metabolism, hormonal status, and receptor distribution across sexes and species(Kruse et al., 2019, Linher-Melville et al., 2023). Our study focused on female Sprague Dawley rats, and the robust analgesia observed, coupled with eventual tolerance

development, suggests that HP-β-CD does not eliminate sex-related variability but may mitigate some drawbacks observed with less efficient carriers. A direct comparison across sex and species, using HP-β-CD, could provide valuable insights into how improved delivery systems interact with biological factors to shape THC's pharmacological profile.

While the analgesic effects of THC are often associated with CB1 receptor-mediated modulation of pain pathways and potential interactions with dopaminergic and opioid systems, previous studies have shown that THC's influence on dopamine release is region-specific and dose-dependent (Tseng and Craft, 2001). Our neurochemical findings (as described in the broader results), showing increased dopamine levels in the PAG but not in the NAc or PFC, support the hypothesis that THC's analgesic action may involve selective neural circuits. The PAG is a critical hub in descending pain modulation, and dopamine release there could synergize with cannabinoid-mediated disinhibition of antinociceptive pathways, enhancing analgesic outcomes (Kearse and Green, 2000).

By improving oral bioavailability, the HP-β-CD complex may ensure sufficient THC concentrations reach the PAG and other pain-relevant structures rapidly and consistently, thereby enhancing the acute analgesic response. The lack of dopamine changes in reward-related regions like the NAc could suggest that while analgesic pathways are facilitated, reinforcing or psychoactive properties may not be equally amplified. This dissociation, if reproducible in future studies, could be therapeutically beneficial, potentially lowering the abuse liability commonly associated with THC. Our data pave the way for further research and potential clinical applications. Enhancing THC's oral bioavailability via HP-β-CD could increase therapeutic efficacy at lower doses, minimize side effects, and improve the predictability of oral cannabinoid treatments. Such improvements are paramount in chronic pain management where oral cannabinoid therapies are increasingly considered but often hindered by inconsistent absorption, variable responses, and rapid tolerance formation.

Our study also demonstrates that Δ9-THC significantly influences hepatic lipid metabolism, particularly by modulating de novo lipogenesis (DNL). Administration of 1 mg/kg dose of THC, led to a significant increase in DNL in the liver.

One potential mechanisms through which THC might exert its effects on lipid metabolism is via activation of type 1 cannabinoid receptors (CB1Rs). Activation of CB1Rs has been shown to stimulate hepatic DNL, likely through interactions with nuclear receptors such as sterol regulatory element-binding proteins (SREBP1), which promote lipid synthesis. Concurrently, this activation downregulates pathways associated with FA oxidation, including those regulated by PPARs (Osei-Hyiaman et al., 2005, O'Sullivan et al., 2021b). Previous studies have indicated that CB1 activation can negatively regulate carnitine palmitoyltransferase 1 (CPT1) through the PPARα signaling pathway, resulting in reduced FA oxidation and enhanced lipid storage in the liver (Wei et al., 2018). Furthermore, CB1R plays a role in regulating PPARα expression, a critical regulator of lipid metabolism in hepatocytes and evidence suggests a relationship between CB1R and PPARα. Inhibition of hepatic CB1R, through either pharmacological approaches or genetic knockout, has been shown to upregulate PPARα expression and activity, suggesting an inverse regulatory relationship between these pathways (Azar et al., 2020). Available evidence suggests that most cannabinoids (both endocannabinoid and phytocannabinoid as 9-THC), can directly or indirectly activate PPARs via distinct molecular signaling pathways (Pistis, M., O'Sullivan, S.E., 2017. Advances in Pharmacology 80:291e328). Moreover, CB1R may indirectly influence PPARα by modulating hepatic levels of AEA and eCB-like molecules such as OEA and PEA (Azar et al., 2020) known to be potent endogenous ligands of PPARα [M. Melis, G. Carta, M. Pistis, S. Banni, CNS Neurol. Disord.: Drug Targets 12 (1) (2013) 70–77; O'Sullivan, S.E., 2016. British Journal of Pharmacology 173:1899e1910] (Melis et al., 2013, O'Sullivan, 2016). Interestingly, our findings showed that administration of THC at a dose of 1 mg/kg increased hepatic levels of NAEs, including AEA, PEA, OEA and DHEA, all of which serve as endogenous ligands for PPARα. A significant positive correlation was observed between PPARα gene expression and increasing THC doses. Additionally, the elevated PEA and OEA levels persisted even with a 3mg/kg treatment, suggesting that this may induce PPARα gene expression to counteract THC's negative effect on hepatic lipid metabolism.

Administration of a higher dose of THC (3 mg/kg) led to a reduction in AEA levels, potentially due to CB1 receptor downregulation, a phenomenon consistent with tolerance mechanisms observed in chronic cannabis use. This observation aligns with previous studies

on rodents, where prolonged cannabinoid exposure resulted in decreased CB1 receptor density and activity (Romero et al., 1998). These findings suggest that the pharmacological tolerance to THC after chronic exposure is likely based on pharmacodynamic adaptations rather than pharmacokinetic changes. Receptor downregulation may lead to diminished activation of CB1-associated signaling pathways, which could explain the observed decrease in AEA levels following higher doses of THC. Additionally, the decline in AEA may be partially attributed to increased catabolism via fatty acid-amide hydrolase (FAAH). This could result from cytoplasmic fatty acid binding proteins (FABPs), particularly FABP1, which plays a key role in the intracellular transport and metabolism of Δ9-THC and its metabolites. Although Δ9-THC shares the same CB1 signaling pathway as the endocannabinoids, AEA and 2-AG, the molecular details of its intracellular trafficking remain unclear. Within the blood, Δ9-THC is highly bound to serum lipoproteins and albumin (Dingell et al., 1973). However, over 90% of Δ9-THC undergoes rapid hepatic clearance and metabolism within the liver (Huestis, 2007, Grotenhermen, 2003). In the hepatocytes, Δ9-THC is localized primarily to the endoplasmic reticulum (wherein Δ9-THC oxidative enzymes are localized) and to the nucleus (wherein Δ9-THC regulates transcriptional activity of nuclear receptors in lipid and drug metabolism (Dingell et al., 1973). Metabolism of THC occurs by hydroxylation and oxidation reactions catalyzed the cytochrome P450 (CYP450) enzymes (Watanabe et al., 1993). The first step involves hydroxylation of THC to its primary metabolite 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC). This metabolite retains high CB1 receptor affinity and may even be slightly more psychoactive than the parent THC compound itself (Lemberger et al., 1973). Subsequently, 11-OH-THC undergoes further oxidation by CYP450s to the inactive secondary metabolite 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) (see Fig. 6 for THC metabolic pathway schematic). The second step of THC metabolism involves glucuronidation of THC-COOH by UDP-glucuronoyltransferases (UDP-GT), conferring sufficient aqueous solubility for bioelimination through urine, sweat, and feces (Huestis, 2007). Both CYP450s and UDP-GTs are localized to the endoplasmic reticulum of hepatocytes. Given the highly lipophilic nature of cannabinoids, a mechanism required to transport them to intracellular metabolic enzymes is liver-type fatty acid binding protein (FABP) which mediates cannabinoids and

endocannabnoids transport and subsequent inactivation (Elmes et al., 2019b, Huang et al., 2016). The importance of FABP1 as a THC carrier was further confirmed by FABP1-KO mice that exhibited reduced THC clearance rates and a concomitant enhancement of its behavioral effects (Elmes et al., 2019b). Recently mouse FABP1 was shown to interact with phytocannabinoids in vitro with high affinities (Huang et al., 2016).

Overall, the binding affinities for Δ9-THC and its metabolites for FABP1, as well as endogenous ligands, follow this order: fatty acids, 2-AG > Δ9-THC, Δ9-THC−OH, fatty acyl-CoAs, N-acylethanolamides (AEA, PEA, OEA, DHEA) ((Huang et al., 2016).

Therefore, these findings suggest that FABP1 may serve as a major cytosolic hepatocyte binding protein facilitating the transport of Δ9-THC and/or its hydroxylated metabolite, Δ9-OH-THC, to intracellular sites of metabolism and action. Due to FABP1's nearly 10-fold higher affinity for FA, the hepatic FA load could significantly influence the binding of Δ9-THC and even more so Δ9-OH-THC to FABP1.

In our study, we observed elevated total FFA levels following a 3mg/kg THC treatment. This increment may induce a conformational change in FABP1, promoting the redistribution of FABP1-bound ligands into the nucleus, where they can form a complex FABP1-ligand-PPARα, which undergoes further conformational change to facilitate the transfer of ligands to PPARα leading to its activation and subsequent transcription of multiple genes involved in FA metabolism. Moreover, THC induces hepatic accumulation of endocannabinoids in a FABP1-dependent manner, likely indicating competition for FABP1-mediated transport (McIntosh et al., 2018).

Our results also demonstrated an increase in hepatic NAE levels in rat dams treated with 1mg/kg of THC, indicating competition for FABP1 during the transport of NAE through the cytoplasm to the endoplasmic reticulum, where they are degraded by the fatty acid-amide hydrolase (FAAH). However, at a higher THC dose (3mg/kg), there may be a prolonged activation of PPARα gene expression that in turn could increase FABP1 expression. This enhanced FABP1 expression might regulate the altered catabolism of EC via FAAH enzyme leading thus higher degradation of AEA.

In contrast to AEA, the endocannabinoids 2-AG did not show elevated levels following the 1mg/kg treatment. This outcome may be ascribed to the fact that FABP1 has a 10-fold higher binding affinity for 2-AG than for 9-THC (Huang et al., 2016). FABP1 effectively facilitates the 2-AG transport to monoacylglycerol hydrolase (MAGL) targeting it for degradation (Fowler, 2012). Since 2-AG uptake is not primarily driven by intracellular hydrolysis, the continuous transport by FABP1 can lead to increased hepatocyte levels of 2-AG, as shown in 3mg/kg treatment. Moreover, we observed an increased OEA/2-AG ratio in rat dams treated with 1mg/kg of THC, suggesting a more efficient enzymatic degradation of 2-AG compared to AEA and its congeners. This ratio is also considered a potential indicator of the balance between endogenous PPARα and ECS activation, indicating an improved PPARα system activity.

Noteworthy, our data also indicate that 1mg/kg of THC significantly increased DHEA levels. It has been shown that DHEA promotes neurite growth, synaptogenesis, and the expression of glutamate receptor subunits, thereby enhancing glutamatergic synaptic activity and stimulating the development of hippocampal neurons (Kim et al., 2011).

Future studies are required to confirm whether the observed liver metabolic alterations at different THC dosages can be attributed to modifications in the PPARα/endocannabinoid axis, and consequently, the regulation of FABP1 expression and other genes involved in energy metabolism, such as fibroblast growth factor 21 (FGF21), a transcription factor directly regulated by PPARα.

Additionally, further investigation is needed to determine whether these hepatic metabolic changes are reflected in alterations to fatty acid and endocannabinoid profiles in other peripheral tissues and the brain. This is particularly relevant in light of recent findings (Murru et al., 2024), which demonstrated the influence of THC on central metabolism, potentially providing mechanistic insights into the metabolic underpinnings of psychiatric conditions associated with THC treatment.

6. Concluding remarks and future directions:

This study demonstrates that the HP-β-CD formulation significantly enhances the oral bioavailability and therapeutic efficacy of Δ9-THC in female rats, improving solubility, stability, and absorption to achieve robust analgesic effects at low doses while influencing feeding behavior and metabolic parameters in a dose- and time-dependent manner. The biphasic effect on feeding behavior—orexigenic at lower doses and anorexigenic at higher doses—aligns with existing cannabinoid literature, emphasizing the importance of precise dose titration to balance therapeutic benefits (e.g., analgesia, short-term appetite stimulation) and potential drawbacks (e.g., tolerance, catabolic effects). Neurochemical analyses revealed region-specific dopaminergic responses, with increased dopamine in the PAG after acute treatment and in the VTA under chronic administration, highlighting CB1 receptor modulation's role in pain perception and reward pathways. Additionally, locomotor activity suppression and hepatic analyses indicating dose-dependent modulation of lipid metabolism underscore the formulation's systemic effects, including its impact on motor control and metabolic regulation. However, the study's sex-specific scope and limited dose range necessitate further investigation, including extending findings to male rats, exploring additional doses, and conducting detailed time-course studies to clarify biphasic effects, tolerance development, and therapeutic windows. Future research should also focus on molecular mechanisms, such as how HP-β-CD–THC complexes interact with intracellular signaling cascades, transporter proteins, and nuclear receptors, as well as evaluate clinical potential in disease models like chronic pain or metabolic syndrome. Understanding whether enhanced bioavailability alters side-effect profiles, such as anxiety or cognitive impairment, is critical before clinical translation. To conclude, while HP-β-CD shows promise as a vehicle for improving oral THC delivery, combining mechanistic exploration with translational studies will

be essential to optimize cannabinoid-based therapies, maximizing clinical benefits while minimizing risks.

While our findings highlight the potential of using cyclodextrin as a carrier to improve THC's pharmacological effects, certain limitations should be acknowledged. First, this study was conducted exclusively in female rats, which may restrict the generalizability of the results to male populations. Incorporating both sexes would offer a more comprehensive understanding of sex-specific responses and could refine dosing and safety guidelines in clinical contexts. Second, although we measured THC and metabolite concentrations in the liver, a direct comparison with levels in plasma was not yet performed, though planned in next experiments. Future investigations that systematically profile THC and its metabolites across multiple biological matrices would provide a clearer picture of drug distribution and metabolism. Such comparative analyses could illuminate tissue-specific pharmacokinetics and optimize therapeutic approaches for cannabinoid-based treatments. Together, addressing these limitations will be crucial for strengthening the translational value of our findings and advancing our knowledge of cannabinoid pharmacology.

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