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Development and characterization of the CB1-E134G transgenic mouse model to study the role of endogenous pregnenolone in modulating the endocannabinoid system

Pierre-Louis Raux

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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 la Santé

SPÉCIALITÉ – Neurosciences

Par Pierre-Louis RAUX

**Développement et caractérisation du modèle transgénique murin
CB1-E134G pour l'étude du rôle de la pregnénolone endogène dans
la modulation du système endocannabinoïde**

**Development and characterization of the CB1-E134G transgenic
mouse model to study the role of endogenous pregnenolone in
modulating the endocannabinoid system**

Sous la direction de : Monique VALLÉE

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RESEARCH UNIT

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RESUME (FR)

Le système endocannabinoïde (SEC) est un système biologique complexe qui a suscité un intérêt scientifique considérable ces 50 dernières années. La grande majorité de nos connaissances sur le SEC est associée au récepteur aux cannabinoïdes de type 1 (CB1), récepteur à 7 domaines transmembranaires couplé aux protéines G. Le CB1 constitue le récepteur métabotropique le plus abondant dans le cerveau des mammifères et est impliqué dans la régulation de nombreux processus physiologiques. Ainsi, la dérégulation de son activité est associée à l'apparition de nombreuses pathologies. En outre, la récente découverte de mécanismes de régulation endogènes mis en place par l'organisme pour maintenir la charge homéostatique associée au récepteur, constitue un intérêt thérapeutique majeur. Cette étude s'intéresse tout particulièrement à la pregnénolone (PREG), un stéroïde identifié comme modulateur allostérique négatif endogène du CB1. Il a été montré que la suractivation du CB1 par son agoniste exogène et composant psychoactif principal du Cannabis, le Δ^9 -tétrahydrocannabinol (THC), entraîne une augmentation des niveaux centraux de PREG. La PREG se lie ensuite au CB1 au niveau de la région TMH1/TMH8/Hx8 et bloque sélectivement les voies de signalisation médiées par le récepteur. Ainsi, la PREG est impliquée dans une boucle de rétroaction négative protégeant le cerveau des effets toxiques du THC. Néanmoins, nos connaissances actuelles sur l'interaction entre la PREG et le CB1 demeurent partielles. En effet les outils pharmacologiques actuels sont largement insuffisants pour identifier de nouveaux rôles de la PREG endogène dans la modulation des fonctions médiées par CB1. Afin de mieux appréhender ce sujet, nous rapportons ici le développement et la caractérisation d'une nouvelle lignée de souris transgéniques porteuses d'une mutation ponctuelle « faux-sens » dans le gène codant pour CB1 (*Cnr1*). Cette mutation entraîne la substitution E134G visant à perturber le site de liaison allostérique de la PREG sur le récepteur. Les objectifs généraux de ce travail de thèse ont ainsi eu pour but de décrire les phénotypes des souris CB1-E134G mâles et femelles à un niveau basal, dans des conditions expérimentales connues pour induire une activation endogène du CB1, et en réponse à l'administration de cannabinoïdes exogènes. Nos résultats n'indiquent pas d'altération majeure dans les phénotypes comportementaux des souris mutantes CB1-E134G par rapport aux souris témoins « Wild-type » (WT), au niveau basal et après une activation endogène du récepteur, suggérant que la PREG endogène ne régule pas cette activité CB1. Cependant, nous observons une baisse considérable de l'expression de la protéine CB1 dans le cerveau des souris CB1-E134G naïves, suggérant l'existence d'un mécanisme compensatoire pour prévenir la surcharge d'activation du récepteur suite à l'absence de liaison de la PREG. Par ailleurs, nous notons une efficacité accrue des cannabinoïdes exogènes chez les souris CB1-E134G par rapport aux souris WT, au niveau comportemental, somatique et neurochimique.

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De façon surprenante, la PREG semble réguler préférentiellement l'activité du CB1 associé à la mitochondrie par rapport au CB1 associé à la membrane plasmique, impliqués respectivement dans les effets locomoteurs et antinociceptifs des cannabinoïdes. Ainsi, ces données confirment que la PREG représente un mécanisme de défense contre la suractivation du CB1 et suggèrent que cette boucle de rétroaction négative implique préférentiellement les récepteurs associés à la mitochondrie. Ainsi, la PREG agirait pour protéger des effets secondaires moteurs des cannabinoïdes tout en conservant les effets à potentiel thérapeutique, comme l'analgésie. En conclusion, l'utilisation de ce nouveau modèle transgénique pourrait ouvrir de nouvelles voies pour étudier les mécanismes d'action de la PREG en tant que modulateur allostérique négatif du CB1 et ainsi offrir de nouvelles perspectives thérapeutiques.

Mot clés:

Neuroendocrinologie, Pregnenolone, Cannabinoïdes, CB1, Modèle génétique murin, Comportement

ABSTRACT (EN)

The endocannabinoid system (ECS) is a complex neuromodulatory network that has attracted tremendous research interest over the past 50 years. The vast majority of our knowledge of ECS-mediated signaling is associated with the type 1 cannabinoid receptor (CB1R), a seven-transmembrane domain G-protein coupled receptor. CB1R is expressed in various organs and tissues, with the highest levels found in the brain, where it is the most abundant metabotropic receptor in mammals and is intimately involved in the regulation of a plethora of biological functions. Because CB1R controls such a broad range of physiological processes, dysregulation of CB1-mediated signaling has been implicated in the pathogenesis of many central and peripheral disorders. Thus, CB1R represents an exciting therapeutic target for the development of pharmacotherapies in the context of neuropsychiatric and metabolic diseases. From this perspective, the discovery of endogenous regulatory mechanisms to prevent an imbalance in CB1R activity represents a promising therapeutic strategy to restore the CB1R-associated homeostatic load. In particular, the steroid pregnenolone (PREG) has been identified as an endogenous CB1-negative allosteric modulator with neuroprotective effects. Interestingly, CB1 overactivation by the exogenous CB1 agonist Δ^9 -tetrahydrocannabinol (THC), the primary psychoactive component of *Cannabis sativa*, has been shown to upregulate central PREG levels, establishing an endogenous negative feedback loop between the steroid and the CB1R. Indeed, PREG is able to bind to the TMH1/TMH8/Hx8 region of the CB1R and selectively inhibit CB1-mediated signaling pathways to protect against the acute and toxic effects of THC. However, our current understanding of the interaction between PREG and CB1R remains elusive, and the limited pharmacological tools available are insufficient to identify novel roles for PREG in modulating CB1-mediated signaling and functions. To further explore this topic, we report the development and characterization of a novel transgenic mouse line carrying a missense point mutation in the CB1-encoding gene (*Cnr1*), resulting in the amino acid substitution E134G, aimed at disrupting the PREG allosteric binding site on CB1. The general objectives of this work are to describe the phenotypes of male and female CB1-E134G mice at baseline, under experimental conditions known to induce endogenous CB1R activation, and in response to exogenous cannabinoid administration. Analyses show little to no change in the behavior of mutant CB1-E134G mice compared to their wild-type (WT) littermates at baseline and after endogenous CB1 activation, suggesting that endogenous PREG does not regulate this CB1 activity. However, CB1 protein was found to be drastically downregulated in the brain of naive CB1-E134G mutants, suggesting a compensatory mechanism to prevent CB1R activation overload and a role for PREG in CB1R dynamics *in vivo*. In addition, CB1-E134G mice exhibited enhanced efficacy of exogenous cannabinoids at the behavioral, somatic, and neurochemical

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levels compared to WT mice. Interestingly, PREG was found to preferentially regulate striatonigral mitochondria-associated CB1R over plasma membrane-associated CB1R involved in cannabinoid-mediated locomotor effects and antinociception, respectively. Collectively, these data confirm that PREG represents a defense mechanism against CB1 overactivation and suggest that this regulatory loop may involve specific intracellular subsets of receptors to maintain homeostasis. Furthermore, it can be hypothesized that PREG represents a molecular switch to bias the action of cannabinoids toward therapeutically relevant analgesia by inhibiting motor side effects, suggesting the therapeutic potential of PREG in cannabis use disorders. Finally, future use of this model may provide new opportunities to elucidate the mechanism of action of PREG as a CB1 negative allosteric modulator and new therapeutic perspectives.

Key words:

Neuroendocrinology, Pregnenolone, Cannabinoids, CB1, Transgenic mouse model, Behavior

RESUME ETENDU (FR)

Le système endocannabinoïde (SEC) est un système biologique complexe qui a suscité un intérêt considérable de la part des chercheurs au cours des 50 dernières années. Son identification découle de l'isolement et de l'élucidation des composants phytochimiques de la plante *Cannabis sativa* (i.e., phytocannabinoïdes), et en particulier du principal ingrédient psychoactif responsable des effets intoxicants du cannabis, le Δ^9 -tétrahydrocannabinol (THC). De par sa nature lipidique, il a d'abord été postulé que le THC produisait ses effets intoxicants en perturbant les lipides membranaires de manière aspécifique. Cependant, une série de découvertes au cours des années 1980 ont conduit à l'identification de sites de liaisons au THC dans le cerveau du rongeur de laboratoire, compatibles avec l'existence d'un récepteur sensible aux phytocannabinoïdes. Ce récepteur, appartenant à la superfamille des récepteurs à sept domaines transmembranaires couplés à des protéines G (RCPG), a ainsi été officiellement dénommé "récepteur aux cannabinoïdes de type 1" (CB1). Suite à l'identification du récepteur CB1, d'autres acteurs moléculaires endogènes fonctionnellement liés aux effets des phytocannabinoïdes ont été décrits, y compris un second récepteur aux cannabinoïdes (CB2) et les ligands endogènes à ces récepteurs ("endocannabinoïdes" ; eCBs), principalement le 2-arachidonoyglycérol (2-AG) et l'anandamide (AEA). Ainsi, les récepteurs CB1, CB2, les eCBs et les enzymes responsables de la biosynthèse et de l'inactivation des eCBs constituent le SEC.

Bien que des années de recherche aient révélé la complexité croissante du SEC avec l'identification de centaines de partenaires moléculaires associés à ce système biologique, la grande majorité de nos connaissances sur la signalisation intracellulaire mise en jeu au sein du SEC est associée au récepteur CB1. Le CB1 est exprimé dans divers organes et tissus, mais les niveaux les plus élevés se trouvent dans le cerveau, où il est l'un des récepteurs métabotropiques les plus abondants chez les mammifères. Plus précisément, le récepteur CB1 est majoritairement exprimé à la membrane plasmique de neurones pré-synaptiques, où il inhibe la libération de neurotransmetteurs, participant ainsi au contrôle de la plasticité neuronale. En plus de sa localisation pré-synaptique, le récepteur CB1 est fonctionnellement couplé à des organites intracellulaires, tels que la membrane externe de la mitochondrie, où il inhibe l'activité de la chaîne respiratoire et la phosphorylation oxydative, contrôlant ainsi l'économie énergétique de la cellule. Ainsi, le récepteur CB1 exerce un rôle prédominant dans la modulation de l'activité cellulaire.

De par son abondance dans le cerveau et ses nombreuses fonctions cellulaires, le récepteur CB1 est impliqué dans la régulation de nombreux processus biologiques tels que les comportements liés aux émotions, la mémoire et l'apprentissage, les circuits de la

récompense, la motricité, la nociception et la balance énergétique. Aussi, parce qu'il contrôle un tel éventail de fonctions physiologiques, la dérégulation de son activité est impliquée dans l'étiologie de nombreuses pathologies, ce qui en fait une cible thérapeutique majeure. De fait, l'identification de mécanismes biologiques endogènes contrôlant l'activité de ce récepteur et permettant de maintenir les processus de l'homéostasie associée au SEC représente une stratégie thérapeutique intéressante. Parmi ces mécanismes biologiques, la pregnénone (PREG), un stéroïde endogène dérivé du cholestérol, a été identifié comme modulateur allostérique négatif du récepteur CB1. Notamment, il a été montré que la sur-activation du récepteur CB1 par le THC, impliqué dans les désordres associés à l'intoxication et l'addiction au cannabis, augmente la production cérébrale de PREG. De façon rétroactive, la PREG se fixe alors au récepteur au niveau d'une poche de liaison allostérique située à l'interface des régions TMH1/TMH8/Hx8, entraînant l'inhibition sélective des voies de signalisation médiées par le récepteur CB1 et protégeant ainsi des effets aigües et toxiques du THC.

Au cours des dernières années, les propriétés neuroprotectrices de la PREG ont généré un intérêt clinique majeur, avec le développement d'analogues synthétiques non métabolisables de la PREG, offrant ainsi une découverte thérapeutique significative pour le traitement des troubles liés à l'usage du cannabis. Néanmoins, la contribution endogène de la PREG dans la modulation de l'activité du récepteur CB1 et des rôles physiologiques du SEC reste méconnue. Aussi, une telle étude est nécessaire pour mieux comprendre le rôle fonctionnel endogène de la PREG dans la régulation de l'activité du récepteur CB1, mais également pour mieux appréhender le potentiel thérapeutique des analogues synthétiques de la PREG en clinique. Pour répondre à cet objectif de recherche et afin de contourner les limitations techniques associées à la manipulation directe des niveaux centraux de PREG chez le rongeur, nous proposons dans cette étude une approche méthodologique alternative originale reposant sur l'invalidation du site de liaison de la PREG sur le récepteur CB1.

Cette approche est basée sur l'identification *in silico* d'un acide aminé critique pour la stabilisation de la PREG dans sa poche de liaison allostérique. Plus précisément, les analyses *in silico* ont permis de prédire l'existence d'une liaison hydrogène entre le groupe cétone de la PREG et le résidu glutamate E.1.49 situé dans le premier domaine transmembranaire du récepteur CB1 humain à la position 133 de la méthionine. Des études consécutives menées *in vitro* ont confirmé que la substitution de ce résidu par une glycine, ne formant pas de liaison hydrogène, empêche les effets modulateurs de la PREG sur l'activité du récepteur. Par conséquent, nous proposons ici d'appliquer cette stratégie de mutagenèse chez la souris en substituant le résidu E.1.49 du récepteur CB1 murin à la position 134 de la méthionine, afin de générer un mutant chez lequel la PREG ne peut pas se fixer au récepteur CB1. L'objectif général de ce travail de recherche a donc été de décrire les phénotypes moléculaires et

comportementaux de souris mutantes CB1-E134G mâles et femelles adultes dans divers paradigmes expérimentaux.

En premier lieu, nous avons cherché à fournir une caractérisation générale de la lignée CB1-E134G en conditions basales afin d'explorer de potentielles conséquences fonctionnelles de la mutation et de l'absence de liaison de la PREG au récepteur CB1 au cours du développement. Dans un deuxième temps, nous avons cherché à étudier les effets moléculaires et comportementaux de l'administration de cannabinoïdes exogènes, dont le THC, afin de confirmer la validité apparente de notre modèle, c'est-à-dire la susceptibilité phénotypique associée à une suractivation du récepteur CB1 et à la perte des effets neuromodulateurs de la PREG. Ce deuxième objectif a également eu pour but d'aborder la contribution endogène de la PREG dans la régulation de l'activité du récepteur CB1 en conditions « supra physiologiques » induites par l'administration de cannabinoïdes exogènes. Enfin, afin d'étudier les fonctions endogènes et physiologiques de la PREG dans le maintien de la charge homéostatique associée au récepteur CB1, nous avons soumis les souris mutantes CB1-E134G à divers challenges environnementaux, ayant pour but de manipuler de façon différentielle l'activité du SEC.

Les principaux résultats de ces travaux de recherche indiquent une baisse drastique des niveaux d'expression du récepteur CB1 dans le cerveau des souris mutantes CB1-E134G par rapport à leurs homologues sauvages. Cette baisse est associée à une hypersensibilité somatique, comportementale et neurochimique en réponse aux cannabinoïdes exogènes. L'ensemble de ces résultats confirme la perte de la régulation allostérique endogène de la PREG sur l'activité du récepteur CB1 chez les mutants CB1-E134G. Par ailleurs, bien que les souris CB1-E134G soient globalement plus sensibles aux effets des cannabinoïdes exogènes, certains de ces effets demeurent similaires chez les mutants par rapport aux souris sauvages. En particulier, nos résultats indiquent une dichotomie apparente entre les effets analgésiques des cannabinoïdes, qui ne sont pas affectés chez les mutants par rapport aux sauvages, et leurs effets moteurs, qui sont amplifiés. Ces données suggèrent en outre que, de manière endogène, la PREG régule spécifiquement certains effets délétères des drogues cannabimimétiques sans altérer leurs effets bénéfiques. De façon très intéressante, nos résultats suggèrent également que la PREG n'agit pas sur tous les récepteurs CB1, mais de façon biaisée (préférentielle) sur les récepteurs CB1 associés à la mitochondrie, responsables des effets moteurs des cannabinoïdes, et pas sur les récepteurs CB1 situés à la membrane plasmique, responsables de leurs effets analgésiques.

En parallèle des effets différentiels des cannabinoïdes chez les souris CB1-E134G, nous montrons que la libération de dopamine sous-corticale induite par le THC est augmentée

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chez les souris mutantes par rapport aux souris sauvages. Ces effets étant associés aux propriétés renforçantes du cannabis et représentant un des substrats moléculaires impliqués dans les troubles liés à la prise de cannabis, nous postulons que ces souris présentent une plus grande propension à développer une addiction aux drogues cannabimimétiques. Aussi, de futures expériences seront réalisées dans cette perspective afin d'étudier la motivation à utiliser de telles drogues, et les endophénotypes comportementaux associés à la perte de contrôle. Enfin, bien que les animaux mutants CB1-E134G soient caractérisés par une absence globale de phénotypes comportementaux en conditions basales, l'étude des animaux en réponse à divers challenges environnementaux a révélé l'occurrence de patterns d'adaptation spécifiques, se traduisant notamment par une baisse plus forte de la glycémie chez les mutants en réponse à un jeûne alimentaire. Ces patterns d'adaptation pouvant indiquer un rôle potentiel de la PREG dans la régulation des mécanismes physiologiques gouvernés par les récepteurs CB1, de futures études seront menées pour explorer plus en détail ces processus.

Ainsi, notre travail a permis de valider le modèle murin CB1-E134G comme pouvant être un outil approprié pour l'étude de la régulation endogène de la PREG sur le récepteur CB1 et des mécanismes moléculaires impliqués dans les interactions stéroïdes-endocannabinoïdes. En outre, nous émettons l'hypothèse que la PREG agit comme un commutateur moléculaire qui oriente l'action des cannabinoïdes vers des effets présentant un intérêt thérapeutique (i.e., analgésie), tout en inhibant leurs effets secondaires moteurs. Cette étude renforce ainsi d'avantage l'intérêt clinique des analogues synthétiques de la PREG pour leur utilisation dans le contexte de l'addiction au cannabis et des effets intoxicants de la consommation de cannabis chez l'homme, et ouvre de nouvelles perspectives dans l'étude des effets de la PREG sur l'activité du récepteur CB1 en tant que modulateur allostérique.

Mots clés:

Neuroendocrinologie, Pregnenolone, Cannabinoïdes, CB1, Modèle murin transgénique, Comportement

INTRODUCTION

1. The Endocannabinoid System (ECS)

1.1. From Cannabis to the ECS

Since its discovery in the late 20th century, the endocannabinoid system (ECS) has been at the center of groundbreaking discoveries that shed light on its essential role in human pathophysiology. To understand the history of the ECS, one must trace its origins back to the plant that gave it its name, *Cannabis sativa*, and its early use by ancient civilizations.

The cannabis plant, also known as hemp or marijuana, is believed to be native to Western and Central Asia (Small 2015). According to a recent biogeographic study, wild cannabis may have originated on the eastern Tibetan plateau about 28 million years ago and then spread to the west (Europe, present-day Russia) by 6 million years ago and to the east (present-day China) by 1.2 million years ago (McPartland, Hegman, and Long 2019). By the end of the Pleistocene Epoch (11,700 BP), Cannabis hemp had spread throughout Eurasia and was available for humans to harvest and domesticate (Tarasov et al. 2007). The earliest evidence of harvesting and cultivation dates back to 8500 and 6000 BP, respectively, making hemp one of the oldest crops used by humans (Small 2015). Cannabis has been valued primarily as a source of fiber (for making rope and textiles) and food (edible seeds) rather than for its euphoric effects. Indeed, wild hemp has low levels of psychoactive compounds (the most abundant being Δ^9 -tetrahydrocannabinol, THC), and it is still debated as to when, where, and how the plant was first cultivated for its intoxicating properties (Small 2015; Crocq 2020).

Some of the earliest evidence for the use of cannabis as a mind-altering plant was discovered at the Yanghai Tombs (Xinjiang, Western China), where archaeologists have unearthed cannabis macro-remains from burial sites dating back to 350 BC. Phytochemical analyses of the plant revealed high levels of THC and cannabiol (CBN, an oxidative degradation product of THC), suggesting, but not confirming, shamanic use of the plant (Russo et al. 2008). More recently, excavations at the Jirzankal cemetery (Pamir Mountains, western China) revealed the presence of braziers containing high-THC/high-CBN cannabis residues. Radiocarbon analysis dated the burials to 500 BC, thus providing the earliest solid archaeological evidence for the ritualized use of potent cannabis (Ren et al. 2019). At both the Yanghai and Jirzankal sites, cannabis residues had much higher THC levels than wild hemp. Humans may have deliberately bred high-potency cannabis for its psychoactive properties, but it has also been suggested that high THC levels in cannabis may result from adaptive changes in response to natural stressors, such as the high altitudes of the western Chinese plateaus (Small 2015; Ren et al. 2019).

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In addition to its use as a psychostimulant in ritual and religious practices, cannabis was rapidly used for medical purposes to treat pain, constipation, fever, skin diseases, and nausea, among others (Crocq 2020). Some of the earliest records of the use of cannabis for its medicinal properties are found in China. In particular, ancient Chinese have reported the plant's virtues in the *Shénnóng běncǎo jīng* (神農本草經) ("Compendium of 365 Medicinal Herbs"), the world's oldest pharmacopeia compiled between 221 BC and 220 AD, but likely based on oral traditions dating back to around 2000 BC (Pisanti and Bifulco 2019; Crocq 2020). In India, cannabis was widely used as both a recreational drug and as a medicine and maintained a direct link with religious practices, as mentioned in the Atharva Veda (अथर्ववेद), a collection of sacred texts, in which cannabis is referred to as a "divine ingredient" (Crocq 2020). Over the centuries, human migrations and trade routes spread the use of the cannabis plant to Western Europe, the Middle East, and Africa. Mentions of medicinal and recreational cannabis use can be found in ancient Egypt, Greece, and the Roman Empire, thus reflecting its spread. Later, the European colonization of the Americas and the transatlantic slave trade ended the global spread of cannabis (Lozano 2001; Pisanti and Bifulco 2019; Crocq 2020).

In the 19th century, the rise of Orientalism in European centers of learning and their colonial outposts led to a revival of scholarly interest in ancient Oriental manuscripts and the rediscovery of the medicinal and psychoactive properties of cannabis (Pisanti and Bifulco 2019). The revival of interest in cannabis in Europe is primarily attributed to two physicians: William Brooke O'Shaughnessy (1808-1889) and Jacques-Joseph Moreau de Tours (1804-1884). While traveling in India, O'Shaughnessy noticed that the local cannabis plant (which he named "Indian cannabis" or *Cannabis indica*) produced significant euphoric effects as compared to the variety grown in Europe for fiber production (*Cannabis sativa*). Interested in the pharmacological properties of the plant, O'Shaughnessy reported its therapeutic (especially analgesic) and psychoactive properties, which contributed to the growing popularity of the plant in Europe (Booth 2005; Pisanti and Bifulco 2019). Following in O'Shaughnessy's footsteps, Moreau de Tours, a French psychiatrist, investigated the psychotropic properties of cannabis in the hope of elucidating the mechanisms underlying mental disorders for cannabis "produced disorganization of ideas and temporal distortions" (Crocq 2020). The use of cannabis quickly expanded beyond therapeutic applications, and the reintroduction of cannabis into European societies led to its spread as a recreational drug (Pisanti and Bifulco 2019) in addition to its use medical use for pain relief, appetite stimulation, and sleep improvement in modern Western pharmacopeia (Booth 2005). However, in the early 20th century, the discovery of new molecules with better pharmacological profiles and non-psychoactive properties (such as aspirin), along with the growing concerns about the safety profile of Cannabis, led to the gradual decline of its use as a medicine. Cannabis was later

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classified as a substance of abuse, limiting its use primarily to basic research (Pisanti and Bifulco 2019; Crocq 2020).

More than 8,000 years have separated the first human contact with cannabis from the first discoveries of its phytochemical constituents. Of the hundreds of plant cannabinoids (phytocannabinoids) present in the *Cannabis sativa* plant, the first to be isolated from an oil extract in the late 19th century was cannabinol (CBN), a mildly psychoactive derivative of THC whose structure was later identified in the 1930s (Pertwee 2006). In 1940, a second non-psychoactive phytocannabinoid called cannabidiol (CBD) was identified, and its structure was elucidated in 1963 (Pertwee 2006). The discovery of CBD was quickly eclipsed by the isolation and elucidation of the structure of Δ^9 -tetrahydrocannabinol (THC) by R. Mechoulam in 1964, which proved to be the principal psychoactive ingredient responsible for the intoxicating effects of cannabis (Gaoni and Mechoulam 1964). This landmark discovery laid the foundation for the study of a new endogenous modulatory network. Because of its lipidic nature, it was initially thought that the psychoactivity of THC was the result of its ability to disrupt membrane lipids (Pertwee 2006), but further research in the 1980s indicated that the mechanism of action of THC was consistent with the modulation of the activity of a membrane receptor (A. C. Howlett and Fleming 1984; A. C. Howlett 1985; A. C. Howlett, Qualy, and Khachatrian 1986). Finally, 24 years after the identification of THC and thanks to the development of synthetic cannabinoid analogs, W.A. Devane formally discovered this receptor in the rat brain, named "type 1 cannabinoid receptor" (CB1) (W. A. Devane et al. 1988), later cloned in 1990 (Matsuda et al. 1990). Only four years later, the same scientist identified an endogenous ligand capable of binding to cannabinoid receptors, arachidonylethanolamine (AEA), also called "anandamide" after the Sanskrit word "Ānanda" (आनन्द), which means "bliss, pleasure" (W. Devane et al. 1992). A second cannabinoid receptor, the type 2 cannabinoid receptor (CB2), and a second endogenous ligand, 2-arachidonoylglycerol (2-AG), were subsequently identified (Munro, Thomas, and Abu-Shaar 1993; Mechoulam et al. 1995; Sugiura et al. 1995).

Since then, this endogenous receptor system, consisting of cannabinoid receptors, their endogenous ligands (endocannabinoids, eCBs), and all the enzymatic machinery involved in their synthesis and degradation, has become known as the "endocannabinoid system" (ECS). Over the years, hundreds of lipid mediators, more than 20 metabolic enzymes and receptors (nuclear receptors, ion channels, deorphanized GPCRs), and endogenous allosteric modulators have been found to interact with the ECS, forming the "extended ECS", also referred to as the endocannabinoidome or endocannabidome (eCBome) (Vincenzo Di Marzo 2020). The ever-increasing complexity of the ECS/eCBome raises the question of the importance of these new players in regulating physiological functions, their role in pathological

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alterations, and their significance for drug discovery, thus opening a new era in cannabinoid research (Ishiguro 2022).

1.2. Classical cannabinoid receptors

1.2.1. The type-1 cannabinoid receptor (CB1)

After the identification of the first phytocannabinoids and the main psychoactive component of *Cannabis sativa*, THC, the question arose as to how these molecules produced their effects. Howlett and colleagues showed that cannabimimetic compounds decreased the enzymatic activity of adenylate cyclase (AC; a membrane-bound enzyme involved in intracellular signaling) in a dose-dependent and stereoselective manner (i.e., (R)-(-)-enantiomers, which occur naturally in the *Cannabis* plant, produced more potent effects than their (S)-(+)-isoform). These effects of cannabimimetic drugs strongly suggested the involvement of a receptor rather than an aspecific mechanism of action, and evidence showed that the mechanism of action of THC was consistent with modulation of the activity of a seven-transmembrane domain (7TM) G-protein coupled receptor (GPCR) (A. C. Howlett and Fleming 1984; A. C. Howlett 1985). Indeed, the same authors showed that the cannabinoid-induced decrease in AC activity was blocked by pertussis toxin (PTX), an exotoxin inhibiting the activity of Gi signaling protein subunits coupled to GPCR, demonstrating the requirement of a functional Gi-coupled receptor in the action of cannabimimetic drugs (A. C. Howlett, Qualy, and Khachatryan 1986). In 1988, Devane and colleagues evidenced the existence of a cannabinoid receptor in the rat brain using tritium-labeled CP-55,940 ([³H]-CP-55,040), a synthetic cannabimimetic drug (W. A. Devane et al. 1988). Shortly after that, Matsuda and colleagues reported the identification of a cDNA from a rat cerebral cortex cDNA library, the translated sequence of which revealed a 473 amino acid protein belonging to the 7TM GPCR super-family, which was formally identified as the first cannabinoid receptor (type-1 cannabinoid receptor or CB1) (Matsuda et al. 1990). Homologs of CB1 receptors were subsequently identified in humans (Gérard et al. 1990) and mice (Chakrabarti, Onaivi, and Chaudhuri 1995), as well as in other mammalian vertebrates, non-mammalian vertebrates (such as amphibians and birds), and some invertebrate species (bony fish) (Elphick 2012). Interestingly, the CB1 amino acid sequence is highly conserved among species (with approximately 97 to 99% homology between humans, mice, and rats), suggesting that CB1-mediated signaling is a well-conserved system in evolution (Kano et al. 2009).

While the CB1 receptor is widely distributed in the body and present in a variety of organs, including the gastrointestinal tract, liver, pancreas, and muscle (Piazza, Cota, and Marsicano 2017), its highest expression levels are found in the central nervous system (CNS),

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and it is considered to be one of the most highly expressed GPCRs in the mammalian brain (Kano et al. 2009) ([Figure 1](#)). The earliest study of CB1 expression in the CNS was performed by Herkenham and colleagues using radiolabeled [3H]-CP-55,040 and reported that the CB1 expression pattern is highly conserved among the species studied (i.e., human, rhesus monkey, and rat) and is expressed at different levels in distinct brain regions (Herkenham et al. 1990). Brain regions with the highest levels of CB1 are the innermost layers of the olfactory bulb, hippocampus (molecular layer of the dentate gyrus and CA3), lateral striatum, globus pallidus, entopeduncular nucleus (or internal globus pallidus, GPi, in primates), substantia nigra (pars reticulata), and the molecular layer of the cerebellum. Moderate CB1 expression is found in the cerebral cortex, septal area, amygdala, ventromedial hypothalamus, target nuclei of the midbrain and hindbrain (lateral subnucleus of the interpeduncular nucleus, parabrachial nucleus, nucleus of the solitary tract), and spinal horn. Finally, the lowest levels of CB1 expression are found in the thalamus, other nuclei of the brainstem, and the ventral horn of the spinal cord (Herkenham et al. 1990; Kano et al. 2009).

Subsequent cloning of the CB1 cDNA allowed more detailed analysis of CB1 expression at the cellular level by *in situ* hybridization for CB1 mRNA and immunohistochemistry for CB1 protein (Matsuda et al. 1990). While CB1 was found in virtually all major cell types in the brain, including astroglial cells, oligodendrocytes, and microglia (Han 2012; Mato et al. 2009; Araujo, Tjoa, and Saijo 2019), it is primarily expressed in neurons, but at different levels depending on the neuronal population and its location. For example, one of the largest neuronal pools of CB1 receptors is found in cortical GABAergic interneurons, whereas cortical glutamatergic neurons express CB1 at lower levels (G. Marsicano and Lutz 1999). In addition to GABA and glutamate, CB1 is associated with other major neurotransmitter systems, including serotonergic, noradrenergic, and cholinergic neurons (Lutz 2020). Interestingly, CB1 expression levels are not proportional to CB1-mediated signaling. While CB1 is more abundant in GABAergic interneurons, G protein activation, as measured by [35S]GTPγS binding assays, is more efficient in glutamatergic neurons and accounts for the majority of CB1-dependent signaling within a given region, demonstrating a dichotomy between expression levels and functionality (Steindel et al. 2013).

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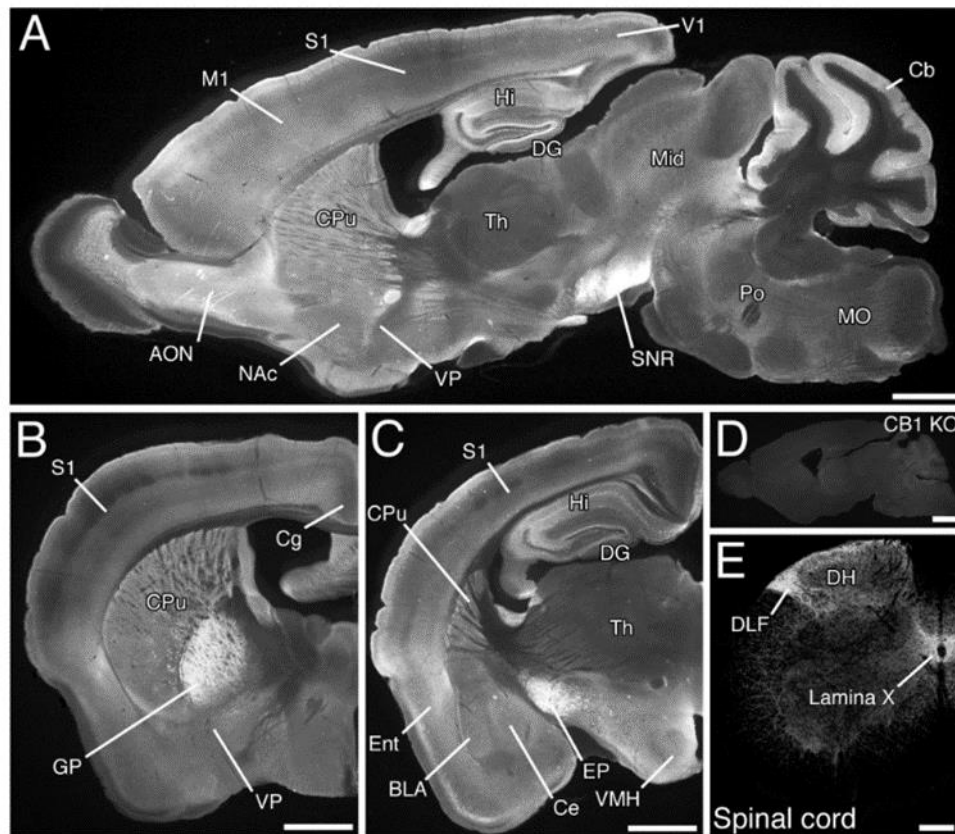


Figure 1: Distribution of CB1 receptors in the central nervous system and spinal cord of adult mice.

A-D: Overall distribution in parasagittal (**A** and **D**) and coronal (**B** and **C**) brain sections of wild-type (**A-C**) and full CB1 Knock-out mice (**D**) immunolabeled with a polyclonal antibody directed against the C-terminal domain of the mouse CB1 receptor. **Abbreviations:** AON, Anterior olfactory nucleus; BLA, Basolateral amygdala; Cb, Cerebellum; Cg, Cingulate cortex; Cpu, Caudate putamen; DG, Dentate gyrus; DH, Dorsal horn; DLF, Dorsolateral funiculus; Ent, Entorhinal cortex; EP, Entopeduncular nucleus; GP, Globus pallidus; Hi, Hippocampus; M1, Primary motor cortex; Mid, Midbrain; MO, Dendate molecular layer; NAc, Nucleus accumbens; Po, Pons; S1, Primary somatosensory cortex; SNR, Substantia nigra pars reticulata; Th, Thalamus, VMH, Ventromedial hypothalamus; VP, Ventral pallidum.

Adapted from [Kano et al., 2009](#).

In addition to its broad distribution across brain structures and cell types, CB1 can also be viewed in terms of its subcellular localization. One of the most important breakthroughs in cannabinoid research was the discovery that CB1 receptors are located on the plasma membrane of presynaptic nerve terminals. The presynaptic localization of CB1 was first demonstrated on cortical GABAergic axon terminals using immunogold labeling coupled with electron microscopy ([Katona et al. 1999](#)) and has since been identified in other major neurotransmitter systems that express CB1, including glutamatergic long-range projection neurons ([Katona and Freund 2008](#)). This discovery allowed the subsequent description of the

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mechanisms underlying cannabinoid-mediated signaling, in particular, the retrograde modulation of synaptic transmission (i.e., the release of endogenous CB1 ligands at postsynaptic terminals that reach presynaptic CB1 receptors to modulate neurotransmitter release) and the role of CB1 in the pleiotropic modulation of physiological functions, including memory, movement, emotion, and pain, among others ([See chapter 2](#)) (Lutz 2020).

Although the presynaptic localization of the CB1 receptor has long been recognized, there is evidence for a postsynaptic localization of CB1 receptors, suggesting an autocrine mechanism of action for cannabinoid signaling in addition to the classically described retrograde signaling (Bacci, Huguenard, and Prince 2004). However, anatomical evidence that postsynaptic CB1 receptors are expressed at the plasma membrane is still lacking, and it has been suggested that this receptor pool may coincide with intracellular subsets of CB1 receptors (Arнау Busquets-Garcia, Bains, and Marsicano 2018). Indeed, CB1 receptors have been detected in late endosomal/lysosomal compartments in the context of steady-state and agonist-induced internalization, where they can mediate signaling (Thibault et al. 2013; Rozenfeld and Devi 2008). Postsynaptic localization of CB1 receptors could also be attributed to another intracellular subset of CB1 receptors, the mitochondria-associated CB1 receptors (mtCB1). Indeed, immunogold labeling coupled with electron microscopy and functional assays revealed the presence of CB1 receptors localized to the outer mitochondrial membrane of neurons in the CA1 subfield of the hippocampus, where they mediate the reduction of oxygen consumption by exogenous and endogenous cannabinoids (Bénard et al. 2012). mtCB1 receptors have also been detected in the periphery in sperm and muscle at relatively higher levels than in the brain (Mendizabal-Zubiaga et al. 2016), in cerebral regions other than the hippocampus, including the basal ganglia (Soria-Gomez et al. 2021), and astrocytes (Jimenez-Blasco et al. 2020). In the brain, the presence of mtCB1 has implications for the regulation of memory processes (Hebert-Chatelain et al. 2016), sociability (Jimenez-Blasco et al. 2020), motor functions (Soria-Gomez et al. 2021), and most likely the regulation of food intake (M. Koch et al. 2015), thus shedding light on this new subset of receptors in the regulation of physiological and pathological behavioral responses.

1.2.2. The type-2 cannabinoid receptor (CB2)

While the central distribution of the CB1 receptor is consistent with the psychoactive properties of cannabinoids and their effects on cognitive and motor functions, cannabimimetic drugs exert a wide range of other effects, including analgesia, anti-inflammation, and immunosuppression. Therefore, it has been proposed that the non-psychoactive effects of cannabimimetic drugs may be mediated in part by receptors other than CB1 or by non-receptor protein targets (Kano et al. 2009).

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In 1993, just three years after the cloning of the CB1 receptor, Munro and colleagues reported the identification of a peripheral cannabinoid receptor called the type 2 cannabinoid (CB2) receptor. While searching for novel GPCRs expressed in a human myeloid cell line (a bone marrow cell lineage that can differentiate into blood cells such as granulocytes and macrophages), the authors identified a human cDNA encoding a GPCR consisting of 360 amino acids and sharing 44% homology with the human CB1 receptor (Munro, Thomas, and Abu-Shaar 1993). The homology between the receptors increased to 68% for transmembrane residues proposed as the binding site for cannabinoid ligands. The authors then showed that this novel GPCR was indeed able to bind the synthetic cannabinoids WIN 55,212-2 and CP 55,940, the phytocannabinoids CBN, THC, and the hydroxy metabolite of THC (11-OH-THC), among others. In contrast to the CB1 receptor, *in situ* hybridization analyses did not initially reveal the presence of the CB2 receptor in the rat brain, but they did find a restricted expression in the marginal areas of the spleen, a region mainly populated by macrophages (Munro, Thomas, and Abu-Shaar 1993).

Human CB1 and CB2 receptors share more sequence similarity than any other mammalian GPCR, suggesting that they are derived from a common ancestral gene, making them paralogs (i.e., gene copies created by a duplication event within the same genome) (Elphick 2012). In addition to human CB2 receptors, clones have been isolated from other species, including mice (Shire et al. 1996) and rats (S. M. Brown, Wager-Miller, and Mackie 2002), and orthologous genes encoding for CB2 can be found in most vertebrate species, including non-mammalian vertebrates and bony fish (Elphick 2012). However, it should be noted that the CB2 receptor shares less amino acid identity between species than the CB1 receptor, with, for example, only 82% homology between humans and mice (versus 97% for CB1), which may lead to species-dependent CB2 pharmacology (Allyn C. Howlett and Abood 2017).

Since its identification, the CB2 receptor has been well described to be expressed in peripheral organs and cells with a role in immunity (e.g., spleen, tonsils, thymus, macrophages, and leukocytes) (Allyn C. Howlett and Abood 2017) but also in pulmonary endothelial cells, testes, and osteocytes (Atwood and Mackie 2010). However, its putative expression in the brain has been debated over the past decades. While early studies reported the absence of CB2 expression in the healthy CNS, advances in the sensitivity and specificity of RNA and protein detection, coupled with the description of tissue-specific isoforms of the receptor, have challenged this dogma (Jordan and Xi 2019).

Recent studies have reported CB2 expression in the healthy brain, in microglia (the resident immune cells of the brain), consistent with a role for the CB2 receptor in inflammation, but also in neurons of the hippocampal formation, brainstem, and cerebellum (Atwood and

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Mackie 2010; Jordan and Xi 2019). However, CB2 expression in the healthy brain is low compared to central CB1 expression, with some qPCR and *in situ* hybridization analyses showing expression levels close to the detection threshold (Atwood and Mackie 2010). Furthermore, the lack of CB2 receptor homology between species and the lack of functional evidence for central CB2 activity under physiological conditions requires caution in interpreting the data and in considering whether CB2 receptors are present at functionally relevant levels in the healthy human brain under physiological conditions (Atwood and Mackie 2010). Despite conflicting data on CB2 expression under physiological conditions, it is more evident that central CB2 mRNA and protein are upregulated in the brain in response to various threats and pathological conditions, such as ischemia-induced hypoxia, chronic pain, drug addiction, Alzheimer's disease, and multiple sclerosis, underscoring its role in neuroprotection (Atwood and Mackie 2010).

1.3. Endogenous ligands – endocannabinoids

1.3.1. From phyto- to endocannabinoids: an overview

Cannabinoid receptors were first identified for their ability to bind and respond to phyto- and synthetic cannabinoids. Given the wide range of effects these substances elicit, it was promptly postulated that cannabinoid receptors would regulate various physiological functions such as movement, memory, emotion, and pain (among others). These physiological roles ultimately required the existence of endogenous ligands to activate cannabinoid receptors to produce their effects. Several endogenous cannabinoid ligands have since been identified and, in analogy to phytocannabinoids, have been collectively termed "endocannabinoids" (eCBs). Like their plant counterparts, eCBs are lipidic in nature and are primarily derived from long-chain polyunsaturated fatty acids (PUFAs) such as arachidonic acid (Kano et al. 2009; M. G. Cascio and Marini 2015).

The first eCB was identified in 1992 and named anandamide (AEA) (W. Devane et al. 1992). AEA was first isolated from porcine brain and was able to competitively inhibit the binding of tritium-labeled HU-243 (a synthetic CB1 receptor agonist) and produce cannabimimetic effects (e.g., inhibition of the electrically evoked twitch response of the mouse *vas deferens*). Three years later, a second eCB, 2-arachidonoylglycerol (2-AG), was similarly identified in the dog intestine (Mechoulam et al. 1995; Sugiura et al. 1995). Although AEA and 2-AG were initially thought to have similar pharmacological profiles (i.e., affinity, potency, and efficacy), further investigation revealed that 2-AG was more potent than AEA. Thus, 2-AG behaves as a full agonist at cannabinoid receptors (i.e., a drug capable of producing a maximal biological response), whereas AEA behaves as a partial agonist (i.e., a drug that weakly

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activates a receptor, thus producing a submaximal biological response). Both AEA and 2-AG display little selectivity between CB1/CB2 receptors ([Console-Bram, Marcu, and Abood 2012](#)).

In addition to AEA and 2-AG, other PUFAs have been identified as eCBs, including 2-arachidonylglycerol ether (noladine ether), O-arachidonoyl-ethanolamine (virodhamine), and N-arachidonoyl-ethanolamine (NADA) ([Porter et al. 2002](#)). While noladine ether is considered a potent and selective CB1 agonist, virodhamine acts as an endogenous CB1 antagonist/inverse agonist and CB2 full agonist, and NADA is a partial CB1/CB2 agonist with low selectivity ([Console-Bram, Marcu, and Abood 2012](#)). However, the pharmacological properties of these eCBs and their biological effects *in vivo* have been less extensively studied ([V. Di Marzo 2008](#)).

Besides PUFAs, short-chain fatty acids (SCFAs) such as docosahexaenoyl-ethanolamide (DHEA) and eicosapentaenoyl-ethanolamide (EPEA) have also been proposed to act as eCBs, although they exert both cannabinoid receptor-dependent and independent effects ([M. G. Cascio and Marini 2015](#)). Finally, lipid mediators that share common biosynthetic pathways with eCBs, defined as 'eCB-like' compounds, have been described to interact with the ECS and act as 'entourage molecules'. These eCB-like mediators will be discussed in more detail when addressing the concept of endocannabinoids ([See 1.4.3](#)) ([M. G. Cascio and Marini 2015](#)). Among all lipid mediators acting at cannabinoid receptors, AEA and 2-AG have been extensively studied and are considered to be the main eCBs. Therefore, the following sections will mainly focus on the biosynthesis and degradation pathways of AEA and 2-AG.

1.3.2. Synthesis of endocannabinoids

One of the leading concepts surrounding the biosynthesis of eCBs is that their production is "on demand". Unlike classical neurotransmitters, which are "pre-synthesized" and stored in secretory vesicles, eCBs are produced in response to a given signal from phospholipid precursors present in biological membranes and are immediately released from cells ([Lu and Mackie 2021](#)). 2-AG and AEA are typically produced following increased intracellular Ca^{2+} influx, either due to cell depolarization or mobilization of intracellular Ca^{2+} stores following GPCR (Gq/11) activation ([V. Di Marzo 2008](#)). Of interest, it has also been reported that AEA can be sequestered in adiposomes (lipid droplets), although it remains unclear whether this represents a dynamic reservoir for the activity-dependent release of AEA or contributes to AEA inactivation by degradative enzymes ([S. Oddi et al. 2008](#); [Kaczocha et al. 2010](#)). After their synthesis, eCBs (mainly 2-AG) act as retrograde neurotransmitters (i.e., they are released from the postsynaptic element and travel "backward" to the presynaptic element) to activate CB1 receptors ([Kano et al. 2009](#)).

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Although they are metabolic derivatives of arachidonic acid, AEA and 2-AG have different biosynthetic pathways. The main biosynthetic pathway of AEA consists of two steps. Briefly, a calcium-dependent N-acyltransferase (NAT) transfers the acyl chain of an arachidonic acid-containing glycerophospholipid to the hydroxyethyl group of phosphatidylethanolamine (PE) to form the metabolic intermediate N-arachidonoyl-phosphatidylethanolamine (NAPE). NAPE is then hydrolyzed by a NAPE-specific phospholipase D-type enzyme (NAPE-PLD) to produce the N-acylethanolamine (NAE) anandamide (AEA) (Vincenzo Di Marzo et al. 1994; V. Di Marzo 2008; M. G. Cascio and Marini 2015). Interestingly, studies conducted in NAPE-PLD knockout mice revealed little to no change in endogenous AEA levels, suggesting that NAPE-PLD-independent biosynthetic pathways for AEA exist (Leung et al. 2006). These alternative pathways involve the recruitment of different enzymes to produce AEA from various precursors and metabolic intermediates (e.g., N-acyl-lysophosphatidylethanolamine, glycerophosphoarachidonoyl-ethanolamide, phospho-AEA) (M. G. Cascio and Marini 2015) ([Figure 2](#)).

Unlike AEA, 2-AG synthesis is more redundant and can occur via multiple lipid metabolic pathways. However, in the central nervous system (CNS), 2-AG is primarily synthesized in a two-step process. First, phosphatidylinositol 4, 5-bisphosphate (PIP₂), an arachidonic acid-containing membrane phospholipid, is hydrolyzed by a phospholipase C β -type enzyme (PLC β) to form 1, 2-diacylglycerol (DAG). DAG is then converted to 2-AG by the enzyme diacylglycerol lipase (DAGL) (Bisogno et al. 2003; M. G. Cascio and Marini 2015; Murataeva, Straiker, and Mackie 2014). Because 2-AG is also a metabolic intermediate in the production of other lipids, it is much more abundant in tissues than AEA (M. G. Cascio and Marini 2015) ([Figure 2](#)).

Of concern, two isoforms of DAGL have been described: DAGL α and DAGL β . While DAGL β is more abundant in the developing brain, DAGL α is mainly expressed in the adult brain and localizes to postsynaptic terminals (V. Di Marzo 2008). Most interestingly, postsynaptic DAGL α is complementary to CB1-expressing presynaptic elements, suggesting a refined organization of ECS-associated elements at the synapse and a central role for 2-AG in CB1-mediated retrograde signaling (Yoshida et al. 2006; Katona et al. 2006; Wang and Ueda 2009). In contrast, NAPE-PLD is predominantly expressed in presynaptic terminals, suggesting that NAPE-PLD-dependent AEA synthesis is unlikely to be involved in retrograde neurotransmission (Nyilas et al. 2008).

1.3.3. Transport of endocannabinoids

Once synthesized on demand, eCBs exert most of their effects by acting as retrograde messengers (i.e., from postsynaptic to presynaptic nerve terminals) and binding to CB1

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receptors localized on the surface of presynaptic elements. However, due to their lipid nature, it has been hypothesized that the diffusion of eCBs in aqueous environments (the synaptic cleft) requires efficient transport systems (M. G. Cascio and Marini 2015). Furthermore, a key feature of eCB signaling is precise temporal and spatial release, which requires termination of eCB signaling by degradative enzymes (Kano et al. 2009). eCB catabolic enzymes are primarily intracellular, which ultimately requires eCBs to re-enter the cell for degradation (Lu and Mackie 2021).

Some data support the existence of carrier-mediated transport of eCBs ([Figure 2](#)). Indeed, the uptake of eCBs is rapid, temperature-dependent, and saturable, consistent with a facilitated transport mechanism. However, the putative protein responsible for this mechanism, termed "endocannabinoid membrane transporter" (EMT), has not yet been identified, and its existence is supported only by indirect evidence (M. G. Cascio and Marini 2015). Several candidate proteins have been proposed for AEA transport, including the fatty acid binding proteins FABP5 and FABPP7 (Kaczocha, Glaser, and Deutsch 2009), heat shock protein 70 (Hsp70), and albumin (Sergio Oddi et al. 2009). Although most evidence for facilitated transport of eCBs has been found for AEA, some studies have suggested that 2-AG transport may follow similar mechanisms (Chicca et al. 2012).

Conflicting reports argue against the existence of EMT and in favor of the passive diffusion of eCBs across membranes following an intracellular/extracellular gradient, but this hypothesis has several weaknesses. Indeed, the polar nature of eCBs would limit their passage across cell membranes, which is inconsistent with the rapid uptake of these lipid mediators (Lu and Mackie 2021). Finally, passive diffusion of eCBs would cease once equilibrium between the intracellular and extracellular spaces is reached, preventing further release of eCBs upon cell activation (M. G. Cascio and Marini 2015).

1.3.4. Degradation of endocannabinoids

As mentioned above, the degradation of eCBs is essential for the spatiotemporal dynamics of eCB signaling. As for their synthesis, AEA and 2-AG are metabolized by specific enzymes with distinct subcellular localization (M. G. Cascio and Marini 2015) ([Figure 2](#)).

Fatty acid amide hydrolase (FAAH) is the primary enzyme responsible for the hydrolysis of AEA (Benjamin F. Cravatt et al. 1996). Specifically, FAAH targets the amide bond of AEA, forming arachidonic acid and ethanolamine as degradation products, but also has selectivity for other fatty acid amides (e.g., eCB-like mediators, [see section 1.4.3](#)) and fatty acyl esters, including 2-AG (V. Di Marzo et al. 1998) and virodhamine (Steffens et al. 2005). Despite the broad substrate selectivity of the enzyme, immunohistochemical analyses have revealed a

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complementary distribution of CB1 receptor and FAAH proteins. FAAH is enriched in brain regions with high CB1 receptor expression, including the neocortex, hippocampus, and cerebellum (M. G. Cascio and Marini 2015), and is mainly expressed in the somata and dendrites of neurons that are postsynaptic to CB1-expressing axon fibers (Egertová, Cravatt, and Elphick 2003). The localization of FAAH suggests that it regulates the postsynaptic accumulation of AEA, thereby influencing the dynamics of eCB signaling. A second isoform of FAAH, FAAH-2, has also been identified, but its expression is restricted to the periphery, limiting the regulation of synaptic transmission to FAAH (Wei et al. 2006). Although FAAH is responsible for the majority of AEA degradation in the brain (B. F. Cravatt et al. 2001), N-acylethanolamine-selective acid amidase (NAAA) can also hydrolyze AEA but has a lower affinity for this substrate than other fatty acid amides (Katayama et al. 1999; Ueda, Tsuboi, and Uyama 2013) ([Figure 2](#)).

In contrast to AEA, 2-AG can be degraded by several hydrolytic pathways. As mentioned above, FAAH could metabolize both AEA and 2-AG, although the role of FAAH in 2-AG metabolism is controversial (V. Di Marzo 2008). Rather than FAAH, the hydrolase enzyme monoacylglycerol lipase (MAGL) is involved in the inactivation of 2-AG (T. P. Dinh et al. 2002; Thien P. Dinh, Kathuria, and Piomelli 2004). Like FAAH, MAGL has poor substrate selectivity and can recognize other 2-acylglycerols and esterified PUFAs but differs from FAAH in its subcellular localization (V. Di Marzo 2008). Indeed, MAGL localization in the brain coincides with presynaptic nerve terminals expressing CB1 receptors (Savinainen, Saario, and Laitinen 2012) and is complementary to 2-AG-synthesizing enzymes. Thus the localization of these metabolic enzymes is consistent with a role for 2-AG in CB1-mediated retrograde signaling (Gulyas et al. 2004; T. P. Dinh et al. 2002). Although MAGL is considered the major enzyme involved in the catabolism of 2-AG, it accounts for only 50% to 80% of the total 2-AG hydrolyzing activity in the rat and mouse brain (Thien P. Dinh, Kathuria, and Piomelli 2004; Blankman, Simon, and Cravatt 2007). The remaining fraction is mainly hydrolyzed by two α/β -hydrolase domains containing protein-6 (ABHD6) and -12 (ABHD12). In contrast to MAGL, ABHD6 is located in the postsynaptic dendrites of neurons, whereas ABHD12 is mainly expressed in microglia, suggesting that they have access to different pools of 2-AG and may have different functions *in vivo* (Kano et al. 2009) ([Figure 2](#)).

In addition to these classical hydrolytic pathways, both AEA and 2-AG can be degraded by oxidation of their arachidonate moiety catalyzed by 12- and 15-lipoxygenases (LOXs), cytochrome p450 oxygenases, and cyclooxygenase-2 (COX-2) (V. Di Marzo 2008). It is interesting to note that while some oxidation products remain moderately active at cannabinoid receptors, it has been suggested that others become active at new, as yet unidentified,

receptors and may contribute to the transition to a new type of signaling (V. Di Marzo 2008; Lu and Mackie 2021).

1.4. The extended endocannabinoid system

1.4.1. The emerging concept of endocannabinoidome

Classically, the ECS is defined as a neuromodulatory network consisting of the cannabinoid receptors CB1 and CB2, eCBs (mainly AEA and 2-AG), and their metabolic enzymes. However, this canonical view of the ECS is challenged by its overlap with metabolic pathways involved in the synthesis of other lipid mediators, its promiscuity with multiple receptor and protein targets, and the existence of endogenous molecules that regulate the activity of these receptors (Vincenzo Di Marzo 2020; Veilleux, Di Marzo, and Silvestri 2019; Cristino, Bisogno, and Di Marzo 2020) ([Figure 2](#)). This highly complex endocannabinoid-related network, defined as the "endocannabinoidome" (eCBome), is of great clinical importance. Indeed, due to the high redundancy in eCB metabolism, therapeutic approaches based on pharmacological manipulation of eCB levels (e.g., using inhibitors targeting synthesis and degradation enzymes) without affecting other related lipid mediators often prove complicated. Moreover, direct pharmacological manipulation of CB1/CB2 activity by classical agonist/antagonists has been associated with several drawbacks (e.g., the withdrawal from the market of the anorexigenic CB1 antagonist SR141716, also called rimonabant) due to disruption of endogenous signaling (Taylor 2009). Therefore, a better understanding of the eCBome is critical to the development of new drugs that modulate eCB signaling with safer pharmacological profiles. Accordingly, the following sections will outline the main features of the eCBome, focusing on the non-canonical targets of cannabinoids, eCB-like mediators, and endogenous allosteric modulators of cannabinoid receptors.

1.4.2. Non CB1/CB2 cannabinoid-responsive receptors

The identification of the two metabotropic cannabinoid receptors, CB1 and CB2, has allowed extensive documentation and characterization of cannabinoid actions at central and peripheral levels. However, a growing body of evidence suggests that the actions of cannabimimetic drugs, as well as the physiological regulation of synaptic transmission by eCBs, are not solely attributable to CB1 and CB2 receptors and are subject to cannabinoid receptor-independent mechanisms. Of interest, cannabinoids have been shown to modulate the activity of voltage- and ligand-gated ion channels, ion-transporting membrane proteins, nuclear receptors, and non-CB1 non-CB2 GPCRs (De Petrocellis et al. 2017; Kano et al. 2009; Cristino, Bisogno, and Di Marzo 2020) ([Figure 2](#)).

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Among the most studied receptors in the broader endocannabinoidome are the ionotropic receptors belonging to the transient receptor potential (TRP) channel superfamily, which are functionally diverse (i.e., in their activation mechanisms, voltage dependence, and pharmacological properties) and non-selective cation channels involved in cellular Ca^{2+} homeostasis (De Petrocellis et al. 2017). In particular, the first member of the transient receptor potential vanilloid subfamily, TRPV1, has been described as a target for cannabinoids. TRPV1 was initially described as a receptor for the vanilloids capsaicin and resiniferatoxin (i.e., compounds containing a vanillyl or vanilloyl group), which are natural irritants and neurotoxins found in chili peppers and resin spurge (*Euphorbia resinifera*), respectively (Szallasi and Blumberg 1991). In addition to vanilloids, TRPV1 responds to physical and chemical stimuli, including noxious heat ($>43^{\circ}\text{C}$) and low pH, and is expressed predominantly in neurons associated with nociception in the dorsal root ganglia, suggesting an essential role in sensory perception (Caterina et al. 1997). Although this is true, TRPV1 has also been detected in neurons unrelated to nociception, particularly postsynaptic GABAergic and glutamatergic terminals and neuronal somata of the hippocampus and cerebellum (Storozhuk, Moroz, and Zholos 2019). Indeed, its role in mediating short- and long-term synaptic plasticity has been demonstrated, with implications for the physiological regulation of memory, food intake, and mood, among others (Cristino, Bisogno, and Di Marzo 2020). Interestingly, TRPV1 can be activated by AEA (along with the AEA non-hydrolyzable analog methanandamide) (Ross et al. 2001) and can induce Ca^{2+} -dependent internalization of AMPA receptors and long-term depression (LTD) (Chávez, Chiu, and Castillo 2010). This strongly supports the interaction between the endocannabinoid and vanilloid systems and the ability of eCBs to induce synaptic plasticity in a CB1-independent manner.

Some studies have also reported that AEA, and to some extent, 2-AG, can modulate the activity of other members of the TRP family, in particular TRPV2-4 of the vanilloid subfamily, TPRA1 of the ankyrin subfamily, and TRPM1,8 of the melastatin subfamily (De Petrocellis et al. 2017). However, most of these studies were performed in heterologous cells overexpressing these channels, and the reported effects were achieved at high concentrations of eCBs in the micromolar range, ultimately undermining the physiological relevance of these results *in vivo*. Moreover, in contrast to TRPV1, eCB-mediated activation of TRP channels, in particular TRPV4, may be the result of an indirect action requiring the hydrolysis of eCB to arachidonic acid (H. Watanabe et al. 2003). In addition, eCBs can also modulate the activity of non-TRP ionotropic channels, including the ligand-gated N-methyl-D-aspartic acid (NMDA), γ -aminobutyric acid type A (GABA_A) and glycine (Gly) receptors, as well as voltage-gated Ca^{2+} and ATP-sensitive K^+ channels. However, their mechanisms of action vary widely depending

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on the target and experimental conditions and include CB1-dependent, CB1-independent, and TRPV1-dependent mechanisms (De Petrocellis et al. 2017; Vincenzo Di Marzo 2020).

In addition to ion channels, peroxisome proliferator-activated receptors (PPARs) are considered an extension of the ECS. PPARs are a family of ligand-activated nuclear receptors/transcription factors and consist of three distinct isoforms designated PPAR α , β/δ , and γ (also known as NR1C1, NR1C2, and NR1C3, respectively) (Iannotti and Vitale 2021). All three PPAR isoforms are relatively ubiquitous, with main expression sites found in peripheral tissues with a high rate of fatty acid metabolism (e.g., liver, brown adipose tissue, gastrointestinal tract, myocardium, and skeletal muscle) and in the CNS in all major cell types, with highest localization in the basal ganglia and hippocampal subfields. Consistent with their expression sites, PPARs are involved in metabolic homeostasis, neuroprotection, and anti-inflammatory processes (Iannotti and Vitale 2021). Several endogenous PPAR ligands, including eCBs, have been reported. In particular, AEA can bind both PPAR α (Sun et al. 2007) and PPAR γ (Bouaboula et al. 2005), although with higher affinity for the α isoform, and activate the transcriptional potency of these receptors to induce neuroprotection. The two eCBs noladine ether and virodhamine, also activate PPAR α but are less potent than AEA (Sun et al. 2007). 2-AG has been shown to promote PPAR γ activation, although its effects are most likely mediated by one of its COX-2-derived metabolites (Raman et al. 2011).

Finally, there is evidence that eCBs and related lipid mediators may signal through non-CB1 non-CB2 cannabinoid-related GPCRs. In particular, the deorphanized GPR55 (or LPI1) and GPR18 have been described as targets of eCBs, synthetic cannabinoids, and phytocannabinoids (Morales and Reggio 2017). Both GPCRs share low amino acid sequence homology with classical cannabinoid receptors: GPR55 shares only 13% and 14% and GPR18 only 13% and 8% sequence identity with CB1 and CB2 receptors, respectively (Morales and Reggio 2017). Furthermore, the pharmacology of these receptors differs significantly from that of cannabinoid receptors. Although GPR55 is widely expressed in the brain and periphery, often colocalizing with cannabinoid receptors (Balenga et al. 2011), it exhibits differential G protein coupling, including G α 12, 13, 12/13, q/11, whereas CB1 and CB2 are primarily coupled to G α i/o proteins (Morales and Reggio 2017). In addition, the phospholipid lysophosphatidylinositol (LPI) has been reported as the primary endogenous GPR55 ligand, but other compounds, including 2-AG, AEA, noladine ether, and virodhamine, also activate the GPR55 receptor. However, unlike cannabinoid receptors, other lipid mediators have been found to activate GPR55, and discrepancies have been reported regarding the effects of synthetic cannabinoids, including the lack of activity of WIN55,212-2 and the CB1 antagonist AM251 which behaves as a GPR55 agonist (Ryberg et al. 2008; Morales and Reggio 2017).

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On the other hand, GPR18 is mainly expressed in lymphoid tissues, among other peripheral tissues and organs, and can signal through Gai/o and Gαq/11 (Morales and Reggio 2017). N-arachidonoylglycine (NAGly) has been proposed as the primary endogenous GPR18 ligand, but AEA has also been reported as a GPR18 agonist (Rajaraman et al. 2016), however not 2-AG (Yin et al. 2009). Overall, due to the pharmacological discrepancies observed for the effects of cannabinoids at these receptors and the lack of understanding of their relationship with the ECS *in vivo*, none of these GPCRs have been classified as the "type 3 cannabinoid receptor" (CB3). In addition to GPR55 and GPR18, other deorphanized GPCRs, including GPR3, 6, 12, 110, and 119, have been proposed as putative cannabinoid receptors, either because of their phylogenetic proximity to CB1 and CB2 or their ability to respond to eCB-like mediators (Morales and Reggio 2017; Lee et al. 2015; Syed et al. 2012), but our current knowledge of the interaction of these GPCRs with the ECS is largely incomplete.

1.4.3. Endocannabinoid-like mediators

Over the past decades, several endogenous lipid mediators have been identified, and their involvement in various biological processes and their mechanisms of action have been investigated. These lipid mediators include eCBs and their structural congeners, the non-eCBs N-acylethanolamines (NAEs), and mono-acylglycerols (2-MAGs), referred to as "eCB-like mediators" (H. S. Hansen, Kleberg, and Hassing 2015) ([Figure 2](#)). Unlike eCBs, eCB-like mediators cannot activate classical cannabinoid receptors, but they share common metabolic pathways. For example, the formation of AEA is often associated with that of other NAEs, most commonly oleoylethanolamide (OEA), palmitoylethanolamide (PEA), and linoleoylethanolamide (LEA). The enzyme NAPE-PLD is primarily responsible for the formation of NAEs (Tsuboi et al. 2011), while FAAH and NAAA mediate their inactivation (Long et al. 2011; Ueda, Tsuboi, and Uyama 2010). On the other hand, the synthesis of 2-MAGs, including 2-oleoylglycerol (2-OG), 2-palmitoylglycerol, and 2-linoleoylglycerol, is separate from 2-AG because it requires the enzymatic activity of the lipoprotein lipase (Young and Zechner 2013); however the same catabolic enzyme mediates their hydrolysis, namely MAGL (V. Di Marzo 2008). Although eCB-like mediators do not target CB1 and CB2 receptors like eCBs, they have been shown to activate some of the previously described atypical cannabinoid-sensitive receptors. Of interest, OEA and PEA have been shown to exert anti-inflammatory effects by activating the transcription factor PPARα (Fu et al. 2003; LoVerme et al. 2005). OEA and 2-OG can also target the deorphanized GPR119 with possible implications for the regulation of energy homeostasis (Overton et al. 2006; K. B. Hansen et al. 2011). Overall, eCB-like mediators may regulate inflammation, pain, and energy balance, but our current knowledge of their physiological relevance *in vivo* remains incomplete. This lack of knowledge is partly due to the redundancy in the synthesis and degradation of eCBs and eCB-like mediators and the

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activation of common receptor targets, making it challenging to assess their specific role in the modulation of biological processes.

Conversely, it must be considered that pharmacological manipulation of eCB levels by stimulating or inhibiting the activity of eCB metabolic enzymes may result in the upregulation of structural analogs of eCBs, which in turn may lead to off-target effects. From a therapeutic perspective, although the discovery of the extended ECS may provide many opportunities for the development of multi-target drugs, this complex and intricate biological system has also hindered the development of ECS-targeting drugs with clinical safety and efficacy profiles, thus limiting their success rate (for review see [Vincenzo Di Marzo 2018](#)). In this line, the discovery and characterization of more sophisticated endogenous regulatory mechanisms, such as positive and negative allosteric sites in cannabinoid receptors, may provide a solution for the development of highly specific drugs with low side effects.

1.4.4. Endogenous allosteric modulators of CB1/CB2

Cannabinoid receptors are highly expressed in various organs and tissues and are involved in the control of a wide range of biological processes. Therefore, they are endowed with endogenous regulatory mechanisms to tune their activity (i.e., increase or decrease signaling) and maintain ECS-mediated homeostasis ([Piazza, Cota, and Marsicano 2017](#)). Several endogenous allosteric modulators have been described among the regulatory mechanisms involved in tuning cannabinoid receptor activity ([Figure 2](#)).

Allosteric modulators bind to receptors at allosteric sites distal to the classical receptor/endogenous substrate interface (or orthosteric site), thereby affecting receptor signaling ([Cheng and Jiang 2019](#)). Based on their effects, allosteric modulators are classified as positive allosteric modulators (PAMs), negative allosteric modulators (NAMs), or neutral/silent allosteric modulators (SAMs) ([Burford et al. 2011](#)). PAMs increase receptor signaling by enhancing the binding affinity and/or efficacy of the orthosteric agonist, whereas NAMs decrease receptor signaling by inhibiting the binding affinity and/or efficacy. However, SAMs do not affect the affinity or efficacy of orthosteric agonists but can act as competitive antagonists at the allosteric binding site of PAMs and NAMs. Of note, some allosteric compounds have intrinsic activity (i.e., they can activate the receptor in the absence of an orthosteric ligand) and are referred to as "allosteric agonists" ([Burford et al., 2011](#)). It should also be noted that the observed properties of an allosteric modulator (NAM, PAM, or SAM) ultimately depend on the specific orthosteric agonist being modulated and may differ for other agonists (probe dependence). In addition, analogous to orthosteric agonists that can induce different functional effects at the same receptor (in terms of magnitude of response and the

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signaling pathways activated), allosteric modulators may exhibit different properties depending on the functional readout (functional selectivity) (Burford et al. 2011).

Endogenous allosteric modulation of cannabinoid receptors was first reported for pepcans, a family of endogenous peptides (Bauer et al. 2012). Pepcans are described as N-terminally extended peptides of the α -hemoglobin-derived "RDV-hemopressin" (RVDPVNFKLLSH) and are named pepcan-12 (RVDPVNFKLLSH) to pepcan-23 (ALSDLHAHKLRVDPVNFKLLSH) according to the length of their amino acid sequence (Bauer et al. 2012). Among the pepcan family, pepcan-12 (RDV-hemopressin) is the most abundant in the rat brain and has been described as a CB1-NAM. Namely, pepcan-12 partially inhibited the binding of the tritium-labeled CB1 agonists WIN 55,212-2 and CP55, 940 and increased the dissociation rate constant of CP55, 940, consistent with negative allosteric modulation. In addition, pepcan-12 decreased orthosteric agonist-induced cAMP signaling, [³⁵S]GTP γ S binding, and CB1 receptor internalization, indicating potent CB1-NAM activity (Bauer et al. 2012). An independent study further confirmed pepcan-12 activity as a CB1-NAM by examining its effects on eCB-mediated depolarization-induced suppression of excitation (DSE) in cultured autaptic hippocampal neurons. This study showed that pepcan-12 attenuated DSE without directly inhibiting CB1 activity (Straiker et al. 2015). More recently, pepcan-12 was reported to act as a CB2-PAM (Petrucci et al. 2017). In contrast to CB1 receptors, pepcan-12 increased orthosteric CB2 agonist binding, [³⁵S]GTP γ S binding, and cAMP signaling induced by synthetic agonists and 2-AG (Petrucci et al. 2017). Pepcan-12 is most highly expressed in noradrenergic neurons in the CNS and the adrenal medulla in the periphery (Hofer et al. 2015), and its expression is upregulated by lipopolysaccharide (LPS)-induced endotoxemia and ischemia-reperfusion injury, suggesting a protective role of pepcan-12 under pathophysiological conditions (Petrucci et al. 2017).

Shortly after the identification of pepcan-12 as a CB1-NAM, another endogenous CB1 allosteric modulator, lipoxin A4 (LXA4), was identified (Pamplona et al. 2012). LXA4 is an endogenous bioactive metabolite of arachidonic acid primarily involved in anti-inflammation by activating peripheral LXA receptors, and it has been reported to act as a CB1-PAM with neuroprotective effects. Indeed, intracerebral infusion of LXA4 could produce cannabimimetic effects in an LXA-independent but CB1-dependent manner. Interestingly, LXA4 alone did not displace the tritium-labeled CB1-selective orthosteric antagonist SR141716A (rimonabant or SR) but increased the binding and potency of AEA at CB1 receptors. The increased binding affinity and potentiation of CB1-mediated signaling were independent of AEA metabolism, as LXA4 did not increase central AEA levels or reduce FAAH enzymatic activity. Together, these data are consistent with CB1-positive allosteric modulation by LXA4 (Pamplona et al. 2012).

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Furthermore, enhancement of CB1 signaling by LXA4 resulted in neuroprotection against β -amyloid-induced memory impairment, highlighting the importance of allosteric modulators in regulating GPCR activity (Pamplona et al. 2012). In this study, LPX4 did not alter the binding or efficacy of 2-AG, and its effects on eCB-mediated signaling were specific to AEA. Interestingly, another study reported that LXA4 did not enhance but inhibited eCB-mediated DSE, which is more consistent with a NAM profile than a PAM profile (Straiker et al. 2015). Since eCB-mediated DSE is mainly attributed to 2-AG signaling, LXA4 may have PAM-like effects on AEA-mediated signaling but NAM-like effects on 2-AG-mediated signaling, illustrating the aforementioned principle of "probe dependence".

Finally, the (neuro)steroid pregnenolone (PREG) was identified as a second endogenous CB1-NAM with a specific mechanistic profile (Vallée et al. 2014). Like all steroid hormones, PREG is derived from cholesterol metabolism and is synthesized in steroidogenic tissues and organs. While classical steroidogenic sites are located in the periphery (e.g., adrenal glands, gonads, placenta, and muscles, among others), the CNS has also been described as a source of steroid production (namely "neurosteroids") (See Chapter 3) (Vallée 2016). Interestingly, acute administration of THC and the synthetic CB1 agonists HU210 and WIN 55,212-2 increased central but not peripheral PREG levels in rodents. The increase in PREG levels was abolished by co-administration of THC with the CB1 antagonist AM251 and in CB1 knockout mice and was not recapitulated by administration of the CB2 agonist JWH-133, favoring a CB1-dependent mechanism (Vallée et al. 2014). In turn, PREG attenuated some cannabimimetic effects, suggesting an autocrine/paracrine loop involved in the modulation of CB1 activity (Vallée et al. 2014; A. Busquets-Garcia et al. 2017). More recently, PREG was shown to rescue hyperdopaminergic states associated with prenatal THC exposure (Frau et al. 2019). *In vitro*, PREG did not inhibit the binding affinity of CB1 orthosteric agonists, THC-induced decrease in cAMP levels, and [35S]GTP γ S binding but inhibited CB1-dependent modulation of the ERK/MAPK pathway and ADP-stimulated mitochondrial respiration, indicating functional selectivity (Vallée et al. 2014; Gamage et al. 2017). The allosteric properties of PREG were further confirmed by the identification of the TMH1/TMH8/Hx8 region of the CB1 receptor as a putative PREG binding site, which has been validated *in vitro* by a mutagenesis approach (Vallée et al. 2014).

Some studies have reported discrepancies in the effects of PREG, including slight displacement of tritium-labeled SR and lack of effect on THC-induced ERK/MAPK activation, eCB-mediated DSE, and THC-induced suppression of GABAergic synaptic transmission (Khajehali et al. 2015; Straiker et al. 2015; Krohmer et al. 2017). A possible explanation for the lack of PREG effects reported in these studies may be the high probe dependence of PREG to THC and synthetic CB1 agonists such as WIN55,212-2, although this needs to be

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investigated in future experiments. In addition, it should be noted that PREG has unique mechanistic properties, and the lack of effects may be due to functional selectivity rather than probe dependence. Finally, because PREG serves as a precursor for the synthesis of other steroids (i.e., it can be rapidly metabolized by steroidogenic enzymes) and because the application of CB1 agonists can dynamically regulate PREG levels, pharmacokinetic assays may prove necessary as controls. For example, it may be necessary to verify whether in vitro studies use steroidogenic competent cells to account for potential confounding effects of endogenously released PREG or metabolization of exogenously applied PREG. Nevertheless, the functional selectivity of PREG represents a unique mode of action and synthetic PREG derivatives (i.e., which are not metabolized and better absorbed than PREG itself), a new class of specific signaling inhibitors of CB1 receptors (CB1-SSi) (developed by the biotech company *Aelis Farma*, www.aelisfarma.com), show promise for clinical application, particularly in pathological conditions characterized by excessive activation of CB1 receptors, including cannabis use disorder (CUD) ([Haney et al. 2023](#)).

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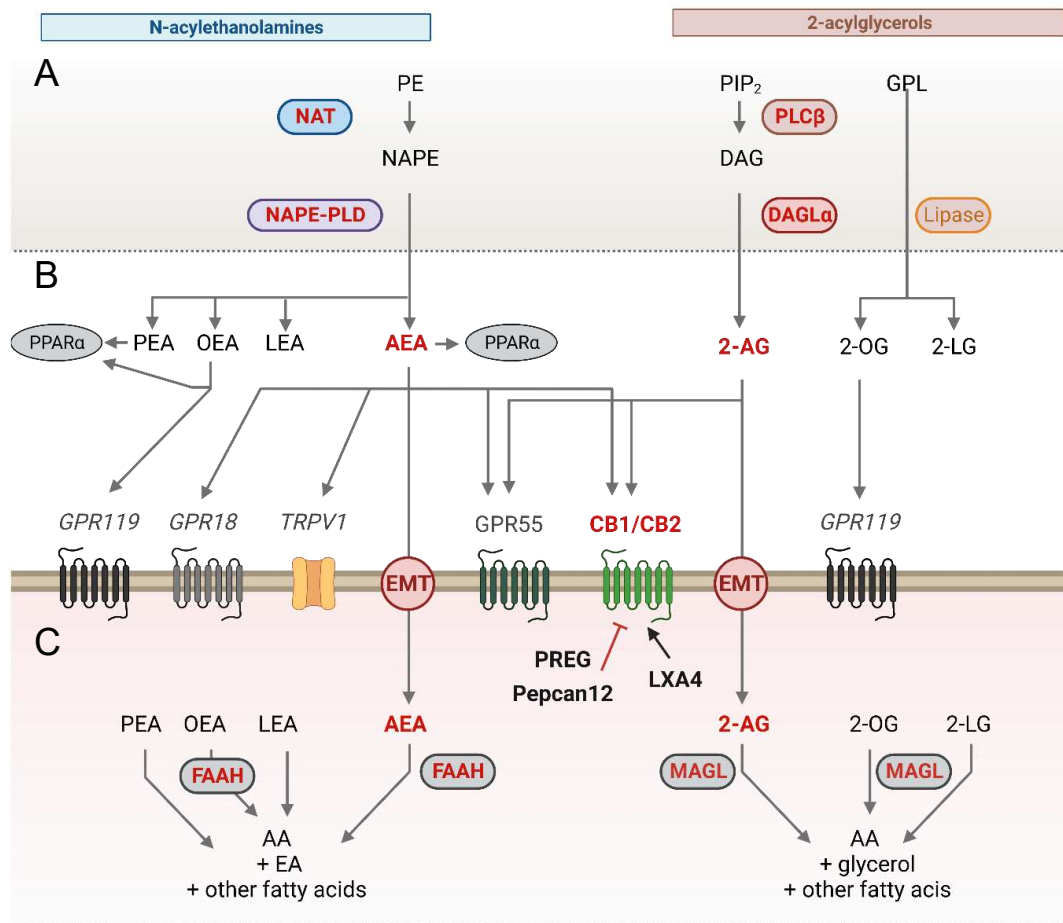


Figure 2: The endocannabinoid system and related lipid mediators and molecular targets.

A) The top figure shows the main synthetic pathways leading to the synthesis of N-acylethanolamines and 2-acylglycerols, including endocannabinoids (eCBs), AEA and 2-AG, and their lipid congeners. **B)** The middle figure represents the major molecular targets of endocannabinoids and related lipids. eCBs act at the classical cannabinoid receptors, CB1 and CB2, along with other G-protein coupled receptors, ionotropic receptor targets, and transcription factors. Some of these molecular targets are shared with their lipid congeners. The existence of an eCB membrane transporter has been hypothesized for the reuptake of eCBs leading to the termination of signaling. **C)** The lower figure shows the main degradation pathways of eCBs, which are also shared with their lipid congeners. In addition to degradation, endogenous allosteric modulators (PREG, Pepcan12, LXA4) have been identified for CB1 receptors and participate in the modulation of signaling by either downregulating (red line) or potentiating (black arrow) receptor activity. The classical components of the endocannabinoid system are highlighted in red (A to C).

Abbreviations: 2-LG, 2-linoleoylglycerol; 2-OG, 2-oleoylglycerol; AA, arachidonic acid; DAG, diacylglycerol; DAGL α , diacylglycerol lipase α ; CB1, cannabinoid type 1 receptor; EA, ethanolamide; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; GPL, glycerophospholipid; GPR18, 55, 119, G protein-coupled receptor 18, 55, 119; LEA, linoleoyl ethanolamide; LX4, lipoxin A4; MAGL, monoacylglycerol lipase; NAPE, N-acylphosphatidylethanolamine; NAPE-PLD, NAPE-specific phospholipase D; NAT, N-acyltransferase; OEA, oleoylethanolamide; PPAR α , peroxisome proliferator-activated receptor α ; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC β , phospholipase C β ; PREG, pregnenolone; TRPV1, transient receptor potential vanilloid type 1.

Adapted from [Cristino et al., 2020](#).

2. CB1 signaling and functions in physiopathology

The ECS has been described as a pleiotropic modulatory system essential for maintaining homeostasis, and its role in the etiology of many neuropsychiatric and metabolic disorders has attracted tremendous research interest (Lutz 2020). Despite the increasing complexity of the ECS with the discovery of new receptor targets for eCBs and additional lipid mediators, the vast majority of our knowledge of endocannabinoid signaling is associated with the CB1 receptor. CB1 belongs to the superfamily of seven-transmembrane domain GPCRs (7TM-GPCRs) and is classified in the class A (rhodopsin-like) subfamily based on sequence homology and functional similarity (Foord et al. 2005). Class A represents the largest and most diverse GPCR subfamily in humans and shares a highly conserved structure that includes seven transmembrane helical domains with a ligand-binding domain and a G-protein binding region located at the extracellular and intracellular ends of the helix bundle, respectively (Q. Zhou et al. 2019). Like most GPCRs, the coding region of the CB1 encoding gene (*Cnr1*) is intronless, implying that its transcription results in the formation of a single major mRNA. However, splice variants of CB1 receptors with modified N-terminals have been identified and originate from the non-coding regions of *Cnr1* (Sabine Ruehle et al. 2017), as well as post-transcriptional modifications that are mainly located within the C-terminal domain (Allyn C. Howlett, Blume, and Dalton 2010). Thus, these modifications may affect receptor function and lead to differences in CB1-mediated signaling. In addition to the functional consequences of CB1 variants at the transcriptional and translational levels, a large body of literature has shown that the high heterogeneity of CB1-mediated signaling also depends on the cellular and subcellular localization of the receptor, along with a variety of effector proteins to transduce the signal into the cell (Arnau Busquets-Garcia, Bains, and Marsicano 2018). This heterogeneity of CB1 signaling is particularly relevant for understanding CB1-mediated functions at physiological and supraphysiological or pathological levels, with the discovery of new mechanisms of action providing a better understanding of the effects of endocannabinoid signaling and exogenous cannabinoids targeting CB1 receptors. Thus, the following chapter will focus exclusively on CB1 receptors with respect to their signaling at the molecular and cellular levels and their implication in the modulation of a plethora of biological functions.

2.1. Cellular CB1 signaling mediated by Gi/o proteins and synaptic functions

2.1.1. Suppression of neurotransmission via Gi/o proteins

Activation of CB1 receptors is primarily associated with inhibition of neurotransmitter release. In particular, several electrophysiological and biochemical analyses have shown that CB1 receptors inhibit the release of glutamate, GABA, glycine, acetylcholine, norepinephrine, dopamine, serotonin, and cholecystokinin (CCK) (Kano et al. 2009). Although the mechanisms

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underlying the suppression of neurotransmitter release may differ among neurotransmitters, cell types, and brain regions, the canonical effects of CB1 are primarily attributed to its presynaptic localization and activation of multiple intracellular signaling pathways, including modulation of second messengers, ion channels, and signal transduction protein phosphorylation cascades (Kano et al. 2009). ([Figure 4](#) for the visual summary).

These signaling pathways are typically associated with the activation of heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins), which are the primary effectors of GPCR signaling and regulate various biochemical functions. G-proteins are composed of three subunits, α , β , and γ , with the β/γ subunits being tightly associated and functioning as a single molecular entity. G-protein activity is typically triggered by agonist binding to the GPCR, resulting in the exchange of guanosine diphosphate (GDP) bound to $G\alpha$ for guanosine triphosphate (GTP) and subsequent dissociation of the $G\alpha$ subunit from the $G\beta/\gamma$ dimer, which then functions as two functional subunits to signal different cellular pathways (Syrovatkina et al. 2016). G-proteins are classified into four families based on the functional similarities of their $G\alpha$ subunit: 1) $G_{\alpha s}$, which stimulate adenylyl cyclase to convert ATP into cyclic AMP (cAMP); 2) $G_{\alpha i}$ (more commonly referred to as "Gai/o"), which inhibit adenylyl cyclase to reduce intracellular cAMP levels; 3) $G_{\alpha q}$, which activates β -isoforms of phospholipase C (PLC- β) to convert phosphatidylinositol biphosphate (PIP₂) to inositol trisphosphate (IP₃), leading to an increase in intracellular Ca²⁺ levels; 4) $G_{\alpha 12}$, which interacts with various proteins including, but not limited to, non-receptor tyrosine kinases (Syrovatkina et al. 2016).

A large body of evidence indicates that CB1 receptors are primarily coupled to G proteins of the Gai/o subfamily. Indeed, agonist-induced CB1 activation triggers the inhibition of adenylyl cyclase activity, leading to a reduction in cAMP levels and protein kinase A (PKA) activity. These effects are sensitive to pertussis toxin, confirming the involvement of Gi/o proteins (Kano et al. 2009). In addition, activation of CB1 receptors modulates several ion channels in a cAMP-dependent or -independent manner. Cannabinoid agonists modulate the voltage sensitivity of rapidly inactivated A-type potassium (KA⁺) channels, increasing voltage-gated A-type potassium currents (Deadwyler et al. 1993). These effects depend on the inhibition of adenylyl cyclase and PKA activity via Gai/o subunits, leading to dephosphorylation of KA⁺ channels and subsequent activation of outward potassium currents (Deadwyler et al. 1993; Mu et al. 2000). Cannabinoids also activate G protein-coupled inwardly rectifying potassium (GIRK) channels, which are involved in the hyperpolarization and maintenance of membrane resting potential (Mackie et al. 1995). Conversely, CB1 receptors inhibit the activation of voltage-gated Ca²⁺ channels through $G\beta/\gamma$ subunits (Araque et al. 2017), including P/Q-type (Mackie et al. 1995), N-type (Pan et al., 1996) and L-type calcium channels (Endoh 2006). Taken together, CB1 modulation of ion channels reduces neuronal

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excitability by maintaining the negative resting membrane potential and reducing the likelihood of neurotransmitter release.

In addition, CB1 coupling to Gi/o proteins has been shown to modulate other intracellular signaling pathways, including the MAPK extracellular signal-regulated kinase (ERK) pathway, which is involved in cell proliferation, differentiation, and survival, among others functions ([Wortzel and Seger 2011](#)). Rapidly following agonist stimulation (0-5min), Gi/o β/γ subunits disengage to transactivate multiple receptor tyrosine kinases, leading to the activation of the Ras/Raf/MEK/ERK pathway. This pathway is under the control of protein kinase A (PKA), whose activity is dependent on cAMP levels and inhibits Raf-induced phosphorylation of MEK/ERK. Due to the inhibition of adenylate cyclase by Gai/o subunits and the subsequent reduction in cAMP accumulation, PKA activity is downregulated, thus blunting its inhibition of ERK1/2 phosphorylation ([Dalton and Howlett 2012](#)). Finally, many other signaling pathways involved in the regulation of gene expression, cell survival, and metabolism (e.g., Jun (c-Jun N-terminal) kinase (JNK), p38-kinase and Akt-mammalian target of rapamycin (mTOR)) are activated upon the binding of cannabinoids to CB1 receptors and mediate signal transduction, but do not necessarily rely on G-protein activation ([A. C. Howlett 2005](#)).

2.1.2. Retrograde CB1 signaling and synaptic plasticity

Since the discovery of CB1 receptors and their endogenous ligands, eCBs, it has been well described that eCB-mediated signaling is involved in the modulation of short- and long-term plasticity. In particular, the action of eCBs on synaptic plasticity is mainly based on retrograde signaling. Namely, eCBs are produced and released "on demand" by postsynaptic neurons either phasically (i.e., in an activity-dependent manner) or tonically (i.e., under basal conditions). Subsequently, eCBs travel across the synaptic cleft to activate Gi/o-coupled CB1 receptors localized on the plasma membrane of presynaptic neurons to mediate suppression of neurotransmitter release via eCB-mediated short-term depression (eCB-STD) or long-term depression (eCB-LTD) ([Kano et al. 2009](#)) ([Figure 3](#)).

- *Retrograde CB1 signaling and short-term depression (STD)*

The discovery that eCB-mediated retrograde signaling is involved in STD stems from the identification of depolarization-induced suppression of inhibition (DSI). Indeed, depolarization-induced suppression of GABAergic inhibitory inputs from Purkinje cells and CA1 pyramidal cells was identified by measuring the transient suppression of spontaneous inhibitory postsynaptic potentials (IPSCs) following repeated postsynaptic depolarization

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(Llano, Leresche, and Marty 1991; Pitler and Alger 1992). In an attempt to elucidate the mechanisms underlying this form of synaptic plasticity, it was discovered that Ca^{2+} entry into the postsynaptic compartment is necessary for DSI to occur. Thus, it was proposed that depolarization-induced Ca^{2+} entry through voltage-gated Ca^{2+} channels, which causes an increase in intracellular postsynaptic Ca^{2+} , induces DSI (Llano, Leresche, and Marty 1991; Vincent and Marty 1993). Furthermore, DSI was associated with a decrease in the frequency of miniature IPSCs in the presence of tetrodotoxin (i.e., in the absence of a presynaptic action potential) but an increase in the amplitude of GABA-induced currents, suggesting that DSI is expressed presynaptically by inhibiting GABA release, but not by postsynaptic changes (Llano, Leresche, and Marty 1991). Thus, it was postulated that DSI depends on postsynaptic intracellular Ca^{2+} levels that cause the release of a retrograde transmitter to induce presynaptic changes. This putative retrograde transmitter was later identified as eCBs with the observation that 1) CB1 antagonists completely block DSI (T. Ohno-Shosaku, Maejima, and Kano 2001; Wilson and Nicoll 2001); 2) DSI occurs at cannabinoid-sensitive synapses (T. Ohno-Shosaku, Maejima, and Kano 2001); 3) DSI does not depend on postsynaptic vesicular release, which is required for classical neurotransmitters (Wilson and Nicoll 2001). A phenomenon similar to DSI was subsequently identified at excitatory synapses in Purkinje cells, termed depolarization-induced suppression of excitation (DSE), and recapitulated the critical features of DSI, namely postsynaptic Ca^{2+} entry, CB1 dependence, and non-vesicular release of the retrograde transmitter (Kreitzer and Regehr 2001) ([Figure 3](#)).

Ca^{2+} alone is sufficient to induce DSI/DSE at high concentrations (Wilson and Nicoll 2001) and can accumulate in postsynaptic neurons from voltage-gated Ca^{2+} but also from Ca^{2+} -permeable ionotropic receptors (Takako Ohno-Shosaku et al. 2007) and in some cases from intracellular Ca^{2+} stores (Isokawa and Alger 2005). In addition, Ca^{2+} -driven DSI/DSE have been shown to be prolonged after inhibition of the 2-AG catabolic enzyme MAGL but not the AEA catabolic enzyme FAAH (Hashimotodani, Ohno-Shosaku, and Kano 2007; Makara et al. 2005; Straiker and Mackie 2005), suggesting that 2-AG, but not AEA, is primarily responsible for Ca^{2+} -induced eCB-STD. Therefore, many studies have investigated whether 2-AG synthesizing enzymes, particularly $\text{PLC}\beta$ and DAGL, are involved in this phenomenon. Despite conflicting results, the current model of Ca^{2+} -driven eCB-STD is thought to require 2-AG synthesis, presumably in a $\text{PLC}\beta$ -independent but DAGL-dependent manner. This leads to the release of 2-AG from postsynaptic neurons to suppress neurotransmitter release via presynaptic CB1 receptors localized in inhibitory or excitatory terminals, thereby mediating DSI or DSE, respectively (Kano et al. 2009) ([Figure 3](#)).

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In addition, some metabotropic receptors have been shown to induce eCB-STD at basal Ca^{2+} levels, the most studied being the type 1/5 glutamate (mGlu1/5) and type 1/3 acetylcholine (mACh1/3) receptors. All of these receptors are known to stimulate PLC β activity via Gq/11 proteins, suggesting that they can induce 2-AG production by activating the PLC β /DAGL synthesis pathway (Kano, 2009). Indeed, studies using PLC β knockout mice (Hashimotodani, Ohno-Shosaku, and Kano 2007; Kano et al. 1998; M. Watanabe et al. 1998) and pharmacological inhibitors of DAGL (Galante and Diana 2004; Hashimotodani, Ohno-Shosaku, and Kano 2007) strongly suggest that both enzymes are required for mGluR- and mAChR-driven eCB-STD. Therefore, activation of postsynaptic Gq/11-coupled receptors stimulates 2-AG synthesis via the classical two-step pathway mediated by PLC β and DAGL, which can then travel to presynaptic CB1 receptors to suppress neurotransmission. In this case scenario, 2-AG can travel to homosynaptic terminals (i.e., those that release glutamate or acetylcholine), mediating DSE, or to neighboring heterosynaptic terminals, including inhibitory terminals, mediating DSI. Both Ca^{2+} - and receptor-driven eCB-STD may occur synergistically at the synapse, with postsynaptic elevation of Ca^{2+} levels potentiating the effect of metabotropic receptor activation on eCB release, likely due to the Ca^{2+} -dependent nature of PLC β . Finally, in all cases, eCB-STD (DSE/DSI) is terminated by hydrolysis of 2-AG by MAGL localized in presynaptic compartments (Kano et al. 2009) ([Figure 3](#)).

- *Retrograde CB1 signaling and long-term depression (LTD)*

2-AG can be released post-synaptically to induce LTD, presumably by a similar mechanism to STD (i.e., Ca^{2+} -driven release, receptor-driven release, or both). LTD is typically induced by repetitive afferent stimulation, with or without postsynaptic depolarization, but can also be triggered by postsynaptic firing. Although the mechanisms underlying eCB-mediated LTD are not fully understood, it requires sufficient postsynaptic Ca^{2+} influx for sustained mobilization of eCB and subsequent activation of presynaptic CB1 receptors to induce the prolonged suppression of neurotransmission (Takako Ohno-Shosaku and Kano 2014). Notably, although the induction of eCB-LTP requires the transient activation of CB1 receptors, presumably for downstream metabolic processes to occur, once established, this phenomenon becomes CB1 independent (Chevalleyre and Castillo 2003; Chevalleyre, Takahashi, and Castillo 2006). Intracellular metabolic processes may include the downregulation of cAMP levels via Gi/o coupled CB1 receptors and subsequent inhibition of PKA activity (Castillo 2012). In addition, CB1 activation alone is not sufficient to induce LTD but may require additional mechanisms, including Ca^{2+} influx into presynaptic terminals via the concomitant activation of presynaptic NMDA receptors and presynaptic voltage-gated calcium channels (Nevian and Sakmann 2006). In particular, Ca^{2+} influx has been proposed to contribute to the activation of

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intracellular mediators, including the Ca^{2+} -sensitive phosphatase calcineurin (CaN), which may promote dephosphorylation of unidentified presynaptic targets, together with reduced PKA-dependent phosphorylation, to mediate the sustained reduction in transmitter release (Heifets, Chevaleyre, and Castillo 2008). Further associations with the presynaptic proteins RIM1 α and RAB3B (Chevaleyre et al. 2007; Tsetsenis et al. 2011), the dopamine D2 receptor, and the muscarinic acetylcholine M2 receptor have been proposed to ensure selective induction of eCB-LTD at active synapses (Takako Ohno-Shosaku and Kano 2014). ([Figure 3](#)).

The other major eCB, AEA, also contributes to synaptic plasticity, although to a different extent than 2-AG. Indeed, several studies have reported that AEA contributes to high-frequency stimulation-induced eCB-LTD, whereas 2-AG contributes to low-frequency stimulation-induced eCB-LTD (Lerner et al. 2010). In addition, although AEA may act at presynaptic CB1 receptors, accumulating evidence shows that AEA also elicits LTP via postsynaptic TRPV1 channels, thereby inducing Ca^{2+} influx (Grueter, Brasnjo, and Malenka 2010; Chávez, Chiu, and Castillo 2010). Thus, it has been proposed that 2-AG mediates both eCB-STD (DSE/DSI) and eCB-LTP via CB1 receptors, whereas AEA mediates eCB-LTP mainly via TRPV1 (Puente et al. 2011). Finally, it has been proposed that 2-AG may function phasically (i.e., in an activity-dependent manner) to transmit a rapid and transient retrograde signal, whereas AEA mediates a tonic eCB tone (Takako Ohno-Shosaku and Kano 2014) ([Figure 3](#)).

See figure 3 next page

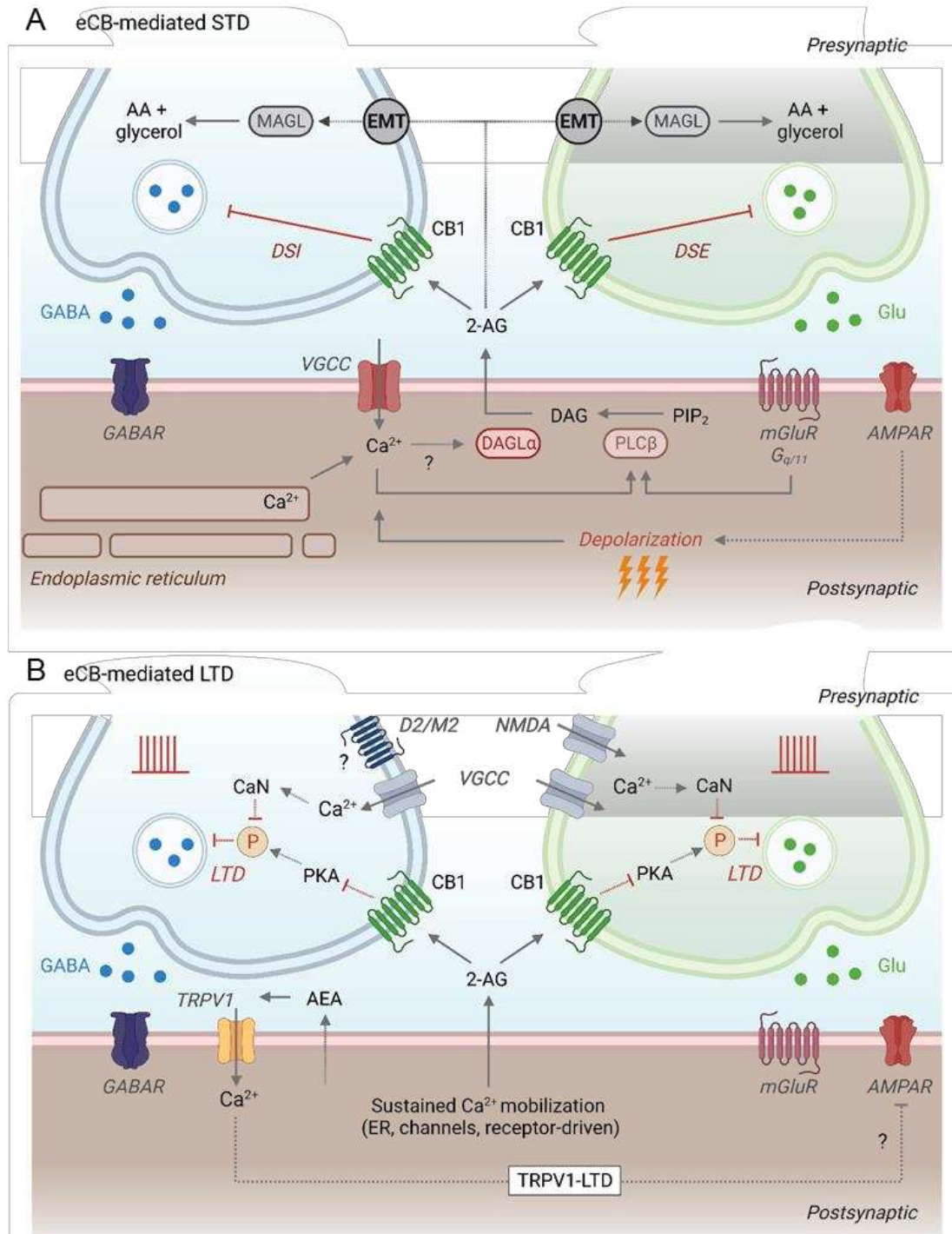
Figure 3: CB1-mediated synaptic plasticity.

A) eCB-mediated short-term depression (STD). The production of 2-AG is induced by large increase in intracellular Ca^{2+} following depolarization-induced activation of VGCC and/or Gq/11-coupled receptors such as mGluR. Both mechanisms can act synergistically by activating PLC β followed by the enzymatic action of DAGL α to yield 2-AG. 2-AG then acts on presynaptic CB1 receptors to suppress neurotransmitter release, resulting in DSI at inhibitory synapses or DSE at excitatory synapses. Excitatory synapses that release glutamate also activate AMPAR receptors, which cause an increase in intracellular Ca^{2+} via VGCC, resulting in the production of 2-AG, which can act at homosynaptic or heterosynaptic synapses. 2-AG is then taken up and hydrolyzed by MAGL. **B)** eCB-mediated long-term depression (LTP). 2-AG, produced by sustained intracellular Ca^{2+} elevation, suppresses neurotransmission via presynaptic CB1 receptors. Within the presynaptic elements, CB1 receptor activity, including inhibition of PKA, is combined with other factors, including presynaptic depolarizing activity, Ca^{2+} elevation via VGCC and NMDA receptors, activation of Ca^{2+} -dependent CaN, activation of D2 receptors, all presumably acting by blocking phosphorylation of an unidentified protein target. On the other hand, AEA synthesized tonically at basal Ca^{2+} levels produces LTP via TRPV1 channels that modulate the activity of AMPAR receptors.

Abbreviations: AA, arachidonic acid; CaN, calcineurin; D2, dopamine type 2 receptor; DAG, diacylglycerol; DAGL α , diacylglycerol lipase α ; DSE, depolarization-induced excitation suppression; DSI, depolarization-induced inhibition suppression; CB1, cannabinoid type 1 receptor; EMT, endocannabinoid membrane transporter; ER, endoplasmic reticulum; GABAR, GABA receptor; Glu, glutamate; M2, muscarinic type 2 receptor; mGluR, metabotropic glutamate receptor; MAGL, monoacylglycerol lipase; PIP2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PLC β , phospholipase C β ; TRPV1, transient receptor potential vanilloid type 1; VGCC, voltage-gated calcium channel.

Adapted from [Hashimotodani et al., 2007](#).

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2.2. Emerging mechanisms of action

2.2.1. Cellular CB1 non-canonical Gi/o-protein signaling

Advances in the understanding of CB1-mediated signaling suggest that the canonical Gai/o coupling is not an "intrinsic" property of CB1 receptors but that they can regulate a broader repertoire of G proteins with varying efficacy depending on environmental (cell type, subcellular localization) and pharmacological conditions ([Arнау Busquets-Garcia, Bains, and Marsicano 2018](#)).

As such, Gas is one of the best-characterized secondary signaling pathways of the CB1 receptor. In particular, CB1 was first observed to elicit Gas-like signaling upon co-stimulation with the dopamine type 2 receptor (D2) (i.e., a GPCR primarily coupled to Gai/o proteins). In primary rat striatal neurons, stimulation with D2 or CB1 agonists produced the expected inhibition of cAMP accumulation, whereas stimulation with both agonists reversed the inhibitory signal in a concentration-dependent manner ([Glass and Felder 1997](#)). In addition, in both primary cells and a cell line stably expressing CB1 receptors, pretreatment with pertussis toxin, which inhibits Gi/o activity, was able to unmask Gs-like signaling upon CB1 agonist stimulation ([Glass and Felder 1997](#); [Bonhaus et al. 1998](#); [Scotter et al. 2010](#)). Consistent with these findings, it was demonstrated that cannabinoid-induced depression of synaptic transmission was shown to be switched to stimulation upon activation of D2 receptors in the rodent globus pallidus, affecting motor control and highlighting the physiological relevance of non-canonical CB1 Gs signaling ([Caballero-Florán et al. 2016](#)).

This ability of GPCRs to signal through multiple G proteins is sometimes referred to as "promiscuous G protein coupling" ([Maudsley, Martin, and Luttrell 2005](#)) and may depend on several factors. One hypothesis is that CB1 receptors exhibit Gs-like coupling when the cellular pool of Gai/o is depleted (e.g., by G protein sequestration by D2 receptors or pharmacological inhibition by pertussis toxin). An alternative hypothesis is that heterodimerization of CB1 with other receptors (e.g., D2) ([Bagher et al. 2016](#); [Marcellino et al. 2008](#)) leads to changes in protein conformation and, thus, receptor signaling. However, these two hypotheses are not mutually exclusive. Recently, CB1 receptor availability at the membrane has been identified as a novel determinant in the functional switch from Gi/o to Gs signaling. Indeed, increased secretion of nascent CB1 protein in a cell line stably transfected with the pplss-3HA-CB1 construct resulted in accumulation of cellular cAMP upon CB1 stimulation, in contrast to control cells stably transfected with control 3HA-CB1. Furthermore, supplementation with Gi/o protein reversed the stimulatory effects of pplss-3HA-CB1 on cAMP, suggesting that receptor availability is indeed a determinant in the switch from Gi/o to Gs, falling under the "Gi/o

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exhaustion" hypothesis (Finlay et al. 2017). Importantly, this study demonstrates that CB1 coupled to Gs proteins exhibits significant agonist bias (i.e., the receptor responds differently depending on the agonist tested), which adds to the complexity of G-protein-mediated CB1 signaling and may have implications for the effects of cannabinoid signaling *in vivo* (Finlay et al. 2017) ([Figure 4](#)).

In addition, CB1 coupling to G proteins may differ depending on the cell type. One of the most striking examples of this phenomenon occurs in astrocytes, where the CB1 receptor is thought to couple to Gq proteins to modulate intracellular Ca⁺ levels. In astrocytes, intracellular Ca²⁺ is released from the endoplasmic reticulum (ER) following the production of inositol triphosphate (IP₃) by PLC and subsequent activation of IP₃ receptors (Eraso-Pichot et al. 2022). IP₃ production can be induced by a number of Gq protein-coupled receptors, including CB1 receptors. This is shown by the study of the application of cannabinoids to mouse brain slices, which leads to increased intracellular Ca²⁺ in astrocytes (M. Navarrete and Araque 2008). This effect was not recapitulated in slices obtained from CB1 knockout mice and was blocked by CB1 antagonists but not by pertussis toxin, suggesting a CB1-mediated but not Gi/o-mediated mechanism of action. However, the effects of cannabinoids on Ca²⁺ levels in astrocytes were blunted by a PLC inhibitor, favoring a Gq-mediated response (M. Navarrete and Araque 2008). In addition, cannabinoid regulation of astroglial Ca²⁺ is thought to play an essential role at the tripartite synapse. Indeed, eCB-dependent activation of CB1 receptors in astrocytes can trigger the release of various gliotransmitters (Eraso-Pichot et al. 2022), which can then act on neighboring or distant neurons (Han 2012; Martín et al. 2015), thus providing an example of CB1-mediated functions beyond retrograde signaling.

More generally, studies aimed at investigating the families and subtypes of G proteins activated by CB1 receptors, using GTPγS assays and co-immunoprecipitation have shown that CB1 receptors in the mouse cortex are coupled not only to classical Gi/o proteins but also to Gq and G12 proteins (Diez-Alarcia et al. 2016; Prather et al. 2000). Most intriguingly, it has also been reported that endogenous and exogenous CB1 ligands can recruit G protein binding in a biased manner, preferentially affecting specific signaling pathways (Diez-Alarcia et al. 2016). In this regard, it has been proposed that heterogeneity in CB1 coupling to G-protein and the associated biased action of cannabinoid ligands may depend, at least in part, on cell type-specific expression of the receptor (Arnau Busquets-Garcia, Bains, and Marsicano 2018). Finally, it should be noted that CB1 coupling to G proteins differs in efficacy between neuronal subpopulations. For example, although CB1 receptors are more abundant in cortical GABAergic neurons than in cortical glutamatergic neurons, CB1-mediated G-protein activation is stronger in glutamatergic than in GABAergic neurons, suggesting additional heterogeneity

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in the efficacy of CB1 receptor G-protein-dependent signaling (Steindel et al. 2013). However, the mechanisms underlying these functional differences remain poorly understood.

Therefore, CB1 receptor expression is not stochastically related to the efficacy of G-protein-mediated signaling and differs from canonical Gi/o signaling depending on the recruitment of different G-protein subtypes based on complex and intricate factors (e.g., cell type, receptor number, and dimerization). This further supports the idea that CB1 coupling to Gi/o proteins is not an intrinsic property of the receptor and that CB1 is functionally diverse in its mechanism of action, which may, in part, support the pleiotropic action of the receptor in the brain.

2.2.2. Cellular CB1 signaling mediated by β -arrestins

In addition to G-protein-mediated signaling, CB1 has been shown to interact with other effector proteins to transduce signaling into the cell (Figure 4). Like most class A GPCRs, CB1 receptors can interact with β -arrestins (Leo and Abood 2021); in particular, β -arrestin 1 (also known as arrestin 2) and β -arrestin 2 (also known as arrestin 3), which are ubiquitously expressed in most mammalian tissues and cells (Chutkow et al. 2010). β -Arrestins were initially identified as proteins involved in the desensitization of GPCRs (Freedman and Lefkowitz 1996). In this model, agonist-induced receptor activation triggers the dissociation of the G protein subunits (α and β/γ), leading to the recruitment of soluble second messengers. GPCR signaling is then terminated by a series of serine/threonine phosphorylations of the receptor by kinase proteins, including the GPCR kinase (GRK) family, and the subsequent binding of β -arrestins to desensitize the receptor by preventing further interaction with G proteins. In a second step, β -arrestins can also mediate internalization of the receptor into clathrin-coated pits, from which receptors can either be dephosphorylated and recycled to the membrane or targeted for lysosomal degradation if ligand-induced stimulation persists (Freedman and Lefkowitz 1996; Yu et al. 1993).

Although this canonical model of GPCR signaling remains true, subcellular signaling does not end there. In fact, there is increasing evidence that β -arrestins, in addition to their role in receptor desensitization and internalization, become part of a multi-protein signaling complex, thereby inducing a novel G-protein-independent signaling cascade (Luttrell et al. 1999). In particular, the discovery that molecular effectors other than G proteins can transduce signals has reshaped our understanding of GPCR activity with the emergence of two key concepts: receptor signaling pluridimensionality and ligand bias. Briefly, the specific interaction of GPCRs with G proteins and other effectors (e.g., β -arrestins) allows the formation of preferential signaling pathways that may depend on cell type, tissue, and expression of signal

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transducers, thereby defining receptor function. In addition, different ligands can alter the conformational equilibrium of the receptor to direct specific interactions with downstream effectors. Together, these processes point to a new paradigm, defined as "biased" signaling, that is particularly relevant for understanding GPCR biological function and drug development ([van Gastel et al. 2018](#)).

The CB1 receptor has been shown to interact with both β -arrestin 1 and 2, although their functional interactions in receptor desensitization, internalization, and signaling have not been clearly elucidated ([Ibsen et al. 2019](#)). β -Arrestin 2 has typically been implicated with CB1 receptor desensitization and internalization. Indeed, mutant mice lacking β -arrestin 2 exhibit attenuated tolerance to the CB1 agonist THC, accompanied by decreased CB1 downregulation and impaired desensitization of agonist-stimulated GTP γ S binding. Using siRNA technology, β -arrestin 2 was also found to be critical for CB1 receptor internalization induced by the synthetic PAM ORG27569 ([Ahn et al. 2013](#)). Furthermore, phosphorylation of a serine/threonine cluster located at the extreme "distal" C-terminus of the receptor by GRK is required for β -arrestin 2 translocation. Consequently, loss of phosphorylation of the CB1 C-terminal domain results in decreased receptor internalization and increased signaling ([Jin et al. 1999](#); [Daigle, Kwok, and Mackie 2008](#)).

On the other hand, β -arrestin 1 is primarily associated with receptor signaling, and its binding to GPCRs leads to phosphorylation of the MAPK/ERK pathway ([DeWire et al. 2007](#)). Accordingly, ORG27569-induced ERK1/2 and MERK1/2 phosphorylation was attenuated by β -arrestin 1 siRNA but not by pertussis toxin, demonstrating β -arrestin 1-dependent, but G-protein-independent, signaling downstream of CB1 receptors ([Ahn et al. 2013](#)). In addition, loss of translocation of β -arrestin 2 in the absence of phosphorylation of the distal C-terminal domain of CB1 was observed to increase interaction with β -arrestin 1. Thus, it was proposed that the reduced rate of internalization of CB1 would allow the receptor to interact with β -arrestin 1, resulting in increased ERK1/2 signaling ([Delgado-Peraza et al. 2016](#)). However, a recent study did not observe β -arrestin 1 binding to CB1 receptors when C-terminal phosphorylation is impaired. In addition, β -arrestin 1 and 2 appeared to have similar phosphorylation requirements to interact with CB1 receptors, suggesting that the reported potentiation of ERK1/2 signaling may be due to increased G-protein activity resulting from reduced interaction with both arrestin subtypes ([Manning et al. 2023](#)). Therefore, the mechanism by which agonist-induced CB1 activation recruits β -arrestins and how CB1 ligands exhibit functional selectivity for G- or β -arrestin-mediated signaling ([Laprairie et al. 2014](#)) remains unclear. It should also be noted that β -arrestin-mediated activation of the ERK1/2 pathway is temporally distinct from G-protein-mediated activation. Indeed, whereas G-protein-

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mediated ERK1/2 phosphorylation occurs within the first five minutes of stimulation, β -arrestins trigger a second wave of signaling that occurs later, between then and thirty minutes (Nogueras-Ortiz and Yudowski 2016).

2.2.3. Other proteins interacting with cellular CB1 receptors

In addition to β -arrestins, CB1 signaling can be further modified by several accessory proteins, including (but not limited to) the cannabinoid receptor interacting protein 1a (CRIP1A), G-protein-associated sorting proteins (GASP), and Src homology3-domain growth factor receptor-bound 2-like endophilin inter-acting protein 1 (SGIP1) (Allyn C. Howlett, Blume, and Dalton 2010). CRIP1A binds the distal C-terminal tail of the CB1 receptor and was initially characterized for its ability to reverse CB1-mediated inhibition of Ca^{2+} channels in superior cervical ganglion neurons (Niehaus et al. 2007). Since then, the interaction between CRIP1A and CB1 receptors has been shown to affect G-protein coupling and, thereby, signaling via the cAMP and ERK1/2 pathways. Specifically, N18TG2 neuronal cells stably transfected with CRIP1A show attenuation of CP55940-induced inhibition of cAMP accumulation and reduced basal, but not agonist-stimulated, phosphorylation levels of ERK1/2 compared to wild-type cells. In contrast, CP55940-induced inhibition of cAMP accumulation was enhanced in CRIP1A knockdown cells, as were basal and CP55940-stimulated ERK1/2 phosphorylation (Blume et al. 2015). In addition, CRIP1A overexpression was associated with a general suppression of functional Gi/o protein coupling as assessed by $\text{GTP}\gamma$ binding assays (Blume et al. 2015). Taken together, these data are consistent with a role for CRIP1A in dampening CB1-dependent G protein activation, leading to suppression of cAMP inhibition and ERK1/2 phosphorylation. Additionally, CRIP1A has been shown to reduce agonist-induced CB1 internalization and β -arrestin recruitment, suggesting that CRIP1A and β -arrestins mediate opposing effects on CB1 receptor trafficking (Blume et al. 2015). Interestingly, CRIP1A and β -arrestins do not co-immunoprecipitate with each other, which may be due to the competitive binding of both proteins to phosphorylation sites located on the C-terminal tail of CB1 receptors (Blume et al. 2017).

The role of GASP1 in regulating CB1 activity has been linked to the termination of the G-protein signaling cycle. Indeed, the interaction of GASP1 with CB1 has been demonstrated in co-immunoprecipitation studies as well as in pull-down assays (Martini et al. 2007). In particular, upon agonist stimulation, the CB1 receptor is internalized and colocalizes with GASP1 in lysosomes, suggesting that GASP1 regulates post-endocytic targeting of the receptor to the lysosome for degradation (Tappe-Theodor et al. 2007). This hypothesis is supported by the depletion of GASP1 by viral delivery of dominant-negative cGASP1-AAV in primary neuronal cultures and the subsequent attenuation of agonist-induced CB1 efflux from

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the plasma membrane (Tappe-Theodor et al. 2007). Furthermore, mice injected with dominant-negative cGASP1-AAV show impaired tolerance to the analgesic effects of CB1 agonists and reduced downregulation of CB1 receptor expression (Tappe-Theodor et al. 2007). At the structural level, GASP1 has been proposed to compete with G proteins due to similar binding sites on GPCRs (particularly the helix 8 domain in the case of CB1 coupled to Gi/o proteins), thereby exerting its role in terminating G protein signaling (Allyn C. Howlett, Blume, and Dalton 2010). Finally, in contrast to GASP1, SGIP1 has been identified as a CB1-interacting protein at the C-terminal tail that prevents endocytosis of CB1 receptors. SGIP1 colocalizes with CB1 receptors in neurites of primary neuronal cultures, increases receptor surface stability in transfected HEK293 cells, and blocks CB1 internalization in the absence of agonists, thus suggesting an effect on constitutive endocytosis of the receptor (Hájková et al. 2016). At the molecular level, SGIP1 was observed to affect CB1 signaling in a biased fashion. SGIP1 does not affect G-protein signaling but reduces agonist-induced ERK1/2 phosphorylation, likely by preventing β -arrestin-mediated activation of the ERK pathway from internalized receptors (Hájková et al. 2016). However, the molecular mechanisms of action of SGIP1 in modulating CB1 trafficking and signaling pathways remain unclear and require further investigation for clarification.

2.2.4. Subcellular CB1 signaling

The CB1 receptor, like most GPCRs, is classically defined as a plasma membrane receptor protein that mediates signaling from extracellular ligands into the cell. While this is true, increasing evidence suggests that GPCRs are functionally active within the cell, either by maintaining signaling from endosomes after internalization or by functional targeting in various organelles (Jalink and Moolenaar 2010; Arnau Busquets-Garcia, Bains, and Marsicano 2018) ([Figure 4](#)).

Although CB1 signaling has been primarily associated with its localization to the plasma membrane of presynaptic neuronal terminals (Kano et al. 2009), a large proportion of receptors are localized to endosomes, either through steady-state internalization due to constitutive activity of the receptor (i.e., signaling in the absence of ligand) or through agonist-induced internalization (Thibault et al. 2013). In addition, while newly synthesized CB1 receptor proteins translocate from their biosynthetic compartment to the plasma membrane, the vast majority of receptors never reach the membrane but associate with the adaptor protein AP-3 for trafficking to the lysosomal compartment (Rozenfeld and Devi 2008). Interestingly, intracellular CB1 receptors found in endosomal/lysosomal compartments were found to be functionally coupled to Gi/o proteins and mediate signal transduction through the activation of the MAPK/ERK pathway (Rozenfeld and Devi 2008). Another report indicated that endocytosed CB1 does not

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directly contribute to this intracellular receptor pool or redistribute to the plasma membrane but is primarily targeted for degradation ([Grimsey et al. 2010](#)), suggesting that intracellular CB1 receptors have a distinct function from membrane receptors. Furthermore, a study reported that intracellular injection of AEA into HEK293 cells activates the intracellular pool of CB1 receptors, resulting in Ca^{2+} mobilization from endolysosomes, further demonstrating that endolysosome-targeted CB1 receptors are functional ([Brailoiu et al. 2011](#)).

Furthermore, the most striking evidence for functional CB1-mediated signaling at the intracellular level comes from the discovery of CB1 receptors associated with mitochondrial membranes (mtCB1) ([Aquila et al. 2010](#); [Bénard et al. 2012](#); [Mendizabal-Zubiaga et al. 2016](#)). The effects of cannabinoids on mitochondrial functions were reported as early as the 1970s, including inhibition of the activity of enzymes involved in the respiratory chain complex ([Bartova and Birmingham 1976](#); [Chari-Bitron and Bino 1971](#); [Schurr and Livne 1975](#)), changes in mitochondrial ultrastructure ([Bino, Chari-Bitron, and Shahar 1972](#)), and an overall decrease in mitochondrial respiration ([Mahoney and Harris 1972](#)). However, due to their lipidic nature, cannabinoids were initially thought to mediate unspecific changes in the mitochondrial membrane, thereby affecting the function of the organelle. The first evidence for the functional presence of CB1 receptors in mitochondria came from peripheral tissues, in particular from spermatozoa. It has been reported that sperm cells are equipped with CB1 receptors along with the typical enzymatic machinery of the ECS ([Schuel et al. 1994](#); [Maccarrone 2005](#)). In addition, studies have reported that the eCB AEA reduces sperm motility, a highly energy-consuming process, by reducing mitochondrial functions ([Rossato et al. 2005](#)). Therefore, in the 2010s, the ultrastructural localization of CB1 receptors in sperm cells was investigated by transmission electron microscopy coupled with immunogold analysis and revealed the presence of CB1 receptors on the mitochondrial membranes of the sperm head, supporting the effects of cannabinoids on bioenergetic functions ([Aquila et al. 2010](#)).

Just two years later, similar ultrastructural analyses coupled with functional assays revealed the presence of a subcellular fraction of CB1 receptors associated with neuronal mitochondria (approximately 15% of total cellular CB1 receptors) in the mouse hippocampus ([Bénard et al. 2012](#)). Although present at lower levels than in sperm cells ([Mendizabal-Zubiaga et al. 2016](#)), neuronal mtCB1 receptors have been found in both GABAergic and glutamatergic neurons of the hippocampal subfield and directly control cellular respiration and energy production. Indeed, exogenous and endogenous cannabinoids have been shown to reduce mitochondrial respiration in purified mitochondria from the brains of wild-type but not CB1 knockout mice ([Bénard et al. 2012](#)). On a mechanistic level, several reports have shown that mitochondria contain downstream effectors of G-protein signaling, including soluble adenylylase (sAC) and PKA ([Zippin et al. 2003](#); [Ryu et al. 2005](#)). Thus, cAMP can be produced in

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mitochondria to activate PKA, leading to the phosphorylation of complex I of the respiratory/OXPHOS chain and subsequent energy production ([Acin-Perez et al. 2009](#); [R. Chen et al. 2004](#)).

Interestingly, cannabinoids were found to mediate the inhibition of cAMP accumulation in purified mitochondria, along with reduced activity of cAMP-dependent PKA and complex I activity ([Bénard et al. 2012](#)). A subsequent study reported that these effects were blocked by pertussis toxin and inhibition of sAC activity, suggesting that mtCB1 is functionally coupled to Gi/o proteins. In addition, mtCB1 decreases PKA-dependent phosphorylation of mitochondrial proteins, including the complex I subunit NDUF52, linking mtCB1 signaling to the reduction of mitochondrial respiration ([Hebert-Chatelain et al. 2016](#)). It should be noted, however, that the mechanisms of action by which mtCB1 induces a reduction in oxygen consumption are still open to question. In particular, whereas the effects of cannabinoids on cellular respiration are blocked by pertussis toxin, sAC is generally considered to mediate G-protein-independent signaling, as it lacks the classical structural features that allow membrane-bound AC to interact with G proteins ([Steegborn 2014](#)). In this context, mitochondria may possess unique signaling complexes that may provide an indirect link between G proteins and sAC ([Arnau Busquets-Garcia, Bains, and Marsicano 2018](#)). In addition to neurons, mtCB1 has been identified in astroglial mitochondria from different subfields of the mouse hippocampus ([Gutiérrez-Rodríguez et al. 2018](#); [Jimenez-Blasco et al. 2020](#)) and in white adipocyte mitochondria ([Pagano Zottola et al. 2022](#)) ([Figure 4](#)).

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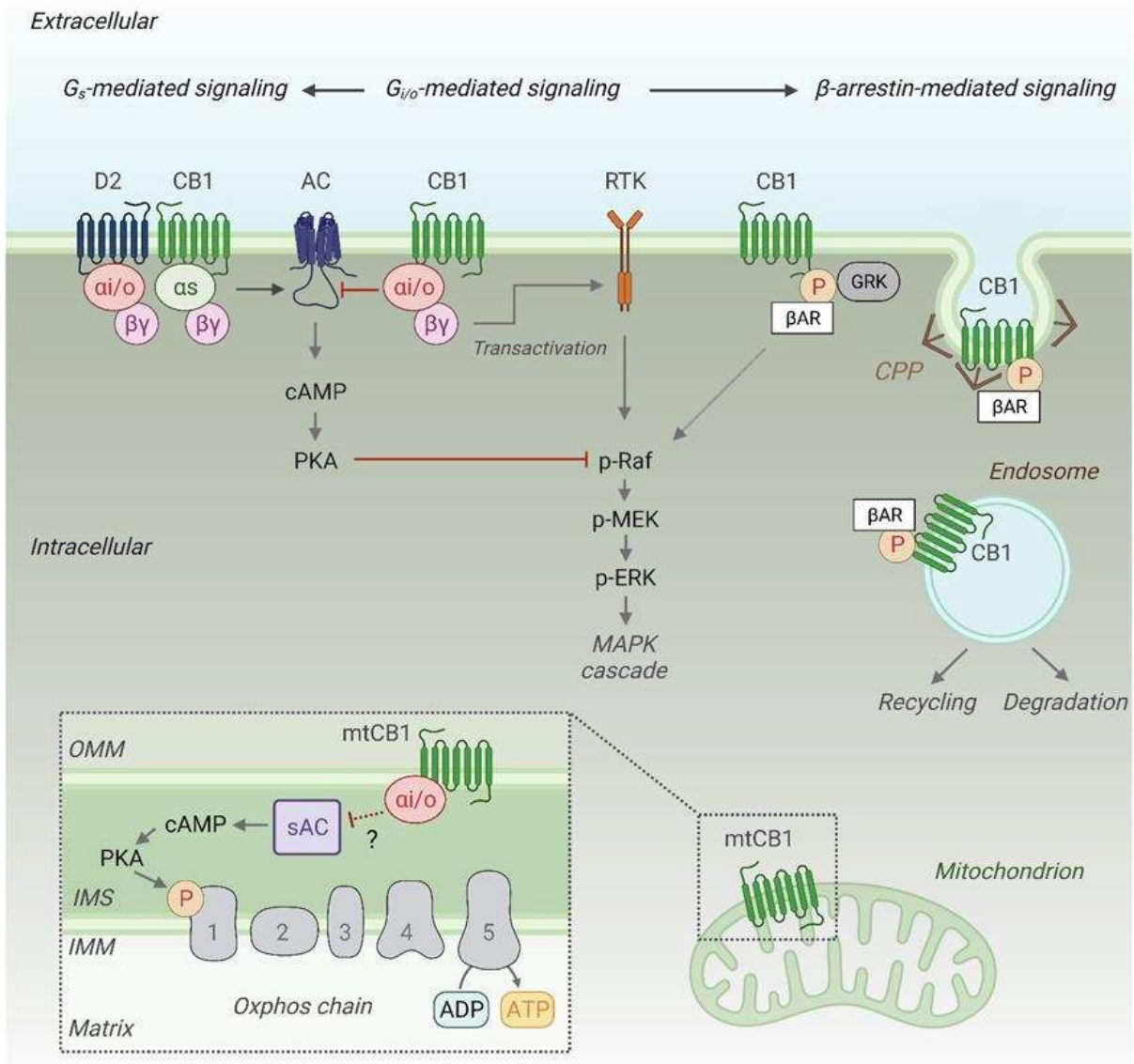


Figure 4: CB1-mediated signaling pathways via G- and/or β -arrestin proteins at the plasma and subcellular membranes.

CB1 is typically coupled to $G_{i/o}$ proteins. $G_{i/o}$ subunits inhibit the activity of adenylyl cyclase, thereby blunting cAMP production and PKA activity. $G\beta\gamma$ subunits can transactivate receptor tyrosine kinases, leading to activation of the MAPK cascade. Under certain conditions (e.g., dimerization with D2 receptors), CB1 can couple G_s subunits, leading to activation of adenylyl cyclase. CB1 can be phosphorylated by GRKs, allowing the recruitment of β -arrestins that activate MAPK pathways and induce receptor internalization. Internalized CB1 receptors can still mediate signaling and are subject to membrane recycling or degradation. At the subcellular level, CB1 receptors are functionally coupled to mitochondria where they inhibit soluble adenylyl cyclase activation via $G_{i/o}$ proteins, thereby blunting phosphorylation of complex I of the OxPhos chain and reducing ATP production. **Abbreviations:** AC, Adenylyl cyclase; ADP, Adenosine bisphosphate; ATP, Adenosine trisphosphate; β AR, β -arrestins; CB1, Cannabinoid type-1 receptor; CPP, Clathrin-coated pits; cAMP, Cyclic adenosine monophosphate; D2, Dopamine type-2 receptor; GRK, G protein-coupled receptor kinase; IMM, Inner mitochondrial membrane; IMS, Intermembrane space; mtCB1, Mitochondrial CB1 receptor; OMM, Outer mitochondrial membrane; OxPhos, Oxidative phosphorylation; P, Phosphate/phosphorylation of CB1 receptor; PKA, Protein kinase A; RTK, Receptor tyrosine kinase; sAC, Soluble adenylyl cyclase.

Adapted from Dalton et al., 2012; Busquets-Garcia et al., 2018.

2.3. CB1 functions in physiopathology

2.3.1. The importance of CB1 receptor localization

The CB1 receptor is considered to be the most abundant GPCR in the mammalian brain and is also expressed at peripheral levels in a variety of other organs and tissues. Thus, it is not surprising that the widespread distribution of CB1 receptors in the body regulates a variety of biological functions, both at the physiological level and in pathological conditions. At the physiological level, CB1 receptors are predominantly involved in modulating processes such as emotional behaviors (Lafenêtre, Chaouloff, and Marsicano 2007; Maldonado, Cabañero, and Martín-García 2020), memory and learning (Kruk-Slomka et al. 2017), reward processing (Spanagel 2020), motor function (Morera-Herreras et al. 2012), nociception (Finn et al. 2021), and energy balance (Piazza, Cota, and Marsicano 2017). Interestingly, CB1 activation has surprising consequences and does not always follow a linear rule where a given CB1 agonist produces a single behavioral outcome. Instead, low doses of a CB1 agonist can produce one effect, while higher doses produce the opposite, resulting in a bidirectional regulation of behavior (Arnau Busquets-Garcia et al. 2015). The biphasic control of CB1 receptors on biological functions often lies in the balance between excitatory glutamatergic and inhibitory GABAergic inputs. Indeed, several pharmacological studies coupled with genetic deletion of CB1 receptors in different cell types indicate that low doses of CB1 agonists preferentially activate "glutamatergic" CB1 receptors, whereas high doses activate "GABAergic" CB1 receptors, resulting in the biphasic action of cannabinoids (Bellocchio et al. 2010; Arnau Busquets-Garcia et al. 2015; Metna-Laurent et al. 2012; Rey et al. 2012). Moreover, the behavioral consequences of CB1 activation are critically linked to their subcellular localization, with the discovery of intracellular pools of receptors, particularly mtCB1, providing new insights into cannabinoid-mediated effects (Arnau Busquets-Garcia, Bains, and Marsicano 2018). Therefore, in the following sections, we will provide some examples of the main features of CB1 activation depending on the cellular and subcellular localization of the receptors and their consequences for behavioral modulation.

2.3.2. CB1-related emotional behaviors

CB1 cell type-dependent modulation of behavior can be illustrated by emotional responses, which include anxiety-related behaviors and fear responses. Anxiety is defined as an innate state of arousal associated with the anticipation of impending and potentially harmful events that allows an individual to initiate appropriate behavioral and physiological responses (e.g., avoidance, increased heart rate, and decreased motor activity) to avoid potentially dangerous or harmful situations. However, chronic and exaggerated anxiety can become detrimental on a physical and psychological level and eventually lead to the development of

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anxiety-related disorders (Thibaut 2017). Low doses of cannabinoids are known to induce anxiolytic effects, while high doses induce anxiogenic effects (Rubino et al. 2008; Lutz et al. 2015). Furthermore, the complete deletion of CB1 receptors in knockout mice (CB1-KO) results in anxiety-like behaviors under aversive conditions (Jacob et al. 2009). This is partially recapitulated by the conditional deletion of CB1 receptors on cortical glutamatergic neurons, with Glu-CB1-KO mice showing reduced exploratory behavior, suggesting anxiety-like phenotypes (Jacob et al. 2009; Rey et al. 2012; Lafenêtre, Chaouloff, and Marsicano 2009), but not by conditional deletion of CB1 receptors on cortical GABAergic neurons, with GABA-CB1-KO mice showing the opposite phenotype (Rey et al. 2012; Lafenêtre, Chaouloff, and Marsicano 2009; Häring et al. 2011). These findings are consistent with the reported low "anxiolytic" and high "anxiogenic" doses of cannabinoids that mediate their effects via "glutamatergic" and "GABAergic" CB1 receptors, respectively (Rey et al. 2012), suggesting that CB1 regulates anxiety-like states in a bidirectional manner.

Whereas anxiety is an innate state elicited by potential future threats, fear is classically defined as a state of arousal in response to perceived or imminent threats that allows an individual to initiate active (e.g., fleeing) or passive (e.g., freezing) behaviors. Furthermore, fear can be either innate (e.g., fear of natural predators) or acquired (e.g., learning that a stimulus represents a threat) (Steimer 2002). Most studies in rodents investigating the neural mechanisms of fear rely on learned fear responses modeled by cued fear conditioning. Briefly, animals learn the association between a neutral/conditioned stimulus (CS) (e.g., a visual or acoustic cue) and a "fear"/unconditioned stimulus (US) (e.g., an electric shock) until the presentation of the CS alone is capable of eliciting a fear response, which in rodents is most commonly recapitulated by freezing behavior, characterized by the absence of any movement other than breathing (Wotjak 2019). The fear memory is consolidated within hours of CS-US pairing but can be further modulated by environmental conditions. Indeed, brief exposure to the CS alone triggers reconsolidation of the fear memory trace, whereas repeated exposure to the CS alone results in a decrease in the evoked fear response (also known as "extinction") (Wotjak 2019). CB1-KO mice show no significant alterations in fear memory acquisition and consolidation but are characterized by an impairment in fear extinction (Giovanni Marsicano et al. 2002). Subsequent studies have shown that "glutamatergic" CB1 receptors are necessary but not sufficient for fear extinction (Dubreucq et al. 2012; S. Ruehle et al. 2012), whereas "GABAergic" CB1 receptors are not essential for the expression of fear extinction (Dubreucq et al. 2012; Llorente-Berzal et al. 2015). Although the CB1 receptor has been found to mediate extinction via habituation-like (i.e., non-associative) processes (Kornelia Kamprath et al. 2006; K. Kamprath et al. 2009), another key determinant of the behavioral consequences of CB1 activity on fear expression lies in the temporal expression of passive (e.g., freezing) and active

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(e.g., rearings, digging) fear coping strategies. Indeed, the dominant freezing responses adopted by CB1-KO in classical fear conditioning are likely due to the deletion of "glutamatergic" CB1 receptors. Conversely, deletion of "GABAergic" CB1 receptors results in active coping behaviors and facilitates active avoidance learning ([Metna-Laurent et al. 2012](#)). Thus, CB1 receptors exert bimodal control over fear-coping strategies to shape emotional responses.

2.3.3. CB1-related memory processes

In addition to innate emotional states and acquired emotional memories, CB1 receptors have also been implicated in the modulation of non-emotional memories in a cell-type dependent manner. The hippocampus, a critical region involved in memory formation with high levels of CB1 receptors ([Kano et al. 2009](#)), is involved in mediating the amnesic effects of cannabinoids ([Lundqvist 2005](#); [Arnau Busquets-Garcia et al. 2015](#)). The effects of cannabinoids are often associated with an imbalance between excitatory and inhibitory transmission. For example, THC administration in rodents suppresses GABA release, resulting in increased glutamatergic transmission ([Pistis et al. 2002](#)). Consistent with this, studies using conditional CB1 knockout mice have reported that THC-induced long-term memory deficits are reduced in GABA CB1 KO mice and attenuated by pharmacological inhibition of NMDA receptors, suggesting that the amnesic effects of cannabinoids are mediated via CB1 receptors located on GABAergic axon terminals, thereby potentiating glutamatergic transmission ([Puighermanal et al. 2013](#)). In addition to GABAergic and glutamatergic neurons, other players have been identified in mediating the acute effects of cannabinoids on memory impairment. Indeed, mice lacking CB1 receptors, specifically on astrocytes, do not exhibit cannabinoid-induced impairments in short-term working memory. This suggests that activation of astroglial CB1 receptors is required for cannabinoid-induced short-term memory impairment ([Han et al. 2012](#)). Several mechanisms have been proposed for the regulation of memory by astroglial CB1 receptors. Indeed, CB1 in astrocytes may contribute to the formation of adult neurons in the hippocampus (i.e., adult neurogenesis) ([Sultan et al. 2015](#)), but also to the metabolic coupling between neurons and astrocytes ([Arnau Busquets-Garcia, Bains, and Marsicano 2018](#)).

Interestingly, mtCB1 receptors have been found to directly affect bioenergetic processes by reducing mitochondrial respiration, and their activation is required to mediate acute cannabinoid-induced memory impairment in mice ([Hebert-Chatelain et al. 2016](#)). A study also reported that activation of mtCB1 in astrocytes attenuated glucose metabolism and lactate production in the mouse brain through a reduction in mitochondrial respiratory chain activity and generation of reactive oxygen species by astrocytes ([Jimenez-Blasco et al. 2020](#)). The

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reduction in lactate production subsequently affected neuronal functions, resulting in impaired behavioral responses in social interaction assays (Jimenez-Blasco et al. 2020). Thus, it could be speculated that the acute amnesic effects of cannabinoids mediated by mtCB1 may occur in part by decoupling metabolic processes between neurons and astrocytes. Finally, mtCB1 has recently been reported to mediate stress-induced memory impairment. Namely, mtCB1 receptors in noradrenergic neurons are recruited for stress-induced impairment of memory consolidation, whereas mtCB1 receptors associated with hippocampal GABAergic interneurons inhibit stress-induced memory retrieval (Skupio et al. 2023). Thus, these findings, together with the above-mentioned effects on fear extinction, suggest that CB1 receptors are critically involved in "forgetting" through complex processes depending on their cellular and subcellular localization.

2.3.4. CB1-related regulation of pain and movement

A large body of evidence suggests that cannabinoids have a high therapeutic potential for pain-related disorders. Indeed, cannabinoids regulate nociceptive thresholds, and hypoactivation of the ECS is associated with hyperalgesia (i.e., increased pain sensitivity) and chronic pain (Richardson, Aanonsen, and Hargreaves 1997). Although many studies have attributed an essential role to the CB2 receptor in pain management via anti-inflammatory processes (Anthony et al. 2020), activation of the CB1 receptor also results in a decrease in both nociceptive pain and inflammatory hypersensitivity (Starowicz and Finn 2017). The growing interest in cannabis-based pain management is, therefore, not surprising (Donvito et al. 2018). However, cannabis is known to cause significant side effects, including cognitive and motor dysfunction, raising questions about the safety profile of such therapies. Indeed, CB1 activation triggers impairments in motor control (Blázquez et al. 2020; Monory et al. 2007), and cannabis use is associated with cataleptic-like effects (i.e., impaired ability to initiate movement), which are a significant safety concern (Martin et al. 2017).

Moreover, one of the main evaluation criteria to determine whether a pharmacological agent is a CB1 agonist or not is based on a classic preclinical model called the "cannabinoid-induced tetrad", which aims to evaluate four prototypical phenotypes induced by acute administration of cannabinoids. The tetrad includes the evaluation of two signs of motor impairment, hypolocomotion (i.e., decrease in spontaneous locomotor activity) and catalepsy (i.e., impaired ability to initiate movement), as well as hypothermia (i.e., decrease in body temperature) and analgesia (i.e., decrease in pain sensitivity) (Metna-Laurent et al. 2017). Thus, the analgesic effects of cannabinoids are often inseparable from their undesirable motor side effects, and understanding the neurobiological underpinnings that regulate the beneficial (e.g., analgesia) and adverse (e.g., catalepsy) effects of cannabis is therefore highly relevant to the development of safe-profile cannabinoid-derived drugs. In this regard, a recent study

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has reported that pharmacological activation of CB1 receptors within the striatonigral circuits induces both analgesia and catalepsy in mice. Surprisingly, the analgesic effects mediated by CB1 activation were associated with receptors located at the plasma membrane, whereas catalepsy was associated with mtCB1 receptors (Soria-Gomez et al. 2021). Thus, cannabinoids exert different effects by affecting distinct subcellular processes within the same neuronal circuits, further emphasizing the importance of CB1 receptor localization for physiological functions.

2.3.5. CB1-related energy homeostasis

CB1 signaling is closely associated with biological systems that regulate energy intake and expenditure, making it a key player in energy homeostasis. In fact, CB1 receptors are expressed in various organs and tissues, most of which are involved in the regulation of energy homeostasis. In particular, CB1 receptors are expressed in the gastrointestinal tract, liver, pancreas, muscles, and adipocytes (Izzo et al. 2015; Kunos and Osei-Hyiaman 2008; Pagotto et al. 2006; Silvestri and Di Marzo 2013). In addition, the CB1 receptor is expressed centrally in limbic areas that control motivational aspects of food intake, such as the nucleus accumbens, and in hypothalamic nuclei that regulate food intake and peripheral metabolic processes (Giovanni Marsicano and Kuner 2008). Since the majority of eCB signaling is mediated via central and peripheral CB1 receptors, the question arises as to the functions of CB1 in modulating energy homeostasis.

Global CB1 signaling is typically associated with orexigenic effects (i.e., increased food intake). Indeed, cannabinoids induce eating in fed rodents (Williams and Kirkham 1999), and this effect is enhanced with palatable food, suggesting that CB1 receptors promote the incentive value of food and override satiety signals (Higgs, Williams, and Kirkham 2003). In contrast, pharmacological inhibition and global genetic deletion of CB1 receptors tend to reduce food intake, especially under fasting conditions and in obesity models (D. Zhou and Shearman 2004; Bellocchio et al. 2010; Cota et al. 2003). Interestingly, CB1 signaling has also been shown to control food intake in a bidirectional manner. Indeed, low to moderate doses of CB1 agonists induce an increase in food intake (i.e., hyperphagia), whereas high doses can induce a decrease in food intake (i.e., hypophagia) (Bellocchio et al. 2010). Like the CB1-mediated functions discussed above, this bimodal regulation of food intake relies on the excitatory/inhibitory balance. Indeed, when tested under fasted conditions, conditional mice mutant for CB1 receptors showed that "glutamatergic" CB1 receptors promote food intake while "GABAergic" CB1 receptors reduce food intake (Bellocchio et al. 2010). This also suggests that CB1 receptors on glutamatergic neurons are likely responsible for the typical anxiogenic functions of CB1 signaling and, thus, that CB1 control of glutamate is the primary

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driver of food intake. Interestingly, CB1 receptors on GABAergic neurons have been shown to control the motivation to run over the motivation for palatable food in an operant conditioning paradigm (Muguruza et al. 2019). Thus, CB1 receptors on glutamatergic and GABAergic neurons appear to have opposing functions in energy balance by promoting energy intake and energy expenditure, respectively.

CB1 signaling regulates energy balance through many other mechanisms, including regulation of lipid synthesis in the liver, fat accumulation in adipose tissue, glucose metabolism, sensory perception (Piazza, Cota, and Marsicano 2017), and control of the gut-brain axis (Berland et al. 2022). The coordinated functions of the ECS, and CB1 signaling in particular, have been recapitulated in an elegant review by P.-V. Piazza, D. Cota, and G. Marsicano. The authors propose that CB1 signaling is a critical determinant of coordinated exostatic processes (Piazza, Cota, and Marsicano 2017). In short, the authors emphasize that energy homeostasis is the result of a balance between a "homeostatic" or "endostatic" system and a "non-homeostatic" or "exostatic" system. Essentially, the endostatic system responds to internal energy status and aims to meet the immediate needs of the organism ("I eat when I am hungry"). In contrast, the exostatic system responds to the presence of food in the environment and promotes the accumulation of energy to anticipate future needs ("I eat when I see food"). In this case scenario, the exostatic system refers to an evolutionarily conserved process that favors sufficient caloric storage and promotes survival during periods of scarce energy sources. However, as humans evolved and food production and availability stabilized, individuals with higher exostatic drive became more at risk for overeating and developing obesity and related metabolic problems (Piazza, Cota, and Marsicano 2017). The ECS, and in particular CB1 receptors, is a good candidate for such an exostatic system.

2.3.6. Endogenous breaks of CB1 signaling and therapeutic opportunities

The CB1 receptor is a very complex receptor whose functions are associated with the modulation of various biological systems. Therefore, the dysregulation of CB1-mediated activity can have dangerous consequences at multiple levels, as evidenced by the reported role of CB1 receptors in the etiology of many psychiatric and metabolic disorders (Piazza, Cota, and Marsicano 2017; Thibaut and Hoehe 2020; Spanagel 2020; Maldonado, Cabañero, and Martín-García 2020). Nevertheless, CB1 signaling is associated with sophisticated endogenous regulatory mechanisms to maintain homeostasis. As mentioned above, the functions of the CB1 receptor are largely dependent on its cellular and subcellular localization. In this line, the main "cannabinoid drive" seems to rely mainly on CB1 receptors located on the glutamatergic terminals of neurons, since the phenotype of Glu-CB1 KO mice often confirms that of total CB1-KO, and because "glutamatergic" CB1 receptors are preferentially recruited

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to lower doses of cannabinoids ([Bellocchio et al. 2010](#); [Arnau Busquets-Garcia et al. 2015](#); [Dubreucq et al. 2012](#); [Metna-Laurent et al. 2012](#); [Lafenêtre, Chaouloff, and Marsicano 2009](#); [Rey et al. 2012](#)). Conversely, GABA-CB1-KO mice often exhibit the opposite phenotype to GLU-CB1-KO, and "GABAergic" CB1 receptors are recruited to higher doses of cannabinoids. Thus, CB1 receptors on GABAergic neurons may represent an endogenous "break" to counteract the cannabinoid drive mediated by "glutamatergic" CB1 receptors above a certain threshold ([Piazza, Cota, and Marsicano 2017](#)). Moreover, the cost-benefit ratio associated with cannabinoids and cannabis use depends in part on the subcellular localization of CB1 receptors, with different subsets of receptors exerting multimodal control over physiological functions ([Soria-Gomez et al. 2021](#)).

Although this cellular and subcellular specificity in CB1-mediated signaling represents an exciting opportunity for future research in the cannabinoid field, other endogenous regulatory mechanisms may serve more pragmatic therapeutic purposes. This is particularly true for endogenous allosteric modulators, including the neurosteroid PREG, which acts as a CB1-PAM and CB1-SSi, whose function has been shown to downregulate excessive CB1 activation and counteract toxic effects associated with acute cannabinoid administration ([Vallée et al. 2014](#); [A. Busquets-Garcia et al. 2017](#)). Interestingly, PREG production from CB1 activation appears to be largely dependent on CB1 receptors on GABAergic neurons ([Vallée et al. 2014](#)), so it is tempting to speculate that PREG may be released in a paracrine manner to downregulate the activity of CB1 receptors on glutamatergic neurons, thus acting synergistically with "GABAergic" CB1 receptors ([Raux and Vallée 2022](#)). However, future investigations should address this question, starting with the endogenous role of PREG in the regulation of CB1-mediated functions.

3. Neurosteroids and their impact on CNS function

3.1. Biosynthesis and metabolism of steroids

3.1.1. Steroid hormones: An overview

Steroid hormones represent a large class of hormonal mediators that regulate a wide range of physiological processes from development to adulthood, including sexual differentiation, reproduction, metabolism, stress response, immunity, and memory (Miller and Auchus 2011; P. Porcu et al. 2016; Schiffer et al. 2019; Chakraborty, Pramanik, and Mahata 2021). Due to their common origin in cholesterol, all steroid hormones share a similar "tetracyclic" carbon skeleton or cyclopentane-perhydrophenanthrene 4-ring structure (Bacila, Elder, and Krone 2019). Although steroid hormones are hydrophobic and lipo-soluble compounds typically found in lipid fractions extracted from animals, they are not considered "true lipids" (i.e., they do not contain a fatty acid chain) and constitute a separate family of chemical compounds (i.e., steroids) (Kater, Giorgi, and Costa-Barbosa 2022). Furthermore, despite their structural similarities, steroid hormones are a highly heterogeneous group from a functional point of view and are classified according to their physiological effects and biochemical properties (Bacila, Elder, and Krone 2019). Thus, steroid hormones are referred to as pregnane, androstane, and estrane for the cholesterol derivatives whose structure contains 21 (C21), 19 (C19), and 18 (C18) carbon atoms, respectively (Kater, Giorgi, and Costa-Barbosa 2022) and can be further divided into five major classes of steroid hormones. Pregnanes give rise to the synthesis of progestagens (or progestogens, e.g., progesterone) and the corticoid family, which includes glucocorticoids (e.g., cortisol/corticosterone) and mineralocorticoids (e.g., aldosterone). Finally, androstanes and estranes give rise to the sex hormones androgens (e.g., testosterone) and estrogens (e.g., estradiol), respectively (J. Hu et al. 2010; Kater, Giorgi, and Costa-Barbosa 2022) ([Figure 5](#)).

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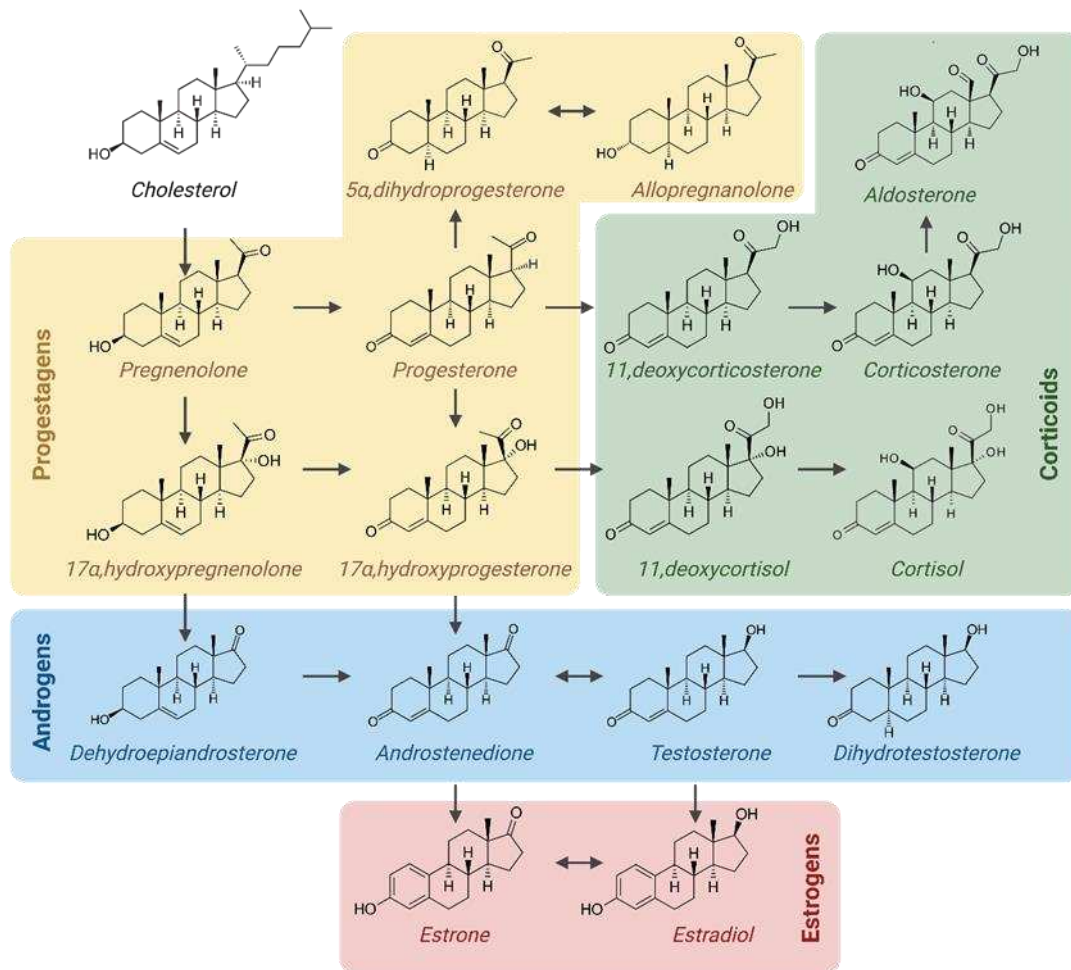


Figure 5: Main steroid synthesis pathways from cholesterol including progestagens, corticoids, androgens and estrogens. Double arrows indicate reversible reactions.

Adapted from [Raux et al., 2021](#).

All the major classes of steroid hormones are derived from a common steroid precursor, pregnenolone (PREG), whose synthesis from cholesterol is the first and rate-limiting step in the formation of other steroid hormones (steroidogenesis) ([Vallée 2016](#)). Therefore, an organ or tissue capable of steroidogenesis (also referred to as steroidogenic tissue) is defined by its ability to initiate the mobilization of cholesterol from various sources and transport it to a competent steroidogenic enzyme localized in the mitochondria, P450_{scc} (i.e., cholesterol side chain cleavage enzyme), for the *de novo* production of PREG ([Vallée 2016](#)). From PREG, several other steroidogenic enzymes then act to synthesize downstream steroid products, mainly cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes. Specifically, P450 enzymes mediate carbon-carbon bond cleavage and hydroxylation reactions in a unidirectional/irreversible manner. In contrast, two HSDs can either drive the dehydrogenation/oxidation of hydroxysteroids to form ketosteroids (i.e., a steroid containing a

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ketone group) or, conversely, promote the reduction of ketosteroids to hydroxysteroids, thus acting as a reductase (Miller and Auchus 2011). Thus, the synthesis of specific steroid hormones and steroidogenic pathways ultimately depends on a distinct repertoire of enzymes in a given organ or tissue.

Classical steroidogenic tissues are localized in the periphery and include the adrenal cortex, gonads (ovaries and testes), and placenta (Schiffer et al. 2019) but can be extended to newly described peripheral steroidogenic sites such as adipocytes (J. Li et al. 2014), gastrointestinal tissues (Bouguen et al. 2015; Mohibbi et al. 2017), skin (A. T. Slominski, Manna, and Tuckey 2015), retina (C. Cascio et al. 2015), and skeletal muscle (Vechin et al. 2023). In particular, the identification of other sites of steroid production is supported by the observation that they express at least the cholesterol side-chain cleavage enzyme and are thus capable of *de novo* PREG production, along with other enzymes involved in the conversion of PREG to downstream steroids and the detection of steroid content (J. Li et al. 2014; Bouguen et al. 2015; Tchernof et al. 2015; Diviccaro et al. 2020; Vechin et al. 2023). In addition to peripheral tissues, the central and peripheral nervous systems were shown to be capable of *de novo* steroidogenesis in the 1980s and 1990s, leading to the concept of "neurosteroids" synthesized "by the nervous system, for the nervous system" with implications for the modulation of synaptic plasticity (Corpéchet et al. 1981; Baulieu 1997; Mensah-Nyagan et al. 2008; P. Porcu et al. 2016).

In order to appreciate the importance of steroids in the modulation of physiological processes, and in particular, the emerging role of neurosteroids in the tuning of brain activity, the following sections will outline the mechanisms underlying the *de novo* production of steroids from cholesterol at all sources, including the delivery of cholesterol to the mitochondria and its subsequent cleavage by the P450_{scc} enzyme to yield the "mother hormone" PREG, and finally the specificities of neuro-steroidogenesis.

3.1.2. Cholesterol supply for steroidogenesis

Steroidogenic tissues and organs are defined by their unique ability to utilize cholesterol as the source material for the mitochondrial biosynthesis of PREG, the precursor of all steroid hormones. Thus, the modulation of steroidogenesis is closely linked to the cellular cholesterol economy and its availability for subsequent metabolic processing (Figure 6).

Cholesterol is an essential component of cellular bilayer membranes and interacts with phospholipid molecules to modulate membrane biophysical properties, including membrane fluidity and permeability to polar molecules (Simons and Vaz 2004). Interestingly, cholesterol is heterogeneously distributed among cell membranes. The plasma membrane is highly

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enriched in cholesterol, accounting for 20-25% of the total lipid fraction and thus representing the highest pool of membrane cholesterol in the cell. In contrast, the endoplasmic reticulum (ER) has low levels of cholesterol (about 1% of total cell cholesterol), but the amount of cholesterol in the membrane lipid fraction increases along the ER to the trans-Golgi network (i.e. the cell's primary secretory sorting station), which is involved in membrane transport and the cholesterol homeostatic machinery (Coxey et al. 1993; Mukherjee et al. 1998; Ikonen 2008). The outer (OMM) and inner (IMM) mitochondrial membranes have relatively low cholesterol content compared to other bilayer membranes. Therefore, an additional supply of cholesterol to the mitochondria is required to initiate and maintain steroidogenesis (Shi et al. 2022; Kraemer, Shen, and Azhar 2017). Although cholesterol allows the cell to function properly, excess cholesterol can have detrimental effects, including over-arrangement of phospholipids, leading to membrane rigidity. Intercalation of cholesterol into biological membranes is thus a dynamic process, and the influx, storage, and efflux of cholesterol are tightly regulated by cells. When the amount of cholesterol exceeds the complexing capacity of the plasma membrane, excess cholesterol can be mobilized to other cellular compartments. This fraction of cholesterol is often referred to as the "active" or "free" fraction of cholesterol (i.e., in the sense that it is accessible and not tightly bound to membranes) and constitutes the substrate for steroidogenesis (Miller and Bose 2011).

The cholesterol reservoir for steroidogenesis can originate from several sources, including endogenous or exogenous/dietary sources, which are best described for peripheral steroidogenic tissues (e.g., adrenals and gonads). Like all nucleated cells, steroidogenic cells can synthesize cholesterol *de novo* from acetyl-CoA within the endoplasmic reticulum (ER) via the mevalonate pathway. Briefly, two units of acetyl-CoA are converted to acetoacetyl-CoA by acetyl-coenzyme A acetyltransferases (ACAT or thiolase), and the addition of a third unit of acetyl-CoA forms 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) via the enzyme HMG-CoA synthase. HMG-CoA is then converted to mevalonate by the enzyme HMG-CoA reductase, which is the rate-limiting step in the *de novo* production of cholesterol in the ER (Shi et al. 2022; Kraemer, Shen, and Azhar 2017). Mevalonate then undergoes a series of transformations to squalene, which is then processed to lanosterol by the enzyme lanosterol synthase encoded by the *Lss* gene, and finally to cholesterol (Shi et al. 2022). Cholesterol synthesized *de novo* in the ER can be transferred to other cellular compartments to replenish pre-existing cholesterol pools, including mitochondrial membranes, or esterified as cholesteryl esters (CEs) by cholesterol acyltransferase (ACAT) for storage in lipid droplets (Ikonen 2008).

The contribution of *de novo* cholesterol synthesis is typically estimated at 60-70% of total body cholesterol, with the remaining 30-40% derived from diet (Shi et al. 2022; Ikonen 2008). However, the high demand for steroidogenic cells in the periphery requires a substantial

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influx of cholesterol, mainly from exogenous sources (Miller and Auchus 2011). Dietary cholesterol is absorbed from food by enterocytes in the small intestine and transported to the liver as CEs on lipoproteins called "chylomicrons", that circulate through the blood and lymph. In the liver, CEs and exogenous lipids are packaged by hepatocytes into very low-density lipoproteins (VLDLs), which in turn are processed into low-density lipoproteins (LDLs), primarily through the release and metabolism of triglycerides in peripheral tissues. LDLs can then deliver CEs to cells via the endocytic pathway (Ikonen 2008). First, LDLs bind to LDL receptors expressed on the plasma membrane, leading to their internalization into clathrin-coated pits (Ikonen 2008). Then, after endocytosis and delivery to early sorting endosomes, the LDL receptors are recycled back to the plasma membrane, and the LDLs are transferred to later acidic endocytic compartments (Kovanen et al. 1979; Möbius et al. 2003). Finally, CEs are hydrolyzed by a lysosomal acid lipase (LAL) to yield free cholesterol, which can be trafficked to the ER for transport to other cellular compartments or reesterified in lipid droplets (Kraemer, Shen, and Azhar 2017) ([Figure 6](#)).

An alternative source of cholesterol can be provided by high-density lipoproteins (HDL), which are involved in reverse cholesterol transport. This process refers to the removal of excess cholesterol from extrahepatic tissues into HDLs and its metabolism by the liver into bile acids for excretion in the small intestine (Ikonen 2008). HDLs are also involved in the delivery of cholesterol to steroidogenic tissues, particularly the adrenal and gonadal glands (Shen, Azhar, and Kraemer 2018), where they deliver CEs to cells via a specialized pathway called the "selective cholesteryl ester uptake pathway". In contrast to LDL receptor-mediated cholesterol influx, the selective pathway allows the transfer of large amounts of CEs to the plasma membrane and intracellular compartments in a non-endocytic manner (i.e., without internalizing the lipoprotein particle itself) (Shen, Azhar, and Kraemer 2018). Although the exact mechanism underlying this form of CE delivery remains somewhat controversial, the early step of the selective pathways requires the binding of HDLs to the plasma membrane scavenger receptor B1 (SR-B1) (Kraemer, Shen, and Azhar 2017). Furthermore, unlike CEs derived from the endocytic pathway, the hydrolysis of CEs delivered by the selective pathway is mediated by hormone-sensitive lipase (HSL), encoded by the *Lipe* gene, rather than LAL to yield free cholesterol (Kraemer, Shen, and Azhar 2017). While rodents preferentially use HSL-derived cholesterol for steroidogenesis (particularly adrenal steroidogenesis), this selective pathway is thought to play a minor role in human steroidogenesis (Miller and Auchus 2011). Furthermore, the contribution of LDL- and HDL-mediated cholesterol delivery to steroidogenic cells is limited to the periphery, as the brain is isolated from this system by the blood-brain barrier (BBB). Therefore, these mechanisms are not involved in the formation of neurosteroids (see [section 3.2.1](#)) ([Figure 6](#)).

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Excess cholesterol, regardless of its origin, can be stored as CE in cytoplasmic lipid droplets via the enzyme ACAT. Interestingly, under physiological conditions, steroidogenic cells accumulate more CE-enriched lipid droplets than any other cell type (Shen, Azhar, and Kraemer 2016). Moreover, lipid droplets are highly dynamic structures that traffic with other organelles, including the ER and the mitochondria, underlying their importance as a source of cholesterol for steroidogenesis (Murphy, Martin, and Parton 2009; Kraemer, Shen, and Azhar 2017). This pool of CEs is mainly accessible through the enzymatic activity of HSL, although other enzymes may be involved in the hydrolysis of cytoplasmic CEs (Kraemer 2007). Finally, the plasma membrane has been proposed as a source of cholesterol for steroidogenesis. Indeed, when the complexing capacity of the membrane is exceeded, cholesterol can be excreted via reverse cholesterol transport in HDLs, translocated to lipid droplets in esterified form for future use, or provide a direct substrate for steroidogenesis (Venugopal et al. 2016; Deng et al. 2019) (Figure 6).

3.1.3. Delivery of cholesterol to the outer mitochondrial membrane

As mentioned above, cholesterol accumulates in the cell and is redistributed to different cellular compartments according to cellular needs. To enable steroidogenesis, cholesterol must first be transported from the resident pool to the OMM. Lipid transfer between subcellular membranes generally occurs by vesicular trafficking, facilitated diffusion in an aqueous environment by soluble lipid transfer proteins, and across membrane contact sites by membrane-bound lipid transfer proteins. Although the intracellular trafficking of cholesterol in steroidogenic cells is not fully understood, mitochondria are generally excluded from the vesicular trafficking network, so steroidogenic 'free' cholesterol is most likely delivered via membrane contact sites and soluble lipid transfer proteins (Elustondo, Martin, and Karten 2017) (Figure 6).

The best described cholesterol-binding proteins belong to the Steroidogenic Acute Regulatory Protein-related lipid transfer (START) domain superfamily. The START domain refers to a conserved amino acid sequence that forms a hydrophobic cleft (Iyer, Koonin, and Aravind 2001) and defines a family of proteins capable of binding lipid ligands, including sterols, phospholipids, bile acids, and ceramides (Barbara J. Clark 2020). In mammals, STAR domain proteins are divided into six families, two of which have been reported to affect intracellular cholesterol trafficking and homeostasis: the cholesterol-specific and membrane-bound StarD1/D3 subfamily and the sterol-binding and cytosolic StarD4 subfamily (consisting of StarD4/D5/D6) (Barbara J. Clark 2020). Of interest, START domain proteins of the D1/D3 and D4 subfamilies have been shown to transport cholesterol to the mitochondria *in vitro*, and their ectopic expression in steroidogenic cell lines increases steroid hormone production

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([Watari et al. 1997](#); [Tuckey et al. 2002; 2004](#); [Soccio et al. 2005](#)). Another group of lipid-binding proteins, namely the oxysterol-binding proteins-related proteins (ORP), act as lipid sensors and can exchange cholesterol at ER-Golgi membrane contact sites ([Goto, Charman, and Ridgway 2016](#)). However, there is no direct evidence for a role of ORPs in mediating cholesterol transfer directly to the mitochondria, and their contribution to steroidogenesis remains questionable ([Elustondo, Martin, and Karten 2017](#)).

Cholesterol delivery to the OMM may originate from membrane contacts with the ER, also known as 'mitochondria-associated membranes' (MAM), where ordered lipid domains provide dynamic interactive properties between organelles ([C. King et al. 2020](#)). Interestingly, cholesterol is more abundant in MAMs than in other regions of the ER, and it has been suggested that cholesterol levels may influence the connectivity between the ER and mitochondria ([Fujimoto, Hayashi, and Su 2012](#)). In particular, a recent study has highlighted the role of the enzyme ACAT in the accumulation of cholesterol in MAMs and the connectivity between the ER and mitochondria ([Harned et al. 2023](#)). Furthermore, tropic hormone stimulation can increase the number of ER-mitochondria contact sites in steroidogenic cell lines ([Issop et al. 2015](#)), and several proteins localized to MAMs have been proposed to regulate mitochondrial cholesterol import from the ER and steroidogenesis ([Elustondo, Martin, and Karten 2017](#)). These proteins include the sigma-1 receptor, a transmembrane protein involved in ER calcium homeostasis, as evidenced by interfering RNA-mediated knockdown of sigma-1, which leads to reduced PREG synthesis in steroidogenic cell lines ([Marriott et al. 2012](#)). Furthermore, co-immunoprecipitation studies indicate that sigma-1 interacts with voltage-dependent anion channel 2 (VDAC2), a protein of the OMM, suggesting that sigma-1 and VDAC2 in the MAM coordinate cholesterol import into the mitochondria ([Marriott et al. 2012](#)).

In addition to sigma-1 and other MAM proteins, StarD1 has also been proposed to be involved in the transfer of cholesterol from the ER to the OMM. StarD1, more formally known as the steroidogenic acute regulatory protein (StAR), contains an N-terminal mitochondrial targeting leader sequence and regulates the delivery of cholesterol from the OMM to the IMM, where the first and rate-limiting step in steroidogenesis, the conversion of cholesterol to PREG, occurs ([B. J. Clark et al. 1994](#); [Caron et al. 1997](#)). Given the subcellular localization of StarD1, it was hypothesized that the protein could also accept cholesterol from membranes near the mitochondria. However, studies supporting this hypothesis have mainly been performed in non-steroidogenic cells, undermining the role of StarD1 in cholesterol import into the OMM for steroidogenesis ([Elustondo, Martin, and Karten 2017](#)).

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Cholesterol uptake from circulating lipoproteins and trafficking through the endocytotic pathway represents a significant source of cholesterol for the cell. Therefore, the transfer of cholesterol from late endosomes to mitochondria may provide a steady supply for steroidogenesis (Elustondo, Martin, and Karten 2017). Although the mechanisms leading to cholesterol transfer between endosomes and mitochondria are poorly understood, several lines of evidence suggest that internalized cholesterol can be transported through the ER (Miller and Bose 2011) or via direct contact sites between endosomes and mitochondria (Kennedy, Charman, and Karten 2012) ([Figure 6](#)).

Finally, cholesterol import into the OMM may occur via contact sites between lipid droplets and mitochondria or via cytosolic cholesterol transfer mediated by soluble proteins. Lipid droplets are in close contact with the ER, which, as mentioned above, may provide a common pathway for cholesterol delivery from endosomes and cytosolic CEs to mitochondria (Goodman 2008). However, direct contact sites between lipid droplets and mitochondria have been identified by ultrastructural analyses (Murphy, Martin, and Parton 2009; Sturmey, O'Toole, and Leese 2006), which may be facilitated by interacting proteins (Y. Lin et al. 2016). On the other hand, cytosolic cholesterol transport may be supported by the StarD4 protein family. In contrast to StarD1/D3, StarD4/D5/D6 are soluble proteins that contribute to the dynamic distribution of cholesterol between membranes. Because of their dynamic nature and cytosolic localization, members of the StarD4 subfamily have been proposed as cholesterol transport proteins involved in loading cholesterol into the OMM (Miller and Auchus 2011) ([Figure 6](#)).

3.1.4. Cholesterol transport from the OMM to the IMM

Regardless of how cholesterol is delivered to the OMM, the subsequent steps involve the import of cholesterol to the IMM for enzymatic conversion to PREG by P450_{scc}. The translocation of cholesterol from the OMM to the IMM requires the synthesis and interaction of several OMM proteins that form a cholesterol transport complex called the "transduceosome complex". This complex includes StarD1/StAR, whose mechanism of action has been extensively studied but remains incompletely understood (Miller 2017). Overexpression of StarD1 in steroidogenic cell lines is sufficient to induce steroidogenesis (B. J. Clark et al. 1994). In contrast, mutation of the protein is responsible for human congenital lipoid adrenal hyperplasia (CLAH), a pathology characterized by defective steroidogenesis (Caron et al. 1997), highlighting the biological activity of StarD1 as a rate-limiting step in the formation of steroid hormones. StarD1 is synthesized upon hormonal stimulation as a 37kDa cytoplasmic precursor containing an N-terminal mitochondrial targeting sequence typical of matrix proteins and a C-terminal START cholesterol-binding domain. StarD1 then undergoes a series of post-

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translational modifications, including phosphorylation by protein kinase A (PKA) and subsequent cleavage to a 30kDa 'mature' intra-mitochondrial form (Miller and Auchus 2011).

The cytosolic 37kDa form of StarD1 has a shorter half-life than the mature 30kDa form, suggesting that the intra-mitochondrial protein is the biologically active moiety (Elustondo, Martin, and Karten 2017). However, studies have shown that deletion of the N-terminal domain of StarD1 supports steroidogenesis without mitochondrial import (Arakane et al. 1996) and that StarD1 is biologically active when anchored to the OMM but not to the OMM and matrix sides of the IMM (H. S. Bose, Lingappa, and Miller 2002). Furthermore, the biological activity of StarD1 is inversely proportional to its rate of entry into the mitochondria (H. S. Bose, Lingappa, and Miller 2002). Taken together, these data suggest that StarD1 is functionally active when located on the cytosolic side of the OMM and becomes inactive when it reaches the mitochondrial matrix to which it is targeted. In this model, targeting StarD1 to the mitochondria may allow it to focus its activity on the OMM, while maturation to the 30kDa protein may prevent overproduction of steroid hormones (Elustondo, Martin, and Karten 2017). However, StarD1 translocation to the mitochondrion may serve purposes other than the termination of steroidogenesis. Indeed, studies have shown that re-expression of N-terminally truncated StarD1 cannot restore steroidogenesis in StarD1 knock-out mice, whereas re-expression of the full-length protein results in normal steroidogenesis (Sasaki et al. 2008). In addition, intra-mitochondrial processing of StarD1 appears to be necessary for optimal cholesterol transport, allowing the import of 400 molecules of cholesterol/StarD1/min (Artemenko et al. 2001). Although the precise mechanisms underlying cholesterol import into the IMM by StarD1 remain unknown, maturation of StarD1 in the mitochondrial matrix may maintain cholesterol influx rather than suppress it.

StarD1 works with several other OMM proteins to trigger cholesterol transfer across mitochondrial membranes, but the exact composition of the cholesterol "transduceosome complex" has not yet been elucidated. The translocator protein (TSPO) (formerly known as the peripheral benzodiazepine receptor or PBR) was one of the first candidate proteins identified for this complex. The role of TSPO in mediating cholesterol movement in mitochondria was primarily supported by high expression levels in steroidogenic organs (e.g., adrenal and gonad) (Anholt et al. 1985; Antkiewicz-Michaluk, Guidotti, and Krueger 1988; Morohaku, Phuong, and Selvaraj 2013) and the ability of TSPO ligands, including the selective agonist PK-11195, to upregulate steroidogenesis *in vitro* and *in vivo* (Mukhin et al. 1989; Verleye et al. 2005; Liere et al. 2017; Lejri et al. 2019). Furthermore, TSPO is enriched in the OMM (Anholt et al. 1986), and a consensus cholesterol recognition amino acid (CRAC) sequence has been identified in the C-terminal region of TSPO (Georges et al. 2021; H. Li et al. 2001), consistent

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with a role in regulating steroid hormone production. It has therefore been proposed that TSPO may act as a cholesterol transport channel, presumably through protein oligomerization (i.e., the binding of monomers to form a macromolecular complex) (Elustondo, Martin, and Karten 2017). However, recent data have called into question the role of TSPO in modulating steroidogenesis. In particular, it has been reported that TSPO knock-out does not affect steroidogenesis in mice and cell lines (Tu et al. 2014) and that PK-11195 stimulates steroidogenesis in a TSPO-independent manner (Tu et al. 2015). More recently, a comprehensive analysis of the steroid profile in TSPO knock-out mice reported little to no change in central and peripheral steroid hormone concentrations and steroidogenic enzymes under basal conditions (Liere et al. 2023). Although TSPO may be involved in intracellular cholesterol transport (Falchi et al. 2007), it may not be essential for steroidogenesis (El Chemali, Akwa, and Massaad-Massade 2022).

Other proteins interacting at the OMM/IMM have also been proposed to be involved in the transfer of cholesterol to the IMM. These proteins include the acyl-coenzyme A binding domain containing 3 (ACBD3) and the voltage-dependent anion channel (VDAC-1) located at the OMM, and the ATPase family AAA domain-containing protein 3A (ATAD3A) located at the IMM. Indeed, several multiprotein complexes have been identified, providing evidence for interactions between ACBD3/StarD-1/VDAC-1 and TSPO (Liu, Rone, and Papadopoulos 2006) and ATAD3A/TSPO/VDAC-1 and P450scc (Rone et al. 2012). Briefly, a simplified mechanism linking these proteins may involve (I) StarD1 binding to VDAC-1 (M. Bose et al. 2008); (II) ACBD3 serving as an anchoring protein for PKA for the phosphorylation and subsequent maturation of StarD1 (Elustondo, Martin, and Karten 2017); (III) ATAD3A providing contact sites between the IMM and the OMM and bringing StarD1 closer to P450scc (M. Bose et al. 2008).

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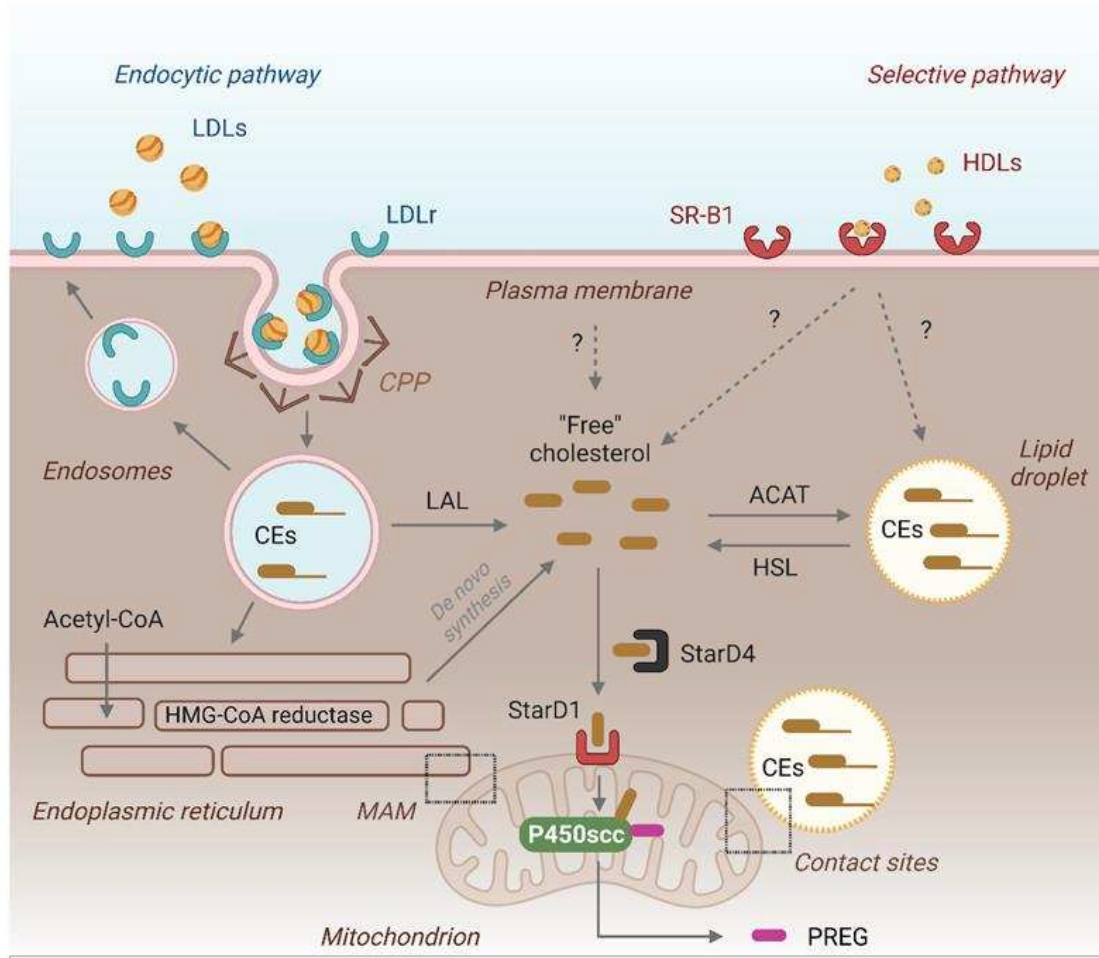


Figure 6: Key features of the cellular cholesterol economy for steroidogenesis.

Steroidogenic cells can take up cholesterol in esterified form from circulating LDLs via the LDL receptor and store it in endosomes, or from HDLs via SR-B1 receptors and target it to lipid droplets. Free cholesterol can then be produced by the action of LAL enzyme in endosomes or HSL in lipid droplets. Steroidogenic cells can also synthesize cholesterol *de novo* in the endoplasmic reticulum. Cholesterol-enriched plasma membranes may also be a source of free cholesterol. Regardless of the source, free cholesterol can be bound to StarD4 for cytoplasmic transport to mitochondria or imported via membrane contacts between mitochondria and ER or lipid droplets. StarD1 accepts cholesterol at the outer mitochondrial membrane for transfer to P450scc at the inner mitochondrial membrane, which metabolizes it to pregnenolone. Excess cholesterol can be re-esterified in lipid droplets by the enzyme ACAT. **Abbreviations:** ACAT, Acyl-CoA cholesterol acyltransferase; CEs, Cholesterol esters; HDLs, High-density lipoproteins; HSL, Hormone-sensitive lipase; LAL, Lysosomal acid lipase; LDLs, Low density lipoproteins; LDLr, LDL receptor; MAM, Mitochondria associated membranes; P450scc, Cholesterol side-chain cleavage enzyme; PREG, Pregnenolone; SR-B1, Scavenger receptor class B type 1.

Adapted from [Miller and Auchus, 2011](#).

3.1.5. Cleavage of cholesterol by P450scc

P450scc belongs to a family of oxidative enzymes called cytochrome P450s, which includes over 50 enzymes in humans, and is encoded by the CYP11A1 gene located on chromosome 15q23-q24 and approximately 30 kb in length (Sparkes, Klisak, and Miller 1991; Miller and Auchus 2011). The P450scc enzymes, along with a few other cytochrome P450s, are targeted to the mitochondria and are referred to as "type 1", while the vast majority are targeted to the ER and are referred to as "type 2" (Miller and Auchus 2011). Like StarD1, P450scc is targeted to mitochondria by a mitochondrial leader peptide, which is then cleaved upon entry of the enzyme (Chung et al. 1986; Morohashi et al. 1987). However, unlike StarD1, the mitochondrial environment is required for the proper function of the enzyme, and P450scc is only active when bound to the matrix side of the IMM (Churchill and Kimura 1979; Black et al. 1994).

Genetic deletion of the CYP11A1 gene or loss-of-function mutations result in loss of steroidogenesis, indicating that P450scc is required for the initiation of steroidogenesis and that its activity represents the rate-limiting step in steroid hormone production (M.-C. Hu et al. 2002; Kim et al. 2008). Therefore, steroidogenic cells are defined by the expression of P450scc and can produce steroids *de novo*, in contrast to other cells that can modify pre-existing steroids but are not steroidogenic cells *stricto sensu*. P450scc mediates the conversion of cholesterol to PREG via a series of three reactions that require electron transport from nicotinamide adenine dinucleotide phosphate (NADPH), resulting in the 22-hydroxylation of cholesterol, the 20-hydroxylation of 22R-hydroxycholesterol, and the C20-C22 bond cleavage of 20R,22R-hydroxycholesterol, leading to the formation of PREG (Miller and Auchus 2011). This last step is referred to as the side-chain cleavage event and gives P450scc its name (i.e., the suffix "scc" stands for "side-chain cleavage"). P450scc can also cleave the side-chain of other hydroxysterols such as 7-dehydrocholesterol to form 7-dehydropregnenolone, and hydroxylate vitamin D3 (A. Slominski et al. 2005; A. T. Slominski et al. 2009; Tuckey et al. 2008).

Furthermore, while StarD1 is primarily involved in the acute regulation of steroidogenesis by delivering cholesterol to the OMM, the chronic regulation of steroidogenesis is quantitatively determined by P450scc expression (Miller and Bose 2011). Finally, after synthesis, PREG is subjected to different metabolic pathways to form downstream steroid hormones or for degradation, depending on the tissue, species, and their enzymatic repertoire (P450 enzymes and HSD) (Vallée 2016).

3.2. Neurosteroids: relevance for neuroprotection and neuromodulation

3.2.1. Discovery and specificities of neurosteroidogenesis

The discovery of the brain as a source of steroid synthesis was pioneered by the group led by E-E. Baulieu. They found that: (1) Rat brain levels of steroids, especially dehydroepiandrosterone (DHEA), progesterone, and PREG, were unaffected by the removal of peripheral sources of steroids (i.e., adrenals and gonads), (Corpéchet et al. 1981); (2) some steroids were present at higher levels in the brain than in the periphery (Corpéchet et al. 1983); (3) the formation of steroids in the brain was independent of peripheral endocrine glands (Robel and Baulieu 1985). These data paved the way for the demonstration that the brain is capable of *de novo* steroidogenesis, leading to the emerging concept of "neurosteroids" produced "by the nervous system, for the nervous system" (Baulieu 1998). Furthermore, local synthesis of neurosteroids has been demonstrated in the spinal cord and peripheral nervous system (PNS) (Giatti, Garcia-Segura, and Melcangi 2015; Melcangi, Panzica, and Garcia-Segura 2011; Mensah-Nyagan et al. 2008; Patte-Mensah et al. 2006), and this process is shared across species, from mammals (including humans) to birds, amphibians, and fish, highlighting the importance of neurosteroid biosynthesis (Weill-Engerer et al. 2002; Tsutsui and Yamazaki 1995; Mensah-Nyagan et al. 1994; Sakamoto, Ukena, and Tsutsui 2001).

Neurosteroids are essentially produced by the same mechanisms as peripheral steroid hormones, although neurosteroidogenesis (i.e., local brain steroidogenesis) has some unique features. Like peripheral steroid hormones, locally synthesized neurosteroids are derived from the metabolism of cholesterol, which is particularly abundant in the brain, accounting for 25% of total cholesterol in the human body and 15% of brain dry weight (Ito and Yokoyama 2003). Brain cholesterol is predominantly (99.5%) in the unesterified form, with 70% in myelin and 30% in the plasma membrane of astrocytes and neurons (Björkhem and Meaney 2004). While peripheral steroidogenesis is derived from endogenous and exogenous sources of cholesterol, the brain is isolated from dietary cholesterol import by the blood-brain barrier (BBB), which prevents circulating plasma lipoproteins (e.g., HDL) from entering the CNS. Therefore, the brain is an autonomous organ in terms of cholesterol economy, and brain cholesterol homeostasis is thought to depend mainly on endogenous biosynthesis (Dietschy and Turley 2001). In addition, the brain has its own lipoprotein system, mainly glial cell-derived HDL, which enables cholesterol transport in the cerebrospinal fluid (CSF) (Roheim et al. 1979; Pitas, Boyles, Lee, Hui, et al. 1987; Pitas, Boyles, Lee, Foss, et al. 1987; Borghini et al. 1995; S. Koch et al. 2001). Finally, it should be noted that steroids detected in the CNS and PNS may originate from local synthesis by cholesterol conversion (i.e., neurosteroids) or from circulating steroid precursors. In fact, the vast majority of steroid hormones produced by peripheral

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steroidogenic tissues, with the exception of sulfated steroids, can cross the BBB and be metabolized in the brain by various neurosteroidogenic enzymes. Thus, blood-borne steroids are functionally and biochemically indistinguishable from brain-derived steroids, and virtually all five major classes of steroids (i.e., progestogens, mineralocorticoids, glucocorticoids, androgens, and estrogens) can be detected in the brain (Baulieu, Robel, and Schumacher 2001).

In addition to the quantitative detection of neurosteroids in mammalian and non-mammalian brain tissue and CSF, further evidence for neurosteroidogenesis has been provided by studies of the expression of steroidogenic enzymes. In particular, the expression of enzymes involved in cholesterol transport and conversion to PREG, namely StarD1 and P450scc, together with enzymes responsible for the conversion of PREG and its downstream metabolites, have been demonstrated in the brain (Furukawa et al. 1998; Ukena et al. 1998; Mensah-Nyagan et al. 1999; Mellon, Griffin, and Compagnone 2001). Particular attention has been paid to the mRNA and protein expression of P450scc because the enzyme catalyzes the rate-limiting step in steroid hormone formation and defines the steroidogenic status of a cell. However, detection of the steroidogenic enzyme has often proven difficult.

The synthesis of neurosteroids in the central and peripheral nervous systems is predominantly attributed to glial cells, particularly oligodendrocytes. Early studies showed that oligodendrocytes have elevated mRNA and protein levels of P450scc compared to neurons and astrocytes (Jung-Testas et al. 1989; Zwain and Yen 1999). Consistently, oligodendrocytes have been reported to be more proficient at converting cholesterol to PREG (R. C. Brown, Cascio, and Papadopoulos 2001), suggesting that neurosteroid production is primarily derived from this specific cell type. However, it is worth noting that P450scc expression has also been detected in astrocytes, neurons, and possibly microglia, with some studies even reporting higher or similar levels of this enzyme compared to oligodendrocytes (Y. C. Lin and Papadopoulos 2021). At the regional level, P450scc mRNA has been identified in several rodent brain regions, including the cerebral and cerebellar cortices, hippocampus, midbrain, and pons (Kimoto et al. 2001; S. R. King et al. 2002). In humans, P450scc mRNA has been documented in brain regions such as the frontal and temporal lobes, hippocampus, and cerebellum (Stoffel-Wagner 2003). Immunohistochemical data for P450scc protein detection are more variable in both rodents and humans, and their reliability remains controversial (Y. C. Lin and Papadopoulos 2021). In addition to P450scc, StarD1 has been found to be widely distributed in neurons and glial cells, particularly in subfields of the hippocampal formation, and co-expressed with P450scc in neuronal subpopulations in mouse and human brains (S. R. King et al. 2002). On the other hand, TSPO is differentially expressed by neuronal cells, with

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higher levels found in glia compared to neurons (El Chemali, Akwa, and Massaad-Massade 2022).

Of interest, the reported discrepancies in P450scc expression at cellular and regional levels may be due to several technical drawbacks. P450scc mRNA levels in the brain are relatively low compared to peripheral steroidogenic tissues such as the adrenal glands. Therefore, classical *in situ* hybridization techniques may not provide an accurate picture of the cell type expressing P450scc, and more robust neuroanatomical characterization of the enzyme may require the future use of new probes coupled to signal amplification systems designed to improve the signal-to-noise ratio. In addition, the potential lack of reliability of immunohistochemical data may be due to poor antibody specificity for P450scc and cross-reactivity with other cytochrome P450 enzymes. Alternatively, an emerging hypothesis suggests that PREG production in the brain may follow P450scc-independent mechanisms, undermining the importance of P450scc expression in the brain. In this regard, a recent study has reported the involvement of another mitochondrial P450 enzyme in PREG production in human glial cells (Y. C. Lin et al. 2023). However, further investigation is required to determine the significance of this alternative synthetic pathway for neurosteroidogenesis *in vivo*.

3.2.2. Mechanisms of action of neurosteroids

The enzymatic repertoire in a given cell type and brain region determines the amount of *de novo* synthesized neurosteroids and the conversion of blood-borne hormonal steroids that cross the BBB, which can then act as neuromodulators to regulate brain-related functions (Longone 2011). In the nervous system, steroids exert their actions through either "classical" or "non-classical" receptors localized in both glial and neuronal compartments (Garcia-Segura and Melcangi 2006; Melcangi, Garcia-Segura, and Mensah-Nyagan 2008) ([Figure 7](#)).

The classical action of steroids in the body, including the nervous system, is related to their genomic action at steroid hormone receptors. Namely, steroids enter cells through the plasma membrane and bind to receptors localized mainly in the cytoplasm (e.g., androgen receptors (AR) and glucocorticoid receptors (GR)) or in the nucleus (e.g., estrogen receptors σ and β (ER σ/β)). Steroid hormone receptors can then act as transcription factors by translocating to the nucleus (in the specific case of cytoplasmic receptors) and binding to specific DNA recognition sequences (steroid response elements, SREs) to modulate the transcription of specific genes, thereby regulating cellular functions (Levin and Hammes 2016). This classical model of steroid function thus requires gene transcription and translation of the newly produced mRNA into proteins, which can be detected in cells within minutes to an hour (Clayton 2000). However, most proteins undergo post-translational modifications and must be transported to their site of action to become fully functional, requiring a longer time course that

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can last from several hours to days. Therefore, these effects develop relatively slowly and may persist long after the steroid has disappeared from the brain (McEwen 1991) ([Figure 7](#)).

Interestingly, more than 50 years of research has shown that steroids can produce immediate (within seconds to minutes) changes in neuronal excitability, thus ruling out a genomic mode of action. Indeed, in addition to interacting with classical intracellular receptors, steroids can rapidly modulate brain excitability by binding stereoselectively and with high affinity to membrane receptors (McEwen 1991; Rupprecht 2003; Tuem and Atey 2017). Accordingly, the term "neuroactive steroid" has been coined for neurosteroids and blood-borne steroids that can act rapidly at the membrane level to modulate neuronal function (Paul and Purdy 1992).

One of the earliest indications of nongenomic steroid action was the ability of certain pregnane steroids to induce rapid sedation and anesthesia that could not be explained by gene regulation (Selye 1942). The molecular mechanisms underlying these rapid effects began to be elucidated in the 1980s, when the synthetic steroidal anesthetic alfaxalone was shown to prolong the inhibitory postsynaptic conductance of neurons (Scholfield 1980) and to potentiate the effects of GABA_A receptor agonists (Harrison and Simmonds 1984). Because alfaxalone is structurally related to certain endogenous pregnane steroids, it was quickly speculated that endogenous steroids and their metabolites might also interact with plasma membrane receptors, including GABA_A receptors, to modulate brain activity. Subsequently, some progesterone (PROG) metabolites, in particular 3 α ,5 α -THPROG (5 α -pregnan-3 α -ol-20-one or allopregnanolone, ALLOP), were identified as PAMs of the GABA_A receptor (Harrison et al. 1987; Callachan et al. 1987). In addition, these endogenous metabolites have been shown to exhibit stereoselectivity toward GABA_A receptor subtypes, particularly those containing the δ subunit (δ -GABA_AR), and produce anxiolytic, analgesic, anticonvulsant, and sedative effects in addition to their anesthetic effects at higher doses (Belelli et al. 2022). Thus, the rapid neuromodulatory properties of steroids have attracted research interest in drug development for psychiatric conditions. ALLOP remains one of the most studied neuroactive steroids due to its high potency as a GABA_A receptor PAM and has been identified as an important therapeutic target. In particular, treatment with the ALLOP synthetic analog brexanolone has been shown to improve depression scores in patients suffering from postpartum depression and is the first drug approved by the Food and Drug Administration (FDA) for the treatment of this condition (Leader, O'Connell, and Vandenberg 2019; Dacarett-Galeano and Diao 2019).

Although GABA_A receptors are the primary molecular targets of neuroactive steroids, other receptors have been shown to mediate their rapid effects (Tuem and Atey 2017). In particular, PREG-sulfate (PREG-S) is known to be an allosteric modulator of the NMDA and

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AMPA subtypes of ionotropic glutamate receptors. PREG-S can act as either a PAM or a NAM at NMDA receptors, depending on the receptor subunit composition (Wu, Gibbs, and Farb 1991; Gibbs, Russek, and Farb 2006), and as a NAM at AMPA receptors (Cameron et al. 2012). DHEA and DHEA-S have also been proposed as allosteric modulators of NMDA and AMPA receptors. However, there is currently no direct evidence for the binding of these steroids to ionotropic glutamatergic receptors, and it has been proposed that their effects are mediated through the activation of σ 1 receptors located in the ER (T. Maurice et al. 2001; Schverer et al. 2018). In addition, although classical steroid receptors are predominantly located in the cytoplasm and nucleus of the cell, a small fraction is located in membranes, particularly the plasma membrane. This fraction, which accounts for approximately 5% of all steroid receptors, can interfere with the cell signaling machinery to promote rapid membrane-induced responses (McEwen 1991; Levin and Hammes 2016). Finally, steroids have recently been reported to interact with another family of membrane receptors. Namely, PREG acts as a CB1 NAM and signal-specific inhibitor, opening new perspectives in the functional interactions between the steroid and the ECS (Vallée et al. 2014; A. Busquets-Garcia et al. 2017).

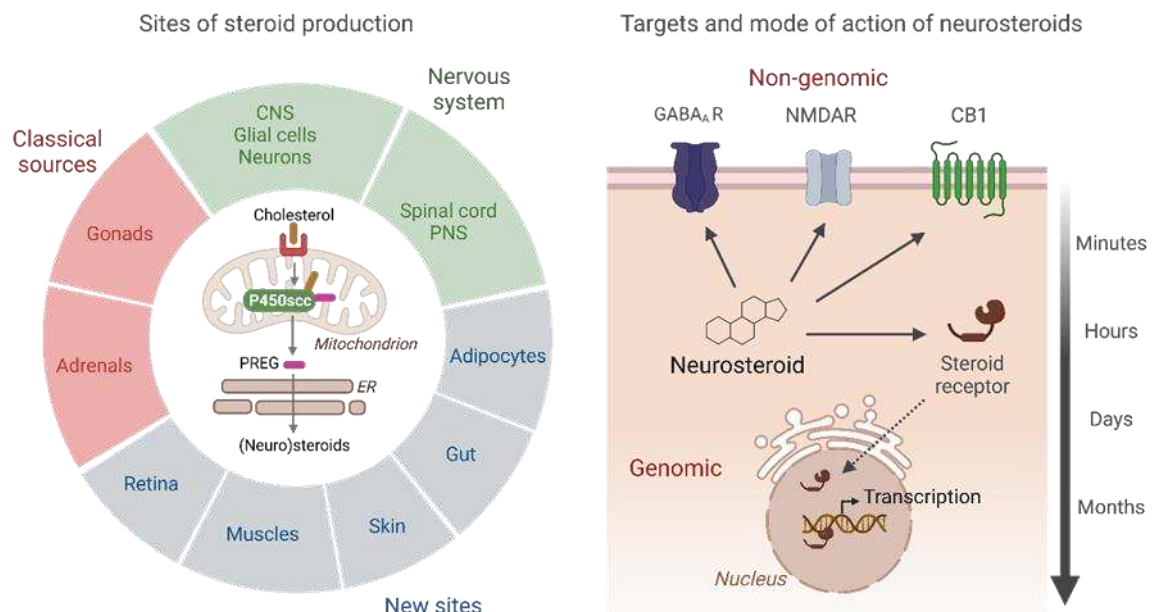


Figure 7: Sites of steroidogenesis and mode of action of neurosteroids.

Caption on next page

Figure 7: Sites of steroidogenesis and mode of action of neurosteroids.

A) Recapitulation of the classical peripheral sources of steroids, sources within the nervous system, and newly discovered peripheral sources that initiate at least the conversion of cholesterol to pregnenolone. **B)** Neurosteroids can act either on classical genomic targets, steroid receptors, which then translocate to the nucleus to induce changes in gene transcription, or on atypical non-genomic targets, including plasma membrane receptors (in particular GABA_AR, NMDAR and CB1). The time course of neurosteroid effects varies depending on the target, with neurosteroids inducing rapid changes (within minutes to hours) by acting at the plasma membrane, or delayed/persistent changes (within hours to months) by acting via classical steroid receptors.

Abbreviations: CB1, Cannabinoid type-1 receptor; CNS, Centre nervous system; ER, Endoplasmic reticulum; GABA_AR, GABA_A receptor; NMDAR, NMDA receptor; P450_{scc}, Cholesterol side-chain cleavage enzyme; PNS, Peripheral nervous system; PREG, Pregnenolone.

3.3. Pregnenolone: an emerging neurosteroid

3.3.1. Role of pregnenolone in CNS physiopathology

Steroid levels in the body are not fixed but are dynamically regulated in response to various environmental and internal factors to produce their biological effects. Thus, changes in metabolite levels can have physiological and pathophysiological implications when endogenous variations of steroids fall into the maladaptive range, either by downregulation or upregulation of their "optimal" physiological levels. In this perspective, it is noteworthy that the development of highly specific and selective analytical methods is crucial to study the steroid metabolome and the fluctuations of steroid levels under physiopathological conditions. Indeed, steroids whose levels are altered under certain conditions may serve as biomarkers and potential therapeutic targets with clinical prospects (Stephenson, Hoeflerlin, and Chalfant 2017; Matias et al. 2023). In this sense, PREG, as the first intermediate of steroidogenesis, represents one of the most abundant steroids in the body, and its endogenous levels are modulated in different conditions (Vallée 2016). Therefore, the following section will briefly outline some examples of changes in PREG levels in physiopathological conditions, mainly in the CNS, highlighting the potential role of PREG in modulating biological functions.

Among their various roles in modulating brain activity, steroids have been extensively described as mediators of the stress response. While stress refers to a broad range of physiological responses to noxious and challenging stimuli aimed at maintaining homeostasis, the intensity, and chronicity of exposure to stressors are associated with an increased risk of maladaptive behaviors and vulnerability to psychiatric disorders (Sinha 2008; Goh and Agius 2010). Of interest, steroids and neurosteroids modulate the physiological and pathophysiological effects of stress-related functions, mainly through the action of glucocorticoids and positive GABA_A allosteric modulators (e.g., ALLO) on the hypothalamic-pituitary-adrenal (HPA) axis, and thus participate in the etiology of stress-related disorders

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(Barbaccia et al. 2001; Higashi, Takido, and Shimada 2005; Vallée 2016; Tomaselli and Vallée 2019). PREG, among other neurosteroids, has attracted attention as a potential mediator of the stress response due to the modulation of its endogenous levels in response to stressors. Indeed, significant increases in PREG have been reported in the frontal cortex of rodents subjected to acute swim stress compared to other steroids and to plasma PREG levels (Vallée et al. 2000; Vallée 2014). Furthermore, plasma PREG levels are increased in monkeys after pharmacological activation of the HPA axis (Patrizia Porcu et al. 2006). More recently, it has been confirmed that PREG levels are elevated in response to acute swim stress; however, the increase in PREG levels was observed not only in the frontal cortex but also in the hypothalamus, amygdala, hippocampus, brainstem, and plasma (Sze and Brunton 2021). In addition, although the increase in PREG levels was robust compared to other steroids, some of its metabolites, including PROG derivatives, were also upregulated following swim stress (Sze and Brunton 2021).

Preclinical and clinical data have consistently linked PREG levels to stress-related disorders, including mood disorders and addiction. Indeed, it has been reported that unmedicated patients diagnosed with anxiety-depressive disorders have lower cerebrospinal fluid (CSF) levels of PREG compared to healthy volunteers (George et al. 1994). In addition, trilostane, a competitive inhibitor of the main PREG-metabolizing enzyme, rescued depressive-like phenotypes in mice, most likely by increasing brain PREG levels (Espallergues et al. 2012). Similarly, co-administration of the antidepressants olanzapine and fluoxetine increases hippocampal PREG levels in rats (Marx et al. 2006). Although the effects of PREG may be related to a general increase in steroidogenesis, the discovery of specific molecular targets for PREG with implications for mood disorders (Massimiliano Bianchi and Baulieu 2012) argues for a distinct biological role of the steroid (see [section 3.3.2](#)).

In addition, it has previously been shown that rodent PREG levels, primarily in the brain and much less so in plasma, are elevated in response to the acute administration of major drug classes, including cocaine, morphine, nicotine, alcohol, and, most notably, THC. In contrast, other downstream neurosteroids, including ALLOP and DHEA, were relatively unaffected (Vallée et al. 2014). Consistently, recent studies have reported that orally administered PREG reduces stress- and alcohol-cue-induced craving and normalizes HPA axis activity and stress-induced autonomic arousal (e.g., heart rate and blood pressure) in patients diagnosed with cocaine (Milivojevic et al. 2022) and alcohol use disorders (Milivojevic et al. 2023). However, it should be noted that PREG was administered at supraphysiological doses (300 or 500 mg/day); which are known to increase plasma levels of PREG, but also other steroid metabolites, such as ALLOP (Sripada et al. 2013). As the authors did not include a comprehensive steroid metabolomic analysis of patient plasma samples, future investigations

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should therefore address whether or not PREG administered at the reported doses increases blood levels of downstream metabolites that may account for the beneficial effects of PREG on alcohol and cocaine craving. Among the major classes of drugs of abuse tested for their effects on neurosteroid levels, THC was the most potent drug in increasing brain levels of PREG compared to other drugs (e.g., cocaine and alcohol) (Vallée et al. 2014). Given that THC is the most abundant psychoactive component of cannabis, and that administration of PREG blunts THC-mediated effects and addictive properties of CB1 agonists in rodent models (Vallée et al. 2014), it was promptly postulated that PREG may exert protective effects against cannabis intoxication and cannabis use disorders (Tomaselli and Vallée 2019). A recent synthetic analog of PREG, AEF0117, encouragingly showed promising results in a double-blind, placebo-controlled, crossover phase 2a study in subjects with cannabis use disorder (CUD). Indeed, AEF0117 significantly reduced patients' subjective positive effects of cannabis and cannabis self-administration (CUD.ClinicalTrials.gov identifiers: NCT03325595, NCT03443895, and NCT03717272) (Haney et al. 2023).

In addition to the classic landmarks of CUD, cannabis with high THC content is associated with the onset of acute psychotic-like states and is considered a risk factor for the development of schizophrenia in susceptible individuals (van Amsterdam, Brunt, and van den Brink 2015). Interestingly, endogenous serum levels of PREG are decreased in patients with schizophrenia (M. Ritsner et al. 2007). In contrast, PREG levels in postmortem brain samples are higher in schizophrenic patients, possibly reflecting the effects of classical antipsychotics such as clozapine and olanzapine, which dramatically increase PREG levels in rodents (Barbaccia et al. 2001; Marx et al. 2006). Conversely, PREG blocked a broad range of acute THC-induced endophenotypes associated with psychotic-like states, including cognitive impairment, somatosensory gating, and reality testing (i.e., a behavioral paradigm designed to capture THC-induced positive-like symptoms) (A. Busquets-Garcia et al. 2017). Chronic administration of PREG also restored the intrinsic and synaptic properties of dopamine neurons and mesolimbic transmission to prevent acute THC-induced deficits in prepulse inhibition (PPI) in a rodent model of prenatal cannabis exposure (PCE) (Frau et al. 2019). From a clinical perspective, administration of PREG has been shown to alleviate some symptoms of schizophrenia, including improvement in negative symptoms, functional capacity, reduction in positive symptom scores, and improvement in attention and working memory performance in schizophrenic individuals (Marx et al. 2009; M. S. Ritsner et al. 2010).

Overall, the alteration of PREG levels in the disorders described here strongly suggests that PREG may serve as an endogenous biomarker and therapeutic tool for these pathologies, with the development of synthetic PREG analogs offering several advantages in clinical trials, including better oral bioavailability and lack of metabolization to downstream steroids (Vallée

2016). Nevertheless, further research is needed to better understand the mechanisms of action of PREG to elucidate which molecular targets are involved in the beneficial and therapeutic effects of PREG in psychiatric disorders.

3.3.2. Molecular targets of PREG

PREG is a steroid with specific properties, being the first intermediate in cholesterol metabolism at all sources of steroidogenesis and the precursor of all steroid hormones. While classical neuroactive steroids (e.g., ALLOP, DHEA, DHEA-S, and PREG-S) act on GABA_A, NMDA, and AMPA receptors to rapidly modulate neuronal excitability, PREG itself has little to no affinity for these receptors (Park-Chung et al. 1999; Weaver et al. 2000). Furthermore, unlike PROG, sex hormones, and corticosteroids, no corresponding nuclear receptor has yet been identified for PREG (Schverer et al. 2018). Thus, PREG has long been considered a steroid precursor with limited biological activity *per se*. This assumption has gradually been refuted as new molecular targets for PREG have been identified, leading to renewed interest in its role in physiology and pathophysiology.

One of the first reports suggesting the existence of a PREG binding site on membrane receptors was made in the late 1980s by identifying a link between steroids and σ 1 receptors (Su, London, and Jaffe 1988). σ 1 receptors are widely distributed centrally and peripherally, with the highest expression levels found in cortico-limbic areas, particularly the hippocampus (Alonso et al. 2000; Phan et al. 2003). In the brain, σ 1 is expressed in neurons, ependymocytes, and oligodendrocytes (Alonso et al. 2000; Palacios et al. 2003; Teruo Hayashi and Su 2004), where it is targeted to the ER membrane and is involved in modulating Ca²⁺ homeostasis (Hanner et al. 1996; T. Hayashi and Su 2001). Although no true ligands for the receptor have been identified, several compounds, including the psychomimetic benzomorphan SKF-10,047, psychostimulants such as cocaine and amphetamines, and cytochrome P450 inhibitors have been found to interact with σ 1 (Tangui Maurice, Grégoire, and Espallergues 2006). In addition, some steroids have been shown to bind σ 1 receptors. In particular, PROG was identified as a potent σ 1 ligand by inhibiting the binding of SKF-10,047 (T. Maurice, Roman, and Privat 1996) along with PREG-S and DHEA, which also led to a significant reduction in SKF-10,047 binding *in vivo*. These effects were consistent with the identification of a steroid binding site on the σ 1 receptor, which has been proposed to play a role in synaptic plasticity (e.g., via modulation of NMDA-evoked firing activity) and to mediate the neuroprotective effects of steroids (Tangui Maurice, Grégoire, and Espallergues 2006). Of concern, PREG was also found to inhibit SKF-10,047 binding to σ 1 receptors *in vitro* but with less efficacy than PROG or its sulfated derivative (Tangui Maurice, Grégoire, and Espallergues 2006). Thus, although PREG can act as a σ 1 ligand, the functional significance of this

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interaction is undermined by its lack of efficacy *in vitro* and has not been further investigated *in vivo* in favor of more potent steroid ligands.

In the early 2000s, a second PREG-binding site was discovered in the rat brain cytosol and identified as microtubule-associated protein 2 (MAP2) (Murakami et al. 2000). Neuronal microtubules are primarily composed of tubulin, which interacts with several other proteins called "microtubule-binding proteins" (MTBPs) that control the stabilization and destabilization of microtubule assembly, the formation of microtubule networks (cross-linkers) and access to the free end of microtubules, thereby affecting filament elongation (capping proteins). Microtubule-associated proteins then refer to the subset of MTBPs that interact with microtubules during polymerization and depolymerization events and play an essential role in neurite formation and growth (Goodson and Jonasson 2018). In particular, MAP2 is the predominant cytoskeletal regulator in neuronal dendrites, reducing the frequency of depolymerization events to allow for microtubule maintenance and growth, thereby affecting synaptic function (DeGiosio et al. 2022). Interestingly, PREG can bind MAP2 proteins with high affinity and increase the rate and extent of MAP2-induced microtubule assembly in rat brain extracts (Murakami et al. 2000). The effects of PREG are recapitulated by 3 β -methoxypregnenolone (MePREG, also known as MAP4343), a synthetic and non-metabolizable analog of PREG that can bind MAP2 and stimulate microtubule assembly *in vitro*, but not by the metabolites of PREG, confirming PREG-specific effects (Fontaine-Lenoir et al. 2006). In addition, both PREG and MePREG were able to protect against the effects of Nocodazole, a microtubule-disrupting agent, by preventing neurite retraction in cultured cells (Fontaine-Lenoir et al. 2006). From a clinical perspective, alterations in hippocampal MAP2 expression have been proposed to contribute to the etiology of depressive disorders, with the selective serotonin reuptake inhibitor (SSRI), fluoxetine affecting the microtubule cytoskeletal system in the rat hippocampus (M. Bianchi, Hagan, and Heidbreder 2005; Massimiliano Bianchi et al. 2009). In this line, MePREG was able to reverse "depressive-like" phenotypes in rats in a more durable and effective manner than Fluoxetine (Massimiliano Bianchi and Baulieu 2012), and is being studied in humans (Isabella Barbiero, Bianchi, and Kilstrup-Nielsen 2022; Daftary et al. 2017) (ClinicalTrials.gov identifier: Drug resistant depression. NCT03870776).

In addition to MAP2, PREG can bind to a second microtubule-binding protein, CLIP170. CLIP170 is a linker protein that cooperates with various molecular partners to regulate microtubule dynamics, thereby contributing to neuronal morphogenesis and motility (Isabella Barbiero, Bianchi, and Kilstrup-Nielsen 2022). For example, CLIP170 localizes to the cell body and dendrites, regulating dendritogenesis, and is enriched in the axon growth cone, promoting axon formation (Neukirchen and Bradke 2011). PREG has been shown to bind to CLIP170, inducing its active conformation and interaction with partner proteins, leading to microtubule

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polymerization and cell migration (Weng et al. 2013). From a clinical perspective, the functional interaction between PREG and CLIP170 has been proposed as a therapeutic approach for CDKL5 deficiency disorder (CDD). CDD is an X-linked neurodevelopmental encephalopathy characterized by early-onset refractory epilepsy and cognitive and motor developmental delay. Genetic models, including CDKL5 knock-out (KO) mice and primary hippocampal neurons derived from CDKL5 KO mice, have shown significant alterations in dendritic length and complexity and reduced axon extension (Tang et al. 2017; I. Barbiero et al. 2020). Since CDKL5 interacts with CLIP170 to promote binding to microtubules, it was investigated whether PREG could rescue microtubule-associated defects in CDKL5 deficiency models. Indeed, PREG and MePREG were found to promote axon length, dendrite formation, and dendritic spine maturation in primary CDKL5 KO neurons (I. Barbiero et al. 2020). Although the precise molecular mechanisms underlying the beneficial effects of PREG and MePREG are still under investigation, it is believed that they depend in part on the activation of CLIP170 by promoting its function in microtubule assembly.

Consistent with its role in regulating microtubule dynamics for dendritic and axonal growth, PREG has been reported to regulate cell division. Cholesterol has previously been shown to actively regulate mitosis (H. W. Chen, Kandutsch, and Waymouth 1974; Fernández et al. 2004), but the effects of its metabolites have been relatively understudied. For example, one study reported that PREG could bind to the protein Shugoshin 1 (sSgo1), which protects centriole cohesion in mitosis (Hamasaki et al. 2014). Paired centrioles play a critical role in organizing the microtubules that form the two poles of the bipolar spindle during cell mitosis. These centrioles disengage or separate from each other as the cell divides. Thus, maintaining the tight association of centrioles as a pair is essential because it allows for the precise timing of centriole separation to ensure accurate cell division (Hamasaki et al. 2014). Because PREG promotes prostate cancer cell growth (Grigoryev et al. 2000), the authors suggest that the protective effects of PREG on centriole cohesion may be one of the cell survival strategies for certain types of cancer (Hamasaki et al. 2014). Thus, inhibition of PREG interaction with sSgo1 may provide future strategic opportunities to induce cancer cell death and warrants further investigation.

Finally, PREG was identified as a CB1-NAM and signaling-specific inhibitor (Vallée et al. 2014). Briefly, brain levels of PREG are upregulated in rodents by acute administration of CB1 agonists, including the phytocannabinoids THC and the synthetic cannabinoid WIN55, 212-2. Thus, CB1 activation triggers a negative feedback mechanism in which PREG binds to the receptor to downregulate its hyperactivity. The specific binding of PREG to the CB1 receptor was investigated by computational analysis, which revealed a dedicated binding pocket in the TMH1/TMH7/Hx8 region of the receptor, confirmed by an *in vitro* mutagenesis

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approach (discussed in detail in the [section 3.3.3](#)). This discovery provides the first evidence for pregnenolone interaction with a GPCR and its role in rapid modulation of neuronal activity. Together with the previously described targets and mechanisms of action, this finding underscores that PREG is indeed neuroactive and not devoid of biological activity.

Moreover, the identification of this new molecular target for PREG falls within the research framework of the steroid/ECS interface. Indeed, critical interactions between steroids and the ECS have emerged, with steroids proposed to fine-tune endocannabinoid tone both centrally and peripherally and *vice versa*. Notably, functional crosstalk between the two families of lipid messengers has implications for a variety of pathophysiological processes ranging from stress-related disorders to drug addiction, reproduction, and cancer ([Balsevich, Petrie, and Hill 2017](#); [Maccarrone 2005](#); [Santoro et al. 2021](#); [Proto et al. 2012](#)).

3.3.3. Pregnenolone as a CB1-NAM

The discovery that PREG levels are upregulated by acute administration of synthetic and plant-derived cannabinoids, thereby engaging a negative autocrine/paracrine feedback loop with CB1 receptors, has shed light on the therapeutic potential of PREG in downregulating hyperactivity states of the most abundant GPCR in the brain ([Raux et al. 2021](#)). Specifically, the downregulation of PREG synthesis by the P450_{scc} enzyme inhibitor aminoglutethimide (AMG) exacerbated the classical tetrad effects induced by THC in rodents (i.e., hypolocomotion, hypothermia, catalepsy, and analgesia). These effects were partially rescued by co-administration of AMG with PREG, suggesting a neuroprotective role of upregulation of PREG synthesis against the acute and toxic effects of cannabinoids ([Vallée et al. 2014](#)). Furthermore, PREG administration was able to counteract some other classical effects of THC, namely THC-induced hyperphagia in food-restricted and *ad libitum*-fed rodents and THC-induced memory impairment in a novel object recognition task ([Vallée et al. 2014](#)). Most interestingly, PREG was able to antagonize THC-induced dopamine release in the nucleus accumbens (NAc), a neurochemical signature common to most drugs of abuse ([Di Chiara and Imperato 1988](#)), and reduced motivation for the CB1 agonist WIN 55-512-2 in a cannabinoid self-administration model ([Vallée et al. 2014](#)), highlighting the therapeutic potential of PREG for CUD.

The neuroprotective effects of PREG against CB1 receptor hyperactivity are associated with a specific mechanism of action. Indeed, PREG acts as a CB1-NAM and signaling-specific inhibitor by downregulating the MAPK/ERK pathway but not the cAMP pathways, thus exhibiting functional selectivity. In addition, PREG does not competitively displace CB1 ligand binding but interacts with a specific allosteric pocket. Indeed, computational experiments using the Forced-Biased Metropolis Monte Carlo simulated annealing (MMC) program for ligand-

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protein binding (M. Clark et al. 2006) in a human CB1R model (Marcu et al. 2013) have predicted that PREG binds to CB1 at a specific binding pocket formed by the transmembrane domains TMH1 and TMH7 associated with the C-terminal intracellular helix 8 (Hx8) (Vallée et al. 2014). In this model, each polar group of PREG interacts through hydrogen bonding with the glutamic acid residue E.1.49 (for the ketone group) and the arginine residue R7.65 (for the hydroxyl group) on the TMH1, 7 and Hx8 regions, respectively ([Figure 8](#)).

Interestingly, sequence analysis of GPCR receptors using Uniprot (www.uniprot.org) and GPCRDB (www.gpcr.org/7tm/) indicates that the occurrence of glutamic acid at position 1.49 is quite rare among human class A GPCR sequences, with most class A GPCRs having a glycine at position 1.49. Therefore, to confirm the predicted docking of PREG to CB1, it was hypothesized that mutation of E1.49 to a non-hydrogen-bonding residue such as glycine would critically reduce PREG interaction with the receptor. The relative position of this residue on the TMH bundle of CB1 would not be expected to affect receptor function *per se* (Vallée et al. 2014).

This assumption is based primarily on the identification of the structural aspect of class A GPCRs with their activity. GPCRs can be roughly divided into three functional parts: 1) the extracellular domain, which includes the N-terminal domain and extracellular loops; 2) the transmembrane domain, which includes the 7 α -helices; 3) the intracellular domain, which includes the C-terminal domain and intracellular loops (Venkatakrisnan et al. 2013). The extracellular domain of a GPCR is typically involved in modulating ligand binding kinetics and shapes the route of ligand entry into the binding pocket (Venkatakrisnan et al. 2013). Although water-soluble ligands can enter their receptor directly through an opening formed in the extracellular domain, hydrophobic ligands such as cannabinoids have been proposed to diffuse laterally in the lipid bilayer and enter their binding sites through an opening formed by transmembrane domains (Hanson et al. 2012). In the case of CB1, the extracellular loops also form a "hydrophobic lid" that may help stabilize ligand binding (Marcu et al. 2013; Ahn et al. 2009). Within the transmembrane domain, topologically equivalent residues from TMH3, TMH6, and TMH7 have been identified as ligand contact sites in nearly all class A GPCRs (Venkatakrisnan et al. 2013). Finally, the intracellular domain of GPCRs is involved in G-protein interaction and selectivity. In particular, CB1 intracellular loops mediate G-protein binding, selectivity for inhibitory over stimulatory proteins, and can be phosphorylated, thereby modulating receptor activity (X. Chen et al. 2010; Shim, Khurana, and Kendall 2016) ([Figure 8](#)).

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Because the 1.49 point mutation is located in the TMH1 domain, it is unlikely to affect ligand binding affinity by causing conformational changes within the extracellular domain or by disrupting direct ligand interaction with highly conserved amino acid residues. In addition, the mutation is topographically distant from intracellular regulatory sites associated with signal transduction, making it unlikely to affect G-protein coupling alone. Therefore, *in vitro* experiments were performed in cells transfected with either the wild-type human CB1R (hCB1wt) or the mutant human CB1R (hCB1Rp.E1.49G) in which the glutamate (E) residue at position 1.49 has been replaced by a glycine (G). The activity of both receptors was compared by THC-induced decrease in mitochondrial respiration. THC reduced respiration similarly in both cells transfected with hCB1wt and hCB1Rp.E1.49G. However, the inhibitory effect of PREG on THC was absent in cells transfected with hCB1Rp.E1.49G, in contrast to what was observed in cells transfected with hCB1wt, confirming the binding site of PREG on the CB1 receptor (Vallée et al. 2014).

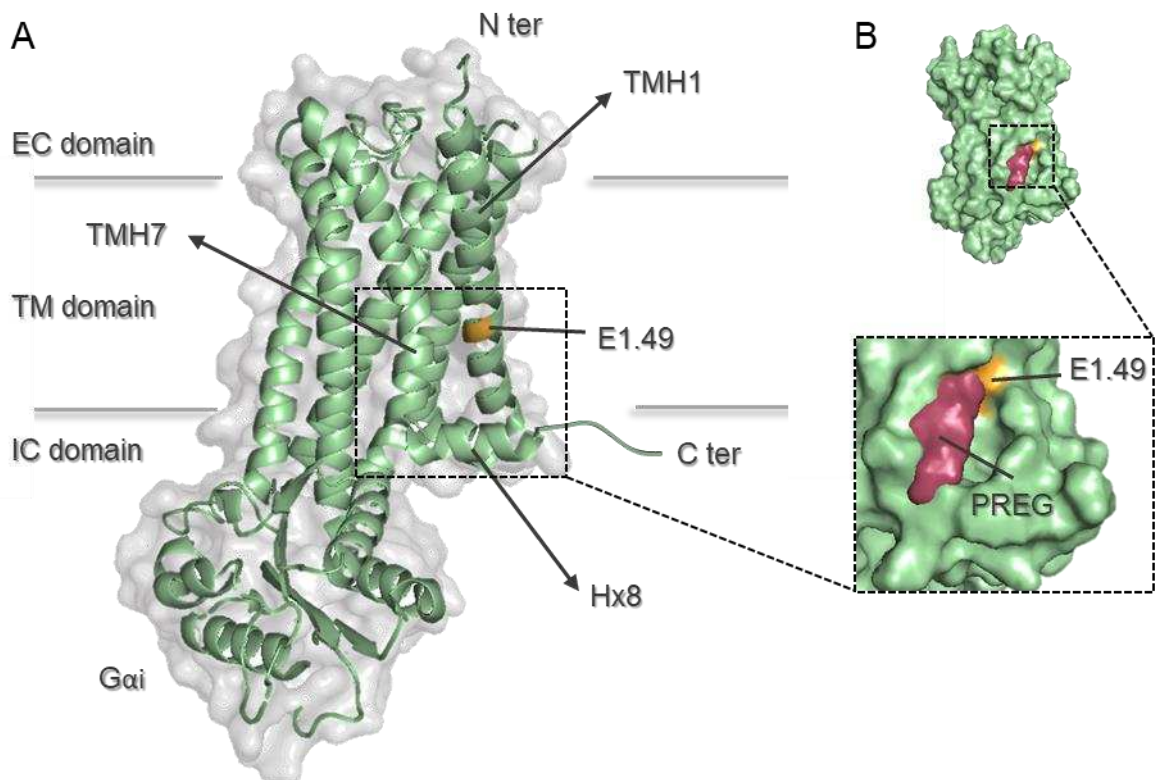


Figure 8: Representation of the E1.49 residue within the human CB1 receptor and docking of PREG to its allosteric binding site.

A) Three-dimensional representation of the human CB1 receptor in light green and the position of the glutamate residue E1.49 highlighted in orange. **B)** Zoom on the pregnenolone (PREG) binding site within the TMH1/TMH7/Hx8 region of the CB1 receptor.

Abbreviations: EC, extracellular; TM, transmembrane; IC, intracellular; Hx8, helix 8; N ter, N-terminus; C ter, C-terminus; PREG, pregnenolone.

3.3.4. Pregnenolone and CB1-related disorders

The neuroprotective effects of PREG were further confirmed by examining its effects on THC-induced psychotic-like states, which represent a growing health concern associated with cannabis use (A. Busquets-Garcia et al. 2017). PREG was able to block the cognitive impairments associated with acute THC administration, including working-like memory deficits, as well as THC-induced sociability deficits and impairment of somatosensory gating, as assessed by prepulse inhibition (PPI). In addition, PREG was able to block THC-induced hyperlocomotion and impairment of mental sensory representation (reality testing) (i.e., a behavioral paradigm designed to assess positive symptoms of psychosis) (A. Busquets-Garcia et al. 2017). Thus, PREG has significant therapeutic potential for the treatment of cannabis-induced acute psychotic-like states (CIAPS).

Despite its promising potential for CUD and CIAPs, PREG itself is not a viable option for pharmacotherapy due to several drawbacks. In fact, PREG has a short half-life and low oral bioavailability and is rapidly converted to downstream steroid metabolites, which may lead to adverse/unwanted effects (Vallée 2016). Therefore, a new synthetic analog of PREG, AEF0117, was developed and tested in a phase 2a proof-of-concept study in volunteers with CUD. AEF0117 was well tolerated, showed no toxicity (*in vitro* and *in vivo*), and did not exacerbate the classical effects of CB1 antagonists (e.g., reduced food intake and increased anxiety/depressive-like behaviors, among others) (Haney et al. 2023). AEF0117 recapitulated the previously observed effects of PREG on THC-induced intoxication, psychotic-like effects, and cannabinoid self-administration, confirming PREG-like biological activity. In CUD subjects, AEF0117 reduced positive cannabis perception and cannabis self-administration, demonstrating a first-in-class safe and potentially efficacious treatment for CUD (Haney et al. 2023).

More recently, PREG was found to rescue the hyperdopaminergic states induced by prenatal THC exposure (Frau et al. 2019). Specifically, the authors examined endophenotypes associated with dopaminergic function in the male and female offspring of THC-exposed dams in a rat model of prenatal cannabis exposure (PCE). Results indicate that male, but not female, PCE offspring exhibit behavioral sensitivity to THC, including THC-induced alterations in somatosensory gating (PPI) and hyperlocomotion. Behavioral alterations in male PCE offspring were associated with functional changes in dopamine signaling. Indeed, PCE enhanced the pacemaker and evoked activity of ventral tegmental area (VTA) dopaminergic neurons, which was associated with reduced synaptic inhibition and potentiation of excitatory inputs to dopamine neurons. Accordingly, THC-induced dopamine release in the nucleus accumbens (NAc) was increased in male PCE offspring compared to the control group.

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PREG, administered chronically for fifteen days during postnatal weeks three and four, was able to restore the synaptic properties of dopamine neurons from PCE animals and alleviate the deficits in dopamine signaling and associated THC-induced behavioral and neurochemical alterations, including PPI and dopamine release in the NAc. In an elegant control experiment, the authors co-administered PREG with the P450_{scc} enzyme inhibitor trilostane, which did not affect the neuroprotective effect of PREG. Thus, these findings are consistent with an effect of PREG *per se* and not its downstream metabolites (Frau et al. 2019). It should be noted, however, that the mechanism by which PREG exerts its effects on PCE offspring is currently unknown. Although it could be speculated that the aforementioned effects of PREG are related to its activity as a CB1-NAM, this protocol did not use co-administration of PREG with THC in dams or PCE offspring, and there is no direct evidence for the action of PREG at CB1 receptors. Thus, further evidence is needed to confirm direct action at CB1 receptors

It has also been reported that the cognitive deficits induced by an obesogenic diet in rodents are associated with decreased levels of PREG in the hypothalamus (Ramírez et al. 2022). Intra-hypothalamic administration of PREG was able to rescue memory deficits induced by an obesogenic diet by affecting hippocampal long-term potentiation. In addition, circulating PREG levels in cerebrospinal fluid were positively correlated with cognitive performance in obese patients, suggesting that PREG protects against cognitive impairments associated with metabolic diseases (Ramírez et al. 2022). Knowing the role of hypothalamic endocannabinoid signaling in obesity and related disorders (Miralpeix et al. 2021), and the ability of PREG to block cannabinoid-induced memory deficits (Vallée et al. 2014), it can be postulated that the above-mentioned role of PREG on obesity-related cognitive disorders may involve CB1 receptors (Bellocchio and Marsicano 2022). However, the mechanisms by which PREG affects the cognitive status of metabolically unhealthy animals have not been investigated, and it is unclear whether PREG exerts its effects via CB1 receptors or other intracellular molecular targets. Thus, future investigations are needed to determine the potential importance of negative allosteric modulation by PREG in diet-induced cognitive changes.

In conclusion, although the above data have examined the ability of PREG to counteract the acute effects of exogenous cannabinoids, primarily the phytocannabinoid THC, it remains unclear why the brain has established this endogenous regulatory feedback loop between PREG and CB1 receptors. Therefore, it is worthwhile to investigate the role of endogenous PREG in modulating CB1 functions at physiological and pathophysiological levels.

OBJECTIVES AND METHODOLOGICAL APPROACHES

1. General Aim

As detailed in the introduction, PREG has been identified as a NAM and signaling-specific inhibitor of the CB1 receptor that protects against hyperactivation states induced by exogenous cannabinoids (Vallée et al. 2014; A. Busquets-Garcia et al. 2017; Frau et al. 2019). In recent years, the specific neuroprotective properties of PREG in modulating CB1 activity have attracted clinical interest, with the development of the synthetic PREG analog AEF0117 offering new hope for the treatment of cannabis use disorder (CUD) (Haney et al. 2023). However, the contribution of the endogenous PREG-CB1 negative feedback loop to the modulation of CB1 activity remains elusive. In particular, it remains unknown whether PREG can fine-tune CB1-mediated functions under physiological conditions and whether the action of PREG on CB1 activity prevents the transition from physiological to pathological behaviors during hyperactivation states of the receptor. This is particularly relevant because CB1 plays a critical role in the regulation of most behavioral functions involved in maintaining homeostasis (Lutz 2020).

To better understand the functional role of the PREG-CB1 loop, one strategy may be to manipulate PREG levels and examine the resulting consequences on the CB1-mediated functions. Several pharmacological (e.g., steroid enzyme inhibitors) and genetic (e.g., CRISPR and RNAi-mediated knockdown) tools are available to address the endogenous contribution of neurosteroids in modulating receptor activity *in vivo* (Baston and Leroux 2007; Leykauf et al. 2023). With respect to PREG, such approaches include targeting the P450scc enzyme to block the formation of PREG from cholesterol. Conversely, inhibition of the P450c17 and 3 β -HSD enzymes, which are responsible for the conversion of PREG to DHEA and progesterone, respectively, can increase endogenous PREG levels (Espallergues et al. 2012). Although useful, these approaches lack specificity in their effects because most steroid enzymes mediate more than one conversion reaction in steroid biosynthesis (Miller and Auchus 2011). On the other hand, PREG itself can be administered, but the use of PREG is associated with poor bioavailability, short biological half-life, and rapid *in vivo* metabolism. Synthetic and non-metabolizable analogs of PREG that are being developed for clinical prospects can overcome most of the disadvantages associated with PREG administration *in vivo* by improving the biodistribution of the steroid and preventing metabolic conversion (Isabella Barbiero, Bianchi, and Kilstrup-Nielsen 2022; Haney et al. 2023). However, such compounds may differ from PREG in their action on the CB1 receptor and, more importantly the use of such compounds precludes the study of endogenous steroid signaling.

Objectives and methodological approaches

For all of the above reasons, we sought to use an alternative approach to investigate the endogenous PREG-CB1 loop with respect to endogenous PREG levels and steroid metabolism. This approach is based on the specific knockdown of the PREG allosteric binding pocket on the CB1 receptor using a mutagenesis approach previously validated on the human CB1 receptor *in vitro* (Vallée et al. 2014). We then translated this approach from the human CB1 receptor to the murine receptor by developing a new CB1 mutant mouse line (**CB1^{E134G} mice**) (Raux et al. 2021).

Therefore, the overall goal of this work was to investigate the behavioral and molecular phenotypes of this new mouse line in order to unravel novel functions for PREG in modulating CB1 activity at the endogenous level.

The following section outlines the development of this new CB1^{E134G} mouse line and describes the specific goals for characterizing the mutant mice.

2. Methodological approach: development of the CB1E134G mouse line

2.1. Translation of the research strategy from human to mouse

The identification of the PREG-CB1 allosteric binding pocket was performed using the Forced-Biased Metropolis Monte Carlo (MMC) simulated annealing program for ligand-protein binding (M. Clark et al. 2006) in a human CB1R model (Marcu et al. 2013). This model predicted a hydrogen bond between the ketone group of PREG and the glutamic acid residue E.1.49 located within the first transmembrane domain of the human CB1 receptor at position 133 from methionine ([Figure 8](#)) (Vallée et al. 2014). Substitution of residue E.1.49 with a non-hydrogen-bonding glycine (G.1.49) by a missense mutation in the human CB1 receptor successfully prevented PREG modulation of CB1 activity *in vitro* (Vallée et al. 2014), confirming the assignment of the PREG-CB1 binding site. Sequence alignment between human (P21554) and mouse (P47746) CB1 protein sequences using Uniprot (www.uniprot.org) indicates that residue E1.49 is located at position 134 of the amino acid sequence in the mouse receptor ([Figure 9](#)). Therefore, the following strategy was used to generate a missense mutation resulting in the E134G mutation in the open reading frame of the mouse gene encoding the CB1 receptor.

Objectives and methodological approaches

```
P21554|CB1_HUMAN MKSILDGLADTTFRITITDLLYVGSNDIQYEDIKGDMSKLGYPQKFPL 50
P47746|CB1_MOUSE MKSILDGLADTTFRITITDLLYVGSNDIQYEDIKGDMSKLGYPQKFPL 50

P21554|CB1_HUMAN TSFRGSPFQEKMTAGDNPQLVPA.DQVNITEFYNKSLSSFENEENIQCG 99
P47746|CB1_MOUSE TSFRGSPFQEKMTAGDNSPLVPA.GDTTNITEFYNKSLSSFENEDNIQCG 100

                                TM1                                133
P21554|CB1_HUMAN ENFMDIECFMVLNPSQQLAIAVLSLTLGFTFTVLENLLVLCVILHSRSLRC 149
P47746|CB1_MOUSE ENFMDMECFMILNPSQQLAIAVLSLTLGFTFTVLENLLVLCVILHSRSLRC 150

                                TM2                                134                                TM3
P21554|CB1_HUMAN RPSYHFIGSLAVADLLGSVIFVYSFIDFHVFHRKDSRNVFLFKLGGVTAS 199
P47746|CB1_MOUSE RPSYHFIGSLAVADLLGSVIFVYSFVDFHVFHRKDSPNVFLFKLGGVTAS 200

                                TM4
P21554|CB1_HUMAN FTASVGSFLTAIDRYISIHRLPLAYKRIVTRPKAVVAFCLMWTIAIVIAV 249
P47746|CB1_MOUSE FTASVGSFLTAIDRYISIHRLPLAYKRIVTRPKAVVAFCLMWTIAIVIAV 250

                                TM5
P21554|CB1_HUMAN LPLLGWNCCKLQSVCSDFPHIDETYLFWIGVTSVLLLFIVYAYMYILW 299
P47746|CB1_MOUSE LPLLGWNCCKLQSVCSDFPLIDETYLFWIGVTSVLLLFIVYAYMYILW 300

                                TM6
P21554|CB1_HUMAN KAHSHAVRMIQRGTQKSI IHTSEDGKQVTRPDQARMDIRLAKTLVLIL 349
P47746|CB1_MOUSE KAHSHAVRMIQRGTQKSI IHTSEDGKQVTRPDQARMDIRLAKTLVLIL 350

                                TM7
P21554|CB1_HUMAN VVLIICWGPLLAIMVYDVF GKMNKLIKTVFAFCSMLCLLNSTVNP I IYAL 399
P47746|CB1_MOUSE VVLIICWGPLLAIMVYDVF GKMNKLIKTVFAFCSMLCLLNSTVNP I IYAL 400

P21554|CB1_HUMAN RSKDLRHAFRSMFSPCEGTAQPLDNSMGSDCLHKHANNAAASVHRAAESC 449
P47746|CB1_MOUSE RSKDLRHAFRSMFSPCEGTAQPLDNSMGSDCLHKHANNATASMHRAAESC 450

P21554|CB1_HUMAN IKSTVKIAKVTMSVSTDTSAEAL 472
P47746|CB1_MOUSE IKSTVKIAKVTMSVSTDTSAEAL 473
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Figure 9: Sequence alignment between human and mouse CB1 receptor.

Amino acid positions between the human and mouse CB1 receptor are numbered from methionine to residues 472 and 473 for human and mouse, respectively. Residues that differ between human and mouse are shown in red. The dot indicates the absence of the corresponding amino acid between human and mouse. Transmembrane domain 1 (TM1), indicated by the yellow box, contains residue E1.49 at position 133 in the human CB1 receptor and position 134 in the mouse CB1 receptor sequence, indicated by the red box. Transmembrane domains 2 to 7 (TM2-TM7) are indicated by the blue boxes.

2.2. Generation of the CB1^{E134G} mouse line

The CB1^{E134G} knock-in mouse line was generated at the Center for Immunophenomics (CIPHE, Marseille) using a previously described flox-stop strategy (S. Ruehle et al. 2013; Soria-Gomez et al. 2021). Briefly, the E134G point mutation was introduced by modifying a pSV plasmid consisting of an SV40 promoter-driven neomycin resistance (Neo) coding sequence, an HSV-TK polyadenylation sequence, three additional AATAAA polyadenylation

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sequences, and a Lox-Stop-Lox (LSL) cassette in the 5'UTR region of the gene encoding CB1 (*cnr1*). This modification was achieved by homologous recombination in a competent bacterium using a synthetic insert containing a GGG codon encoding a glycine (E) 134 instead of a GAG codon encoding a glutamate (G) 134 (missense mutation "E134G"). The complete targeting construct was assembled by adding a 5' negative selection cassette (upstream of the stop cassette) for homologous recombination in embryonic stem (ES) cells. The targeting construct (21553bp) was linearized and electroporated into v6.5 (C57BL/6 (F) × 129/sv (M)) ES cells as described previously (S. Ruehle et al. 2013). After electroporation, ES cells were cultured under selective conditions, with the introduction of the DT-A cassette limiting random integration of the targeting vector in favor of clones that recombined at the target locus. Resistant clusters were transferred to 96-well plates for screening by Southern blot analysis using previously validated hybridization probes (S. Ruehle et al. 2013) to control for proper 5' and 3' homologous recombination at the target locus. ES clones validated for injection were subjected to PCR (forward primer: 5'-TGTGTGAATCGATAGTACTAAC-3'; reverse primer: 5'-TGGATATGTACCTGTTCGATG-3') and sequencing to confirm the correct insertion of the E134G point mutation in the coding region of *cnr1*.

ES clones with correct homologous recombination and E134G point mutation were injected into Balb/cN blastocysts to generate chimeric mice. Chimeric females were systematically sacrificed, and only chimeric males with chimerism greater than or equal to 50% were maintained. Chimeric male founder mice were then mated to C57BL/6N females. Genetic transmission in the heterozygous F1 offspring was confirmed by biopsies and PCR, allowing characterization and differentiation of the wild-type (WT) allele (forward primer: 5'-CAAGAAATGAGAACCGTGTC-3'; reverse primer: 5'-GTTCTCCTTGAACGATGAGA-3') and the CB1^{E134G} mutant allele before deletion of the LSL cassette (forward primer: 5'-TGTGTGAATCGATAGTACTAAC-3'; reverse primer: 5'-GTTCTCCTTGAACGATGAGA-3'), generating 543bp and 462bp amplicons respectively. The heterozygous F1 progeny were then backcrossed for at least seven generations to generate Stop-CB1^{E134G} mice on a C57BL/6N background ([Figure 10A](#)).

Crossing STOP-CB1^{E134G} mice with a mouse line expressing CRE recombinase under the cytomegalovirus (CMV) promoter B6.C-Tg (CMV-cre) 1Cgn/J (The Jackson Laboratory, JAX stock #006054) allowed excision of the LSL cassette. The heterozygous CB1^{E134G} male progeny were then backcrossed to C57BL/6N females for at least two generations to eliminate the Cre allele ([Figure 10B](#)). The presence of the Cre allele was determined by PCR (forward primer: 5'-CGGCATGGTGCAAGTTGAATA-3'; reverse primer: 5'-GCGATCGCTATTTCCATGAG-3'). CB1^{E134G} mice and their WT littermates were genotyped by qPCR using the same primer pair (forward primer: 5'-CCTGGCACCTCTTTCTCAGT-3';

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reverse primer: 5'- GTTCTCCTTGAACGATGAGA -3'), which generated a 333 bp product in WT mice and a longer 367 bp product in mutants due to the residual LoxP site of the LSL sequence. Consistent with previously reported CB1-deficient mouse lines ([Giovanni Marsicano et al. 2002](#); [Monory et al. 2007](#); [S. Ruehle et al. 2013](#); [Soria-Gomez et al. 2021](#)), CB1^{E134G} mutants showed no gross morphological defects, postural changes, or clinical abnormalities (seizures, respiratory distress, digestive distress, dental abnormalities, nodules), and Mendelian frequencies reached standard values (approximately 50% for heterozygotes and 25% each for WT and mutants) (APAFIS #16454).

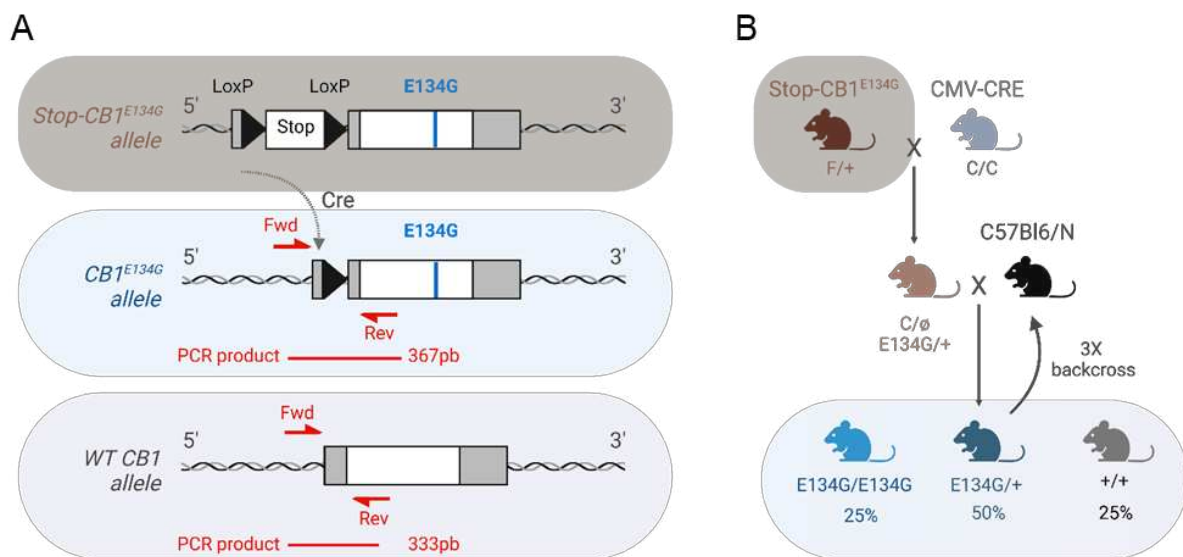


Figure 10: Generation of the CB1^{E134G} mouse line.

A. From top to bottom: Schematic representation of the Stop-CB1^{E134G} allele after homologous recombination with the targeting vector (top), the CB1^{E134G} allele generated by excision of the lox-stop-lox (LSL) cassette by Cre recombinase (middle), and the wild-type (WT) CB1 allele (bottom). Forward (Fwd) and reverse (Rev) primers used to genotype CB1^{E134G} mutant mice and their WT littermates are indicated. Black triangles, LoxP sites; white box (stop), stop cassette; gray boxes, untranslated regions of the exon containing; white box (CB1), the entire open reading frame encoding the CB1 receptor. **B.** Schematic of crossing and germline transmission to generate the CB1^{E134G} mouse line. Heterozygous Stop-CB1^{E134G} mice are indicated as floxed (F)/+; homozygous mice expressing Cre recombinase under the cytomegalovirus (CMV) promoter are indicated as C/C; heterozygous mice generated by crossing Stop-CB1^{E134G} with CMV-CRE mice after excision of the LSL cassette are indicated as C/ø for the Cre allele and E134G/+ for the CB1 allele. CB1^{E134G} heterozygous mice produced by backcrossing to C57BL/6N background are indicated as E134G/+, mutant and WT homozygous are indicated as E134G/E134G and +/+, respectively.

3. Specific aims

3.1. To study the phenotype of naive CB1^{E134G} mice at the molecular and behavioral level

We sought to investigate the phenotype of mutant mice carrying the long-lived CB1^{E134G} point mutation to determine whether this missense has any functional effect. To this end, naive male and female CB1^{E134G} mutant mice were compared to their WT littermates in molecular and behavioral assays.

Molecular analyses: Using a combination of reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), Western immunoblotting and gas and liquid chromatography tandem mass spectrometry (GC-MS/MS and LC-MS/MS), we investigated the effect of the CB1^{E134G} point mutation on the major components of the endocannabinoid system (ECS) and the steroidogenic system. We analyzed the expression of cannabinoid receptors, enzymes involved in the metabolism of endocannabinoids (eCBs) and steroids, and quantified eCB and steroid analytes in several brain areas of mutant mice and their WT controls.

Ongoing molecular analyses: Further analyses are aimed at investigating the effect of the CB1^{E134G} point mutation on receptor function. To confirm that the mutation does not affect receptor activity *per se*, radioligand and [³⁵S]-GTPγS binding assays are currently being performed by our collaborators at IMG Pharma (Spain) ([Soria-Gomez et al. 2021](#)). Analyses are being performed on membranes extracted from the brains of mutant CB1^{E134G} mice and their WT controls to investigate the effect of the point mutation on receptor availability, ligand affinity and functional G-protein binding. In parallel, we implanted a primary cortical cell culture model derived from the CB1^{E134G} mouse to further investigate the effect of PREG on CB1-mediated signaling and receptor dynamics.

Behavioral analyses: Mice were tested with a battery of standard behavioral tests based on the animal's spontaneous behavior related to memory and emotional processes ([Crawley 2008](#)). Episodic-like memory was assessed in naive mice using a modified version of the novel object recognition task ([Da Cruz et al. 2020](#)), while working and spatial reference memory were tested by assessing spontaneous and forced alternation in a Y-maze apparatus. Anxiety-like behavior was assessed using the elevated plus maze, elevated zero maze, and open field tests, while depressive-like behavior was assessed using the anhedonia-based sucrose preference test.

3.2. To study the effects of exogenous cannabinoid administration in $CB1^{E134G}$ mice

In this second aim, we sought to investigate the effects of exogenous cannabinoid administration in mutant mice and their WT controls. We examined the sensitivity of male and female $CB1^{E134G}$ mice to the primary psychoactive component of cannabis, THC, using a combination of the tetrad assay (typically used to assess the behavioral effects of CB1 agonists, (Metna-Laurent et al. 2017) and *in vivo* intracerebral microdialysis coupled with dopamine assay (Cathala et al. 2019). In particular, these experiments were used to determine the "face validity" of our model, e.g. the phenotypic and pathophysiological similarity to the dysfunction associated with CB1 overactivation (van der Staay, Arndt, and Nordquist 2009). Indeed, genetic deletion of the PREG-CB1 allosteric binding site should recapitulate the sensitization to the behavioral and neurochemical effects of THC previously observed using pharmacological approaches (Vallée et al. 2014). In addition, this model allows us to address the contribution of endogenously produced PREG to the regulation of CB1 activity during hyperactivity states.

Based on the results obtained with the tetrad assays, we addressed the putative specificity of endogenous PREG for specific CB1 receptor subsets by measuring the effect of the exogenous CB1 agonist WIN 55,212-2 on ADP-stimulated mitochondrial respiration in brain extracts from $CB1^{E134G}$ and $CB1^{WT}$ mice (Soria-Gomez et al. 2021). In addition, we investigated the subcellular localization of CB1 receptors involved in PREG production after acute THC administration by gas chromatography tandem mass spectrometry (GC-MS/MS) using the $CB1^{E134G}$ mouse model in addition to the DN22-CB1 knock-in mice lacking mitochondria-associated CB1 receptors (Hebert-Chatelain et al. 2016; Soria-Gomez et al. 2021).

3.3. To study the effect of endogenous CB1 stimulation in $CB1^{E134G}$ mice

3.3.1. To study energy balance on chow and high-calorie diet in $CB1^{E134G}$ mice

Hyperactivation of CB1 signaling is typically associated with metabolic changes and susceptibility to diet-induced obesity and comorbidities (Vincenzo Di Marzo and Matias 2005; Cota 2008; Pucci et al. 2021). Preliminary data from our laboratory indicate that PREG levels are increased during metabolic challenges, including a fasting challenge (unpublished data), suggesting that PREG may regulate endocannabinoid-mediated CB1 signaling. Therefore, we sought to investigate the contribution of the endogenous PREG-CB1 negative feedback loop to energy balance under standard chow conditions as well as under a high-fat diet to address, first, the endogenous regulation of CB1-related metabolic functions and, second, whether

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PREG may be an interesting pharmacological candidate for the treatment of obesity and related comorbidities, as has been shown for other pathologies associated with abnormal CB1 signaling ([Haney et al. 2023](#)).

Standard chow diet analyses: We first examined metabolic phenotypes in male CB1^{E134G} and WT littermate controls maintained on a standard chow diet. We used a combination of *in vivo* body composition analysis, indirect calorimetry, and other metabolic assessments such as intraperitoneal glucose and insulin tolerance tests, classically used in preclinical research to study glucose metabolism ([Bowe et al. 2014](#)). We also assessed animal motivation for wheel running and palatable food to address the rewarding properties of physical activity and food palatability, consistent with the balance between energy expenditure and energy intake ([Muguruza et al. 2019](#)).

Metabolic challenge analyses: In addition to the chow diet analyses, mice were subjected to metabolic challenges including a fasting refeeding challenge and acute exposure to a high fat diet (HFD) during indirect calorimetry analyses, along with in-cage food intake and locomotor activity measurements. Finally, we examined the effect of diet-induced obesity in mutant and WT mice by placing mice on an HFD for approximately 3 months. Food consumption and body weight were monitored, and the mice were subjected to a series of metabolic tests to investigate whether the CB1^{E134G} point mutation had any effect on the overall metabolic status of the animals.

3.3.2. On-going experiments

This second part aims to investigate whether the PREG-CB1 regulatory loop occurs under endogenous CB1 overactivation when endocannabinoid levels are induced. In addition to previous physiological modulators of eCBS tonus, such as fasting, we will use here a pharmacological approach using selective inhibitors of the degradation enzymes of AEA and/or 2-AG, and an environmental challenge under stressful conditions.

Pharmacological manipulation of endocannabinoid (eCB) levels: Using pharmacological inhibitors of eCB (AEA and 2-AG) degradation enzymes (FAAH and MAGL, respectively) in conjunction with gas and liquid chromatography tandem mass spectrometry (GC-MS/MS and LC-MS/MS) and behavioral paradigms, we will investigate the effect of endogenous stimulation of CB1 receptors by elevated eCB levels. First, we will determine whether AEA and 2-AG increase PREG production by stimulating CB1 receptors, as previously observed with exogenous CB1 ligands ([Vallée et al. 2014](#)). In addition, we will determine if AEA and 2-AG are equally effective in stimulating PREG production or if there are differences between the two eCBs that may indicate different and specific roles in the endogenous PREG-

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CB1 regulatory loop. These approaches will therefore be combined with analyses of the behavioral effects of eCB degradation enzyme inhibitors in CB1^{E134G} mice to investigate whether endogenously produced PREG can counteract the effects of eCBs after their endogenous levels have been increased pharmacologically.

Fear memory and stress: Since CB1 receptor activation has been linked to modulation of the stress response (Morena et al. 2016), and brain PREG levels are dynamically regulated by exposure to stressful stimuli (Vallée et al. 2000; Vallée 2014), we will examine whether PREG forms casual associations with CB1 receptor activity during the stress response to shape behavior. To this end, we will use previously validated behavioral paradigms that have revealed a role for CB1 receptors in mediating fear extinction (Giovanni Marsicano et al. 2002) and the amnesic effects of stress (Skupio et al. 2023). More generally, we will compare the behavioral and physiological effects of stress between CB1^{E134G} mice and their WT littermates. Finally, we will investigate whether stress-induced PREG production is associated with CB1 receptor activation using gas chromatography tandem mass spectrometry (GC-MS/MS).

MATERIAL AND METHODS

1. Animals

All experimental procedures and animal husbandry were performed in strict compliance with the European Directive 2010/63/EU and French laws on animal experimentation and were approved by the local ethics committee (CE50) of the University of Bordeaux and the French Ministry of Agriculture and Forestry (approval project numbers APAFIS #16454, #22030, #32638, and #34991). Male and female adult mice were housed in separate rooms under standard conditions (controlled temperature and hygrometry of $22 \pm 2^\circ\text{C}$ and 50-60%, respectively) with a 12 h/12 h light/dark cycle (lights on at 7:00 AM for males and 12:00 AM for females) and free access to food (A04-10 pellets, Scientific Animal Food & Engineering) and water *ad libitum*, unless otherwise specified. Experiments were performed during the light phase of the light-dark cycle, unless otherwise stated. Wild-type (CB1^{WT}) and mutant ($\text{CB1}^{\text{E134G}}$) littermate male and female mice (2-4 months old) were obtained and genotyped as described in the methods section (see section [Generation of the CB1E134G mouse line](#)). Both male and female homozygous mice were used for experiments, except for those performed in calorimetric chambers and on a high-fat diet (**Aim 3: Investigation of the effect of endogenous CB1 stimulation in CB1E134G mice: energy balance**). Heterozygotes were used for breeding only, except for primary culture experiments where homozygotes were bred together to obtain genotypically homogeneous embryos. After weaning, mice from the same litter were grouped by sex and litter (3 to 8 per cage). One week before the start of the study, mice were individually housed in close-fitting clear plastic cages (33x15x13 cm). For enrichment, a cellulose nesting square was placed in each cage, and a cardboard tunnel was placed in each housing cage and used to handle the animals. Male DN22-CB1-KI (DN22-CB1) mice and their WT littermates (2-4 months old) were kindly provided by the team of Giovanni Marsicano (Endocannabinoids and Neuroadaptation, Neurocentre Magendie, INSERM U1215, Bordeaux, France) and were obtained, bred and genotyped as described ([Soria-Gomez et al. 2021](#)). Male C57BL/6 N mice (8 weeks old) were obtained from Janvier (Janvier Labs, Le Genest-Saint-Isle, France). Both DN22-CB1-KI and C57BL6/N mice were maintained under the same housing conditions as the $\text{CB1}^{\text{E134G}}$ mouse line.

2. Drugs

THC was purchased as Dronabinol resin from THC Pharm GmbH (Frankfurt, Germany, ref. THC-1295S 250 - Dronabinol DAC 2008) and prepared as stock solutions at 50 mg/ml in ethanol and stored at -20°C in sealed amber glass vials. WIN55-212-2 (St. Louis, USA), Pregnenolone (PREG) (Acros Organics, New Jersey, USA), URB597, JZL184, and JZL195 (all from Bertin Technologies, France) were purchased as powder.

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For *in vivo* experiments, THC was freshly prepared before the experiments and dissolved in a mixture of 2% ethanol, 3% Tween80, and 95% saline in amber glass vials. The vehicle of THC contained the same amount of solvents. THC and vehicle solutions were stirred at room temperature for 1 hour before use and vortexed well before intraperitoneal administration at a volume of 10 ml/kg (1.5, 3, 6, 10, 12, or 15 mg/kg). THC was always injected 30 min before behavioral testing and biological sampling ([Vallée et al. 2014](#); [Metna-Laurent et al. 2017](#)). URB597, JZL184, and JZL195 were freshly prepared before experiments and dissolved in a mixture of 2.5% cremophor, 2.5% DMSO, and 95% saline in amber glass vials. Vehicle solutions contained equal amounts of solvents. Solutions were stirred at 37°C for 1 hour prior to use and vortexed well prior to intraperitoneal administration at a volume of 10 ml/kg. URB597 was prepared at a dose of 10mg/kg and injected 1 hour prior to biological sampling. JZL184 and JZL195 were prepared at a dose of 40 and 20mg/kg, respectively, and injected 2 hours prior to biological sampling.

For *in vitro* experiments, WIN and PREG were dissolved in DMSO, and the final DMSO concentration did not exceed 0.001%. An equivalent DMSO solution was used for control experiments. Doses and concentrations of the drugs were chosen based on previously published data or preliminary experiments ([Hebert-Chatelain et al. 2016](#); [Arnau Busquets-Garcia et al. 2015](#); [Vallée et al. 2014](#)).

3. Tissue and Plasma Collection for Molecular and Biochemical Analyses

Mice were sacrificed by decapitation. Trunk blood was collected in EDTA-coated tubes and centrifuged at 2000 rpm for 20 min (4°C). Supernatant plasma was collected in 2 ml Eppendorf tubes and rapidly frozen on dry ice. Brain structures were rapidly dissected on ice using a coronal mouse brain matrix (RBM-2000C, ASI Instruments, Warren, USA), collected in 2ml Eppendorf tubes, and frozen on dry ice. Plasma and brain samples were stored at -80°C until analysis.

4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using a standard acid guanidinium thiocyanate/phenol/chloroform protocol and purified by incubation with Turbo DNA-free (Fischer Scientific) ([Chomczynski and Sacchi 1987](#)). Purity and concentration of RNA samples were determined from OD260/280 readings using the ND1000 UV spectrophotometer (Thermo Scientific), and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab Chip Kit on the Bioanalyzer 2100 (Agilent Technologies). cDNA was synthesized from 2 µg RNA using Maxima RT (Fischer Scientific) with a mixture of random primers (Fischer Scientific) and oligo(dT)18 primers (Fischer Scientific). Transcript-specific primers were generated using

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Primer Express software (Applied Biosystems) based on GenBank sequence information, verified by NCBI BLAST search, and custom synthesized (Eurogentec). In addition, the primers were designed according to the MIQE parameters (Bustin et al. 2009). The sequences of all primer pairs are listed in **Table 1**. Each primer set was tested for the absence of primer dimer artifacts and multiple products by melting curve analysis and gel electrophoresis. The amplification efficiency of each set was determined by qPCR using repeated dilution series of cDNA. Only primers with an efficiency of ~2 were used.

Table 1: Primer sequences

Transcript	Forward primer	Reverse primer	Amplicon size	Accession N°
<i>Abhd4</i>	5'-TATCCTTATCAGCCACAGAATGCT-3'	5'-TGGAGGCCATGGTGACAGAT-3'	71bp	NM_134076
<i>Abhd6</i>	5'-CAGACTGGAGGGTCACACCAA-3'	5'-CCAAGGGTTTTAGTAGAACAGC-3'	71bp	NM_025341
<i>Abhd12</i>	5'-GCCTTCCTCAGCTGCCCT-3'	5'-GAAGTGCAGATCCAGGTGCC-3'	72bp	NM_024465
<i>Cnr1</i>	5'-GTCGATCTTAGACGGCCTTGC-3'	5'-TTGAGCCCACGTAGAGGAGGT-3'	71bp	NM_007726
<i>Cnr2</i>	5'-TGTGTGGGTGAGGCATGCT-3'	5'-CACTTGCTTGGTACACCGGAA-3'	71bp	NM_009924
<i>Cyp11a1</i>	5'-ATGACCTGCAGTGGAACCT-3'	5'-ACTTCAGCCCGCAGCATC-3'	71bp	NM_019779
<i>Dagla</i>	5'-GGTCTGCTCGTGTCTC-3'	5'-TGCAGCCACAACAGTTGTCTTC-3'	84bp	NM_198114
<i>Daglb</i>	5'-TTGTAGGCCAGCCCATGG-3'	5'-GCCTTGACAGCCCACTAAGG-3'	71bp	NM_144915
<i>Faah</i>	5'-CGGCCATCTTGAGGGTCAT-3'	5'-GGCTGCAGTGCAGAGCG-3'	71bp	NM_010173
<i>Lipe</i>	5'-CCTTCAGACAGCCCGAGA-3'	5'-AAGTTGACATCAGAGGGTGTGA-3'	71bp	NM_010719
<i>Lss</i>	5'-AGAGCAGGCTAGCTCCAGTTC-3'	5'-GCATGCCTTAATCCCAGCA-3'	71bp	NM_146006
<i>Mgll</i>	5'-TCACACTTCCCTTTCTCCTGAT-3'	5'-GTGACAAACCAGTGACCCACTGT-3'	71bp	NM_011844
<i>Napepld</i>	5'-GAACGGCTTGGCATAGCT-3'	5'-GACCAGACCACAATCACATGG-3'	72bp	NM_178728
<i>Plcg1</i>	5'-CGGCAAGGTGCAAGTTTTG-3'	5'-AGGTTGCTGGTGTGGCTTATT-3'	71bp	NM_021280
<i>Star</i>	5'-GGTGTGAAAGACCCATGTGT-3'	5'-TGTTTGTGAAGGCGCTTCT-3'	71bp	NM_011485
<i>Tspo</i>	5'-TGGCTCTACATAGTCTGAAAGA-3'	5'-TGTAGAGACCAAGGGAACCAT-3'	71bp	NM_009775
<i>Actb</i>	5'-TGACCGAGCGTGGCTACA-3'	5'-CATAGCACAGCTTCTCTTTGATGC-3'	78bp	NM_007393
<i>Eef1a1</i>	5'-TGAACCATCCAGGCCAAATC-3'	5'-GCATGCTATGTGGGCTGTGT-3'	71bp	NM_010106
<i>Gapdh</i>	5'-TCAAGAAGGTGGTGAAGCAG-3'	5'-TGGGAGTTGCTGTTGAAGTC-3'	70bp	NM_008084
<i>Nono</i>	5'-CTGTCTGGTGCATTCTGAACTAT-3'	5'-AGCTCTGAGTTTCTTTCCCATG-3'	72bp	NM_023144
<i>Ubc</i>	5'-AGACAGAGCTACCTTCTCACCA-3'	5'-CCCATCACACCAAGAACAAG-3'	71bp	NM_019639

The quantification cycle (Cq) value was measured using the second derivative maximum method implemented in the Light Cycler® Real-Time PCR System (Roche Applied Science). A no template control was performed for each primer set, and Cq values with a difference < 6 cycles were discarded. PCR conditions and Light Cycler® 480 SYBR Green I Master Mix (Roche Applied Science) were used in a reaction of 10 µL using transcript-specific primers (0.6 µM) and 2 µL cDNA corresponding to 4 ng total RNA input. An initial denaturation step at 95 °C for 5 min was followed by 45 cycles of 95 °C for 15 s, 61 °C for 15 s. PCR data were exported and analyzed using the GEASE (Gene Expression Analysis Software Environment) IT tool developed at the Neurocentre Magendie. The relative gene expression levels of the transcripts were analyzed by the comparative Cq method (2- $\Delta\Delta$ CT) implemented in GEASE, as described in detail by (Livak and Schmittgen 2001), using two reference genes for normalization. Based on twelve genes selected as potential reference genes, we used the geNorm software package (Vandesompele et al. 2002) to find the most stable genes and to determine how many reference genes should be used as a minimum for normalization. The reference genes used for the analysis were *Actb* (β -Actin), *Eef1a1* (eukaryotic translation

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elongation factor 1 alpha 1), *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *Nono* (non-POU domain-containing, octamer binding protein), *Ubc* (Ubiquitin C).

5. Protein extraction and western immunoblotting

Cells collected by scraping 6-well plates and frozen brain tissue were homogenized on a Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) using RIPA lysis buffer (10% Tris-buffered saline, 1% Triton X-100, 5% Na-deoxycholate 10%, 1% sodium dodecyl sulfate 10%, 0.4% EDTA 0.5M). Homogenization was performed at 5000 rpm, twice for 30 s plus a 10 s break. Cell debris was removed by three consecutive centrifugations at 10,000 rpm (4°C), and the supernatant was collected. Protein concentrations were measured using a Pierce BCA protein assay kit (Thermo Scientific). Proteins were diluted in Runblue LDS sample buffer (Euromedex), heat denatured at 37°C for 10 minutes, and then subjected to Western blot analysis. 10 to 15 µg of total proteins were separated on Mini Protean TGX precast electrophoresis gels 4-15% (Bio-Rad Laboratories, USA) at 200 V constant for 50 min in Tris-Glycine-SDS buffer (Euromedex France). Proteins were transferred to PVDF membranes (Immobilon P, Millipore, USA). Transfer was performed at 4 °C for 1 h in Tris/glycine/10% methanol buffer at 100 V. For CB1 Western immunoblotting, the membrane was first blocked for 60 minutes at room temperature in TBS/T-milk, Tris-buffered saline (TBS) containing 5% non-fat dry milk powder, 0.05% Tween 20. The membrane was then incubated overnight at 4°C in the same TBS/T-milk solution containing a rabbit anti-CB1 polyclonal antibody (# ab23703, Abcam, France) at 1/5000 dilution. The membranes were then washed twice for 7 minutes with TBS/T and incubated for 1 hour at room temperature in TBS/T containing anti-rabbit IgG-HRP (#7074S, 1/5000, Cell Signaling Technology, USA) secondary antibody at 1/5000 dilution. After TBS/T and TBS washes, the signal was detected by chemiluminescence (Immobilon Forte Western HRP Substrate, Millipore, USA). The x-ray films (BioMax MR film, Kodak, USA) used for chemiluminescence signal detection were scanned in transmission mode using a GS-800 scanner (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. The same membrane was reused for control hybridization with monoclonal anti- α -tubulin (#NR356, 1/50000, Amersham, USA) and detected with anti-mouse IgG-HRP secondary antibody (#7076, 1/20000, Cell Signaling Technology, USA) ([Di Franco et al. 2022](#)).

6. Endocannabinoid measurements by LC/MS-MS

Plasma and brain levels of AEA and 2-AG were determined as previously described ([Matias et al. 2023](#)). Briefly, samples were homogenized with chloroform/methanol/Tris-HCl (50 mM, pH 7.5, 2:1:1, vol/vol) containing internal deuterated standards (AEA-d4 and 2-AG-d5), and the dried lipid extract was purified by solid phase extraction (SPE C18 Agilent,

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France). Mass spectral analyses were performed on a TSQ Quantum triple quadrupole instrument (Thermo-Finnigan) equipped with an atmospheric pressure chemical ionization source and operating in positive ion mode. AEA and 2-AG levels were determined by isotope dilution using a calibration curve. Endocannabinoid levels were then normalized to fresh tissue weight.

7. Steroid measurements by GC/MS-MS

Plasma and brain levels of pregnenolone (PREG), allopregnanolone (ALLOP), dehydroepiandrosterone (DHEA), and 17-hydroxyprogesterone (17-OHPROG) were determined as previously described (Matias et al. 2023). Briefly, plasma samples were homogenized in methanol/H₂O (75/25, v/v, 500 µl), and brain samples in methanol/H₂O (75/25, v/v, 1 ml/100 mg tissue) containing deuterated internal standards (PREG-d₄, ALLOP-d₄, DHEA-d₅, 17-OHPROG-d₈). After centrifugation (10000 rpm, 10 min, 4°C), the supernatant of the brain homogenate was removed. Plasma and brain samples were then purified by solid phase extraction (SPE) using reverse phase C18 columns (Varian, France). The eluted steroid fraction was evaporated to dryness at 50°C under a nitrogen stream in preparation for derivatization. Dried methanol extracts of biological samples and standards were derivatized in a two-step procedure to provide adequate sensitivity, selectivity, and reproducibility for the simultaneous measurement of endogenous steroid levels. The derivatized samples were injected directly into a GCMS-QP2010 mass spectrometer through an autosampler. The instrument was operated in negative ion chemical ionization (NICI) mode. Endogenous steroid levels were determined by isotope dilution using a calibration curve and normalized to the weight of fresh tissue.

8. Novel-object recognition memory task

Recognition memory was assessed using the hippocampal-dependent novel object recognition task (Puighermanal et al. 2013; Hebert-Chatelain et al. 2016). This task was performed using an L-shaped maze made of dark blue Plexiglas with two corridors (35 cm and 30 cm long for the outer and inner V-walls, respectively, 7 cm wide and 15 cm high walls) set at a 90° angle (Noldus, Wageningen, The Netherlands) and under dim light intensity (25 lux). The task consisted of three consecutive daily trials of 10 minutes each ([Figure 11](#)). Day 1 (Habituation): Mice were placed at the intersection of the two arms and allowed to explore the maze freely. Day 2 (Acquisition): Two identical objects were placed at the end of each arm. Day 3 (Retrieval): A novel object, different in shape, color, and texture, was placed at the end of one arm, while the familiar object remained at the end of the other arm. The position of the novel object and the pairings of novel and familiar objects were randomized. Exploration of each object was scored offline by an observer blinded to genotype. Exploration was defined

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as the time the mouse spent with its nose pointing at the object at a distance of less than 1 cm, whereas climbing or chewing on the object was not considered exploration (Da Cruz et al. 2020). Mice that showed a significant place preference during the acquisition trial were excluded from the analysis to avoid confounding factors. Memory performance was assessed using the discrimination index. The discrimination index was calculated as the difference between the time spent exploring the novel object (TN) and the familiar object (TF) divided by the total exploration time (TN+TF): $\text{discrimination index} = (TN - TF) / (TN + TF)$. Memory performance was compared to a chance level set up at 0. The total exploration time of the two objects during the retrieval trial was used as a control.

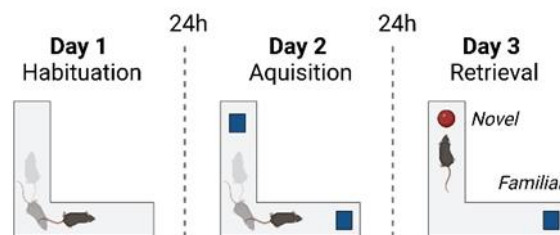


Figure 11: Schematic diagram of the experimental protocol of the NOR task.

The three phases of the 24h-delay test include habituation, acquisition and retrieval.

9. Working-like memory task

Working-like memory was assessed using the spontaneous alternation task (Prieur and Jadavji 2019). This task was performed in a Y-shaped maze made of white Plexiglas with three equidistant and identical arms (40 cm long, 8 cm wide, and 15 cm high walls) at a 120° angle and under low light intensity (80 lux). The task consisted of a single trial lasting 8 min. Mice were placed in the center of the three arms and allowed to explore the maze freely. The sequence of arm entries was scored offline by an observer blinded to genotype. An arm entry was considered complete when mouse's hind paws were completely placed in the arm. Working-like memory performance was assessed by the percentage of spontaneous alternations, defined as consecutive entries into three arms on overlapping triplet sets. For example, entries into arms 1, 3, and 2 would be considered an alternation, whereas entries into arms 1, 2, and 1 would not be considered an alternation. Accordingly, the percentage of alternations was calculated as the $(\text{number of triplets performed} \times 100) / (\text{number of triplets possible} (= \text{number of entries} - 2))$. Working-like memory performance was compared to a chance level set at 50%. A minimum of 10 entries was required for statistical analysis. The total number of arm entries was used to control for locomotion.

10. Spatial reference memory task

Spatial memory was assessed using a modified version of the Y-maze task (forced alternation) (Kraeuter, Guest, and Sarnyai 2019). This task was performed in a Y-shaped maze made of white Plexiglas with three equidistant and identical arms (40 cm long, 8 cm wide, and 15 cm high walls) at an angle of 120° and under low light intensity (80 lux). Visual cues were placed on the end wall of two arms, referred to as the test arms, while the third arm was referred to as the departure arm. The task consisted of two consecutive trials of 8 min each. Trial 1 (Acquisition): Mice were placed in the departure arm and allowed to explore the maze with one of the test arms closed during the session. Trial 2 (Retrieval): After a 1-hour inter-trial interval (ITI) during which mice were removed from the maze and placed in their home cage, mice were returned to the departure arm and allowed to explore all test arms by removing the barrier. Running distance within all three arms of the maze was measured using a video tracking device with a ceiling-mounted camera connected to a monitoring computer in a room adjacent to the testing room. Data were then analyzed using video tracking software (Ethovision XT, Noldus, Wageningen, The Netherlands). Spatial memory performance was assessed using a distance ratio calculated as the percentage of distance within the novel arm (NA) divided by the total distance within the NA and familiar arm (FA): $(NA/NA+FA) \times 100$. Spatial memory performance was compared to the chance level, which was set at 50%. The total number of arm entries was used as a control.

11. Elevated-Plus maze test

Anxiety-like behavior was assessed using the elevated plus maze test (Crawley 2008; Komada, Takao, and Miyakawa 2008). The test was conducted in a cross-shaped apparatus made of dark gray Plexiglas, consisting of four elevated arms (37 cm long, 6 cm wide, and 100 cm above the floor). A 60 cm high wall enclosed two opposite arms, while the other two arms were open with a very thin 0.5 cm wall (Noldus, Wageningen, The Netherlands). The arms were connected by a square central platform (6cm²). Both the central platform and the open arms were dimly lit at 25 lux, while the remaining closed arms were dimly lit at 5 lux. The task consisted of a single trial lasting 5 minutes. Mice were placed on the central platform facing an open arm and allowed to freely explore the maze. The number of arm entries and the time spent in the open and closed arms were measured using a video tracking device with a ceiling-mounted camera connected to a monitoring computer in a room adjacent to the testing room. Data were then analyzed using video tracking software (Ethovision XT, Noldus, Wageningen, The Netherlands). Anxiety-like behavior was assessed by measuring the percentage of time spent in open arms (OA) divided by the time spent in both open and closed arms (CA): $(OA/OA+CA) \times 100$. The total number of arm entries was used to control for locomotion.

12. Elevated-zero maze test

Anxiety-like behavior was assessed using the elevated zero maze test (Shepherd et al. 1994). The test was conducted in a dark gray Plexiglas apparatus consisting of a circular path (50 cm in diameter, 6 cm wide, and elevated 100 cm above the floor) divided into four equal quadrants. A 60 cm high wall enclosed two opposite quadrants, while the other two quadrants were open (Noldus, Wageningen, The Netherlands). The open quadrants were dimly lit at 15 lux, while the remaining closed quadrants were dimly lit at 5 lux. The task consisted of a single 5-minute trial. Mice were placed in one of the closed quadrants facing an open quadrant and allowed to freely explore the maze. Total distance traveled and time spent in the open and closed quadrants were measured using a video tracking device with a ceiling-mounted camera connected to a monitoring computer in a room adjacent to the experimental room. Data were then analyzed using video tracking software (Ethovision XT, Noldus, Wageningen, The Netherlands). Anxiety-like behavior was assessed by measuring the percentage of time spent in open quadrants/areas (OA) divided by the time spent in both open and closed quadrants/areas (CA): $(OA/OA+CA) \times 100$. Total running distance was used to control for locomotion.

13. Sucrose preference test

Anhedonia-like behavior was assessed using the sucrose preference test (Willner et al. 1987; Pothion et al. 2004). Two 50 ml bottles containing water and 2% (wt/vol) sucrose, respectively, were made available to the mice ad libitum for four days to assess sucrose preference. Each day, the position of the bottles in the cage was rotated to avoid place preference. Water and sucrose intake was monitored based on the difference in bottle weight, which was used to calculate daily and total fluid intake.

14. Cell culture

Primary cortical cultures were prepared from CB1^{WT} and CB1^{E134G} E17.5 embryos. Briefly, shortly after pregnant mice were sacrificed by cervical dislocation, fetal cortices were dissected under a binocular loupe in Hank's balanced salt solution medium (Gibco, France) containing penicillin-streptomycin (Gibco, France) and dissociated in 2% papain (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes at 37°C. Cells (3.5x10⁶ cells/ml) were then plated on poly-L-lysine/laminin-coated 6-well plates for Western blots and 12-well plates with coverslips for immunocytochemistry using Neurobasal plus medium (Gibco, Paisley, UK) supplemented with 5% b27 plus supplement, 2.5% GlutaMAX and 10% horse serum (all from Gibco, France). One hour after plating, the culture medium was removed and replaced with Neurobasal plus medium containing 5% b27 plus supplement, 2.5% GlutaMAX, 1.5% horse

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serum, and AraC (0, 100, 500 or 1000nM) (Gibco, France) to minimize glial growth. Serum and AraC were removed after 4 days in vitro (DIV4). Primary cortical cultures were used at DIV8. Cells were maintained at 37°C and 5% CO₂ throughout the experiments.

15. Immunocytochemistry

Primary cells seeded on 12-well plates were fixed on day 8 in vitro with phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% formalin for 15 minutes at room temperature (RT). Plates were washed three times with PBS and then stored in PBS containing 0.02% sodium azide at 4°C. For immuno-labeling, coverslips were washed 3 times for 5 minutes each with PBS to remove sodium azide and saturated with a solution containing PBS + 0.1% Triton + 2% normal goat serum (all from Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at RT. Cells were then incubated overnight at 4°C with chicken anti-MAP2 (1:1000, COVANCE BioLegend) and mouse anti-GFAP Cy3 conjugate (1:400, Sigma C9205) in the saturating solution. The signal was detected with goat anti-chicken 488 (1/1000, Invitrogen). Coverslips were mounted on slides with Aquapolymount and DAPI mounting medium (FISHER Scientific) and imaged using a Leica DM6000 epifluorescence microscope (Leica, Germany) with a 20x fluorescence objective. Three wells were analysed per experimental condition.

16. Intracerebral microdialysis and dopamine assay

Surgical and perfusion procedures were performed as previously described ([Cathala et al., 2022](#)) with minor modifications. Microdialysis probes (CMA/11, cuprophan, 240 µm outer diameter, Carnegie Medicin, Phymep, France) were 1 mm in length. Stereotaxic coordinates were chosen according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin 2001). Mice were weighed, anesthetized with 3% isoflurane (CSP, Cournon d'Auvergne, France), and placed in a stereotaxic frame. The microdialysis probe was implanted in the right NAc (coordinates in mm, relative to the interaural point: anteroposterior (AP) +5.1, lateral (L) - 0.5, ventral (V) +0.8). After surgery, the percentage of isoflurane was adjusted to 1.5% until the end of the experiment. The probes were perfused at a constant flow rate (1µl/min), using a microperfusion pump (CMA 111, Carnegie Medicin, Phymep), with artificial cerebrospinal fluid containing (in mM): 147 NaCl, 4 KCl, 2.2 CaCl₂, pH 7.4. Pharmacological treatments were performed 120 minutes after the start of perfusion (stabilization period). Dopamine (DA) efflux was monitored for 120 min after the last drug injection. Dialysates were collected every 15 min in a refrigerated fraction collector (MAB 85 Microbiotech, Phymep). At the end of each experiment, mice were deeply anesthetized with an overdose of pentobarbital (Exagon, 200mg/kg, Centravet), and the brain was removed and fixed in NaCl (0.9%)/paraformaldehyde (10%) solution. Probe location in the target region was determined histologically on serial

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coronal sections (60 μm) stained with cresyl violet, and only data from mice with correctly implanted probes were included in the results.

After collection, the dialysate samples were immediately analyzed with a high-performance liquid chromatography apparatus (Alexys UHPLC/ECD Neurotransmitter Analyzer, Antec, The Netherlands) equipped with an autosampler (AS 110 UHPLC cool 6-PV, Antec). The mobile phase (containing (in mM) 100 phosphoric acid, 100 citric acid, 0.1 EDTA.2H₂O, 4.6 octane sulfonic acid.NaCl plus 4.5% acetonitrile, adjusted to pH 6.0 with NaOH solution (50%)) was delivered at a flow rate of 0.075 ml/min using an LC 110S pump (Antec) through an Acquity UPLC BEH column (C18; 1 x 100 mm, particle size 1.7 μm ; Waters, Saint-Quentin en Yvelines, France). Detection of DA was performed using an electrochemical detector (DECADE II, Antec) with a VT-03 glassy carbon electrode (Antec) set at +460 mV versus Ag/AgCl. The output signals were recorded on a computer (Clarity, Antec). Under these conditions, the retention time for DA was 4-4.5 min, and the sensitivity was 50 pM with a signal-to-noise ratio of 3:1. DA levels in each sample were expressed as a percentage of the average baseline level calculated from the three fractions prior to drug administration. Basal dopamine levels were expressed in nM corresponding to the mean values of the baseline levels.

17. THC-induced tetrad

Behavioral and somatic effects of THC were assessed in mice using the THC-induced tetrad procedure as previously described ([Metna-Laurent et al. 2017](#)). Locomotor activity was assessed in an actimeter consisting of eight boxes (22 cm long, 12 cm wide, 18 cm high walls) equipped with infrared sensors placed 3 cm above the box floor to detect locomotor activity in the horizontal plane (Imetronic, Pessac, France). The cages were illuminated with low light intensity (25 lux). The actimeter was connected to a monitoring computer equipped with software for simultaneous management of all eight boxes and data analysis (Imetronic, Pessac, France). Body temperature was measured with a flexible rectal probe (Ret 3; Physitemp, France) monitored by a Thermalert monitoring thermometer (Physitemp, France). Catalepsy was assessed in modified housing cages (33 cm long, 15 cm wide, and 13 cm high walls) equipped with a horizontal bar 1 cm in diameter fixed across the width at 3.5 cm from the cage floor. Analgesia was assessed by means of a hot plate equipped with a timer with a temperature fixed at $52 \pm 1^\circ\text{C}$ (reference BIO-CHP, Bioseb, France).

Mice were tested sequentially for all four parameters as follows ([Figure 12](#)). Mice were weighed, and their basal body temperature was measured prior to intraperitoneal administration of THC or its vehicle. Thirty minutes after injection, mice were placed in the actimeter for 15 minutes to assess horizontal locomotor activity, measured as the total number of break cells. Five minutes after the end of the actimeter session, a second measurement of

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body temperature was taken to calculate a delta between pre- and post-injection temperature (Δ temperature= post-injection temperature - pre-injection temperature). Approximately 20 min after injection, mice were tested for catalepsy by placing their forepaws on the horizontal bar. The latency to move off the bar was recorded for a maximum of four consecutive trials or a cut-off time fixed at 600 s. The maximum latency achieved was used for analysis. Finally, approximately 60 to 80 min after injection, the mice were placed in the hot plate apparatus. The latency for the onset of the first signs of pain (licking, shaking, and jumping) was measured with a cut-off time of 60 s to avoid tissue damage.

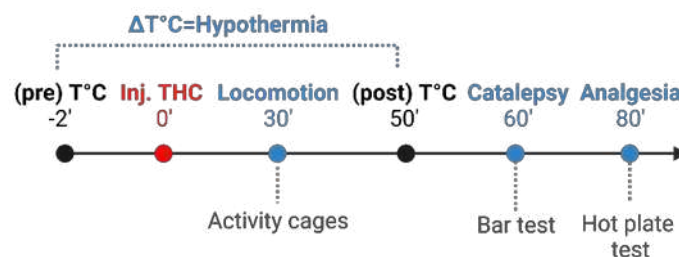


Figure 12: Schematic diagram of the tetrad protocol.

Sequential timeline (in minutes) of the tetrad, before and after the THC injection, including body temperature, locomotion, catalepsy, and analgesia measures.

18. Mitochondrial respiration measurements

Mitochondrial respiration in substantia nigra extracts was measured as previously described (Soria-Gomez et al. 2021). Briefly, immediately after cervical dislocation, mouse brains were extracted, and the substantia nigra was rapidly dissected on ice using a coronal brain matrix and homogenized in 450 mL Miro5 buffer without taurine supplementation (Makrecka-Kuka et al., 2015) using a Politron homogenizer (11,000 rpm 3-5 s). After brief centrifugation, the supernatant was treated with saponin at a final concentration of 12.5 mg/ml. Respiration analysis was performed using a 2K Oroboros device (Makrecka-Kuka et al., 2015). Briefly, 100 ml of lysate was added to each chamber, and complex I-dependent respiration was induced by the addition of malate (2 mM), pyruvate (5 mM), and glutamate (10 mM) (MPG) (Makrecka-Kuka et al., 2015). We then applied DMSO or WIN55, 212-2 at final concentrations of 0.1 and 1 μ M, followed 5 minutes later by 1.25 mM ADP. Each measurement of OCR in the ADP condition was normalized to the values before ADP injection, and the effect of WIN 55,212-2 was expressed as a percentage of the vehicle condition. Only samples for which the ADP/MPG ratio in the vehicle was equal to or greater than 1.5 were retained for analysis. For PREG pretreatment, the drug was dissolved in DMSO, which was also used as the vehicle for PREG, and injected together with the lysates at a final concentration of 500nM.

19. Body composition analysis

Body composition analysis was performed on conscious mice using a nuclear magnetic resonance whole-body composition analyzer (Echo MRI 900; EchoMedical Systems, Houston, TX, USA) as previously described ([Cardinal et al. 2014](#)). Briefly, mice were weighed and placed in the Echo MRI using a motion restraint to allow for proper measurements. All measurements were performed in duplicate at the same time of day under free-feeding conditions. Data for fat and lean mass were extracted for analysis. Fat mass and lean mass were expressed as a percentage of body weight at the time of analysis.

20. Glucose metabolism in standard diet and diet-induced obesity

Glucose and insulin tolerance tests were performed on separate groups of mice fed either a standard chow diet (A04-10 pellets, Scientific Animal Food & Engineering) or a high-fat diet (HFD) containing 60% of calories from fat (60% HFD, Research Diets, 5.24 kcal/g, New Brunswick, NJ). Both male and female mice were used for the chow diet experiments, whereas only males were used for the diet-induced obesity (DIO) experiments. For the glucose tolerance test (GTT), mice were fasted for 15 h and injected intraperitoneally with D-glucose solution (Sigma Aldrich, St. Louis, MO) at a concentration of 2 g/kg lean mass in the chow diet group and 1.5 g/kg lean mass in the DIO group at a volume of 10 ml/kg. For the insulin tolerance test (ITT), mice were fasted for 6 h and injected intraperitoneally with insulin solution (Humulin, Lilly, France) at a concentration of 0.5 UI/kg lean mass in the chow diet group and 0.75 UI/kg lean mass in the DIO group at a volume of 10 ml/kg. Blood samples were collected from the tail vein, and glucose was measured using glucose sticks (OneTouch, Vita, France) at baseline, 15, 30, 45, 60, 90, and 120 min after administration of glucose or insulin. For the GTT experiments, glucose-stimulated insulin secretion (GSIS) was assessed at baseline and 15 min after glucose administration by collecting blood from the tail vein in 0.5 ml Eppendorf tubes. Insulin was then measured using mouse insulin ELISA (10-1247-01, Mercodia) according to the manufacturer's instructions. Glucose and insulin concentrations were determined based on previous experiments ([Cota et al. 2009](#)). GTT and ITT data were expressed as blood glucose in mg/dl or as a percentage of baseline.

21. In-cage locomotor activity, food and drink intake

In-cage locomotor activity, food, and water intake were measured in light, temperature, and humidity-controlled calorimetric chambers (TSE Systems GmbH, Germany). Mice were acclimated to the chambers for 3 days, and measurements were taken automatically every 20 min. Food and water consumption were measured continuously using integrated scales inside the cages. For feeding pattern analysis, a meal consisted of the consumption of at least 0.03

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g of food separated by at least 10 min from the next feeding episode. After the baseline analysis, mice were fasted for 24h followed by refeeding with standard chow (A04-10 pellets, Scientific Animal Food & Engineering) starting 20 min before the start of the dark period of the light-dark cycle. One week after the fasting-refeeding challenge, mice were switched to a high-fat diet containing 60% of calories from fat (60% HFD, Research Diets, 5.24 kcal/g, New Brunswick, NJ). The first 24 hours of exposure to this HFD were recorded in calorimetric chambers, and the mice were then returned to their home cage and maintained on the HFD for approximately 4 months. O₂ consumption and CO₂ production were also measured during recording in calorimetric chambers to calculate gas exchange, respiratory quotient, and energy expenditure. However, we reported a batch effect on lean mass in CB1^{E134G} mice that affected these parameters, and data were not included in the present results.

Upon return to their home cage, body weight, and HFD consumption were recorded weekly. Feed efficiency was calculated by dividing body weight gain for a given period by total caloric intake for that period ([Castellanos-Jankiewicz et al. 2021](#)). Animals that chewed their food made it impossible to quantify their intake and were therefore excluded from the food intake and feed efficiency analyses. GTT, GSIS, and ITT analyses were then performed on the DIO group.

22. Operant running protocol

Motivation for wheel running was assessed as previously described ([Muguruza et al. 2019](#)). Briefly, nose-poke (NP) performance could be either 'active' (leading to cue light illumination and wheel unlocking) or 'inactive' (having no consequence). The left/right allocation of active/inactive NP ports was counterbalanced across animals during the experiments. All devices in the operant chambers were connected to a computer that recorded the number of active/inactive NPs, the number of running sequences, and the running duration/distance covered during each sequence. Experiments were conducted during the active (dark) phase of the mice's light/dark cycle. After a familiarization period, mice were tested in the fixed ratio 1 (FR1) schedule of reinforcement, where a single active NP was sufficient to simultaneously illuminate the cue light over the port for 10 seconds and unlock the running wheel for 60 seconds. NPs in the inactive port were counted but had no effect. At the end of the 60 seconds, the wheel light turned off, and the brake applied, requiring the mouse to step off the wheel and make another NP to unlock it. NPs made in the active port while the wheel was already unlocked had no effect. Mice completed 5 daily FR1 sessions of 60 minutes each. Mice then progressed to the FR3 schedule of reinforcement, which required 3 consecutive active NPs to unlock the wheel. As with FR1, mice completed 5 daily FR3 sessions of 60 minutes each. The day after the final FR3 session, mice were tested under a linear

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progressive ratio (PR) schedule of reinforcement in which the number of active NP required to unlock the running wheel increased by 3 between each rewarded step (3, 6, 9, etc.; PR3). A 15-minute time limit was imposed between 2 consecutive steps. By quantifying the number of NPs corresponding to the last reinforced ratio (i.e., the breakpoint level), the PR session makes it possible to estimate reward motivation ([Hodos 1961](#)).

23. Operant feeding protocol

Motivation for palatable food was assessed as previously described ([Muguruza et al. 2019](#)). Briefly, prior to the experiments, animals were habituated to the 20 mg chocolate pellets used in the operant chambers ("Dustless Precision Pellets," F05301; Plexx, for BioServ) by providing 5 pellets per day in their home cages for 3 days. Daily food consumption and body weight of each mouse were recorded daily for 1 week before the mice were given a restricted amount of food to maintain their body weight at 90% of their free-feeding weight. Food was always provided 60-90 minutes after the daily completion of the operant conditioning session to minimize the possibility of interactions between free-feeding and operant behavior. Nose-poke (NP) performance could be either "active" (resulting in cue light illumination and a single pellet delivery) or "inactive" (having no consequence). The number of active/inactive NPs, the number of pellets delivered, and the number of pellets consumed were recorded. As in the operant running protocol, mice underwent habituation followed by FR1, FR3, and PR schedules of reinforcement. In contrast to the operant running protocol, only 4 FR1 and FR3 sessions were required to reach performance stability, and the sessions lasted 30 min each to avoid satiety. In addition, there was no time limit between two consecutive trials in the PR schedule of reinforcement, consistent with the short duration of the session.

24. Acute stress and blood sampling

The corticosterone response to acute swim stress was measured in mice. An initial blood sample was collected from the animals to measure basal corticosterone levels beginning two hours after the start of the light period of the light/dark cycle. Blood was collected from mice using the submandibular vein (cheek pouch) method. Briefly, mice were restrained with the lateral surface of the head facing the operator. A small pouch was made in the cheek approximately midway between the ear and the mandible using a 21G gauze needle. Blood drops were quickly collected in EDTA-coated tubes and kept on ice until the following steps. Bleeding was rapidly stopped by applying light pressure to the cheek with a sterile gauze pad for approximately 5 seconds. 20 min after the first blood collection, mice were subjected to forced swim stress for 10 min. They were placed in a 15 cm diameter, 21 cm high cylinder filled with water at 24°C to a depth of approximately 15 cm. After stress exposure, the mice were removed from the water, dried with a paper towel, and returned to their home cage. Three

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additional blood samples were collected at 10, 60, and 120 min after the end of stress, alternating between the left and right cheek of the mice. Blood sampling was performed carefully to avoid sampling more than 10% of the total blood volume of the mice. Blood samples were rapidly centrifuged at 2000 rpm for 20 min (4°C) after collection, and the supernatant plasma was stored in 0.5 ml Eppendorf tubes at -80°C until analysis.

25. Adrenal sampling

Mice were quickly decapitated, and the abdominal cavity was cut open. The adrenal glands were dissected, and the fat surrounding the glands was removed for subsequent adrenal weight measurements.

26. Corticosterone assay

Corticosterone was measured from mouse plasma samples using the Arbor Assays K014-H1 ELISA kit (DetectX®, Arbor Assays™, Eisenhower Place Ann Arbor, MI, USA) in the 50 µl (20.9 pg/ml sensitivity) format according to the manufacturer's instructions. Briefly, plasma samples collected before and after the acute stress procedure were vortexed and diluted 1:100 and 1:200 in assay buffer, respectively. The supplied corticosterone standard was used to generate a standard curve for the assay by performing serial dilutions in the assay buffer. Standards and samples were added in duplicate to a 96-well microplate coated with donkey anti-sheep IgG antibody. Then 25µl of corticosterone peroxidase conjugate and 25µl of sheep polyclonal corticosterone antibody were then added to the wells, and the plate was agitated at 100 rpm for 1 hour at room temperature. After 1 hour, excess corticosterone peroxidase conjugate was washed off, and 100µl of TMB substrate was added to the wells for 30 minutes at room temperature. After 30 minutes, 50µl of stop solution was added to the wells, and the signal was detected using a plate reader at 450nm (POLARstar Omega). Corticosterone concentrations were determined from the standard curve using myassay.com.

27. Fear conditioning and extinction

Cued fear conditioning and extinction sessions were performed as previously described ([Giovanni Marsicano et al. 2002](#)). On the day of conditioning, mice were placed in conditioning chambers (Imetronic, Pessac, France). After a 180-s habituation period, a 20-s tone (9 kHz, 80dB) was presented, followed by a 2-s electric foot shock (0.7mA). Mice were returned to their home cages 60 s after shock application. On days 1, 2, 3, 6, and 20 after conditioning, animals were placed in transparent Plexiglas cylinders that were different from the conditioning context, and after a 180-s blank period, a 60-s tone was presented (extinction trials). After another 60 s, the animals were returned to their home cage. Freezing behavior (defined as the absence of all movements except respiration) was quantified from video recordings by a

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trained observer blinded to genotype. Data were normalized as a percentage of the total freezing response for day 1 and as a percentage of freezing observed on day 1 for subsequent extinction sessions (days 2 to 20).

28. Statistical analyses

Results are expressed as mean \pm SEM. Graphs and statistical analyses were performed using GraphPad Prism 7 software (La Jolla, CA, USA) and STATISTICA (StatSoft GmbH, Hamburg, Germany). Data sets were tested for normal distribution using the D'Agostino-Pearson test or the Shapiro-Wilk test, depending on sample size. If the data were normally distributed, 2-group comparisons were performed using the Student's t-test, and multifactorial analyses were performed using 2-way or 3-way ANOVA, depending on the experimental design. Analysis of variance was followed by appropriate post hoc multiple comparisons. Data that did not follow a normal distribution or where the experimental design precluded the use of parametric statistics (e.g., cut-off) were analyzed using nonparametric Mann-Whitney and Kruskal-Wallis tests for 2-group comparisons and analyses of variance, respectively. The significance level was set at $p < 0.05$ for all data sets. Detailed statistical results are presented in [APPENDIX I. STATISTICAL TABLE](#).

RESULTS

1. Investigation of molecular and behavioral phenotypes of CB1^{E134G} mice at baseline

1.1. Rationale

The CB1^{E134G} point mutation was generated to study the regulatory functions of the neurosteroid PREG in modulating CB1 receptor activity by selectively perturbing its allosteric binding site on the receptor, as previously reported (Vallée et al. 2014). This mutation successfully prevented the allosteric modulation of PREG on CB1 receptors, as assessed *in vitro* by the THC-induced decrease in ADP-stimulated mitochondrial respiration (Vallée et al. 2014). In addition, the relative position of the mutated amino acid residue on the TMH bundle of the CB1 receptor was chosen on the basis that it would not affect receptor functions *per se*, but would provide specific insights into the effects of endogenous PREG release on CB1 activity (Vallée et al. 2014). Based on these *in vitro* studies, we developed the CB1^{E134G} mouse model to better understand the PREG-CB1 negative feedback loop *in vivo* and to address the endogenous functions of the steroid on CB1 activity and functions.

Early development and expansion of the CB1^{E134G} mouse line revealed no gross effects on viability and morphology (data not shown, see section [Generation of the CB1E134G mouse line](#)). As part of the ongoing development of this new model, we first sought to provide a general characterization of the mutant male and female mice compared to their CB1^{WT} (Wild type, WT) littermates. Specifically, we performed molecular and behavioral analyses to determine whether the CB1^{E134G} point mutation bears any functional effects, and, in a mirror image, whether endogenous PREG has an effect on CB1 activity. These analyses were first performed in naive animals to provide a baseline picture of the phenotype of mutant mice compared to WT controls. At the molecular level, we characterized the endocannabinoid and steroidogenic systems in the mutant mice with respect to the PREG-CB1 regulatory loop. Gene expression of proteins involved in the formation of endocannabinoids (eCBs) and (neuro) steroids, as well as cannabinoid receptors, was analyzed by RT-qPCR. If significantly different between mutant and WT mice, RT-qPCR analyses were followed by semi-quantitative protein detection by Western immunoblotting. In addition, eCBs and (neuro) steroid analytes were directly detected and quantified by spectral analysis (LC-MS/MS and GC-MS/MS, respectively). At the behavioral level, we analyzed general motor, cognitive, and emotional functions known to be regulated by cannabinoids, mainly through activation of the CB1 receptor (S. Ruehle et al. 2012; Morera-Herreras et al. 2012; Wotjak 2005; Kruk-Slomka et al. 2017). Genetic manipulation of the CB1 receptor (e.g., by complete or partial deletion in genetically engineered mice) can lead to phenotypic changes at baseline and thus reveal the

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contribution of receptor activity to a given biological function (F. Navarrete et al. 2020; Albergaria et al. 2020). Since the general effects of the CB1^{E134G} point mutation and the relative contribution of PREG in modulating CB1-dependent functions in all endogenous activation states are unknown, the general description of the behavioral phenotype of CB1^{E134G} mice is an important starting point for further investigation of the endogenous PREG-CB1 regulatory loop.

1.2. Results

1.2.1. CB1 expression is downregulated at protein but not mRNA level in CB1^{E134G} mice

To investigate whether the CB1^{E134G} point mutation has any effect on CB1 receptor expression, we first performed RT-qPCR analyses in discrete brain areas of naive male and female CB1^{E134G} mice to analyze the expression of the CB1-encoding gene (*cnr1*) at the mRNA level ([Figure 13A](#)). *Cnr1* expression was found to be upregulated in the nucleus accumbens (NAc) and dorsal striatum (CPu) of mutant mice compared to their WT littermates, but not in other regions analyzed, including the prefrontal cortex (PFC), hypothalamus (HTHAL), hippocampus (HPC), and cerebellum (CB) ([Figure 13A-B](#)). In contrast, the expression of the CB2-encoding gene (*cnr2*) was unchanged between mutant and WT mice. Thus, these results suggest that the CB1^{E134G} point mutation at the level of the PREG binding site specifically upregulates CB1 expression in some brain areas of mutant mice. We therefore investigated whether these changes at the mRNA level translate into an increase in CB1 protein expression. To this end, we performed total CB1 protein analysis in mouse brain regions by semi-quantitative Western immunoblotting. In contrast to RT-qPCR, Western blot analysis revealed a dramatic decrease in CB1 protein in naive CB1^{E134G} mice compared to their WT littermates in virtually all brain regions analyzed, including the NAc, CPu, and one of their major associated input-output regions, the ventral midbrain (VMB) ([Figure 13C](#)). This downregulation was present in protein extracts from both male and female mice and ranged from approximately 70% to 80% reduction ([Figure 13D](#) and [Figure 13E](#), respectively). The discrepancies between mRNA and protein suggest a post-translational compensation effect of the mutation leading to an overall downregulation of CB1 protein. Of interest, this compensation could be the result of the loss of endogenous allosteric modulation of PREG on the CB1 receptor, which would further suggesting that endogenous PREG can regulate CB1 protein levels ([see DISCUSSION](#)).

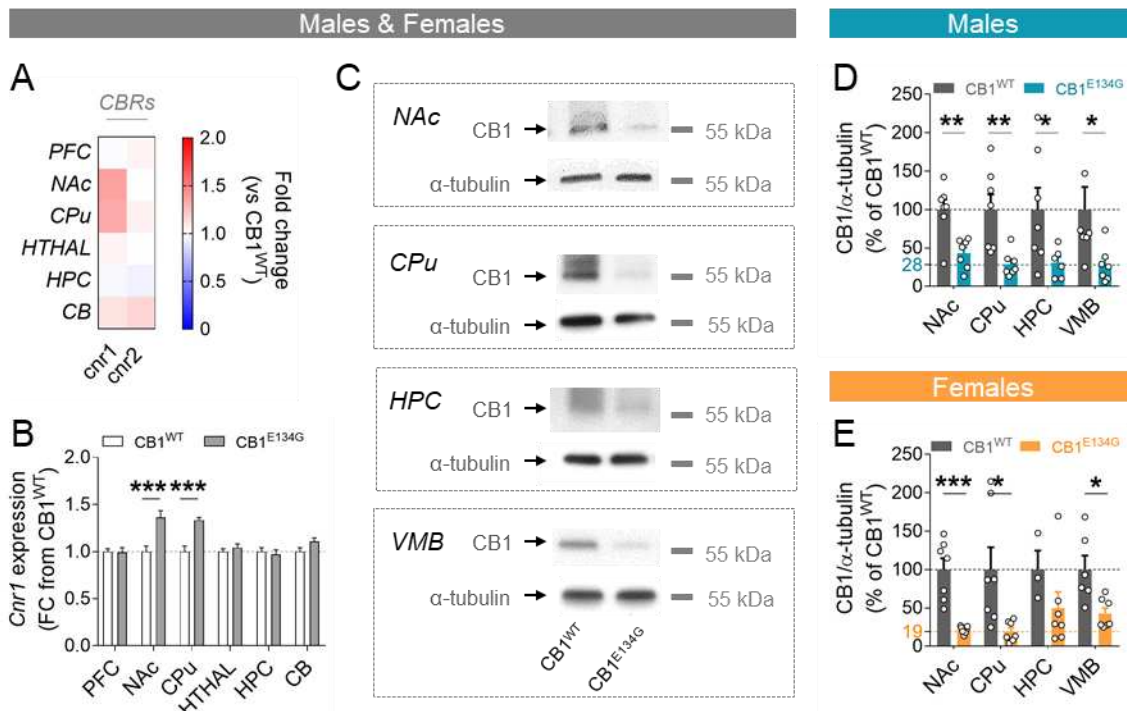


Figure 13: CB1 expression at the mRNA and protein level in the brain of CB1^{wt} and CB1^{E134G} mice.

(A) qPCR profiling of genes encoding the cannabinoid receptors (CBRs) CB1 (cnr1) and CB2 (cnr2) in different brain regions of male and female CB1^{E134G} mice, expressed as fold change (FC) from CB1^{wt} mice. The fold-change value for each gene is indicated by the color scale, with red indicating a value >1 and blue indicating a value <1. (B) Detailed FC values of cnr1. (C) Representative Western blots of CB1 and α-tubulin proteins in different brain regions of CB1^{wt} and CB1^{E134G} mice. (D-E) CB1 protein expression levels in various brain regions of male and female mice, respectively, expressed as a percentage of the CB1/α-tubulin ratio of CB1^{wt} mice. α-Tubulin was used as a loading control. X-ray films were quantified by densitometry. Data are mean (A) and mean ± SEM (B, D, E) (n=16 animals per group for qPCR analysis and n=3-7 for Western blots). *p<0.05, **p<0.01, and ***p<0.001 for 2-group comparison by Student's t-test or non-parametric Mann-Whitney test (B, D, E).

Abbreviations: PFC, prefrontal cortex; NAc, nucleus accumbens; CPu, caudate putamen (dorsal striatum); HTHAL, hypothalamus; HPC, hippocampus; CB, cerebellum; VMB, ventral midbrain.

1.2.2. Endocannabinoid and steroid levels are not affected in CB1^{E134G} mice

One of the key interests underlying the development of the CB1^{E134G} mouse line is to preserve endogenous cannabinoid and steroid signaling to study the role of the PREG-CB1 negative feedback loop in contrast to traditional pharmacological approaches. Therefore, we sought to investigate whether the CB1^{E134G} point mutation has any off-target effects on endocannabinoid and steroid metabolism. In this line, we performed RT-qPCR analyses in the brain of male and female CB1^{E134G} mice to investigate the expression of genes encoding enzymes responsible for the synthesis and degradation of the two major eCBs, AEA and 2-AG

Results

([Figure 14A-B](#)). No major differences in gene expression were observed between CB1^{E134G} and their WT littermates ([Figure 14B](#)).

In addition, we performed spectral analysis to quantify brain and plasma levels of the two major endocannabinoids, AEA and 2-AG, in CB1^{E34G} and CB1^{WT} mice. AEA levels were slightly elevated in the NAc and plasma of male CB1^{E34G} mice compared to WT controls, but not in other brain regions analyzed ([Figure 14C](#)). 2-AG levels were unaffected in the brain and plasma of male CB1^{E134G} mice compared to WT littermates ([Figure 14D](#)), and both AEA and 2-AG levels were similar in the brain and plasma of mutant and WT female mice ([Figure 14C-D](#)).

Consistent with the analysis of the eCB profile of CB1^{E134G} mice, we then tested whether the mice differ in basal steroidogenesis. To this end, we performed RT-qPCR analysis in the brain of male and female CB1^{E134G} and CB1^{WT} mice to examine the expression of genes encoding enzymes that are critically involved in the formation of steroids ([Figure 15A](#)). No major differences in gene expression were observed between CB1^{E134G} and their WT littermates ([Figure 15B](#)). In addition, spectral analysis was used to quantify brain ([Figure 15C-J](#)) and plasma ([Figure 16](#)) levels of PREG and some of its closest related metabolites: dehydroepiandrosterone (DHEA), 17-hydroxy-progesterone (17OHPROG), and allopregnanolone (ALLOP). PREG levels were slightly upregulated in the hypothalamus of male CB1^{E134G} mice compared to their WT control ([Figure 15C](#)). No other major changes were detected for PREG levels in other brain areas and for DHEA, 17OHPROG, and ALLOP ([Figure 15C-F](#)). In females, no changes in PREG, DHEA, and 17OHPROG levels were observed ([Figure 15G-J](#)). However, we observed a trend for increased elevations of ALLOP levels in virtually all brain regions analyzed, which were significant in the PFC and NAc ([Figure 15J](#)). Plasma steroid levels were globally unaffected by the CB1^{E134G} mutation in both male and female mice ([Figure 16](#)).

Overall, the RT-qPCR analyses coupled with the detection of eCB and steroid levels in mouse brain and plasma suggest that the mutation has virtually no major effect on these metabolites. Also, the significant variations observed in discrete brain areas of mutant mice compared to their WT littermates may be due to batch effect and should be further investigated to confirm the reported variations.

Results

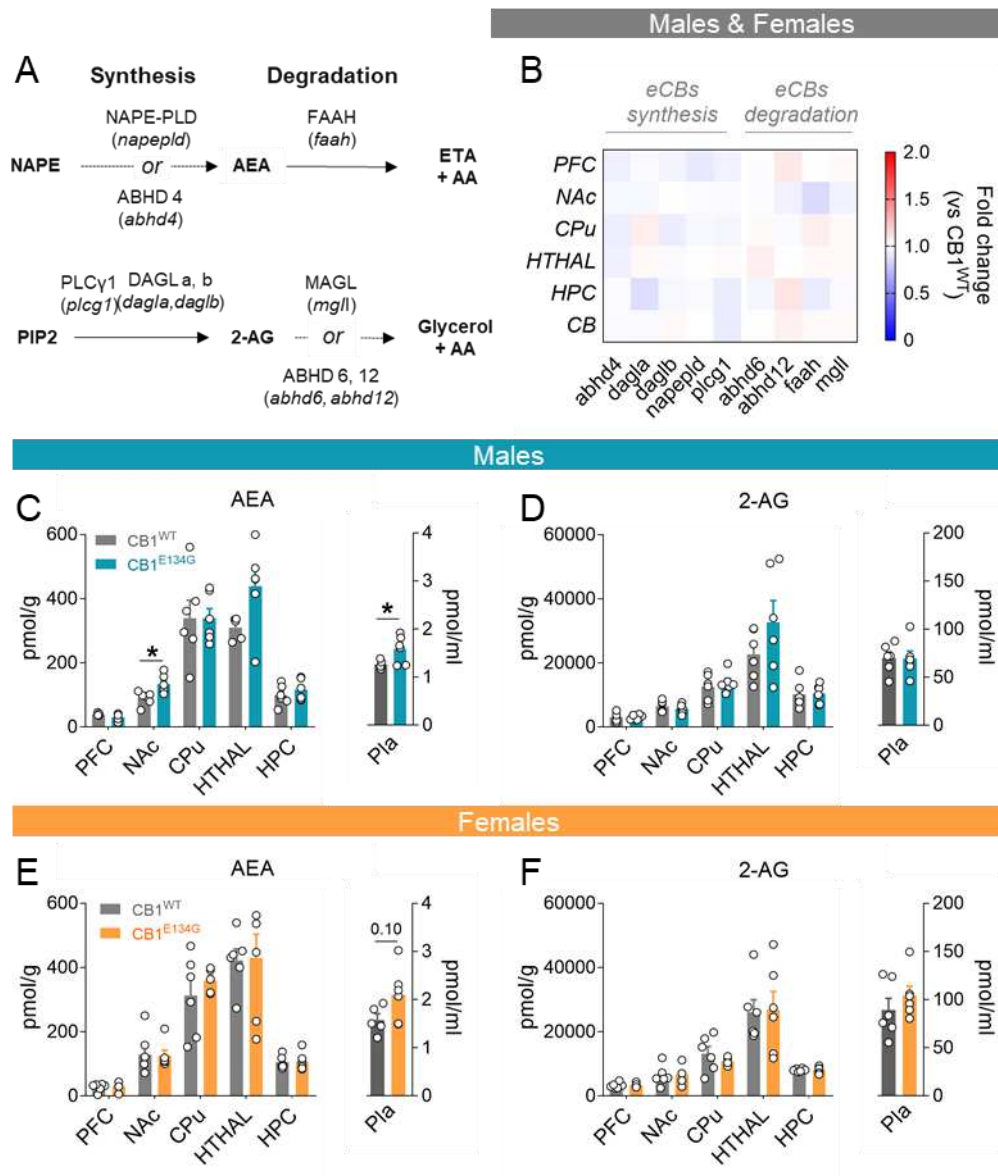


Figure 14: Quantification of eCBs in brain and plasma of CB1^{WT} and CB1^{E134G} mice.

(A) Diagram of the canonical pathway for endocannabinoid (eCB, anandamide, AEA and 2-arachidonoylglycerol, 2-AG) synthesis and degradation. (B) qPCR profiling of genes encoding eCB synthesis and degradation enzymes in different brain regions of male and female CB1^{E134G} mice, expressed as fold change (FC) from CB1^{WT} mice. The fold-change value for each gene is indicated by the color scale, where red indicates a value >1 and blue indicates a value <1. (C) AEA and (D) 2-AG levels in different brain regions and plasma of male CB1^{WT} and CB1^{E134G} mice. (E) AEA and (F) 2-AG levels in different brain regions and plasma of female CB1^{WT} and CB1^{E134G} mice. Data are mean \pm SEM (n=5-6 animals per group). *p<0.05 for 2-group comparison by Student's t-test (C). Abbreviations: PFC, prefrontal cortex; NAc, nucleus accumbens; CPu, caudate putamen (dorsal striatum); HTHAL, hypothalamus; HPC, hippocampus; Pla, plasma; see text for genes encoding synthesis and degradation enzymes of AEA and 2-AG.

Results

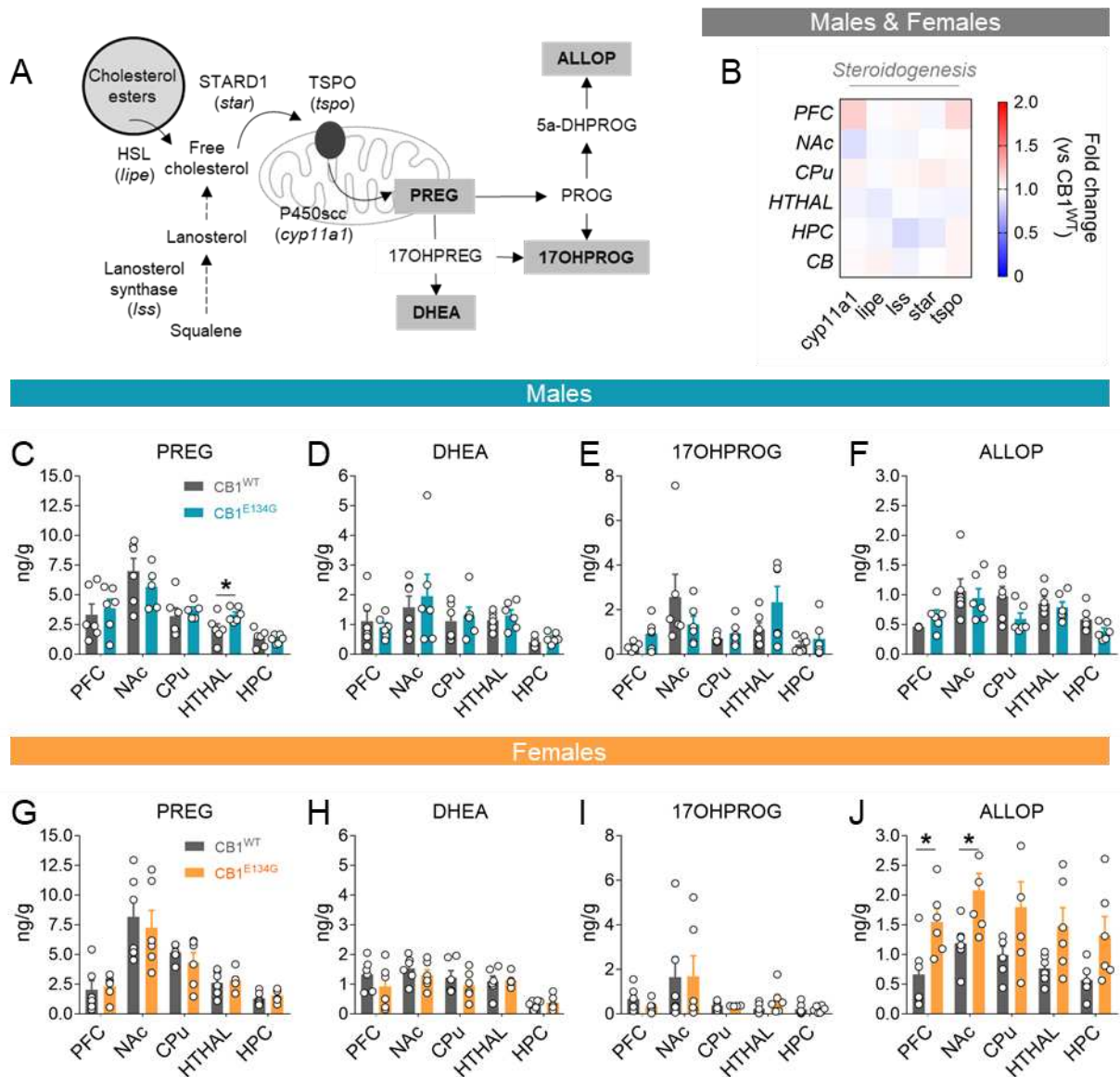


Figure 15: Quantification of neurosteroids in the brain of $CB1^{WT}$ and $CB1^{E134G}$ mice.

(A) Schematic of the canonical pathway for the synthesis of PREG and metabolites from cholesterol. Analyzed neurosteroids are highlighted in gray. **(B)** qPCR profiling of genes encoding enzymes involved in steroidogenesis in different brain regions of male and female $CB1^{E134G}$ mice, expressed as fold change (FC) from $CB1^{wt}$ mice. The fold-change value for each gene is indicated by the color scale, with red indicating a value >1 and blue indicating a value <1 . **(C-F)** Quantification of pregnenolone (PREG), dehydroepiandrosterone (DHEA), 17-hydroxy-progesterone (17OHPROG), and allopregnanolone (ALLOP), respectively, in different brain regions of male $CB1^{wt}$ and $CB1^{E134G}$ mice. **(G-J)** Quantification of PREG, DHEA, 17OHPROG, and ALLOP, respectively, in different brain areas of female $CB1^{wt}$ and $CB1^{E134G}$ mice. Data are mean \pm SEM ($n=4$ to 6 animals per group). * $p<0.05$ for 2-group comparison by Student's t-test or non-parametric Mann-Whitney test (**C, J**). **Abbreviations:** *Cyp11a1*: cytochrome P450_{scc} gene; *lipo*: hormone sensitive lipase gene; *lss*: lanosterol synthase gene; *star*: STARD1 gene, *tspo*: translocator protein gene; PFC, prefrontal cortex; NAc, nucleus accumbens; CPu, caudate putamen (dorsal striatum); HTHAL, hypothalamus; HPC, hippocampus.

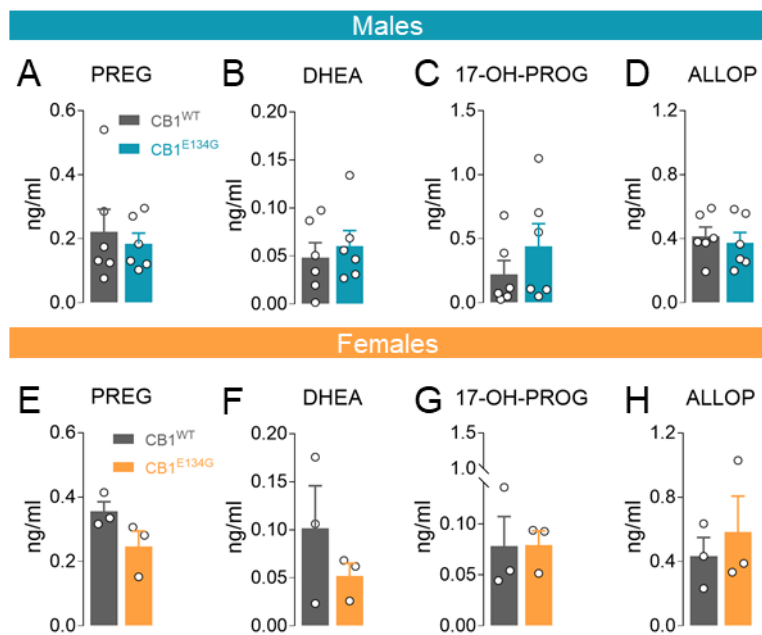


Figure 16: Quantification of steroids in the plasma of CB1^{WT} and CB1^{E134G} mice.

Quantification of pregnenolone (PREG), dehydroepiandrosterone (DHEA), 17-hydroxyprogesterone (17OHPROG) and allopregnanolone (ALLOP), respectively, in the plasma of male (A-D) and female (E-H) CB1^{WT} and CB1^{E134G} mice. Data are mean \pm SEM (n=3 to 6 animals per group).

1.2.3. CB1^{E134G} mice have unaltered memory at baseline

To investigate whether the CB1^{E134G} point mutation bears any functional effect on memory performance, male and female mice were assessed for episodic-like recognition memory, working-like memory, and spatial reference memory ([Figure 17](#)). Recognition memory was assessed using the L-shaped maze version of the novel object recognition (NOR) task ([Da Cruz et al. 2020](#)). Memory performance was expressed as a recognition index that, if above or below 0, indicates increased exploration time with the novel and familiar objects, respectively, while near 0 reflects similar exploration time within the two objects. Total exploration time during retrieval was used to control for proper object exploration. During the acquisition phase, male and female mice of both genotypes showed the same exploration time between the two identical objects, thus controlling for place preference (data not shown). In the retrieval phase, all groups were successful in discriminating the novel object during the retrieval phase of the NOR procedure, as indicated by a discrimination index significantly greater than 0. CB1^{E134G} and their WT littermates performed similarly in the task regardless of sex, and the total amount of time spent exploring the objects was unaffected in the mutants ([Figure 17A-D](#)).

Results

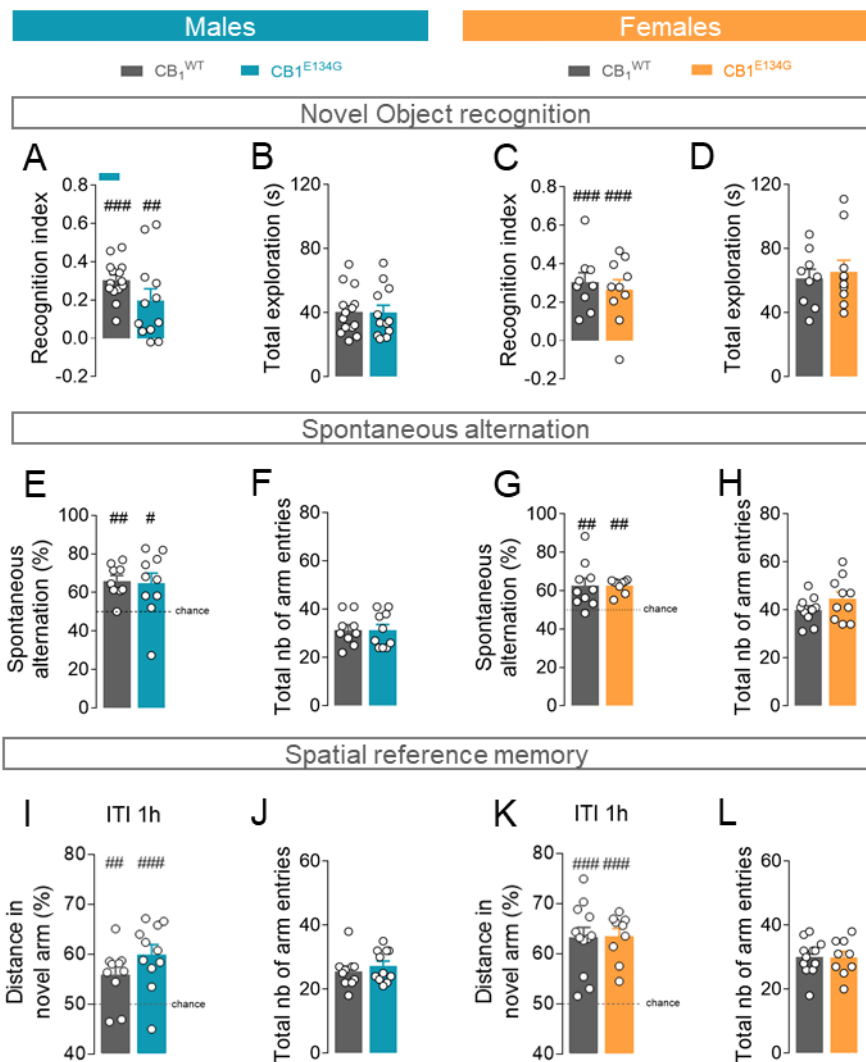


Figure 17: Memory performance of CB1^{WT} and CB1^{E134G} mice.

(A) Recognition memory assessed by the novel object recognition test, expressed as a recognition index and (B) total object exploration control expressed in seconds in male CB1^{wt} and CB1^{E134G} mice. (C-D) Recognition index and total object exploration control, in female CB1^{wt} and CB1^{E134G} mice, respectively. (E) Working-like memory as assessed by the Y-maze test, expressed as a percentage of spontaneous alternations and (F) total number of arm entries in male CB1^{wt} and CB1^{E134G} mice. (G-H) Spontaneous alternation and total number of arm entries, in female CB1^{wt} and CB1^{E134G} mice, respectively. (I) Spatial reference memory assessed in a modified version of the Y-maze test with a 1-hour inter-trial interval (ITI) between training and test sessions, expressed as the distance ratio between the novel and the familiar arm and (J) total number of arm entries in male CB1^{wt} and CB1^{E134G} mice. (K-L) Distance ratio and total number of arm entries, in female CB1^{wt} and CB1^{E134G} mice respectively. Data represent mean \pm SEM (n=9 to 14 animals per group). # p <0.05 ## p <0.01 and ### p <0.001 for group comparison at chance level by one-sample t-test (A, C, E, G, I, K).

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We also tested the mice for working-like memory and spatial reference memory in a Y-maze apparatus. Working-like memory was assessed by measuring the percentage of spontaneous alternations between the three arms of the mice, which, if greater than 50%, indicates normal memory performance. The total number of arm entries during the test session was used to control for locomotion. CB1^{E134G} and their WT littermates showed similar working memory performance as indicated by spontaneous alternation rates above chance and locomotion independent of sex ([Figure 17E-H](#)). Finally, we assessed mice for spatial reference memory in a modified version of the Y-maze (forced alternation) by measuring the percentage of running distance within the novel arm, with a 1-hour inter-trial interval between the acquisition and retrieval sessions. Normal spatial reference memory was defined when the percentage of distance within the novel arm was greater than 50%. The total number of arm entries during the test session was also used to control for locomotion. Again, CB1^{E134G} and their WT littermates of both sexes showed similar memory performance and locomotion ([Figure 17I-L](#)). Thus, the CB1^{E134G} point mutation has no functional effect on memory performance in mice at baseline.

1.2.4. CB1^{E134G} mice have unaltered emotional behaviors

To investigate whether the CB1^{E134G} point mutation has a functional effect on emotional behaviors, male and female mice were tested in a classical behavioral paradigm to measure anxiety- and depression-related behaviors ([Figure 18](#)). The elevated plus maze (EPM), elevated zero maze (EZM), and open field (OF) are based on the natural tendency of rodents to avoid open spaces and were used as a general index of anxiety by measuring the time spent in the open arms and areas of the EPM and EZM, respectively, and the time spent in the central area of the OF. Total number of arm entries for the EPM and total running distance for the EZM and OF were used to control for locomotion. For both males and females, CB1^{E134G} and their WT littermates spent a similar amount of time in the open arms of the EPM, and the total number of arm entries was also similar between genotypes ([Figure 18A-D](#)), although there was a trend toward an increased number of visits in mutant females ([Figure 18D](#)). In all groups, the time spent in open arms ranged from 5% to 10% of the total arm explorations under low aversive test conditions, indicating relatively high anxiety-like states in mice regardless of genotype and sex. Similar to the EPM, time spent in anxiogenic areas and total running distance in the EZM ([Figure 18E-H](#)) and OF ([Figure 18I-L](#)) tests were similar between mutants and their WT controls in both males and females.

Results

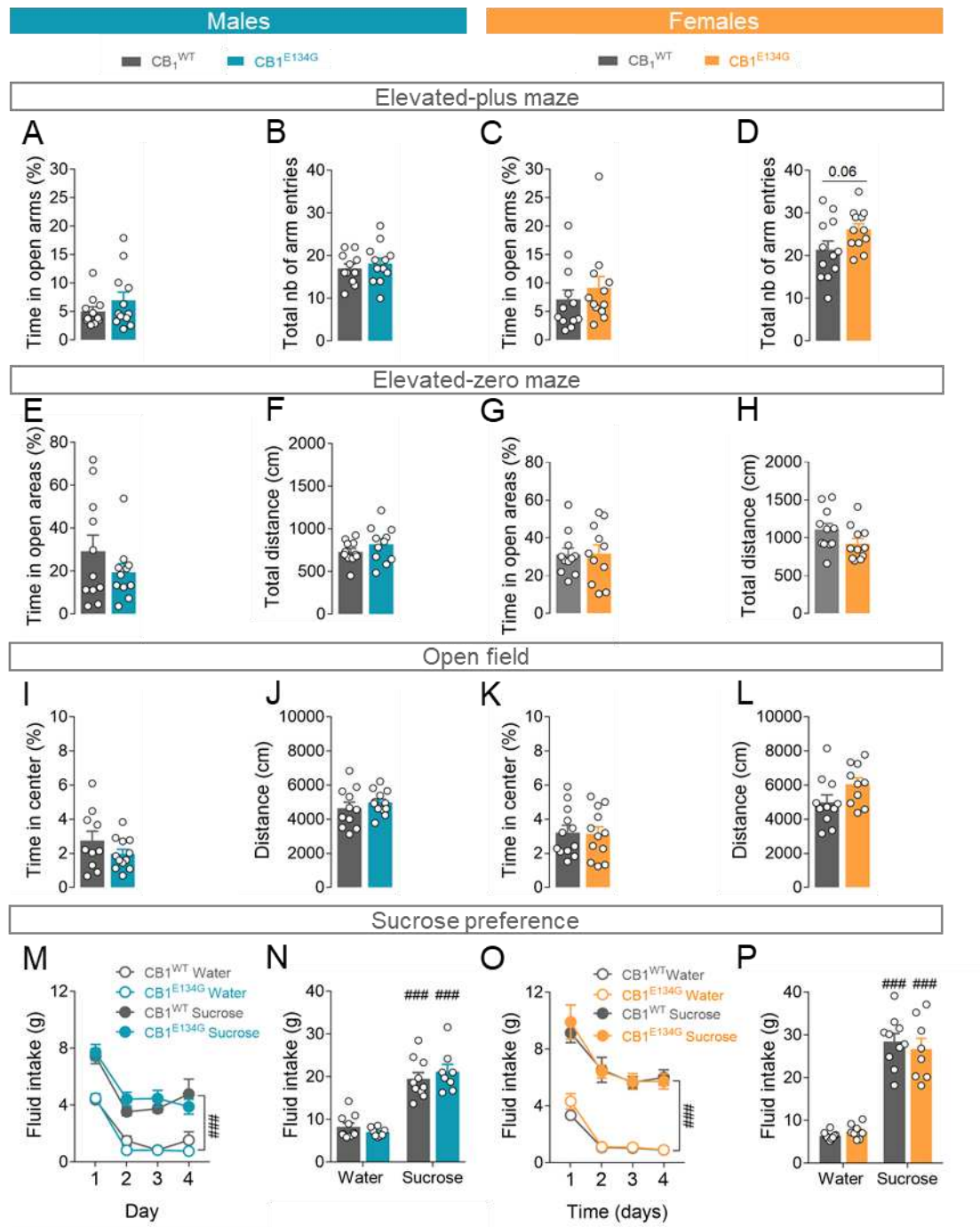


Figure 18: Emotional behavior in CB₁^{WT} and CB₁^{E134G} mice.

(A-B) Percentage of time spent in the open arms of an elevated-plus maze and total number of arm entries in the maze, respectively, in male and (C-D) female CB₁^{WT} and CB₁^{E134G} mice. (E-F) Percentage of time spent in the open areas of an elevated-zero maze and total running distance in cm, respectively, in male and (G-H) female CB₁^{WT} and CB₁^{E134G} mice. (I-J) Percentage of time spent in the central area of an open field and total running distance in cm, respectively, in male and (K-L) female CB₁^{WT} and CB₁^{E134G} mice. (M-N) Daily water and sucrose intake in g and total intake in g in male and (O-P) female CB₁^{WT} and CB₁^{E134G} mice. Data represent mean ± SEM (n=8 to 12 animals per group). ###p<0.001 for main water vs sucrose intake in 3-way ANOVA (M and O) and by Sidak's multiple comparisons post-hoc test (N and P).

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We also assessed sucrose preference in mice as an index of depressive-like behaviors associated with anhedonia in a two-bottle choice paradigm ([Figure 18M-P](#)). Mice were given the choice between water and a 2% sucrose solution *ad libitum* for 4 consecutive days. The position of the two bottles was rotated daily to avoid place preference in fluid intake. All mice showed a preference for sucrose over water, as shown by the measurement of daily and total fluid intake ([Figure 18M-P](#)). CB1^{E134G} mice consumed the same amount of sucrose as their WT littermates. Taken together, these data indicate that the CB1^{E134G} point mutation has no functional effect on emotional behavior in mice at baseline.

1.3. Ongoing experiments

1.3.1. Activity of the mutant CB1^{E134G} receptor

To fully characterize the effect of the CB1^{E134G} point mutation on receptor activity and function, ongoing analyses are aimed at deciphering the pharmacological properties of the mutant receptor compared to the WT receptor. Our data clearly show a drastic reduction of CB1 receptor expression *in vivo*, in virtually all brain regions of mutant mice. Spectral analyses show that this downregulation is not the mere consequence of increased eCB signaling via the central elevation of AEA and 2-AG levels in the brain of mutant mice compared to their WT littermates. Since the CB1^{E134G} point mutation prevents PREG from binding to the CB1 receptor, and since PREG downregulates CB1 receptor activity, we hypothesize that this loss of regulation results in a long-lasting downregulation of receptor levels. In this line, the downregulation of CB1 receptor levels in mutants would represent a form of compensation to maintain normal CB1-mediated signaling, which is further consistent with the lack of effect of phenotypic changes in mice under basal conditions. To support this hypothesis, we are collaborating with the biotechnology company IMGpharma (Zamudio, Spain) to investigate whether the mutation causes alterations in CB1 agonist binding and agonist-induced G protein coupling, by radio ligand binding and [35S] GTPγS assays, as previously described for CB1 in WT mice ([Soria-Gomez et al. 2021](#)). Since the CB1^{E134G} point mutation is not located in the orthosteric ligand and G-protein recognition domains, these parameters should not be affected. This would further confirm the absence of an intrinsic effect of the mutation on receptor activity and emphasize that the observed downregulation of CB1 levels *in vivo* is associated with the lack of PREG binding itself ([see DISCUSSION](#)).

1.3.2. Effect of the endogenous PREG-CB1 loop on subcellular signaling

The development of the CB1^{E134G} mouse line provides an important opportunity to investigate the molecular mechanisms underlying the PREG-CB1 regulatory loop at the signaling level. To this end, we have developed a primary cortical cell culture model derived from the CB1^{E134G} mouse line ([Figure 19](#)). This model has the advantage of allowing the optimization of high-throughput assays for semi-quantitative detection of phosphoproteins by AlphaLisa ([Zanese et al. 2020](#)) and forskoline-stimulated cAMP levels ([Yoon et al. 2020](#)) in particular to study the allosteric properties of PREG and to characterize the CB1^{E134G} mutant receptor at the functional level. The development of this *in vitro* model consisted first of optimizing the culture protocol in cells derived from the cortex of E17.5 WT embryos by determining an optimal dose of cytosine arabinoside (AraC) to deplete non-neuronal dividing cells, thus enriching wells in the post-mitotic fraction to extract neuronal signaling and avoid confounding signaling events. Immunocytochemical analysis revealed that AraC at a dose of 500nM significantly reduced the number of non-neuronal cells without affecting the total number of neurons, thus enriching the neuronal fraction within the wells ([Figure 19A-B](#)). Representative images of the cellular mat in the absence and presence of AraC 500nM are shown ([Figure 19C-D](#)). Interestingly, both mixed and neuronal cultures recapitulated the downregulation of total CB1 protein expression in cultures derived from E17.5 mutant embryos compared to WT controls ([Figure 19E-F](#)). This suggests that the downregulation of CB1 protein is a conserved process between *in vivo* and *in vitro* conditions, which may allow us to extrapolate results obtained from primary cortical neurons to CB1^{E134G} mice. Furthermore, this model will be useful not only to study not only the effect of endogenous PREG on receptor signaling, but also on the putative mechanisms leading to CB1 protein downregulation in mutants ([See DISCUSSION](#)).

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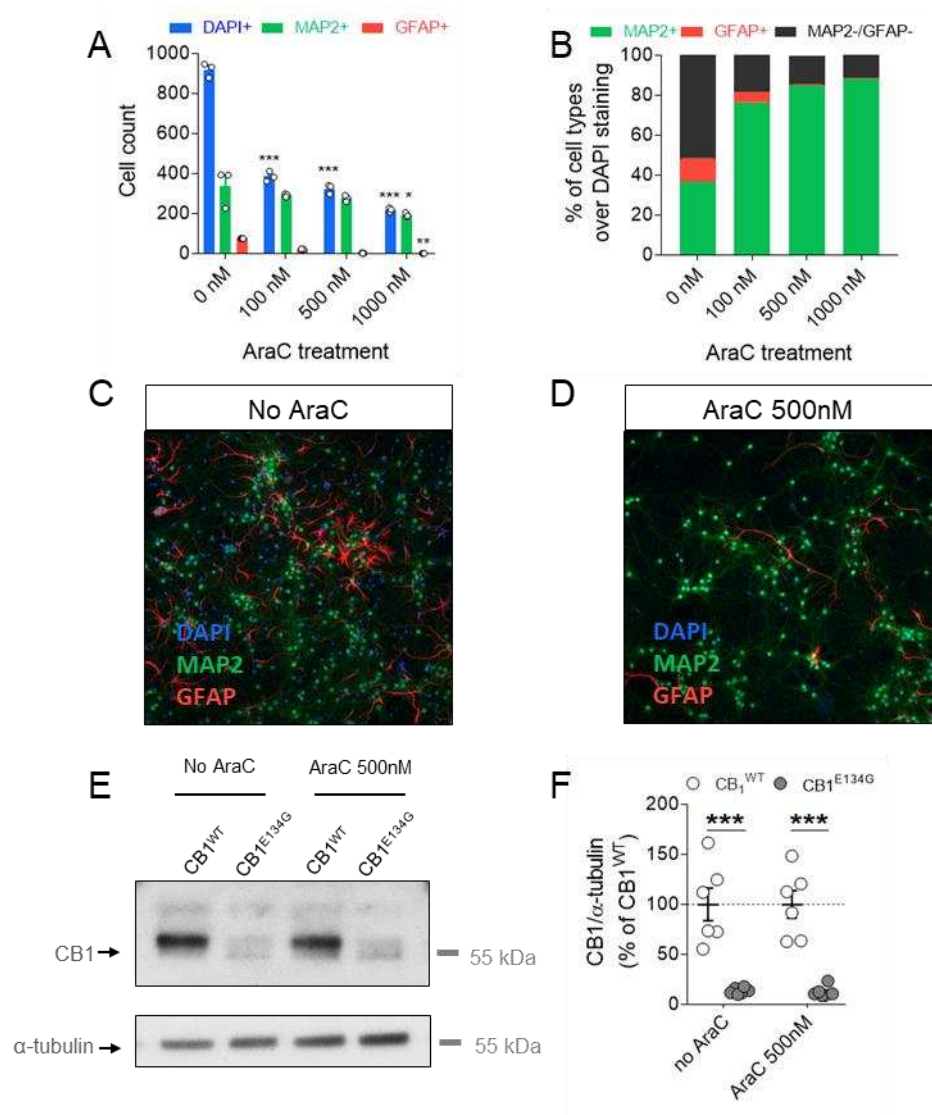


Figure 19: Development of a primary cortical cell culture model derived from the CB1^{E134G} mouse line.

(A) Total number of DAPI+, neuronal marker MAP2+ and astrocytic marker GFAP+ cells in primary cortical cultures derived from CB1^{wt} mice, cultured in the absence or presence of cytosine arabinoside (AraC) (100 to 1000nM). **(B)** Proportion of MAP2+, GFAP+ and MAP2/GFAP- cells, in primary cortical cultures derived from CB1^{wt} mice cultured in the absence or presence of AraC (100 to 1000nM), expressed as a percentage of cells over DAPI staining. **(C-D)** Representative Immunostaining for DAPI (blue), MAP2 (green), and GFAP (red) in primary cortical cultures derived from CB1^{wt} mice, cultured in the absence or presence of AraC 500nM. **(E)** Representative Western blots of CB1 and α -tubulin proteins in primary cortical cultures derived from CB1^{wt} and CB1^{E134G} mice cultured in the absence or presence of AraC 500nM. **(F)** CB1 protein expression levels in primary cortical cultures derived from CB1^{wt} and CB1^{E134G} mice cultured in the absence or presence of AraC 500nM, expressed as a percentage of the CB1/ α -tubulin ratio of CB1^{wt} mice in each AraC condition. α -Tubulin was used as a loading control. X-ray films were quantified by densitometry. Data are \pm SEM (n=3-6 culture wells per condition). *p<0.05, **p<0.01, ***p<0.001 for parametric Fisher's or non-parametric Dunn's post hoc comparisons with 0nM AraC **(A)** and genotype comparison by Tukey's multiple comparison post hoc test **(F)**.

1.4. Conclusion

In this first aim, we used molecular and behavioral assays to characterize the effect of the CB1^{E134G} mutation in male and female mice at baseline, to gain insight into the endogenous function of the PREG-CB1 loop. We report an unexpected and strong downregulation of CB1 at the protein but not the mRNA level, suggesting that the CB1^{E134G} point mutation affects receptor dynamics, leading to post-translational modulation of CB1 levels. However, the observed downregulation of CB1 protein expression is not associated with alterations in eCB and (neuro) steroid metabolism, nor with functional alterations in memory and emotional behavior in mutants compared to their WT control. The reported decrease in CB1 protein levels is particularly striking. Indeed, the CB1^{E134G} point mutation, located in the first transmembrane region of the receptor, was previously selected by *in silico* modeling coupled with *in vitro* assays on the basis that it would not affect receptor function *per se* (Vallée et al. 2014). The mutation is not present in the ligand and G protein binding domains (Venkatakrisnan et al. 2013) or in the intracellular C-terminal domain, which is a hotspot for CB1 accessory protein interaction (Venkatakrisnan et al. 2013). Thus, it can be speculated that CB1 downregulation is the direct consequence of the loss of PREG binding to its allosteric site within the TM1/TM7/hx8 region, thus affecting receptor structure and activity and leading to possible compensation. While further information on receptor activity will be provided by the ongoing functional binding and cellular signaling assays from *ex vivo* and *in vitro* samples, respectively, we investigated whether CB1^{E134G} leads to increased cannabinoid efficacy *in vivo* to further validate the loss of PREG function at CB1^{E134G} mutant receptors.

2. Investigation of the effect of exogenous cannabinoid administration in CB1^{E134G} mice

2.1. Rationale

The identification of the negative feedback loop linking PREG to CB1 receptors is based on several lines of pharmacological evidence. First, it has been shown that acute and systemic administration of THC in rodents increases brain production of PREG via classical steroidogenic pathways and in a CB1-dependent manner (Vallée et al. 2014). This suggests that PREG levels are dynamically regulated by CB1 hyperactivation states. Second, pharmacological inhibition of P450_{scc} activity by aminoglutethimide (AMG) as a means to block PREG synthesis exacerbated the behavioral and somatic effects of THC as assessed by the cannabinoid tetrad procedure (Vallée et al. 2014; Metna-Laurent et al. 2017). Thus, the CB1-dependent increase in PREG production exerts neuroprotection against the toxic effects of cannabinoids (Vallée et al. 2014). Finally, exogenous administration of PREG is able to counteract most of the known effects of exogenous cannabinoids (Vallée et al. 2014; A.

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[Busquets-Garcia et al. 2017](#); [Frau et al. 2019](#)). Since PREG mediates its effects at CB1 receptors through its TM1/TM7/hx8 allosteric binding site and requires interaction with the E1.49 residue at position 134 in rodent CB1 receptors, we hypothesized that the CB1^{E134G} mutation would result in increased sensitivity to exogenous cannabinoids.

Studying the effects of exogenous cannabinoids in the CB1^{E134G} mouse model serves two purposes. First, the enhanced effects of cannabinoids in CB1^{E134G} mice compared to their WT littermates should validate the face validity of our model, i.e., by supporting the absence of PREG regulation of CB1 hyperactivation (since in WT mice the observed outcome results from both the effect of exogenous cannabinoids and the regulatory action of PREG, whose levels are increased). Second, it may serve to describe a translational model to study the consequences of cannabis-like intoxication in a model with a greater predisposition to develop cannabis use disorders. In addition, studying the effects of exogenous cannabinoids may help unravel the PREG-CB1 regulatory loop at the circuit and subcellular levels by distinguishing effects that rely on cannabinoid-mediated activation of specific pools of CB1 receptors ([Arnau Busquets-Garcia et al. 2015](#)).

2.2. Results

2.2.1. THC produces dichotomous cataleptic/analgesic effects in CB1^{E134G} mice

To validate the loss of PREG binding to CB1 receptors in CB1^{E134G} mice and as a means to study the PREG-CB1 loop, we investigated the susceptibility of CB1^{E134G} mice to cannabinoids at the behavioral and somatic levels using the well-characterized cannabinoid tetrad model. This model is considered a prototypical signature of cannabinoid intoxication, consisting of the sequential analysis of hypolocomotion, hypothermia, catalepsy, and analgesia induced by the acute and systemic administration of THC ([Metna-Laurent et al. 2017](#)). In particular, it has been previously shown that blockade of endogenous PREG synthesis by the P450scc enzyme inhibitor, aminoglutethimide (AMG) enhances the THC-induced tetrad and that this effect is reversed by PREG administration, suggesting that PREG is involved in a negative feedback loop regulating the hyperactivation of CB1 receptors by exogenous cannabinoids ([Vallée et al. 2014](#)). Therefore, we examined the effects of intraperitoneal THC administration at doses ranging from low (1.5 mg/kg) to high (10 mg/kg; typically used to induce the tetrad response) in male and female CB1^{E134G} mice compared to their WT littermates ([Figure 20](#)).

We found that male CB1^{E134G} mice were more sensitive to THC-induced hypolocomotion, hypothermia, and catalepsy ([Figure 20A-C](#)) compared to their WT littermates. Catalepsy was the most affected parameter, as evidenced by a drastic increase in

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latency to move in the catalepsy bar test at 1.5 mg/kg in mutants, whereas this dose produced limited effects in WT mice ([Figure 20G](#)). Surprisingly, THC-induced analgesia was unaffected in mutants compared to WT mice ([Figure 20D](#)), even at the higher dose of 15 mg/kg (data not shown). Similar observations were made in female mice ([Figure 20E-H](#)), with increased sensitivity to the behavioral and somatic effects of THC in mutants compared to WT mice and, again, a robust dichotomous effect between catalepsy and analgesia ([Figure 20G-H](#)).

In addition, we examined the brain production of PREG and downstream metabolites following acute and systemic administration of THC at doses ranging from 1.5 mg/kg to 15 mg/kg ([Figure 21A](#)). Male CB1^{E134G} mice and WT controls showed a similar increase in PREG levels of approximately 57% maximum following THC administration ([Figure 21A](#)), while downstream metabolites were relatively unaffected by the treatment ([Figure 21B-D](#)). Similarly, in females, THC-induced PREG production was unaffected in mutants ([Figure 21E](#)), and downstream metabolite levels did not change following drug administration ([Figure 21F-H](#)), although reported DHEA brain levels were lower in mutant females compared to their WT littermates ([Figure 21F](#)). Strikingly, THC-induced PREG production was much higher in females than in males, as evidenced by an approximately 340% increase ([Figure 21E](#)).

Results

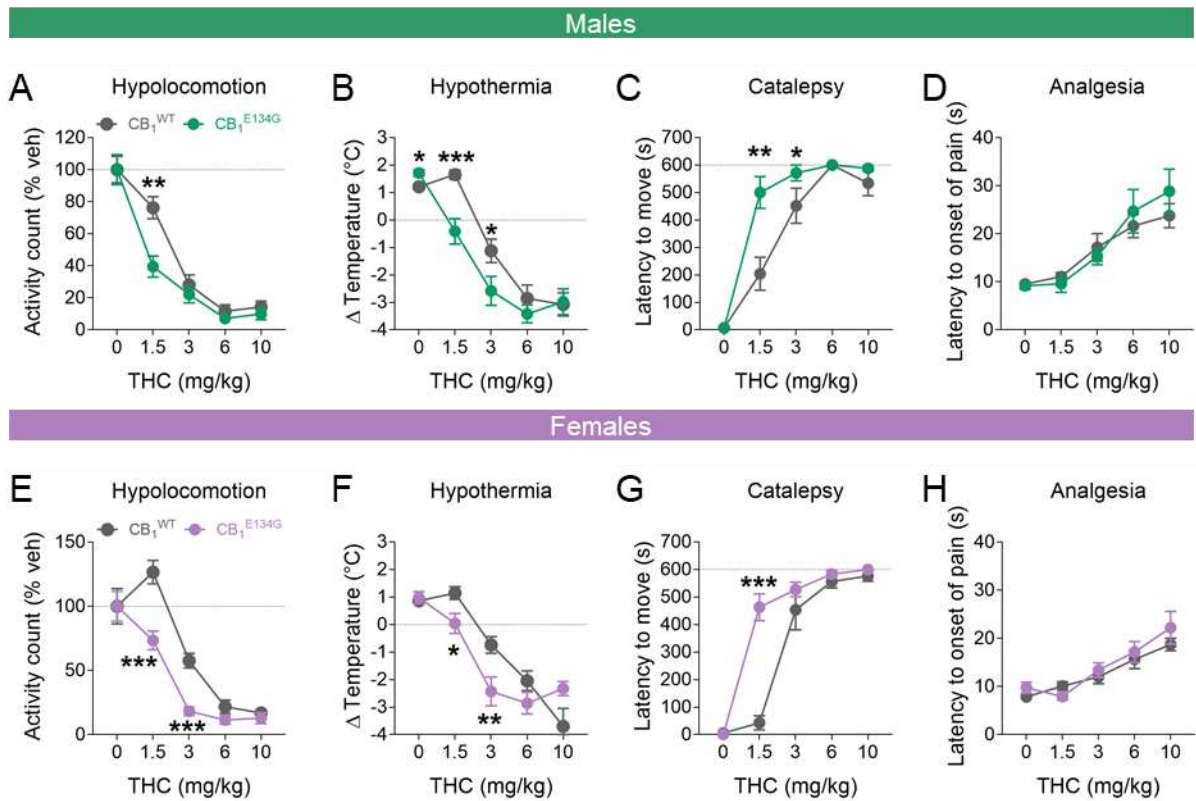


Figure 20: THC-induced tetrad in $CB1^{WT}$ and $CB1^{E134G}$ mice.

(A) Hypolocomotion (expressed as a percentage of the activity count of the vehicle-treated groups), (B) hypothermia (expressed as the delta between before and after drug administration), (C) catalepsy (measured as the latency to move with a threshold of 600 s) and (D) analgesia (measured as the latency to show first signs of pain with a threshold of 60 s) were assessed for doses of THC ranging from 1.5 to 10mg/kg or its vehicle administered by the intraperitoneal route in male mice. (E-F) Reciprocal analyses in female mice. Data represent mean \pm SEM ($n=8$ to 10 animals per group and dose). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ for 2-group comparison by Mann-Whitney's test (A, B, C, E, F, G).

Taken together, these data suggest that the increased sensitivity of mutant mice to THC at the behavioral and somatic levels is not due to a deficiency in central PREG production but rather to the absence of PREG binding to CB1 receptors, thus providing face validity to our model. It could also be hypothesized that the contribution of PREG to the endogenous regulation of CB1 receptor activity may differ significantly between males and females, as suggested by the apparent difference in CB1-dependent PREG increase. In addition, the lack of difference in THC-induced PREG increase between mutant and WT mice strongly suggests that the PREG-CB1 negative feedback loop does not directly contribute to the modulation of CB1-dependent PREG production. Most interestingly, these data also suggest that endogenous PREG differentially regulates THC-induced phenotypes (i.e., hypothermia and motor impairment versus antinociception).

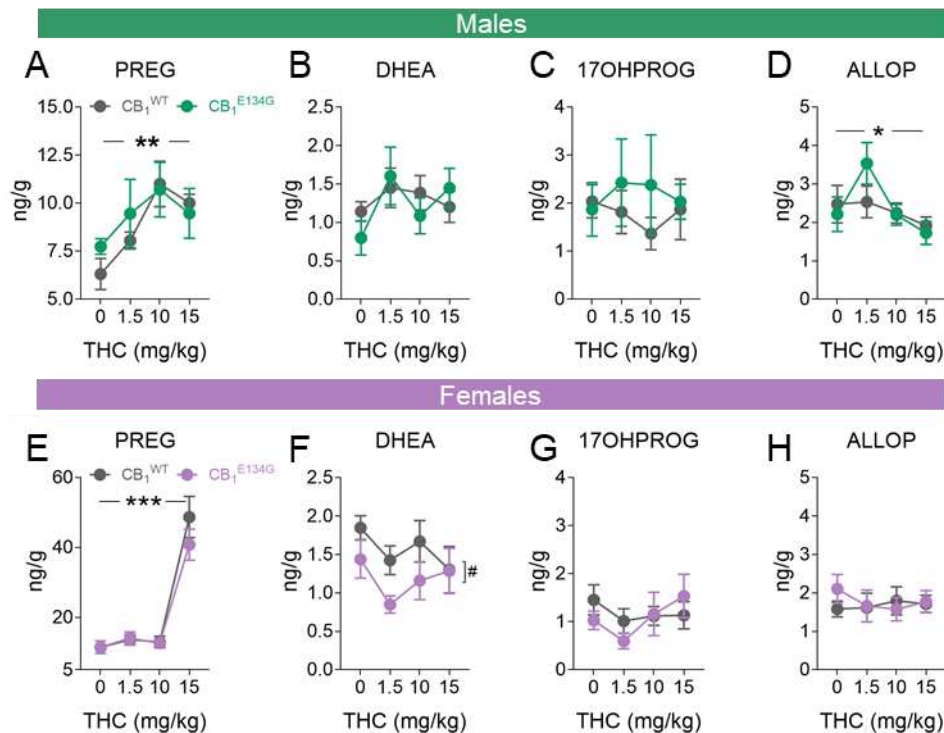


Figure 21: Quantification of neurosteroids in the brain of $CB1^{WT}$ and $CB1^{E134G}$ mice in response to acute THC administration.

(A-D) Quantification of pregnenolone (PREG), dehydroepiandrosterone (DHEA), 17-hydroxyprogesterone (17OHPROG) and allopregnanolone (ALLOP), respectively, in the dorsal striatum (CPu) of male and female (E-H) $CB1^{WT}$ and $CB1^{E134G}$ mice. (E-H). Data represent mean \pm SEM (n=4 to 6 animals per group). * $p < 0.05$ and ** $p < 0.01$ for main treatment effect (A, D, E) and # $p < 0.05$ for main genotype effect by 2-way ANOVA.

2.2.2. Endogenous PREG can regulate mtCB1 activity from SN homogenates

The present findings suggest that PREG may preferentially modulate the activity of specific receptor pools, as it has recently been reported that activation of CB1 receptors at different subcellular locations within the same neuronal circuit can mediate distinct behaviors. In particular, pharmacological activation of CB1 within striatonigral (SN) circuits elicits both the cataleptic and analgesic effects of THC via mitochondrial-associated CB1 receptors (mtCB1) and plasma membrane CB1 receptors (pmCB1), respectively (Soria-Gomez et al. 2021) (Figure 22A). Furthermore, it has been reported *in vitro* that exogenous PREG administration inhibits THC-induced decreased ADP-stimulated mitochondrial respiration (Vallée et al. 2014). Therefore, based on the reported dichotomous effects of THC on catalepsy and analgesia in $CB1^{E134G}$ mice, and because PREG is endogenously produced from classical steroidogenic pathways within mitochondria (Vallée 2016), we propose that endogenous PREG may

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preferentially regulate striatonigral mtCB1 activation over pmCB1 receptors. To explore this hypothesis, we investigated the PREG-CB1 interaction on the putative mechanism of mtCB1R-induced catalepsy (Soria-Gomez et al. 2021), namely by assessing *ex vivo* mitochondrial respiration in the substantia nigra (SN) ([Figure 22B](#)).

We investigated the effects of the synthetic CB1 agonist, WIN 55,212-2 (WIN) at the previously reported effective dose of 1 μ M (Soria-Gomez et al. 2021) and at the lower dose of 0.1 μ M, consistent with the increased susceptibility of CB1^{E134G} mice to cannabinoids. Oxygen consumption assays in SN homogenates showed that WIN 0.1 μ M decreased ADP-stimulated mitochondrial respiration in CB1^{E134G} mice but not in CB1^{WT} mice ([Figure 22C](#)). In contrast, WIN 1 μ M similarly decreased ADP-stimulated mitochondrial respiration in CB1^{E134G} and CB1^{WT} mice ([Figure 22C](#)). Notably, the effect of WIN 1 μ M was abolished by pre-treatment with exogenous PREG at 500nM in SN homogenates from CB1^{WT} but not CB1^{E134G} mice ([Figure 22D](#)).

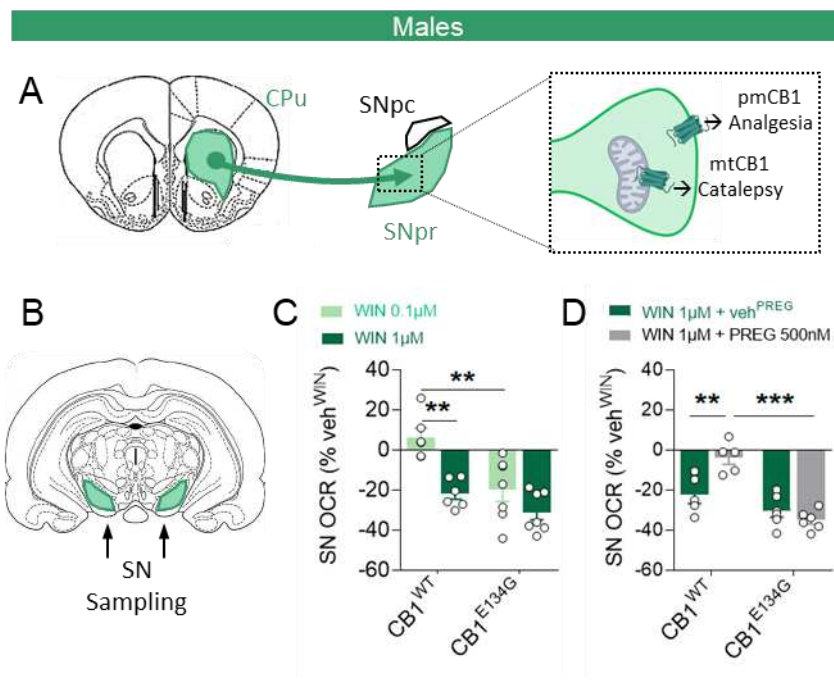


Figure 22: Mitochondrial respiration in substantia nigra extracts from CB1^{WT} and CB1^{E134G} mice.

(A) Schematic representation of striatonigral terminals mediating the analgesic and cataleptic effects of THC via plasma membrane (pmCB1) and mitochondria-associated CB1 (mtCB1) receptors, respectively. CPu, dorsal striatum; SNpc, substantia nigra pars compacta; SNPr, substantia nigra pars reticulata. (B) Anatomical representation of substantia nigra (SN) samples for mitochondrial respiration analysis. (C) WIN effect on mitochondrial respiration measured as oxygen consumption rate (OCR) expressed as a percentage of the vehicle-treated group in substantia nigra extracts from male CB1^{WT} and CB1^{E134G} mice. (D) Effect of PREG on WIN-induced decrease in mitochondrial respiration in substantia nigra extracts from male CB1^{WT} and CB1^{E134G} mice. ** $p < 0.01$ and $p < 0.001$ for multiple comparisons by Sidak's post hoc test (C and D).

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Collectively, these data strongly suggest that endogenous PREG mediates a regulatory effect on striatonigral mtCB1 receptors and further confirm the absence of PREG regulation of CB1 receptor activity in CB1^{E134G} mice. Together with the reported phenotypes of CB1^{E134G} mice on THC-induced tetrad, endogenous PREG may thus represent a molecular gatekeeper that biases cannabinoid action toward therapeutically relevant analgesia by inhibiting cataleptic-like side effects through the preferential regulation of subcellular pools of CB1 receptors.

2.2.3. mtCB1 receptors do not contribute to THC-induced PREG production

Acute and systemic administration of cannabinoids, including THC and WIN, induces an increase in brain production of PREG in rodents, thereby triggering the PREG-CB1 negative feedback loop (Vallée et al. 2014). Previously reported and preliminary data from our laboratory indicate that the cannabinoid-induced PREG increase is no longer observed in the NAc (Vallée et al. 2014) and striatum (unpublished data) of mutant D1R-CB1 KO mice, suggesting that CB1 receptor expression in D1R striatal neurons is required for local PREG production.

Given that endogenously produced PREG can regulate mtCB1 activation, we further investigated the subcellular location of cannabinoid PREG production in the striatonigral circuit. To this end, we took advantage of the development of the DN22-CB1 knock-in (KI) mutant mouse, in which the CB1 receptor gene is replaced by the coding sequence of the DN22-CB1 protein, which lacks the first 22 amino acid residues, resulting in the exclusion of CB1 receptors at the mitochondrial level (Hebert-Chatelain et al. 2016; Soria-Gomez et al. 2021). Specifically, DN22-CB1-KI do not exhibit THC-induced catalepsy (Soria-Gomez et al. 2021) and other major adverse effects associated with cannabinoids, including impairment of hippocampal-dependent memory consolidation (Hebert-Chatelain et al. 2016). Male DN22-CB1-KI and their WT littermates were systemically injected with a high dose of THC (12 mg/kg), and brain levels of PREG and most direct metabolites (DHEA, 17-OH-PROG, and ALLOP) were quantified by spectral analysis. THC induced a similar increase in striatal PREG levels in both WT and DN22-CB1-KI mice ([Figure 23A](#)), while levels of downstream PREG metabolites were not affected ([Figure 23B-D](#)).

This suggests that PREG production is not stimulated by activation of mtCB1 receptors, but is likely derived from plasma membrane receptor pools. Since endogenous PREG does not regulate its production after CB1 activation and preferentially regulates mtCB1 receptor activation over pmCB1 receptors in striatonigral neurons, it can be hypothesized that endogenous PREG levels are increased by pmCB1 receptor activation but selectively inhibit mtCB1 receptor activity in striatonigral circuits.

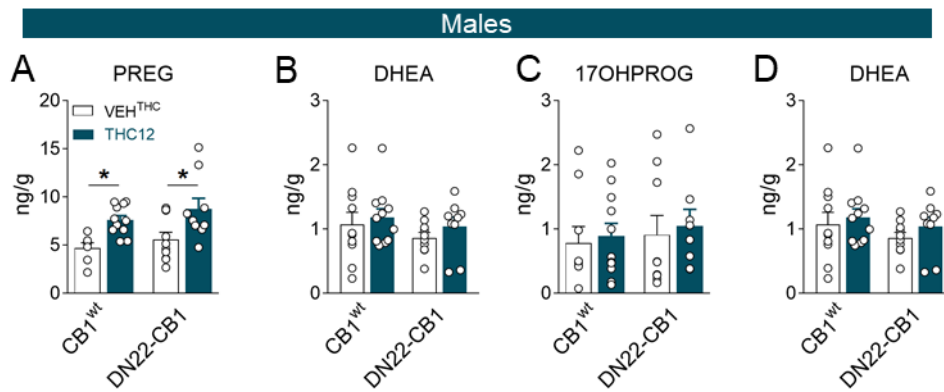


Figure 23: Quantification of neurosteroids in the brain of DN22-CB1-KI mice and their $CB1^{WT}$ littermates in response to acute THC administration.

Quantification of pregnenolone (PREG) (**A**), dehydroepiandrosterone (DHEA) (**B**), 17-hydroxyprogesterone (17OHPROG) (**C**) and allopregnanolone (ALLOP) (**D**), respectively, in the dorsal striatum (CPu) of male DN22-CB1-Ki (DN22-CB1) mice and their $CB1^{WT}$ littermates. Data represent mean \pm SEM (n=6 to 11 animals per group). * $p < 0.05$ for treatment comparisons by Sidak's multiple comparison post hoc test (**A**).

2.2.4. THC-induced mesoaccumbal DA transmission is increased in $CB1^{E134G}$ mice

Mesoaccumbal dopamine (DA) release has been implicated in mediating the reinforcing effects of drugs of abuse (Di Chiara and Imperato 1988), including cannabinoids. Furthermore, it has been previously reported that PREG inhibits THC-induced DA release in the rodent NAc by reducing the firing activity of ventral tegmental area (VTA) neurons and attenuates the reinforcing effects of CB1 agonists in a cannabinoid self-administration model (Vallée et al. 2014). Therefore, we investigated whether the $CB1^{E134G}$ mice susceptibility to cannabinoids would also translate into increased mesoaccumbal DA transmission. To this end, we assessed the susceptibility of male and female $CB1^{E134G}$ mice to THC by examining THC-induced DA outflow in the NAc (Figure 24). Analyses were performed in anesthetized mice using intracerebral microdialysis by implanting a probe in the right NAc (stereotaxic coordinates relative to the interaural point: AP: +5.1, L:-0.5, V: +0.8) (Figure 24A). We examined the effects of intraperitoneal THC administration at 1.5 mg/kg and 3 mg/kg, as these doses were effective in revealing the hypersensitivity of $CB1^{E134G}$ mice to THC in the tetrad model and in eliciting behavioral and somatic responses in WT controls. First, we observed that baseline DA levels were similar in the NAc of male $CB1^{E134G}$ and $CB1^{WT}$ mice (Figure 24B), indicating that disruption of PREG binding to CB1 receptors does not tonically affect basal mesoaccumbal dopamine transmission. As expected, THC administered at 1.5 mg/kg transiently increased DA levels in the NAc of male $CB1^{E134G}$ mice (Figure 24C) but not in WT controls. In contrast, THC administered at 3 mg/kg increased DA release in the NAc indistinguishably between male $CB1^{E134G}$ and WT mice (Figure 24D).

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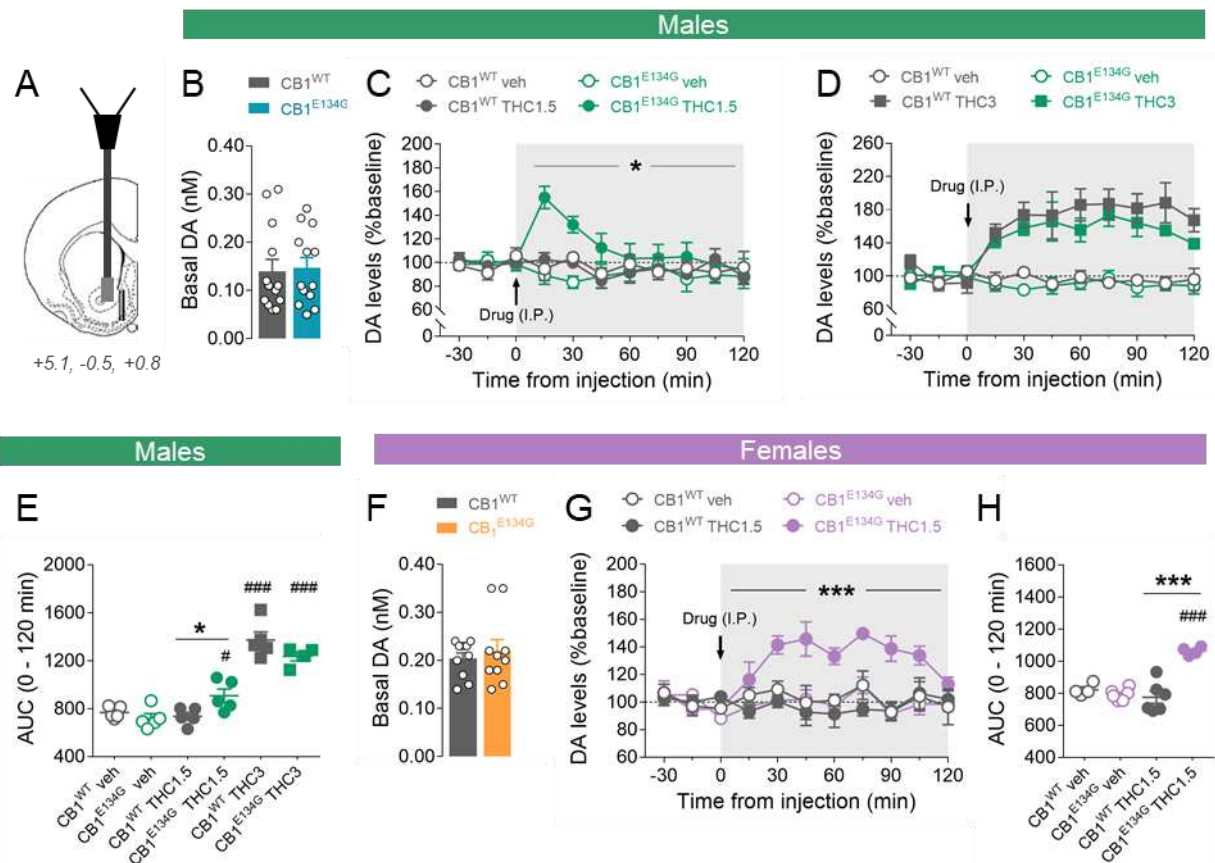


Figure 24: Neurochemical effects of THC on dopamine (DA) outflow in the nucleus accumbens (NAc) of CB1^{WT} and CB1^{E134G} mice.

(A) Anatomical localization of the microdialysis probe within the NAc. The indicated stereotaxic coordinates are AP, L, V (mm) from the interaural point. (B) Average DA levels measured in the NAc male mice at baseline, 30min before drug administration. (C) Time course (samples every 15 minutes) of the effect of THC at 1.5mg/kg (THC1.5), (D) 3mg/kg (THC3) or vehicle (veh) on DA outflow in the NAc of male mice expressed as a percentage of the baseline calculated from the three samples before drug administration by intraperitoneal (I.P) route. (E) Area under the curve (AUC) from the time point of drug injection to the end of sample collection (0-120 min) in male mice. (F) Average DA levels measured in female mice at baseline, 30min before drug administration. (G) Time course (samples every 15 minutes) of the effect of THC at 1.5mg/kg (THC1.5) or vehicle (veh) on DA outflow in the NAc of female mice expressed as a percentage of the baseline calculated from the three samples before drug administration by the I.P. route. (H) AUC from the time point of drug injection to the end of sample collection 0-120 min) in female mice. Data represent mean \pm SEM (n=4 to 6 animals per group). * $p < 0.05$, *** $p < 0.001$ for main effect of genotype x treatment x time in 3-way ANOVA (C and G) and genotype comparisons by Sidak's multiple comparison post hoc test (E and H). # $p < 0.05$ ### $p < 0.001$ for group comparisons with their respective vehicle by Sidak's multiple comparison post hoc test (E and H).

Similar to males, basal mesoaccumbal DA transmission was unaffected in female CB1^{E134G} mice compared to their WT controls (Figure 24F), and THC administered at 1.5 mg/kg significantly increased DA outflow only in CB1^{E134G} mice, but in a more prolonged

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manner compared to males ([Figure 24G](#)). Thus, these data suggest that CB1^{E134G} mice are more susceptible to the neurochemical effects of THC, which are associated with the reinforcing properties of the drug. Interestingly, the time course of THC effects on DA outflow was even more pronounced in females compared to males, suggesting that the relative contribution of endogenous PREG in modulating THC-induced mesoaccumbal DA is more pronounced in females. Therefore, it can be hypothesized that CB1^{E134G} mice may be more susceptible to reinforcing effects of cannabinoids in recently validated self-administration models ([Cajiao-Manrique, Maldonado, and Martín-García 2023](#)), with increased susceptibility in females compared to males

2.3. Ongoing experiments

Several experiments are underway to strengthen our current results and the validity of our model. To further confirm that the sensitivity of CB1^{E134G} mutant mice to cannabinoids is due to the absence of endogenous regulation of PREG at CB1 receptors, we aim to perform a tetrad experiment using the P450scc inhibitor AMG (50mg/kg) co-administered with THC (1.5mg/kg). Based on previous data ([Vallée et al. 2014](#)), we expect AMG to increase the susceptibility of WT mice to THC by blocking THC-induced PREG production. The 1.5 mg/kg dose of THC is particularly relevant because it is highly effective in mutants but not in WT, and AMG should therefore elicit comparable efficacy of THC in CB1^{WT} and CB1^{E134G} mice. In contrast, we expect AMG to have no effect in KI, as the lack of binding to the receptor should mask any effect on brain production of PREG.

In addition, to confirm the absence of PREG effects in SN extract from CB1^{E134G} mice on mitochondrial respiration, we aim to validate the loss of PREG effect *in vivo*. In a similar approach, we will administer PREG (6 mg/kg) together with THC (10 mg/kg) in the tetrad procedure. In this case scenario, the dose of 10 mg/kg THC is relevant because this dose induces the maximum tetrad effect that is indistinguishable between mutant and WT mice. Furthermore, based on published results ([Vallée et al. 2014](#); [A. Busquets-Garcia et al. 2017](#)), this dose of PREG should (at least partially) reverse the effects of THC in CB1^{WT} mice, but have no effect in CB1^{E134G} mutant mice.

Finally, additional intracerebral microdialysis coupled with DA assays will be performed to confirm that the reported results following THC administration and increased mesoaccumbal DA transmission are not due to mere changes in DA signaling, including recapture via the dopamine transporter (DAT) at the presynaptic level in DA neurons (in the NAc). To this end, we will administer cocaine (15 mg/kg) to anesthetized mice and measure mesoaccumbal DA transmission. Although the lack of change in DA levels in response to cocaine between mutant CB1^{E134G} mice and CB1^{WT} may indicate that DA signaling *per se* is unaltered by the mutation,

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some modifications could be observed. Indeed, CB1 receptors within GABAergic neurons have previously been shown to control cocaine-induced dopamine release in the nucleus accumbens (Martín-García et al. 2016). Therefore, changes in cocaine-induced dopamine release in CB1^{E134G} mice compared to CB1^{WT} controls may also reflect interactions between endogenous PREG production and the neuronal population of CB1 receptors associated with the reinforcing effects of cocaine.

2.4. Conclusion

In this second aim, we validated our hypothesis regarding the increased sensitivity of CB1^{E134G} mice to cannabinoids in the cannabinoid tetrad procedure and THC-induced mesoaccumbal DA release. In addition, we verified the lack of effect of PREG on the mutant receptor by *ex vivo* mitochondrial respiration analyses. Interestingly, we report that although CB1^{E134G} mice are generally more susceptible to the toxic effects of cannabinoids, the analgesic effect of THC is unaffected, suggesting that endogenous PREG-CB1 regulation affects specific subsets of effects associated with CB1 stimulation. In particular, these effects are highly dependent on the cellular and subcellular localization of the receptor (Arnau Busquets-García et al. 2015). This is true for the cataleptic motor side effects and analgesic effects of THC, which are mediated by plasma membrane and mitochondrial resident pools of the CB1 receptor, respectively, within D1 striatonigral circuits (Soria-Gomez et al. 2021). Because THC-induced analgesia and catalepsy are differentially affected in CB1^{E134G} mutant mice, and because endogenous PREG can regulate the molecular substrate responsible for THC-induced catalepsy (i.e., mitochondrial respiration), we propose that the endogenous PREG-CB1 loop preferentially regulates mtCB1 activity over pmCB1 activity, at least within striatonigral circuits (see [Figure 31](#)).

Finally, because THC-induced mesoaccumbal DA release is increased in CB1^{E134G} mice compared to their WT controls, we proposed that CB1^{E134G} would be more sensitive to the reinforcing effects of cannabinoids. Future experiments should address this issue using behavioral models of cannabis use disorder (A. Busquets-García et al. 2017; Cajiao-Manrique, Maldonado, and Martín-García 2023) and decipher whether CB1^{E134G} mice can be used to study the consequences of cannabis-like intoxication as a model with a greater predisposition to develop cannabis use disorder.

3. Investigation of the effect of endogenous CB1 stimulation in CB1^{E134G} mice: energy balance

3.1. Rationale

Since the cloning of the CB1 receptor and the subsequent description of the endocannabinoid system (ECS), research efforts have shed light on the intimate relationship of this system with energy metabolism. Indeed, it has been well described that exogenous cannabinoids can regulate food intake ([Bellocchio et al. 2010](#)) and that CB1 receptor activation is associated with potentiation of energy accumulation in the body thanks to the coordinated action of central and peripheral mechanisms ([Piazza, Cota, and Marsicano 2017](#); [Muguruza et al. 2019](#); [Gangarossa et al. 2023](#)). In addition, the association between ECS activity and metabolic disorders, including obesity, has long been recognized. In particular, the major eCBs, AEA and 2-AG, are upregulated in rodent models of obesity ([Vincenzo Di Marzo and Matias 2005](#)) and classical CB1 antagonists show a clear anti-obesity effect ([Quarta et al. 2010](#); [Christensen et al. 2007](#)). However, the use of such antagonists has been associated with serious drawbacks, including neuropsychiatric side effects ([Quarta and Cota 2020](#)).

Thus, new molecules with a safer profile of action that are known to regulate ECS activity, particularly CB1 receptors, may represent a promising alternative for the treatment of obesity and its associated comorbidities. This may be the case for PREG and its synthetic analog derivatives, which have been shown to regulate excessive CB1 activation ([Vallée et al. 2014](#); [A. Busquets-Garcia et al. 2017](#); [Haney et al. 2023](#)). Notably, while eCBs have been described in the literature as orexigenic compounds (i.e., promoting food intake) ([Gatta-Cherifi et al. 2012](#)), PREG has been associated with opposite anorexigenic effects (i.e., reducing food intake) ([Vallée et al. 2014](#)). In rodents, deficits in hypothalamic PREG production have been associated with cognitive impairment induced by an obesogenic diet ([Ramírez et al. 2022](#)), while cerebrospinal fluid PREG levels in metabolically unhealthy obese patients are inversely correlated with body mass index (BMI) and positively correlated with cognitive scores ([Ramírez et al. 2022](#)). In addition, plasma PREG levels have been shown to be inversely correlated with BMI and body fat percentage in humans ([Drummen et al. 2020](#)).

Therefore, we took advantage of the development of the CB1^{E134G} mouse line to investigate the putative effect of the endogenous PREG-CB1 regulatory loop in modulating energy balance and protecting against diet-induced obesity. To this end, we examined the energy balance phenotypes of male CB1^{E134G} mice and their WT littermates by performing analyses of metabolic parameters including body composition analysis, indirect calorimetry, and glucose metabolism on a standard chow diet. Additionally, we examined the effect of the CB1^{E134G} point mutation on the incentive drive for exercise and food-related pleasure by

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addressing the motivation for wheel-running and palatable food intake (Muguruza et al. 2019). We then subjected mice to metabolic challenges, including fasting-refeeding challenges and acute exposure to a high-fat diet (HFD), to further activate the ECS (Bellocchio et al. 2010; Kirkham et al. 2002; Kuipers et al. 2019; Lau et al. 2017), and finally examined metabolic changes in mice maintained on the HFD for 3 months.

3.2. Results

3.2.1. Food intake and energy homeostasis are not affected in $CB1^{E134G}$ mice on chow diet

We investigated the metabolic consequences of the $CB1^{E134G}$ point mutation in male mice maintained on a standard chow diet with 72.4% calories from carbohydrates, 19.3% from proteins and 8.4% from lipids. Prior to all subsequent analyses, the mice were systematically weighed and their body composition (fat and lean mass) was assessed *in vivo* using a quantitative magnetic resonance body composition method. Food and water intake, locomotor activity and peripheral substrate utilization were assessed by longitudinal indirect calorimetry. Finally, glucose metabolism was assessed by intraperitoneal glucose and insulin tolerance tests (Figure 25). No major differences in body weight or body composition were observed in $CB1^{E134G}$ mutant mice compared to their WT littermates (Figure 25A). However, it should be noted that we previously observed some batch effect on body composition with increased lean mass in mutants tested by indirect calorimetry, thus affecting basal metabolic rate and energy expenditure during longitudinal recording of mice in calorimetry chambers (see below). Time course assessment of food intake in calorimetry chambers revealed no genotype effect on food intake during either the active (dark) or inactive (light) periods (Figure 25B). Furthermore, detailed analyses of meal patterns during the nocturnal period revealed no changes in the number, size, or duration of meals (Figure 25C). Similar to food intake, water intake and locomotor activity were similar between mutants and their WT control during all periods of the light-dark cycle (Figure 25D). When considering peripheral substrate utilization using the respiratory exchange ratio (RER) (Gangarossa et al. 2023), all mice, regardless of genotype, preferentially utilized glucose substrates during the dark period ($RER \approx 1$) and lipids during the light period ($RER \approx 0.7$) (Figure 25F). As mentioned above, mutant mice tested by indirect calorimetry showed a batch-dependent increase in lean mass, leading to an overall increase in energy expenditure and basal metabolic rate. These differences were largely abolished after Ancova corrections for energy expenditure versus lean mass (data not shown).

In addition, mice were subjected to the glucose tolerance test (GTT) and insulin tolerance test (ITT), two metabolic assays that evaluate glucose tolerance and insulin sensitivity, respectively. Mice were fasted for 15 hours prior to intraperitoneal injection of

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glucose (2 mg/kg of lean mass) and their glycaemia was measured. Interestingly, CB1^{E134G} mice had lower blood glucose under fasted conditions compared to their WT control ([Figure 25G](#)). However, both mutant and WT mice showed similar tolerance to glucose, although a significant interaction effect was observed when expressing blood glucose as a percentage of baseline, with a non-significant trend for increased blood glucose levels at the peak, 15 minutes after injection ([Figure 25H](#)). In parallel with GTT, plasma insulin was quantified at baseline and 15 minutes after glucose administration. As expected, plasma insulin was increased after glucose compared to baseline, but was not affected by genotype ([Figure 25I](#)), suggesting that the reported effect on glycaemia under fasting conditions is not due to increased circulating insulin levels in mutants.

In line with these observations, we then performed an ITT. Mice were fasted for 6 h prior to testing, and again a trend toward lower glycaemia was observed in the mutants ([Figure 25J](#)). ITT analyses revealed a trend toward lower blood glucose levels in mutants compared to WT controls after systemic insulin administration, but this difference was abolished when blood glucose levels were expressed as a percentage of baseline ([Figure 25K](#)). Furthermore, the decay of the glucose rate in the ITT (KITT) was equivalent between genotypes ([Figure 25L](#)), indicating that insulin sensitivity is similar in mutant and WT mice. Overall, we report that the CB1^{E134G} mutation does not affect food intake and energy homeostasis under standard chow conditions. However, mutant mice seem to have a tendency to hypoglycemia under fasted conditions, independent of insulin levels and sensitivity.

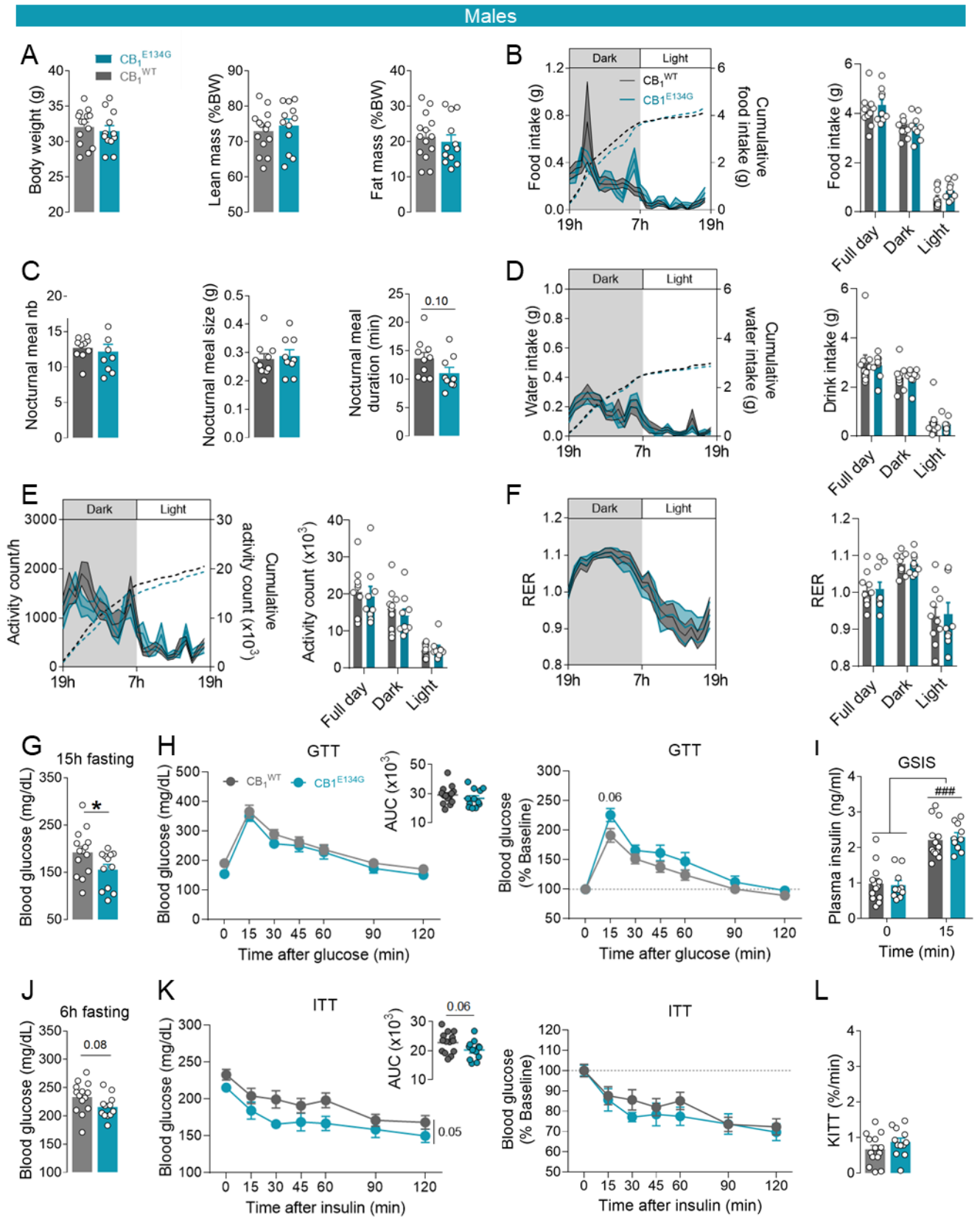
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Figure 25: Metabolic characterization of chow-fed CB1^{WT} and CB1^{E134G} male mice.

(A) Body weight (BW) and body composition expressed as percentage of lean and fat mass in CB1^{WT} and CB1^{E134G} mice. (B) Food intake and cumulative food intake in g during the light-dark cycle in CB1^{WT} and CB1^{E134G} mice. (C) Analysis of nocturnal feeding pattern in CB1^{WT} and CB1^{E134G} mice. (D) Water intake and cumulative water intake in g during the light-dark cycle in CB1^{WT} and CB1^{E134G} mice. (E) Locomotor activity and cumulative locomotor activity during the light-dark cycle in CB1^{WT} and CB1^{E134G} mice. (F) Respiratory exchange ratio (RER) during the light-dark cycle in CB1^{WT} and CB1^{E134G} mice. (G) Glycaemia after 15 hours of fasting prior to (H) glucose tolerance test (GTT) assessed as the time course of glycaemia following intraperitoneal administration of 2 g glucose per kg lean mass in CB1^{WT} and CB1^{E134G} mice, expressed in mg/dl and as a percentage of the baseline (I) Glucose-stimulated insulin secretion (GSIS) assessed by quantification of plasma insulin at baseline (15 h fasting) and 15 h after glucose administration in CB1^{WT} and CB1^{E134G} mice. (J) Glycaemia after 6 h of fasting prior to (K) insulin tolerance test (ITT) assessed as the time course of glycaemia following intraperitoneal administration of 0.5 UI of insulin per kg of lean mass in CB1^{WT} and CB1^{E134G}, expressed in mg/dl and as a percentage of the baseline. (L) Decrease in glucose rate per minute during ITT (KITT) in CB1^{WT} and CB1^{E134G}. Data represent mean \pm SEM (n=8 to 14 animals per group). *p<0.05 for 2-group comparison by Student's t-test (G) and ###p<0.001 for main time effect in 2-way ANOVA (I).

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Figure 25: Metabolic characterization of chow-fed CB1^{WT} and CB1^{E134G} male mice.



3.2.2. Motivation for wheel running and palatable food is not affected in *CB1^{E134G}* mice

Energy balance is a tightly regulated process in which energy expenditure and energy intake must be coordinated to maintain the metabolic state of the individual. In the case scenario of obesity, this balance is dysregulated in favor of caloric intake, which may or may not be associated with physical inactivity (Piazza, Cota, and Marsicano 2017; Muguruza et al. 2019). In line with the investigation of the role of the endogenous PREG-CB1 negative feedback loop in the modulation of energy balance, we wondered whether mutant mice would show any phenotype on the incentive drive for exercise and palatable food intake. Therefore, we examined the effect of the *CB1^{E134G}* mutation on motivation for two rewards, namely wheel running and palatable food. To investigate the putative phenotypes induced by the *CB1^{E134G}* mutation on wheel running motivation, male mice were conditioned under FR1 and FR3 schedules of reinforcement as previously described (Muguruza et al. 2019) (Figure 26A). Both mutants and their WT littermates learned to perform the operant response by discriminating between active and inactive nose pokes to unlock access to the wheel, with a moderate but significant tendency for mutants to acquire the operant response better than WT mice (Figure 26B). However, mutant and WT mice did not differ when tested under a PR schedule of reinforcement the day after the final FR3 session (Figure 26C). Furthermore, the reported operant response observations were not associated with changes in wheel running performance as assessed by running duration and running distance per sequence under FR and PR schedules of reinforcement (Figure 26D-F). It should be noted, however, that the mutant mice exhibited a moderate but significant decrease in running duration per sequence associated with a non-significant trend toward decreased running distance per sequence (Figure 26F) compared to their WT littermates under the PR session.

Similarly, mice were tested for motivation to palatable food as described by Muguruza and collaborators (Muguruza et al. 2019) (Figure 26G). Again, all animals learned to perform the operant response (Figure 26H) under FR1 and FR3 schedules of reinforcement, but unlike wheel running, there was no genotype difference across sessions. In addition, mutant and WT mice did not differ when tested under a PR schedule of reinforcement, either when fed *ad libitum* or under fasted conditions (Figure 26I). Finally, feeding performance, as assessed by the amount of pellets delivered and consumed under FR and PR schedules of reinforcement, was not affected by genotype (Figure 26J-K). Collectively, these data indicate that the *CB1^{E134G}* point mutation does not affect motivation for wheel running and palatable food, suggesting that endogenous PREG does not modulate CB1 receptor activity that regulates the balance between feeding and exercise (Muguruza et al. 2019).

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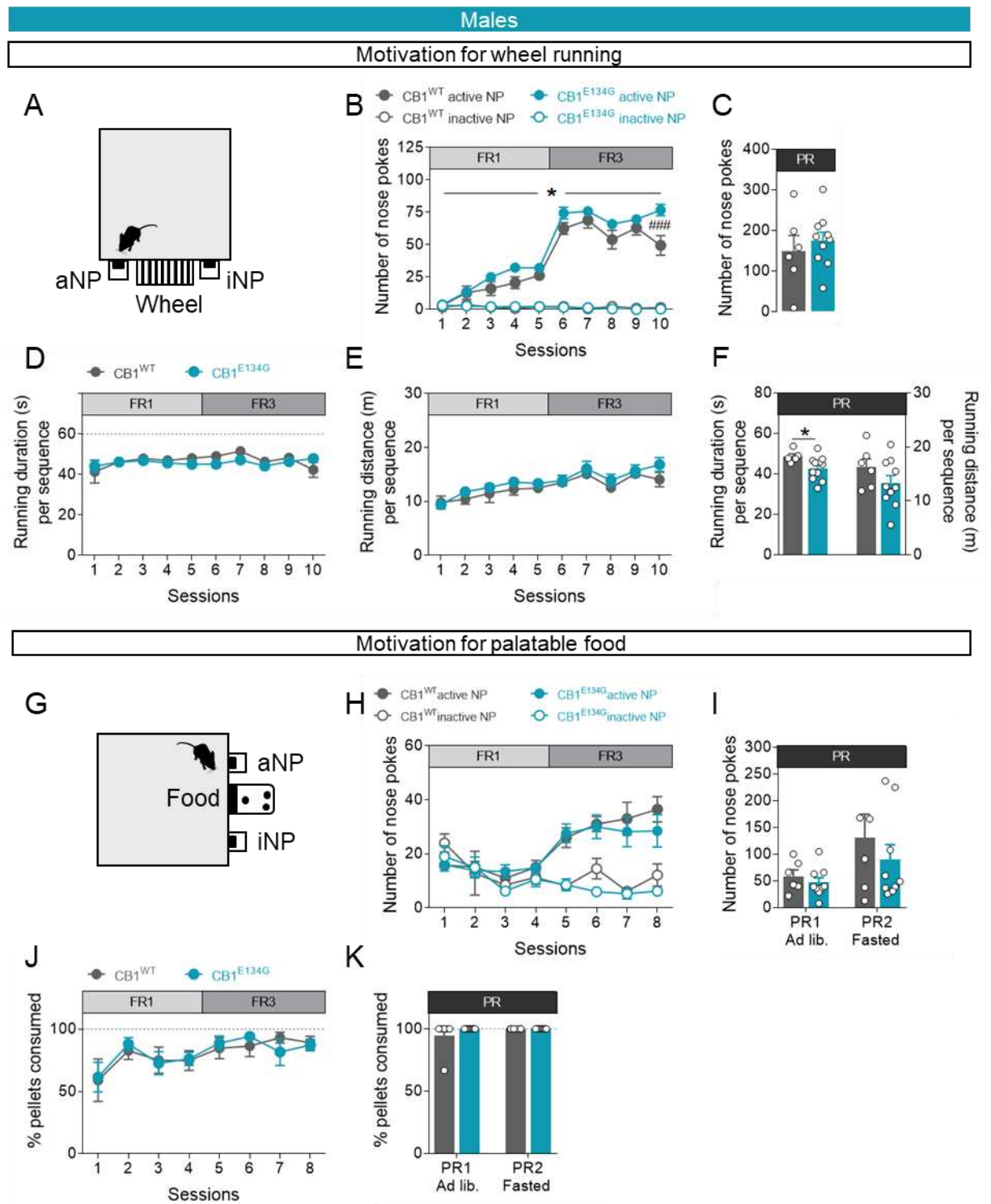


Figure 26: Motivation for wheel running and palatable food in male $CB1^{WT}$ and $CB1^{E134G}$ mice.

Caption on next page.

Figure 26: Motivation for wheel running and palatable food in male CB1^{WT} and CB1^{E134G} mice.

(A) Operant chamber setup with active/inactive nose poke (aNP/iNP) ports with or without wheel lockout. (B) Operant responses for wheel running performed by CB1^{WT} and CB1^{E134G} mice measured as number of NPs under fixed ratio FR1 and FR3 sessions. (C) aNP performance of CB1^{WT} and CB1^{E134G} mice under a progressive ratio (PR) session. (D-E) Running duration and distance per sequence of CB1^{WT} and CB1^{E134G} mice under FR sessions. (F) Running duration and distance per sequence of CB1^{WT} and CB1^{E134G} mice under PR session. (G) Operant chamber setup with aNP/iNP ports with or without food access. (H) Operant responses to palatable food performed by CB1^{WT} and CB1^{E134G} mice measured as number of NPs under fixed ratio FR1 and FR3 sessions. (I) aNP performance of CB1^{WT} and CB1^{E134G} mice under progressive ratio session in *ad libitum* fed conditions (PR1) and progressive ratio session in fasted conditions (PR2). (J) Food consumption of CB1^{WT} and CB1^{E134G} mice under FR1 and FR3 sessions expressed as the percentage of pellet consumed over total pellet released by aNP. (K) Food consumption of CB1^{WT} and CB1^{E134G} mice during PR1 and PR2 sessions. Data represent mean \pm SEM (n=6 to 10 animals per group). *p<0.05 for overall genotype effect in 2-way ANOVA (B) and 2-group comparison by Student's t-test (F). ### p<0.001 for genotype effect on aNP performance by Sidak's multiple comparison post hoc test (B).

3.2.3. Acute HFD but not fasting moderately increase food intake in CB1^{E134G} mice

We further investigated the metabolic phenotype of CB1^{E134G} mice compared to their WT littermates under metabolic challenge. First, mice were subjected to a 24 h fasting challenge followed by standard chow refeeding. Food was reintroduced 20 min before the start of the dark period of the light-dark cycle and food intake, locomotor activity and peripheral substrate utilization were measured in calorimetry chambers. Both mutants and their WT controls exhibited hyperphagia (increased food intake) after the fasting period, consuming an average of 6 g of food pellets during the 24 h after refeeding, compared to an average of 4 g under free-feeding conditions ([Figure 27A](#)). Analysis of food intake during the refeeding period and meal patterns during the nocturnal/active period showed no change between mutant and WT mice ([Figure 27A-B](#)). Similarly, locomotor activity and RER were unaffected in mutant mice under both fasting and refeeding conditions in CB1^{E134G} mice compared to WT mice ([Figure 27C-D](#)), with both groups preferentially using carbohydrates as an energy source after refeeding (RER \approx 1) during dark and light periods ([Figure 27D](#)).

In addition, we exposed mice to a high calorie diet with 60% calories from fat. The first 24 h of exposure to this diet was analyzed similarly to the fasting-refeeding challenge, with the HFD introduced 20 min before the start of the dark period of the light-dark cycle and food intake. Upon exposure to the HFD, mutant mice increased their food intake compared to their WT controls, which was significant throughout the 24h of exposure but not when divided into light and dark periods ([Figure 27E](#)). This was accompanied by an increase in the size of

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nocturnal meals, but not in the number and duration of meals ([Figure 27F](#)). On the other hand, locomotor activity during HFD was not affected by genotype ([Figure 27G](#)), and RER indicated a switch to lipid utilization in both light and dark periods (RER≈0.7) independent of genotype. Notably, energy expenditure was slightly increased in mutants under both fasting-refeeding conditions and acute HFD exposure due to the aforementioned batch-dependent lean mass effect, but this was attenuated by Ancova corrections (data not shown). Taken together, these data suggest that the CB1^{E134G} mutation slightly increases food intake during HFD diet exposure, but not following a fasting-refeeding challenge, and does not affect energy expenditure in either challenge.

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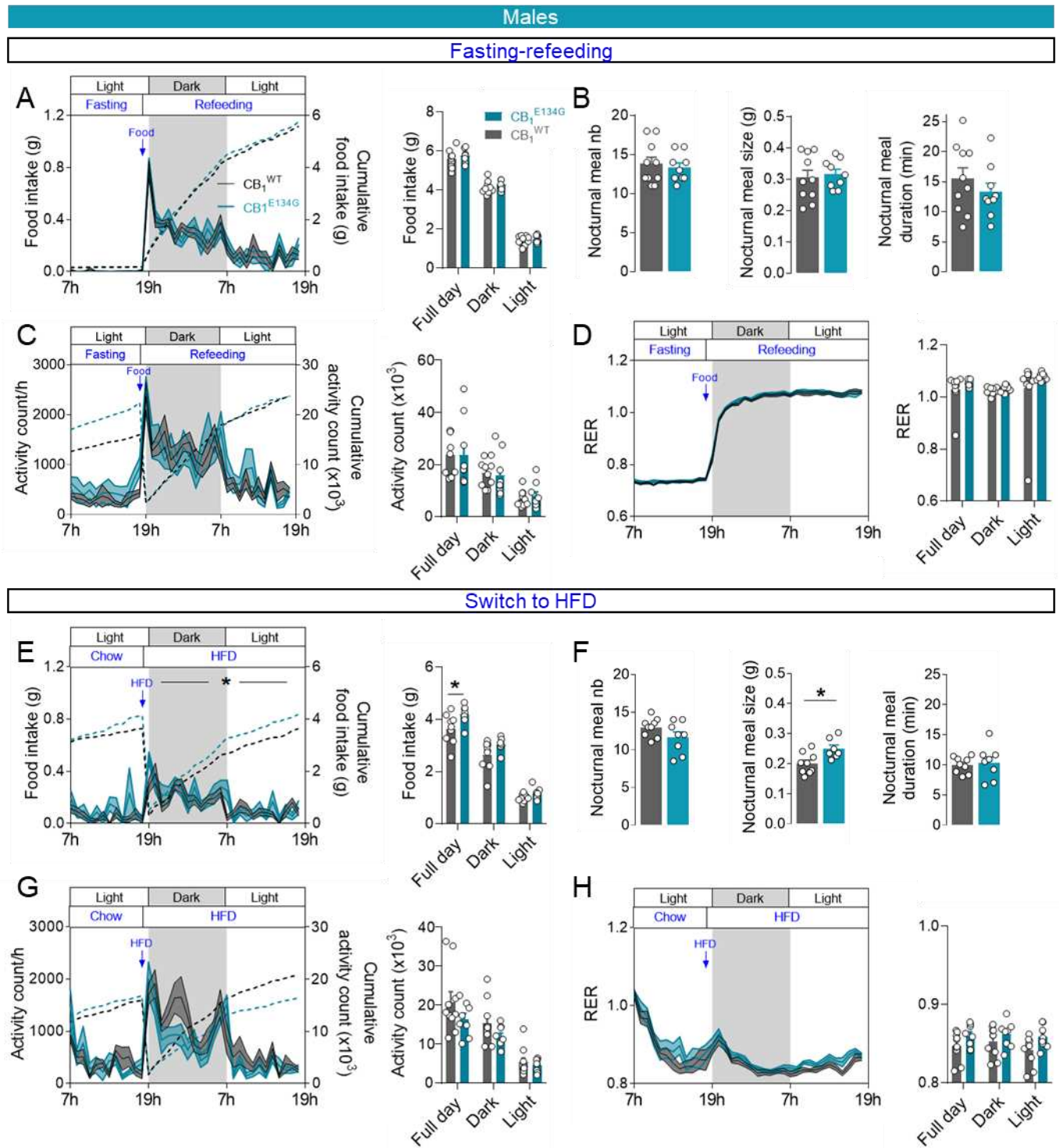


Figure 27: Metabolic effects of fasting-refeeding and acute HFD exposure in CB₁^{WT} and CB₁^{E134G} male mice.

(A) Food intake and cumulative food intake in g during the light/dark cycle, (B) analysis of the nocturnal eating pattern, (C) locomotor activity and cumulative locomotor activity during the light/dark cycle, and (D) respiratory exchange ratio (RER) during the light/dark cycle in CB₁^{WT} and CB₁^{E134G} mice during a 24 h fasting-refeeding challenge. (E-H) Reciprocal analyses during a 24 h acute high fat diet (HFD) challenge. Data represent mean ± SEM (n=8 to 14 animals per group). *p<0.05 for main effect of genotype by 2-way ANOVA (E) and 2-group comparison by Student's t-test (F).

3.2.4. Diet-induced obesity differentially affects glucose metabolism in *CB1^{E134G}* mice

To investigate whether the *CB1^{E134G}* point mutation has any effect on metabolic changes in response to an obesogenic environment, mice were maintained on a high calorie diet with 60% calories from fat for approximately three months, referring to a mouse model of diet-induced obesity (Cota et al. 2008; Bajzer et al. 2011). The mice were weighed and their body composition analyzed prior to switching to the HFD (control chow diet), and their body weight and food intake were monitored weekly over the course of the experiments ([Figure 28](#)). Long-term exposure to the HFD resulted in a similar increase in body weight between mutants and their WT controls, as measured by change in body weight ([Figure 28A](#)). Changes in body composition were assessed after 3 months on HFD and revealed an increase and decrease in the percentage of fat and lean mass, respectively, compared to baseline analyses on chow diet, regardless of genotype ([Figure 28B](#)). Consistent with these results, cumulative food intake was unchanged in mutants compared to WT controls ([Figure 28C](#)). In addition, we performed GTT and ITT assays to examine the comorbidities of diet-induced obesity, as obesity is often associated with metabolic related disorders in humans (Engin 2017). Mice were fasted for 15 h prior to GTT analysis and blood glucose was measured. Interestingly, blood glucose was lower in fasted *CB1^{E134G}* mice compared to their WT controls ([Figure 28D](#)), which is consistent with similar effects reported in mutants on a chow diet ([Figure 27](#)). Notably, the hypoglycemic effects of fasting in mutant mice appeared to be enhanced on the HFD compared to the chow diet. Consistent with this hypoglycemic phenotype, glycaemia was significantly lower in mutants compared to their WT littermates following intraperitoneal injection of glucose at 1.5 mg/kg of lean mass. This effect was abolished when blood glucose was expressed as a percentage of baseline ([Figure 28E](#)). Thus, the *CB1^{E134G}* mutation appears to exert a protective effect on glucose metabolism under HFD, although this effect is not related to glucose tolerance *per se*. Similar to what was observed in the chow diet, the effect of the mutation on glycaemia is independent of circulating insulin ([Figure 28F](#)).

Finally, mice were fasted for 6h and tested for ITT. Again, glycaemia was significantly lower in mutants compared to WT mice on HFD ([Figure 28G](#)), whereas only a trend was observed in chow-fed mice after 6h fasting ([Figure 27](#)). Blood glucose was significantly lower in mutants compared to WT after intraperitoneal insulin administration, and as with GTT, this effect was abolished when blood glucose levels were expressed as a percentage of baseline ([Figure 28I](#)). Furthermore, although the decay of the glucose rate in the ITT (KITT) appeared to be slightly higher in mutants, this effect was not significant ([Figure 28J](#)), indicating similar insulin sensitivity between genotypes on HFD. Thus, although the mutation has no effect on body weight gain, body composition, and food intake under HFD, mutant mice are more

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sensitive to fasting-induced hypoglycemia, which confers protective effects on glucose metabolism independent of glucose tolerance and insulin sensitivity *per se*.

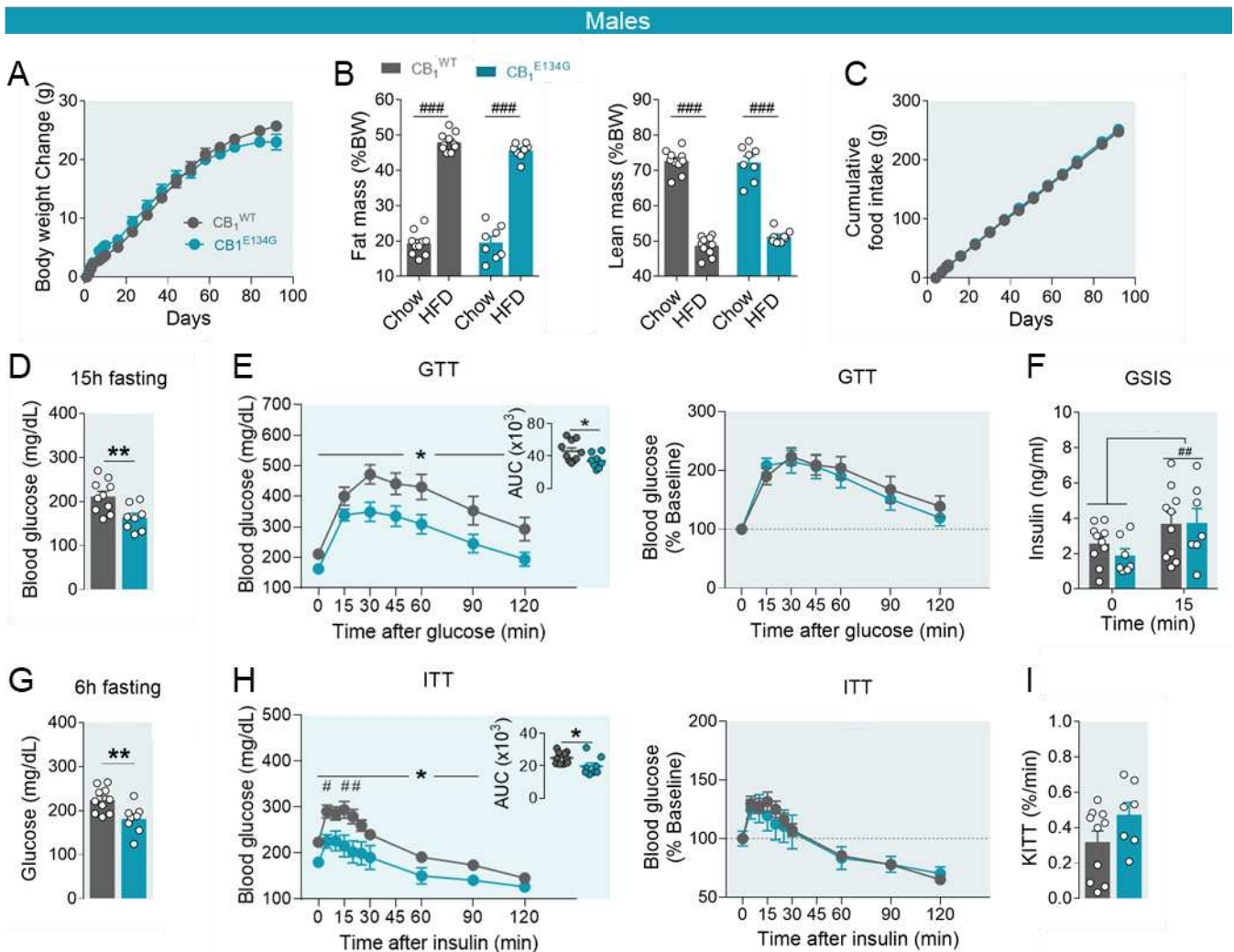


Figure 28: Effect of diet-induced obesity in male $CB1^{WT}$ and $CB1^{E134G}$ mice.

(A) Body weight change on high fat diet (HFD) in $CB1^{WT}$ and $CB1^{E134G}$ mice. **(B)** Body composition expressed as percentage of fat and lean mass in $CB1^{WT}$ and $CB1^{E134G}$ mice, on chow diet and after 3 months on HFD. **(C)** Cumulative food intake on HFD in $CB1^{WT}$ and $CB1^{E134G}$ mice. **(D)** Glycaemia after 15 h of fasting prior to **(E)** glucose tolerance test (GTT), assessed as the time course of glycaemia following intraperitoneal administration of 1.5 g glucose per kg of lean mass in $CB1^{WT}$ and $CB1^{E134G}$ mice expressed in mg/dL and as a percentage of baseline **(F)** Glucose-stimulated insulin secretion (GSIS) assessed by quantification of plasma insulin at baseline (15 h fasting) and 15 h after glucose administration in $CB1^{WT}$ and $CB1^{E134G}$ mice. **(G)** Glycaemia after 6 h of fasting prior to **(H)** insulin tolerance test (ITT) assessed as the time course of glycaemia following intraperitoneal administration of 0.75 UI of insulin per kg of lean mass in $CB1^{WT}$ and $CB1^{E134G}$ mice expressed in mg/dL and as a percentage of baseline. **(I)** Decay in glucose rate per minute during ITT (KITT) in $CB1^{WT}$ and $CB1^{E134G}$ mice. Data represent mean \pm SEM (n=8 to 14 animals per group). * $p < 0.05$ for 2-group comparison by Student's t-test (**D**, **G** and GTT/ITT AUC (upper inserts **E**, **H**)) and main genotype effect by 2-way ANOVA (**E** and **H**), **##** $p < 0.01$ for main treatment effect by 2-way ANOVA (**F**) and **###** $p < 0.001$ for diet effect by Sidak's multiple comparison post hoc test (**B**).

3.3. Conclusion

In this third aim, we investigated the putative role of the endogenous PREG-CB1 regulatory loop on energy balance. Analyses revealed no major changes induced by the CB1^{E134G} point mutation in body composition, food intake, and peripheral substrate utilization (e.g., glucose or lipid substrates), either on standard chow or during metabolic challenges, including fasting-refeeding and high-fat diet (HFD) exposure. Although HFD consumption was slightly increased in CB1^{E134G} mutant mice during the first 24 h of exposure, this effect appeared to be transient and was not observed during chronic exposure to the diet. This transient increase is unlikely to be due to changes in motivation for palatable food as assessed by operant condition protocols. In addition, motivation for voluntary physical activity was similar between mutants and their WT controls. Collectively, these data suggest that the endogenous PREG-CB1 regulatory loop is not involved in the modulation of energy balance.

Interestingly, however, the mutant mice displayed a phenotype related to glucose metabolism. On fasting, mutant mice had lower plasma glucose levels than WT controls, and this effect was enhanced on HFD challenge. This effect was not associated with increased plasma insulin levels or insulin sensitivity in either diet condition, and conferred a somewhat protective effect on mutant mice in the glucose tolerance test by reducing absolute levels of glycaemia, but not by affecting glucose tolerance *per se*. These data are consistent with unpublished results from our laboratory showing that exogenous chronic PREG administered at 2 and 5 mg/kg increases glycaemia in mice without affecting insulin release and sensitivity. Thus, the PREG-CB1 regulatory loop may control glucose homeostasis through insulin-independent mechanisms. This observation requires further investigation ([see DISCUSSION](#)).

4. Investigation of the effect of endogenous CB1 stimulation in CB1^{E134G} mice: Ongoing experiments

4.1. Rationale

Consistent with the previously reported effect of exogenous PREG administration in rodents (Vallée et al. 2014; A. Busquets-Garcia et al. 2017; Frau et al. 2019), we have shown that CB1^{E134G} mice lacking the endogenous PREG-CB1 regulatory loop are more sensitive to exogenous CB1 overactivation (e.g., by THC and WIN 55-212.2). However, these mice show little to no phenotypic changes compared to their WT littermates under most of the physiopathological conditions tested. Thus, the question remains as to what is the physiological role of PREG in modulating CB1 activity and why the CB1^{E134G} point mutation lacks functional effects in most behavioral paradigms.

The lack of phenotypic changes in CB1^{E134G} mice may be due to the requirement of sufficient CB1 activation to trigger the endogenous PREG-CB1 loop, which was not achieved under our experimental conditions, as well as the specificity of this loop for certain cannabinoid ligands. To address this issue, we propose to induce endogenous overstimulation of CB1 activity using pharmacological inhibitors of the major eCB degradation enzymes, FAAH and MAGL for AEA and 2-AG, respectively. These inhibitors have the advantage of enhancing and maintaining eCB tone by preventing AEA and 2-AG degradation relative to the endogenous distribution of metabolizing enzymes (Long et al. 2009; A Busquets-Garcia 2011). Specifically, the use of these inhibitors will allow us to determine: 1) whether endogenous overactivation of CB1 receptors by eCBs can trigger brain production of PREG; 2) whether AEA and 2-AG are equally involved in the PREG-CB1 regulatory loop; 3) whether endogenously produced PREG can counteract the behavioral effects induced by increased eCB signaling; and 4) whether endogenous PREG preferentially regulates AEA- or 2-AG-mediated effects, or both.

A complementary explanation may also lie in the specificity of the PREG-CB1 regulatory loop for certain neuronal circuits and receptor pools, as evidenced for example for mtCB1 receptors associated with the D1 striatonigral pathway mediating the cataleptic effects of THC (see Aim 2: [section 3.2](#)). Therefore, in parallel to the above pharmacological experiments, we propose to further investigate the phenotype of CB1^{E134G} mice in other experimental paradigms. In particular, we propose to study the effects of stress on the behavior of CB1^{E134G} mutant mice and their WT littermates. Indeed, there is a bidirectional regulation between the stress response and the ECS (Hillard and Liu 2014; Morena et al. 2016), and CB1 receptor activation has been implicated in the behavioral effects associated with exposure to stressful events, including extinction of aversive memories (Giovanni Marsicano et al. 2002) and stress-

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induced amnesia (Skupio et al. 2023), some of which have been shown to rely on mitochondrial CB1 receptor pools (Skupio et al. 2023). Most interestingly, brain PREG levels are also dynamically regulated after acute stress exposure (Vallée et al. 2000; Vallée 2014). Thus, we wonder whether increased brain PREG levels in response to stress contribute to CB1-mediated functions.

4.2. Preliminary data

4.2.1. 2-AG but not AEA stimulates brain PREG production

As mentioned above, our goal is to investigate the relationships between eCBs and the PREG-CB1 regulatory loop. One of the strategies we have chosen is based on the pharmacological manipulation of eCB levels by targeting their metabolic enzymes. In a first set of preliminary experiments, we selected three pharmacological inhibitors of eCB degradation to increase brain levels of AEA and/or 2-AG. We tested the effect of these inhibitors on brain PREG production in male C57BL6/N mice ([Figure 29](#)) to determine if 1) eCBs can enhance brain production of PREG in a manner similar to exogenous cannabinoids (Vallée et al. 2014) and if 2) AEA and 2-AG are equally effective in stimulating PREG production.

To this end, we used URB597 (10 mg/kg) and JZL184 (40 mg/kg) to inhibit the enzymatic activity of FAAH and MAGL, respectively, and the dual FAAH/MAGL inhibitor JZL195 (20 mg/kg) (A Busquets-Garcia 2011; Long et al. 2009) ([Figure 29A](#)). We tested the efficacy of the three pharmacological inhibitors by quantifying AEA and 2-AG levels by mass spectrometry. As expected, both URB597 and JZL195, but not JZL184, increased brain AEA levels compared to vehicle-treated animals, consistent with their effect on the FAAH enzyme ([Figure 29B](#)). Conversely, JZL184, ZL195, but not URB597, increased 2-AG levels, consistent with their effect on the MAGL enzyme ([Figure 29C](#)). We also quantified PREG levels in the brain of the same animals injected with the inhibitors. Only JZL184 and JZL195 significantly increased brain PREG levels compared to vehicle-treated animals ([Figure 29D](#)). By performing linear regression analysis between brain levels of either AEA or 2-AG and PREG levels in all animals, we observed that 2-AG levels, but not AEA levels, were positively correlated with brain PREG levels ([Figure 29E-F](#)). Taken together, these data suggest that PREG production may be endogenously controlled by 2-AG, but not by AEA. Although these data are preliminary and should be confirmed in the future, they may indicate a differential role of AEA and 2-AG in the endogenous PREG-CB1 regulatory loop.

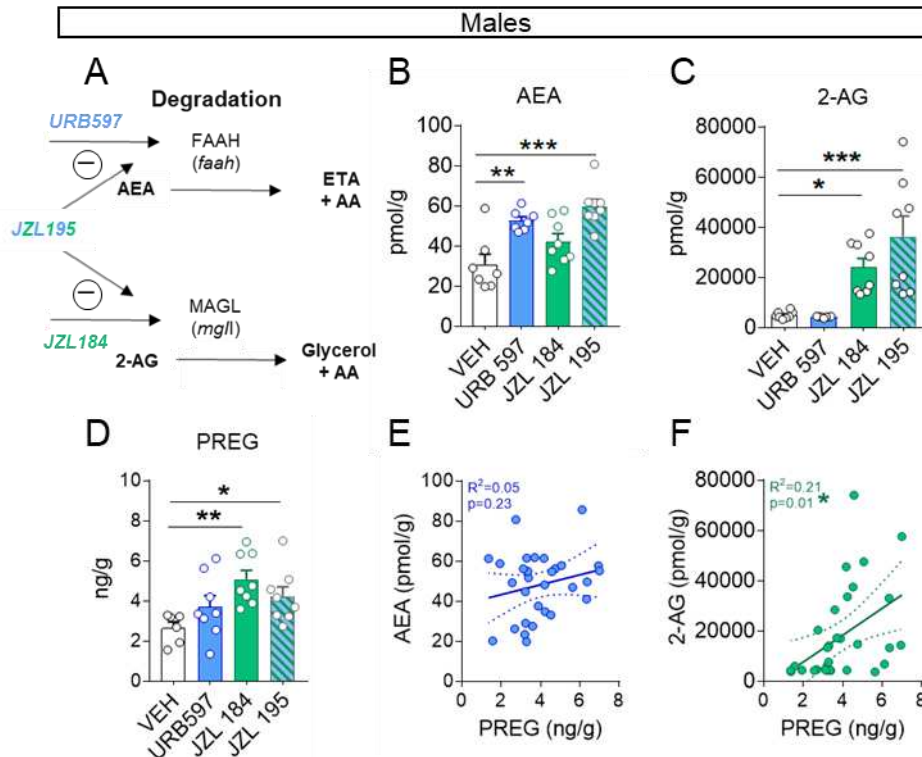


Figure 29: Effect of eCB degradation inhibitors on brain eCB and PREG levels in male C57BL6/N mice.

(A) Schematic of the major pathways of endocannabinoid (eCB, anandamide, AEA and 2-arachidonoylglycerol, 2-AG) degradation and targets of eCB degradation inhibitors. URB597 targets the AEA degrading enzyme FAAH. JZL184 targets the 2-AG degrading enzyme MAGL. JZL195 is a dual inhibitor targeting both FAAH and MAGL. (B) AEA, (C) 2-AG and (D) PREG brain levels in C57BL6/N mice treated with URB597 (10mg/kg), JZL184 (40mg/kg), JZL195 (20mg/kg) or their vehicle (VEH). (E) Linear regression and correlation between AEA and (F) 2-AG brain levels with PREG brain levels. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for Dunnett's multiple comparison post hoc test relative to Veh-treated group (B, C, D) and deviation from zero (F).

4.2.2. Fear extinction may be facilitated in $CB1^{E134G}$ mice

Since CB1 receptor activation has been linked to modulation of the stress response (Morena et al. 2016), and PREG levels are dynamically regulated by exposure to stressful stimuli (Vallée et al. 2000; Vallée 2014), we wonder whether PREG can modulate CB1-mediated effects of stress, including stress coping ability and fear memory.

We first investigated whether the $CB1^{E134G}$ mice differ in the HPA axis activation by measuring corticosterone (CORT) levels at baseline and in response to acute swim stress (Figure 30). CORT measured at baseline (t-30min) prior to stress exposure showed no difference between $CB1^{E134G}$ mutant mice and their WT littermates in either males or females (Figure 30A-B). Similarly, the increase in CORT levels after stress exposure, measured as the delta between peak (t+10min) and baseline (t-30min), and the time course of plasma CORT

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levels did not differ between genotypes in either sex ([Figure 30A-B](#)). Consistently, the adrenal weights of CB1^{E134G} mice were similar to those of WT mice in both sexes ([Figure 30C;E](#)). Thus, the CB1^{E134G} point mutation, and, to a lesser extent, the endogenous PREG-CB1 regulatory loop, do not appear to affect the endocrine effect of stress, although complementary targets of the HPA axis (e.g., ACTH and CRF) should be analyzed, along with the effect of chronic stress exposure.

In an independent experiment, we tested mice for cued fear conditioning and fear extinction, as CB1 receptors have been well described to be required for extinction of the fear response ([Giovanni Marsicano et al. 2002](#); [Kornelia Kamprath et al. 2006](#)). Acquisition of the fear memory trace was similar between mutants and their WT controls in both males and females, as evidenced by similar levels of freezing during exposure to the conditioned stimulus (CS) 1 day after conditioning ([Figure 30D; F](#)). Mice were then re-exposed to the CS for several days to assess extinction of the fear memory. Interestingly, although male WT mice failed to extinguish the fear memory, as indicated by the lack of decrease in their freezing behavior, mutant CB1^{E134G} mice were capable of extinction ([Figure 30D](#)). This effect was not observed in females, as both mutant and WT mice failed to extinguish the fear memory, although mutant mice always exhibited lower levels of freezing than their WT littermates during all extinction sessions ([Figure 30F](#)). Although these preliminary data suggest that the extinction protocol used in our mouse model may not be the most appropriate, as WT animals fail to reduce freezing behavior in response to CS re-exposure, they may also indicate that extinction is facilitated in mutants. Since CB1 receptors are required for extinction of fear memories ([Giovanni Marsicano et al. 2002](#)), our extinction data would be consistent with increased CB1 activity, in this case due to the lack of PREG binding to the receptor. To test this hypothesis, other extinction protocols are currently being tested in CB1^{E134G} mice ([Wotjak 2019](#)).

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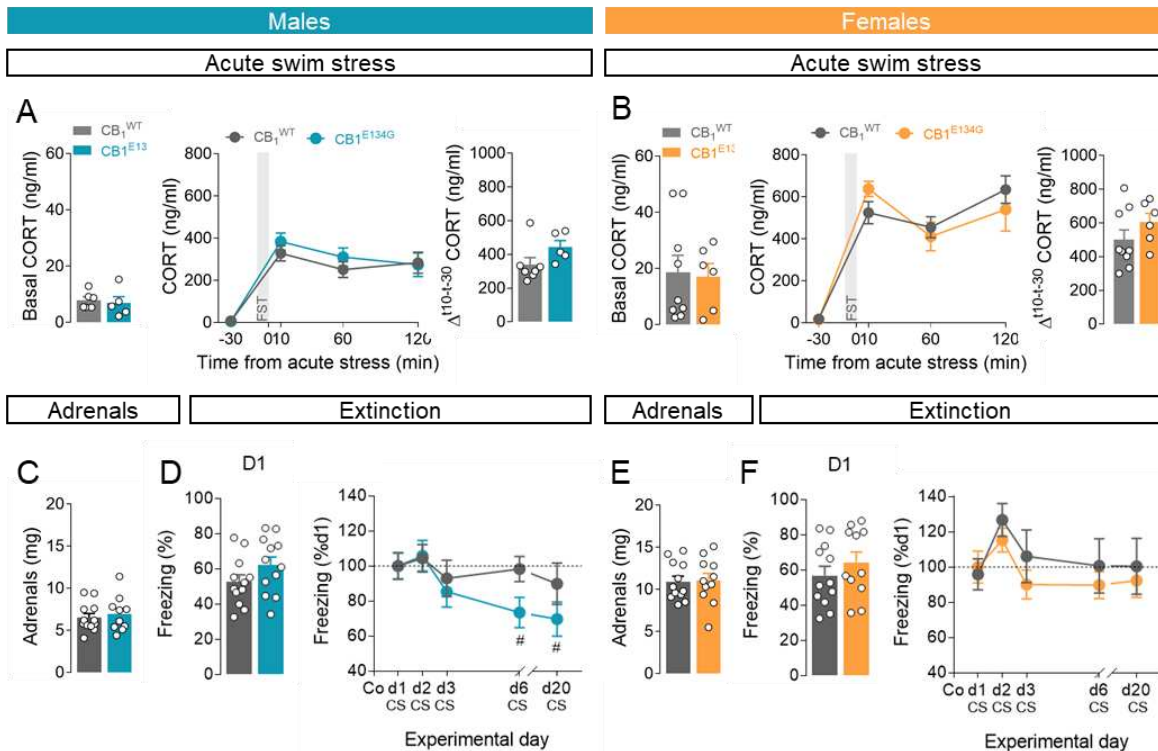


Figure 30: Endocrine effects of stress and fear memory in $CB1^{WT}$ and $CB1^{E134G}$ mice.

(A-B) Basal plasma corticosterone (CORT) levels, CORT levels in response to acute swim stress, and delta CORT levels before and after stress in (A) male and (B) female $CB1^{WT}$ and $CB1^{E134G}$ mice. (C) Adrenal weights in male $CB1^{WT}$ and $CB1^{E134G}$ mice. (D) Percentage of freezing response on day 1 (D1) after cued fear conditioning (Co) and time course of freezing response during extinction sessions (d1-d20) in male $CB1^{WT}$ and $CB1^{E134G}$ mice. (E) Adrenal weights in female $CB1^{WT}$ and $CB1^{E134G}$ mice. (F) Percentage of freezing response during at D1 after Co and time course of freezing response during extinction sessions (d1-d20) in female $CB1^{WT}$ and $CB1^{E134G}$ mice. # $p < 0.05$ for Dunnett's multiple comparison post hoc test vs. D1 (D).

4.3. Conclusion

Pharmacological findings using inhibitors of eCB degradation suggest that eCBS, particularly 2-AG, may stimulate PREG production, although future experiments should be performed to verify the absence of effect of AEA by performing a dose-response and time-course response of all three inhibitors mentioned, as well as other FAAH inhibitors (such as PF-3845; (Bedse et al. 2018)). Nevertheless, these preliminary results suggest that the endogenous PREG-CB1 loop is likely recruited by endogenous activation of the CB1 receptor. In addition, endogenous activation may be involved in the regulation of fear memory formation as assessed by our extinction data; however, future studies will be conducted to investigate whether $CB1^{E134G}$ mice do indeed exhibit an extinction phenotype ([see DISCUSSION](#)).

DISCUSSION

1. CB1^{E134G} mice: advantages and limits of the model

The present work aimed to describe the novel CB1^{E134G} mouse model in order to study the endogenous PREG-CB1 negative feedback loop. Specifically, this mouse model was developed based on the previously reported effect of the CB1^{E133G} mutation of the human CB1 receptor, which invalidates the allosteric binding site of PREG on the CB1 receptor *in vitro* (Vallée et al. 2014). By translating this mutagenesis approach from the human CB1 receptor to the murine CB1 receptor, we sought to investigate the role of PREG in modulating CB1 activity *in vivo* while preserving endogenous cannabinoid and steroid signaling, which has significant advantages over traditional pharmacological approaches.

The data presented above indicate that the CB1^{E134G} point mutation does not affect the endogenous production of endocannabinoids and steroids, as evidenced by the lack of overall change in spectral analyses between mutant mice and their littermate controls. These results are critical because the absence of alterations in AEA and 2-AG, but also PREG levels in CB1^{E134G} mice, strengthens the use of the model over the use of pharmacological agents to study the endogenous regulatory loop between PREG and its receptor target CB1. In addition, we hypothesized that CB1^{E134G} mutant mice would exhibit increased sensitivity to exogenous cannabinoid administration due to the lack of negative modulation of PREG on cannabinoid-induced CB1 hyperactivity. As expected, we observed hypersensitivity of CB1^{E134G} mice to the primary psychoactive component of cannabis, THC, in the THC-induced tetrad protocol and on THC-induced mesoaccumbal dopamine (DA) transmission. We also report that the effects of the synthetic CB1 agonist WIN 55,212-2 (WIN) on mitochondrial respiration are enhanced in substantia nigra extracts from CB1^{E134G} mice compared to WT controls. Most importantly, we show that the effects of WIN are reversed by exogenous PREG administration in WT but not CB1^{E134G} samples, confirming the absence of allosteric modulation of PREG on CB1 activity in the mutant. Taken together, these data support a mouse model in which PREG cannot regulate CB1 receptor activity, and in which the reported behavioral and molecular effects of the mutation reflect the contribution of endogenously produced PREG to CB1-mediated signaling.

Interestingly, the use of this model allowed us to reveal the differential contribution of endogenously produced PREG in regulating the activity of subcellular pools of CB1 receptors. In particular, our data indicate that endogenous PREG preferentially regulates the activity of striatonigral mtCB1 receptors, which are associated with the cataleptic effects of THC, over pmCB1 receptors, associated with the analgesic effects of THC (Soria-Gomez et al. 2021). This is in contrast to previously reported data showing that pharmacological inhibition of PREG

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synthesis via the P450_{scc} enzyme inhibitor aminoglutethimide (AMG) potentiated THC-induced catalepsy as well as analgesia and was reversed by exogenous PREG administration (Vallée et al. 2014). This further highlights the advantage of the CB1^{E134G} model over classical pharmacological manipulation of steroids, which, in addition to the risk of inducing unwanted changes in downstream metabolite levels, does not always recapitulate the refinement of GPCR signaling modulation at the subcellular level. Thus, we propose that the CB1^{E134G} mouse line is a suitable and promising tool for the study of endogenous PREG-CB1 regulation and the elucidation of subcellular circuits underlying the functions of the (neuro) steroid in cannabinoid signaling.

Unexpectedly, however, we report that the CB1^{E134G} point mutation causes a drastic downregulation of CB1 expression at the protein but not the mRNA level in the brain of mutant mice compared to their WT controls, suggesting compensation mechanisms at the post-transcriptional level. Although this compensation in CB1 protein does not preclude the validity of our model, since CB1^{E134G} mice remain hypersensitive to exogenous overstimulation of CB1 activity, it is necessary to understand how and why this compensation occurs. Indeed, this downregulation of CB1 protein certainly compensates for an increase in receptor activity, which may be the result of the lack of PREG binding to the receptor. Therefore, this compensation may represent a limitation for the elucidation of the endogenous roles of the PREG-CB1 negative feedback loop by masking the contribution of PREG to CB1-mediated functions at the behavioral and systemic level. In this perspective, one should remain cautious when interpreting the results of the current study regarding the absence of phenotype of CB1^{E134G} mice in the context of endogenous cannabinoid signaling (e.g., energy metabolism). In the meantime, this downregulation may also indicate that the role of PREG in modulating CB1 activity may be greater than originally thought and represents an exciting research avenue for the study of PREG on CB1 receptor dynamics. Therefore, the following section will outline a conceptual framework and research proposal for the future investigation of the molecular mechanisms underlying the downregulation of the mutant CB1^{E134G} protein and its consequences at the behavioral level.

2. Which CB1 receptors are involved in the downregulation process?

The present study shows that CB1 receptors are downregulated in CB1^{E134G} mutant mice compared to WT controls. Before addressing the molecular mechanisms underlying this downregulation, the first question that comes to mind is which CB1 receptors are downregulated. Indeed, as described in the introduction, the current view of CB1 receptor localization is quite complex and includes the plasma membrane as well as subcellular localization, in particular mitochondria-associated CB1 receptors. Furthermore, CB1 receptors

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are heterogeneously distributed across cell types and brain regions, and receptor functionality does not follow a linear rule in which expression levels correlate with signaling outcome, further highlighting the complexity behind receptor localization (Arнау Busquets-Garcia, Bains, and Marsicano 2018). Our current data do not address the complexity associated with the localization of CB1 receptors, as Western immunoblotting was performed on total protein extract. However, the downregulation of the mutant CB1^{E134G} protein was recapitulated in all brain regions analyzed, regardless of sex, strongly suggesting that this is a generalized phenomenon.

In addition, although Western immunoblotting allows only semi-quantification of the detected proteins, we observed a drastic reduction of CB1 proteins in mutant extracts (approximately 70 to 80%). For comparison, the exclusion of mtCB1 receptors by deletion of the first 22 amino acids of the receptor sequence in DN22-CB1-KI mice barely affects the total number of CB1 receptor particles as measured by immunogold labeling coupled to electron microscopy (Soria-Gomez et al. 2021). Therefore, it is virtually impossible that the mitochondrial pool of CB1 receptors alone is affected by the present decrease. Alternatively, we hypothesize that the present compensation of CB1 protein levels in CB1^{E134G} mutant mice affects all CB1 receptors, regardless of their cellular and subcellular localization. To test this hypothesis, we propose to perform additional Western immunoblotting analyses on samples obtained by differential centrifugation to distinguish the plasma membrane from the mitochondrial pool of CB1 receptors (Liao et al. 2020) and by Magnetic-Activated Cell Sorting (MACS) to distinguish different brain cell populations (Holt, Stoyanof, and Olsen 2019). Finally, since the analysis of CB1 expression was focused on the brains of mutant and WT mice, we propose to extend these analyses to peripheral tissues, which are known to express significant and functional levels of CB1 receptors (Piazza, Cota, and Marsicano 2017).

3. What mechanisms are involved in the downregulation process?

3.1.1. Insight on structure-activity relationships

Beyond the cellular and subcellular pools of receptors affected by CB1 protein downregulation in CB1^{E134G} mutant mice, the following questions arise: How and why does this compensation occur? Single and double point mutations can have drastic effects on CB1 receptor activity depending on their localization (Scott et al. 2013; Ahn et al. 2013; Wickert et al. 2018). However, as mentioned in the introduction, the 1.49 glutamate residue (position 133 in humans and 134 in mice) was selected and tested for PREG allosterism because mutation at this site is unlikely to affect receptor function *per se*. (Figure 8). Namely, because the CB1^{E134G} point mutation is located in TMH1, it is unlikely to affect ligand binding affinity by causing conformational changes within the extracellular domain or by disrupting direct ligand

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interaction with highly conserved amino acid residues. In addition, the mutation is topographically distant from intracellular regulatory sites associated with signal transduction, making it unlikely that the mutation alone can affect G-protein coupling.

To confirm these assumptions, our collaborators at IMGpharma (Zamudio, Spain) are performing radio-ligand binding assays and [35S]GTP γ S assays to compare receptor binding affinity and functionality, respectively, in membrane extracts obtained from CB1^{E134G} mice and their WT littermates. If confirmed, the lack of difference in radiolabel binding between CB1^{E134G} mutant and WT receptors would also be consistent with the lack of effect of PREG on CB1 orthosteric ligand binding (Vallée et al. 2014). On the other hand, although changes in G protein coupling are not expected in the mutant receptor, if observed, such differences may be the direct consequence of the point mutation or the consequence of the loss of PREG binding to the receptor. Since the effect of PREG on G-protein coupling has never been investigated, the ongoing study will also include a dose-response curve with PREG on [35S]GTP γ S binding in both mutant and WT receptors.

It is important to note that the TMH1 region does not undergo major conformational changes upon GPCR activation. Moreover, because it is the first transmembrane region translated by ribosomes, it may play an important role in receptor folding and in membrane insertion (Venkatakrisnan et al. 2013). Thus, mutations within consensus regions within the TMH1 domain may indirectly affect receptor function (e.g., ligand binding and G protein coupling affinity). Although future structure-relationship analyses dedicated to the CB1 receptor could reveal an essential role of the 1.49 glutamate residue on receptor conformation, our current knowledge does not allow us to conclude on the possible changes in receptor conformation leading to protein downregulation in CB1^{E134G} mutant mice.

3.1.2. Putative effect of PREG on CB1 receptor internalization and degradation

An alternative hypothesis that may explain the observed downregulation of CB1 protein in mutant mice is based on the absence of allosteric regulation of CB1 activity by PREG. In particular, the CB1^{E134G} point mutation was designed to reduce the interaction of the ketone end of PREG with the CB1 receptor, which, based on computational modeling, is thought to limit PREG stabilization within the TMH1/TMH7/hx8 region (Vallée et al. 2014). Interestingly, it has been proposed that PREG binding to this exosite limits TMH7 movement, thereby modulating β -arrestin-mediated signaling (Hurst et al. 2019). B-arrestins have primarily been described as modulators of GPCR signaling by participating in the internalization of the receptor into clathrin-coated pits, which may ultimately lead to its degradation in lysosomes (Freedman and Lefkowitz 1996; Yu et al. 1993; Ahn et al. 2013). In addition, CB1 ligands,

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including the endogenous agonist 2-AG, have been shown to bias CB1-signaling in favor of β -arrestin 2 signaling thereby promoting receptor internalization (Laprairie et al., 2014). Therefore, it can be hypothesized that the downregulation of CB1 protein in CB1^{E134G} mutant mice results from the lack of PREG-mediated stiffening of the TMH1/TMH7/hx8 region, leading to increased recruitment of internalizing and signaling proteins. This hypothesis is also supported by elements of the literature indicating that the endogenous CB1-NAM pepcan-12 attenuates agonist-induced internalization of the CB1 receptor (Bauer et al. 2012), further highlighting the action of allosteric modulators in controlling receptor dynamics.

Consistently, the present downregulation of the CB1 receptor in mutant mice would not only represent a form of compensation to counterbalance the effect of increased signaling associated with TMH7 lability, but would also suggest that one of the endogenous roles of PREG is to maintain CB1 receptor expression at a "steady state" level by stabilizing it at the membrane. To test this hypothesis, we propose to investigate several mechanisms and molecular pathways *in vitro* using 1) primary cortical neuron cultures derived from CB1^{E134G} and WT embryos (E17.5) and 2) the human HEK293 cell line transiently transfected with a plasmid containing either the *cnr1* sequence encoding the human CB1^{E133G} mutant receptor or the human WT receptor (as described in (Vallée et al. 2014)). Interestingly, these two cellular models recapitulate the downregulation of CB1 expression observed *in vivo* (see 1.3.2 for primary culture; ongoing analyses for transfected HEK293 cells), which supports their use for studying the intracellular signaling pathways associated with the CB1 receptor (e.g., functionality assay), along with the molecular events leading to the downregulation of the mutant CB1 protein.

First, we propose to test whether complete blockade of CB1 activity rescues the downregulation of CB1 protein in mutants. To this end, mutant and WT cultures and cell lines will be treated with the CB1 orthosteric inverse agonist rimonabant (SR141716; SR), proteins will be extracted, and total CB1 expression will be analyzed by Western immunoblotting. Partial or complete rescue of CB1 protein in mutant cells to levels comparable to WT cells will indicate that the observed downregulation of CB1 is indeed due to increased receptor signaling. In parallel to this experiment, we propose to test the effect of PREG on agonist-induced downregulation of CB1 receptor protein (Blair et al. 2009; Smith et al. 2015). To this end, cells will be treated with the synthetic CB1 agonist WIN 55-212, 2 in the absence or presence of PREG. If our hypothesis is correct, we should first confirm the downregulation of CB1 protein in response to WIN 55-212, 2 application, and co-treatment with PREG should limit the downregulation of the receptor in WT cells but not in mutant cells. In addition to the lack of effect of PREG in mutant cells, it is also possible that WIN application causes an increased rate of CB1 protein downregulation compared to WT cells. Together with the results from SR

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treatments, this would further suggest that the lack of PREG-mediated regulation of CB1 activity in mutants is responsible for the downregulation of CB1 protein expression. In addition to proteomic analysis by Western immunoblotting, the above experiments should include RT-qPCR to verify the expression of *cnr1* in all experimental conditions to verify that the effect of SR or PREG is related to post-translational but not transcriptional mechanisms.

To investigate the downregulation of CB1 protein in mutants at a more mechanistic level, we propose to use additional pharmacological approaches *in vitro*. Since agonist-induced activation of CB1 receptors can target the protein for degradation in lysosomes (Rozenfeld 2011), we propose to treat mutant and WT cells, both transfected HEK293 cells and cortical primary cells, with the lysomotropic agent chloroquine (Martini et al. 2007). Consistent with our hypothesis, the addition of the proteolysis inhibitor chloroquine should prevent degradation of the mutant CB1 receptor and partially or completely rescue the protein at levels comparable to WT cells. Although lysosomes are the major recycling and degradation pathways for integral membrane proteins, including the CB1 receptor, we cannot exclude the existence of alternative degradation pathways. Such pathways may include multiprotein enzymatic complexes such as the proteasome, which is more involved in the proteolysis of soluble cytoplasmic proteins. Therefore, we propose to test a proteasome inhibitor (e.g. MG132) in addition to the lysomotropic agent chloroquine.

In addition to degradation, molecular events upstream of lysosomal degradation could be investigated, including endocytosis of the receptor. In particular, the CB1 receptor can be endocytosed via clathrin-coated pits (CCP), but also via membrane lipid rafts coupled to caveolae-mediated endocytosis (Wickert et al. 2018). To distinguish by which endocytic mechanism the mutant CB1 receptor is preferentially internalized before degradation, CPP and caveolae inhibitors can be used *in vitro* (e.g., PitStop2TM and M β CD, respectively). It is worth noting that in the event that PREG has no effect on receptor degradation upon agonist-induced stimulation, this series of pharmacological experiments would remain informative as to which downstream mechanisms (i.e., internalization and degradation) lead to the degradation of the mutant CB1 protein.

It is noteworthy that the development of additional constructs may be informative to study membrane receptor dynamics in human HEK293 cells. Indeed, the above experiments propose to study the effects of different pharmacological treatments on total CB1 receptor levels. Such experiments can be performed at different time points after application of the treatments in the culture media, but provide poor temporal and no spatial resolution. Although immunocytolabeling of CB1 receptors coupled with confocal imaging could improve the lack of spatial resolution associated with total protein analyses, it would still be limited on the time

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scale. Thus, the development of fusion protein constructs, such as CB1-eGFP chimeras (Leterrier et al. 2004) containing the E133G substitution (in the case of the human CB1 receptor) transfected into HEK293 cells, may allow us to perform live imaging experiments to study the effects of PREG on the endocytic cycling of the CB1 receptor. Of interest, care should be taken to decide where to localize eGFP between the N- and C-terminal domains of the receptor to avoid an occluding effect of the construct on cellular signaling.

4. Investigation of the CB1^{E134G} protein downregulation *in vivo* and implication for cannabis intoxication

In addition to the aforementioned *in vitro* experiments, additional *in vivo* analyses can be performed to test the hypothesis that the mutant CB1^{E134G} protein is downregulated due to the lack of PREG binding to the receptor, resulting in increased signaling, internalization, and degradation of CB1. Similar to pharmacological treatment in mutant and WT cultures and transfected cell lines, we propose to chronically administer the CB1 inverse agonist rimonabant (SR141716; SR) to CB1^{E134G} mice and their WT littermates to test whether blockade of CB1 activity can rescue the downregulation of protein in mutants. In addition to protein detection by Western immunoblotting, RT-qPCR can be added to specify the effect of rimonabant at the post-transcriptional level.

Conversely, we propose to test the effect of chronic THC administration in CB1^{E134G} mice and their WT littermates to investigate 1) behavioral tolerance to THC and 2) effect on CB1 protein (Tai et al. 2015). Based on the phenotype of CB1^{E134G} mice during exogenous overactivation states induced by cannabinoids, together with the downregulation of CB1 protein, we hypothesize that chronically administered THC will induce increased or early tolerance associated with increased downregulation of CB1 protein in mutants compared to their WT control. To this end, we propose to treat mice with THC at a high dose (10 or 15 mg/kg) once daily for one week and test them in the tetrad procedure. Alternatively, mice could be sacrificed at different time points to study the effect of chronic treatment on CB1 expression levels. If confirmed, this hypothesis will further support a role for PREG in maintaining steady state levels of CB1 upon receptor activation. Given that CB1^{E134G} mice are more sensitive to the effects of THC on mesoaccumbal dopamine transmission, a neurochemical signature associated with the reinforcing effects of drugs, and that tolerance is a risk factor participating to the etiology of addiction (Colizzi and Bhattacharyya 2018), this would imply that CB1^{E134G} mice are at greater risk for developing addiction-like features for cannabinoid drugs. This could be further tested in a mouse model of cannabinoid addiction that allows the study of motivation for cannabinoid drugs and compulsivity associated with use, despite aversive consequences (Cajiao-Manrique, Maldonado, and Martín-García 2023). In this line, CB1^{E134G} mice could

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therefore serve as a model to study endophenotypes associated with cannabis use disorders (CUD).

In addition, it would be interesting to investigate the effect of PREG at a more mechanistic level by examining the interactions of the mutant CB1^{E134G} and CB1^{WT} receptors with accessory and signaling proteins, including CRIP1A, GASP1, SGIP1, and β arrestins (Allyn C. Howlett, Blume, and Dalton 2010; Blume et al. 2015; 2017). Indeed, these proteins regulate CB1 trafficking and signaling by interacting with the receptor at the C-terminal domain (as described in the Introduction), which is in close proximity to the TMH1/TMH7/hx8 region that forms the PREG allosteric binding pocket. In particular, CRIP1A has been proposed to downregulate CB1 activity while reducing agonist-induced internalization by competing with β -arrestins for the C-terminal domain (Blume et al., 2015; 2017). Similarly, SGIP1 has been shown to reduce β -arrestins-mediated activation of the MAPK pathway (Hájková et al. 2016) which is known to be selectively modulated by PREG (Vallée et al. 2014). Therefore, it could be hypothesized that the absence of PREG within the TMH1/TMH7/hx8 region affects receptor interaction with accessory proteins, namely by favoring the recruitment and activity of β arrestins over CRIP1A and SGIP1. Conversely, GASP1 has been implicated in the termination of CB1-mediated signaling and the development of tolerance to cannabinoids by regulating receptor targeting to the lysosome for degradation (Tappe-Theodor et al. 2007). Thus, it could be speculated that GASP1 recruitment is enhanced in the absence of PREG interaction with the CB1 receptor, thereby contributing to the present downregulation of the CB1 proteins in CB1^{E134G} mutants to present receptor overactivation. Therefore we propose to investigate these interactions by coimmunoprecipitation studies in brain extract from CB1^{E134G} mice and WT controls at baseline and in response to CB1 agonist stimulation. In particular, changes between CB1^{E134G} and CB1^{WT} receptor revealed by coimmunoprecipitation studies would provide valuable information on the transducing effectors responsible for PREG-mediated effects.

5. Subcellular effect of endogenous PREG and implication for physiopathology

A key finding of the present work lies in the identification of differential consequences of the CB1^{E134G} mutation on THC-mediated effects. Indeed, we observed that THC-induced motor effects, particularly catalepsy, were enhanced in CB1^{E134G} mutant mice compared to their WT littermates. However, the beneficial antinociceptive effects of THC were unaffected in the mutants. These findings provide two important pieces of information. First, because of the generalized downregulation of the CB1^{E134G} mutant protein (discussed above), it can be argued that the increased sensitivity of the CB1^{E134G} mutant is not specifically related to the loss of PREG binding to the receptor, but rather is a consequence of the mutation *per se*

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leading to hyper-responsiveness to ligands (e.g., through changes in ligand binding or receptor coupling to signaling proteins). If this were the case, all THC-induced effects would have been affected to the same extent in mutant mice compared to WT. Thus, the lack of effect of the mutation on THC-induced analgesia provides a "negative control" in favor of increased sensitivity due to the absence of an allosteric modulator of PREG on CB1 receptor activity and further supports the hypothesis mentioned above that the CB1^{E134G} mutant protein is downregulated due to the absence of PREG binding.

Second, since THC-induced catalepsy and analgesia are mediated by the same neuronal circuits but at different subcellular levels ([Soria-Gomez et al. 2021](#)), it was promptly postulated that the endogenous PREG-CB1 regulation was also compartmentalized. Namely, we hypothesized that endogenously produced PREG would act preferentially on mitochondria-associated CB1 receptor (mtCB1) rather than plasma membrane-associated CB1 receptors (pmCB1) within D1 striatonigral circuits. This hypothesis was supported by subsequent experiments suggesting that endogenous PREG regulates the putative mechanism underlying THC-induced catalepsy, namely mitochondrial respiration within substantia nigra. Moreover, the absence of effects of exogenous PREG application on mitochondrial respiration in mutant but not WT extracts strengthen the former observation. In addition, since PREG is endogenously synthesized from cholesterol at mitochondria level, these results suggest that the (neuro) steroid acts preferentially within close proximity of its production site ([Miller and Auchus 2011](#)).

Still at the subcellular level, we further searched for the localization of CB1 receptors that may underlie the brain production of PREG and the PREG-CB1 negative allosteric feedback loop. For this purpose, we used the DN22-CB1-KI mutant mouse line ([Soria-Gomez et al. 2021](#)), in which CB1 receptors are deleted exclusively at the mitochondrial level ([Hebert-Chatelain et al. 2016](#); [Soria-Gomez et al. 2021](#)). We observed that THC-induced PREG production was not affected in DN22-CB1-KI mice compared to their WT controls, suggesting that PREG production is not supported by mtCB1, but likely by pmCB1 receptors. Thus, the pool of receptors involved in triggering the PREG-CB1 loop is likely different from those targeted by this negative feedback mechanism ([see Figure 31](#)). Overall, these data suggest that PREG functions as a molecular switch in order to bias cannabinoids effect towards therapeutically relevant analgesia, by inhibiting cataleptic-like side effect. This further points out the therapeutic potential of PREG and its synthetic derivatives in relation to cannabis use disorders ([Haney et al. 2023](#)).

It should be noted, however, that although PREG may preferentially affect mtCB1 activity due to local synthesis and proximity to sites of action, this does not exclude an effect

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of PREG on pools of receptor, under certain conditions and in other brain circuits. In particular, based on the strong downregulation of CB1 protein in CB1^{E134G} mutant mice and the putative interaction of PREG with CB1-associated regulatory proteins, it can be further hypothesized that PREG can regulate the activity of internalized CB1 receptors and their cellular fate (i.e. by promoting recycling over degradation), in addition to the mitochondrial receptor pool.

Thus, future investigations of the molecular mechanisms involved in the PREG-CB1 regulatory loop may help to specify which cellular and subcellular compartments are involved in the neuroprotective effect of the (neuro) steroid with respect to cannabinoid signaling. This may include the use of other CB1 genetic mouse models, such as DN22-CB1-KI mice, together with pharmacological and genetic tools for biological readout of cannabinoid signaling (e.g. synaptic transmission, signaling cascades, mitochondrial activity). These studies should include analyses under physiological and supraphysiological activation of the ECS, e.g. by exogenous cannabinoids and inhibitors of eCB degradation enzymes, to investigate not only which receptor pools but also which cannabinoid ligands are involved in the PREG-CB1 loop.

CONCLUSION

The aim of this study was to develop and characterize the novel CB1^{E134G} mutant mouse model to study the endogenous role of the (neuro)steroid PREG in the control of CB1 receptor-associated homeostatic load. Namely, our strategy was based on the genetic disruption of the allosteric binding pocket of PREG on the receptor in the TMH1/TMH8/Hx8 region. First, we report that CB1 protein is unexpectedly and drastically downregulated in the brain of naive CB1^{E134G} mutants compared to their WT littermates, suggesting that compensatory mechanisms occur to prevent CB1 activation overload. This compensatory mechanism is unlikely to be the result of structure-dependent changes associated with the relative position of the mutation within the receptor sequence, but rather the consequence of uncoupling between PREG and the CB1 receptor. Thus, we propose that endogenously produced PREG may regulate CB1 receptor dynamics by an as yet unknown mechanism, which may include modulation of receptor internalization and/or degradation. Accordingly, experiments will be performed in the near future to investigate this hypothesis.

In addition, we report that CB1^{E134G} mutant mice exhibit enhanced efficacy of exogenous cannabinoids at the behavioral, somatic, and neurochemical levels compared to WT mice. Interestingly, cannabinoid-induced analgesia was not affected, strongly suggesting that PREG preferentially regulates mitochondria-associated CB1 receptors that mediate the adverse motor effects of cannabinoids, but not plasma membrane receptors that mediate their beneficial analgesic effects. Consistently, we found that endogenously produced PREG can indeed regulate the putative mechanism linking subcellular CB1 signaling to catalepsy, namely mitochondrial respiration. Future studies will further investigate the functional relationships between PREG and subcellular CB1 signaling and their implications for physiopathology. Finally, although mutant mice did not show major changes in their behavior or physiology under basal conditions or experimental conditions known to recruit CB1 receptor activity, suggesting no role for PREG in modulating this activity, the lack of changes may be due to the aforementioned compensatory mechanisms or a true lack of function under such conditions. In this perspective, experiments based on concomitant pharmacological manipulation of eCB-mediated signaling and exploration of new experimental paradigms will address these questions. In conclusion, we propose CB1^{E134G} as a suitable model to study the endogenous modulatory functions of PREG targeting CB1 receptors, opening exciting research avenues in the understanding of the functioning of the ECS and extended molecular partners

CONCLUSION

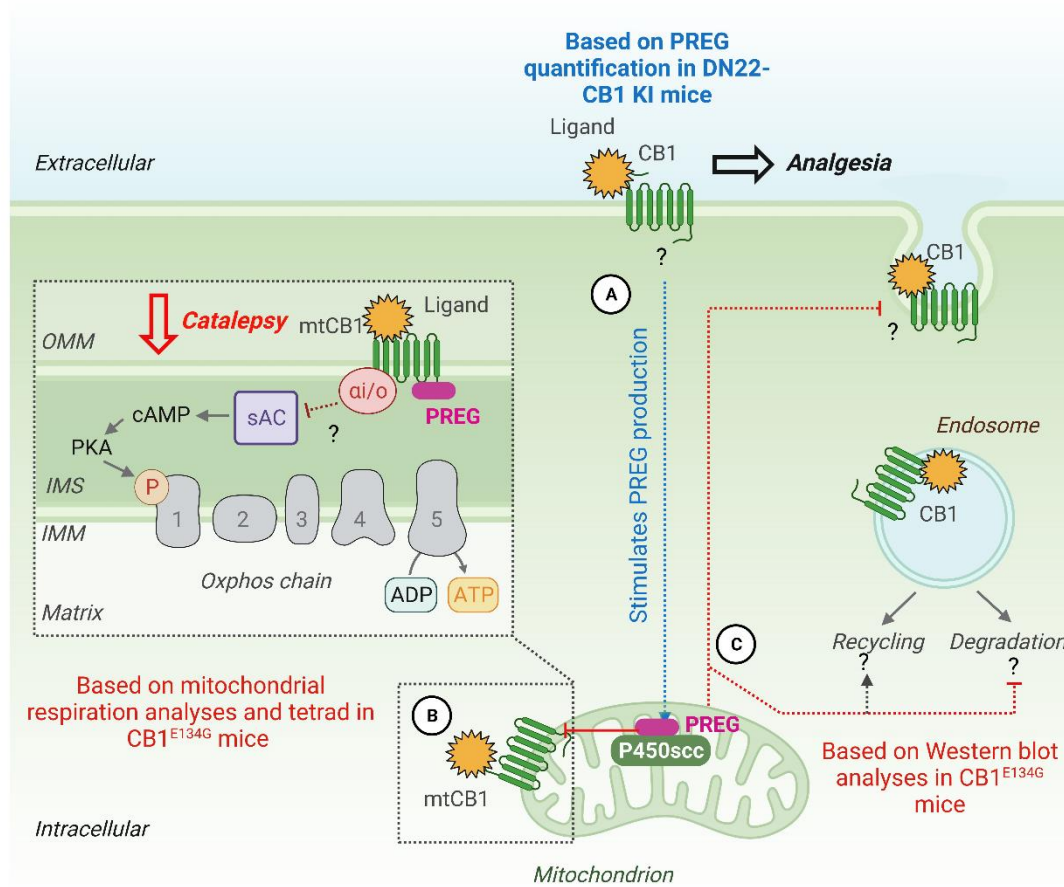


Figure 31: Schematic representation of the results.

A) Based on PREG quantification in the brain of DN22-CB1-KI mice lacking mitochondrial-associated receptors, we report that plasma membrane but not mitochondrial CB1 receptors are responsible for cannabinoid-induced PREG production via yet uncharacterized signaling events.

B) Based on tetrad and mitochondrial respiration experiments in CB1^{E134G} mice, we report that endogenously produced PREG can regulate mitochondrial receptor activity and thus protect against the cataleptic effect of cannabinoids. Conversely, PREG does not appear to regulate the activity of the plasma membrane pool of receptors mediating cannabinoid-induced analgesia.

C) Based on Western blot analyses of CB1 protein expression in the brain of CB1^{E134G} mice, we hypothesize that endogenous PREG may act on CB1 receptor dynamics, either by preventing receptor internalization upon ligand-induced activation AND/OR by promoting recycling to the membrane over degradation.

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CB1, cannabinoid type 1 receptor; IMM, inner mitochondrial membrane; IMS, intermembrane space; mtCB1, mitochondria-associated CB1; OMM, outer mitochondrial membrane; OxPhos, oxidation phosphorylation chain; P450scc, cholesterol side chain cleaving enzyme; PKA, protein kinase A; PREG, pregnenolone; sAC, soluble adenylyl cyclase.

LIST OF ABBREVIATIONS

2-AG: 2-arachidonoylglycerol

2-MAG: mono-acylglycerol

2-OG: 2-oleoylglycerol

7TM-GPCR: seven-transmembrane domain GPCR

ABHD12: α/β -hydrolase domains containing protein-12

ABHD6: α/β -hydrolase domains containing protein-6

AC: adenylate cyclase or adenylyl cyclase

ACAT : acyl-CoenzymeA cholestérol acyltransférase

ACBD3: acyl-coenzyme A binding domain containing 3

AEA: arachidonylethanolamine or anandamide

ALLO: 5 α -pregnan-3 α -ol-20-one or allopregnanolone,

AMG : aminoglutethimide

AMPA: (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate *receptor*

AR: androgen receptor

ATAD3A: ATPase family AAA domain-containing protein 3A

BBB: blood-brain barrier

cAMP: cyclic adenosine monophosphate

CaN: calcineurin

CB1: type-1 cannabinoid receptor

CB1-SSi: specific signaling inhibitors of CB1 receptors

CB2: type-2 cannabinoid receptor

CBD: cannabidiol

CBN: cannabinol

CCK: cholecystokinin

CDD: CDKL5 deficiency disorder

CE: cholesteryl ester

CIAPS: cannabis-induced acute psychotic-like state

CLAH: human congenital lipid adrenal hyperplasia

CLIP170: cytoplasmic linker protein 170

Cnr1: CB1 encoding gene

CNS: central nervous system

CNS: central nervous system

COPII: coat protein II

COX-2: cyclooxygenase-2 ()

CRAC: consensus cholesterol recognition amino acid sequence

LIST OF ABBREVIATIONS

CRIP1A: cannabinoid receptor interacting protein 1a
CS: conditioned stimulus
CSF: cerebrospinal fluid
CUD: cannabis use disorder
D2: dopamine type 2 receptor
DA: dopamine
DAG: 1,2-diacylglycerol
DAGL: diacylglycerol lipase
DHEA: dehydroepiandrosterone
DHEA: docosahexaenoyl-ethanolamide
DSE: depolarization-induced suppression of excitation
DSI: depolarization-induced suppression of inhibition
eCBome: endocannabinoidome or endocannabidome
eCBs: endocannabinoids
ECS: endocannabinoid system
EMT: endocannabinoid membrane transporter
EPEA: eicosapentaenoyl-ethanolamide
ER: endoplasmic reticulum
ERK: extracellular signal-regulated kinase ()
ER σ/β : estrogen receptors σ and β
FAAH: Fatty acid amide hydrolase
FABP: fatty acid binding protein
FDA: Food and Drug Administration
GABA_A: γ -aminobutyric acid type A
GASP: G-protein-associated sorting proteins
GDP: guanosine diphosphate
GIRK: G protein-coupled inwardly rectifying potassium
GIRK: G protein-coupled inwardly rectifying potassium
Gly: glycine
GPCR: G-protein coupled receptor
GPi : internal globus pallidus
GR: glucocorticoid receptor
GTP: guanosine triphosphate
hCB1Rp.E1.49G: mutant human CB1 receptor in which the glutamate (E) residue at position 1.49 has been replaced by a glycine (G)
hCB1wt: wild-type human CB1 receptor
HDL: high-density lipoprotein

LIST OF ABBREVIATIONS

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzymeA
HPA: hypothalamic-pituitary-adrenal
HSD: hydroxysteroid dehydrogenase
HSL: hormone-sensitive lipase
IMM: inner mitochondrial membrane
IP₃: inositol 1,4,5-trisphosphate
IPSC: spontaneous inhibitory postsynaptic potential
JNK: c-Jun N-terminal kinase
KO: knockout
LAL: lysosomal acid lipase
LDL: low-density lipoprotein
LEA: linoleoylethanolamide
LOXs: lipoxygenases
LPI: lysophosphatidylinositol
LTD: long-term depression
LXA4: lipoxin A
MAGL: hydrolase enzyme monoacylglycerol lipase
MAM: mitochondria-associated membrane
MAP2: microtubule-associated protein 2
MAPK: mitogen-activated protein kinase
MENTAL: MLN64 N-terminal domain
MePREG: 3 β -methoxypregnenolone or MAP4343
MTBP: microtubule-binding proteins
mtCB1: mitochondria-associated CB1 receptors
mTOR: *mammalian target of rapamycin*
NAAA: acylethanolamine-selective acid amidase
NAc: nucleus accumbens
N-acylethanolamine: NAE
NADA: N-arachidonoylethanolamine
NAM: negative allosteric modulator
NAPE: N-arachidonoyl-phosphatidylethanolamine
NAPE-PLD: NAPE-specific phospholipase D-type enzyme
NAT: N-acyltransferase
NMDA: N-methyl-D-aspartic acid
NPC: Niemann-Pick type C disease-related protein
OEA: oleoylethanolamide
OMM: outer mitochondrial membrane

LIST OF ABBREVIATIONS

ORP: oxysterol-binding protein
P450_{scc}: cholesterol side-chain cleavage enzyme cytochrome P450
PAM: positive allosteric modulators
PBR: peripheral benzodiazepine receptor
PCE: prenatal cannabis exposure
PE: phosphatidylethanolamine
PEA: palmitoylethanolamide
PIP₂: phosphatidylinositol bisphosphate (
PKA: protein kinase A
PLC β : phospholipase C β -type enzyme
PNS: peripheral nervous system
PPAR: peroxisome proliferator-activated receptor
PPI: prepulse inhibition
PREG: pregnenolone
PREG: pregnenolone
PREG-S: PREG-sulfate
PROG: progesterone
PTX: pertussis toxin
PUFAs: long-chain polyunsaturated fatty acids
RIM1 α : Rab3-interacting molecule-1 α
sAC: soluble adenylate cyclase
SAM: neutral/silent allosteric modulator
SCAP: SREBP cleavage activating protein
SCFAs: short-chain fatty acids (
SGIP1: Src homology3-domain growth factor receptor-bound 2-like endophilin inter-acting
protein 1
SR: SR141716 or rimonabant
SR-B1: plasma membrane scavenger receptor B1
SRE: steroid response element
SREBP: sterol regulatory element binding protein
sSgo1: Shugoshin 1
SSRI: selective serotonin reuptake inhibitor
StarD1: steroidogenic acute regulatory protein (StAR)
START: Regulatory Protein-related lipid transfer (
STD: short-term depression
THC: Δ^9 -tetrahydrocannabinol,
TRP: transient receptor potential channel

LIST OF ABBREVIATIONS

TSPO: translocator protein

US: unconditioned stimulus

VDAC-1: voltage-dependent anion channel 1

VDAC-2: voltage-dependent anion channel 2

VLDL: very low-density lipoprotein

VTA: ventral tegmental area

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APPENDIX I. STATISTICAL TABLE

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Figure	Comparisons	n	Normality test	Analysis	Factor analyzed	p -value	
13	B	ΔΔCl PFC - CB1WT vs CB1E134G (M+F)	16	YES	Unpaired t test	Genotype	t=0.148 df=30 p=0.88
		ΔΔCl NAC - CB1WT vs CB1E134G (M+F)	16	YES	Unpaired t test	Genotype	t=3.87 df=30 p<0.001
		ΔΔCl CPu - CB1WT vs CB1E134G (M+F)	16	YES	Unpaired t test	Genotype	t=4.64 df=30 p=0.001
		ΔΔCl HTHAL - CB1WT vs CB1E134G (M+F)	16	YES	Unpaired t test	Genotype	t=0.89 df=30 p=0.38
		ΔΔCl HPC - CB1WT vs CB1E134G (M+F)	16	YES	Unpaired t test	Genotype	t=0.479 df=30 p=0.64
	D	ΔΔCl CB - CB1WT vs CB1E134G (M+F)	16	YES	Unpaired t test	Genotype	t=1.89 df=30 p=0.07
		WB NAC - CB1WT vs CB1E134G (M)	7	YES	Unpaired t test	Genotype	t=3.772 df=12 p=0.003
		WB CPu - CB1WT vs CB1E134G (M)	7	YES	Unpaired t test	Genotype	t=3.382 df=12 p=0.005
		WB HPC - CB1WT vs CB1E134G (M)	6-7	NO	Mann Whitney test	Genotype	64, 27, 6 p=0.03
		WB VMB - CB1WT vs CB1E134G (M)	7	NO	Mann Whitney test	Genotype	69, 36, 8 p=0.04
	E	WB NAC - CB1WT vs CB1E134G (F)	7	YES	Unpaired t test	Genotype	t=5.33 df=12 p<0.001
		WB CPu - CB1WT vs CB1E134G (F)	7	YES	Unpaired t test	Genotype	t=2.729 df=12 p=0.02
		WB HPC - CB1WT vs CB1E134G (F)	3-7	YES	Unpaired t test	Genotype	t=3.544 df=7 p=0.009
		WB VMB - CB1WT vs CB1E134G (F)	6-7	YES	Unpaired t test	Genotype	t=3.088 df=11 p=0.01
	14	C	AEA PFC - CB1WT vs CB1E134G (M)	5-6	YES	Unpaired t test	Genotype
AEA NAC - CB1WT vs CB1E134G (M)			6	YES	Unpaired t test	Genotype	t=2.875 df=10 p=0.0165
AEA CPu - CB1WT vs CB1E134G (M)			6	YES	Unpaired t test	Genotype	t=0.01981 df=10 p=0.9846
AEA HTHAL - CB1WT vs CB1E134G (M)			5-6	YES	Unpaired t test	Genotype	t=2.148 df=9 p=0.0602
AEA HPC - CB1WT vs CB1E134G (M)			6	YES	Unpaired t test	Genotype	t=0.8961 df=10 p=0.3912
AEA Pla - CB1WT vs CB1E134G (M)			5-6	YES	Unpaired t test	Genotype	t=2.395 df=9 p=0.0402
D		2-AG PFC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.01317 df=10 p=0.9897
		2-AG NAC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.8818 df=10 p=0.3986
		2-AG CPu - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.6529 df=10 p=0.5285
		2-AG HTHAL - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=1.348 df=10 p=0.2073
		2-AG HPC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.1392 df=10 p=0.8921
		2-AG Pla - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.03482 df=10 p=0.9729
E		AEA PFC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.1478 df=10 p=0.8854
		AEA NAC - CB1WT vs CB1E134G (F)	5-6	YES	Unpaired t test	Genotype	t=0.6715 df=9 p=0.5187
		AEA CPu - CB1WT vs CB1E134G (F)	5-6	YES	Unpaired t test	Genotype	t=0.7911 df=9 p=0.4492
		AEA HTHAL - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.0771 df=10 p=0.9401
		AEA HPC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.2231 df=10 p=0.8279
		AEA Pla - CB1WT vs CB1E134G (F)	5-6	YES	Unpaired t test	Genotype	t=1.843 df=9 p=0.0984
F		2-AG PFC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.4112 df=10 p=0.6896
		2-AG NAC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.1797 df=10 p=0.861
		2-AG CPu - CB1WT vs CB1E134G (F)	5-6	YES	Unpaired t test	Genotype	t=0.9559 df=9 p=0.3641
		2-AG HTHAL - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.13 df=10 p=0.8991
		2-AG HPC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.01377 df=10 p=0.9893
		2-AG Pla - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.9496 df=10 p=0.3647
15	C	PREG PFC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.4389 df=10 P=0.6
		PREGNAC - CB1WT vs CB1E134G (M)	5-6	YES	Unpaired t test	Genotype	t=2.676 df=9 P=0.2
		PREG CPu - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=2.595 df=10 P=0.2
		PREG HTHAL - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=2.373 df=10 P=0.03
		PREG HPC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.2707 df=10 P=0.8
	D	DHEA PFC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.5093 df=10 P=0.6
		DHEA NAC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.4499 df=10 P=0.6
		DHEA CPu - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.4529 df=10 P=0.7
		DHEA HTHAL - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.7876 df=10 P=0.4
		DHEA HPC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=1.427 df=10 P=0.2
	E	17-OH-PROG PFC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=1.8 df=9 P=0.1
		17-OH-PROG NAC - CB1WT vs CB1E134G (M)	6	NO	Mann Whitney test	Genotype	46, 32, 11 P=0.3
		17-OH-PROG CPu - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.9402 df=10 P=0.4
		17-OH-PROG HTHAL - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	33, 45, 12 P=0.4
		17-OH-PROG HPC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.6459 df=10 P=0.5
	F	ALLOP PFC - CB1WT vs CB1E134G (M)	4-6	YES	Unpaired t test	Genotype	t=1.476 df=8 P=0.2
		ALLOP NAC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.4705 df=10 P=0.6
		ALLOP CPu - CB1WT vs CB1E134G (M)	6	NO	Mann Whitney test	Genotype	53, 25, 4 P=0.2
		ALLOP HTHAL - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0.4567 df=10 P=0.7
		ALLOP HPC - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=1.972 df=10 P=0.07
	G	PREG PFC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.3317 df=10 P=0.7
PREGNAC - CB1WT vs CB1E134G (F)		6	YES	Unpaired t test	Genotype	t=0.4371 df=10 P=0.7	
PREG CPu - CB1WT vs CB1E134G (F)		5-6	YES	Unpaired t test	Genotype	t=0.8459 df=9 P=0.4	
PREG HTHAL - CB1WT vs CB1E134G (F)		6	YES	Unpaired t test	Genotype	t=0.3185 df=10 P=0.7	
PREG HPC - CB1WT vs CB1E134G (F)		6	YES	Unpaired t test	Genotype	t=0.8216 df=10 P=0.4	
H	DHEA PFC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=1.121 df=10 P=0.3	
	DHEA NAC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.9071 df=10 P=0.4	
	DHEA CPu - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.8743 df=10 p=0.4	
	DHEA HTHAL - CB1WT vs CB1E134G (F)	5-6	YES	Unpaired t test	Genotype	t=0.1769 df=9 P=0.9	
	DHEA HPC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.3492 df=10 P=0.7	
I	17-OH-PROG PFC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=1.411 df=10 P=0.2	
	17-OH-PROG NAC - CB1WT vs CB1E134G (F)	6	NO	Mann Whitney test	Genotype	41, 37, 16 P=0.8	
	17-OH-PROG CPu - CB1WT vs CB1E134G (F)	4-5	YES	Unpaired t test	Genotype	t=0.1857 df=7 P=0.8	
	17-OH-PROG HTHAL - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=1.453 df=10 P=0.17	
	17-OH-PROG HPC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=0.04142 df=10 P=0.9	
J	ALLOP PFC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=2.76 df=10 P=0.02	
	ALLOP NAC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=2.705 df=10 P=0.02	
	ALLOP CPu - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=1.802 df=10 P=0.1	
	ALLOP HTHAL - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=2.196 df=10 P=0.05	
	ALLOP HPC - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=2.186 df=10 P=0.05	

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Figure	Comparisons	n	Normality test	Analysis	Factor analyzed	p-value	
16	A PREG plasma (ng/ml) - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0,4916 df=10 P=0.6	
	B DHEA plasma (ng/ml) - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0,5471 df=10 P=0.5	
	C 17-OH-PROG plasma (ng/ml) - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=1,059 df=10 P=0.3	
	D ALLOP plasma (ng/ml) - CB1WT vs CB1E134G (M)	6	YES	Unpaired t test	Genotype	t=0,4871 df=10 P=0.6	
	E PREG plasma (ng/ml) - CB1WT vs CB1E134G (F)	6	YES	Unpaired t test	Genotype	t=1,922 df=4 P=0.1	
	F DHEA plasma (ng/ml) - CB1WT vs CB1E134G (F)	3	YES	Unpaired t test	Genotype	t=1,08 df=4 P=0.3	
	G 17-OH-PROG plasma (ng/ml) - CB1WT vs CB1E134G (F)	3	NO	Mann Whitney test	Genotype	10, 11, 4 P>0.99	
	H ALLOP plasma (ng/ml) - CB1WT vs CB1E134G (F)	3	YES	Unpaired t test	Genotype	t=0,5966 df=4 P=0.6	
17	A NOR Recognition index - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=1,684 df=24 p=0.11	
	B NOR Tot. Expl. Time (s) - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=0,073 df=24 p=0.94	
	C NOR Recognition index - CB1WT vs CB1E134G (F)	9-10	YES	Unpaired t test	Genotype	t=0,5229 df=17 p=0.61	
	D NOR Tot. Expl. Time (s) - CB1WT vs CB1E134G (F)	9-10	YES	Unpaired t test	Genotype	t=0,4385 df=17 p=0.6666	
	E YM spontaneous alternation - CB1WT vs CB1E134G (M)	9-10	YES	Unpaired t test	Genotype	t=0,19 df=17 p=0.85	
	F YM Nb arm entries - CB1WT vs CB1E134G (M)	9-10	YES	Unpaired t test	Genotype	t=0,01404 df=17 p=0.989	
	G YM spontaneous alternation - CB1WT vs CB1E134G (F)	9-10	YES	Unpaired t test	Genotype	t=0,01308 df=17 p=0.99	
	H YM Nb arm entries - CB1WT vs CB1E134G (F)	10	YES	Unpaired t test	Genotype	t=1,401 df=18 p=0.1783	
	I YM ITI 1h distance ratio - CB1WT vs CB1E134G (M)	10-11	YES	Unpaired t test	Genotype	t=1,505 df=19 p=0.1489	
	J YM ITI 1h Nb arm entries - CB1WT vs CB1E134G (M)	10-11	NO	Mann Whitney test	Genotype	101, 130, 46 p=0.5424	
	K YM ITI 1h distance ratio - CB1WT vs CB1E134G (F)	9-12	YES	Unpaired t test	Genotype	t=0,09094 df=19 p=0.9285	
	L YM ITI 1h Nb arm entries - CB1WT vs CB1E134G (F)	9-12	YES	Unpaired t test	Genotype	t=0,07955 df=19 p=0.9374	
18	A EPM % time in open arms - CB1WT vs CB1E134G (M)	11-12	NO	Mann Whitney test	Genotype	121, 155, 55 p=0.53	
	B EPM Tot. Nb arm entries - CB1WT vs CB1E134G (M)	11-12	YES	Unpaired t test	Genotype	t=0,67 df=21 0.51	
	C EPM % time in open arms - CB1WT vs CB1E134G (F)	12	NO	Mann Whitney test	Genotype	131, 169, 53 p=0.29	
	D EPM Tot. Nb arm entries - CB1WT vs CB1E134G (F)	12	YES	Unpaired t test	Genotype	t=1,948 df=22 p=0.06	
	E EZM % time in open areas - CB1WT vs CB1E134G (M)	11	NO	Mann Whitney test	Genotype	131, 122, 56 p=0.8	
	F EZM Tot. distance - CB1WT vs CB1E134G (M)	11	YES	Unpaired t test	Genotype	t=1,184 df=20 p=0.25	
	G EZM % time in open areas - CB1WT vs CB1E134G (F)	11	YES	Unpaired t test	Genotype	t=0,09822 df=20 p=0.92	
	H EZM Tot. distance - CB1WT vs CB1E134G (F)	11	YES	Unpaired t test	Genotype	t=1,715 df=20 p=0.1	
	I OF % time in center - CB1WT vs CB1E134G (M)	10-12	YES	Unpaired t test	Genotype	t=1,334 df=20 p=0.1972	
	J OF Tot. distance - CB1WT vs CB1E134G (M)	11-12	YES	Unpaired t test	Genotype	t=0,8539 df=21 p=0.4028	
	K OF % time in center - CB1WT vs CB1E134G (F)	12	YES	Unpaired t test	Genotype	t=0,1434 df=22 p=0.8873	
	L OF Tot. distance - CB1WT vs CB1E134G (F)	10-11	YES	Unpaired t test	Genotype	t=1,821 df=19 p=0.0844	
	N SP Total fluid intake - CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Interaction Drink Genotype	F (1, 16) = 1,386 P=0,26 F (1, 16) = 104,9 P<0,001 F (1, 16) = 0,02324 P=0,88	
	P SP Total fluid intake - CB1WT vs CB1E134G (F)	8-10	YES	2way ANOVA	Interaction Drink Genotype	F (1, 16) = 0,8036 P=0,38 F (1, 16) = 161,5 P<0,001 F (1, 16) = 0,05155 P=0,82	
	19	Cell count DAPI - AraC treatment	3	Yes	1way ANOVA	Treatment	0,3606 (3, 8) P<0,001
		Cell count MAP2- AraC treatment	3	No	Kruskal-Wallis	Treatment	7.2 P<0,001
Cell count GFAP - AraC treatment		3	No	Kruskal-Wallis	Treatment	10.4 P<0,001	
F Western Blot CB1 - AraC Treatment - CB1WT vs CB1E134G		6	YES	2way ANOVA	Interaction Treatment Genotype	F (1, 20) = 0,001878 P=0,97 F (1, 20) = 0,001878 P=0,97 F (1, 20) = 65,4 P<0,001	

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Figure	Comparisons	n	Normality test	Analysis	Factor analyzed	p-value	
20	A	Hy polocomotion (%veh) - CB1WT (M)	8-10	NO	Kruskal-Wallis	Treatment	41.7 P<0,0001
		Hy polocomdion (%veh) - CB1E134G (M)	8-10	NO	Kruskal-Wallis	Treatment	37.8 P<0,0001
	B	Hy pothermia (delta) - CB1WT (M)	8-10	N/A	Kruskal-Wallis	Treatment	46 P<0,0001
		Hy pothermia (delta) - CB1E134G (M)	9-10	N/A	Kruskal-Wallis	Treatment	35.6 P<0,0001
	C	Catalepsy (s) - CB1WT (M)	8-10	N/A	Kruskal-Wallis	Treatment	41.4 P<0,0001
		Catalepsy (s) - CB1E134G (M)	9-10	N/A	Kruskal-Wallis	Treatment	37.8 P<0,0001
	D	Analgesia (s) - CB1WT (M)	8-10	N/A	Kruskal-Wallis	Treatment	32.8 P<0,0001
		Analgesia (s) - CB1E134G (M)	9-10	N/A	Kruskal-Wallis	Treatment	32.6 P<0,0001
	E	Hy polocomotion (%veh) - CB1WT (F)	8-10	NO	Kruskal-Wallis	Treatment	44.7 P<0,0001
		Hy polocomotion (%veh) - CB1E134G (F)	8-10	NO	Kruskal-Wallis	Treatment	35.8 P<0,0001
	F	Hy pothermia (delta) - CB1WT (F)	7-10	N/A	Kruskal-Wallis	Treatment	43 P<0,0001
		Hy pothermia (delta) - CB1E134G (F)	8-10	N/A	Kruskal-Wallis	Treatment	34.1 P<0,0001
	G	Catalepsy (s) - CB1WT (F)	8-10	N/A	Kruskal-Wallis	Treatment	40.7 P<0,0001
		Catalepsy (s) - CB1E134G (F)	7-10	N/A	Kruskal-Wallis	Treatment	31.6 P<0,0001
	H	Analgesia (s) - CB1WT (F)	9-10	N/A	Kruskal-Wallis	Treatment	35.6 P<0,0001
		Analgesia (s) - CB1E134G (F)	8-10	N/A	Kruskal-Wallis	Treatment	28.7 P<0,0001
21	A	PREG (ng/g) - CB1WT vs CB1E134G (M)	6-8	YES	2way ANOVA	Interaction Treatment Genotype	F (3, 42) = 0,5751 P=0,6346 F (3, 42) = 5,502 P=0,0028 F (1, 42) = 0,4988 P=0,4839
	B	DHEA (ng/g) - CB1WT vs CB1E134G (M)	5-9	YES	2way ANOVA	Interaction Treatment Genotype	F (3, 53) = 0,86 P=0,4677 F (3, 53) = 1,907 P=0,1397 F (1, 53) = 0,1316 P=0,7182
	C	17-OH-PROG (ng/g) - CB1WT vs CB1E134G (M)	5-9	YES	2way ANOVA	Interaction Treatment Genotype	F (3, 51) = 0,3988 P=0,7544 F (3, 51) = 0,06342 P=0,9789 F (1, 51) = 1,025 P=0,3162
	D	ALLOP (ng/g) - CB1WT vs CB1E134G (M)	5-9	YES	2way ANOVA	Interaction Treatment Genotype	F (3, 52) = 1,114 P=0,3519 F (3, 52) = 3,346 P=0,0260 F (1, 52) = 0,1939 P=0,6616
	E	PREG (ng/g) - CB1WT vs CB1E134G (F)	7-10	YES	2way ANOVA	Interaction Treatment Genotype	F (3, 61) = 0,9515 P=0,4215 F (3, 61) = 62,07 P<0,0001 F (1, 61) = 0,9114 P=0,3435
	F	DHEA (ng/g) - CB1WT vs CB1E134G (F)	7-10	YES	2way ANOVA	Interaction Treatment Genotype	F (3, 61) = 0,542 P=0,6554 F (3, 61) = 1,639 P=0,1896 F (1, 61) = 5,02 P=0,0287
	G	17-OH-PROG (ng/g) - CB1WT vs CB1E134G (F)	8-10	YES	2way ANOVA	Interaction Treatment Genotype	F (3, 58) = 0,7732 P=0,5137 F (3, 58) = 0,9453 P=0,4248 F (1, 58) = 0,1955 P=0,6600
	H	ALLOP (ng/g) - CB1WT vs CB1E134G (F)	8-10	YES	2way ANOVA	Interaction Treatment Genotype	F (3, 61) = 0,4871 P=0,6925 F (3, 61) = 0,1515 P=0,9283 F (1, 61) = 0,1988 P=0,6572
22	C	OCR (%vehWIN) - CB1WT vs CB1E134G	6-7	YES	2way ANOVA	Interaction Treatment Genotype	F (1, 22) = 3,49 P=0,08 F (1, 22) = 15,46 P<0,001 F (1, 22) = 18,94 P<0,001
	D	OCR (%vehWIN) + PREG - CB1WT vs CB1E134G	5-6	YES	2way ANOVA	Interaction Treatment Genotype	F (1, 18) = 13,04 P=0,002 F (1, 18) = 36,88 P<0,001 F (1, 18) = 4,655 P=0,04

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Figure	Comparisons	n	Normality test	Analysis	Factor analyzed	p-value
23	A PREG (ng/g) - CB1WT vs DN22-CB1-KI (M)	7-11	YES	2way ANOVA	Interaction	F (1, 33) = 0,0295 P=0,8646
					Treatment	F (1, 33) = 15,5 P=0,0004
					Genotype	F (1, 33) = 1,74 P=0,1958
	B DHEA (ng/g) - CB1WT vs DN22-CB1-KI (M)	9-11	YES	2way ANOVA	Interaction	F (1, 36) = 0,0525 P=0,82
Treatment					F (1, 36) = 1,49 P=0,23	
Genotype					F (1, 36) = 1,02 P=0,32	
C 17-OH-PROG (ng/g) - CB1WT vs DN22-CB1-KI (M)	8-11	YES	2way ANOVA	Interaction	F (1, 33) = 0,00443 P=0,95	
				Treatment	F (1, 33) = 0,326 P=0,57	
				Genotype	F (1, 33) = 0,238 P=0,63	
D ALLOP (ng/g) - CB1WT vs DN22-CB1-KI (M)	8-11	YES	2way ANOVA	Interaction	F (1, 35) = 1,2 P=0,28	
				Treatment	F (1, 35) = 0,92 P=0,34	
				Genotype	F (1, 35) = 0,68 P=0,42	
24	A Basal DA (nM) - CB1WT vs CB1E134G (M)	13	YES	Unpaired t test	Genotype	t=0,2127 df=24 P=0,8
	E AUC DA - CB1WT vs CB1E134G (M)	5	YES	2way ANOVA	Interaction	F (2, 23) = 5,898 P=0,009
					Treatment	F (2, 23) = 83,38 P<0,001
	F Basal DA (nM) - CB1WT vs CB1E134G (M)	10	YES	Unpaired t test	Genotype	F (1, 23) = 0,1037 P=0,92 t=0,6114 df=18 P=0,5
H AUC DA - CB1WT vs CB1E134G (M)	4-6	YES	2way ANOVA	Interaction	F (1, 16) = 34,12 P<0,001	
				Treatment	F (1, 16) = 17,11 P<0,001	
A	Body weight (g) - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	F (1, 16) = 22,78 P<0,001 t=0,57 df=24 P=0,57
	Lean mass %BW - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=0,62 df=24 P=0,54
	Fat mass %BW - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=0,6092 df=24 P=0,54
B	Food intake (g) 24h - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (23, 408) = 1,176 P=0,2622
					Time	F (23, 408) = 9,221 P<0,0001
	Genotype	F (1, 408) = 0,212 P=0,6455				
Food intake (g) / period - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (2, 34) = 1,35 P=0,27	
				Day period	F (2, 34) = 375 P<0,001	
Genotype	F (1, 17) = 0,475 P=0,50					
C	Nocturnal meal nb - CB1WT vs CB1E134G (M)	9-10	NO	Mann Whitney test	Genotype	109, 91, 36 P=0,49
	Nocturnal meal size (g) - CB1WT vs CB1E134G (M)	9-10	NO	Mann Whitney test	Genotype	93, 96, 38 P=0,61
	Nocturnal meal duration (min) - CB1WT vs CB1E134G (M)	9-10	YES	Unpaired t test	Genotype	t=1,752 df=17 P=0,10
D	Water intake (g) 24h - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (23, 408) = 0,4517 P=0,9876
					Time	F (23, 408) = 13,03 P<0,0001
	Genotype	F (1, 408) = 0,2294 P=0,6322				
Water intake (g) / period - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (2, 34) = 0,205 P=0,82	
				Day period	F (2, 34) = 298 P<0,001	
Genotype	F (1, 17) = 0,102 P=0,75					
E	Activity count/h 24h - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (23, 391) = 1,418 P=0,0968
					Time	F (23, 391) = 19,31 P<0,0001
	Genotype	F (1, 17) = 0,111 P=0,7431				
Activity count/h / period - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (2, 34) = 0,751 P=0,48	
				Day period	F (2, 34) = 113 P<0,001	
Genotype	F (1, 17) = 0,111 P=0,74					
F	RER 24h - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (23, 391) = 0,2849 P=0,9996
					Time	F (23, 391) = 35,17 P<0,0001
	Genotype	F (1, 17) = 0,00995 P=0,9217				
RER / period - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (2, 34) = 0,0223 P=0,98	
				Day period	F (2, 34) = 66,9 P<0,001	
Genotype	F (1, 17) = 0,00995 P=0,92					
G	Glycaemia mg/dL 15h fasting - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=2,065 df=24 P=0,0499
H	GTT Glycaemia mg/dL - CB1WT vs CB1E134G (M)	12-14	YES	2way ANOVA	Interaction	F (6, 144) = 0,662 P=0,6805
					Time	F (6, 144) = 128 P<0,0001
	Genotype	F (1, 24) = 1,1 P=0,3054				
AUC GTT - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=0,9197 df=24 P=0,3669	
				Interaction	F (6, 144) = 2,52 P=0,0237	
GTT % basal- CB1WT vs CB1E134G (M)	12-14	YES	2way ANOVA	Time	F (6, 144) = 127 P<0,0001	
				Genotype	F (1, 24) = 2,11 P=0,1592	
I	GSIS Insulin ng/ml - CB1WT vs CB1E134G (M)	10-13	YES	2way ANOVA	Interaction	F (1, 42) = 0,1999 P=0,6571
					Time	F (1, 42) = 84,15 P<0,0001
	Genotype	F (1, 42) = 0,03343 P=0,8558				
J	Glycaemia mg/dL 6h fasting - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=1,84 df=24 P=0,08
K	ITT Glycaemia mg/dL - CB1WT vs CB1E134G (M)	12-14	YES	2way ANOVA	Interaction	F (6, 144) = 0,704 P=0,6470
					Time	F (6, 144) = 22,2 P<0,0001
	Genotype	F (1, 24) = 4,26 P=0,0500				
AUC ITT - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=1,936 df=24 P=0,06	
				Interaction	F (6, 144) = 0,7237 P=0,6312	
ITT % basal- CB1WT vs CB1E134G (M)	12-14	YES	2way ANOVA	Time	F (6, 144) = 22,13 P<0,0001	
				Genotype	F (1, 24) = 0,5266 P=0,4750	
L	KITT %/min - CB1WT vs CB1E134G (M)	12-14	YES	Unpaired t test	Genotype	t=1,258 df=24 P=0,2207

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Figure	Comparisons	n	Normality test	Analysis	Factor analyzed	p-value				
27	A	Food intake (g) fasting 24h - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (23, 391) = 0,876 P=0,6317			
						Time	F (23, 391) = 21,25 P<0,0001			
						Genotype	F (1, 17) = 1,085 P=0,3123			
	A	Food intake (g) fasting /period - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (2, 32) = 0,391 P=0,68			
						Day period	F (2, 32) = 2226 P<0,001			
	B	Nocturnal meal nb fasting - CB1WT vs CB1E134G (M)	9-10	NO	Mann Whitney test	Genotype	t=0,4376 df=17 P=0,66			
		Nocturnal meal size (g) fasting - CB1WT vs CB1E134G (M)	9-10	NO	Mann Whitney test	Genotype	t=0,4028 df=17 P=0,69			
		Nocturnal meal duration (min) fasting - CB1WT vs CB1E134G (M)	9-10	YES	Unpaired t test	Genotype	t=0,9251 df=17 P=0,36			
	C	Activity count/h 24h fasting- CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Interaction	F (23, 391) = 1,164 P=0,2740			
						Time	F (23, 391) = 13,5 P<0,0001			
	C	Activity count/h fasting / period - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Genotype	F (1, 17) = 2,336e-001 P=0,9962			
						Interaction	F (2, 34) = 0,362 P=0,70			
	D	RER 24h fasting - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Day period	F (2, 34) = 99,2 P<0,001			
						Genotype	F (1, 17) = 2,34e-005 P>0,99			
						Interaction	F (23, 368) = 0,7503 P=0,7921			
	D	RER / period fasting - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Time	F (23, 368) = 242,8 P<0,0001			
						Genotype	F (1, 16) = 0,4466 P=0,5135			
						Interaction	F (2, 34) = 0,717 P=0,50			
	E	Food intake (g) HFD 24h - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Day period	F (2, 34) = 1,7 P=0,20			
						Genotype	F (1, 17) = 1,24 P=0,28			
Interaction						F (23, 322) = 1,602 P=0,0412				
E	Food intake (g) HFD /period - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Time	F (23, 322) = 9,188 P<0,0001				
					Genotype	F (1, 14) = 4,814 P=0,0456				
					Interaction	F (2, 28) = 1,94 P=0,16				
F	Nocturnal meal nb HFD - CB1WT vs CB1E134G (M)	9-10	NO	Mann Whitney test	Day period	F (2, 28) = 395 P<0,001				
					Genotype	F (1, 14) = 4,81 P=0,05				
					Interaction	t=1,584 df=15 P=0,13				
F	Nocturnal meal size (g) HFD - CB1WT vs CB1E134G (M)	9-10	NO	Mann Whitney test	Genotype	t=2,853 df=14 P=0,01				
					Nocturnal meal duration (min) HFD - CB1WT vs CB1E134G (M)	9-10	YES	Unpaired t test	Genotype	t=0,3646 df=15 P=0,72
									Interaction	F (23, 368) = 1,829 P=0,0120
G	Activity count/h 24h HFD- CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Time	F (23, 368) = 18,54 P<0,0001				
					Genotype	F (1, 16) = 1,788 P=0,1999				
					Interaction	F (2, 32) = 2,58 P=0,09				
G	Activity count/h HFD / period - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Day period	F (2, 32) = 137 P<0,001				
					Genotype	F (1, 16) = 1,79 P=0,20				
					Interaction	F (23, 368) = 1,048 P=0,4034				
H	RER 24h HFD - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Time	F (23, 368) = 31,24 P<0,0001				
					Genotype	F (1, 16) = 2,592 P=0,1270				
					Interaction	F (2, 32) = 2,87 P=0,07				
H	RER / period HFD - CB1WT vs CB1E134G (M)	9-10	YES	2way ANOVA	Day period	F (2, 32) = 8,55 P=0,001				
					Genotype	F (1, 16) = 2,63 P=0,12				

APPENDIX I. STATISTICAL TABLE

Figure	Comparisons	n	Normality test	Analysis	Factor analyzed	p-value	
28	A	Body weight change HFD- CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Interaction	F (18, 288) = 2,74 P<0,001
						Time	F (18, 288) = 512 P<0,001
						Genotype	F (1, 16) = 0,0685 P=0,80
	B	Fat mass %BW pre post HFD- CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Interaction	F (1, 16) = 1,51 P=0,24
						Time	F (1, 16) = 604 P<0,001
						Genotype	F (1, 16) = 0,885 P=0,36
	C	Lean mass %BW pre post HFD- CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Interaction	F (1, 16) = 1,72 P=0,21
						Time	F (1, 16) = 388 P<0,001
						Genotype	F (1, 16) = 1,09 P=0,31
	D	Cumulative food intake (g) HFD- CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Interaction	F (15, 240) = 0,524 P=0,93
	E	GTT Glycaemia mg/dL - CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Time	F (15, 240) = 40,10 P<0,001
						Genotype	F (1, 16) = 0,158 P=0,70
						Interaction	F (1, 16) = 0,158 P=0,70
	F	GTT % basal HFD - CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Genotype	t=3,041 df=16 P=0,007
Interaction						F (6, 96) = 1,36 P=0,2389	
Time						F (6, 96) = 44,9 P<0,0001	
G	GTT % basal HFD - CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Genotype	F (1, 16) = 5,69 P=0,0298	
					Interaction	t=2,27 df=16 P=0,03	
					Time	F (6, 96) = 1,05 P=0,3980	
H	GSIS Insulin ng/ml - CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Time	F (6, 96) = 49,6 P<0,0001	
					Genotype	F (1, 16) = 0,0849 P=0,7746	
					Interaction	F (1, 15) = 0,8724 P=0,37	
I	Glycaemia mg/dL 6h fasting HFD - CB1WT vs CB1E134G (M)	8-10	YES	Unpaired t test	Genotype	F (1, 15) = 13,92 P=0,002	
					Interaction	F (1, 15) = 0,203 P=0,66	
					Time	t=3,024 df=16 P=0,008	
J	ITT Glycaemia mg/dL HFD - CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Interaction	F (9, 144) = 3,35 P=0,0009	
					Time	F (9, 144) = 72 P<0,0001	
					Genotype	F (1, 16) = 6,83 P=0,0188	
K	AUC ITT HFD - CB1WT vs CB1E134G (M)	8-10	YES	Unpaired t test	Genotype	t=2,342 df=16 P=0,03	
					Interaction	F (9, 144) = 1,111 P=0,3585	
					Time	F (9, 144) = 69,55 P<0,0001	
L	ITT % basal HFD - CB1WT vs CB1E134G (M)	8-10	YES	2way ANOVA	Genotype	F (1, 16) = 0,1172 P=0,7366	
					Interaction	t=1,627 df=15 P=0,12	
					Time	F (1, 16) = 0,1172 P=0,7366	
29	B	AEA eCBs inhibitors - C57BL6/N	7-8	YES	1way ANOVA	Treatment	0,7086 (3, 26) P<0,0001
	C	2-AG eCBs inhibitors - C57BL6/N	7-8	NO	Kruskal-Wallis test	Treatment	23,06 P<0,0001
	D	PREG eCBs inhibitors - C57BL6/N	7-8	YES	1way ANOVA	Treatment	0,458 (3, 26) P=0,23
	E	Linear regression PREG - AEA - C57BL6/N	7-8			Deviation 0	1,515 1,28 P=0,23
	F	Linear regression PREG - 2-AG - C57BL6/N	7-8			Deviation 0	7,535 1,29 P=0,01
	30	A	Basal CORT - CB1WT vs CB1E134G (M)	5-6	YES	Unpaired t test	Genotype
Interaction							F (2, 30) = 0,4496 P=0,6421
Time							F (2, 30) = 2,297 P=0,1179
B		CORT kinetics 10-120 - CB1WT vs CB1E134G (M)	8-9	YES	2way ANOVA	Genotype	F (1, 15) = 0,7265 P=0,4074
						Interaction	t=1,735 df=10 p=0,1134
						Time	t=1,735 df=10 p=0,1134
C		CORT delta +10/-30 - CB1WT vs CB1E134G (M)	5-7	YES	Unpaired t test	Genotype	74, 46, 25 p=0,8639
						Interaction	F (2, 34) = 2,173 P=0,1293
						Time	F (2, 34) = 5,602 P=0,0079
D		Basal CORT - CB1WT vs CB1E134G (F)	6-9	NO	Mann Whitney test	Genotype	F (1, 17) = 0,09259 P=0,7646
						Interaction	t=1,249 df=13 p=0,2338
						Time	t=1,249 df=13 p=0,2338
E		CORT kinetics 10-120 - CB1WT vs CB1E134G (F)	8-9	YES	2way ANOVA	Genotype	t=0,1503 df=21 p=0,88
						Interaction	t=0,1503 df=21 p=0,88
	Time					t=0,1503 df=21 p=0,88	
F	Adrenals g - CB1WT vs CB1E134G (F)	11-12	YES	Unpaired t test	Genotype	t=0,1503 df=21 p=0,88	
					Interaction	t=1,552 df=22 P=0,13	
					Time	t=1,552 df=22 P=0,13	
G	% Freezing to the tone D1 - CB1WT vs CB1E134G (M)	12	YES	Unpaired t test	Genotype	t=1,552 df=22 P=0,13	
					Interaction	F (4, 88) = 1,91 P=0,12	
					Time	F (4, 88) = 5,852 P<0,001	
H	Extinction Freezing %D1- CB1WT vs CB1E134G (M)	12	YES	2way ANOVA	Genotype	F (1, 22) = 1,026 P=0,32	
					Interaction	t=0,5184 df=20 p=0,61	
					Time	t=0,5184 df=20 p=0,61	
I	Adrenals g - CB1WT vs CB1E134G (M)	10-12	YES	Unpaired t test	Genotype	t=0,956 df=21 P=0,35	
					Interaction	t=0,956 df=21 P=0,35	
					Time	t=0,956 df=21 P=0,35	
J	% Freezing to the tone D1 - CB1WT vs CB1E134G (F)	12	YES	Unpaired t test	Genotype	t=0,956 df=21 P=0,35	
					Interaction	F (4, 80) = 0,5457 P=0,70	
					Time	F (4, 80) = 4,436 P=0,003	
K	Extinction Freezing %D1- CB1WT vs CB1E134G (F)	12	YES	2way ANOVA	Genotype	F (1, 20) = 0,4522 P=0,51	
					Interaction	F (1, 20) = 0,4522 P=0,51	
					Time	F (1, 20) = 0,4522 P=0,51	

APPENDIX II. Raux et al., J Neuroendocrinol. 2021

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INVITED REVIEW

New perspectives on the role of the neurosteroid pregnenolone as an endogenous regulator of type-1 cannabinoid receptor (CB1R) activity and function

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Abstract

Pregnenolone is a steroid with specific characteristics, being the first steroid to be synthesised from cholesterol at all sites of steroidogenesis, including the brain. For many years, pregnenolone was defined as an inactive precursor of all steroids because no specific target had been discovered. However, over the last decade, it has become a steroid of interest because it has been recognised as being a biomarker for brain-related disorders through the development of metabolomic approaches and advanced analytical methods. In addition, physiological roles for pregnenolone emerged when specific targets were discovered. In this review, we highlight the discovery of the selective interaction of pregnenolone with the type-1 cannabinoid receptor (CB1R). After describing the specific characteristic of CB1Rs, we discuss the newly discovered mechanisms of their regulation by pregnenolone. In particular, we describe the action of pregnenolone as a negative allosteric modulator and a specific signalling inhibitor of the CB1R. These particular characteristics of pregnenolone provide a great strategic opportunity for therapeutic development in CB1-related disorders. Finally, we outline new perspectives using innovative genetic tools for the discovery of original regulatory mechanisms of pregnenolone on CB1-related functions.

KEYWORDS

allosteric regulation, biased signalling, GPCR, neurosteroids, pregnenolone, type-1 cannabinoid receptor (CB1R)

1 | INTRODUCTION

Pregnenolone is defined as the "mother hormone", being the precursor of all the so-called steroid hormones, including progesterone and hydroxylated metabolites (e.g., allopregnanolone), dehydroepiandrosterone (DHEA), corticoids, and the sex hormones (androgens and oestrogens) (Figure 1). The biosynthesis of specific steroids matches the repertoire of expressed steroidogenic enzymes in a steroidogenic tissue or cell.¹

De novo synthesis of pregnenolone from cholesterol and its downstream steroid hormones is a typical feature of all steroidogenic

sources, including the classical steroidogenic tissues, such as the gonads, adrenals and placenta. However, other tissues can also be classified as steroidogenic tissues because they express the enzyme (CYP11A1) at least, which initiates steroid synthesis by converting cholesterol to pregnenolone. This is the case for adipocytes,^{2,3} gastrointestinal tissue,^{4,5} retina⁶ and skin with subcutis.⁷ Furthermore, it has been well documented that the brain is a steroidogenic tissue, for which the concept of neurosteroids has emerged as one of the most innovative and pioneering in the field of neuroendocrinology.

The initial discovery of the neurosteroid concept originated from the detection of steroids in the rodent brain with no peripheral sources

of steroids.^{8,9} This concept was further validated by studies on the expression of steroidogenic enzymes for synthesis and metabolism, as well as on neurosteroid content in brain tissues and cells. In this context, de novo steroid synthesis in the brain has been reported to be well conserved across species, from amphibians to mammals, including humans.¹⁰⁻¹⁵ In addition, the mitochondria in the brain, as in the other steroidogenic cells, the adrenals, gonads and placenta, comprise important delivery sites for intracellular cholesterol and cholesterol transport to the mitochondrial inner membrane, which is the rate-limiting step for steroid synthesis, with the production of pregnenolone being critical for the subsequent production of all steroid hormones.^{16,17}

It has been suggested that steroids act as neuromodulators to regulate brain-related functions.^{18,19} Further challenges lie ahead in proving these regulations, such as detecting neurosteroids in discrete brain areas linked to brain function,²⁰ as well as fully characterising the effect of steroids on specific receptor functions in the brain.²¹ In particular, neurosteroids could bind as neuromodulators to G-protein-coupled receptors (GPCRs), which are most commonly involved in neuromodulation and constitute the largest, most ubiquitous family of membrane receptors. Here, we review recent findings concerning the discovery of pregnenolone function as a negative allosteric modulator of the type-1 cannabinoid receptor (CB1R), the major GPCR in the mammalian brain, as well as the subsequent regulation of the homeostasis of the endocannabinoid system (ECS), providing a new translational potential for pregnenolone.²²

2 | PREGNENOLONE: A NEUROSTEROID WITH UNIQUE CHARACTERISTICS

2.1 | Neurosteroids synthesis: an overview

2.1.1 | Mitochondrial production of pregnenolone from cholesterol

Cholesterol is an essential and tightly regulated constituent of membranes, maintaining membrane fluidity. Cholesterol surplus to the complexing capacity of the membrane lipids, referred to as the "active" or "free" cholesterol, constitutes the pool of available substrate for steroidogenesis after its uptake to intracellular membranes.²³

To initiate and sustain steroidogenesis, a constant supply of cholesterol must be available within the cell and it has to be delivered to the cleavage site in the inner mitochondrial membrane. The cholesterol used in steroidogenic cells, such as adrenals and gonads, derives from a number of sources, including de novo synthesis of cellular cholesterol, lipoprotein-derived cholesteryl esters and hydrolysis of cholesteryl esters stored in lipid droplets, with free cholesterol being produced from cholesteryl esters by hormone-sensitive lipase, encoded by the *LIPE* gene.^{7,24} Brain cholesterol, however, originates from local synthesis in neural and glial cells because the blood-brain barrier prevents the entry of circulating lipoproteins carrying cholesterol.²⁵

Two transport processes can occur for intracellular cholesterol supplies. First, cholesterol provided by cellular stores, including lipid

droplets and other cellular membranes, is mobilised to the outer mitochondrial membrane. Second, cholesterol is transferred from the outer to the inner mitochondrial membrane.²⁶ The coordination processes for the mobilisation of cholesterol in steroidogenic cells to the outer and mitochondrial membrane may involve the steroidogenic acute regulatory (StAR) protein in conjunction with the translocator protein (18-kDa) TSPO (formerly known as peripheral benzodiazepine receptor) together with TSPO-associated proteins.²⁷ StAR is widely distributed in specific populations of neurones and glial cells. StAR expression in the brain has been found in pyramidal and granule cells, as well as in the dentate gyrus of the hippocampus. A much broader distribution of StAR-positive cells was detected with neuronal and glial staining in the striatum, cerebellum, pons and thalamus. StAR protein was also detected, to a lesser extent, in neurones in the preoptic area and the arcuate nucleus of the hypothalamus.²⁴ Moreover, the coexpression of StAR and the cholesterol side-chain cleavage enzyme, P450_{scc}, as encoded by the *CYP11A1* gene, has been established in neuronal subpopulations in mouse and human brains.²⁸ Because StAR-mediated transport appears to be low under basal conditions, it was proposed that plasma membrane cholesterol may also be transported via START (StAR-related lipid transfer) proteins that contain a lipid-binding domain homologous to the C-terminus of StAR.²⁹ TSPO is abundant in all steroidogenic cells and it has been shown that TSPO drug ligands stimulate the formation of steroids.²⁷

Although these proteins in the mitochondrial membrane play a key role in neurosteroidogenesis, the regulatory mechanisms underlying the neurosteroid biosynthesis in the brain remain unclear.

2.1.2 | Neurosteroidogenesis enzymes

Evidence has emerged concerning the existence of steroid synthesis mechanisms in the brain that are similar to, but independent of, those present in peripheral tissues. Over time, a broader distribution of the enzymatic machinery for steroidogenesis has been identified, providing evidence for local, paracrine actions of brain-derived steroids in brain tissues.^{18,30}

Many of the enzymes necessary for steroidogenesis occur in brain neurones and glia cells in the central and peripheral nervous systems, with astrocytes being the most active steroidogenic cells in the brain.^{15,31-33} 21 Carbon pregnenolone is produced from 27 carbon cholesterol, which is converted to 20 α ,22(α or β)-dihydroxycholesterol through two NADPH-dependent hydroxylases. Then, the cytochrome side chain cleavage enzyme P450_{scc}, or *CYP11A1* enzyme, catalyses C20-C22 bond-cleavage in the 20,22-hydroxylated cholesterol, leading to pregnenolone. Brain astrocytes and neurones express P450_{scc}, or *CYP11A1* enzyme, which converts cholesterol to pregnenolone.^{34,35} In particular, P450_{scc} has been reported within the hippocampal neurones of rats.³⁶ Pregnenolone can then be processed through multiple metabolic pathways, depending on the demands and needs of the cells. First, pregnenolone can be transformed either to progesterone or to 17-hydroxy pregnenolone and DHEA. Pregnenolone and DHEA,

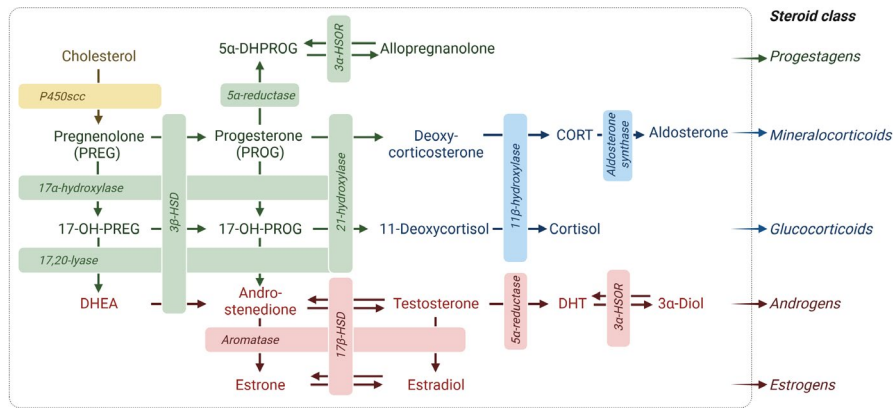


FIGURE 1 Main steroid synthesis pathways from pregnenolone. Cholesterol is converted into pregnenolone by the cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone then serves as a precursor for the synthesis of other progestagens and other steroids, including mineralocorticoids, glucocorticoids, androgens and oestrogens. Abbreviations: 3 α -diol, 3 α -androstenediol; 3 α -HSOR, 3 α -hydroxysteroid oxidoreductase; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; 5 α -DHPROG, 5 α -dihydroprogesterone; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase; CORT, corticosterone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; PREG, pregnenolone; PROG, progesterone. Adapted from Hanukoglu (1992). Created using BIOENDER (<https://biorender.com>)

may be metabolised into 7 α -hydroxylated derivatives.^{37,38} Another alternative is sulphation of the C3 hydroxyl group of pregnenolone into 3 β -sulphated derivative by sulphotransferase enzymes in the rodent and human brain.³⁹ However, the presence of sulphated pregnenolone, which has been reported in postmortem human brain tissue, is a matter of debate in the rodent brain.⁴⁰ It is unlikely that the sulphated form of pregnenolone crosses the blood-brain barrier (BBB) because this requires the presence of transporter proteins. Moreover, although pregnenolone sulphate could be taken up across the BBB in rodents, it would be rapidly metabolised in favour of free pregnenolone,⁴¹ which is concordant with the low levels of pregnenolone sulphate measured in rodent brain with highly sensitive mass spectrometry methods.⁴²⁻⁴⁵ The 3 β -hydroxysteroid dehydrogenase (3 β -HSD) enzyme required for the conversion of pregnenolone to progesterone is expressed in the rat and human brain.⁴⁶⁻⁴⁹ Two enzymes, 5 α -reductase and 3 α -hydroxysteroid oxidoreductase that convert progesterone to dihydroprogesterone and to its active metabolite, allopregnanolone, were then localised in principal glutamatergic output neurones in the cortex, hippocampus, and amygdala of mice, as well as in the human brain.^{31,50} The conversion in the brain of pregnenolone into DHEA by 17-hydroxylase/17,20 lyase (P450c17) is challenging because no expression of P450c17 has been detected in rat brain glia,⁵¹ although adult rat brain and neurones and astrocytes in culture have been shown to convert pregnenolone to DHEA.⁴⁰ In the human brain, postmortem studies report that P450c17 was not expressed in the temporal lobe and limbic system of the adult human⁵² but was present in the developing human fetal nervous system, suggesting that DHEA may be synthesised locally.⁵³ Finally, in rat and human brain, DHEA can be converted into androstenedione and then into testosterone, which can be metabolised into oestradiol by aromatase.^{50,54,55} Similarly, evidence has shown that oestradiol can be synthesised from pregnenolone in the rat hippocampus.⁵⁶

In summary, the tissue-specific expression of steroidogenic enzymes allows the modulation of active steroid levels locally; thus, measuring local steroid concentrations rather than the circulating levels would appear to be a more accurate indicator of the steroid action within a specific tissue.

2.2 | Local measurement of neurosteroids and pregnenolone

Steroid metabolome approaches have recently been promoted with the development of state-of-the-art quantification methods. Metabolomics can be defined as the investigation of the global metabolite profile in a system (cell, tissue or organism) and its application to research into mental disorders has become prominent in recent years.⁵⁷ In metabolomic research, one of the most widely used analytical techniques is mass spectrometry (MS), which focuses on identifying selected metabolites known to be involved in a particular metabolic pathway, such as steroid metabolites.⁵⁸

Validation of chromatography coupled with MS methods have been successful in the simultaneous quantification of steroid metabolites in different biological matrices, including plasma, serum, saliva, urine, hair, cerebrospinal fluid and brain regions in animals and/or humans. The development of these methods has been extensively published and reviewed.⁵⁹⁻⁶⁸ Local measurement of steroid metabolites in human fluids and in specific areas of the rodent brain has helped to uncover the role of some of them in physiological functions and brain-related diseases. A comparison between local brain and circulating concentrations further emphasises the concept of neurosteroids.

Pregnenolone was detected in human post-mortem brain tissue at considerably higher concentrations than those typically observed in serum or plasma.^{69,70} Moreover, in response to stress challenges,

higher levels of brain pregnenolone than those in the blood of adult rodents have been measured using gas chromatography coupled with MS.^{71,72} Similarly, the acute administration of drugs, including cocaine, amphetamine, alcohol, nicotine and the psychoactive component of cannabis, Δ^9 -tetrahydrocannabinol (THC), was shown to induce a specific increase in pregnenolone in brain areas to a much greater extent than in rodent plasma.⁷³ Furthermore, alterations in pregnenolone concentration related to pathological aspects of the central nervous system (CNS) have been reported in animals and humans.^{74,75} Briefly, in humans, increased levels of postmortem pregnenolone have been reported in brain regions of patients with Alzheimer's disease compared to non-cognitively impaired control subjects,^{70,76} as well as in subjects with schizophrenia and bipolar disorder compared to control subjects.⁶⁹ In addition, an increase in pregnenolone levels, associated with a decrease in progesterone metabolites, was observed in the cerebrospinal fluid (CSF) and plasma of male subjects with multiple sclerosis, an inflammatory and demyelinating disease of the CNS with relevant neurodegenerative aspects.⁷⁷ By contrast, decreased pregnenolone has been found in the CSF of patients suffering from anxiety and depressive disorders⁷⁸ or postmenstrual syndrome,⁷⁹ as well as in the serum of subjects diagnosed with schizophrenia.^{80,81}

In terms of animal research, although many studies have reported neurosteroid alterations under various circumstances involving stress and drug challenges, as well as in relation to cognitive and/or anxiety/depression states,⁷⁵ few studies have focused on pregnenolone. One of the main reasons for this lack of attention to pregnenolone is our limited knowledge of its cellular and molecular targets.

2.3 | Cellular and molecular targets of pregnenolone

Pregnenolone is inactive with respect to modulating the ionotropic receptors, including the GABA_A and NMDA receptors that are the classical targets of neurosteroids, such as allopregnanolone and the sulphated forms of pregnenolone and DHEA⁸²⁻⁸⁶ (Table 1).

TABLE 1 Reported molecular targets for pregnenolone

Targets	Acronym	Models	Reported effect (effective concentration)	References
Ionotropic receptors	GABA _A	GABA-mediated synaptic responses in rat hippocampal neurons	Inactive	Park-Chung et al (1999) ⁸⁴
	NMDA	NMDA-mediated Ca ²⁺ influx in rat hippocampal neurons	Inactive	Weaver et al (2000) ⁸⁶
Ligand-operated protein	σ 1	Binding assay in rodent brain tissue	Inactive or low affinity	Monnet & Maurice (2006); Su et al (1988) ^{87,88}
Microtubules	MAPs	Culture of rat neurons	Binding to MAP2 and CLIP-170 (30–60 nM)	Murakami et al (2000); Weng et al (2013) ^{91,92}
GPCR	CB1R	cell lines expressing the human CB1R	Binding to a specific allosteric site of CB1R (10 nm–1 μ M)	Vallée et al (2014) ⁷³

Abbreviations: CB1R, type-1 cannabinoid receptor; MAP, microtubule-associated protein GPCR, G-protein coupled receptor; σ 1, sigma-1.

Pregnenolone, in contrast to its sulphate derivative, has been described as being inactive or as having a very low affinity on sigma-1 (σ 1) receptor.^{87,88} σ 1 receptors are expressed in many organs, including the nervous system, and are located in specific microdomains of the endoplasmic reticulum called mitochondrial associated membranes.⁸⁹ σ 1 receptor can influence synaptic functions through modulation of NMDA receptor activity and can remove the negative-regulation of NMDA receptors by CB1R.⁹⁰ Moreover, pregnenolone, along with DHEA, can interact with the microtubule-associated proteins (MAPs), in particular MAP2 and CLIP-170.^{91,92} Pregnenolone or DHEA are able to bind, with affinity in the nanomolar range, to the amino-terminal region of MAP2 protein, which stimulates MAP2-driven microtubule assembly. Finally, a novel mechanism of action of pregnenolone involving the GPCR, CB1R, has recently been demonstrated.⁷³ In vitro experiments have revealed that pregnenolone (1 μ M) can block the THC-induced increase in Erk1/2^{MAPK} phosphorylation in Chinese hamster ovary (CHO) cells expressing the human CB1 receptor (hCB1R) and the THC-induced decrease in mitochondrial and cellular respiration in HEK293 cells transfected with the hCB1R.⁷³ In addition, a binding pocket in the TMH1/TMH7/Hx8 lipid region of the CB1R has been identified for pregnenolone⁷³ using the Forced-Biased Metropolis Monte Carlo simulated annealing program (MMC)⁹³ and was validated in vitro.⁷³ Pregnenolone was no longer able to prevent the THC-induced decrease in cellular respiration in cells transfected with a mutant hCB1R containing a point mutation that prohibits binding of the ketone end of pregnenolone to CB1R.⁷³

3 | CB1R ACTIVITY AND FUNCTION

3.1 | Brief description of the ECS

The discovery of the principal psychoactive constituent of *Cannabis sativa*, Δ^9 -THC⁹⁴ in the mid-1960s led to the identification of the CB1R. Although it was initially assumed that Δ^9 -THC worked via nonspecific mechanisms, this assumption was refuted by the discovery of transmembrane proteins that were able to bind cannabinoids

and were therefore called "cannabinoid receptors".⁹⁵ The CB1R was first identified in the rat brain and is dominantly expressed in the central nervous system,^{95,96} whereas the type-2 cannabinoid receptor (CB2R) was identified at the periphery where it is contained in immune cells and tissues.^{97,98}

Taken together, CB1R and CB2R belong to the family of GPCRs and are activated by endogenous lipid compounds, endocannabinoids (eCBs), of which the two best described are *N*-arachidonylethanolamine (AEA, anandamide)⁹⁹ and 2-arachidonoylglycerol (2-AG).^{100,101} The ECS, comprising eCBs, their synthesis and degradation enzymes, and target receptors, is a signalling system involved in the fine-tuning of many physiological functions. Therefore, deregulation of eCBs-mediated signalling may contribute to the aetiology of disorders such as neuropsychiatric and metabolic disorders.¹⁰²⁻¹⁰⁵ In this context, unravelling the endogenous regulatory mechanisms that help to maintain the physiological activity of the ECS is highly relevant for the development of promising therapeutic approaches.

3.2 | CB1R distribution

CB1R is considered the most abundant GPCR in the mammalian brain. Its distribution across brain regions is highly heterogeneous and parallels the well-known effects of cannabinoids on motor functions, cognition, emotional behaviours and reward processing.⁹⁸ The highest CB1R expression levels are observed in the basal ganglia, cerebellum and corticolimbic areas. Lower yet physiologically relevant CB1R levels are also detected in the thalamus, hypothalamus, brainstem and spinal cord.¹⁰⁶⁻¹¹⁰

Central CB1Rs are primarily contained in neurones,¹¹¹ mainly in cortical GABAergic interneurons and cortical glutamatergic neurones.¹¹² Nevertheless, CB1R is associated with other neurotransmitter systems (e.g., serotonergic, cholinergic, noradrenergic and dopaminergic systems)^{111,113,114} and non-neuronal cell types (astrocytes, oligodendrocytes and microglia).¹¹⁵⁻¹¹⁷ In addition to being a "central receptor", CB1R is also largely expressed in peripheral tissues and organs such as the terminals of sensory nerves, as well as the gastrointestinal tract, pancreas, muscles, liver and adipose tissue.¹¹⁸⁻¹²²

Besides its systemic and cellular localisation, CB1R is also distributed in several subcellular compartments where it plays distinct roles. Central CB1Rs are mainly detected in presynaptic terminals where they control the retrograde suppression of neurotransmission and ensure the excitatory/inhibitory balance within the brain. Briefly, eCBs are synthesised *de novo* in postsynaptic cells upon intracellular Ca²⁺ increase and activate presynaptic CB1Rs leading to the transient or long-lasting suppression of neurotransmitter release (i.e., GABA or glutamate).¹²³

Several lines of evidence indicate that CB1Rs are not solely involved in retrograde signalling. It has been reported that CB1Rs are present in endosomal compartments in the context of internalisation and axonal trafficking and, more recently, at the outer mitochondrial membrane.^{114,124} Indeed, mitochondrial CB1Rs (mtCB1Rs) can inhibit the soluble adenylate cyclase and the OXPHOS mitochondrial

respiratory chain, thereby regulating bioenergetic processes within the brain.¹²⁴ Although the discovery of mtCB1Rs provides new insights into the behavioural effects of cannabinoids, it will also undoubtedly shed light on new mechanisms of action of eCBs-mediated signalling at the subcellular level^{125,126} (Figure 2A).

3.3 | Endocannabinoids: endogenous ligands of CB1R

CB1R are activated by endogenous lipid messengers called eCBs, which are typically produced *de novo* upon neuronal activation rather than stored in secretory vesicles, and can signal in a retrograde manner (i.e., from postsynaptic neurones to presynaptic neurones).¹²² Their biosynthesis occurs through the cleavage of phospholipid precursors, and their lipid nature allows them to move quickly within biological membranes. However, their diffusion within aqueous environments (e.g., cytosol and extracellular space) requires efficient transport systems to activate distant cannabinoid receptors and for their intracellular inactivation.¹²⁷

The cellular reuptake of eCBs is also facilitated by uncharacterised eCBs membrane transporters that participate in the termination of eCBs-mediated signalling.^{128,129} The two main eCBs are AEA and 2-AG, which are partial and full agonists of CB1Rs, respectively.¹³⁰ Although they are both derived from arachidonic acid, AEA and 2-AG display distinct biosynthesis and degradation pathways.¹²⁷

AEA formation in neurones is initiated by the enzyme *N*-acyltransferase to yield *N*-arachidonoyl-phosphatidylethanolamine (NAPE) from phospholipid precursors and enriches membrane pools thereof. NAPE is then hydrolysed into AEA by a NAPE-specific phospholipase D.¹³¹ The inactivation of AEA is primarily catalysed by the enzyme fatty acid amine hydrolase, located on intracellular membranes of postsynaptic neurones. However, multiple oxidation pathways may participate in the intracellular deactivation of AEA.¹³²⁻¹³⁴

2-AG is the most abundant eCB in the brain and an intermediate in lipid metabolism.^{127,135} It is primarily involved in the retrograde inhibition of neurotransmission and is synthesised upon depolarisation of postsynaptic neurones. Specifically, phosphatidylinositol is hydrolysed by a phospholipase C into diacylglycerol (DAG). The hydrolysis of DAG by DAG lipase then leads to the formation of 2-AG.^{127,136-138} 2-AG travels to presynaptic neurones retrogradely and is hydrolysed by the monoacylglycerol lipase (MAGL). Although MAGL is primarily responsible for the termination of 2AG signalling, the α/β -hydrolase domain 6 and domain 12 enzymes can degrade 2-AG to avoid its excessive accumulation in postsynaptic neurones and the extracellular space, respectively.¹³⁹⁻¹⁴¹ As for AEA, alternative pathways may participate in the inactivation of 2-AG.¹⁴²

3.4 | CB1R activity

CB1R displays both basal and agonist-induced signalling and internalisation. Although the basal activity of CB1Rs has been mainly described

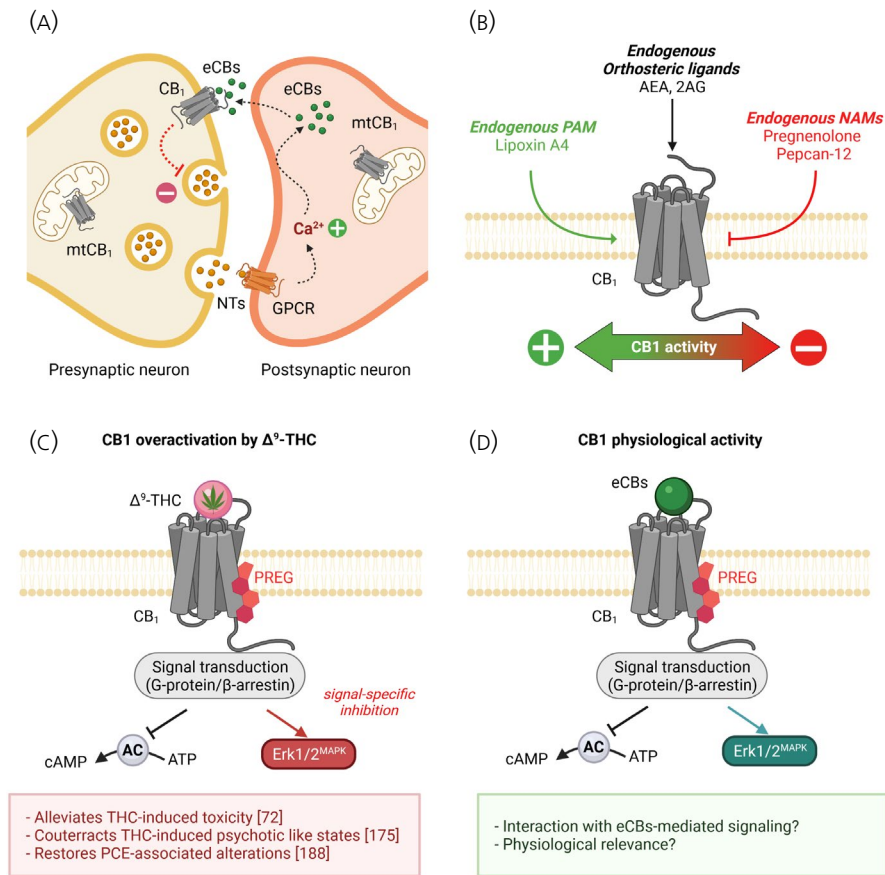


FIGURE 2 Pregnenolone is an endogenous allosteric modulator of the type-1 cannabinoid receptor (CB₁R). A, Simplified overview of the endocannabinoid system (ECS) in neurons. CB₁Rs are present at presynaptic plasma membranes and outer mitochondrial membranes. Presynaptic CB₁Rs are primarily involved in the retrograde suppression of neurotransmission. B, The main endogenous orthosteric ligands of CB₁R are AEA and 2-AG, which activate the CB₁R via their orthosteric binding site. The endogenous NAMs and PAMs of CB₁R can, respectively, negatively and positively modulate CB₁ activity via distinct allosteric binding sites. C, In the presence of high doses of Δ⁹-THC, pregnenolone binds to CB₁R on a dedicated allosteric pocket and acts as a signal-specific inhibitor of the Erk1/2^{MAPK} pathway, resulting in the blockade of Δ⁹-THC-induced toxic outcomes. D, The role of pregnenolone in regulating the activity of eCBs-mediated CB₁ signalling needs to be addressed to depict the physiological functions that pregnenolone-CB₁ regulation may fulfill. Abbreviations: 2-AG, 2-arachidonoylglycerol; AC, adenylyl cyclase; AEA, anandamide; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CB₁, type-1 cannabinoid receptor; eCBs, endocannabinoids; Δ⁹-THC, Δ⁹-tetrahydrocannabinol; Erk1/2^{MAPK}, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase; GPCR, G-protein coupled receptor; mtCB₁, mitochondria-associated CB₁; NAM, negative allosteric modulator; NTs, neurotransmitters; PAMs, positive allosteric modulators; PCE, prenatal cannabis exposure; PREG, pregnenolone. Created using BIOENDER (<https://biorender.com>)

as an agonist-independent or constitutive activity, some studies have suggested that retrograde eCB release participate in the persistent activation of presynaptic CB₁R.¹⁴³ Moreover, the tonic activity of ECS may arise not only from the endogenous release of eCBs onto CB₁Rs, but also from the presence of CB₁Rs in a constitutively active state.¹⁴⁴ In addition, CB₁ activity is mediated by ligand-receptor interaction and the specificity of the CB₁ GPCR that involves biased signalling will be addressed, as well as the different functions induced by ligand binding to CB₁R orthosteric or allosteric sites.

3.4.1 | Constitutive activity

Distinguishing between endogenous ligand activity and the constitutive activity of a receptor in an intact biological system is quite challenging.^{145,146} However, it is generally accepted that the CB₁

GPCR behaves constitutively in most systems.^{143,144,147,148} The ability of the GPCR to mediate a signal response in the absence of an agonist will define its constitutive activity.¹⁴⁹

A constitutive activity is based on a two-state model of a receptor, including two interchangeable conformations: a constitutively active "on" state and a constitutively inactive "off" state. In this model, an agonist will increase the number of receptors in the active state, whereas an inverse agonist will shift the equilibrium from the active to the inactive state, and a neutral antagonist will not affect any state in the model.¹⁴⁴ CB₁R constitutive activity was first described by comparing 'basal' G protein activation and G protein regulated signal transduction in cells expressing recombinant CB₁Rs compared with CB₁R-deficient host cells.¹⁵⁰⁻¹⁵²

Then, further identification of *in vivo* and *in vitro* effects of several inverse agonists (including Rimobant or SR141716A, AM251, AM281 or LY320135) on CB₁Rs confirmed the constitutive activity

for CB1Rs.¹⁴⁴ However, the interpretation of these studies must take into consideration the local production of eCBs, which may exert autocrine and paracrine stimulation of CB1Rs. Thus, the term 'basal endocannabinoid system tone' or signal transduction 'in the absence of exogenously applied agonists' has been put forward as being more accurate than 'constitutive activity'.¹⁴⁹

Interestingly, the development of neutral competitive CB1R antagonists (such as AM4113, NESS0327, LH21, O-2050) and the comparison of their effects with those of inverse agonists should allow for the distinction between the tonic activity of endocannabinoid release at CB1Rs and the presence of CB1Rs in a constitutively active state. In this context, it has been revealed that constitutive CB1 activity has a pivotal function in the tonic control of hippocampal GABA release.¹⁵³ Moreover, constitutive CB1 activity also regulates GABAergic and glutamatergic neurotransmission in the ventral tegmental area and basolateral amygdala of the mouse brain, suggesting that this constitutive activity is significantly involved in anxiety and motivation-related functions such as for reward.¹⁵⁴ These findings indicate that constitutive CB1 activity not only occurs in artificial systems, but also can also regulate neurotransmission in native brain tissue.

In addition, it has been demonstrated that the effect of inverse agonists, such as rimonabant (SR141716A) can be attributed to a mechanism of action involving the suppression of constitutive CB1R activity. Thus, the negative modulation of the constitutive CB1 activity is certainly linked to the serious adverse psychiatric effects (anxiety, depression and suicidality) associated with inverse agonists such as rimonabant.^{155,156}

It is noteworthy that the constitutive activity of CB1 certainly depends not only on the experimental models used, but also on the expression of the gene coding for the CB1R. Thus, the regional distribution of CB1R expression explains the great diversity of constitutive responses.

3.4.2 | Biased signalling

CB1Rs, similar to most GPCRs, are coupled to G proteins and β -arrestins, initiating multiple intracellular signalling pathways and different transduction mechanisms. Biased agonism, also called "functional selectivity", refers to the selective activation of specific signalling cascades by distinct GPCR agonists resulting in dedicated physiological responses.¹⁵⁷

GPCRs interact with a host of elements in their environment that modify the specificity, selectivity, and time course of signalling. Accordingly, the ligand-receptor-environment complex will dictate the signalling capacity of the system.¹⁵⁸ The expression of each actor in this complex will engage a single receptor to differentially activate multiple signalling pathways in a brain structure-selective manner. In this paradigm, the agonist ligands will favour a conformation of receptor that will be engaged in specific signalling.¹⁵⁹ This functional selectivity or ligand bias has clearly been demonstrated for CB1 signalling where the CB1R can adopt multiple conformations depending on agonist occupancy. One of the first series of studies revealing this

ligand bias identified a ligand preferentially coupling to $G\alpha$ proteins at CB1R in Sf9 insect cell membrane preparations, demonstrating agonist-selective G protein signalling by the CB1R.¹⁶⁰ Further investigations demonstrated the existence of functional selectivity both in vitro and in vivo, showing that distinct cannabinoid agonists can display different abilities to regulate diverse components of dopamine neurotransmission system in vivo.¹⁶¹ Interestingly, the expression level of the CB1R has recently been identified as a novel determinant of the signalling outcome, and this effect has been shown to be dependent on the CB1 agonist used and the expression level of the $G\alpha i$ protein.¹⁶²

Apart from G proteins, arrestin proteins are essential components of multiple GPCR signalling cascades involving kinases or phosphatases.¹⁶³ CB1Rs can thus recruit β -arrestin-1 (arrestin-2) and β -arrestin-2 (arrestin-3) for signalling, and the coupling of CB1R to β -arrestin may affect the localisation of receptors at the surface, their internalisation, recycling and degradation.¹⁶⁴

Studies of GPCRs and arrestin proteins may present conflicting results as a result of the different methodological approaches used, and research on structural features associated with cannabinoid receptor- β -arrestin interactions is limited, although several mutational studies have explored the importance of C-terminal residues for binding of β -arrestins to CB1R.¹⁶⁵

3.4.3 | Modulation of CB1 activity via allosteric sites

The study of allosteric modulation is a relatively new concept in GPCR research that emerged in the early 1990s. Two binding sites on the GPCR were then defined for a ligand. First, the orthosteric binding site, which is defined as the primary binding site recognised by the endogenous ligand, and, second, the allosteric sites, which are topographically and structurally distinct from the orthosteric sites.

From a mechanistic point of view, allosteric ligands potentiate (positive allosteric modulators; PAM) or inhibit (negative allosteric modulators; NAM) receptor activation by an orthosteric ligand.¹⁶⁶

Allosteric ligands can alter the functional response of orthosteric ligands, and allow for receptor subtype selectivity, receptor trafficking and/or even elicit a signalling response in the absence of an orthosteric ligand.¹⁶⁷ The combined effects can lead to overall allosteric modulation on receptor function.¹⁶⁸

Several classes of allosteric modulators, which bind to CB1R allosteric binding sites, have been discovered. CB1R allosteric modulators have been widely reviewed elsewhere.^{22,168,169} To date, approximately nine classes of CB1R allosteric modulators have been identified, including lipoxin A4, ZCZ011, pepcan-12, Org27569 and PSNCBAM-1, and, more recently, pregnenolone. Among them, several have been endogenously detected, such as lipoxin A4, which was first described as a PAM¹⁷⁰ but also as a NAM,¹⁷¹ as well as NAMs pepcan-12¹⁷² and pregnenolone.⁷³ (Figure 2B). Here, we focus more particularly on pregnenolone in modulating CB1R activity and functions.

As previously described, the endogenous neurosteroid pregnenolone can bind to a specific binding pocket on the CB1R.⁷³ From a

mechanistic perspective, pregnenolone did not alter (up to 100 μM) the equilibrium binding of the radiolabelled CB1 receptor agonists [^3H]CP55,940 and [^3H]WIN 55,212-2, and did not decrease (up to 1 μM) Δ^9 -THC-induced cAMP reduction, although it did alter CB1 signalling with a non-competitive decrease in the phosphorylation of Erk1/2^{MAPK} 73 (Figure 2C). Overall, the effects of pregnenolone converge to a NAM action on CB1R. Furthermore, pregnenolone did not increase the binding of CB1 agonists, in contrast to the effects of the allosteric modulators, Org27569 and PSNCBAM-1, suggesting that pregnenolone binds to a distinct allosteric site. Another functional study of pregnenolone on CB1 showed that pregnenolone dose-dependently decreased [^3H]SR141716A binding but, because the displacement of [^3H]SR141716A was incomplete at the maximum concentration of pregnenolone that could be used in the assay, it was not possible to conclude whether the action of pregnenolone was competitive or allosteric.¹⁷³ Moreover, in contrast of the previously described data,⁷³ pregnenolone did not modulate CB1 agonist-mediated Erk1/2^{MAPK} phosphorylation in CHO-hCB1 cells.¹⁷³ Furthermore, it was also found that pregnenolone did not attenuate the suppression of depolarisation-induced excitation induced by 2-AG in autaptic hippocampal neurones.¹⁷¹ However, in this model, Straiker et al¹⁷¹ also showed that lipoxin A4 acted as a NAM on CB1Rs rather than a PAM. Because it was previously reported that lipoxin A4 exhibited probe-dependence favouring AEA over 2-AG,¹⁷⁰ it is possible that the above reported discrepancies arise from the probe-dependence of pregnenolone. Finally, although one study has reported the lack of pregnenolone effects on cannabinoid-induced attenuation of GABAergic and glutamatergic transmission *ex vivo*,¹⁷⁴ a subsequent survey has shown that pregnenolone (10 μM) could modulate CB1-mediated suppression of neurotransmission through the Erk1/2^{MAPK} signalling cascade.¹⁷⁵

The property of pregnenolone as an endogenous NAM of the CB1R was further confirmed through *in vivo* studies. Namely, pregnenolone was able to attenuate a complete spectrum of cannabinergic effects induced by the CB1 agonist Δ^9 -THC in mice.^{73,176}

In conclusion, modulation of CB1R by pregnenolone offers interesting potential for the relief of CB1-related diseases, although the mechanism of action of pregnenolone on CB1 requires further exploration. It should also be noted that pregnenolone binds to a specific CB1R site in the TMH1/TMH7/Hx8 transmembrane region, located in the C-terminal region of the CB1 receptor.⁷³ In this region, it has been proposed that cannabinoid receptor interaction protein 1a (CRIP1a) could interact with CB1R,¹⁷⁷ suggesting potential functional interactions of pregnenolone with CRIP1a.²²

3.5 | CB1R function modulation and therapeutic opportunity

3.5.1 | Advantages of biased signalling and allosteric modulation

The insight that G protein- and arrestin-dependent signals can be dissociated using pathway-selective "biased" agonists is gaining

importance in the field of drug discovery for GPCRs.¹⁵⁸ This biased signalling represents an interesting therapeutic opportunity to target specific pathways that only bring about the desired effects. Indeed, the basic principle of ligand bias is the selective activation of signalling pathways that mediate therapeutic effects and are free of adverse effects, which relies on these pathways being distinct.¹⁶⁵

Historically, drug discovery approaches have focused on identifying ligands that can compete with endogenous ligands at the orthosteric sites. However, compared to orthosteric ligands, allosteric ligands present several advantages for clinical research and drug discovery.¹⁷⁸ Allosteric ligands exhibit increased selectivity of receptor subtypes and modulate specific signalling pathways (biased-signalling) that reduce off-target and on-target side effects from interferences. They also have a maximum ("ceiling") effect, so that increasing the dose does not enhance the allosteric response, thus avoiding overdose. Finally, allosteric modulators of CB1R should induce fine-tuning of CB1 signalling, under conditions where endocannabinoids are produced and released "on demand". Thus, the functional selectivity of CB1Rs might generate a pharmacologically improved therapeutic effect, with reduced on-target adverse effects compared to the orthosteric ligand.

In summary, ligand-mediated biased signalling and allosteric modulation of CB1Rs offer pharmacological approaches that could potentially be used to develop improved CB1 drugs by modulating only therapeutically relevant CB1 signalling pathways.¹⁷³ In line with this, the discovery that pregnenolone is a biased endogenous allosteric signalling modulator is highly relevant to CB1-dependent functions and affords new and exciting opportunities for the development of pregnenolone analogues with strong therapeutic benefits.

3.5.2 | Pregnenolone function and proof of concept studies

As mentioned previously, the CB1R is widely distributed within the body, thus highlighting its pivotal role as a modulator of physiological functions. Such functions include cognition and memory,¹⁷⁹ emotional behaviours,¹⁸⁰ energy metabolism,¹⁰² motor functions,¹⁸¹ thermoregulation, and pain processing.¹⁸² Despite the wide range of functions involving CB1Rs, one core feature of CB1-mediated signalling is responsiveness to external and internal stimuli. It is generally accepted that CB1 activation functions as an adaptive response to preserve homeostasis.¹⁰⁴ However, repeated challenges (e.g., chronic stress, high calorie diet) may contribute to the deregulation of CB1 activity and the onset and etiology of various disorders.^{102,103} Moreover, the acute or chronic disruption of endogenous CB1 signalling by exogenous cannabinoids may produce deleterious effects as a result of the receptor's over-activation.¹⁸³

Our research group first demonstrated that pregnenolone was able to attenuate the complete spectrum of tetrad cannabinergic effects (locomotor suppression, hypothermia, catalepsy and analgesia) induced by the CB1 agonist Δ^9 -THC in mice. Furthermore, pregnenolone was able to normalise the release of dopamine (DA) induced

by Δ^9 -THC in the nucleus accumbens shell (NAcS) of rats, a feature shared by all drugs of abuse,¹⁸⁴ and to reduce motivation for the self-administration of the CB1 agonist WIN 55,512-2 in an operant conditioning paradigm.⁷³ The action of pregnenolone on CB1Rs was related to a drastic increase in pregnenolone production by CB1 agonists,⁷³ resulting in unforeseen feedback control of CB1 activity in a paracrine/autocrine manner.

It has also been proposed that pregnenolone could compensate for some mechanisms of THC addiction, such as restoring the activity of CB1Rs located on GABAergic interneurons of the ventral tegmental area, leading to the normalisation of DA release.¹⁸⁵ In particular, the reported alleviating effects on THC-intoxication were specific to pregnenolone and not its downstream steroids.⁷³

Taken together, these data highlight the therapeutic potential for pregnenolone against cannabis use disorder (CUD), for which no pharmacological treatment was available¹⁸⁶ until the recent development of C3-17 pregnenolone synthetic analogues, named AEF compounds, by the biotechnology company *Aelis Farma* (WO2012/160006A1; WO2014/083068A1; WO2019/162328A1 patents). Specifically, those compounds display a better therapeutic profile than pregnenolone, with increased half-life, no conversion into steroids, and good bioavailability and administration per os. One of the lead AEF compounds is currently in a phase II clinical trial for CUD indications and shows very promising results (ClinicalTrials.gov; Identifier NCT03717272; Effect of AEF0117 on Subjective Effects of Cannabis in CUD Subjects).

Along with CUD, cannabinoid intoxication has long been associated with the onset of psychosis. In particular, it has been reported that early-life exposure to cannabis is correlated to a greater risk of developing psychotic disorders during adulthood, whereas cannabinoids can elicit transient psychotic symptoms in healthy subjects.¹⁸⁷ Although the relationship between cannabis and psychosis is still being debated, CB1Rs have emerged as potential targets for antipsychotic drugs. However, the main concerns remain the severe side effects of CB1 pharmacological blockade.¹⁸⁸ In this context, Busquets-Garcia et al¹⁷⁶ have reported promising effects of pregnenolone against Δ^9 -THC-induced psychotic-like behaviours in mice. In particular, they showed that pregnenolone was able to antagonise a wide range of effects elicited at different doses of Δ^9 -THC, including impairments in cognitive functions, somatosensory gating and social interaction.¹⁷⁶

Furthermore, pregnenolone administration is associated with an amelioration of psychosis-related symptoms in humans and murine models of schizophrenia. However, it is not known whether CB1Rs mediate those effects and whether the high doses of pregnenolone used induced an increase in downstream steroids, such as allopregnanolone, which is a potent modulator of GABA_A receptors.⁷⁵

More recently, Frau et al¹⁸⁹ have reported the neuroprotective effects of pregnenolone against the neurological alterations associated with prenatal cannabis exposure (PCE). In a rat model of PCE, they showed that the adolescent male offspring of dams exposed to Δ^9 -THC display increased behavioural sensitivity to Δ^9 -THC-induced somatosensory gating alterations. These behavioural changes were

causally linked to a dysfunction in the dopaminergic system, producing increased activity of dopaminergic neurones within the ventral tegmental area and increased DA release in the NAcS in response to Δ^9 -THC. Encouragingly, the chronic administration of pregnenolone normalised the firing properties of dopaminergic neurones, DA release and somatosensory gating alterations in PCE animals.¹⁸⁹ Furthermore, the pharmacological inhibition of the enzyme 3 β -HSD did not impair the neuroprotective action of pregnenolone, hence confirming that pregnenolone but not its metabolites were involved in these effects.¹⁸⁹

4 | CONCLUSIONS AND PERSPECTIVES

In conclusion, the above-reported studies encourage the idea that the mechanisms of action of pregnenolone on CB1Rs as a negative "signalling-specific" allosteric modulator can be used as a therapeutic means to block THC-induced addiction and psychotic-like states. Moreover, although pregnenolone could antagonise THC-induced hyperdopaminergic states, it did not produce hypodopaminergic phenotypes alone, nor did it have any per se effects at the doses used in vivo.^{73,176,189} Instead, the neuroprotective effects of pregnenolone appeared to be state-dependent, which may confer significant therapeutic advantages such as a safe profile of action, unlike CB1 antagonists.¹⁹⁰ This strengthens the proof of concept for a suitable therapeutic profile of signal-specific inhibitors of excessive CB1 signalling, such as the recently developed synthetic analogues of pregnenolone.

Further studies are needed to determine whether pregnenolone is a suitable therapeutic candidate for other CB1-related pathologies associated with changes in ECS components, such as bipolar disorders, stress-related disorders, attention-deficit/hyperactivity disorders and eating disorders^{102,103} and whether changes in pregnenolone levels might represent suitable biomarkers for such pathologies.

Finally, although the aforementioned in vivo studies have examined the ability of pregnenolone to counteract the effects of exogenous cannabinoids, we still do not know how pregnenolone and CB1R interact with one another in physiological conditions and how endogenously-produced pregnenolone may help to maintain ECS-mediated homeostasis (Figure 2C–D). In this specific research frame, pharmacological tools such as P450scc and 3 β -HSD inhibitors or pregnenolone synthetic analogues may impede the interpretation of data in the long run because they will likely produce changes in steroid levels and function in non-physiological ranges. Therefore, the development of a mouse model including a mutated CB1R unable to bind pregnenolone, as shown in our in vitro model,⁷³ should allow us to elucidate new endogenous mechanisms of CB1 regulation by pregnenolone. Genetic tools are indeed key to unravelling new CB1-mediated functions, such as exploring the in vivo effects of mtCB1 receptors in DN22-CB1 and DN22-CB1-KI mice.^{125,191} Targeting the pregnenolone-CB1 binding pocket may represent an exciting opportunity to shed light on new functions for steroids with respect to regulating GPCR activity.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Pierre-Louis Raux: Methodology; Resources; Writing-original draft; Writing-review & editing. **Guillaume Drutel:** Writing – review & editing. **Jean-Michel Revest:** Writing – review & editing. **Monique Vallée:** Conceptualisation; Investigation; Project administration; Resources; Supervision; Validation; Writing – original draft; Writing – review & editing.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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APPENDIX III. Raux & Vallée, *J Neuroendocrinol.* 2022

Cross-talk between neurosteroid and endocannabinoid systems in cannabis addiction

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Abstract

Steroids and endocannabinoids are part of two modulatory systems and some evidence has shown their interconnections in several functions. Homeostasis is a common steady-state described in the body, which is settled by regulatory systems to counterbalance deregulated or allostatic set points towards an equilibrium. This regulation is of primary significance in the central nervous system for maintaining neuronal plasticity and preventing brain-related disorders. In this context, the recent discovery of the shutdown of the endocannabinoid system (ECS) overload by the neurosteroid pregnenolone has highlighted new endogenous mechanisms of ECS regulation related to cannabis-induced intoxication. These mechanisms involve a regulatory loop mediated by overactivation of the central type-1 cannabinoid receptor (CB1R), which triggers the production of its own regulator, pregnenolone. Therefore, this highlights a new process of regulation of steroidogenesis in the brain. Pregnenolone, long considered an inactive precursor of neurosteroids, can then act as an endogenous negative allosteric modulator of CB1R. The present review aims to shed light on a new framework for the role of ECS in the addictive characteristics of cannabis with the novel endogenous mechanism of ECS involving the neurosteroid pregnenolone. In addition, this new endogenous regulatory loop could provide a relevant therapeutic model in the current context of increasing recreational and medical use of cannabis.

KEYWORDS

endocannabinoid system, neuromodulation, neurosteroids, pregnenolone, type-1 cannabinoid receptor

1 | INTRODUCTION

Neurosteroids have been investigated for almost 40 years^{1–3} and were discovered in the 1980s as steroids synthesized “by the brain for the brain”.^{4–6} One of their main characteristics is their de novo production on-demand in the brain, which is common to all species, all the way from amphibians to mammals up to and including humans.^{7–11} The first step, which can be defined as the rate-limiting step in their synthesis, involves the transport of cholesterol into the mitochondria mediated by the action of steroidogenic acute regulatory protein (StAR) and translocator protein 18 kDa (TSPO)^{12–14} and

its conversion to pregnenolone catalyzed by CYP11A1 (a P450 side-chain cleaving enzyme, P450sc).^{15–17} Subsequently, several pathways of pregnenolone metabolism, initially in the endoplasmic reticulum, trigger the synthesis of steroid families, including progestagens, corticoids, androgens and estrogens.^{18,19} Neurosteroid production occurs primarily in neurons and astrocytes in several brain areas, as first indicated by P450sc regionalization for pregnenolone synthesis.^{18,20,21} In addition to the localization of their production, some neurosteroids have aroused scientific interest for their action not on their receptors, but on neuromodulator receptors, mainly GABA_A and the NMDA receptors,^{22–24} which has conferred them a

neuromodulator status and thus a crucial role in the regulation of brain functions.^{20,25,26}

Another family of lipid neuromodulators that has gained the interest of the scientific community over the last 20 years arises from the discovery that the brain can produce endogenous analogs of phytocannabinoids, a class of naturally occurring molecules derived from the *Cannabis sativa* plant, of which Δ^9 -tetrahydrocannabinol (THC) is the major psychoactive component.²⁷ Those messengers, named “endocannabinoids” (short for endogenous cannabinoids) (eCBs) were identified in the 1990s as endogenous ligands of the two main cannabinoid receptors, type 1 (CB1R) and type 2 (CB2R) cannabinoid receptors,²⁸ formerly described with respect to binding exogenous cannabinoids, such as THC.²⁷

Subsequently, the discovery of the on-demand synthesis of eCBs in the postsynaptic membrane and their action on retrograde synaptic signaling led to a new perspective on how messengers can modulate neuronal activity²⁹ and opened up new fields of cannabinoid research, from the functioning of synaptic plasticity to cognition and motivation functions. The two main studied eCBs include *N*-arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol, which are synthesized from phospholipid precursors and are rapidly degraded into the cell by distinct enzymes: fatty acid amide hydrolase and monoacylglycerol lipase, respectively.^{30,31} The endocannabinoid system (ECS) thus includes CB1R and CB2R, the eCBs, and their synthesis and degradation enzymes. ECS function is a phylogenetically widespread feature in vertebrates³² and has been described in specific regions of the rodent brain and in different (sub)cellular sites, which accounts for its broad involvement in brain function.³³

Both the neurosteroid system and the ECS are involved in brain-modulating processes, and their imbalance can lead to brain dysfunction. In the present study, we address an unlikely and unexpected interaction between neurosteroids and the ECS, focusing on pregnenolone and CB1R function in cannabis addictive-like behavior.

2 | NEUROSTEROIDS AND ENDOCANNABINOIDS AS NEUROMODULATORS: NEW PERSPECTIVES

Neuromodulators can be defined as endogenous molecules or neuronal messengers that modulate neuronal activity upon stimulation. They have been observed in several species, from the nematode *Caenorhabditis elegans* to mammals.^{34,35} They can be involved in mediating local (autocrine or paracrine) neuromodulation or in a hormonal mode on neuronal targets distant from their release site.³⁶

Two examples of well-described neuromodulatory systems are the GABAergic and glutamatergic neuronal systems, which underlie the excitability properties of neurons and control the cellular inhibitory and excitatory balance, respectively.³⁷ These two systems work together to shape adaptive responses to external stimuli and are involved in the maintenance of physiological balance. Hence, deregulation of this equilibrium can contribute to the pathophysiology of brain disorders.

In this context, some neurosteroids are referred to as paracrine and/or autocrine neuromodulators³⁸ and they have been shown to modulate GABA_A and NMDA receptor activity with a similar affinity to that of

the endogenous neurotransmitter ligands GABA and glutamate, respectively.³⁹ This is the case for the sulfated derivative of pregnenolone, but not pregnenolone, which modulates GABA_A and NMDA receptors negatively and positively, respectively.⁴⁰ In addition, the progesterone metabolites allopregnanolone and pregnanolone act as positive allosteric modulators of the GABA_A receptor by facilitating the effects of GABA.⁴¹

On the other hand, the neuromodulatory actions of eCBs involve mainly a retrograde signaling mechanism, (i.e., release from the post-synaptic site to act on the pre-synaptic compartment), which can result in the inhibition of the transmitter release at both excitatory and inhibitory synapses. Indeed, the eCBs, by acting on CB1Rs localized on presynaptic GABAergic and glutamatergic neurons,^{28,42} can modulate synaptic plasticity by fine-tuning the activity of the aforementioned neurons, a process referred to as depolarization-induced suppression of inhibition or excitation, respectively.^{29,43–45} The CB1Rs are among the most abundant G-protein coupled receptors (GPCRs) in the brain⁴⁶ and are expressed in the brain to similar protein amounts as GABA_A and NMDA receptors. CB1Rs are highly present in sensory and motor brain areas, including the basal ganglia, substantia nigra, globus pallidus, cerebellum and hippocampus, which underlies their role in cognition and motivation.⁴⁶ In addition, eCBs interact with other neuromodulators, such as dopamine (DA) and serotonin (5-HT).⁴⁷ This interaction can occur through the release of eCBs from DA and 5-HT neurons, which regulates glutamatergic and GABAergic afferents. This has been documented, for example, for the mesolimbic dopaminergic system, in which DA neurons in the ventral tegmental area projecting to the nucleus accumbens (NAc) receive GABAergic inputs and 5-HT/glutamate inputs from the dorsal raphe nucleus. As a result, eCBs can fine-tune the activity of the mesolimbic DA system by acting on pre-synaptic CB1Rs in GABAergic and glutamatergic neurons, thereby modulating excitatory and inhibitory signaling, respectively.^{48,49} This suggests that eCBs play a crucial role in controlling adaptive behaviors and maintaining reward system homeostasis, positioning the ECS as a key modulatory network in the brain.^{50,51}

In this neuromodulation framework, the putative interactions between neurosteroids modulating GABA_A and NMDA receptors together with ECS could prove relevant to maintaining the activity of the neuronal networks involved in reward homeostasis. Here, however, we propose the neurosteroid pregnenolone as a key player in the interconnection between steroid and endocannabinoid systems, with the discovery of a regulatory loop between pregnenolone and the CB1R.

3 | DISCOVERY AND EVIDENCE OF THE REGULATORY LOOP BETWEEN PREGNENOLONE AND CB1R

3.1 | CB1 activation and pregnenolone production

3.1.1 | The actors of brain pregnenolone production

General steroid biosynthesis derives from approximately 2% of the daily cholesterol pool in humans, acquired from the diet from de novo

synthesis from acetyl-coenzyme A.⁵² In the brain, it has been proposed that the source of cholesterol rather originates from de novo synthesis because the blood–brain barrier is not permeable to cholesterol lipoproteins.⁵³ One of the rate-limiting factors for steroidogenesis is the available amount of cholesterol in astrocytes and neurons. First, the free cholesterol pool in the cytosol is related to the action of the hormone-sensitive lipase/cholesteryl esterase (HSL), encoded by the LIPE gene, which is responsible for hydrolyzing cholesteryl esters located in lipid droplets.⁵⁴ HSL protein and its enzymatic activity have been detected in the mouse brain at neuronal synapses.⁵⁵ The proteins StAR and TSPO were described to be respectively involved in the delivery of cholesterol and its transport from the outer membrane to the inner membrane of mitochondria.⁵⁶ StAR mRNA and protein were detected in brain glial and neuronal cells and co-localization with P450scc was observed in the mouse and human cerebral cortex.⁵⁷

In steroidogenic tissues, including the brain, cholesterol is the unique substrate of the P450scc (i.e., CYP11A1) enzyme, which catalyzes its conversion to pregnenolone. The structural organization of cholesterol, in particular the C20–C22 region of its side chain, allows the recognition and binding to the catalytic domain of the enzyme. CYP11A1 is involved in three consecutive reactions from cholesterol to pregnenolone, including the hydroxylation of C22 and C20, and then the cleavage of the C22–C20 bond. The expression of P450scc mRNA has been detected in several rodent brain areas, including the cortex, amygdala, hippocampus and midbrain,⁵⁸ as well as in humans.⁵⁹ Human P450scc is encoded by a single gene on chromosome 15, the CYP11A1 gene.⁶⁰ Its expression has been reported in the human temporal lobe, frontal lobe and hippocampus, although it is much lower than in the adrenals, expressing high CYP11A1 mRNA levels.^{61,62}

3.2 | Effect of acute THC on steroidogenesis

In rodents, we found that acute THC, through a CB1-dependent mechanism, dramatically and dose-dependently (with a plateau at 9 mg kg⁻¹ in rats and 12 mg kg⁻¹ in mice) increased pregnenolone levels in several brain areas and peripheral tissues, without altering downstream steroidal metabolites, suggesting that pregnenolone is the specific steroid target of THC.⁶³ Pregnenolone levels were especially high in CB1-enriched areas of the brain, including the frontal cortex, nucleus accumbens, dorsal striatum, thalamus, hippocampus and cerebellum.² This effect also appeared to be drug-specific because other drugs of abuse (such as cocaine, nicotine, amphetamine and alcohol) induced a much smaller increase in pregnenolone at doses corresponding to their respective median effective dose for most of the unconditioned behavioral effects.⁶³ This is consistent with the reported dose-dependent increase in cerebrocortical levels of pregnenolone, as well as the downstream steroids, progesterone and allopregnanolone, following acute administration of nicotine or amphetamine in rats.^{64,65}

By screening the phosphorylation status of proteins involved in steroidogenesis, we identified that acute THC administration was able

to promote steroid synthesis in the NAc of the adult male rat by increasing the amounts of phosphorylated of HSL and P450scc, without affecting the StAR or TSPO proteins.⁶³

Overall, these data demonstrate that the activation of CB1R by THC stimulates the two key steps of neurosteroidogenesis, that is the availability of free cholesterol and the synthesis of pregnenolone from cholesterol, which results in increased pregnenolone brain levels.

3.3 | Effects of pregnenolone on CB1-mediated addiction-related functions

Pregnenolone has been shown to prevent the effect of CB1 activation at several stages that underlie the mechanisms of cannabis-related addictive behavior. From a neuromodulatory perspective, *ex vivo* electrophysiology experiments have shown that pregnenolone alters the effects of CB1 activation on glutamate synaptic plasticity. Pregnenolone, acting at the level of presynaptic glutamatergic neurons, attenuated the effect of THC inhibition on glutamate release as determined by the measurement of excitatory postsynaptic currents in rodent brain (i.e., NAc) slices.⁶³ This downward effect was approximately 34%, whereas another study reported a smaller decrease (approximately 15%).⁶⁶ In addition, pregnenolone also down-regulates the glutamatergic response to a synthetic CB1 agonist in CA1 hippocampal slices because pregnenolone can block the WIN55,512-2-induced decrease in field excitatory postsynaptic currents.⁶⁷

At the cellular and neurochemical scales, the regulatory action of pregnenolone was assessed with respect to the stimulatory effect of THC on striatal DA neurotransmission. The activation of CB1R by endocannabinoids and exogenous cannabinoids, including THC, in animals has been shown to enhance the firing of mesolimbic DA neurons and ventral striatal DA levels.^{68–71} THC also increased DA levels in the ventral striatum of humans as shown by positron emission tomography scans in healthy subjects.⁷² Using *in vivo* microdialysis coupled to electrophysiology,⁷³ acute pregnenolone inhibited the THC-induced increase in the firing activity of DA neurons located in the ventral tegmental area and the associated increase in DA output in the NAc of adult mice.⁶³ In addition, subacute treatment with pregnenolone in postnatal rats (postnatal day 5–13) subjected to prenatal THC exposure has been shown to prevent hyperdopaminergic alterations.⁶⁹ Therefore, pregnenolone may modulate the effects of THC on the electrical and neurochemical activities of striatal dopaminergic neurons, suggesting that pregnenolone may restore the balance of the mesolimbic DA network, conceivably by counteracting the inhibition of GABA release induced by CB1 activation on presynaptic GABAergic neurons.²

From a behavioral perspective, THC and CB1 agonists exhibit reward properties by implementing self-administration behavior in animals.⁷⁴ In a mouse model of self-administration of the CB1 agonist, WIN 55,212-2,⁷⁵ pregnenolone significantly reduced the amount of drug intake and motivation for the drug.⁶³ Furthermore, to address the action of pregnenolone on cannabis use disorders, the models of cannabis-induced acute psychotic states (CIAPS) in mice⁷⁶ and

prenatal cannabis exposure (PCE) in rats⁶⁹ have been investigated. The CIAPS and PCE models are of clinical relevance because exposure to THC acutely during the teenage years or throughout the pre-birth period may increase the likelihood of developing subsequent psychotic-like symptoms and cognitive deficits.⁷⁷⁻⁷⁹ In the CIAPS or PCE model, pregnenolone inhibits several THC-induced psychotic-like symptoms including somatosensory gating and deficits in memory.^{69,76}

Together, these data strongly suggest that pregnenolone may reduce the development of cannabis abuse by acting on cannabis-related behavioral alterations and the underlying mechanisms.

3.4 | Pregnenolone action on CB1R

3.4.1 | Pregnenolone acts as an endogenous signaling-specific negative allosteric modulator on CB1R

One general consequence of allosteric modulation is the stabilization of the receptor in a given conformation, which can lead to a change in protein-protein interactions and thus affect receptor activation. In general, negative and positive allosteric modulators (NAMs and PAMs, respectively) bind to an allosteric site on the receptor that is distinct from the endogenous ligand-binding site (orthosteric site) and may influence the binding of the ligand and/or signaling pathways, resulting in the altered affinity and/or efficacy for the agonist.⁸⁰ If we consider the case of NAMs, they have specific properties that differ from orthosteric antagonists and give them benefits for therapeutic development. This includes a probe-specific effect, which means that NAMs produce different effects depending on the ligands of the receptor. Furthermore, NAMs do not completely block the binding of the natural agonist. This feature may be an advantage from a therapeutic point of view because it allows the maintenance of normal receptor activity towards endogenous ligands.

In the case of the CB1R, several allosteric modulators have been described, among which three are classified as endogenous allosteric modulators, including pregnenolone.^{19,81,82} Several pieces of evidence have demonstrated that pregnenolone acts as a NAM on CB1R. First, it did not alter the equilibrium binding of CB1R ligands, such as WIN55,212-2 or CP55,940, to hCB1R in Chinese hamster ovary cells.⁶³ In addition, the behavioral effects of THC were increased by blocking the synthesis of pregnenolone by the administration of an inhibitor of P450_{sc} in mice, supporting a decrease in the potency and/or affinity of THC by pregnenolone.⁶³ Finally, pregnenolone involves biased modulation of CB1 signaling in response to THC on hCB1R in HEK293 cells, specifically by inhibiting the activation of the ERK1/2^{MAPK} pathway without altering the G-protein pathway involved in THC-induced cAMP decrease.⁶³

Further examination of the CB1-mediated biased action of pregnenolone remains to be investigated across multiple signaling cascades, including action on ERK1/2^{MAPK}, because one study reported no effect of pregnenolone on the activation of ERK1/2^{MAPK} induced

by THC or WIN55,212-2 in Chinese hamster ovary-hCB1R cells.⁸³ Moreover, the lack of effect of pregnenolone described on depolarization-induced suppression of excitation induced by 2-arachidonoylglycerol in hippocampal neurons⁸⁴ may suggest a ligand-dependent action of pregnenolone.

3.4.2 | Pregnenolone binds to a specific binding site on CB1R

Computational experiments, using the forced-biased Metropolis Monte Carlo simulated annealing program for ligand-protein binding⁸⁵ between pregnenolone and a CB1R model,⁸⁶ have revealed that pregnenolone binds to a specific binding pocket formed by the transmembrane domains, TMH1 and TMH7, associated with the C-terminal intracellular helix 8.⁶³ Each polar group of pregnenolone interacts with residue E.1.49 (for the ketone group) or R7.65 (hydroxyl group), on the TMH1, 7 and Hx8 regions, respectively. The localization of this binding site has been confirmed by an *in vitro* experiment measuring mitochondrial respiration in cells transfected with a mutant human CB1R (hCB1R^{p.E1.49G}), in which the glutamate (E) residue in position 1.49 has been substituted by a glycine (G). In these cells, the inhibitory effect of pregnenolone on the THC-induced decrease in cellular respiration was absent, in contrast to that observed in cells transfected with a wild-type hCB1R.⁶³

Pregnenolone binding to the TMH1/TMH7/Hx8 site, likely results in rigidity of the intracellular end of TMH7, a region that is required to move upon activation of class A GPCRs.⁸⁷ Thus, the identified binding site of pregnenolone is in agreement with its action as a negative allosteric modulator on the CB1R.

3.5 | How does the PREG-CB1 regulatory loop work?

The postulated model of the PREG-CB1 regulatory loop involves several steps. When excessive activation of CB1 signaling arises during THC intoxication (as a result of high-dose THC in mice or cannabis use/abuse in humans), an imbalance of brain systems may occur, such as in the DA mesolimbic network, known to be involved in the cannabis addictive process. The CB1R signal may serve as a homeostasis sensor, which activates the production of pregnenolone in several brain areas, including the NAC, via the activation of HSL and P450_{sc}. In turn, a feedback mechanism is engaged via the binding of pregnenolone to CB1R at an allosteric binding pocket, which suppresses the effect of THC on specific CB1 signaling pathways (involving a biased activity at the Erk1/2^{MAPK} pathway), and on THC-mediated outcomes, thereby correcting the homeostatic imbalance. It emerges from this model that the interaction between the neurosteroid pregnenolone and the ECS system orchestrates many behaviors in health and disease that are intrinsic features of body homeostasis. A broad illustration of this model is depicted in Figure 1.

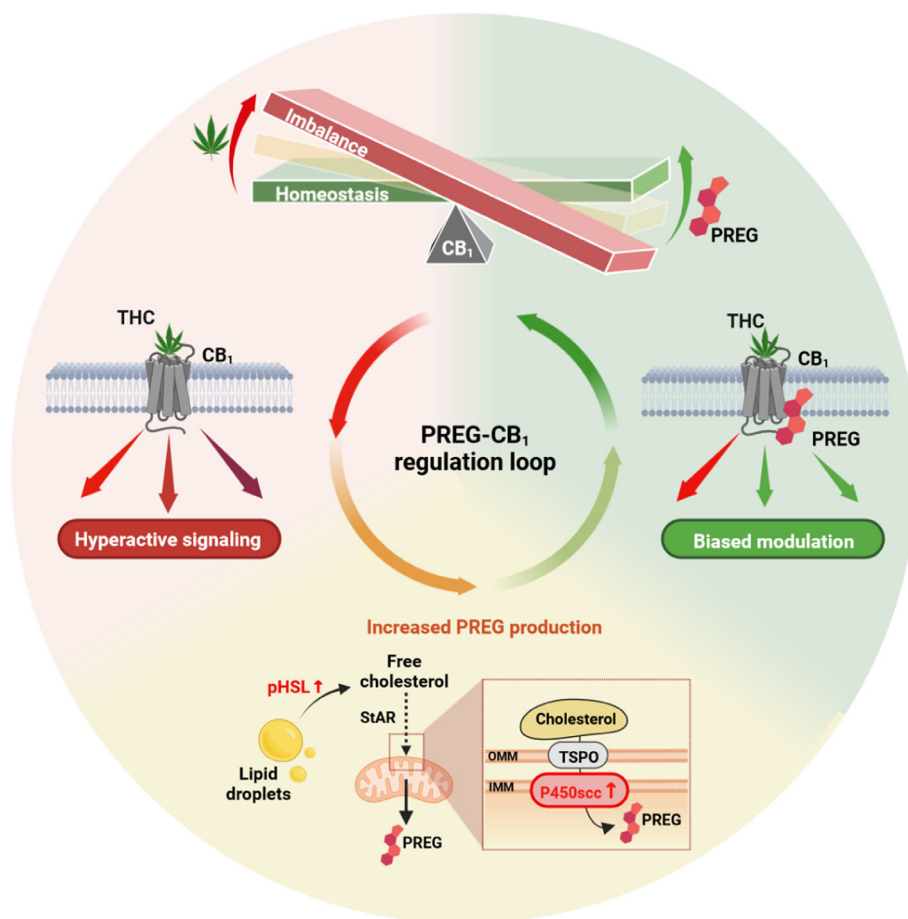


FIGURE 1 A postulated model for the PREG-CB1 regulatory loop: THC is responsible for the intoxicating effects of cannabis through the dysregulation of CB1-mediated homeostasis, leading to hyperactive receptor signaling. This dysregulation triggers an endogenous regulation loop, starting with the THC-induced increase in the amount of phosphorylated HSL in the brain, leading to the mobilization of free cholesterol from lipid droplets serving as the available pool of cholesterol for de novo steroidogenesis. Interaction of cholesterol together with StAR and TSPO allows its transport to the mitochondria and translocation from the outer to the inner mitochondrial membrane for the onset of steroid synthesis. Within the mitochondria, THC administration increases p450scc levels, the enzyme responsible for the synthesis of pregnenolone from cholesterol, hence elevating pregnenolone brain production and levels. Pregnenolone then exerts negative feedback on the CB1 receptor by docking on a dedicated allosteric binding pocket on the receptor. This results in the biased modulation of CB1 intra-cellular signaling pathways, allowing blockade of THC outcomes and restoration of CB1-mediated homeostasis. Abbreviations: CB1, cannabinoid type 1 receptor; HSL, hormone-sensitive lipase; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; p450scc, cholesterol side-chain cleavage enzyme; pHSL, phosphorylated hormone-sensitive lipase; PREG, pregnenolone; StAR, steroidogenic acute regulatory protein; THC, Δ^9 -tetrahydrocannabinol; TSPO, translocator protein.

4 | PREG-CB1R LOOP IN HOMEOSTASIS PROSPECTIVE

The above reports demonstrate that the production of pregnenolone can be upregulated by CB1R activation in the brain. Although additional regulatory mechanisms need to be evaluated further, we have identified two effectors/sensors of modulation, including HSL and P450scc. The first involves the availability of free cholesterol required for steroidogenesis with the increase of HSL responsible for the hydrolysis of cholesterol esters. HSL has been reported at neuronal synapses with a wide distribution within the mouse brain and has been proposed to play a key role in the neuroactive lipid homeostasis related to normal brain function.⁵⁵ Mitochondrial P450scc enzyme

(CYP11A1) is the second target for CB1-mediated pregnenolone increase. P450scc is responsible for the initiation of steroidogenesis with the conversion of cholesterol to pregnenolone. A significant decrease in free GABA levels and a slight reduction in free glutamate levels have been reported in the cerebrospinal fluid of subjects with homozygous P450scc disruption,⁸⁸ suggesting that P450scc may play a role in the inhibitory/excitatory homeostasis process in the brain. Thus, it can be hypothesized that the impact of increased HSL and P450scc may involve the action of pregnenolone on CB1Rs.

Our findings also support a novel role for pregnenolone via its action through the CB1R GPCR. It is more likely that pregnenolone operates as either an autocrine or paracrine neuromodulator than an endocrine one. Indeed, pregnenolone is produced within mitochondria

in neurons and astrocytes,⁸⁹ yet the mitochondrial CB1R (mtCB1R) has recently focused all attention first with the discovery of its presence on the outer membrane of mouse brain neuronal mitochondria,⁹⁰ described as a G protein-coupled receptor containing the first 22 amino acids of the CB1 protein.⁹¹ In addition, mtCB1Rs have been involved in neuronal energy metabolism and CB1-mediated synaptic plasticity such as depolarization-induced suppression of inhibition⁹⁰, as well as in the regulation of memory.⁹¹ Finally, mtCB1Rs in astrocytes are involved in glucose metabolism and THC-induced deficits in social behavior.⁹² Further studies are needed to demonstrate a direct action of pregnenolone on mtCB1. We first assessed the action of pregnenolone on in vitro mitochondrial respiration using a human CB1R (hCB1R) mutated at the PREG binding site, preventing its binding to the CB1R. In doing so, we found that, in cells transfected with intact hCB1R, PREG blocked the THC-induced decrease in cellular and mitochondrial respiration, whereas this effect was abolished in cells transfected with mutated hCB1R.⁶³ Ongoing in vivo experiments translating this genetic strategy into mice are exploring the effects of endogenous pregnenolone on CB1-mediated behavior.

In terms of homeostasis gain and an evolutionary perspective, the body has set up an ingenious device such that the disruption of ECS signaling by exogenous THC is attenuated by an endogenous inhibitory modulator produced locally in the brain. The fact that CB1Rs localized in D1 striatal GABAergic neurons have been shown to mediate THC-induced pregnenolone production⁶³ suggests that pregnenolone serves as a neuroprotective messenger mediated by GABAergic CB1Rs. This has relevance with the regulatory action of GABAergic CB1Rs during excessive ECS stimulation, as provided by the biphasic function of ECS mediated by the balance between glutamatergic and GABAergic CB1Rs in response to low and high doses of THC, respectively.^{93–96} Thus, pregnenolone can serve as an endogenous “brake” during uncontrolled ECS activation⁹⁷; however, it is not a brake *stricto sensu*, such as how the orthosteric CB1 antagonist operates (i.e., by completely shutting down the activity of ECS), but rather a “slowing down mechanism”, which is more likely to be recruited after high degrees of CB1 activation.

It is noteworthy that pregnenolone, which acts as a NAM by binding to a specific allosteric binding pocket without altering the competitive binding of CB1 ligands, does not tend to alter the equilibrium established by the action of eCBs. Furthermore, inhibition of CB1-specific signaling by pregnenolone, which more likely acts through β -arrestin-1 mediated pathways because it does not change cAMP response mediated by a G protein, is not expected to have a significant impact on the fine-tune of ECS on homeostasis. Together, both properties of pregnenolone may represent promising therapeutic advantages by blunting adverse signaling side effects.^{51,82,98} This is critical in the context of the current increase in medical and recreational cannabis use and cannabis use disorders, whereas, at the same time, the perceived risk of cannabis exposure is decreasing.^{99,100}

In conclusion, the neurosteroid pregnenolone and eCBs are typical examples of endogenous neuromodulators of the CB1R metabotropic GPCR, involving second messenger signaling cascades, which can be translated into broad and diverse signals. Nevertheless,

despite the diversity of CB1 functions that could arise from these signals, it is more likely that the pregnenolone-CB1R regulatory loop is involved in particular functions given the specific brain region and (sub)cellular localization of the CB1R and the functional selectivity of the CB1R that engages a ligand-specific response leading to an allosterically biased signaling response. This review has outlined the role of the PREG-CB1R regulatory loop in CB1-related intoxication and addiction, which can be applied to a broader homeostatic process. Ongoing and future research will help to elucidate whether this cooperative neuromodulation duo participates in fine-tuning ECS function in response to physiological and other pathophysiological inputs.

AUTHOR CONTRIBUTIONS

Pierre-Louis Raux: Visualization; writing – review and editing. **Monique Vallée:** Conceptualization; writing – original draft; writing – review and editing.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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