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Cannabinoid modulation of behavioral processes : from adolescence to adulthood

Christina Ioannidou

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE

DOCTEUR DE L'UNIVERSITÉ DE BORDEAUX

École Doctorale des Sciences de la Vie et de Santé
Spécialité - Neurosciences

Par Christina IOANNIDOU

CANNABINOID MODULATION OF BEHAVIORAL PROCESSES:
From Adolescence to Adulthood

Sous la direction de Giovanni MARSICANO

Soutenue le 16/12/2021

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MODULATION DES PROCESSUS COMPORTEMENTAUX PAR LE SYSTEME ENDOCANNABINOÏDE: De l'adolescence à l'âge adulte

Le système endocannabinoïde (SEC) est un système modulateur impliqué dans la régulation de fonctions cérébrales et processus comportementaux. Le SEC est la cible du principal composant psychoactif du cannabis, le Δ^9 -tétrahydrocannabinol (THC), qui exerce la plupart de ses effets psychotomimétiques en se liant aux récepteurs cannabinoïdes de type 1 (CB1) du cerveau. Le cannabis est le psychotrope le plus largement utilisé dans le monde, les adolescents et les jeunes adultes ayant le taux de consommation le plus élevé. L'adolescence est une période sensible du développement, au cours de laquelle le cannabis peut facilement affecter la maturation du cerveau, entraînant des conséquences à long terme. L'association entre la consommation de cannabis à l'adolescence et des effets négatifs persistants est depuis longtemps étudiée en neurosciences, des études identifiant une série de troubles neuropsychiques - les troubles psychotiques étant parmi les plus fréquents. Cependant, les mécanismes reliant l'exposition aux cannabinoïdes aux effets comportementaux à long terme sont complexes, et les études dans ce domaine restent peu concluantes.

Les modèles précliniques où les animaux sont exposés au THC pendant les périodes de développement (dont l'adolescence) sont essentiels pour évaluer l'impact de la consommation de cannabis. En utilisant un modèle d'exposition au THC à l'adolescence chez la souris, le premier objectif de ma thèse était de caractériser le profil comportemental à l'âge adulte de souris mâles et femelles. Dans ce but, une batterie de tests comportementaux a été réalisée à l'âge adulte permettant d'étudier une possible altération des fonctions motrices, sociales et cognitives. De plus, afin d'évaluer les altérations possibles des représentations sensorielles mentales, caractéristiques majeures des symptômes psychotiques (i.e. hallucinations et délires), nous avons étudié l'effet de l'exposition au THC à l'adolescence dans un protocole d'apprentissage associatif d'ordre supérieur, i.e. reposant sur les représentations sensorielles internes. Nos résultats révèlent des effets dépendants du sexe, l'exposition au THC à l'adolescence perturbant les fonctions motrices, sociales et de mémoire à court terme chez les souris mâles, et l'apprentissage et la mémoire associative chez les souris femelles.

Le deuxième objectif de cette étude était d'explorer les mécanismes neurobiologiques potentiels qui sous-tendent les effets de l'exposition au THC à l'adolescence. Nous avons analysé le niveau d'expression des récepteurs CB1 et ses voies de signalisation, et nous avons observé des changements dépendants du sexe et de la région. Nos études récentes ont révélé que l'instabilité du complexe I (CI)

mitochondrial par déphosphorylation spécifique de sa sous-unité NDUFS4 dans les astrocytes constitue le mécanisme conduisant aux déficits sociaux à long terme induits par le THC. Au cours de ma thèse, nous avons démontré que la phosphorylation de NDUFS4 est diminuée par l'exposition au THC à l'adolescence chez les souris mâles, mais pas chez les souris femelles, suggérant que le statut de phosphorylation de cette sous-unité du CI pourrait être responsable des effets comportementaux dépendant du sexe. En particulier, l'expression d'une forme active de NDUFS4 dans les astrocytes a prévenu les effets de l'exposition au THC à l'adolescence sur la locomotion et les interactions sociales évaluées chez la souris mâle adulte. Ces résultats soutiennent donc l'idée que des altérations mitochondriales au sein des astrocytes seraient impliquées dans les effets comportementaux à long terme de l'exposition au THC à l'adolescence, soulignant le rôle de la bioénergétique cellulaire dans la régulation comportementale.

Enfin, un objectif supplémentaire de mon travail a été l'exploration du rôle physiologique des récepteurs CB1 dans les processus d'apprentissage associatif d'ordre supérieur reposant sur les représentations mentales. Ces processus peuvent être adaptés aux études précliniques des états psychotiques, et les théories de l'apprentissage associatif ont fourni le cadre dans lequel les phénomènes médiés par les représentations peuvent être étudiés chez les animaux, en utilisant des tâches comportementales de conditionnement d'ordre supérieur. De précédents résultats ont établi l'importance du contrôle des représentations sensorielles par les récepteurs CB1 dans le cerveau. Ici, nous avons étudié le rôle de différentes populations de récepteurs CB1 dans l'apprentissage médié par les représentations, ainsi que dans l'apprentissage par renforcement direct, en utilisant des lignées de souris mutantes conditionnelles spécifiques qui ciblent les récepteurs CB1 dans les cellules exprimant les récepteurs à la dopamine D1, ainsi qu'associé aux mitochondries.

Dans l'ensemble, le présent travail ajoute des résultats importants à la littérature existante sur les effets de l'exposition aux cannabinoïdes à l'adolescence, et décrit un nouveau mécanisme moléculaire responsable de certains d'entre eux, tout en contribuant à faire progresser la compréhension du rôle des récepteurs CB1 dans les processus d'apprentissage associatif d'ordre supérieur.

Mots clés : adolescence, récepteurs CB1, comportement, psychose, THC, mitochondries, apprentissage associatif, apprentissage médié par la représentation

CANNABINOID MODULATION OF BEHAVIORAL PROCESSES:

From Adolescence to Adulthood

The endocannabinoid system (ECS) is a crucial modulatory system involved in the regulation of diverse brain functions and behavior. The ECS is the target of the main psychoactive component of cannabis, Δ^9 -tetrahydrocannabinol (THC), which exerts many of its psychotomimetic effects by binding to brain cannabinoid type-1 (CB1) receptors. Cannabis is currently the most widely used psychoactive substance worldwide, with a highest consumption rate observed among adolescents and young adults. Adolescence comprises a sensitive developmental period, during which cannabinoid drugs can easily disrupt the normative trajectory of brain maturation, giving rise to long-term consequences. Indeed, the association between adolescent cannabis use and persistent negative outcomes has long been the focus of neuroscience research, with numerous studies associating these outcomes with a range of neuropsychiatric disorders—psychotic disorders being some the most prominent. However, the mechanisms linking cannabinoid exposure to long-term behavioral effects are complex, and results in the field remain inconclusive.

Animal models exposed to THC during developmental periods (e.g. adolescence) are therefore critical to assess the impact of cannabis use. By using a model of adolescent THC in mice, the first aim of this study was to characterize the specific adult behavioral profile of male and female mice. To this end, a set of psychotic-like behavioral processes related to motor, social and cognitive functions were evaluated in adulthood. Moreover, in order to assess possible alterations in mental sensory representations, which more accurately represent the distinctive features of the core psychotic symptoms (i.e. hallucinations and delusions), we investigated the effect of adolescent THC in a representation-mediated learning protocol. Resembling cannabis-induced psychotic states in humans, impairments in this protocol have been described after acute administration of THC, however the effect of adolescent THC has never been investigated. Altogether, our findings reveal sex-dependent effects, with adolescent THC mainly disrupting motor, social and short-term memory functions in male mice, and associative learning and memory in female mice.

The second aim of this study was to explore potential neurobiological mechanisms underlying the effects of adolescent THC. Therefore, we performed analysis of the CB1 receptor status and signaling targets (such as the ERK and CREB proteins), observing sex- and region-dependent changes. Our recent findings have shown that mitochondrial Complex I (CI) instability through the specific dephosphorylation of its NDUFS4 subunit in astrocytes is the mechanism leading to persistent THC-

induced social deficits. Here, we demonstrated that NDUFS4 phosphorylation is specifically decreased by adolescent THC in male, but not in female mice, suggesting that the phosphorylation status of this CI subunit might be responsible for the sex-specific behavioral effects. Notably, astroglial expression of a phosphomimetic mutant form of NDUFS4 was able to prevent the adolescent THC-induced behavioral effects on locomotion and social interaction in adult male mice. These results strongly support the idea that mitochondrial alterations in astrocytes are causally involved in the enduring behavioral effects of adolescent THC, highlighting the emerging role of cellular bioenergetics in the regulation of behavior.

Finally, an additional objective of my work was dedicated to the exploration of the physiological role of CB1 receptors in representation-mediated learning processes. These processes may be uniquely suited for preclinical investigations of psychosis, and associative learning theories have provided the framework, through which representation-mediated phenomena can be studied in animals, using higher-order conditioning behavioral tasks. Earlier findings have already established the importance of the CB1-dependent control of sensory representations in the brain. Here, we have investigated the role of different CB1 receptor populations in representation-mediated learning, as well as in direct reinforcement learning, using specific conditional mutant mouse lines that target the CB1 receptors in dopamine D1-positive cells, and also in mitochondria.

Overall, the present work adds important findings to the existent literature of adolescent cannabinoid effects, and describes a novel molecular mechanism causally mediating some of them, while also contributing in advancing the understanding of CB1 receptors' role in higher-order associative learning processes.

Keywords: adolescence, CB1 receptors, behavior, psychosis, THC, mitochondria, associative learning, representation-mediated learning,

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Ioannidou, C., Busquets-Garcia, A., Soria- Gómez, E., Bellocchio, L., Zhao, Z., Julio-Kalajzić, F., Fermigier, A., Mariani, Y., Ferreira, G.* and Marsicano, G*. CB1 Receptors in the Anterior Olfactory Nucleus Control Odor Aversion Memory. (In preparation)

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Ioannidou, C., Busquets-Garcia, A., Ferreira, G. and Marsicano, G. (2021). Neural Substrates of Incidental Associations and Mediated Learning: The Role of Cannabinoid Receptors. Front. Behav. Neurosci. 15:722796

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Jimenez-Blasco, D.*, Busquets-Garcia, A.*, Hebert-Chatelain, E.*, Serrat, R., Vicente-Gutierrez, C., **Ioannidou, C.** et al. (2020). Glucose metabolism links astroglial mitochondria to cannabinoid effects. Nature. 583, 603–608

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Scientific Posters

Gordon Research Conference "Cannabinoid Function in the CNS", Spain (2019)

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Adolescent cannabinoid exposure induces psychotic-like effects in a sex-dependent manner.
Ioannidou C., Bartholomeo M., Cayrel M., Jiménez Blasco D., Busquets-Garcia A., Marsicano G.

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Adolescent cannabinoid exposure induces psychotic-like effects in a sex-dependent manner
Ioannidou C., Bartholomeo M., Cayrel M., Busquets-Garcia A., Marsicano G.

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LIST OF ABBREVIATIONS

1,2-DAG : 1,2-Diacylglycerol.....	31
11-OH-THC : 11-hydroxy-THC	177
2-AG : 2-Arachidonoylglycerol	22
AEA : N-Arachidonylethanolamide	22
AON : Anterior olfactory nucleus	173
ATP : Adenosine Triphosphate	27
CB2 : Cannabinoid Type-2.....	22
CBD :Cannabidiol	4
CBN : Cannabinol	4
CCK : Cholecystokinin	43
CI : Complex I.....	71
CNS : Central Nervous System	35
CR : Conditioned response.....	54
CREB : cAMP response element-binding proteins	97
CS : Conditioned Stimulus.....	79
DAGL : Diacylglycerol Lipase	31
DSE : Depolarization-Induced Suppression of Excitation	25
DSI : Depolarization-Induced Suppression of Inhibition	25
ECS : Endocannabinoid System.....	3
ERK : Extracellular signal-regulated kinases.....	28

FAAH : Fatty Acid Amide Hydrolase.....	32
FABPs : Fatty acid binding proteins	32
GFAP : Glial fibrillary acidic protein.....	104
i.p. : intraperitoneal	45
iLTD : inhibitory long-term depression	26
LTD : Long-term depression.....	26
MAPK : Mitogen-activated protein kinases	28
mCS : Mediated Conditioned Stimulus.....	80
MRI : Magnetic resonance imaging	20
mtCB1 : Mitochondria associated CB1	71
NAPE : N-arachidonyl-phosphatidyl-ethanolamine.....	30
NDUFS4 : NADH dehydrogenase [ubiquinone] iron-sulfur protein 4	164
OXPHOS : Oxidative Phosphorylation.....	162
PET : Positron emission tomography	20
PFC : Prefrontal cortex.....	12
THC : Δ^9 -tetrahydrocannabinol.....	4
UR : Unconditioned response.....	54
US : Unconditioned stimulus	54

Chapter I Introduction

1 Cannabis and Adolescence

Humans have utilized cannabis products in various forms throughout recorded history. The cannabis plant has been one of the earliest plants to be cultivated and used for millennia for its fiber, as food or for religious and recreational purposes. Cannabis has been also used as medicine for thousands of years, alleviating symptoms of many different conditions. Nevertheless, cannabis use is surrounded by numerous controversies and misconceptions regarding its medical use, legalization and long-term health consequences. In this century, we are finally beginning to understand the precise pharmacological mechanisms underlying the effects of cannabis, most of which can be explained through the lens of the endocannabinoid system (ECS).

In the following pages, I will first introduce some general information about cannabis and briefly describe the history and evolution of cannabis use. I will then explore the current consumption patterns of cannabis for recreational purposes, focusing especially in adolescents and young adults. Finally, I will discuss some of the reasons why cannabis use during adolescence should be of concern and I will present an overview of human studies on the long-lasting effects of adolescent cannabis exposure.

1.1 Cannabis and Cannabinoid Use

Cannabis is a genus of flowering plants indigenous to central Asia. Botanically, it belongs to the annual plants of the Cannabaceae family and the Cannabis genus contains only one species, *Cannabis sativa* L., which is divided into three different subtypes: sativa, indica, and ruderalis. Archaeological findings indicate that *Cannabis sativa* was among the first plants that were cultivated by humans, first in Asia and later worldwide. Cannabis has been cultivated for its fibers, its seeds, oils and leaves, as

well as for medicinal and spiritual purposes (Merlin, 2003; Small, 2015). The word cannabis itself most likely originates from a Scythian or Thracian word, with the meaning of 'way to produce smoke', which loaned into Persian as kanab, then into Greek as κάνναβις and subsequently into Latin as cannabis, which was then transformed into hænep and hemp in English (Solymosi and Kofalvi, 2017).

The plant contains more than 400 distinct chemical compounds including over 60, which are specific to the plants of the genus Cannabis and are called phytocannabinoids, to distinguish them from synthetic or endogenous cannabinoids (see below). The most known phytocannabinoid is the Δ 9-tetrahydrocannabinol (THC) (Adams, 1942; Mechoulam and Gaoni, 1965), which is the main psychoactive component of cannabis and is therefore responsible for most of the psychoactive and physical effects of cannabis consumption (Pertwee, 2005). Other active compounds of cannabis are the Δ 8-tetrahydrocannabinol, cannabitol (CBN), and cannabidiol (CBD) (Mechoulam, 1970). Those phytocannabinoids can show additive, synergistic, or antagonist effects with Δ 9-THC and these interactions may often modulate its actions when cannabis is consumed as an herbal mixture (Ashton, 2001). Today, the terms “cannabis” or “marijuana” are commonly used to refer to blends of the dried flowering tops, leaves, and stalks of the mature cannabis plant (Befort, 2016).

People use cannabis or other cannabimimetic drugs because of the several effects they can acutely produce. Thus, cannabis users might seek therapeutic effects and/or pleasant recreational (psychotomimetic) effects. Potentially therapeutic effects encompass anesthesia, airway relaxation, or antiemesis, as well as increased insulin release and an increase in appetite, thirst, food palatability and caloric intake (Solymosi and Kofalvi, 2017). As for the psychotomimetic outcomes of cannabis intoxication, these often include euphoria, relaxation, richer sensory experiences and increased sociability. Cannabinoid consumption, however, can be accompanied by several undesired effects, such as hypothermia, hypolocomotion, catalepsy, tachycardia at systemic level, or disorganization, deficits in attention, increased reaction time, social and emotional withdrawal, altered external and internal perception and sometimes increased anxiety, dysphoria, paranoia, and panic attacks at psychological level (Tart, 1970; Thomas, 1993; Volkow et al., 2014b). It is also worth mentioning that most of these latter effects tend to coincide with schizophrenia-like positive and negative symptoms (D'Souza et al., 2004; Thomas, 1993). Of course, many factors can influence all the aforementioned effects, including the relative content in different phytocannabinoids (e.g. THC, CBD), the mode of consumption, the frequency in the use of cannabis and other drugs, different gene polymorphisms, as well as the internal state of the individual. Regarding the mode of consumption, cannabis is used primarily by the

smoking route, mixed or not with tobacco (“joint”) (Knapp et al., 2019; Russell et al., 2018). Often it is also ingested in the form of edibles. Oral ingestion delays the onset of effects by up to 60–120 minutes and the circulating levels of THC are smaller but longer-lasting than when cannabis is smoked (Barrus et al., 2016; Ohlsson et al., 1980). Unlike other types of drugs that can also be injected, the intravenous use of cannabis is not common, mainly due to its relative insolubility in water.

1.1.1 A Brief History of Cannabis: How One Plant Spread Through the World

The first pieces of evidence of the use of cannabis were found in China, dating approximately 6000 years ago, when the plant was grown for textile and paper extracts from fibers rather than for its psychoactive properties (Li, 1974). Archaeological evidence proved the early medical use of cannabis, which is also extensively documented in written records of several cultures. Some of these records include the book "Pen Ts'ao" or "The Herbal", written by the Chinese emperor Shen Nung, the "Ebers Papyrus" in Egypt, the "Atharva Veda" collection of texts in India, as well as well as in Greek pharmacopeia (Herodotus, Dioskurides) (Rätsch, 2005; Russo, 2007). Indeed, the hemp plant has been historically used in many cultures for its therapeutic properties such as analgesic/anesthetic, anticonvulsant, sedative, antibiotic, appetite stimulant, diuretic and aphrodisiac effects (Zuardi, 2006). The psychotropic properties of Cannabis have also been well documented since long, from around 100 B.C. in China (Merlin, 2003).

In the beginning of the 19th century medicinal cannabis was introduced in Europe (Fankhauser, 2008). The British physician William Brooke O’Shaughnessy introduced cannabis into Western medicine, after observing its use in Calcutta as a muscle relaxant and brought a large supply of cannabis specimens and seeds with him to England, where he generously provided to physicians throughout the country (Mikuriya, 1969; Russo, 2007). In the 1840s, the French psychiatrist Jacques-Joseph Moreau (or "Moreau de Tours") published "Du Hashish et de L’Aliénation Mentale" (translated as "Hashish and Mental Illness"), where he documented the psychomimetic effects of cannabis resin, arguing that cannabis intoxication represents a powerful approach to investigate mental illness, leading to multiple studies on cannabis medical value both in Europe and North America and paving the way to modern psychopharmacology (Moreau, 1845). Indeed, the selling of plant extracts by private laboratories from the second half of the 19th century allowed the experimental identification of several of its physical benefits such as sedation, analgesia, appetite-stimulation and others (Mikuriya, 1969).

During the starting decades of the 20th century however, the medical use of cannabis in the west significantly decreased. One reason for that was the difficulty to obtain consistent and replicable effects, since the many varieties of the plant were extremely different in regard to their efficacy. At that time, the drug was primarily used in the form of different extracts, whose effects were dependent on factors such as the origin of the plant, its age or the mode of its preparation (Zuardi, 2006). In addition, various additional medications appeared at the end of the 19th century, with known efficacy for the treatment of the main indications of cannabis. Vaccines for various infectious diseases were developed while at the same time effective analgesics such as aspirin also appeared. Moreover, the use of hypodermic syringes allowed the injectable use of morphine, and cannabis' use as a sedative was overshadowed by substances such as paraldehyde and barbiturates (Fankhauser, 2002; Zuardi, 2006). Finally, multiple legal restrictions limited the medical use of cannabis (cannabis was registered as an illicit drug by the Geneva Convention in 1925 and by US Federal Law in 1937), decreasing the scientific interest in the properties and mechanisms of action of the plant (Bewley-Taylor et al., 2014; Grinspoon et al., 1997).

However, cannabis reached great social importance in the second half of the 20th century, due to the explosion of its consumption for recreational purposes. Since the 1960s, the use of cannabis rapidly spread among the younger ranges of the population throughout the Western world. This use still remains high until today (EMCDDA, 2021; UNODC, 2021). After many decades of research and significant contributions to the elucidation of the structure of cannabinoids [(Adams, 1942), recently reviewed in (Appendino, 2020)], the chemical structure of THC was eventually fully identified in 1964 by Gaoni and Mechoulam (Gaoni and Mechoulam, 1964), which contributed to a proliferation of studies about the active constituents of cannabis. These findings led to numerous pharmacological studies both in humans and animals showing that THC mediates the main psychoactive properties of the plant (Carlini et al., 1974; Kiplinger et al., 1971). At the same time, Mechoulam et al. completed the synthesis of the pure molecules, established their molecular structures and studied their structure-activity relationships (Mechoulam et al., 1972). This better knowledge of its chemical composition, along with the aforementioned boost in cannabis consumption, contributed to a significant increase in scientific interest for cannabis. The number of publications on cannabis reached their peak in the early- middle 1970s, after which it started to slowly decline during the following two decades. This was likely due to the fact that no specific mechanism of action was discovered yet. Indeed, the scientific interest was renewed in the early 1990s, after the description of specific receptors for cannabinoids in the brain and with the subsequent isolation of anandamide, one of the main endogenous cannabinoids (Martin et al., 1999).

Since then, the number of publications about cannabis has been continuously growing, attesting the great interest in research involving the herb (Liu et al., 2021; Treister-Goltzman et al., 2019) (Figure 1).

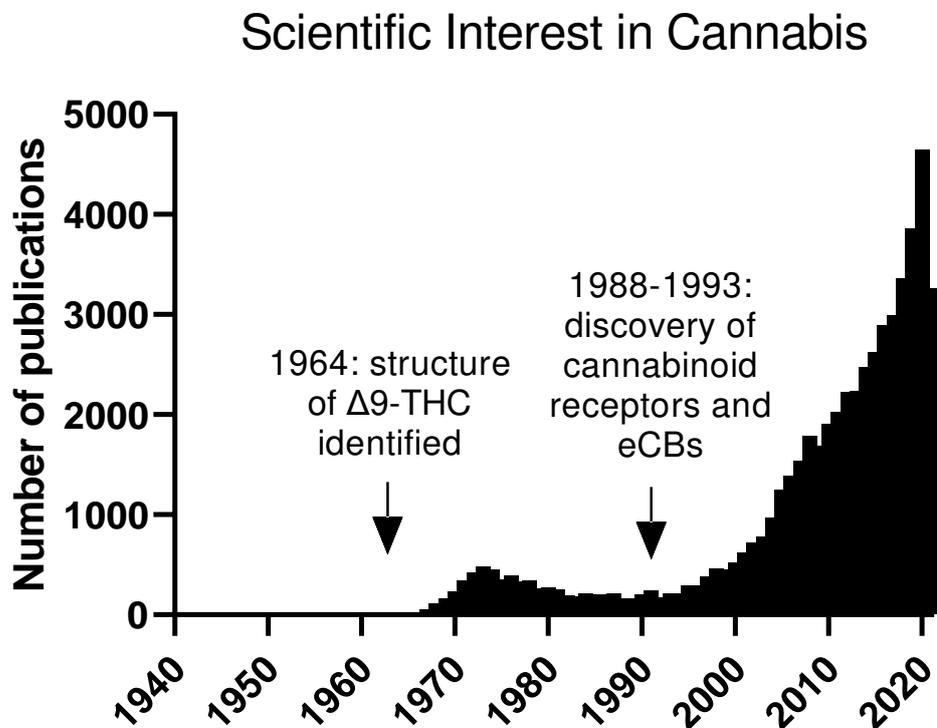


Figure 1. Scientific publications in PubMed containing “cannabinoid”, “cannabis” or “endocannabinoid system” from 1940 to 2021. Key years and discoveries are indicated with the black arrows.

More recently, the clinical use of cannabis-based medical treatments has been rapidly emerging, accompanied by strong debates regarding its legalization and safety concerns (Abuhasira et al., 2018). Pharmaceutical preparations containing different cannabis extracts or synthetic cannabinoids (i.e. synthetic THC called dronabinol as well as a THC-analog known as nabilone) are commercially available in several countries for different medical purposes, for example for treating neuropathic pain in multiple sclerosis, for muscle spasms, for eating disorders or for reducing nausea and vomiting associated with chemotherapy (Borgelt et al., 2013).

In parallel, the recreational use of cannabis is currently being authorized under different legal frames in many countries. This changing legal landscape for the drug has possibly been associated with both increased use and a reduction in the perception of harm, especially in the younger demographics (Cerdá

et al., 2017). Cannabis and related compounds are nowadays the most abused illicit substances worldwide. At the same time, there have been already since the 70s increasing concerns over the link between cannabis use and the risk of psychiatric illness (Kolansky and Moore, 1972; Tennant and Groesbeck, 1972), which has intensified after a series of large scale epidemiological studies and public debate in recent years (Andréasson et al., 1987; Murray et al., 2017).

Moreover, a relatively recent phenomenon is the recreational use of synthetic cannabinoids. Synthetic cannabinoids have been historically synthesized for research purposes in order to investigate the pharmacology of cannabinoid signaling and to develop novel therapeutic agents. In 2008, their recreational use was first recognized, when their presence was identified in products marketed as herbal incense known as “K2”, “Spice” or “Magic Gold” (Auwärter et al., 2009). At that time, the most commonly identified products were derived from the JWH designation (synthesized by John W. Huffman at Clemson University) or CP designation (Pfizer) (Brents and Prather, 2014). Since then, various chemically similar compounds have been identified in such herbal mixtures (Worob and Wenthur, 2019). Synthetic cannabinoids are usually much more potent than THC and have consistently been associated with robust adverse effects including psychotic symptoms and panic attacks, as well as dependence-withdrawal and tolerance (Hermanns-Clausen et al., 2013; Murray et al., 2016; Spaderna et al., 2013)

1.1.2 Epidemiology and Pattern of Cannabis Use

Cannabis continues to be the most widely used psychotropic drug worldwide. Although cannabis consumption might still often be underestimated, the current prevalence has been estimated to approximately 200 million past year users in 2019, corresponding to 4% of the global population. North America has the highest consumption rate at 14.5%, followed by Australia and New Zealand (12.1%) and West and Central Africa (9.4%) (UNODC, 2021) (Figure 2). The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) estimates that in 2021, around a quarter (27.2%) of adults aged 15–64 reported lifetime cannabis use. The prevalence in Europe varies largely depending on the country, with higher prevalence in Mediterranean and Central-Western, and lower in Eastern and Northern European countries (EMCDDA, 2021)

The overall number of people who used cannabis in the past year is estimated to have increased by nearly 18% over the past 10 years (2010–2019), reflecting in part an increase in the global population of about 10% over the same period. This means that, since the 2010s, the past-year prevalence of cannabis use has increased by nearly 5% (UNODC, 2021) (Figure 2). The lack of systematic data in the largest European countries (including France, Germany, and Italy, which make up 40% of the EU population) makes it harder to obtain a conclusive statement for the trend of cannabis use compared to previous years in Europe. However, based on available data, it seems that cannabis use has remained mostly stable in Europe in recent years. On the contrary, the number of users entering treatment for problems related to cannabis has increased by 45% between 2009 and 2019, based on available data from 24 countries (EMCDDA, 2021).

Even though the reason for the diverging trends of cannabis use prevalence and treatment demand in Europe is unclear, it could be explained by several potential factors. Firstly, the increase of cannabis treatment entrants could be the result of changes in cannabis use patterns, with higher frequency of cannabis consumption and larger quantities of cannabis being consumed. General population surveys indicate that around 1.8% of adults aged 15-64 in the European Union are daily or near-daily cannabis users, with most of them (61%) being under 35 (EMCDDA, 2021). Preliminary evidence also suggests that the prevalence of cannabis use disorders might also be associated with cannabis potency levels, through rising THC and decreasing CBD concentrations (Wilson et al., 2019) (Figure 2), or with the increasing use of highly potent synthetic cannabinoids (Murray *et al.*, 2016). In fact, the cannabis sold in Europe right now is much more potent than before, with THC contents ranging between 20% and 28% (EMCDDA, 2021). Lastly, better detection rates of disorders and improved treatment paths, as well as widespread positive changes in the stigma towards mental health, are important factors that could also contribute to the observed trends.

Typically, cannabis use has been more prevalent in men than in women, although more recent studies seem to narrow this sex gap (Chapman et al., 2017). However, there are differences in the motivation for cannabis use, in the age of use onset—with women usually starting later than men—, in the responses to the drug, as well as in the comorbid mental disorders between the sexes (Cooper and Craft, 2018; MischleyLaurie, 2016; Tirado-Muñoz et al., 2020). Still, besides neurobiological factors that could underlie some of these differences, the crucial impact of cultural and socioeconomic factors should not be ignored.

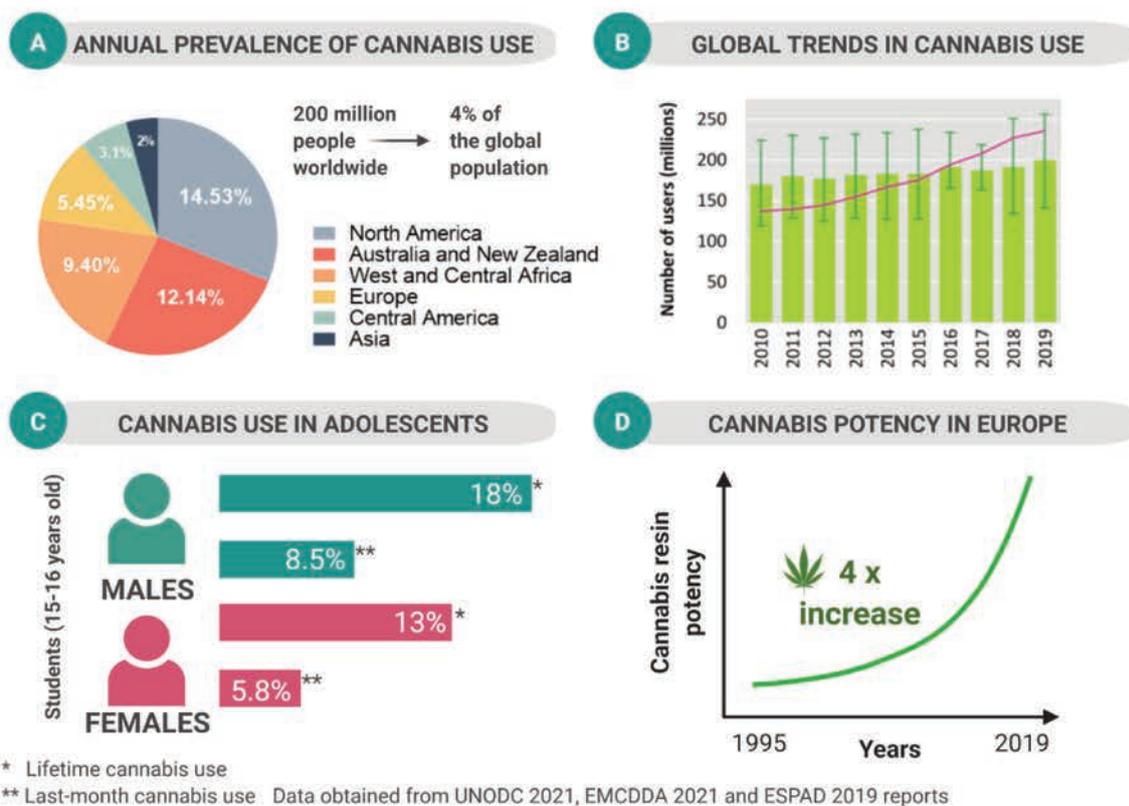


Figure 2. Graphical summary of cannabis epidemiological data. All information represented here are obtained from the following reports: United Nations Office on Drugs and Crime (UNODC) World Drug Report 2021, European Monitoring Centre for Drugs and Drug Addiction.

1.1.3 Cannabis Use during Adolescence

It is well established that the initiation of cannabis use often occurs during adolescence, with heaviest use usually occurring during late adolescence and young adulthood (Coffey and Patton, 2016; Sobesky and Gorgens, 2016; UNODC, 2021). The age of cannabis initiation is of critical importance in understanding the path from recreational to problematic use, as early exposure has been shown to increase the risk of future substance use disorders (Behrendt et al., 2012).

Global comparative data on adolescents' cannabis prevalence has often been difficult to obtain, since adolescent age groups are differently classified across the world and many countries do not perform surveys to track the trends and prevalence. In the United States, the levels of past-year and past-month use of cannabis among high-school students have remained stable over the past 10 years (2011–2020); in 2020, 14.6% of high-school students reported past-month use of cannabis. There has been however a

significant increase in almost daily use of cannabis in the last two recorded years (2019 and 2020). Indeed, in 2020, the daily use of cannabis was estimated at 4.1% among high-school students compared with close to 1% in 1991 (UNODC, 2021). In Europe, based on data from 26 countries that undertook a survey between 2015 and 2020, last-year cannabis use of adults aged 15-34 is estimated at 15.4%. When only 15- to 24-year-olds are considered, this prevalence is even higher, with 19.2% having used the drug in the last year. Furthermore, among the 24 European countries that participated in the 2019 ESPAD survey for middle school students, last-year prevalence of cannabis use had an average of 17.3% (EMCDDA, 2021) (Figure 2). Interestingly, some data suggests that the prevalence of cannabis use in adolescents in low and middle income countries is much lower than in high-income countries (WHO, 2019).

As different factors play different roles in drug abuse by adolescents, it has always been challenging to pinpoint the causes of adolescent cannabis use. These factors often range from peer influence and availability of the drug to family structure (Sefa and Ali, 2020). Mental health problems and stress are also factors that are frequently associated with early initiation of cannabis use (Hawke et al., 2020). However, the widespread increase in availability accompanied by a reduction in perception of harm, renders the need to understand the magnitude of neurobehavioral alterations that can occur due to cannabis use rather urgent. Indeed, the question of safety for adolescent and young users is taken more seriously as more countries have legalized the use of recreational cannabis by adults. Although it remains illegal for minors, there are concerns that the changing legal landscape will result to cannabis products becoming more accessible and attractive to teens. At the same time, the recent debates about medical use of cannabis and measures allowing non-medical use of cannabis in the United States and elsewhere have led adolescents to perceive cannabis as less harmful than they previously had (UNODC, 2021). In Europe, for example, the perception of risk or harm from trying cannabis once or twice among students aged 15–16 declined by nearly half, and the perception of risk or harm from smoking cannabis regularly declined by a quarter over the period 1995–2019 (UNODC, 2021), underlying the clear conflict between the harm perceived by adolescents and the actual potential harm that cannabis products of greater potency could pose to their health.

1.2 Cannabis Use and Adolescence

1.2.1 Adolescence: A Sensitive Period for Brain Development

Adolescence refers to the developmental time period between childhood and adulthood and is generally considered to occur from 12 to 17 years of age (Spear, 2000a). This period represents a critical phase in development, in which major biological, neurobiological, social, and personal transitions are made. The brains of adolescents and young adults undergo substantial change, potentially increasing their vulnerability to the adverse effects of regular substance use on brain development (Bara et al., 2021; Lorenzetti et al., 2019). At the same time, a ‘window of vulnerability’ appears to exist during the adolescent period regarding the onset of certain symptoms of common neuropsychiatric disorders such as anxiety, depression and schizophrenia spectrum disorders (Patton et al., 2016), since many of these mental illnesses are developmental in nature. In fact, mental disorders and substance use disorders often co-occur in adolescence and young adulthood (Hall et al., 2016). This may be because they share risk factors, e.g., shared genetic risks and family environment, or the risk of developing one type of disorder may increase the risk of developing another. For example, cannabis use may increase the risk of psychotic disorders and vice versa, as we will discuss in the following section.

During the adolescent period, the brain undergoes radical functional changes that are associated with a high degree of plastic structural remodeling, including myelination, synaptic pruning, volumetric growth, changes in receptor distribution, and programming of neurotrophic levels. These physiological modifications occur primarily in areas involved in planning and decision-making, such as the prefrontal cortex (PFC) – the cognitive or reasoning area of the brain important for controlling impulses and emotions (Arain et al., 2013; Casey et al., 2008). The brain’s reward system is more active during adolescence than in adulthood, and the adolescent brain is still strengthening connections between its cognitive and emotion-related regions (Sowell et al., 1999). It has been argued that this period involves a shift from a predominantly limbic to a much more frontal cortical control of behavior, with an increase in the inhibitory connections between these two regions (Spear, 2000b). These changes suggest that cognitive control over highly risky behaviors is still under maturation, making teens more inclined to engage in risky behaviors, as is the case for example with early-onset use of illicit drugs (Wilson *et al.*, 2019).

Epidemiological studies have consistently demonstrated that cannabis use in adolescence is associated with vast negative consequences. The onset and magnitude of these consequences however remains under debate and definitive evidence is still elusive. For example, numerous studies have suggested that adolescent cannabis use can be linked to cognitive impairments and increased risk of psychotic disorders (Stringfield and Torregrossa, 2021). However, in almost every domain that researchers have examined, comparing results have been inconclusive regarding the nature and strength of such associations, and there is not a widespread consensus as to whether cannabis directly causes long-term harms, whether it's one of a number of risk factors, or whether it simply correlates with other core causes (Shen, 2020).

1.2.2 Consequences of Adolescent Cannabis Exposure

A big part of research has been examining the effects of adolescent cannabis use in humans, with the majority of studies pointing out at least one or more adverse outcomes related to mental health and overall well-being later in life. The data specifically link early-onset cannabis use with deficits in cognitive processes, an increased likelihood of neuropsychiatric disorders, as well as a transition to other drugs of abuse and dependence in adulthood. Some of these lasting effects of cannabis exposure have been proposed to depend on the exact age of onset, as well as frequency and degree of cannabis use (Blest-Hopley et al., 2018; Ganzer et al., 2016). However, it is important to emphasize again that the literature is not at a consensus, and although many studies have found links between adolescent cannabis use and poor outcomes in adulthood, others show that these are not permanent or may be due to factors other than cannabis use *per se* (Levine et al., 2017).

One important challenge in attributing long-lasting outcomes to cannabis use is that usually regular cannabis users, both adolescents and adults, differ from non-users in many ways that already predict a higher risk of such outcomes (Hall *et al.*, 2016). For example, it is more likely for them to also use alcohol, tobacco, and other illicit drugs or to engage in more risky behaviors (Dhein, 2020; Hall et al., 2020). Moreover, it is important to note that epidemiological studies of chronic cannabis use are rarely able to measure the doses of THC or of other cannabinoids like CBD that cannabis users receive. In the absence of these data, studies define heavy or regular the daily or almost-daily use of cannabis (Hall *et al.*, 2020).

In the following pages, I will summarize evidence from human studies concerning the reported adverse adult outcomes associated with adolescent cannabis use. I will specifically discuss the effects on future substance abuse, cognitive functioning, emotional behavior, and the risk of developing psychiatric disorders like schizophrenia in adulthood. Finally, I will describe some of the structural and functional brain changes related to adolescent cannabis exposure that have been explored through human imaging studies.

Psychosocial outcomes

The regular use of cannabis in adolescence is generally related to poorer educational attainment and educational problems, leading often to socioeconomic consequences (Legleye et al., 2010; Macleod et al., 2004). Recent reviews on the educational outcomes of adolescent cannabis use have found a consistent association between cannabis use and early dropout of school (Dhein, 2020; Hall *et al.*, 2020; Pacheco-Colón et al., 2019). There has been some debate on this association, since one explanation could be that poorer educational performance increases the risk of cannabis use. Early cannabis use has also been associated with unemployment, lower income, or lower work engagement in adulthood (Danielsson et al., 2015; Fergusson and Boden, 2008; Hyggen, 2012). Some of these effects were however overshadowed by confounding factors such as family structure and adverse life course, suggesting that social or genetic factors could also underlie the observed results.

Addiction and future substance abuse

Cannabis use is characterized by the gradual development of tolerance to THC (Ramaekers et al., 2020) and users who seek treatment for cannabis use disorders often report mild, but consistent withdrawal symptoms (including changes in appetite, insomnia, headaches and anxiety) (Budney et al., 2003), posing an important obstacle to abstinence. In 2020, around 111,000 people entering specialized drug treatment in Europe (35% of all treatment demands) named cannabis as their primary problem (EMCDDA, 2021). The age of initiation to cannabis use plays a critical role in the risk of later becoming addicted to cannabis, with 16–17% of those who begin use as adolescents developing addiction (Dhein, 2020).

In line with this, cannabis use among adolescents has long been argued to act as a “gateway drug”, facilitating the later use during adulthood of other drugs (Fergusson et al., 2006). Multiple epidemiological data indicate that early cannabis use is associated with an increased risk of using, abusing or becoming dependent on other illicit substances as well. Adults reporting illicit drug use, such as cocaine, methamphetamine, or heroin, often report prior adolescent cannabis use. This likelihood of future use and dependence increases the earlier a person initiates cannabis and the more they consume it thereafter (Otten et al., 2017; Weinberger et al., 2016).

However, it remains difficult to demonstrate a clear causal link between early cannabis consumption and later heavier drug use since other variables, like the environment or genetic factors, are probably involved. We can briefly discuss three broad explanations when viewing cannabis as a “gateway drug”. First, it makes sense that cannabis users have more practical opportunities to obtain other illicit drugs from the same market that provided them with cannabis. Secondly, early cannabis users might be predisposed to escalate drug use for reasons that are unrelated to cannabis (e.g., higher risk-taking or sensation-seeking behavior) (Levine, Clemenza et al. 2017). Thirdly, it is possible that the pharmacological effects of cannabis can increase an adolescent’s vulnerability to other drugs (Hall *et al.*, 2020). As I will discuss in the second part of this chapter, the adolescent period is critical in the development and maturation of reward related circuitries (Casey *et al.*, 2008) and several studies have established the influence of the ECS on the mesocorticolimbic system, which is heavily implicated in reward processing and addiction (Koob and Le Moal, 2008). Further studies have found that directly enhancing cannabinoid signaling may modulate other neurotransmitter systems (dopamine, opioid, noradrenaline) contributing to drug dependence by priming the brain to the rewarding effects of other drugs (Koob and Volkow, 2016).

Cognitive and Executive Functions

One of the best-known effects of cannabis is its influence on cognitive function, which has been demonstrated in a plethora of studies (Volkow et al., 2014a). Cannabis intoxication produces a range of acute transient and dose-related impairments, including impaired attention and concentration, less accurate decision-making and greater impulsivity as well as deficits in sensory gating, associative learning and set shifting (Ganesh et al., 2018; Ranganathan and D’Souza, 2006). Although some of these acute cognitive effects are reversible, numerous studies have shown that adolescent cannabis use is

specifically associated with poor cognitive outcomes later in adulthood (Crean et al., 2011; Levine *et al.*, 2017). Adults who began cannabis use as adolescents often present impairments in attention, working memory, decision-making, verbal fluency, executive functioning, visuospatial attention, cognitive inhibition and impulsivity (Renard et al., 2016a; Scott et al., 2018). The magnitude of these deficits is proportional to the frequency, dose, and age of onset of use (Renard *et al.*, 2016a).

The permanence of these impairments, however, is in dispute and to date, there are no definitive conclusions about how long do these cannabis effects last. While some studies have reported that cognitive function is never fully restored even in midlife (Curran et al., 2016; Meier et al., 2012), other studies suggest that some of the deficits can diminish after years of abstinence, where the longer this period the greater the improvements (Levine *et al.*, 2017; Tait et al., 2011). Finally, contrasting with other domains, most research into cognition has found that it is only early adolescent users (≤ 15 -16 years of age depending on the study) who are at risk for these deficits, while late-onset adolescent users remain largely unaffected. Moreover, most reports find that the cognitive outcomes among humans affect mostly equally both sexes (Levine *et al.*, 2017)

Affective Disorders

Epidemiological research has described some associations between adolescent cannabis use and an increased likelihood for developing a depressive or anxiety disorder later in adulthood (Gobbi et al., 2019). The risk increases the earlier an individual initiates cannabis use (Hayatbakhsh et al., 2007) or the more frequent the consumption is (Van Laar et al., 2007). Although most data point towards an association between adolescent cannabis use and the presence of a mood or anxiety disorder in adulthood, the direction of causality is not that clear. Affective disorders often surface during adolescence and it can be that for individuals predisposed to these disorders, cannabis is a way to medicate emerging symptoms (Butterworth et al., 2014), although some studies provide evidence against that hypothesis, showing that adolescent depression and anxiety measures do not predict later cannabis use (Gobbi *et al.*, 2019). Overall, the majority of research has found no clear gender differences in affective outcomes, although women seem to be in general more susceptible than men (Levine *et al.*, 2017).

In addition, an increased risk for suicidal tendencies has been associated with cannabis exposure in adolescence. Systematic reviews have found that cannabis use during adolescence was associated with a modest increase in the risk of suicidal ideation and a bigger increase in the risk of suicidal attempts in young adults (Gobbi *et al.*, 2019; Moore *et al.*, 2007), with the strongest effect in those who engaged in heavy cannabis use (Borges *et al.*, 2016). To conclude, it appears that in spite of the difficulty in establishing a causal effect of adolescent cannabis use on long-term emotional well-being, there is a link between age of onset and frequency of use with an increased risk of negative emotional outcomes in later life.

Psychosis

One risk of using cannabis that continues to attract significant attention is the relationship between cannabis and psychosis (Box 1). Indeed, one of the most frequent concerns around adolescent cannabis use refers to the associated risk of psychiatric disorders, particularly psychotic disorders and full-blown schizophrenia, with longitudinal epidemiological studies consistently reporting an association between cannabis use and psychosis (Volkow *et al.*, 2016).

The first robust evidence for adolescent cannabis as a risk factor for psychotic disorders came from the now classic paper on the Swedish Conscripts Study, led by Andreasson and colleagues, in which 45,570 military recruiters (97% of all Swedish males aged 18–20 years) were followed-up for 15 years (Andréasson *et al.*, 1987). The researchers observed a dose–response relationship between cannabis use at the beginning of the military service and hospitalization for a psychotic disorder over the next 15 years, with the highest risk in those who had smoked cannabis more than 50 times. Even after adjusting for potential confounding factors, such as psychiatric illness at the time of recruitment or other drug abuse, the risk remained significantly high (Radhakrishnan *et al.*, 2014). In a later follow up study of the same cohort, Zammit and colleagues replicated the findings, showing that cannabis use was associated with an increase in the risk of developing schizophrenia (Zammit *et al.*, 2002). Since then, the Swedish cohort findings have been supported by multiple subsequent longitudinal studies (Arseneault *et al.*, 2002b; Fergusson and Horwood, 2001; Fergusson *et al.*, 2003; Henquet *et al.*, 2005; Stefanis *et al.*, 2004; Van Os *et al.*, 2002). All studies found a relationship between adolescent cannabis use and the risks of either psychotic disorders or psychotic symptoms. Systematic reviews of these studies found that the effects were more common among regular cannabis users (Moore *et al.*, 2007),

and that the association is even stronger in those who consumed more potent types of cannabis (Marconi et al., 2016). Currently consistent evidence keeps emerging in support of the association between adolescent cannabis use and the risk of psychotic symptoms (D'Souza et al., 2009; Di Forti et al., 2019; Stilo and Murray, 2019). At the same time, psychotic reactions are increasingly being reported following the acute use of synthetic cannabinoids (Castaneto et al., 2014) and accumulating evidence suggests that more chronic psychotic disorders can also occur (Deng et al., 2018). To date, human studies on this issue do not show a strong gender focus, since some of the large longitudinal studies included only male participants (Andréasson *et al.*, 1987), while many of the studies did not selectively adjust for sex in their analysis.

Box 1. The Debate on Cannabis and Psychosis

Cannabis intoxication can produce a range of transient, dose-related, schizophrenia-like symptoms in healthy individuals. But despite these transient psychotic-like experiences during the intoxication period, most individuals who try cannabis do not end up developing persistent psychosis or schizophrenia. Of course, the etiology of schizophrenia is complex and reflects an interaction between genetic vulnerability and environmental contributors, including cannabis use as an important environmental risk factor.

The question of which specific factors account for the vulnerability to the psychotogenic effects of cannabis is still open, with researchers arguing on whether cannabis can directly cause schizophrenia, or if it primarily triggers and/or accelerates schizophrenia progression in a subgroup of individuals already predisposed to developing the disorder (Shen, 2020). Many researchers supporting the latter theory (Hill, 2015), argue that the hypothesis of a causal role is inconsistent with the absence of an increase in the prevalence of schizophrenia —often cited to be around 1% (Saha et al., 2005)—, over a period when cannabis use has dramatically increased. The evidence on that is rather mixed. While one study failed to find any increase in the incidence of psychosis following the increased cannabis use since the 1980s (Degenhardt et al., 2003), another study concluded that it is still too early to detect such increases (Hickman et al., 2007). Moreover, it is difficult to decide if cannabis use has indeed any effects on psychosis incidence because the increase in risk is expected to be relatively modest, while any kind of interpretation is further complicated by changes in diagnostic criteria, psychiatric services, and the quality of administrative available data (Volkow et al., 2016).

Complicating matters even more, the neurobiological mechanisms behind psychotic disorders themselves are still far from fully understood, and many other factors can influence mental health outcomes. Despite all this ambiguity however, there is strong physiological and epidemiological evidence supporting a mechanistic link between cannabis use and psychosis.

Changes in brain structure

Neuroimaging studies, using techniques such as magnetic resonance imaging (MRI) or positron emission tomography (PET), have compared adolescent cannabis users to control non-users on the volumes of specific brain regions. Overall, they have reported differences in the volume of the hippocampus, amygdala, cerebellum, striatum, insula, temporal pole and prefrontal cortex (Lorenzetti *et al.*, 2019). Both increases and decreases in regional volumes have been reported, together with an altered grey and white matter architecture (Bava *et al.*, 2009), suggesting a potential disruption of the pruning process. However, conflicting results have also been described with some recent studies finding no significant differences between adolescent daily cannabis users and non-users regarding global or regional brain volumes or cortical thickness (Scott *et al.*, 2019; Weiland *et al.*, 2015).

Adolescent cannabis use has also been associated with some differences in the structure of prefrontal and temporal brain regions and with weak functional connectivity between regions (Chye *et al.*, 2019; Jacobus *et al.*, 2019; Lichenstein *et al.*, 2017). Some of these impairments in brain connectivity following adolescent cannabis exposure are consistent with many preclinical findings (described in detail in Section 3 of this chapter), indicating that the cannabinoid system plays an important role in synapse formation during brain development (Bara *et al.*, 2021).

2 From Cannabinoids to the Endocannabinoid System

2.1 A General Overview of the Endocannabinoid System (ECS)

The discovery and characterization of the endogenous cannabinoid system represents a great milestone in neuroscience research in the last decades. The ECS is a widespread neuromodulatory system, whose functions are difficult to list one by one since almost all biological processes can involve endocannabinoid signaling in specific conditions (Busquets-Garcia et al., 2018a; Ligresti et al., 2016; Mechoulam and Parker, 2013; Rodríguez de Fonseca et al., 2005). Found in nearly all animals, from primitive species such as Cnidaria to mammals, the early emergence of the ECS in the evolutionary process, indicates its biological importance (Elphick and Egertova, 2001; Salzet and Stefano, 2002; Silver, 2019).

Although discoveries of major neurotransmitter systems occurred many decades ago, the ECS remained unknown until the early 1990s. This was in part due to the lack of research on phytocannabinoids, which was made difficult by legal constraints. Indeed, many of the major phytocannabinoids were not identified, but eminent scientists were significantly contributing to the structural elucidation of cannabinoids [(Adams, 1942; Jacob and Todd, 1940), for a recent review see (Appendino, 2020)]. The publication of the chemical structure of Δ^9 -tetrahydrocannabinol (THC) in 1964 (Gaoni and Mechoulam, 1964), further contributed to a proliferation of studies on the biochemistry, metabolism, and pharmacology of the active constituents of cannabis. However the actual mechanism of action of THC remained unknown for more than 20 years. As THC and most cannabinoids are lipid molecules, early hypotheses suggested that they might act non-specifically by altering cell

membranes properties, which are very sensitive to lipid composition. Despite some evidence supporting this hypothesis, large amount of data seemed to point to more specific mechanisms of action. Indeed, in the late 1980s-early 1990s, the target of THC, namely the “cannabinoid receptor type 1” (CB1), was discovered (Devane et al., 1988) and subsequently cloned (Matsuda et al., 1990). Some years later, the second cannabinoid receptor, namely the “cannabinoid receptor type 2” (CB2) was characterized (Munro et al., 1993).

Following the discovery of the membrane receptors that bind phytocannabinoids, the endogenous ligands of these receptors, the so-called endocannabinoids were quickly identified as well. First, the N-Arachidonylethanolamide (AEA), which is a long-chain fatty acid amide, also known as "anandamide", from the Sanskrit word "ananda" which means bliss (Devane et al., 1992). Then, in 1995 another main endocannabinoid was discovered, the 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995). Endocannabinoids are produced from lipid precursors metabolized by enzymes located in cell membranes. The basic levels of endocannabinoids are often called the “endocannabinoid tone”, which varies based on the specific tissue (Howlett et al., 2011). It is important to highlight here that most components of the ECS are multifunctional, meaning that rather than being an isolated system, the ECS influences, and is influenced by, many other signaling pathways. Indeed, the ECS is interacting with many neurotransmitter and lipid signaling systems, being part of a wide functional network (Cristino et al., 2020; Lutz, 2020).

A multitude of studies on the endocannabinoid system have been published in recent years (Di Marzo and Piscitelli, 2015; Kilaru and Chapman, 2020; Lu and Mackie, 2020; Maccarrone et al., 2014; Meyer et al., 2018) and the details that have emerged on the machinery for endocannabinoid signaling, and the number of physiological and behavioral processes that are regulated by the ECS have remarkably expanded (Busquets-Garcia *et al.*, 2018a; Busquets Garcia et al., 2016).

2.2 Composition and Expression of the ECS

As introduced above, the cannabinoid receptors (CBRs), their endogenous ligands (endocannabinoids) and the proteins responsible for their transport, synthesis and degradation are the main components forming the ECS. The most well studied components are the two cannabinoid receptors and their main endocannabinoid ligands, anandamide and 2-AG. As explained below, the

enzymes involved in the synthesis and degradation of anandamide and 2-AG, also received notable attention due to their ability to modulate endocannabinoid concentration and the resulting signaling responses.

2.2.1 Cannabinoid Receptors: Focus on CB1 receptors

CB1 and CB2 receptors belong to the large family of seven transmembrane domain receptors coupled to G proteins (GPCRs) (Howlett, 2002), featuring the many typical characteristics of GPCRs which makes them highly versatile and adaptable, for example, regarding ligand binding, intracellular signaling coupling, homo- and hetero-dimerization (Lutz, 2020). The homology between CB1 and CB2 receptors is relatively small, even in regions that are well conserved among GPCRs. The main difference between the two receptors is in the extracellular end that is 74 amino acids longer for CB1, compared to CB2 (Howlett, 2002; Shahbazi et al., 2020). Since the main topic of my doctoral research involves the functions of CB1 receptors, the focus of the following pages will be on the CB1 receptor, the main cannabinoid receptor in the nervous system, although some aspects of CB2 receptors may also be addressed.

The discovery and study of the functions of CB1 receptor is undoubtedly one of the most fascinating chapters in the physiology of multicellular organisms (Piazza et al., 2017). While the highest levels of expression are observed in the brain, CB1 receptors are widely distributed in the body and are present in a large number of organs, including muscles (Heyman et al., 2012; Mendizabal-Zubiaga et al., 2016a), the gastrointestinal tract (Izzo et al., 2015), the liver (Kunos and Osei-Hyiaman, 2008), the pancreas (Linari et al., 2009; Silvestri and Di Marzo, 2013), the adipose tissue (Cota et al., 2003) and others. CB2 receptor expression has been mostly described in peripheral immune cells (Maccarrone et al., 2015) and microglia (Tanaka et al., 2020), although it was eventually also detected in neurons (Jordan and Xi, 2019). In the central nervous system, CB2 receptor expression is reported to be 100 to 200 times less abundant than CB1, however in some conditions, such as chronic pain, inflammation or stroke, its expression has been found to be upregulated (Lutz, 2020).

In the brain, CB1 receptor is present in virtually all brain regions, including the basal ganglia, the hippocampus, the limbic system, the cortex, the olfactory system, the cerebellum, and the hypothalamus (Herkenham et al., 1990; Marsicano and Kuner, 2008; Marsicano and Lutz, 1999) (Figure 3) and all major cell types i.e., neurons and glial cells. CB1 receptors are also present in neurons of all the major

neurotransmitter systems (glutamate, GABA, noradrenalin, serotonin, acetylcholine, and possibly also in dopaminergic neurons) (Busquets-Garcia *et al.*, 2018a; Lutz, 2020).

The CB1 receptor expression levels can be varied, depending on the brain region and cell type (Marsicano and Kuner, 2008; Oliveira da Cruz *et al.*, 2016). For example, the central amygdala contains very low levels of CB1 in a few GABAergic neurons, while very high levels of CB1 are detected in hippocampal and neocortical GABAergic interneurons (Lutz, 2020). In general, glutamatergic neurons contain relatively low levels of CB1 receptors (Katona *et al.*, 2006; Monory *et al.*, 2006). Finally, CB1 receptors are also detectable in astrocytes (Gutiérrez-Rodríguez *et al.*, 2018; Han *et al.*, 2012), oligodendrocytes (Ilyasov *et al.*, 2018), and adult neural stem cells (Aguado *et al.*, 2006). Importantly, the relative high or low CB1 receptor expression does not necessarily indicate the functional importance of the receptor in a particular physiological process, demonstrating that, for the ECS, quantity doesn't always equal quality (Busquets-Garcia *et al.*, 2018a; Lutz, 2020).

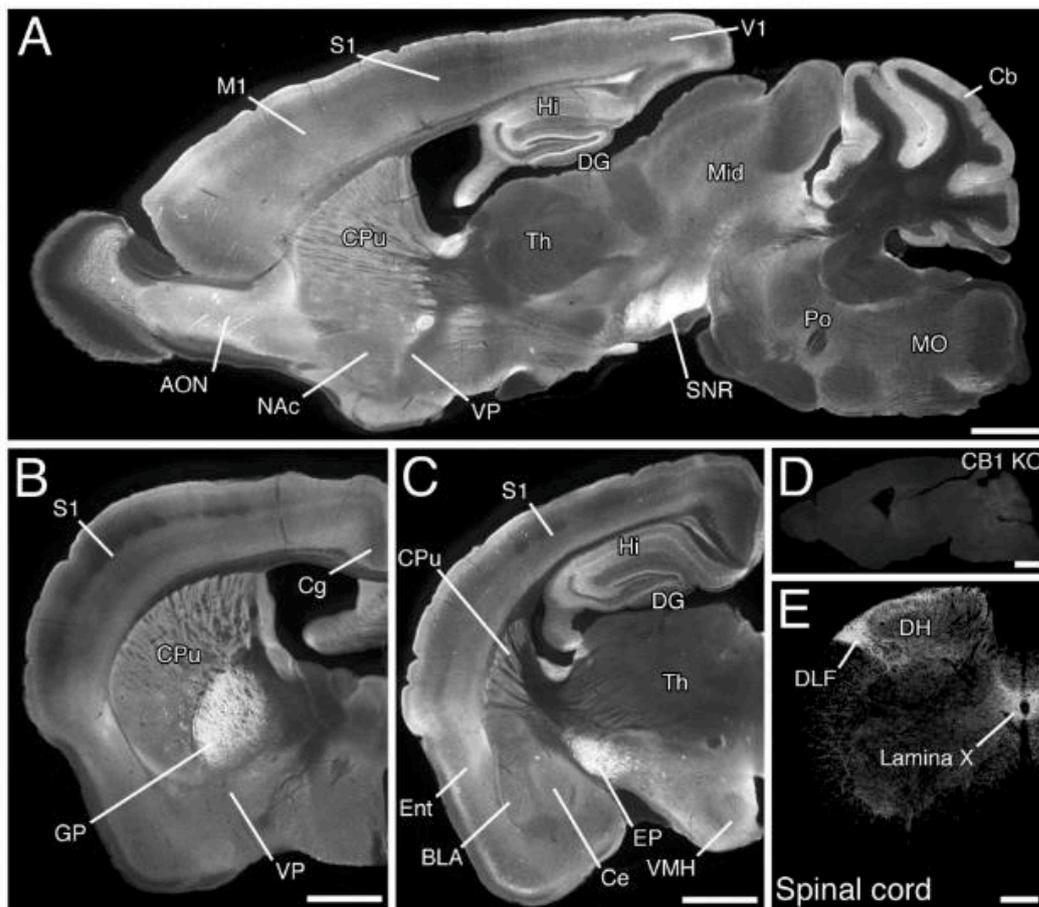


Figure 3. Distribution of CB1 receptors in the adult mouse brain. Immunolabelling of the CB1 receptor protein in sagittal sections of wild-type (A) and CB1-KO mice (D), and in coronal sections of wild type mice (B, C and E). High levels of CB1 expression are found in the anterior olfactory nucleus (AON), neocortex (M1, primary motor cortex; S1, primary somatosensory cortex; V1, primary visual cortex), caudate putamen (CPU), hippocampus (Hi), thalamus (Th), basolateral (BLA) and central (Ce) amygdaloid nuclei, cerebellum (Cb) and spinal cord (E). CB1 protein is absent in the CB1-KO mouse brain (D). Scale bars: 1 mm (A-C, E), 200 μ m (D). From Kano et al., 2009.

Cellular localization and expression of CB1 receptors

CB1 receptors are primarily located at presynaptic terminals (Freund et al., 2003), where their activation can suppress the presynaptic release of many neurotransmitters on a short- or long-term scale. This mechanism is very well studied for glutamatergic and GABAergic synapses, where endocannabinoids generated in the postsynaptic site and retrogradely travelling to the presynaptic site, stimulate CB1 receptors. This results in a short-term decrease in calcium influx at the presynaptic terminal that leads to two forms of short-term synaptic plasticity, namely depolarization-induced suppression of excitation (DSE) at glutamatergic synapses, and depolarization-induced suppression of inhibition (DSI) at GABAergic synapses (Diana and Marty, 2004; Kano et al., 2009; Katona and Freund,

2012; Lutz, 2020). The majority of studies have described 2-AG as the principal retrograde endocannabinoid that mediates DSE and DSI, with the role of anandamide in these processes still not completely understood (Lutz, 2020). Following the discovery of DSI and DSE, endocannabinoids were shown to also mediate long-term forms of synaptic plasticity. Examples of endocannabinoid-mediated long-term depression (LTD) have been reported at excitatory synapses in striatum, nucleus accumbens, neocortex and cerebellum (Gerdeman et al., 2002; Kreitzer and Malenka, 2005; Robbe et al., 2002; Sjöström et al., 2003), as well as at inhibitory synapses in the hippocampus, amygdala and hypothalamus (Bains et al., 2015; Chevaleyre and Castillo, 2003; Marsicano et al., 2002), which are named inhibitory long-term depression (iLTD, I-LTD or LTDi). In contrast to short-term plasticity, LTD induction requires long-lasting mobilization of endocannabinoids that activate CB1 receptors for several minutes, and its maintenance relies on molecular changes, likely at presynaptic level (Castillo et al., 2012). Finally, although neuronal CB1 receptor is predominantly expressed at the presynaptic level, CB1 expression at postsynaptic structures has also been reported in electrophysiological experiments (Bacci et al., 2004; Marinelli et al., 2009a). Moreover, many studies during the last years have shown the crucial importance of astrocytic CB1 receptors in the control of cellular, synaptic, metabolic and behavioral functions of the brain (Covelo et al., 2021; Han *et al.*, 2012; Jimenez-Blasco et al., 2020; Navarrete and Araque, 2008; Navarrete and Araque, 2010; Oliveira da Cruz *et al.*, 2016; Robin et al., 2018).

Intracellular localization of CB1 receptors: receptor trafficking and mitochondrial CB1 receptors

The intercellular trafficking of GPCRs is critical for their regulation. CB1 receptor is synthesized in the soma and from there gets transported to its final membrane location. The distribution of the receptor in different intracellular sites is highly influenced by its activation status. For example, activation with a CB1 receptor agonist increases its localization in endosomes and decreases it at the presynaptic terminals, while treatments with CB1 antagonists can decrease the receptor's presence in endosomes, suggesting that under a dynamic balance, CB1 is consistently activated and internalized in the soma (Lutz, 2020).

Besides receptor trafficking, CB1 has also been detected at mitochondrial membranes of both neurons and glia, and is often described as mitochondria-associated CB1 receptor or mtCB1 receptor (Benard et al., 2012; Gutiérrez-Rodríguez *et al.*, 2018) (Figure 4). MtCB1 can mediate the reduction in mitochondrial oxygen consumption following ligand stimulation, eventually decreasing the production

of adenosine triphosphate (ATP) (Benard *et al.*, 2012; Hebert-Chatelain *et al.*, 2014; Mendizabal-Zubiaga *et al.*, 2016a). Moreover, cannabinoids have been shown to regulate the complex I of the electron transport chain by modulating the activity of the mitochondrial protein kinase A (PKA) and downstream phosphorylation events (Hebert-Chatelain *et al.*, 2016). More recently, we showed that activation of astrocytic mtCB1 receptors inhibits glucose metabolism and lactate production, subsequently altering neuronal functions (Jimenez-Blasco *et al.*, 2020).

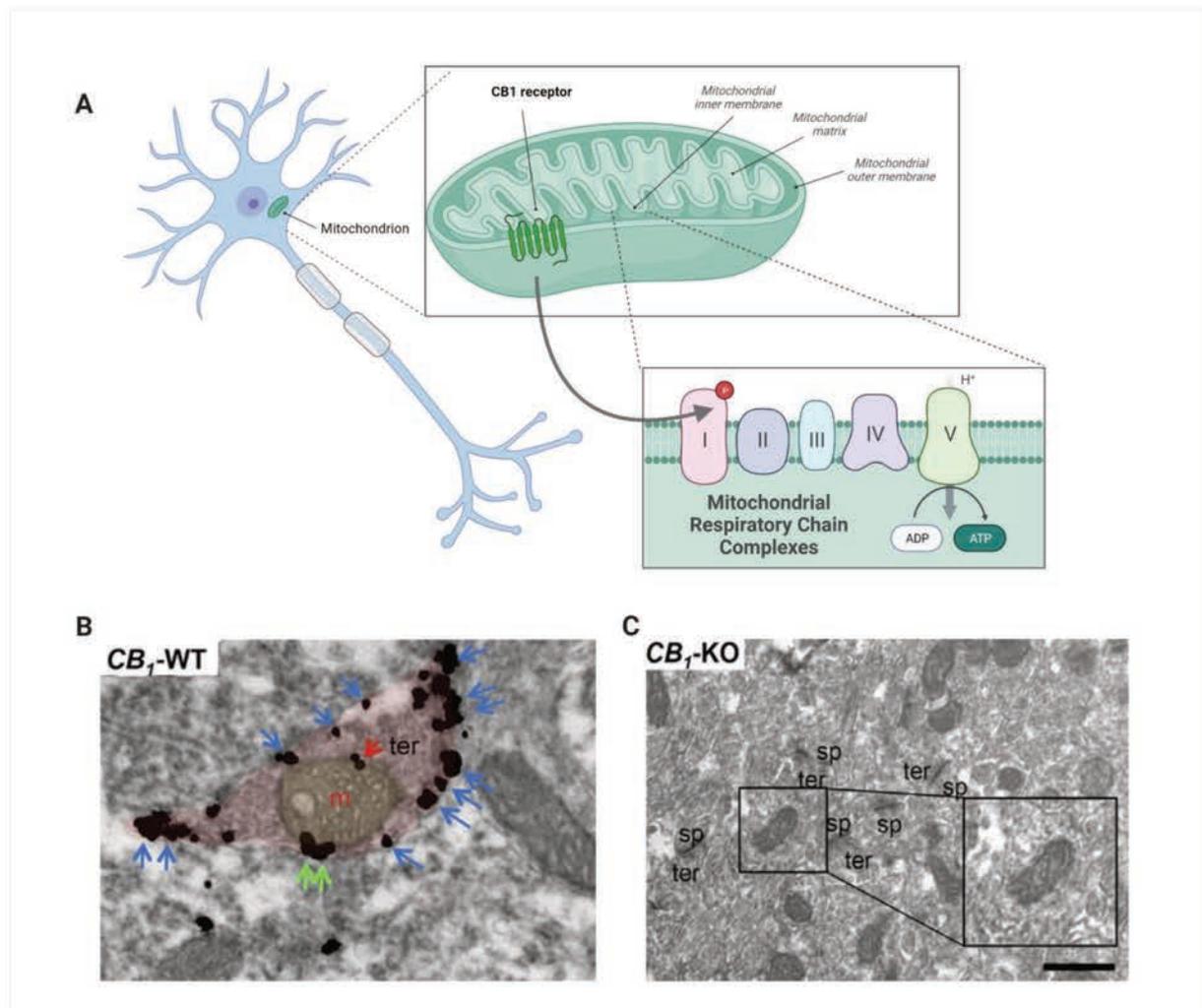


Figure 4. Intracellular localization of CB1 receptors in mitochondrial membranes. (A) Schematic representation of CB1 receptor's presence in mitochondrial outer membrane of neurons and effect of its activation to the mitochondrial electron transport chain. (B, C) From Hebert-Chatelain *et al.*, 2014. Immunoelectron detection of CB1 receptors in neuronal hippocampal mitochondria in CB1-WT (B) and CB1-KO mice (C). CB1 immunoparticles are on presynaptic terminal (ter) membranes (blue arrows) and on mitochondrial (m) membrane segments close to (distance <80 nm; green arrows) or far away from (distance >80 nm; red arrows) other membranes.

CB1 receptor-dependent signaling pathways

The CB1 receptor is one of the most abundant GPCRs in the brain, with protein levels in the same range as of components of major neurotransmitter systems, such as NMDA or GABA_A receptors (Pertwee *et al.*, 2010). As is the case with many GPCRs, CB1 receptors have a rich repertoire of intracellular signaling cascades upon ligand activation and their complexity still leaves much more to be understood about how CB1 receptor signaling gains its high specificity depending on the cellular context (Busquets-Garcia *et al.*, 2018a; Lutz, 2020). CB1 receptors modify cellular activities through activation of G proteins, mainly the inhibitory Gi/o (Howlett, 2002), although evidence for coupling with other G protein subunits, such as Gs or Gq in specific cell types (striatal neurons and astrocytes, for instance) has been accumulated over the last decades (Figure 5) (Díaz-Laviada and Ruiz-Llorente, 2005; McAllister and Glass, 2002; Navarrete and Araque, 2010). Activation of Gi/o by the CB1 receptor leads to a decreased production of cyclic adenosine monophosphate (cAMP) and an inhibition of calcium channels, resulting in decreased calcium influx upon stimulation (Figure 5) (Pertwee *et al.*, 2010). Overall, CB1 receptor signaling depends on the availability of the cell's G protein machinery and differences in the G protein coupling have been proposed as a possible mechanism to explain for example how low levels of CB1 receptors in glutamatergic hippocampal neurons show higher functional activity than the higher levels in GABAergic interneurons (Busquets-Garcia *et al.*, 2018a; Lutz, 2020). Moreover, the regulator of G protein–signaling (RGS) proteins also play an important role in the control of CB1 receptor signaling (Lutz, 2020; O'Brien *et al.*, 2019).

CB1 receptors are able to stimulate different cellular pathways including (among others): the mitogen-activated protein kinases / extracellular signal-regulated kinase (MAPK/ERK), c-Jun N-terminal kinases (JNK), mammalian target of rapamycin (mTOR) and phosphatidylinositol 3-kinases (PI3K) pathways (Turu and Hunyady, 2009). In addition, CB1 receptors signaling can also be mediated by β -arrestin pathways. CB1 receptor couples with β -arrestins, leading to its internalization following ligand activation, but β -arrestins act also as signal transducers for pathways, such as ERK and JNK (Raehal and Bohn, 2014; Turu and Hunyady, 2009).

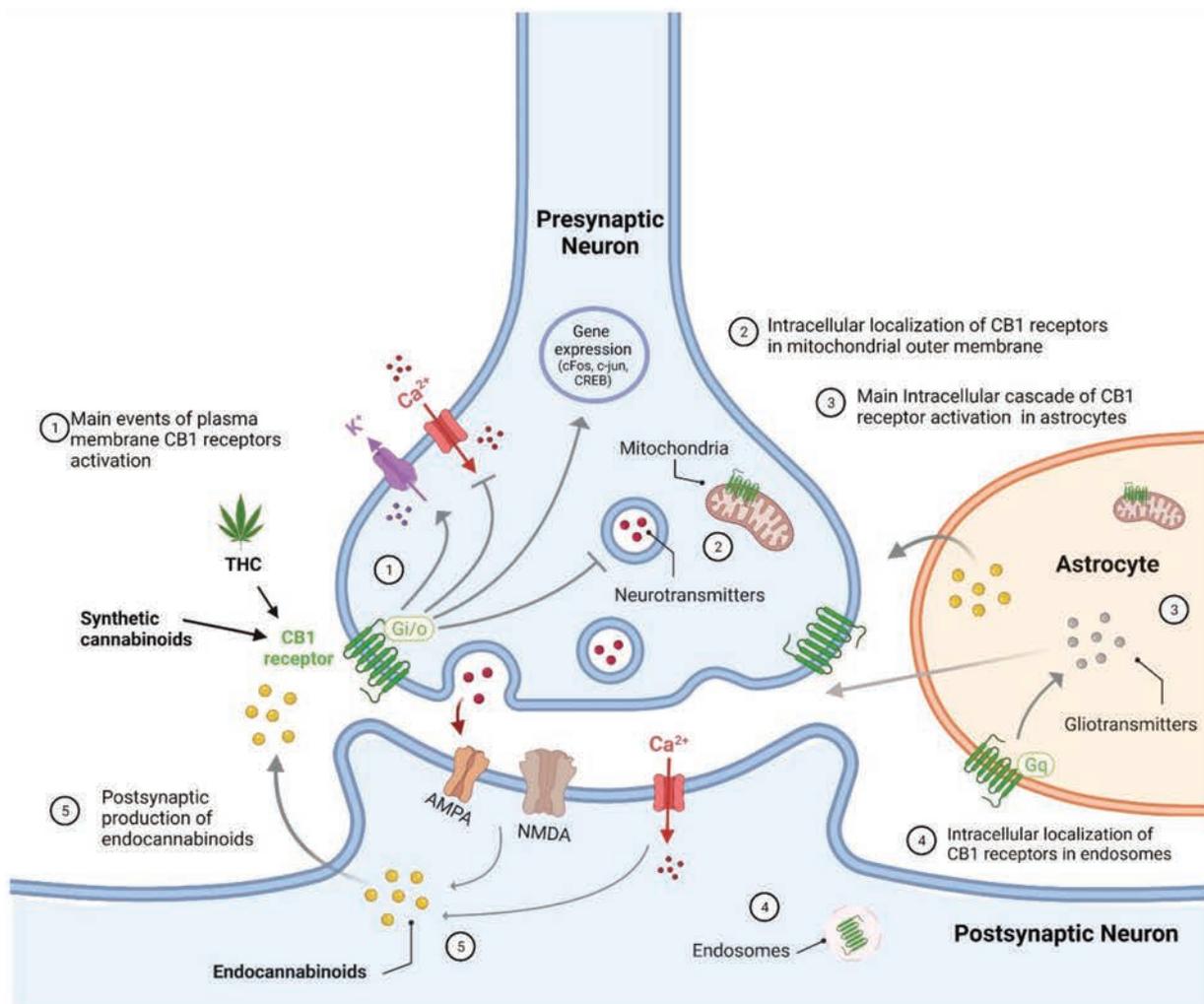


Figure 5. Schematic view of CB1 receptors localization at the synapse and main mechanisms of endocannabinoid action and CB1 receptors activation. CB1 receptors are present at both presynaptic terminals and postsynaptic compartments of neurons and on astrocytes (3), exerting different impacts at the tripartite synapse. They are also present at mitochondrial membranes of both presynaptic and somatodendritic compartments of neurons and of astrocytes (2), as well as endosomes (4). Post-synaptic calcium elevation (depolarization or activation by metabotropic glutamate or muscarinic acetylcholine receptors) induces the synthesis of endocannabinoids (5) that bind to presynaptic CB1 receptors. Activation of the protein Gi/o coupled to CB1 receptor modulates neurotransmitter release and gene expression through several intracellular pathways (1).

2.2.2 Endogenous Ligands-Endocannabinoids

The endocannabinoids are defined as the endogenous ligands for cannabinoid receptors (Di Marzo and Piscitelli, 2015). They are mainly lipids derived from amides or esters of long chain fatty acids (arachidonic acid). The first endocannabinoid to be identified was N-arachidonylethanolamide (AEA) or anandamide (Devane *et al.*, 1992), and the second one, the 2-Arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995). Over the past years, CB1 receptors have been the focus of a number of studies identifying more endogenous orthosteric and allosteric ligands. Although the main endocannabinoids are lipids, hemopressin, an alpha hemoglobin-derived peptide, was found to serve as an inverse agonist of CB1 receptors (Heimann *et al.*, 2007). Peptide derivatives of hemopressin, called pepcans, have also been shown to act as negative allosteric modulators of CB1 receptors (Bauer *et al.*, 2012). Additional endogenous modulators have been found, including the lipids oleamide (Fowler, 2004; Leggett *et al.*, 2004), lipoxin A4 (Pamplona *et al.*, 2012) and pregnenolone (Vallee *et al.*, 2014). Lipoxins and the neurosteroid pregnenolone also act as allosteric modulators, with lipoxin A4 increasing and pregnenolone inhibiting CB1 receptor signaling. Pregnenolone is particularly interesting, because its production in the brain is induced by excessive activation of CB1 receptors. By acting as an allosteric inhibitor, therefore, this neurosteroid represents a clear endogenous molecular inhibitory loop to limit the excessive activation of CB1 receptors (Vallee *et al.*, 2014).

Synthesis, transport and degradation of endocannabinoids often occur in a short period of time and only when cells are under particular conditions. Thus, endocannabinoids actions are often referred to as “on demand”, because they are synthesized *de novo* where and when they are required, and rapid degradation decreases their activity (Piomelli, 2003).

Synthesis of endocannabinoids

The main described biosynthetic pathways of endocannabinoids start from the hydrolysis of membrane phospholipid precursors by specific enzymes. Anandamide is synthesized in the plasma membrane of cells in two steps. The first is the formation of the N-arachidonyl-phosphatidylethanolamine (NAPE) from arachidonic acid and phosphatidylethanolamine by the enzyme N-acyltransferase (NAT). NAPE subsequently gives rise to anandamide, with the action of a specific phospholipase, the NAPE-PLD (Cadas *et al.*, 1997; Okamoto *et al.*, 2007) (Figure 6).

For 2-AG, two synthetic pathways, both of which start from phosphatidylinositol, have been described (Piomelli, 2003). The first one involves the action of the phospholipase C (PLC), which transforms phosphatidylinositol in 1,2-diacylglycerol (1,2-DAG), which is then hydrolyzed into 2-AG, by the enzyme diacylglycerol lipase (DAGL) (Stella et al., 1997) (Figure 6). The second pathway requires the phospholipase A1, which transforms phosphatidylinositol in 2-arachidonoyllysophospholipid, which is further hydrolyzed into 2-AG (Piomelli, 2003).

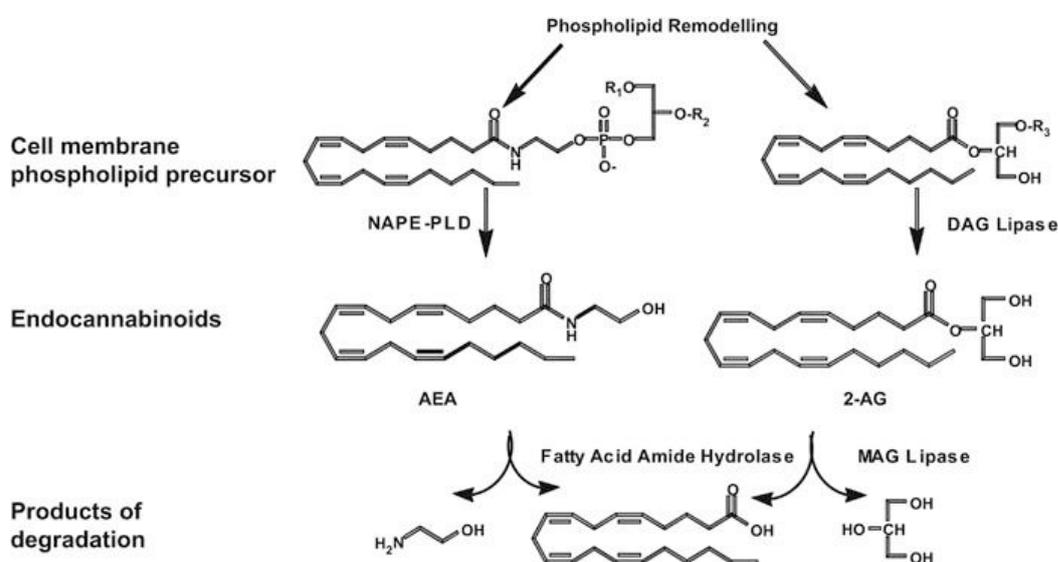


Figure 6. Synthesis and enzymatic degradation of the two main endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG). DAG, diacylglycerol; MAG, monoacylglycerol; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D. From Gatta-Cherifi and Cota, 2016.

Transport of endocannabinoids

As they are lipids, endocannabinoids can move within membranes, and thereby bind and activate cannabinoid receptors in the same cells where they are produced through autocrine-like mechanisms (Bacci *et al.*, 2004; Marinelli et al., 2009b). In parallel, endocannabinoids, as we saw in the previous pages, are thought to primarily act as retrograde signaling molecules at synapses, which implies that endocannabinoids synthesized postsynaptically need to travel through aqueous media, within the cytosol and across the synaptic cleft, in order to activate cannabinoid receptors presynaptically. Many hypotheses have been proposed to explain how the hydrophobic endocannabinoids diffuse in and out of the cell. Several intracellular proteins that bind to anandamide have been reported, including fatty acid

binding proteins (FABPs) (Elmes et al., 2015), as well as an anandamide transporter, a catalytically silent variant of the degrading enzyme FAAH (Fu et al., 2012). It has also been shown that newly synthesized anandamide could also be released into secreted vesicles from microglial cells (Gabrielli et al., 2015). Unlike some existing evidence for the transport of anandamide, the mechanisms of 2-AG transport is still unknown (Di Marzo and Piscitelli, 2015).

Degradation of endocannabinoids

After their synthesis and actions, endocannabinoids are rapidly inactivated by two subsequent mechanisms, cellular reuptake and intracellular enzymatic degradation (Piomelli, 2003). The cellular reuptake accounts for passive diffusion and facilitated transport. The mechanisms of potential facilitated transport still remains mostly elusive, although certain proteins have been proposed as candidates (Beltramo and Piomelli, 2000; Nicolussi and Gertsch, 2015). Once they enter the cell, endocannabinoids are inactivated by specific enzymes, the fatty acid amide hydrolase (FAAH) for anandamide and the monoacylglycerol lipase (MAGL) for 2-AG (Mechoulam and Parker, 2013; Silver, 2019) (Figure 6). At a lower extent, 2-AG can also be hydrolyzed by the serine hydrolase ABHD6 (Marrs et al., 2010) or oxidized by the cytochrome c oxidase subunit II (COX2), a mitochondrial enzyme involved in the electron transport chain (Feledziak et al., 2012). Evidently, the enzymatic degradation of endocannabinoids plays a crucial role in the control of their overall tissue levels. Thus, it has been shown how the genetic deletion of FAAH or MAGL greatly increases endocannabinoid tissue levels (Cravatt et al., 2001; Schlosburg et al., 2010).

2.3 Toolbox to Dissect the Functions of the ECS

The widespread distribution of the components of the ECS and the complex cannabinoid signaling, renders necessary the use of sophisticated methods in order to fully understand its different roles in biological processes and behavior. Accordingly, several tools for the study of the ECS have been developed and discussed in recent years (Di Marzo, 2018; Lutz, 2014; Monory and Lutz, 2009). Such tools vary from cannabinoid receptor agonists, antagonists and allosteric modulators to inhibitors of endocannabinoid biosynthesis and degradation, and to different genetic approaches. Especially when it comes to CB1 receptors, the complexity of their functions due to their broad distribution in several brain regions, circuits and cell types, requires a set of tools in order to obtain interpretable data. Thus, in the following paragraphs, I will summarize some of these tools and approaches commonly used to dissect the role of CB1 receptors in animal behavior. While advanced methods such as cutting edge microscopy, optogenetic and chemogenetic tools, or sophisticated electrophysiological techniques can allow for a highly specific examination of CB1 receptors, here I will keep the focus relevant to my thesis work and on two main approaches – the pharmacological and the genetic one.

Pharmacological tools

Pharmacological tools allow the investigation of the temporal and the spatial function of CB1 receptors. For example, while a mouse is performing behavioral tasks, the local injection of a CB1 antagonist can highlight the CB1-dependent behavioral contribution within a specific brain region. Besides natural ligand components like THC, numerous synthetic cannabinoids have been also used as full agonists with high activity, as well as partial agonists with mild affinity for the receptor. The best characterized synthetic agonists, that present even higher affinity and potency than THC, are WIN 55,212-2, HU-210 and CP55940 (Pertwee *et al.*, 2010). At the same time, selective CB1 receptor antagonists are also often used, with the best characterized being rimonabant (SR141716A) and AM-251 (Pertwee *et al.*, 2010). By acting at orthosteric sites, CB1 receptor antagonists block all G protein dependent signaling pathways (Chorvat, 2013). In contrast, recent allosteric modulators (e.g. pregnenolone and its derivatives) have been described to act as pathway-specific negative modulators. By binding at allosteric sites, they can be used to block excessive CB1 receptor activation in pathological conditions (e.g. models of cannabis addiction or cannabis-induced psychosis) (Busquets-Garcia *et al.*, 2017b; Vallee *et al.*, 2014) (Figure 7).

Overall, pharmacological compounds are powerful tools to activate or inhibit CB1 receptors. However, they intrinsically lack cell type specificity, thereby limiting the information they can provide on specific circuits where CB1 signaling is involved. That is where genetic tools come in handy.

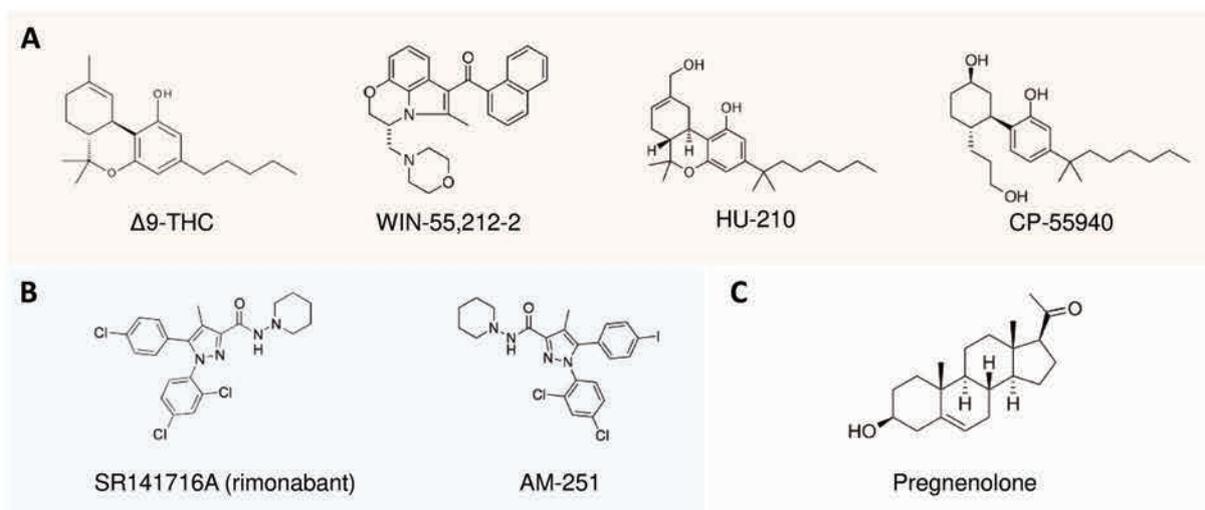


Figure 7. Chemical structure of exogenous ligands of cannabinoid receptors. (A) Natural and synthetic agonists (B) Antagonists (C) Allosteric modulators. Adapted from Piomelli, 2003.

Genetic tools

State-of-the-art genetics has provided a powerful tool to uncover and understand the varied functions of the ECS (Lutz, 2014). Genetic approaches allow understanding the implication of CB1 receptor's function within a particular brain region or a specific cell type, or even within a subcellular compartment. The generation of mouse mutant lines globally lacking CB1 receptors provided a major step to study the specific function of these receptors at the molecular, cellular and behavioral level (Ledent et al., 1999; Marsicano *et al.*, 2002; Zimmer et al., 1999). However, as CB1 receptors are expressed in brain cells and circuits with apparent opposing effects, the constitutive deletion of CB1 does not allow studying its specific contribution to brain functions (Busquets-Garcia *et al.*, 2018a). Thus, researchers need a targeted inactivation of the CB1 receptor gene in particular brain regions and/or cell types (Lutz, 2014). This has been possible thanks to the introduction of two loxP sites surrounding the CB1 gene in mice: one into the intron in front of the exon which encodes CB1 receptor protein and the other into the 3' untranslated region directly after the open reading frame. The small dimensions of the loxP sites

guarantee that the mutation does not alter the expression of the floxed gene. Thus, floxed mouse lines can be considered as phenotypically wild-type (Nagy, 2000). By Cre recombinase-mediated excision, the CB1 receptor sequence flanked by loxP can be removed in order to obtain the inactivated allele in particular brain locations (Lutz, 2014; Marsicano et al., 2003; Marsicano *et al.*, 2002; Vianna et al., 2012). These floxed mice have been crossed with transgenic mice that were expressing the Cre recombinase under the control of specific promoters. The Cre-lox system allows specific recombination in cells containing Cre (Sauer and Henderson, 1988), leading to conditional deletion in specific CB1-expressing cells (conditional mutagenesis). Following this genetic system, multiple cell-type specific CB1 knock-out (CB1-KO) mouse lines have been generated since (Gutierrez-Rodriguez et al., 2017; Han *et al.*, 2012; Monory *et al.*, 2006; Pagano Zottola et al., 2020) (Table 1). It is important to note that both the global and the conditional CB1-KO mice are viable, fertile, they develop normally and they do not suffer any severe deficits.

Additionally, the regional and cell type-specific expression of CB1 can also be accomplished using viral techniques. During the last decades, viruses have been shown to have the potential to mediate the delivery and stable transduction of genes into the central nervous system (CNS). Thus, stereotaxic injections of an adeno-associated virus expressing Cre recombinase (AAV-Cre) into the target brain region of a "floxed" mouse can allow the deletion of the gene of interest in a specific location. Moreover, to avoid the recombination in all cell-types, it is possible to place the viral Cre recombinase under the control of a cell-selective promoter. In the specific case of the CB1 receptor, many studies have shown the efficacy of such viral approaches (Monory *et al.*, 2006), which has been used to specifically delete CB1 in the dentate gyrus of the hippocampus (Monory *et al.*, 2006), to overexpress CB1 in glutamatergic neurons of the hippocampus (Guggenhuber et al., 2010), to obtain mice lacking CB1 in medial habenula neurons (Soria-Gomez et al., 2015), in the anterior olfactory nucleus (Soria-Gomez et al., 2014), the anterior piriform cortex (Terral et al., 2019) and many more.

The aforementioned genetic approaches are clearly very powerful, however it is always important to recognize their limitations. Mammalian organisms have very strong regulative mechanisms to maintain homeostasis, making it very possible that compensatory processes occur, in particular during development, but also during adulthood, with upregulation or downregulation of different proteins. This compensations can then easily hide or distort the observed phenotype and hinder the interpretations (Lutz, 2014). Regarding the limitations of conditional mouse mutants, one such limitation is the undesired ectopic expression of the Cre recombinase, which can cause the deletion of CB1 in cell types

that were expected to be excluded from the recombination. This can lead again to serious misinterpretations of the resulting phenotype. Another possible drawback is the fact that in some cases the transgene expressing the Cre recombinase could cause unspecific effects, making imperative the need of comparing the phenotype of mice expressing solely Cre recombinase (and not the "floxed" alleles) with wild-type animals (Lutz, 2014).

Table 1. Different conditional mutant mouse lines targeting the CB1 receptor gene. Adapted from Monory and Lutz, 2009.

Mutant line	Regulatory elements	Sites of recombination / Deletion of CB1	Reference
CaMK-CB1	CaMKII α -Cre	In principal forebrain neurons	(Marsicano et al. 2003)
Dlx-CB1 also called GABA-CB1-KO	Dlx5/6-Cre	In forebrain GABAergic neurons	(Monory et al. 2006)
Nex-CB1 also called Glu-CB1-KO	Nex-Cre	In cortical glutamatergic neurons	(Monory et al. 2006)
GFAP-CB1	GFAP-CreERT2	Tamoxifen-inducible deletion in astrocytes	(Han et al. 2012)
D1-CB1	D1-Cre	In dopamine receptor D1 expressing neurons	(Monory et al. 2007)
DN22-CB1	-	Exclusion of CB1 from mitochondria (no mtCB1)	(Soria-Gomez et al. 2021)

3 The Endocannabinoid System and the Developing Brain

3.1 Physiology of the Endocannabinoid System during Development

The ECS plays a broad and critical role in numerous developmental processes. Both CB1 receptors and other principal components of the ECS have been detected from the earliest phases of ontogenetic development, when they play an essential role in neuronal development in a number of species, including rodents and humans (Berghuis *et al.*, 2007; Berrendero *et al.*, 1998; Maccarrone *et al.*, 2014; Mato *et al.*, 2003; Rodriguez de Fonseca *et al.*, 1993). CB1 receptors are present and functional already from the early gestational period in both humans and rodents (Berrendero *et al.*, 1998; Berrendero *et al.*, 1999a; Biegon and Kerman, 2001; Buckley *et al.*, 1997; Mato *et al.*, 2003). At these early stages, the ECS is vital for important processes including the proliferation and differentiation of the progenitor cells, for neuronal migration and for axonal guidance (Berghuis *et al.*, 2007).

There is also multiple evidence showing that there are developmental changes within the ECS itself (Meyer *et al.*, 2018). During the perinatal period, a common pattern of CB1 receptor transient expression has been found both in rodents and humans: high densities of CB1 receptors have been observed in white matter neuronal fibers, potentially reflecting CB1 on axons as they grow and migrate to their final site, or their presence on non- neuronal cells (astrocytes and oligodendrocytes) that guide neuronal migration (Mato *et al.*, 2003; Rodriguez de Fonseca *et al.*, 1993).

The ECS reaches its peak activity during adolescence (Meyer *et al.*, 2018), which places it as a key modulator of developmental processes during this period (Hurd *et al.*, 2019) (Figure 8). Although many

of the mechanisms still remain unknown, evidence suggests that the ECS is particularly important for mediating the balance between the excitatory and inhibitory neurotransmission during adolescence (Meyer *et al.*, 2018). Contributing to this heightened activity during adolescence, peaks in the expression of CB1 receptors as well as in the levels of the main endocannabinoids have been reported. In the next part, I will describe in more detail the development of the ECS and its different constituents during the adolescent period.

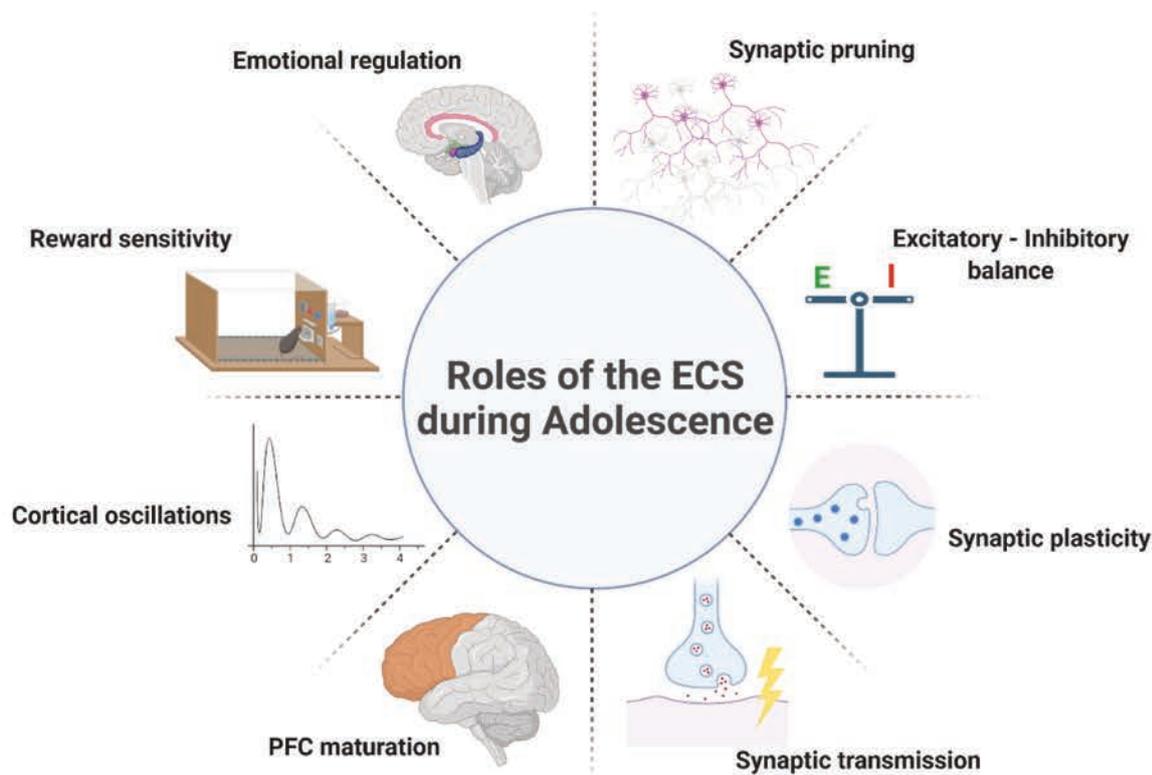


Figure 8. Regulation of various processes and aspects of behavior during adolescence by the endocannabinoid system (ECS). During adolescence, both rodent and human studies have revealed how changes in endocannabinoid signaling contribute to the maturation of corticolimbic circuit populations of neurons, such as mediating the balance between excitatory and inhibitory neurotransmission within the prefrontal cortex. The ECS during adolescence also has a critical involvement in synaptic pruning, a process characteristic of this developmental period needed to establish the ultimate architecture of the mature brain, as well as synaptic function and plasticity. For additional information refer to the main text.

Maturation of the ECS during Adolescence

Evidence on how endocannabinoid signaling changes during adolescence in humans is relatively sparse (Meyer *et al.*, 2018). In humans, CB1 expression in the prefrontal cortex seems to peak quite early, during the neonatal period and infancy, and subsequently slowly decreases until adulthood (Choi *et al.*, 2012). Moreover, elevated 2-AG synthesis has been reported during human adolescence, as well as a gradual increase in the expression of FAAH and of the AEA synthesizing enzyme NAPE-PLD, suggesting increased control of anandamide availability over the time (Long *et al.*, 2012). Considering the practical limitations of this kind of studies for human adolescence, most of the current knowledge has emerged from studies in rodents.

In rodents, the peak expression of CB1 receptors is observed at the beginning of adolescence, around postnatal day (PND) 30, particularly in regions such as the medial prefrontal cortex, the striatum and the dorsal hippocampus, with a following decline until adulthood (approximately PND 70) (Ellgren *et al.*, 2008; Meyer *et al.*, 2018; Rodriguez de Fonseca *et al.*, 1993; Schneider, 2008) (Figure 9). In other cortical areas, such as the sensory motor cortex, comparable declines are not observed until late adolescence (Heng *et al.*, 2011). Similar patterns are observed for the functionality of CB1 in prefrontal and limbic regions, as seen by reductions in CB1 receptor-dependent inhibition of synaptic transmission in PFC during the transition from adolescence to adulthood (Heng *et al.*, 2011).

In parallel, dynamic alterations in the levels of endocannabinoids have also been reported (Figure 9). Regarding the two main endocannabinoids, AEA concentration exhibits a progressive, although fluctuating, increase from early postnatal stages to adulthood, and reaches maximal levels during adolescence, in most of the brain regions that have been studied. For 2-AG, its levels are high both during early and late adolescence, with a notable decrease during mid-adolescence (Berrendero *et al.*, 1999b; Ellgren *et al.*, 2008; Harkany *et al.*, 2008; Hill *et al.*, 2019; Lee *et al.*, 2013). Developmental changes have also been reported in the enzymatic machinery of the ECS. Changes in FAAH activity have been reported during adolescence, which could contribute to the observed fluctuations in AEA levels (Lee *et al.*, 2016) (Figure 9). Less is known regarding the developmental expression of MAGL, but evidence in humans indicate rapid decreases following the onset of adolescence (Long *et al.*, 2012).

The exact mechanisms through which the ECS influences the neurodevelopmental processes of adolescence are still incompletely understood. Across adolescence there are marked increases in synaptic pruning (Andersen *et al.*, 2000; Meyer *et al.*, 2018). Specifically in the PFC, pruning occurs to

a greater degree at excitatory compared to inhibitory synapses, resulting in an increase of prefrontal synaptic inhibition (Dow-Edwards and Silva, 2017). Therefore, the balance of excitation and inhibition has been shown to differ greatly between adolescents and adults (Sturman and Moghaddam, 2011). Evidently, the ECS has a critical contribution to this balance, through the regulation of both glutamatergic and GABAergic terminals (Bossong and Niesink, 2010; Lubman et al., 2015; Marsicano *et al.*, 2003).

Importantly, there are notable sex differences in the timing of ECS development, with CB1 receptor expression levels peaking earlier in female compared to male rodents (Rodriguez de Fonseca et al., 1994). Subsequently, the endocannabinoid activity fluctuates during the estrous and menstrual cycle (Bradshaw et al., 2006b; Cooper and Craft, 2018; El-Talatini et al., 2010; González et al., 2000; Gorzalka and Dang, 2012). Moreover, studies have shown that endocannabinoid-mediated long-term depression (LTD) is also sexually dimorphic, emerging earlier in females than males (Bernabeu et al., 2020; Borsoi et al., 2018). It is therefore possible that a close interaction between the ECS and sex hormones contributes to the cannabinoid changes occurring during adolescence. As a matter of fact, the ECS has been shown in many studies to modulate the release and activity of sex hormones –androgens, estrogen, and progesterone–, as well gonadotropins –the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH)– both in humans and in rodents (Gorzalka and Dang, 2012; Murphy et al., 1998; Rodriguez de Fonseca *et al.*, 1994; Tsutahara et al., 2011; Wenger et al., 2001). Endocannabinoid activity appears to decrease the release of sex hormones in order to maintain physiological levels, and subsequently changes in sex hormone levels can influence endocannabinoid signaling as well (Gorzalka and Dang, 2012).

All findings described above emphasize the dynamic changes in brain endocannabinoid functions during adolescence. These quantitative and qualitative variations in the temporal and regional distribution of its components can contribute to the emergence of a sensitive window, especially during early adolescence, when the corticolimbic circuitry is particularly sensitive to perturbations of endocannabinoid signaling (Fuhrmann et al., 2015). Consistent with this idea, environmental interference with the ECS (e.g., via exposure to exogenous cannabinoids such as with heavy cannabis use) during this period can lead to long-term and persistent neurobiological, and consequently behavioral, effects in adulthood (Blest-Hopley et al., 2020; Portugalov and Akirav, 2021). I will further discuss some of the potential mechanisms by which adolescent cannabis use can impact brain development in the following section.

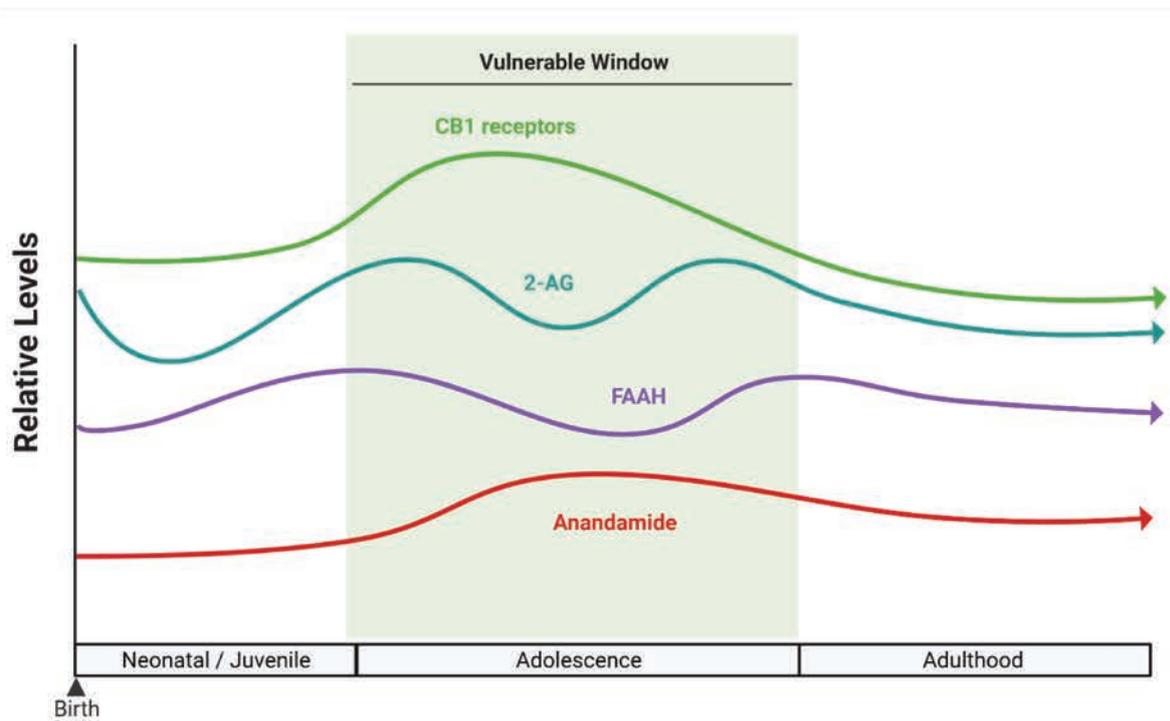


Figure 9. Developmental trajectories of the main components of the ECS. CB1 receptor expression peaks with the onset of adolescence. 2-AG has the highest expression right after birth and may fluctuate throughout adolescence. Anandamide gradually increases during early life and fluctuates during adolescence. FAAH activity fluctuates in reciprocal fashion to anandamide during adolescence. Based on data from Berrendero et al., 1999, Ellgren et al., 2008, Fernandez-Ruiz et al., 2000, Heng et al., 2011, Lee et al., 2013, Rodriguez de Fonseca et al., 1993, Rubino et al., 2015, Wenger et al., 2002. Adapted from Lee et al, 2016.

3.2 Neural Substrates Underlying the Impact of Cannabinoid Exposure during Adolescence

To date, the majority of research on the roles of the ECS during adolescence has focused on the long-term consequences of disrupting the physiological activity of this system during development. Compared to human studies, in preclinical animal studies many complex factors (for example, genetics, environment or THC concentration) can be successfully controlled. Therefore, animal models have been extensively used in the field and demonstrated that adolescent THC exposure can lead to several neuroadaptations (Bara *et al.*, 2021; Stringfield and Torregrossa, 2021), causally linked to behavioral outcomes, relevant to those observed in humans (the behavioral effects in humans were discussed in section 1.4 of this chapter).

In the previous pages, we have seen how the ECS undergoes significant changes during adolescence. Adolescent THC exposure is well documented to alter this normal developmental profile of the ECS. For example, the course of AEA and 2-AG levels throughout the adolescent period are different in THC-exposed animals (Ellgren *et al.*, 2008). It has been shown that adolescent THC exposure leads to a premature peak of AEA levels in the NAc of male rats during mid-adolescence (achieving levels comparable to those of adulthood), while at the same time the normal increase in PFC AEA levels in late adolescence are reduced by escalating, high- dose THC exposure in female animals (Bara *et al.*, 2021). On the contrary, neither moderate nor high THC exposure has been reported to alter the levels of 2-AG levels (Rubino *et al.*, 2015b). Moreover, adolescent THC exposure was also shown to inhibit the normal endocannabinoid-mediated LTD seen in medial PFC (Cuccurazzu *et al.*, 2018).

Adolescent THC exposure has also a significant impact on the CB1 receptor expression and functionality, which often depend of the doses used and the sex of the animals. For example, while a moderate dosing schedule has been found to elevate CB1 levels in the amygdala of THC-exposed animals (Silva *et al.*, 2016), escalating, high doses led to a decrease of CB1 levels in multiple brain regions, including the amygdala, PFC, striatum, hippocampus, thalamus, VTA and cerebellum regardless of sex (Bara *et al.*, 2021; Rubino *et al.*, 2015b; Rubino *et al.*, 2008). Since CB1 is primarily localized in GABAergic terminals, it is expected that inhibitory transmission is also particularly affected in these regions (Molla and Tseng, 2020). These results also relate to functional reductions in CB1 G protein activity in these regions, with the strongest changes observed in females (Rubino *et al.*, 2008).

In addition to the changes in the ECS, adolescent THC exposure also has profound effects on the excitatory and inhibitory balance. CB1 receptors are known to be involved in the modulation of excitatory glutamatergic and inhibitory GABAergic tone in the PFC and other cortical regions (Fortin and Levine, 2007) (see also section 2.2.1 of this chapter). Therefore, exogenous cannabinoid exposure during adolescence can pose a conceivable threat to this modulation. For instance, chronic exposure to escalating THC doses during adolescence has been shown to result in long-lasting down-regulation of the glutamic acid decarboxylase 67 (GAD67), an enzyme involved in GABA synthesis. This effect can be observed in different groups of inhibitory neurons, such as cholecystokinin (CCK) and parvalbumin (PV) positive interneurons, indicating long-lasting alterations in distinct GABAergic cell types (Molla and Tseng, 2020; Renard et al., 2017; Zamberletti et al., 2014). On the other hand, the excitatory tone in the adult PFC is increased following adolescent THC exposure, with higher spontaneous firing of PFC pyramidal neurons (Renard *et al.*, 2017). Moreover, region-dependent changes in overall levels of glutamatergic markers, like AMPA and NMDA receptor subunits, the presynaptic marker synaptophysin, as well as the postsynaptic marker PSD95 have been reported after adolescent THC exposure, together with alterations to their normal developmental trajectory (Bara *et al.*, 2021).

Additionally, the structural maturation of PFC circuits is also shown to be affected by adolescent cannabinoid exposure, with prefrontal pyramidal neurons showing transcriptional changes and early pruning (Miller et al., 2018) as well as decreased dendritic arborization (Renard et al., 2016c). Cannabinoid exposure during adolescence also disrupted cortical oscillations, suppressing them at different bands (Raver et al., 2013). Finally, more neurotransmitter systems have been suggested to be altered after THC exposure, such as the serotonergic system, at least in male animals (Pouliat et al., 2019). An additional neurotransmitter system affected is the dopaminergic, since adolescent THC exposure has been shown to lead to a hyperactive DAergic neuronal activity (Renard *et al.*, 2017) and to decrease CB1 expression in the VTA (Kruse et al., 2019; Rubino *et al.*, 2008).

3.3 Insights from Animal Models of Adolescent Cannabinoid Exposure

Numerous observational studies in humans suggest a link between adolescent cannabis use and poor later outcomes (already discussed in section 1.4). However, since human studies are naturally clouded by several confounding variables, substantial part of research has turned to the use of controlled experimental conditions in animals. Although animal models cannot represent the full phenotypic spectrum of what would be observed in humans, their use in the study of adolescent vulnerability to cannabinoids has several advantages. First of all, experimentally designed cannabinoid exposure protocols can precisely control both the age and the amount of exposure to specific cannabinoids. Secondly, the biological adaptations that arise can be easily measured at different time points after the adolescent exposure, for example during adolescence itself, or at early or late adulthood. Finally, once the specific effects of adolescent cannabinoid exposure are identified, experimental manipulations can be performed to unravel cause-effect relationships, and try to prevent or reverse cannabinoid-induced effects (Renard et al., 2016b).

The majority of animal models used for adolescent cannabinoid exposure involve mice and rats. The advantage of using these two species is that there is already a very good knowledge of their physiology, their genetic background and their behavior. Moreover, the average life span in these species is about 2 years, which allows for developmental studies from the adolescent period to late adulthood in a relatively short time (Tirado-Muñoz *et al.*, 2020). In addition, adolescent rodents exhibit a particular behavioral profile that in a way resembles human adolescent behaviors, including high levels of exploration, novelty and sensation seeking, as well as impulsivity and increased sensitivity to incentives (Viveros et al., 2012).

The most commonly used protocols to evaluate the long-term effects of cannabis use involve the exposure of adolescent rats or mice to THC or different synthetic cannabinoids and, later in life (mainly during adulthood) after a significant withdrawal or washout period, the analysis of different behaviors relevant to cognition and memory, anxiety, depressive-like behavior, responses to drugs of abuse or psychotic like-symptoms, as well as neurophysiological parameters related to the analyzed behaviors (Tirado-Muñoz *et al.*, 2020). In rodents, the beginning of the adolescent period is roughly considered around postnatal day 28 (PND28) and the end around PND60, during which time animals are reaching their full sexual maturity. This period can be further divided into specific phases such as early

adolescence (begins around PND28), middle adolescence (around PND38), and late adolescence (around PND49) (Spear, 2000a) (Figure 10).

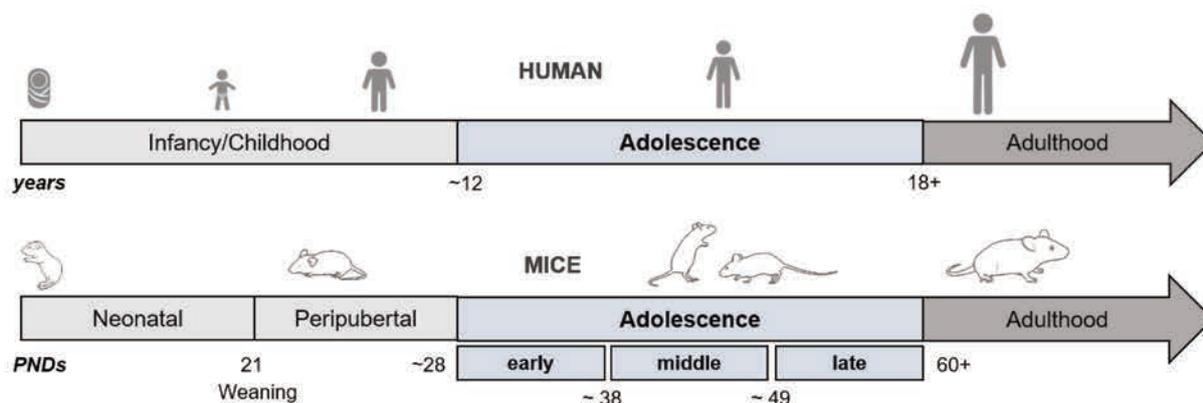


Figure 10. Comparison of human and mouse developmental stages. PNDs, postnatal days

The main cannabinoids that are commonly used in this type of studies are either the natural Δ^9 -THC (THC) or the synthetic cannabinoids WIN55,212-2 (WIN) and CP55,940 (CP) (Renard *et al.*, 2016b), although other synthetic drugs have also been used in some studies (Kevin *et al.*, 2017; Lee *et al.*, 2014). It is important to note here that the three agonists have notable differences in their pharmacological profiles. THC acts as a partial agonist for CB1, while both WIN and CP are full CB1 agonists (Pertwee, 2005). They can also vary in their efficacy, meaning the maximum to which they stimulate G protein-coupled signaling (Childers, 2006), as well as in their specific pattern of G protein subunit activation (Diez-Alarcia *et al.*, 2016) and their metabolism (Grotenhermen, 2003), accounting for often inconsistencies between studies of adolescent cannabinoid exposure. Further inconsistencies between studies can be associated with sex, strain, mode of administration, dosing scheme and frequency of exposure, as well as the specific time frame of the adolescent exposure (Stringfield and Torregrossa, 2021).

Regarding the route of cannabinoid administration, most of the studies utilize systemic injections, mostly intraperitoneal (i.p.) or sometimes subcutaneous. Whereas systemic injections obviously do not possess the same pharmacokinetics as the human used smoking or ingestion routes (Hložek *et al.*, 2017), exposure to single i.p. cannabinoid injections does produce elevated plasma cannabinoid levels, which can cross the blood brain barrier, resulting in activation of CB1 receptors in the brain. Notably, recent studies have also focused on methods that more closely model the human routes of exposure (i.e., smoke,

vapor, or oral consumption) (Abraham et al., 2020; Blaes et al., 2019; Bruijnzeel et al., 2019; Freels et al., 2020; Kruse *et al.*, 2019; McLaughlin, 2018; Nguyen et al., 2020), while some studies have also used intravenous self-administration of cannabinoids during adolescence (Kirschmann et al., 2017). Naturally, the difference in the administration mode plays an important role in the extent of the observed behavioral effects, as it has been demonstrated in many of the studies cited above, although it is also difficult to directly compare doses across these different routes of administration.

Finally, regarding dosage schemes, typically slight deviations in the doses used are found, where for example, for THC injections, doses can range from 0.3 mg/kg to 10 mg/kg (Ellgren *et al.*, 2008; Poulia *et al.*, 2019; Renard *et al.*, 2017). A commonly used exposure paradigm is based on the escalating dosage model, paying resemblance to what would be seen in humans (Lopez-Rodriguez et al., 2014; Poulia *et al.*, 2019; Poulia et al., 2021; Renard *et al.*, 2017; Rubino *et al.*, 2008).

In the following parts, I will describe some of the most commonly used behavioral paradigms to assess the long-term consequences of adolescent cannabinoid exposure and summarize what different studies have reported on each distinct category (Figure 11).

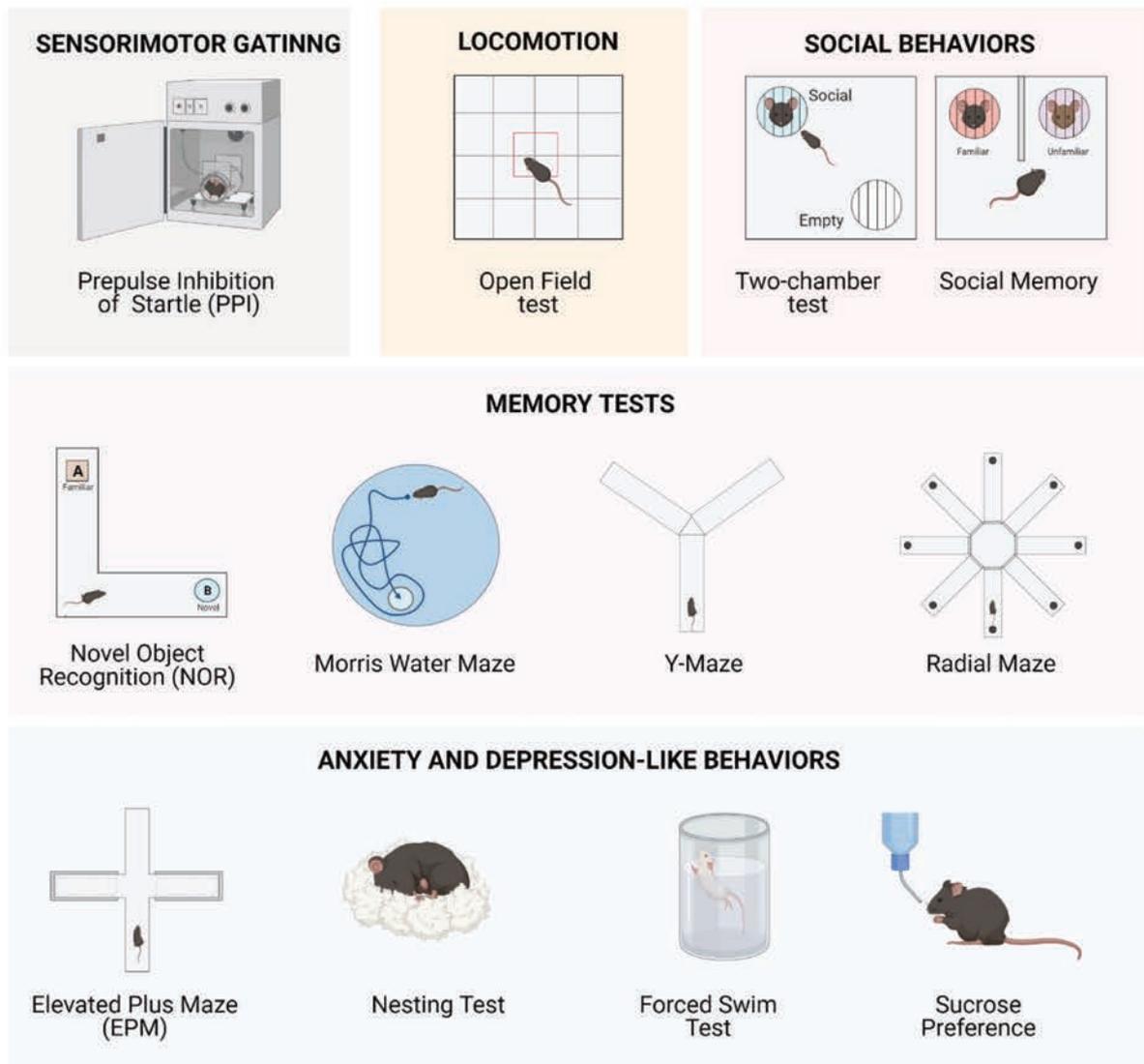


Figure 11. Mouse behavioral tests for the major categories related to adolescent cannabinoid long-term effects. The prepulse inhibition of the startle response (PPI) provides a ready measure of sensorimotor gating deficits related to adolescent cannabis exposure. The psychomotor agitation found in some chronic cannabis users can be modelled in mice by testing locomotor responses in a novel environment as well as the locomotor hyperactivity-inducing effects of psychotomimetic drugs. Social interactions with other mice and social memory can be assessed as a means to study social interaction impairments following chronic cannabis exposure. Impairments in cognition and executive functions are perhaps the most prominent feature of adolescent cannabis use. Learning and memory can be tested in mice using for example the novel object recognition task, the Morris water maze, the Y-maze or radial maze to study recognition memory, spatial memory, working memory and learning. Lastly, measures of anxiety and depressive-like behaviors following adolescent cannabis exposure have been obtained using the elevated plus maze (EPM) task, assessing nest building, assessing the mobility/immobility of mice in the forced swim test as well as using the sucrose preference test to investigate anhedonia-like behavior.

3.3.1 Pre-pulse Inhibition (PPI) of the Startle Response

One of the most frequent tests used in animal studies of adolescent cannabinoid exposure is the pre-pulse inhibition (PPI) of the startle response paradigm, a measure of sensorimotor gating that reflects the ability of an organism to attain information and process it correctly. Loss of normal PPI is also widely accepted as an endophenotype of psychotic disorders with very high translational validity, since it can be assessed in both animals and humans (Ioannidou *et al.*, 2018). Evidence has shown that chronic THC or WIN exposure during middle (PND35 to PND45) or late adolescence (PND40 to PND65), induces persistent PPI deficits in adult animals (decreased PPI) (Renard *et al.*, 2016a; Schneider and Koch, 2003; Wegener and Koch, 2009b). Conversely, other groups have reported no long-term changes in PPI following chronic adolescent THC treatment (Bortolato *et al.*, 2014; Poulia *et al.*, 2021; Scherma *et al.*, 2016). This inconsistency in the results can be attributed to differences in the period of treatment, the cannabinoid agonist or dose used, as well as the genetic background of the animals used.

3.3.2 Locomotion and Drug-induced Hyperlocomotion

With respect to locomotor activity, results obtained from studies examining the spontaneous locomotor activity of rodents following chronic treatment with cannabinoids during adolescence have generally been inconsistent. For example, using the open field test in adulthood after adolescent cannabinoid treatments, studies have reported either hyperlocomotion (Abush and Akirav, 2013; Poulia *et al.*, 2019; Poulia *et al.*, 2021; Wegener and Koch, 2009a), hypolocomotion (Llorente-Berzal *et al.*, 2013; Renard *et al.*, 2017) or no effects (Pickel *et al.*, 2020; Rubino *et al.*, 2008; Schneider and Koch, 2003; Schoch *et al.*, 2018; Shen *et al.*, 2011). Again here, the use of different agonists or the differences in the dosage and regimen chosen could account for the inconsistent results. On the contrary, results have been more consistent when studies measure the locomotor activity that is induced by psychoactive drugs such as amphetamine (AMPH) or phencyclidine (PCP). Adolescent cannabinoid exposure has been found to increase both the locomotor response to AMPH challenge in adulthood (Gomes *et al.*, 2015) and PCP-induced locomotor activity (Zamberletti *et al.*, 2014).

Notably, heightened locomotor activity is another classic preclinical model of psychotic-like deficits that, together with PPI, is believed to model dysregulation of mesocorticolimbic dopaminergic transmission (Peleg-Raibstein *et al.*, 2008; van den Buuse *et al.*, 2005) (Box 2). Given the well-established role of DAergic abnormalities in psychotic and schizophrenia-related symptoms (Kapur *et*

al., 2005), the aforementioned findings are consistent with the hypothesis that adolescent cannabinoid exposure may unbalance dopaminergic circuits, leading to disturbances in dopamine transmission and the observed behavioral symptoms (Kesby et al., 2018b).

Box 2. Modelling Positive Psychotic Symptoms in Animals

The most striking and characteristic symptoms of psychotic disorders are hallucinations and delusions, often referred to as primary or positive (based on their presence, rather than absence) psychotic symptoms. In terms of using animal models to study these psychotic behavioral abnormalities, for many years, preclinical research primarily focused on assessments such as motor stereotypies and hyperactivity (Ellison, 1991; Robbins, 2017). For example, drug-induced hyperlocomotion has been broadly used, and suggested to have some degree of validity for certain psychotic symptoms, mainly due to the dopaminergic neurochemical relevance and the potential to guide the development of antipsychotic drugs (Jones et al., 2011; van den Buuse, 2010). However, it seems evident that changes in overt behavior do not constitute an adequate way to study the mismatch between perception and reality, that is the true hallmark of positive psychotic states, and certainly doing so entails some degree of anthropomorphism (van den Buuse et al., 2005). In fact, the terms in which the symptoms that define psychotic disorders, such as delusions and hallucinations, are currently defined (American Psychiatric, 2013), renders them distinctive to humans and very challenging to model in animals.

To overcome this limitation, studies in recent years have used behavioral procedures in rodents, grounded in associative learning theories, that are designed to measure the accuracy of mental representations of reality, also referred to as "reality testing" (McDannald and Schoenbaum, 2009). In these kind of paradigms, alterations in "reality testing" performance can account for an impairment in perception, that can be relevant to aspects of psychosis (Busquets-Garcia et al., 2017a; Dwyer, 2018). Indeed, impaired "reality testing" has been demonstrated in several pharmacological (Busquets-Garcia et al., 2017b), genetic (Fry et al., 2019; Kim and Koh, 2016), lesion (McDannald et al., 2011) and developmental (Busquets-Garcia *et al.*, 2017b; Koh et al., 2018) animal models of psychosis, underlying the potential benefits of this approach. I will further discuss in more detail the "reality testing" paradigms, and the associative learning background they emerged from, in section 4 "Associative Learning: from Classical to Higher-order Conditioning" of this chapter.

3.3.3 Social Interaction

Considering the important role of the ECS in social behavior (Wei et al., 2017), another paradigm that is frequently used are specific tests to measure the animals' social behavior after adolescent cannabinoid exposure. Results from different studies are quite consistent, with the majority of them reporting an impairment in different social interaction tasks (O'Shea et al., 2006; Poulia *et al.*, 2021; Quinn et al., 2008; Renard *et al.*, 2016a; Renard *et al.*, 2017; Zamberletti *et al.*, 2014), although some studies have reported no effects (Bortolato *et al.*, 2014; Chen and Mackie, 2020; Gleason et al., 2012; Zamberletti et al., 2012).

3.3.4 Cognition, Learning and Memory

An extensive part of the research has focused on the investigation of the long-term effects of adolescent cannabinoid exposure on cognitive functions. However, despite the fact that many studies utilized relatively similar exposure designs either with THC or synthetic agonists, results have been again variable. For example, studies using the novel object recognition (NOR) task to test for recognition memory have reported memory deficits (Abush and Akirav, 2013; Higuera-Matas et al., 2012; Mateos et al., 2011; Quinn *et al.*, 2008; Renard et al., 2013; Renard *et al.*, 2017; Schneider and Koch, 2003; Zamberletti *et al.*, 2012), while some other studies showed no effects (Llorente-Berzal *et al.*, 2013) (Bruijnzeel *et al.*, 2019). Overall, performance in the NOR task was mostly impaired in male rodents, while the same effect in females was not consistent in the different studies (Stringfield and Torregrossa, 2021). Spatial learning and memory have also been tested in adult rodents exposed to cannabinoids as adolescents using the Morris water maze task, the object location task or the radial maze paradigm. Depending on the exact task and the agonist used, spatial memory was reported to be impaired at multiple doses, time points, in both males and females (Stringfield and Torregrossa, 2021).

A recent study also evaluated working memory using the delayed alternating T-maze task, showing that animals exposed to THC during adolescence display impairments in working memory acquisition and consolidation (Chen and Mackie, 2020). In regards to other learning paradigms, recent studies have shown that THC treatment during adolescence can impair reversal learning in a cognitive flexibility paradigm (in combination with ethanol) (Gibula-Tarlowska et al., 2020), reward-predictive cue behaviors (Kruse *et al.*, 2019) and performance in a paired-associates learning task (Abela et al., 2019), indicating long-lasting deficits in associative learning.

3.3.5 Anxiety- and Depression-like Behaviors

Finally, studies have also investigated the presence of adverse long-term effects of adolescent cannabinoid exposure on anxiety phenotypes and on depression-like responses in adulthood. Using the time spent in the center of the open field as a measure of reduced anxiety, studies have found both anxiogenic effects (Renard *et al.*, 2017) and no overall effects (Abush and Akirav, 2013; Bruijnzeel *et al.*, 2019; Rubino *et al.*, 2008).. Interestingly, when low doses of THC or WIN were used during the adolescent exposure, increased center time was reported at adulthood (Lovelace *et al.*, 2015; Poulia *et al.*, 2019; Wegener and Koch, 2009a), suggesting an anxiolytic effect. Similarly adolescent cannabinoid exposure on adult anxiety responses measured using the elevated plus maze (EPM) task, had in many studies anxiogenic-like effects (Scherma *et al.*, 2016; Wegener and Koch, 2009a), while no effects have been reported in other articles (Bortolato *et al.*, 2014; Bruijnzeel *et al.*, 2019; Rubino *et al.*, 2008; Schoch *et al.*, 2018). Overall, it seems that, across multiple studies, higher doses of THC are more likely to have a long-lasting anxiogenic response on separate measures of anxiety, while the results for synthetic agonists are more diverse and strongly depend on the timing of the exposure and the doses (Prenderville *et al.*, 2021; Stringfield and Torregrossa, 2021).

Regarding the effect of adolescent cannabinoid exposure on adult depression-like behaviors, the most commonly used measures are the level of immobility in the forced swim test (FST) or indices of anhedonia in the sucrose preference test. In the case of the FST, adolescent THC seems to have a greater impact in female animals, which show increased immobility (Rubino *et al.*, 2008), although again some studies report no changes in the behavior (Bruijnzeel *et al.*, 2019). For the sucrose preference test, one study found that adolescent cannabinoid exposure led to reduced sucrose preference only in males (Scherma *et al.*, 2016), while another study found the same effect only in females (Rubino *et al.*, 2008), and some studies have failed to observe an effect whatsoever (Abush and Akirav, 2013; Bruijnzeel *et al.*, 2019). To conclude, although results vary depending on the particular conditions and task used, there is evidence that adolescent cannabinoid exposure can affect emotional regulation in adulthood.

4 Associative Learning: from Classical to Higher-order Conditioning

4.1 An Overview of Associative Learning

Well before the birth of modern psychology and neuroscience, philosophers suggested that the way the mind creates ideas is by forming associations between events. Matters experienced would be joined because of their temporal proximity, common spatial locations, or perceived similarity. More complex thoughts would, in turn, be built from these basic associations. Thus, there is a long history that acquired associations are at the core of the way the mind represents the world.

(Fanselow and Wassum, 2015)

The main aim of early cognitive psychology, followed nowadays by cognitive neuroscience, is to explain an animal's behavior in terms of its mental organization of information, including how environmental information are acquired, processed, stored, and acted upon (Shettleworth, 2009). By studying animal cognition, thus, we study how an animal perceives, learns, remembers, and makes decisions. Learning in particular is one of the most important processes in the development of animal behavior and since the late 19th and early 20th century many explanatory proposals and models have aimed at describing the general principles of learning (Dickinson, 2012; Fanselow and Poulos, 2005; Mackintosh, 1974; Shanks, 2009). The study of animal learning has a long history and two main approaches have been followed, leading to what we now know today. The first approach conveys that understanding animal learning demands long-term observation of animals in their natural environment, noting how their behaviors are adapting to their surroundings (evidently tracing back to the pioneering work of Charles Darwin) (Mackintosh, 1974). The second approach focuses on the experimental

investigation of changes in behavior and the conditions under which these changes occur in laboratory settings, aiming at the dissection of the biological mechanisms involved in specific behaviors. Following this approach many scientists began exploring learning behaviors in different species (Lashley, 1951; Skinner, 1965; Tolman, 1948; Watson and Rayner, 1920; Yerkes and Dodson, 1908), with most influential perhaps the work of Russian physiologist Ivan Pavlov.

In a typical classical or Pavlovian conditioning procedure, a neutral conditioned stimulus (CS) is paired with a motivationally significant unconditioned stimulus (US), which produces an unconditioned response (UR). As a result of the pairing of the CS and US, the CS itself comes to evoke a conditioned response (CR). Pavlov formally documented this fundamental learning process, when he noticed that dogs would salivate (CR) in the presence of cues (CS) that predicted their feeding (like in response to a bell tone or when an experimenter would enter the room)(Pavlov, 1927; Pavlov, 1930). Classical Pavlovian conditioning has become, together with instrumental (operant) conditioning (Skinner, 1963), the main experimental paradigms to study learning. The basic distinction between classical and instrumental conditioning refers to the experimental arrangements (how the reinforcer or reward is being delivered), but does not necessarily imply different learning processes (Hull, 1942). In classical conditioning stimuli are presented independently of the actions of the animal, while in instrumental conditioning, the behavioral action of the animal is “instrumental” in obtaining an outcome or in avoiding a punishment (Dickinson, 1994; Thorndike, 1898).

Importantly, both instrumental and classical conditioning involve the formation of associations, either between the animals’ voluntary actions and a specific outcome in the former case, or between a conditioned stimulus with an unconditioned one (stimulus associations) in the latter. Indeed, associative theory accounts for the phenomena of conditioning as well as higher forms of learning, such as higher-order conditioning (see below), mediated learning, reinforcer devaluation, blocking or latent inhibition, and has for many years dominated the history of learning (Skinner, 1950; Thorndike, 1931). Within more recent associative learning models, information takes the form of what is often called a representation of the association between events, following Pavlov’s lead in assuming that pairings of a CS with a US result in the formation of an association between the central representations of the events (Mackintosh, 1975; Pearce and Hall, 1980; Rescorla, 1988; Rescorla and Wagner, 1971). Notably, this approach has created a novel framework for the preclinical investigation of neuropsychiatric disorder symptoms, which were previously thought impossible to model in animals (Box 2). For example, the study of core psychotic symptoms, namely hallucinations and delusions, had for years entailed a high

degree of anthropomorphism that led to polarized opinions regarding their validity, and subsequently very little progress, in terms of an understanding of relevant neural mechanisms (Forrest et al., 2014). However, there are now cognitive theories of hallucinations (Corlett et al., 2019; Powers et al., 2016) and delusions (Feeney et al., 2017; Schmack and Sterzer, 2019), suited for modelling across species (Schmack *et al.*, 2021), using higher-order learning behavioral tasks (Box 3).

To conclude this brief introduction to associative theory, it is important to highlight three core questions that dominate the neuroscientific study of associative learning. As Fanselow and Poulos put it, "at the systems level we ask, what are the brain circuits that mediate between environmental stimuli and acquired behavior? Then, within those circuits, what synapses must undergo modification for learning to occur? The third question is decidedly more molecular: What intracellular events occur at those sites of critical plasticity to confer the necessary changes in the synaptic efficacy that underlies the formation of learning?" (Fanselow and Poulos, 2005). Consequently, what has been recognized for some time, is that the type of the stimuli used, or the kind of action that the animals exert, greatly determine the neural circuits that mediate the observed behavior, suggesting that there is no single broad mechanism for all associative learning. Rather, there are many diverse systems, and although they may share some characteristics, they are just as likely to be different (Fanselow and Poulos, 2005; Fanselow and Wassum, 2015). This fact also calls for specific models and paradigms of conditioning, such as fear conditioning, appetitive conditioning or higher-order conditioning, each of which needs to be considered on its own at the mechanistic level.

Box 3. Relevance of Associative Learning Models in the Study of Psychotic Symptoms

Dating back to Ivan Pavlov (Pavlov, 1932) and Jerzy Konorski (Ellison, 1991; Konorski, 1948; Konorski, 1967), associatively retrieved representations have been implicated in the origin of psychotic symptoms, such as delusions and hallucinations.

These ideas were further developed by the seminal work of Peter Holland and colleagues (Holland, 1981b). Holland showed that if a hungry rat is presented with a tone, and, subsequently, a sucrose solution, the rat will quickly learn that the tone predicts the sucrose. In this case, the tone evokes a highly realistic, sensory representation of the sucrose, which the animal has trouble distinguishing from reality (McDannald and Schoenbaum, 2009). In this case, if the tone gets devaluated, by being for example paired with a nausea-inducing agent, rats will eventually reduce their sucrose consumption (Holland, 1981a). However, this phenomenon is not observed if the animals undergo a lengthier period of the tone-sucrose pairings (Holland, 2005). It seems that, by extending this training, the animals acquire more information about the two stimuli (tone vs expected reward), allowing them to separate their specific sensory features and consequently their associated outcomes. As the two stimuli are indeed separate entities in reality, researchers defined this process as “reality testing”, following the basis of reality monitoring, the ability of individuals to distinguish real from illusory patterns and associations (Johnson and Raye, 1981; McDannald and Schoenbaum, 2009). Interestingly, a few well-established animal models of schizophrenia have been found to be sensitive to these “reality testing” alterations where, despite extended courses of training, these animals will still confuse the cue-evoked anticipation of reward with the actual reward delivery (Busquets-Garcia *et al.*, 2017b; Fry *et al.*, 2019; Kim and Koh, 2016; Koh *et al.*, 2018; McDannald *et al.*, 2011). “It is as if these animals are associatively retrieving aberrant sensory perceptions; in other words, hallucinations” (Corlett and Schoenbaum, 2021). Finally, more evidence on the relevance of associative learning paradigms in the study of psychosis comes from recent findings suggesting that such phenomena involve dopamine signaling (Schmack *et al.*, 2021), which is since long well-known to be altered in psychotic disorders (Kapur *et al.*, 2005; Kesby *et al.*, 2018a).

Another core symptom of psychotic states is delusions, “fixed beliefs that are not amenable to change in light of conflicting evidence” (American Psychiatric, 2013). The recent conceptualization, to a certain extent, of delusions as fixed aberrant beliefs, can also allow

associative learning paradigms to be utilized in order to uncover some of the underlying neural mechanisms of these states. Higher-order conditioning tasks, like sensory preconditioning, for example can provide an assessment of how the brain structures mental representations of the world (Wikenheiser and Schoenbaum, 2016), an ability clearly impacted in those suffering from different forms of delusions. Interestingly, in rodents, the formation of the sensory associations during sensory preconditioning also appears to be governed by dopaminergic signaling (Seitz et al., 2021; Sharpe et al., 2017b).

4.2 Higher-order Conditioning

Although extremely informative in many aspects, classical conditioning alone is not sufficient to represent every-day life situations. In reality, both humans and animals alike, are always surrounded by myriad of random stimuli that, directly or indirectly, could represent crucial elements of future decisions and behaviors. Indeed, a large part of learning processes involves indirect types of learning based often on associations between low salience sensory stimuli, whose either simultaneous or contiguous occurrence is stored because of its future potential value (Bornstein et al., 2017; Shohamy and Wagner, 2008; Wimmer and Shohamy, 2012). As described in the previous pages, in classical, or otherwise termed first-order, Pavlovian conditioning, a conditioned stimulus (CS) of usually low-salience (i.e. with low intrinsic attractiveness or repulsiveness, such as a tone or a light), acquires motivational significance by being paired with either an intrinsically rewarding or an aversive unconditioned stimulus (US). Learning then is evaluated by the change in the animal's behavior as seen by the ability of the CS to elicit a conditioned response (Pavlov, 1927; Rescorla and Wagner, 1971).

However, stimuli can also acquire the ability to elicit conditioned responses, without being directly paired with the US, through processes referred to as higher-order conditioning. In higher-order conditioning a CS (S2) acquires associative strength by being paired with another CS (S1), instead of a US. Both animal and human studies over the last decades, have utilized two separate higher-order conditioning paradigms, second-order conditioning and sensory preconditioning. Their difference is that in second-order conditioning, the S1-S2 pairing can occur after S1 has been paired with the US, whereas in sensory preconditioning S1-S2 pairing precedes the S1-US (Gewirtz and Davis, 2000; Parkes and Westbrook, 2011). In both cases subjects eventually display a conditioned response to a stimulus that was never explicitly paired with the reinforcer and thus higher-order conditioning tasks have been largely used to evaluate forms of non-direct learning (Rizley and Rescorla, 1972) (Figure 12).

I will focus a bit further on sensory preconditioning (SPC) as it is relevant to some of the aims of this doctoral thesis. In a typical experiment of sensory preconditioning, two low-salience stimuli are first presented jointly during a preconditioning phase (S1-S2), then followed by classical conditioning of one of these stimuli by pairing it with a biologically meaningful (high salience) unconditioned reinforcer, like food or a foot shock (S1-US). Finally, exposing the subjects to either of the original stimuli (the one directly associated with the reinforcer and the one never associated) reveals the retrieval of direct and indirect learning, respectively (Brogden, 1939). The fact that the subjects' response to the indirect

preconditioned stimulus (S2) is consistently found to be similar to that evoked by the directly conditioned one (S1), assumes that an association between the two has been formed (Rizley and Rescorla, 1972). Since the introduction of SPC by Brogden (Brogden, 1939), sensory preconditioning has been described across a range of species, from humans and rodents to birds, fish and insects (Hall and Suboski, 1995; Karn, 1947; Kojima et al., 1998; Muller et al., 2000).

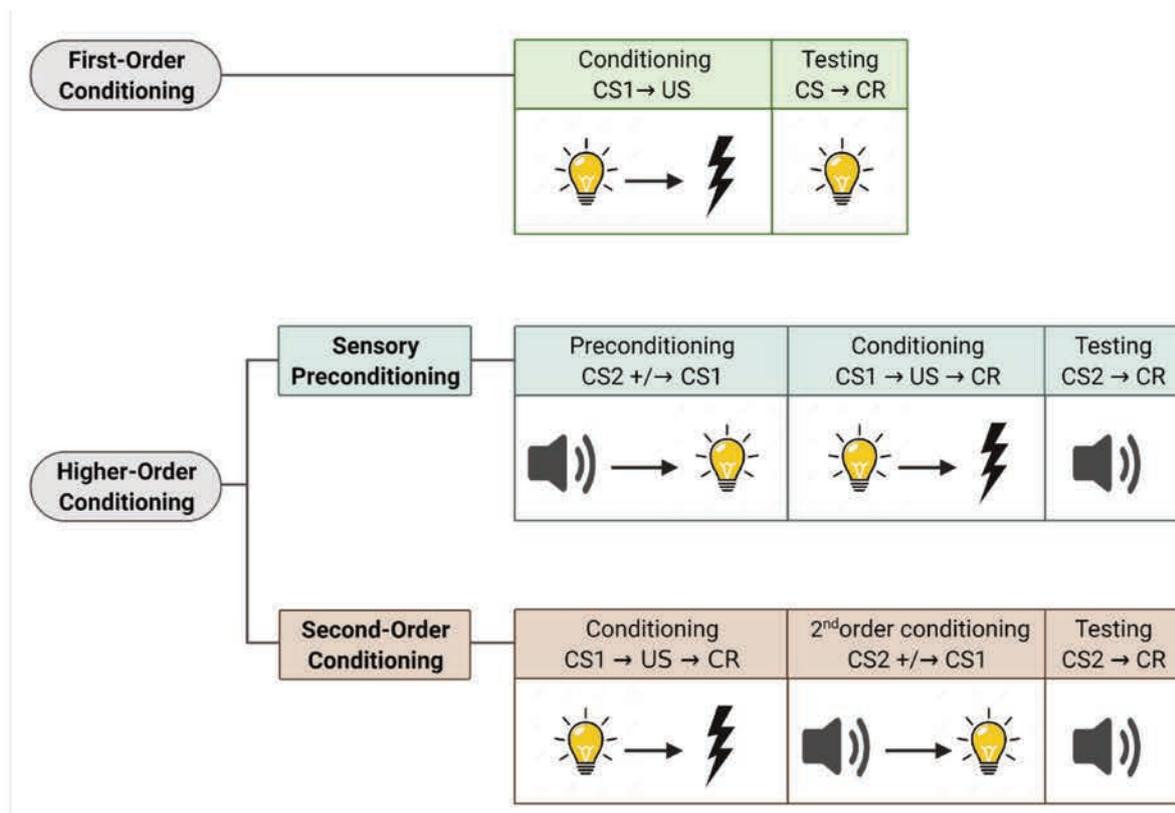


Figure 12. Schematic of classical versus higher-order conditioning procedures. The conditioned stimuli (CS) depicted here are visual (light) and auditory (tone), however different sensory stimuli such as odors, flavors or contexts are also widely used. Similarly, the unconditioned stimulus (US) can vary from an aversive one (electric foot shock as depicted here or agent induce malaise) to an appetitive one (for example food reward).

4.3 The Role of the ECS and CB1 Receptors in Higher-order Conditioning

The ECS, being a major neuromodulatory system, plays a crucial role in cognitive processes and animal models have been used extensively to assess its physiological, and pathological, effects on various stages of learning and memory, including encoding of information, consolidation, retrieval, re-consolidation and extinction (Busquets-Garcia et al., 2015; Kruk-Slomka et al., 2017; Marsicano and Lafenêtre, 2009; Zanettini et al., 2011). Indeed, the ECS, often via CB1 receptor signaling, has been found to be involved in many different forms of direct learning such as fear conditioning through CB1 in the amygdala (Marsicano *et al.*, 2002; Metna-Laurent et al., 2012), conditioned taste aversion through CB1 in insular cortex (Kobilo et al., 2007), conditioned odor aversion through CB1 in medial habenula (Soria-Gomez *et al.*, 2015) or conditioned odor preference through CB1 in the anterior piriform cortex (Terral *et al.*, 2019), among others.

However, despite the fact that CB1 receptor plays crucial roles in almost all the different phases of learning and memory processes (Akirav, 2011; Drumond et al., 2017; Marsicano and Lafenêtre, 2009; Rueda-Orozco et al., 2008), not many studies have addressed the physiological role of cannabinoid signaling in mediated learning, like for example in higher-order learning. This subject matter constitutes the second main thematic of my thesis and some results and conclusions of my relevant research will be discussed in Chapters IV and V, respectively. In the next and final section of this introductory chapter, I will summarize the role of CB1 receptors in higher-order conditioning, and in particular sensory preconditioning, in the form of a recently published review article.



Neural Substrates of Incidental Associations and Mediated Learning: The Role of Cannabinoid Receptors

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The ability to form associations between different stimuli in the environment to guide adaptive behavior is a central element of learning processes, from perceptual learning in humans to Pavlovian conditioning in animals. Like so, classical conditioning paradigms that test direct associations between low salience sensory stimuli and high salience motivational reinforcers are extremely informative. However, a large part of everyday learning cannot be solely explained by direct conditioning mechanisms – this includes to a great extent associations between individual sensory stimuli, carrying low or null immediate motivational value. This type of associative learning is often described as incidental learning and can be captured in animal models through sensory preconditioning procedures. Here we summarize the evolution of research on incidental and mediated learning, overview the brain systems involved and describe evidence for the role of cannabinoid receptors in such higher-order learning tasks. This evidence favors a number of contemporary hypotheses concerning the participation of the endocannabinoid system in psychosis and psychotic experiences and provides a conceptual framework for understanding how the use of cannabinoid drugs can lead to altered perceptual states.

Keywords: CB1, endocannabinoids, higher-order conditioning, sensory preconditioning, incidental learning, incidental associations, mediated learning

INTRODUCTION

In order to make decisions in daily life, we often rely on our previous experiences. We tend to repeat actions that were profitable in the past and, conversely, to avoid those that led to negative consequences. Therefore, the vast majority of learning and memory studies tends to focus on similar situations, where a neutral stimulus (i.e., carrying low salience levels *per se*) is directly associated with a biologically significant, highly salient stimulus (food, electric shock, etc.), producing a new learned response in the individual. However, more often than not, in our

environment we are exposed to novel and ambiguous settings, where direct experience is sparse and where a more flexible approach is required to predict – or guess – how a decision might turn out. In reality, while we engage in a particular activity, we are simultaneously surrounded by many incidental associations that might as crucially influence our future choices, as our direct experiences. Both humans and other animals have been shown to learn about the external world using such associations, often with the involvement of similar neural systems. Contrary to classical conditioning that generally produces solid and long-lasting responses, the memory of incidental associations is intrinsically labile. Rather than providing the individuals with direct information about the external world, it involves a large degree of “ambiguity.” Such ambiguity provides a level of flexibility that may be highly adaptive in changing environments. However, forming a mental association between two stimuli could also prove less beneficial if their co-occurrence is simply by chance and doesn’t represent truly an association. Incidental information is therefore constantly weighed against expectancies and environmental input to test its adherence to reality. Thus, whereas the ability to form incidental associations offers a way to better respond to unpredictable future challenges, a failure to precisely revise them and update them according to incoming information can account for “learning errors.” This can be observed for example in individuals who experience psychotic symptoms like delusions, who can rapidly accept incidental stimuli and events as meaningful and link them in unusual ways.

Understanding the cognitive and neurobiological mechanisms underlying these processes can therefore provide valuable insight both into the complicated abstract ways we learn, as well as into a potential source of cognitive dysfunctions in many mental illnesses. On that account, non-human animal models of incidental learning are crucial in contemporary neuroscientific research.

HIGHER-ORDER CONDITIONING, INCIDENTAL ASSOCIATIONS AND REPRESENTATION-MEDIATED LEARNING

Classical Pavlovian conditioning described how the brain represents dependent relationships between environmental stimuli and still remains the best-characterized associative learning model (Pavlov, 2010). In first-order Pavlovian conditioning, a conditioned stimulus (CS, such as a tone or light) acquires motivational significance by being paired with an intrinsically rewarding or aversive unconditioned stimulus (US, such as food or foot shock). Learning is evaluated by the ability of the CS to elicit a conditioned response (CR) in anticipation of the occurrence of the US. Although traditional views for Pavlovian conditioning described it as the transfer of an unconditioned reflex from the US to the CS, most contemporary learning theories agree that it involves the establishment of associations between internal memory representations of the CS, US, and their relationship (Fanselow and Wassum, 2015).

Although extremely informative, Pavlovian first-order conditioning is not sufficient for representing more ambiguous situations, such as the majority of the ones occurring in every-day life. In fact, a large part of the learning processes to represent our external world involves higher-order conditioning based on associations between low salience sensory stimuli, whose simultaneous or contiguous occurrence is stored because of its potential value for future choices. In higher-order conditioning a CS (S2) acquires associative strength by being paired with another CS (S1), rather than with a US. Two higher-order conditioning paradigms have been mainly used to assess higher-order conditioning in humans and animals, second-order conditioning, and sensory preconditioning. In second-order conditioning, the S1–S2 pairing can occur after S1 has been paired with the US, whereas in sensory preconditioning S1–S2 pairing precedes the S1-US (Gewirtz and Davis, 2000). In both cases subjects eventually display a conditioned response to a stimulus that was never explicitly paired with the reinforcer and thus higher-order conditioning tasks have been largely used to evaluate forms of indirect learning.

Sensory preconditioning in particular represents the most common behavioral protocol for studying incidental associations among relatively neutral or low-salience stimuli. In a typical experiment, two low-salience stimuli are first presented jointly during a preconditioning phase (S1–S2), then followed by classical conditioning of one of these stimuli by pairing it with a biologically meaningful (high salience) unconditioned reinforcer, like food or a foot shock (S1-US). Finally, exposing the subjects to either of the original stimuli (the one directly associated with the reinforcer and the one never associated) reveals the retrieval of direct and indirect memories, respectively (Brogden, 1939). Across a range of species (Karn, 1947; Hall and Suboski, 1995; Kojima et al., 1998; Muller et al., 2000; Wimmer and Shohamy, 2012), subjects’ response to the indirect preconditioned stimulus (S2) is found to be similar to that evoked by the directly conditioned cue (S1), assuming an association between the two has been formed.

Two prominent theoretical accounts are generally applied to explain the cognitive processes that underlie sensory preconditioning: the first one is the “associative chain” model, where the different associations are formed during the first and second phases of training allowing inference at test. In this account, the S1–S2 learning (phase 1) and the S1-US learning (phase 2) occur independently of each other and memories are integrated at the time of the testing, by recalling the two associations in order to infer on-the-fly the outcome that will likely follow (Rizley and Rescorla, 1972; Jones et al., 2012; Sharpe et al., 2017a; Sadacca et al., 2018; Wong et al., 2019; Wang et al., 2020). The second account, does not require memory integration at the time of testing, and refers to a process through which the preconditioned stimuli directly acquire positive or negative value during conditioning, due to a “unified representation” of S1 and S2. Through this process, often termed mediated or representation-mediated learning, presentation of S2 during the second phase of training activates a mental representation of S1, so that that this associatively retrieved memory might become further associated with the experience of the US. Eventually

presentation of the S1 during test, retrieves this mediated S1-US association, and thus, elicits the observed response (Holland, 1981b; Hall, 1996; Wheeler et al., 2008; Wimmer and Shohamy, 2012; Schlichting and Preston, 2015; Lin and Honey, 2016). Representation-mediated learning was originally described by Holland (1981a, 1990), whose work demonstrated that animals can learn not only about directly perceived stimuli, but also about indirect, associatively retrieved representations of that stimuli. Auditory or visual stimuli (Holland, 1981a) or contexts (Dwyer, 1999, 2001) were initially paired with a flavored solution. When the tone, light or context were later paired with a gastric malaise, they served as substitutes for their associated flavor stimuli. This paradigm differs from a classical sensory preconditioning task in that these stimuli (tone, light, or context) did not form any appreciable first-order association with the illness, however, the associatively activated taste representations did support taste-aversion learning.

BRAIN REGIONS INVOLVED IN INCIDENTAL LEARNING

Imaging studies in humans as well as experiments in rodents have provided insights into a network of brain regions that are involved in sensory preconditioning. The orbitofrontal cortex (OFC) has been shown to be necessary for forming value-neutral sensory associations, since both entire and selective inactivation of the OFC impairs inference about previously acquired stimulus-stimulus associations during the testing phase of sensory preconditioning (Jones et al., 2012). Moreover, single-unit recording experiments showed that neural activity in the lateral OFC reflects the acquisition of the associative information during the initial phase of training (Sadacca et al., 2018), and that optogenetic silencing of the OFC during this phase completely eliminates responding to the preconditioned cue during testing (Hart et al., 2020). Other structures, like the perirhinal and retrosplenial cortices have also been implicated. Lesions of the perirhinal cortex or its inactivation during preconditioning abolished sensory preconditioning (Nicholson and Freeman, 2000; Holmes et al., 2013; Wong et al., 2019), whereas chemogenetic silencing of the retrosplenial cortex during the preconditioning phase prevented inference at test without influencing direct conditioning (Robinson et al., 2014).

Interestingly, all aforementioned cortical regions are directly and indirectly interacting with the hippocampus (Agster and Burwell, 2013; Ritchey et al., 2015; Witter et al., 2017). Decades of research have characterized how the hippocampus critically contributes to representing and processing both real and abstract associative information (Port et al., 1987; Manns and Eichenbaum, 2009; Zeithamova et al., 2012; Voss et al., 2017) and many studies have highlighted its importance in sensory preconditioning both in humans (Bornstein and Daw, 2012, 2013; Wimmer and Shohamy, 2012; Shohamy and Turk-Browne, 2013) and in animals (Iordanova et al., 2009, 2011; Wheeler et al., 2013; Lin et al., 2016; Barron et al., 2020). Recent work additionally shows that a crosstalk between

the hippocampus and the orbitofrontal cortex is important for inferring future outcomes during sensory preconditioning (Wang et al., 2020). Notably, in some studies, hippocampal activation has been demonstrated during the testing phase, suggesting its involvement primarily in the retrieval of the sensory-sensory associations (Talk et al., 2002; Barron et al., 2020). However, in other studies, hippocampal activation has been also shown during the conditioning phase of sensory preconditioning, as well as during the initial stimulus-stimulus associations, supporting a widespread hippocampal involvement and suggesting that this brain region may be particularly important not only for retrieval but also for the encoding of the incidental associations between neutral stimuli (Wang et al., 2020). This is consistent with evidence showing that the hippocampus is essentially involved in the acquisition of information, which can then be used by different brain regions to guide flexible behavior (Elliott Wimmer and Büchel, 2019; Schuck and Niv, 2019). In the following paragraphs we argue that one possible mechanism for the formation of low-salience stimulus-stimulus associations in the hippocampus during sensory preconditioning is involving the tight regulation of hippocampal GABAergic interneurons by cannabinoid receptors.

CANNABINERGIC CONTROL OF INCIDENTAL ASSOCIATIONS

Originally discovered as the endogenous targets of the cannabis plant psychotropic derivative Δ^9 -tetrahydrocannabinol (THC), cannabinoid receptors and specifically type 1 cannabinoid receptors (CB1Rs) are key neuromodulatory elements of synapses. Physiologically, cannabinoid receptors are the main targets of endogenous signaling molecules called endocannabinoids, forming, together with the enzymatic machinery for their synthesis and degradation, the so-called endocannabinoid system (ECS) (Piomelli, 2003; Lu and Mackie, 2016). CB1 receptors are likely the most abundant G protein-coupled receptors in the brain, with amounts of protein comparable to NMDA and GABA receptors (Herkenham et al., 1990; Howlett, 2002; Freund et al., 2003). The expression levels of CB1 receptors can drastically differ among different cell types and can diverge between different brain regions (Han et al., 2012; Busquets-Garcia et al., 2018a). In cortical areas such as the hippocampus and neocortex, both glutamatergic principal neurons and GABAergic interneurons contain CB1 receptors, with the latter expressing the highest levels (Marsicano and Lutz, 1999; Marsicano and Kuner, 2008). The ECS has been involved in many forms of direct learning such as fear conditioning through CB1R in the amygdala (Marsicano et al., 2002; Metna-Laurent et al., 2012), conditioned taste aversion through CB1R in insular cortex (Kobilo et al., 2007), conditioned odor aversion through CB1R in medial habenula (Soria-Gomez et al., 2015) or conditioned odor preference through CB1R in the anterior piriform cortex (Terral et al., 2019), among others. Interestingly, the involvement of the ECS in direct conditioning appears to be more prominent in the modulation of the behavioral expression

of the acquired memory, rather than its formation (Kobilo et al., 2007). However, despite the fact that CB1 receptor plays crucial roles in different phases of learning and memory processes (Rueda-Orozco et al., 2008; Marsicano and Lafenêtre, 2009; Akirav, 2011; Drummond et al., 2017), not many studies have addressed the physiological role of endocannabinoid signaling in higher-order learning.

In our previous work (Busquets-García et al., 2018b) we evaluated the role of CB1R during the formation of incidental associations, using two different sensory preconditioning protocols in mice. Mice were first preconditioned by repeated exposure to pairs of low- salience sensory stimuli (pairing of an odor with a taste, or a light with a tone) forming an association between them. On subsequent days, mice were classically conditioned to associate one of these sensory stimuli (but not the other) with either an aversive or an appetitive stimulus. At the time of testing, both the directly conditioned stimulus but also the incidental preconditioned stimulus produced an aversion/preference, indicating the acquisition of both direct learning and mediated learning, respectively. Using this task, we showed that CB1R blockade upon preconditioning impaired the expression of mediated learning, however, CB1R blockade (or activation) at the stage of the testing did not affect the response to the preconditioned cue, strongly arguing for a specific role of endocannabinoid signaling in the initial processing of incidental stimulus-stimulus associations. Importantly, this effect did not appear to be limited to the specific sensory modality of the stimuli – whether those were olfactory and gustatory, or visual and auditory. The involvement of the ECS in different experimental conditions suggests broad common mechanisms underlying higher-order learning processes independently of the sensory modalities used and of the nature (aversive or appetitive) of the reinforcer.

With the hippocampus being a key brain region for sensory preconditioning, we addressed the role of hippocampal CB1R in these processes. In mice lacking CB1Rs selectively in the hippocampus or in forebrain GABAergic interneurons, mediated learning was compromised, yet direct learning was unaffected. Further experiments revealed that CB1Rs in hippocampal GABAergic neurons are indeed crucial for incidental learning, demonstrating a physiological link between hippocampal GABAergic signaling and associative memory between low-salience events. In fact, the paired presentations of the low-salience sensory cues during the initial, preconditioning phase induced a specific protein synthesis-dependent enhancement of hippocampal CB1R expression and facilitated long-term synaptic plasticity at hippocampal inhibitory synapses, suggesting that incidental learning might involve synthesis of new CB1Rs in hippocampal interneurons (Busquets-García et al., 2018b). Interestingly, midbrain dopaminergic signaling has been shown to be both necessary and sufficient for the formation of incidental associations (Sharpe et al., 2017b). Dopamine function is also tightly regulated by and regulating the hippocampus (Lisman and Grace, 2005), and recently CB1 receptors have been identified in a subpopulation of hippocampal D1R-positive interneurons, where they control memory processes (Oliveira da Cruz et al., 2020). Therefore it is possible that endocannabinoids modulate

incidental learning at hippocampal level through dopaminergic circuits, and further research should address this hypothesis.

FROM INCIDENTAL LEARNING TO REALITY TESTING: A ROLE FOR CB1 RECEPTOR SIGNALING

Contrary to classical conditioning between a conditioned stimulus and an unconditioned stimulus that generally produces solid and long-lasting responses, an elemental characteristic of incidental associations between stimuli is that they are intrinsically weak (McDannald and Schoenbaum, 2009). Several studies have shown that, when studied through sensory preconditioning paradigms, the establishment of incidental learning requires a certain amount of training/paired presentations between the preconditioned stimuli. Paradoxically though, extending this training or pairings during preconditioning abolishes its expression (Holland, 2005; Holland et al., 2008; Busquets-García et al., 2017), suggesting that the sensitivity to incidental learning can change as training proceeds. One explanation for this phenomenon suggests that, with moderate preconditioning, animals form a unified mental representation of the different preconditioned stimuli (S1+S2). However, with prolonged exposure to the stimuli, the subjects acquire more information about these stimuli, allowing them to separate their specific sensory features and consequently their associated outcomes (McDannald and Schoenbaum, 2009). As the preconditioned cues are indeed separated entities in reality, researchers defined this process as “reality testing,” following the basis of reality monitoring, the ability of individuals to distinguish real from illusory patterns and associations (Johnson and Raye, 1981; McDannald and Schoenbaum, 2009). An important aim down the road is therefore to unravel the complex biological processes that allow animals to switch from a unified representation of the different stimuli to their discrimination as independent entities (“reality testing”).

Type 1 cannabinoid receptors appear to be a key element of this switch. Our studies using reality testing protocols revealed that cannabinoids could disrupt this fundamental adaptive process, since acute administration of the main psychoactive component of cannabis, THC, was shown to impair reality testing, through activation of hippocampal CB1Rs (Busquets-García et al., 2017). Thus, there is a dual impact of hippocampal CB1R signaling: whereas a *minimal activation* of CB1Rs is required for incidental learning in order to form unified stimuli representations, their *excessive stimulation* impedes testing of the real nature of these representations (reality testing). The data collected so far indicate that there seems to be a descending gradient of CB1R signaling during the switch between incidental learning and reality testing. On one hand, ECS activity has to be sustained at the moment of forming incidental learning, during which individuals collect possible useful information from seemingly unrelated stimuli. On the other hand, CB1R signaling has to be reduced when this potential information is contrasted to reality. In other words, more ECS activity leads to the generation of “open possibilities,” whereas the “closing” of

these possibilities when they do not adhere to reality requires a decrease of CB1R signaling.

CONCLUDING REMARKS: FROM IMAGINATION TO PSYCHOSIS?

The formation of incidental associations can underlie particular human abilities such as imagination and creativity, which are characterized by the ability to assume connections between unrelated phenomena in order to construct new ideas and imagine future scenarios (Schacter et al., 2012; Uddin, 2021). Cannabis use and creativity are also often portrayed as linked (LaFrance and Cuttler, 2017), with their connection culturally and commonly accepted. Cannabis intoxication has been shown to promote divergent thinking, the ability to see connections between distant concepts and reveal something new (Eisenman et al., 1980; Morgan et al., 2010), but at the same time to impair convergent thinking, the ability to reason based on logical inference (Oomen et al., 2018). This disparity could result in connections being made between seemingly unrelated concepts or ideas, which are then linked together and elaborated upon, a characteristic of creative thinking but also of the development of a delusional system, often present in psychiatric conditions such as schizophrenia and psychosis. Interestingly, the reconceptualization of schizophrenia symptoms as aberrant perceptions (hallucinations) (Corlett et al., 2019) and beliefs (delusions) (Feeney et al., 2017), has provided the framework to be studied through associative learning tasks in both humans and animals (Powers et al., 2017; Dwyer, 2018; Koh and Gallagher, 2020). Indeed, impaired “reality testing” was recently demonstrated in several animal models of schizophrenia in a way that mimics psychotic-like percepts (McDannald et al., 2011; Kim and Koh, 2016; Busquets-Garcia et al., 2017; Koh et al., 2018; Fry et al., 2019), with recent evidence suggesting that such phenomena involve dopamine signaling (Schmack et al., 2021).

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Cannabis has been linked to the development of psychotic symptoms since a long time (Zuardi, 2006) and is well known to produce a range of immediate-onset psychotomimetic symptoms (Solymosi and Kofalvi, 2017), while alterations in the endocannabinoid system have also been implicated to the pathogenesis of schizophrenia and similar psychotic disorders (Muller-Vahl and Emrich, 2008). Given the general importance of the endocannabinoid system in the modulation of sensory perception (Soria-Gomez et al., 2014) and the fact that this function is centrally altered in psychotic states, it has been suggested that one important mechanism of cannabinoid-induced psychoses is linked to the alteration of perception of the external world. We therefore argue that the control of cannabinoid receptors over the formation and updating of incidental associations is contributing in orchestrating learning and associative thinking, in a continuum from normal perception to altered perceptual states.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Chapter II Research

Objectives

Objective 1: Investigating the long-term effects of chronic adolescent THC exposure

Adolescence is a critical period of brain development, which is characterized by behavioral changes that might predispose teenagers to experimentation with substances like cannabis. The association between adolescent cannabis use and persistent negative outcomes has long been the focus of neuroscience research. Indeed, numerous studies on its effects on brain maturation and function, cognition, addiction and vulnerability to neuropsychiatric disorders exist, with psychotic disorders being the most prominent focus of research in this context. However, the mechanisms linking cannabinoid exposure to long-term, persistent behavioral effects are complex and results in the field remain inconclusive.

The first aim of my PhD focused on the study of the long-term behavioral (Objective 1.1) and neurobiological (Objective 1.2) consequences of chronic adolescent THC exposure, in male and female mice. This first part of this thesis will be resumed in *Chapter IV - Section 1: Sex-dependent long-term effects of adolescent THC exposure* and is the subject of the following manuscript, which is currently in preparation:

“Metabolic Substrates Underlying the Sex-specific Effects of Adolescent Cannabinoid Exposure”

Christina Ioannidou, Daniel Jimenez-Blasco, Carlos Vicente-Gutierrez, Francisca Julio-Kalajzić,

Gilles Bonvento, Juan P. Bolaños, Arnau Busquets-Garcia* & Giovanni Marsicano*

My contribution to this work was to set up and follow the protocol of chronic THC exposure during adolescence, design, perform and analyze the behavioral experiments and write the manuscript. I also performed the different brain region extractions for samples that were subsequently used for all molecular analysis and participated in the undertaking of Western blot analyses. In addition, I performed the retro-orbital viral delivery in pre-adolescent mice and the subsequent behavioral assessment and viral expression analysis.

1.1 Assessing the behavioral phenotype of adult mice following chronic adolescent THC exposure

To characterize the effects of chronic adolescent THC on the behavior of adult mice, adolescent mice underwent repeated THC injections with two different doses of THC (1 mg/kg or 5 mg/kg) during 10 days (PND 35–45). Following a wash-out / manipulation-free period of a month, a set of psychotic-like behavioral processes related to motor, social and cognitive functions were evaluated in a battery of behavioral tests, including 1) locomotor activity, 2) social interaction, 3) short-term spatial memory and 4) prepulse inhibition of the acoustic startle response. Given that exogenous cannabinoids often have sex-dependent effects on brain and behavior (Cooper and Craft, 2018; Fattore and Fratta, 2010b), and there is evidence that some of the effects of adolescent cannabinoid exposure are sex-dependent as well (Tirado-Muñoz *et al.*, 2020), we tested both male and female mice, allowing for analysis of sex differences in response to the THC treatment.

Moreover, in order to assess possible alterations in mental sensory representations, which more accurately relate to the distinctive features of the core psychotic symptoms (i.e. hallucinations and delusions), we investigated the effect of adolescent THC in a representation-mediated learning protocol. Representation-mediated learning tasks can be used to evaluate a cognitive process of behavioral flexibility, often described as “reality testing”. Grounded in earlier works in associative learning theory, reality testing tasks probe the capacity of rodents to distinguish internally generated representations from external reality, and are thought to be the basis of studying impairments in perception which might underlie the cognitive/perceptual substrates of classic psychotic symptoms, such as hallucinations and delusions (Dwyer, 2018; Feeney *et al.*, 2017; Koh and Gallagher, 2020; McDannald and Schoenbaum, 2009). Resembling cannabis-induced psychotic states in humans, impairments in this protocol have been described after acute administration of THC, which are notably blocked by antipsychotic pre-treatment (Busquets-Garcia *et al.*, 2017b). However, the effect of adolescent THC in this, or similar, higher-order conditioning tasks has never been investigated. Here, utilizing a version of sensory preconditioning that evaluates representation-mediated taste aversion (Busquets-Garcia *et al.*, 2017a), we relied on olfactory and gustatory stimuli to first evaluate the normal formation of mediated learning, as well as the formation of classical reinforcement learning, following the adolescent THC treatment protocol, and subsequently to assess the effect of adolescent THC in reality testing performance.

1.2 Assessing the molecular changes induced by chronic adolescent THC exposure

Following the behavioral study of the effects of adolescent THC exposure, the second aim was to investigate a range of potential long-term biochemical changes, including CB1 receptor expression, as well as downstream targets of CB1 receptor activation signaling, such as the ERK and CREB proteins. Besides their localization at the level of the plasma membrane and the classical signaling pathways associated, CB1 receptors are also associated with mitochondrial membranes, where they control important functions, such as memory formation (Hebert-Chatelain *et al.*, 2016) and social behavior (Jimenez-Blasco *et al.*, 2020). Furthermore, mitochondrial integrity has been recently implicated in the pathophysiology of psychotic disorders (Konradi and Öngür, 2017), supporting the potential role of mitochondria associated CB1 (mtCB1) receptors in psychotic-like effects of adolescent THC exposure. Our recent findings have shown that mitochondrial Complex I (CI) instability in astrocytes is linked to persistent THC-induced behavioral deficits through the de-phosphorylation of the CI subunit NDUFS4 (Jimenez-Blasco *et al.*, 2020). Thus, we aimed at examining the impact of adolescent THC on the modulation of mitochondrial CI proteins including the phosphorylation of the NDUFS4 subunit, and subsequently, establishing whether these potential molecular alterations were causally linked to the THC-induced long-term behavioral effects.

Objective 2: Investigating the role of CB1 receptors in higher-order cognitive processes

The second objective of the thesis was dedicated to the exploration of the physiological role of CB1 receptors in representation-mediated learning processes. As introduced before, these processes may be uniquely suited for preclinical investigations of psychosis, however, they also reflect a large part of learning, which —unlike direct reinforcement learning— is governed by indirect associations between individual sensory stimuli. This type of indirect, or incidental, learning can be easily studied in animals, using higher-order conditioning tasks. Interestingly, earlier findings have already established the importance of the CB1 receptors in the control of incidental learning (Busquets-Garcia et al., 2018b; Ioannidou et al., 2021). Here, we have extended these previous findings by investigating the role of different CB1 receptor populations in representation-mediated learning, using specific conditional mutant mouse lines that target the CB1 receptors in dopamine D1-positive cells, and also in mitochondria.

Objective 3: Extending the behavioral impact of cannabinoid drugs

During the course of my doctoral studies, my research objectives extended through my participation in two additional projects where several cannabinoid-induced behavioral alterations were studied, the results of which will be described in the final sections of Chapter IV. The end result of these two projects is presented in the Annex of this dissertation in the form of the following published articles:

Annex I: Jimenez-Blasco, D., Busquets-Garcia, A., Hebert-Chatelain, E. et al. Glucose metabolism links astroglial mitochondria to cannabinoid effects. *Nature* 583, 603–608 (2020).

Annex II: Oliveira da Cruz*, JF, Ioannidou*, C, Pagano Zottola*, AC, et al. Sex-dependent pharmacological profiles of the synthetic cannabinoid MMB-Fubinaca. *Addiction Biology* 26:e12940 (2021) *co-authorship

Chapter III Materials and Methods

In the following pages I will describe in detail the Materials and Methods used for all the main experiments of my PhD work, the results of which will be described in:

- Chapter IV - Section 1: Sex-dependent long-term effects of adolescent THC exposure and
- Chapter IV - Section 2: Role of CB1 receptors in higher-order cognitive processes

Regarding the Materials and Methods of the results described in Chapter IV - Section 3: *Glucose Metabolism Links Astroglial Mitochondria to Cannabinoid Effects* and Chapter IV - Section 4: *Sex-dependent Pharmacological Profiles of the Synthetic Cannabinoid MMB-Fubinaca*, you can refer to the published articles in **Annex I** and **Annex II**, respectively.

1 Mice

All experiments have been performed according to the French (Décret 2013-118) and European (2010/63/EU) rules on animal experimentation. Approval of animal care and use for these experiments was provided by the Committee on Animal Health and Care of INSERM, and the French Ministry of Agriculture and Forestry (authorization number 3306369) and the French Ministry of Higher Education, Research and Innovation (authorization APAFIS#15502). Maximal efforts were made to reduce the suffering and the number of mice used. Sample sizes were chosen based on similar prior experiments that have produced significant results with a similar number of mice. Animals were housed in groups of 3-5 per cage under standard conditions of temperature and humidity in a day/night cycle of 12/12hr (light on at 7 am). Food and water were available ad libitum in the home cage. For the experiments of adolescent THC treatment, adolescent and adult female and male CB1-floxed mice [mice carrying the “floxed” CB1 gene (CB1^{f/f}) (Marsicano *et al.*, 2003)] were used. For the experiments using higher-order conditioning tasks, naïve male mice and mutant mice, aged 2–4 months, were used. All the mutant mice used were generated as previously described, e.g. deletion of CB1 receptors in dopamine receptor type 1 positive neurons (D1-CB1-KO) (Monory *et al.*, 2007) and in mitochondrial membranes (Pagano Zottola *et al.*, 2020). Briefly, for the generation of D1-CB1-KO, CB1 floxed mice (Marsicano *et al.*, 2003) were crossed with the D1-Cre line (Lemberger *et al.*, 2007), in which the Cre recombinase was placed under the control of the D1 gene (*Drd1a*) regulatory sequences using transgenesis with modified bacterial artificial chromosomes. The pattern of Cre expression recapitulated the expression pattern of the endogenous *Drd1a* (Lemberger *et al.*, 2007). Breeding was performed by mating male Cre-positive

D1-CB1-KO mice with homozygous CB1-flox female mice deriving from a separate colony. 8-9 weeks old naive male D1-CB1-KO and WT were used. DN22-CB1-KI mice were generated using a flox-stop strategy as previously described (Pagano Zottola *et al.*, 2020; Ruehle *et al.*, 2013). Stop cassette was excised via a Cre-deleter mouse strain and knock-in mice were maintained over a C57BL6/N background for several generations before experiments. All animals used in experiments involving mutant mice were littermates. All experiments were performed during the light phase of the cycle (from 9am to 1pm) and all animals were randomly assigned to the experimental groups.

2 Drug Preparation and Administration

THC was purchased from THC-Pharm (Frankfurt, Germany) and dissolved in 4% ethanol, 4% Cremophor-EL and 92% saline. For the adolescent treatment THC 1 mg/kg, THC 5 mg/kg or the corresponding vehicle were administered by once-daily intraperitoneal injections, in a volume of 10 mL/kg, from postnatal day (PND) 35 to PND 45. After completion of the last injection on PND 45, the mice were returned to their home cages without further injections in the 25-day interim from PND 45 to young adulthood at PD70, when behavioral evaluation begun.

3 Behavioral Procedures

All behavioral experiments were conducted during the light phase (from 9am to 3pm). The age of the mice at the beginning of all the behavioral experiments was 10-12 weeks. Figure 13 shows the overall experimental layout and the order and timing for the adolescent THC administration and the behavioral tests.

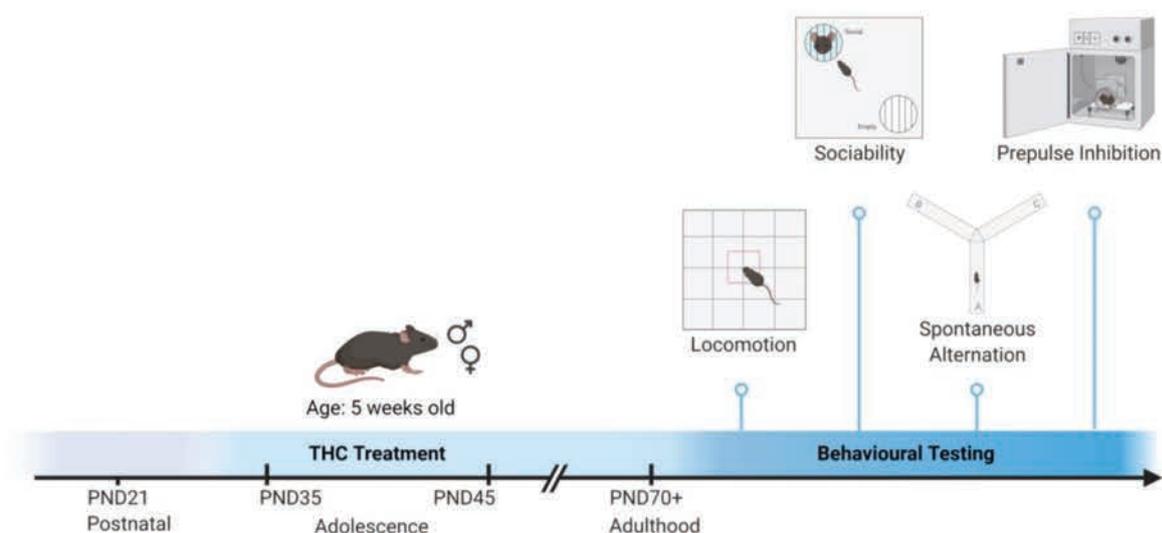


Figure 13. Schematic timeline of the experimental design. Adolescent male and female mice received daily i.p. injections of THC (1 and 5 mg/kg) or the corresponding vehicle from postnatal day (PND) 35 until PND45, followed by a drug-free washout period of a month. Behavioral experiments began after PND70 and lasted approximately two weeks. At the end of the behavioral testing mice were sacrificed and different brain region samples were obtained.

3.1 Open-field test

Mice were placed in an open field Plexiglas box (width, 60 cm; length, 60 cm) the bottom of which was divided into squares. A ceiling-mounted camera recorded mouse movements. Total locomotion was assessed by the number of total squares crossed and the number of vertical rearings, this number being manually counted during 5 minutes by the experimenter in a different room.

3.2 Two-chamber social interaction test

As previously described (Busquets-Garcia *et al.*, 2017b), mice were tested in an open field arena (35 × 35 cm) with two plastic containers (plastic cylinders of 8-cm diameter with holes for odor interaction) in two opposite corners, one of them hosting a mouse (8–10-week-old adult C57BL/6-N of the same sex as the tested mouse), while the other container remained empty. In each corner we defined the ‘social’ and ‘non-social’ zones as an 8-cm area surrounding the containers. For each experimental group, mice were put in the middle of the open field for 5 minutes and the position of the container with the mouse was counterbalanced. A ceiling-mounted camera recorded mouse movements. A social interaction index

was calculated as time spent in the social zone divided by the total time spent in both zones. The total interaction time of the two zones was used to control exploration activity.

3.3 Spontaneous alternation

Spontaneous alternation was assessed in a Y-maze (42 cm long, 8 cm wide, 120° between arms) in a room with 50 lux of light containing a ceiling-mounted camera. Mice were placed in one of the arms for 8 minutes and scored for each entry when all four paws were placed in one of the arms. The percentage of alternation was calculated as $\frac{\text{Number of correct triplets}}{(\text{Total number of arm entries})-2} \times 100$.

3.4 Prepulse inhibition

PPI was measured in a startle chamber (SR-Lab San Diego Instruments, San Diego, CA, USA). As previously described (Ioannidou *et al.*, 2018), mice were placed in the startle chamber and a 70-dB background noise was presented during a 5-minute acclimation period. The PPI session consisted of randomly presented 100 trials: a 120-dB noise trial presented alone (Startle, S), no stimulus trial, prepulse 73-dB trial, prepulse 76-dB trial, prepulse 82-dB trial, prepulse 73-dB + pulse 120-dB, prepulse 76-dB+pulse 120-dB, prepulse 82-dB+pulse 120-dB. The intervals between single trials were randomized between 10 and 30 s. The 100 milliseconds response after the presentation of the 120-dB pulse was analyzed by the PPI setting and we used the maximal response peak to calculate the PPI (% PPI = 100 × (Startle Alone – Prepulse + Startle) / Startle Alone). The startle alone response was used as a control index for the reaction to the startle pulse.

3.5 Odor-Taste Sensory Preconditioning Paradigm: Mediated Learning

A separate batch of naïve adolescent THC-treated (or VEH-treated) male and female mice underwent odor-taste sensory preconditioning a month after the adolescent treatment. The age of the mice at the beginning of the protocol was 10-12 weeks. All phases of the protocol were conducted during the light phase (from 9am to 1pm).

3.5.1 Habituation

Mice were individually housed, acclimated and water deprived in the same room where the whole protocol took place, under controlled temperature (21 ± 1 °C) and humidity ($55 \pm 10\%$) conditions, as described before (Busquets-Garcia et al., 2017a, 2017b). During three consecutive days, all subjects had 1h access to water in their home cage. The water bottle was weighed before and after this period in order to precisely quantify how much the animals drink in each session. The height and angle of the bottles was maintained throughout the protocol, while the position of the bottle (right or left side of the home cage) was counterbalanced during the experimental days.

3.5.2 Preconditioning

Over the following days, the odor-taste pairing was conducted in which animals were exposed to 3 odor-taste pairings, this phase lasting a total of 6 days. Each pairing included two days: on the first day, the subjects had 1h access to a flavored odor-scented water solution containing a new taste (5% sucrose or 5% maltodextrin; Taste 1, T1) and a new odorant [banana (isoamyl acetate, 0.05%) or almond (benzaldehyde, 0.01%); Odor 1, O1] in order to pair T1 with O1. On day two, the animals received the taste and odor not given during the previous day, i.e., Taste 2 (T2) and Odor 2 (O2).

3.5.3 Conditioning

During the next phase of 6 days (conditioning or devaluation phase) O1 (or T1) was devaluated as to become the conditioned stimulus (CS+). On conditioning days 1, 3 and 5, subjects received 1h access to O2 (or T2) followed by i.p. injection of saline (CS-). On conditioning days 2, 4 and 6, they received 1h access to O1 (or T1) immediately followed by an i.p. injection of the malaise-inducing agent lithium chloride (LiCl, 0.3 M, 1% body weight). The LiCl injection is performed just after removal of the bottles. A few minutes after injection, animals are observed to experience dizziness and greatly reduced

locomotion. The different odor/taste combinations were counterbalanced between each group. After this conditioning, subjects were given a recovery day in which they only received water during 1h.

3.5.4 Mediated and Direct Aversion Testing

On the next 2 days, mediated and direct aversions were assessed using a 1h two-choice test. Mediated aversion was always evaluated on the first test day with a choice between the stimulus T1 (or O1) previously associated with the CS+ but never directly paired with LiCl (thereby called mediated CS+, mCS+) and the stimulus T2 (or O2) previously associated with the CS- (mediated CS-, mCS-). On the second test day, the direct aversion was evaluated with a choice between the CS+ and CS-. The placement of each bottle is counterbalanced to avoid any position bias in task performance. At the beginning of this test session a ‘forced sampling’ procedure is used to ensure that the mice sample both fluids. Both bottles are presented: when the mouse has licked one of the bottles this bottle is removed until the mouse approaches and licks the second bottle. After this, the second bottle is also momentarily removed and finally, both bottles are then presented simultaneously. The order of presentation of the bottles during forced sampling is also counterbalanced to avoid any order bias. Aversion is revealed by lower consumption of mCS+ than mCS- (mediated aversion) or of CS+ than CS- (direct aversion). Data are presented as absolute liquid intake and an aversion index is also calculated using the following formula: $\frac{(mCS-) - (mCS+)}{(mCS-) + (mCS+)}$ or $\frac{(CS-) - (CS+)}{(CS-) + (CS+)}$ for mediated or direct aversion respectively.

For the experiments assessing the role of CB1 receptors in mediated learning formation, wild-type and mutant mice underwent the odor-taste sensory preconditioning protocol as described above. The age of the mice at the beginning of the protocol was 8-12 weeks. All phases of the protocol were conducted during the light phase (from 9am to 1pm).

3.5.5 Odor-Taste Sensory Preconditioning Paradigm: Reality Testing

Two new batches of naïve adolescent THC-treated (or VEH-treated) male mice underwent the odor-taste sensory preconditioning using a more extensive number of pairings (6 odor-taste pairings) a month after the adolescent treatment. According to previous studies (Busquets-Garcia *et al.*, 2017a; Busquets-Garcia *et al.*, 2017b), control animals with intermediate training (3 pairings) during preconditioning develop mediated aversion. However, if the training sessions are increased (6 pairings), the animals do not present mediated aversion anymore, a phenomenon described as reality testing, which is notably abolished following administration of psychotogenic drugs (e.g., amphetamine or cannabinoids)

(Busquets-Garcia *et al.*, 2017b), reflecting the kind of perceptual alterations that are the hallmarks and early features of positive psychotic-like symptoms. The experimental procedure followed here was identical to the one described before (section 1.3.5), with the only difference being the duration of the preconditioning phase.

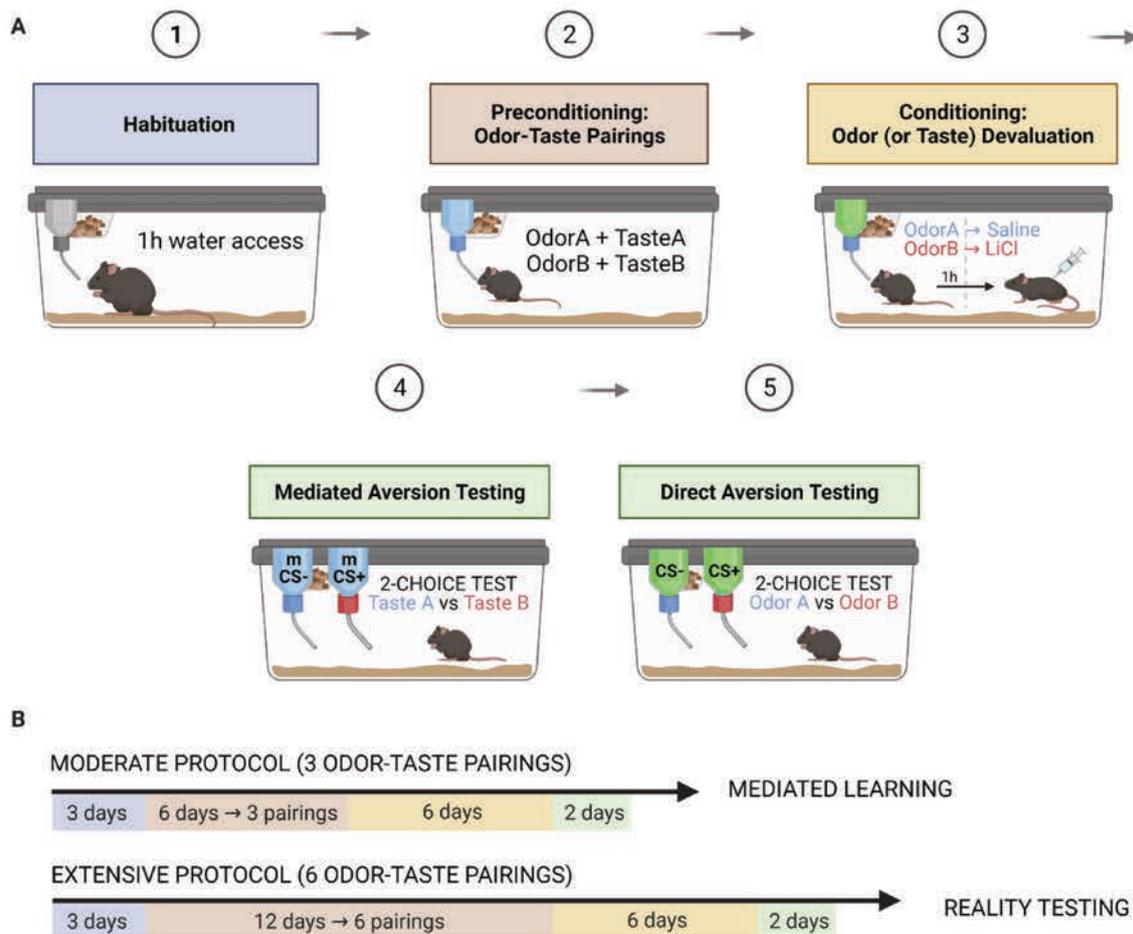


Figure 14. Schematic representation of the odor-taste preconditioning paradigm used to assess mediated learning and reality testing in mice. (A) Graphical representation of the five phases of the odor-taste preconditioning paradigm to assess mediated taste aversion and direct odor aversion. For a more detailed description of each phase refer to the main text. (B) Description of the moderate and extensive versions of the protocol in order to evaluate basal mediated learning and mediated learning in the context of reality testing.

3.5.6 Odor Perception

Mice were water deprived and then habituated to 1h access to water for three consecutive days. The day after they underwent an olfactory detection test. The test lasted 1h and consisted in a simultaneous

choice between two bottles: one containing an odorized water solution [banana (isoamyl acetate, 0.05%) or almond (benzaldehyde, 0.01%)] and the other containing plain water. Naive mice with normal odor perception preferred water over odorized water (Bailey and Westbrook 2007). The left/right position of the odorized solution alternated between subjects, so that half of the mice in each group received it on the left side of the cage and the other half on the right side.

4 Intravenous injections of adeno-associated viral vectors

We used an AAV infection approach using the AAV-PHP.eB capsid (Chan et al., 2017) to efficiently transduce our viral vectors to the central nervous system after intravenous injection. Young male mice (PND25) were briefly anaesthetized with isoflurane (Virbac, France) at 5% in an anesthesia chamber. $5E+10$ viral genomes of AAV vector (AAV-PHP.eB-gfa-ABC1D-GFP or AAV-PHP.eB-gfa-ABC1D-NDUFS4-PM) in a volume of $50\mu\text{L}$ were injected with a 0.5 mL 28G insulin syringe into the center of the retro-orbital venous sinus. After injection, each mouse was returned to its cage. The total duration of the procedure lasted less than 10 minutes, including anesthesia and recovery, for each mouse. The retro-orbital intravenous route of AAV delivery to the CNS was chosen over others based on its high success rate (Yardeni et al., 2011) and because it can be easily performed at juvenile mice.

To show representative images of this approach, mice were perfused transcardially with 20 mL PBS pH 7.4 following by 50 mL of cold formalin solution neutral buffered 10% (approx. 4% formaldehyde, Sigma-Aldrich). The brains were cryoprotected by immersing them in a 30% sucrose solution in PBS pH 7.4 for 3-5 days. Sagittal brain sections ($30\mu\text{m}$) were cut on a cryostat (CM1950S Leica), serially collected and stored at $-20\text{ }^{\circ}\text{C}$ until analysis. The sections were incubated overnight at 4°C with rabbit anti GFAP primary antibody (1:500; DAKO) diluted in a blocking solution containing in PBS pH7.4: 0.3% Triton X-100 (Sigma-Aldrich) and 10% normal donkey serum (Merck). After rinsing, the brain slices were incubated for 2 h at room temperature with fluorescent secondary antibody donkey anti rabbit Alexa Fluor 647 (Invitrogen) and finally mounted with Fluoromount-G (Electron Microscopy Sciences). Images were acquired with an epifluorescence Leica DM6000 microscope (Leica, Germany).

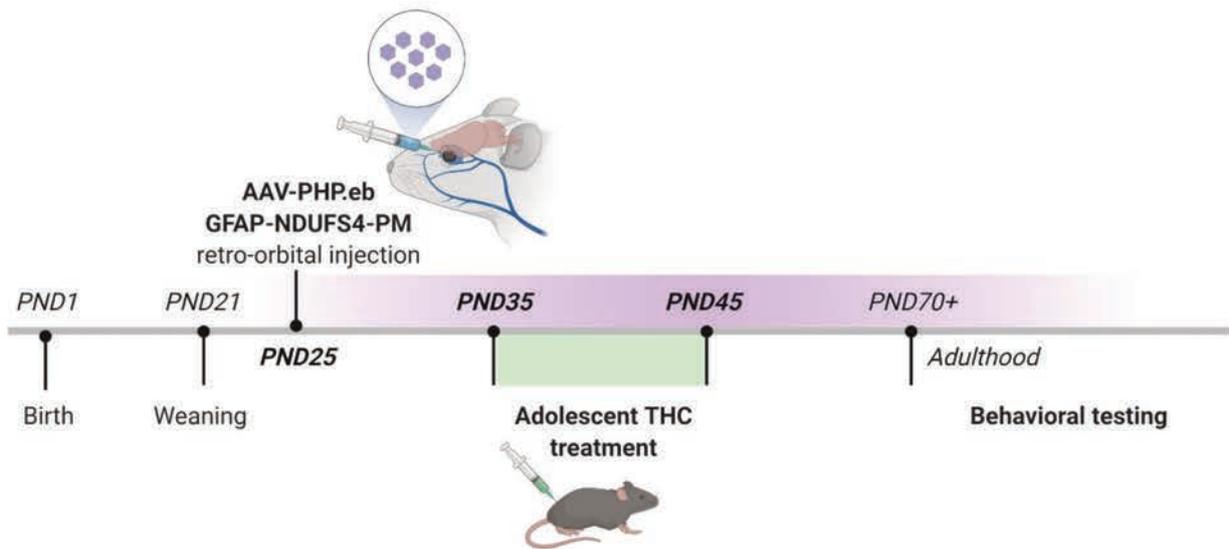


Figure 15. Schematic timeline of experimental design for the intravenous injections of adeno-associated viral vectors. The GFAP-GFP and GFAP-NDUFS4-PM viral vectors were retro-orbitally injected in postnatal day (PND) 25 old mice. The adolescent THC treatment started at PND35 and lasted until PND45, as described before, followed by a drug-free washout period of a month. Behavioral experiments began after PND70 and at the end mice were perfused and brain samples were extracted.

5 Western blot analysis of CB1 receptor and associated proteins expression

Brain tissues were extracted, frozen in dry ice and kept in -80°C . Samples were homogenized in lysis buffer (0.05M Tris-HCl pH 7.4, 0.15M NaCl, 0.001M EDTA, 10% Glycerol, 1% Triton X-100), supplemented with protease and phosphatase inhibitors purchased from Roche (Basel, Switzerland), using the Tissue Lyser (Quiagen, Hilden, Germany). After a 10-minute incubation at 4°C , samples were centrifuged at $17000\times g$ for 20 minutes at 4°C to remove insoluble debris. The protein contents of the total solubilized fractions were determined with Roti-Nanoquant protein quantitation assay, following manufacturer's instruction (Carl Roth, Karlsruhe, Germany). Protein extracts were mixed with denaturing 4x Laemmli loading buffer and warmed for 30 minutes at 37°C .

Samples were analyzed on 4%–20% precast polyacrylamide gels (Bio-Rad, Hercules, California) and transferred onto PVDF membranes $0.45\mu\text{m}$ (Merk Millipore, Billerica, MA). Membranes were blocked in a mixture of Tris-buffered saline and polysorbate 20 (20mM Tris-HCl pH 7.6, 150mM NaCl, 0.05% Tween 20) containing 5% of non-fat milk (or BSA to detect phosphorylated proteins) for 1 hour at room temperature (RT). For immunoblotting we used antibodies against CB1 receptor (ab23703; 1:200, 1h RT, Abcam, Cambridge, UK), phospho - cAMP response element-binding protein (pCREB, Ser133, #9198; 1:1000, overnight 4°C , Cell Signaling Technology Danvers, MA), CREB (#9104; 1:1000 overnight 4°C , Cell Signaling Technology Danvers, MA), pERK (phospho-p44/42 MAPK, #4370; 1:2000, overnight 4°C , Cell Signaling Technology, Danvers, MA), ERK (p44/42 MAPK, #9102; 1:2000 overnight 4°C , Cell Signaling Technology Danvers, MA, USA) and tubulin (sc-69969; 1:5000, 30 minutes RT, Santa Cruz Biotechnology, Dallas, Texas).

Bound primary antibodies were detected with HRP-linked antibodies (1:2000, Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence detection (Clarity Western ECL Substrate, Bio-Rad, Hercules, California). The optical densities of immunoreactive bands were quantified by the Image Lab software (Bio-Rad, Hercules, California) after acquisition on ChemiDoc Touch (Bio-Rad, Hercules, California).

6 Assessment of mitochondrial complex I subunits

6.1 Mitochondria isolation and solubilization

Mitochondria were obtained according to a previously published protocol (Acín-Pérez et al., 2008). Briefly, cells (12-100 millions) were collected, cell pellets frozen at -80 °C and homogenized (10 strokes) in a glass-teflon Potter-Elvehjem, in Buffer A (sucrose 83 mM; MOPS 10 mM; pH 7.2). The same volume of Buffer B (sucrose 250 mM; MOPS 30 mM) was added to the sample, and the homogenate was centrifuged (1000 g, 5 minutes) to remove unbroken cells and nuclei. Centrifugation of the supernatant was then performed (12000 g, 2 minutes) to obtain the mitochondrial fraction, which was washed in Buffer C (sucrose 320 mM; EDTA 1 mM; Tris-HCl 10 mM; pH 7.4). Mitochondria were suspended in Buffer D (6-aminohexanoic acid 1M; Bis-Tris-HCl 50 mM; pH 7.0). Solubilization of mitochondria was performed with digitonin at 4 g/g (5 minutes in ice). After a 30 minutes centrifugation at 13000 g, the supernatant was collected.

6.2 Primary antibodies for western blotting

Immunoblotting was performed using goat polyclonal anti-NDUFS1 (sc-50132, Santa Cruz Biotechnology, 1/500), mouse monoclonal anti-ATPB (1/1000, MS503, MitoSciences), rabbit polyclonal anti-phospho Ser173 NDUFS4 (1/100, provided by D. De Rasmio) (De Rasmio et al., 2012), mouse monoclonal NDUFS4 (1/800, ab87399, Abcam.) and mouse monoclonal anti-NDUFA9 (1/1000, ab14713, Abcam).

6.3 Western blotting

Brain tissues (prefrontal cortex and hippocampus) from male and female mice were lysed in RIPA buffer (2% sodium dodecylsulphate, 2 mM EDTA, 2 mM EGTA and 50 mM Tris pH 7.5), supplemented with phosphatase inhibitors (100 µM phenylmethylsulfonyl fluoride, 50 µg/mL antipain, 50 µg/mL pepstatin, 50 µg/mL amastatin, 50 µg/mL leupeptin, 50 µg/mL bestatin, 1 mM o-vanadate, 50 mM NaF, and 50 µg/mL soybean trypsin inhibitor) and boiled for 5 minutes. Extracts were centrifuged at 13,000xg for 5 minutes at 4°C, and aliquots of lysates (50µg protein, unless otherwise stated) were subjected to sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis on a 8, 10 or 12% acrylamide gel (MiniProtean®, Bio-Rad) including PageRuler Plus Prestained Protein Ladder (Thermo). The resolved proteins were transferred electrophoretically to nitrocellulose membranes (Hybond-ECL, Amersham

Bioscience Europe GmbH, Barcelona, Spain). Membranes were blocked with 5% (w/v) low-fat milk in 20 mM Tris, 500 mM NaCl, and 0.1% (w/v) Tween 20, pH 7.5, for 1 h. After blocking, membranes were immunoblotted with primary antibodies at dilutions ranging from 1:100 to 1:1000 overnight at 4°C. After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnologies), goat anti-mouse IgG (Santa Cruz Biotechnologies), or rabbit anti-goat IgG (Santa Cruz Biotechnologies) (all at 1:10,000 dilution), membranes were immediately incubated with the enhanced chemiluminescence kit WesternBright ECL (Advansta) for 2 minutes or Immobilon Western Chemiluminiscent HRP Substrate (Merck Millipore; Darmstadt, Germany) for 2 minutes, before exposure to Fuji Medical X-Ray film (Fujifilm), and the autoradiograms scanned. Biologically independent replicates were always performed, and a representative western blot is shown.

7 Statistical analysis

Data are expressed as mean \pm SEM or single data points and were analyzed with Prism 9.0 (Graphpad Software). Data were checked for normality using the Shapiro–Wilk test. Repeated or unpaired statistical analyses were obtained with Student’s t test or ANOVA (one-way or two-way) to compare two or multiple groups. For ANOVA, only when significant interaction effects of main factors were detected, post-hoc analyses (Sidak’s) were performed. When normality could not be calculated for all groups, non-parametric tests (Mann Whitney test and Wilcoxon) were also used. Significance was set at $p \leq 0.05$. Most behavioral experiments were repeated two times in independent groups of mice with similar results. Predefined exclusion criteria were set before the initiation of behavioral tests. Group sizes indicate independent animals and any outliers in the behavioral tests were removed from the data sets based either on the predefined exclusion criteria or following statistical tests for outliers (Grubbs’ test). For western blot analysis, the average value of the control group was set to “100,” and experimental group measures were reported relative to control values. Statistical details for each quantitative experiment are illustrated in Supplementary Table 1. Graphical figures created with Prism 9.0 (Graphpad Software) and biorender.com.

Chapter IV Results

1 Sex-dependent long-term effects of adolescent THC exposure

1.1 Chronic THC exposure during adolescence led to long-lasting sex-dependent behavioral deficits

Adolescent male and female mice were treated with two different doses of THC (1 mg/kg or 5 mg/kg respectively, from now on indicated as THC1 and THC5) during 10 days (PND 35–45). The body weight of the animals was daily evaluated during the course of the THC treatment and THC treatment had no effect on body weight or overall growth of mice of either sex (data not shown). THC-treated groups did not differ in weight from vehicle-treated groups neither during adolescence, nor in adulthood (data not shown). When mice reached adulthood [considered as PND70 onwards, (Spear, 2000a)], the effect of the adolescent THC administration on a battery of behavioral tasks known to be affected by acute cannabinoid administration in rodents and often used as correlates of psychotic-like effects (Busquets-Garcia *et al.*, 2017b; Powell and Miyakawa, 2006), including locomotion, social interactions and spontaneous alternation.

Locomotion

The open field test was used to assess generalized locomotion and exploratory behavior in a novel environment in adult male and female mice (Seibenhener and Wooten, 2015) (Figure 16A). In males, a significant increase in the total locomotor activity was found for both the THC1 and THC5 groups, as compared to the vehicle (Figure 16B), while no significant changes were observed in females (Figure

16C). Analyzing separately the horizontal locomotor activity and the vertical rearing responses during the 5 minutes in the open field, male THC-treated groups showed both increased horizontal locomotion, and increased vertical rearing, while female THC-treated groups did not differ from vehicle for neither horizontal nor vertical locomotor activity.

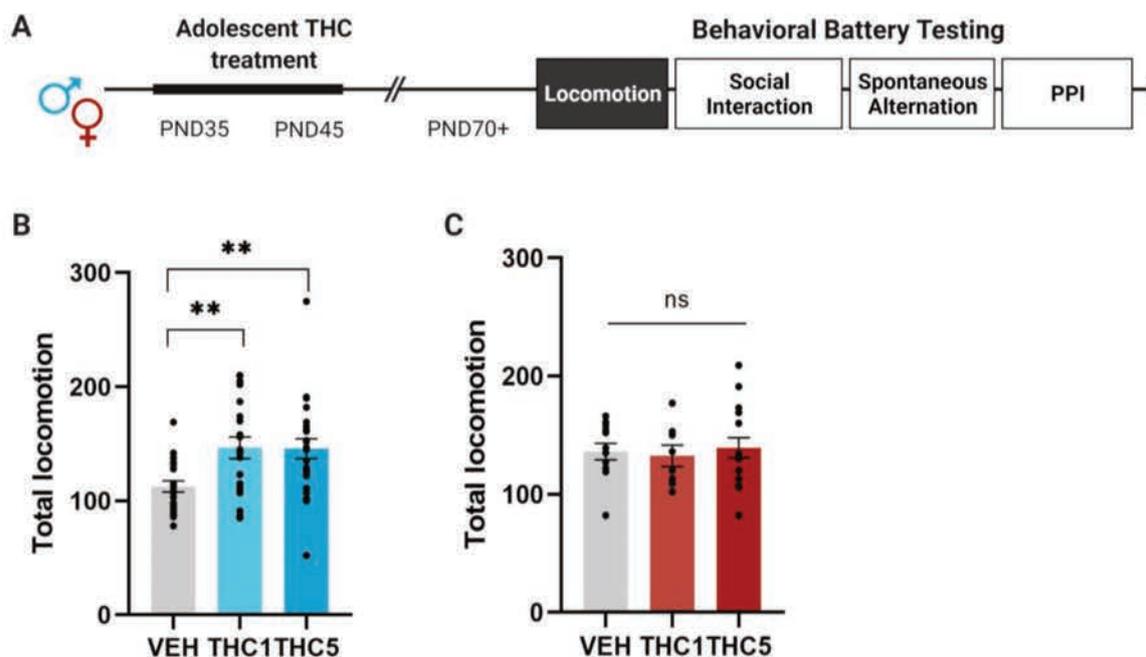


Figure 16. Adolescent THC treatment enhances locomotion in adult male but not female mice. (A) Schematic timeline of experimental design. (B, C) Effect of adolescent THC (1 and 5 mg/kg) or vehicle treatment in the spontaneous locomotor activity of adult male (B) and female (C) mice. All data is presented as mean \pm S.E.M. ** $P < 0.01$ compared to mice exposed to vehicle treatment. For statistical details and n , refer to Supplementary Table 1.

Social interaction

The social behavior of the mice was assessed in the social interaction task (Figure 17A). A social interaction index in the two-chamber test was calculated by dividing the time animals spent exploring the social zone by the total time spent in both the social and non-social zones, as described before (Chapter III). Adolescent THC treatment led to an impairment in social interactions in male mice, as indicated by the decreased interaction index for both THC-treated groups (Figure 17B), while no significant difference between the THC groups and the vehicle group was observed for female animals (Figure 17C). To control for the general exploratory activity of the different groups, the total time exploring both the social and non-social zones during the 5-minute test was evaluated. The total

exploration was not significantly different between the THC groups and vehicle in males (Figure 17D), while in female mice, the THC1 group showed a significant decrease in the exploration time compared to the THC5 group ($P=0.0210$) (Figure 17E).

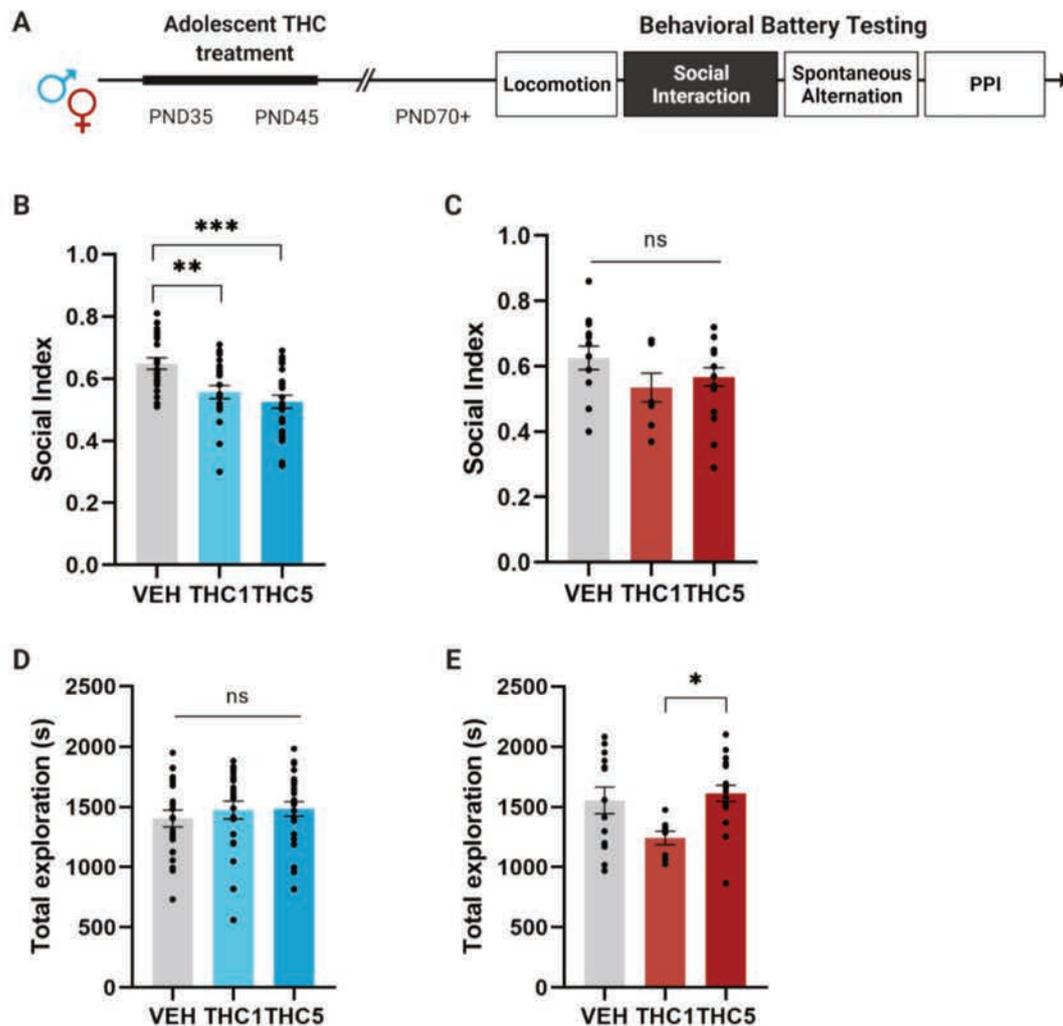


Figure 17. Adolescent THC treatment impairs social interaction in adult male but not female mice. (A) Schematic timeline of experimental design. (B, C) Effect of adolescent THC (1 and 5 mg/kg) or vehicle treatment in the social interaction task in adult male (B) and female (C) mice. (D, E) Impact of the two different THC doses of the adolescent THC treatment in the total exploration of the animals in the social interaction task, as measured by the total interaction time (seconds) in both social and non-social compartments in male (D) and female treated mice (E). All data is presented as mean \pm S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to mice exposed to vehicle treatment. For statistical details and n, refer to Supplementary Table 1.

Spontaneous alternation

Mice were then tested in the spontaneous alternation task using a Y-maze in order to evaluate short-term spatial memory (Hughes, 2004) (Figure 18A). Performance in the task was impaired following the adolescent THC treatment in male mice, with a significant effect only for the higher dose ($P=0.0194$ for "VEH vs. THC5") (Figure 18B). No differences in performance were observed between the THC-treated groups and the vehicle group for females (Figure 18C). The total number of entries in all three arms was not affected by the adolescent THC treatment in neither of the sexes (Figure 18D, E).

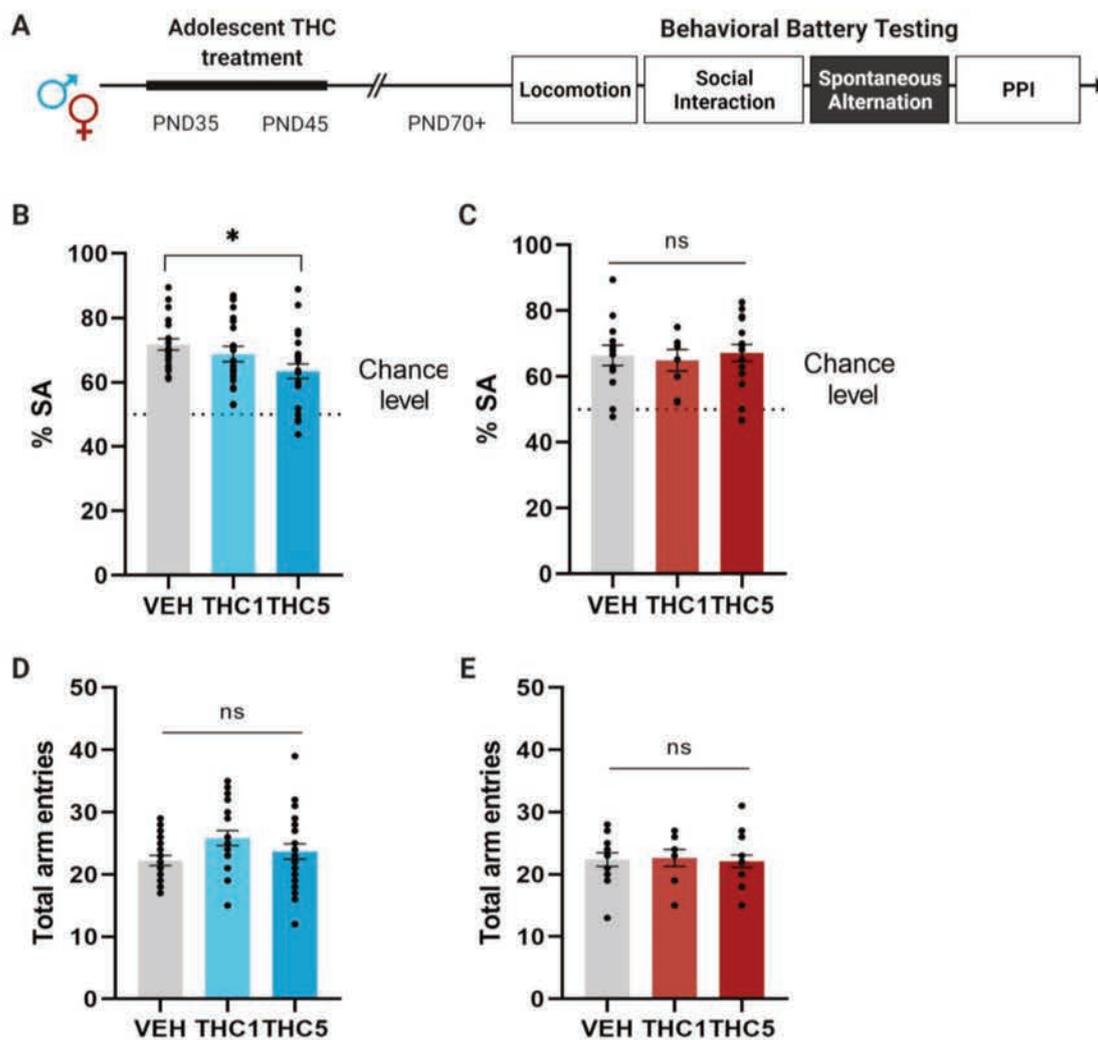


Figure 18. Adolescent THC treatment impairs short-term spatial memory in adult male but not female mice. (A) Schematic timeline of experimental design. (B, C) Effect of adolescent THC (1 and 5 mg/kg) or vehicle treatment in the spontaneous alternation task in adult male (B) and female (C) mice. (D, E) The two different THC doses of the adolescent THC treatment did not impact the total exploration of the animals in the spontaneous alternation task, as measured by the number of total alternations during the task, in neither male (D), nor female treated mice (E). All data is presented as mean \pm S.E.M. *

P<0.05 compared to mice exposed to vehicle treatment. For statistical details and n, refer to Supplementary Table 1.

Overall, these results indicate that THC administration during adolescence has a clear impact in locomotion, social behavior and short-term spatial memory in adult male mice, but not in their female littermates.

1.2 Chronic THC exposure during adolescence did not impact PPI in adult male and female mice

We then analyzed the prepulse inhibition of the startle response (PPI) to study the effect of adolescent THC on sensorimotor gating (Ioannidou *et al.*, 2018) (Figure 19A). We found no significant differences between the vehicle- and THC-treated groups when analyzing the PPI at three different prepulse (PP) intensities (PP73, PP76, PP82) in either sexes (Figure 19B, C). Moreover, vehicle- and THC-treated mice did not show differences in the acoustic startle response amplitude, independently of the sex (Figure 19D, E).

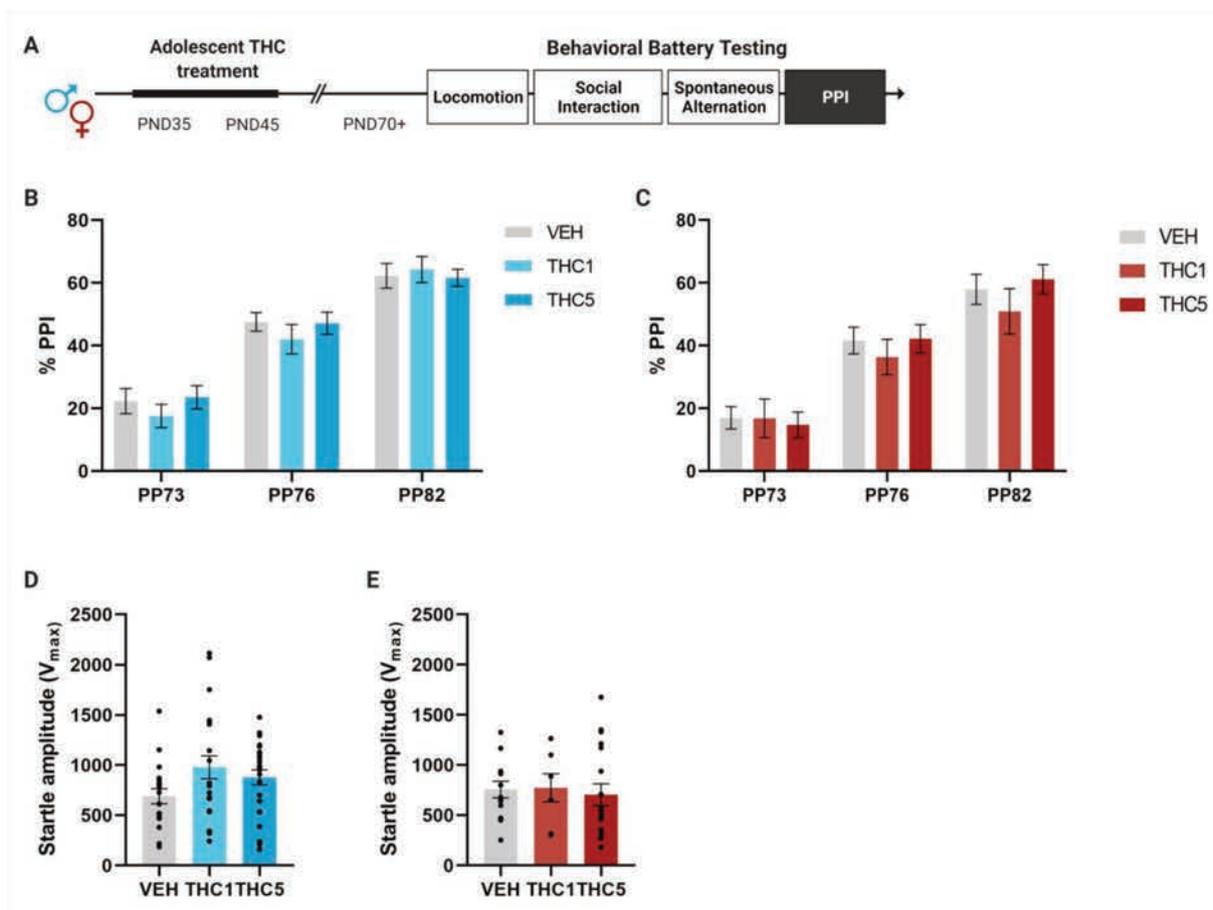


Figure 19. Adolescent THC treatment did not impact sensorimotor gating in adult male and female mice. (A) Schematic timeline of experimental design. (B, D) Adolescent THC (1 and 5 mg/kg) did not have an effect in the prepulse inhibition of the startle response at three different prepulse intensities (73, 76, and 82 dB) in neither adult male (B), nor female mice (D). (C, E) Adolescent THC (1 and 5 mg/kg) did not impact the amplitude of the startle response in the PPI task in neither male (C) nor female (E) treated animals. Data presented as mean \pm S.E.M. For statistical details and n, refer to Supplementary Table 1.

1.3 Molecular changes induced by chronic THC exposure during adolescence

Following the behavioral characterization, I aimed at investigating a range of potential long-term biochemical effects of adolescent THC. We first isolated brain samples of adult mice following adolescent THC treatment. Samples of the hippocampus and prefrontal cortex were extracted from male and female mice at the end of the behavioral battery testing. The regions of interest were chosen according to their high expression of CB1 receptors (Marsicano and Lutz, 1999), as well as their relevance to the behavioral alterations we observed (De Melo et al., 2005; Rubino et al., 2009a). First, we sought to identify potential direct changes on CB1 receptors expression induced by adolescent THC

treatment. Proteins involved in major CB1 signaling cascades were also quantified in order to further examine the neurobiological status reflecting the behavioral effects observed, including the activation of extracellular signal-regulated kinases (ERK) and of cAMP response element-binding proteins (CREB), which are phosphorylated/activated upon CB1 activation (Galve-Roperh et al., 2002; Lazenka et al., 2013).

Moreover, we have recently shown that THC effects on social interaction are tightly linked to mitochondrial activity, through the phosphorylation of the mitochondrial complex I subunit NDUFS4 [(Jimenez-Blasco *et al.*, 2020), Section 3 of this Chapter]. Since adolescent THC administration led to deficits in social interaction in male treated animals, we hypothesized that complex I proteins, including NDUFS4 phosphorylation might also be affected after the treatment. To assess this hypothesis, brain samples from both adolescent and adult male and female animals were extracted and NDUFS4 phosphorylation was quantified.

1.3.1 CB1 receptor down-regulation in the hippocampus of adult male, but not female, mice after adolescent THC treatment

At the end of the behavioral characterization, we assessed the expression of CB1 and related signaling proteins in hippocampal brain samples. We focused on the hippocampus based on the relative abundance of CB1 receptors in this region and the relevance to the behavioral tasks that were used. CB1 receptor expression was decreased in the hippocampus of adolescent THC-treated adult male mice, with the post-hoc test revealing a significant difference between the vehicle and THC5 groups ($P= 0.0032$) (Figure 20A). On the contrary, no differences were observed between controls and THC-treated groups in adult female animals (Figure 20B).

A separate batch of male mice underwent adolescent THC treatment (here treated only with the THC dose of 5 mg/kg) and hippocampal brain samples were extracted directly at the end of the adolescent THC treatment (PND46). Notably, a significant decrease of CB1 receptor protein expression was observed in the hippocampus of these mice (Figure 20C), suggesting that the THC-induced reduction of hippocampal CB1 receptors expression is observed from adolescence and conserved at adulthood.

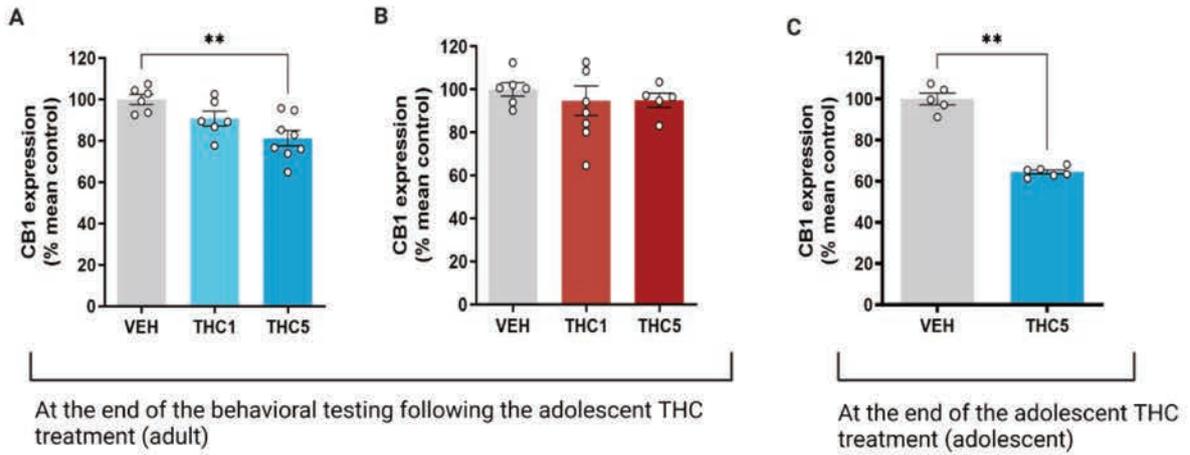


Figure 20. Effects of adolescent THC treatment on hippocampal CB1 receptor levels. Optical densitometric quantification of CB1 expression levels following adolescent THC treatment, in the hippocampus of adult male and female mice (A, B, respectively) and adolescent male mice (THC). Data are expressed as mean \pm SEM. ** $P < 0.01$ as compared to vehicle treated mice. For statistical details and n, refer to Supplementary Table 1.

1.3.2 Altered ERK and CREB phosphorylation in the hippocampus of adult male and female mice after adolescent THC treatment

The phosphorylation state of extracellular signal-regulated kinases (ERK) and of cAMP response element-binding proteins (CREB) were also assessed as markers of the CB1 receptors activation signaling cascades (Galve-Roperh *et al.*, 2002; Lazenka *et al.*, 2013). One-way ANOVA revealed a significant reduction in ERK phosphorylation of THC-treated mice in hippocampus after adolescent THC exposure compared to vehicle, reflecting specifically a reduction in the THC1 group (Figure 21A). On the contrary, in the hippocampus of female THC-treated mice, an increase in ERK phosphorylation was observed in the THC1 group (Figure 21B). Regarding the phosphorylation of CREB, the adolescent THC significantly increased phospho-CREB in both male and female mice (Figure 21C, D).

The total protein levels of ERK and CREB after the adolescent THC treatment were unaffected in adult male mice (Figure 22A, B), however in females the THC5-treated groups showed increased total levels of both proteins (Figure 22C, D).

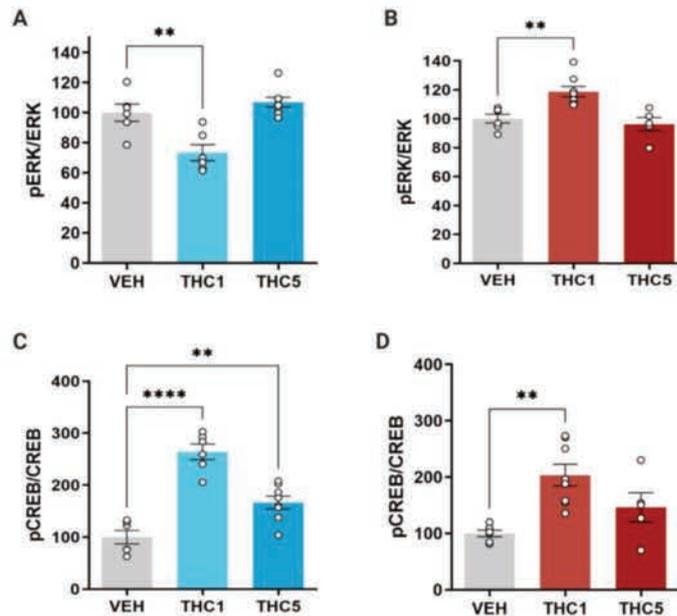


Figure 21. Molecular alterations in the hippocampus of adult male and female mice following adolescent THC treatment. Optical densitometric quantification of ERK and CREB phosphorylation levels in adolescent treated male (A, C) and female mice (B, D). Data are expressed as mean \pm SEM. ** $P < 0.01$ and **** $P < 0.0001$ as compared to vehicle treated mice. For statistical details and n, refer to Supplementary Table 1.

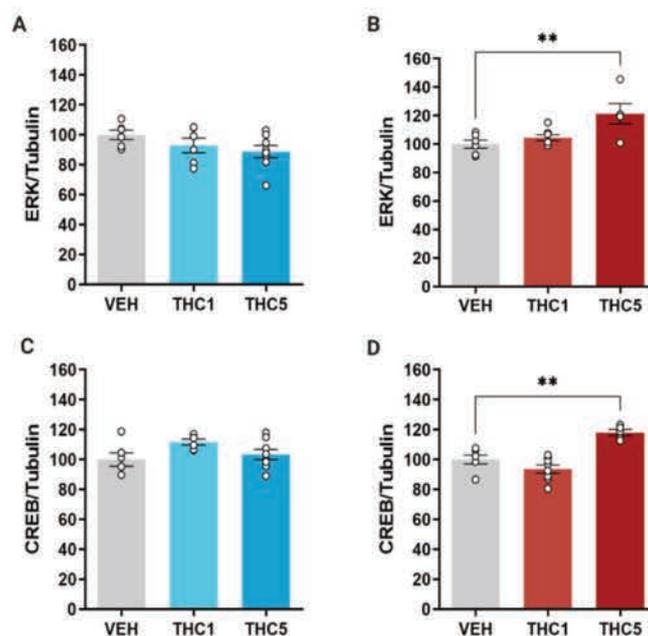


Figure 22. Total protein levels of ERK and CREB in adolescent treated male (A, C) and female mice (B, D). Data are expressed as mean \pm SEM. ** $P < 0.01$ as compared to vehicle treated mice.

1.3.3 Sex differences on NDUFS4 phosphorylation after adolescent THC treatment

The data showed so far indicates that the adolescent THC treatments specifically reduce adult levels of hippocampal CB1 receptor protein in male mice. However, the CB1 receptor-dependent signaling pathways investigated (ERK and CREB) displayed scattered alterations in both males and females. It is therefore unlikely that these "classic" CB1 receptor-dependent signaling pathways might explain the differential behavioral effects of adolescent THC in adult male *versus* female mice. Therefore, we investigated less conventional recently described signaling pathways activated by CB1 receptor agonism. Our recent findings show that mitochondrial complex I instability in astrocytes is linked to persistent THC-induced social deficits through the de-phosphorylation of the complex I subunit NDUFS4 (Jimenez-Blasco *et al.*, 2020). Thus, I asked whether adolescent THC might affect the levels of NDUFS4 phosphorylation, in a similar manner as after acute THC administration in adulthood (Jimenez-Blasco *et al.*, 2020).

We quantified NDUFS4 protein and its phosphorylation levels at two different time points following the adolescent THC treatment, either at the end of the adolescent treatment (PND46, 24h after last

injection, from now on described as the "Adolescent" group) or after an abstinence of a month, when animals reached adulthood (PND75, from now on described as the "Adult" group). Since the most prominent behavioral deficits reported in the previous part were found in those animals chronically treated with the highest dose of the drug during adolescence (THC 5 mg/kg), we only kept this dose for the experiments described here.

Age was accompanied with differences in the basal levels (i.e. vehicle-treated) of phosphorylation of the NDUFS4 subunit at Ser173 (NDUFS4-Ser173) in a brain region- and sex-dependent manner (Figure 23). Interestingly, however, adolescent THC treatment significantly decreased the hippocampal and prefrontal cortex levels of NDUFS4-Ser173 phosphorylation of male adolescent mice (Figure 24A, B). In contrast, both hippocampal and prefrontal cortex samples of THC-treated female mice did not display any alteration of NDUFS4-Ser173 phosphorylation (Figure 24C, D). However, the effect of the THC treatment in adolescent male mice was completely abolished in the adults (Figure 24A, B), suggesting a transient effect of adolescent THC, while no effect was observed in adult female mice (Figure 24C, D).

These data indicate that NDUFS4-Ser173 phosphorylation is specifically reduced by adolescent THC treatment in male, but not female mice, suggesting that this molecular pathway might be responsible of the later behavioral effects of the treatment.

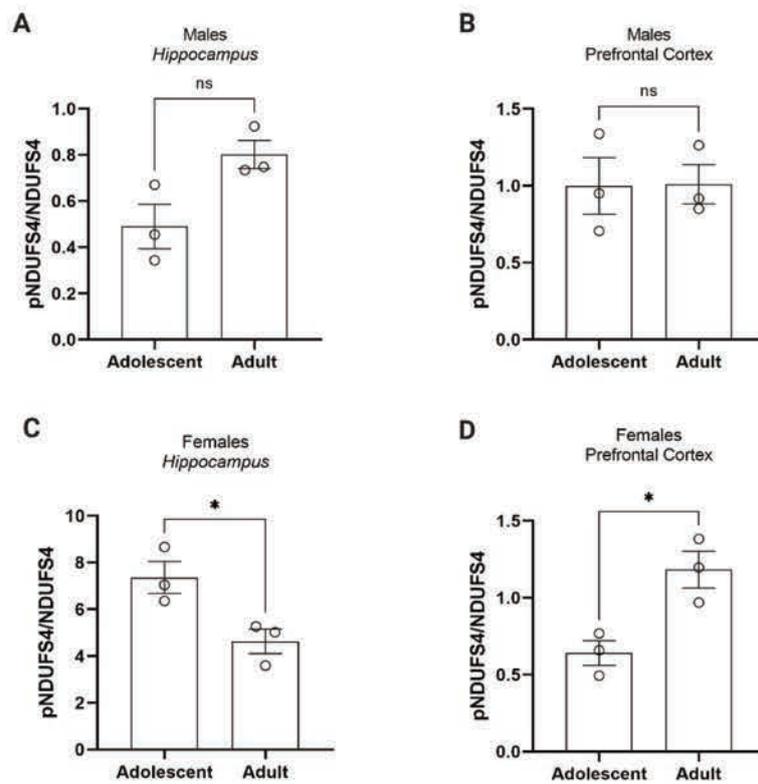


Figure 23. Basal NDUF54-Ser173 phosphorylation in the hippocampus and prefrontal cortex of adolescent vehicle treated male and female mice. Data is presented as mean \pm S.E.M. * $P < 0.05$, unpaired t tests.

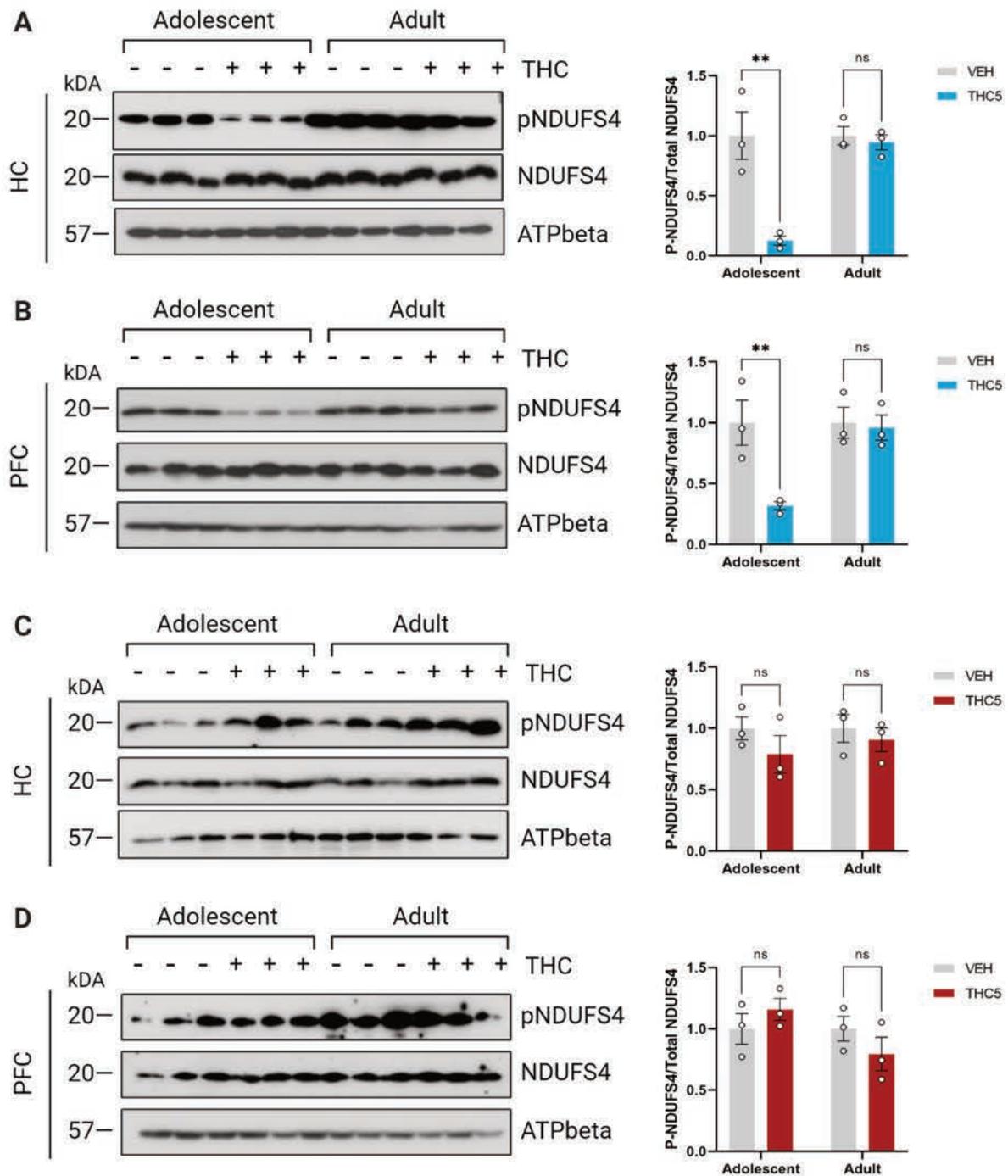


Figure 24. Adolescent THC treatment reduces the phosphorylation of NDUFS4 in adolescent male, but not female, mice. Effect of adolescent THC treatment on pNDUFS4 in the hippocampus (HC) and prefrontal cortex (PFC) of male and female mice (A, B and C, D, respectively). In the right panels the effect of THC is normalized to the respective vehicle group for each condition. Data are expressed as mean \pm SEM. ** $P < 0.01$ as compared to vehicle treated mice. For statistical details and n , refer to Supplementary Table 1.

1.4 Astroglial NDUFS4-PM overexpression prevents adolescent THC-induced behavioral effects in adult male mice

To assess the direct involvement of NDUFS4-Ser173 phosphorylation in the adolescent THC-mediated behavioral effects and to assess the cell type where this occurs, we overexpressed a phosphomimetic mutant of NDUFS4 in which the Ser173 residue was replaced by a Glu residue (NDUFS4-PM) (Jimenez-Blasco et al., 2020). We retro-orbitally injected an AAV-PHP.eB viral vector that expresses NDUFS4-PM under the control of the human Glial Fibrillary Acidic Protein (GFAP) promoter (AAV PHP.eB gfaABC1D-NDUFS4-PM; here called AAV-GFAP-NDUFS4-PM) in pre-adolescent (PND25) male mice. GFAP is a marker of mature astrocytes, therefore, the overexpression of the NDUFS4-PM construct is highly targeted to astrocytes. The mice then underwent chronic adolescent THC treatment, as previously described (daily i.p. injections of 5 mg/kg THC during 10 days, from PND 35–45) and after a month they were tested in the social interaction task, locomotion in the open field and spontaneous alternation in the Y-maze (Figure 25A). At the end of the behavioral testing, mice were sacrificed and viral expression in astrocytes was confirmed in the AAV-Control group carrying a fluorescent protein. Interestingly, the effects of the adolescent THC treatment in social interaction (Figure 25B), as well as locomotor activity (Figure 25B) were completely abolished in the AAV-GFAP-NDUFS4-PM group, whereas only a non-significant trend was observed in the spontaneous alternation task (Figure 25D). Representative images of the viral expression by retro-orbital injection are shown for the AAV-Control group (Figure 26A). In the NDUFS4-PM groups the viral infection could not be assessed since the viral construct used here did not contain a tag or fluorescent protein. Notably, the astroglial overexpression of NDUFS4-PM had no effect *per se* on the baseline behavior of the vehicle-treated group in any of the behavioral tasks (Figure 26). Altogether, these data strongly suggest that at least some of the "psychotic-like" effects caused by chronic THC administration during adolescence are due to a transient reduction of astroglial NDUFS4-Ser173 phosphorylation.

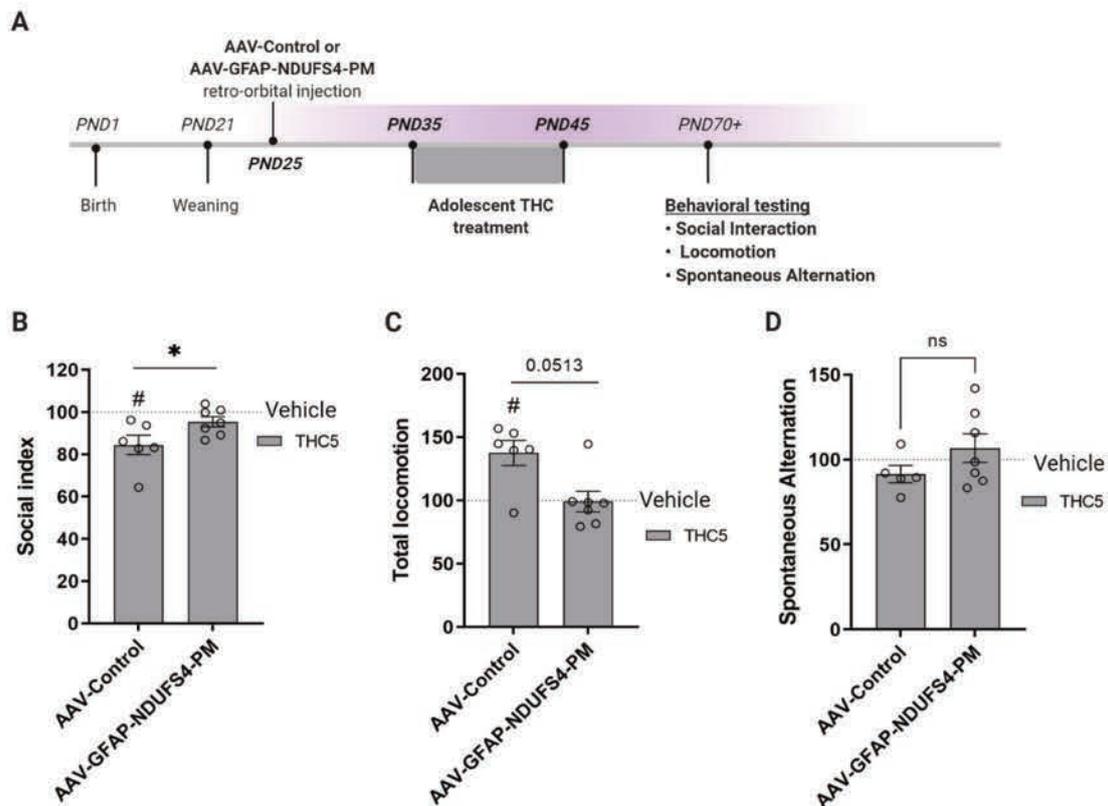


Figure 25. NDUFS4-PM expression in astrocytes blocks the adolescent THC-induced impairment in social interaction and the adolescent THC-induced hyperlocomotion in adult male mice. (A) Schematic timeline of experimental design. (B-D) Effect of viral expression of GFAP-NDUFS4-PM by retro-orbital injection on the adolescent THC-induced decrease of social interaction (B), hyperlocomotor activity (C) and spontaneous alternation performance (D) in adult male mice. Data are expressed as mean \pm SEM, normalized to the corresponding vehicle groups. * $P < 0.05$ (as compared to AAV-Control), # $P < 0.05$ (as compared to vehicle treated mice). For statistical details and n , refer to Supplementary Table 1.

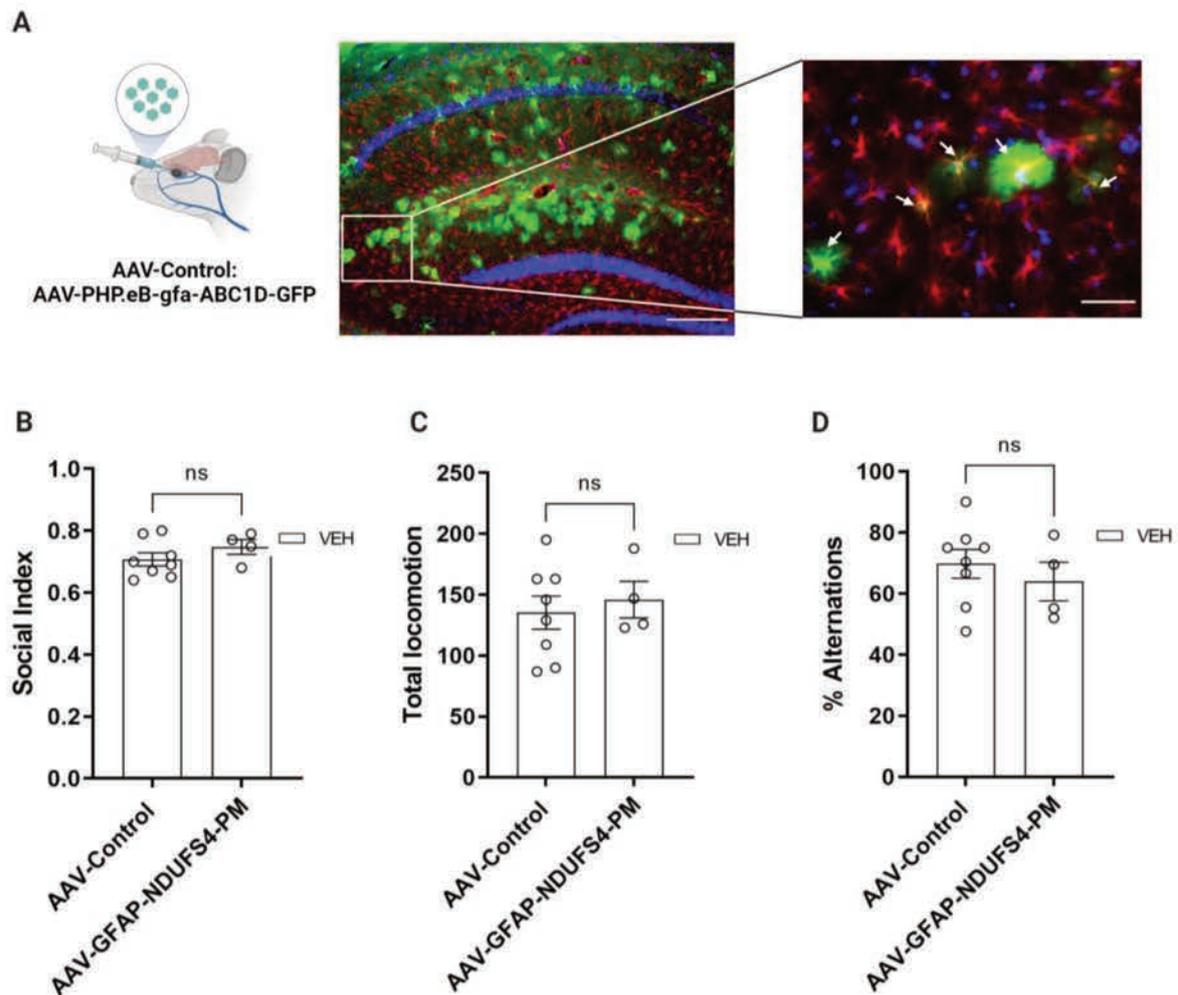


Figure 26. Effect of astroglial expression of NDUFS4-PM on the behavior of the vehicle-treated group. (A) Representative images showing expression of AAV-GFAP-GFP in the hippocampus of adult male mice. GFAP (red), GFP (green) and DAPI (blue) in sagittal section. Scale bar, 200 mm and 40 μ m. (B-C) The performance of adult mice treated with vehicle during adolescence did not differ between the AAV-Control and AAV-GFAP-NDUFS4-PM expressing groups in the social interaction test (B), locomotor activity (C) or in the spontaneous alternation task (D). Data is presented as mean \pm S.E.M.

1.5 Effects of Adolescent Cannabinoid Exposure in Higher-order Cognitive Processes

1.5.1 Adolescent THC exposure did not impact mediated and direct learning in adult male mice

In the following experiments we aimed at assessing for the first time the impact of chronic adolescent THC exposure in higher-order learning in adulthood, using a version of sensory preconditioning that

evaluates representation-mediated taste aversion (Busquets-Garcia *et al.*, 2017a) (for a detailed description of the protocol refer to the respective section in Chapter III Materials and Methods).

Following the adolescent THC treatment and after a month of washout and manipulation-free period, adult animals underwent an odor-taste sensory preconditioning task. First, adult male mice, treated with THC during adolescence, were exposed to a preconditioning phase with three odor-taste pairings (sucrose and maltodextrin as tastes; banana and almond as odors), followed by a conditioning phase consisting in the devaluation of one of the odor stimuli (Figure 27A). Animals in normal conditions consume lower amounts of the solution containing the taste or odor that were indirectly (preconditioned) or directly devaluated, indicating the typical formation of mediated and direct aversion learning (Busquets-Garcia *et al.*, 2018b; Busquets-Garcia *et al.*, 2017b). For the adolescent treated mice, both the vehicle group and the two THC-treated groups demonstrated an intact mediated aversion to the preconditioned stimulus (taste), as well as an aversion to the directly conditioned stimulus (odor) (Figure 27B, C). Two-way ANOVA analysis for total consumption, with treatment and conditioning as between-subjects factors revealed a significant main effect of conditioning both for mediated taste [$F(1, 27) = 21.21, P < 0.0001$] and direct odor [$F(1, 27) = 35.57, P < 0.0001$] aversion, and no effect of the THC treatment or interaction between the two factors.

Finally, the total liquid intake that the mice consumed did not differ between the vehicle and THC-treated groups during the two choice tests (Figure 28) or during the other phases of the protocol (data not shown).

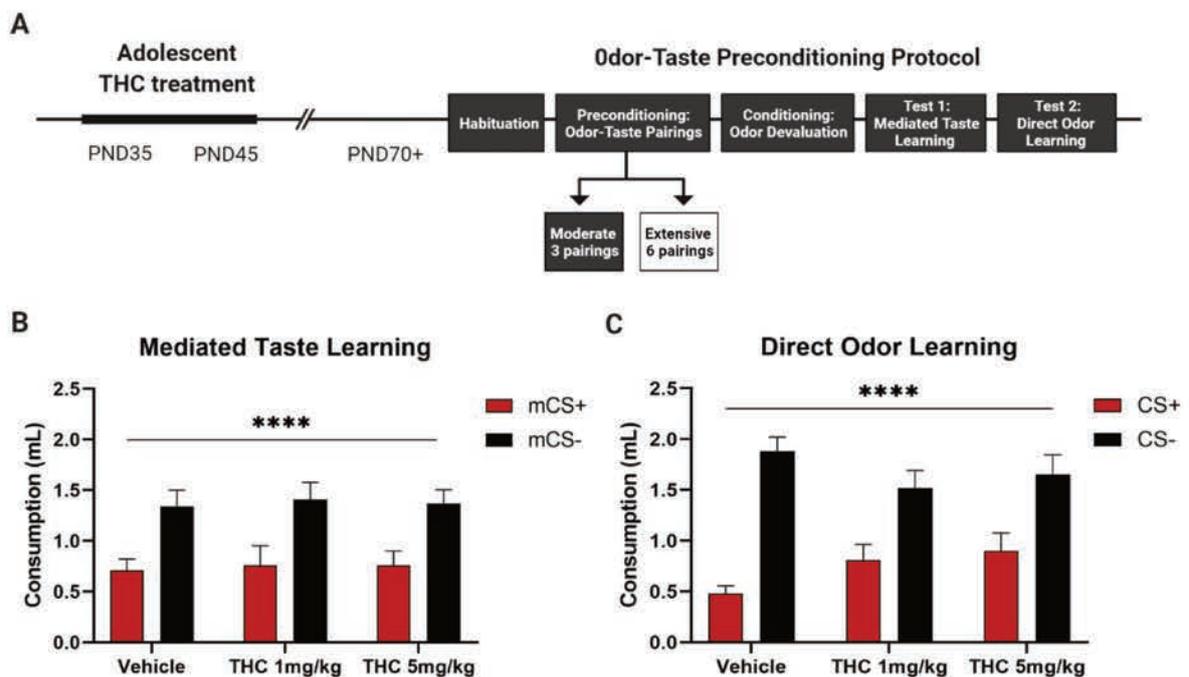


Figure 27. Mediated and direct learning in adult male mice following adolescent THC treatment. (A) Schematic timeline of experimental design. (B, C) Effect of adolescent THC treatment in mediated taste (B) and direct odor (C) aversion in adult male mice. **** $P < 0.0001$ for the main effects of the mCS and CS. For statistical details and n , refer to Supplementary Table 1.

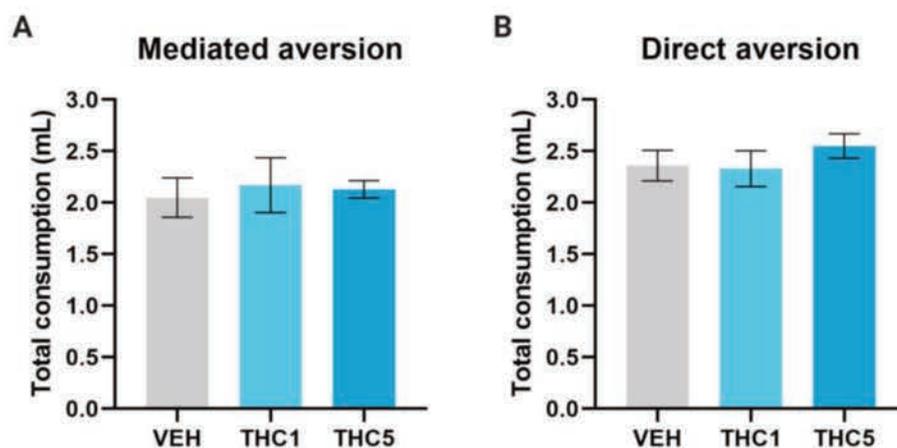


Figure 28. Total liquid consumption of adult male mice following adolescent THC treatment during mediated taste (A) and direct odor (B) learning tests. No significant differences. Data is presented as mean \pm S.E.M.

1.5.2 Adolescent THC exposure led to impairment in mediated and direct learning in adult female mice

The described odor-taste preconditioning protocol had been established and previously tested only in male mice (Busquets-Garcia *et al.*, 2018b; Busquets-Garcia *et al.*, 2017b). Therefore, before addressing the effect of the adolescent THC treatment on mediated and direct learning in female animals, it was necessary to first test the protocol's efficiency in naïve female mice. For this reason, a total of 33 female wild-type C57BL/6 mice were purchased and used in the odor-sensory preconditioning protocol. The female mice were exposed to a preconditioning phase with three odor-taste pairings (sucrose and maltodextrin as tastes; banana and almond as odors), followed by a conditioning phase consisting in the devaluation of one of the two stimuli. In half of the mice the conditioning phase accounted for the devaluation of the odor stimulus, while in the other half for the devaluation of the taste stimulus. Consequently, in the first group mediated taste and direct odor learning were evaluated, and in the second group mediated odor and direct taste were assessed at the testing phase (Figure 29A). Regarding the consumption of the tastes or odors that were indirectly devaluated, female mice displayed a trend of lower consumption that almost reached levels of significance ($P=0.0888$ and $P=0.0885$, when odors and tastes were devaluated, respectively) (Figure 29B, C), partly supporting the formation of a mediated aversion learning, though not as profound as the one displayed by male animals undergoing the same protocol. Moreover, as expected, the female mice consumed lower amounts of the tastes or odors that were directly devaluated, indicating the formation of a reliable direct aversion learning with both stimuli (Figure 29D, E), although the direct aversion was more robust when taste was devaluated (Figure 29E).

We therefore proceeded to evaluate the effect of adolescent THC treatment on the formation of mediated and direct learning in adult female mice (Figure 30A). Contrary to what was observed in male mice, the adolescent THC treatment led to impairments in both mediated and direct learning. Two-way ANOVA analysis for total consumption, with treatment and conditioning as between-subjects factors showed no significant interaction or main effects of the treatment and the conditioning for mediated taste aversion, while for direct odor aversion, a significant main effect of conditioning [$F(1, 29) = 21.25$, $P < 0.0001$] was observed. However, separate paired *t* test analysis between mCS+ and mCS- (for mediated aversion) or CS+ and CS- (for direct aversion), revealed a trend towards or a clear impairment in the formation of mediated aversion in the THC1 and THC5 treated groups ($P=0.5770$ and $P=0.9818$, respectively) (Figure 30B). A similar impairment was observed for the formation of direct odor aversion in the THC5 treated group ($P=0.2347$) (Figure 30C). Importantly, the total liquid intake that the mice

consumed did not differ between the vehicle and THC-treated groups during the two choice tests (Figure 31) or during the other phases of the protocol (data not shown).

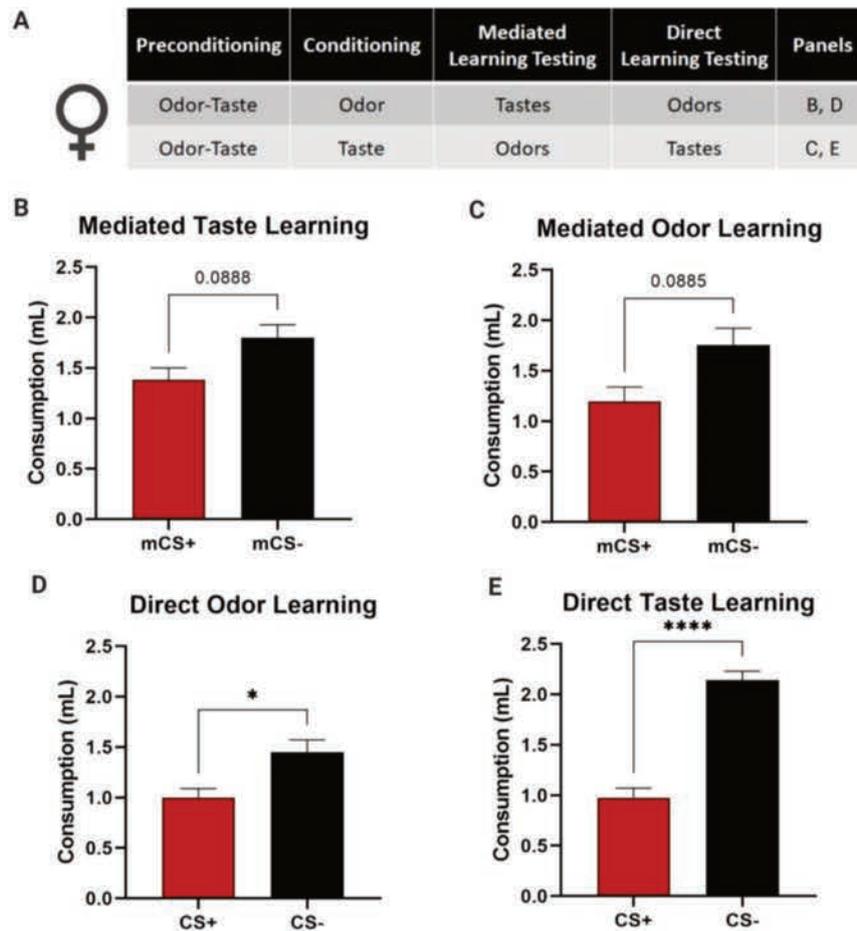


Figure 29. Mediated and direct learning in naïve adult female mice. (A) Schematic table of the odor-taste sensory preconditioning protocol. (B, C) Liquid consumption under conditions of mediated taste (B) or odor (C) aversion. (D, E) Liquid consumption under conditions of direct odor (D) or taste (E) aversion. ** $P < 0.01$; **** $P < 0.0001$. For statistical details and n , refer to Supplementary Table 1.

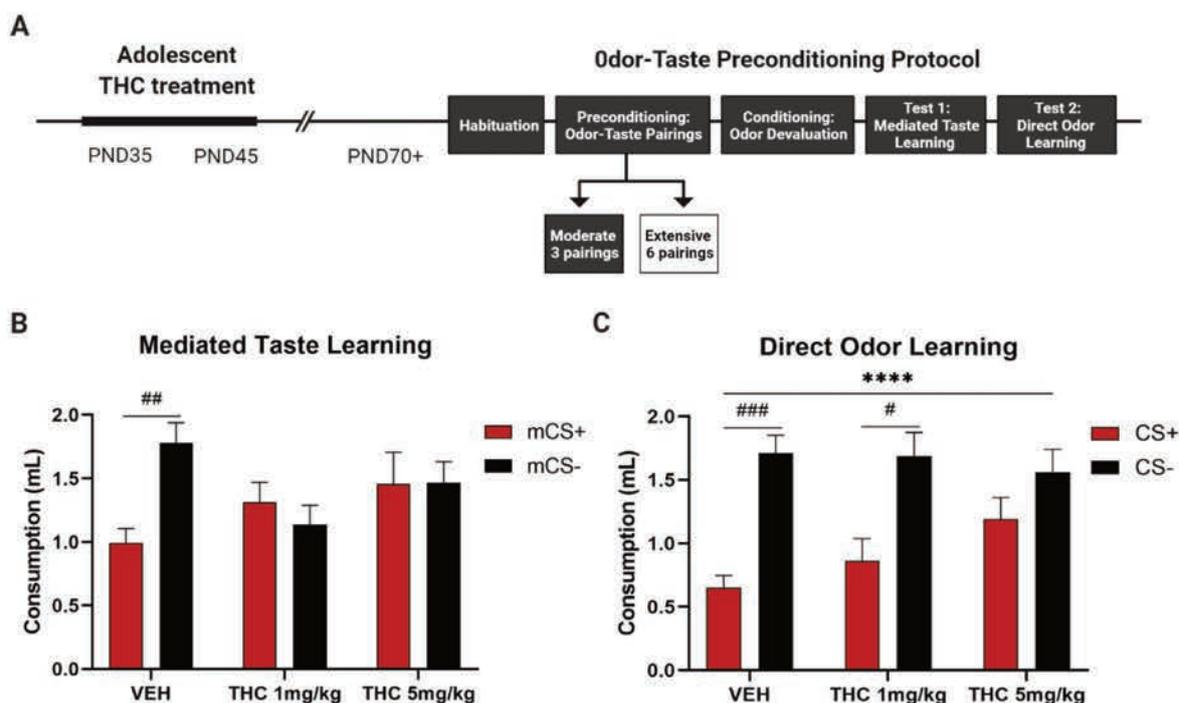


Figure 30. Mediated and direct learning in adult female mice following adolescent THC treatment. (A) Schematic timeline of experimental design. (B, C) Effect of adolescent THC treatment in mediated taste (B) and direct odor (C) aversion in adult female mice. **** $P < 0.0001$ for the main effect of the CS. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ (mCS+ versus mCS- or CS+ versus CS-). For statistical details and n, refer to Supplementary Table 1.

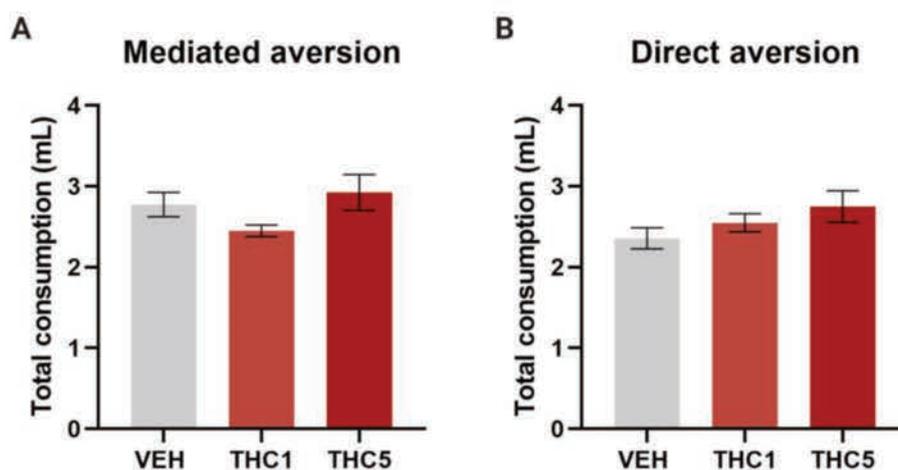


Figure 31. Total liquid consumption of adult female mice following adolescent THC treatment during mediated taste (A) and direct odor (B) learning tests. No significant differences. Data is presented as mean \pm S.E.M.

1.5.3 Adolescent THC exposure did not impact reality testing performance in adult male mice

In the previous sections it was shown that in the representation-mediated learning task used here (mentioned as Odor-Taste Preconditioning Protocol, see the respective section in the Materials and Methods chapter for more details), control animals with a moderate amount of training (3 odor-taste pairings) during preconditioning develop mediated aversion to the preconditioned stimulus. Notably, it has been demonstrated that if the training sessions are increased (to 6 or even 9 odor-taste pairings), the animals do not present mediated aversion anymore, a behavioral change also described as “reality testing” and thought to be the basis of studying impairments in perception which might underlie the cognitive/perceptual substrates of traditional psychotic symptoms, such as hallucinations and delusions (Busquets-Garcia *et al.*, 2017a; Busquets-Garcia *et al.*, 2017b; Kim and Koh, 2016; Koh and Gallagher, 2020; McDannald and Schoenbaum, 2009) (refer to the Box 2 and Box 3 sections of Chapter I for more details).

An association between adolescent cannabis use and a greater risk of developing psychotic symptoms or psychotic disorders later in life has long been established in humans (Arseneault *et al.*, 2002a; Malone *et al.*, 2010; Marconi *et al.*, 2016). Thus, paradigms that evaluate reality testing performance represent a way to model potential psychotic-like behavior in adult mice treated with THC during adolescence. Here, we evaluated for the first time reality testing performance, in adult mice following adolescent THC administration. For evident reasons, the formation of a mediated aversion to the preconditioned stimulus with a moderate amount of training is a prerequisite to proceed to the evaluation of reality testing. Therefore we only performed the protocol with an extensive amount of training (6 pairings) in male adolescent THC-treated mice, as female adolescent THC-treated mice displayed impairment in the formation of mediated and direct learning. Moreover, since most robust behavioral alterations in previous experiments with adolescent THC-treated mice were observed with the higher dosage scheme of 5 mg/kg, we performed the following experiments using only this dose.

Following the adolescent THC treatment and after a month of a manipulation-free period, adult male mice underwent the odor-taste sensory preconditioning task. First, the animals were exposed to a preconditioning phase with six odor-taste pairings, followed by a conditioning phase consisting in the devaluation of one of the odor stimuli, as described before ((Busquets-Garcia *et al.*, 2017a) (Figure 32A). Interestingly, both the vehicle group and the THC-treated group demonstrated the expected absence of a mediated aversion to the preconditioned stimulus (taste), indicating a normal reality testing

performance (Figure 32B). Two-way ANOVA analysis for the liquid consumption during the mediated taste aversion test, with treatment and conditioning as between-subjects factors, showed no significant effects of conditioning or of treatment, neither an interaction between the two factors. Regarding the direct odor learning test, both groups developed a normal aversion, as indicated by the lower consumption of the solution containing the odor that was previously directly devaluated (Figure 32C). Two-way ANOVA analysis for the liquid consumption during the direct odor aversion test, revealed a significant main effect of conditioning [$F(1, 41) = 77.98, P < 0.0001$] and no effect of the treatment or an interaction between the two factors. The total liquid intake that the mice consumed did not differ between the vehicle and THC group during the two choice tests (Figure 33) or during the other phases of the protocol (data not shown).

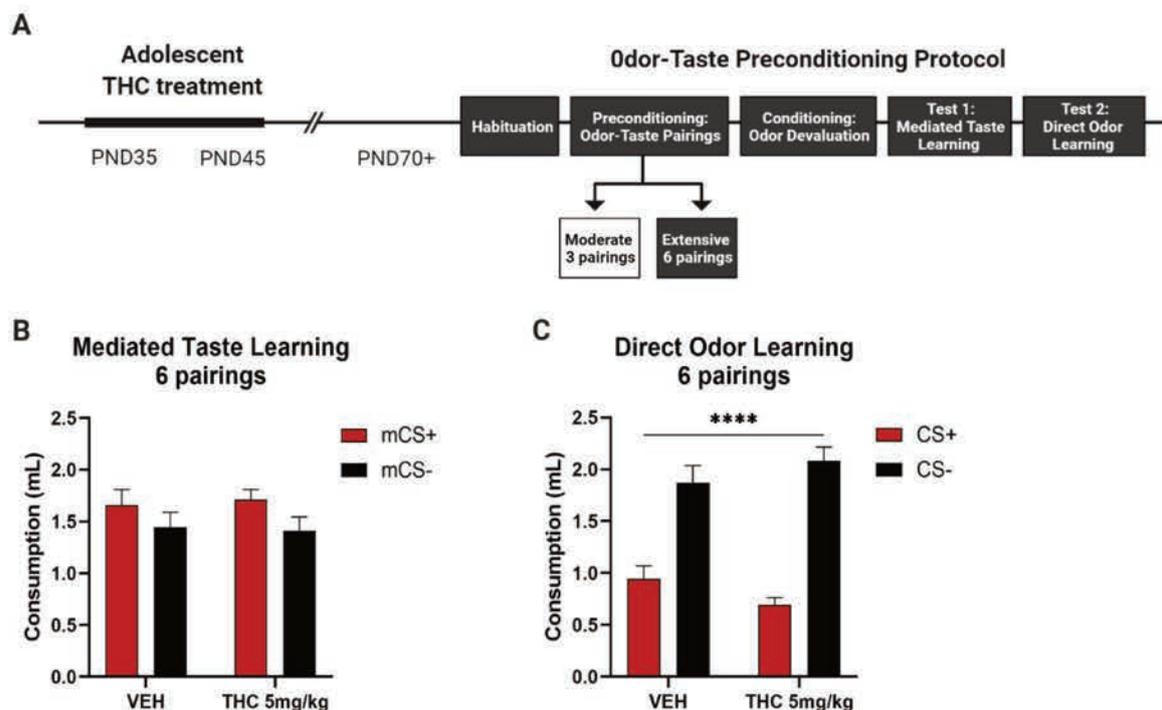


Figure 32. Reality testing protocol in adult male mice following adolescent THC treatment. (A) Schematic timeline of experimental design. (B, C) Effect of adolescent THC treatment in mediated taste (B) and direct odor (C) aversion in adult male mice. **** $P < 0.0001$ for the main effects of the CS. For statistical details and n, refer to Supplementary Table 1.

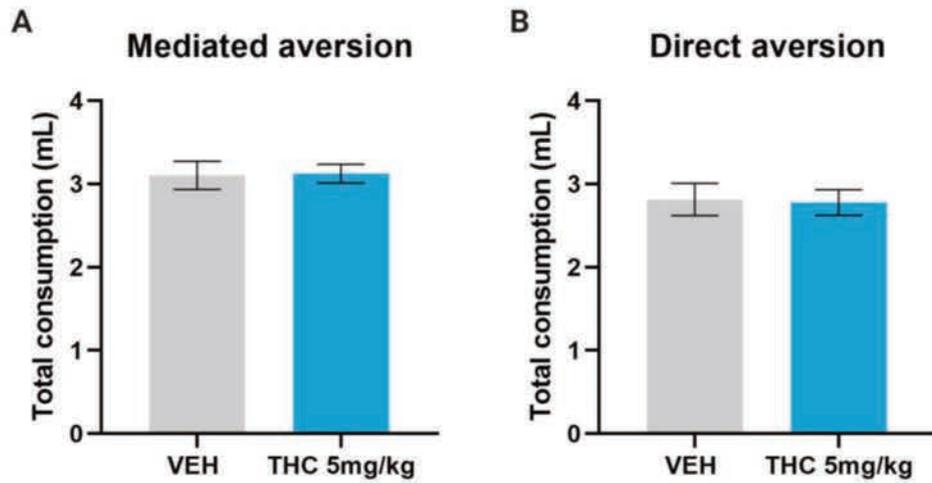


Figure 33. Total liquid consumption of adult male mice following adolescent THC treatment in the reality testing task during mediated taste (A) and direct odor (B) learning tests. No significant differences. Data is presented as mean \pm S.E.M.

1.5.4 Adolescent THC exposure did not sensitize adult male mice to the acute THC-induced impairment in reality testing

A secondary hypothesis for the impact of adolescent THC treatment in reality testing performance is that instead of a direct effect on the mediated learning, the chronic THC administration during adolescence could result in a greater sensitivity to the acute THC-induced impairment in reality testing. It has been previously demonstrated that different psychotogenic drugs, including THC, can acutely lead to deficits in reality testing task performance. When THC was administered at a dose of 1 mg/kg i.p. before Test 1 (mediated learning test), it blocked the effect of the extended pre-conditioning of six pairings, re-establishing the mediated aversion typical of three pairings (Busquets-Garcia *et al.*, 2017b). Thus, I hypothesized that a lower dose, which produces no effect in naïve animals, would result in an impairment of reality testing performance in adolescent THC-treated animals.

To test this hypothesis, a preliminary dose–response experiment was performed to determine the highest ineffective dose of THC not interfering with reality testing performance in adult naïve animals. Three different doses of THC were administered in the mice by i.p. injection 2 hours before the evaluation of the mediated aversion test: 0.1 mg/kg, 0.3 mg/kg and 1 mg/kg (Figure 34A). The results revealed that 1 mg/kg THC blocked the effect of the extended pre-conditioning of six pairings, re-establishing the mediated aversion and confirming previous findings, while 0.1 and 0.3 mg/kg THC did not have any effect. Two-way ANOVA analysis for the liquid consumption during the mediated taste aversion test, with THC and conditioning as between-subjects factors, showed no significant effects or interaction. Individual paired t test analysis between mCS+ and mCS- revealed the absence of mediated aversion for the vehicle, THC0.1 and THC0.3 groups (P values of 0.5142, 0.2056 and 0.2617, respectively), while mediated aversion was re-established in the THC1 group (P=0.0192) (Figure 34B). Accordingly, 0.3 mg/kg THC was chosen as the highest sub-effective dose to evaluate the acute THC effect on reality testing in adolescent THC-treated animals.

Importantly, none of the different doses of THC affected the display of direct odor aversion, as indicated by the lower consumption of the solution containing the odor that was previously directly devaluated (Figure 34C). Two-way ANOVA analysis for the liquid consumption during the direct odor aversion test, revealed a significant main effect of conditioning [$F(1, 33) = 62.51, P < 0.0001$] and no effect of THC or interaction between the two factors. Moreover, the acute THC treatment did not affect the total liquid intake of the mice during the two choice tests (Figure 35).

As previously described, a small batch of adult male mice ($n=7$ per group) that received adolescent THC treatment during 10 days underwent the odor-taste sensory preconditioning task after a month of washout/manipulation-free period. The animals were first exposed to the preconditioning phase with six odor-taste pairings, followed by a conditioning phase consisting in the devaluation of one of the odor stimuli. THC was acutely administered at 0.3 mg/kg i.p. 2 hours before the first (mediated learning test) (Figure 36A). Both the adolescent vehicle and the THC-treated group demonstrated a loss of mediated aversion to the preconditioned stimulus (taste), indicating a normal reality testing performance, and an absence of an acute THC-induced effect (Figure 36B). Two-way ANOVA analysis for the liquid consumption during the mediated taste aversion test, with adolescent treatment and conditioning as between-subjects factors, showed no significant main effects, nor an interaction. Importantly, the acute THC administration did not impact direct odor aversion, with animals from both groups consuming lower consumption of the solution containing the odor that was previously directly devaluated (Figure 36C). Two-way ANOVA analysis for the liquid consumption during the direct odor aversion test, showed a significant effect of the conditioning [$F(1, 12) = 12.15, P=0.0045$]. The acute THC administration did not affect the total liquid intake of the mice during the two choice tests (Figure 37).

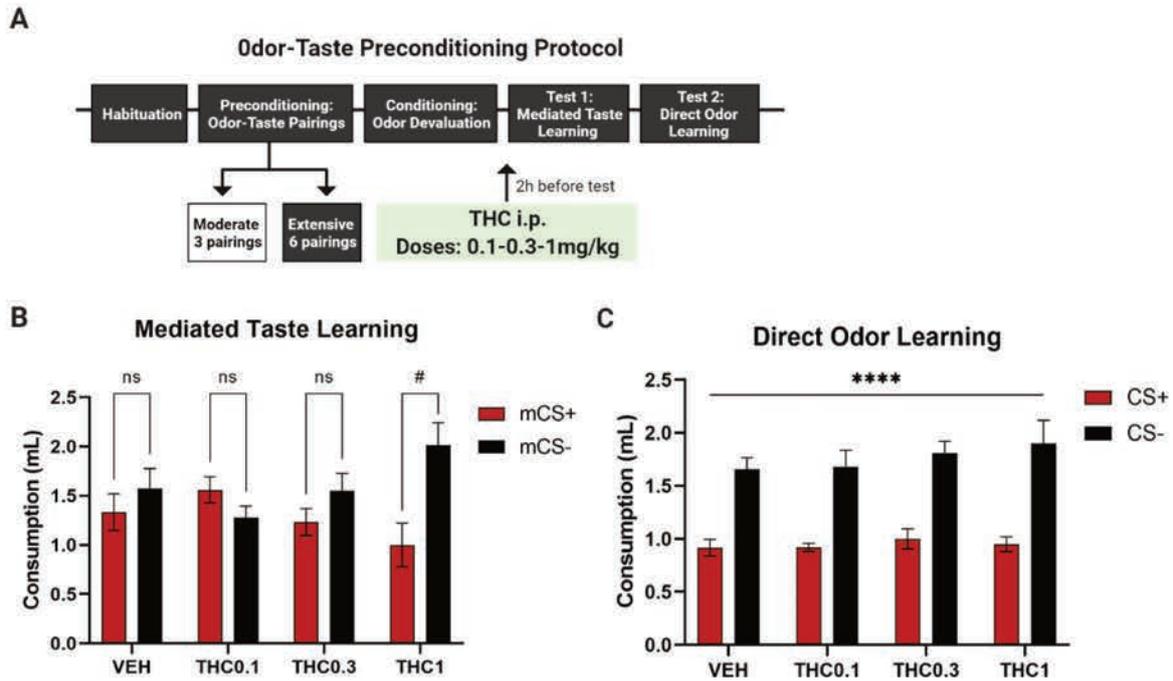


Figure 34. Effect of THC in the reality testing task. (A) Schematic representation of the experimental protocol. (B, C) Effect of acute THC administration at 0.1, 0.3 and 1 mg/kg before Test 1 in the following mediated taste (B) and direct odor (C) learning tests in adult naïve male mice. **** $P < 0.0001$ for the main effects of the CS. # $P < 0.05$; ns, not significant (mCS+ versus mCS). For statistical details and n, refer to Supplementary Table 1.

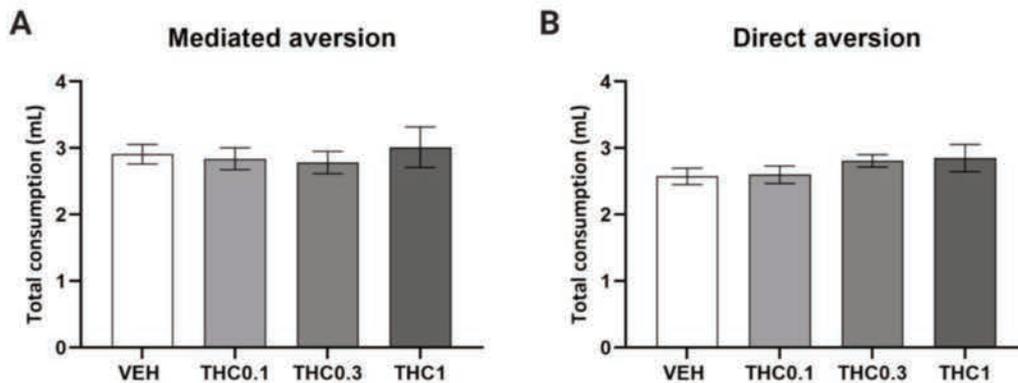


Figure 35. Effect of different THC doses administered 2h before the mediated learning test in the total liquid consumption during the mediated taste (A) and direct odor (B) tests in adult male mice. No significant differences. Data is presented as mean \pm S.E.M.

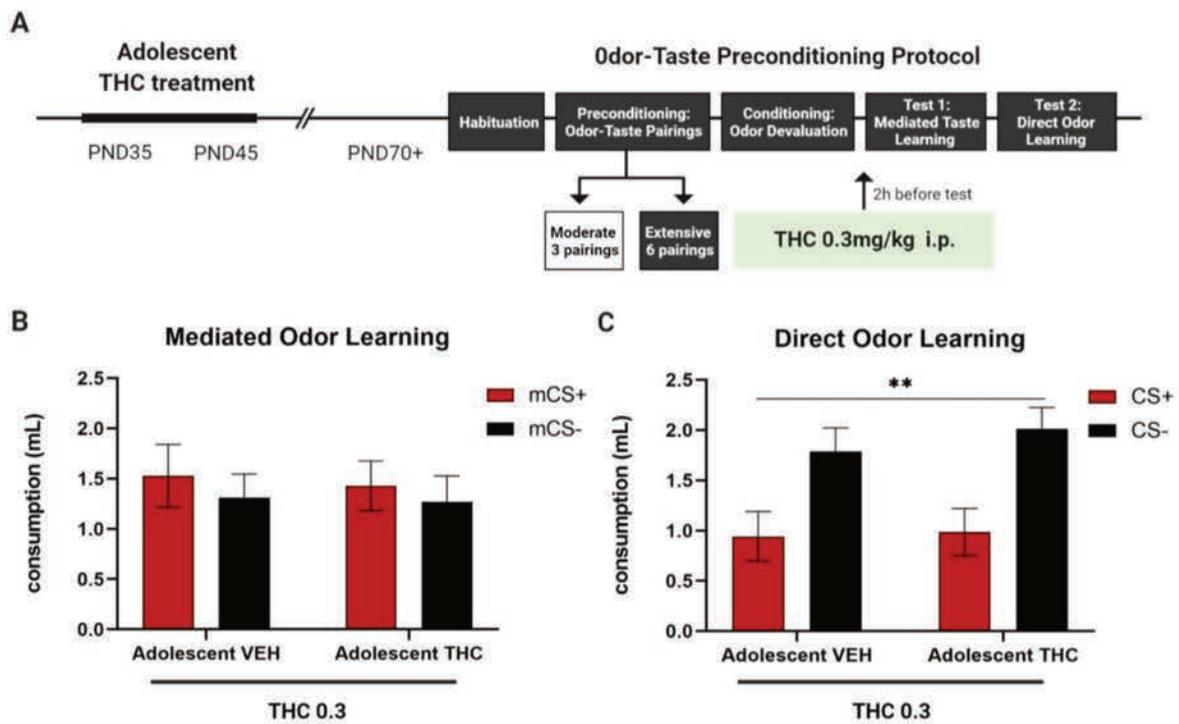


Figure 36. Adolescent THC treatment does not lead to sensitization to the acute THC-induced impairment in the reality testing task. (A) Schematic timeline of experimental design. (B, C) Effect of the acute THC administration at 0.3 mg/kg before Test 1 in the following mediated taste (B) and direct odor (C) learning tests in adult male mice following the adolescent THC treatment. ** $P < 0.01$ for the main effects of the CS. For statistical details and n , refer to Supplementary Table 1.

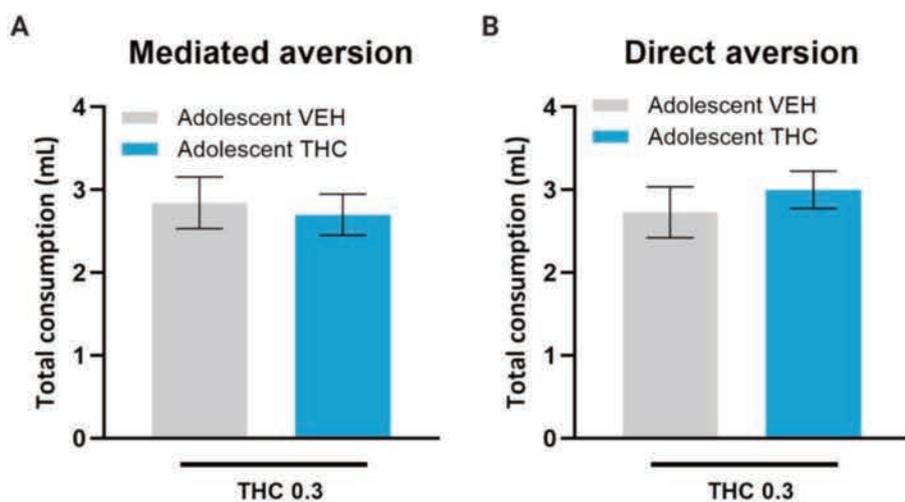


Figure 37. Effect of THC 0.3 mg/kg administered 2h before the mediated learning test in the total liquid consumption during the mediated taste (A) and direct odor (B) tests in adult male mice following the adolescent THC treatment. No significant differences. Data is presented as mean \pm S.E.M.

2 Role of CB1 receptors in higher-order cognitive processes

The endocannabinoid system, as a major brain modulatory system, controls a plethora of different functions mainly *via* the CB1 receptor-dependent modulation of neuronal and glial cell activity. Notably, CB1 receptor activity plays crucial roles in different phases of learning and memory processes (Drumond *et al.*, 2017; Marsicano and Lafenêtre, 2009; Rueda-Orozco *et al.*, 2008), however, studies on the role of CB1 receptors in higher-order learning are relatively sparse. We have previously demonstrated that CB1 receptors are necessary for the formation of mediated learning and that more specifically CB1 located in GABAergic neurons of the hippocampus is both necessary and sufficient for mediated learning (Busquets-Garcia *et al.*, 2018b). The role of CB1 receptors in other locations (e.g. other brain cell types or in subcellular compartments), however, is still unexplored. In the following sections, I will describe the results I obtained investigating the role of different CB1 receptor populations in representation-mediated higher-order learning, using specific conditional mutant mouse lines that target the CB1 receptors in the D1-positive brain cells and also in the mitochondria.

2.1 CB1 receptors in D1-positive cells are necessary for mediated taste learning

A cross-talk between the endocannabinoid system and the dopaminergic system has been reported to modulate cognitive functions, such as learning and memory processing in many brain regions (Bloomfield *et al.*, 2016; Hermann *et al.*, 2002; Oliveira da Cruz *et al.*, 2020; Terzian *et al.*, 2011). CB1 receptors are densely expressed in neuronal populations that express dopamine D1 receptors, like in D1-positive medium spiny neurons of the striatum (Monory *et al.*, 2007), a region involved in aversive learning (Delgado *et al.*, 2008; Liljeholm and O'Doherty, 2012). A subpopulation of hippocampal CB1

interneurons has also been described to express D1 receptors (Gangarossa et al., 2012; Puighermanal et al., 2017), and was recently found to control object recognition memory, suggesting that CB1 receptor signaling in these cells determines a functional link between dopaminergic and GABAergic control of memory consolidation (Oliveira da Cruz *et al.*, 2020).

Therefore, I opted to investigate the role of CB1 receptors in the dopamine D1-expressing neurons in higher-order learning. For this purpose, conditional CB1 mutant mice, bearing a deletion of the CB1 receptor gene in cells expressing dopamine D1 receptors (D1-*CB1* knock-out mice) (Monory *et al.*, 2007), were submitted to the odor-taste preconditioning paradigm. D1-*CB1* wild-type and knock-out mice were exposed to a preconditioning phase with three odor-taste, followed by a conditioning phase consisting in the devaluation of one of the odor stimuli (Figure 38A). We observed that wild-type mice consumed lower amounts of the tastes or odors that were indirectly (preconditioned) or directly devaluated, indicating the formation of reliable mediated and direct aversion learning, respectively. Interestingly, D1-*CB1* knock-out mice displayed impaired mediated taste aversion (Figure 38B), while still maintaining normal expression of direct learning (Figure 38C). Two-way ANOVA analysis for the liquid consumption during the mediated taste aversion test, with genotype and conditioning as between-subjects factors, showed a significant main effect of conditioning [$F(1, 30) = 6.782, P=0.0142$] and an interaction between the two factors [$F(1, 30) = 6.939, P=0.0132$], thereby demonstrating that this subpopulation of CB1 receptors is specifically required for mediated taste learning. The total liquid intake during the tests did not differ between the wild-type and the knock-out animals (Figure 39).

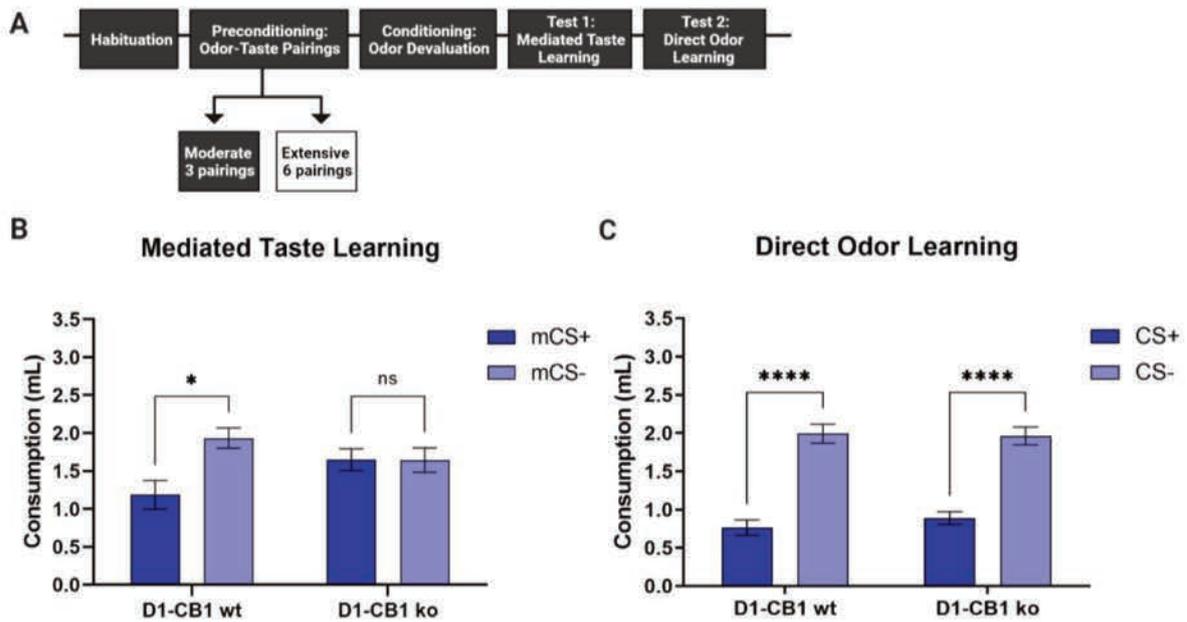


Figure 38. Mediated and direct learning in D1-CB1 KO mice. (A) Schematic representation of the experimental protocol. (B, C) Liquid consumption under conditions of mediated taste (B) and direct odor (C) aversion in D1-CB1 WT and KO mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant (mCS+ versus mCS- or CS+ versus CS-). For statistical details and n, refer to Supplementary Table 1.

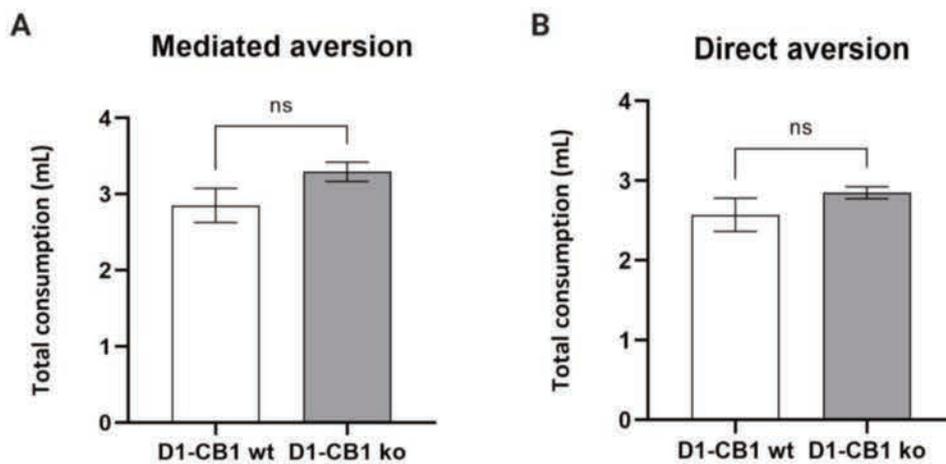


Figure 39. Total liquid consumption of D1-CB1-WT and D1-CB1-KO mice during mediated taste (A) and direct odor (B) learning tests. No significant differences. Data is presented as mean \pm S.E.M.

2.2 Mitochondria-associated CB1 receptors are necessary for direct odor learning, but not for direct taste learning

Besides their classical location at plasma membranes, CB1 receptors are also found intracellularly (Sarnataro *et al.*, 2005; Thibault *et al.*, 2013). Among other intracellular locations, a significant proportion of CB1 receptors has been observed in association with mitochondrial membranes of different cells (mtCB1) (Gutierrez-Rodriguez *et al.*, 2017; Hebert-Chatelain *et al.*, 2014; Koch *et al.*, 2015; Mendizabal-Zubiaga *et al.*, 2016a). In the brain, mtCB1 receptors can regulate food intake (Koch *et al.*, 2015), social behavior (Jimenez-Blasco *et al.*, 2020), and, particularly important in the present context, memory formation processes (Hebert-Chatelain *et al.*, 2016), through the modulation of mitochondrial energy metabolism. In order to further explore the roles of mtCB1 receptors, a mouse model lacking this localization of the receptors has been recently generated (Pagano Zottola *et al.*, 2020; Soria-Gomez *et al.*, 2021). Briefly, it was previously shown that a mutant version of the CB1 protein lacking the first 22 amino acids (named DN22-CB1 mutant) is neither anatomically nor functionally associated with mitochondrial membranes, while maintaining its other functions (e.g., at the plasma membranes) (Hebert-Chatelain *et al.*, 2016). Thus, a new knock-in mutant mouse line (DN22-CB1-KI) was generated, where the wild-type CB1 receptor gene is replaced by the coding sequence of the DN22-CB1 mutant protein. The levels of plasma membrane CB1 are not affected by the mutation, however, the density of mtCB1 receptors and the percentage of CB1-expressing mitochondria reach levels that are undistinguishable from global CB1 knock-out mice. The DN22-CB1-KI mice are viable and fertile (Pagano Zottola *et al.*, 2020; Soria-Gomez *et al.*, 2021), and display normal recognition and fear memory (currently unpublished data).

This mutant line has therefore allowed me to explore whether mtCB1 receptor signaling participates in the formation of higher order learning and memory in the odor-taste preconditioning paradigm. DN22-CB1-KI mice and their wild-type littermates were exposed to a preconditioning phase with three odor-taste pairings (sucrose and maltodextrin as tastes; banana and almond as odors), followed by a conditioning phase consisting in the devaluation of one of the stimuli (Figure 40A). Both mediated taste and mediated odor learning seemed to be impaired in the DN22-CB1 group, with no significant differences between the mediated CS+ and CS- consumption (Figure 40B, C). It is important to note here that their wild-type counterparts failed to display a clear mediated aversion, with a trend that almost

reached significance for the mediated taste aversion (Figure 40B) and no difference between the mediated CS+ and CS- consumption in the case of mediated odor aversion (Figure 40C).

Interestingly, direct odor aversion was clearly impaired in the DN22-CB1 group. Two-way ANOVA analysis for the liquid consumption during the direct odor aversion test, with genotype and conditioning as between-subjects factors, revealed a main effect of conditioning [$F(1, 16) = 16.53, P=0.0009$], as well as a significant interaction between the two factors [$F(1, 16) = 10.21, P=0.0056$] (Figure 40D). On the contrary, no group difference was observed in the formation of direct taste aversion (Figure 40E), suggesting that mtCB1 receptors are specifically required for direct odor, but not taste, learning. The total liquid intake during the tests did not differ between the wild-type and the knock-in animals (Figure 41).

The odor performance of the mutant mouse line had not been previously described, so in order to exclude an innate impairment in odor perception in these mice, we performed an olfactory detection test. A new batch of DN22-CB1 wild-type and knock-in mice received a 2-choice test between an odorized water solution (banana or almond) and non-odorized plain water (Figure 42A). Naive mice with normal odor perception have been shown to prefer water over the odorized water (Bailey and Westbrook, 2007). Both wild-type and DN22-CB1-KI mice displayed a clear preference for the non-odorized water over both odors (Figure 42B), suggesting that olfactory perception in DN22-CB1-KI mice was not impaired.

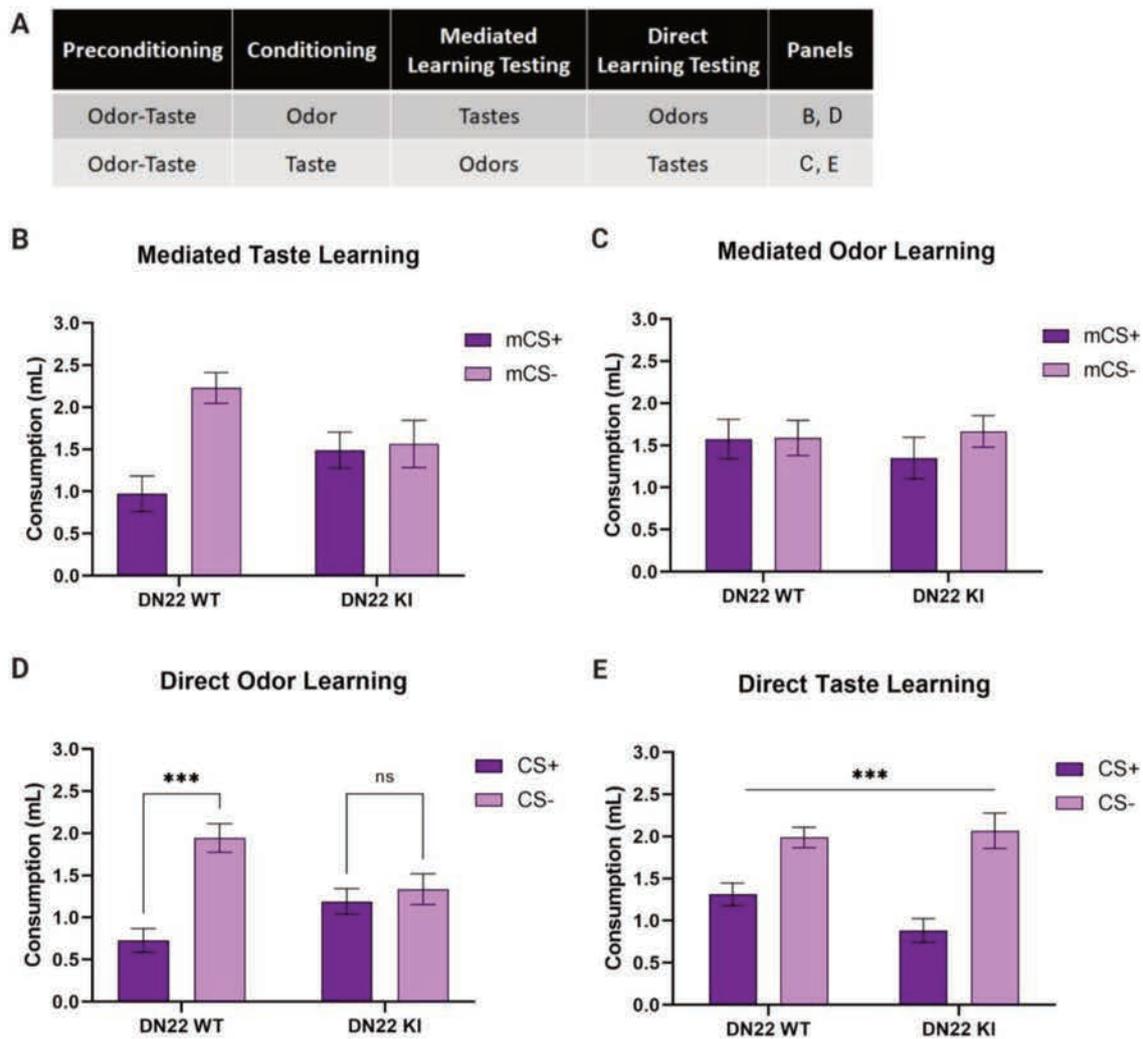


Figure 40. Mediated and direct learning in DN22-CB1 KI mice. (A) Schematic table of the odor-taste sensory preconditioning protocol. (B, C) Liquid consumption under conditions of mediated taste (B) or odor (C) aversion. (D, E) Liquid consumption under conditions of direct odor (D) or taste (E) aversion. *** $P < 0.001$ for the main effects of the CS (E) and for CS+ versus CS-(B). For statistical details and n , refer to Supplementary Table 1.

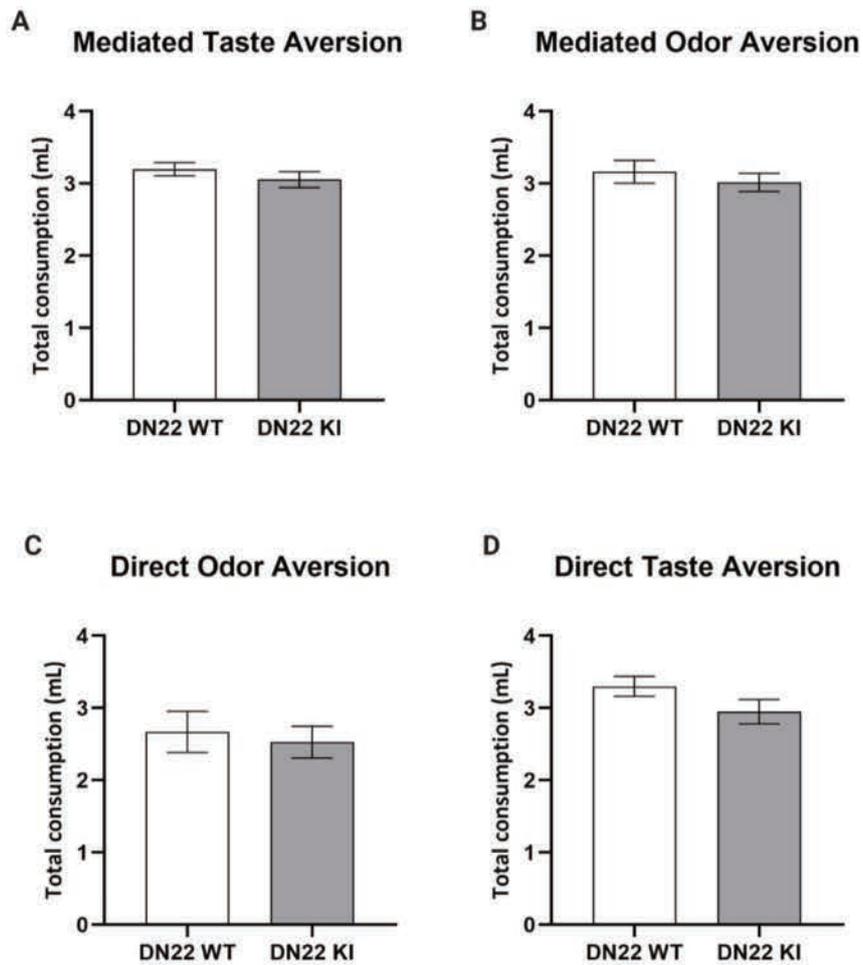


Figure 41. Total liquid consumption of DN22 WT and DN22 KI mice during mediated taste and odor (A, B), and direct odor and taste (C, D) learning tests. No significant differences. Data is presented as mean \pm S.E.M.

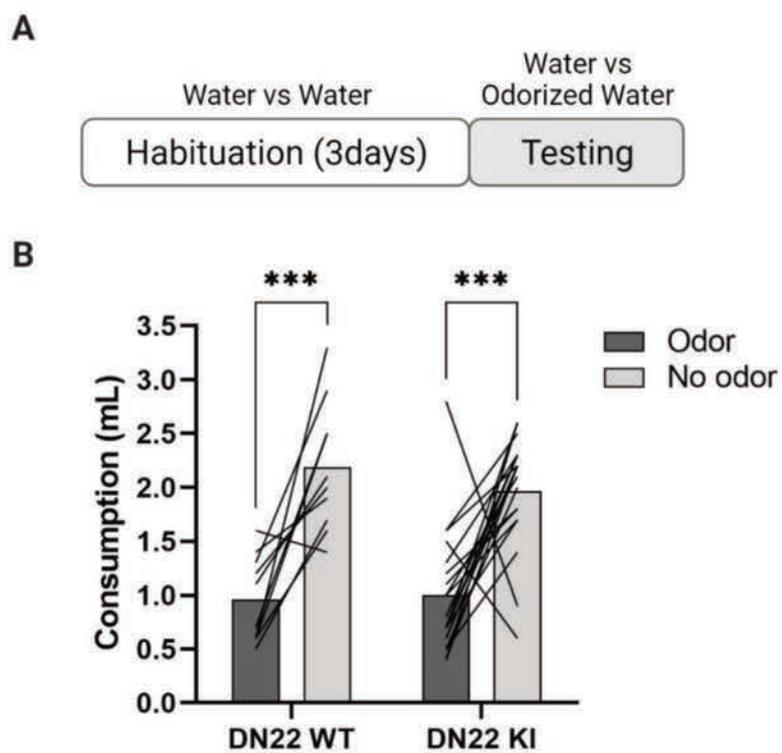


Figure 42. Odor perception in DN22-CB1-KI mice. (A) Schematic representation of the experimental protocol. (B) Consumption of odorized and non-odorized water solution during the odor detection test in DN22-CB1 wild-type and knock-in mice. *** $P < 0.001$ (odor versus no odor). For statistical details and n, refer to Supplementary Table 1.

3 Glucose Metabolism Links Astroglial Mitochondria to Cannabinoid Effects

Besides the objectives addressed in the two previous sections, an important part of my PhD work involved my participation in crucial behavioral experiments during the review process for the publication of the article entitled “Glucose Metabolism Links Astroglial Mitochondria to Cannabinoid Effects” (**Annex I**). Specifically, I designed and performed behavioral experiments combined with local infusions of specific drugs in mice, as well as analyzed the data obtained from those experiments. Together with the rest of the authors, I gave input, edited and approved the final manuscript.

In this section, I will briefly summarize some of the relevant results from this work:

“Glucose metabolism links astroglial mitochondria to cannabinoid effects”

Daniel Jimenez-Blasco*, Arnau Busquets-Garcia*, Etienne Hebert-Chatelain*, Roman Serrat, Carlos Vicente-Gutierrez, **Christina Ioannidou**, Paula Gómez Sotres, Irene Lopez-Fabuel, Monica Resch, Eva Resel, Dorian Arnouil, Dave Saraswat, Marjorie Varilh, Astrid Cannich, Francisca Julio-Kalajzic, Itziar Bonilla-Del Río, Angeles Almeida, Nagore Puente, Svein Achicallende, Maria-Luz Lopez-Rodriguez, Charlotte Jollé, Nicole Déglon, Luc Pellerin, Charlène Josephine, Gilles Bonvento, Aude Panatier, Beat Lutz, Pier-Vincenzo Piazza, Manuel Guzmán, Luigi Bellocchio, Anne-Karine Bouziers-Sore, Pedro Grandes, Juan P. Bolaños# and Giovanni Marsicano#

*Share first authorship

#Co-senior and co-corresponding authors

It is well known that the crosstalk between neurons and glia is critical for the precision of neurotransmission in the execution of specific behaviors (Oliveira et al., 2015; Perea et al., 2014) (Halassa and Haydon, 2010). At the same time, brain metabolism is also fundamental for the regulation of behavioral functions (Watts et al., 2018). However, the high complexity with which these parameters interact is often difficult to depict. This work establishes a cell-type-specific mechanistic framework, by revealing that mitochondrial G protein-coupled receptors can affect the bioenergetics of specific cell types and determine intercellular metabolic interactions, thereby regulating animal behavior. Specifically, we show that astroglial mtCB1 receptors regulate glycolysis and lactate production through a precise molecular cascade to eventually govern the metabolic state of neurons and control social interaction in mice.

3.1 Mitochondria-associated CB1 receptors are present in astrocytes

The classical view of CB1 receptors expression placed these receptors at the plasma membrane, where they convert extracellular signals into intracellular responses. However, this view has been revised over the years with the identification of intracellular CB1 receptors. One of the first pieces of evidence came from the observation that cannabinoids can activate CB1 receptors localized in endosomes/lysosomes (Thibault *et al.*, 2013), where they can trigger G protein-dependent signaling (Rozenfeld and Devi, 2008). The most complete evidence so far however, came with the identification of CB1 receptors on neuronal mitochondrial membranes (called mtCB1 receptors) (Benard *et al.*, 2012). Further experiments demonstrated that mtCB1 receptors mediate the reduction of oxygen consumption caused by exogenous and endogenous cannabinoids, underlying the contribution of cannabinoid signaling to bioenergetic processes (Hebert-Chatelain *et al.*, 2016). Here, we extended these findings, by identifying functional mtCB1 receptors in astrocytes in the mouse hippocampus, prefrontal cortex, nucleus accumbens and piriform cortex (FIG). Similar to what is observed in neurons, the activation of astroglial mtCB1 receptors, reduced astrocytic oxygen consumption through the soluble adenylyl cyclase and the protein kinase A (PKA)-dependent phosphorylation of OXPHOS proteins.

3.2 Biochemical dissection of how mtCB1 receptors hamper glucose metabolism, resulting in altered neuronal functions

We then showed how the activation of astroglial mtCB1 receptors reduces the phosphorylation of the mitochondrial complex I subunit NDUFS4, which decreases the stability and activity of complex I. This leads to a reduction in the generation of reactive oxygen species by astrocytes and affects the glycolytic production of lactate through the hypoxia-inducible factor 1 pathway, eventually resulting in neuronal redox stress (see below).

A) mtCB1 receptors destabilize mitochondrial complex I in astrocytes by reducing the phosphorylation of NDUFS4

We showed that mtCB1 activation by THC in astrocytes disrupts the activity of mitochondrial complex I, the first component of the OXPHOS chain 4, which is critical for NADH binding and oxidation. This occurred through the disruption of its N-module, which is critical for the assembly and stability of the complex. Specifically, THC-induced activation of mtCB1 reduced the phosphorylation of the NDUFS4 subunit of complex I, at its Ser173 residue. A series of in vitro experiments with a phosphomimetic mutant construct of NDUFS4 (in which the Ser173 residue was replaced by a Glu residue, thereby activating the protein) confirmed the critical role of reduced NDUFS4 phosphorylation in destabilizing astrocytic energy metabolism.

B) Complex I destabilization affects glycolysis and lactate production through astroglial ROS

The complex I N-module, which is specifically destabilized by mtCB1 activation, contains a binding pocket for oxygen molecules that accepts electrons from NADH(H⁺) to generate ROS (Mimaki et al., 2012). As expected, the activation of astrocytic mtCB1 receptors by THC diminished ROS levels in vitro, an effect that was reversed by restoring the activity of NDUFS4. Downstream, the hypoxia-inducible factor 1 (HIF-1) transcriptional cascade is known to detect ROS and to adapt the cell metabolism accordingly (Chandel et al., 1998; Patten et al., 2010; Shadel and Horvath, 2015). Here, nuclear HIF-1 levels, as well as its promoter activity were reduced by THC, an observation completing the mechanism of the mtCB1 → complex I → ROS → HIF-1 cascade. As HIF-1 signaling

physiologically stimulates glycolysis, it was expected that THC reduces glycolytic activity and, ultimately, lactate production in astrocytes. In line with this, we found that stimulation of mtCB1 by THC did decrease lactate production in astrocytes.

C) Consequences on neuronal function

Lactate that is produced by astrocytes is a key energetic support to sustain neuronal activity and functions (Barros, 2013; Pellerin et al., 2007). We therefore demonstrated that the THC-mediated decrease in lactate formation by astrocytes has deleterious effects on neurons. When we co-cultured neurons alongside astrocytes that had previously been exposed to THC, these neurons showed a decrease in mitochondrial membrane potential and an increase in mitochondrial ROS compared with neurons co-cultured alongside untreated astrocytes, indicating bioenergetic and oxidative stress. Neurons purified from THC-treated mice also had increased mitochondrial ROS levels compared with those from vehicle-treated animals, and showed signs that they were undergoing apoptotic cell death. All of these negative effects were reversed by lactate delivery to the neurons.

3.3 Astroglial mtCB1 receptors and cannabinoid modulation of social behavior

One of the key parts of this work, and especially relevant to the context of this thesis, is the effect of the described molecular mechanisms on mouse behavior. I will therefore describe some of the principal experiments in which I participated in and how the results of these experiments link all the previously described molecular processes to behavior, and specifically to the THC-induced impairment of mouse social behavior.

In one of the first experiments performed, we demonstrated that THC administration (at 10 mg/kg, i.p.) had a persistent effect over 24 hours, leading to impairments in the spontaneous alternation and social interaction tasks (Figure 43, e-g), but not in PPI, locomotion or anxiety-like behavior (Figure 43, a-d). Notably, the social interaction deficit was abolished in conditional mutant mice that lack CB1 specifically in cells (mainly astrocytes) that express glial fibrillary acidic protein (GFAP), known as GFAP-CB1-KO mice (Figure 43, e-g).

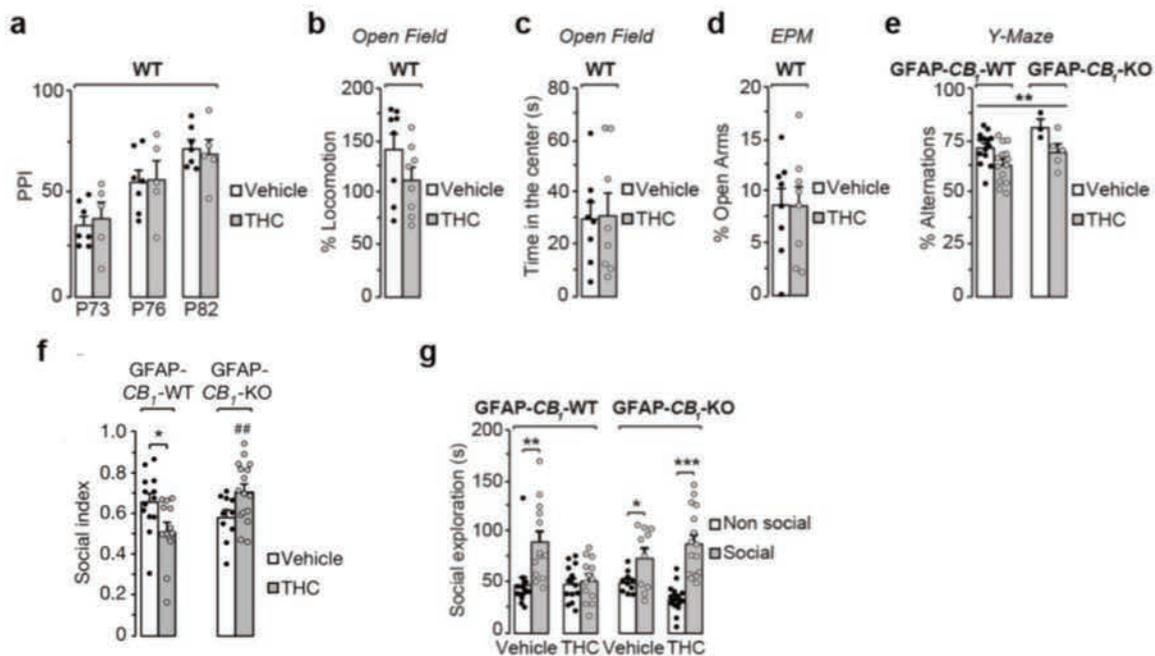


Figure 43. Behavioral effects of THC administration. (a–d) The administration of THC (10 mg per kg, i.p.) did not affect the prepulse inhibition (PPI) of the startle response (a), the locomotion in the open field (b), the anxiety-like responses in the open field (c) or in the elevated plus maze (d). (e) THC induced spontaneous alternation impairment in both GFAP-CB₁-WT and GFAP-CB₁-KO mice. ** $P < 0.01$ (vs vehicle). (f) THC induced impairment of social interaction in GFAP-CB₁-KO mice. * $P < 0.05$ (versus vehicle or GFAP-CB₁-WT); ## $P < 0.01$ (versus wild-type THC). (g) THC effects on the exploration times of social and non-social compartments in GFAP-CB₁-KO mice and GFAP-CB₁-WT littermates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (versus non-social). For supplementary figures, statistical details and additional information refer to Annex I.

We then proceeded to stereotactically inject a viral vector that expressed NDUFS4-PM under the control of the human GFAP promoter (AAV-GFAP-NDUFS4-PM) in both the hippocampus and the prefrontal cortex of wild-type mice. The astroglial expression of NDUFS4-PM in both brain regions abolished the THC-induced impairment of social interaction (Figure 44, b). Moreover, this manipulation totally blocked the selective degradation of the N-module of complex I and its activity induced by intraperitoneal injection of THC, demonstrating that also in vivo THC destabilize astroglial complex I by decreasing the phosphorylation of NDUFS4.

Next, we showed that direct intracerebroventricular (ICV) injection of lactate abolished the THC-induced social interaction impairments without exerting any significant effect *per se* on the social

behavior of control mice (Figure 44, a), suggesting that the same mechanisms that were described *in vitro* also underlie the THC-induced effect on social behavior.

In order to investigate even further the role of lactate in THC-induced impaired social interaction, we virally expressed a short hairpin RNA (shRNA) that reduces the expression of the monocarboxylate transporter 2 (MCT2), through which lactate shuttles between astrocytes and neurons, in the hippocampus and prefrontal cortex. Decreased MCT2 expression resulted in a decrease in social interactions and thereby occluded the THC effect (Figure 44, b), reinforcing the role of lactate transport from astrocytes to neurons in this behavior.

Finally, we explored whether relieving the astroglial mtCB1-dependent neuronal bioenergetic stress could compensate for the social impairment induced by THC. Local injection of a viral vector that induces the neuronal expression of a mitochondrial form of the ROS scavenger enzyme catalase26 (AAV-Syn-mitoCAT) into the hippocampus and prefrontal cortex did not alter social interactions *per se*, but it abolished the THC-induced impairment (Figure 44, b). Altogether, these data indicate that excessive ROS production in neurons, induced by an astroglial mtCB1-dependent decrease of lactate, mediates the cannabinoid-induced impairment of social interactions.

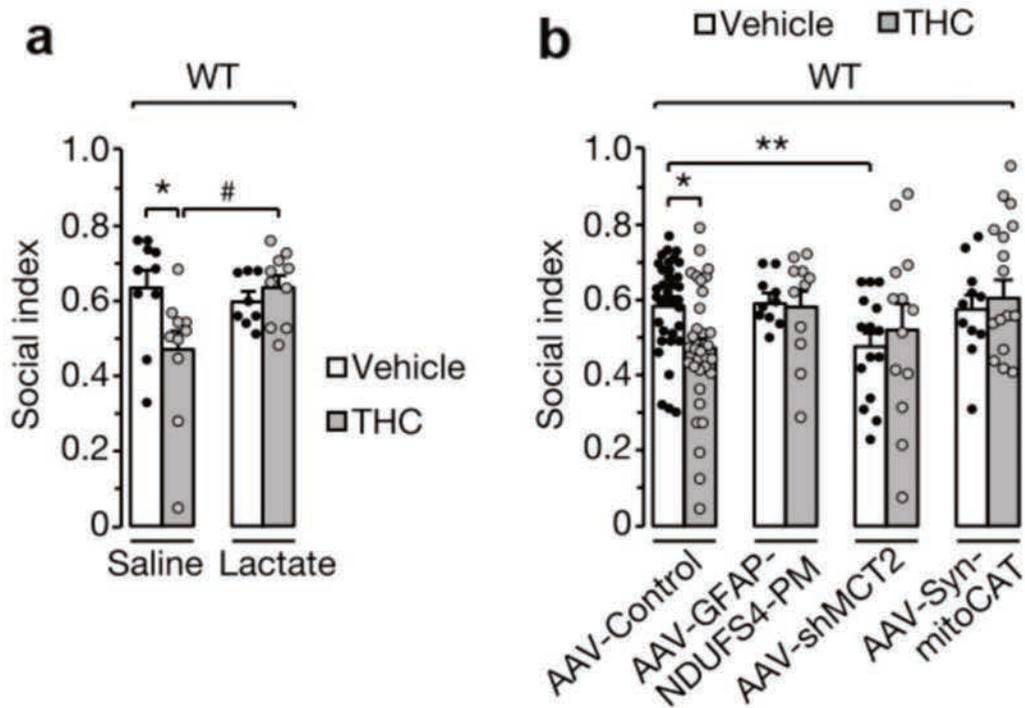


Figure 44. Astroglial mtCB1 activation decreases the production of lactate and mediates the THC-induced impairment of social behavior. (a) ICV infusion of lactate blocks THC-induced social impairment. * $P < 0.05$ (versus vehicle); # $P < 0.05$ (versus THC saline). (b) Effects of THC on social interaction in mice that were infused with AAV-control, AAV-GFAP-NDUFS4-PM, AAV-shMCT2 or AAV-Syn-MitoCAT. * $P < 0.05$; ** $P < 0.01$ (versus AAV-control). All data are expressed as mean \pm SEM. For supplementary figures, statistical details and additional information refer to Annex

4 Sex-dependent Pharmacological Profiles of the Synthetic Cannabinoid MMB-Fubinaca

The last section of this chapter will be dedicated to briefly describe the results obtained from a parallel project I was involved in, where we investigated the pharmacology and action of the synthetic cannabinoid MMB-Fubinaca. In the last decade, the use of synthetic cannabinoids for recreational reason, has been associated with damaging consequences for public health and multiple adverse effects including psychotic symptoms and panic attacks, as well as dependence-withdrawal and tolerance (Hermanns-Clausen *et al.*, 2013; Murray *et al.*, 2016; Spaderna *et al.*, 2013). Most of these compounds present a much higher potency compared to THC and their pharmacology is often not fully understood. The synthetic cannabinoid MMB-Fubinaca (also known as AMB-Fubinaca or FUB-AMB) is a great example of this, with relatively limited research around it. MMB-Fubinaca has been recognized as a serious threat following vast intoxication incidents (Adamowicz *et al.*, 2019; Adams *et al.*, 2017). It consists of an amino acid derived indazole-3-carboxamides or indole-3-carboxamides, which is similar to other synthetic cannabinoids, but its mechanisms of action was remaining relatively unknown.

Using behavioral, molecular, pharmacological, and *in vivo* electrophysiological approaches combined with genetic mouse models, we characterized the pharmacological profile of MMB Fubinaca. This work revealed that MMB-Fubinaca induces G protein functional coupling both in mouse and human native tissue. Moreover, it suggested sex differences and CB1-receptor dependency regarding the MMB-Fubinaca-mediated behavioral and *in vivo* electrophysiological effects. The findings of this study were published in the form of the manuscript entitled “Sex-dependent Pharmacological Profiles

of the Synthetic Cannabinoid MMB-Fubinaca” (**Annex II**). My participation consisted in performing and analyzing behavioral experiments in male and female mice, that revealed a sex-difference in the behavioral effects of the drug, as well as experiments in CB1 knock-out mice that demonstrated that MMB-Fubinaca exerts its effects through CB1 receptors. I also participated in the writing and revision of the final manuscript.

In the following pages, I will summarize the behavioral findings described in this work:

“Sex-dependent Pharmacological Profiles of the Synthetic Cannabinoid MMB-Fubinaca”

José F. Oliveira da Cruz*, Christina Ioannidou*, Antonio C. Pagano Zottola*, Carolina Muguruza*, Paula Gomez-Sotres, Monica Fernandez, Luis F. Callado, Giovanni Marsicano# and Arnau Busquets-Garcia#

* Share first authorship

Share senior authorship

4.1 In vivo sex-dependent effects of MMB Fubinaca.

After characterizing the MMB-Fubinaca-induced CB1 receptor-dependent signaling effects (refer to **Annex II**), we investigated some of the in vivo behavioral effects of this drug. Activation of CB1 receptors in animal models of cannabinoid exposure is responsible for a battery of specific behavioral phenotypes, the so-called “cannabinoid tetrad” (Schreiber et al., 2019; Wiley et al., 2015). The cannabinoid tetrad represents the most common cannabinoid-dependent set of behavioral effects, for which changes in locomotion, body temperature, catalepsy, and nociception are analyzed (Metna-Laurent et al., 2017).

The acute administration of MMB-Fubinaca (0.1 and 0.3 mg/kg; i.p.) 45 minutes before testing was able to induce hypolocomotion, enhanced catalepsy, antinociception and a decrease of body temperature in both male and female mice (Figure 45). Notably, except for the effects on locomotion, the MMB-Fubinaca effects were stronger in male compared to female mice (Figure 45). A baseline difference between male and female mice were observed in locomotor activity in the open field, but not basal differences were observed in the rest of the behavioral tests. Indeed, in locomotion, MMB-Fubinaca induced an effect of similar magnitude in male (75.7% for 0.1 mg/kg and 97.2% for 0.3 mg/kg) and female (54.5% for 0.1 mg/kg and 94.8% for 0.3 mg/kg) mice.

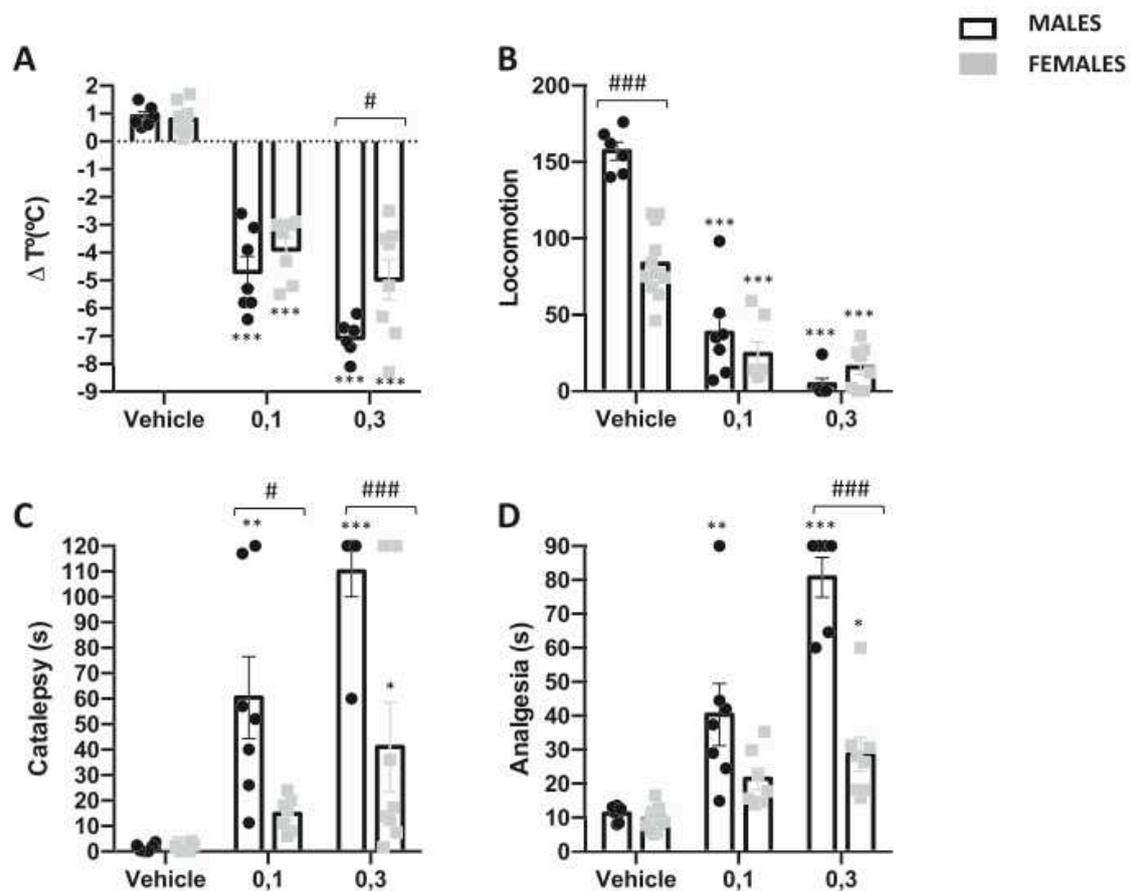


Figure 45. In vivo sex-dependent effects of MMB-Fubinaca. The cannabinoid tetrad induced by MMB-Fubinaca was assessed in male and female C57BL6N mice 45 minutes after the injection of vehicle or two different doses of MMB-Fubinaca (0.1 and 0.3 mg/kg, i.p.). Both doses produced clear cannabinoid-like effects such as hypothermia (A), hypolocomotion (B), catalepsy (C), and analgesia (D) with particular sex-dependent effects. Data are expressed as mean \pm SEM ($n = 6-12$ per group). * $p < .05$; ** $p < .01$; *** $p < .001$ compared with vehicle treated mice. # $p < .05$; ### $p < .001$ compared with male mice

4.2 CB1 receptors are necessary for the tetrad effects induced by MMB-Fubinaca

No alterations in body temperature, locomotion, catalepsy, or nociception were observed in CB1 knock-out mice (CB1^{-/-}), indicating that MMB-Fubinaca effects depend on CB1 receptor activation (Figure 46). Altogether, these data show that MMB-Fubinaca induces its tetrad effects via activation of CB1 receptors.

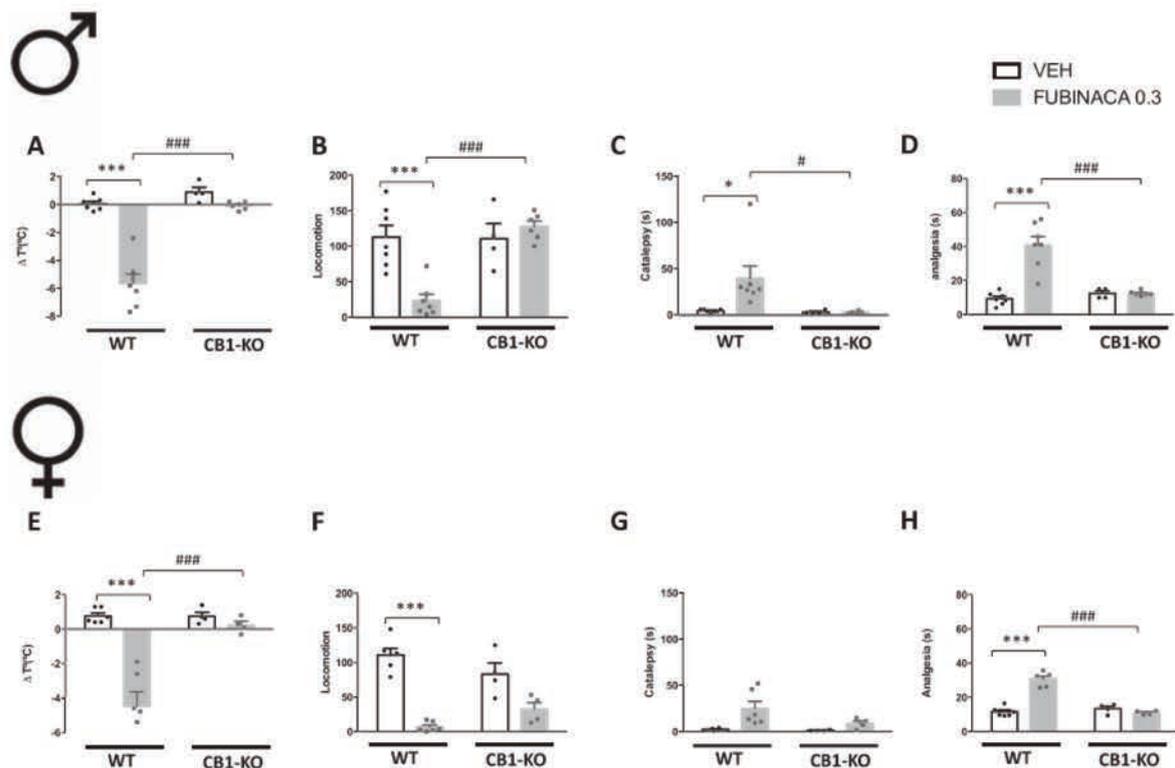


Figure 46. CB1 receptors are necessary for the tetrad effects induced by MMB-Fubinaca. MMB-Fubinaca was administered 45 minutes before the tetrad test in both CB1^{-/-} and CB1^{+/+} mice. Note that the cannabinoid-like effects in temperature (A, E), locomotion (B, F), catalepsy (C, G), and analgesia (D, H) induced by MMB-Fubinaca were not present in mice lacking CB1 receptors both in males and in females. Data are expressed as mean \pm SEM (n = 6–12 per group). *p < .05; ***p < .001 compared with vehicle treated mice. #p < .05; ###p < .001 compared with WT mice

5 Supplementary

Supplementary Table 1. Statistical analysis related to main figures

	Conditions	N	Analysis (post-hoc test reported in figures)	Factors analyzed	Passed normality test? ($\alpha=0.05$)	F-ratios	P-values
Figure 16B	Effect of adolescent THC in the spontaneous locomotor activity of adult male mice	22-22-24	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 65) = 5.734	P=0.0051
Figure 16C	Effect of adolescent THC in the spontaneous locomotor activity of adult female mice	13-8-18	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 35) = 0.1427	P=0.8675
Figure 17B	Effect of adolescent THC in the social interaction task in adult male mice	22-22-25	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 66) = 9.802	P=0.0002
Figure 17C	Effect of adolescent THC in the social interaction task in adult female mice	13-8-18	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 36) = 1.521	P=0.2321
Figure 17D	Effect of adolescent THC on the total exploration in the social interaction task in adult male mice	22-22-25	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 66) = 0.4061	P=0.6679
Figure 17E	Effect of adolescent THC on the total exploration in the social interaction task in adult female mice	13-8-18	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 36) = 4.103	P=0.0248
Figure 18B	Effect of adolescent THC in the spontaneous alternation task in adult male mice	22-22-25	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 66) = 4.002	P=0.0229
Figure 18C	Effect of adolescent THC in the spontaneous alternation task in adult female mice	13-8-18	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 36) = 0.1252	P=0.8827
Figure 18D	Effect of adolescent THC on the total exploration in the spontaneous alternation task in adult male mice	22-22-25	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 66) = 2.503	P=0.0896
Figure 18E	Effect of adolescent THC on the total exploration in the spontaneous alternation task in adult female mice	13-8-18	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 36) = 0.04858	P=0.9526
Figure 19B	Effect of adolescent THC in the PPI task in adult male mice	22-22-25	Two-way ANOVA repeated measures	VEH vs THC1 vs THC5 PP73 vs PP76 vs PP82	Yes	Interaction F (4, 132) = 1.778	P=0.1371
						PP effect F (1.856, 122.5) = 347.4	P<0.0001
						THC effect F (2, 66) = 0.2216	P=0.8019
Figure 19C	Effect of adolescent THC in the PPI task in adult female mice	13-7-18	Two-way ANOVA repeated measures	VEH vs THC1 vs THC5 PP73 vs PP76 vs PP82	Yes	Interaction F (4, 70) = 1.088	P=0.3692
						PP effect F (1.722, 60.26) = 142.7	P<0.0001
						THC effect F (2, 35) = 0.2445	P=0.7844
Figure 19D	Effect of adolescent THC in the startle amplitude in adult male mice	20-22-24	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 63) = 2.517	P=0.0888
Figure 19E	Effect of adolescent THC in the startle amplitude in adult female mice	13-7-18	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 35) = 0.1059	P=0.8998

Figure 20A	Effect of adolescent THC in hippocampal CB1 expression in adult male mice	6-6-8	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 17) = 7.610	P=0.0044
Figure 20B	Effect of adolescent THC in hippocampal CB1 expression in adult female mice	6-8-5	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 16) = 0.2758	P=0.7625
Figure 20C	Effect of adolescent THC in hippocampal CB1 expression in adult male mice	5-6	Unpaired t test	VEH vs THC5	Yes	t=12.76, df=9	P <0.0001
Figure 21A	Effect of adolescent THC in hippocampal ERK phosphorylation in adult male mice	6-6-8	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 17) = 14.62	P=0.0002
Figure 21B	Effect of adolescent THC in hippocampal ERK phosphorylation in adult female mice	6-8-5	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 16) = 11.22	P=0.0009
Figure 21C	Effect of adolescent THC in hippocampal CREB phosphorylation in adult male mice	6-6-8	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 17) = 34.79	P<0.0001
Figure 21D	Effect of adolescent THC in hippocampal CREB phosphorylation in adult female mice	6-8-5	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 16) = 8.495	P=0.0031
Figure 22A	Effect of adolescent THC in hippocampal total ERK levels in adolescent treated male mice	6-6-8	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 17) = 1.898	P=0.1803
Figure 22B	Effect of adolescent THC in hippocampal total ERK levels in adolescent treated female mice	6-8-5	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 16) = 7.292	P=0.0056
Figure 22C	Effect of adolescent THC in hippocampal total CREB levels in adolescent treated male mice	6-6-8	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 17) = 2.728	P=0.0939
Figure 22D	Effect of adolescent THC in hippocampal total CREB levels in adolescent treated male mice	6-8-5	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 16) = 19.07	P<0.0001
Figure 23A	Basal NDUFS4-Ser173 phosphorylation in the hippocampus of adolescent vehicle treated male mice	3-3	Unpaired t test	Adolescent vs Adult	N too small	t=2.738, df=4	P= 0.0520
Figure 23B	Basal NDUFS4-Ser173 phosphorylation in the prefrontal cortex of adolescent vehicle treated male mice	3-3	Unpaired t test	Adolescent vs Adult	N too small	t=0.05017, df=4	P= 0.9624
Figure 23C	Basal NDUFS4-Ser173 phosphorylation in the hippocampus of adolescent vehicle treated female mice	3-3	Unpaired t test	Adolescent vs Adult	N too small	t=3.169, df=4	P= 0.0339
Figure 23D	Basal NDUFS4-Ser173 phosphorylation in the prefrontal cortex of adolescent vehicle treated female mice	3-3	Unpaired t test	Adolescent vs Adult	N too small	t=3.768, df=4	P= 0.0196
Figure 24A	Effect of adolescent THC treatment on pNDUFS4 in the hippocampus of male mice	3-3-3-3	Two-way ANOVA	VEH vs THC5 Adolescent vs Adult	N too small	Interaction F (1, 8) = 13.51	P=0.0063
						THC effect F (1, 8) = 17.32	P=0.0032
						Age effect F (1, 8) = 13.51	P=0.0063
Figure 24B	Effect of adolescent THC treatment on pNDUFS4 in	3-3-3-3	Two-way ANOVA	VEH vs THC5 Adolescent vs Adult	N too small	Interaction F (1, 8) = 6.656	P=0.0326

	the prefrontal cortex (PFC) of male mice					THC effect F (1, 8) = 8.431	P=0.0326
						Age effect F (1, 8) = 6.656	P=0.0198
Figure 24C	Effect of adolescent THC treatment on pNDUFS4 in the hippocampus (HC) of female mice	3-3-3-3	Two-way ANOVA	VEH vs THC5 Adolescent vs Adult	N too small	Interaction F (1, 8) = 0.2530	P=0.6285
						THC effect F (1, 8) = 1.702	P=0.2283
						Age effect F (1, 8) = 0.2530	P=0.6285
Figure 24D	Effect of adolescent THC treatment on pNDUFS4 in the prefrontal cortex (PFC) of female mice	3-3-3-3	Two-way ANOVA	VEH vs THC5 Adolescent vs Adult	N too small	Interaction F (1, 8) = 2.528	P=0.1505
						THC effect F (1, 8) = 0.04187	P=0.8430
						Age effect F (1, 8) = 2.528	P=0.1505
Figure 25B	Effect of adolescent THC treatment on social interaction in adult mice expressing AAV-GFAP-NDUFS4-PM	6-7	Mann Whitney test	AAV-Control vs AAV-GFAP-NDUFS4-PM	No	-	P=0.0513
Figure 25C	Effect of adolescent THC treatment on locomotor activity in adult mice expressing AAV-GFAP-NDUFS4-PM	6-7	Unpaired t test	AAV-Control vs AAV-GFAP-NDUFS4-PM	Yes	t=2.204, df=11	P=0.0498
Figure 25D	Effect of adolescent THC treatment on spontaneous alternation in adult mice expressing AAV-GFAP-NDUFS4-PM	6-7	Unpaired t test	AAV-Control vs AAV-GFAP-NDUFS4-PM	Yes	t=1.399, df=11	P=0.1922
Figure 26A	Effect of astroglial expression of NDUFS4-PM on the behavior of the vehicle-treated group in the social interaction test	8-4	Mann Whitney test	AAV-Control vs AAV-GFAP-NDUFS4-PM	No	-	P= 0.7758
Figure 26B	Effect of astroglial expression of NDUFS4-PM on the behavior of the vehicle-treated group in locomotor activity	8-4	Unpaired t test	AAV-Control vs AAV-GFAP-NDUFS4-PM	Yes	t=1.154, df=10	P= 0.2752
Figure 26C	Effect of astroglial expression of NDUFS4-PM on the behavior of the vehicle-treated group in the spontaneous alternation task	8-4	Unpaired t test	AAV-Control vs AAV-GFAP-NDUFS4-PM	Yes	t=0.7201, df=10	P=0.4880
Figure 27B	Mediated taste learning in adult male mice following adolescent THC treatment	10-10-10	Two-way ANOVA repeated measures (Sidak)	VEHvsTHC1vsTHC5 mCS+ vs mCS-	Yes	Interaction F (2, 27) = 0.007126	P=0.9929
						mCS+ vs mCS- F (1, 27) = 21.21	P<0.0001
Figure 27C	Direct odor learning in adult male mice following adolescent THC treatment	10-10-10	Two-way ANOVA repeated measures (Sidak)	VEHvsTHC1vsTHC5 CS+ vs CS-	Yes	Interaction F (2, 27) = 1.958	P=0.1607
						CS+ vs CS- F (1, 27) = 35.57	P<0.0001
Figure 28A	Total liquid consumption during mediated taste learning test in adult male mice following adolescent THC treatment	10-10-10	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 27) = 0.09780	P=0.9072
Figure 28B	Total liquid consumption during direct odor learning test in adult male mice following adolescent THC treatment	10-10-10	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 27) = 0.6416	P=0.5343

Figure 29B	Mediated taste learning in adult female mice	16	Paired t test	mCS+ vs mCS-	Yes	t=1.820, df=15	P=0.0888
Figure 29C	Direct odor learning in adult female mice	16	Paired t test	CS+ vs CS-	Yes	t=2.364, df=15	P=0.0320
Figure 29D	Mediated odor learning in adult female mice	17	Paired t test	mCS+ vs mCS-	Yes	t=1.814, df=16	P=0.0885
Figure 29E	Mediated taste learning in adult female mice	16	Paired t test	CS+ vs CS-	Yes	t=7.669, df=15	P<0.0001
Figure 30B	Mediated taste learning in adult female mice following adolescent THC treatment	12-8-12	Two-way ANOVA repeated measures (Sidak)	VEHvsTHC1vsTHC5 mCS+ vs mCS-	Yes	Interaction F (2, 29) = 2.843	P=0.0746
						mCS+ vs mCS- F (1, 29) = 1.344	P=0.2557
Figure 30C	Direct odor learning in adult female mice following adolescent THC treatment	12-8-12	Two-way ANOVA repeated measures (Sidak)	VEHvsTHC1vsTHC5 CS+ vs CS-	Yes	Interaction F (2, 29) = 1.803	P=0.1827
						CS+ vs CS- F (1, 29) = 21.25	P<0.0001
Figure 31A	Total liquid consumption during mediated taste learning test in adult female mice following adolescent THC treatment	12-8-12	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 29) = 1.653	P=0.2090
Figure 31B	Total liquid consumption during mediated taste learning test in adult female mice following adolescent THC treatment	12-8-12	One-way ANOVA (Dunnett)	VEH vs THC1 vs THC5	Yes	F (2, 29) = 1.669	P=0.2061
Figure 32B	Mediated taste learning after 6 pairings in adult male mice following adolescent THC treatment	20-23	Two-way ANOVA repeated measures (Sidak)	VEH vs THC5 mCS+ vs mCS-	Yes	Interaction F (1, 41) = 0.07448	P=0.7863
						mCS+ vs mCS- F (1, 41) = 2.734	P=0.1059
Figure 32C	Direct odor learning after 6 pairings in adult male mice following adolescent THC treatment	20-23	Two-way ANOVA repeated measures (Sidak)	VEH vs THC5 CS+ vs CS-	Yes	Interaction F (1, 41) = 3.113	P=0.0851
						CS+ vs CS- F (1, 41) = 77.98	P<0.0001
Figure 33A	Total liquid consumption during mediated taste learning test in adult male mice following adolescent THC treatment in the reality testing task	20-23	Unpaired t test	VEH vs THC5	Yes	t=0.1050, df=41	P= 0.9169

Figure 33B	Total liquid consumption during direct odor learning test in adult male mice following adolescent THC treatment in the reality testing task	20-23	Unpaired t test	VEH vs THC5	Yes	t=0.1517, df=41	P= 0.8802
Figure 34B	Effect of THC in the mediated taste learning after 6 pairings	12-5-12-8	Wilcoxon tests	mCS+ vs mCS-	No	THC1	P=0.0625
Figure 34C	Effect of THC in the direct odor learning after 6 pairings	12-5-12-8	Wilcoxon tests	CS+ vs CS-	No	VEH	P=0.001953
						THC0.1	P=0.062500
						THC0.3	P=0.000488
						THC1	P=0.007813
Figure 35A	THC dose response in the total liquid consumption during the mediated taste test in adult male mice	12-5-12-8	Kruskal-Wallis test	VEH vs THC0.1 vs THC0.3 vs THC1	No	-	P= 0.8638
Figure 35B	THC dose response in the total liquid consumption during the direct odor test in adult male mice	12-5-12-8	Kruskal-Wallis test	VEH vs THC0.1 vs THC0.3 vs THC1	No	-	P= 0.4408
Figure 36B	Effect of THC in the mediated taste learning after 6 pairings in adult male mice following the adolescent THC treatment	7-7	Two-way ANOVA repeated measures (Sidak)	VEH vs THC mCS+ vs mCS-	Yes	Interaction F(1,12)=0.008190	P=0.9294
						mCS+ vs mCS- F (1, 12) = 0.3460	P=0.5673
Figure 36C	Effect of THC in the direct odor learning after 6 pairings in adult male mice following the adolescent THC treatment	7-7	Two-way ANOVA repeated measures (Sidak)	VEH vs THC CS+ vs CS-	Yes	Interaction F(1,12) =0.1196	P=0.7355
						CS+ vs CS- F (1, 12) = 12.15	P=0.0045
Figure 37A	THC effect in the total liquid consumption during the mediated taste test after 6 pairings in adult male mice following the adolescent THC treatment	7-7	Unpaired t test	VEH vs THC5	Yes	t=0.3572, df=12	P= 0.7271
Figure 37B	THC effect in the total liquid consumption during the direct odor test after 6 pairings in adult male mice following the adolescent THC treatment	7-7	Unpaired t test	VEH vs THC5	Yes	t=0.7126, df=12	0.4897
Figure 38B	Mediated taste learning in D1-CB1 KO mice	14-18	Two-way ANOVA repeated measures (Sidak)	WT vs KO mCS+ vs mCS-	Yes	Interaction F (1, 30) = 6.939	P=0.0132
						mCS+ vs mCS- F (1, 30) = 6.782	P=0.0142
Figure 38C	Direct odor learning in D1-CB1 KO mice	14-18	Two-way ANOVA repeated	WT vs KO CS+ vs CS-	Yes	Interaction F (1, 30) = 0.3169	P=0.5777

			measures (Sidak)			CS+ vs CS- F (1, 30) = 68.62	P<0.0001
Figure 39A	Total liquid consumption during mediated taste learning in D1-CB1-KO mice	14-18	Unpaired t test	WT vs KO	Yes	t=1.784, df=31	P= 0.0842
Figure 39B	Total liquid consumption during direct odor learning in D1-CB1-KO mice	14-18	Unpaired t test	WT vs KO	Yes	t=1.346, df=31	P= 0.1880
Figure 40B	Mediated taste learning in DN22-CB1 KI mice	7-11	Two-way ANOVA repeated measures (Sidak)	WT vs KI mCS+ vs mCS-	Yes	Interaction F (1, 16) = 3.002	P=0.1024
						mCS+ vs mCS- F (1, 16) = 3.785	P=0.0695
Figure 40C	Mediated odor learning in DN22-CB1 KI mice	8-6	Two-way ANOVA repeated measures (Sidak)	WT vs KI mCS+ vs mCS-	Yes	Interaction F (1, 12) = 0.2559	P=0.6221
						mCS+ vs mCS- F (1, 12) = 0.2996	P=0.5941
Figure 40D	Direct odor learning in DN22-CB1 KI mice	7-11	Two-way ANOVA repeated measures (Sidak)	WT vs KI CS+ vs CS-	Yes	Interaction F (1, 16) = 10.21	P=0.0056
						CS+ vs CS- F (1, 16) = 16.53	P=0.0009
Figure 40E	Mediated taste learning in DN22-CB1 KI mice	8-6	Two-way ANOVA repeated measures (Sidak)	WT vs KI CS+ vs CS-	Yes	Interaction F (1, 12) = 1.918	P=0.1913
						CS+ vs CS- F (1, 12) = 25.63	P=0.0003
Figure 41A	Total liquid consumption during mediated taste test in DN22 KI mice	7-11	Unpaired t test	WT vs KI	Yes	t=0.9320, df=16	P= 0.3652
Figure 41B	Total liquid consumption during mediated odor test in DN22 KI mice	8-6	Unpaired t test	WT vs KI	Yes	t=0.6846, df=12	P= 0.5066
Figure 41C	Total liquid consumption during direct odor test in DN22 KI mice	7-11	Unpaired t test	WT vs KI	Yes	t=0.4040, df=16	P= 0.6916
Figure 41D	Total liquid consumption during direct taste tests in DN22 KI mice	8-6	Unpaired t test	WT vs KI	Yes	t=1.631, df=12	P= 0.1289
Figure 42B	Odor perception in DN22-CB1 KI mice	10-19	Wilcoxon tests	Odor vs No odor	No	WT	P=0.003906
						KI	P=0.002480

Chapter V Discussion

1 Long-term Impact of Adolescent Cannabinoid Exposure

The first main objective of this PhD thesis was to investigate the long-term behavioral and neurobiological effects of chronic adolescent THC exposure in male and female mice. In the first section of this chapter, I will further discuss the results obtained during my PhD, providing potential interpretations, addressing how my findings relate to previous research, and finally proposing some perspectives and future directions.

1.1 Summary of main results

My results showed that THC treatment during adolescence exerted enduring sex-dependent effects in adulthood on locomotor activity, social interaction and spatial working memory. These effects were observed only in male mice, along with neurobiological alterations in CB1 receptors expression in the hippocampus. I also tested, for the first time, the impact of adolescent THC exposure on higher-order learning in adulthood, using a version of sensory preconditioning that evaluates representation-mediated taste aversion (Busquets-Garcia *et al.*, 2017a). The results showed that adult male mice treated with THC during adolescence were able to (i) express a normal aversion response to a directly conditioned stimulus (odor), (ii) display an aversion response to the indirectly conditioned stimulus (taste), as evidenced by the normal mediated aversion during the test and (iii) perform normal reality testing. Interestingly, preliminary results also showed that adolescent THC treatment is likely not sufficient to increase the adult animal's sensitivity to the acute THC-induced impairment of reality testing (Busquets-Garcia *et al.*, 2017b), although these results should be replicated and further investigated before reaching

final conclusions. In contrast to male animals, we found that adult female mice treated with THC during adolescence failed to express direct or mediated learning, suggesting that memory processes of female mice are more sensitive to THC effects during adolescence than the ones of males.

In the attempt to investigate the mechanisms involved in these behavioral effects of adolescent THC exposure, we also investigated a range of potential long-term molecular effects, including downstream targets of CB1 receptor signaling such as the ERK and CREB proteins, where we observed sex- and region-dependent changes. However, we were not able to causally link these "classic" CB1 receptor-dependent signaling pathways with the behavioral effects of adolescent THC in adult male and female mice. Therefore, we investigated less conventional recently described signaling pathways modulated by the CB1 receptor activation. Our recent findings have shown that mitochondrial complex I instability through the de-phosphorylation of the complex I subunit NDUFS4 in astrocytes is the mechanism leading to persistent THC-induced social deficits (Jimenez-Blasco *et al.*, 2020). Here, we demonstrated that NDUFS4 phosphorylation is specifically decreased by adolescent THC treatment in male, but not in female mice, suggesting that the phosphorylation status of NDUFS4 might be responsible for the later sex-specific behavioral effects of the treatment. Indeed, my data show that viral-mediated astroglial expression of a phosphomimetic mutant of NDUFS4 was able to prevent the adolescent THC-induced behavioral effects on locomotion and social interaction in adult male mice. These results strongly support the idea that some of the persistent effects caused by chronic THC exposure during adolescence are due to a transient reduction of astroglial NDUFS4 phosphorylation. This work adds novel interesting behavioral findings to the existent literature of adolescent cannabinoid effects. Most importantly, however, these data represent the first demonstration of a molecular mechanism causally mediating some of these behavioral effects. Indeed, one of the biggest problems in this type of experiments is the temporal distance between treatment and observed effects, making it very difficult to go beyond merely descriptive data and to establish causal relationships between observed molecular modifications and behavioral effects. The striking result that the expression of a phosphomimetic NDUFS4 fully prevents locomotor and social effects of adolescent THC in adults represents a clear indication that mitochondrial alterations in astrocytes are causally involved in these effects. Certainly, this is just a first step and further research in this direction is necessary, in the hope of leading to a better understanding of the neural perturbations associated with adolescent cannabinoid exposure. In the following pages, I will discuss in more detail these results, their implications and future perspectives.

1.2 Sex-dependent behavioral phenotypes following adolescent THC exposure

Adolescent THC treatment

During my research work, I have used repeated THC injections in adolescent male and female mice, followed by behavioral and biochemical analysis in adulthood, about 4 weeks after the last THC injection. The use of THC instead of other synthetic cannabinoid compounds (WIN 55,212 or CP-55,940) was chosen in part based on the fact that humans who consume cannabinoid products are primarily exposed to THC. Importantly, THC acts on CB1 receptors as a partial agonist, whereas CP-55,940 and WIN 55,212 have higher relative intrinsic activity and they are likely to trigger different cellular adaptations than THC.

The choice of the dose(s) used for adolescent THC treatments varies greatly among studies, with schedules ranging from 0.3 mg/kg to 10 mg/kg (Pouliat *et al.*, 2021; Rubino *et al.*, 2008). A common scheme, used across multiple studies, builds on an escalating dose exposure (Bruijnzeel *et al.*, 2019; Pouliat *et al.*, 2021; Renard *et al.*, 2017; Rubino *et al.*, 2008; Scherma *et al.*, 2016), similar to what might be observed in humans (Bagot *et al.*, 2015; Gunn *et al.*, 2020; Taylor *et al.*, 2017). Here, we have chosen two different dosage schemes of daily i.p. injections, 1 mg/kg and 5 mg/kg. The i.p. route of administration was chosen over alternative routes— that more closely model human use (i.e., smoke, vapor, or oral consumption) — to allow comparison with previous studies in the literature, as well as for experimental feasibility. The two doses used (1 mg/kg and 5 mg/kg) were chosen to evaluate in the same experimental design the long-term effects of both a low and a moderate/high THC exposure scheme. Moreover, in adult rodents these doses have been shown to acutely produce distinct, sometimes opposite, effects in different behavioral responses (Busquets-Garcia *et al.*, 2017b; Han *et al.*, 2012; Puighermanal *et al.*, 2009), therefore we wanted to investigate whether similar phenotypic differences would arise following chronic adolescent treatment.

Importantly, even though there are no studies directly comparing THC pharmacokinetics in rodents and humans, previous studies have reported that an i.p. injection of either of these doses in rodents acutely raises serum concentrations to levels comparable to acute cannabis intoxication in humans (Beiersdorf *et al.*, 2020; Klein *et al.*, 2011; Torrens *et al.*, 2020). In addition, after daily chronic use of THC in rodents, the amount of plasma THC remains stable (Klein *et al.*, 2011). Therefore, the THC doses used here seem relevant to the recreational use in adolescent people, especially considering the

steady increase of THC potency in cannabis products (Wilson *et al.*, 2019). The behavioral findings showed that adolescent THC treatment led to several alterations in adult male animals, including increased locomotor activity, reduced social interaction and deficits in spatial working memory, none of which were present in adult female mice. These behavioral tests were chosen as they are commonly used to mimic psychotic-like effects of drugs or treatments (Powell and Miyakawa, 2006).

Locomotor activity

THC-treated male mice, but not female, travelled a longer distance and exhibited increased rearing behavior when compared to vehicle-treated controls. Cannabinoids are classically known to produce potent effects on motor functions and the high expression pattern of CB1 in areas controlling motor behavior, such as the basal ganglia and the cerebellum is consistent with these effects (Herkenham *et al.*, 1990). It is well documented that acute cannabinoid administration affects locomotion in a dose-dependent biphasic way within the basal ganglia (de Fonseca *et al.*, 1998). While high doses of THC induce hypo-locomotion and catalepsy (Chaperon and Thiebot, 1999), an increase in motor activity has been associated with low doses of THC (Busquets-Garcia *et al.*, 2017b; Sañudo-Peña *et al.*, 2000). Previous studies using rodent models of adolescent cannabinoid use have also reported an increased locomotion after different schemes of adolescent WIN (Abush and Akirav, 2013; Wegener and Koch, 2009a) or THC treatment (Poulia *et al.*, 2019; Poulia *et al.*, 2021) in male mice. At the same time, many studies in both sexes have failed to show an effect in adult locomotion following adolescent THC exposure (Chen and Mackie, 2020; Murphy *et al.*, 2017; O'Tuathaigh *et al.*, 2010; Rubino *et al.*, 2008; Shen *et al.*, 2011) or reported a reduction in open-field activity (Llorente-Berzal *et al.*, 2013; Renard *et al.*, 2017). However, it is important to note that most of these inconsistencies in the results can be attributed to major differences in the period of treatment, the cannabinoid agonist or the dose used, as well as the species and genetic background of the animals used. Overall, the results presented here support that two separate dosage schemes (1 mg/kg and 5 mg/kg) of adolescent THC exposure, affect spontaneous motor activity in adult mice in a sex-specific manner. As already discussed in the introductory chapter (see Chapter I, Box 2), locomotor hyperactivity in response to drugs or novelty is a measure that has often been used as an approach to study positive psychotic symptoms, such as hallucinations and delusions, in rodents (van den Buuse, 2010). Therefore, the effects of THC on locomotor activity described here might represent evidence for a psychotic-like phenotype following adolescent THC treatment. However, as commented above and as I will further discuss in this chapter,

there is an urgent need for behavioral tasks that better model the hallmarks of positive psychotic symptoms.

Social interaction

Short- and long-term alterations in social behavior have been described following adolescent exposure to cannabinoid agonists, which is not surprising given the important role of the ECS in the modulation of brain circuits involved in social behavior (Wei *et al.*, 2017). In our experiments, adolescent treatment with either 1 or 5 mg/kg THC resulted in a decrease of social interaction in adult male, but not female, mice. Previous studies have reported similar results in male rodents following adolescent THC (Quinn *et al.*, 2008; Renard *et al.*, 2016a; Renard *et al.*, 2017; Zamberletti *et al.*, 2014). However, a recent study in male and female mice did not report any effect on social interactions in the 3-chamber test in mice receiving chronic adolescent THC (Chen and Mackie, 2020). In addition, another study using the synthetic cannabinoid CP55,940 in rats reported an attenuation of sociability in both sexes when tested soon after the treatment, but this effect was no longer present in young adulthood (Cossio *et al.*, 2020). Importantly, these discrepancies can be attributed to the different administration protocols when it comes to the dose, duration and time-frame chosen as well as to the behavioral protocols and observational parameters used to assess social behavior.

Finally, following the rationale of a psychotic-like phenotype as a consequence of the adolescent THC treatment, it is important to take into account, that social deficits constitute one of the main “negative symptoms” of psychotic disorders— including besides social withdrawal, apathy, anhedonia and a general blunting of emotional responses, all of which are defined by the absence of normal functions, thus referenced as negative symptoms, also in contrast to the main positive psychotic symptoms (Carbon and Correll, 2014; Miyamoto and Nitta, 2014; Stefanis *et al.*, 2004). Interestingly, several studies in humans have found gender differences in negative symptoms of schizophrenia, showing that men have significantly higher levels of negative and disorganization symptoms than women (Cowell *et al.*, 1996; Ochoa *et al.*, 2012; Riecher-Rössler and Häfner, 2000; Shtasel *et al.*, 1992), further supporting our current findings on the sex-differentiation in the effects of adolescent THC exposure.

Spontaneous alternation

The effect of adolescent THC on short-term spatial memory was assessed in the Y-maze alternation task (Kraeuter et al., 2019). Short-term memory in this task was impaired in male THC-treated animals, while no effect was observed in the female groups. Adolescent cannabinoid exposure in male rodents has been previously reported to result in disruption of spatial working memory in adulthood in different tasks such as the radial maze (Rubino et al., 2009b) or the Morris water maze (Abboussi et al., 2014; Abush and Akirav, 2013). Using the Y-maze, some studies have also shown similar impairments in female treated rats (Rubino et al., 2015a) or mice (O'Tuathaigh and Waddington, 2010). However, it's also noteworthy that working memory is one of the best characterized cognitive domains that are affected in psychotic disorders (Bora and Murray, 2014; Forbes et al., 2009; Goldman-Rakic, 1994). Although it has been used in multiple different studies (Cleal et al., 2021; Kraeuter *et al.*, 2019), the value of spontaneous alternation as a true measure of working memory (rather than simple short-term memory) is still debatable (Hughes, 2004), however our results account, at least at some level, for the cognitive deficits that are present in psychotic patients.

Prepulse inhibition (PPI)

Prepulse inhibition of the acoustic startle response has been widely used as a measure of sensorimotor gating mechanisms, which are altered under psychotic-like conditions. Deficits in PPI have been reported in several neuropsychiatric disorders (Geyer et al., 2001; Kohl et al., 2013), including cannabis-induced psychosis (Morales-Munoz et al., 2017; Winton-Brown et al., 2015), and it is well known that acute cannabinoid treatment affects PPI in mice and rats (Busquets-Garcia *et al.*, 2017b; Nagai et al., 2006; Schneider and Koch, 2002; Wegener et al., 2008). In our experimental conditions, the adolescent THC treatment did not modify PPI, similar to the findings of other groups after adolescent cannabinoid treatment in rats (Bortolato *et al.*, 2014; Llorente-Berzal *et al.*, 2013; Poulia *et al.*, 2021; Scherma *et al.*, 2016; Silva *et al.*, 2016). In contrast, some previous reports have shown PPI impairment in males after adolescent exposure to THC (Renard *et al.*, 2016a) or WIN (Gleason *et al.*, 2012; Schneider and Koch, 2003; Wegener and Koch, 2009a), as well as in adult females after adolescent treatment with CP 55,940 (Llorente-Berzal et al., 2011). It has been argued that the potency of the cannabinoid agonist used during the adolescent treatment (THC versus synthetic agonists) can affect the observed effect on PPI (Silva *et*

al., 2016). Similarly, the time of testing seems to also be an important parameter, suggesting a transient nature of the deficit in sensorimotor gating (Abela *et al.*, 2019).

Associative learning: Direct odor aversion and odor-mediated taste aversion

Higher-order learning paradigms, such as the odor-taste sensory preconditioning paradigm used here, allow us to study both mediated and direct associative learning. First, we can evaluate an animal's response to a directly conditioned stimulus (direct learning). In this case, we were able to study if animals treated with THC during adolescence were affected in direct odor aversion. At the same time, we could evaluate the response of the animals to a stimulus (in this case a taste) that was indirectly conditioned, through its previous association with the directly conditioned odor. This is referred as a mediated learning response, to be distinguished from the direct conditioned response, and since it is in fact mediated by an associative representation of the odor, hence odor-mediate taste aversion. Direct and mediated responses are also described as a response to the conditioned and preconditioned stimulus, respectively (Batsell and Batson, 1999; Hart *et al.*, 2020; Sharpe *et al.*, 2017a). However, I will keep using the terms direct and mediated in consistence with our previous publications (Busquets-Garcia *et al.*, 2018b; Busquets-Garcia *et al.*, 2017a; Busquets-Garcia *et al.*, 2017b).

Adult male mice, treated with THC during adolescence, displayed both a reliable mediated and direct aversion, similar to their vehicle-treated littermates, indicating that adolescent THC treatment does not have an impact on these two types of memory at adulthood. To our knowledge, no previous studies have evaluated the impact of adolescent cannabinoid treatment on aversive olfactory memory. Some studies have however reported impairments in fear memory. For example, studies using THC or WIN have shown that adolescent cannabinoid treatment can impair contextual fear conditioning (Gleason *et al.*, 2012; Tomas-Roig *et al.*, 2016), while another study reported no effect of adolescent THC in the passive avoidance task (Rubino *et al.*, 2009b). In addition, this work represents the first attempt to study the impact of adolescent cannabinoid treatment on higher-order conditioning paradigms, although we found no long-term alterations. Of course, adolescence is a critical period for the development of higher-order cognitive functions (Yurgelun-Todd, 2007) and future studies should further add to these results, investigating for example if similar observations emerge under different sensory modalities. It is important for instance, to evaluate if intact mediated aversion in males is present irrespectively of whether odor or taste are devaluated during conditioning.

Contrary to what was observed in males, the adolescent THC-treated female groups failed to show mediated aversion. At the same time, direct aversion was also impaired in females following adolescent treatment with the highest dose of 5 mg/kg THC. Interestingly, aversive olfactory memory has not been previously addressed in adult female mice following adolescent THC treatment. However, the fact that females appear more sensitive than males to the effects of THC in this task could reflect a stronger cannabinoid impact in emotional-related behaviors in females compared to males. Indeed, studies have shown that female rodents display pronounced anxiety or depressive-like responses following adolescent cannabinoid treatment, in contrast with males (Tirado-Muñoz *et al.*, 2020). These differences might also be in line with a suggested greater susceptibility for divergent psychiatric vulnerability in humans, where females with cannabis use show greater susceptibility for depression, while males appear more prone to addiction-like behaviors (Rubino *et al.*, 2012). Moreover, gender differences in psychotic symptoms have also been described, with some studies suggesting that women present higher levels of affective symptoms than men (Cotton *et al.*, 2009; Ochoa *et al.*, 2012). Importantly, in our behavioral battery we did not include tasks directly assessing the affective state of the mice, but we might speculate that this domain could be more strongly impaired in female than male mice. Future experimental studies could shed more light on such differences.

Reality testing: a paradigm to study alterations of mental sensory representation in mice

All the behavioral alterations discussed above can be seen as representing different aspects of relevant human deficits following chronic cannabinoid exposure during adolescence. As mentioned in the introductory part of this PhD thesis, adolescent cannabis use, notably chronic and heavy use, has been associated with both social (Macleod *et al.*, 2004) and cognitive impairments (Scott *et al.*, 2018) later in adulthood. However, one of the most prominent and frequently addressed consequences of adolescent cannabis use is the potential risk for the development of persistent psychotic symptoms and even full-blown psychotic disorders like schizophrenia. We have already discussed how the modeling and investigation of psychotic-symptoms, like hallucinations and delusions, can be especially challenging when it comes to laboratory animals (Chapter 1, Box 2), and how the recent shift to paradigms and interpretations rooted on associative learning principles can provide a promising way to understand the underlying mechanisms of these symptoms across species (Chapter 1, Box 3).

“Reality testing” is the process through which individuals verify the correspondence between the internal representations of meaningful stimuli, and their real presence and meaning in the external world (McDannald and Schoenbaum, 2009). Likewise, impaired reality testing might correspond to an inability to distinguish between a present event and an internal representation of an absent event (Kim and Koh, 2016). This is of course a peculiar human ability, but evidence exists that animals can construct complex internal representations of their world as well. Notably, this kind of ability can be observed in some of the normal phenomena in associative learning, like in the case of mediated learning, where one stimulus (e.g. an odor, a taste, a light or a tone) comes to activate an internal representation of a second stimulus with which it is paired), and this representation can serve for acquisition of new learning (conditioned response). For instance, in the results discussed right above, we saw how an odor-evoked representation of the taste could substitute for the taste itself in the acquisition of an aversion. One of the possible explanations for this phenomenon (Ioannidou *et al.*, 2021) suggests that the taste stimulus might directly acquire negative value during conditioning, due to a “unified representation” of taste and odor, in a way that eventually, presentation of the taste alone during test elicits the observed response (Lin and Honey, 2016; Schlichting and Preston, 2015; Wheeler *et al.*, 2008; Wimmer and Shohamy, 2012).

Interestingly, three consecutive odor-taste preconditioning pairings are sufficient, as we saw, to induce a reliable mediated aversion, suggesting that the mice formed a unique mental representation of the two previously associated stimuli (odor and taste). However, it has been shown that if the preconditioning “training” is extended to six, or even nine, pairings before the selective conditioned devaluation, the formation of mediated aversion is suppressed, indicating that the extended training during preconditioning induced a mental representation of the stimuli as two separated entities (Busquets-Garcia *et al.*, 2017b). This flexibility in adapting the meaning of stimuli to changing conditions can be seen as a prerequisite of reality testing. Considering that the stimuli are separate in reality, one can argue that extended training allows animals to acquire additional information that enables the “reality testing” of the independent meaning of the two stimuli (Busquets-Garcia *et al.*, 2017b; Kim and Koh, 2016; McDannald and Schoenbaum, 2009; McDannald *et al.*, 2011), implying a correct mental representation of the values of the stimuli. In other words, what reality testing tasks assess is the ability to adapt the internal representation of stimuli to their “real” presence and meaning (McDannald *et al.*, 2011; Saddoris *et al.*, 2005; Wheeler *et al.*, 2013). Previous work from the lab has shown that acute THC administration can impair reality testing, while CB1 receptor antagonists, such

as rimonabant, were shown to block the THC-induced reality testing impairment, suggesting a strong role of CB1 receptor activation.

Here I evaluated the performance of adult mice in the reality testing protocol after the adolescent THC treatment. As female mice already presented impaired learning after three preconditioning pairings, it was decided to assess the reality testing after six preconditioning pairings only in male mice for this work. Contrary to our original hypothesis, our results showed that adolescent THC-treated mice did not display any impairment in the reality testing paradigm. Much alike their adolescent vehicle-treated littermates, mice exposed to the chronic adolescent THC treatment displayed an absence of mediated aversion, suggesting a correct mental representation of the independent nature of the two stimuli. Importantly, the establishment of a direct aversion both in the vehicle-treated and THC-treated groups was intact, indicating that no underlying impairment in basal reinforcement learning was responsible for the absence of mediated learning. To our knowledge, this is the first study to evaluate the behavioral performance in a reality testing paradigm after adolescent THC exposure.

A recent study, that evaluated the persistent effects of adolescent THC exposure on a paired-associates learning task in rats, suggested that cognitive impairments produced by adolescent THC may be transient in nature. They showed that rats exposed to THC during adolescence initially displayed impaired learning of the task, but this deficiency was less evident as time passed, and their performance was seemingly unaffected in a more challenging version of the task (Abela *et al.*, 2019). So, although associative learning and information processing during adolescence seems to be vulnerable to THC exposure, the impairments that arise could represent an interval of vulnerability in young adulthood that recovers over time, as opposed to one that persists indefinitely (Abela *et al.*, 2019). If this hypothesis is correct, it would be important to explore in future studies whether impairments in reality testing could appear earlier, if the animals are tested for example during adolescence or young adulthood, and then recover in later adulthood.

The importance of the endocannabinoid system in the ability to shift from a previously learned response to a similar, yet different, new one has been previously documented. Both THC and CB1 receptor antagonists have been shown to impair behavioral flexibility (Egerton *et al.*, 2005; Hill *et al.*, 2006) and CB1 knockout mice have presented deficits, for instance, in their ability to locate the position of a moved platform in the reversal phase of the Morris water maze (Varvel and Lichtman, 2002). Reversal learning requires a degree of flexibility, and in order to be successful animals have to actively

suppress previously learned responses in order to perform the newly appropriate ones, similarly perhaps to how animals in reality testing have to suppress their previous learning about the unified stimuli association in order to learn the independent nature of the two stimuli. In this sense, an older study evaluating reversal learning in the active place avoidance task in rats administered with THC during adolescence, showed that animals treated with THC during early adolescence made more errors on the reversal trial, but in animals treated during late adolescence there were no significant differences (Harte and Dow-Edwards, 2010). Therefore, possibly the exact age of the adolescent THC treatment could play an important role in later effects in the execution of complex discrimination and reversal tasks or, in our case, reality testing, with early adolescence being a time period of heightened vulnerability to these effects.

Our second hypothesis regarding the impact of adolescent THC treatment in reality testing performance was that instead of a direct effect on the mediated learning, the chronic THC administration during adolescence could result in a greater sensitivity to the acute THC-induced impairment in reality testing in adult mice. As previously mentioned, different psychotogenic drugs, including THC, can acutely lead to deficits in reality testing task performance (Busquets-Garcia *et al.*, 2017b) and adolescent THC exposure has been shown to increase sensitivity to the effects of THC and other drugs (Cadoni *et al.*, 2015; Ellgren *et al.*, 2007; Gomes *et al.*, 2015; Higuera-Matas *et al.*, 2008; Tomasiewicz *et al.*, 2012; Zamberletti *et al.*, 2014). Therefore, we speculated that a low THC dose, which normally produces no effect in naïve animals, could result in an impairment of reality testing performance in adolescent THC-treated animals. In our results, we report that adolescent THC exposure did not sensitize reality testing performance to the acute effect of THC. Considering, however, the preliminary nature of this experiment, it is not possible to draw conclusions from these preliminary data yet and additional future experiments are required.

1.3 CB1 receptor expression in the hippocampus following adolescent THC

Adolescent THC exposure led to CB1 receptor downregulation in the hippocampus of male mice, while no alterations were observed in female mice. In the hippocampus, most CB1 receptors are located on cholecystokinin (CCK)-positive GABAergic interneurons (Marsicano and Lutz, 1999), and their stimulation is capable of enhancing the activation of pyramidal through a decrease in GABA inhibitory tone (Ohno-Shosaku et al., 2001; Wilson et al., 2001). Our current findings would indicate a decreased disinhibition of pyramidal neurons, potentially leading to disruption of hippocampal functions. Moreover, these results agree with previous studies demonstrating widespread desensitization of CB1 receptor-mediated G protein activation and down-regulation of CB1 receptor binding sites after long-term cannabinoid administration (Rubino *et al.*, 2008; Sim-Selley, 2003; Sim-Selley and Martin, 2002). Indeed, a previous study examining the effects of chronic treatments with high doses of THC or WIN showed long-lasting decreases in CB1 receptors (Sim-Selley et al., 2006). This adaptation is supported also by the finding that repeated cannabis use also produces CB1 receptors down regulation in humans (Hirvonen et al., 2012).

One potential mechanism of the observed CB1 receptor downregulation can be the transcriptional regulation of the receptor's expression. However, we have found no changes in CB1 receptor mRNA in the hippocampus neither directly after the chronic THC treatment, nor after a one-month abstinence period (data not shown). Other studies have also reported similar findings (Rubino et al., 1994; Sim-Selley *et al.*, 2006), suggesting that alternative mechanisms, such as post-transcriptional modifications leading to persistent internalization or degradation of the receptor, possibly contribute to the long-lasting down-regulation of hippocampal CB1 receptors caused by chronic cannabinoid administrations.

1.4 Modulation of ERK and CREB activation by adolescent THC

CB1 receptor activation can modulate multiple downstream signaling pathways. Of particular interest is the activation of the extracellular signal-regulated kinase (ERK), which has been related to neuronal adaptive processes and learning and memory formation (Giovannini, 2006; Kushner et al., 2005; Peng et al., 2010). Notably, we observed a decrease of ERK phosphorylation in male mice following adolescent THC treatment with the lowest dose (1 mg/kg), while female mice treated with the same dose showed increased levels of phospho-ERK. Interestingly, the activation of the ERK pathway

appears essential for some forms of LTP in hippocampus (Winder et al., 1999) and for the regulation of gene expression in the context of synaptic plasticity (Impey et al., 1999). It is therefore possible that these sex-dependent changes on hippocampal ERK phosphorylation and their potential consequences on gene regulation (via phosphorylation of transcription factors) or on intracellular signaling may participate in the different behavioral phenotypes found in male and female mice, through alterations in synaptic plasticity.

Considering that the major role of ERK lies in the regulation of gene expression, we also examined a downstream ERK target such as the cyclic AMP response element binding protein (CREB) transcription factor. Localized within the nucleus, CREB is crucial for stimulus–transcription coupling, and is best known for its roles in learning and memory (Carlezon et al., 2005; Silva et al., 1998). Here, we showed that both male and female mice expressed increased levels of phospho-CREB, following adolescent THC exposure. In line with this, previous studies have shown that both acute and chronic THC exposure can result in a significant activation of CREB in the hippocampus (Derkinderen et al., 2003; Rubino et al., 2004). Notably, the activation of the CREB signaling pathway in the hippocampus has been shown to play an important role in spatial memory formation (Mizuno et al., 2002), and increases in CREB function have been shown to facilitate memory (Josselyn et al., 2001; Yin et al., 1995)- even if the underlying mechanisms are not completely understood. Taking this into account, our present findings, suggesting that the increased CREB activation after adolescent THC could underlie the impairment in spatial working memory in male mice, seems contradictory with the role of CREB in learning and memory processes. However, one possible explanation is that an over- stimulation of the cAMP/PKA/CREB pathway can actually produce memory deficits, following the notion that memory function requires an optimal range of CREB activation: stimulation of the pathway can facilitate memory if activity is low, but it can be disruptive if activity is too high (Davis et al., 1995). Moreover, evidence suggests that CREB activity has very different roles – both beneficial and detrimental – depending on the brain region involved (Carlezon *et al.*, 2005), therefore it would be important to assess whether differences in CREB activation after adolescent THC exposure follow a similar direction in other brain regions.

Overall, our results indicate that the ERK/CREB pathway may contribute to the initiation of a gene expression cascade in response to the chronic adolescent THC exposure. However, the degree of relevance of each of these protein alterations to the long-lasting behavioral effects of adolescent THC

requires further experiments. For example, more research is needed to know why enhanced CREB levels in females are not translated in spatial working memory impairment as we observed in males.

1.5 Mitochondria: the missing link between adolescent THC exposure and persistent behavioral effects?

As described in the first chapter of this PhD thesis, CB1 receptors are the critical signal transduction components of the ECS and usually operate as Gi/a-coupled GPCRs at the cell plasma membrane (Howlett, 2002). However, CB1 receptors have been also shown to localize at or very close to mitochondrial membrane (named mtCB1 receptors) (Benard *et al.*, 2012; Lipina *et al.*, 2014; Mendizabal-Zubiaga *et al.*, 2016b), where they regulate energy metabolism and mitochondrial functions in both neurons and glia (Benard *et al.*, 2012; Hebert-Chatelain *et al.*, 2016; Hebert-Chatelain *et al.*, 2014; Jimenez-Blasco *et al.*, 2020).

By regulating numerous cellular processes, mitochondria exert a plethora of functions that are particularly crucial for one of the most energy requiring organs of the body, such as the brain (Picard and McEwen, 2014). Proper mitochondrial function is critical for maintaining energy homeostasis and is responsible for generating most of cellular energy in the form of ATP. ATP production is obtained through oxidative phosphorylation (OXPHOS), a process requiring the action of a set of respiratory enzyme complexes, known as the electron transport chain. The electron transport chain consists of 5 enzyme complexes embedded in the mitochondrial inner membrane: complex I (CI, ubiquinone oxidoreductase), complex II (CII, succinate dehydrogenase), complex III (CIII, cytochrome bc1 complex), complex IV (CIV, cytochrome c oxidase) and complex V (CV, ATP synthase) (Chaban *et al.*, 2014). Cannabinoid receptor agonists, such as THC or WIN55,212-2 have been shown to inhibit complexes I, II, and III of the respiratory chain (Singh *et al.*, 2015; Wolff *et al.*, 2015) and a series of recent evidence suggests that activation of mitochondrial CB1 receptors can directly impact a cell's ability of oxidative phosphorylation and neuronal energy production (Costa and Colleoni, 2000; Fišar *et al.*, 2014; Hebert-Chatelain *et al.*, 2016; Hebert-Chatelain *et al.*, 2014). Data collected so far indicate that decreased phosphorylation of Complex I (CI) subunits represents the main target of mtCB1-dependent alterations of mitochondrial functions (Benard *et al.*, 2012; Hebert-Chatelain *et al.*, 2014; Jimenez-Blasco *et al.*, 2020). CI is the largest complex of the respiratory chain and consists of multiple

conserved subunits that form an L-shaped structure. This structure consists of a hydrophilic peripheral arm that protrudes into the mitochondrial matrix, and is constituted by the N module (for NADH oxidation) and Q module (for ubiquinone reduction), and also a membrane embedded arm, lying perpendicular to the peripheral arm, that constitutes the proton pumping P module of the complex (Mimaki *et al.*, 2012) (Figure 47). Lots of efforts have been made to better understand the function of CI, as its dysfunction is the most common OXPHOS disorder in humans and observed in many diseases (Pagliarini *et al.*, 2008).

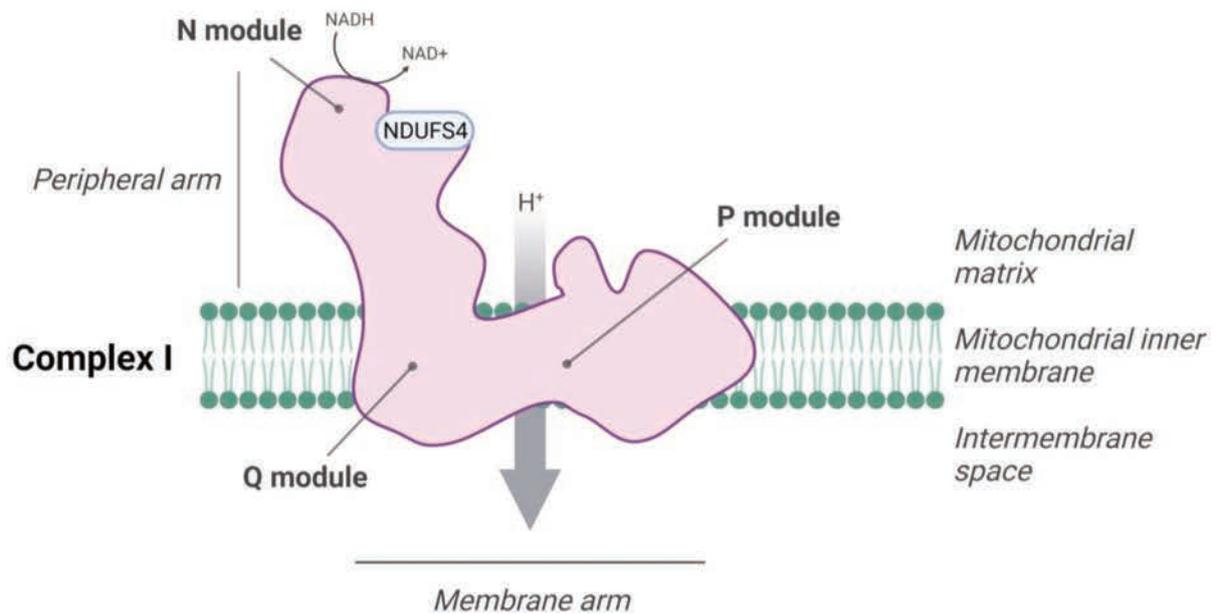


Figure 47. Schematic representation of mitochondrial complex I structure. Complex I has an L-shaped structure with a hydrophilic peripheral arm protruding into the matrix and a hydrophobic arm embedded in the mitochondrial inner membrane. It is composed of three conserved functional modules: the NADH dehydrogenase module (N module), the electron transfer module (Q module) and the proton translocation module (P module). The NDUFS4 subunit is located in the peripheral arm, on the matrix side of the inner mitochondrial membrane, and is involved in the assembly and stability of complex I.

Recently, we demonstrated that activation of astroglial mtCB1 receptors by THC prevents the phosphorylation of the NADH dehydrogenase [ubiquinone] iron-sulfur protein 4 (NDUFS4) subunit of CI. NDUFS4 is a nuclear encoded protein that is located on the matrix side of the inner mitochondrial membrane and is essential for maintaining the proper function of CI, by ensuring the stability of its N module (Kahlhöfer et al., 2017) (Figure 47). In our work, we showed the importance of NDUFS4 phosphorylation at its Ser173 residue for CI stability, thus indicating that the THC-induced reduction in its phosphorylation destabilizes CI. Subsequently, this leads to a reduction in the generation of reactive oxygen species by astrocytes and affects the glycolytic production of lactate, eventually resulting in neuronal redox stress [(Jimenez-Blasco *et al.*, 2020), Chapter IV-Section 3] (Figure 48).

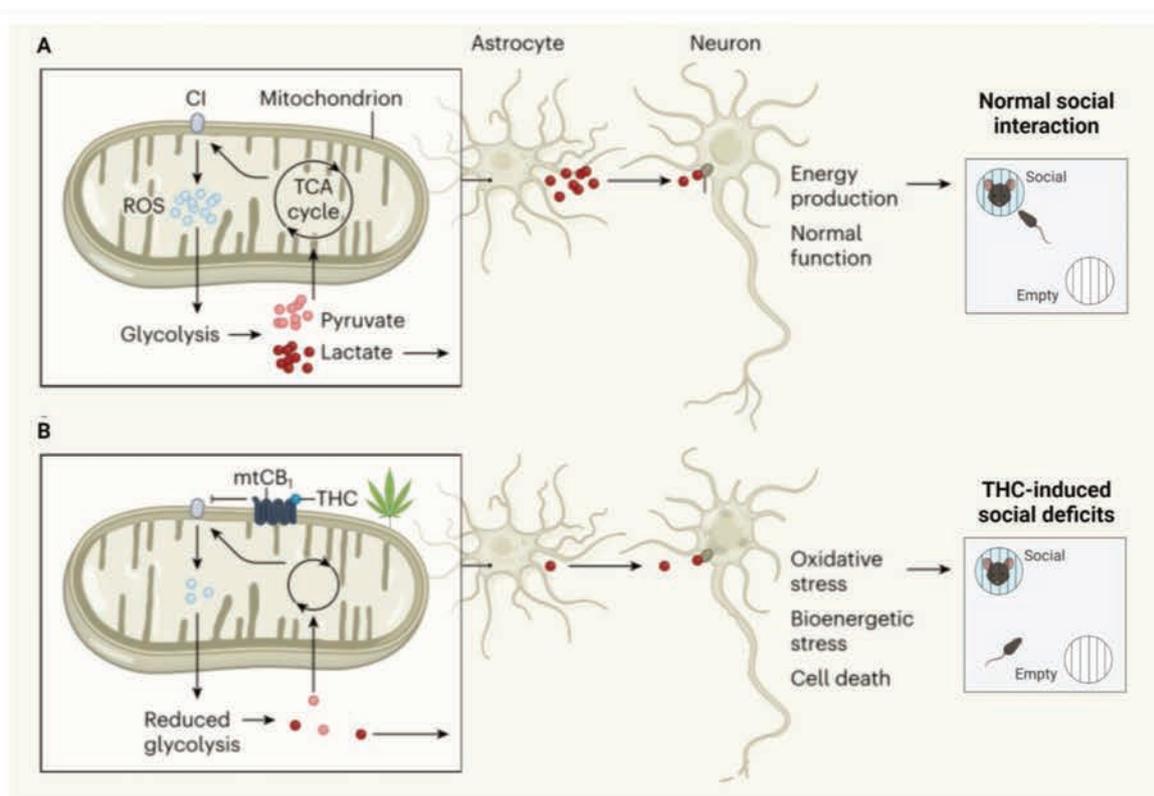


Figure 48. Glucose metabolism links astroglial mitochondria to cannabinoid effects. (A) Mitochondrial complex I activity leads to the production of reactive oxygen species (ROS). ROS promote glycolysis, in which pyruvate and lactate are generated. Pyruvate is metabolized in mitochondria through the tricarboxylic acid (TCA) cycle, which feeds back into oxidative phosphorylation. Lactate is typically shuttled from astrocyte cells and into neurons. In neurons, lactate is used to generate energy, enabling normal neuronal functions and circuits involved in social behavior. (B) THC-induced activation of mtCB1 receptors in astrocytes, disrupts the activity of complex I, down-regulating this metabolic pathway and decreasing the amount of lactate that is shuttled to neurons. This causes oxidative and bioenergetic stress in the neurons, as well as neuron death, and results the persistent THC-induced social deficits in mice. Adapted from Magistretti, 2020.

Clearly, mitochondrial function can be highly affected by acute exogenous cannabinoids, such as THC (Athanasίου *et al.*, 2007; Fišar *et al.*, 2014; Hebert-Chatelain *et al.*, 2016; Singh *et al.*, 2015). Yet, the impact of chronic adolescent THC exposure on metabolic and/or mitochondrial processes is still in the early stages of investigation. In the face of the previous evidence from the laboratory, we hypothesized that adolescent chronic THC treatment could disrupt brain bioenergetics altering the mitochondrial respiratory chain. Indeed, the results indicate that the phosphorylation of the NDUFS4 subunit of CI is specifically decreased by adolescent THC treatment in male, but not female mice, suggesting that this molecular pathway might be responsible for the later sex-specific behavioral effects. Interestingly, this reduction of NDUFS4 phosphorylation was fully recovered in adult male mice. Our published data show that an acute single administration of THC (at 10 mg/kg) in adult male mice causes a pronounced reduction of the phosphorylation of the NDUFS4 subunit, which persists up to 24h later (Jimenez-Blasco *et al.*, 2020). In the adolescent THC experiments, samples were obtained 24 hours after the last of the chronic THC treatment (at PND46). Therefore, we cannot exclude the possibility that the observed reduction in NDUFS4 phosphorylation is due to the persistent 24h effect of the last THC injection. Further experiments should clarify the nature of this effect by obtaining brain samples at different time points during and after the adolescent THC treatment. However, two interesting observations can arise from the present data. First, if the observed reduction in phosphorylation levels is due to the acute effect of the last injection, it would indicate that even a lower dose than the previously described 10 mg/kg, is sufficient to affect mitochondrial proteins at least 24 hours after administration (here the chosen dose for the adolescent THC treatment was 5 mg/kg). Second, this would suggest that even after 10 consecutive treatment days, there is no development of tolerance to the effects of THC on NDUFS4. In this sense, it is well described that following repetitive exposure to exogenous cannabinoids, a high degree of tolerance to their behavioral and pharmacological effects often develops (González *et al.*, 2005), including CB1 receptor downregulation and desensitization (Sim-Selley, 2003), as well as a downregulation of several other proteins (Butovsky *et al.*, 2006; Kittler *et al.*, 2000; Martin *et al.*, 2004; Rubino *et al.*, 2006). In contrast, it has been shown that there is no tolerance to some deleterious effects of THC on memory (Puighermanal *et al.*, 2009) and it would be worthwhile to explore if such a profound downregulation of NDUFS4 phosphorylation is linked to the cognitive deficits induced by cannabinoids.

Yet, the most interesting part of our results is clearly the absence of this profound effect on NDUFS4 phosphorylation in female treated mice. Our previous studies have not assessed the effect of acute THC

in female mice, therefore this is the first time we observe such sex-dependent molecular changes in mitochondrial proteins. Interestingly, sexual dimorphism in mitochondrial function has been previously described (Demarest and McCarthy, 2015; Ventura-Clapier et al., 2017), including differences in oxidative stress (Borrás et al., 2003) and lipid metabolism (Acáz-Fonseca et al., 2017). Estrogens have been shown to increase mitochondrial function (Klinge, 2008), and one study showed that brain mitochondria from female rodents exhibit greater functional capacity compared to males, especially in aged animals (Guevara et al., 2011). It is therefore important to evaluate in future experiments potential basal differences between males and females in the expression of NDUFS4 and phosphoNDUFS4 levels, as well as CI assembly. Indeed, some preliminary data from our collaborators suggest that astrocytes from female mice appear to have a more disassembled CI than males in basal conditions (unpublished data), possibly due to a lower degree of NDUFS4 phosphorylation in females. Therefore, the underlying mechanisms leading to the sex-dependent effect of chronic adolescent THC in mitochondrial functions remain to be meticulously explored.

Yet, although the exact mechanistic pathway is not yet uncovered, strong evidence suggests that the reduction of NDUFS4 phosphorylation, following the adolescent THC exposure in male mice, is causally related to the persistent behavioral effects of adolescent THC. Indeed, my data show that the astroglial expression of a phosphomimetic mutant form of NDUFS4 is able to prevent the adolescent THC-induced effects in locomotion and social interaction in adult male mice, strongly suggesting that at least some of the long-term behavioral effects caused by chronic THC exposure during adolescence are due to a transient reduction of astroglial NDUFS4 phosphorylation.

For a better understanding of the social deficits induced by adolescent THC, we would need to assess when exactly do these deficits emerge – whether it is already short time after the THC treatment in adolescence or if they only manifest themselves later into adulthood. Studies until now have consistently reported social deficits induced by adolescent cannabinoid exposure when animals were tested after variable wash-out periods (Quinn *et al.*, 2008; Renard *et al.*, 2016a; Renard *et al.*, 2017; Zamberletti *et al.*, 2014), but not many have investigated social behaviors during, or immediately after, the adolescent exposure. Yet, a recent study using the synthetic cannabinoid CP55,940 in rats reported an attenuation of sociability soon after the treatment, although they did not report the maintenance of these impairments into young adulthood (Cossio *et al.*, 2020). Undoubtedly, further research employing our regimen of treatment, but evaluating the social behaviors at different time points can clarify the dynamics of behavioral changes induced by adolescent cannabinoid treatment. Interestingly, the astroglial expression

of the NDUFS4 phosphomimetic did not only prevent the adolescent THC-induced impairment in social interaction in adult male mice, but also the adolescent THC-induced hyperlocomotion phenotype in the same mice. This suggests the possibility for a common mechanism connecting cannabinoid-induced mitochondrial dysfunctions during adolescence with the latter behavioral alterations observed. It is important to mention here that adolescent THC did not affect the locomotion of mice during the social interaction test in any of our experiments, indicating that the hyperlocomotion effect might be more associated with the animals' motor response to novelty, rather than alteration on the motor function *per se*. Related to this assumption, a recent study showed that adolescent THC exposure in rats influenced the pattern of behavioral habituation to the open field, with THC-treated animals displaying a rather gradual-delayed reduction in motor habituation compared with the normal and sharp reduction of the vehicle-treated ones (Poulia *et al.*, 2019).

Of course, future research is necessary in order to further elucidate the exact biological steps, connecting the reduction in NDUFS4 phosphorylation during adolescence with the behavioral effects in adulthood. Based on our previous experience however, increased oxidative stress in neurons resulting from metabolic disruptions in astrocytes appears to be a likely candidate. It has been shown that CB1 receptor activation leads to an increase of ROS production in vitro (Donadelli *et al.*, 2011; Rajesh *et al.*, 2010) and in vivo (Koch *et al.*, 2015), and it is known that mitochondria are producing the largest ROS amount in the cell (Massaad and Klann, 2011), which are often created through the “leaking” of electrons at the level of CI (Chen *et al.*, 2003). In this sense, it has been known that both women and female rodents are more “protected” against redox stress as compared to males, due to the higher expression of antioxidant enzymes, as well as the antioxidant properties of estrogen (Kander *et al.*, 2017). Such differences could potentially account for the different molecular and behavioral effects that adolescent THC exposure exerted in male and female mice.

To conclude, it is clear that the disruption of mitochondrial function appears as a new promising direction for investigating cannabinoid effects. Moreover, the prominent role of CB1 receptors in new and previously neglected locations, including neuronal and astroglial mitochondria, highlights, one more time, the extent and diversity of cannabinoid signaling and reveals how the nearly ubiquitous expression of the endocannabinoid system is able to determine highly specific functions (and dysfunctions) in the brain (Busquets-Garcia *et al.*, 2018a).

2 The Role of CB1 Receptors in Associative Learning and Memory Processes

In the second main objective of this work, I aimed at characterizing the role of specific CB1 receptor subpopulations in the control of associative learning. As already discussed in Chapter I, the way animals and humans adapt to changes in the environment is largely through the storage and encoding of past experiences. Although these past experiences are sometimes encoded as direct associations, our daily choices are not always dictated by stimuli that have been directly associated with a reinforcer, whose salience is transferred to a previously neutral stimulus in what we might describe as direct reinforcement learning. Rather, they are often triggered by stimuli that have not been directly associated with a reinforcer, but that were previously associated with other stimuli paired with a specific aversive or rewarding meaning (Bornstein *et al.*, 2017; Parkes and Westbrook, 2011; Wimmer and Shohamy, 2012). The underlying learning processes of such behaviors are what we often refer to as incidental learning. The underlying mechanisms of how the brain encodes and stores complex incidental learning processes have been slowly, but steadily, gaining popularity in the neuroscience field, as they might represent the most evolutionarily advanced way to increase the chances of predicting future events and flexibly adapting behavior to an ever-changing environment (Ioannidou *et al.*, 2021; Lin and Honey, 2016).

Incidental learning can be studied in humans and animals through higher-order conditioning paradigms, such as sensory preconditioning. As described above (Chapter I, Section 4), sensory preconditioning entails presenting subjects with two low-salience stimuli, simultaneously or in close succession, such that an association can form between them, but without any transfer of salience. The formation of such incidental associations during preconditioning can be revealed if for example the second stimulus is then paired with an unconditional stimulus (US). Subsequently, the first stimulus

comes to elicit a conditioned response that is not directly attributed to the US but is mediated by the retrieved association with the second stimulus, hence called a representation-mediated or simply mediated conditioned response.

CB1 receptor signaling, has been found to be involved in many forms of direct reinforcement learning, including fear conditioning (Marsicano *et al.*, 2002; Metna-Laurent *et al.*, 2012), conditioned taste aversion (Kobilo *et al.*, 2007), conditioned odor aversion (Soria-Gomez *et al.*, 2015) or conditioned odor preference (Terral *et al.*, 2019), among others. However, despite the fact that CB1 receptor plays crucial roles in almost all the different phases of learning and memory processes (Akirav, 2011; Drumond *et al.*, 2017; Marsicano and Lafenêtre, 2009; Rueda-Orozco *et al.*, 2008), the involvement of cannabinoid signaling in incidental learning represents a novel trajectory for future studies.

Here, we have employed an odor-taste preconditioning paradigm to investigate (i) the role of dopamine D1 receptors and (ii) mitochondria-associated CB1 (mtCB1) receptors in the formation of such incidental associations, as well as in direct reinforcement learning. Below, I will summarize and discuss the main results and future perspectives from this line of work.

2.1 Dopamine and CB1 receptor interactions in the formation of incidental learning

The results obtained by testing the D1-CB1 knock-out mice in our established sensory preconditioning task, suggest that CB1 receptors in dopamine D1-positive cells are necessary for the formation of mediated learning. These results confirm previous studies showing a potential functional crosstalk between the endocannabinoid and the dopaminergic systems in the modulation of learning and memory functions.

We have previously showed that the formation of mediated learning requires CB1 receptors activity on hippocampal GABAergic interneurons (Busquets-Garcia *et al.*, 2018b), but so far, the exact nature of these neurons has not been identified. Recently, it was discovered that a small subpopulation of inhibitory interneurons expressing both D1 and CB1 receptors exists in the hippocampus, where it controls late consolidation of novel object recognition memory and associated synaptic plasticity, by moderating local inhibitory GABAergic activity (Oliveira da Cruz *et al.*, 2020). The data presented here demonstrate that mutant mice lacking CB1 expression specifically in D1-positive cells were able to express direct conditioned responses in the odor-taste preconditioning task, however they displayed no mediated responses, indicating lack of mediated learning. These results suggest that the small subpopulation of CB1 receptors expressed in D1-positive neurons is required for the formation of indirect learning, implying that not only endocannabinoids, but also dopamine likely plays a role in this process. Generally, the activation of D1 or CB1 receptors leads to neuronal excitation or inhibition, respectively. Thus, it is possible to hypothesize that a fine-tuned regulation of neuronal excitability by dopamine and endocannabinoids physiologically controls this form of incidental learning.

Dopaminergic transmission through D1 receptors has been long implicated in the modulation of different types of memory (i.e. recognition, spatial or aversive) (El-Ghundi *et al.*, 1999; El-Ghundi *et al.*, 2001; Lemon and Manahan-Vaughan, 2006). In addition, midbrain dopaminergic signaling has been linked to the formation of indirect associations in a different sensory preconditioning paradigm (Sharpe *et al.*, 2017b). As D1 receptors are expressed in different neuronal populations, including striatal medium spiny neurons and hippocampal interneurons, it is important to investigate if a specific regional D1/CB1-expressing cell population is specifically involved in mediated learning. For this purpose, a “rescue” approach can be adopted (Oliveira da Cruz *et al.*, 2020), where we are able to obtain viral re-expression of the CB1 gene in the cells that carry its deletion in mutant mice. This strategy can be applied to the main brain regions involved in sensory preconditioning such as cortical areas (e.g. retrosplenial,

perirhinal or orbitofrontal cortices) or to the brain regions clearly expressing D1-positive cells such as the hippocampus (where the CB1/D1-positive neurons were shown to impact memory and where CB1 receptors control mediated learning) and striatum. Overall, the rescue of the impaired phenotype described here in the D1-CB1-KO mice can decipher the brain region where D1/CB1-positive cells control mediated learning.

Another open question that remains to be answered is if the phenotype observed in the D1-CB1-KO mice is limited to the mediated learning about a gustatory stimulus, or if it extends to olfactory stimuli and even other sensory modalities. In fact, we have previously shown that the control of mediated learning by CB1 receptors is not restricted to one sensory modality, but it appears consistent whether the stimuli presented in the preconditioning task are odors, tastes, visual or auditory cues (Busquets-Garcia *et al.*, 2018b). More research is required to investigate if this is the case for the role of the CB1/D1-positive neurons.

2.2 MtCB1 receptors in the control of odor aversion memory

The results presented in the previous chapter showed that mutant mice lacking mtCB1 receptors (DN22-CB1-KI) (Pagano Zottola *et al.*, 2020; Soria-Gomez *et al.*, 2021) failed to express a direct conditioned response to the devaluated odor stimulus (and therefore a mediated response to the associated preconditioned taste stimulus). On the contrary, the DN22-CB1-KI mice expressed a normal conditioned response to the devaluated taste stimulus, suggesting that mtCB1 receptors are specifically required for direct odor-, but not taste-mediated, learning. Olfactory information is a fundamental driver of rodent behavioral functions, including feeding, social relationships, reproduction, the ability to perceive rewarding or aversive olfactory cues in the environment and retrieve its potential meaning, among others (Smeets and Dijksterhuis, 2014; Sullivan *et al.*, 2015). Importantly, we confirmed that the DN22-CB1-KI mice could successfully detect the odor stimuli in the concentrations used in our odor-taste preconditioning paradigm, indicating that a general impairment of olfactory perception is not responsible for the absence of an aversive response to the conditioned odor. The sensitivity of these mice to the nauseating effect of the unconditioned stimulus, namely the injections of a lithium chloride (LiCl) solution, was also not altered, as indicated by the expected reduction in locomotor activity following the LiCl injections (data not shown). These observations strongly suggest that the impaired response of the DN22-CB1-KI mice reflect a specific alteration of odor aversion memory processes, rather than basal perceptual dysfunctions.

Whether mtCB1 receptors are involved during the formation, consolidation or retrieval of the odor memory will need to be further investigated, as the spectrum of CB1 receptor involvement in different phases of learning and memory is wide (Busquets-Garcia *et al.*, 2018b; Oliveira da Cruz *et al.*, 2020; Terral *et al.*, 2019) (Marsicano and Lafenêtre, 2009).

In rodents, the formation of long-term conditioned odor aversion depends to a substantial degree on olfactory regions (Kobayakawa *et al.*, 2007; Li and Liberles, 2015). An important processing station in the olfactory pathway, the anterior olfactory nucleus (AON), is also an important region for odor learning, as well as for storing odor memory representations (Aqrabawi and Kim, 2020; Levinson *et al.*, 2020). The AON controls odor information through bidirectional projections towards the olfactory bulb and the piriform cortex (Russo *et al.*, 2020). In the AON, CB1 receptors are highly expressed in GABAergic interneurons, whereas the majority of pyramidal glutamatergic neurons display moderate-to-low levels of CB1 mRNA (Marsicano and Lutz, 1999; Monory *et al.*, 2006). CB1 receptors have been

specifically described in glutamatergic corticofugal fibers (CFF) coming from the AON and targeting granule cells (GCs) of the OB (Soria-Gomez *et al.*, 2014). Interestingly, previous results have suggested the involvement of CB1 receptors in the AON in direct odor learning (unpublished data). Similarly, experiments using conditional mutant mice lacking CB1 receptors from cortical glutamatergic neurons (Bellocchio *et al.*, 2010; Monory *et al.*, 2006) showed a specific impairment of conditioned odor aversion, but no impairment in conditioned taste aversion in the same paradigm (unpublished data). These experimental observations shape the hypothesis that mtCB1 receptors in the pyramidal glutamatergic neurons of the AON could be specifically controlling odor aversion memory in mice. However, this hypothesis remains to be directly tested and it constitutes one of the perspectives of future studies on mtCB1 receptor's cellular and behavioral functions.

3 General Discussion

In the third and final section of this chapter, I will address and discuss some prevailing overarching themes that are observed throughout my thesis work including some of the potential caveats and limitations. I will close this chapter with an overview of the general impact of this doctoral study, for basic neuroscience research and potentially future translational approaches.

3.1 Sex-dependent differences in cannabinoid effects

Although the importance of accounting for sex as a biological variable in neuroscience research is now evident (Cahill, 2006), in many studies there is still a substantial bias to not include female rodents or to not report the sex of the subjects (Flórez-Vargas et al., 2016; Shansky and Murphy, 2021). This often lurks the risk of results obtained using only one sex (usually males) to be broadly generalized to the other sex. In addition, there are studies where data from both sexes are pooled together with important caveats such as that sex-specific effects might be covered and wrong conclusions might be drawn. Not including female rodents has been often justified due to the variable nature of female data caused by hormonal fluctuations associated with the female reproductive cycle. However recent studies have debunked these concerns showing that even when females are used in experiments without taking into consideration the estrous cycle, their data are not more variable than those of males, and this is true from behavioral to electrophysiological, neurochemical, and histological measures (Beery, 2018; Prendergast et al., 2014).

As already described, a big part of my work involved the investigation of cannabinoid effects — either the long-lasting effects of adolescent THC exposure (Chapter IV, Section 1) or the acute effects of the synthetic cannabinoid MMB-Fubinaca (Chapter IV, Section 4)— in both male and female mice.

Research on sex differences in cannabinoid signaling has greatly increased in the last years, and although systematic studies including both biological sexes in humans are still lacking, preclinical studies have provided some evidence on the possible underlying mechanisms for the observed sexual dimorphisms (Craft et al., 2013; Fattore and Fratta, 2010a; Tirado-Muñoz *et al.*, 2020). In the following paragraphs, I will discuss some factors that may underlie the observed sex differences within the framework of my work.

In the first place, it is clear that there is a sex-dependent component in the effects of adolescent THC exposure, both regarding the behavioral effects, as well as different molecular and metabolic parameters. As indicated in the previous section (Section 1 of this chapter), there is a large number of studies describing sexual dimorphisms in adolescent cannabinoid outcomes and it is difficult to establish when exactly these sex differences emerge or through which mechanisms. To start with, the onset of the adolescent period, when the physical markers of puberty typically appear, arguably differs between sexes. Female rodents are thought of reaching puberty around postnatal day (PND) 30–40, while in males it appears later, approximately around PND 40–50 (Schneider, 2008; Vetter-O'Hagen and Spear, 2012). Therefore, the timespan of the adolescent THC exposure (here during mid-adolescence) could interfere with slightly different stages of brain maturation in males and females, which might explain subsequent behavioral differences in adulthood.

Moreover, the endocannabinoid system itself is characterized by several differences between the sexes. For example, differences in CB1 receptor density between male and female rodents may be associated with distinct behavioral phenotypes. In adult rats, males show higher levels of CB1 expression than females in brain regions such as the hippocampus, the prefrontal cortex, the amygdala, the striatum and the cerebellum (Farquhar et al., 2019; Llorente-Berzal *et al.*, 2013; Mateos *et al.*, 2011; Paola Castelli et al., 2014). In turn, female rats have been shown to exhibit a pattern of higher G protein coupling and CB1-mediated G protein activation, at least in the hippocampus (Mateos *et al.*, 2011). Importantly, some of these differences seem to arise during adolescence, with one study showing that adolescent male rats were expressing higher CB1 density when compared with females of the same age (Marco et al., 2009). Similar findings have been observed in the mouse brain, with the expression of CB1 mRNA being higher in males than in females in several brain regions, including the hippocampus, striatum and the orbital, insular, cingulate, piriform and visual cortices, while higher expression in females was observed in the hypothalamus (Liu et al., 2020). Notably, some human *in vivo* findings are

in agreement with rodent studies, showing higher CB1 availability and CB1 binding in males compared to females in multiple brain areas (Laurikainen et al., 2019; Van Laere et al., 2008).

In addition, cannabinoid metabolism can also underlie sex differences in cannabinoid effects. For instance, THC is differently metabolized in males as compared to females. While THC metabolism in male rats results in the formation of 11-hydroxy-THC (11-OH-THC), but also of relatively large amounts of several other, mostly inactive, metabolites, in females 11-OH-THC is the primary metabolite (Narimatsu et al., 1991). Consequently, females reach more elevated brain concentrations of 11-OH-THC than males, even if the brain concentrations of THC are similar (Wiley and Burston, 2014). 11-OH-THC is an active metabolite and therefore can lead to prolonged cannabinoid effects in females versus males (Tseng and Craft, 2001; Tseng et al., 2004). Moreover, adolescent female rats have been shown to exhibit an even more pronounced metabolism of THC to 11-OH-THC compared to adult females, especially after repeated THC administration (Wiley and Burston, 2014). In mice, some early studies showed that 11-OH-THC is also a major metabolite of THC, however, these studies were performed only in male mice (Borys and Karler, 1979; Harvey and Brown, 1991). Therefore, whether similar metabolic sex differences are also present between male and female mice is still unknown.

Lastly, sex hormones seem to also play an important role in cannabinoid sensitivity (Struik et al., 2018). The estrous cycle for example in female rodents has been reported to influence cannabinoid effects (Cooper and Craft, 2018). One reason for that can be that CB1 receptor levels and pattern of expression, as well as its affinity for different cannabinoid ligands, appears to change throughout the cycle (De Fonseca et al., 1994; Liu *et al.*, 2020). Moreover, higher levels and fluctuations during the ovarian cycle have been found for AEA, but not for 2-AG, in the anterior pituitary gland of females (González *et al.*, 2000). Overall, recent findings suggest that hormonal differences can explain some sex-dependent behavioral effects. Thus, it will be interesting to investigate in the future the impact of sexual hormones on the sex-specific effects of cannabinoids.

Besides the sex-dependent effect of adolescent THC exposure, sex differences were also observed in the acute effects of the synthetic cannabinoid MMB-Fubinaca, with our results showing a higher sensitivity for MMB-Fubinaca in male than in female mice, at both the behavioral and the synaptic level. As discussed above, sex differences in the density and function of CB1 receptors in specific brain regions (Farquhar *et al.*, 2019; Rosas et al., 2018) and through specific cell-type mechanisms (de Salas-Quiroga et al., 2020) might explain in part these effects, together with the interaction of sex hormones

with CB1 receptor expression (Rodriguez de Fonseca *et al.*, 1994) and endocannabinoid levels (Bradshaw *et al.*, 2006a). Another possibility explaining sex-biased effects might be related to the sex differences on brain dopaminergic activity, as it is well known that cannabinoids can act on specific cell types of the reward system (Melis and Pistis, 2007; Volkow *et al.*, 2017). In future studies, an extended analysis of potential baseline sex differences on synaptic activity might also prove useful to better understand the mechanisms mediating these cannabinoid-dependent behavioral effects in male and female mice.

To conclude, the discovery of sex-dependent differences in preclinical studies can stimulate future research in order to explore human sex differences regarding cannabis effects. Right now, there is a clear need for more sex-focused studies, both in humans and animals, in order to fill the knowledge gap regarding the influence of sex in cannabinoid effects, especially taking into account the important clinical relevance for cannabis-based therapies and related disorders.

3.2 Limitations in behavioral assessment

Behavioral assessment in rodents is one of the cornerstones of neuroscience research. We have all witnessed how over the last few decades, the automation of many behavioral measurements has provided the accurate tracking of an animal's behavior. This has been revolutionary for many testing setups. Examples include from complex home cage monitoring systems that can continuously measure the activities of a mouse, to elaborate operant conditioning setups and more. Today, technology has moved even further, with supervised and unsupervised machine learning tools enabling the detection and quantification of complex behaviors, with ease and high accuracy, revealing information on subtle behavioral changes, often undetectable by humans (Mathis and Mathis, 2020; von Ziegler *et al.*, 2021).

However, the implementation of these latest tools is still slow and presents some barriers in the neuroscience field as they do not have an easy access and, most of the times, knowledge in computer science is required along with programming skills and/or experience in deep learning architecture (Ching *et al.*, 2018). Evidently, this also applies to the present work, which could have been majorly benefitted by an in-depth behavioral analysis of multiple variables. As I described, the way behavioral responses were evaluated in my PhD thesis projects reflects the more traditional way of measuring behavior until now. Many assessments were based on non-automated scoring of observed behaviors,

like the manual counting of the distance travelled in the open field, explorations times in the social interaction task or alternations between arms explored in the Y-maze. Of course, some behavioral responses entailed higher level of automated analysis than others (e.g., PPI versus locomotion), while others might have not benefitted much from evaluating additional parameters (e.g. conditioned aversions).

Yet, as more efforts are continuously made to lower the entry barrier for these advanced technologies, further discoveries from already obtained data is not impossible. Keeping the example of my thesis work, all behavioral assessments were recorded by a ceiling-mounted camera, which recordings can be used in the future to generate additional data. Even if limitations in format quality and transferability are real, more user-friendly softwares are emerging and computational training is easily attainable, creating an opportunity for future analyses and interpretations.

3.3 Limitations in statistical analysis

When analyzing behavioral (and even molecular) rodent data, several limitations are worth to be briefly discussed. In particular, these include issues regarding small sample sizes, assumptions for the use of appropriate statistical tests, influence of outliers or individual variability in behavioral responses. In this section, I would briefly describe these limitations that can be taken into account for future similar behavioral approaches using animal models.

Relatively small sample sizes is of course one of the most obvious constraints for behavioral experiments, as for both ethical and practical reasons, it is often hard to acquire an ideal sample size. This limitation tends to bring up subsequent statistical issues, since small samples are inherently met with low statistical power. Indeed, in the experiments described in this thesis manuscript no formal power analysis was calculated, and in some of the experiments small group sizes (roughly $n < 10$) was an important concern in the analysis of the obtained data. Therefore, it is very likely that effects of small or moderate sizes—which could potentially be biologically important—are not detected, while a low power also reduces the likelihood of a statistically significant result to reflect a true effect (Button et al., 2013). Another important limitation of small samples is often the inability to verify certain assumptions that different statistical tests require. Most importantly, it is hard to evaluate whether observations belong to a normally distributed population, which is a critical assumption for most parametric tests.

Here, when it was impossible to statistically verify assumptions about normality (i.e. when none of the known normality tests were significant), non-parametric statistical tests were preferred. Finally, with small sample size, the importance of single data points is relatively high, as the influence of a single outlier can drastically affect the observed results. In the results presented here, outliers were removed from the data sets and excluded from analysis, only if they were the result of a measurement error or data entry error, or following statistical tests for outliers (Grubbs' test).

A common mistake I would like to highlight, which is also present in the reporting of some of the results presented in this work, is the treatment of non-significant P-values (e.g. $P=0.06$) as nearly significant, when it is accompanied by an observed trend for a behavioral response. It is true that the use of the term “trend” to describe “almost significant” differences can promote a misunderstanding of P values and be misleading, and this is the reason I clarify its use in this section. In a broader view, the use of P values itself has a long history and debate around its interpretation (Halsey et al., 2015). A concern that is often discussed about the null hypothesis testing and P value approach is that it imposes a binary conclusion about the existence or non-existence of effects. The problem in this case, is that non-significant results might be wrongly interpreted as evidence for the absence of a “real” biological effect of the variables that we are investigating (i.e. negative result). However in reality, a non-significant result might be just a “no result” at all, for which no interpretations can be made until further observations are included. One way to counteract this binary approach, could have been the reporting of effect sizes when appropriate, as effect size is much closer to what we would naturally consider “significant”, as compared to statistical significance (Sullivan and Feinn, 2012).

Moreover, in experimental designs like the one used here, including multiple factors (sex, drug effects, age, diverse behavioral measures), standard ANOVA analyses cannot address the cumulative effects of these factors or the proportion of variance that could be accounted for by each separate variable. This kind of study might therefore highly benefit from statistical approaches that reduce the dimensionality of the final datasets, such as principal component analysis (PCA) and factor analyses (Budaev, 2010; Keeley and McDonald, 2015). Using PCA, future analyses of the collected data can further decipher which variables contribute to individual differences and enhance interpretability.

Lastly, I would like to stress the fact that although classical (frequentist) statistics were chosen for the analysis of the data obtained here, this is not the only option. In fact, Bayesian statistics, when applied to behavioral data, can offer a number of advantages over classical analysis, including an avoidance of

the pitfalls inherent to significance testing based on P-values. However, as Bayesian approaches often require advanced mathematical and computational knowledge in order to be applied, as well as appropriate data sets, they were not considered for the specific objectives of my thesis work.

3.4 Significance and impact

In the last decades, views towards cannabis are continuously changing, with more policies in favor of legalization, and an intense investigation into the medicinal potential of cannabinoid compounds. At the same time studies suggest an increase in cannabis use, accompanied by a reduction in the perception of harm, especially in younger demographics. Consequently, there are increased concerns about possible harms associated with cannabis use, particularly during the vulnerable period of adolescence. Adolescence is a critical period of brain development and is also characterized by behavioral changes that might predispose teenagers to experimentation with substances like cannabis. The association between adolescent cannabis use and potentially persistent negative outcomes has long been the focus of neuroscience research, with numerous studies on its effects on brain maturation and function, cognition, addiction and vulnerability to neuropsychiatric disorders, with psychotic disorders being the most prominent. A variety of factors such as sex, the type of cannabinoid compound, dosing, age span etc. complicate the picture and underscores the need for more preclinical studies. This being said, the present work adds to the existent literature of adolescent cannabinoid effects and creates new questions and directions for future research. Importantly, my work provides a novel physiological mechanism involved in the behavioral effects of adolescent THC, which highlights the emerging role of mitochondrial functions and cellular bioenergetics. This is a first step to a better understanding of how adolescent cannabinoid exposure might affect neural functions through changes in brain cell metabolism. Moreover, as the importance of sex in the different effect of cannabinoids becomes more and more evident, my work adds valuable insights into underlying mechanisms of such differences.

Another critical aspect of my work, that I would like to highlight, is the employment of associative learning paradigms in the study of psychotic-like behavior, related, in the context of the present results, with adolescent THC exposure. The past years have seen a revolution in our understanding of the basic principles underlying associative learning, which has enabled the study of behavioral states that were previously thought from difficult to impossible to study, especially in animal models. The

reconceptualization of psychotic symptoms, like hallucinations and delusions, in terms that are detached from solely humanistic traits, but instead encompass basic processes of the brain, such as perception, learning and predictions, has already offered important insights (Corlett *et al.*, 2019; Corlett and Schoenbaum, 2021; Feeney *et al.*, 2017). And although modern studies of higher-order learning and reality testing tasks in this context are still in their beginning, I expect that some of the findings described here might lead to a broader interest in their use. At the same time, a further exploration of higher-order learning processes can inform about the way the brain constructs complex representations of the external world, in a setting that can be readily translated from animals to humans, and vice versa (Dwyer, 2018; Schmack *et al.*, 2021). For instance, the formation of incidental associations can underlie particular human abilities, such as imagination and creativity, which are all characterized by the ability to assume connections between unrelated phenomena in order to construct new ideas and imagine future scenarios (Schacter *et al.*, 2012; Uddin, 2021). Deconstructing terms like creativity into their basic building blocks will allow basic neuroscience research to advance the understanding of core brain processes.

Finally, the present study demonstrates once more the differential influence of CB1 receptors activity in both physiological and pathological processes. As evidenced before, when it comes to studying CB1 receptors, “the where matters”, with CB1 receptors in different regions, cell populations and intracellular compartments controlling different aspects of animal behavior (Busquets-Garcia *et al.*, 2018a; Busquets-Garcia *et al.*, 2015; Robledo-Menendez *et al.*, 2021; Terral *et al.*, 2020). In the present and previous works, I described evidence of CB1 receptors associated with mitochondrial membranes (mtCB1) in the modulation of odor learning, of astroglial mtCB1 receptors in the modulation of social behavior (Jimenez-Blasco *et al.*, 2020), as well as of hippocampal CB1 receptors, but also CB1 receptors in dopaminergic D1 cells, in the control of indirect, incidental learning (Busquets-Garcia *et al.*, 2018b). Overall, future work on how the endocannabinoid system guides behavior can surely contribute to our knowledge of brain processes and can drive innovative therapeutic strategies for related pathologies.

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Annex I

Glucose metabolism links astroglial mitochondria to cannabinoid effects

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Astrocytes take up glucose from the bloodstream to provide energy to the brain, thereby allowing neuronal activity and behavioural responses^{1–5}. By contrast, astrocytes are under neuronal control through specific neurotransmitter receptors^{5–7}. However, whether the activation of astroglial receptors can directly regulate cellular glucose metabolism to eventually modulate behavioural responses is unclear. Here we show that activation of mouse astroglial type-1 cannabinoid receptors associated with mitochondrial membranes (mtCB₁) hampers the metabolism of glucose and the production of lactate in the brain, resulting in altered neuronal functions and, in turn, impaired behavioural responses in social interaction assays. Specifically, activation of astroglial mtCB₁ receptors reduces the phosphorylation of the mitochondrial complex I subunit NDUFS4, which decreases the stability and activity of complex I. This leads to a reduction in the generation of reactive oxygen species by astrocytes and affects the glycolytic production of lactate through the hypoxia-inducible factor 1 pathway, eventually resulting in neuronal redox stress and impairment of behavioural responses in social interaction assays. Genetic and pharmacological correction of each of these effects abolishes the effect of cannabinoid treatment on the observed behaviour. These findings suggest that mtCB₁ receptor signalling can directly regulate astroglial glucose metabolism to fine-tune neuronal activity and behaviour in mice.

The energy demands of astrocytes are almost exclusively met by glycolysis, whereas those of neurons mainly rely on oxidative phosphorylation (OXPHOS)⁸. In this context, mitochondrial complex I is assembled into supercomplexes to increase OXPHOS efficacy in neurons, whereas the majority of astroglial complex I is free, which results in low OXPHOS efficacy in these cells⁹. Thus, astrocytes produce higher levels of mitochondrial reactive oxygen species (mROS)⁹, which control the hypoxia-inducible factor (HIF) cascade and the expression of key

enzymes for the metabolism of glucose¹⁰. However, the potential effect of this molecular cascade on animal behaviour is unknown.

The type-1 cannabinoid receptor (CB₁) is the main target of Δ^9 -tetrahydrocannabinol (THC)—the psychoactive component of *Cannabis sativa*—and of other (endo)cannabinoids. CB₁ is widely expressed in the brain and regulates several behavioural responses¹¹. Whereas high levels of CB₁ are found in neurons that control many cannabinoid-induced effects, previous studies have underlined that

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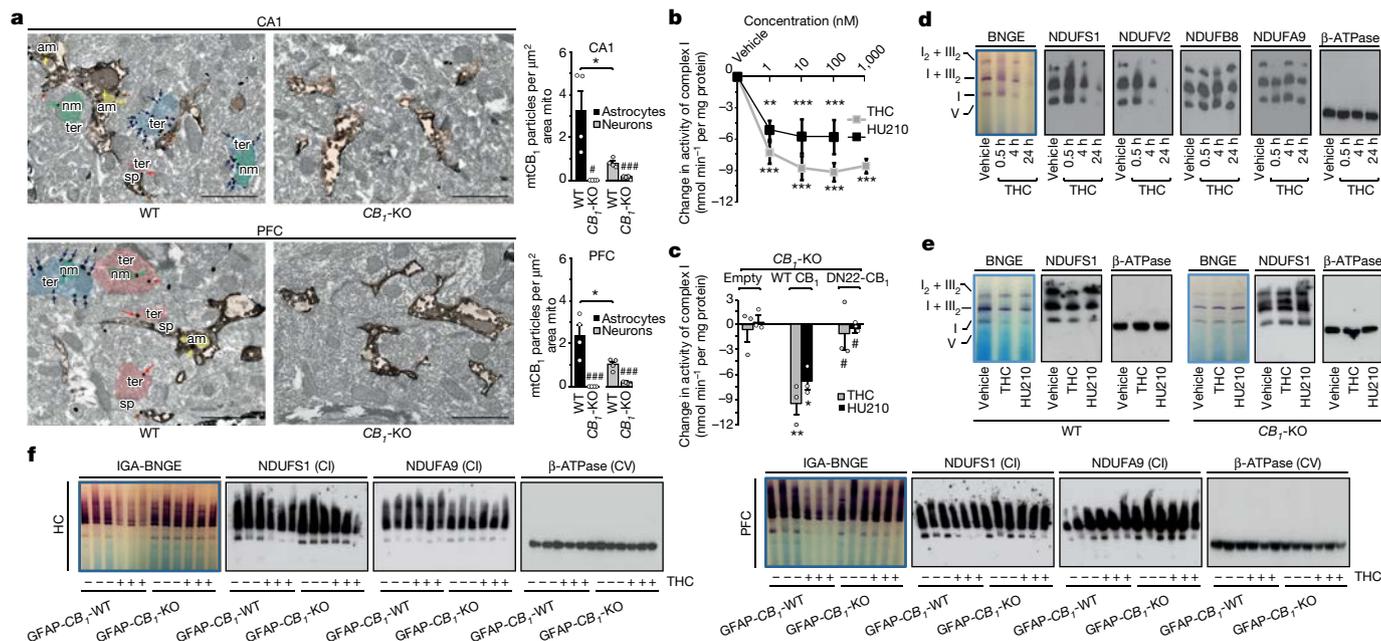


Fig. 1 mtCB₁ activation inhibits the activity of complex I by destabilization of the N-module. **a**, Left, detection of CB₁ receptors on mitochondrial membranes of astroglia and neurons in the hippocampus (CA1 region) and the PFC of wild-type and *CB₁*-KO mice (*n* = 4 mice). am, astrocytic mitochondria; nm, neuronal mitochondria; sp, spine; ter, terminal. CB₁-positive inhibitory terminals are marked in blue, astrocytes in brown, excitatory terminals in red, astrocytic mitochondria in yellow and neuronal mitochondria in green. Asterisks indicate astrocytic processes; coloured arrows point to CB₁ particles at colour-matching subcellular compartments and mitochondria. Scale bars, 1 μm. Right, quantification of mtCB₁ on mitochondrial membranes (mean ± s.e.m.). *P* values by one-way analysis of variance (ANOVA); **P* < 0.05 (wild-type astrocytic versus wild-type neuronal mitochondria); #*P* < 0.05, ###*P* < 0.001 (wild type versus *CB₁*-KO). **b**, Change in the activity of complex I in wild-type astrocytes when treated with THC (1 μM) or HU210 (50 nM). **c**, Change in the activity of complex I in *CB₁*-KO astrocytes that were transfected with empty vector or with plasmids that express wild-type CB₁ or DN22-CB₁, and treated with THC (1 μM) or HU210 (50 nM). Data in **b**, **c** are expressed as the

normalized baseline change in complex I activity (mean ± s.e.m.); **b**, 22.9 ± 1.3 nmol min⁻¹ per mg protein; **c**, 26.6 ± 1.4, 23.7 ± 2.2 and 21.3 ± 2.7 nmol min⁻¹ per mg protein for empty vector, wild-type CB₁ and CB₁-DN22, respectively. *P* values by one-way ANOVA; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (versus vehicle); #*P* < 0.05 (versus wild-type CB₁). *n* = 3 independent experiments (**b**, **c**); Methods. **d**, Effects of THC treatment for the indicated times on the activity of complex I (measured by blue native gel electrophoresis (BNGE)) and the expression of NDUFS1, NDUFB8, NDUFA9 and β-ATPase in wild-type astrocytes. **e**, Effects of THC or HU210 treatment on the activity of complex I (BNGE) and the expression of NDUFS1 and β-ATPase in wild-type or *CB₁*-KO astrocytes. **f**, Effects of THC treatment (10 mg kg⁻¹) on the activity of complex I (measured by in-gel NADH dehydrogenase activity (IGA) after BNGE; IGA-BNGE) and the expression of NDUFS1, NDUFA9 and β-ATPase in the hippocampus (HC) and PFC of GFAP-*CB₁*-WT and GFAP-*CB₁*-KO mice. CI, complex I; CV, complex V. For original gels, see Supplementary Fig. 1; for statistical details, see Supplementary Table 1.

astroglial CB₁ modulates the communication between astrocytes and neurons at the synaptic and behavioural level^{11–13}. The presence of functional mtCB₁ in different tissues, including the brain, underlies the contribution of cannabinoid signalling to bioenergetic processes^{14–19}. By modulating several mitochondrial functions, brain mtCB₁ affects synaptic transmission and behaviour¹⁵. However, whether astroglial mtCB₁ and the associated bioenergetic processes can affect behavioural responses has not yet been investigated.

Astroglial mtCB₁ regulates brain OXPHOS

In addition to the presence of mtCB₁ in neurons^{14,15,18,20}, CB₁ protein was specifically detected in close juxtaposition to astroglial mitochondria in the mouse hippocampus¹⁹, prefrontal cortex, piriform cortex and nucleus accumbens (Fig. 1a, Extended Data Fig. 1a). The cell-permeable CB₁ receptor agonist 1,1-dimethylheptyl-11-hydroxy-Δ⁸-tetrahydrocannabinol (HU210), but not a cell-impermeable biotinylated version of HU210 (HU210-biotin)¹⁴, reduced oxygen consumption in cultured mouse astrocytes (Extended Data Fig. 1b), suggesting that intracellular CB₁ is required for this effect of HU210. Activation of mtCB₁ reduces the mitochondrial activity of soluble adenylyl cyclase and the protein kinase A (PKA)-dependent phosphorylation of OXPHOS proteins¹⁵. Treatment with KH7, an inhibitor of soluble adenylyl cyclase, abolished the effect of HU210 on oxygen

consumption in cultures of mouse astrocytes (Extended Data Fig. 1c). The reduction in respiration that was induced by the CB₁ agonists THC and WIN55,212-2 (WIN) in purified mitochondria from whole brains of wild-type mice^{14,15} was abolished both in *CB₁*-knockout (KO) mice, which lack the *CB₁* gene (also known as *Cnr1*) globally, and in conditional mutant mice that lack *CB₁* specifically in cells (mainly astrocytes) that express glial fibrillary acidic protein (GFAP) (GFAP-*CB₁*-KO mice)²¹ (Extended Data Fig. 1d, e). Thus, astrocytes contain functional mtCB₁ receptors.

MtCB₁ destabilizes complex I

To ascertain whether astroglial mtCB₁ controls mitochondrial bioenergetics, cultured mouse astrocytes were incubated for 24 h with THC or HU210, which reduced the activity of complex I (Fig. 1b), but not that of other OXPHOS complexes or citrate synthase (Extended Data Fig. 2a), in a dose-dependent manner. To investigate the specific involvement of mtCB₁, we transfected astrocytes derived from *CB₁*-KO mice with plasmids that express either wild-type CB₁ or DN22-CB₁—a mutant form of CB₁ that is functional but is excluded from the mitochondria¹⁵. Re-expression of wild-type CB₁, but not DN22-CB₁, rescued the effect of THC and HU210 on the activity of complex I (Fig. 1c). As the structural conformation of complex I is pivotal to its enzymatic activity^{22,23}, we next assessed its stability. Treatment with THC or HU210

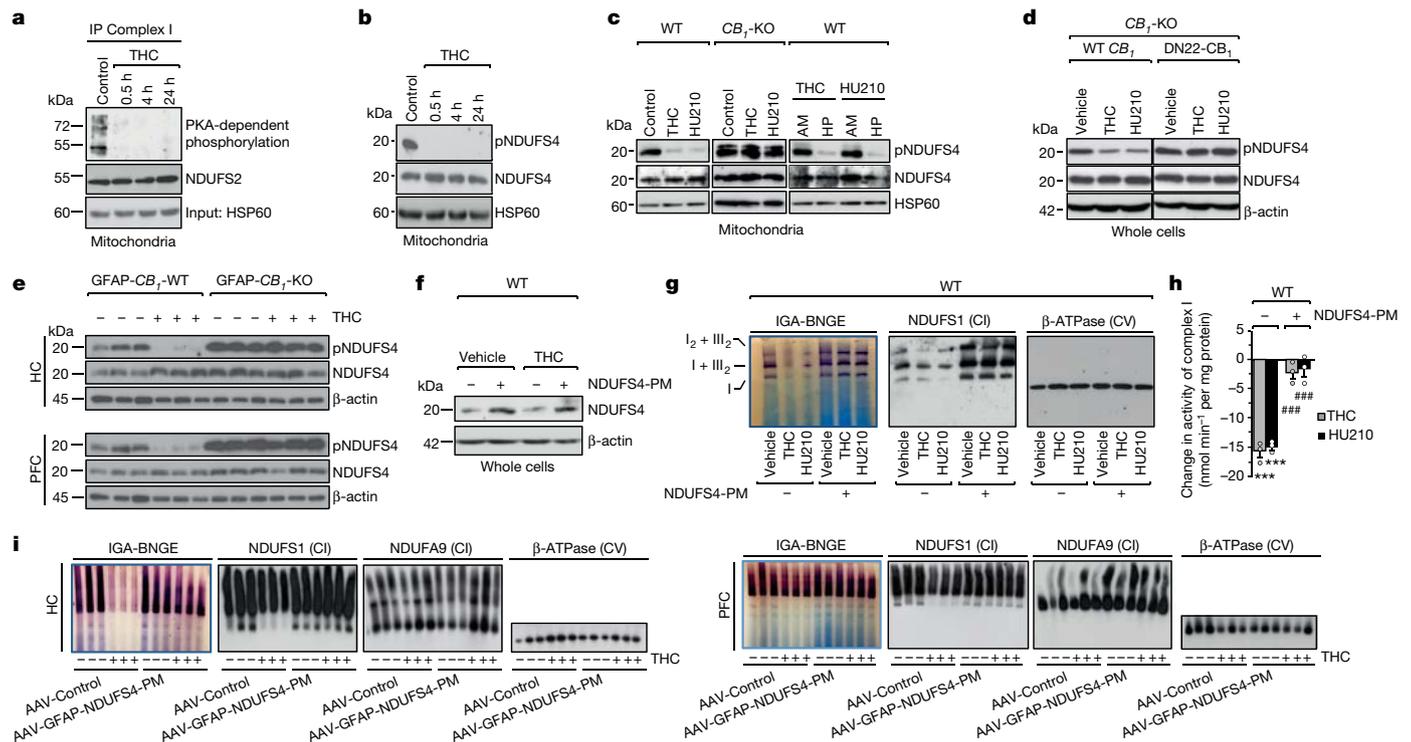


Fig. 2 | Astroglial mtCB₁ activation decreases the level of phosphorylated NDUFS4 and inhibits the activity of complex I. **a, b**, Effects of THC treatment (1 μM) for the indicated times on PKA-mediated phosphorylation and NDUFS2 expression in complex I-immunoprecipitated (IP) proteins (**a**) and on the levels of phosphorylated NDUFS4 (pNDUFS4) (**b**) in isolated mitochondria from wild-type astrocytes. **c**, Effects of THC (1 μM) or HU210 (50 nM) treatment on the levels of pNDUFS4 in mitochondria from wild-type astrocytes (left), *CB₁*-KO astrocytes (middle) or wild-type astrocytes in the presence of 1 μM AM251 (AM) or 10 μM haemopressin (HP) (right). **d**, Effects of THC or HU210 treatment on pNDUFS4 in *CB₁*-KO astrocytes that were transfected with plasmids that express wild-type *CB₁* or DN22-*CB₁*. **e**, Effects of THC treatment (10 mg kg⁻¹) on pNDUFS4 in the hippocampus and PFC of *GFAP-CB₁*-WT and *GFAP-CB₁*-KO mice.

f, Expression of NDUFS4-PM in wild-type astrocytes treated with THC. **g**, Effects of NDUFS4-PM expression on the activity of complex I and the expression of NDUFS1 and β-ATPase in wild-type astrocytes after treatment with vehicle, THC or HU210. **h**, Effects of THC or HU210 treatment on the activity of complex I in astrocytes that were transfected with empty vector or that express NDUFS4-PM. Data are mean ± s.e.m. (*n* = 3 independent experiments). *P* values by one-way ANOVA; *****P* < 0.001 (versus control conditions); ###*P* < 0.001 (versus empty vector). **i**, Effects of NDUFS4-PM expression in astrocytes of the hippocampus and PFC on the THC-induced decrease of complex I activity and NDUFS1 or NDUFA9 expression. HSP60 and β-actin were used as loading controls (**a–f**). For original gels, see Supplementary Fig. 1; for statistical details, see Supplementary Table 1.

reduced the activity of complex I in cultured astrocytes in a time- and *CB₁*-dependent manner, as well as decreasing the protein abundance of NDUFS1 and NDUFS2—which are subunits of the N-module of the matrix arm of complex I^{22,23}—but not that of NDUFB8 and NDUFA9, which are subunits of the inner-membrane-embedded P-module^{22,23} (Fig. 1d, e, Extended Data Fig. 2b–e). Administration of THC (10 mg per kg body weight by intraperitoneal injection) induced a destabilization of complex I in the hippocampus and prefrontal cortex (PFC) of *GFAP-CB₁*-WT mice (that is, wild-type littermates of *GFAP-CB₁*-KO)—but not of *GFAP-CB₁*-KO mice—24 h after injection, with a decrease in the abundance of the N-module NDUFS1 and NDUFS2 subunits but not the P-module NDUFA9 subunit (Fig. 1f, Extended Data Fig. 2f, g). Thus, *in vitro* and *in vivo* pharmacological activation of astroglial *CB₁* receptors causes a selective degradation of the N-module of complex I, which is responsible for the binding and oxidation of NADH(H⁺)^{22,23}.

MtCB₁ reduces the phosphorylation of NDUFS4

We next investigated the signalling mechanism that leads to complex I instability. Incubation with THC rapidly and persistently inhibited PKA-mediated phosphorylation of complex I proteins in cultured astrocytes, without affecting the Q-module NDUFS2 subunit (Fig. 2a, Extended Data Fig. 3a). As phosphorylation of the NDUFS4 subunit at Ser173 (NDUFS4-Ser173) determines the stability and assembly of the N-module of complex I²⁴, we next assessed whether stimulation of the

CB₁ receptor alters this phosphorylation site in astrocytes. Treatment with THC or HU210 decreased the phosphorylation of NDUFS4-Ser173 in astrocytes from wild-type mice, but not *CB₁*-KO mice (Fig. 2b, c, Extended Data Fig. 3b, c). This effect was blocked by the cell-permeable *CB₁* antagonist AM251, but not the cell-impermeable antagonist haemopressin¹⁴ (Fig. 2c, Extended Data Fig. 3c). Re-expression of *CB₁*, but not DN22-*CB₁*, in *CB₁*-KO astrocytes rescued the effects of THC and HU210 on NDUFS4-Ser173 phosphorylation (Fig. 2d, Extended Data Fig. 3d). Both hippocampus and PFC samples of THC-treated *GFAP-CB₁*-WT mice showed a strong reduction of NDUFS4-Ser173 phosphorylation (Fig. 2e, Extended Data Fig. 3e). Basal NDUFS4-Ser173 phosphorylation was higher in *GFAP-CB₁*-KO mice than in *GFAP-CB₁*-WT littermates (Fig. 2e, Extended Data Fig. 3e). However, the effect of THC was abolished in the mutant mice (Fig. 2e, Extended Data Fig. 3e). Thus, NDUFS4-Ser173 dephosphorylation involves the activation of astroglial *CB₁* receptors.

To assess the involvement of NDUFS4-Ser173 phosphorylation in mtCB₁-dependent effects, we generated a phosphomimetic mutant of NDUFS4 in which the Ser173 residue was replaced by a Glu residue (NDUFS4-PM) (Fig. 2f, Extended Data Fig. 3f). Expression of NDUFS4-PM in cultured astrocytes abolished the decrease in NDUFS1 levels and complex I activity that was induced by THC or HU210, without affecting the activity of citrate synthase (Fig. 2g, h, Extended Data Fig. 3g, h). We then stereotactically injected a viral vector that expresses NDUFS4-PM under the control of the human *GFAP* promoter (AAV-gfa-ABC₁D-NDUFS4-PM-IRES-mRuby; here called AAV-GFAP-NDUFS4-PM)²⁵ in both the

hippocampus and the PFC of wild-type mice. The astroglial expression of NDUFS4-PM in both brain regions (Extended Data Fig. 4) totally blocked the selective degradation of the N-module of complex I and its activity induced by intraperitoneal injection of THC (Fig. 2i, Extended Data Fig. 3i). Thus, cannabinoids destabilize astroglial complex I by decreasing the phosphorylation of NDUFS4 in a mtCB₁-dependent manner.

MtCB₁ regulates astroglial mROS

The complex I N-module, which is specifically destabilized by mtCB₁ activation, contains a binding pocket for O₂ molecules that accepts electrons from NADH(H⁺) to generate mROS²³. Notably, a 24-h incubation with THC or HU210 decreased the production of mROS in astrocytes from wild-type mice but not from CB₁-KO mice (Fig. 3a, Extended Data Fig. 5a, b) in a manner that depended on the presence of intracellular CB₁ and mtCB₁ (Fig. 3b, c). Expression of NDUFS4-PM in astrocytes did not alter the levels of mROS per se (Extended Data 5c), but it abolished the decrease in mROS that was induced by THC or HU210 (Fig. 3d). Fluorescence-activated cell sorting (FACS) analysis (Extended Data Fig. 5d) of GFAP-positive cells acutely isolated from the hippocampus and PFC (Extended Data Fig. 5e) revealed that a similar reduction of mROS was induced 24 h after THC treatment (Fig. 3e). Viral intravenous administration²⁶ of a plasmid to express the NDUFS4-PM mutant in a large number of astrocytes did not significantly alter mROS levels in the hippocampus and PFC (Extended Data Fig. 5f), but it abolished the effect of THC (Fig. 3f). Thus, the cannabinoid-induced reduction of astroglial mROS is due to destabilization of the N-module of complex I through reduced phosphorylation of NDUFS4-Ser173.

Effects of MtCB₁ on glycolysis and lactate

In resting conditions, astrocytes produce higher amounts of mROS than do neurons⁹. The transcription factor hypoxia-inducible factor 1 (HIF-1) is known to sense mROS and to adapt the cell metabolism accordingly^{27–30}. Treatment with THC or HU210 decreased, in a CB₁-dependent manner, the nuclear levels of the HIF-1 regulatory subunit HIF-1α, as well as the promoter activity of HIF-1 and the expression of HIF-1 target genes in cultured mouse astrocytes (Fig. 3g, Extended Data Fig. 6a–c). Similarly, THC decreased the expression of HIF-1α in both the hippocampus and the PFC of GFAP-CB₁-WT mice, but not GFAP-CB₁-KO littermates (Fig. 3h, Extended Data Fig. 6d). Astroglial expression of NDUFS4-PM abolished the THC-induced decrease of HIF-1α both in culture (Fig. 3i, Extended Data 6e) and in hippocampus and PFC samples from THC-treated mice (Fig. 3j, Extended Data Fig. 6f). Thus, the mtCB₁-dependent decrease in NDUFS4-Ser173 phosphorylation, the destabilization of the N-module of complex I and the reduced generation of mROS eventually lead to the downregulation of the HIF-1 pathway in astrocytes.

The HIF-1 pathway stimulates glycolysis in cells³¹. Incubation of cultured mouse astrocytes with THC or HU210 for 24 h decreased the release of lactate—which typically reflects glycolytic activity³²—in a dose- and CB₁-dependent way (Fig. 4a, b). THC or HU210 treatment strongly decreased the rate of glycolysis³³ in cultured astrocytes in a mtCB₁-dependent manner (Fig. 4c, d). Expression of NDUFS4-PM, which restored the cannabinoid-mediated reduction of HIF-1, abolished the THC- or HU210-induced decrease of lactate release (Fig. 4e). In addition, overexpression of HIF-1α (Extended Data Fig. 6g, h) blunted the THC- or HU210-induced decrease of lactate release (Fig. 4f). Together, these results indicate that downregulation of the HIF-1 pathway is responsible for the decreased glycolytic activity and lactate release induced by activation of mtCB₁ in astrocytes.

Lactate that is produced by astrocytes is a key energetic support to sustain neuronal activity and functions^{1,34}. Drug-naïve neurons co-cultured for 24 h with astrocytes that were previously treated with

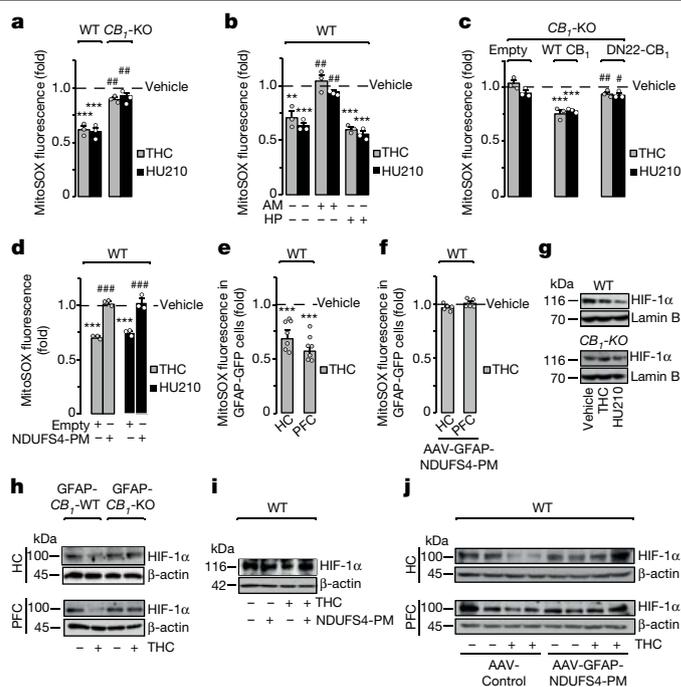


Fig. 3 | MtCB₁-dependent destabilization of complex I reduces levels of mROS and leads to downregulation of the astroglial HIF-1 pathway. **a**, Effects of THC (1 μM) or HU210 (50 nM) treatment on mROS levels (detected using the fluorescent probe MitoSOX) in wild-type and CB₁-KO astrocytes (*n* = 3). *P* values by two-way ANOVA; ****P* < 0.001 (versus vehicle); ##*P* < 0.01 (versus wild type). **b**, Effects of AM251 (1 μM) or haemopressin (10 μM) treatment on the cannabinoid-induced decrease in mROS in wild-type astrocytes (*n* = 3). *P* values by one-way ANOVA; ***P* < 0.01, ****P* < 0.001 (versus vehicle); ##*P* < 0.01 (versus cannabinoid agonist). **c**, Effects of THC or HU210 treatment on mROS levels in CB₁-KO astrocytes that were transfected with empty vector or that express wild-type CB₁ or DN22-CB₁ (*n* = 3). *P* values by two-way ANOVA; ****P* < 0.001 (versus vehicle); **P* < 0.05, ##*P* < 0.01 (versus wild-type CB₁). **d**, Effects of NDUFS4-PM expression on the cannabinoid-induced decrease of mROS in wild-type astrocytes (*n* = 3). *P* values by one-way ANOVA; ****P* < 0.001 (versus vehicle); ###*P* < 0.001 (versus cannabinoid agonist). **e**, Effects of THC treatment (10 mg kg⁻¹) on mROS levels in astrocytes from the hippocampus and PFC (*n* = 7). *P* values by two-sided *t*-test; ****P* < 0.001 (versus vehicle). **f**, Effects of viral expression of NDUFS4-PM by retro-orbital injection on the THC-mediated decrease in mROS in astrocytes from the hippocampus and PFC (*n* = 5). **g**, Effects of THC (1 μM) or HU210 (50 nM) treatment on the nuclear levels of HIF-1α in wild-type and CB₁-KO astrocytes. **h**, Effects of THC treatment (10 mg kg⁻¹) on the levels of HIF-1α in the hippocampus or PFC of GFAP-CB₁-WT and GFAP-CB₁-KO mice. **i**, Effects of astroglial expression of NDUFS4-PM on the THC-induced decrease of HIF-1α in astrocytes. **j**, Effects of astroglial expression of NDUFS4-PM on the THC-induced decrease of HIF-1α in the hippocampus or PFC. In **a–f**, data are mean ± s.e.m. and *n* represents the number of independent experiments. Lamin B (**g**) and β-actin (**h–j**) were used as loading controls. For original gels, see Supplementary Fig. 1; for statistical details, see Supplementary Table 1.

THC or HU210 showed intracellular neuronal bioenergetic and redox stress, as judged by a decrease in mitochondrial membrane potential (Δψ_m) (Extended Data Fig. 7a, b) and an increase in mROS generation (Fig. 4g). Supplementing these co-cultures with lactate protected neurons against cannabinoid-induced stress (Fig. 4h, Extended Data Fig. 7c). FACS analyses (Extended Data Figs. 5d, 7d) revealed that intraperitoneal injection of THC increased the levels of mROS and apoptosis in freshly isolated neurons from the hippocampus and PFC of wild-type mice 24 h after treatment (Fig. 4i, Extended Data Fig. 7e). These effects were rescued by intracerebroventricular (ICV) supplementation of lactate (Fig. 4i, Extended Data Fig. 7e) or by viral expression of NDUFS4-PM in astrocytes (Fig. 4j, Extended Data Fig. 7f–h). Thus, the functional

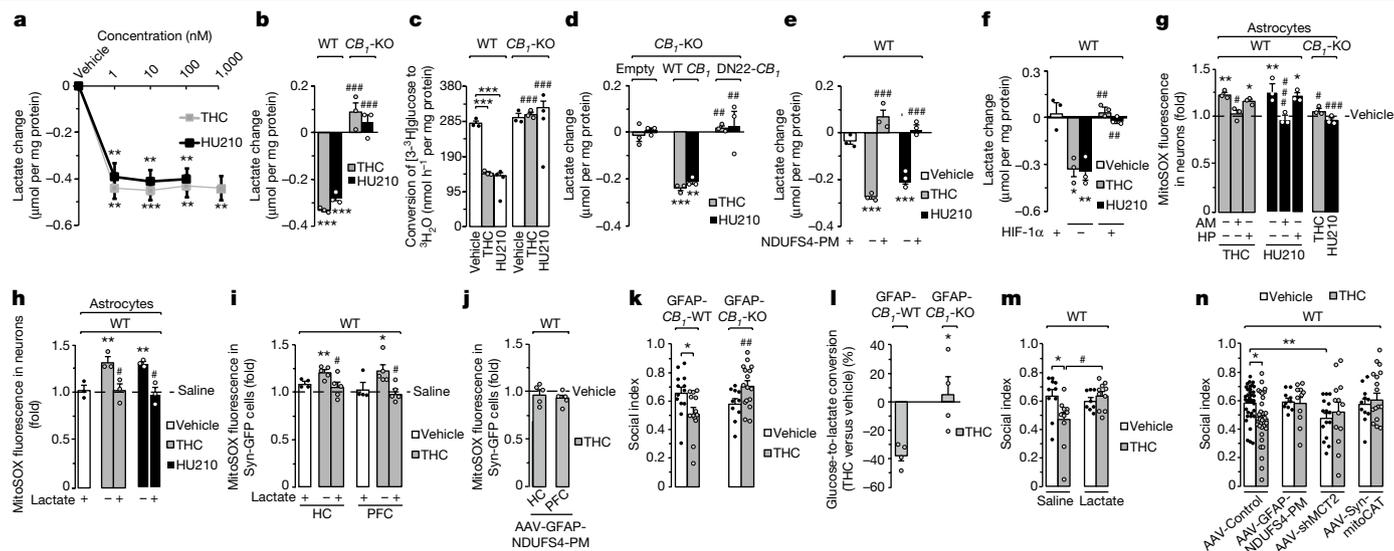


Fig. 4 | Astroglial mtCB₁ activation decreases the production of lactate and mediates the THC-induced impairment of social behaviour. **a**, Change in the production of lactate in cultured wild-type astrocytes after treatment with THC (1 μM) or HU210 (50 nM) (*n* = 3). ***P* < 0.01, ****P* < 0.001 (versus vehicle). **b, c**, Effects of THC or HU210 treatment on lactate production (**b**) and glycolysis (**c**) in wild-type and CB₁-KO astrocytes (*n* = 3). ****P* < 0.001 (versus vehicle); ###*P* < 0.001 (versus wild type). **d**, Change in the production of lactate in CB₁-KO astrocytes that were transfected with empty vector or that express wild-type CB₁ or DN22-CB₁ after treatment with THC or HU210 (*n* = 3). ***P* < 0.01, ****P* < 0.001 (versus vehicle); ###*P* < 0.01 (versus wild-type CB₁). **e, f**, Effects of overexpression of NDUFS4-PM (**e**) or HIF-1α (**f**) on the cannabinoid-induced decrease in lactate release (*n* = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (versus vehicle); #*P* < 0.01, ###*P* < 0.001 (versus empty vector). **g**, Effects of THC or HU210 treatment on mROS levels in neurons that were co-cultured with wild-type or CB₁-KO astrocytes (*n* = 3). **P* < 0.05, ***P* < 0.01 (versus vehicle); #*P* < 0.05, ###*P* < 0.001 (versus cannabinoid). **h**, Effects of lactate supplementation (2 mM) on mROS levels in neurons that were co-cultured with astrocytes pretreated with THC or HU210 (*n* = 3). ***P* < 0.01 (versus saline);

#*P* < 0.05; (versus cannabinoid). **i**, Effects of ICV infusion of lactate (100 mM) on THC-induced increase in mROS in neurons from the hippocampus or PFC (*n* = 5). **P* < 0.05, ***P* < 0.01 (versus saline); #*P* < 0.001 (versus cannabinoid). **j**, Effects of viral expression of NDUFS4-PM by retro-orbital injection on the THC-induced increase in mROS in neurons from the hippocampus or PFC (*n* = 5). **k, l**, Effects of THC treatment (10 mg/kg) on social interaction (**k**) and brain glucose-to-lactate conversion (**l**) in GFAP-CB₁-KO and GFAP-CB₁-WT mice (**k**, GFAP-CB₁-KO vehicle, *n* = 10; GFAP-CB₁-KO THC, *n* = 16; GFAP-CB₁-WT, *n* = 16; **l**, *n* = 4). **P* < 0.05 (versus vehicle or GFAP-CB₁-WT); #*P* < 0.01 (versus wild-type THC). **m**, ICV infusion of lactate blocks THC-induced social impairment (for *n* numbers, see Extended Data Fig. 9). **P* < 0.05 (versus vehicle); #*P* < 0.05 (versus THC saline). **n**, Effects of THC on social interaction in mice that were stereotactically infused with AAV-control, AAV-GFAP-NDUFS4-PM, AAV-shMCT2 or AAV-Syn-MitoCAT (for *n* numbers, see Extended Data Fig. 9). **P* < 0.05; ***P* < 0.01 (versus AAV-control). All data are mean ± s.e.m.; *n* represents the number of independent experiments in **a–j** and the number of mice in **k–n**. *P* values by one-way ANOVA (**a**), two-way ANOVA (**b–k, m, n**) or unpaired two-sided *t*-test (**l**). For statistical details, see Supplementary Table 1.

viability of neurons is affected by the mtCB₁-induced downregulation of the HIF-1 pathway and the consequent reduction of glycolytic activity and lactate release in neighbouring astrocytes.

Astroglial mtCB₁ and social behaviours

To investigate the potential effects of these molecular mechanisms on mouse behaviour, we first tested whether typical short-term behavioural effects of cannabinoids persisted over 24 h after intraperitoneal injection of THC (10 mg per kg body weight). The drug had no persistent effect on prepulse inhibition of startle responses (PPI), locomotion or anxiety-like behaviour (Extended Data Fig. 8a–d), but it led to impairments in spontaneous alternation and social interaction (Fig. 4k, Extended Data Fig. 8e–g, Supplementary Videos 1–10). Notably, only the social interaction deficit was abolished in GFAP-CB₁-KO mice (Fig. 4k, Extended Data Fig. 8e–g). Whereas the rate of conversion of glucose to lactate in the brain did not differ between GFAP-CB₁-WT and GFAP-CB₁-KO littermates (GFAP-CB₁-WT, 50.7 ± 7.2%; GFAP-CB₁-KO mice, 53.1 ± 7.8% (mean ± s.e.m); *P* > 0.9), intraperitoneal injection of THC reduced this value in GFAP-CB₁-WT mice but not in GFAP-CB₁-KO littermates (Fig. 4l, Extended Data Fig. 8h), indicating that cannabinoids decrease lactate conversion via astroglial CB₁. Notably, ICV injection of lactate⁴ abolished the THC-induced social interaction impairments without exerting any significant effect per se on the social behaviour of control mice (Fig. 4m, Extended Data Fig. 9a–e). Astroglial expression of NDUFS4-PM (Extended Data Fig. 4) in the hippocampus and PFC

abolished the THC-induced impairment of social interactions (Fig. 4n, Extended Data Fig. 9f–h). These data suggest that the same mechanisms that lead to reduced lactate supply and the metabolic effects that we observed also underlie THC-induced social effects.

Downstream effects of lactate decrease

In addition to its metabolic roles, lactate can act through the specific G-protein-coupled receptor GPR81 (ref. ³⁵). The ICV infusion of two saturating doses of the GPR81 agonist 3,5-dihydroxybenzoic acid (DHBA)³⁶ before the sociability test did not block the effect of THC on social interactions (Extended Data Fig. 9i–k), which probably excludes the involvement of these receptors. Metabolically, lactate shuttles from astrocytes to neurons through monocarboxylate transporters (MCTs), predominantly neuronal MCT2 (ref. ³⁷). To further investigate the role of lactate in THC-induced impaired social behaviour, we virally expressed a short hairpin RNA (shRNA) that reduces the expression of MCT2 (ref. ³⁸) in the hippocampus and PFC. Decreased MCT2 expression resulted in a decrease in social interactions and thereby occluded the THC effect (Fig. 4n, Extended Data Fig. 9f–h), reinforcing the role of lactate shuttling between astrocytes and neurons in this behaviour.

Next, we investigated whether relieving the astroglial mtCB₁-dependent neuronal bioenergetic stress might compensate for the social effects induced by THC. Local injection of a viral vector that induces the neuronal expression of a mitochondrial form of the ROS scavenger enzyme catalase²⁶ (AAV-Syn-mitoCAT) into the hippocampus

and PFC did not alter social interactions per se, but it abolished the THC-induced impairment (Fig. 4n, Extended Data Figs. 9f–h, 10). Together, these data indicate that excessive mROS production in neurons, induced by an astroglial mtCB₁-dependent decrease of lactate, mediates the cannabinoid-induced impairment of social interactions.

Our data reveal that mitochondrial G-protein-coupled receptors can affect the bioenergetics of specific cell types and determine intercellular metabolic interactions, thereby regulating animal behaviour. Specifically, we show that mtCB₁ regulates astroglial glycolysis and lactate production through a precise molecular cascade to eventually govern the metabolic state of neurons and control social interactions (Extended Data Fig. 11). Metabolic processes are thus markedly different between astrocytes and neurons^{9,26} and they do not merely affect steady-state bioenergetics of the brain, but also have a key signalling function to regulate behavioural responses.

Therefore, the apparently obvious statement that brain metabolism is fundamental for the regulation of behavioural functions actually hides high levels of complexity, owing to the fine heterogeneity of different mechanisms that the brain uses to transform energy^{39,40}. This work establishes a cell-type-specific mechanistic framework that will have to be taken into account in studies of the pharmacological and pathophysiological functions of the endocannabinoid system and of the bioenergetic brain processes that regulate animal behaviour.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-020-2470-y>.

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Methods

Mice

All experimental procedures were approved by the Committee on Animal Health and Care of INSERM and the French Ministry of Agriculture and Forestry (authorization numbers, A501350, A3310035, C33063096) and by the Bioethics Committee of the University of Salamanca in accordance with the Spanish legislation (RD53/2013). Male C57BL/6-N mice purchased from Janvier or bred in the University of Salamanca, *CB₁*-KO male mice bred at the Complutense University of Madrid and male inducible GFAP-*CB₁*-KO mutant mice and their wild-type (GFAP-*CB₁*-WT) littermates²¹ bred at the Neurocentre Magendie were used in this study. As previously shown, to obtain the deletion of the astroglial CB₁ receptor, tamoxifen was daily injected (1 mg/kg, intraperitoneally (i.p.)) for 8 days. Three weeks after the last injection of tamoxifen we performed the *in vivo* experiments in both GFAP-*CB₁*-KO and GFAP-*CB₁*-WT mice. The age of the mice at the beginning of all the behavioural experiments was 8–12 weeks. All experiments were performed during the first part of the light phase (09:00. to 14:00). Experimenters were always blinded to treatments and genotypes.

Reagents

For *in vitro* experiments sodium L-lactate (2 mM; L7022, Sigma-Aldrich) was dissolved in culture medium. THC (1–1,000 nM, THC Pharm), WIN (100 nM, Sigma-Aldrich), HU210 (1–100 nM, Tocris), HU210-biotin²⁴ (1 μM), AM251 (1 μM; ab120088, Abcam), SR141716A (1 μM; 0923, Tocris), haemopressin (5–10 μM; 3791, Tocris) and KH7 (5 μM, Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO; maximum final DMSO concentration was 0.1%). The doses and concentrations of the different reagents were chosen from previous published data^{14,15,20,41} or from preliminary experiments as specified in the main text and/or figure legends.

For *in vivo* administrations, sodium L-lactate (100 mM; 71718, Sigma-Aldrich) and the specific GPR81 agonist 3,5-DHBA (100 nM and 1 mM; 4599, Sigma-Aldrich) were dissolved in saline. THC (10 mg/kg, i.p.) was purchased from THC-Pharm and dissolved in 4% ethanol, 4% Cremophor-EL and 92% saline. THC was injected in a volume of 10 ml/kg.

Virus generation

AAV-gfa-ABC₁D-IRES-mRuby, AAV-hSyn-IRES-mRuby, AAV-gfa-ABC₁D-NDUFS4-PM-IRES-mRuby and AAV-hSyn-MitoCAT-IRES-mRuby viral constructs were generated using standard molecular cloning techniques described for other viral constructs used before in the laboratory^{15,25}. In brief, the coding sequence of NDUFS4-PM²⁴ and MitoCAT²⁶ were cloned into gfa-ABC₁D-GFP (Addgene plasmid #50473) or AAV-hSyn-GFP (Addgene plasmid #50465), respectively. The GFP sequence inside the vector was replaced by NDUFS4-PM or MitoCAT coupled to the IRES-mRuby sequence for correct visualization of transfected cells. Viral particles (AAV8 serotype) were produced by polyethylenimine transfection of HEK293T cells (obtained from ATCC, CRL-3216, lot number #62729596) and subsequent iodixanol density gradient purification as previously described^{15,25}. The AAV2/DJ-CBA-mCherry-miR30E-shMCT2⁴² was provided by L. Pellerin.

Cell cultures and co-cultures

Primary cultures of brain cortical neurons were prepared from C57BL/6 mice of 15.5 days of gestation. Cell suspensions were seeded at 2.5×10^5 cells/cm² in different size plastic plates previously coated with poly-D-lysine (10 μg/ml) and incubated in Neurobasal A (Life Technologies) supplemented with 5.5 mM of glucose, 2 mM of glutamine, 0.25 mM of pyruvate and 2% MAO (Minus Antioxidant) B27 supplement (Life Technologies). Cells were incubated at 37 °C in a humidified 5% CO₂-containing atmosphere. At 72 h after plating, the medium was

replaced and the neurons were used for the experiments on day 7 *in vitro* (DIV).

Primary cultures of brain cortical astrocytes were prepared from either C57BL/6 wild-type or *CB₁*-KO⁴³ neonatal mice of 0–1 days old. Cell suspensions were seeded at 2.5×10^5 cells in 175-cm² plastic flasks and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Non-astroglial cells were detached after 7 DIV by shaking the flasks at 200 rpm overnight and discarding the supernatant. The remaining attached, astrocyte-enriched cells were re-seeded in different size plastic plates and further incubated for 7 DIV for the experiments.

For the astrocyte–neuron co-cultures, astrocytes were re-seeded on inserts (0.4-μm pore size, Millipore Merck) and incubated for 5 DIV in DMEM containing 10% FBS. Then, cells were washed with phosphate-buffered saline (PBS, pH 7.4) and further incubated for 24 h in Neurobasal A containing glucose (5.5 mM), glutamine (2 mM), pyruvate (0.25 mM) and MAO-B27 supplement (2%), either in the absence or in the presence of THC (1 μM) or HU210 (50 nM). After this period, inserts containing the cells were thoroughly washed with PBS, placed on the top of 6 DIV neuronal culture and incubated for 24 h in Neurobasal A containing glucose (5.5 mM), glutamine (2 mM), pyruvate (0.25 mM) and MAO-B27 supplement (2%) in the absence of THC or HU210.

Plasmid construction and site-directed mutagenesis

The N-terminal deletion of the first 22 amino acids (66 base pairs) in the mouse CB₁ receptor coding sequence to obtain the DN22-CB₁ mutant was achieved by polymerase chain reaction (PCR). In brief, for DN22-CB₁ a forward primer hybridizing from the 67th base starting from ATG was coupled to a reverse primer hybridizing to the end of the coding sequence, including the TGA stop codon. To guarantee proper translation of the construct, the forward primer included an ATG codon upstream of the hybridizing sequence. The cDNA for DN22-CB₁ (ref. ¹⁵) was amplified using HF Platinum DNA polymerase (Invitrogen) and inserted into a PCRII-Topo vector (Invitrogen) according to the manufacturer's instructions. The absence of amplification mismatches was then verified by DNA sequencing. The primers used were: forward 5'-**ATGGTGGGCTCAAATGACATTCAG**-3' (in bold the inserted ATG), reverse 5'-**TCACAGAGCCTCGGCAGACGTG**-3' (stop codon in bold). The cDNA sequence for CB₁ or DN22-CB₁ was inserted into a modified version of the pcDNA3.1 mammalian expression vector using BamHI-EcoRV according to standard cloning procedures. CB₁ or DN22-CB₁ was fused to GFP by using the pEGFP-N1 vector (Addgene) according to the manufacturer's instructions for control of transfection efficiency.

The phosphomimetic version of NDUFS4 was synthesized from the full-length human NDUFS4 sequence (provided by S. Papa)²⁴. The NDUFS4 cDNA constructs were engineered to contain 5' HindIII and 3' XhoI and cloned in a pcDNA3.1(+) vector with the T7 promoter. cDNA constructs were then subjected to site-directed mutagenesis on Ser173 (the phosphorylation site of PKA), which was replaced by a glutamic acid residue to obtain the Ser173Glu phosphomimetic version of NDUFS4 (NDUFS4-PM) using the QuikChange XL kit (Stratagene), followed by DpnI digestion. The forward and reverse oligonucleotides designed for this mutation were, 5'-GGAACAAAAGAACAAGAGTAGAAACAAAATAG-3' and 5'-CTATTTGTTTCTACTCTTGTCTTTTGTCC-3' (the Glu codon is indicated in bold).

The full-length cDNA encoding human HIF-1α was a gift from S. Cadenas.

Cell transfections and treatments

All cell transfections with plasmids were performed using Lipofectamine LTX + Reagent Plus (Invitrogen) following the manufacturer's instructions. Astrocytes were transfected with 0.16–1.6 μg/ml of the plasmids and, after 1 h, cells were incubated with the drugs (THC, HU210, AM251, SR141716A or haemopressin) for another 24 h.

Subcellular fractionation

To fractionate cytosol from nucleus, cells were washed with cold PBS containing 1 mM MgCl₂, collected with cytosolic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, NP-40 0.1%, v/v, 1.5 M sucrose and protease and phosphatase inhibitors cocktail, pH 7.9), triturated with a micropipette to promote cell lysis, left on ice for 30 min and vortexed for 10 s. After checking cell lysis under a light microscope, extracts were centrifuged at 830g for 10 min and the cytosolic fraction (supernatant) was removed and boiled for 5 min. Lysis of the nuclei was performed by resuspending the nuclear pellet in nuclear buffer (50 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl mM, 0.5 mM NaCl, 1 mM EDTA, NP-40 1%, v/v and protease and phosphatase inhibitor cocktail, pH 7.9), triturating with a micropipette, leaving on ice for 2 h, vortexing (10 s), boiling (5 min) and sonicating (5 min).

Blue native gel electrophoresis

To assess the mitochondrial respiratory complexes, digitonin-solubilized mitochondria (10–50 µg) were loaded in NativePAGE Novex 3–12% gels (Life Technologies, New York, USA). After electrophoresis, in-gel NADH dehydrogenase activity (IGA-BNGE) was evaluated⁹ and the individual complex I and complex I-containing supercomplex (SC) bands identified. Individual complex I or complex I-containing SC bands were excised from the gel and denatured in 1% SDS (containing 1% β-mercaptoethanol) for 1 h. The proteins contained in the gel slices were separated electrophoretically, followed by western blotting against specific antibodies. Direct transfer of BNGE was also performed, when indicated, after soaking the gels for 20 min (4 °C) in carbonate buffer (10 mM NaHCO₃, 3 mM Na₂CO₃·10H₂O, pH 9.5–10). Protein transfer to PVDF membranes was carried out at 300 mA and 60 V for 1.5 h at 4 °C in carbonate buffer.

Electron microscopy

Electron microscopy was performed following a published protocol⁴⁴. Coronal hippocampal CA1, prefrontal (prelimbic) cortex (PFC), nucleus accumbens and piriform cortex containing vibrosections of mice were cut at 50 µm and collected in 0.1 M phosphate buffer (PB) (pH 7.4) with 0.1% sodium azide at room temperature. They were transferred and preincubated in a blocking solution of 10% bovine serum albumin (BSA), 0.1% sodium azide and 0.02% saponin prepared in Tris–hydrogen chloride-buffered saline 1× (TBS), pH 7.4 for 30 min at room temperature. Then, the wild-type and *CB₁*-KO tissue sections were incubated with the primary guinea pig polyclonal anti-*CB₁* receptor antibody (2 µg/ml, #CB1-GP-Af530, Frontier Institute; RRID: AB_2571593) and rabbit polyclonal anti-A522 (EAAT1 [GLAST]) antibody (0.3 µg/ml, Ab#314, provided by N. C. Danbolt; RRID: AB_2314561) diluted in 10% BSA/TBS containing 0.1% sodium azide and 0.004% saponin on a shaker for 2 days at 4 °C. Then, the tissue was incubated after several washes in 1% BSA/TBS with the biotinylated anti-rabbit (1:200, BA-1000; RRID: AB_2313606) and the 1.4 nm gold-conjugated anti-guinea pig IgG (Fab' fragment) (1:100, Nanoprobes; goat; #2055) secondary antibodies in 1% BSA/TBS with 0.004% saponin for 3 h at room temperature, washed in 1% BSA/TBS and subsequently incubated in the avidin–biotin complex (1:50; PK-7100, Vector Labs) diluted in the washing solution for 1.5 h. After washing the sections in 1% BSA/TBS overnight at 4 °C, they were post-fixed with 1% glutaraldehyde in TBS for 10 min and washed in double-distilled water. Then, the gold particles were silver-intensified with a HQ Silver kit (Nanoprobes) for about 12 min in the dark, washed in 0.1 M PB (pH 7.4) and immediately afterwards incubated in 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide prepared in 0.1 M PB for 3 min. Finally, the sections were osmicated (1% osmium tetroxide, in 0.1 M PB pH 7.4) for 20 min, washed in 0.1 M PB (pH 7.4), dehydrated in graded alcohols (50–100%) to propylene oxide and plastic-embedded in Epon resin 812. Fifty-nanometre ultrathin sections were cut with a diamond knife (Diatome USA), collected on nickel mesh grids, stained

with 2.5% lead citrate and examined with a JEOL JEM 1400 Plus electron microscope. Tissue preparations were photographed using a digital camera coupled to the electron microscope. Minor adjustments in contrast and brightness were made to the figures using Adobe Photoshop (Adobe Systems).

Semi-quantification of the *CB₁* receptor immunogold and immunoperoxidase staining. The pre-embedding immunogold and immunoperoxidase methods were simultaneously applied and repeated three times on the sections obtained from each of the three individual wild-type and *CB₁*-KO mice studied⁴⁴. Immunogold-labelled resin-embedded sections were first visualized under the light microscope to select portions with good and reproducible *CB₁* receptor immunolabelling.

Then, three to four semi-thin sections (1-µm thick) were cut with a histo diamond knife (Diatome USA) and stained with 1% toluidine blue. To further standardize the conditions, only the first 20 ultrathin sections (50-nm thick) were cut and collected onto the grids. We randomly took 4 × 4 assemblies of 16 electron micrographs (×8,000) from each brain region (1,000 µm²), of the wild-type and *CB₁*-KO mice. Sampling was always carefully and accurately carried out in the same way for all the mice studied and experimenters were blinded to the samples during *CB₁* receptor quantification.

Positive EAAT1 (GLAST) astrocytic processes were identified by the presence of DAB immunodeposits. Positive *CB₁* receptor labelling was considered if at least one immunoparticle was within around 30 nm from the outer mitochondrial membranes. Furthermore, only particles on mitochondrial membrane segments far away from other astrocytic membranes (distance ≥ 80 nm) and well distinct from the astrocytic intermediate filaments or any other intracellular organelle membranes were considered^{20,44}. *CB₁* receptor particles per area of astrocytic or neuronal mitochondria were counted in wild-type and *CB₁*-KO mice. A one-way ANOVA was used for comparisons between cell types and *t*-test analysis was used to compare genotypes.

Immunoblotting

Primary antibodies for western blotting. Immunoblotting was performed using mouse monoclonal anti-lamin B (1:100) (sc374015, Santa Cruz Biotechnologies), mouse monoclonal anti-β-actin (1:30,000) (A5441, Sigma-Aldrich), rabbit polyclonal anti-Hsp60 (1:1,000) (ab46798, Abcam), mouse monoclonal anti-β-ATP (1:1,000) (MS503, MitoSciences), mouse monoclonal anti-HIF-1α (MAB1536, R&D Systems), mouse monoclonal anti-complex I immunocapture (1:500) (ab109798, Abcam), rabbit polyclonal anti-phospho-(Ser/Thr) PKA substrate (1:1,000) (9621, Cell Signalling), goat polyclonal anti-NDUFS1 (1:500) (sc-50132, Santa Cruz Biotechnology), rabbit monoclonal anti-NDUFV2 (1:1,000) (ab183715, Abcam), mouse monoclonal anti-NDUFA9 (1:1,000) (ab14713, Abcam), mouse monoclonal anti-NDUFS2 (1:800) (ab110249, Abcam), mouse monoclonal anti-NDUF8 (1:500) (ab110242, Abcam), rabbit polyclonal anti-phospho-Ser173 NDUFS4 (1:100) (provided by D. De Rasmio)⁴⁵ and mouse monoclonal NDUFS4 (1:800) (ab87399, Abcam).

Secondary antibodies for western blotting. Immunoblotting was performed using horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG or rabbit anti-goat IgG (1:10,000; Santa Cruz Biotechnologies).

Determination of protein concentrations

Protein concentrations were determined in the cell suspensions, lysates or in parallel cell culture incubations after solubilization with 0.1 M NaOH. Protein concentrations were determined with the BCA protein assay kit (Pierce) following the manufacturer's instructions, using BSA as standard.

Western blotting. Cells and subcellular fractions or brain tissues (prefrontal cortex and hippocampus) from GFAP-*CB₁*-WT and GFAP-*CB₁*-KO

were lysed in RIPA buffer (2% sodium dodecylsulfate, 2 mM EDTA, 2 mM EGTA and 50 mM Tris pH 7.5), supplemented with protease and phosphatase inhibitor cocktail (100 μ M phenylmethylsulfonyl fluoride, 50 μ g/ml antipain, 50 μ g/ml pepstatin, 50 μ g/ml amastatin, 50 μ g/ml leupeptin, 50 μ g/ml bestatin, 1 mM orthovanadate, 50 mM NaF and 50 μ g/ml soybean trypsin inhibitor) and boiled for 5 min. Extracts were centrifuged at 13,000g for 5 min at 4 °C, and aliquots of lysates (50 μ g protein, unless otherwise stated) were subjected to SDS-PAGE on a 8, 10 or 12% acrylamide gel (MiniProtean, Bio-Rad) including PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific). The resolved proteins were transferred electrophoretically to nitrocellulose membranes (Hybond-ECL, Amersham Bioscience Europe). Membranes were blocked with 5% (w/v) low-fat milk in 20 mM Tris, 500 mM NaCl and 0.1% (w/v) Tween 20, pH 7.5, for 1 h. After blocking, membranes were immunoblotted with primary antibodies at dilutions ranging from 1:200 to 1:1,000 overnight at 4 °C. After incubation with the secondary antibodies described above (all at 1:10,000 dilution), membranes were immediately incubated with the enhanced chemiluminescence kit WesternBright ECL (Advansta) for 2 min or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) for 5 min, before exposure to Fuji Medical X-Ray film (Fujifilm), and the autoradiograms were scanned. Biologically independent replicates were always performed, and a representative western blot is shown.

Mitochondrial measurements

Mitochondria isolation and solubilization. Purified brain mitochondria were isolated as previously described from GFAP-*CB₁*-KO mice and their GFAP-*CB₁*-WT littermates^{14,15,20,41}. In brief, forebrain tissue was collected and homogenized in isolation buffer (250 mM D⁽⁺⁾-sucrose, 10 mM Tris-base (pH 7.6, HCl), 1 mM EDTA (EDTA), protease inhibitors (Roche), 10 mM Na₃VO₄, 2 mM NaF). Homogenates were centrifuged at 1,500g for 5 min. Then, the supernatant was centrifuged at 12,500g for 5 min and the pellet was kept. The cycle of centrifugation was kept, and the final pellet was added on top of a 12–7% Ficoll400 gradient for ultracentrifugation (100,000g for 1h). The obtained purified brain mitochondria were kept on ice for no more than 3 h.

Mitochondria were obtained from cell cultures according to a previously published protocol⁴⁶. In brief, cells (12 million–100 million) were collected, cell pellets frozen at –80 °C and homogenized (10 strokes) in a glass-teflon Potter-Elvehjem, in Buffer A (saccharose 83 mM; MOPS 10 mM; pH 7.2). The same volume of Buffer B (saccharose 250 mM; MOPS 30 mM) was added to the sample, and the homogenate was centrifuged (1,000g, 5 min) to remove unbroken cells and nuclei. Centrifugation of the supernatant was then performed (12,000g, 2 min) to obtain the mitochondrial fraction, which was washed in Buffer C (saccharose 320 mM; EDTA 1 mM; Tris-HCl 10 mM; pH 7.4). Mitochondria were suspended in Buffer D (6-aminohexanoic acid 1 M; Bis-Tris-HCl 50 mM; pH 7.0). Solubilization of mitochondria was performed with digitonin at 4 g/g (5 min in ice). After a 30 min centrifugation at 13,000g, the supernatant was collected.

Immunoprecipitation of complex I. For immunoprecipitation of complex I, digitonin-solubilized mitochondria (3–10 μ g) were incubated with complex I agarose-conjugated dynabeads (Life Technologies) overnight at 4 °C in an orbital roller. Proteins were eluted from the beads by heating at 70 °C for 10 min in Laemmli's buffer (5% β -mercaptoethanol, 2%; SDS, 0.05%; Bromophenol Blue, 10%; glycerol and 60 mM Tris-HCl). Eluted proteins were separated directly by SDS-PAGE (12% gel). For the input, 3 μ g of mitochondria lysates was loaded. Western blot was performed using anti-phospho-(Ser/Thr) PKA substrate, anti-NDUFS2 and anti-Hsp60 (input) antibodies.

Oxygen consumption of purified brain mitochondria and primary astrocytes. As previously described¹⁵, oxygen consumption of brain mitochondria and intact cells was measured using a Clark-type oxygen

electrode (Hansatech) at 37 °C. Purified mitochondria (75–100 μ g) were suspended in 500 μ l of respiration buffer (75 mM mannitol, 25 mM sucrose, 10 mM KCl, 10 mM Tris-HCl pH 7.4, 50 μ M EDTA). Then, pyruvate (5 mM), malate (2 mM) and ADP (5 mM) were successively added to measure complex I-dependent respiration. Drugs (THC and WIN) were directly added into the chambers. The experiments using primary cultures of brain cortical astrocytes were performed on 2×10^6 cells per ml in growth medium. Intact astrocytes were incubated with drugs (HU210, HU210-biotin, KH7) for 1 h directly in T175-cm² flasks. Astrocytes were then transferred into the chamber and basal respiration was recorded.

Detection of mROS. mROS were detected using the fluorescent probe MitoSOX (Life Technologies). Cells, astrocytes and neurons in primary culture or brain cell suspensions, were incubated, respectively, with 2 or 3 μ M of MitoSOX for 30 min at 37 °C in a 5% CO₂ atmosphere in HBSS buffer (NaCl 134.2 mM; KCl 5.26 mM; KH₂PO₄ 0.43 mM; NaHCO₃ 4.09 mM; Na₂HPO₄·2H₂O 0.33 mM; glucose 5.44 mM; HEPES 20 mM; CaCl₂·2H₂O 4 mM; pH 7.4). Cells were then washed with PBS, and the fluorescence was assessed by a FACSCalibur flow cytometer (15 mW argon ion laser tuned at 488 nm; CellQuest software, Becton Dickinson Biosciences) in arbitrary units. In all cases, antimycin A (10 μ M, Sigma-Aldrich), which increases mROS, was used as a positive control.

Assessing H₂O₂. For H₂O₂ assessments, AmplexRed (Life Technologies) was used. Cells grown on 96-well plates were washed with PBS and incubated in KRPG buffer (NaCl 145 mM; Na₂HPO₄ 5.7 mM; KCl 4.86 mM; CaCl₂ 0.54 mM; MgSO₄ 1.22 mM; glucose 5.5 mM; pH 7.35) in the presence of 9.45 μ M AmplexRed containing 0.1 U/ml of horseradish peroxidase. Luminescence was recorded for 2 h at 30-min intervals using a Varioskan Flash (Thermo Fisher Scientific) spectrofluorometer (excitation 538 nm; emission 604 nm). Slopes were used for calculations of the rates of H₂O₂ formation.

Detection of mitochondrial H₂O₂ with pHyPer-dMito by epifluorescence microscopy. The pHyPer-dMito plasmid (Evrogen) expresses a modified version of the domain OxyR from *Escherichia coli*, which is sensitive to oxidation by H₂O₂. After oxidation, a conformational change renders the protein fluorescent⁴⁷. The excitation wavelength (λ_{exc}) reaches its maximum value at 420 and 500 nm, whereas the emission wavelength (λ_{em}) stays at 516 nm. The production of H₂O₂ by mitochondria in intact cells was assessed using this approach and analysed by epifluorescence microscopy. Astrocytes grown on glass coverslips were transfected with pHyPer-dMito and, after 1 h, treated with THC or HU210 and incubated for a further 24 h. Cells were then fixed with 4% (v/v in PBS) paraformaldehyde for 20 min and incubated with DAPI (30 μ M; Sigma-Aldrich). Coverslips were washed and mounted in SlowFade light antifade reagent (Molecular Probes) on glass slides for fluorescence microphotographs at $\times 40$ magnification using a Nikon Eclipse Ti-E. The fluorescence intensities were quantified in at least five microscopic fields using the ImageJ 1.48V software.

Mitochondrial membrane potential. The mitochondrial membrane potential ($\Delta\psi_m$) was assessed using the MitoProbe DiIC₁ (1,1',3,3,3',3'-hexamethylindodicarbo-cyanine iodide, 50 nM) Assay Kit for flow cytometry (Life Technologies). For this purpose, cells, neurons in primary culture or brain cell suspensions were incubated with the dye at 37 °C for 15 min. $\Delta\psi_m$ values were expressed in arbitrary units. Cells were incubated with CCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; 10 μ M) for 15 min and analysed to define the depolarized value ($0 \Delta\psi_m$).

Specific activity of mitochondrial respiratory complexes. Cells were collected and suspended in PBS (pH 7.0). After three cycles of freezing and thawing, to ensure cellular disruption, complex I, complex

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II–III, complex IV and citrate synthase activities were determined. Rotenone-sensitive complex I (NADH-ubiquinone oxidoreductase) activity⁴⁸ was measured in KH_2PO_4 (20 mM, pH 7.2) in the presence of 8 mM MgCl_2 , 2.5 mg/ml BSA, 0.15 mM NADH and 1 mM KCN. Changes in absorbance at 340 nm (30 °C) ($\epsilon = 6.81 \text{ mM}^{-1} \text{ cm}^{-1}$) were recorded after the addition of 50 μM ubiquinone and 10 μM rotenone. Complex II–III (succinate-cytochrome c oxidoreductase) activity⁴⁹ was determined in the presence of 100 mM phosphate buffer, plus 0.6 mM EDTA(K^+), 2 mM KCN and 200 μM of cytochrome c. Changes in absorbance were recorded (550 nm; 30 °C) ($\epsilon = 19.2 \text{ mM}^{-1} \text{ cm}^{-1}$) after the addition of 20 mM succinate and 10 μM antimycin A. For complex IV (cytochrome c oxidase) activity, the first rate constant (k (min^{-1} per mg protein)) of cytochrome c oxidation was determined⁵⁰ in the presence of 10 mM phosphate buffer and 50 μM of reduced cytochrome c; absorbance was recorded every minute at 550 nm, 30 °C ($\epsilon = 19.2 \text{ mM}^{-1} \text{ cm}^{-1}$). Citrate synthase activity⁵¹ was measured in the presence of 93 mM of Tris-HCl, 0.1% (v/v) triton X-100, 0.2 mM acetyl-CoA and 0.2 mM DTNB; the reaction was started with 0.2 mM of oxaloacetate and the absorbance was recorded at 412 nm (30 °C) ($\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Flow cytometric analysis of apoptotic cell death

Cells, neurons in primary culture or brain cell suspensions were stained with APC-conjugated annexin-V and 7-amino-actinomycin D (7-AAD) (Becton Dickinson Biosciences) in binding buffer (100 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2), according to the manufacturer's instructions, to determine quantitatively the percentage of apoptotic cells by flow cytometry. Cells were analysed, in three replicates per condition, on a FACSCalibur flow cytometer (15 mW argon ion laser tuned at 488 nm; CellQuest software, Becton Dickinson Biosciences). Annexin⁺ and 7-AAD⁻ cells were considered apoptotic. The analyser threshold was adjusted on the flow cytometer channel to exclude most of the subcellular debris to reduce the background noise owing to the neurite disruption during neuronal detaching. Data were expressed as fold change.

Quantitative PCR with reverse transcription analysis

This analysis was performed in total RNA samples, purified from astrocytes using a commercially available kit (Sigma-Aldrich), using the Power SYBR Green RNA-to- C_T 1-Step kit (Applied Biosystems). Reverse transcription was performed for 30 min at 48 °C, and PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C plus 1 min at 55 °C using the following forward and reverse primers, respectively (Thermo Fisher Scientific), 5'-TCTCAGAGCGCTCAAGACAA-3' and 5'-GATGGCACGAACCTGTAGCA-3' (*Hklf*), 5'-CACCTTCTTCAAAGTCCCG-3' and 5'-ATGCTGTCAACTCCACCG-3' (*Glut3*), 5'-GGGTGTGAACCAAGGAAAT-3' and 5'-GACTGTGGTCATGACCCCTT-3' (*Capdh*) and 5'-CAACGTACACTTCATGATG-3' (*Actb*). The mRNA abundance of each transcript was normalized to the β -actin mRNA abundance obtained in the same sample. The relative mRNA levels were calculated using the $\Delta\Delta\text{C}_t$ method, and were expressed as the fold change between sample and calibrator.

Rate of glycolysis and lactate concentration

The glycolytic flux was assessed as previously described³³. In brief, attached intact cells were washed with PBS and incubated in the presence of 5 $\mu\text{Ci/ml}$ of D-[3-³H]glucose in 2 ml of a Krebs–Henseleit buffer (11 mM Na_2HPO_4 , 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.3 mM CaCl_2 ; pH 7.4) supplemented with 5.5 mM D-glucose at 37 °C with shaking in a sealed flask. Before sealing the flask with a rubber cap, a 1.5-ml Eppendorf tube containing 1 ml of water (for ³H₂O trapping) was fixed inside the flask by holding it from the flask tab using a rib, and the flask atmosphere was gassed with a O₂/CO₂ (95/5) mixture for 20 s. Flasks were incubated in the air-thermostated chamber of an orbital shaker (Forma Benchtop Orbital Shaker, Model 420, Thermo Fisher Scientific). In preliminary experiments (data not shown), we observed that ³H₂O collection in the holding tube was linear with time

for up to 120 min; therefore, all remaining incubations were performed for this period. The reactions were stopped by adding 0.2 ml of 20% (v/v) HClO₄ through the rubber cap, and flasks were further incubated for 4 days to allow equilibration of ³H₂O between water and the incubation medium. In preliminary experiments using ³H₂O, we observed that the ³H₂O equilibrated between both compartments was 28% (hence reflecting an actual 70% of recovery efficiency). This efficiency value (28% for ³H₂O), was considered for the calculations. Results were expressed as nmol of D-[3-³H]glucose incorporated into ³H₂O per h and per mg protein (rate of glycolysis). Lactate concentrations were measured in the culture medium spectrophotometrically by determining the increments in absorbance of the samples at 340 nm in a mixture containing 1 mM NAD⁺ and 22.5 U/ml lactate dehydrogenase in 0.25 M glycine/0.5 M hydrazine/1 mM EDTA buffer at pH 9.5³³.

Luciferase assay

To analyse HIF-1 promoter activity, we performed a luciferase assay by using a plasmid vector encoding the luciferase full-length cDNA under the control of a minimal promoter containing three copies of the hypoxia response element (pRE- $\Delta\text{tk-LUC-3xHRE}$, donated by E. Berra.). Wild-type or *CB₁*-KO mice astrocytes were transfected with pRE- $\Delta\text{tk-LUC-3xHRE}$ or with the empty vector at 14 DIV. After 24 h, cells were lysed and luciferase activity was determined using a Luciferase Assay System kit (Promega Biotech Iberica,) following the manufacturer's instructions. Luminescence was assessed in 96-well opaque plates (BD Falcon) using a Varioskan Flash (Thermo Fisher Scientific) spectrofluorometer. Values were normalized to those found in the cells transfected with empty vector. At least five wells were recorded per condition in each experiment ($n = 3$ experiments) and the mean values are shown.

NMR approach

Infusion. Male GFAP-*CB₁*-KO mutant mice and their GFAP-*CB₁*-WT littermates²¹ mice treated with vehicle or THC (10 mg/kg, i.p.) 24 h before, were used for these experiments. The mice were anesthetized with an intraperitoneal injection of a mixture containing ketamine and medetomidine (17%/83%, Virbac/Domitor, 0.2 ml/100 g body weight) before infusion of the labelled substrate in the tail vein for 1 h. The infusate solution contained [¹⁻¹³C]glucose (enriched at 99%, from Euriso-top, 750 mM). The infusate flow was monitored to obtain a time-decreasing exponential from 15 ml/h to 1.23 ml/h during the first 25 min after which the rate was kept unchanged. The blood samples were immediately mixed with 13 μl perchloric acid (11.6 M) and then centrifuged. At the end of the experiment, a sample of blood was removed; mice were rapidly euthanized by cerebral-focused microwaves (5 KW, 1 s, Sacron8000, Sairem), the only way to immediately stop all enzymatic activities and to avoid post-mortem artefacts such as anaerobic lactate production. Their brain was then rapidly removed after cutting the dome of the skull with a microcircular saw and dipped in liquid nitrogen.

Brain perchloric acid extracts. The frozen cerebral tissues were weighted and pulverized under liquid nitrogen with a mortar and pestle. A volume of 2 ml of 0.9 M perchloric acid was then added drop by drop at the nitrogen surface and the frozen droplets immediately pulverized. The mixture was transferred to a Dounce's homogenizer and homogenized at 4 °C after thawing. The suspension was then centrifuged at 4000g for 10 min. The supernatant was neutralized with KOH, centrifuged to eliminate perchlorate salts and freeze-dried. For nuclear magnetic resonance (NMR) spectroscopy, each sample was dissolved in 700 μl D₂O.

NMR spectroscopy. ¹H-NMR spectroscopy was conducted on a Bruker DPX500 wide-bore spectrometer. Spectra were acquired at 4 °C and the 90° flip angle was measured for each sample. Used parameters were: 8 s relaxation delay, 5,000 Hz sweep width and 32 K memory size, water

suppression (homonuclear presaturation). The carbon-13 specific enrichment (^{13}C -SE) of carbon position 3 for lactate (^{13}C -SE lactate C3) and that of carbon position 1 for glucose (^{13}C -SE glucose C1) were calculated based on the satellite peak areas resulting from the heteronuclear spin-coupling patterns on spectra (sera and brain samples).

Surgical procedures

Surgery for cannula implantation. Stereotaxical surgeries, performed as previously described^{15,52}, were aimed at implanting guide cannulas (Plastics One) targeting the lateral ventricles (ICV) with the following coordinates: antero-posterior (AP, -0.2), medio-lateral (ML, +/- 0.9) and dorso-ventral (DV, -2). Lactate or saline were injected using a peristaltic pump (PHD 22/2000 Syringe Pump Infusion, Harvard Apparatus, flow rate: 0.5 $\mu\text{l}/\text{min}$). The volume injected for ICV injections was 2 μl .

Surgery for viral administration. Stereotaxical surgeries, performed as previously described^{15,53}, were aimed for viral infusions in the HC and PFC. AAV vectors were injected with the help of a microsyringe attached to a pump (UMP3-1, World Precision Instruments). Mice were injected with AAV-gfa-ABC₁D-IRES-mRuby, AAV-hSyn-IRES-mRuby or AAV2/DJ-CBA-mCherry (control), AAV-gfa-ABC₁D-NDUF S4-PM-IRES-mRuby, AAV2/DJ-CBA-mCherry-miR30e-shMCT2 and AAV-hSyn-MitoCAT-IRES-mRuby viral vectors directly into the prefrontal cortex (2 injections of 1 μl per side), with the following coordinates: AP +2.9 (1st injection) and +2 (2nd injection), ML +/-1.3 (1st injection) and +/-1.5 (2nd injection), DV -2; and the hippocampus (2 injections of 1 μl per side), with the following coordinates: AP -2, ML +/-1.5, DV -2 (1st injection) and -1.5 (2nd injection), according to Paxinos and Franklin⁵⁴. Mice were used for the social interaction approach five weeks after injections for optimal expression of the viruses. mCherry or mRuby expression was checked by epifluorescence (see below) in mice used in the behavioural experiments as shown by representative images in Extended Data Figs. 4, 10c, 11. Despite the low expression of GFAP in neocortical areas⁵⁵, the use of the reduced promoter gfa-ABC₁D allowed good astroglial expression of the viruses in both HC and PFC.

Intravenous injections of AAVs. We used an adeno-associated virus (AAV) infection approach to distinguish adult astrocytes in vivo. To do this, we used the AAV-PHP.eB capsid, which efficiently transduces the central nervous system, containing GFP under the control of the short astrocyte-specific GFAP promoter (gfa-ABC₁D-GFP or gfa-ABC₁D-NDUF S4-PM) or neuron-specific synapsin promoter (hSyn-GFP). Mice were briefly anesthetized with sevoflurane (Sevorane, AbbVie) at 6% for initiation followed by around 3% for maintenance in air with supplement O₂ and NO₂ (0.4 and 0.8 l/minute, respectively) using a gas distribution column (Hersill H-3, Spain) and a vaporizer (InterMed Penlons Sigma Delta). A 50- μl aliquot of a PBS solution containing 0.1% Pluronic F-127 (Sigma-Aldrich) and AAV-PHP.eB-gfa-ABC₁D-GFP (5×10^{10} vector genomes, vg) or AAV-PHP.eB-hSyn-GFP (2×10^{10} vg) or AAV-PHP.eB-gfa-ABC₁D-NDUF S4-PM (5×10^{10} vg) was administered in the venous sinus by retro-orbital injection. The total duration of the procedure per mouse was less than 10 min, including anaesthesia and recovery. Three weeks after infection, brains were extracted and a single-cell suspension was achieved by trypsinization and smooth mechanical resuspension⁹. Despite the low expression of GFAP in neocortical areas⁵⁵, the use of the reduced gfa-ABC₁D promoter allowed good astroglial expression of the viruses in both HC and PFC. The cell suspension was incubated with different probes as described above, to assess mROS, mitochondrial membrane potential and apoptosis in GFP⁺ cells by flow cytometry.

To show representative images of this approach, three weeks following retro-orbital injection of AAVs PHP.eB-gfaABC1D-GFP or PHP.eB-hSyn-GFP, mice were euthanized by an overdose of pentobarbital and perfused transcardially with 100 ml of cold 4% paraformaldehyde (PFA) solution in phosphate buffer (PB, 0.1 M, pH 7.4). The brains were

cryoprotected in a 30% sucrose solution in PB for 24 h. Sagittal brain sections (35 μm) were cut on a freezing microtome, serially collected and stored at -20 °C until analysis. The slices were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) and blocked with 4.5% normal goat serum (Sigma-Aldrich) for 30 min and were incubated overnight at room temperature with the following primary antibodies: Phgdh (1:500, rabbit; Frontier Institute) or NeuN (1:500, mouse; Chemicon). After rinsing, the brain slices were incubated for 3 h at room temperature with fluorescent secondary 594 Alexa Fluor-conjugated antibody (Invitrogen) and mounted with FluorSave reagent (Calbiochem). Images were acquired by confocal microscopy (Leica SP8).

Immunohistochemistry and fluorescence detection

After the behavioural experiment, mice were anesthetized with chloral hydrate (400 mg/kg body weight), transcardially perfused first with phosphate-buffered solution (PB 0.1 M, pH 7.4) and then with 50 ml of 4% formaldehyde to fix tissues. After brain extraction, brain was embedded with sucrose 30% for 3 days, frozen and kept at -80 °C. Serial brain coronal cryosections were cut at 40 μm . Hippocampus and PFC sections were pre-incubated in a blocking solution of 10% donkey serum, 0.02% sodium azide and 0.3% Triton X-100 prepared in 0.1 M PB for 1 h at room temperature. All the antibodies were prepared in the blocking solution.

Staining of S100 β -mRuby. Free-floating sections were blocked with 3% H₂O₂ for 30 min and then incubated with a mix of primary antibodies: mouse monoclonal S100 β (1:1,000, Sigma-Aldrich) and rabbit polyclonal DsRed (1:1,000, Takara Bio) overnight at 4 °C. After several washes, the slices were placed for 2 h with a mix of secondary antibodies: HRP-anti mouse (1:500, Cell Signaling) and anti-rabbit Alexa Fluor 594 (1:500, Thermo Fisher Scientific). Sections were incubated with TSA FITC (1:300, 10 min, Perkin Elmer), washed, mounted, dried and coverslipped. The images were taken and analysed with an epifluorescence Leica DM6000 microscope (Leica, France).

Staining of NeuN-mRuby. Free-floating sections were incubated with a mix of primary antibodies: mouse NeuN (1:500, Sigma-Aldrich) and rabbit polyclonal DsRed (1:1,000, Takara Bio) overnight at 4 °C. After several washes, the slices were incubated for 2 h with a mix of secondary antibodies: anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (1:500, Thermo Fisher Scientific). Sections were washed, mounted, dried and coverslipped. The images were taken and analysed with an epifluorescence Leica DM6000 microscope (Leica, France).

Behavioural procedures

Prepulse inhibition. Prepulse inhibition (PPI) was measured in a startle chamber (SR-Lab San Diego Instruments) as described previously in the laboratory⁵². Male mice were placed in the startle chamber and a 70-dB background noise was presented during a 5-min acclimation period. The PPI session consisted of randomly presented 100 trials: a 120-dB noise trial presented alone (Startle, S), no stimulus trial, prepulse 73-dB trial, prepulse 76-dB trial, prepulse 82-dB trial, prepulse 73-dB + pulse 120-dB, prepulse 76-dB + pulse 120-dB, prepulse 82-dB + pulse 120-dB. The intervals between single trials were randomized between 10 and 30 s. The 100-ms response after the presentation of the 120-dB pulse was analysed by the PPI setting and we used the maximal response peak to calculate the PPI (% PPI = 100 \times (Startle Alone - Prepulse + Startle)/ Startle Alone). THC administration (10 mg/kg, i.p.) was performed 24 h before starting the PPI experiment.

Two-chamber social interaction test. As previously described⁵², male mice were tested in an open field (35 \times 35 cm) arena with two plastic containers (plastic cylinders of 8-cm diameter with holes for odour interaction) in two opposite corners, one of them hosting a mouse (8-10-week-old adult male C57BL/6-N) and the other container

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remaining empty. In each corner we defined the 'social' and 'non-social' zones as an 8-cm area surrounding the containers. For each experimental group, mice were put in the middle of the open field for 5 min and the position of the container with the mouse was counterbalanced. A ceiling-mounted camera recorded mouse movements. Male mice were used and THC (10 mg/kg, i.p.) or its vehicle were administered 24 h before the task. A social interaction index was calculated as time spent in the social zone divided by the total time spent in both zones⁵².

Direct social interaction test. Cannulated adult male mice were tested in 35.5 × 23.5 × 19 cm cages at 20 ± 2 lux with fresh bedding and video recorded using a Blackfly S digital camera. Each subject mouse was matched with a stimulus mouse in age and weight, which was habituated to the testing cage for 10 min the day prior. Subject mice were injected intraventricularly with 2 µl of saline or lactate (100 mM) solutions in an adjacent room just before the test. The test (adapted from a previous study⁵⁶) consists of 3 trials preceded by a 5-min habituation to the testing cage, during which subject mice were allowed to explore the empty testing cage freely. Immediately afterwards, a stimulus male mouse was introduced in the cage for 1-min direct interaction. After each trial, the stimulus mouse was moved back to its home cage during the 2-min intertrial time. We repeated this for 3 trials with an intertrial time of 2 min. Social behaviours were manually scored offline by a blinded experimenter. Mice that fought in more than one trial were excluded from the analysis. THC (10 mg/kg, i.p.) or its corresponding vehicle were administered 24 h before the task.

Open-field test. Mice were placed in an open field (width, 60 cm; length, 60 cm) the bottom of which was divided into squares. A ceiling-mounted camera recorded mouse movements. Total number of crossed squares and the time spent in the centre of the open field were measured manually during 5 min by the researcher in a different room. Mice were treated with THC (10 mg/kg) or vehicle 24 h before the experiment.

Elevated plus maze. The elevated plus maze consisted of four elevated arms (height, 66 cm; length, 45 cm; width, 10 cm). The arms were arranged in a cross-like configuration, with two opposite arms being enclosed by 50-cm high walls and the two other arms being open. The four arms were connected by a squared central platform (10 cm × 10 cm). Both the central platform and the open arms were under bright illumination (100–120 lux), whereas the closed arms were under weak illumination (30 lux). Each mouse was placed on the central platform, facing an open arm. The time spent on the open arms were recorded for 5 min in mice receiving vehicle or THC (10 mg/kg, i.p.) 24 h before the experiment.

Spontaneous alternation. Spontaneous alternation was assessed in a Y-maze (42 cm long, 8 cm wide, 120° between arms) in a room with 50 lux of light containing a ceiling-mounted camera. Mice were placed in one of the arms for 8 min and scored for each entry when all four paws were placed in one of the arms. GFAP-*CB₁*-WT and GFAP-*CB₁*-KO mice were treated with an injection of THC (10 mg/kg) or vehicle 24 h before entering the Y-maze. The percentage of alternation was calculated as: [number of correct triplets/(total number of arm entries – 2)] × 100.

Statistical analysis

No statistical methods were used to predetermine sample size. All results are expressed as the mean values ± s.e.m. For the electron microscopy experiments, we used ANOVA analysis to compare all experimental groups for each brain region followed by Bonferroni test. For the in vitro experiments, to compare the values between genotypes we used a Student's *t*-test. For the multiple values comparisons, we used a one-way or two-way ANOVA followed by Bonferroni test. For the NMR experiments we used a non-paired *t*-test between the two experimental groups. For the behavioural experiments, we used a

two-way ANOVA followed by Bonferroni test (for example, social index) or paired *t*-test (for example, exploration time) when an interaction was found. A one-way ANOVA was also used to analyse possible differences between control groups. All the statistical analysis was performed using SPSS v.16.0 or GraphPad Prism software for Macintosh. In all cases, *P* < 0.05 was considered significant and the exact *P* value is shown except when the statistical software is just providing a range. When normalization was required in some in vitro measurements, the statistics were performed on the raw data presented in the source data files. Representative immunoblot or immunochemistry images represent experiments repeated at least three times with similar results. All molecular experiments were repeated three to five times and behavioural experiments were also repeated two or three times in independent groups of mice with similar results. Additional statistical details and original gels can be found in the Supplementary Information.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All data generated in this manuscript are included within the paper (and its Supplementary Information files). For any further inquiries about our work please contact the corresponding authors. Source data are provided with this paper.

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Author contributions J.P.B. and G.M. conceptualized and supervised the study. D.J.-B., E.H.-C., C.V.-G., R.S., I.L.-F., M.R.-B., D.S. and A.A. performed and supervised in vitro experiments in cell and astrocyte cultures and ex vivo analysis of brain tissue; A.B.-G., C.I. and P.G.-S. performed behavioural experiments and surgical procedures in mice; E.R. and M.G. provided some CB₁-KO mice to the group of J.P.B.; D.A. and A.P. performed electrophysiological experiments not shown in the manuscript; M.V. and F.J.-K. performed mouse perfusion and immunohistochemistry experiments; A.C. and L.B. produced some of the viral constructs used (for example, Syn-MitoCAT); I.B.-D.R., N.P., S.A. and P.G. performed and supervised electron microscopy experiments; M.-L.L.-R. provided pharmacological tools (HU210-Biotin); C. Jollé, N.D. and L.P. provided specific viral constructs to modulate the MCT2 transporter; C. Josephine and G.B. provided data and viral vectors for mouse retro-orbital injections; B.L. and P.-V.P. provided conceptual ideas; and A.-K.B.-S. performed in vivo NMR experiments. D.J.-B., A.B.-G., E.H.-C., M.G., J.P.B. and G.M. wrote the paper with input from all authors.

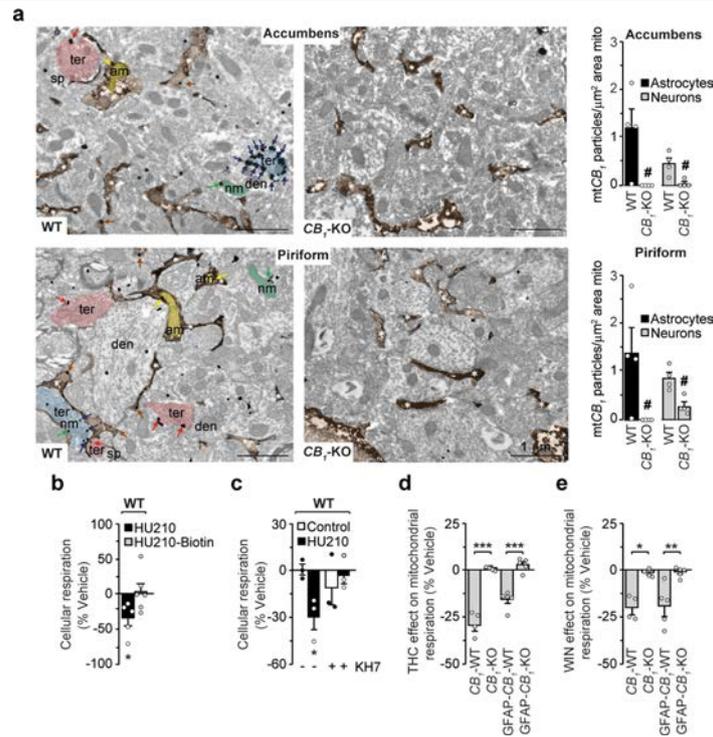
Competing interests The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41586-020-2470-y>.

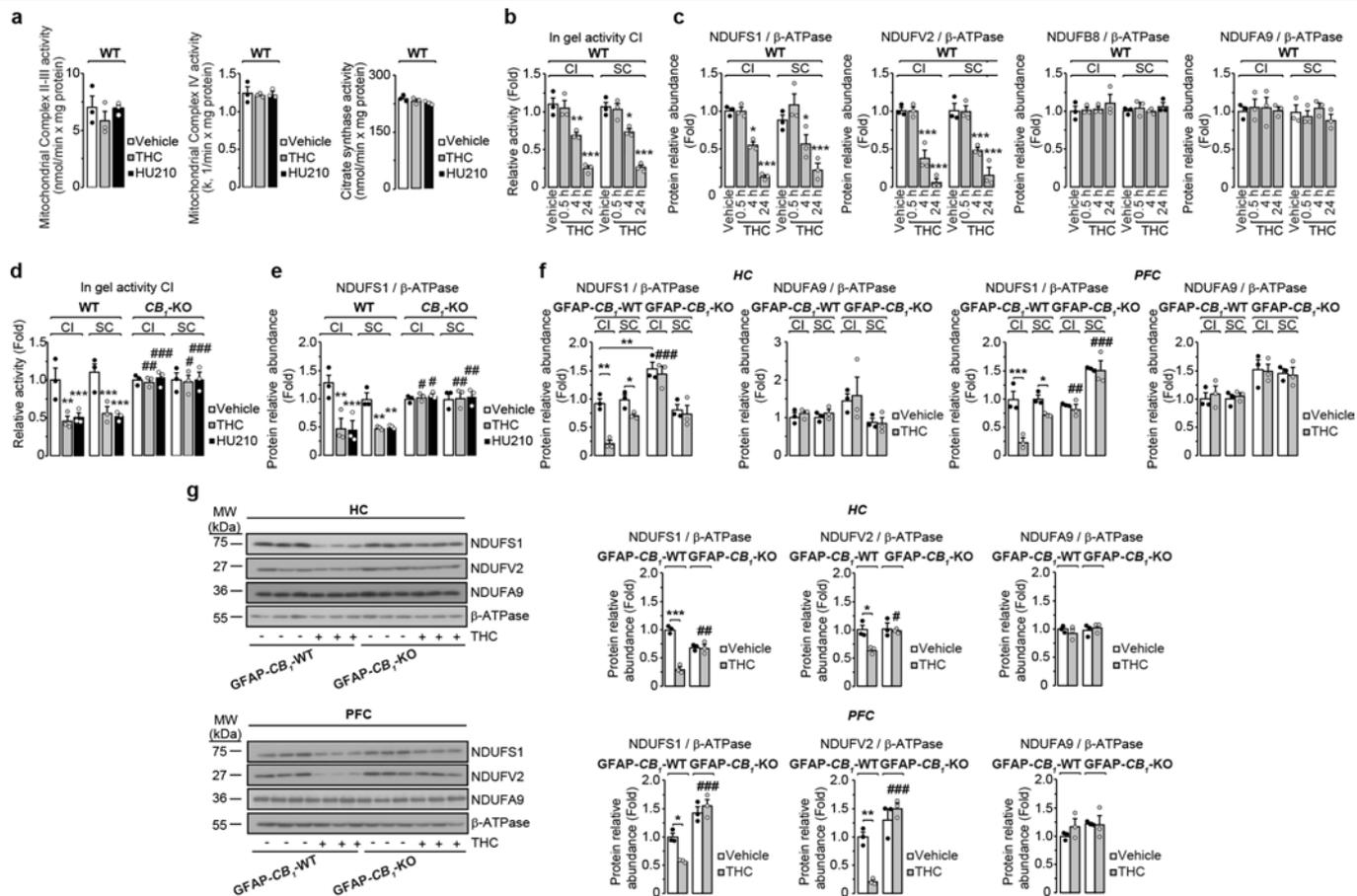
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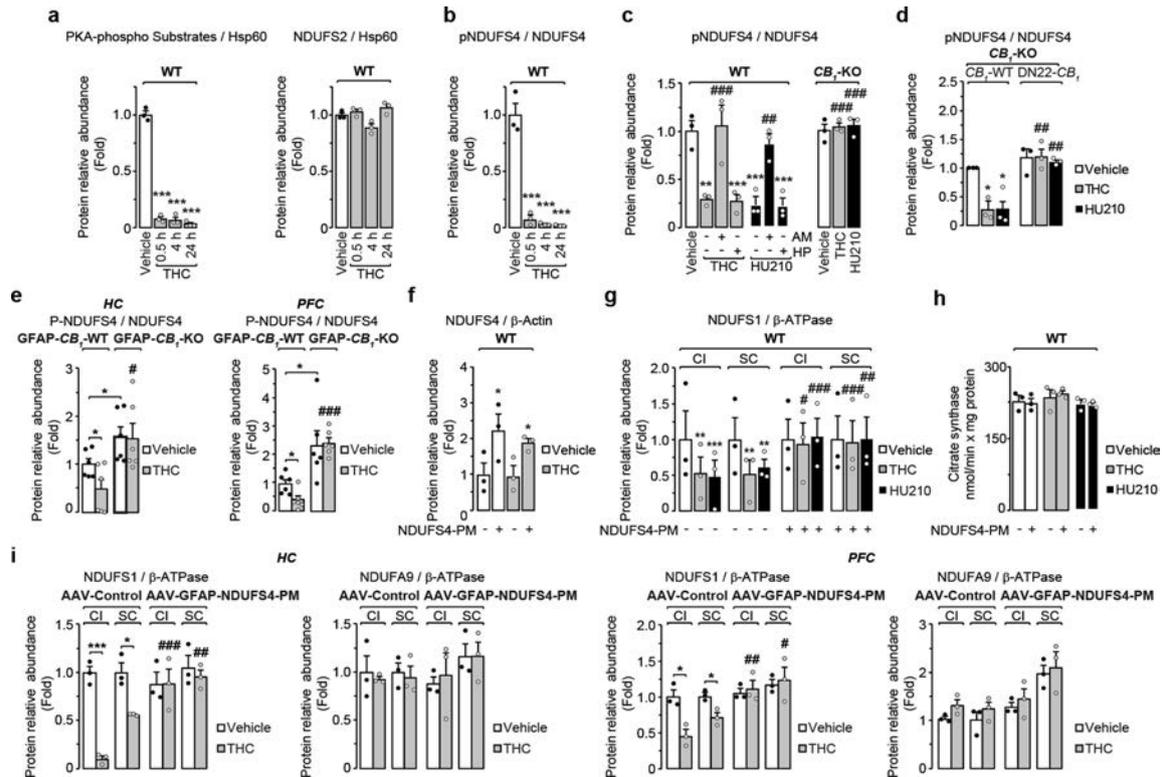
Extended Data Fig. 1 | Role of astroglial mtCB₁ in the cannabinoid-induced decrease of respiration in forebrain mitochondria. **a**, Detection of CB₁ receptors on astroglial and neuronal mitochondrial membranes in the nucleus accumbens and piriform cortex of wild-type and CB₁-KO mice ($n = 4$). am, astrocytic mitochondria; nm, neuronal mitochondria; sp, spine; ter, terminal. CB₁-positive inhibitory terminals are marked in blue, astrocytes in brown, excitatory terminals in red, astrocytic mitochondria in yellow and neuronal mitochondria in green. Asterisks indicate astrocytic processes; coloured arrows point to CB₁ particles at colour-matching subcellular compartments and mitochondria. Scale bars, 1 μm. #, $P < 0.05$ (vs WT). **b**, Effects of the CB₁ receptor cell permeant and impermeant agonists HU210 (1 μM, $n = 5$) and

HU210-Biotin (1 μM, $n = 6$), respectively, on cellular respiration of primary mouse cortical astrocyte cultures. *, $P < 0.05$ (versus HU210-Biotin). **c**, sAC inhibitor KH7 (5 μM, $n = 3$) effects on the HU210-induced reduction of cellular respiration in astrocyte cultures. *, $P < 0.05$ (versus control). **d**, **e**, THC (800 nM) (**d**) and WIN (100 nM) (**e**) effects on respiration of purified brain mitochondria from CB₁-KO ($n = 4$) and GFAP-CB₁-KO ($n = 5$) mice and their respective WT littermates (CB₁-WT and GFAP-CB₁-WT). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (versus WT conditions). Data are expressed as mean ± s.e.m. and are analysed by one-way ANOVA in **a**, **d** and **e**; two-way ANOVA in **c** and unpaired two-sided Student's *t*-test in **b**. *n* represents number of mice in **a**, **d** and **e** and independent experiments in **b** and **c**. Statistical details, Supplementary Table 2.



Extended Data Fig. 2 | Activation of mtCB₁ receptors inhibits complex I activity by destabilizing the N-module of complex I. **a**, THC (1 μ M) or HU210 (50 nM) effects on complex II–III (CII–III; left), complex IV (CIV; centre) and citrate synthase (right) activities in WT astrocyte cultures ($n = 3$). **b**, **c**, Quantification ($n = 3$) of gels represented in Fig. 1c, reporting the effects of THC on **(b)** mitochondrial complex I (CI) activity and **(c)** expression (normalized to β -ATPase) of the complex I subunits NDUFS1, NDUFV2, NDUFB8 and NDUFA9 in complex I and super-complexes (SC) of cultured astrocytes from WT mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (versus Vehicle). **d**, **e**, Quantification ($n = 3$) of gels represented in Fig. 1d, reporting THC or HU210 effects on **(d)** complex I activity and **(e)** expression (normalized to β -ATPase) of the complex I subunit NDUFS1 in complex I and SC of WT and CB₁-KO mice. **, $P < 0.01$; ***, $P < 0.001$ (versus Vehicle); #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (versus WT). **f**, Relative quantification ($n = 3$) of BNGE represented in Fig. 1e, reporting the

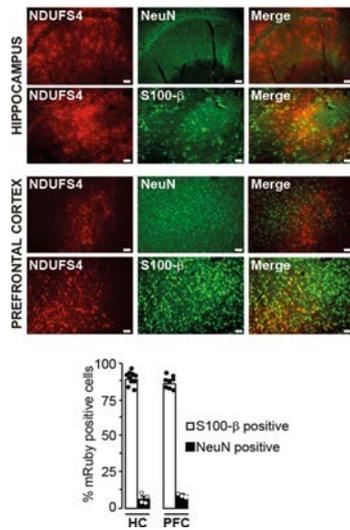
in vivo THC effects (10 mg/Kg, 24h before) on complex I activity and expression (normalized to β -ATPase) of the complex I subunit NDUFS1 and NDUFA9 in complex I and SC of HC (left panels) or PFC (right panels) from GFAP-CB₁-WT and GFAP-CB₁-KO mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ##, $P < 0.01$; ###, $P < 0.001$ (versus WT). **g**, Representative Western Immunoblottings (left) and relative quantification of these gels ($n = 3$) (right) showing the effects of in vivo THC treatment (10 mg/Kg, 24h before) on expression of the complex I subunits NDUFS1, NDUFV2 and NDUFA9 of HC or PFC from GFAP-CB₁-WT and GFAP-CB₁-KO mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (versus WT). Data are expressed as mean \pm s.e.m. and are analysed by one-way ANOVA in **a–c** and two-way ANOVA in **d–g**. n represents number of independent experiments. Original gels, Supplementary Fig. 1; Statistical details, Supplementary Table 2.



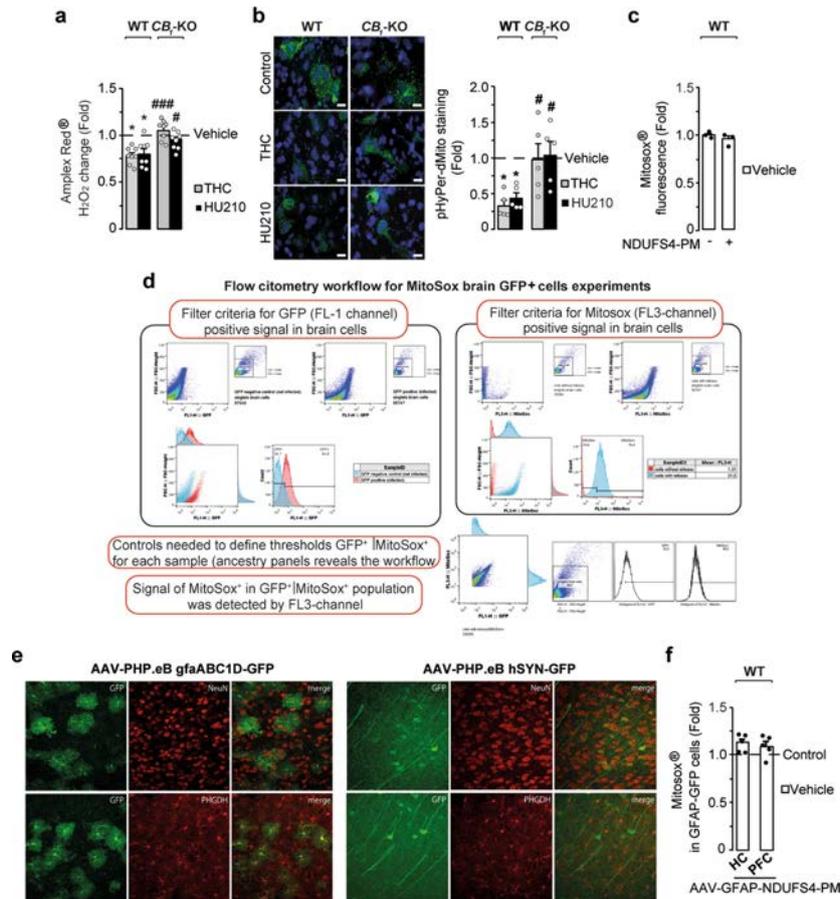
Extended Data Fig. 3 | Activation of astroglial mtCB₁ receptors decreases NDUFS4-Ser173 phosphorylation to inhibit complex I activity.

a, Quantification ($n = 3$) of Fig. 2a, reporting the THC (1 μ M) effects on PKA-dependent phosphorylation of complex I proteins (left) and on NDUFS2 levels in WT astrocytes (right). ***, $P < 0.001$ (versus Vehicle). **b**, Quantification ($n = 3$) of Fig. 2b, reporting the THC effects on pNDUFS4 in WT astrocytes. ***, $P < 0.001$ (versus Vehicle). **c**, Quantification ($n = 3$) of Fig. 2c, reporting THC or HU210 effects on pNDUFS4 levels in WT and CB₁-KO astrocytes. **, $P < 0.01$; ***, $P < 0.001$ (versus Vehicle). ##, $P < 0.01$; ###, $P < 0.001$ (versus WT-THC). **d**, Quantification ($n = 3$) of Fig. 2d, reporting THC or HU210 effects on pNDUFS4 levels in CB₁-KO astrocytes (empty, CB₁-WT, DN22-CB₁). *, $P < 0.05$ (versus Vehicle). ##, $P < 0.01$ (versus WT). **e**, Quantification ($n = 3$) of Fig. 2e, reporting THC (10 mg/Kg, 24h before) effects on pNDUFS4 of HC (left) or PFC (right) from GFAP-CB₁-WT and GFAP-CB₁-KO mice. *, $P < 0.05$ (versus Vehicle). #, $P < 0.05$; ###, $P < 0.001$ (versus WT). **f**, Quantification ($n = 3$) of Fig. 2f, reporting the

expression of NDUFS4-PM in WT astrocytes with or without THC treatment. *, $P < 0.05$ (versus Vehicle). **g**, Quantification ($n = 3$) of Fig. 2g, reporting NDUFS4-PM expression in WT astrocytes effects on complex I activity, NDUFS1 levels and β-ATPase expression after 24h incubation with vehicle, THC or HU210. **, $P < 0.01$; ***, $P < 0.001$ (versus Vehicle). #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (versus empty). **h**, THC or HU210 effects on citrate synthase activity of WT astrocytes in the presence or absence of NDUFS4-PM ($n = 3$). **i**, Quantification of Fig. 2i, reporting AAV-GFAP-NDUFS4-PM effects on complex I activity, NDUFS1 and NDUFA9 levels and -ATPase expression in HC (left) or PFC (right) 24h after THC treatment (10 mg/Kg). *, $P < 0.05$; ***, $P < 0.001$ (versus Vehicle). #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (vs AAV-Control). Data are expressed as mean \pm s.e.m. and are analysed by one-way ANOVA in **a**, **b** and two-way ANOVA in **c**-**i**. n represents number of independent experiments. Statistical details, Supplementary Table 2.

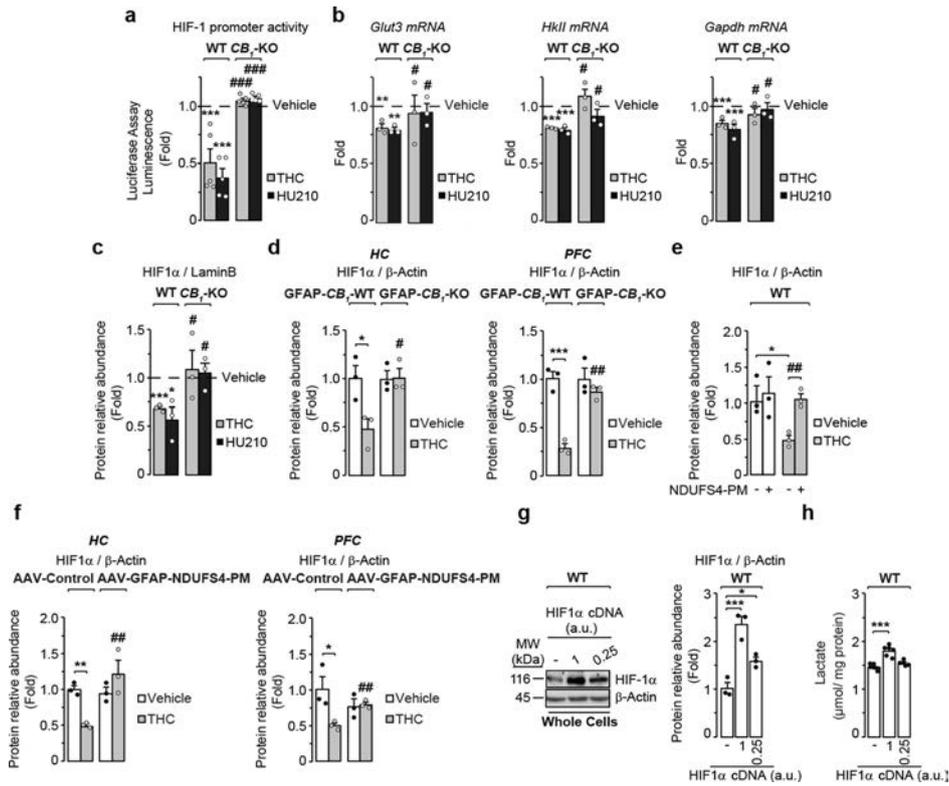


Extended Data Fig. 4 | Astroglial expression of NDUF54-PM in the hippocampus and PFC. Top, immunofluorescence micrographs showing the expression of AAV-gfa-ABC₁D-NDUF54-PM (fused to mRuby, red) in neurons (staining with NeuN marker, green) or astrocytes (staining with an anti-S-100β antibody, green) of the HC and the PFC. Note the large overlapping in the merged images when using the S-100-β antibody (HC, *n* = 10 mice; PFC, *n* = 8) but not the neuronal NeuN marker (*n* = 5 mice; PFC, *n* = 3). Bottom, graph quantification showing the percentage of mRuby positive cells (NDUF54-PM positive) that colocalize with the astroglial marker S-100-β or the neuronal one NeuN. Data are expressed as mean ± s.e.m. Scale bars, 40 μm.



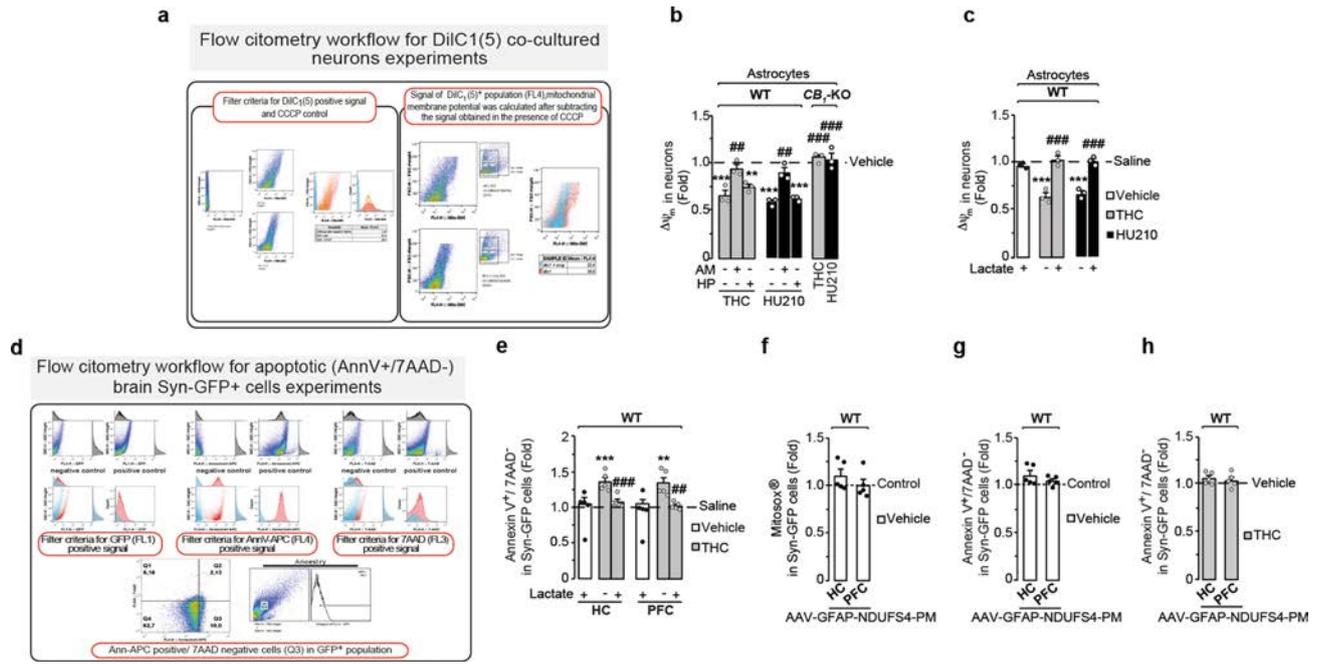
Extended Data Fig. 5 | MtCB₁-dependent effects on mROS and mitochondrial membrane potential in astrocytes. **a**, THC (1 μ M) or HU210 (50 nM) effects on mROS in *CB₁*-WT and *CB₁*-KO astrocytes ($n = 8$) as revealed by Amplex Red fluorescence. *, $P < 0.05$ (versus Vehicle); #, $P < 0.05$; ###, $P < 0.001$ (versus WT). **b**, pHyPer-dMito micrographs (left) and quantification (right) 24h after THC (1 μ M) or HU210 (50 nM) treatments in *CB₁*-WT and *CB₁*-KO astrocytes ($n = 5$). Scale bar: 40 μ M. *, $P < 0.05$ (versus Vehicle); #, $P < 0.05$ (versus WT). **c**, Effects on mROS of NDUFS4-PM transfection per se in astrocytes from *CB₁*-WT ($n = 3$). **d**, MitoSOX schematic example of the gating strategy and workflow for the determination of mROS in GFP+ cells by flow cytometry. **e**, Left,

immunofluorescence micrographs showing expression of GFP three weeks after retro-orbital injection of the AAV-PHP.eB gfaABC1D-GFP. GFP is expressed in PHGDH-positive astrocytes but not in NeuN-positive neurons ($n = 3$). Right, immunofluorescence micrographs showing expression of GFP three weeks after retro-orbital injection of the AAV-PHP.eB hSYN-GFP. GFP is expressed in NeuN-positive neurons and not in PHGDH-positive astrocytes ($n = 3$). **f**, AAV-gfa-ABC1D-NDUFS4-PM infusion effects on the levels of mROS in HC and PFC ($n = 5$). Data are expressed as mean \pm s.e.m. and are analysed by two-way ANOVA. n represents number of independent experiments. Statistical details are in Supplementary Table 2.



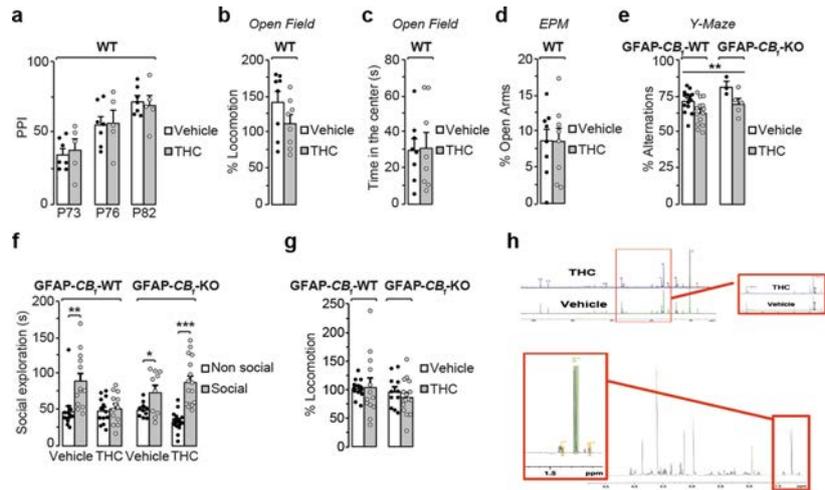
Extended Data Fig. 6 | Activation of astroglial mtCB, receptors decreases glycolysis and lactate release by attenuating the HIF-1 pathway. **a**, THC (1 μ M) or HU210 (50 nM) effects on HIF-1 promoter activity in WT and CB_1 -KO astrocytes ($n=5$). $***, P < 0.001$ (versus Vehicle); $###, P < 0.001$ (versus WT). **b**, THC or HU210 effects ($n=3$) on mRNA levels of different HIF-1 targets (*Glut3*, *HkII* and *Gapdh*) in WT and CB_1 -KO astrocytes. $** , P < 0.01$; $***, P < 0.001$ (versus Vehicle); $#, P < 0.05$ (versus WT). **c**, Quantification ($n=3$) of Fig. 3g, reporting THC and HU210 effects on HIF-1 α nuclear expression in WT and CB_1 -KO astrocytes. $* , P < 0.05$; $***, P < 0.001$ (versus Vehicle); $#, P < 0.05$; (versus WT). **d**, Quantification ($n=3$) of Fig. 3h, reporting THC effects (10 mg/Kg, 24h before) on HIF-1 α protein levels in HC (left) or PFC (right) from GFAP- CB_1 -WT and GFAP- CB_1 -KO mice. $* , P < 0.05$; $***, P < 0.001$ (versus Vehicle); $#, P < 0.05$; $##, P < 0.01$ (versus WT). **e**, Quantification ($n=3$) of Fig. 3i, reporting NDUFS4-PM

expression effects on the THC-induced HIF-1 α expression decrease in WT astrocytes. $* , P < 0.05$ (versus Vehicle); $##, P < 0.01$ (versus empty). **f**, Quantification ($n=3$) of Fig. 3j, reporting the effect of local infusion of AAV-GFAP-NDUFS4-PM on the THC-mediated decrease of HIF-1 α in HC (left) and PFC (right) from WT mice. $* , P < 0.05$; $** , P < 0.01$ (versus Vehicle); $##, P < 0.01$ (versus AAV-Control). **g**, Western immunoblotting (left) and quantification ($n=3$, right) showing the HIF-1 α overexpression obtained by transfecting astrocytes with 1 or 0.25 μ g plasmid DNA, respectively. $* , P < 0.05$; $***, P < 0.001$ (versus empty). **h**, Effect of HIF-1 α overexpression ($n=6$) on lactate release from WT astrocytes. $***, P < 0.001$ (versus empty). Data are expressed as mean \pm s.e.m. and are analysed by one-way ANOVA in **g-h** and two-way ANOVA in **a-f**. n represents number of independent experiments. Statistical details, Supplementary Table 2.



Extended Data Fig. 7 | Determination of mitochondrial membrane potential and apoptosis in neurons. **a**, Schematic and representative example of the gating strategy and workflow for the determination of mitochondrial membrane potential (DiIC1(5)) in neurons co-cultured with astrocytes by flow cytometry. **b**, Effects on cultured neurons co-cultured with astrocytes from *CB1*-WT or *CB1*-KO mice previously treated with THC (1 μM) or HU210 (50 nM) on mitochondrial membrane potential (ΔΨ_m). **, *P* < 0.01; ***, *P* < 0.001 (versus Vehicle); ##, *P* < 0.01; ###, *P* < 0.001 (versus WT-cannabinoids). **c**, Effects of lactate supplementation (2 mM) on the decreased mitochondrial membrane potential (ΔΨ_m). ***, *P* < 0.001 (versus Saline); ###, *P* < 0.001; (versus cannabinoids). **d**, Schematic and representative example of the gating strategy and workflow for the determination of apoptosis (AnnV+/7AAD) in brain GFP+ cells by flow cytometry. **e**, Effects of ICV

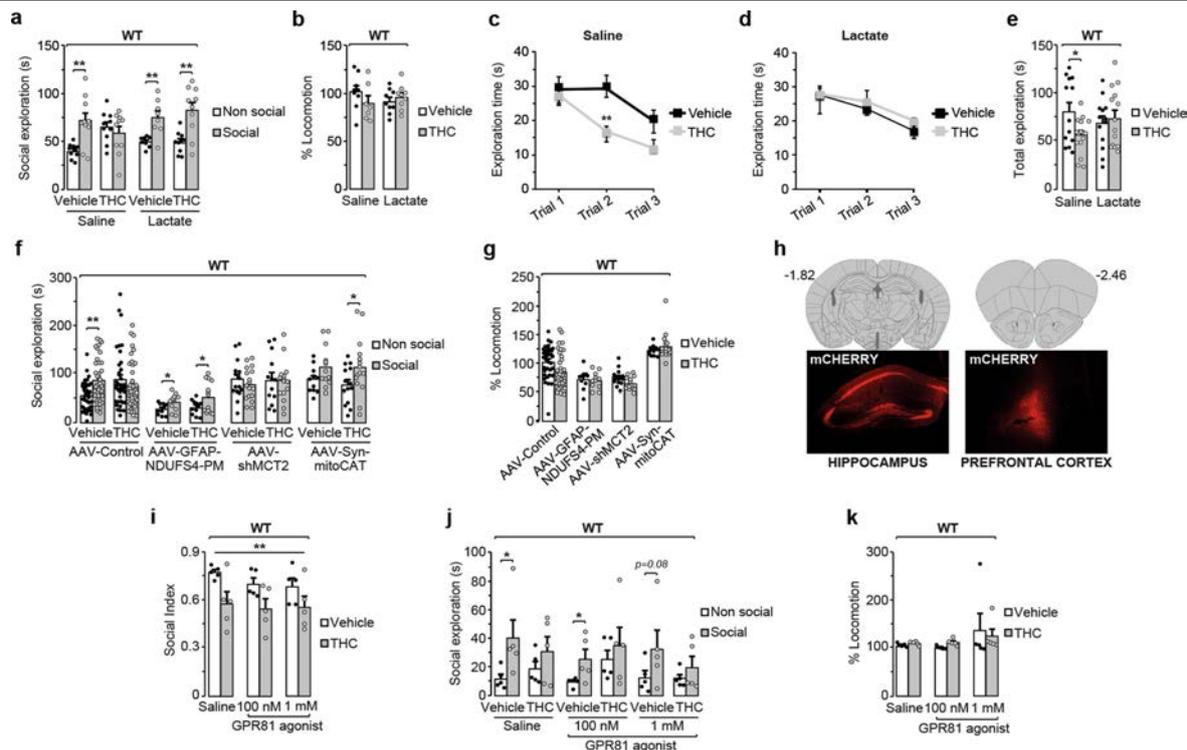
lactate supplementation (100 mM) on the THC-induced increase of apoptotic cell death, in Syn-positive cells (neurons) from PFC or HC (*n* = 5). **, *P* < 0.01; ***, *P* < 0.001 (versus Saline); ##, *P* < 0.01; ###, *P* < 0.001 (versus THC). **f**, Effect of the intravenous administration of AAV-gfa-ABC₁D-NDUFS4-PM on the neuronal levels of mROS as revealed by MitoSOX analysis by fluorescence-activated cell sorting (*n* = 5) and the apoptotic cell death in neurons as revealed by cell sorting flow cytometry (*n* = 5) (**g**). **h**, Effects of retro-orbital injection of AAV-PHP.eB-gfa-ABC₁D-NDUFS4-PM on the THC-induced increase of apoptotic cell death, in Syn-positive cells (neurons) from PFC or HC (*n* = 5). Data are expressed as mean ± s.e.m. and are analysed by one-way ANOVA in **b** and two-way ANOVA in **c** and **e**. *n* represents number of independent experiments. Statistical details, Supplementary Table 2.



Extended Data Fig. 8 | Behavioural and metabolic effects of THC

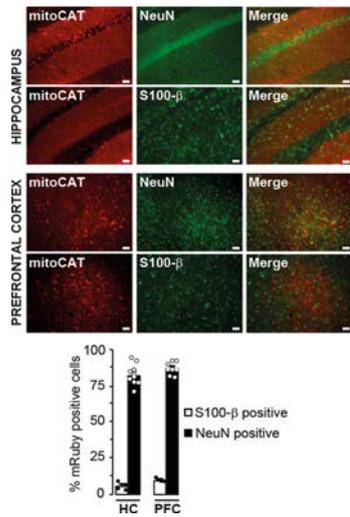
administration in GFAP-*CB₁*-KO mice. **a-d**, The administration of THC (10 mg per kg, i.p.) did not affect the prepulse inhibition (PPI) of the startle response (**a**, Veh, $n = 7$; THC, $n = 5$), the locomotion assessed in the Open Field (**b**, $n = 8$), the anxiety-like responses assessed in the Open Field (**c**, $n = 8$) or in the elevated plus maze (**d**, $n = 8$). **e**, THC induced spontaneous alternation impairment in both in GFAP-*CB₁*-WT ($n = 15$) and GFAP-*CB₁*-KO mice ($n = \text{Veh}, n = 3$; THC, $n = 5$). $**P < 0.01$ (versus Vehicle). **f**, THC effects (Fig. 4k) on the exploration times of social and non-social compartments in GFAP-*CB₁*-KO mice (Veh, $n = 11$; THC, $n = 16$) and GFAP-*CB₁*-WT ($n = 13$) littermates. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (versus non-social). **g**, Locomotor effects of THC in the sociability test in GFAP-*CB₁*-KO mice (Veh, $n = 11$; THC, $n = 16$) and GFAP-*CB₁*-WT littermates

($n = 13$). **h**, Top, typical ¹³C-NMR spectra of brain perchloric acid extracts of a control mouse treated with Vehicle (green) or THC (blue). 1: Alanine C3; 2: Lactate C3; 3: Glutamine C3; 4: Glutamate C3; 5: Glutamine C4; 6: Glutamate C4; 7: GABA C2; 8: Glutamine C2; 9: Glutamate C2; 10: Ethylene glycol (external standard); 11: α Glucose C1; 12: β Glucose C1. Right, zoom showing the difference in the incorporation of carbone-13 into carbone 2 compared to carbone 4 between vehicle- and THC-treated. Black arrow represents the height of carbone-2 peak. Bottom, typical raw ¹H-NMR spectra of brain perchloric acid extracts of vehicle treated mouse. Data are expressed as mean \pm s.e.m. and are analysed by two-way ANOVA in **e**, **f**. n represents number of mice. Experiments shown in **h**, **i** were repeated in four independent experiments. Statistical details, Supplementary Table 2.

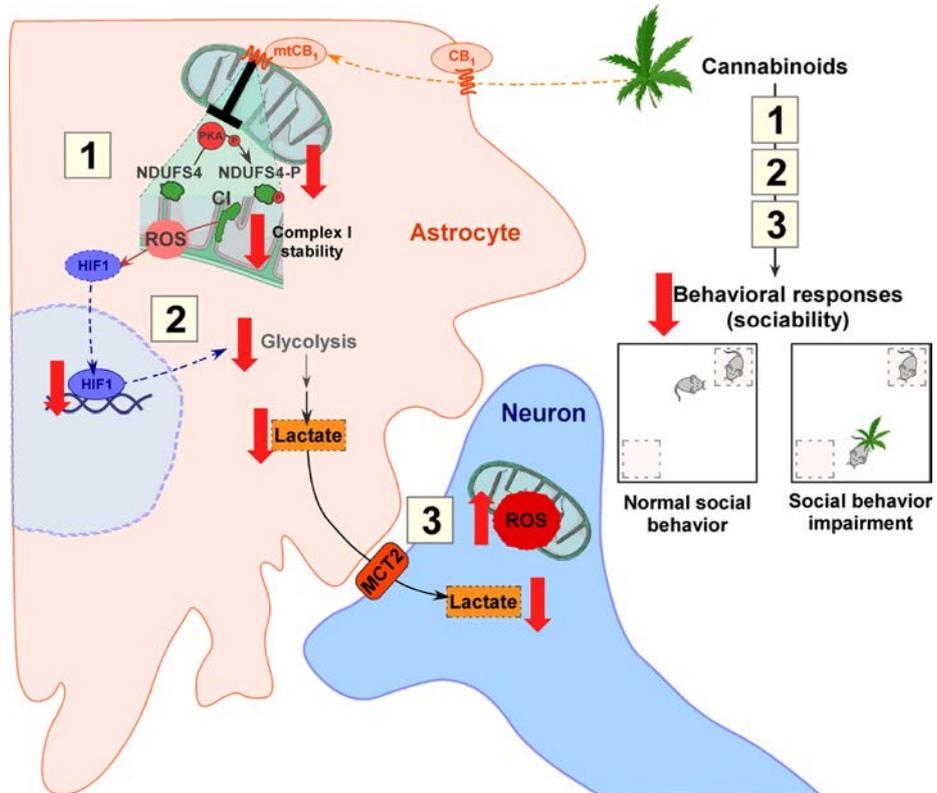


Extended Data Fig. 9 | Mechanisms that underlie the THC-induced impairment of social interaction. **a**, THC effects (Fig. 4m) on exploration times of social and non-social compartments (Sal-Veh, $n = 10$; Sal-THC, $n = 8$; Lac-Veh, $n = 10$; Lac-THC, $n = 10$). **, $P < 0.01$ (versus non-social). **b**, Locomotor effects of THC in the sociability test in mice receiving saline or lactate (Sal-Veh, $n = 9$; Sal-THC, $n = 7$; Lac-Veh, $n = 9$; Lac-THC, $n = 9$). **c**, **d**, Effects of THC on the direct social interaction test on mice treated ICV with saline (**c**, Veh-Sal, $n = 13$; THC-Sal, $n = 16$) or lactate (**d**, Veh-Lac, $n = 13$; THC-Lac, $n = 14$). **, $P < 0.01$ (versus Vehicle). **e**, Overall social exploration through the 3 trials of the direct social interaction task in mice receiving Vehicle (Veh-Sal, $n = 13$; Veh-Lac, $n = 13$) or THC (THC-Sal, $n = 16$, HC-Lac, $n = 14$) under lactate or Saline (total exploration, 3 trials together). *, $P < 0.05$ (versus vehicle). **f**, THC effects (Fig. 4n) on exploration times of social and non-social compartments (Control-Veh, $n = 37$; Control-THC, $n = 38$; NDUSF4-Veh, $n = 10$; NDUSF4-THC, $n = 11$; MCT2-Veh, $n = 15$; MCT2-THC, $n = 13$; mitoCAT-Veh, $n = 11$; mitoCAT-THC, $n = 14$) in mice injected with the different viral vectors. *, $P < 0.05$; **, $P < 0.01$ (versus Non-social). **g**, Locomotor effects of THC administration in the sociability test in mice

injected with the different viral vectors (Control-Veh, $n = 37$; Control-THC, $n = 38$; NDUSF4-Veh, $n = 10$; NDUSF4-THC, $n = 11$; MCT2-Veh, $n = 15$; MCT2-THC, $n = 13$; mitoCAT-Veh, $n = 11$; mitoCAT-THC, $n = 14$). In **f**, **g**, different batches of mice infused with different control viral vectors (Methods) were pooled as One-way ANOVA analysis of social indices shown in Fig. 4n indicated no statistical differences between control groups ($P = 0.357$). **h**, Representative images of HC and PFC for the different viral vectors infused in HC and PFC. Here, we are showing the expression of mCherry after infusion of AAV2/DJ-CBA-mCherry-miR30E-shMCT2 in these brain regions. **i**, Central (ICV) supplementation of GPR81 receptor agonist (100 nM and 1 mM, $n = 5$) did not rescue the social impairment induced by THC. **, $P < 0.01$ (main effect of THC). **j**, THC effects on the exploration times of social and non-social compartments in the sociability test shown in **i** ($n = 5$). *, $P < 0.05$ (versus non-social). **k**, Locomotor effects of THC administration in the sociability test in mice receiving saline or the GPR81 receptor agonist just before the test ($n = 5$). Data are expressed as mean \pm s.e.m. and are analysed by two-way ANOVA in **a**, **c**, **e**, **f**, **i**, **j**. n represents number of mice. Statistical details, Supplementary Table 2.



Extended Data Fig. 10 | Neuronal expression of mitochondrial catalase in the hippocampus and PFC. Top, immunofluorescence micrographs showing the expression of AAV-hSyn-mitoCAT (fused to mRuby, red) in neurons (staining with an anti-NeuN antibody) or astrocytes (staining with S100- β , green) of HC and PFC. Note the large overlapping in the merged images when using the NeuN antibody (HC, $n = 8$ mice; PFC, $n = 8$) but not the astroglial S100- β marker (HC, $n = 4$ mice; PFC, $n = 4$). Bottom, graph quantification showing the % of mRuby positive cells (neurons) that colocalize with the NeuN antibody. Data are mean \pm s.e.m. Scale bars, 40 μ m.



Extended Data Fig. 11 | Astroglial $mtCB_1$ receptor activation impairs social interactions by hampering the metabolism of lactate in the brain and altering neuronal functions through a complex I–mROS–HIF-1 pathway.

(1), Activation of astroglial $mtCB_1$ receptors reduces PKA-dependent phosphorylation of the mitochondrial complex I subunit NDUFS4, which disrupts the assembly and activity of complex I to attenuate the levels of

mitochondrial reactive oxygen species (mROS). (2) The decrease in mROS levels leads to a HIF-1 α -dependent reduction in the glycolytic production of lactate. (3) The diminished release of lactate from astrocytes to neurons through MCT2 results in neuronal bioenergetic and redox stress. All of these processes lead to deficits in social interaction. This scheme was created by the authors with the use of some free images from Servier Medical Art.

Annex II

Sex-dependent pharmacological profiles of the synthetic cannabinoid MMB-Fubinaca

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Abstract

Synthetic cannabinoids have emerged as novel psychoactive substances with damaging consequences for public health. They exhibit high affinity at the cannabinoid type-1 (CB₁) receptor and produce similar and often more potent effects as other CB₁ receptor agonists. However, we are still far from a complete pharmacological understanding of these compounds. In this study, by using behavioral, molecular, pharmacological, and electrophysiological approaches, we aimed at characterizing several *in vitro* and *in vivo* pharmacological effects of the synthetic cannabinoid MMB-Fubinaca (also known as AMB-Fubinaca or FUB-AMB), a particular synthetic cannabinoid. MMB-Fubinaca stimulates CB₁ receptor-mediated functional coupling to G-proteins in mouse and human brain preparations in a similar manner as the CB₁ receptor agonist WIN55,512-2 but with a much greater potency. Both drugs similarly activate the CB₁ receptor-dependent extracellular signal-regulated kinase (ERK) pathway. Notably, *in vivo* administration of MMB-Fubinaca in mice induced greater behavioral and electrophysiological effects in male than in female mice in a CB₁ receptor-dependent manner. Overall, these data provide a solid pharmacological profiling of the effects of MMB-Fubinaca and important information about the mechanisms of action underlying its harmful impact in humans. At the same time, they reinforce the significant sexual dimorphism of cannabinoid actions, which will have to be taken into account in future animal and clinical studies.

KEYWORDS

MMB-Fubinaca, AMB-Fubinaca, FUB-AMB

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Giovanni Marsicano and Arnau Busquets-Garcia share senior authorship.

1 | INTRODUCTION

The use of novel psychoactive substances, primarily used for recreation, constitutes an important health hazard to modern societies.¹ Harmful synthetic substitutes of natural cannabinoid drugs (i.e. Δ^9 -tetrahydrocannabinol [THC] from the plant *Cannabis sativa*) with better pharmacological and pharmacokinetic profiles have surged in the past decades.² Due to their intense psychoactive effects, some of these synthetic cannabinoids have been listed high among commonly used drugs of abuse. These substances can interact with different type-1 (CB₁) or type-2 (CB₂) cannabinoid receptors in a similar manner as natural cannabinoids.³ However, they often display greater toxicity and detrimental behavioral effects,⁴ although little is known about their *in vivo* pharmacological and behavioral properties.

The recently discovered synthetic cannabinoid MMB-Fubinaca (also known as FUB-AMB or AMB-Fubinaca)³ has been recognized as a serious threat following a vast intoxication incident that took place in New York City in 2016.⁵ MMB-Fubinaca consists of an amino acid-derived indazole-3-carboxamides or indole-3-carboxamides, which is similar to other synthetic cannabinoids being classified as putative CB₁ receptor agonist.^{3,6-8} However, it is yet to be demonstrated, based on modern genetic tools (i.e., animal models lacking CB₁ protein), that CB₁ receptors are necessary for the reported effects.

Sex is an important biological variable to be considered in human and animal studies. Previous studies have revealed important sex-related differences in CB₁-dependent effects. For instance, both natural and synthetic cannabinoids^{6,9} can induce several responses, including alterations in adolescence,¹⁰⁻¹⁴ addiction,¹⁵⁻¹⁷ antinociception and locomotion,¹⁸ among others. However, due to their particularly high potency and their specific pharmacological profile, more preclinical research is needed to understand the sex-dependent effects of synthetic cannabinoids such as MMB-Fubinaca.

Using behavioral, molecular, pharmacological, and *in vivo* electrophysiological approaches combined with genetic mouse models, this study aimed at characterizing the pharmacological profile of MMB-Fubinaca. This work reveals that MMB-Fubinaca induces G-protein functional coupling both in mouse and human native tissue. Moreover, it suggests sex differences and CB₁-receptor dependency regarding the MMB-Fubinaca-mediated behavioral and *in vivo* electrophysiological effects. Overall, this study provides a better understanding on the pharmacological profile of the synthetic cannabinoid MMB-Fubinaca between species and sexes.

2 | MATERIAL AND METHODS

2.1 | Animals

Experiments were approved by the local ethical committee of the University of Bordeaux (approval number 501350-A) and the French Ministry of Agriculture and Forestry (authorization number 3306369). Mice were maintained under standard conditions (food and water *ad libitum*; 12-/12-h light/dark cycle, light on 7 a.m.). Behavioral

experiments were performed between 9 a.m. and 2 p.m., and electrophysiological experiments between 9 a.m. and 7 p.m. C57BL/6-N mice were purchased from Janvier (France). Male and female wild-type (CB₁^{+/+}) and knock-out (CB₁^{-/-}) mice (2-4 months old) were obtained, bred, and genotyped as previously described.¹⁹ Experimenters were always blind to treatments and genotypes.

2.2 | Drugs

For *in vivo* treatments, MMB-Fubinaca (also known as FUB-AMB or AMB-Fubinaca) and Rimonabant were obtained from Cayman Chemical (#9001960 and #9000484; Michigan, United States). Both drugs were dissolved in a mixture of 4% ethanol, 4% cremophor (Sigma-Aldrich, #C5135, St. Louis, MO, United States), and 0.9% physiological saline solution. Vehicle solutions contained the same amount of solvents and all drugs were freshly prepared before each experiment.

For *in vitro* experiments ([³⁵S]GTP γ S binding assays), MMB-Fubinaca, Rimonabant (Merck Life Science-Sigma-Aldrich, SML0800, Madrid, Spain) and WIN 55,512-2 (Biogen Cientifica-Tocris, TO-1038, Madrid, Spain) were dissolved in dimethyl sulfoxide (DMSO), which was also used as vehicle (final DMSO concentration in the assay 6%). For the ERK phosphorylation assay, MMB-Fubinaca was dissolved in acetonitrile and WIN 55,512-2 (Sigma-Aldrich, #W102, St. Louis, MO, USA) in DMSO.

2.3 | Brain cellular membranes preparation

Human brain samples from 16 subjects (eight males and eight females) who died by sudden and violent causes were obtained at autopsy in the Basque Institute of Legal Medicine (Bilbao, Spain). All subjects were determined to be free of psychiatric or neurological disorders based on medical histories and postmortem tissue examinations. Samples from the prefrontal cortex (Brodmann's area 9) and hippocampus were dissected at the time of autopsy and immediately stored at -70°C until assay. The postmortem delay (PMD) of the samples ranged from 9 to 37 h, and the age at time of death ranged from 29 to 68 years. No statistical differences were found between the mean PMD and mean age at death between males and females (Table 1). The study was developed in compliance with policies of research and ethical review boards for postmortem brain studies. On the other hand, brain samples from 10 male and 10 female mice (C57BL6/N, 8-10 weeks old) were also used. The cortex and hippocampus were dissected and immediately stored at -70°C until assay. For each brain region, male and female tissue (human and mice) samples were pooled separately to obtain the enriched fractions of plasma membranes.

Tissue samples were homogenized using a Teflon-glass grinder (IKA Labortechnik, Satufen, Germany) at 1500 rpm (10 up-and-down strokes) in 30 volumes of homogenization buffer (1 mM EGTA, 3 mM MgCl₂, 1 mM DTT, and 50 mM Tris-HCl, pH 7.4) supplemented with 0.25 M sucrose. The crude homogenate was centrifuged for 5 min at 1000 \times g at 4 $^{\circ}\text{C}$, and the supernatant layer was recentrifuged for

TABLE 1 Human samples characteristics. Values represent mean \pm SEM

	N	PMD, h	Age, years
Males	8	16.6 \pm 3.2	50.0 \pm 4.7
Females	8	17.3 \pm 2.7	49.3 \pm 4.6

10 min at 40 000 \times g (4°C). The resultant pellet (P₂ fraction) was washed twice in 10 and 5 volumes of homogenization buffer, respectively, and recentrifuged in similar conditions. Protein content was measured according to Bradford's method using BSA as standard. Samples were aliquoted in order to have a protein content of 1 mg and then centrifuged in a benchtop centrifuge (EBA 12 R, Hettich Instruments, Tuttlingen, Germany) at highest speed (14 000 rpm) during 15 min at 4°C. The supernatant layer was carefully discarded, and the pellets stored at -70°C until assay. The tissue samples of the eight experimental conditions (two species, two genders, and two brain regions) were processed in parallel on the same day.

The day of the experiment, the membrane pellets were defrosted (4°C), thawed, and resuspended in 11 ml of incubation buffer containing 1 mM EGTA, 3 mM MgCl₂, 100 mM NaCl, and 50 mM Tris-HCl, pH 7.4, reaching a final protein concentration of 0.09 mg/ml approximately. The real final protein content was measured after the experiment according to Bradford's method.

2.4 | MMB-Fubinaca and WIN 55,512-2 stimulated [³⁵S]GTPγS binding assays

The binding assays were carried out in a final volume of 250 μ l in 96-well plates, containing 1 mM EGTA, 3 mM MgCl₂, 100 mM NaCl, 0.2 mM DTT, 50 μ M GDP, 50 mM Tris-HCl at pH 7.4, and 0.5 nM [³⁵S]GTPγS. Stimulation curves were carried out by incubating increasing concentrations of the CB₁ receptor agonist WIN 55,512-2 or MMB-Fubinaca (10⁻¹²-10⁻⁴ M; nine concentrations by duplicate; three independent experiments). Additionally, the CB₁ receptor antagonist Rimonabant (10 μ M) was coincubated with a saturating concentration of each of both agonists (10 μ M) to test the specificity of CB₁-receptor dependent G-protein activation. The incubation was started by addition of the membrane suspension (18 μ g of membrane proteins per well) and was performed at 30°C for 120 min with shaking (450 rpm). Incubations were terminated by rapid filtration under vacuum (1450 FilterMate Harvester, PerkinElmer) through GF/C glass fiber filters (Printed Filtermat A) presoaked in ice-cold incubation buffer (1 mM EGTA, 3 mM MgCl₂, 100 mM NaCl, 0.2 mM DTT, 50 mM Tris-HCl at pH 7.4). The filters were then rinsed three times with 300- μ l ice-cold incubation buffer, air dried (20°C, 120 min), and counted for radioactivity (4 min) by liquid scintillation spectrometry using a MicroBeta TriLux counter (PerkinElmer). Nonspecific binding of the radioligand was defined as the remaining [³⁵S]GTPγS binding in the presence of 10- μ M unlabeled GTPγS, and the basal binding, as the signal in the absence of agonist.

2.5 | Cell culture and transfection

HEK293T cells (ATCC, Manassas, VA) were grown in DMEM high glucose Glutamax, supplemented with 10% fetal bovine serum, 2 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, nonessential amino acids (0.1 mM of Glycine, L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic Acid, L-Proline, and L-Serine), 10 mM HEPES, 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA) and maintained at 37°C in the presence of 5% CO₂. Cells were seeded in 6-well plate at a density of 500 000 cells/well and, 24 h after, transfected with control plasmid mCherry (mCherry) or CB₁ cDNA coupled with mCherry (CB₁) cloned in pcDNA 3.1 (+). The transfections were carried out using polyethylenimine (Polysciences, Warrington, PA).

2.6 | ERK phosphorylation assay

This assay has been performed as described before with some modifications.²⁰ Cells transfected to express mCherry or CB₁ were allowed to recover for 48 h and then starved overnight in serum-free DMEM high glucose Glutamax before the treatment. Cells were incubated at 37°C with WIN 55,512-2 (dissolved in DMSO) or MMB-Fubinaca (dissolved in acetonitrile) at 0.01, 0.1, and 1 μ M or vehicle for 10 min. The medium was removed, and the plates were snap frozen in liquid nitrogen and stored at -80°C before preparation for western blot.

Lysis buffer (0.05M Tris HCl pH 7.4, 0.15M NaCl, 0.001M EDTA, 10% Glycerol, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, Saint Louis, MO, USA) was added to each well. Cells were recovered by scraping and centrifuged at 12 500 g at 4°C for 5 min to remove insoluble debris. The protein content was determined with Roti-Nanoquant protein quantification assay, following manufacturer's instruction (Carl Roth, Karlsruhe, Germany). Protein extracts were mixed with denaturing 4 \times Laemmli loading buffer and boiled for 5 min at 95°C. Samples (30 μ g per lane) were loaded on 4-20% precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes 0.45 μ m (Merk Millipore, Billerica, MA, USA). Membranes were soaked in a mixture of Tris-buffered saline and polysorbate 20 (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20) containing 5% of nonfat milk for 1 h at room temperature (RT). For immunoblotting, antibodies against pERK (phospho-p44/42 MAPK, #4370; 1:2000, overnight 4°C, Cell Signaling Technology, Danvers, MA) and ERK (p44/42 MAPK, #9102; 1:2000 overnight 4°C, Cell Signaling Technology Danvers, MA, USA) have been used. Bound primary antibodies were detected with HRP-linked antibodies (1:2000, 1h RT, Cell Signaling Technology, Danvers, MA, USA) using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). Labeling was quantified using the Image Lab software (Bio-Rad, Hercules, CA, USA) after acquisition on ChemiDoc Touch (Bio-Rad, Hercules, CA, USA). pERK levels normalized to the amount of total ERK in the same sample have been expressed as a percentage of vehicle treated cells.

2.7 | Tetrad assessment in mice

Body temperature was measured, by using a rectal probe and monitored by a thermalert monitoring thermometer, in male and female $CB_1^{+/+}$ and $CB_1^{-/-}$ mice just before an acute intraperitoneal (i.p.) injection of MMB-Fubinaca (0.1 or 0.3 mg/kg) or vehicle. Around 45 min later, animals underwent a battery of behavioral tests (see below). Temperature was measured again at the end of the experiment.

Locomotion was measured in an open field system (box size 100 × 100 × 30 cm, 100 lux, videotracking system, divided by squares) during 5 min, and the number of total squares crossed was calculated as locomotion value.

Catalepsy was measured by placing the forepaws of mice on a 1-cm diameter bar fixed horizontally at 3.5 cm from the bench surface. The latency to fall was recorded. Four different attempts were performed per mouse. The cut-off was 2 min.

Analgesia was measured using a hot plate meter at $52^\circ\text{C} \pm 0.1^\circ\text{C}$. The time until mice showed the first sign of discomfort (paw licking or jumping) was recorded. The cut-off time was 2 min.

2.8 | *In vivo* electrophysiology in anesthetized mice

Experiments were performed as described before.²¹ Mice were anesthetized in a box containing 5% Isoflurane (VIRBAC, France) before being placed in a stereotaxic frame (Model 900, Kopf instruments, CA, USA) in which 1.0% to 1.5% of Isoflurane was continuously supplied via an anesthetic mask during the whole duration of the experiment. The body temperature was maintained at $\sim 36.5^\circ\text{C}$ using a homeothermic system (model 50-7087-F, Harvard Apparatus, MA, USA), and the state of anesthesia was assessed by mild tail pinch. Before surgery, 100 μl of the local anesthetic lurocaine (vetoquinol, France) was injected in the scalp region. After ensuring the correct alignment of the head (Bregma and Lambda evaluation), two holes were drilled in the skull for electrode placement. A glass recording electrode was inserted in CA1 stratum radiatum (A/P -1.5 , M/L -1.0 , DV 1.20) whereas a concentric stimulating bipolar electrode (Model CBARC50, FHC, ME, USA) was placed in the CA3 region (A/P -2.2 , M/L -2.8 , D/V -1.3 [20° insertion angle]). The recording electrode (tip diameter = 1–2 μm , 4–6 $\text{M}\Omega$) was filled with a 2% pontamine sky blue solution in 0.5M sodium acetate. At first, the recording electrode was placed by hand until it reached the brain surface and then to the final depth using a hydraulic micropositioner (Model 2650, KOPF instruments, CA, USA). The stimulation electrode was placed in the correct area using a standard manipulator. Both electrodes were adjusted to find the area with maximum response. *In vivo* recordings of evoked field excitatory postsynaptic potentials (fEPSPs) were amplified 1000 times and filtered (low pass at 1 Hz and high pass 3000 Hz) by a DAGAN 2400A amplifier (DAGAN Corporation, MN, USA). fEPSPs were digitized and collected online using a laboratory interface and software (CED 1401, SPIKE 2; Cambridge Electronic Design, Cambridge, United Kingdom). Test pulses were generated through an isolated constant current stimulator (DS3, Digitimer, Hertfordshire,

United Kingdom) triggered by the SPIKE 2 output sequencer via CED 1401 and collected every 2 s at a 10-kHz sampling frequency and then averaged every 180 s. Test pulse intensities were typically between 40 and 250 μA with a duration of 50 μs . Basal stimulation intensity was adjusted to 30–50% of the current intensity that evoked a maximum field response. After baseline fEPSPs were recorded for at least 20 min, we administered vehicle or MMB-Fubinaca (0.3 mg/kg) in male and female $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. All responses were expressed as percent from the average responses recorded during the 15-min before high frequency stimulation (HFS). HFS was induced by applying three trains of 100 Hz (1 s each), separated by a 20-s interval. fEPSP were then recorded for a period of 30 or 39 min, depending on the experiment. At the end of each recording, the position of the electrodes was marked (recording area: iontophoretic infusion of the recording solution during 180 s at $-20 \mu\text{A}$; stimulation area: continuous current discharge over 20 s at $+20 \mu\text{A}$), and histological verification was performed *ex vivo* as previously described.²¹

2.9 | Statistical analysis

The pharmacological parameters of the stimulation curves of the [^{35}S]GTP γS binding, the maximal effect (E_{max}), and the concentration of the drug that determines the half maximal effect (EC_{50}) were obtained by nonlinear analysis using GraphPad Prism software Version 5.0. The points fit to a concentration-response curve (standard slope). The pharmacological parameters E_{max} and pEC_{50} are expressed as means \pm SEM. The statistical comparison of the data sets was performed in GraphPad Prism software Version 5.0 by a coanalysis of the curves. This statistical analysis can be found in Tables 2, 3 and 4. For ERK phosphorylation assay, two-way ANOVA has been performed together with a Sidak's multiple comparison test. For the behavioral experiments as well as for *in vivo* electrophysiology experiments, two-way analysis of variance (ANOVA) was performed and when interaction was significant, Bonferroni's post-hoc analysis was used.

3 | RESULTS

3.1 | CB_1 receptor-mediated G-protein activation induced by MMB-Fubinaca in native tissue

To understand the activity profile of MMB-Fubinaca, we first performed [^{35}S]GTP γS functional assays in mouse and human native brain tissues, comparing the specific activity of the drug with the cannabinoid agonist WIN 55,512-2. G-protein-coupled CB_1 receptors activation leads to the exchange of GDP by GTP at the G-protein α -subunit (G_α). Thus, in a [^{35}S]GTP γS binding assay, the level of G-protein activation following cannabinoid agonist receptor binding is determined by the binding of the GTP nonhydrolyzable analog [^{35}S]GTP γS to G_α subunits. The nonlinear regression coanalysis of the dose-response curves allows the determination of the agonist

TABLE 2 Comparison between MMB-Fubinaca and WIN 55,512-2 of the pharmacological parameters obtained in [³⁵S]GTPγS binding assays performed in male and female, mouse and human brain membrane preparations

	Males			Females		
	MMB-Fubinaca	WIN55,512-2	F (DFn, DFd)	MMB-Fubinaca	WIN55,512-2	F (DFn, DFd)
CTX Mouse						
E _{max}	121.8 ± 1.9	125.2 ± 1.7	1.31 (1,105)	119.8 ± 1.3	123.2 ± 1.8	2.39 (1,93)
pEC ₅₀	8.7 ± 0.3 ^{***}	6.8 ± 0.2	13.55 (1,105)	8.7 ± 0.2 ^{***}	6.5 ± 0.2	31.47 (1,93)
CTX Human						
E _{max}	161.5 ± 2.7	154.0 ± 4.1	2.17 (1,106)	160.1 ± 3.8	147.1 ± 5.3	3.75 (1,104)
pEC ₅₀	8.1 ± 0.2 ^{***}	6.2 ± 0.2	43.98 (1,106)	8.0 ± 0.2 ^{***}	6.6 ± 0.3	13.80 (1,104)
HC Mouse						
E _{max}	127.7 ± 1.3	129.8 ± 1.6	1.03 (1,114)	132.1 ± 2.9	133.0 ± 3.9	0.04 (1,113)
pEC ₅₀	8.2 ± 0.2 ^{***}	6.8 ± 0.1	35.93 (1,114)	7.9 ± 0.3 ^{**}	6.6 ± 0.3	8.24 (1,113)
HC Human						
E _{max}	156.6 ± 2.6	153.5 ± 3.9	0.49 (1,112)	151.3 ± 2.7	145.8 ± 2.8	2.01 (1,113)
pEC ₅₀	8.1 ± 0.1 ^{***}	6.7 ± 0.2	36.67 (1,112)	8.2 ± 0.2 ^{***}	6.7 ± 0.2	33.78 (1,113)

The pharmacological parameters are obtained from nonlinear regression coanalyses of three independent experiments ran in duplicates. Values are represented as the mean ± SEM (CTX: cortex; HC: hippocampus).

^{***}*p* < .001 versus WIN 55,512-2 (F-test after coanalysis).

^{**}*p* < .01 versus WIN 55,512-2 (F-test after coanalysis).

TABLE 3 Comparison between mouse and human brain membrane preparations in males and females of the pharmacological parameters obtained in [³⁵S]GTPγS binding assays for MMB-Fubinaca and WIN 55,512-2

	Males			Females		
	Mouse	Human	F (DFn, DFd)	Mouse	Human	F (DFn, DFd)
CTX MMB-Fubinaca						
E _{max}	121.8 ± 1.9	161.5 ± 2.7 ^{***}	75.79 (1,108)	119.8 ± 1.3	160.1 ± 3.8 ^{***}	34.71 (1,96)
pEC ₅₀	8.7 ± 0.3	8.1 ± 0.2	0.64 (1,108)	8.7 ± 0.2	8.0 ± 0.2	0.71 (1,96)
CTX WIN55,512-2						
E _{max}	125.2 ± 1.7	154.0 ± 4.1 ^{***}	38.97 (1,103)	123.2 ± 1.8	147.1 ± 5.3 ^{***}	15.95 (1,101)
pEC ₅₀	6.8 ± 0.2	6.2 ± 0.2	3.09 (1,103)	6.5 ± 0.2	6.6 ± 0.3	0.0004 (1,101)
HC MMB-Fubinaca						
E _{max}	127.7 ± 1.3	156.6 ± 2.6 ^{***}	99.47 (1,114)	132.1 ± 2.9	151.3 ± 2.7 ^{***}	23.35 (1,115)
pEC ₅₀	8.2 ± 0.2	8.1 ± 0.1	0.09 (1,114)	7.9 ± 0.3	8.2 ± 0.2	0.86 (1,115)
HC WIN55,512-2						
E _{max}	129.8 ± 1.6	153.5 ± 3.9 ^{***}	31.39 (1,112)	133.0 ± 3.9	145.8 ± 2.8 ^{**}	7.41 (1,111)
pEC ₅₀	6.8 ± 0.1	6.7 ± 0.2	0.32 (1,112)	6.6 ± 0.3	6.7 ± 0.2	0.09 (1,111)

The pharmacological parameters are obtained from nonlinear regression coanalyses of three independent experiments ran in duplicates. Values are represented as the mean ± SEM (CTX: cortex; HC: hippocampus).

^{***}*p* < .001 versus mouse (F-test after coanalysis).

^{**}*p* < .01 versus mouse (F-test after coanalysis).

pharmacological parameters potency (pEC₅₀) and efficacy (E_{max}). All dose-response curves were best fitted to a sigmoidal curve with a standard slope (slope factor 1; Figure 1). No significant differences were found in the E_{max} values obtained for MMB-Fubinaca compared with WIN 55,512-2 when tested in the same brain membrane preparations (Table 2 and Figure 1). However, the pEC₅₀ values of MMB-Fubinaca were significantly higher compared with those of WIN 55,512-2 in both human and mouse brain membranes, representing

almost two orders of magnitude higher potency of MMB-Fubinaca compared with WIN 55,512-2 in all experimental conditions (Table 2 and Figure 1).

The respective potencies observed were similar between human and mouse membrane preparations in the brain areas tested (Table 3). Nevertheless, the E_{max} values obtained in humans were always significantly higher as compared with those obtained in mice (Table 3). Importantly, we have included brain membrane preparations from

TABLE 4 Comparison between males and females in mouse and human brain membrane preparations of the pharmacological parameters obtained in [³⁵S]GTP_γS binding assays for MMB-Fubinaca and WIN 55,512-2

	Mouse			Human		
	Males	Females	F (DFn, DFd)	Males	Females	F (DFn, DFd)
CTX MMB-Fubinaca						
E _{max}	121.8 ± 1.9	119.8 ± 1.3	0.60 (1,97)	161.5 ± 2.7	160.1 ± 3.8	0.09 (1,107)
pEC ₅₀	8.7 ± 0.3	8.7 ± 0.2	0.002 (1,97)	8.1 ± 0.2	8.0 ± 0.2	0.05 (1,107)
CTX WIN55,512-2						
E _{max}	125.2 ± 1.7	123.2 ± 1.8	0.61 (1,101)	154.0 ± 4.1	147.1 ± 5.3	1.04 (1,103)
pEC ₅₀	6.8 ± 0.2	6.5 ± 0.2	0.98 (1,101)	6.2 ± 0.2	6.6 ± 0.3	1.32 (1,103)
HC MMB-Fubinaca						
E _{max}	127.7 ± 1.3	132.1 ± 2.9	1.89 (1,117)	156.6 ± 2.6	151.3 ± 2.7	2.08 (1,112)
pEC ₅₀	8.2 ± 0.2	7.9 ± 0.3	0.60 (1,117)	8.1 ± 0.1	8.2 ± 0.2	0.31 (1,112)
HC WIN55,512-2						
E _{max}	129.8 ± 1.6	133.0 ± 3.9	0.65 (1,110)	153.5 ± 3.9	145.8 ± 2.8	2.84 (1,113)
pEC ₅₀	6.8 ± 0.1	6.6 ± 0.3	0.32 (1,110)	6.7 ± 0.2	6.7 ± 0.2	0.08 (1,113)

The pharmacological parameters are obtained from nonlinear regression coanalyses of three independent experiments ran in duplicates. Values are represented as the mean ± SEM (CTX: cortex; HC: hippocampus).

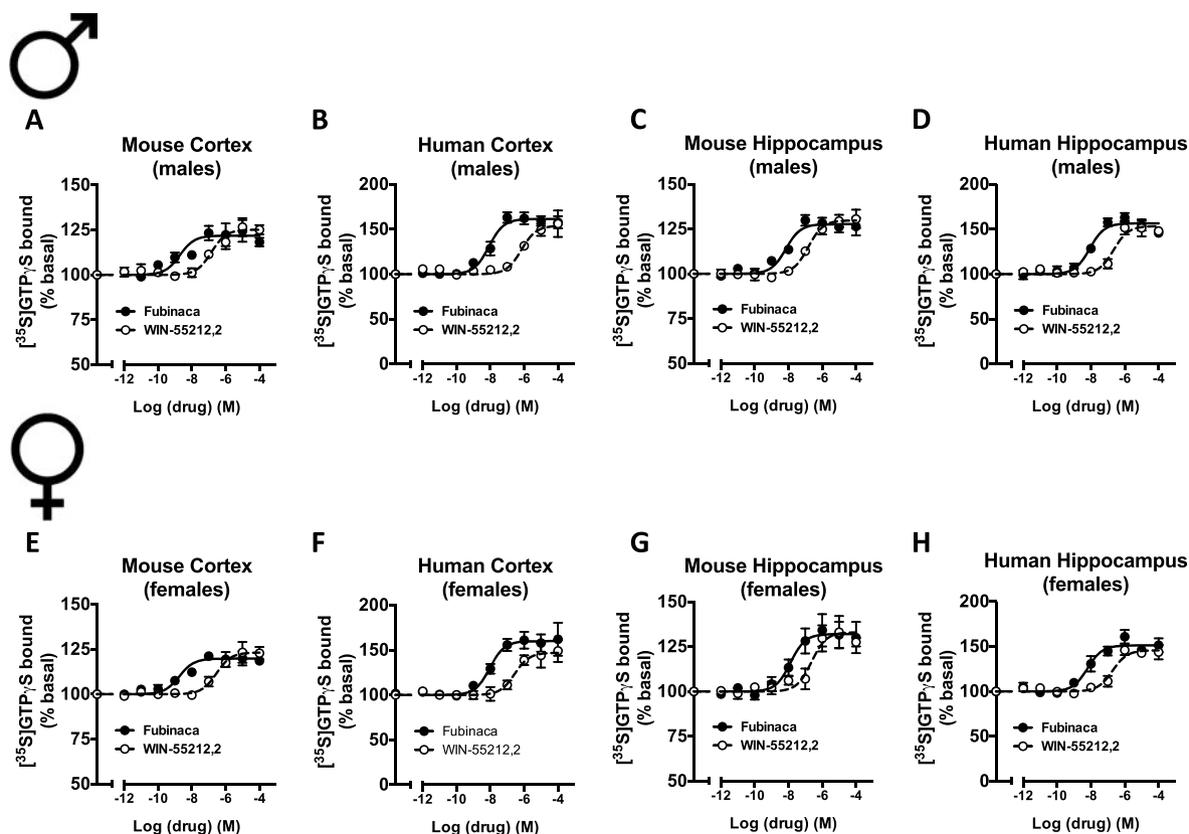


FIGURE 1 G-protein stimulation induced by MMB-Fubinaca. Concentration-response curves of MMB-Fubinaca and WIN 55,512-2 stimulated [³⁵S]GTP_γS specific binding over basal in cortical (A, B, E, F) and hippocampal (C, D, G, H) membrane preparations from mouse (A, C, E, G) and human (B, D, F, H) brains. In total, eight experimental preparations (A-H) were included (each preparation comprise a pool of 8-10 samples). In all experimental conditions, MMB-Fubinaca showed a greater potency (curve shifted to the left) compared with WIN55,212-2. Values are expressed by mean ± SEM of three independent experiments run in duplicate. Statistical details are found in Tables 2-4

male and female mice and humans, which did not differ on [³⁵S]GTPγS basal binding values, to test the potential influence of sex on the potency and efficacy. As shown in Table 4 and Figure 1, no significant differences were found neither on the potency nor on the efficacy of MMB-Fubinaca or WIN 55,512-2 between male and female mice or humans.

As a proof of cannabinoid specificity, the activation of G-proteins induced by MMB-Fubinaca and WIN 55,512-2 at a saturating concentration (10 μM) was blocked by the CB₁ antagonist Rimobant (10 μM; Figure 2), suggesting a CB₁ receptor-dependent effect. Indeed, Rimobant did not only completely reverse the effect of MMB-Fubinaca and WIN 55,512-2 but also decreased the basal stimulation.

3.2 | MMB-Fubinaca activates ERK pathway in a CB₁-dependent manner

The previous results indicate that MMB-Fubinaca has a cannabinoid-like profile, and it is much more potent than WIN 55,512-2 in terms of CB₁ receptor-mediated G-protein activation. To further characterize its pharmacological profile, we used an *in vitro* approach to study the activation of extracellular signal-regulated kinases (ERK), which are phosphorylated/activated upon CB₁ activation.²² Human embryonic kidney cells transfected with a control plasmid (HEK-mCherry) or to express CB₁ receptor (HEK-CB₁) were exposed to increasing doses of MMB-Fubinaca and WIN 55,512-2 (Figure 3), and the ratio between the phosphorylated ERK and total protein was analyzed. Both compounds induced a CB₁-dependent activation of the ERK

pathway in HEK-CB₁ but not in HEK-mCherry. No significant differences between the cannabinoid agonists were observed, but these results provide additional strong evidences for the ability of MMB-Fubinaca to modulate ERK pathway (Figure 3), similarly to what has been previously observed by other CB₁ receptor agonists.⁷

3.3 | MMB-Fubinaca induces CB₁ receptor-dependent behavioral effects in male and female mice

Following the characterization of the MMB-Fubinaca-induced CB₁ receptor-dependent signaling effects, we moved to study some of the *in vivo* behavioral effects of this drug. Activation of CB₁ receptors is responsible for specific behavioral phenotypes in animal models of cannabinoid exposure.^{23,24} The so-called “cannabinoid tetrad” represents the most common cannabinoid-dependent set of behavioral effects, for which changes in locomotion, body temperature, catalepsy, and nociception are analyzed.²⁵ The acute administration of MMB-Fubinaca (0.1 and 0.3 mg/kg; i.p.) in C57BL/6N mice was able to induce hypolocomotion, enhanced catalepsy, antinociception, and a decrease of body temperature in both male and female mice (Figure 4A–D). Notably, except for the effects on locomotion, the MMB-Fubinaca effects were stronger in male compared with female mice (Figure 4A–D). Unexpected baseline differences between male and female mice were observed in locomotor activity but not in the other behaviors. Indeed, in locomotion, MMB-Fubinaca induced an effect of similar magnitude in male (75.7% for 0.1 mg/kg and 97.2% for 0.3 mg/kg) and female (54.5% for 0.1 mg/kg and 94.8% for 0.3 mg/kg) mice. Importantly, no alterations in body temperature,

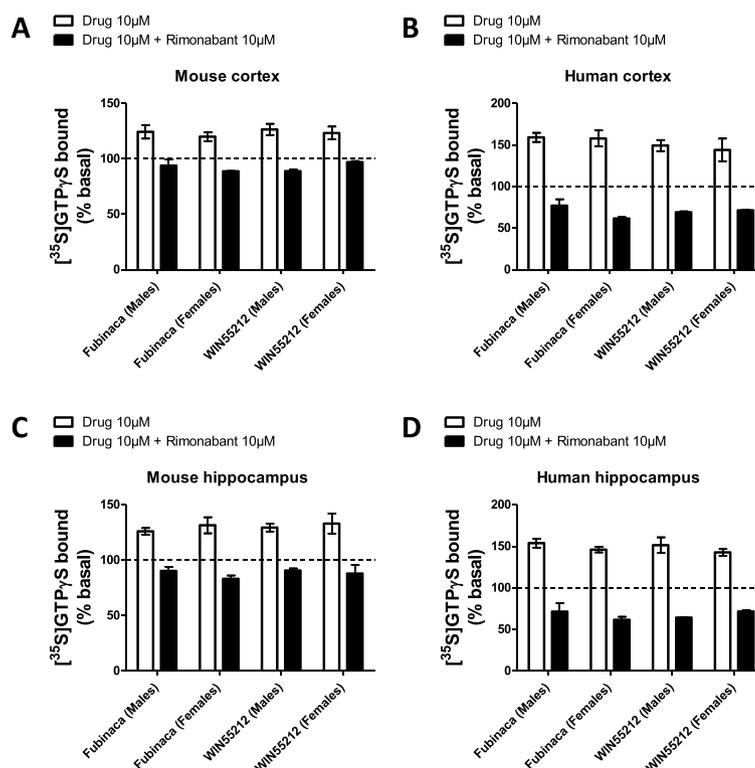


FIGURE 2 G-protein stimulation induced by MMB-Fubinaca is blocked by the CB₁ antagonist Rimobant. Effect of the coinubation of MMB-Fubinaca or WIN55,212-2 (saturating concentration: 10 μM) with the CB₁ receptor antagonist Rimobant (10 μM) on [³⁵S]GTPγS specific binding over basal in cortical (A, B) and hippocampal (C, D) membrane preparations from mouse (A, C) and human (B, D) brains. Note that in all experimental conditions, the coinubation with Rimobant blocked the stimulation of [³⁵S]GTPγS binding induced by both agonists. Values are expressed by mean ± SEM of one to three independent experiments run in duplicate. Student's t test: **p* < .05; ***p* < .01; ****p* < .001

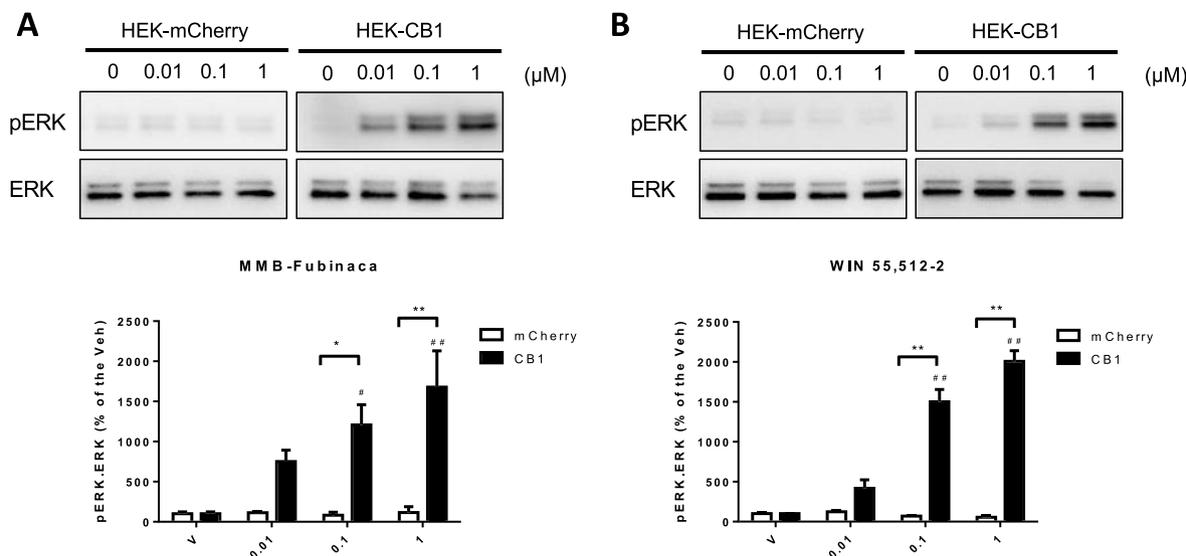


FIGURE 3 *In vitro* effect of MMB-Fubinaca (A) and WIN 55,512-2 (B) at the final concentration of 0.01, 0.1, or 1 μM have been used to treat HEK cells transfected to express CB₁ (HEK-CB₁) or mCherry (HEK-mCherry). Both compounds induce ERK phosphorylation via CB₁ receptor. Data are expressed as mean ± SEM (n = 3–5 per group). *p < .005; **p < .0001; compared with HEK-mCherry. #p < .005; ###p < .0001 compared with the vehicle

locomotion, catalepsy, or nociception were observed in CB₁^{-/-} mice, indicating that MMB-Fubinaca effects depend on CB₁ receptor activation (Figure 5). Altogether, these data show that MMB-Fubinaca induces its tetrad effects via activation of CB₁ receptors.

3.4 | MMB-Fubinaca induces a CB₁ receptor-dependent transient depression of *in vivo* hippocampal synaptic transmission in male and female mice

In order to further characterize the *in vivo* pharmacological profile of MMB-Fubinaca, we investigated the induction of hippocampal long-

term depression of synaptic transmission, which has been shown to be altered by cannabinoid agonists.²⁶ We used *in vivo* electrophysiology under anesthesia to record evoked hippocampal field excitatory post synaptic potentials (fEPSP) in the stratum radiatum region after electrical stimulation in the Shaffer collateral fibers.²¹ Notably, we observed that MMB-Fubinaca (0.3 mg/kg) induced a transient depression of synaptic transmission in both male (Figure 6A–C) and female mice (Figure 6D–F). This effect was dependent on CB₁ receptors, as it was not present in male and female CB₁^{-/-} mice (Figure 6A–F). The depression observed in control mice was elicited shortly after the treatment, became more pronounced at around 15 minutes post-injection (Figure 6C,F) and returned to baseline levels within 90 min

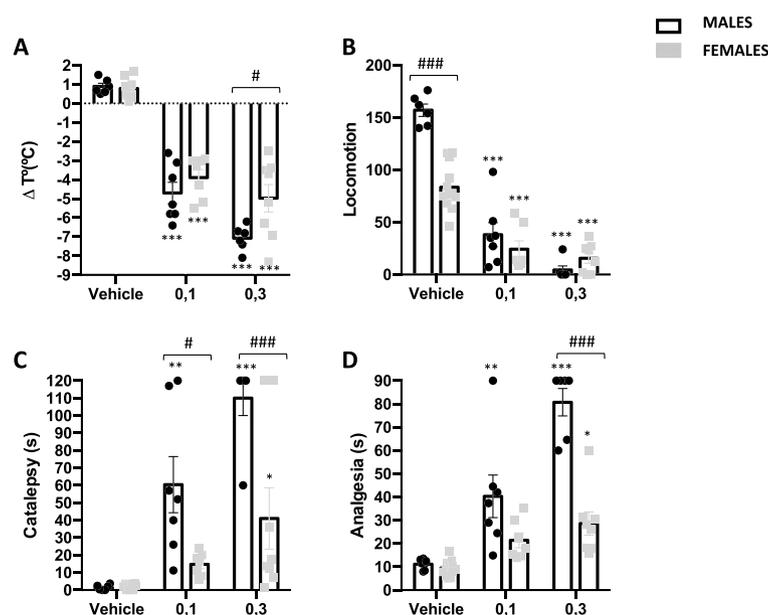


FIGURE 4 *In vivo* sex-dependent effects of MMB-Fubinaca. The cannabinoid tetrad induced by MMB-Fubinaca was assessed in male and female C57BL/6N mice 45 min after the injection of vehicle or two different doses of MMB-Fubinaca (0.1 and 0.3 mg/kg, i.p.). Both doses produced clear cannabinoid-like effects such as hypothermia (A), hypolocomotion (B), catalepsy (C), and analgesia (D) with particular sex-dependent effects. Data are expressed as mean ± SEM (n = 6–12 per group). *p < .05; **p < .01; ***p < .001 compared with vehicle treated mice. #p < .05; ###p < .001 compared with male mice

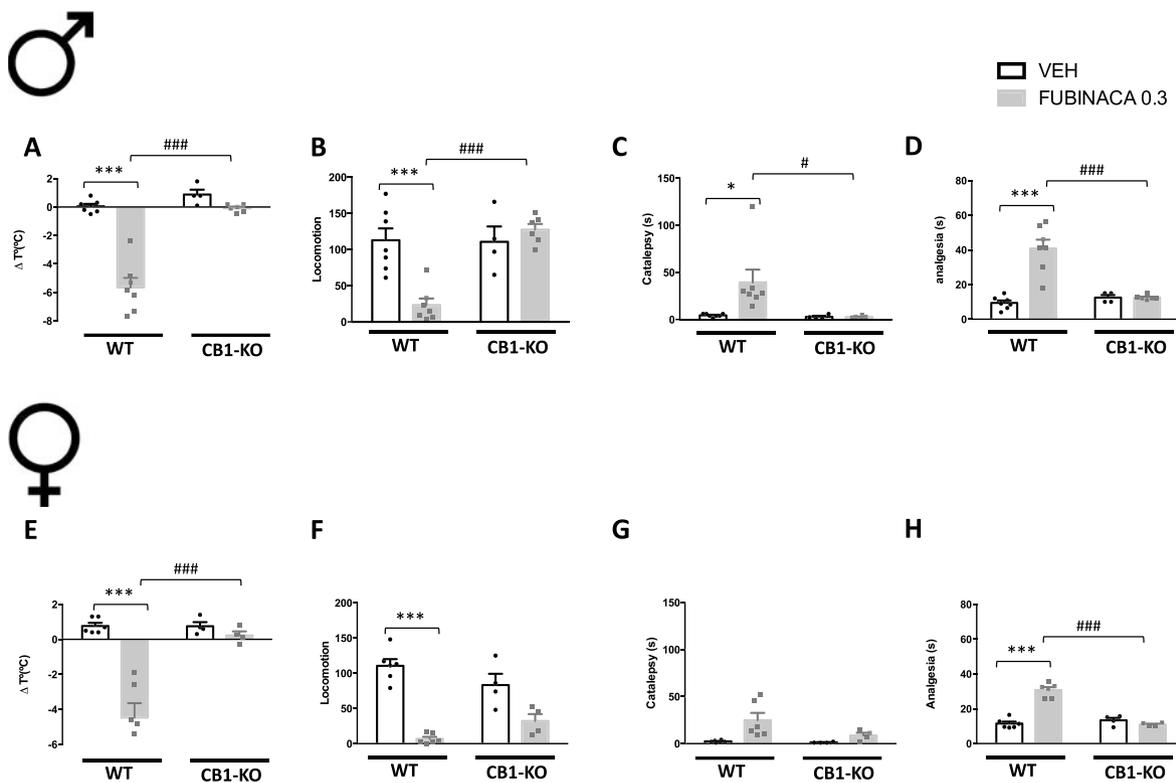


FIGURE 5 CB₁ receptors are necessary for the tetrad effects induced by MMB-Fubinaca. MMB-Fubinaca was administered 45 min before the tetrad test in both CB₁^{-/-} and CB₁^{+/+} mice. Note that the cannabinoid-like effects in temperature (A, E), locomotion (B, F), catalepsy (C, G), and analgesia (D, H) induced by MMB-Fubinaca were not present in mice lacking CB₁ receptors both in males and in females. Data are expressed as mean ± SEM (*n* = 6–12 per group). **p* < .05; ****p* < .001 compared with vehicle treated mice. #*p* < .05; ###*p* < .001 compared with WT mice

postinjection (Figure 6C,F). Interestingly, the MMB-Fubinaca-induced synaptic depression was much more pronounced in male mice as compared with females (Figure 6G–I), indicating the presence of a sex-dependent modulatory mechanism in these hippocampal effects.

4 | DISCUSSION

The recreational use of synthetic cannabinoids has been associated with adverse neurologic, cardiovascular, renal, pulmonary side effects, and in some cases even death.²⁷ Animal studies have reported several behavioral effects induced by synthetic cannabinoids,^{24,28–30} including a decrease on locomotion, antinociception, hypothermia, and increased catalepsy.^{23,24,31} In the present study, by using several approaches ranging from *in vitro* to *in vivo* analyses, we provide an extensive pharmacological profiling of MMB-Fubinaca (also known as FUB-AMB or AMB-Fubinaca)³ addressing different CB₁-mediated effects across different and independent experimental settings in male and female mice, as well as in human brain samples. Interestingly, we characterized the G-protein functional coupling and the ERK signaling effects induced by MMB-Fubinaca, and we found sex differences and CB₁-receptor dependent behavioral and electrophysiological alterations.

Novel synthetic cannabinoids have been shown to exert high agonist activity at human CB₁ receptors in cultured cells.³⁰ In particular, MMB-Fubinaca was shown to exert high affinity for human CB₁ and CB₂ receptors producing higher maximal effects in [³⁵S]GTPγS binding assays and a greater activation of β-arrestins than other cannabinoid agonists such as THC,^{6,7} which acted as a partial agonist in these functional assays. To go one step further, we have evaluated [³⁵S]GTPγS functional activity *ex vivo* in male and female human and mouse brain tissues comparing MMB-Fubinaca and WIN55,512-2. Both cannabinoids exhibit CB₁ receptor-dependent agonist activity profile; however, MMB-Fubinaca exerts about two orders of magnitude more potency than WIN55,512-2 in CB₁-receptor-mediated G-protein activation. The higher potency of MMB-Fubinaca is in agreement with previous findings in cells using a variety of functional assays (including [³⁵S]GTPγS).^{3,6,7,32} Thus, we might also expect higher maximal effect and potency of MMB-Fubinaca compared with THC at this functional level in native brain tissue. The pharmacological profile of MMB-Fubinaca in brain human and mouse native tissues shows pattern similarities in both species. Interestingly, MMB-Fubinaca and WIN 55,512-2 display higher E_{max} values in human cortical and hippocampal membrane preparations as compared with those in mice. These results suggest that *in vivo* CB₁ receptor-dependent maximal effects in humans might be even

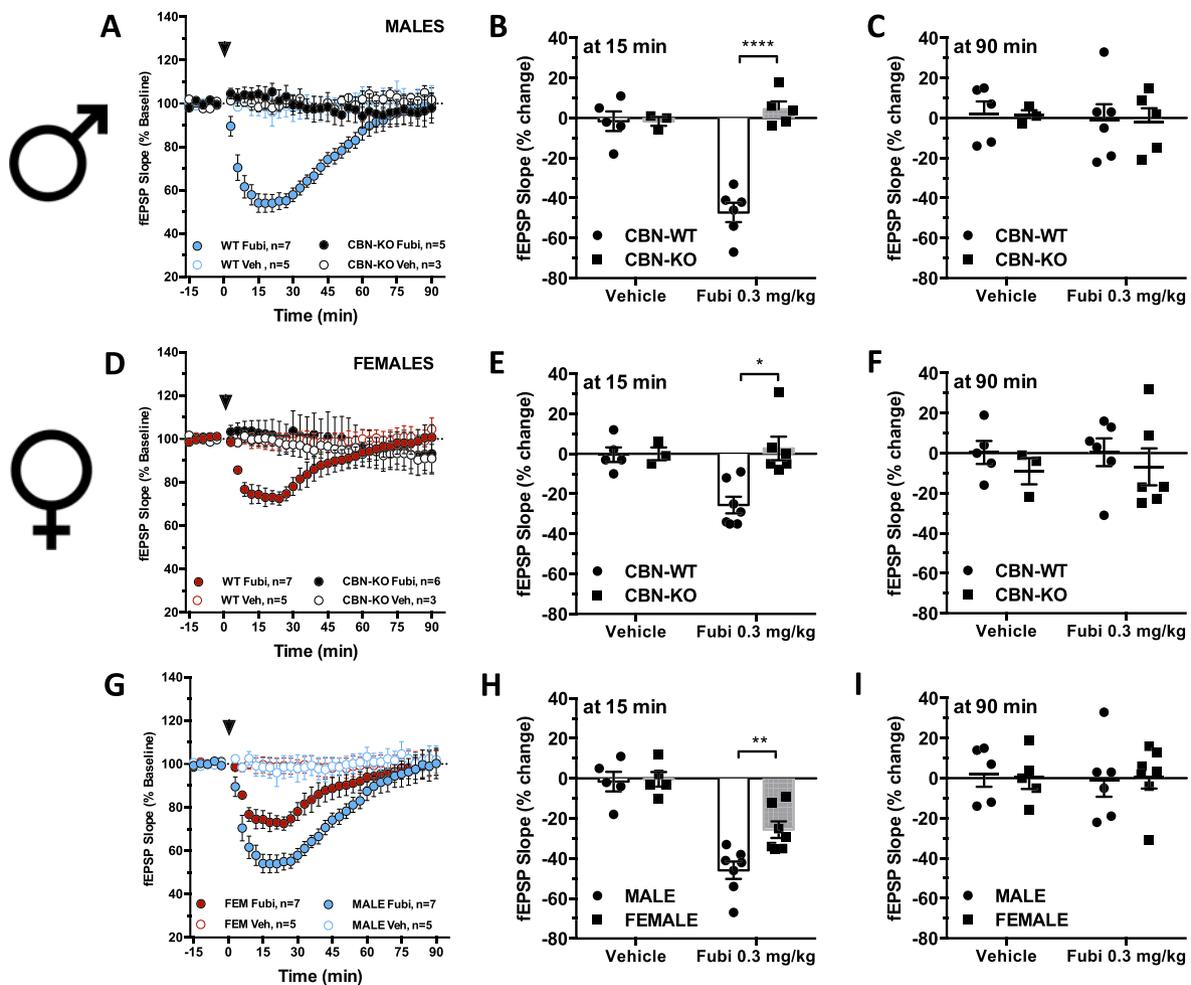


FIGURE 6 MMB-Fubinaca induces a transient modulation of hippocampal synaptic transmission in male and female mice. (A–C) MMB-Fubinaca induces a transient depression in male $CB_1^{+/+}$ but not in $CB_1^{-/-}$ mice. (A) Summary plots of recorded evoked fEPSP from $CB_1^{+/+}$ and $CB_1^{-/-}$ mice expose to vehicle or MMB-Fubinaca (Fubi, 0.3 mg/kg, i.p.). (B) Bar histograms representing the % of change in evoked field potentials 15 and (C) 90 min after vehicle or MMB-Fubinaca (Fubi, 0.3 mg/kg, i.p.). (D–F) MMB-Fubinaca induces a transient depression in female $CB_1^{+/+}$ but not in $CB_1^{-/-}$ mice. (D) Summary plots of recorded evoked fEPSP from female $CB_1^{+/+}$ and $CB_1^{-/-}$ mice expose to vehicle or MMB-Fubinaca (Fubi, 0.3 mg/kg, i.p.). (E) Bar histograms representing the % of change in evoked field potentials 15 and (F) 90 min after control or MMB-Fubinaca (Fubi, 0.3 mg/kg, i.p.). (G–I) MMB-Fubinaca induces a higher transient depression in male $CB_1^{+/+}$ as compared with females. (G) Summary plots of recorded evoked fEPSP from $CB_1^{+/+}$ and $CB_1^{-/-}$ male and female mice expose to vehicle or MMB-Fubinaca (Fubi, 0.3 mg/kg, i.p.). (H) Bar histograms representing the % of change in evoked field potentials 15 and (I) 90 min after vehicle or MMB-Fubinaca (Fubi, 0.3 mg/kg, i.p.). Data, mean \pm SEM. * $p < .05$, ** $p < .01$, *** $p < .0001$

higher than those observed in mice, offering valuable information for future translational studies.

CB_1 receptors modulate several signaling pathways, including ERK, p38, and c-Jun N-terminal kinase.³³ The activation of ERK signaling pathway has been linked to the molecular alterations associated with cannabinoid intoxication.³⁴ In this context, previous findings have characterized the signaling effects of MMB-Fubinaca including its impact on cAMP levels, pERK activation, or β -arrestins translocation.^{6,7} Using an *in vitro* genetic approach (i.e., CB_1 receptor transfection in HEK cells), our work confirms and extends these previous results by showing that MMB-Fubinaca induces activation of the ERK signaling pathway in a CB_1 receptor-dependent manner (i.e., effect observed only in HEK cells expressing CB_1 receptors). In our conditions, although we found differences in the [³⁵S]GTP γ S assay, we

were not able to see a significantly greater effect of MMB-Fubinaca compared with WIN 55,512-2 on ERK signaling as previously reported.⁷ This discrepancy could be attributed to different experimental procedures with different sensitivities used between studies.

MMB-Fubinaca intoxication in mouse models has been shown to induce cannabinoid-like behavioral effects such as seizures, hypocomotion, or hypothermia.^{3,6,24,28,29,35} Interestingly, a sex-dependent effect of MMB-Fubinaca has been described to be blocked by CB_1 antagonists in a paradigm assessing addictive-like behavior.³⁶ However, previous studies only used pharmacological tools and did not address CB_1 dependency by using specific mutant mice lacking CB_1 receptors. In this regard, our results show that MMB-Fubinaca induces clear CB_1 -dependent behavioral effects in male and female mice.

Natural and synthetic cannabinoid agonists have been shown to induce a specific *in vivo* cannabinoid-induced LTD (CB-LTD).²⁶ However, it remained to be addressed if novel synthetic cannabinoids such as MMB-Fubinaca can induce similar CB₁-dependent synaptic plasticity effects. Interestingly, acute administration with MMB-Fubinaca induces a robust *in vivo* transient decrease of synaptic transmission in the hippocampus of CB₁^{+/+} but not in CB₁^{-/-} mice, suggesting that CB₁-dependent mechanisms also underlie these pharmacological effects. This effect, which is greater in male compared with female mice, is similar in amplitude as the one of THC previously described in male mice.³⁷ However, whereas THC and the synthetic cannabinoid HU-210 induced a sustained *in vivo* CB-LTD, the decrease in synaptic transmission induced by MMB-Fubinaca is reversible, suggesting that distinct CB₁ receptor agonists can differentially modulate synaptic plasticity independently of the potency of the drug. Future studies will address the mechanisms underlying these interesting pharmacological profile differences across different cannabinoid agonists. The observed sex differences could be explained considering the larger hippocampal volume or synapse number in male compared with female rodents.³⁸ However, the explanation might be more intriguing as it is known that CB-LTD is dependent on astroglial CB₁ receptors,²⁶ and there is currently no evidence about the sex-dependent distribution of these receptors in mice. Thus, a proper analysis combining number of synapses, number of active astroglial processes, and/or CB₁ receptor levels in these compartments would be needed to define the anatomical differences possibly underlying the MMB-Fubinaca-induced sex differences.

Ex vivo [³⁵S]GTPγS binding assays revealed no sex differences in the potency and maximal effects of MMB-Fubinaca, whereas sex differences were found in the behavioral and electrophysiological experiments. Several possibilities might explain this discrepancy. First, whereas [³⁵S]GTPγS assays reflect events immediately after agonist binding to the receptor (G-protein activation), they do not address downstream pathways. Thus, G-protein activation might be similar between sexes, but important differences might exist in downstream effectors, thereby contributing to the behavioral and electrophysiological dissimilarities observed. Second, in these experimental conditions, we mainly analyze inhibitory G-protein modulation, but other G-proteins might be involved in the *in vivo* effects of MMB-Fubinaca as it has been shown for other cannabinoid drugs.³⁹ Moreover, MMB-Fubinaca might have different sex-dependent pharmacokinetic profiles and metabolite generation,^{32,40} thereby potentially explaining the differential effects. In short, whereas our results exclude differences at the level of G-protein activation, future studies will determine the mechanisms underlying the sex-dependent effects of MMB-Fubinaca.

The study of sex-dependent effects of cannabinoid drugs is growing and has been extensively discussed in the field.^{9,41} In particular, several studies have revealed the impact of sex differences on CB₁-dependent effects during adolescence,¹⁰⁻¹⁴ addiction,¹⁵⁻¹⁷ antinociception and locomotion,¹⁸ among others. Accordingly, previous studies also showed sex differences in tetrad behaviors induced by THC.^{42,43} In addition, the sex-biased impact of MMB-Fubinaca

has recently started to be evaluated.³⁶ Our results show a higher sensitivity for MMB-Fubinaca in male than in female mice, at both behavioral and synaptic levels. However, future research is needed to elucidate the mechanisms underlying these sex differences, which are not fully understood. In this context, biochemical evidence showed sex-dependent differences in the density and function of CB₁ receptors in specific brain regions^{9,44-46} and through specific cell-type mechanisms.⁴⁶ Indeed, sex differences in the endocannabinoid system seem to emerge early in development,¹³ and sex hormones can interact with CB₁ receptor expression⁴⁷ and endocannabinoid levels⁴⁸ in an estrous cycle-dependent manner in female rodents. Another possibility explaining sex-biased effects might be related to the sex differences on brain dopaminergic activity as it is well known that cannabinoids can act on specific cell types of the reward system.⁴⁹

Our results suggest the existence of sex-dependent effects induced by MMB-Fubinaca. However, we must acknowledge some limitations and open questions that emerge from our study. First, the impact of gonadal hormones on the sex differences observed was not directly assessed in the present study. The role of sex hormones on behavior in general,⁵⁰ or in cannabinoid-induced sex-dependent effects⁹ in particular, has been widely discussed. Future experiments must address whether testosterone and/or estrogens are responsible or contribute for such sex differences in MMB-Fubinaca-induced *in vivo* effects. Second, the behavioral responses evaluated in our work represent only a part of the wide spectrum of cannabinoid-induced effects. In this study, we have used the classic tetrad procedure to characterize the *in vivo* effects of MMB-Fubinaca, but future studies are necessary to address its effects on other behavioral aspects such as cognition, addiction, or psychotic-like symptoms. Moreover, an extended analysis of potential baseline sex differences on synaptic activity (e.g., input-output curves using ex vivo electrophysiology, population spikes using *in vivo* electrophysiology) might be useful to better understand the mechanisms mediating these behavioral effects in male and female mice. Finally, an extended analysis of the MMB-Fubinaca effects on other brain regions will be necessary to complete the pharmacological profile of MMB-Fubinaca.

Overall, this work provides new information to better understand the pharmacological profile of the potent synthetic cannabinoid MMB-Fubinaca.^{3,5,6} By using human native tissue, male and female mutant mice and the evaluation of *in vivo* hippocampal synaptic plasticity, our work adds valuable information to better understand how this synthetic cannabinoid exerts its deleterious effects.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Conceived the idea: A.B-G, G.M. *Performed experiments:* C.M. (^{35}S GTP γ S assays); A.C.P.Z. (*in vitro* ERK analysis); C.I., P.G-S, M. F (behavioural experiments); J.F.O.d.C (*in vivo* electrophysiology). *Supervised experiments and analyzed data:* A.B-G, G. M, L.F.C. *Wrote the manuscript:* A.B-G, J.F.O.d.C, C.I., A.C.P.Z, C.M. *Edited and approved the manuscript:* All co-authors

DATA AVAILABILITY STATEMENT

The raw data and original gels relative to figures will be available upon request to the corresponding author.

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