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Role of pregnenolone derivative AEF0117 on the regulation of CB1 signaling that mediates behavioral effects of THC

Giovanni Tomaselli

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Presented by Giovanni TOMASELLI

**Role of the pregnenolone derivative AEF0117 on the regulation of CB1
signaling that mediates behavioral effects of THC**

Under the supervision of Dr. Monique Vallée

Defended in September the 21st, 2020

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THÈSE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

**DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX**

SCIENCE DE LA VIE ET DE LA SANTÉ

NEUROSCIENCE

Par Giovanni TOMASELLI

**Rôle du dérivé de la pregnénolone, l'AEF0117, dans la régulation de la
signalisation CB1 impliquée dans les effets comportementaux du THC**

Sous la direction de : Dr. Monique Vallée

Soutenu le 21 Septembre 2020

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Invité

*“If I have seen further it is by
standing on the shoulders of Giants”*

Sir Isaac Newton

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Tomaselli, G., Zanese, M., Roullot-Lacarrière, L., Grel, A., Vallée, M., and Revest, JM. *Exploring intracellular signaling pathways using AlphaLISA SureFire Ultra technology*, 04.2018 Neurocentre Magendie symposium

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G. Tomaselli, A. Busquets-Garcia, V. Lalanne, M. Metna, A. Grel, G. Marsicano, P.V. Piazza, J.M. Revest, M. Vallée. *Synthetic pregnenolone-like compound AEF0117 blocks the hyperlocomotion induced by a low dose of THC in mice*. 25.07.2019, Castelldefels, Gordon Research Conference.

G. Tomaselli*, M. Zanese*, V. Roullot-Lacarrière, A. Grel, J.M. Revest*, M. Vallée* (*equal contribution). *Alpha technology as a powerful tool to detect brain intracellular signaling events*. 24.05.2019, Marseille Neurofrance.

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ABBREVIATIONS

2-AG	2-arachinodoilglycerol
2-AGE	noladin ether
5-HIAA	5-hydroxyindolacetic acid
5-HT	serotonin
5-HT _x	serotonin receptor
7TM	seven transmembrane receptor
ABHD	mammalian alpha beta hydrolase domain
AC	adenylyl cyclase
ACh	acetylcholine
ADHD	attention deficit hyperactivity disorder
AEA	anandamide
AIPP	acute, immediate, persistent psychosis
Akt	protein kinase B
AMG	aminoglutethimide
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
Amy	amygdala
aPKC	atypical protein kinase C
ATP	adenosine triphosphate or acute, transient psychosis
Atp	acute transient psychosis
AUD	alcohol use disorder
Avp	vasopressin
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BG	basal ganglia
BLA	basolateral amygdala
BSN	brainstem nuclei

cAMP	3',5'-cyclic adenosine monophosphate
CB	cerebellum
CB1	type-1 cannabinoid receptor
CB1-SSi	CB1 Signaling Specific Inhibitor
CB2	type-2 cannabinoid receptor
CBD	cannabidiol
CBN	cannabinol
CDPP	chronic, delayed, persistent psychosis
CHO	chinese hamster ovary
CNS	central nervous system
COMT	catechol-O-methyltransferase
CPU	caudate-putamen
CUD	cannabis use disorder
CX	cortex
CYP450 _{scc}	cytochrome P450 side-chain cleavage
D ₁	dopamine receptor type 1
D ₂	dopamine receptor type 2
DA	dopamine
DAG	diacylglycerol
DAGL or DGL	diacylglycerol lipase
DAT	dopamine transporter
DHEA	dehydroepiandrosterone
dHpc	dorsal hippocampus
DNA	deoxyribonucleic acid
DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
DSM	diagnostic and Statistical Manual of Mental Disorders
E	epinephrine
eCBs	endocannabinoids
ECS	endocannabinoid system

eIF	eukaryotic translation initiation factor
Erk	extracellular signal–regulated kinases
FAAH	fatty acid amide hydrolase
FC	frontal cortex
FDA	food and drug administration
FIT	found in Tor
GABA	γ -aminobutyric acid
GABA _A	ionotropic GABA receptor
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GIRK	G protein-coupled inwardly-rectifying potassium channels
Glu	glutamate
GP	globus pallidus
GPCR	G protein-coupled receptor
GP _L	lateral globus pallidus
GP _M	medial globus pallidus
GPR55	G protein-coupled receptor 55
GRK	G protein-coupled receptor kinase
GSK3 β	glycogen synthase kinase 3 β
GSM	glycine Modulator Sites
GTP	guanosine triphosphate
HEK	human embryonic kidney 293 cells
HL60	human leukemia cell line
Hpc	hippocampus
HTS	high throughput screening
Hyp	hypothalamus
IP ₂	inositol 4,5 bisphosphate
IP ₃	inositol 3,4,5 triphosphate
JNK	c-Jun N-terminal kinases
JWH-018	spice

KO	knock out
LC	locus coeruleus
LHb	lateral habenula
LSD	lysergic acid diethylamide
LTD	long-term depression
LTP	long-term potentiation
MAGL	monoacylglycerol lipase
MAM	monoacetylmorphine
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MCtx	motor cortex
mGluR	metabotropic glutamate receptor
MLR	mesencephalic locomotor region
MNK	MAPK interacting protein kinases
MSN	medium spiny neurons
mTOR(C)	mammalian target of rapamycin (complex)
NAAA	N-acylethanolamine acid amide hydrolase
NAc	nucleus accumbens
NAE	N-acylethanolamine
NAPE-PLD	N-acyl phosphatidylethanolamine-specific phospholipase D
NAT	N-acetyltransferase
NE	norepinephrine
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate receptor
NMRI	nuclear magnetic resonance imaging
NRI	norepinephrine reuptake inhibitor
Oxt	oxytocin
PAG	periaqueductal gray
Pepcan	peptide endocannabinoid
PFC	prefrontal cortex

PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinases
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP ₃	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PKD-1	protein kinase D-1
PLC	phospholipase C
PLD	phospholipase D
PND	postnatal day
PNS	peripheral nervous system
PP2A	phosphatase 2A
PPAR	peroxisome proliferator-activated receptors
pPCtx	posterior parietal cortex
PPI	prepulse inhibition
PREG	pregnenolone
PTEN	prime Time Entertainment Network
PVN	paraventricular nucleus
RF	medial reticular formation
RNA	ribonucleic acid
RPS6	ribosomal Protein S6
Ser (also S)	serotonine
SNr	substantia nigra
SON	supraoptic nucleus
StAR	Steroidogenic acute regulatory protein
STAT	signal transducer and activator of transcription
STN	subthalamic nucleus
Str	striatum
Tha	thalamus
THC	Δ^9 -tetrahydrocannabinol
Thr (also T)	Threonine

TMH	transmembrane helix
TrpV1	transient receptor potential cation ch. subfamily V member 1
VCtx	visual cortex
VGCC	voltage-gated calcium channel
vHpc	ventral hippocampus
VMAT	vesicular monoamine transporter
VR1	vanilloid type 1 receptor
VTA	ventral tegmental area
WT	wild type

Abstract (English)

Cannabis sativa is among the most widely-abused drugs worldwide. THC, its main psychoactive component, is a risk factor in several mental pathologies, such as cannabis use disorder, addiction, and psychosis. Being a biphasic drug, high doses of THC cause hypoactivity and aversion, whereas low doses of THC cause hyperactivity and reward. THC acts on the type-1 cannabinoid receptor (CB1), one of the most abundant G-protein-coupled receptors (GPCRs) in the brain, whose signaling is biased, meaning that different transducers can trigger specific pathways in different conditions. Biased signaling has proven to be extremely relevant in drug discovery, while understanding CB1 signaling in pathological conditions is essential for cannabinoid-based drug development. It is known that different doses of THC lead to different behavioral, cellular, and molecular outcomes. However, the link between those phenomena has never been investigated.

For therapeutic purposes, PREG-like CB1-SSi compounds have thus been synthesized that share the same PREG therapeutic potential, but cannot be metabolized into downstream steroids. The CB1-SSi studied in this work was the CB1-SSi lead compound, AEF0117.

The first aim of the current work was to understand the intracellular signaling pathways following low, medium, and high doses of THC, leading to three distinct known behavioral outputs in mice: hyperlocomotion, asociability, and hypolocomotion, respectively.

The second aim of the thesis was to understand the mechanism of action of AEF0117, and its ability to mimic PREG in blocking the behavioral and molecular effects of THC at low, medium, and high doses.

The doctoral dissertation is divided into five main parts. **The introduction** serves to preface the concepts of the endocannabinoid system, as well as cannabis abuse in humans and the behavioral outcomes of THC in mice, including hyperlocomotion, asociability, and hypolocomotion. The state of the art of CB1 signaling involving the biased CB1 system is

described with particular emphasis on CB1 Signaling Specific Inhibitors (CB1-SSi), in particular endogenous pregnenolone (PREG), and its synthetic analogue, the CB1-SSi lead compound, AEF0117.

The article Zanese, Tomaselli et al., 2020 (published in J. Neurosci. Methods) presents validation of the high-throughput analytical technique (AlphaLISA) of choice in this study for detecting protein phosphorylation in brain tissue lysates.

The article Tomaselli et al. (to be submitted) is devoted to studies of the low dose of THC that causes hyperlocomotion, with the discovery of its related intracellular CB1 signaling pathway, along with the signaling transducer involved in the CB1-rich brain areas relevant for locomotor activity (NAc, Str, CB). The main data revealed that THC via CB1 recruits the β -Arrestin1-PI3K-Akt-GSK3 β signaling pathway that leads to hyperlocomotion. Furthermore, both PREG and AEF0117 were able to block the THC-induced hyperlocomotion and altered signaling in mice.

The third part of the data presents studies on the effects of THC at the medium and high doses that induce asocial behavior and hypolocomotion, respectively. Each dose of THC induced specific alterations in the CB1 intracellular signaling pathways in the most CB1-rich brain areas, and treatment with AEF0117 rescued both behaviors.

The general discussion then addresses conclusions and perspectives, highlighting the role of specific CB1 pathways in THC-induced addiction and psychosis, and proposes a mechanism of action for CB1-SSi compounds, including AEF0117.

Resumé (Français)

Le cannabis sativa est l'une des drogues les plus consommées dans le monde. Le THC, sa principale composante psychoactive, représente un facteur de risque de plusieurs pathologies mentales, telles que le trouble de la consommation de cannabis, la dépendance et la psychose. Étant une drogue biphasique, les fortes doses de THC provoquent une hypoactivité et une aversion, tandis que les faibles doses de THC provoquent une hyperactivité et une récompense. Le THC agit sur le récepteur aux cannabinoïdes de type 1 (CB1), l'un des récepteurs couplés aux protéines G les plus abondants dans le cerveau, dont la signalisation est biaisée; ce qui signifie que différents transducteurs peuvent véhiculer une voie spécifique en fonction de différentes conditions. Il a été prouvé que la signalisation biaisée est extrêmement pertinente dans la découverte de médicaments et la compréhension de la signalisation CB1 dans des conditions pathologiques est essentielle pour le développement de médicaments à base de cannabinoïdes. Même si il a été montré que différentes doses de THC entraînent des effets comportementaux, cellulaires et moléculaires différents, le lien entre ces phénomènes n'a jamais été étudié.

En outre, il a été découvert que le neurostéroïde pregnénolone (PREG) est un modulateur allostérique endogène biaisé du CB1, en étant un inhibiteur de signalisation spécifique du récepteur CB1 (CB1-SSi) et capable de bloquer plusieurs comportements déclenchés par le THC. Comme la PREG est un précurseur de stéroïde, son administration à forte dose peut induire la production de stéroïdes en aval, avec d'éventuels effets secondaires.

Ainsi, dans un but thérapeutique, des composés CB1-SSi analogues à la PREG ont été synthétisés, ayant le même potentiel thérapeutique que la PREG, mais sans être métabolisés en d'autres stéroïdes. Le CB1-SSi étudié ici est le composé CB1-SSi leader, l'AEF0117.

Le premier objectif de ce travail était de comprendre les voies de signalisation intracellulaires selon des doses faibles, moyennes et élevées de THC, conduisant à trois sorties

comportementales distinctes chez la souris, respectivement l'hyperlocomotion, l'associabilité et l'hypolocomotion. **Le second objectif** était de comprendre le mécanisme d'action de l'AEF0117 et sa capacité à bloquer les effets comportementaux et moléculaires du THC à faibles, moyennes et fortes doses.

La thèse est divisée en cinq parties principales. **L'introduction** sert à préfacier les concepts du système endocannabinoïde, ainsi que l'abus de cannabis chez l'homme et les effets comportementaux du THC chez la souris, comme l'hyperlocomotion, l'associabilité et l'hypolocomotion. L'état de l'art de la signalisation CB1 impliquant le système CB1 biaisé est décrit avec un accent particulier sur les inhibiteurs spécifiques de la signalisation CB1 (CB1-SSi), en particulier la pregnénolone endogène (PREG), et son analogue synthétique, le composé CB1-SSi, l'AEF0117.

L'article Zanese, Tomaselli et al, 2020 (publié dans J. Neurosci. Methods) porte sur la validation de la technique analytique à haut débit (AlphaLISA) de choix dans cette étude pour la détection de la phosphorylation des protéines dans les lysats de tissu cérébral.

L'article de Tomaselli et al. (à soumettre) est consacré à l'étude de faible dose de THC qui provoque l'hyperlocomotion chez la souris. Les principales données ont révélé que le THC via CB1 recrute la voie de signalisation β -Arrestin1-PI3K-Akt-GSK3 β , dans les zones du cerveau riches en CB1 et pertinentes pour l'activité locomotrice (NAc, Str, CB), qui conduit à l'hyperlocomotion. En outre, PREG et AEF0117 peuvent bloquer l'hyperlocomotion et les modifications de la signalisation CB1 induite par le THC.

La troisième partie représente les études sur les effets du THC à des doses moyennes et élevées qui induisent respectivement un comportement asocial et une hypolocomotion. Chaque dose de THC induit des altérations spécifiques des voies de signalisation intracellulaires CB1 et le traitement avec l'AEF0117 reverse les deux comportements.

La discussion générale aborde ensuite les conclusions et perspectives, en soulignant le rôle des voies CB1 spécifiques dans la dépendance et la psychose induites par le THC, et propose un mécanisme d'action pour les composés CB1-SSi, dont l'AEF0117.

Introduction

I An overview of the endocannabinoid system and exogenous cannabinoids

The endocannabinoid system (ECS) is canonically composed of the type 1 and 2 cannabinoid receptors (CB1 and CB2 respectively), the endocannabinoids, and of the enzymes responsible for the synthesis (e.g., NAPE, DAGL) and degradation (e.g., FAAH, MAGL) of the endocannabinoids (Di Marzo and Piscitelli, 2015) (Fig. 1). In addition, the GPR55 orphan receptor is now considered the type 3 cannabinoid receptor (Moriconi et al., 2010; Ross, 2009; Sharir and Abood, 2010; Yang et al., 2016). The cannabinoid receptors that are expressed the most, CB1 and CB2, are more abundant in the central nervous system (CNS) and periphery, respectively, while GPR55 is relatively abundant in the brain, even though it has been found in peripheral tissues as well (Hu et al., 2011). More recently, it has been shown that endocannabinoids can target other receptors than CB1 and CB2, such as peroxisome proliferator-activated receptors (PPARs) and transient receptor potential (TRP) channels (Lu and Mackie, 2016).

However, it has been demonstrated that more actors can interact strictly with the endocannabinoid system. They are endogenous regulators of the cannabinoid receptors, such as

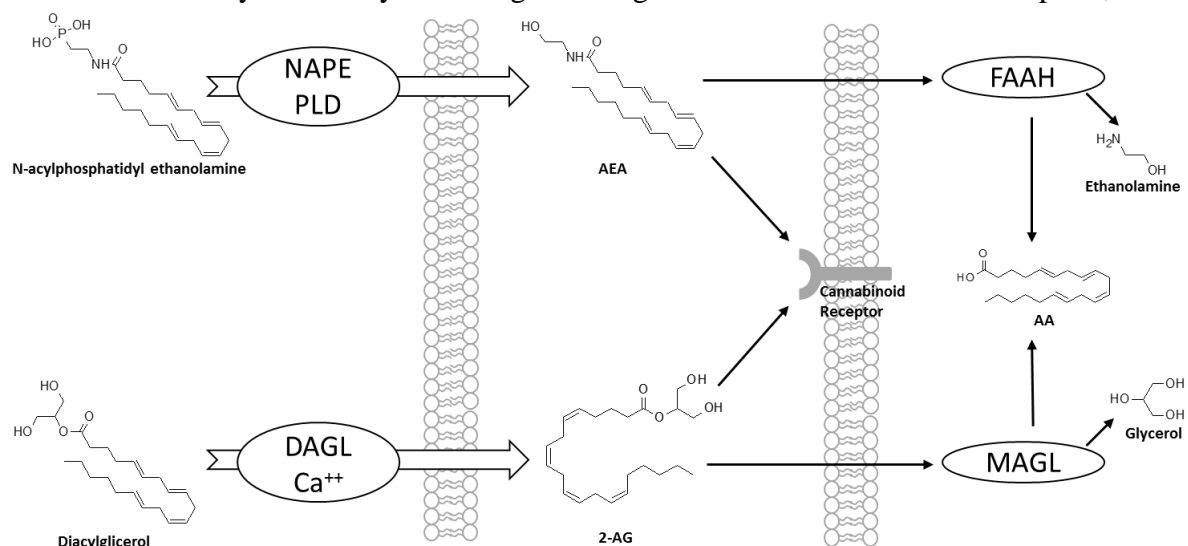


Figure 1: The endocannabinoid system comprises the endocannabinoids, the enzymes responsible for their synthesis and metabolism, and the cannabinoid receptors. Here is represented the "life span" of the two most abundant endocannabinoids. N-acylphosphatidyl ethanolamine is metabolized by the N-acyl phosphatidylethanolamine phospholipase D (NAPE PLD) to anandamide (AEA), while diacylglycerol is converted to 2-arachidonoylglycerol (2-AG) by the Diacylglycerol lipase (DAGL). Both AEA and 2-AG can bind to a cannabinoid receptor (CB1 or CB2), of which 2-AG is a full agonist, and AEA is a partial agonist, and AEA can bind other receptors such as the Vanilloid Type 1 receptor (VR1) with even higher affinity or the GPR55.

the steroid pregnenolone, a negative allosteric modulator of CB1 (Vallée et al., 2014), the peptide RDV-haemopressin, a CB1-positive and CB2-negative allosteric modulator (Petrucci et al., 2017), and the re-uptake system of 2-AG (Bartholomäus et al., 2019; Nicolussi and Gertsch, 2015; Reynoso-Moreno et al., 2017).

I.1 Endocannabinoid system: role in the PNS and CNS

In the Central Nervous System (CNS), the Endocannabinoid System (ECS) controls many functions, such as brain development (Fride et al., 2009), learning and memory (Azad et al., 2008; Li et al., 2019), metabolism (Bellocchio et al., 2008), motor behavior (Katsidoni et al., 2013), food behavior and energy balance (Cota, 2007; Cota et al., 2006), circadian cycles (Vaughn et al., 2010), fear extinction (Kamprath et al., 2006), mood control (Hill and Gorzalka, 2009), social behavior (Wei et al., 2017), body temperature (Metna-Laurent et al., 2017), pain perception (Fine and Rosenfeld, 2013), and reward (Friemel et al., 2014). In the periphery, the ECS controls the immune response (Cluny et al., 2012), lipogenesis (Cota et al., 2003), bone mass (Ofek et al., 2006), cardiovascular system (Cota, 2007), reproduction (Maccarrone, 2008) and male fertility (Battista et al., 2008), the gastrointestinal system (Galiazzo et al., 2018), the skin health (Río et al., 2018), among other functions.

I.2 Cannabinoid receptors, neurotransmission, and (endo)cannabinoids

In 1990, Matsuda et al. discovered an orphan G-protein-coupled receptor (GPCR) in the rat cerebral cortex, named SKR6. This receptor, encoded by the *Cnr1* gene, was responsive to THC, the main psychoactive component of cannabis Sativa, and it was renamed the cannabinoid receptor of type-1 (CB1). Three years later, Munro and colleagues (1993) discovered a GPCR in the leukemic cell line HL60 and identified it as CB2. CB1 has been found in both the CNS and PNS, and in particular at the presynaptic terminals, and soon after its discovery, it was found to be one of the most abundant GPCR in the brain, along with the GABA and NMDA

receptors (TSOU et al., 1998). CB1 was then discovered to retrogradely control neuronal transmission in processes like cognition and memory, motor functions, analgesia, circadian cycles, appetite, and others; CB2 receptors encoded by the *Cnr2* gene, however, have been found mainly in immune cells and regulate immune functions (Howlett, 2002). It is known that amino acid similarity between the CB1 and CB2 receptors is about 44% (Munro et al., 1993). However, taking into account only the intracellular portion, this similarity rises to 68% (Cabral and Griffin-Thomas, 2009). A final putative cannabinoid receptor is the orphan GPR55. It was discovered and cloned in the late '90s (Sawzdargo et al., 1999). This receptor has been identified in several brain regions and is a target of endocannabinoids, but it shows relatively low amino-acid similarities with CB1 and CB2 (13.5 and 14.4% respectively) (Yang et al., 2016). The endogenous ligands that bind onto the cannabinoid receptors are the endocannabinoids: endogenous lipidic molecules that bind onto the cannabinoid receptors. The endocannabinoids *N*-arachidonylethanolamide (anandamide, AEA) and 2-arachidonolglycerol (2-AG) are the most abundant, the most widely studied, and the best characterized.

- Type 1 and 2 cannabinoid receptors: a brief overview.

The CB1 receptor: when it was characterized for the first time, the CB1 receptor was described as a 7TM receptor coupled to a trimeric $G_{i/o}$ protein (Howlett, 1985), with a classical $G_{i/o}$ pathway, but more recent studies have demonstrated that other signaling transducers may be recruited by CB1 (Turu and Hunyady, 2010). CB1 receptor structure reflects the typical GPCR. It is composed of seven transmembrane α -helices linked to each other by three intracellular and three extracellular loops, and an amphipathic helix. A V-shaped loop of the CB1 N-terminus allows access to the binding pocket, sited in the hydrophobic portion of the receptor, only from the extracellular site (Al-Zoubi et al., 2018; Hua et al., 2016) (Fig. 2).

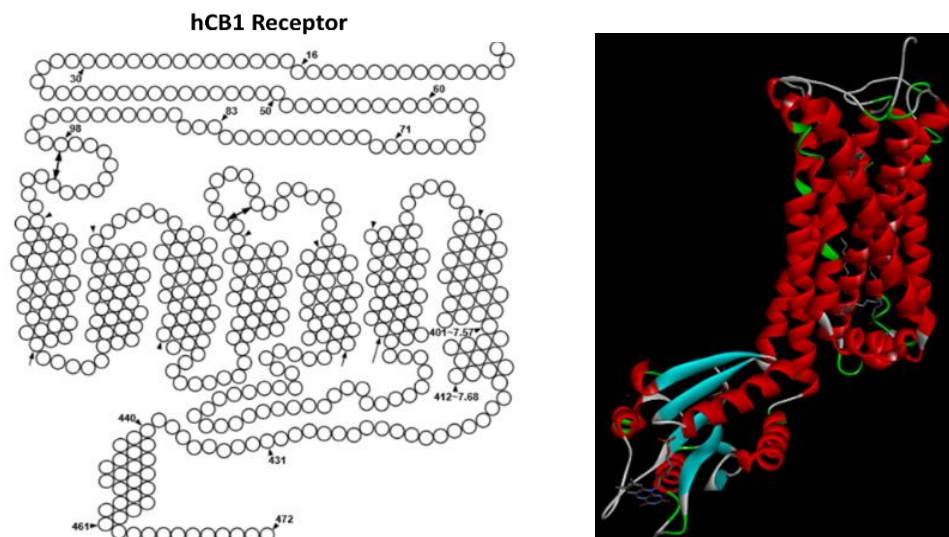


Figure 2: Human CB₁ receptor structure. Being a G protein-coupled receptor, the CB₁ receptor presents seven-transmembrane α -helices, three intracellular and three extracellular loops that link the loops, and an amphipathic helix. A V-shaped loop of the CB₁ N-terminus allows the access to the binding pocket, sited in the hydrophobic portion of the receptor, only from the extracellular site.
Picture kindly provided by Dr. Reggio, Dr. Hurst, and Dr. Al-Zoubi

The cannabinoid receptor of type 1 is highly expressed in the whole brain. In rodents, it has been shown that the areas expressing CB₁ the most are, as shown in Fig. 3, the olfactory bulb (OB), hippocampus (Hpc), caudate-putamen (CPU), globus pallidus (GP), amygdala (AMG), and cerebellum (CB); the cingulate cortex, neocortex, septum, substantia nigra (SNr), ventral tegmental area (VTA), and nucleus tractus solitarius present a lower, but still rather high content of CB₁; while a lower, but still ample expression of the CB₁ receptor can be found in the nucleus accumbens (NAc), olfactory tubercle (OT), thalamus (Tha), periaqueductal gray (PAG), dorsal raphe nucleus (DRN), and locus coeruleus (LC) (Fig. 3). The CB₁ receptor is most likely found in presynaptic position. However, the presence of the CB₁ receptor has been demonstrated in somatodendritic position, where it allows self-inhibition of the neuron (Bacci et al., 2004; Marinelli et al., 2009), and intracellularly on the mitochondria surface, where it regulates the cell metabolism (Bénard et al., 2012). CB₁ has been detected on GABAergic (the most representative neurons expressing CB₁), glutamatergic and serotonergic neurons, and although the presence of CB₁ has not been fully characterized on cholinergic, noradrenergic, and dopaminergic neurons, it has been established that cannabinoids may regulate cholinergic,

adrenergic, and dopaminergic transmission (Busquets-Garcia et al., 2016). Additionally, it has been discovered that CB1 is present not only in neuronal cells, but also in glial cells, and more in particular astroglial cells (Busquets-Garcia et al., 2016). Furthermore, the pioneering work of Bénard and colleagues (2012) assessed the presence of the CB1 receptor in the membrane of mitochondria. Although the presence of CB1 on mitochondria is relatively low, it has been demonstrated that it may be of central importance for several functions.

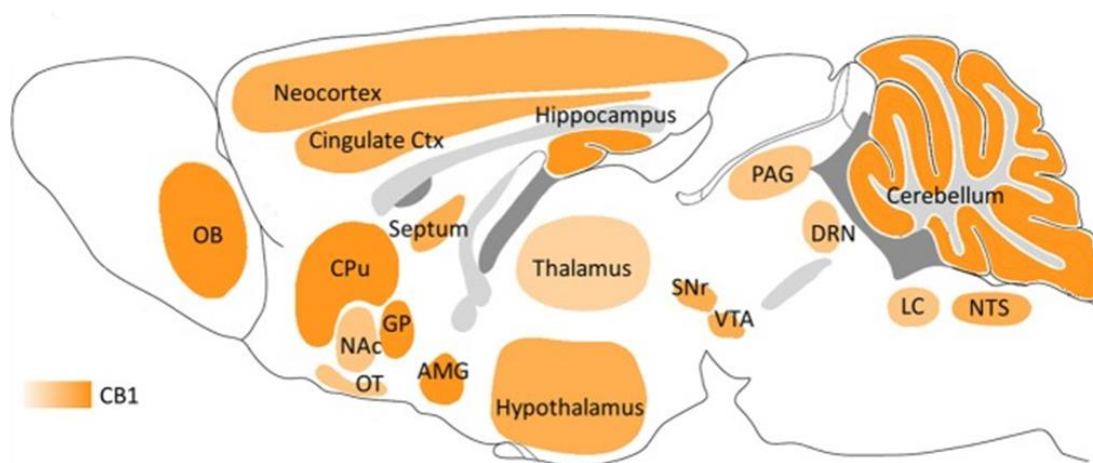


Figure 3: Schematic representation of the main areas expressing CB1 in the mouse brain. The distribution of CB1 follows the color gradient, which is low for a light color and high for a dark color. AMG, amygdala; CPU, caudate-putamen; Ctx, cortex; DRN, dorsal raphe nucleus; GP, globus pallidus; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal gray; PVT, paraventricular nucleus of thalamus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; TMN, tuberomammillary nucleus; VTA, ventral tegmental area. Picture kindly provided by Dr. África Flores. Material from the article Flores et al., *Frontiers in Neuroscience*, 2013

The CB2 receptor, like CB1, is a G-protein-coupled receptor, and is usually bonded to a G_i protein (Mnpoetra et al., 2014). However, it has been discovered that in leukocytes, it is coupled to a G_s protein (Saroz et al., 2019). Although it was initially accepted that the CB2 receptor was expressed in the periphery only, it has since been demonstrated that it can be found in the brain as well: in contrast to the CB1 receptor, that can be found in the neuronal and astroglial cells, the CB2 receptor is present almost only in microglia (Cabral et al., 2008; Pertwee, 2006), with few known exceptions: for instance, low expression of the CB2 receptor was found in the brainstem neurons (Sickle et al., 2005).

- **Cannabinoid transmission:**

The cannabinoid transmission in the CNS and PNS is retrograde. Retrograde transmission occurs when the postsynaptic neuron releases the neurotransmitter in the synaptic cleft, which, in turn, targets the presynaptic neuron. In 1992, Pitler and Alger discovered that when hippocampal pyramidal cells depolarized strongly, a transient short-term inhibition of GABAergic synaptic inputs was produced and projected on these cells that were detected as a decrease in the amplitude of the synaptic current in the depolarized neuron. It was then called retrograde transmission because this depolarization-induced suppression of inhibition (DSI) had a postsynaptic origin and a presynaptic effect (Fig. 4a).

Almost ten years later, Wilson and Nicoll (2001) discovered that in pyramidal cells that received intense stimulation, endocannabinoids were released by the postsynaptic neurons, and spread through the synaptic cleft to the terminals of the GABAergic interneurons, where they acted on the CB1 receptor, preventing GABA release. Retrograde endocannabinoid signaling also occurs to produce depolarization-induced retrograde signaling of interneuronal inhibitory GABAergic synapses in cerebellar Purkinje cells (Kreitzer and Regehr, 2001a), neocortical pyramidal cells (Trettel et al., 2004), and substantia nigra neurons (Yoshida et al., 2002). It has also been demonstrated that cannabinoids were responsible for depolarization-induced suppression of excitation (DSE) in glutamatergic synaptic inputs released on cerebellar Purkinje cells (Kreitzer and Regehr, 2001b) (Fig. 4b). Later on, it was discovered that endocannabinoid signaling was implicated in long-term depression (LTD) as well in both excitatory (Gerdeman et al., 2002) and inhibitory (Castillo et al., 2012) synapses. In particular, it was discovered that in the cerebellum, when parallel cells fire on Purkinje cells, the latter release endocannabinoids, which, through CB1, block the firing of the former for many seconds (Brown et al., 2003). Furthermore, artificial activation of mGluR on postsynaptic Purkinje cells causes a release of endocannabinoids in the synaptic cleft (Brenowitz and Regehr, 2005), and the mGluR-

dependent LTD in Purkinje cells could have been blocked by CB1 antagonists (Safó and Regehr, 2005).

Endocannabinoids can facilitate LTD in various brain regions, including the hippocampus (Chevaléyre and Castillo, 2003), cerebellum (Brown et al., 2003; Safó and Regehr, 2005), nucleus accumbens (Robbe et al., 2002), striatum (Gerdeman et al., 2002), and amygdala, (Marsicano et al., 2002).

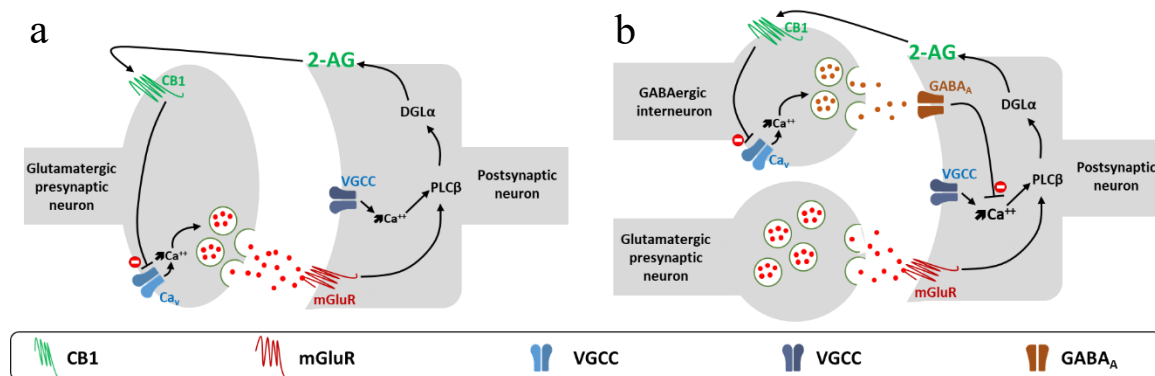


Figure 4: DSI and DSE. **a)** depolarization-induced suppression of excitation (DSE) happens when the excitatory presynaptic neuron (glutamatergic) fires its neurotransmitter on the postsynaptic neuron. This intense signal repeated in time brings the postsynaptic release of the endocannabinoid 2-AG, which retrogradely targets the presynaptic neuron's synaptic bouton. Once CB1 is activated, it blocks neurotransmitter release, stopping the excitatory signal. **b)** depolarization-induced suppression of inhibition (DSI) represents the classical and discovered the endocannabinoid's first function in the brain. When a strong excitatory (glutamatergic) signal is directed onto the Purkinje cells of the cerebellum or pyramidal cells in the hippocampus, this signal can be dampened by GABA release from an adjacent GABAergic interneuron. The postsynaptic neuron, to reinforce the excitatory signal, releases the endocannabinoid 2-AG that retrogradely activates the presynaptic CB1 on the GABAergic presynaptic bouton, thus blocking GABA release, and allowing a stronger excitatory signal

- (Endo)cannabinoids and their enzymes

The endocannabinoids are lipid-based molecules derived from arachidonic acid that can diffuse freely throughout the phospholipidic bilayer and target the cannabinoid receptors, where they show different degrees of efficacy as agonists on the different receptors. The most abundant endogenous endocannabinoids are Anandamide (AEA) and 2-Arachinodoilglycerol (2-AG). Moreover, different endocannabinoids have slight but still significantly different roles in cannabinoid transmission, and different endocannabinoids show cross-talk between their pathways and functions. 2-AG is considered the principal endocannabinoid neurotransmitter, being expressed 1000-fold more in the brain than AEA (Maccarrone et al., 2008), and a full

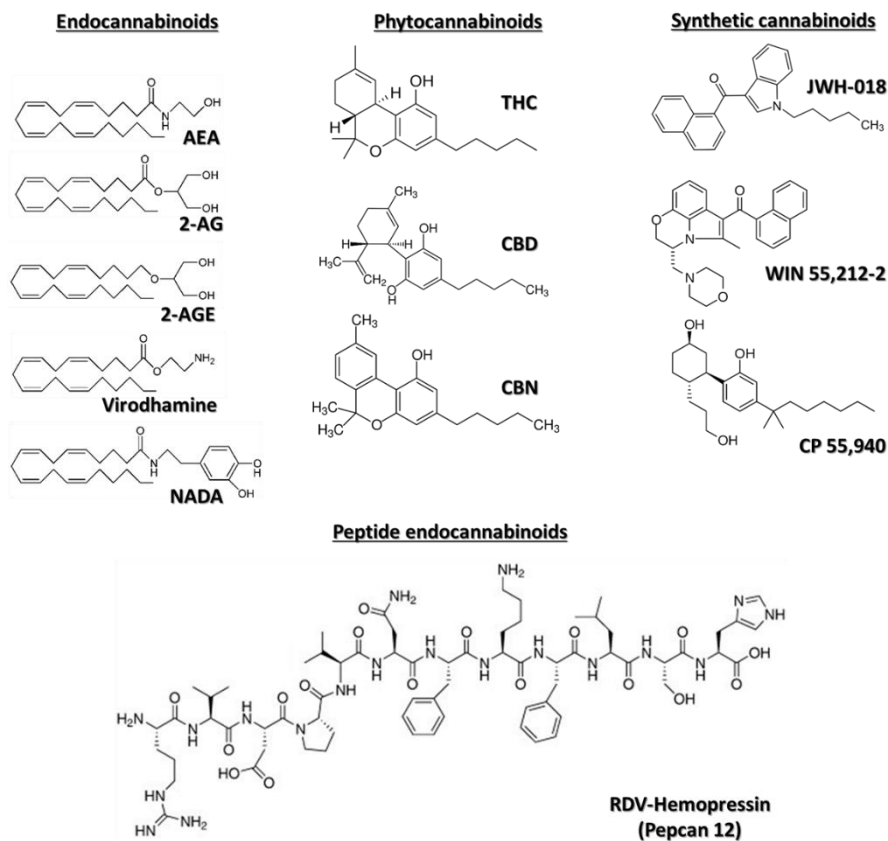


Figure 5: Endocannabinoids, phytocannabinoids, synthetic cannabinoids, and pecans. Among the endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most representative. Although the full agonist 2-AG occurs 1000 fold more than the partial agonist AEA, both are part of an equilibrium in which AEA regulates the efficacy of the production of 2-AG. Other derivatives of arachidonic acid, proposed as endocannabinoids, are N-arachidonoyl dopamine (NADA), virodhamine, and noladin ether (2-AGE). Even if the cannabis *Sativa* plant produces more than eighty different phytocannabinoids, THC and CBD are undoubtedly the most iconic cannabinoids, especially for recent events and policies on legalization worldwide. In contrast, other phytocannabinoids, such as cannabinal, exert no effect in the cell's physiology. THC is a partial orthosteric agonist of CB1, while CBD is a CB1 negative allosteric modulator. The synthetic cannabinoids shown are the JWH-018, the "spice," a potent illegal agonist of both CB1 and CB2, used as a recreational drug of abuse and research purposes WIN55,212-2 that is a full agonist of CB1 and an agonist of PPAR α and PPAR γ as well. Peptide endocannabinoids are peptides active on the cannabinoid receptors CB1 and CB2. RDV-Hemopressin, an N-terminally extended form of hemopressin, acts as a positive allosteric modulator of the CB1 receptor.

agonist of both CB1 and CB2 (Gonsiorek et al., 2000). By contrast, AEA is a partial agonist to CB1 and displays an even lower affinity for CB2 (Reggio, 2010).

Furthermore, AEA can act on other receptors than CB1 and CB2: the Vanilloid Type 1 receptor (VR1), known as transient receptor potential cation channel subfamily V member 1 (TrpV1), or capsaicin receptor, which is a non-selective cation channel (Chávez et al., 2010; Heyman and Rang, 1985). The primary function of VR1 in the peripheral nervous system is to produce nociception. The capsaicin molecule, acting on the VR1, causes the sensation of heat and pain

(Caterina, 2000). Interestingly, the primary role of AEA through VR1 is excitatory and not inhibitory.

Other endogenous molecules, however, have been proposed as being endocannabinoids as well, such as N-arachidonoyl dopamine (NADA), virodhamine and noladin ether (2-AGE) (Fig. 5).

Anandamide (AEA) (for a review, see Muccioli et al., 2010): N-acylphosphatidylethanolamines (NAPEs) are the main precursors of N-acylethanolamines (NAEs), the chemical class to which anandamide (AEA) belongs. NAPE biosynthesis occurs by transfer of an SN-1 acyl chain from 1,2-diacylglycerophospholipid to phosphatidylethanolamine, through an N-acyl transferase enzyme (NAT). Three main possible pathways may then occur: the canonical pathway oversees a NAPE-selective phospholipase D (NAPE-PLD) that hydrolyzes the phosphodiester bond, forming the NAE, in this case anandamide. Interestingly, even though AEA is produced on demand, NAPE-PLD seems to be constitutively active. The non-canonical pathways that lead to biosynthesis of AEA pass through two intermediate compounds: the enzyme sPLA₂ ABHD4 hydrolyzes an acylglycerol chain. The second acylglycerol group is then hydrolyzed by the lyso-LPD enzyme to obtain the NAE. In the last biosynthetic mechanism to produce AEA, phospholipase C (PLC) hydrolyzes

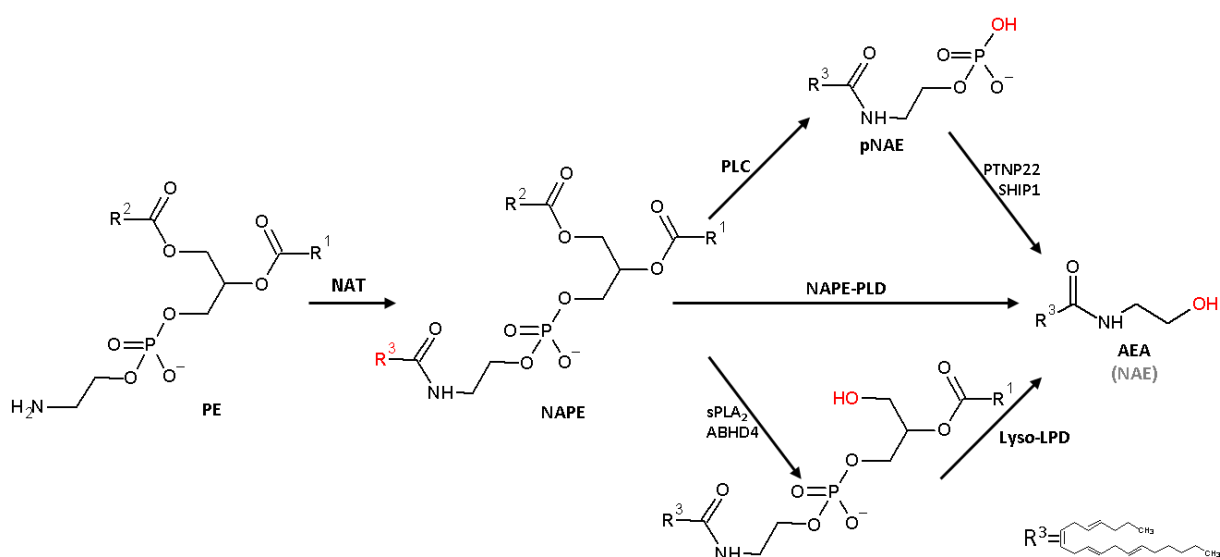


Figure 6: Biosynthesis of anandamide (AEA). PE, phosphatidylethanolamines; NAT, N-acyl transferase enzyme; NAPE, N-acylphosphatidylethanolamines; NAPE-PLD, NAPE-selective phospholipase D; PLC, phospholipase C; AEA, Anandamide; NAE, N-acylethanolamines.

NAPE into phospho-NAE (p-NAE) and diacylglycerol. Thanks to the phosphatase PTPN22 SHIP1, p-NAE is then dephosphorylated into NAE, and thus AEA (Fig. 6).

2-Arachidonoylglycerol (2-AG) (for a review, see Muccioli et al., 2010): 2-AG is known to be produced on demand in both the CNS and the periphery. The stimuli that can trigger 2-AG production may be due to cell depolarization or to metabotropic signals. The biosynthesis of 2-AG starts with the production of diacylglycerol (DAG) from phosphatidylinositol through phospholipase C enzymatic activity, and then through DAG hydrolysis by DAG lipase (DAGL) (Fig. 7).

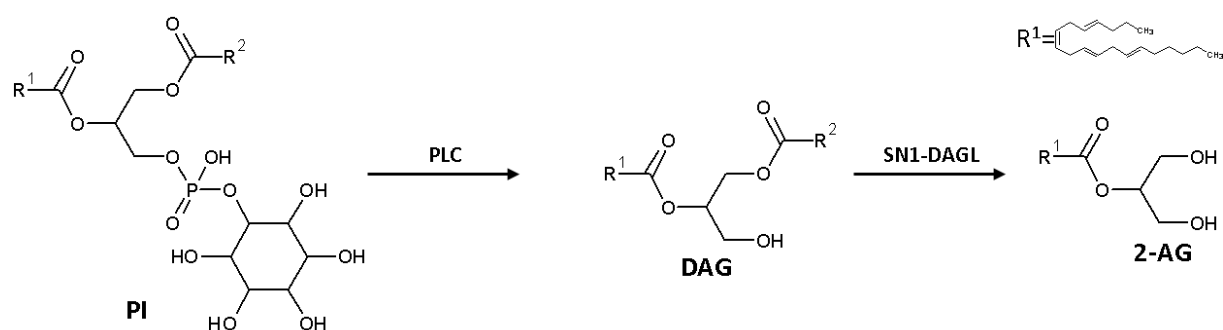


Figure 7: Biosynthesis of 2-Arachidonoylglycerol (2-AG) from phosphatidylinositol (PI). PLC, phospholipase C; DAG, diacylglycerol; DAGL, DAG lipase.

Although endocannabinoids are released in the extracellular matrix, in order to interact with their receptor targets, they are processed and metabolized inside the cell. Although endocannabinoids are lipophilic, and thus do not technically need any transporter to diffuse through the membrane, there are two mechanisms to accelerate this process. One is the presence of endocannabinoid transporters that pump cannabinoids actively from the extracellular to the intracellular compartment (for a review see Nicolussi and Gertsch, 2015), while a second mechanism is the "sink" effect, in which the degradation of the endocannabinoid in the intracellular compartment creates a gradient that will increase diffusion towards the cytosol throughout the membranes. Several enzymes can degrade the endocannabinoids, e.g., Cox2

which is an unspecific enzyme that can convert AEA into prostaglandin 2EA (PGH₂-EA) and 2-AG in PGH₂-G. The other enzymes that metabolize AEA are the fatty acid amide hydrolase (FAAH) 1 and 2, and the N-acyl ethanolamine acid amide hydrolase (NAAA), which converts AEA into arachidonic acid. 2-AG is converted into arachidonic acid by the monoacylglycerol lipase, and alpha/beta-Hydrolase domain containing 6 and 12 (ABHD 6/12) (Fig. 8; for a review see Nicolussi and Gertsch, 2015).

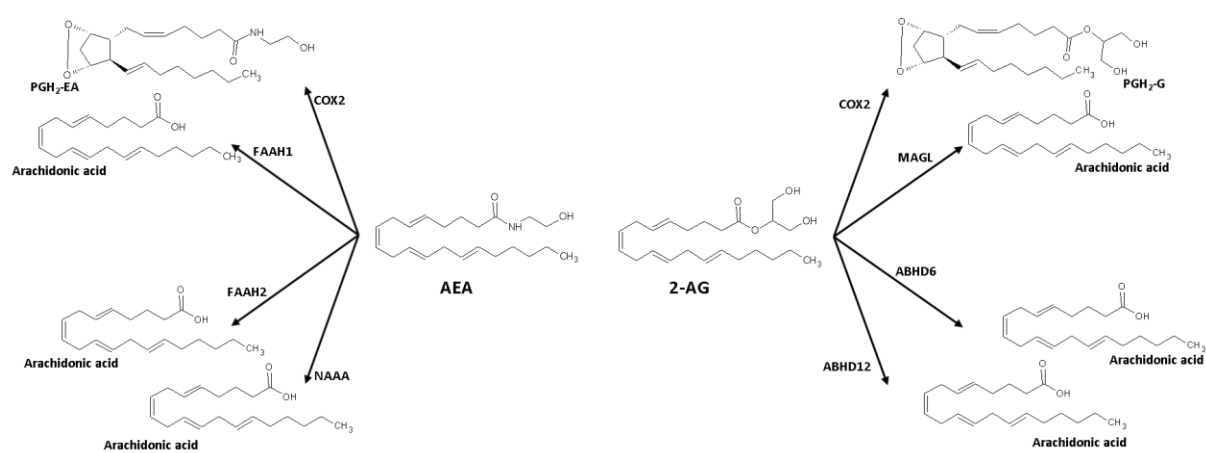


Figure 8: Endocannabinoids (2-AG and AEA) degradation. FAAH, fatty acid amide hydrolase; NAAA, N-acyl ethanolamine acid amide hydrolase; ABHD6/12, alpha/beta-Hydrolase domain containing 6 and 12; MAGL, monoacylglycerol lipase.

- Exocannabinoids

The exogenous molecules that are able to bind to and activate CB1 and CB2 receptors (exocannabinoids) are divided into phytocannabinoids (cannabinoids that can be found in plants), such as THC and CBD which are phytocannabinoids extracted from *Cannabis Sativa*, and synthetic (artificially-produced) cannabinoids that have been produced extensively. Some of them, such as WIN55,212-2 and CP 55,940, are used mainly for research purposes, while the potent CB1 agonist JWH-018, for example, has been used as a recreational drug (Elmore and Baumann, 2018) (Fig. 8). It is infamous nowadays in a mix of synthetic cannabinoids named “spice”, also known as the “zombie drug” (Doward, 2017).

Δ^9 -tetrahydrocannabinol (THC): THC is the most representative psychoactive phytocannabinoid of *Cannabis Sativa* and one of the most abused illicit drugs around the world. The most common way to self-administer THC is by smoking marijuana cigarettes. The content of THC in marijuana may vary considerably, but it has been demonstrated that nowadays it may be approximately 10-12% (ElSohly et al., 2016), of which about 50% degrades through pyrolysis during the act of smoking. The plasmatic peak is reached within a few minutes from the first inhalation (McGilveray, 2005). When administered orally, for example for medical purposes, THC is almost absorbed completely (~88-96%). However, the plasmatic peak may be reached within hours (Sharma et al., 2012). When smoked, THC is absorbed rapidly through the lungs and quickly reaches a high concentration in the blood. On average, 90% of the THC that passed through the lungs stays within the erythrocytes. THC systemic bioavailability varies according to several factors: depth of inhalation, number of puffs, and duration of breath-hold. In habitual and occasional users of cannabis, the systemic bioavailability of THC on average is ~23-27% and ~10-14%, respectively (Sharma et al., 2012). Once in the stream, THC may diffuse throughout the blood brain barrier (BBB), given its lipophilic characteristics, and exert its psychoactive effects. THC metabolism, unlike for the endocannabinoids, occurs mostly in the liver, where THC is subjected to several biotransformations that involve allylic oxidation, epoxidation, decarboxylation, and conjugation. The cytochrome P₄₅₀ (CYP) 2CP hydroxylates THC in position 11, producing the first metabolites 11-hydroxy-THC (11-OH-THC), which is still active and can still pass through the BBB, then the inactive 11-nor-9-carboxyl-THC (THC-COOH) (McGilveray, 2005, Sharma et al., 2012). At this point, THC-COOH is conjugated to glucuronic acid, and then it is excreted through the urine (Fig. 9; Sharma et al., 2012).

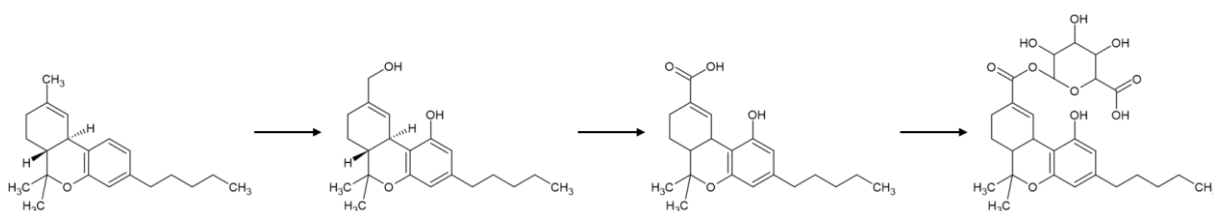


Figure 9: THC metabolism.

II Cannabis and Δ^9 -tetrahydrocannabinol

II.1 Cannabis Sativa history, in brief

Cannabis sativa (cannabis) is an Asian indigenous plant and is among the most ancient cultivated plants in human history. In China, the oldest evidence of cannabis cultivation for fibers dates back to 4,000 BC (Li, 1974). The first use of cannabis as a medical drug is dated to around 2,700 BC, for the purpose of healing rheumatic pain, malaria, and the female reproductive system (Touw, 1981). Furthermore, the founder of Chinese surgery, Hua T'o, used a compound of cannabis and wine as an anesthetic (Li, 1974). In modern-era China, cannabis seeds are still used as described in Hua T'o's medicine for constipation (Touw, 1981). In *pen-t's'ao ching*, a Chinese book of agriculture and medicinal plants, written between 200 and 250 AD, there is the first known evidence of cannabis used as a psychoactive drug, (Li, 1977). Over time, shamanist religions in India and central Asia assigned religious value to this plant (Touw, 1981). Evidence of cannabis use for religious, textile, traditional, and recreational purposes has been found during human history (for a review on this topic see Zuardi, 2005). However, during the second half of the 20th-century, cannabis for hedonistic purposes had an enormous impact on western society. In 1967 in the United States, the percentage of the population that had used cannabis at least once was about 5%, while in 1982, the percentage had risen to 64% (Zuardi, 2005). After the discovery of the endocannabinoid system and the consequent (partial) understanding of the cannabis action mechanism and its healing potential, Δ^9 -tetrahydrocannabinol and cannabidiol, the two main active components of cannabis have been accepted in Canada, the United Kingdom, and Europe and put on the market as treatments for neuropathic pain and multiple sclerosis (Zuardi, 2005). In recent periods, cannabis has been demonstrated to be one of the most commonly-abused illegal drugs around the world, thanks also to its reputation as a "soft drug," with a political debate around marijuana legalization taking hold worldwide from the '90s (Monckeberg B, 2014). Recreational use of marijuana has

been accepted recently in several states, including eleven states of the United States of America and Canada. To date, as the market in marijuana has grown exponentially, marijuana itself has passed through several processes of domestication in order to enrich its THC content (ElSohly et al., 2016).

II.2 Cannabis addiction and cannabis use disorder

II.2.1 Epidemiology of cannabis addiction

All the known definitions of addiction (Piazza and Deroche-Gamonet, 2013) may be summarized as follows: *"Drug addiction can be defined as a chronically relapsing disorder, characterized by a compulsion to seek and take the drug, loss of control in limiting intake, and the emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) when access to the drug is prevented"* (Koob and Volkow, 2016). Cannabis and more particularly Δ^9 -tetrahydrocannabinol (THC), is no exception to this definition. THC is the main psychoactive component contained in *Cannabis Sativa*, one of the most commonly-used illicit drugs abused worldwide (Statistical Bulletin 2018). Although THC is a lipophilic molecule able to mimic the effect of the endogenous cannabinoid via its action on CB1 and CB2 receptors, its psychoactive effects are mainly dependent on its activity on CB1 in the brain.

THC consumption represents a fundamental challenge for the healthcare systems of almost every nation. In 2006, it was estimated that there were 140-160 million cannabis users worldwide, ten times more than cocaine and opium users (Agrawal and Lynskey, 2006). Cannabis use is widespread all over the world, with a prevalence in Oceania (9.1-14.6% of users), North America (10.8%), Western and Central Europe (7%), and Western and Central Africa (5.2-13.5%) (United Nations Office on Drugs and Crime, 2015, 2012; WHO, 2015). Furthermore, it has been estimated that ~10% of those who have used cannabis at least once in their life will develop an addiction to it (Wagner and Anthony, 2002). More recently, several studies have demonstrated how the second-most abundant cannabinoid in *Cannabis sativa*,

cannabidiol (CBD) which is not psychoactive, acting as a CB1 negative allosteric modulator (Chung et al., 2019; Laprairie et al., 2015), can partially protect the brain from THC intoxication (Niesink and van Laar, 2013; Zuardi, 2008; Zuardi et al., 1982). During the last few decades, it has been demonstrated that the potency of cannabis, expressed in THC content, has increased (Cascini et al., 2012), while CBD content has decreased (Hardwick and King, 2008), further exacerbating both the psychoactive and adverse effects of THC on cannabis users. Very interestingly, several works have indicated that people who abuse cannabis tend to adjust the number, frequency, and intensity of puffs from the cannabis cigarette that they smoke to self-administer a relatively low amount of THC at each session, regardless of cannabis potency (Korf et al., 2007; van der Pol et al., 2014). This seems to suggest that it is the THC/CBD ratio, and not the absolute concentration of THC, that is the most important factor to be taken into account in order to consider cannabis potency (Lafaye et al., 2017). Cannabis abuse causes several adverse effects. The acute effects in the short term are memory and learning impairment (Nestor et al., 2008), impaired motor coordination (Hall and Degenhardt, 2009) and, if taken at high doses of THC, psychotic-like events (Wilkinson et al., 2014). The long-term effects of Cannabis abuse are dependence on the substance, and the incremented probability of developing chronic psychosis disorder in people who are already predisposed to it (Hall and Degenhardt, 2008; Müller-Vahl and Emrich, 2008; Veling, 2008).

About 10% of people who use cannabis are at severe risk of developing substance use disorder (Volkow et al., 2014). A substance – or drug – use disorder can be defined as a set of mental, physical, behavioral, and social symptoms related to drug addiction (American Psychiatric Association, 2013). Drugs that can cause drug use disorder are several and are classified as alcohol, phencyclidine, inhalants, stimulants, cannabis, opioids, tobacco, sedatives, hypnotics, and anxiolytics. The drug use disorder relative to cannabis is named cannabis use disorder (CUD), and it has been identified in and added to the latest versions of the DSM (American

Psychiatric Association, 2013). Cannabis use disorder symptoms usually appear after 24h of abstinence, last for approximately one week, and disappear almost entirely after 15 days, although some symptoms (sleep issues) may last longer (Budney et al., 2004). Symptoms that are often observed in CUD are anxiety, irritability, depressed mood, restlessness, disturbed sleep, gastro-intestinal symptoms and decreased appetite (American Psychiatric Association, 2013; Weinstein and Gorelick, 2011; Ramesh and Haney, 2015). Until the beginning of the 2000s, there were few or no pharmacological treatments for CUD, but in recent years, due to the extensive spread of cannabis consumption, the number of treatments that are proposed and accepted has soared. However, the first help proposed to those who request help is psychosocial treatment: the primary therapies are cognitive behavioral therapy, motivational enhancement therapy, contingency management, and family-based therapies (Ramesh and Haney, 2015). With these therapies, it is possible to achieve between 19 and 37% of 12-month abstinence, on average. In order to improve this success rate, pharmacological therapies have been used. Rimonabant, a specific CB1 antagonist, has significantly reduced cravings and consumption of cannabis after 15 days of therapy in humans (Huestis et al., 2007, 2001). However, rimonabant represents a high-risk factor for depression, and was withdrawn from the market for that reason (Scott, 2008). Alternatively, it has been shown that oral usage of synthetic cannabinoids and analogs to THC can bring potential benefits, alleviate withdrawal, and allow continued abstinence from smoking cannabis (Levin and Kleber, 2008). In other cases, in order to diminish the symptoms, opiate antagonists, COMT inhibitors, NRI, anxiolytics, mood stabilizers, and anti-depressants have been used (Weinstein and Gorelick, 2011; Ramesh and Haney, 2015). It should be noted that all the pharmacological therapies currently in use target the symptoms, and the ECS is not targeted by most of the therapies.

II.2.2 A brief overview of the circuitry of addiction: focus on CB1

The circuitry of addiction is a complex tangle of several nodes that interact with each other. Several brain regions are involved in this process (ventral tegmental area, nucleus accumbens, prefrontal cortex, basolateral amygdala, ventral hippocampus, thalamus, globus pallidus, striatum, and cerebellum) and at least three main central neurotransmitter systems

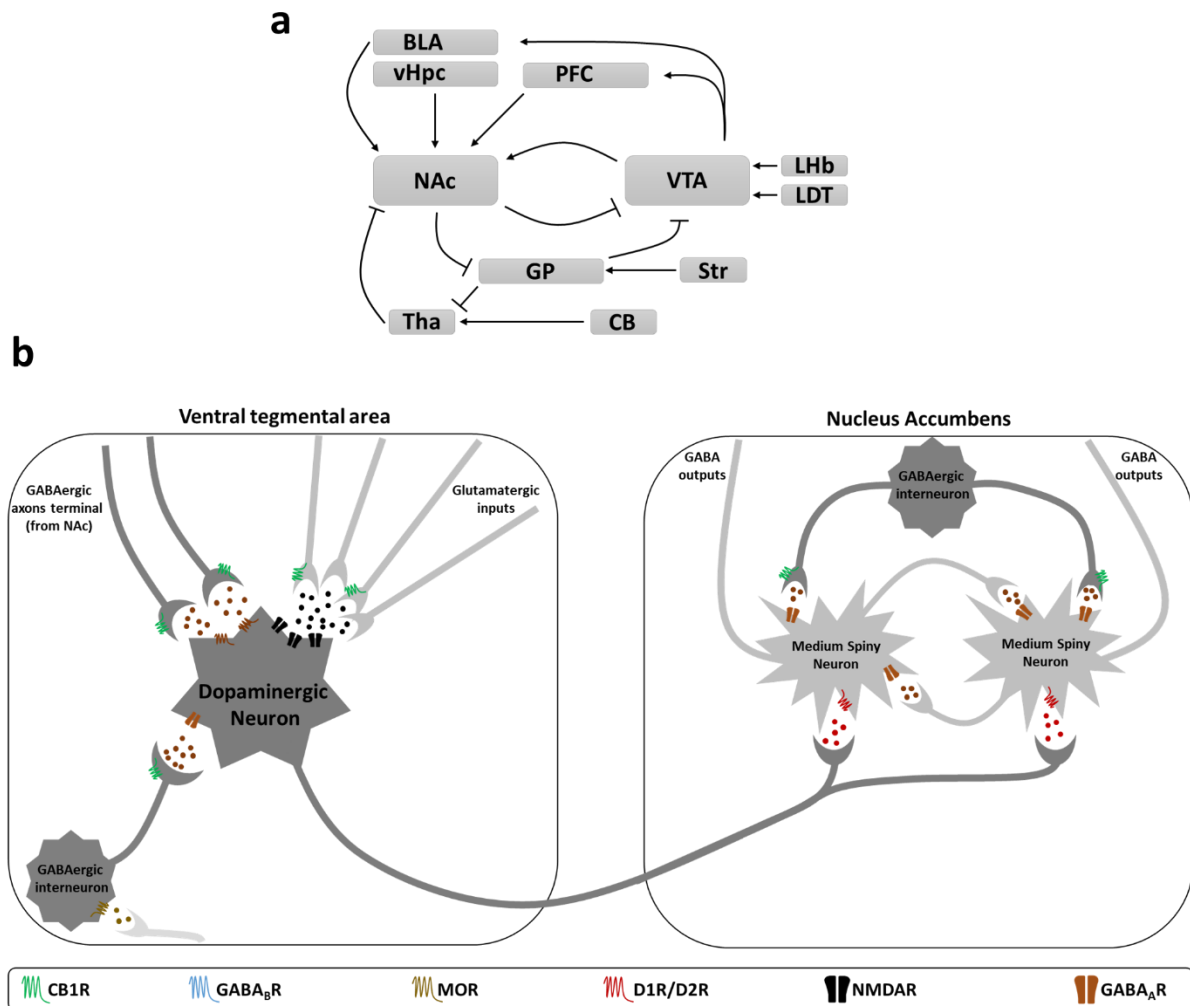


Figure 10: Circuitry of reward and VTA-NAc projections. a) most of the structures involved in the circuitry of addiction. b) the circuitry of addiction in the segment of VTA-NAc. Dopaminergic neurons in the ventral tegmental area receive inputs from glutamatergic and gabaergic neurons. In particular, some of the GABAergic terminals come from the VTA's interneurons, while others are from the nucleus accumbens, and work as negative feedback in case of firing sustained for too long. The GABAergic interneurons have been studied in depth for being the primary target in the opioidergic signal, and it is known that CB1 is expressed in the synaptic boutons of all the neuron types present in the VTA, but the GABAergic neurons are the neurons that express it the most. In case of utilization of cannabinoids, it would block the GABA release, allowing an intense firing of the dopaminergic neuron, and blocking the negative feedback from the NAc. Once the dopaminergic neuron fires in the NAc, the neuron target is a particular GABAergic neuron, named medium spiny neuron (MSN). In the NAc, those neurons receive the dopamine and produce the reward in the brain. GABAergic interneurons regulate the excitation/inhibition steady-state to avoid a signal too much intense. The presynaptic boutons of those GABAergic interneurons present the CB1 receptor, disrupting this steady-state and promoting the excitation over the inhibition. Altogether, several critical points in the VTA-NAc projection can be targeted by cannabinoids to promote addiction. CB1R, cannabinoid receptor type 1; GABA_A/B, GABA receptor type A/B; MOR, μ opioid receptor; D1/D2R, dopamine receptors type 1/2; NMDAR, N-methyl-D-aspartate receptor for glutamate.

(dopaminergic, glutamatergic, and GABAergic) (Fig. 10). The endocannabinoid system is implicated in the reward circuitry and the endocannabinoids, which target the CB1 receptor that is present in GABAergic interneurons, in which the aforementioned areas are rich, ultimately facilitate the signaling of reward (Parsons and Hurd, 2015)

Ventral tegmental area (VTA): the VTA is composed almost entirely of dopaminergic (~60%) and GABAergic (~30%) neurons (Nair-Roberts et al., 2008). It has been demonstrated that VTA releases dopamine in target areas, and more particularly in the nucleus accumbens (NAc), which is involved in reward and thus addiction (Oliva and Wanat, 2016; Volkow and Morales, 2015). Interestingly, VTA does not play a role in reward and addiction only. It has been demonstrated that any stimulus that causes reward allows a selective release of dopamine from the VTA to the NAc, in a system named the mesolimbic dopamine system, while an unpleasant stimulus causes the release of dopamine from the VTA to the mPFC (Lammel et al., 2011). Furthermore, it has been demonstrated that lateral habenula (LHb) can regulate VTA: the laterodorsal tegmentum (LDT) firing in the VTA triggers the release of dopamine to the NAc, causing reward, while the LHb firing on the VTA causes release in the mPFC, with aversive effects (Lammel et al., 2012).

Nucleus accumbens (NAc): if one brain structure were to be considered the quintessential structure of reward, and thus addiction, it would surely be the NAc. Many studies have demonstrated the central role of this structure in addiction and reward (Di Chiara, 2002; Quintero, 2013; Russo et al., 2010; Scofield et al., 2016). The nucleus accumbens is divided into two subregions, the core and shell. Almost all of the neuron population in both the core and shell of the NAc is composed of medium spiny neurons (MSN) (Calipari et al., 2016). The MSNs are GABAergic neurons, classified on the basis of their dopamine receptor expression as D1-type or D2-type (Gerfen et al., 1990). The nucleus accumbens shell and core both express MSN presenting D1 or D2 receptors. In the shell, ~40% of the MSN present both D1 and D2

receptors (Yager et al., 2015). Even though a large amount of modulatory input in the NAc comes from the VTA, other glutamatergic inputs come from the basolateral amygdala (BLA), prefrontal cortex (PFC), ventral hippocampus (vHpc), and mediodorsal thalamus (MTD) (Sesack and Grace, 2010). CB1 is quite abundant in the nucleus accumbens as well (Pickel et al., 2004). The role of CB1 within the nucleus accumbens has been linked several times to dopamine release, and thus addiction (Polissidis et al., 2013). A lack of CB1 receptors in the nucleus accumbens has been demonstrated to cause decreased morphine-induced dopamine release (Mascia et al., 1999), to reduce intake of alcohol (Hungund et al., 2003), and a blockade of CB1 receptors led to inhibition of cocaine relapse in rats (Xi et al., 2006).

The prefrontal cortex (PFC): the activation of glutamatergic input to the NAc from the PFC is important in addictive behavior (Kalivas, 2004). PFC-NAc glutamatergic circuits, in mouse models of addiction, are remodeled, and those modifications play a central role in drug-seeking behavior (Lasseter et al., 2010). The prefrontal cortex is rich in the CB1 receptor. It has been demonstrated that CB1 in the prefrontal cortex is quite involved in addictive behavior. FAAH deletion in mice caused alcohol intake to increase. Coherently, rimonabant can reduce the compulsion toward alcohol, demonstrating that the ECS has a role in reward within this structure (for a review Parolaro et al., 2007)

The basolateral amygdala (BLA) and ventral hippocampus (vHpc): the vHpc is the area that integrates spatial/contextual information coming from the dorsal hippocampus (dHpc) and emotional information that comes from the BLA (Fanselow and Dong, 2010; Kutlu and Gould, 2016; Qi et al., 2018). Furthermore, the vHpc projects onto the NAc and this connection is implicated in both reward and aversive behavior (LeGates et al., 2018). CB1 is especially rich in BLA (Katona et al., 2001) and Hpc (Freundt-Revilla et al., 2017), where it has been demonstrated to regulate long-term potentiation/depression (Chevaleyre and Castillo, 2003; Marsicano et al., 2002)

Striatum (Str), Globus pallidus (GP), and Thalamus (Tha): the thalamus projects onto the NAc directly, and onto the prefrontal and frontal cortex. The Str projects onto the Tha via the GP, and the Tha projects onto the NAc, and PFC (Haber and McFarland, 2001). It has been found that in the addicted human brain, lower thalamic grey matter shrinks. Such a phenomenon is associated with an increased compulsion for drugs of any kind (Huang et al., 2018). CB1 is rich in basal ganglia (Chaves-Kirsten et al., 2013), especially in the globus pallidus and striatum.

Cerebellum (CB): more recent studies have shown how the CB is implicated in the circuitry of addiction (Strick et al., 2009). It has been demonstrated that the cerebellum is implicated in reward and learning (Thoma et al., 2008) and that lesions of the cerebellum cause a decrease in appetitive operant conditioning (Bauer et al., 2011). The CB receives inputs from the prefrontal and associative cortices via the pontine nuclei. Furthermore, it has been proven that spontaneous activity in the NAc is predictive of cerebellar activity (Cauda et al., 2011), demonstrating functional connectivity even without a direct connection between the two structures. The CB is one of the most CB1-rich structures in the brain (Kawamura et al., 2006). Furthermore, the CB has been suggested as being a central area in cannabis withdrawal (Tzavara et al., 2000).

II.3 Cannabis and psychosis

- Pathophysiology of psychosis: a brief overview

Psychosis is one of the most severe mental illnesses and can be classified as a syndrome or group of symptoms. Patients that experience psychosis undergo a "break" with reality, usually associated with disorganized behavior, hallucinations, and delusions. Schizophrenia is a common psychiatric disorder (~0.5-1% of incidence), that affects male and female patients equally, although it appears that men develop the psychotic symptomatology at an earlier stage of their life than females (Naqvi et al., 2005).

Schizophrenia causes long-lasting psychosis, characterized by impairment of perception and expression of reality. It is a cause of disability, social isolation, and decreased life expectancy. Schizophrenic symptoms can be divided into three main branches: positive symptoms, characterized by delusion, hyperactive state, auditory hallucinations, disorganized thoughts, chaotic speech; negative symptoms, characterized by hypoactivity, anhedonia, blunted emotion, poverty of speech, lack of motivation; and cognitive dysfunctions, characterized by motivational and executive function deficit (American Psychiatric Association, 2013; Andreasen, 1995). It has been established that schizophrenia, and psychosis more generally, is caused by a combination of genetic (Kallmann, 1946; Ripke et al., 2014) and environmental (Fearon and Morgan, 2006) factors with common comorbidities like depression (Fineberg et al., 2016), anxiety (Huppert and Smith, 2005), and drug abuse (Hambrecht and Häfner, 1996). The expectancy of a significant recovery from schizophrenia is about 30% (Müller-Vahl and Emrich, 2008).

There are several theories that aim to understand the pathophysiology of psychosis and schizophrenia.

The dopamine hypothesis: psychosis emerges from dysfunctions within the PFC and prefrontal-subcortical network formation (Weinberger, 1987). According to this theory, the dysregulation of the D1 receptors causes the negative symptoms and treatment-resistant cognitive deficits, while D2 dysregulation gives rise to the positive symptoms (Laruelle et al., 1996). Several studies have pointed out that schizophrenic and psychotic patients exhibit poor performance in working memory, abstract reasoning, and cognitive flexibility tasks (Prentice et al., 2008). These functions are strictly regulated in the PFC by dopamine, and by mesocortical dopaminergic projections (Eriksson et al., 2015; Manoach, 2003). Furthermore, several studies have pointed out that D1 receptor activity in PFC follows a Gaussian-shaped curve, in which both up- and down- dysregulation of D1 receptors cause impairment to working memory

(Vijayraghavan et al., 2007). Positive psychotic symptoms like delusion have been correlated to hyperactivity in the PFC, and diminished activation of the thalamic and striatal network (Larivière et al., 2017); while auditory hallucinations in schizophrenic patients have been reported as altered thalamocortical connectivity between Hpc and Tha (Amad et al., 2014), and Hpc, Str, and Tha hyperactivity (Silbersweig et al., 1995), and visual hallucinations have been associated with abnormal activity of the occipital lobe, Str, and Tha (Braun et al., 2003). Anosognosia (or loss of insight), a typical symptom of psychosis, has been associated with alteration in cortico-striatal networks (McMurtray et al., 2014) (Fig. 11).

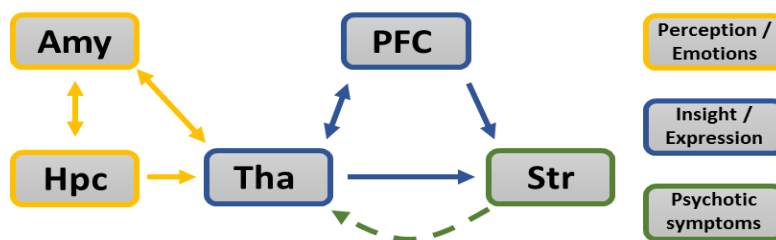


Figure 11: General scheme representing the structures interactions involved in schizophrenia in the dopamine hypothesis. Amygdala (Amy), Hippocampus (Hpc), Thalamus (Tha), Prefrontal cortex (PFC), and Striatum (Str).

The serotonin hypothesis: the serotonin hypothesis was the first to be proposed chronologically. In 1923, German psychiatrist Beringer proposed the use of mescaline as a treatment model of psychosis, although the action mechanism of mescaline was unknown at the time. In 1956, Berzel et al. proposed the use of LSD as a model of psychosis (Breier, 1995; Crow, 1980; Whitaker et al., 1981). However, in schizophrenic patients, with the exception of the acute phase of psychosis, the most common hallucinations are auditory (dopaminergic) and not visual (serotonergic) (Amad et al., 2014; Bracha et al., 1985; McCABE et al., 1972; Morrison et al., 2000; Teeple et al., 2009). Furthermore, drugs that act on the serotonergic system, except when administered in high doses, do not cause the typical social and emotional withdrawal that has been observed in psychotic and schizophrenic patients (Gouzoulis-Mayfrank et al., 1998). With the development of the dopaminergic model of psychosis, the serotonergic model has gradually been abandoned.

The glutamate hypothesis: ketamine, an anesthetic drug that blocks the NMDA receptor (Sleigh et al., 2014), was able to reproduce almost the whole effect of positive psychotic symptoms in healthy patients, including hallucinations, and to exacerbate psychotic events in schizophrenic patients (Honey et al., 2008; Pomarol-Clotet et al., 2006). In addition, like dopamine psychosis models, ketamine was able to enhance subcortical dopamine release (Kegeles et al., 2000).

The GABA hypothesis: the neurocircuitry behind the theory of psychosis is still to be clarified entirely, but the most accredited theory is that cortical GABA interneurons cause alterations in gamma-band oscillations (Jadi et al., 2016), that are actively involved in the synchronization of neuronal populations during working memory and attention. Furthermore, the NMDA hypofunctions in cortical interneurons allow a higher basal activity of glutamatergic projections on other brain areas.

- **Cannabinoid-induced psychosis**

The ability of cannabis to alter the mental state of those who abuse it is nothing new. It has been well known since the Persian Empire. However, in modern times, it was a French psychiatrist who pointed out the psychotic effects of cannabis abuse for the first time (Moreau, 1973). Over time, efforts have been made to achieve a better understanding of the etiopathology of THC-induced psychosis and cannabis abuse, and cannabis dependence has long been proposed as a possible trigger for psychotic syndromes (Brook, 1984). Fergusson et al. (2003) showed that the abuse of cannabis and its dependence over time increased psychotic-like symptoms in adolescents and young adults. However, it has been suggested that cannabis alone is probably neither necessary nor sufficient to develop schizophrenia (Gage et al., 2016), although high doses of CB1 agonists (e.g., THC or spice) can cause individual transient and acute psychotic states. D'Souza et al. (2016) proposed a classification of THC-induced psychosis based on the time-lapse between THC administration and the incidence of psychotic events: acute transient

psychosis (ATP), the most common THC-induced psychosis, that lasts for the time of THC intoxication, usually minutes or hours; acute, immediate, and persistent psychosis (AIPP), that starts with THC intoxication, but can endure for days or weeks, and in general needs medical attention; and chronic, delayed, persistent psychosis (CDPP), that appears years after the abuse of THC, and is the most severe case of THC-induced psychosis (Fig. 12).

Acute transient psychosis (ATP): it is well known that during the period of cannabis intoxication, it is possible to experience psychotic symptoms that, in most cases, are self-remitting (Coyne, 2015). It has been observed that for mild to severe doses of THC, the incidence of positive or negative symptoms of psychosis is equally common. An effect similar to THC has been reported for any active cannabinoid, such as spice (Favrat et al., 2005; Papanti et al., 2013).

Acute, immediate, persistent psychosis (AIPP): new synthetic THC-like drugs that are more potent than usual cannabis are making the problem more acute. Spice and K2, two of the new generation of synthetic cannabinoids, have been reported to cause AIPP more commonly than THC (Roberto et al., 2016; Spaderna et al., 2013; Sweet et al., 2018). These drugs caused more substantial psychotic effects, such as both visual and auditory hallucinations, catatonia, paranoia, dissociation, aggression, depersonalization, and several locomotor effects, that made people that used those drugs act like a "zombie" (Doward, 2017; Farell-Roig et al., 2018; Power, 2019; Williams, 2018). Usually, psychotic states regress with the end of the intoxication, and do not reappear unless the drug is taken again. AIPP is a psychosis that starts with cannabis intoxication, usually after very high doses of THC or any other CB1 agonist, and lasts for days or even weeks. It starts with effects typical of high doses of THC, such as delusions, hallucinations, amnesia, depersonalization, and has been reported as a phenomenon all over the world (Arendt et al., 2005). However, studies have underlined that AIPP patients are more likely to develop schizophrenia (Niemi-Pynttäre et al., 2013). Furthermore, in a study carried out in

northern Europe, ~50% of patients that were hospitalized for acute cannabinoid-induced psychosis, were diagnosed with schizophrenia in long-lasting follow-up (Arendt et al., 2005).

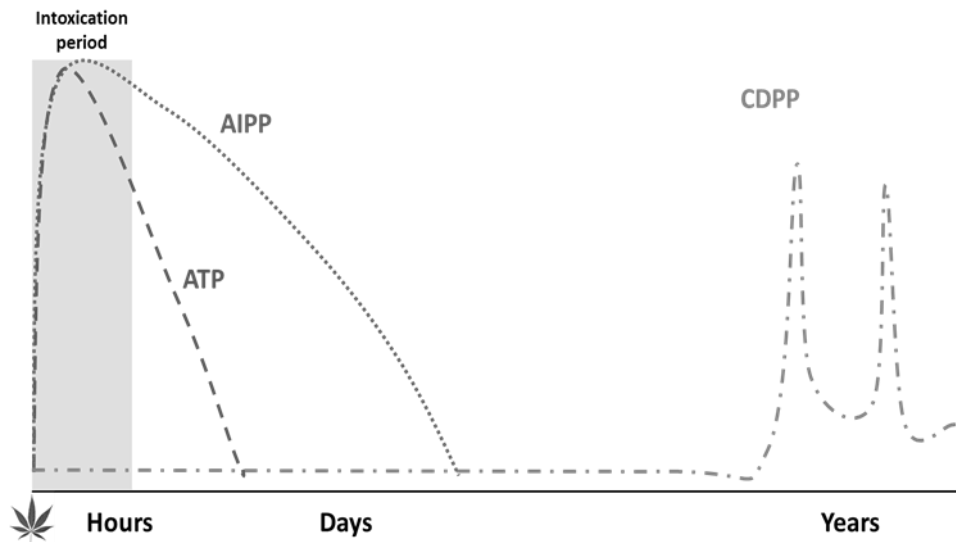


Figure 12: Short- and long-term effect of THC on different psychosis types: Acute, transient psychosis (ATP) that lasts for minutes or a few hours from the cannabis intake, and does not need medical attention. Only after marijuana use, this psychosis is reached. Acute, immediate, persistent psychosis (AIPP) begins as the ATP but lasts for days or weeks. Unless the risk of self-harm, there is no or little need for medical attention. After the end of the crisis, it should not represent if the drug is not retaken. Chronic delayed persistent psychosis (CDPP), also known as THC-induced schizophrenia. Needs medical attention, and can appear years after the period of marijuana intake. It can last years, and there is considerable variability in the subject that experiences this form of THC-induced psychosis.

Chronic, delayed, persistent psychosis (CDPP): it is well known that THC or more general cannabinoid abuse during childhood or adolescence is related to schizophrenia diagnosis in adulthood (Malone et al., 2010; Renard et al., 2014; Rubino and Parolaro, 2014). The first work that pointed out this correlation was conducted in Sweden in 1987. In this work, it was pointed out that heavy cannabis users among a cohort of 18-20 years old had a six-fold probability of developing schizophrenic spectrum disorder compared to non-cannabis users (Sven Andréasson et al., 1987). It has been demonstrated that THC abuse during the prenatal, early-stage, and adolescence periods can cause mild to severe modifications in neurotransmission in the brain (Bossong and Niesink, 2010; Malone et al., 2010). It has been reported that cannabis abuse in adolescent rats led to alterations in the PFC that led to psychotic-

like phenotype (Zamberletti et al., 2014), and that prenatal exposure to THC resulted in altered indolamine transmission with psychotic-like behavior in adult rats (Molina-Holgado et al., 1997).

Furthermore, in schizophrenic patients, a significantly higher level of the endocannabinoid AEA has been found in the cerebrospinal fluid, but not in the plasma (Giuffrida et al., 2004; Leweke et al., 1999). A dopamine-eCB model was proposed after the discovery that THC administration increased DA activity in the mesolimbic area, while indirectly increasing ACh release in the hippocampus and prefrontal cortex (Pisanu et al., 2006; Verrico et al., 2003). In this model, the higher activity of the D₂ receptor is thought to be balanced by the eCB, and if that is the case, those patients whose levels of eCBs rise more will suffer fewer schizophrenic symptoms. However, THC consumption may disrupt eCB production, leading to higher D₂ receptor activity and exacerbating the psychotic symptoms (Giuffrida et al., 2004).

II.4 Cannabis and sociability

The definition of sociability includes all the sets of behaviors that bring individuals of the same group together (e.g. cooperation, mating, playing), or keep them apart (e.g., social dominance, fighting, rivalry) (Caldwell, 2012). Sociability is a complex function that is regulated by several neurohormones and different systems within the brain, which brings everything together in an understanding of the social surrounding of the individual (Caldwell, 2012). In people affected by schizophrenia or autism spectrum disorder, it is not uncommon to observe reduced if not absent sociability (Barak and Feng, 2016; Dodell-Feder et al., 2015). A significant number of different brain areas cooperate in developing healthy social behavior, in an intricate balance between introspection, insight, and extroversion, and processing expression in other individuals (Barak and Feng, 2016). During brain development (Fernández-Ruiz et al., 1999) and through neural plasticity (Dow-Edwards and Silva, 2017), the endocannabinoid system regulates this complex behavior (Caldwell, 2012; Fairless et al., 2012; Lesch and Waider, 2012). More

particularly, schizophrenic people exhibit a very characteristic condition of social withdrawal that involves social anhedonia, amotivation, and acognition (American Psychiatric Association, 2013). Furthermore, as mentioned before, a correlation exists between schizophrenia and cannabis abuse (D'Souza et al., 2016).

In humans, it has been shown in the literature that marijuana biphasically modulates social behavior. In Tart's (1970) work, subjects answering a questionnaire confirmed that when using marijuana, they (the vast majority of the subjects) felt more willing to bond to other people. In this study, it was also reported that cannabis consumption made feel people more confident about their social intuition, but even so, less able to play social games. However, acute marijuana administration can harm social conversation (Higgins and Stitzer, 1986), and augment coercion in the absence of verbal exchanges in small groups (Foltin and Fischman, 1988). Haney et al. (1999) confirmed that cannabis abuse led to social withdrawal and that cannabis withdrawal led to anxiety and irritability. Salzman and colleagues (1976) tested marijuana output on hostile behavior within groups. In their work, they divided the patients into two groups, the marijuana and placebo groups. A short story was told to each group to see whether they would agree or not to the interpretation of the story, but were told in any case that the interpretation was inadequate. Members from the placebo group responded aggressively towards each other, while members from the marijuana group tended to be more cooperative. The fact that THC causes a decrease in aggressivity is consistent with the finding that marijuana abusers show a shrunk amygdala with functional magnetic resonance imaging (Gorka et al., 2014).

III Animal model of THC addiction, psychosis, and sociability

III.1 THC dose-response curve

The THC dose-response curve comprises different kinds of behavioral outputs, most of which are carried out by the CB1 receptor. From the lowest to the highest doses used in experimentation and published in the literature, the trend is clear: lower doses cause excitation, while higher doses cause inhibition and depression. Some of those effects on behavior are biphasic, namely low and high doses show opposite effects (e.g., locomotor and food-related behaviors).

Every species has different sensitivity to THC and cannabinoids, so the specific dose of THC that induces specific behaviors cannot be stated in general. However, across different breeds of mice and rats, it has consistently been found that when progressively increasing the dose of THC, a vast number of different behaviors can be observed, of which some of the most studied are: hyperlocomotion (Busquets-Garcia et al., 2017b; Polissidis et al., 2013; Sulcova et al., 1998) and hyperphagia (Vallée et al., 2014) for low doses, and asocial behavior (Busquets-Garcia et al., 2017b; Malone et al., 2009), hypophagia (Riedel et al., 2009), hypolocomotion (Busquets-Garcia et al., 2017b; Polissidis et al., 2013; Sulcova et al., 1998) and cognitive and memory impairment (Busquets-Garcia et al., 2017b; Vallée et al., 2014) for a middle-range of doses, while at the highest doses it causes the common cannabinoid tetrad effects, including hypolocomotion, catalepsy, analgesia, and hypothermia (Metna-Laurent et al., 2017) and pre-pulse inhibition impairment (Busquets-Garcia et al., 2017b) (Fig. 13).

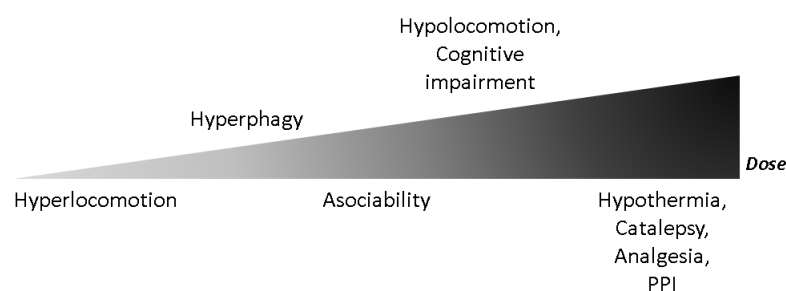


Figure 13: Common behavioural effects of THC at increasing doses in mice

The cannabinoid system modulates systems differently, according to the dose delivered. Polissidis and colleagues (2013) showed that the CB1 agonist WIN 55,212-2 at low and high doses in rats was able to cause two different profiles of neurotransmitter release in the prefrontal cortex, striatum, and nucleus accumbens. The low dose of WIN 55,212-2 (0.1 mg/kg) increased dopamine in the striatum and decreased glutamate in the nucleus accumbens of rats. The high dose of WIN 55,212-2 (1 mg/kg) caused an increase of dopamine in the prefrontal cortex, striatum and accumbens, a sharp increase of glutamate in the prefrontal cortex, and a decrease of glutamate in the striatum and nucleus accumbens. It is possible to explain this plethora of different CB1-dependent effects because CB1 is present in many different cell types and cell compartments, as mentioned before (Busquets-Garcia et al., 2016; Monory et al., 2007).

Interestingly, in cortical glutamatergic neurons of conditional CB1-KO mice, the binding of CP55,940 decreased by ~20%, but G protein activation decreased by ~50% (Steindel et al., 2013), showing that CB1 activity is not the same in every cell type, and some presented more intense activity than others, a discovery that is in line with the definition of cell-type specific signaling by Peters and Scott (2009), in which they showed that the same agonist may trigger different intracellular events in different cell types. Considering that the CB1 receptor is much more expressed in cortical GABAergic interneurons than in glutamatergic neurons, a relatively low decrease in agonist binding was expected. On the other hand, the G protein activation decrease by ~50% points to much stronger G protein activation by CB1 in glutamatergic than in GABAergic neurons. Furthermore, the CB1 receptor recruits different signaling transducers (for a review see Busquets-Garcia et al., 2017 and Turu and Hunyady, 2010). It has been demonstrated that CB1 binds to Gi (Howlett and Fleming, 1984) and Gq (Sugiura et al., 1996) proteins, and a CB1-dependent Gs activation has been shown (Calandra et al., 1999). Moreover, CB1 can recruit and activate β -Arrestins as well (Nogueras-Ortiz and Yudowski, 2016). To understand the different intracellular mechanisms that lead to CB1-dependent behaviors, it is

essential to understand the mechanisms through which CB1 can sort different outcomes in different conditions, namely the CB1 cell-type specific signaling and CB1 biased signaling.

III.2 THC addiction in an animal model

Whether cannabis (and thus THC) can cause addiction in animals is still under debate. Even though there is strong evidence of THC reward effects in humans (Hart et al., 2005), the reward in animals has been less evident in several models of drug abuse (Parolaro et al., 2007, 2005), and seems to depend strictly on the experimental conditions and animal model. Furthermore, if compared to abuse of other drugs, cannabinoid withdrawal syndrome appears to be less frequent in humans and almost absent in rodents (Smith, 2002). However, it must be said that the dose choice is critical, since high doses of THC appear to be aversive instead of reinforcing (Lepore et al., 1995; Sañudo-Peña et al., 1997).

Furthermore, even though it has been proven to be an extremely arduous task to obtain self-administration of THC in rodents, in several experiments, mice and rats self-administered other cannabinoids easily (Fadda et al., 2006; Justinova et al., 2005; Martellotta et al., 1998; Vallée et al., 2014), demonstrating that under the right conditions, cannabinoids are reinforcing for animals too. One other measurement of the addictive power of a drug is spontaneous locomotion activity (Dunnett and Robbins, 1992). Typically, a rodent responding to a psychostimulant drug with increased locomotor activity in a novel environment is more prone to self-administer the said drug (Deroche et al., 1997; Piazza et al., 1989). The vast majority of psychoactive drugs of abuse tend to increase locomotion (Geyer and Paulus, 1992; Piazza et al., 2000; Wise, 1988). However, cannabinoid regulation of locomotor activity in rodents is biphasic (Katsidoni et al., 2013; Polissidis et al., 2010, 2013), meaning that high doses of THC cause hypolocomotion and catalepsy, while low doses of THC cause hyperlocomotion. Interestingly, cannabinoid reward is biphasic as well, a fact that has been stated in both mice (Katsidoni et al., 2013) and rats (Spiller et al., 2019), showing a parallel with human behavior,

in which it has been demonstrated that unconsciously, cannabis users tend to intake only a small amount of THC, regardless of cannabis potency and THC content (van der Pol et al., 2014).

THC and opiates share similar patterns of addiction and withdrawal to some extent. Like opiates, acute administration of THC at high doses in mice causes analgesia and catalepsy, and reduces locomotion; in mice, abstinence from cannabinoids causes increased corticotrophin in the nucleus accumbens with withdrawal symptoms that follow the paradigm of the cycle of dependence (Koob and Moal, 1997). Furthermore, in mice treated chronically with THC cannabinoids, a similar compensation mechanism to that of opiates has been found in the cAMP pathway (Hutcheson et al., 1998). However, while it was shown that the brain area most involved in opiates withdrawal is the locus coeruleus (Rasmussen, 1991), it would seem that the central area in cannabinoid withdrawal is the cerebellum (Tzavara et al., 2000; Wise et al., 2011).

III.3 THC and locomotion

III.3.1 Locomotion: an overview

Locomotion, the ability to move from one place to another, is a complex function that requires both central and peripheral functions to work in coordination. The nervous system has a central role in the process of motor control and the integration of the necessary sensory information (Cech and Martin, 2012). Locomotion is generated in the brain; the signal is then sent through the motoneurons to the muscles, which contract to allow the movement, and thus locomotion. The descending tracts are the pathways by which motor signals are sent from the brain to lower motor neurons. The descending motor pathway is divided into two groups: pyramidal and extrapyramidal systems.

III.3.1.1 The pyramidal system

The pyramidal system includes the corticobulbar and corticospinal tracts. The corticobulbar tract conducts impulses from the brain to the cranial nerves. These nerves control the muscles of the face and neck. The corticospinal tract sends the signal from the brain to the spinal cord. This tract is involved in voluntary movement, and it comprises motor fibers, made up of pyramidal neurons (primary motor cortex, supplementary motor area, premotor cortex, somatosensory cortex, parietal lobe, and cingulate gyrus), and ending in the pons Varolii, medulla oblongata, and the anterior horns of the spinal cord. Those fibers all together form the pyramidal tract, forming the crossed pyramidal tract.

While the pyramidal system may directly innervate motor neurons of the spinal cord or brainstem representing the voluntary movement, the extrapyramidal system is involved in the regulation and modulation of anterior horn cells, fulfilling an indirect control.

Extrapyramidal tracts have been found in the reticular formation of the pons and medulla. They are part of the extrapyramidal system, basal ganglia, and cerebellum. The different tracts of the extrapyramidal system are *i)* the rubrospinal tract, responsible for large muscle movement, originating in the magnocellular red nucleus of the midbrain and descending in the brainstem tegmentum; *ii)* the pontine reticulospinal tract that regulates the anti-gravity extensor muscles, originating in the caudal pontine reticular nucleus and projecting to the lamina VII and VIII of the spinal cord; *iii)* the medullary reticulospinal tract that inhibits the excitatory axial extensor muscles of movement, originating from the gigantocellular nucleus and descending in the spinal cord; *iv)* the lateral vestibulospinal tract that helps to maintain balance posture, originating in the lateral, superior and inferior vestibular nuclei; and *v)* the tectospinal tract that coordinates head and eye movements, and connects the midbrain tectum and the cervical regions of the spinal cord. (Guyton and Hall, 2006) (Fig.14). Interestingly, a significant number of the most CB1-rich areas are strongly implicated in locomotion as well (Fig. 14).

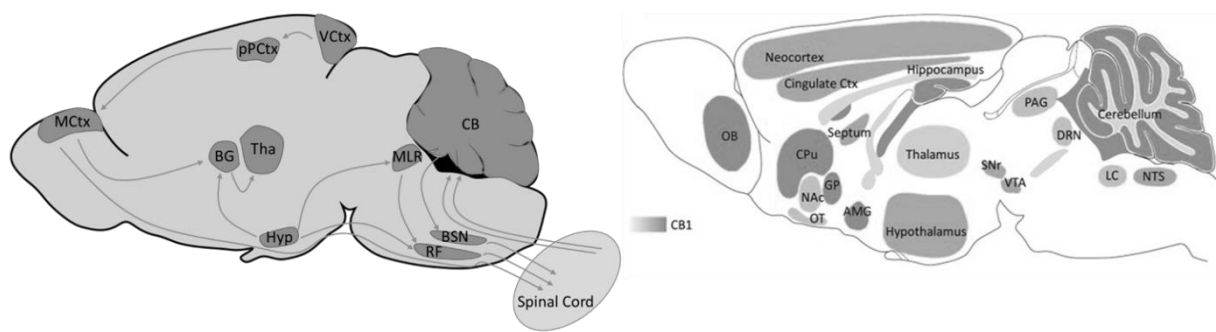


Figure 14: The extrapyramidal structures involved in locomotion in the CNS compared with the CB1 density in the mouse brain. Motor cortex (MCtx), posterior parietal cortex (pPctx), visual cortex (VCtx), basal ganglia (BG), thalamus (Tha), hypothalamus (Hyp), mesencephalic locomotor region (MLR), brainstem nuclei (BSN), medial reticular formation (RF), cerebellum (CB); olfactory bulb (OB), caudate-putamen (CPu), globus pallidus (GP), amygdala (AMG); substantia nigra (SNr), the ventral tegmental area (VTA), nucleus tractus solitarius (NTS), nucleus accumbens (NAc), olfactory tubercle (OT), thalamus, periaqueductal gray (PAG), dorsal raphe nucleus (DRN), and the locus coeruleus (LC).

The name "extrapyramidal system" is used to indicate all the structures involved in locomotion but that do not directly take part in the corticospinal-pyramidal system. The pathways that are included are the basal ganglia, cerebellum, reticular formation of the brain stem, vestibular nuclei, and red nuclei. For this work purposes, only the basal ganglia and cerebellum will be studied in more depth.

Basal ganglia: like the rest of the extrapyramidal system, the basal ganglia are accessory motor systems, whose activity is almost always associated with the activity of the cerebral cortex: the cerebral cortex is the area from and to which the basal ganglia receive and send information. The basal ganglia consist of several structures: both the dorsal striatum (caudate nucleus and putamen) and ventral striatum (nucleus accumbens and olfactory tubercle), the globus pallidus (GP), substantia nigra, and subthalamic nucleus (Albin et al., 1989; Garcia-Rill, 1986; Heimer, 1983). The connection that exists between the basal ganglia, and between the basal ganglia and other structures, is quite complex: two main circuits are activated, the direct and indirect pathways of the basal ganglia (Fig. 15; Kravitz et al., 2010). In the direct pathway, the cortex activates the striatum (glutamatergic signal), which in turn inhibits the medial globus pallidus (GABAergic signal). The medial globus pallidus (GP_M) is constitutively active, and

sends a GABAergic, and thus inhibitory, signal to the thalamus. The thalamus is no longer inhibited and sends an excitatory signal to the cortex. In the indirect pathway, the cortex sends the signal to the striatum, that inhibits the lateral globus pallidus (GP_L), which is constitutively active and in normal conditions sends a constant GABAergic signal to the subthalamic nucleus (STN), which is constitutively active and sends a glutamatergic (excitatory) signal to the GP_M, and at this point the direct and indirect pathways merge (Calabresi et al., 2014; Smith et al., 1998) (Fig. 15). The basal ganglia are essential to execute the most delicate movements (such as writing, using little materials) (Wichmann and DeLong, 1996). Since both cerebellum and basal ganglia are accessory areas for movement, they interact and “work with” other structures. For example, the basal ganglia help the cortex execute subtle movements and learned patterns of movement, and help plan the multiple, parallel, and sequential movement patterns needed for a single task.

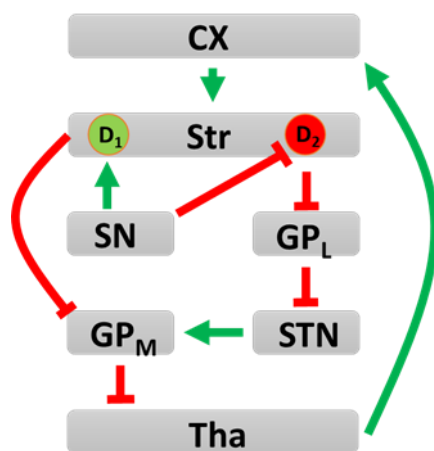


Figure 15: The basal ganglia loop: In the scheme are represented the cortex (CX), the striatum (Str), the substantia nigra (SN), lateral globus pallidus (GPL), medial globus pallidus (GPM), subthalamic nucleus (STN), and the thalamus (Tha). The basal ganglia pathway is a loop, in which the structure from which originates the signal (CX) is ultimately stimulated by the last structure to be activated (Tha). In this way, the same signal, originating from the CX, strengthens the whole pathway through the loop through both the direct and indirect pathways. D1 is the dopamine receptor type 1, while D2 is the dopamine receptor type 2.

Lastly, new research has pointed to a possible and probable interaction between the basal ganglia and cerebellum, two main structures that have long been considered anatomically and physiologically independent of each other. Bostan and Strick (2018) demonstrated that the subthalamic nucleus of the basal ganglia is a source of several projections onto the cerebellar cortex, and that the dentate nucleus of the cerebellum projects to the striatum.

Cerebellum: the cerebellum has long been known as the “silent area”, since electrical stimulation of this area *in vivo* did not cause any relevant conscious sensation (see for review, Mitoma et al., 2018). However, cerebellar damage or exportation causes severe abnormal body movement (Spencer et al., 2003). During quick muscular activity (e.g., running, typing, talking), cerebellar activity soars (Thach, 1968). The primary cerebellar function is to sequence, adjust, and regulate motor activities while they are being executed. The cerebellum receives constant and continuous input from both brain areas involved in the voluntary movement and peripheral areas involved in the same movement. The cerebellum receives that information, integrates it and sends the corrected and adjusted movement information back to the motor system. Within a fraction of a second, the cerebellum can also receive, process, and send back "corrected" signals for the next sequential movement. Being such an integrative structure, it receives several input pathways from other brain areas. Those activations of the antagonist's muscle can be learned and improved with time and exercise.

The afferent pathways that feed the cerebellum can arrive from both the CNS and periphery. It interacts with the brain stem to correct the postural reflexes (Takakusaki, 2017). The cerebellum operates with the cerebral cortex to enhance strength, and to end any movement, it activates antagonist muscles to stop the movement at the intended point (Frysinger et al., 1984).

From the cerebral motor, premotor, and somatosensory cortices, it receives inputs through the corticopontocerebellar pathway. It originates in the motor cortices, passes through the pontile nuclei and pontocerebellar tracts, and ends in the lateral divisions of the contralateral cerebellar hemispheres. From the inferior olive (that receives inputs from the cerebral cortex, basal ganglia, and spinal cord), the olivocerebellar tract innervates the whole cerebellum. From the vestibular apparatus and the brain stem vestibular nuclei, the vestibulocerebellar fibers innervate the cerebellum's flocculonodular lobe. From the brain stem reticular formation, the reticulocerebellar fibers innervate the vermis of the cerebellum. From the periphery, it receives

input from four tracts per each side of the cord. The two most important of those tracts are the dorsal spinocerebellar tract and the ventral spinocerebellar tract. Both tracts send information to the cerebellum from the periphery about muscle contraction, state of tension of tendons, and proprioceptor information. The efferent pathways of the cerebellum originate in the deep cerebellar nuclei (dentate, interposed, and fastigial). All three cerebellar deep nuclei receive inputs from the cerebellar cortex and deep sensory afferent tracts to the cerebellum. Signals that arrive in the cerebellum are split and directed to both a target area of the cerebellar cortex overlying the deep nuclei and to the deep nuclei themselves. At this point, the cerebellar cortex sends a GABAergic inhibitory input to the corresponding cerebellar deep nucleus. A signal is then sent to a target area of the brain (Voogd and Glickstein, 1998).

The cerebellar cortex has three main layers: the molecular layer formed of dendritic arborization of Golgi and Purkinje cells, parallel and climbing fibers; the Purkinje cell layer that is formed of the bodies of the Purkinje cells; and the granule cell layer, where there are the granule cells and the bodies of the Golgi cells.

The neuronal population of the cerebellum is variegated. The cell types present in the cerebellum are Purkinje cells (GABAergic) (Konnerth et al., 1990), Golgi cells (GABAergic) (Schulman and Bloom, 1981), granule cells (glutamatergic) (Chadderton et al., 2004), basket cells (GABAergic) (Southan et al., 2000), stellate cells (GABAergic) (Llano and Gerschenfeld, 1993), Lugaro cells (mainly GABAergic) (Lainé and Axelrad, 2002), and unipolar brush cells (Glutamatergic) (Mugnaini and Floris, 1994). The architecture of the cerebellum is complex and relies mostly on GABAergic transmission.

The cerebellum usually regulates all levels of muscle control. It controls the stretch reflex and if a muscle tries to lift a load that is too heavy, a signal is sent to the cerebellum which will respond, enhancing the reflex to resist the load (Higgins, 1987).

III.3.2 Drug-induced locomotion

Stimulant drugs and hyperlocomotion: The most widely-studied system relating to locomotion is the dopaminergic system (Brown et al., 1985; Eilam et al., 1991, 1991; Gold et al., 1988; Hartesveldt et al., 1992; Mogenson and Wu, 1991; Speciale et al., 1986; Whishaw and Dunnett, 1985; Wu et al., 1993). The depletion of central catecholamines causes akinesia, catalepsy, and abnormal posture. Even though the catecholamines are epinephrine (E), norepinephrine (NE), and dopamine (DA) altogether, these effects have been proven to be entirely reversed by the assumption of L-Dopa alone (Ahlenius et al., 1973), demonstrating that the akinetic effect of anticatecholaminergic drugs was indeed attributable to the dopaminergic, and not the adrenergic and noradrenergic system. Furthermore, more selective drugs inhibiting dopaminergic receptors, such as spiroperidol or high doses of haloperidol, induce catalepsy in mammals (Asper et al., 1973; Sanberg, 1980). On the contrary, drugs that enhance dopaminergic transmission in the brain (e.g., amphetamine, cocaine, and apomorphine) show strong motor activation. In animal models, there are several ways of measuring locomotor activity, including observation in open fields, where data are handily recorded, automated actimetry cages and circular corridors that count photocell breaks, and running wheels among the others. Such a wide variety of different means to measure locomotor activity can enlighten us about different aspects of motor behavior. However, as a general rule, drugs that enhance dopaminergic signals show a coherent and reproducible locomotor increase between alternative measurements (for a review, see Robbins, 1977). The gold standard among the drugs that cause dopaminergic-induced hyperlocomotion are surely amphetamines, even though their pharmacological action stimulates the release of both dopamine and noradrenaline by inhibiting the catecholamine reuptake system and forcing the release of dopamine. Interestingly, low-to-moderate doses of amphetamines cause hyperlocomotion in rodents, but when increasing the dose, locomotion declines and the rodents show stereotypy instead (Fritts et al., 1997; Yates et

al., 2007). Once again, it has been shown that the effect of stimulant drugs on locomotion and stereotypy seems to be dependent on the dopaminergic system, rather than on the adrenergic system (Creese and Iversen, 1975): it is dopaminergic, and not adrenergic activity that causes the drug-induced hyperlocomotor effect. Dopamine acts on the dopamine receptors, seven-transmembrane receptors divided into two main subclasses: D₁-like (D₁ and D₅) (Tiberi et al., 1996) and D₂-like (D₂, D₃, and D₄) (Andersen et al., 1990). D₁-like receptors are coupled to G_{s/olf} proteins, while D₂-like receptors are coupled to G_{i/o} proteins (Beaulieu and Gainetdinov, 2011). In the brain, a high density of D₁ receptor has been found in the striatum, substantia nigra, nucleus accumbens, olfactory bulb, amygdala, and frontal cortex, while low levels can be found in the hippocampus, cerebellum and thalamic areas (Rankin and Sibley, 2010). The D₂ receptor has been found in the striatum, accumbens, olfactory tubercle, substantia nigra, ventral tegmental area, hypothalamus, cortical areas, amygdala and hippocampus (Beaulieu and Gainetdinov, 2011). Both D₁ and D₂ receptors can be found within the medium spiny neurons (MSN) of the striatum, and the neurons are classified based on dopamine receptor expression (D₁ or D₂ neurons). In particular, the MSN that express the D₁ receptor are part of the direct striatonigral pathway, while the neurons that express D₂ are part of the striatopallidal pathway. Interestingly enough, D₁ and D₂ receptors may form a heterodimer, which instead recruits G_q protein (Beaulieu and Gainetdinov, 2011). The D₂ receptor presents a bias in its signaling. It can recruit β-Arrestin 2 to trigger PP2A-Akt-GSK3β signaling within the cell (Beaulieu et al., 2007, 2005, 2004). This β-Arrestin 2-dependent signaling has been proved to be the cause of hyperlocomotion and reward induced by psychostimulant drugs (Beaulieu et al., 2005; Miller et al., 2014).

Depressant drugs and hypolocomotion: Several studies point to the central role of the dopaminergic system in hypokinetic (hypolocomotor) behavior too. Reserpine is an antipsychotic drug that is known to cause Parkinson's-like symptoms in rodents, and whose

mechanism of action consists in blockading the vesicular monoamine transporter (VMAT) that is responsible for the uptake of monoamine from the cytosol into the synaptic vesicle. Monoamines prevented from entering the synaptic vesicles are degraded by monoamine oxidase (MAO), impairing the dopaminergic signal as well. Reserpine can cause severe hypolocomotion and catalepsy in rats, which is proven to be reversed by MAO inhibitors and dopaminergic agonists (Colpaert, 1987). Nitric oxide is known to be a negative regulator of locomotion, and the nitric oxide donor, sodium nitroprusside, causes hypolocomotion in mice. Interestingly, this effect is blocked by the adenosine receptor antagonist theophylline. Furthermore, it has also been demonstrated that sodium nitroprusside increased the extracellular levels of adenosine in the brain, and this effect was especially strong within the striatum. The hypolocomotor effect of nitric oxide was proven to be dependent especially on the adenosine A_{2A} receptor, which is known to regulate D₂R. Adenosine A_{2A} receptor inhibition was proven to rescue the hypolocomotor phenotype of D₂R deficient mice (Aoyama et al., 2000; Dall'Igna et al., 2001). Altogether, the dopaminergic system is implicated in hypolocomotion as well, and drugs that negatively modulate dopaminergic transmission cause hypolocomotion.

III.3.3 THC- induced hyperlocomotion

THC shows behavioral effects that are often biphasic, *i.e.*, high and low doses of THC show opposite effects on a broad set of behaviors, such as memory and cognition, response to stress, and locomotion (Calabrese and Rubio-Casillas, 2018; Katsidoni et al., 2013; Rey et al., 2012; Sañudo-Peña et al., 2000; Sulcova et al., 1998). It is known that the endocannabinoid system plays a role in motor control, motor coordination, and spontaneous activity.

Locomotor activity is one THC-induced biphasic behavior. The biphasic effects of THC on locomotion are time-dose dependent, and even though the most commonly-studied THC-induced effects in mice on locomotion are hypolocomotion and catalepsy (hypoactive behaviors) observed with high doses of THC, several studies have shown how low doses of

THC and other cannabinoids on both mice and rats cause hyperlocomotion (hyperactive behavior) (Davis et al., 1972; Katsidoni et al., 2013; Paydar et al., 2018; Renard et al., 2018). Sulcova et al. (1998) demonstrated that anandamide could cause a biphasic locomotor effect, meaning that high doses of anandamide produced hypolocomotion in mice, while low doses incremented both horizontal and vertical locomotion. Furthermore, although high doses of THC slow cerebral metabolism in several brain structures, low doses of THC enhanced metabolism in the same brain structures (Margulies and Hammer, 1991). Sañudo-Peña et al. (2000) showed a curve of activity following several doses of THC in rats. As expected, high doses of THC caused lower activity (both horizontal and vertical), while low-doses of THC triggered hyperlocomotion (both horizontal and vertical) in male Sprague–Dawley rats (Sañudo-Peña et al., 2000). As confirmation, male Sprague–Dawley rats treated with low doses of THC or WIN 55,212-2 showed hyperlocomotion, and rimonabant was able to block both the former (Katsidoni et al., 2013) and the latter (Polissidis et al., 2013). CBD, a negative modulator of CB1, can block hyperlocomotion (along with a set of other psychotic-like behaviors) caused by chronic administration of THC in C57BL6 mice (Todd et al., 2017).

Several models have been proposed to explain this THC-induced hyperlocomotion. CB1 is abundant in the basal ganglia, of which the Substantia Nigra, the Globus Pallidus, and the Striatum are particularly rich of CB1 (Herkenham et al., 1991). Sañudo-Peña and Walker (1998) proposed a model in which the basal ganglia play a central role in THC-induced locomotion. In their model, cannabinoids act within the basal ganglia loop on the Str, GP, SNr, and subthalamic nucleus, on both the direct and indirect pathways, obtaining an overall increase in locomotion. Busquets-Garcia et al., interestingly, demonstrated that following low doses of THC (0.3 mg/kg), C57BL6N mice showed a steady increase in locomotor reactivity to a novel environment, a test predictive of both addiction and psychosis (Busquets-Garcia et al., 2017; Gancarz et al., 2011; Kalinichev et al., 2004).

III.3.4 THC-induced hypolocomotion

As mentioned previously, THC causes a biphasic effect on locomotion and reward (Katsidoni et al., 2013), meaning that low and high doses present opposite effects. Hypolocomotion is part of a set of behavioral outputs that can be observed in the short term after acute administration of high doses of THC and other cannabinoids (Metna-Laurent et al., 2017). As stated before, locomotion is, in most cases, related to dopaminergic release. Bloomfield and colleagues (2016), in an excellent review, clearly explained what is known about the interaction between cannabinoid and dopaminergic systems. The CB1 receptor is abundant in the most crucial dopaminergic pathways, especially in the ones that regulate locomotion and reward, e.g., the striatum (Herkenham et al., 1991), which are positioned in presynaptic glutamatergic and GABAergic nerve terminals. It has also been demonstrated that, as a general rule, both the most common endocannabinoids, AEA and 2-AG, cause a dopamine increase in the nucleus accumbens. Howes and Osgood (1974), and Bloom and Dewey (1978) discovered through *in vitro* studies that THC increased dopamine release and synthesis in rodent cells.

Interestingly, THC has been demonstrated to present a biphasic effect on dopamine transmission as well: low doses of THC promote the conversion of tyrosine into dopamine, while high doses of THC decrease dopamine release (Poddar and Dewey, 1980). *In vivo*, it has been demonstrated that systemic acute administration of THC increases dopamine release in the prefrontal cortex, striatum, and nucleus accumbens in rats (Chen et al., 1990; Ng Cheong Ton et al., 1988; Pistis et al., 2002, Vallée et al., 2014). However, CB1 receptors have rarely been found in dopaminergic neurons, except in the ventral tegmental area (Wenger et al., 2003). For these reasons, it has also been proposed that the hypolocomotor output of CB1 agonists may be dependent upon cortical glutamatergic neurons (Polissidis et al., 2010), through indirect modulation of dopaminergic transmission within the nucleus accumbens, striatum, and hippocampus, as proposed by Cannizzaro and colleagues (2006). Interestingly, several studies

have pointed out that the behavioral outputs of dopamine follow an inverted U curve: low dopamine and high dopamine both cause lowered locomotion, as excessively high doses of dopaminergic drugs, such as amphetamines, cause reduced locomotion and increased stereotypy in rodents (Dunnett and Robbins, 1992; Randrup and Munkvad, 1967). Polissidis et al., (2013), through a correlative study of behavior and neurotransmitter release, showed that WIN 55,212-2 modulated the release of glutamate and dopamine in the striatum, nucleus accumbens, and prefrontal cortex, as well as biphasically modulating locomotion in rats: low doses of WIN 55,212-2 caused hyperlocomotion, while high doses caused hypolocomotion. However, in the nucleus accumbens and striatum, WIN 55,212-2 produced increased release of dopamine and decreased release of glutamate, while it caused an increase of both neurotransmitters in the prefrontal cortex. Controversially, this effect has been observed to be linear (the trend was more definite at stronger doses) and did not show any biphasic trend, contrary to the previous findings of Poddar and Dewey (1980). The authors did not focus on this discrepancy. From this work, however, it is possible to deduce that hyperlocomotion seems to depend more on the dopaminergic system, while hypolocomotion seems to depend more on the glutamatergic system. Cannabinoids may thus regulate hyperlocomotion and hypolocomotion through two different and isolated mechanisms. However, dopamine release following THC administration has led to discordant conclusions in the literature (Barkus et al., 2011; Bossong et al., 2009, 2009; Castañeda et al., 1991; Stokes et al., 2009; Yasuko Sakurai-Yamashita et al., 1989), and quite interestingly, in Vallée et al., (2014) work, doses of THC of less than 0.6 mg/kg were not sufficient to detect any increase in dopamine in the VTA of rats, while this dose induced hyperlocomotion.

III.4 THC and social behavior

III.4.1 Social behavior: an overview

Characterized as it is by great complexity, social behavior is still not completely understood. However, a number of different works have pointed to several structures that are deeply involved in sociability. Felix-Ortiz and Tye (2014) discovered that modulating input from the amygdala to the ventral hippocampus could modulate social behavior in mice. By silencing the BLA projections in the ventral hippocampus through optogenetic manipulation of the ventral hippocampus, they were able to disrupt sociability in mice. In Kolb and Nonneman's (1974) work, it was shown that lesions in different brain areas led to different social deficits. For instance, lesions to both the hippocampus and amygdala eliminated shock-induced aggression, while amygdala lesions caused diminished contact in a large arena, but had virtually no effect on rats in a small arena (see also Eichelman, 1971; Jonason and Enloe, 1971). Septal lesions had a less significant impact, as they decreased the amplitude of shock-induced aggression, but did not affect contact either in a small or large arena, while other works have shown increased contact in rats in open field tests in the same condition (Jonason and Enloe, 1971), and increased shock-induced aggression (Eichelman, 1971) in rats. Lesions of the orbital frontal and medial frontal cortex increased the shock-induced aggression: orbital frontal lesion reduced the contact between rats in the open field test significantly, while the medial frontal lesion did not (see also Kolb et al., 1974).

Ferdman and colleagues showed that the prefrontal cortex in rats plays a critical role in the development of normal sociability. They demonstrated that rats isolated after weaning show social impairment during adulthood, as well as shorter dendrites and sparser spines in prefrontal regions (Ferdman et al., 2007). Moreover, Tõnissaar and colleagues (2004) showed a correlation between the serotonin levels in the prefrontal cortex and sociability in rats.

Furthermore, the frontal cortex is linked to the facilitation but not to the initiation of social play in rats (Vanderschuren, 1997).

The striatum is also closely involved in sociability. It has been demonstrated that the striatum is responsible for the so-called social reward, a reward that works through the "standard" reward circuitry and is triggered by sociability (Schultz, 2004). These functions inside the striatum are carried out by the nucleus accumbens. It has been demonstrated that oxytocin and dopamine action in the NAc have a crucial role in the partner decision in voles (Liu and Wang, 2003), parental care, affection, social attachment (Young et al., 2001), and social reward (Dölen et al., 2013), while hypoactivity in the nucleus accumbens is correlated to social isolation (Wallace et al., 2009).

Furthermore, Walsh and colleagues (2018) demonstrated that in a mouse autism model, serotonin release in the nucleus accumbens improved sociability. Interestingly, Shonesy and colleagues (2018) found an important link between the cannabinoid system and the striatal pathway. It was also demonstrated that by depleting the primary synthetic enzyme of 2-AG, the DAGL α from dopamine D1 receptor-expressing and adenosine receptor-expressing MSN, 2-AG plays a key role in the D1 direct striatal pathway and sociability (Shonesy et al., 2018). From the pioneering work of Carta et al. (2019), the cerebellum's active role in sociability emerged. They discovered the existence of excitatory projections from the cerebellar nuclei to the VTA. As this cerebellum activity on the VTA was enough to cause place preference conditioning, they concluded that this pathway was rewarding. Even though blocking this pathway was aversive, it completely blocked social behavior in the three chambers sociability test, actively supporting the cerebellum's role in social behavior (Carta et al., 2019). Furthermore, in humans, it has been observed that patients with cerebellar diseases show impairments on the Reading the Mind in the Eyes Task, a test of Emotion Attribution, which is essential for social cognition (Hoche et al., 2016).

III.4.2 THC-induced unsocial behavior

As in humans, THC impairs sociability in rodents as well. High doses of THC disrupt the sociability of THC-treated rats, while low doses of THC reduce exploratory social behavior (e.g., sniffing). This effect, however, has been confirmed for the psychoactive component of cannabis, THC, while CBD did not show any impact on sociability at low or high doses (van Ree et al., 1984). When rats were treated with CP 55,940, a CB1 agonist that is ~45 times more potent than THC, robust social withdrawal was observed 24h post-administration (Genn et al., 2004). However, when rats were treated with CP 55,940 after a social interaction test, and then re-tested 24h later, their sociability had increased, showing that under specific conditions, cannabinoids may cause a biphasic effect on rodent sociability. In a chronic study, the administration of CP 55,940 in adolescent female rats caused memory impairment, and significant social withdrawal (O'Shea et al., 2004). Similarly, it was demonstrated in perinatal, adolescent, and young adult male rats that CP 55,940 caused anxiety, memory impairment, and social withdrawal similar to those observed in adolescent female rats (O'Shea et al., 2004). Another synthetic CB1 agonist, WIN55,212-2, causes sociability deficits in adolescent rats (Schneider et al., 2008). Interestingly, while direct CB1 agonists such as THC, CP 55,940, or WIN55,212-2 can impair sociability in rodents, URB597, an indirect CB1 agonist which inhibits the hydrolysis of anandamide, enhanced social play (Trezza and Vanderschuren, 2008), demonstrating once again the biphasic effect of CB1 agonists on behavior, and most likely a functional selectivity in cannabinoids. The CB1 antagonist rimonabant blocked both direct cannabinoid agonist-induced social impairment and indirect cannabinoid agonist-induced social enhancement effects. Furthermore, the same group discovered that AM404, a FAAH inhibitor, reduced social behavior in rats, while VDM11, an inhibitor of cannabinoid reuptake, enhanced social interaction (Trezza and Vanderschuren, 2009). In C57BL6N mice, THC at a dose of 3 mg/kg caused asocial behavior in a similar manner (Busquets-Garcia et al., 2017).

IV. Cannabinoid receptor type-1: bias, signaling, and modulation

IV.1 CB1 classic signaling

Being a GPCR, CB1 is a metabotropic receptor coupled to a specific signaling transducer (a protein activated directly by the receptor, that in turn activates a protein cascade), usually G proteins. G proteins are a family of trimeric proteins (formed by α , β , and γ subunits), constitutively bound to a molecule of GDP when in inactive state. After that, an agonist binds the receptor, the latter modifies its tridimensional shape and allows the exchange of GDP with GTP, activating the G protein. The G protein, when activated, splits into α monomer and $\beta\gamma$ dimer. G proteins, and consequentially GPCR, can be divided in three main families: inhibitory ($G_{i/o}$), stimulant (G_s), or $G_{q/11}$. The first G protein coupled to CB1 to be identified was a $G_{i/o}$. The $\alpha_{i/o}$ subunit reaches adenylyl cyclase (AC) and inhibits the production of cyclic adenosine monophosphate (cAMP), while the $\beta\gamma$ dimer enhances the activity of G protein-coupled inwardly-rectifying potassium channel (GIRK) (Azad et al., 2008; Henry and Chavkin, 1995) and blocks the voltage-gated calcium channel (VGCC) (Gebremedhin et al., 1999) (Fig. 16a). However, while the most classic $G_{i/o}$ protein signaling does correspond to CB1, newer evidence shows a higher degree of complexity, meaning that CB1 signaling is biased. In fact, CB1 is able to bind to a G_q protein, responsible for phospholipase C (PLC) activation (Fig. 16b), and to a G_s protein, responsible for the activation of adenylyl cyclase (AC) and production of cyclic AMP (cAMP) (Fig. 16c) as well (Turu and Hunyady, 2010).

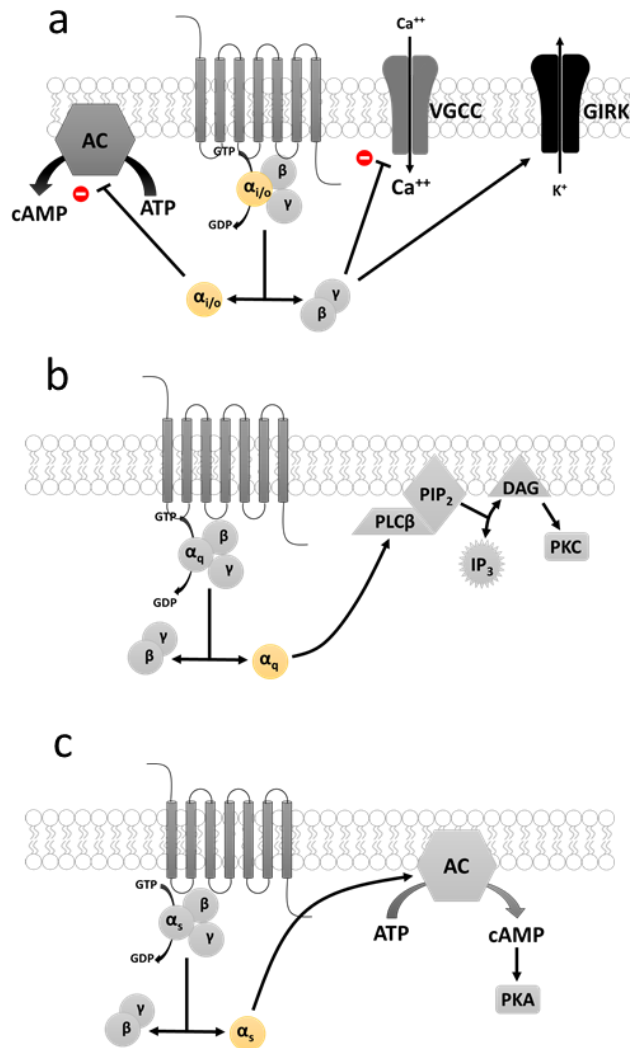


Figure 16: G proteins associated with CB1: When CB1 was discovered, it was thought that the only signaling he carried out the most classical **a)** *Gi* pathway, in which, after that, the endocannabinoid binds the CB1 receptor, the G protein linked to it exchange a GTP with a GDP. The trimeric G protein splits between the subunit α_i and the dimer $\beta\gamma$. α_i , a GTPase enzyme, blocks the adenylyl cyclase (AC), blocking the conversion of ATP in cAMP. In contrast, the $\beta\gamma$ dimer blocks the entrance of Ca^{++} by blocking the voltage-gated calcium channel (VGCC) and augmenting potassium's expulsion through the G's activation protein-coupled inwardly-rectifying potassium channels. Altogether the main effects of the signaling in the cell are inhibitory. It is now accepted that the CB1 receptor can bind **b)** *Gq* protein as well, in which, after the receptor activation, GDP/GTP exchange and G protein separation in the α_q monomer and $\beta\gamma$ dimer, the α_q subunit recruits and activates the PLC β . PLC β , activated, migrates to the inner surface of the phospholipidic bilayer, in which is located the phosphatidylinositol 4,5-bisphosphate (PIP $_2$), and converts it in diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP $_3$). IP $_3$ and DAG are two second messengers able to activate the Inositol trisphosphate receptor (InsP $_3$ R), localized on the endoplasmic reticulum surface, and responsible for releasing Ca^{2+} in the cell, and for the facilitation of the translocation of PKC from the cytosol to the plasma membrane respectively. Lastly, several evidences pointed to **c)** *Gs* recruitment following CB1 activation, in which after the GDP/GTP exchange and the α_s and $\beta\gamma$ dimer separation, the α_s subunit causes the activation of the AC, with the consequent increase of cAMP and PKA activation.

IV.2 Biased signaling: an overview

Until the introduction of the concept of biased signaling, the most accepted pharmacological theory affirmed that the interactions between ligand and receptor were governed by three important properties: affinity, potency, and efficacy (Salahudeen and Nishtala, 2017). The affinity can be defined as the extent to which a drug binds to its receptors at a given drug concentration; the potency, strictly related to the affinity is the measure of necessary amount of the drug that produces an effect of a given magnitude, usually denoted as the median effective concentration (e.g. EC_{50}); the efficacy is the ability of the drug to cause a pharmacologic response when it occurs the interaction with the receptor.

According to the definition above, the efficacy of a ligand measures the stimulus per receptor molecule induced by a ligand. Briefly, a full agonist leads to a maximal response from the receptor, simultaneously and maximally stimulating the response that the receptor can give, while the partial agonists possess lower intrinsic efficacy and lead to different degrees of submaximal responses (Kenakin, 1985). In this model, a receptor can be imagined as a switch that turns to the activated state (R^* , “on”) when it is hit by a ligand, while otherwise it is in its inactive state (R , “off”) (Mallipeddi et al., 2017). Under this classification, different agonists binding the same receptor will give responses that will be quantitatively different, but qualitatively the same. Yet in the last twenty years, studying signal response to agonists that act on the same receptor, it has been observed that the classical concept of intrinsic affinity has its flaws, and it can be strictly dependent on the context and the system in which the receptor is found. In certain cases, it has been observed that the same receptor, when activated by different agonists or in different cell types, can give responses that are both quantitatively and qualitatively different. On several occasions, scientists encountering different signaling to what they were expecting have coined terms to explain the phenomenon, such as *ligand-directed trafficking of receptor stimulus* (Chilmonczyk et al., 2014), *biased agonism* (Charfi et al., 2014),

protean agonism (Jansson et al., 1998), *differential engagement* (Manning, 2002), *biased signaling* (Ahn et al., 2013), *functional selectivity* (Zhou and Bohn, 2014), and *biased receptor signaling* (Kenakin, 2019), to name but a few. Examples of bias receptor signaling can be observed in 5-HT₂, μ -Opioid, β 2-Adrenergic, V2 vasopressin receptors, among others (Smith et al., 2018; Urban et al., 2007). In relatively recent periods, it has been accepted that CB1 receptors also present biased signaling (Ahn et al., 2012, 2013; Al-Zoubi et al., 2019; Ibsen et al., 2017; Khajehali et al., 2015; R.-W. Wu et al., 2011).

A bias in receptor signaling can be observed when different agonists can cause qualitative differences in receptor signaling by recruiting different signaling transducers. In fact, while it was commonly accepted before the discovery of biased signaling that a family of GPCR could engage only one family of signaling transducer (and more particularly a G protein), nowadays it is known that a receptor can have more than one signaling transducer (different families of G protein or β -Arrestins) to carry on its signaling (Seyedabadi et al., 2019). One very important signaling transducer that has been increasingly studied in biased agonism and signaling is β -

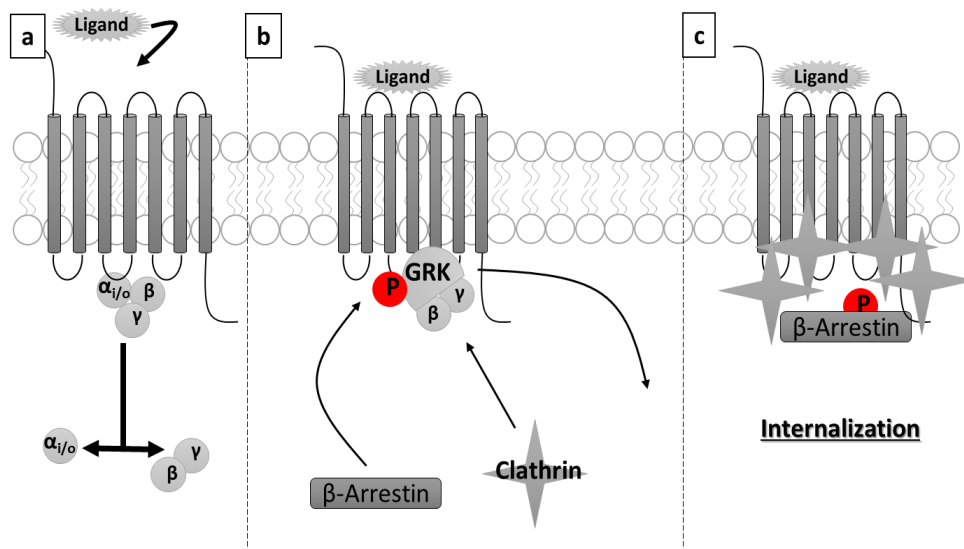


Figure 17: Desensitization of GPCR signal through β -Arrestin. **a)** After that the ligand binds the receptor, a molecule of GDT bond to the α subunit of the G protein is exchanged with a molecule of GTP. The α subunit is an enzyme with GTPase activity. The α subunit acts on its target, but when the GTP is consumed and converted in GDP, its activity finishes and returns to the receptor. **b)** The $\beta\gamma$ subunit, meanwhile, recruits the G protein-coupled receptor kinase (GRK) that phosphorylates a specific epitope of the receptor. This phosphorylation recruits β -Arrestin, that binds the phosphorylated epitope, and recruits, in turn, the clathrins. **c)** The clathrins aggregate on the receptor and the membrane buds inwardly in a process called opsonization, leading to the internalization of the receptor, and thus the end of the signal.

Arrestin, a very versatile actor in cell signaling, which has been discovered relatively recently and can direct the recruitment, activation, and scaffolding of several downstream targets (Lefkowitz and Shenoy, 2005).

β -Arrestins are a sub-family of Arrestins. β -Arrestins 1 and 2 (Arrestins 2 and 3 respectively) are ubiquitous proteins, and in time have been largely studied for their role in receptor internalization (Gurevich and Gurevich, 2013; Jones and Hinkle, 2005; Kohout, 2003) and desensitization (Dawson et al., 1993; Lohse et al., 1992; Pippig et al., 1993). After prolonged signaling (Fig. 17a), the G protein-coupled receptor kinase (GRK) is recruited by the $\beta\gamma$ dimer, and phosphorylates a residue of the receptor (Fig. 17b) recruiting β -Arrestin, the clathrins, structural proteins that allow receptor internalization and the signal to cease (Fig. 17c) (Luttrell and Lefkowitz, 2002).

Furthermore, it has been demonstrated that β -Arrestin 1 and 2 can actively recruit different protein kinases, such as PI3K (Povsic et al., 2003), phosphatase 2A (PP2A) (Beaulieu et al., 2005), Akt (Cianfrocca et al., 2010), MEK (Ma and Pei, 2007) or Erk (Eishingdrelo et al., 2015). However, while that is true as general rule, for CB1 receptors it has been demonstrated that only β -Arrestin 1 is responsible for signal transduction, while β -Arrestin 2 serves rather for receptor internalization and desensitization (Laprairie et al., 2014; Noguera-Ortiz and Yudowski, 2016).

Recently, Smith et al (2018) proposed a model in which three main kinds of bias are taken into account. In this model, three specific situations can be observed in case a bias occurs in the signaling: the ligand bias, the receptor bias, and the system bias (Fig. 18). In the ligand bias model, the different agonist may alter the receptor transduction of the signal, allowing the recruitment of different signaling transducers (Fig 18b).

In the receptor bias model, the receptor may be altered through mutation or differential splicing, which may modify the affinity of the receptor to the signaling transducers. In this case, the

receptor may recruit a non-canonical signaling transducer (Fig. 18c). In the system bias model, augmented expression of one of the signaling transducers increments the probability of recruiting that transducer (Fig. 18d).

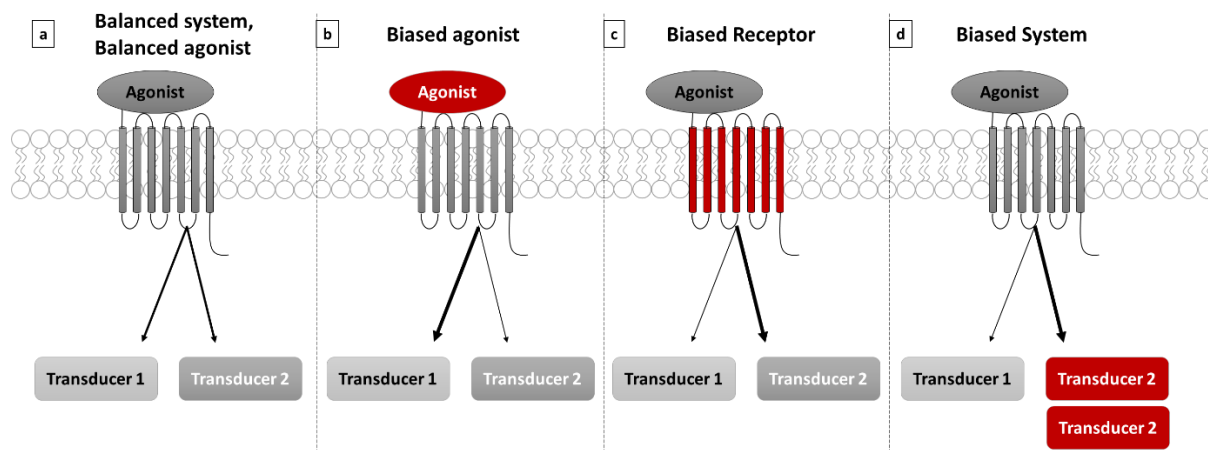


Figure 18: Three models of biased signaling. **a)** balanced system, balanced agonist: nor the system, nor the receptor, nor the agonist present any bias. In this case, the response to the two transducers is balanced. **b)** biased agonism: the receptor binds the two signaling transducers, G protein, and β arrestin, and the ligand induces conformational changes that “force” the receptor to activate one transducer more frequently than the other. **c)** Receptor bias: in this case, the bias is not attributable to the agonist, balanced, but to the receptor. A mutation of the receptor, or a differential splicing, can bring this kind of bias to the receptor, recruiting one transducer over the other when activated. **d)** System bias or apparent bias: in this case, both the ligand and the receptor are balanced. However, there is more availability of one transducer over the other. For this reason, at the moment in which the signal is sustained, the most abundant transducer is the most recruited transducer.

IV.3 Functional selectivity in CB1 receptor

It is known that CB1 receptors have been coupled to a large number of different signaling pathways, a fact that increases the potential of ligand/system selective pathway activation through different signaling transducers.

Glass and Northup (1999) noticed for the first time that CB1 receptors can bind with different kinds of $G_{i/o}$ subunits, and that different agonists showed different affinities for one subunit over the others. For instance, it was demonstrated that synthetic cannabinoid HU-210 produced maximal activation of both G_i and G_o , while the THC phytocannabinoid showed submaximal activation of G_i and G_o . Furthermore, synthetic cannabinoid WIN 55.212-2 has been proven to cause high activation for the three subtypes of $G\alpha_i$ proteins ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$), while the

tricyclic cannabinoid desacetyllevonantradol activated $G\alpha_1$ and $G\alpha_2$ but not $G\alpha_3$. The MAPK pathway may be implicated in CB1 intracellular signaling through $G_{i/o}$ proteins. Studies suggest that a treatment with a CB1 agonist (*e.g.*, THC and methanandamide) induces an increase in ERK1/2 phosphorylation. This effect has been shown to be blocked by pertussis toxin, a toxin that blocks $G_{i/o}$ protein in primary cultures of E7 telencephalic neurons, HEK cells, and in CD1 mouse striatal cells (Asimaki and Mangoura, 2011; Daigle et al., 2008a; Valjent et al., 2001). Other G proteins than $G_{i/o}$ have also been proven to be associated with CB1 receptors.

In Chinese hamster ovary (CHO) cells transfected with CB1 receptors and pre-treated with pertussis toxin, CP 55,940 (a synthetic cannabinoid that mimics the agonism effect of THC) treatment caused an accumulation of cAMP in the cell. This process was blocked by SR 141716A (Rimonabant, a CB1 antagonist) (Abadji et al., 1999; Calandra et al., 1999), demonstrating that it was indeed a CB1-dependent process, and suggesting G_s involvement in the CB1 intracellular signaling pathway.

Furthermore, it has been demonstrated that WIN 55,212-2 causes intracellular Ca^{2+} release via the $G\alpha_q$ protein of the CB1 receptor in HEK cells (Lauckner et al., 2005). Other cannabinoids used in the same conditions, however, did not show such $G\alpha_q$ activation (Lauckner et al., 2005). Moreover, HEK cells transfected with CB1 responded to WIN55,212-2 with an increased intracellular concentration of Ca^{2+} . This process has been proven to be G_q protein dependent (Lauckner et al., 2005). G_q protein stimulates the activity of phospholipase C β (PLC β) that converts phosphatidylinositol (3,4,5)-trisphosphate (PIP $_3$) into inositol (3,4,5)-trisphosphate (IP $_3$) and diacylglycerol (DAG). IP $_3$ binds to the IP $_3$ -sensitive Ca^{++} channel, located on the surface of the endoplasmic reticulum, allowing its opening, and thus the increase of Ca^{++} in the cytosol, while DAG recruits the protein kinase C (PKC) from the cytosol, facilitating its migration to the membrane (Berridge, 1986). CB1 seems to activate different signaling transducers in a time-dependent manner. More particularly, CB1 activation seems to follow

three distinct waves: G protein, followed by β -Arrestin, followed by a third activation of G protein or β -Arrestin (Nogueras-Ortiz and Yudowski, 2016). In the work of Laprairie and colleagues (2014), it was clear that there is a strong signaling bias due to different agonists. For instance, THC tended to activate the β -Arrestin pathway more effectively than endocannabinoids, while endocannabinoids activated G_i and G_q proteins more effectively than THC. The synthetic cannabinoid CP55,940 was the only cannabinoid tested to be able to activate G_s .

Allosteric modulators of the CB1 receptors may cause a modification on the three-dimensional structure of the receptor by not binding to the orthosteric site. For instance, the CB1-positive allosteric modulator ORG27569 has been suggested to be able to cause a bias in the signaling by activating the Erk1/2 pathway through β -Arrestin 1, demonstrating that β -Arrestin proteins can act as signaling transducer for the CB1 receptor (Ahn et al., 2013; Khajehali et al., 2015; Nogueras-Ortiz and Yudowski, 2016). In Ahn's work (2013), it was shown that the role of β -Arrestin 1 was to carry out the MAPK signaling pathway, while β -Arrestin 2 promoted receptor internalization. It has also been discovered that β -Arrestin can play a central role in PI3K-Akt signaling as well (DeWire et al., 2007).

Biochemical analyses point to the fact that CB1, as well as other GPCRs, can signal in three distinct waves (Lohse and Calebiro, 2013): the first wave consists of G protein activation, the second wave consists of β -Arrestin 1 activation, with a second signaling starting from CB1 and propagating from the cell surface (Flores-Otero et al., 2014), and then the third wave emerges from microsomal CB1 receptors (Rozenfeld and Devi, 2008).

IV.4 CB1 signaling pathways

Under different conditions, CB1 activation has been demonstrated to be able to cause the activation of the signaling pathway: MAPK (of which most commonly Erk1/2^{MAPK}) (Asimaki and Mangoura, 2011; Bosier et al., 2008; Rubino et al., 2005; Rueda et al., 2000; R.-W. Wu et al., 2011), Akt-GSK3 β (Ozaita et al., 2007; Trazzi et al., 2018; R.-W. Wu et al., 2011), and Akt-mTORC1 (Raptor) (Blázquez et al., 2015; Puighermanal et al., 2009). For the purposes of this work we took into account the aforementioned signaling, as summarized in Fig.19.

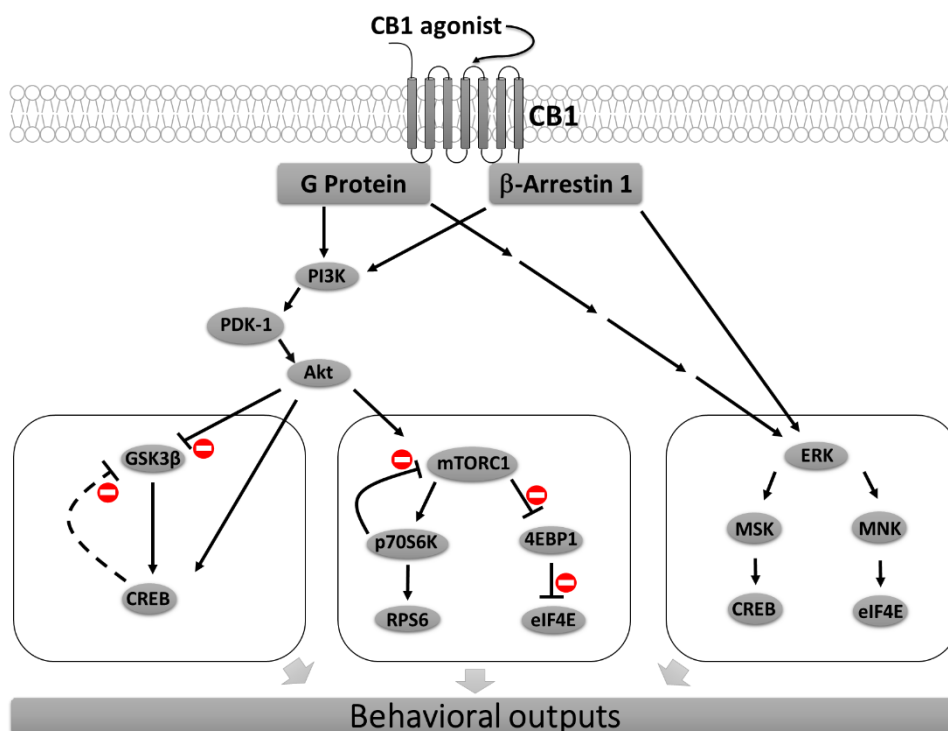


Figure 19: Schematic representation of CB₁-dependent intracellular signaling pathways. In this work it has been taken into account the contribution of the G-proteins and β -Arrestins for further analyses. The signaling pathways analysed are the Akt/GSK3 β ; the Akt/mTORC2; and the Erk1/2^{MAPK} pathways.

IV.4.1 Mitogen-Activated Protein Kinase signaling pathway at CB1 receptor

Mitogen-Activated Protein Kinase (MAPK) pathways regulate cell proliferation, growth, movement, differentiation, and death. The MAPK cascade is organized hierarchically: MAPKs are activated by MAPK kinases (MAPKK) that in turn are activated by MAPKK kinases (MAPKKK), that are phosphorylated and activated by monomeric G-protein or other kinases.

These pathways lead to activation of the MAPK, that are extracellular signal-regulated kinases (Erk) 1/2 and 5, c-Jun N-terminal kinase (JNK), and p38 MAPK. (Fig. 20).

One of the first CB1 signaling pathways to have been identified is the MAPK cascade through the $G_{i/o}$ protein. In CHO cells transfected with human CB1 (*hCB1*) receptor, after treatment with WIN 55,212-2, the levels of p44/42 (Erk1/2) MAPK increased markedly. Moreover, pre-treatment with pertussis toxin or rimonabant blocked the increased phosphorylation of MAPKs, demonstrating that it was indeed both CB1 and G_i dependent (Bouaboula et al., 1995).

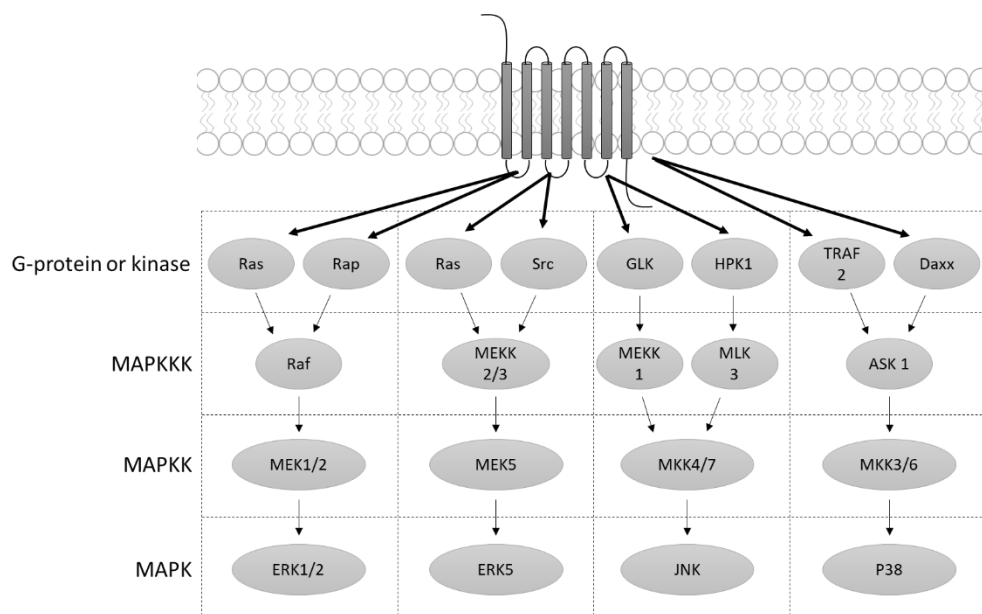


Figure 20: Hierarchical organization of mitogen activated protein kinases: the receptor activates a monomeric G protein or other kinases that in turn phosphorylate and activate the MAPKKK. The MAPKKK activates the MAPKK, that, in turn activates the MAPK.

Furthermore, in mice treated with SL327, an MEK inhibitor, several THC-induced behavioral effects were rescued (e.g. development of tolerance to THC and hypolocomotion), demonstrating the relevance of this target among the CB1 signaling pathways (Rubino et al., 2005). Derkinderen et al. (2003) demonstrated that AEA, THC, and WIN 55,212-2 were able to make Erk 1/2 phosphorylation soar in the hippocampus and that CB1 KO mice showed no cannabinoid-induced increase of phosphorylated Erk1/2. However, in CHO cells transfected with CB1 receptor, the PI3K inhibitor wortmannin did not decrease Erk1/2 phosphorylation, showing that the MAPK pathway did not pass through PI3K (Derkinderen et al., 2003). Rueda

and colleagues (2000) demonstrated that on CHO cells transfected with rat CB1 (rCB1) receptors, THC, AEA, 2-AG, and the synthetic cannabinoids CP-55,940, HU-210, and methanandamide, sharply increased phosphorylation of JNK1/2. In all cases, rimonabant, pertussis toxin, the PI3K inhibitor wortmannin, and a Ras farnesyltransferase inhibitor peptide blocked the effect of JNK1/2 hyperphosphorylation (Rueda et al., 2000). Lastly, p38 MAPK activation has also been observed following THC administration in rat and mouse hippocampus. Rimonabant blocked the p38 MAPK signal and THC was ineffective in CB1 KO mice (Derkinderen et al., 2001).

Pathophysiologic role of the Erk1/2 MAPK pathway: MAPKs are very well-conserved evolutionary proteins that allow the transduction of extracellular signals into intracellular responses (Lewis et al., 1998), and are involved in a plethora of different cell functions. One of the first roles of Erk1/2 MAPK to be discovered was cell proliferation control, because of the rapid Erk1/2 phosphorylation in response to mitogenic stimulation (Nakamura et al., 1983). Later, Kahan et al. (1992) discovered that in hamster fibroblast, the activation of Erk1 (p44 MAPK) followed two waves of activation after a mitogenic stimulus: a first phase of intense and transient (5-10 min) kinase activity, and a second phase of lower but sustained kinase activity, that lasted during the G1 phase. It has been proposed that the two isoforms of Erk could compensate for each other's loss, if necessary, making their role seem redundant (Frémin et al., 2015). However, even when Erk1 KO mice were viable, fertile, and normal-sized, it was evident that those mice had strongly impaired thymocyte maturation (Pagès et al., 1999). Further studies demonstrated the importance of MAPK unequivocally, and in particular of Erk1/2, when the stimulation of protein synthesis through tyrosine kinase receptor was blocked by blocking the MAPK cascade with MEK1/2 inhibitors (Servant et al., 1996). Further studies demonstrated the involvement of Erk1/2 in cardiac hypertrophy (Bueno et al., 2000),

spermatogenesis (Suzuki et al., 2014), angiogenesis (Shin et al., 2016), cancer proliferation (Kohno and Pouyssegur, 2006), apoptosis (Cagnol and Chambard, 2010) and several others.

In the CNS, MAPKs are of central importance in several key physiological and pathological roles. In the CNS, the MAPK pathways aid the differentiation of neuronal cells, and are negatively controlled by proteins such as Spred1, that inhibits the MAPK pathway and avoids premature cell differentiation, maintaining the cell structure and architecture. Without such control, the unregulated MAPK pathway would bring premature cell differentiation and, for example, abnormal cortical development and proliferation (Phoenix and Temple, 2010). Erk1/2 proved to be essential in long-term potentiation (LTP) and long-term depression (LTD) (Impey et al., 1999). English and Sweatt (1997) demonstrated that when rat hippocampal slices were treated with an MEK inhibitor, LTP could not be achieved, demonstrating the necessity of Erk2 (p42 MAPK) for hippocampal LTP induction. Thiels et al. (2002) demonstrated that Erk signaling and the consequent activation of the transcription activator Elk-1 were necessary for LTP to happen. Notably, similarly to LTP, the Erk isoform needed to gain an LTD in the CA1 was Erk2. MAPK-Erk signaling is also involved in learning and memory. Inhibiting MEK in rat hippocampus, the animals showed impaired long-term memory in the object recognition test (Kelly et al., 2003). As this is the MAPK signaling involved in neuronal plasticity, it seemed most likely to be involved in drug addiction, which is considered a neuroadaptive disorder. Valjent et al. (2000) demonstrated that after cocaine administration, the Erk1/2 pathway in the striatum was activated strongly, and that by blocking the Erk pathway, it was possible to recover normal locomotion.

Moreover, when the animals were pre-treated with MEK inhibitors, the rewarding effects of cocaine were abolished, demonstrating the central relevance of Erk1/2 in drug addiction (Valjent et al., 2000). The blockade of MAPK signaling was also able to block the addictive effects of MDMA and amphetamine (Pascoli et al., 2014), and alcohol (Agoglia et al., 2015).

Altered MAPK pathways have also been found in several mental pathologies, such as Parkinson's disease (Bohush et al., 2018), Alzheimer's disease (Kirouac et al., 2017), and schizophrenia (Funk et al., 2012; Kyosseva et al., 1999) among the others.

IV.4.2 Akt-mTORC1 signaling pathway

The mammalian Target of Rapamycin (mTOR) is a highly-conserved signaling pathway across the species, which serves as a central regulator of growth and metabolism in all eukarya (Wullschlegel et al., 2006). mTOR can form two complexes: mTORC1 (Raptor) and mTORC2 (Rictor), which are structurally and functionally different from each other. Different nutrients, growth factors, stressors (Sengupta et al., 2010), or Akt signaling pathways (Dan et al., 2014; Haar et al., 2007) can activate mTORC1. Interestingly enough, the system that underlies mTOR phosphorylation is not yet fully understood. Seven phosphorylation sites have been identified on mTOR proteins: Ser1264, that can be found in both mTORC1 and 2 and increase mTORC1 activity when phosphorylated (Acosta-Jaquez et al., 2009); Ser1415, which is specific to mTORC1 and increases its activity, is an IKK α target following Akt activation (Dan et al., 2014); Ser2159 and Thr2164, that increase mTORC1 activation (Ekim et al., 2011); while Thr2446, Ser2448, and Ser2481 are considered FIT (Found In TOR) domains. Originally, it was thought that the Ser2448 and Ser 2481 epitopes were specific to mTORC1 and 2 respectively (Copp et al., 2009), but recent studies have found that the Ser2481 epitope was phosphorylated in mTORC1 (Soliman et al., 2010), and that even if it seemed to have no effect on its kinase activity, the Ser2448 epitope was phosphorylated in mTORC2 (Rosner et al., 2010). The Ser2481 epitope has been identified as an mTOR autophosphorylation residue (Acosta-Jaquez et al., 2009; Soliman et al., 2010). Initially, it was proposed that mTOR was activated by Akt through phosphorylation of Ser2448 (Hay and Sonenberg, 2004), within the PI3K-Akt-mTORC1 pathway. However, although the pathway has been shown to be correct, mTORC1 activation has been demonstrated to be rather more complex. Akt can be activated by

PDK-1 (Dangelmaier et al., 2014), which can be phosphorylated and activated directly by PI3K, by mTORC2 (Dan et al., 2016; Foster and Fingar, 2010; Hagiwara et al., 2012; Oh and Jacinto, 2011), or by PIP₃, a PI3K metabolic product (Hemmings and Restuccia, 2012), while PKD-1 can inactivate PI3K and thus the Akt signal (Lee et al., 2011; Ni et al., 2013). Akt, once activated, can phosphorylate and inhibit two proteins of the mTOR complex 1, named PRAS40 and TSC1/2. PRAS40 is a constitutively-active inhibitor of mTOR, while TSC1/2 is a constitutively active protein that, by stimulating the hydrolysis of GDP, regulates Rheb, a protein of the mTOR complex 1 that inhibits mTOR activity as well. Finally, Deptor, another protein of the mTOR complex 1, separates from the cluster, to be degraded. Inhibiting the inhibitor, Akt activates mTORC1 (Haar et al., 2007) (Fig. 21). *In vitro* experiments done in islets (from adult CB1 KO and WT mice), have shown that rimonabant-treated cells have a substantial increase in p70S6K and RPS6. Pre-incubation with rapamycin blocked this effect, proving the involvement of mTORC1 (Bermudez-Silva et al., 2016) (Fig. 21). Several studies have shown how mTORC1 can be activated following CB1 activation *in vivo* as well. Díaz-Alonso et al. (2015) demonstrated that the development of the cortex in mice is due to mTOR activation via CB1, while genetic inactivation of the *Cnr1* gene reduced the phosphorylation of RPS6 significantly. In another work, Puighemanal et al. (2009) showed that the amnesic effects of THC in mouse hippocampus depend on the mTORC1-p70S6K-RPS6 signaling pathway. However, they demonstrated that even though the mTORC1 cascade in this context depends on THC, and therefore on CB1 activation, mTORC1 protein phosphorylation and CB1 activation did not colocalize in the same cells. It was proposed that CB1, located on GABAergic neurons, blocked GABA release, and the increased mTORC1 phosphorylation resulting from NMDAR activation, in other neurons. However, pre-treatment with rimonabant or the administration of THC in CB1 KO mice did not show any THC-induced effect on mTOR signaling, demonstrating that mTOR activation was CB1-dependent, albeit with an indirect pathway.

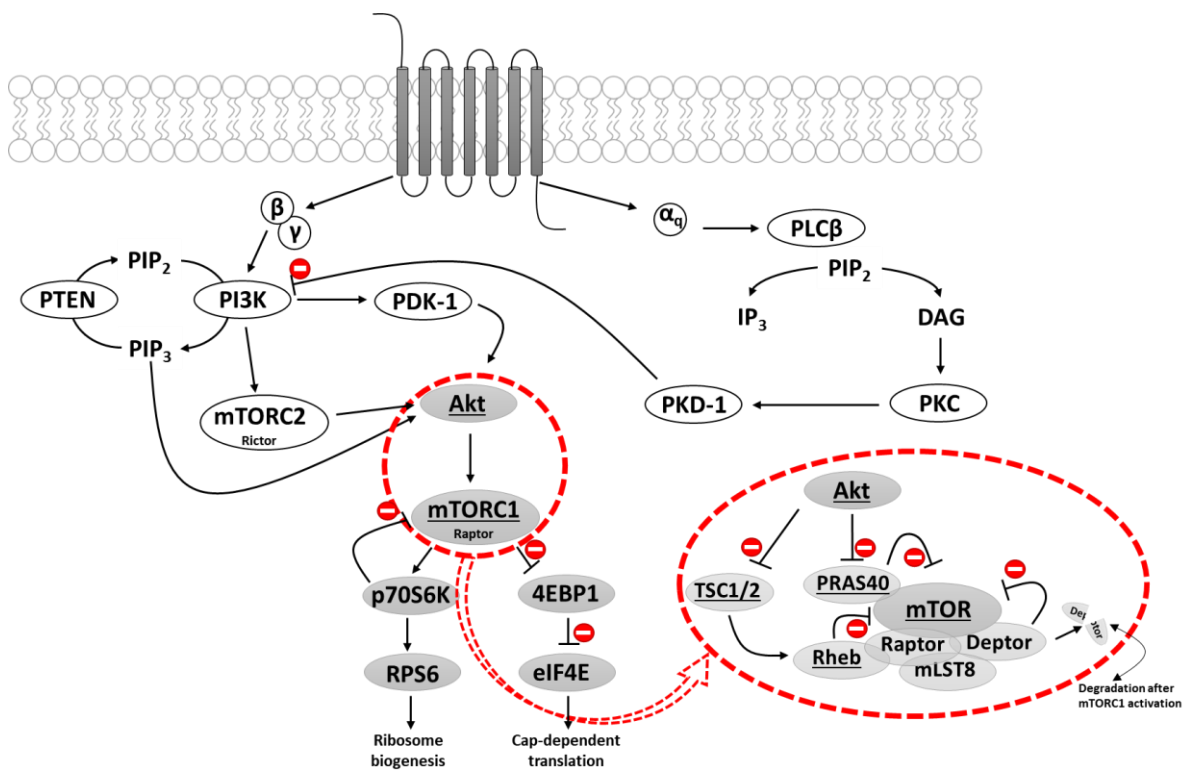


Figure 21: Akt-mTORC1 intracellular signaling pathway. Receptors can indirectly activate Akt through PI3K (through the PI3K product PIP₃, or the targets PDK-1 and mTORC2), while can indirectly inactivate Akt through G_q protein, that, activating the PLC β , that augment the production of DAG, that in turn activate PKC, which activates PKD-1 that inhibits PI3K blocking the Akt signaling. Once activated, Akt can phosphorylate and inhibit PRAS40, a constitutively active protein that binds and inhibits mTOR. Once that PRAS40 is phosphorylated, and thus inactive, deptor, another protein of the mTORC1, is inhibited and leaves the complex to be degraded. mTORC1, active, can phosphorylate and activate p70S6K, which phosphorylates and activates RPS6, promoting the ribosome biogenesis. Another target of mTORC1 is 4EBP1, a constitutively active protein that binds eIF4E. Once 4EBP1 is phosphorylated and inactivated, it leaves the complex with eIF4E, free to be phosphorylated and activated, and promotes the cap-dependent translation.

Pathophysiologic role of Akt-mTORC1 pathway: the discovery of the mammalian target of rapamycin started with the discovery of rapamycin itself, discovered in the Easter Islands (Rapa Nui in native language). In 1975, Sehgal identified rapamycin for the first time, a molecule produced from soil bacteria that seemed to be useful as an antifungal drug (Sehgal et al., 1975; Vézina et al., 1975). Later, rapamycin was discovered to be an immunosuppressant in humans, and the FDA accepted its candidacy as a drug for this function. However, despite its evident pharmacological activity, the mechanism of action remained unknown at the time. It was in the early '90s that mTOR, the mammalian target of rapamycin, was identified for the first time (Heitman et al., 1991). The mechanism of action of rapamycin has been discovered

to block cell activity in the G1 phase of the cell cycle in fungi, while in humans it blocks the transition from the G1 to the S phase in T-lymphocytes (Magnuson et al., 2012).

Nowadays, it is established that mTORC1 can regulate cell growth and proliferation by regulating protein synthesis (Wang and Proud, 2006). mTORC1 can recruit protein from the eukaryotic initiation factors (eIFs), in particular eIF4E that mediates the translation initiation (*i.e.*, recruitment of the mRNA to a small ribosome subunit) (Borden, 2016). mTORC1 activates eIF4E by phosphorylating and inhibiting the eukaryotic initiation factor 4E-binding protein (4EBP1), that is constitutively active and binds the eIF4E, inactivating it (Jossé et al., 2016; Müller et al., 2013). Another mTOR downstream pathway that regulates protein synthesis is RPS6: mTORC1 can phosphorylate and activate p70S6K and activate RPS6 consequentially. RPS6, when phosphorylated, can bind to the 40S ribosomal subunit. The binding site of RPS6 is close to the mRNA/tRNA binding site of the 40S ribosomal subunit, which underlines the role of RPS6 in selecting the mRNA to be transferred (Yoshizawa, 2004). Since the higher the levels of RPS6 phosphorylation, the greater the efficiency of translation of mRNA with a 5'-terminal oligopyrimidine tract (TOP mRNA), it is believed that the RPS6 mechanism of action increases the affinity of ribosomes to the TOP mRNA (Ruvinsky and Meyuhas, 2006).

In addition, it is known that one of the roles of mTORC1 in the CNS is to regulate energy balance and food intake (Catania et al., 2011, Weijenberg et al., 2013), glucose and lipid metabolism (Mao and Zhang, 2018). mTORC1 dysfunction plays a central role in food intake (Morton et al., 2006), and its contextual overactivation in the periphery and hypoactivity in the CNS have been observed in obese individuals (for a review see Catania et al., 2011). mTORC1 can receive stimuli based on internal cell signalization, nutrient factors, and growth factors. It has already been shown that Akt signaling can activate mTORC1. AMP-activated protein kinase (AMPK) is a protein kinase that is sensitive to the cell energy balance. It is sensitive to the levels of ATP, and when the ATP/AMP ratio decreases, AMPK activates in response and

promotes catabolic pathways to generate ATP (Mihaylova and Shaw, 2011). Under nutrient starvation conditions, AMPK regulates mTORC1 by phosphorylating Raptor and thus blocking mTORC1 itself, preventing cell growth and preserving the energy of the cell (Jewell and Guan, 2013). mTORC1 can be regulated by growth factors, such as the insulin and insulin-like growth factor1 receptor signaling that promote the activation of a cascade that ends with Akt phosphorylation, and so mTORC1 activation (Bond, 2016). Furthermore, a recent work demonstrates that mTORC1 regulates the oxidative metabolism in the POMC neurons, and that this process regulates food intake in C57BL6J mice (Haissaguerre et al., 2018; Mazier et al., 2019).

Given its role in cell growth and proliferation, mTOR has been studied in depth in cancer and mTORC1 was found to be hyperactivated in tumor cells (Guertin and Sabatini, 2005; Hay, 2005; Zoncu et al., 2011). Furthermore, a link has been demonstrated between mTORC1 activity and diabetes, with its inhibition increasing hyperglycemia drastically in patients treated with mTOR inhibitors (Vergès and Cariou, 2015; Zoncu et al., 2011).

More recently, mTORC1 dysregulation has been investigated in depth in neurosciences, as a possible cause of several pathologies. In Fragile X syndrome, among the several dysregulated intracellular signaling pathways, mTORC1 showed aberrant, hyperactivated signaling in the hippocampus (Sharma et al., 2010). mTOR has been demonstrated to play roles in several neurodegenerative diseases, such as Parkinson's disease (Malagelada et al., 2006), Huntington's disease (Pryor et al., 2014), and Alzheimer's disease (Pei and Hugon, 2008). It has also been demonstrated that mTOR plays a role in neuropsychiatric disorders: decreased mTORC1 activity has been suggested in depression, and its rescue may lead to anti-depressant effects (Abelaira et al., 2014). It has also been suggested that mTOR could play a role in schizophrenia. A mutation of the *Akt1* gene is associated with a higher probability of developing schizophrenia or psychosis (Thiselton et al., 2008). Akt is indeed a key target upstream from

mTORC1, and it has been demonstrated that depletion of p-Akt^{Ser473} activity led to a decreased prepulse inhibition (PPI) (Siuta et al., 2010), a psychotic-like symptom, in an animal model (Parwani et al., 2000). Some evidence suggests that in schizophrenia, mTOR signaling is severely disrupted (for a review see Gururajan and Buuse, 2014). Lastly, an mTORC1 signaling alteration has been correlated to cannabis and cocaine-induced behaviors. In particular, the THC-induced memory deficit observed at the novel object recognition test following acute treatment with THC has been demonstrated to enhance phosphorylation of mTOR via CB1 in the hippocampus of mice (Puighermanal et al., 2009). Cocaine administration activates mTORC1 in the NAc and VTA, and by blocking the mTORC1 pathway, is possible to block the cocaine-induced locomotor sensitization (Wu et al., 2011).

IV.4.3 Akt-GSK3 β signaling pathway

GSK3 β is a constitutively-active kinase. As GSK3 β is constitutively active, when phosphorylated on Ser9/21 by Akt it passes to its inactive state (Beurel et al., 2015). The PI3K-Akt-GSK3 β axis is a common pathway that leads to different effectors: GSK3 β is part of the Akt pathway and is known to regulate more than 100 different protein effectors and channels (Beurel et al., 2015). GSK3 β can correctly “choose” its downstream target by recognizing it through an epitope phosphorylated four amino acids before the epitope target (as in the example of CREB phosphorylation by GSK3 β , Doble, 2003). It is not the case that GSK3 β can lead to so many different, and sometimes opposite outcomes. In fact, most downstream targets of GSK3 β , in order to be recognized and phosphorylated by the latter, must already be "primed" with a pre-phosphorylated residue four-amino acids removed from the GSK3 phosphorylating site. An example may be CREB, which is phosphorylated by GSK3 β on Ser133, but to be recognized, it needs to be already phosphorylated on Ser129 (Fig. 22) (Jope and Roh, 2006).

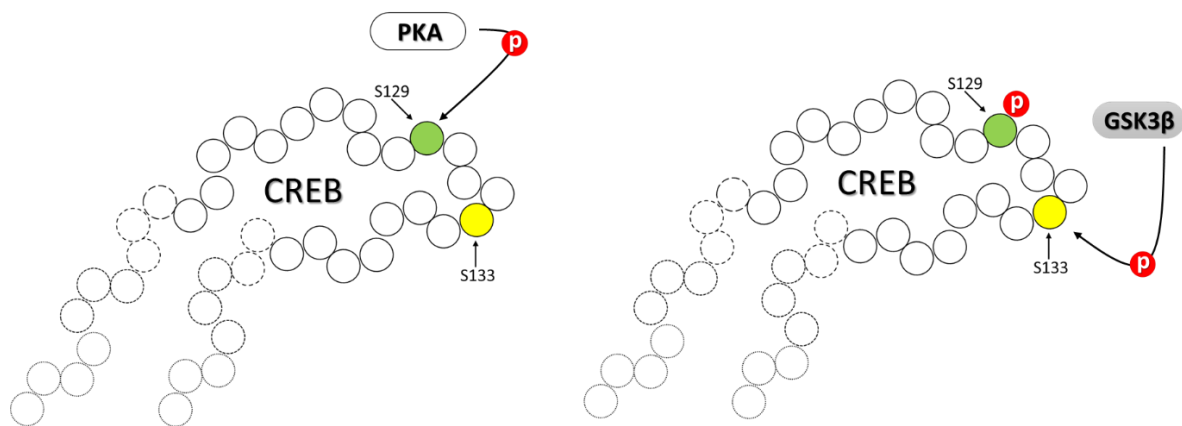


Figure 22: Example of GSK3 β mechanism of action: regulation of GSK3 β downstream protein CREB. CREB needs to be phosphorylated in position Serine 133 in order to be activated by GSK3 β . However, even imagining that GSK3 β would be unphosphorylated and thus activated, it would not recognize CREB. So, PKA needs to be active, which can recognize the n-4 epitope of CREB, where n is the epitope target for GSK3 β (Ser133), and so n-4 is, in this case, the position Serine 129. This phosphorylation does not affect CREB activity, but allow GSK3 β to recognize, phosphorylate, and thus active CREB.

Most of the 100 GSK3 β targets are regulated through double phosphorylation, meaning that GSK3 β activation may lead to entirely different cellular outcomes (Wu and Pan, 2010).

The involvement of GSK3 β in CB1 signaling is already known. Sanchez et al. (2003) demonstrated that in human prostate malignant epithelial PC-3 cells treated with THC, PI3K-Akt signaling was recruited in a CB1-dependent manner. Ozaita et al. (2007) showed that in CD-1 mice, high doses of THC administration caused hyperphosphorylation of Akt and GSK3 β in the hippocampus, cerebellum, striatum, and cortex and that rimonabant was able to block this effect. Furthermore, this THC-induced Akt-GSK3 β hyperphosphorylation in was shown to be PI3K dependent, and MAPK independent. Wang et al. (2009) showed that electroacupuncture in SD rats induced endocannabinoid release in the brain and protected from ischemic stroke. This cannabinoid-mediated protection seemed to be due to phosphorylation of the Akt-GSK3 β signaling, which might have played a neuroprotective role (Wei et al., 2014). Other studies have demonstrated that some of the downstream pathways to GSK3 β are involved in cannabinoid signaling. The Wnt/ β -Catenin pathway, for example, can be regulated by cannabinoids and cause anti-anxiety and anti-depressant effects (Nalli et al., 2019). Moreover,

both THC and CBD were able to decrease the levels of NFκBBV-2 in murine cells, a target that is phosphorylated and activated by GSK3β (Kozela et al., 2010). Melas et al. (2018) demonstrated that in adolescent rats, eIF2α was significantly decreased after WIN 55,212-2 administration. Furthermore, on PC12 cells treated with WIN 55,212-2, eIF2B was strongly upregulated as a result. Interestingly, eIF2α, and consequently eiF2B, are both GSK3β downstream targets (Melas et al., 2018). Moreover, it has been discovered that THC administration in both humans and mice cause a sharp decrease in STAT3 (STAT3 and STAT5 are the only STATs activated by GSK3β) (Chang et al., 2017). In human glioma parietal cells, it has been demonstrated that STAT3 was activated via CB1 and that rimonabant reversed this effect (Ciaglia et al., 2015).

Also, in rat hippocampal slices, treatment with AEA caused a substantial increase in CREB activity (Isokawa, 2009). GSK3β can phosphorylate and activate p-CREB, a transcription

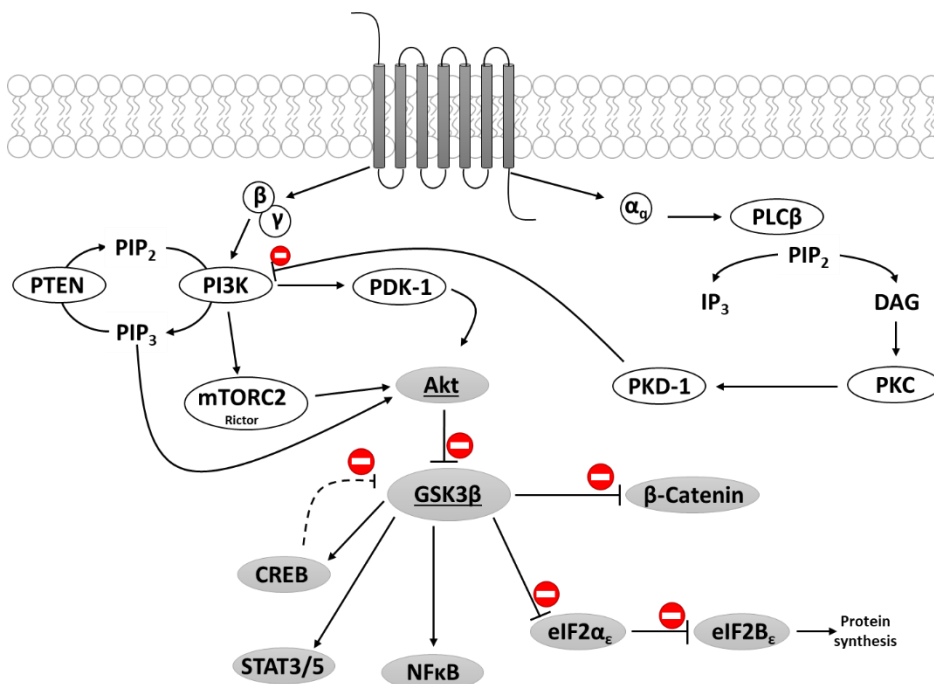


Figure 23: Akt-GSK3β intracellular signaling pathway. The receptor can indirectly activate or inactivate Akt through PDK1, mTORC2, and PIP3, and PKD-1, respectively. Once phosphorylated, and thus activated, Akt can phosphorylate GSK3β. GSK3β is constitutively active, and thus, once that Akt phosphorylates it, it switches to its inactive form. GSK3β, however, regulates many targets. Among them, STAT3/5, NFκB, and CREB are activated by phosphorylation (and so indirectly inactivated by Akt), while GSK3β inactivates other targets, such as eIF2α and β-Catenin through phosphorylation. Furthermore, when the CREB signal is too intense, and its phosphorylation rises sharply, through negative feedback, it is not completely clear if it phosphorylates or inhibits GSK3β.

factor, by phosphorylating its epitope Serine 133 (Ahn et al., 2005; Beurel et al., 2015), while hyperphosphorylation of CREB, through a pathway that has not been completely defined, can lead to GSK3 β phosphorylation, and thus inactivation (Beurel et al., 2015) (Fig. 23).

It must be underlined, however, that although β -catenin, eIF2 α , NF κ B, STAT3/5, and CREB are all downstream proteins of both the CB1 and GSK3 β pathways, it has not been demonstrated that CB1 activation of those targets is via GSK3 β .

The pathophysiologic role of the Akt-GSK3 β pathway: glycogen synthase kinase 3 β (GSK3 β) is a protein kinase that was initially described as a regulator actor in glycogen biosynthesis. The main actor that produces glycogen is glycogen synthase, a constitutively-active protein which exists in two different forms: un-phosphorylated, and phosphorylated. During the catabolic process of glycogen production, glycogen synthase is un-phosphorylated. During the anabolic processes, GSK3 β , which is un-phosphorylated and active, phosphorylates and inactivates the glycogen synthase, stopping the anabolic processes and facilitating catabolic ones (Cohen, 1986; Pelley, 2007).

GSK3 β , however, plays a part in a significant number of physiological functions other than glycogen synthesis. In stem cells, Wnt signaling, and also its negative regulator GSK3 β , are involved in maintenance of pluripotency. Inhibition of GSK3 β activity in stem cells enhances adhesion and transcriptional activity through β -catenin, and capability to bind E-cadherin, increasing cell adhesion capability (Sineva and Pospelov, 2010). Colosimo et al. (2010) discovered in *Drosophila* that a lack of GSK3 β led to increased adhesion, through the atypical protein kinase C (α PKC) that regulates and stabilizes the adhesion junction proteins.

GSK3 β is a very complex kinase. Its activation has already been found in opposite behaviors or biological effects. For example, GSK3 β was found to be both a tumor promoter and a tumor suppressor, depending on the type of tumor. In several tumors, such as the colon and liver tumors, overexpressed GSK3 β was detected. However, GSK3 β suppression of Wnt/ β -catenin has been demonstrated to have tumor-suppressor potentiality in breast cancer (McCubrey et al., 2014). Furthermore, the GSK3 β pathway has a crucial role in neuronal polarization. GSK3 β has a pivotal role in polarizing the cytoskeletal organization, in preparing and promoting the asymmetrical axon extinction, and branching (Kim et al., 2011).

GSK3 β is involved in neurodegenerative pathologies as well. There is a clear link between GSK3 β and Alzheimer's disease: GSK3 β can phosphorylate the tau protein, and thus contribute to one of the main factors that lead to Alzheimer's (Ishiguro et al., 1993).

Furthermore, in Alzheimer's patients, GSK3 β was colocalized with neurofibrillary tangles (Yamaguchi et al., 1996). GSK3 β can also regulate the amyloid precursor protein (APP), increasing production of amyloid β protein (Su et al., 2004). Since amyloid β protein can inhibit the activity of PI3K (Takashima et al., 1996), it establishes positive feedback in which the more the amyloid β protein is expressed, the less GSK3 β is phosphorylated and the more active it is, allowing even more massive production of amyloid β protein. GSK3 β dysregulation can also lead to Parkinson's-like behavioral and physiological symptoms. Credle et al. (2015) discovered that mice overexpressing *h*GSK3 β showed shrunk substantia nigra, accumulation of α -Synuclein, and tau protein. GSK3 β plays a role in hyperactive behavior. In attention-deficit/hyperactivity disorder (ADHD), and more generally in the dopamine-dependent hyperactive behaviors, non-canonical GPCR signaling has been discovered in which, in the striatum and nucleus accumbens, the dopamine receptor of type 2 signaling transducer is β -Arrestin 2 that allows Akt de-phosphorylation on threonine 308 through the phosphatase 2A (PP2A) protein, thus preventing GSK3 β phosphorylation causing its activation (Beaulieu et al., 2005, 2004) (Fig. 24). However, Yen et al. (2015) demonstrated that in amphetamine-treated mice, GSK3 β was hyperphosphorylated in the prefrontal cortex and was thus inhibited. Although it has been demonstrated that inhibition of GSK3 β rescues the hyperactive and hyperlocomotor behaviors (Enman and Unterwald, 2012), it has been discovered that the D1 receptor, through the G $\beta\gamma$ subunits, activates PI3K, which phosphorylates Akt, which in turn phosphorylates and inactivates GSK3 β (Beaulieu et al., 2011). However, Ozaita and colleagues (2007), showed that the THC-dependent increase in GSK3 β phosphorylation in mice was not

affected by D1 or D2 inhibitors, meaning that THC most likely did not act on dopaminergic signaling under those conditions.

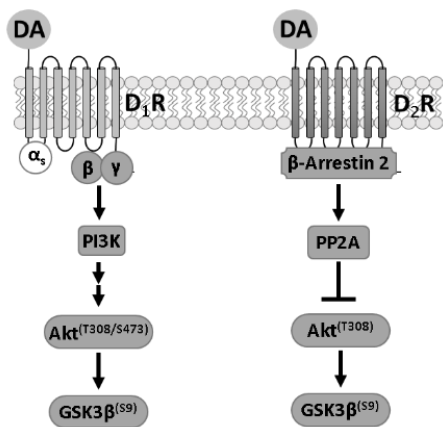


Figure 24: Schematic representation of the dopaminergic phosphorylation of GSK3β. Dopamine binds to the dopamine receptor type 1 (D1) or type 2 (D2). The D1 receptor, through the βγ subunits, activates PI3K, which in turn phosphorylates and activates Akt, which phosphorylates GSK3β, resulting in its inactivation. Receptor D2 recruits and activates β-Arrestin 2 as a signaling transducer, activating phosphatase 2A (PP2A). PP2A then de-phosphorylates Akt on the threonine epitope 308. Usually, Akt is a regulator of GSK3β and inhibits its activity by phosphorylating it. Ultimately, since PP2A inactivates Akt, and GSK3β is therefore constitutively active, this pathway results in the activation of GSK3β.

GSK3β is also a suitable target in bipolar disorder: lithium, one of the most widely-used mood stabilizers (Chiu et al., 2013), blocks GSK3β activity, which has been demonstrated to play an essential role in bipolar disorder (Gould and Manji, 2005). Furthermore, a mutation in the *GSK3B* gene (the gene encoding for hGSK3β protein) may explain the non-responsiveness to lithium of some patients (Iwahashi et al., 2014). Another relevant pathological role of GSK3β and its pathway is in schizophrenia. It has already been shown how Akt1 can be deeply involved in schizophrenia and psychotic disorders (Thiselton et al., 2008), and it has been shown that mTORC1, a downstream target of Akt, may play a role in it (for a review see Gururajan and Buuse, 2014). However, GSK3β is an Akt downstream target, and there is some strong evidence of its involvement in psychosis and schizophrenia. Emamian et al. (2004) demonstrated that the *AKT1* gene could be a potential schizophrenia susceptibility gene. p-Akt1^{T308} and p-GSK3β^{Ser9} have been found to be severely diminished in schizophrenic patients, and haloperidol, a typical antipsychotic medical drug, both rescued the behavioral symptoms and normalized the phosphorylation of Akt1 and GSK3β (Emamian et al., 2004).

GSK3β plays a central role in addiction, too: for example, in CD-1 mice, cocaine administration has been proven to decrease Akt^{Thr308} and GSK3β^{Ser9} phosphorylation by almost 90% but

showed no effect on Akt^{Ser473} phosphorylation in the caudate putamen, striatum and nucleus accumbens, which is consistent with D₂ signaling. This effect was entirely blocked by pre-treatment with D₂R antagonists, and pre-treatment with a GSK3β antagonist prevented the development of cocaine-induced place preference (Miller et al., 2014). In C57BL/6J mice, it has been demonstrated that ethanol regulates GSK3β in the prefrontal cortex.

IV.5 Modulation of CB1: orthosteric ligands and allosteric modulators

All receptors, metabotropic or ionotropic, are the "gates" that allow information to pass from the extracellular matrix to the cytosol. To this end, information is sent to the cell surface in the form of molecules, named ligands that show affinity towards intramembranary proteic structures which allow the information to enter the cell through metabolic or electric processes. Those ligands usually bind the receptor onto a specific portion named the orthosteric site. The orthosteric (from Greek *ortho* – correct and *steric* – space) site is the natural binding site of their ligands. Agonists are ligands that have an affinity towards the orthosteric site, and they can activate the receptor when they bind onto it. When a receptor shows maximal activity after a ligand binds to it, the latter is named a full agonist; if the receptor shows an intermediate level of activity, the ligand is a partial agonist, while when a receptor shows no activity after the ligand binds to it, the latter takes the name of antagonist. In any case, all the orthosteric agonists will always trigger the same response from the receptor, and so the same signal transduction (Nussinov and Tsai, 2012). In many cases, there are several systems to regulate the activity of a receptor (see the schematic overview in Fig. 25).

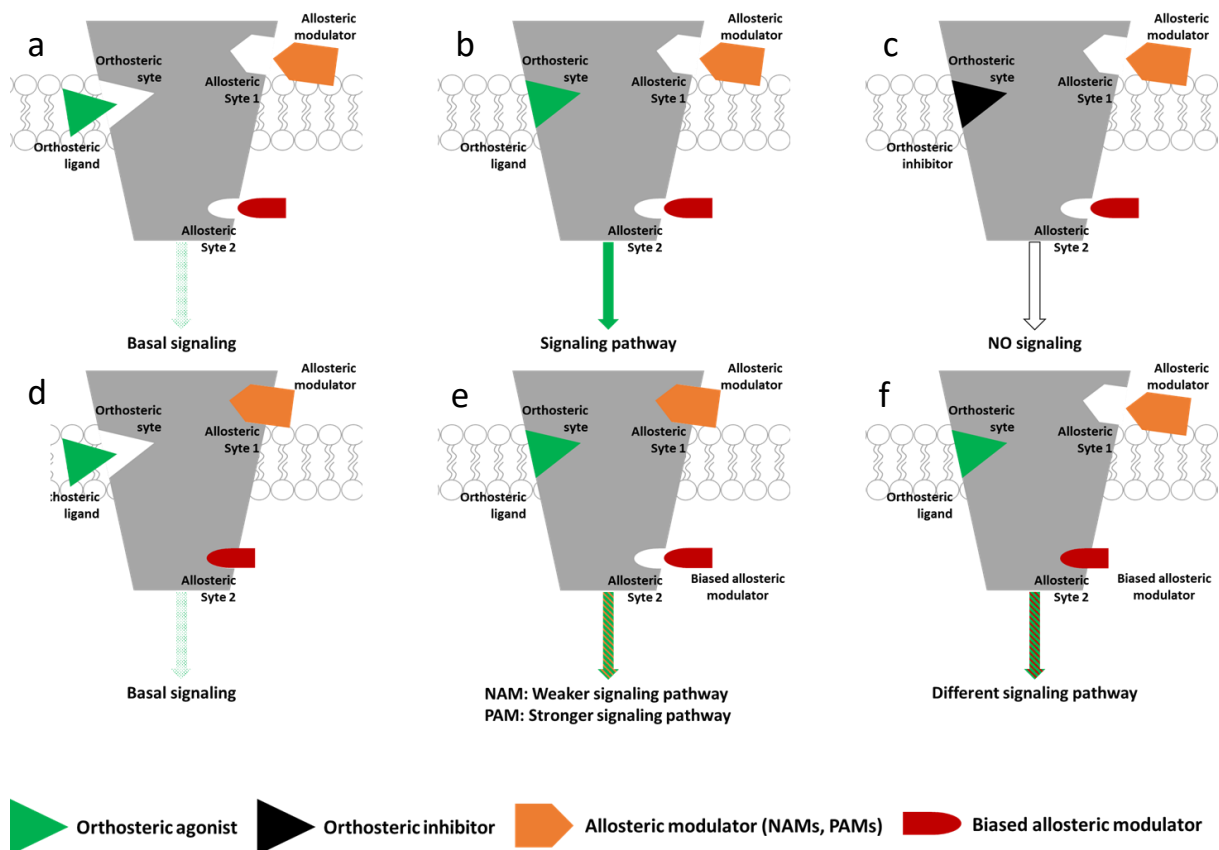


Figure 25: Orthosteric agonism and allosteric (biased) modulation:

a) On one receptor, several pockets can be bound by different types of ligands. The orthosteric pocket is the binding site, usually located in the receptor's extracellular part, for endogenous (and if present, exogenous) ligands. Then, there are the allosteric pockets that are bound by allosteric modulators: these sites can be located in any part of the receptor (extracellular, intracellular, and intramembrane). Usually, even in the absence of its agonist, a receptor, or in the presence of a small amount of agonist, has a slight activity, called the basal signal.

b) When the agonist binds to the orthosteric site, the receptor responds by activating one or more signal transducers, to activate the "canonical" transduction.

c) When an antagonist binds to the orthosteric site, no signal can occur, not even the basal signal

d) When allosteric modulators and biased allosteric modulators bind to the allosteric site, no change is observed, and the receptor continues to produce the basal signal.

e) However, if the agonist binds to the orthosteric site, an allosteric modulator binds to the allosteric site, the response of the receptor is modulated. If the allosteric modulator is a positive allosteric modulator (PAM), the receptor increases the "canonical" transduction response, whereas if the modulator is a negative allosteric modulator (NAM), the receptor reduces the transducer recruitment. If under normal conditions, the receptor recruits more than one signaling transducer, NAMs, and PAMs increase or decrease all signaling transducers' recruitment equally.

f) A biased allosteric modulator binds another allosteric pocket. Suppose this occurs when the agonist binds to the orthosteric site. In that case, the receptor will modify its response by recruiting other signaling transducers, or it may affect the different transducers in different ways (e.g., by decreasing the recruitment of one transducer and increasing the recruitment of the other).

The allosteric modulators are ligands that bind to an allosteric site (from Greek *allos* – other and *steric* – space), which is different from the orthosteric site in that the agonist does not bind to it. When the allosteric modulator binds to the allosteric site, it increases (positive allosteric modulators, PAM) or diminishes (negative allosteric modulators, NAM) the affinity of the orthosteric agonist to the orthosteric site (Bertrand and Gopalakrishnan, 2007; Milligan and Smith, 2007). Another way of modulating the response of the receptor is through the biased allosteric modulator.

While the allosteric modulator modifies the affinity of the receptor to its natural ligand, and so modifies the intensity of the transmission, the biased allosteric modulator modifies the affinity of the receptor to its signaling transducers, modifying not only the intensity of the response, but also the quality of intracellular signaling (Trinh et al., 2018). Developing ligands that target allosteric sites in the receptor may indeed be useful in the development of new drugs. While the orthosteric ligands are relatively aspecific (one single ligand can usually bind to the whole subfamily of the same receptor), allosteric modulation is usually specific to a single sub-type (Dopart et al., 2018).

Furthermore, allosteric modulation usually has a wider therapeutic window and follows the physiology of the system better, since it can only affect receptor activity in the presence of the orthosteric ligand. For example, up until the '60s, the anxiolytic drugs of choice were barbiturates, which are GABA_A agonists that act at low doses as positive allosteric modulators of the same receptor (Löscher and Rogawski, 2012). In 1963, however, Hoffmann-La Roche marketed a benzodiazepine, diazepam, marketed as *Valium*, and from that moment on, barbiturates were replaced by benzodiazepine. Benzodiazepines also act on the GABA_A receptor but are only positive allosteric modulators of said receptor. While an over-dose of barbiturates often proved fatal, benzodiazepine presented a much wider therapeutic window, and consequently, much lower side effects (Olsen and DeLorey, 1999). This happened because,

while barbiturates alone could activate the GABA_A receptor, benzodiazepine could act only in the presence of the endogenous ligand GABA. CB1 receptors have several allosteric modulators, too: RVD-Haemopressin (pepcan 12) and cannabidiol (CBD) have been discovered to be respectively endogenous and exogenous negative allosteric modulators of the CB1 receptors (Bauer et al., 2012; Laprairie et al., 2015). Pepcan 12 is a peptide endocannabinoid able to bind both to the CB1 and the CB2 receptor (Petrucci et al., 2017). In particular, pepcan 12, an endogenous oligopeptide composed of twelve amino acids discovered in mouse brain and plasma, lowered the affinity between the synthetic CB1 agonist CP55,940 and the CB1 receptor in CHO-K1 and HEK293 transfected with *hCB1* (Bauer et al., 2012), while CBD was able to reduce CB1 activity after THC and 2-AG treatment in *STHdh^{Q7/Q7}* and CHO transfected with *hCB1* cells (Laprairie et al., 2015). The synthetic compounds ORG27569, ORG29647, ORG27759, are three indoles developed by Organon Research, of which ORG27569 is considered the prototypical allosteric modulator of CB1, and thus has been the most widely studied. ORG27569 has been considered a NAM for a long time, although it seemed to lack efficacy in counteracting the effects of orthosteric agonists of CB1 (Osborne et al., 2019). It was later established that this compound was indeed a CB1 negative allosteric modulator, but was time-dependent and pathway-specific and was shown to act as a biased allosteric modulator (Ahn et al., 2013; Banister et al., 2019; Gamage et al., 2016).

A recent discovery by Vallée and colleagues (2014) showed that pregnenolone, which is already known to be a steroid precursor, acted on the CB1 receptor as a negative allosteric modulator. In their work, they showed that after THC injection, pregnenolone levels soared in the brain, but this steep increase in pregnenolone was not followed by any peak in downstream steroid, strongly suggesting pregnenolone activity. Pregnenolone was produced as a response to CB1 activation and, through a negative feedback, was able to modify part of the CB1 signaling, although abolishing the behavioral outcome (Vallée et al., 2014). This revolutionary discovery

will be discussed later and provided evidence of the nature of pregnenolone nature as a negative biased allosteric modulator of the CB1 receptor, whose endogenous role was apparently to protect the brain from cannabinoid intoxication.

V CB1-Signaling Specific Inhibitors (CB1-SSi)

V.1 Pregnenolone: the precursor of the steroids

V.1.1 Steroids: an overview

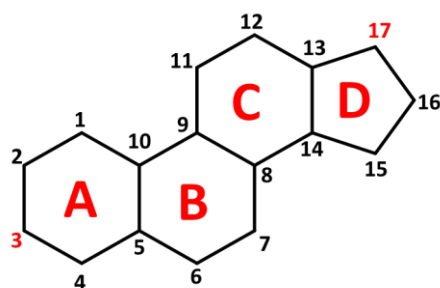


Figure 26: General structure of steroids. There are four rings, A, B, C are cyclohexanes, while D is a cyclopentane. On position 3 and 17, residues can be recognized and modified by the metabolic enzymes to produce downstream steroids. In position 4-5 and 5-6, there could be a double bond.

The steroids are bioactive organic compounds with a characteristic four-ring structure (three six-member cyclohexane rings and one five-member cyclopentane ring, Fig. 26), derived from cholesterol and performing two main functions: increasing membrane fluidity and triggering molecular signaling in cells (Huggins et al., 1954; Shoolery and Rogers, 1958; Suchowsky and Baldratti, 1964). Hundreds of different bioactive steroids exist in nature and among them, those in vertebrates are classified in three main groups, based on their production site: corticosteroids (Alejandro and Burton, 1951; Burton and Henry, 1951), sex steroids, and neurosteroids. The corticosteroids, a class that includes all the steroids produced in the adrenal cortex of vertebrates, are divided into two sub-classes: the glucocorticoids (e.g., cortisol) which are mostly involved in stress-related behavior (Barton et al., 1985) and immune regulation (Espelid et al., 1996), and mineralocorticoids (e.g., aldosterone), which are mostly involved in the regulation of electrolyte balance (Kenyon et al., 1984). The sex steroids or gonadocorticoids

are the hormones that interact with vertebrate androgen or estrogen receptors and are divided into three sub-classes: progestogens (e.g., progesterone), which are involved in the female ovarian cycle (Weinbauer et al., 2008) and represent the general steroid precursor class; androgens (e.g., testosterone), involved in developing and maintaining the male secondary characteristics (McBride et al., 2016); and estrogen (e.g., estradiol) that contributes in developing and maintaining the female secondary characteristics (Miles-Richardson et al., 1999). Furthermore, there are the neurosteroids, described as steroids produced *de novo* within the CNS independently from the periphery, which exert a neuromodulator action within the brain that turns out to be rapid, since neurosteroids act on the membrane and not genomic receptors (Reddy, 2010; Vallée, 2016; Vallée et al., 2004, 2003, 2001). Until the early 80's, it was thought that the endocrine system and hormones did not act directly on the CNS, but thanks to the pioneering work of Baulieu, it was initially discovered that DHEA and PREG are produced directly in the brain, independently of peripheral steroidogenesis (Baulieu, 1981; Baulieu and Robel, 1998).

Neurosteroids are steroids produced "on-demand" inside the brain (Schumacher et al., 2009). The most significant difference with the steroid hormones produced in the endocrine glands is that as the neurosteroids are produced *in loco* in the CNS, their action is more like the autocrine or paracrine system (Compagnone and Mellon, 2000). The original idea that steroids were able only to bind to and activate the nuclear steroid receptor has now been surpassed, and several non-genomic targets for neurosteroids have been discovered. It has been discovered that neurosteroids can modulate several membrane receptors, such as GABA_A (Belelli and Lambert, 2005; Lambert et al., 2009) and NMDA (Irwin et al., 1994; Sedláček et al., 2008) receptors. Neurosteroids can be divided into two groups: inhibitory and excitatory (Lambert et al., 2009; Paul and Purdy, 1992; Rupprecht, 2003). Among the inhibitory steroids, the best characterized is allopregnanolone, a potent GABA_A-positive allosteric modulator (Hosie et al., 2006), while

other neurosteroids, such as pregnenolone sulphate, act as a negative allosteric modulator of the same receptor, ultimately resulting as inhibitory and excitatory neurosteroids respectively (Smith et al., 2014). Some steroids are considered proneurosteroids because they do not have any activity *per se*, but can be produced *on demand* directly in the brain, to be metabolized into neuroactive steroids (Tomaselli and Vallée, 2019).

V.1.2. The pathophysiological role of pregnenolone: an overview

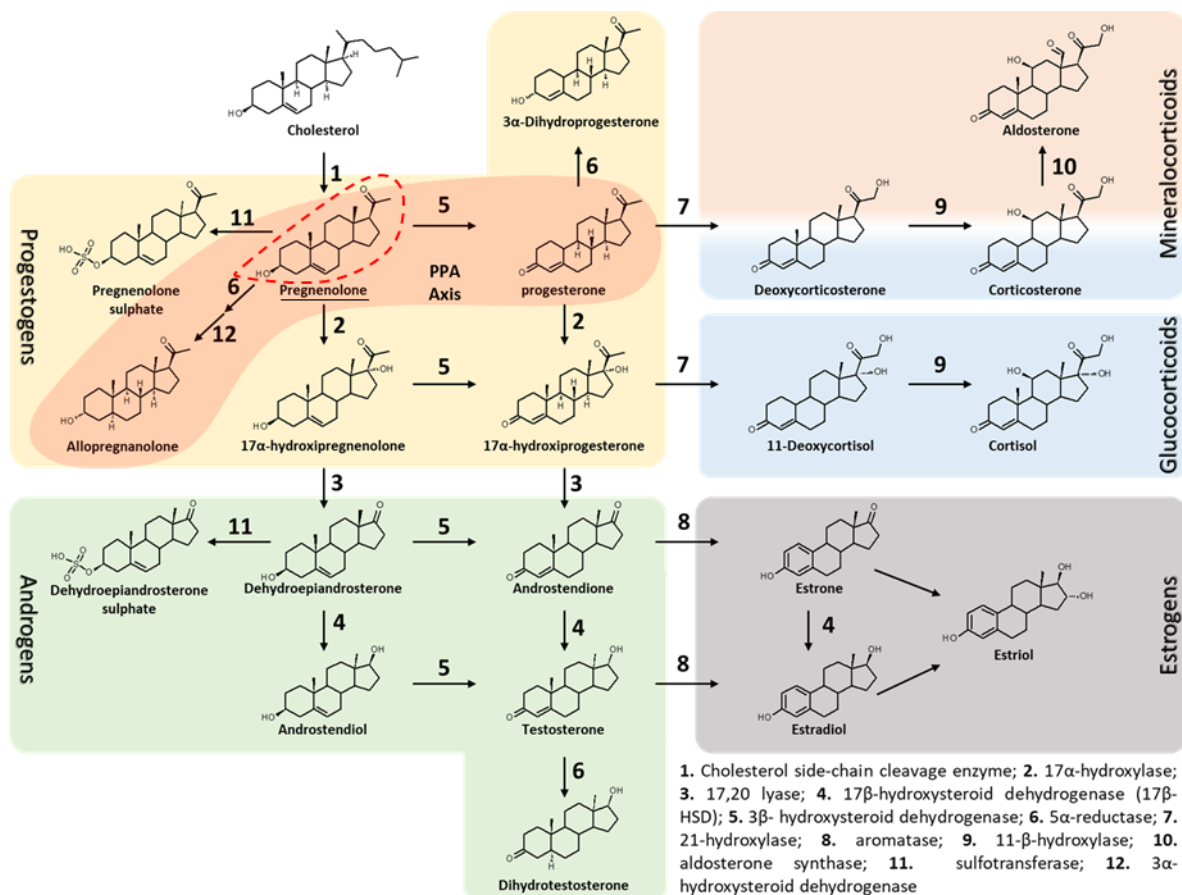


Figure 27: Steroidogenesis. Steroids derive all from the cholesterol that can be produced inside the cell or come from food ingestion. The number of carbons can classify steroids. The progesterogens have 21 carbons and are the first steroids to be produced in the chain. There is the so-called pregnenolone, progesterone, allopregnanolone (PPA) axis, active as neurosteroids in this group. Progesterogens can either lose two carbons and biotransform into androgens (19 carbons), or biotransform into mineralocorticoids and glucocorticoids and then keep the same number of carbons (21 carbons). Androgens can lose another carbon and be biotransform into estrogens (18 carbons).

Pregnenolone (PREG) is the first molecule to be produced in the steroid production chain and the precursor of all the other steroids. To produce steroid hormones, it is first necessary to provide new cholesterol, and hormone-sensitive lipase (HSL) (activated by phosphorylation on

the epitope Ser660) hydrolyzes cholesterol esters to produce free cholesterol. The steroidogenic regulatory protein (StAR) transports the cholesterol inside the mitochondrion, and the cytochrome P450 side-chain cleavage (CYP450_{scc}) converts the cholesterol into PREG (Manna et al., 2013; Stocco et al., 2005). During steroidogenesis, PREG is produced from cholesterol, and then from pregnenolone, while all the other steroids are biosynthesized from different enzymes. PREG is a steroid itself, as well the precursor of all the other steroids (Fig. 27).

PREG is part of the so-called Pregnenolone-Progesterone-Allopregnenolone pathway linked to several brain functions and pathologies (see for review Tomaselli and Vallée, 2019).

Pregnenolone can be considered a biomarker of several mental pathologies, and in several studies, altered levels of pregnenolone in the blood, brain, or cerebrospinal fluid have been correlated to altered brain functions. PREG plays a role in stress-related disorder, psychosis, alcohol use disorders, and cannabis use disorders. Several animal studies on the contribution of neurosteroids to stress-related disorders have focused on the allopregnanolone modulation of GABA_A, underscoring a central role of pregnenolone-progesterone-allopregnanolone in stress-related behaviors (Barbaccia et al., 1994, 1996b, 1996a; Maldonado-Devincci et al., 2014a; Purdy et al., 1991; Roscetti et al., 1997; Vallée et al., 2000; Vallée, 2014). Chronic stress, such as in social isolation, repeated restraint tests or forced swimming tests, causes a significant increase of PREG levels in the brain (Vallée, 2014). Vallée (2014) showed that in chronically-stressed mice, acute stress increased brain levels of PREG. In particular, the brain/plasma ratio following stress tests was much higher for PREG than for allopregnanolone (Park et al., 2017; Vallée, 2014). Furthermore, Sze et al. (2018) showed that in rats, acute stress induced a PREG increase in the brain, while plasma levels remained unaltered, and that this effect appeared to affect female rats more than males, suggesting a sex-specific response to stress. The increment of PREG in the hippocampus and prefrontal cortex showed an antidepressant effect comparable

to the competitive 3 β -HSD (the enzyme that converts PREG into PROG) inhibitor trilostane (Espallergues et al., 2012). In addition, the antipsychotic neuroleptics (e.g., olanzapine, haloperidol, clozapine) used psychosis and bipolar depression produce a hippocampal and cortical increase of PREG in rats (Higashi et al., 2009; Khisti et al., 2002; Marx et al., 2011, 2011, 2006a, 2006b). Interestingly, it has been demonstrated that mouse models of schizophrenia can be rescued by using pregnenolone (Marx et al., 2011; Wong et al., 2012) (Table 1, from Tomaselli and Vallée, 2019).

Disorder	PREG	Treatment	Effect on PREG	Behavioral response
Stress related disorders				
Acute stress	↑brain ¹			
Mild stress	↓brain ²			
Depression-like behaviors	↓brain ³	Trilostane ⁴	↑ ⁴	antidepressant ⁴
		SSRIs ⁵	↑ ⁵	antidepressant ⁵
Anxiety-like behavior	↓brain ^{6,8}	PREG ⁸	N.R.	anxiolytic ⁷
Alcohol intake				
Acute intake	↑brain/plasma ⁹			
Chronic intake	↓brain/plasma ¹⁰	ALLO ¹¹	N.R.	↓(low doses)/↑(high doses) alcohol intake ¹¹
Cannabis intoxication				
THC acute intake	↑brain ¹²	PREG ¹²	↑ ¹²	rescue of THC-induced behaviors ¹²
WIN self-administration	↑brain ¹²	PREG ¹²	↑ ¹²	↓WIN seeking ¹²
Psychotic-like behavior	N.R.	PREG ¹³	N.R.	rescue psychotic-like behaviors ¹³

Table 1 – Brief overview of animal research evidence on the role of the PPA pathway in stress related disorders, alcohol intake and cannabis intoxication.: Bibliography: 1. (Barbaccia et al., 1996a, 1994; Roscetti et al., 1997; Vallée et al., 2000); 2. (Guidotti et al., 2001; Khisti et al., 2000; Serra et al., 2007; Vallée et al., 2014); 3. (Vallée et al., 2014); 4. (Espallergues et al., 2012); 5. (Tohen et al., 2003); 6. (Khisti et al., 2002; Shirayama et al., 2011; Ugale et al., 2004); 7. (Akwa et al., 1999; Engin and Treit, 2007; Frye and Rhodes, 2006; Martín-García and Pallarès, 2005; Picazo and Fernández-Guasti, 1995; Reddy and Kulkarni, 1997; Rodgers and Johnson, 1998; Wieland et al., 1995); 8. (Devroye et al., 2016; Eser et al., 2008; Melchior and Ritzmann, 1994); 9. (O'Dell et al., 2004); 10. (Beattie et al., 2017); 11. (Ford et al., 2005; Morrow et al., 2001); 12. (Piazza et al., 2013, 2012; Vallée et al., 2014); 13. (Busquets-Garcia et al., 2017). Table 1 from Tomaselli and Vallée, 2019.

Clinical data has shown that patients with depression had lower PREG levels in their corticospinal fluid (George et al., 1994), patients with schizophrenia showed lower PREG levels in serum (Marx et al., 2011; Ritsner et al., 2010), while increased PREG levels in plasma have been detected (Cai et al., 2018). Lastly, post-mortem analysis of brain tissue from schizophrenic subjects showed increased levels of PREG (Marx et al., 2006c) (Table 2, from Tomaselli and Vallée, 2019).

Similarly to stress related-disorders, most of the studies on neurosteroids in alcohol use disorders are focused on the role of allopregnanolone and GABA_A, the primary alcohol target, and the levels in brain and blood (Biggio et al., 2007; Gabriel et al., 2004; Maldonado-Devincci et al., 2014b; O’Dell et al., 2004; Porcu et al., 2014; Sanna et al., 2004; VanDoren et al., 2000). However, some evidence has pointed to the role of PREG in AUD as well (O’Dell et al., 2004). Furthermore, PREG administration has been shown to decrease ethanol self-administration in rats (Besheer et al., 2010) (Table 1).

Clinical trials have shown that increased PREG levels are strictly correlated with alcohol liking, but the desire for more alcohol is correlated with the alcohol-dependent allopregnanolone increase (Pierucci-Lagha et al., 2006) (Table 2). Pregnenolone levels are drastically increased following THC administration in rats and mice (Vallée et al., 2014). THC was able to cause synthesis of PREG through two different mechanisms in Wistar rats. The first, quick response was an Erk1/2-dependent increase in levels of cytochrome P450_{scc}, the enzyme responsible for pregnenolone production. The second, later mechanism, was Erk1/2-independent and consisted of a contextual increase in cytochrome P450_{scc} and in phosphorylated hormone sensitive lipase (HSL), which is responsible for the conversion of cholesteryl ester into cholesterol, which is then used for the production of pregnenolone (Yeaman, 2004). This effect turned out to be CB1-

Disorder	PREG	Treatment	Effect on PREG	Clinical response
Stress related disorders				
Depression	↓CSF ¹	3-methoxy PREG ²	none ²	↓depressive symptoms ²
		3β,5α-pregnanolone ³	N.R.	↑ mood ³
Alcohol use disorders				
Alcohol abuse	↑plasma ⁴	3-methoxy PREG ³	none ³	↓alcohol intake ⁵
Cannabis use disorders				
Cannabis dependence	N.R.	AEF0117 (PREG-like) ⁶	none ⁶	↓intake, craving, addiction ⁶
Schizophrenia	↓brain ⁷ ↑plasma ⁸	PREG ⁹	↑ ⁹	↓negative and positive symptoms ⁹
		antipsychotics ¹⁰	↓ ¹⁰	ameliorate symptoms ¹⁰
Bipolar disorder		PREG ¹¹	N.R.	↓depressive symptoms ¹¹

Table 2 – Brief overview of human research evidence on the role of the PPA pathway in stress related disorders, cannabis use disorder and alcohol use disorder.. Bibliography: 1. (George et al., 1994); 2. (Baulieu et al., 2004); 3. (Bixo et al., 2018, 2017); 4. (Koob et al., 2018); 5. (Pierucci-Lagha et al., 2006); 6. (Piazza et al., 2013, 2012); 7. (Marx et al., 2011, 2009; Ritsner et al., 2007, 2010); 8. (Cai et al., 2018; Marx et al., 2006c); 9. (Kreinin et al., 2017; Marx et al., 2014, 2011, 2009; Ritsner et al., 2014, 2010); 10. (Rasmussen et al., 2006); 11. (Brown et al., 2014). Table2 from Tomaselli and Vallée, 2019.

but not CB2-dependent. A CB2 antagonist did not block THC-induced pregnenolone increase, while a CB1 antagonist stopped PREG increase completely. Furthermore, in full CB1 KO mice, THC was no longer able to increase PREG.

PREG showed a negative modulatory effect on motivation in drug seeking and drug intake (Vallée et al., 2014) (Table 1). Preliminary clinical data showed that PREG-like compounds can sharply decrease cannabis intake and craving and, more generally, addiction (Piazza et al., 2013, 2012) (Table 2).

V.2. Pregnenolone is the precursor of the CB1-SSi

Vallée and colleagues (2014) discovered that following THC administration in both rats and mice, PREG levels in the brain increased dramatically in several brain structures (frontal cortex, striatum, nucleus accumbens, hippocampus, thalamus, hypothalamus, ventral midbrain, cortex, cerebellum, spinal cord) (Fig. 28), and in several peripheral organs (kidney, spleen, lung, white adipose tissue), but this increase was not observed in the liver, muscle, intestine or plasma.

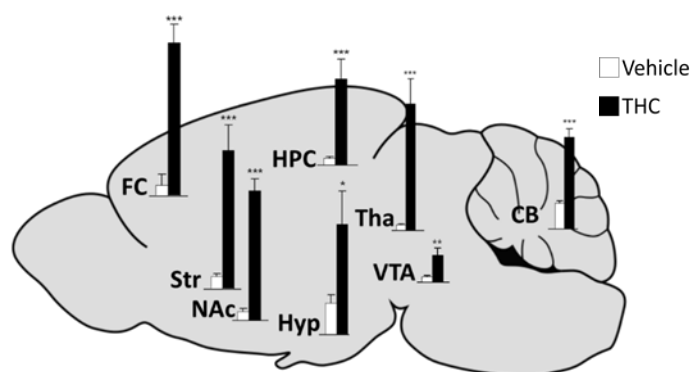


Figure 28: THC administration induces intense production of pregnenolone in several mouse and rat's brain areas: frontal cortex (FC), striatum (Str), nucleus accumbens (NAc), hippocampus (Hpc), thalamus (Tha), hypothalamus (Hyp), ventral tegmental area (VTA), cerebellum (CB).

This effect was indeed specific to CB1 agonists, as such a drastic increase (~1800%) was not recorded for other drugs of abuse (ethanol, nicotine, cocaine, morphine), but could be reproduced by administering other CB1 agonists (HU210, WIN55,212-2) (Vallée et al., 2014). Furthermore, when mice were pretreated with AM251, a CB1 inverse agonist, the THC-

dependent increase in pregnenolone was abolished. Administration of THC at high doses (12 mg/kg) also caused an augmentation of PREG in wild-type mice nucleus accumbens, but not in constitutive or neuron-specific (D1) knockout mice. This increase in PREG is CB1-specific, as when the CB2 agonist JWH-133 was administered in mice, it did not cause any significant alteration of PREG levels (Vallée et al., 2014). In order to understand this process better, and thus to understand the intracellular signaling pathway that led to PREG hyper-production, in the same study, Vallée and colleagues (2014) studied the expression of the enzyme responsible for PREG production. In rats, they observed that THC was able to increase CYP450_{scc} levels rapidly (15 min post-THC), drastically and Erk1/2-dependently. Using the selective MEK1/2 inhibitor SL327, CYP450_{scc} no longer increased. However, an Erk1/2-independent late (30 min post-THC) HSL phosphorylation and CYP450_{scc} activation followed through the production of PREG (Vallée et al., 2014) (Fig. 29).

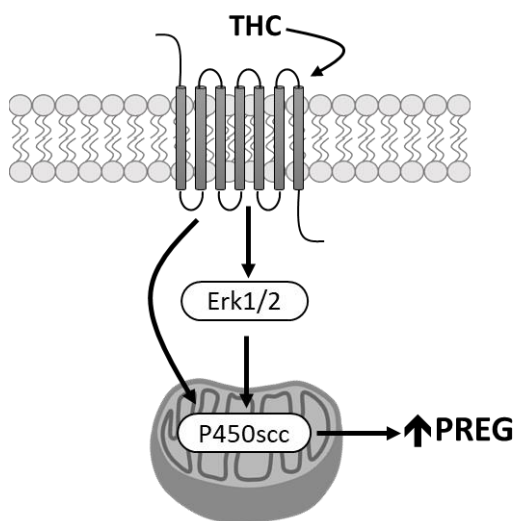


Figure 29: THC-induced production of pregnenolone. Two mechanisms have been identified. The first is a quick response, Erk1/2-dependent, and the second is a slower Erk1/2-independent response.

In vitro studies on HEK and CHO cells transfected with hCB1 have demonstrated that PREG was able to abolish Erk1/2 phosphorylation in both the cell lines and was able to restore the normal mitochondrial cellular respiration, but had no impact on THC-induced cAMP inhibition (Vallée et al., 2014; Welberg, 2014) (Fig. 30).

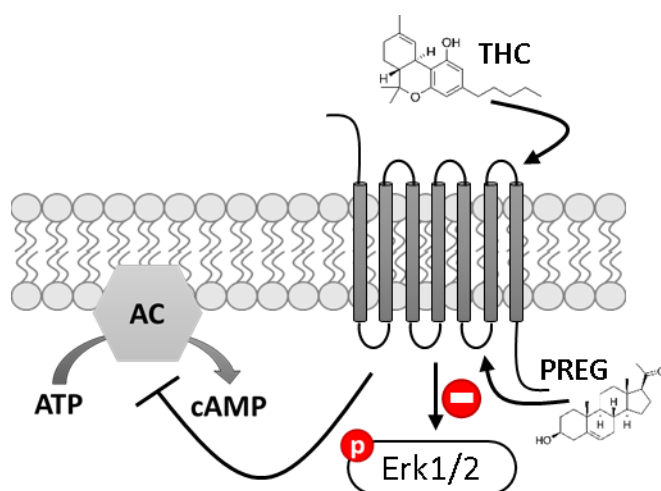


Figure 30: in vitro mechanism of action of PREG on the MAPK pathway. THC in HEK and CHO cells transfected with hCB1 increases the phosphorylation of Erk1/2, and at the same time, blocks the adenylyl cyclase, thereby inhibiting the production of cAMP. PREG binds the CB1 receptor and then blocks the phosphorylation of Erk1/2, but do not interfere with the adenylyl cyclase blockade.

PREG was also able to inhibit several THC-induced behaviors: it inhibited THC (10 mg/kg)-induced tetrad effects (hypolocomotion, hypothermia, catalepsy, and analgesia), abolished THC (10 mg/kg)-induced food-intake, restored THC-induced impaired memory, and in mice trained for WIN55,212-2 self-administration, drastically decreased the number of intakes, demonstrating decreased motivation for drug-seeking (Vallée et al., 2014; Welberg, 2014). Moreover, the pre-treatment of aminoglutethimide (AMG, 50 mg/kg), a selective inhibitor of PREG synthesis, enhanced the THC effects in the tetrad and PREG rescued the effect of AMG, demonstrating that the endogenous role of PREG is indeed to dull the effect of CB1 hyperactivation (Vallée et al., 2014).

Since PREG did not alter the equilibrium binding of [H^3]WIN 55-212,2 with CB1, it has been proposed that it might act not in an orthosteric, but an allosteric position (Vallée et al., 2014). Through the force-biased metropolis Monte Carlo simulated annealing program, it has been possible to discover the allosteric docking site of PREG on the CB1 receptor. Two primary amino acids are responsible for its docking: the glutamate in position 49 of the first transmembrane helix, and the arginine in position 65 of the seventh transmembrane helix (Vallée et al., 2014) (Fig 31). HEK cells with a substitution of the glutamate in position 1.49 with a glycine no longer responded to pregnenolone, but showed no alteration in their response to THC (Vallée et al., 2014).

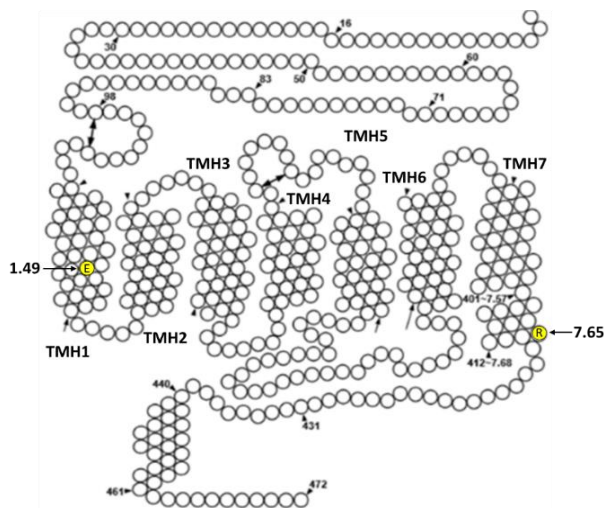


Figure 31: Specific PREG docking site in the CB1 receptor. The 49th amino acid in the first transmembrane helix (TMH) (Glutamate) and the 65th amino acid in the seventh TMH (Arginine) have been identified as the responsible for PREG docking. Disrupting the 1.49 site by a punctual genetic mutation (substituting the glutamate with a glycine), the receptor's functionality was not modified, but PREG lost its effect on the THC effects in HEK cells transfected with mutated hCB1.

For those characteristics of allosteric modulation of a portion of the CB1 signaling, PREG is considered a negative allosteric biased modulator, and the precursor of a new class of drugs, the CB1-Signaling Specific Inhibitors (CB1-SSi).

In Busquets-Garcia et al. (2017), PREG pharmacological potential was tested in a battery of behavior related to schizophrenia and psychosis triggered by THC, which caused different dose-dependent psychotic-like behaviors in mice. In particular, he demonstrated that low doses of THC (0.3 mg/kg) sharply increased locomotor reactivity to a novel environment, considered a positive symptom of psychosis, and that PREG entirely abolished this behavior, while ten-fold higher doses of THC (3 mg/kg) caused a drop in social interaction in the social interaction test, a negative symptom of psychosis, which was also rescued by PREG (Busquets-Garcia et al., 2017).

Some studies have attempted to reproduce the effect of PREG on cell lines treated with THC or endocannabinoids, but without success. For instance, Khajehali et al., (2015;), Straiker et al., (2015) and Gamage et al. (2017) did not explain meticulously the protocol used to dissolve PREG in solution and did not show that the concentration observed in the solution was as expected. The protocol to dissolve PREG is quite complicated (Busquets-Garcia et al., 2017; Vallée et al., 2014) because PREG is a hydrophobic molecule and PREG in suspension can

quickly stick to the plastic walls of a vial. For this reason, it is possible that, due to precipitation and deposition on the walls of the vials, the solution had a much lower concentration of PREG than expected, with no effect on cannabinoid signalization.

Straiker et al. (2015) tried to reproduce the effect of PREG on mouse (CD1) hippocampal neurons treated with the endocannabinoid 2-AG. However, they did not reproduce the effects observed by Vallée et al. (2014) on exocannabinoids. The author himself suggested that the effect of PREG could be protective against exocannabinoids, but not against endocannabinoids, allowing physiological activity and impairing the pathological activity of ECS.

Krohmer et al. (2017) claimed that PREG was not able to block or modify the cannabinoid effect on synaptic transmission in the cerebellum and nucleus accumbens. They were the only ones to declare that the concentration of PREG observed in their solution was appropriate, but while Vallée et al. (2014) observed the EPSC in brain slices from Sprague-Dawley adult rats, Krohmer et al. (2017) used 18-day-old NMRI mice or Wistar rats. Furthermore, the sacrifice, brain slicing, slice preparation, slice stocking, and patch clamping protocols in the two methods were very different, possibly explaining the difference between the results of Vallée et al. (2014) and Krohmer et al. (2017).

V.3 – AEF0117, Synthetic Signaling Specific Inhibitor lead compound

Although PREG has strong potential as a treatment for cannabis abuse, cannabis use disorder and THC-induced psychosis and schizophrenia, it cannot be used as a medical drug. PREG has several drawbacks: a very short half-life (~40 min) (Bélanger et al., 1992), low bioavailability *per os*, and since it is the precursor of all steroids, enzymes can recognize PREG and metabolize it into downstream steroids (Deb et al., 2018), and in this case, the long-term side effect will be similar to steroid abuse.

For those reasons, PREG has been used as a model for a new class of synthetic CB1-SSi. These compounds have been modified in collaboration with Aelis Farma biotech on position C3 and

C17 of PREG, the two positions that can be recognized by metabolic enzymes to produce downstream steroids from PREG (Piazza et al., 2013, 2012) (Fig 32).

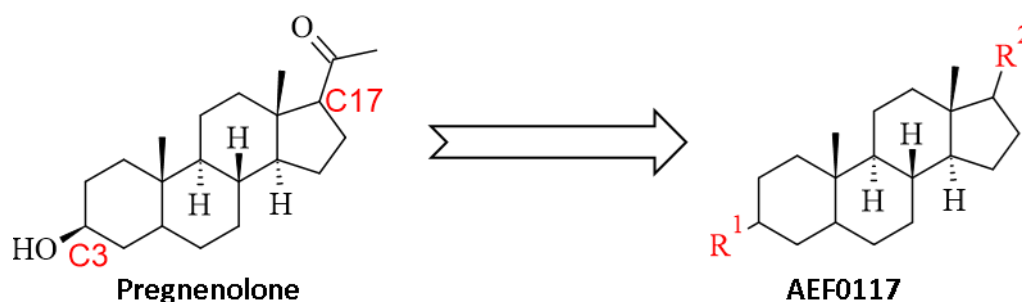


Figure 32: The synthesis of AEF0117 from PREG. The substitution of groups in position C3 and C17 of pregnenolone induces that AEF0117 can no longer be recognized by any of the enzymes that biotransform PREG into downstream steroids. The groups R¹ and R² are confidential.

The lead compound, named AEF0117, has been proven not to be biotransformed by any steroidogenic enzyme. Tests in CHO cells (cell lines expressing all the enzymes for production and biotransformation of steroids) transfected with CB1 receptors demonstrated that, while a sharp increase in allopregnanolone, epiallopregnanolone, pregnenolone, DHEA, and Testosterone was detected following a high dose of pregnenolone treatment, cells treated with AEF0117 did not show any increase in the steroids mentioned above (Piazza et al., 2013). The AEF0117 compound also passed through the blood-brain barrier after oral administration in rats, was not transformed into a significant quantity of downstream metabolites, and did not modify the major body enzymes and transporters. Interestingly, it showed a remarkable therapeutic index. The therapeutic index is obtained through *in vivo* studies, and it is calculated as the ratio of the highest dose of the drug that does not cause any toxic effect to the dose of the drug that leads to the desired pharmacologic effect (Muller and Milton, 2012). The therapeutic index of the AEF0117 has been calculated to be above 7200, meaning that AEF0117 is a utterly safe drug. AEF0117 selectively inhibited the CB1 receptor in a specific manner, mimicking the known effects of pregnenolone *in vitro*, and inhibited a set of unconditioned and conditioned CB1 agonist-induced behaviors. To date, we do not know of any AEF0117 side-

effect that might be comparable to the known CB1 orthosteric antagonists, and unlike the CB1 antagonist rimonabant, AEF0117 did not induce any significant withdrawal signs in cannabinoid-dependent mice (Piazza et al., 2013).

In the first phase of a human clinical trial, AEF0117 did not show any unwanted side effects (NIH, ClinicalTrials.gov Identifier: NCT03325595) and it is currently in the second phase of human experimentation for cannabis use disorder and cannabis abuse (NIH, ClinicalTrials.gov Identifier: NCT03717272).

PREG and AEF0117 proved to be reliable, efficient, and promising drugs, acting on the CB1 receptor without causing any recorded adverse side-effect, but the intracellular mechanism of action that regulates this process is still mostly unknown.

VI Aims

Cannabis Sativa is one of the most common illicit drugs of abuse, and it is estimated that more than 180 million people abuse it worldwide (United Nations Office on Drugs and Crime, 2008). As a risk factor in several mental pathologies (psychosis, depression and anxiety) and in cannabis use disorder (Sven Andréasson et al., 1987; Arendt et al., 2005; Hall and Degenhardt, 2008; Müller-Vahl and Emrich, 2008; Ramesh and Haney, 2015; Wilkinson et al., 2014), THC, the main psychoactive component of *Cannabis Sativa*, represents a crucial public health challenge.

Pregnenolone, an endogenous CB1 modulator, has already been proven to block tetrad, food-related behaviors, memory impairment in rodents (Vallée et al., 2014), hyperlocomotion, and some of the most common psychotic-like THC-induced effects in mice (Busquets-Garcia et al., 2017), and its derivative AEF0117 has not shown any undesirable side-effect (Piazza et al., 2013, 2012; NIH, ClinicalTrials.gov Identifiers: NCT03325595). CB1-SSi, and more particularly AEF0117, could represent a bright possibility for cannabinoid use disorder, psychosis, and schizophrenia (Piazza et al., 2013). However, AEF0117 has so far been tested only for addictive behavior in rodents and primates (Piazza et al., 2013; Vallée et al., 2014), and some of the known THC-induced behaviors (Busquets-Garcia et al., 2017b; Vallée et al., 2014), and the intracellular mechanism of action of both pregnenolone and AEF0117, is mainly unknown.

In order to fully understand the mechanism of action of AEF0117, and by extension of its precursor pregnenolone, it is imperative to understand CB1 signaling as well as possible. It is known that THC can trigger several behavioral outputs, and although several studies have tried to find an explanation for such different biological outcomes, such as the expression of CB1 on different cell-types (Busquets-Garcia et al., 2017a), and biased signaling (Turu and Hunyady,

2010), it is still impossible to clearly predict the signaling pathway that CB1 will recruit under specific circumstances.

This current study aims to study:

- a) The THC-induced CB1 signaling pathway at three primary doses: 0.3, 3, and 10 mg/kg, representing the doses at which pregnenolone is not produced at all, is produced sub-maximally, and reaches its production plateau, that lead in mice to hyperlocomotor, asocial, and hypolocomotor behaviors respectively, and
- b) The intracellular mechanism of action through which the pregnenolone and the AEF0117 block the aforementioned THC-induced behaviors.

The structure of the thesis is organized into three main parts: the journal article Zanese, Tomaselli et al., 2020 aiming to reach the state of the art on a new high-throughput technique that will be used for complete and exhaustive screening of the intracellular signaling pathway. The second part is the article Tomaselli et al. (to be submitted), in which the CB1 signaling pathway following the dose of THC 0.3 mg/kg is studied, and the related mechanism of action of PREG and AEF0117.

The third part on data aims to explore the THC-induced intracellular signaling pathway at doses of THC of 3 and 10 mg/kg, leading to asocial and hypolocomotor behaviors, respectively, in mice, and the relative effect of AEF0117 on asocial behavior.

**Alpha technology: A powerful tool to detect
mouse brain intracellular signaling events**

**Article published in Journal of Neuroscience Methods, Volume
332, 15 February 2020**

The need for a high throughput screening in signaling pathway research

A signaling cascade is a complex event that takes place after that an agonist ligand binds a receptor. The agonist, binding the receptor, gives information that the receptor needs to send to the downstream effectors in the cell to trigger a response (Marks, 2008). However, being one protein only, the receptor would not be able to recruit the effectors in quantity needed to have a significant output. For this reason, between the receptor and the effectors, there is a sophisticated machinery, usually phosphoproteins named kinases. Kinases can amplify the signal exponentially and gain a sufficient response on the effectors (e.g., one receptor-activated two proteins, each protein activates two proteins and so on. In the end several hundred if not thousands of proteins activates the effectors) (Li and Qian, 2003). This machinery works as binary code: proteins can be phosphorylated or not, usually corresponding to the 0 and 1: active and inactive (Marks, 2008; Weber et al., 1999). Studying signaling cascade is a challenging task: correctly identifying a signaling cascade through the semiquantitative analytical detection of phosphoproteins usually requires a certain amount of material and time to analyze the selected targets. This is especially true when the signaling cascade are analyzed in samples obtained from animal tissue, and even more if they come from animal small brain areas. The most common technique used worldwide for this purpose is the western blot that has been proved to be adequate for the task (Pillai-Kastoori et al., 2020; Renart et al., 1979): a technique precise and reproducible enough to gain consistent data in all kind of laboratories around the world. However, the amount of total proteins obtained from mouse brain tissue lysates is scarce when not insufficient to run several tests with the western blot technique. To overcome this problem, it could be possible to use many animals to analyze different targets. Still, in this case, the resulting data would be less robust, not to mention the ethical issue of using too many animals. Another problem is the time that western blot can require to perform a proper screening. In one day of work, it is possible to obtain an average of approximately forty data-

points, working in duplicates, which means twenty samples, a number not sufficient for this project aims. Furthermore, the western blot, although being a very trustworthy technique, present a high variability between the different wells since there are several very delicate steps (such as the membrane washes) and critical moments (such as the running and the washes steps) that can contribute to the partial loss of protein, or an incorrect migration (Beaudet et al., 2008). So the most crucial technical improvement in brain tissue lysates phosphoprotein screening is to reduce the amount of sample needed for each analysis and the consumption of time, increasing the throughput, and, if possible to improve the reproducibility diminishing the most critical steps. For this reason, we have decided to use a new technique that would allow the rapid, sensitive, reproducible, and semiquantitative detection of phosphoproteins in brain tissue lysates. In the market, however, such a method was not still fine-tuned for brain tissue lysate analysis. So we have decided to turn to the Alpha technology, particularly into the AlphaLISA technique, a technique already used in drug discovery and cell culture analyses (Beaudet et al., 2008). We decided to fine-tune the technique ourselves, adapting it for brain tissue lysates (Beaudet et al., 2008).

This work has been published as “*Alpha technology: A powerful tool to detect mouse brain intracellular signaling events*” by M. Zanese*, G. Tomaselli*, V. Roullot-Lacarrière, M. Moreau, L. Bellocchio, A. Grel, G. Marsicano, N. Sans, M. Vallée, J.M. Revest (*equal contribution), Journal of Neuroscience Methods, Volume 332, 15 February 2020. The article oversees the validation of the technique for our research purposes. This article shows that this technique possesses a wide linear range, namely the amplitude of the window of concentration at which the method optimally works. Then we proved that AlphaLISA reproducibility was very high. Even after repeated cycles of freezing and thawing of the sample (that may affect the phosphate group of phosphoproteins), and after performing the analysis in two different time points and at different temperatures, we obtained replicable results, differing only by the

absolute value of the intensity of the output, showing that this semiquantitative technique has a high degree of reproducibility. Another topic that has been shown in the paper is how to normalize our signals using the housekeeping proteins GAPDH and Cofilin total. We showed that GAPDH does work only on murine cell cultures and not in brain tissue lysates, while Cofilin total works fine on both. Then we showed that we were able to observe significant differences in mice treated with a stimulant drug (cocaine) or a depressive drug (high dose of THC), as well as basal differences of signaling in transgenic mice (Ts65Dn). Lastly, we have shown that this technique is well-adapted as well in analyzing cell and synaptosomal fractions. More in particular, in cell fractions (nucleus, microsome, and cytosol), we have shown that an ubiquituary phosphoprotein such as Erk1/2 was significantly present in every cell fraction. By contrast, a typically nuclear phosphoprotein, CREB, was detected only within the cell core. Akt that is known to be mostly cytosolic has been found mostly (~95%) within the cytosol and in little amounts in the core and microsomes. Altogether the data collected and disserted in this article demonstrate that AlphaLISA is a perfectly suited technique in mouse brain signaling pathway research, allowing the rapid, semiquantitative, reproducible detection of phosphoproteins concomitantly from several signaling pathways.

My contribution to this work has been the concept and design of the experiments, the performance of the experiments, the data acquisition, analysis, interpretation, article writing, and the pictures drawing with Marion Zanese under the supervision of Monique Vallée and Jean-Michel Revest.

**Zanese, Tomaselli et al., Journal of Neuroscience Methods,
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Alpha technology: A powerful tool to detect mouse brain intracellular signaling events

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ABSTRACT

Background: Phosphorylation by protein kinases is a fundamental molecular process involved in the regulation of signaling activities in living organisms. Understanding this complex network of phosphorylation, especially phosphoproteins, is a necessary step for grasping the basis of cellular pathophysiology. Studying brain intracellular signaling is a particularly complex task due to the heterogeneous complex nature of the brain tissue, which consists of many embedded structures.

New method: Overcoming this degree of complexity requires a technology with a high throughput and economical in the amount of biological material used, so that a large number of signaling pathways may be analyzed in a large number of samples. We have turned to Alpha (Amplified Luminescent Proximity Homogeneous Assay) technology.

Comparison with existing method: Western blot is certainly the most commonly used method to measure the phosphorylation state of proteins. Even though Western blot is an accurate and reliable method for analyzing modifications of proteins, it is a time-consuming and large amounts of samples are required. Those two parameters are critical when the goal of the research is to comprehend multi-signaling proteic events so as to analyze several targets from small brain areas.

Result: Here we demonstrate that Alpha technology is particularly suitable for studying brain signaling pathways by allowing rapid, sensitive, reproducible and semi-quantitative detection of phosphoproteins from individual mouse brain tissue homogenates and from cell fractionation and synaptosomal preparations of mouse hippocampus.

Conclusion: Alpha technology represents a major experimental step forward in unraveling the brain phosphoprotein-related molecular mechanisms involved in brain-related disorders.

1. Introduction

One of the keys to unraveling the pathophysiological mechanisms that govern living organisms is to understand how cells communicate with each other, how they integrate incoming signals and what intracellular regulations and/or deregulations are taking place. Of the various regulatory mechanisms available to the cell, posttranslational modifications of proteins and specifically phosphorylations play a central role in changing the functional properties of the proteome, affecting the activity of the cells and controlling many aspects of animal physiology. These include cell fate and neuronal adaptation, transition

to long-term adaptive responses and higher brain functions involving specific behaviors (Han and Martinage, 1992; Walsh et al., 2005). Protein phosphorylation is mediated by the largest class of post-translational modifying enzymes that are called protein kinases but are also known as the kinome (Braconi and Orchard, 2008; Johnson and Hunter, 2005). This posttranslational modification is a highly dynamic reversible process (Mann and Jensen, 2003; Deribe et al., 2010) that drives intracellular signaling. Several authors have shown that the disruption of this dynamic intracellular signaling leads to behavioral impairments (Jovanovic et al., 1996; Barik et al., 2013; Revest et al., 2005; Revest et al., 2014; Revest et al., 2010). When signaling activities

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are deregulated such as those observed for example in cancer, brain-related diseases (e.g. Parkinson, Alzheimer), genetic diseases (e.g. Down syndrome), the consequences gradually move from the molecular to the cellular and finally impact behavior, often with very dire consequences (Martin, 2003; Bossy-Wetzell et al., 2004; Yang et al., 2009; Lemmon and Schlessinger, 2010).

The kinases catalyze phosphorylation reactions by transferring phosphate groups from adenosine triphosphate (ATP) to proteins on serine, threonine or tyrosine residues inducing their activation or inhibition (Deribe et al., 2010). Several methods may be used to measure protein kinase activity (Ni et al., 2006; Johnson and Hunter, 2005; Mann et al., 2002; Oda et al., 2001). Western blot is the most commonly used method to measure the phosphorylation state of proteins, and most laboratories are able to use it. Also known as immunoblotting, it is a semi-quantitative analytical method that may be used to detect the presence of proteins in either tissue homogenates or *in vitro* cell lysates, to evaluate their size, their posttranslational modifications and to compare variations in protein concentrations between different groups (Burnette, 1981; Kurien and Scofield, 2006). Even though Western blot is an accurate and reliable method for analyzing modifications of proteins and their relative abundance, it has two significant disadvantages: it is time-consuming and uses large amounts of samples. These parameters are critical when the goal of the research is to comprehend multi-signaling proteic events. Indeed, molecular mechanisms of protein-mediated physiopathologies can be understood only once the specific molecular targets have been identified. Testing all the targets in a pathway is very time-consuming, and Western blot protocols require many washing steps and periods of incubation that vary according to the abundance of protein and/or antibody affinity. They also require a significant amount of proteic sample and the quantity of lysate obtained from some tissues may not be sufficient for testing all the targets needed. This is particularly the case for brain samples. Therefore, we considered a new, more practical and quicker technique to study brain molecular pathways efficiently.

We used Alpha (Amplified Luminescent Proximity Homogeneous Assay) technology, which was developed by PerkinElmer™ (Eglen et al., 2008). It has several advantages such as a high throughput and a high sensitivity, so a large number of signaling pathways can be studied since sample processing and washing steps are not necessary. However, although this technology is widely used in other fields such as human oncology and *in vitro* targeting (Keshvari et al., 2017; Nunes et al., 2017; Jensen et al., 2017), it has not been used to study posttranslational changes *via* phosphorylation in brain tissue.

Here, we describe a set of experiments demonstrating that Alpha technology is a suitable, fast and sensitive method for detecting phosphorylated proteins in small mouse brain areas.

2. Materials and methods

2.1. Animals and drugs

Animals were housed individually in a temperature- (22 °C) and humidity- (60 %) controlled animal facility under a constant light–dark cycle (light on 7 a.m.–7 p.m.). Food and water were available *ad libitum*. All the animals were quickly sacrificed by decapitation. In order to preserve phosphorylation, brain areas were quickly dissected on ice, placed in dry ice-cold Precellys tubes (Bertin Technologies, Montigny-le-Bretonneux, France) and stored at –80 °C before protein extraction (Revest et al., 2010). Nine-week-old male C57BL/6N (weighing 25–30 g; n = 58) and CD1 mice (weighing 25–30 g; n = 12) were purchased from Janvier Laboratories (France). For synaptosomal preparation ten-thirteen-week-old control littermate mice for the Vangl2 Looptail mutation (n = 12) were kept housed in collective cages (Murdoch et al., 2001). C57BL/6 N mice were sacrificed after an *ip* injection (volume of 10 ml/kg) of vehicle (4 % ethanol, 4 % cremophor, 92 % NaCl 0.9 %; n = 28) or 0.3 mg/kg (n = 22) and 10 mg/kg (n = 6) of Δ^9 -

tetrahydrocannabinol in vehicle solution (THC; #THC-1295S-250, THC PharmGMBH The Health Concept, Germany). 30 min after injection of THC 10 mg/kg, the prefrontal cortex (PFC) and the nucleus accumbens (NAc) of these mice were collected and the proteins extracted in Alpha lysis buffer. For the THC 0.3 mg/kg experiment, mice were sacrificed at three different time points: 15 min (n = 6), 30 min (n = 6) or 55 min (n = 10) after the THC injection. The NAc of these mice was collected and the proteins extracted in Alpha lysis buffer. CD1 mice were sacrificed 10 min after an *ip* injection of 10 ml/kg of vehicle (NaCl 0.9 %; n = 6) or 20 mg/kg of cocaine (Sigma, Arlesles, France; n = 6) in vehicle solution. The striatum of these mice was collected and the proteins extracted in Alpha lysis buffer. Ts65Dn mice (n = 9) were obtained by repeated backcrossing Ts65Dn females to C57BL/6J x C3Sn.BLiA-Pde6b + /DnJ F1 hybrid males. The parental generation was obtained from The Jackson Laboratory (USA). Euploid littermates of Ts65Dn mice served as wild-type (WT) controls (n = 9). The hippocampus of these mice was collected, and the proteins extracted in Alpha lysis buffer.

2.2. Protein extraction from mouse brain tissues and protein quantitation

2.2.1. Total protein homogenates preparation

Total proteins from mice brain structures were extracted in AlphaLISA SureFire Ultra lysis buffer supplemented with protease and phosphatase inhibitors (#P8340 and #P0044, Sigma, USA) using the Precellys 24 homogenizer, which is a benchtop device dedicated to the grinding, lysis, and homogenization of biological samples (Bertin Technologies, Montigny-le-Bretonneux, France) (Revest et al., 2010). The homogenizing protocol of brain samples in lysis buffer was two cycles of 30 s at 5000 rpm with a 10-sec break between the two cycles using ceramic CK14 beads (#03961-1-0032, Bertin Technologies). After homogenization, the samples were centrifuged three times at 10,000 rpm, 10 min at 4 °C, and then the supernatants were quantified. Total proteins quantitation was done using a Direct Detect X spectrometer that analyzes the quantity of amine bonds (Merck Millipore) by mid-infrared spectroscopy. Then the protein samples were stored at –80 °C until use (Revest et al., 2010).

2.2.2. Cellular fractionation preparation

A detailed description of cellular fractionation preparation previously described has been adapted (Muguruzza et al., 2014). Briefly, hippocampus was dissected and immediately stored at –80 °C until assay. Tissue samples were homogenized using an a Teflon-glass grinder (IKA Labor Technik, Satufen, Germany) in 30 volumes of homogenization buffer (1 mM EGTA, 3 mM MgCl₂, 1 mM DTT, and 50 mM Tris-HCl, pH 7.4) supplemented with 0.25 M sucrose. The crude homogenate was centrifuged for 5 min at 1000 × g at 4 °C. The resulting pellet was homogenized a second time and re-centrifuged in the same condition to obtain the nuclear fraction. The supernatant obtained from the first centrifugation layer was re-centrifuged for 10 min at 40,000 × g (4 °C). The resultant supernatant was collected as cytosolic fraction whereas the pellet was washed twice in 10 and 5 volumes of homogenization buffer respectively, and re-centrifuged in similar conditions to obtain the microsomal fraction.

2.2.3. Synaptosomal preparation

Subcellular fractionation samples were prepared from 12 mice after decapitation and performed as described previously (Moreau et al., 2010; Sans et al., 2003). Briefly, about 250 mg of hippocampus were homogenized in 2.5 ml of ice-cold homogenization buffer (0.32 M Sucrose, 0.02 M HEPES, 5 mM EDTA) containing protease inhibitors (#11 873 580 001, Roche). The homogenizer was then rinsed with additional 2 ml of homogenization buffer. The homogenate was centrifuged at 3,800 rpm for 7 min at 4 °C in a 70.1 Ti rotor (Beckmann). The supernatant was removed from the pellet (nucleus) and centrifuged at 12,000 rpm for 20 min at 4 °C then centrifuged at 13,200 rpm for 30

min at 4 °C. The supernatant was centrifuged at 45,175 rpm for 2 h at 4 °C. The supernatant (cytosol) and the pellet (microsomes) were collected. The crude synaptosome-enriched pellet was centrifuged at 12,000 rpm for 20 min at 4 °C and resuspended in 1.5 ml of homogenization buffer. The crude synaptosome-enriched fraction was centrifuged twice at 12,000 rpm for 20 min at 4 °C, lysed by hypo-osmotic shock in water adjusted to 1 mM HEPES and mixed constantly for 30 min at 4 °C before being centrifuged at 19,000 rpm for 20 min. The supernatant was removed from the pellet (synaptosomes) and centrifuged at 49,000 rpm for 2 h at 4 °C. The synaptosomal vesicles-enriched pellet was collected. At each step 80–200 µl of each fraction were collected and immediately frozen in dry ice before storage at –80 °C. Phosphatase inhibitors (#P0044, Sigma, USA) were added just prior to the determination of protein concentrations quantified using the Pierce BCA Kit (#23225, Pierce, ThermoFisher Scientific).

2.3. Western blot analysis

A detailed description of immunoblotting analysis was reported previously (Revest et al., 2005; Revest et al., 2014; Revest et al., 2010; Sarrazin et al., 2009; Kitchner et al., 2004). Briefly, protein sample extracts from mouse prefrontal cortex were prepared in AlphaLISA lysis buffer supplemented with protease and phosphatase inhibitors (Sigma, USA) before being subjected to Western blot experiments. SDS-PAGE-separated proteins were then revealed with rabbit monoclonal antibodies anti-phospho-Erk1/2^{T202/Y204} (#4370; 1/10000) from Cell Signaling Technology (CST; MA, USA) and with rabbit polyclonal antibodies anti-total Erk1/2 (#06-182; 1/50000) from Millipore (MA, USA). CST also provided the secondary antibody: anti-rabbit IgG, HRP-linked antibody (#7074, 1/5000). The signal was then revealed following chemiluminescent detection (#WBLUF0100, Immobilon Forte Western HRP Substrate, Millipore, USA). The X-ray films (BioMax MR film, Kodak, USA) used to detect the chemiluminescent signal were quantified by densitometry (optical density; OD) using a GS-800 scanner coupled with Quantity One software (Bio-Rad, CA, USA).

2.4. AlphaLISA analysis

p-Erk1/2^{T202/Y204} (ALSU-PERK-A500), total Erk1/2 (ALSU-TERK-A500), p-Akt1/2/3^{T308} (ALSU-PAKT-A500), p-Akt1/2/3^{S473} (ALSU-PAKT-B500), p-CREB^{S133} (ALSU-PCREB-A500), GAPDH (ALSU-TGAPD-B500) and Cofilin (ALSU-TCOF-A500) levels were quantified using AlphaLISA SureFire Ultra kits according to PerkinElmer's instructions. Briefly, 10 µl/well of diluted protein samples were transferred to an OptiPlate-384 white opaque microplate (PerkinElmer, USA) and 5 µl of acceptor mix (containing both antibodies and Acceptor beads) were added to wells. The plate was sealed with a clear adhesive film and incubated for 1 h at room temperature. Then 5 µl of donor mix (containing Donor beads) were added to wells under subdued light. The plate was sealed again with a clear adhesive film, covered with foil and incubated for 1 h at room temperature in the dark. Controlled temperature and low light intensity (< 100 lx) are two major parameters to consider (see Results; Fig. 2C). Finally, the plate was read with an Alpha technology-compatible plate reader (EnSpire Alpha plate reader, PerkinElmer) using default AlphaLISA settings. Alpha measurements were performed in duplicates: each sample was systematically transferred in two independent wells and the mean Alpha signal value of both wells was used. Raw data represent the luminescence intensity of the Alpha signal and are expressed as a number of counts. For the Alpha experiment performed with *STHdh*^{Q7/Q7} cells samples, 10 µl of the cell lysates diluted in lysis buffer were used to measure GAPDH. For those made from brain homogenates, the samples were diluted in lysis buffer at the desired concentration: 0.62 µg of total proteins in 10 µl of lysis buffer was used to measure p-Erk1/2^{T202/Y204} and total Erk1/2, 1.25 µg of total proteins in 10 µl of lysis buffer were used to measure Cofilin, and 2.5 µg of total proteins in 10 µl of lysis buffer were used to measure p-

Akt1/2/3^{T308} and p-Akt1/2/3^{S473}.

2.5. In vitro experiment

To check the validity of the GAPDH AlphaLISA kit with cell lysates from a mouse cell line, the *STHdh*^{Q7/Q7} neuronal-derived mouse brain striatal cell line (Coriell Institute, # CH00097) was used (Trettel et al., 2000). *STHdh*^{Q7/Q7} cells were seeded in a 96-well culture plate at the appropriate concentration (20,000 cells/well) in fresh medium (DMEM (#61965, Gibco, USA) + 10 % FBS (#10270, Fisher Scientific, USA) with Penicillin/Streptomycin (#15140, Gibco, USA) and 0.4 mg/ml G418 (#11811, Gibco, USA)) and allowed to grow at 33 °C with CO₂ (5 %). The day after plating, the cells were deprived of FBS, penicillin, streptomycin, and G418 and maintained in 100 µl of DMEM. After 24 h of deprivation, the culture medium was removed and the cells were lysed with 100 µl/well of Alpha lysis buffer supplemented with protease and phosphatase inhibitors (Sigma, USA). The plate was agitated on a plate shaker (350 rpm) for 10 min at room temperature and samples were stored at –80 °C for further Alpha experiments.

2.6. Data analysis

All experiments involving mice were performed according to the protocols approved by the Aquitaine-Poitou Charentes local ethical committee (authorization number APAFIS#10936-201708091043277 v5) in strict compliance with the French Ministry of Agriculture and Fisheries (authorization number A33-063-98) and European Communities Council Directive (2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of mice used, while maintaining reliable statistics. Student's *t*-test was used for pairwise comparisons. A significance level of *p* < 0.05 was used for all statistical analyses. Statistical significance was expressed as * = *p* < 0.05; ** = *p* < 0.01. All values were expressed as mean ± s.e.m.

3. Results

3.1. The principle of Alpha technology

Alpha (Amplified Luminescent Proximity Homogeneous Assay) technology developed by PerkinElmer™ includes both AlphaLISA and AlphaScreen assays (Eglen et al., 2008). The AlphaLISA SureFire Ultra technology used in this study is specifically dedicated to the detection of protein phosphorylation. It is a high-throughput technique based on a sandwich immunoassay that allows the rapid, sensitive and semi-quantitative detection of endogenous phosphoproteins in tissue homogenates and cell lysates. It is a homogeneous assay which means that it is a simple mix and read procedure without any washing step. It relies on Alpha beads (Acceptor and Donor beads) capable of forming a complex with the protein of interest. Streptavidin-coated Donor beads bind a biotinylated antibody that, in turn, binds the target protein in an unphosphorylated epitope. CaptSure®-conjugated Acceptor beads bind another antibody that binds the target protein either on the phosphorylated epitope (for the detection of phosphoproteins) or another unphosphorylated epitope (for the detection of total proteins). In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads with red light at 680 nm generates singlet oxygen molecules that trigger a cascade of energy transfer in the Acceptor beads resulting in light emission when beads are close to each other (within about 200 nm). The amount of emitted light at 615 nm is proportional to the protein content (Fig. 1A).

One of our methodological objectives is to be able to measure many signaling pathways in brain lysates from large experimental groups. These two parameters, namely the number of measured phosphoproteins and the number of samples, make it difficult to use the Western blot technique. Even though it is the most commonly used technique for measuring signaling pathways from *in vivo* samples, it is time-

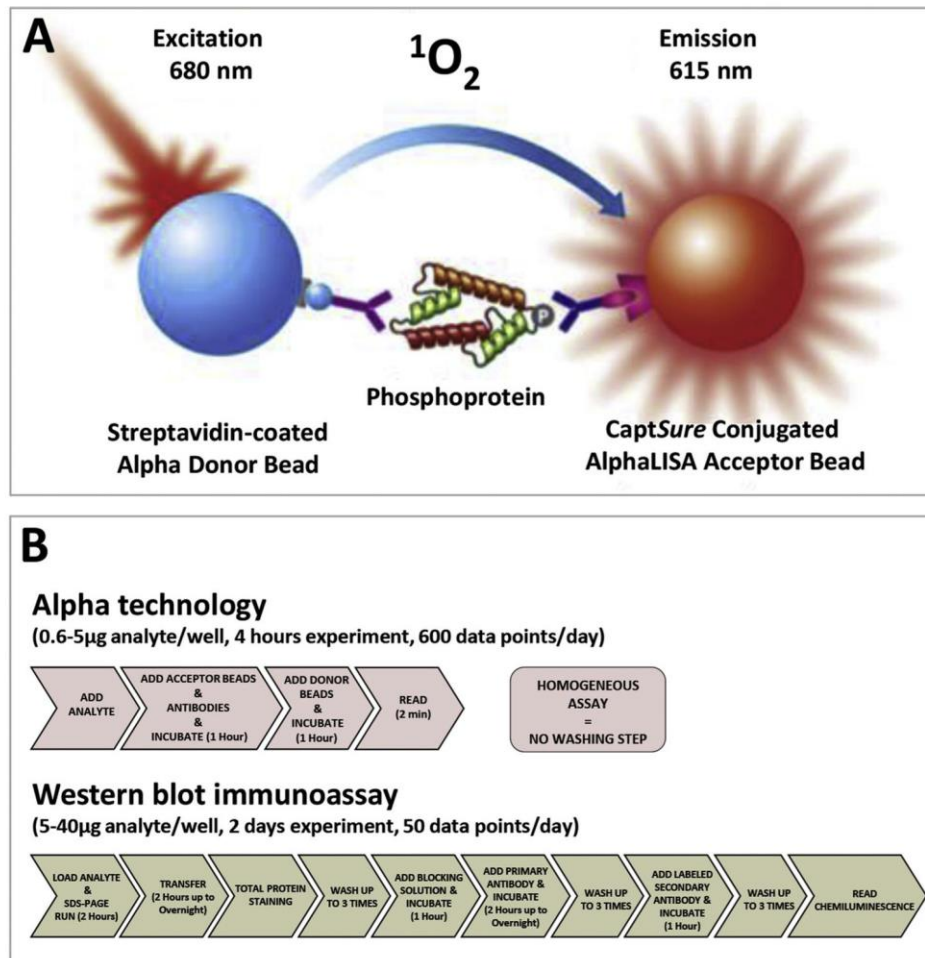


Fig. 1. (A) Schematic representation of the principle of Alpha technology for the quantitative detection of phosphorylated proteins. The sandwich immunoassay is based on streptavidin-coated Alpha Donor beads that bind biotinylated antibodies and on CaptSure®-conjugated AlphaLISA Acceptor beads that immobilize the other selective antibodies, labeled with a CaptSure® tag. When the two selective antibodies bind the phosphoprotein, the Donor and Acceptor beads are brought in close proximity and a cascade of chemical reactions acts to produce a greatly amplified signal. On laser excitation at 680 nm, the Donor bead converts ambient oxygen to singlet oxygen molecules (1O_2) which react with the Acceptor bead resulting in light emission then detected by the Alpha-enabled plate reader. The amount of light emission at 615 nm is directly proportional to the amount of phosphoprotein present in the sample. (B) Comparison of the different methodological steps between Alpha technology and Western blot technique. The experimental requirements and relative performance of each method are presented.

consuming, including many washing steps, and consumes large quantities of biological materials, which is a major problem given the anatomical complexity of brain tissue, which consists of many small brain areas producing a low yield (Fig. 1B). On the other hand, Alpha technology displays all the characteristics to provide quick, efficient and reliable answers (Fig. 1B).

Although Alpha technology was recently used in several bibliographic articles, the measurement of signaling pathways is often restricted to *in vitro* studies (Anavi-Goffer and Ross, 2016; Gauthier et al., 2012) and it has not been used to date for *in vivo* lysates from brain structures.

3.2. Methodological considerations

3.2.1. Protein extraction and quantitation

Alpha experiments, but this also applies to the Western blot technique, are used to measure translational (synthesis, degradation) or posttranslational (e.g. phosphorylation, acetylation, methylation) changes that differentiate experimental groups. For these comparisons to be meaningful, the quantity of starting material must be equivalent between the groups (e.g. same concentration, same number of cells for *in vitro* studies, etc.). An initial consideration is to know how much protein there is in each sample. For the purpose of this study brain tissue lysates were prepared following the same procedure as for Western blot but we replaced the usual lysis buffer with the AlphaLISA lysis buffer. Briefly, we dissected several brain areas (prefrontal cortex, nucleus accumbens, striatum and hippocampus). Proteins were then mechanically extracted in AlphaLISA lysis buffer and total protein

quantitation was assessed using the Direct Detect infrared spectrometer. This quantitation system, which provides accurate results and only requires 2 µl of samples, appeared to be fully compatible with the Alpha buffer (Fig. 2A).

3.2.2. Linear range

When establishing the Alpha assay for tissue lysates, it is important to calibrate the samples such that they are in a linear and responsive range. Indeed, within this range, the Alpha signal which is generated (i.e. the number of counts) is directly proportional to the amount of the measured protein and this is a requirement in order to produce accurate and reproducible results. This is also a concern with the Western blot technique. It is therefore important before comparing experimental conditions to ensure that all the tested proteins, whether they are phosphorylated or not, produce linear responses. This linearity test is performed for all the different targets. To determine the specific amount of total protein per well for each target protein, the experiment consists of a serial dilution of the sample, by diluting the sample by half, starting from 10 µg of proteins per well down to 0.31 µg per well. Under these conditions, the emitted Alpha signal must follow a linear range. The data presented in Fig. 2B provide an example of linearity assessment experiments for the targets p-Erk1/2^{T202/Y204}, p-Akt1/2/3^{T308} and p-Akt1/2/3^{S473}. If the quantity is too high (e.g. for 10 and 5 µg/well), the emitted Alpha signal is affected by the presence of interfering components in the sample matrix; if the quantity is too low it generates signals that are too low. The chosen quantities always lie within the linear range. For example, we used 0.62 µg of total proteins per well to test p-Erk1/2^{T202/Y204} and 2.5 µg for p-Akt1/2/3^{T308} and p-Akt1/2/3^{S473}

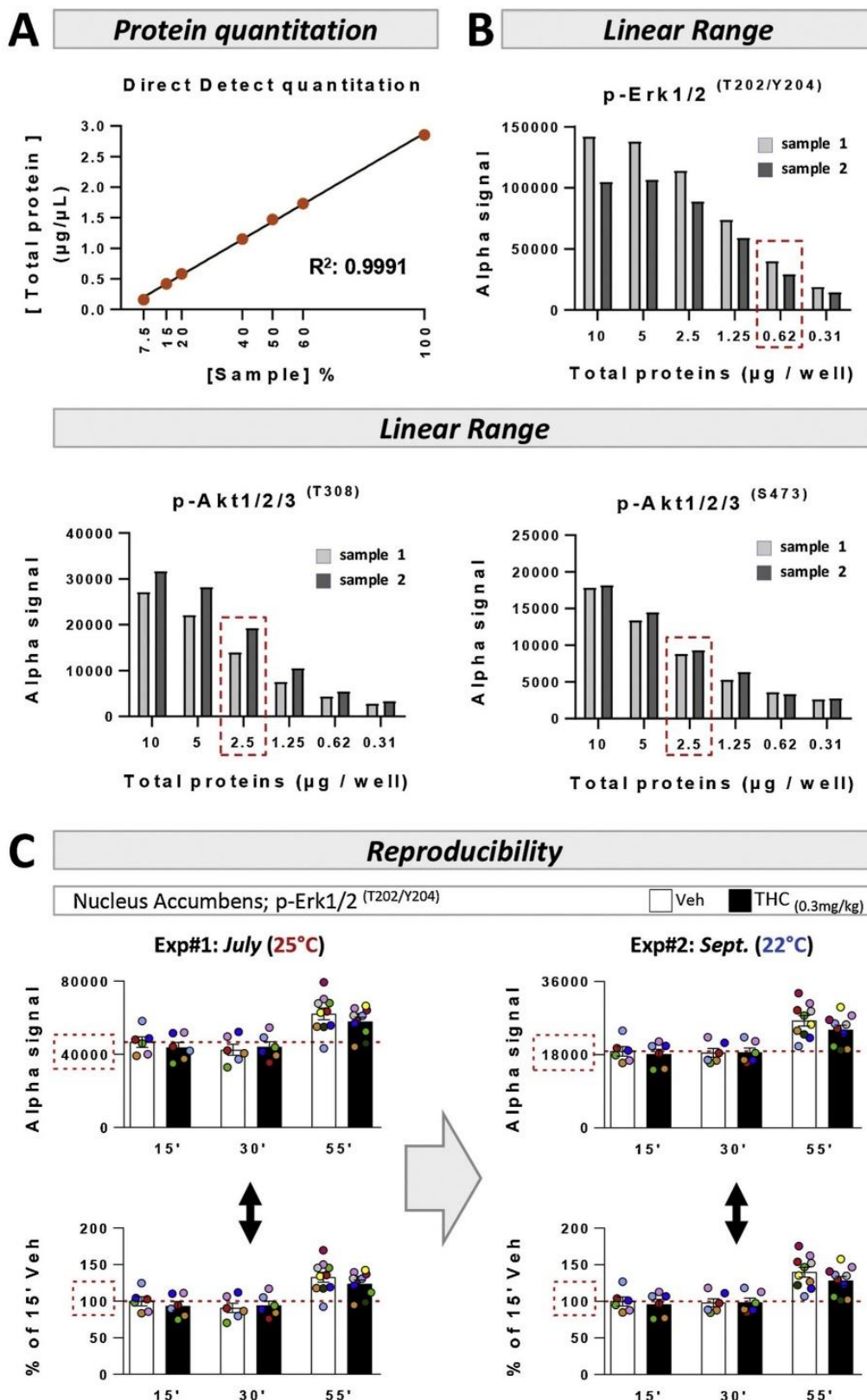


Fig. 2. Methodological considerations when using Alpha technology. (A) Validation of the total protein quantitation method in brain homogenates extracted in Alpha lysis buffer. Example of calibration curve from a serial dilution of a CD1 mouse prefrontal cortex sample to quantify total proteins using Direct Detect infrared spectrometer. (B) Example of linearity assessment experiments for three phosphorylated targets: p-Erk1/2^{T202/Y204}, p-Akt1/2/3^{T308} and p-Akt1/2/3^{S473}. Serial dilutions ranging from 10 µg down to 0.31 µg of total proteins/well from prefrontal cortex protein sample extracts of C57BL/6N mice were analyzed. The chosen quantity (framed in red) lies within the linear range. (C) Reliability also depends on the reproducibility of Alpha experiments. Example of experimental reproducibility for measuring p-Erk1/2^{T202/Y204} target using the same samples at 3-month interval at two different ambient temperatures (25 °C versus 22 °C) from nucleus accumbens protein extracts of C57BL/6N mice treated with THC 0.3 mg/kg or its vehicle and sacrificed at three time points: 15 min (n = 6 per group), 30 min (n = 6 per group), and 55 min (n = 10 per group). Plotted values are means ± sem.

targets (Fig. 2B). Remarkably, we observed that for a same target protein we can use the same quantity of total proteins per well whatever the mouse brain structure.

3.2.3. Reproducibility

Alpha experiments must be reproducible in order to generate reliable results from one experiment to another. To check the reproducibility of our Alpha experiments we used the same samples in two

independent Alpha experiments in which both 1h-incubation periods were done at room temperature according to the kit protocol. We tested samples from 6 experimental groups in July at an ambient temperature of 25 °C (Fig. 2C; Exp#1). The results were presented as raw data (directly expressed in number of counts) or as a percentage of Group 1 (=15' Veh). The same samples were frozen again and retested 3 months later in September (Fig. 2C; Exp#2) at a temperature of 22 °C. The results were also expressed in counts or as a percentage of Group 1 (=15' Veh). The data show that the Alpha signal is temperature-dependent which results in a raw count variations if the temperature of incubation is not kept consistent. However, if the values are expressed as a percentage of the first group (=15' Veh), the relative profile of variation from one experimental group to another is highly reproducible (Fig. 2C).

3.2.4. Normalization

Because of potential experimental biases (e.g. pipetting, variations in concentration measurements), it is important to minimize the effect of loading variations by ensuring that the measurement of the protein of interest can be compared to an internal control such as a housekeeping protein. While there is a wide range of housekeeping proteins available for Western blot experiments (Theillin et al., 1999; Liu and Xu, 2006), the offer is currently very limited for Alpha technology and only three kits, two for measuring GAPDH and one for Cofilin, have been developed by PerkinElmer. One of the GAPDH kits only recognizes human GAPDH so it cannot be used with our murine samples. The second kit is human- and mouse-reactive. It has been tested with *in vivo* murine brain tissue (nucleus accumbens: NAc) lysates (Fig. 3A). As shown in Fig. 3A we were unable to produce a linear signal over a wide range of concentrations so it is not suitable for normalizing brain samples (Fig. 3A). We checked the validity of the kit with *in vitro* lysates from a mouse cell

line, namely *STHdh*^{Q7/Q7} cells (Fig. 3B). The results clearly show that the GAPDH kit can be used to normalize *in vitro* experiments but that the cell lysates should be highly diluted (Fig. 3B). The other kit measuring the Cofilin protein allowed us to determine a linear range from a serial dilution of mouse brain tissues (e.g. Prefrontal Cortex: PFC) (Fig. 3C) so it can be used to normalize our *in vivo* experiments. In cases where the total protein corresponding to the studied phospho-target is available, it can be a substitute for the standard housekeeping protein. For instance total Erk1/2 can be used to normalize raw p-Erk1/2^{T202/Y204} results (Fig. 3D).

3.3. Application of Alpha technology

The validation of our approach first consisted in reproducing with Alpha technology an already published experiment that aimed to measure by immunohistochemistry the activation of the Erk1/2 pathway in the striatum of male CD1 mice in response to an acute injection of cocaine (Valjent et al., 2000). Ten minutes after the intraperitoneal administration of cocaine (20 mg/kg), the striatum was sampled, lysed in AlphaLISA lysis buffer and the pattern of Erk1/2 activation was analyzed by Alpha technology. Our data confirm those previously obtained (Valjent et al., 2000) showing a significant increase in Erk1/2 protein phosphorylation in response to cocaine injection, while there was no difference in total Erk1/2 between the two groups (Veh vs Cocaine) (Fig. 4A). Thus Alpha technology can be used to measure *in vivo* signaling events induced by a pharmacological treatment. We then applied this technology to measure genotype-dependent signaling modifications using a mouse model of genetic disease: the Down syndrome (DS) mouse model Ts65Dn. These mice are trisomic for about two-thirds of the genes orthologous to human chromosome 21 and are a well-characterized model for studying cognition-related

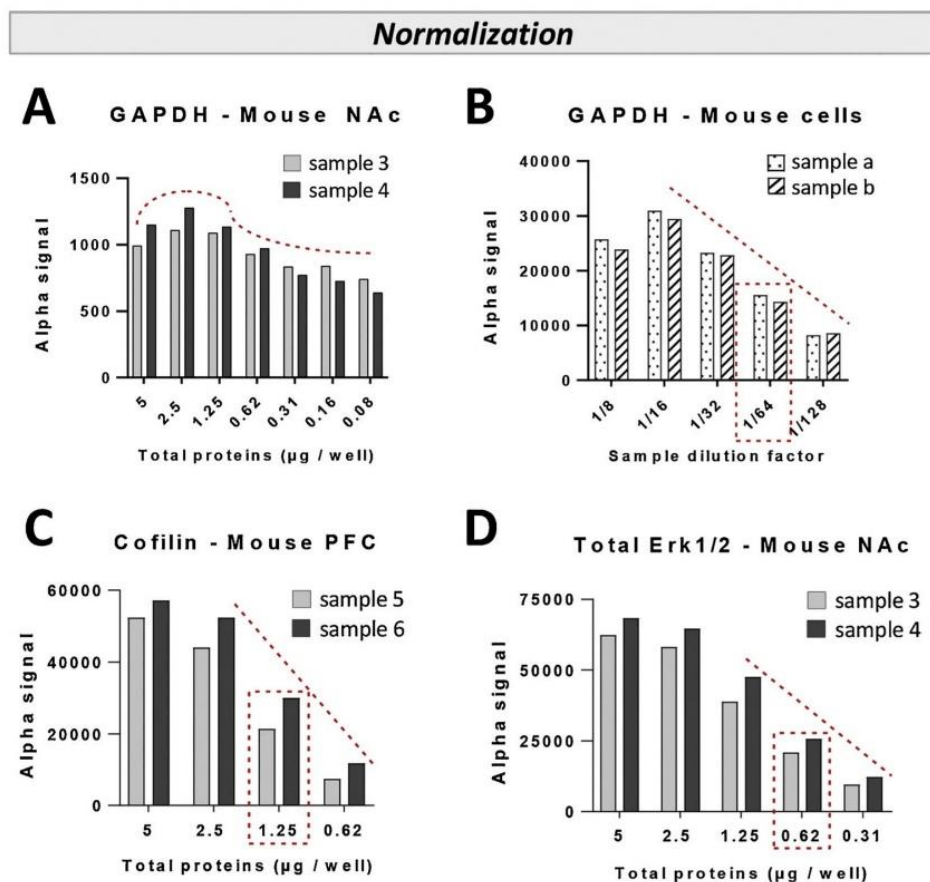


Fig. 3. Normalization of each sample to a housekeeping protein minimizes experimental bias. (A) Mouse brain samples cannot be normalized with GAPDH. Example of linearity test for measuring GAPDH either from *in vivo* (5 µg down to 0.08 µg) (A) or *in vitro* (range from 1/8 down to 1/128 dilution) (B) samples. The results show that the Alpha assay is not suitable for measuring GAPDH from *in vivo* nucleus accumbens protein extract samples of C57BL/6 N mice (A) while it can be used to measure the same protein from *in vitro* *STHdh*^{Q7/Q7} murine cell samples (B). (C) Mouse brain samples can be normalized with Cofilin. Example of linearity test (5 µg down to 0.62 µg) for measuring Cofilin from *in vivo* prefrontal cortex (PFC) protein sample extracts of C57BL/6 N mice. (D) Alternatively the phosphorylated target can be normalized with its corresponding total protein when available. Example of Alpha measurement of total Erk1/2 from *in vivo* nucleus accumbens protein extract samples of C57BL/6 N mice.

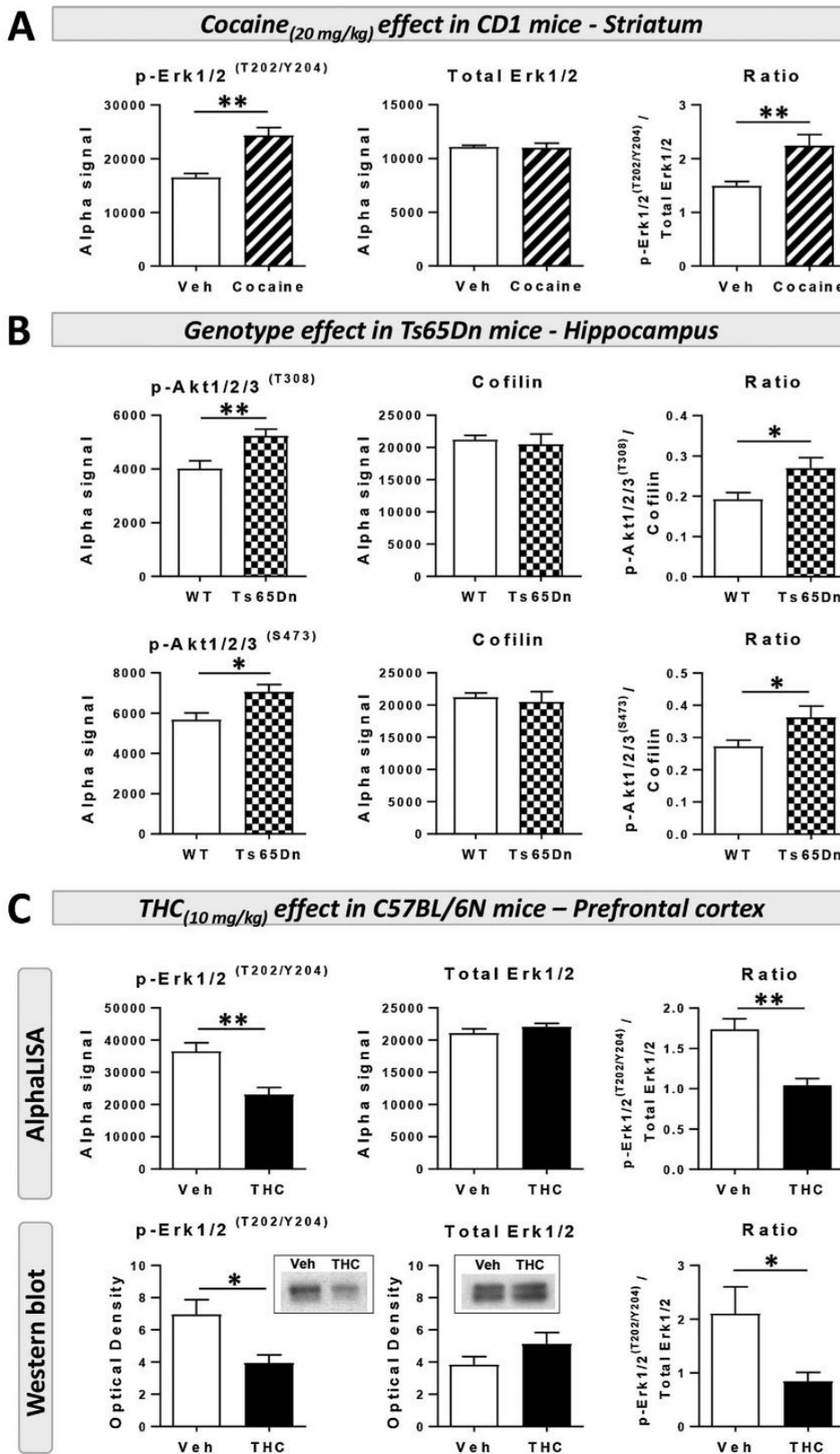
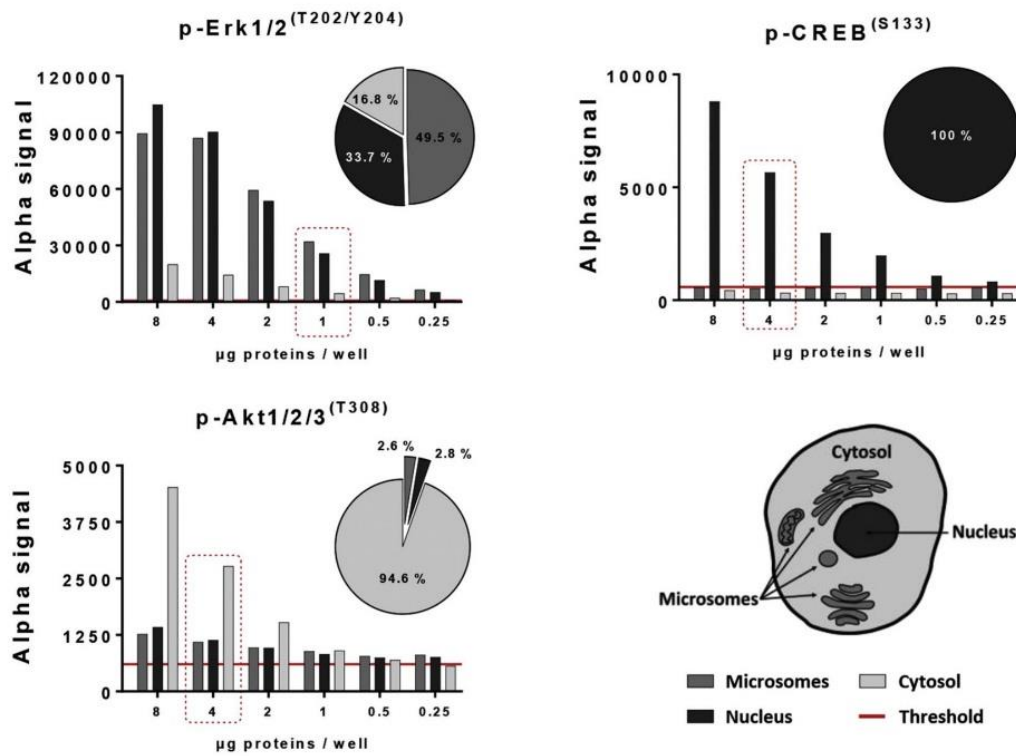


Fig. 4. *In vivo* validation of Alpha technology using protein extracts from different brain structures from several mouse strains. (A) Cocaine effect in the striatum of CD1 mice. Alpha measurement of p-Erk1/2^{T202/Y204}, total Erk1/2 and ratio calculation (p-Erk1/2 over total Erk1/2) from striatum protein extracts of CD1 mice treated with cocaine (20 mg/kg; *ip*; n = 6) or its vehicle (Veh; n = 6). (B) Genotype effect in the hippocampus of Ts65Dn mice. Alpha measurement of p-Akt1/2/3^{T308}, p-Akt1/2/3^{S473}, total cofilin and ratio calculation (p-Akt1/2/3 over total cofilin) from hippocampus protein extracts of Ts65Dn mice (n = 9) and their control littermates (WT; n = 9). (C) THC effect in the prefrontal cortex of C57BL/6 N mice. Comparison of Alpha and Western blot measurements of p-Erk1/2^{T202/Y204}, total Erk1/2 and ratio calculation (p-Erk1/2 over total Erk1/2) from the same prefrontal cortex protein extracts of C57BL/6 N mice treated with THC (*ip*; 10 mg/kg; n = 6) or its vehicle (n = 6). For Alpha experiments, 0.62 µg of total proteins per well were used while 10 µg of total proteins per well were loaded for Western blot analyses. Representative X-ray autoradiographies are shown. Plotted values are means + sem. *p < 0.05; **p < 0.01 in comparison to control groups (Veh or WT).

deficits (Reeves et al., 1995). The signaling events were studied in the hippocampus, which is the main brain structure for the encoding of memory (Revest et al., 2005; Revest et al., 2014; Revest et al., 2010). We targeted the Akt pathway, which has been shown to be activated in

trisomic patients and Ts65Dn mice (Troca-Marin et al., 2012). As expected, we found a significant increase in phosphorylated Akt1/2/3 on threonine 308 and serine 473 both in raw data and after normalization with Cofilin in the hippocampus of Ts65Dn mice compared with

A AlphaLISA detection after cell fractionation



B AlphaLISA detection after synaptosomal preparation

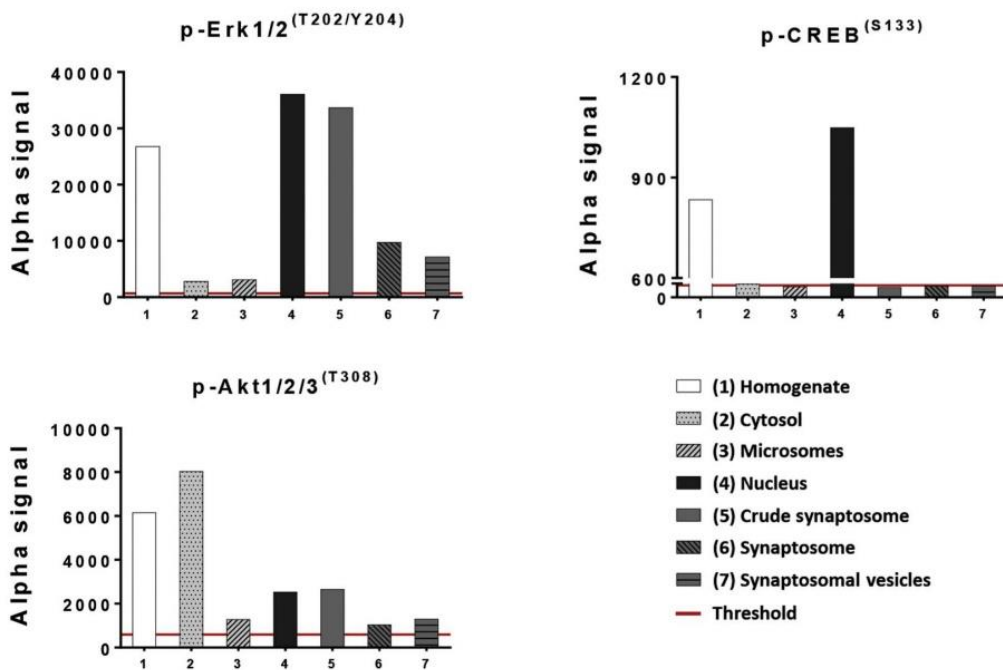


Fig. 5. *In vivo* validation of Alpha technology using protein extracts obtained after cell fractionation and synaptosomal preparations of mouse hippocampus. (A) Alpha measurement of p-Erk1/2^{T202/Y204}, p-Akt1/2/3^{T308} and p-CREB^{S133} after cell fractionation from hippocampus of C57BL/6 N mice (n = 2). Pie chart represents the relative distribution (expressed as a percentage) of each phosphoprotein in the different cell compartments, corrected by the amount of protein found within each compartment (microsomes 21 %, nucleus 18 %, cytosol 61 %). (B) Alpha measurement of p-Erk1/2^{T202/Y204}, p-Akt1/2/3^{T308} and p-CREB^{S133} after synaptosomal preparation obtained from a pool of 12 adult mouse hippocampus. Red line represents the threshold (signal obtained with buffer only).

controls (Fig. 4B).

We also conducted an experiment on male C57BL/6 N mice that received an intraperitoneal injection of THC (10 mg/kg), a CB1 phytocannabinoid agonist known to modulate the Erk1/2 signaling pathway (Busquets-Garcia et al., 2018). Mice were sacrificed 30 min after the injection. The prefrontal cortex was sampled and Erk1/2 phosphorylation levels were measured either by AlphaLISA or by Western blot (Fig. 4C). The overall data were comparable and showed a decrease in Erk1/2 phosphorylation in response to THC, notably with a lower intragroup variation and better significance with Alpha (Fig. 4C). Interestingly the decrease of Erk1/2 phosphorylation in response to THC suggests that the basal signal in the "Veh" condition is not just non-specific signal. It could reflect the endocannabinoid response triggered by basal levels of 2-AG and Anandamide (AEA) on CB1 receptor (Lu and Mackie, 2016; Busquets-Garcia et al., 2018; Di Marzo and De, 2012) or/and CB1 potential constitutive activity (Canals and Milligan, 2008; Costa and Cotecchia, 2005). These results also demonstrated the compatibility of the Alpha lysis buffer with the Western blot technique.

We finally conducted experiments to assess protein phosphorylation after cell fractionation (Fig. 5A) and synaptosomal (Fig. 5B) preparations. Both these preparations yield low quantities of biological materials. The hippocampus of male C57BL/6 N mice sacrificed in basal condition was sampled. We applied a cell fractionation protocol to measure the expression of phosphorylated proteins by AlphaLISA in the three main cell compartments, namely the nucleus, microsomes and cytosol (Fig. 5A). The percentage of each phosphoprotein (p-Erk1/2^{T202/Y204}, p-Akt^{T308}, p-CREB^{S133}) in each cell compartment was calculated within the linear range; namely 1 µg for p-Erk1/2^{T202/Y204}, and 4 µg for both p-CREB^{S133} and p-Akt^{T308}. The results showed a clear detection and distinct distribution of each phosphoprotein. In accordance with the literature, p-Erk1/2^{T202/Y204} is distributed in the three compartments (Shuaib et al., 2016; Brown and Sacks, 2008), p-CREB^{S133} is exclusively nuclear as expected for a transcription factor (Shaywitz and Greenberg, 1999) while p-Akt^{T308} is predominantly cytosolic (Manning and Toker, 2017) (Fig. 5A). We also measured phosphoprotein levels from synaptosomal preparations of mouse hippocampus (Fig. 5B). Consistently, we demonstrated that Erk1/2, Akt and CREB phosphoproteins were distinctly distributed. In particular, p-Erk1/2^{T202/Y204} is found in several fractions, but mainly in the nucleus and crude synaptosomes (synaptosomes and vesicles) and at lower levels in the cytosol and microsomes. P-CREB^{S133} is exclusively nuclear while p-Akt^{T308} is mainly distributed in the cytosol (Fig. 5B). These results also demonstrate that dedicated buffers other than Alpha buffer are compatible with Alpha technology.

4. Discussion

The overall goal of our research is to characterize the biological mechanisms that underlie the shift from physiological behavior to pathological behavior, particularly for addiction and cognitive deficits targeting the CB1 receptor and for stress-related diseases, and especially traumatic memories (Revest et al., 2005; Revest et al., 2014; Revest et al., 2010; Sarrazin et al., 2009; Vallee et al., 2014). These behavioral transitions presuppose molecular changes within the cellular environment. In these processes, changes in phosphorylation states play a major role. However, understanding the molecular mechanisms underlying these brain dysfunctions is a particularly complex process related to the heterogeneous nature of the brain, which is composed of many specific functional structures (Ecker et al., 2017; Jones et al., 2009). In this context, innovative technologies are needed that are not very demanding in terms of biological materials and which allow the rapid analysis of a large number of signaling pathways. Although used in other areas of biology (Eglen et al., 2008; Anavi-Goffer and Ross, 2016; Gauthier et al., 2012), Alpha technology has not been used until now to study brain signaling pathways. To date, the majority of studies addressing brain signaling have used the Western blot technique,

particularly because it has an almost unlimited number of targets at its disposal.

Our current findings show that Alpha technology is particularly suitable for measuring *in vivo* intracellular signaling pathways from individual mouse brain tissues (e.g. prefrontal cortex, nucleus accumbens, striatum, and hippocampus) homogenates but also from cell fractionation and synaptosomal preparations of mouse hippocampus. The protocol is simple and fast since sample processing and washing steps are not necessary, and it can even be automated. Furthermore, it has three main advantages compared to the Western blot technique. First, it requires very little biological material preserving our precious samples. In our hands, about 8-fold less biological material was needed, whatever the targets. This is a major advantage when addressing the rodent brain, which comprises many small structures. In addition, the Alpha lysis buffer is fully compatible with the Western blot technique, so the amount of biological material required is minimized because the same samples can be used in both techniques. The second advantage is that the throughput is substantially increased. For example, while the number of data points per week per experimenter is about 250 with the Western blot technique, it is around 3000 with Alpha, i.e. a 12-fold increase in throughput, with excellent reproducibility. Finally, considering our relatively high throughput (around 500 data points/week), Alpha technology is cost-effective. The costs of Alpha reagents and other consumables are inversely proportional to the throughput, i.e. they decrease as the throughput increases. Even at low throughput, the overall price is quite similar when comparing with Western blot in particular if we include the salary of the experimenter. Indeed processing 600 samples by Western blot takes about 3 weeks on a full-time basis whereas it is easily completed in one day with Alpha technology (Fig. 1B). The latter also offers the possibility of developing and customizing new targets. Alpha technology allows the rapid analysis of the main key pathways, and then since the number of phosphorylated targets validated for Western blot technology is higher, it becomes possible to fine-tune the signaling with Western blot.

In conclusion, Alpha technology, in association or not with the Western blot technique, represents a major step forward in unraveling the brain phosphoprotein-related molecular mechanisms involved in brain-related disorders.

Author contributions

Conception and design of experiments: MZ, GT, JMR. Performed the experiments: MZ, GT, VRL, LB, MM, MV, AG, JMR. Acquisition of data, analysis and interpretation of data: MZ, GT, GM, NS, JMR. Wrote the paper: MZ, GT, JMR.

Declaration of Competing Interest

Authors declare no competing interests and personal relationships with other people or organizations that could inappropriately influence their work.

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PREG-like AEF0117 blocks the THC-induced GSK3 β -dependent hyperlocomotion in mice.

Article in preparation for publication to Current Biology

Inhibition of the effect of a low dose of THC on behavior and CB1 intracellular signaling pathway in mice by the pregnenolone derivative, AEF0117.

A signaling cascade is a complex event that takes place after that an agonist ligand binds a receptor. The agonist, binding the receptor, gives information that the receptor needs to send to the downstream effectors in the cell to trigger a response (Marks, 2008). However, being one protein only, the receptor would not be able to recruit the effectors in quantity needed to have a significant output. For this reason, between the receptor and the effectors, there is a sophisticated machinery, usually, phosphoproteins named kinases, which amplify the signal exponentially and gain a sufficient response on the effectors (e.g., one receptor-activated two proteins, each protein activates two proteins and so on. In the end several hundred if not thousands of proteins activates the effectors) (Li and Qian, 2003). This machinery, usually made of protein kinases, works as binary code: proteins can be phosphorylated or not, usually corresponding to the 0 and 1 (Marks, 2008; Weber et al., 1999). Studying signaling cascade is a challenging task: correctly identifying a signaling cascade through the semiquantitative analytical detection of phosphoproteins usually requires a certain amount of material and time to analyze the selected targets. This is especially true when the signaling cascade are analyzed in samples obtained from animal tissue, and even more if they come from animal small brain areas. The most common technique used worldwide for this purpose is the western blot that has been proved to be adequate for the task (Pillai-Kastoori et al., 2020; Renart et al., 1979): a technique precise and reproducible enough to gain consistent data in all kind of laboratories around the world. However, the amount of total proteins obtained from mouse brain tissue lysates is scarce when not insufficient to run several tests with the western blot technique. To overcome this problem, it could be possible to use many animals to analyze different targets. Still, in this case, the resulting data would be less robust, not to mention the ethical issue of using too many animals. Another problem is the time that western blot can require to perform

a proper screening. In one day of work, it is possible to obtain an average of approximately forty data-points, working in duplicates, which means twenty samples, a number not sufficient for this project aims. Furthermore, the western blot, although being a very trustworthy technique, present a high variability between the different wells since there are several very delicate steps (such as the membrane washes) and critical moments (such as the running and the washes steps) that can contribute to the partial loss of protein, or an incorrect migration (Beaudet et al., 2008). So the most crucial technical improvement in brain tissue lysates phosphoprotein screening is to reduce the amount of sample needed for each analysis and the consumption of time, increasing the throughput, and, if possible to improve the reproducibility diminishing the most critical steps. For this reason, we have decided to use a new technique that would allow the rapid, sensitive, reproducible, and semiquantitative detection of phosphoproteins in brain tissue lysates. In the market, however, such a method was not still fine-tuned for brain tissue lysate analysis. So we have decided to turn to the Alpha technology, particularly into the AlphaLISA technique, a technique already used in drug discovery and cell culture analyses (Beaudet et al., 2008). We decided to fine-tune the technique ourselves, adapting it for brain tissue lysates (Beaudet et al., 2008).

Among the several cannabis-related pathologies, the most common is the cannabis use disorder (CUD) that causes, among other symptoms, cognitive deficits, strong dependency, and paranoia (Agrawal et al., 2014; Hall and Degenhardt, 2008; Hayley et al., 2017), while the most severe effect is psychosis (S. Andréasson et al., 1987; Di Forti et al., 2014; D'Souza et al., 2016, 2009; Favrat et al., 2005). Nowadays, all the strategies for the treatment of CUD treatment have been focused on alleviating the withdrawal without direct interactions with the ECS (e.g., anxiolytics, anti-depressants, mood stabilizers) (Weinstein and Gorelick, 2011), while for THC-induced psychosis, there is, so far, no specific treatment. In animal models, pregnenolone (PREG) was able to rescue several cannabinoid-related behaviors, including WIN 55,212-2 or THC self-

administration in mice or monkeys, respectively (Piazza et al., 2013; Vallée et al., 2014), and several THC-induced positive and negative psychotic-like behaviors (Busquets-Garcia et al., 2017b), such as hyperlocomotor and asocial behavior, respectively. Since PREG can show steroid-related side effects (Vallée, 2016), it was developed a PREG-like class of compounds, the CB1 Signaling Specific inhibitors (Piazza et al., 2013, 2012), among which the AEF0117 showed a strong therapeutic potential for CUD and cannabinoid-induced psychosis, which is currently in phase 2 of clinical trial for CUD (ClinicalTrials.gov Identifier: NCT03717272). AEF0117 was proven to be able to rescue a wide variety of THC-induced behaviors, including memory deficits, cannabinoid self-administration, and asocial behavior (Piazza et al., 2013).

The aim of the current work, that we plan to send to *Current Biology* and formatted accordingly, is to assess the cannabinoid-induced signaling pathway that leads to the hyperlocomotor activity following a low dose of THC in mice and to apprehend if AEF0117 rescues THC-induced behavior and related CB1 signaling pathway, that will help to understand the mechanisms of action of AEF0117 in animal models of addiction and psychosis that can be translated to humans. The importance of focusing on a low dose of THC (0.3 mg/kg in mice) is that in animal models, low THC doses are rewarding, while high doses are aversive (Katsidoni et al., 2013), and the former may indeed simulate the dose of THC in an addiction paradigm. Low doses of THC stimulate the locomotor activity in mice (Busquets-Garcia et al., 2017b), which is often considered predictive of both addiction and positive psychosis (Busquets-Garcia et al., 2017b; Mori et al., 2014). Furthermore, even if it is hard to assess a parallelism between THC doses in humans and rodents. It has been demonstrated that during marijuana smoking session, independently from the THC content within the cannabis cigarettes, cannabis users usually adapt the inhalation frequency and depth to gain constant doses of THC (Casajuana Kögel et al., 2017; van der Pol et al., 2014). Cannabis consumers' preference towards low doses of THC

seems to reflect the aforementioned biphasic effect of THC on reward and aversion in rodents (Katsidoni et al., 2013).

Identifying the THC-induced CB1 signaling that leads to behavioral hyperlocomotion is the crucial first step towards a better understanding of the mechanism of action of PREG and AEF117 and, by extension, its lead compound, PREG.

To this end, our strategy was first to understand the kinetic effects of THC on the CB1 signaling pathway and which brain areas were involved and then to see the impact of PREG and AEF0117 on the most representative time effect for THC.

In the first experiment, we treated mice with THC at the dose of 0.3 mg/kg and 15 or 30 minutes after injection mice were sacrificed, and their brain areas withdrew for further analyses, while another group of mice was subjected to locomotor activity in a novel environment test 45 minutes after injection.

Alphalisa data in the first experiment shows mainly that the phosphorylation of Akt proteins (on the epitopes Ser473 and Thr308) and GSK3 β protein (on the epitope Ser9) was increased within the striatum, nucleus accumbens, and cerebellum 30 minutes after THC injection (see Fig 1c and d in the article and Fig 33a for a schematic summary). These data strongly suggest that the Akt-GSK3 β pathway was related to THC-induced hyperlocomotion. Then, to further prove a causality link between both of them, we used a PI3K (a protein upstream to Akt) inhibitor, GDC-0084, which rescued both THC effects on Akt-GSK3 β signaling and locomotor behavior (see Fig 2 in the article and Fig. 33b for a schematic summary), without showing significant per se effects on GSK3 β phosphorylation or locomotor activity. To deeply understand CB1-related molecular effectors, we then studied the signaling transducer recruited by CB1 receptor to carry on the Akt-GSK3 β pathway. Understanding which CB1 signaling transducer is recruited following low THC doses is essential to understand the bias between the

CB1 signaling. Indeed it has been demonstrated that CB1 signaling is biased (Ibsen et al., 2017). This means that different signaling pathways are activated under different circumstances as a consequence of vary signaling transducer recruitment (see for review, Smith et al., 2018). First, we decided to use an in vitro model. We have chosen the STHdhQ7/Q7 cell line, a murine striatal cell line model that endogenously expresses CB1 receptor. However, we were not able to see any alteration of the Akt-GSK3 β signaling following THC treatment. By looking at the gene expression profile of this cell line, we discovered that this cell line had a CB1 biased system: β -Arrestin 1 was ~50 times less expressed than the Gi protein and was ~5 times less expressed than the β -Arrestin 2. CB1 is indeed able to recruit G proteins (i, q, and s) and β -Arrestins to carry on its intracellular signaling (Nogueras-Ortiz and Yudowski, 2016; Turu and Hunyady, 2010). However, a modification of the expression of the transducers (G proteins and β -Arrestins) may lead to a bias, in which the low ratio of β -Arrestin 1 vs. Gi protein could have caused the preferential recruitment of the most abundant transducer, namely the Gi protein, and be responsible for not seeing a THC effect on Akt-GSK3 β pathway, but instead to observe only the sharp increase of Erk1/2, a known Gi pathway (Goldsmith and Dhanasekaran, 2007). To test this hypothesis, we transfected the cells with ARRB1 gene (the gene encoding for human β -Arrestin 1), and effectively we observed that transfected cells responded to THC with an increase of Akt-GSK3 β phosphorylation, confirming that β -Arrestin1 was involved in THC-induced Akt-GSK3 β modulation via the CB1 receptor. Interestingly those data point out a direct involvement of CB1 receptor, which actively recruits β -Arrestin 1 and then the whole cascade and not a cannabinoid-mediated effect in other systems since STHdhQ7/Q7 cells are a model of murine striatal GABAergic medium spiny neurons. Even though in vivo murine model it is possible to imagine that the effect observed in the structure analyzed may be dependent on the activity of THC on cells upstream the neuronal pathway, in this in vitro model, it is almost

certain that the effect observed following THC treatment is caused directly from the interaction of THC with CB1 receptor.

As the next step, using a genetic approach, we found that β -Arrestin 1 KO mice did not respond to THC. In contrast, their wild-type littermate did, for both hyperlocomotion and GSK3 β hyperphosphorylation 45 and 30 minutes, respectively, after THC injection at the dose of 0.3 mg/kg (see Fig 4 in the article and Fig. 33c for a schematic summary).

Altogether, we have been able to state that β -Arrestin 1 is the signaling transducer that carries on the PI3K-Akt-GSK3 β signaling pathway leading to the THC-induced hyperlocomotion in mice.

Lastly, we have tested the efficacy of the CB1-SSi on the THC-induced hyperlocomotion. To this end, mice were pretreated with pregnenolone or with AEF0117 and then treated with THC. Pregnenolone was able to rescue locomotion in mice, as previously demonstrated (Busquets-Garcia et al., 2017b), and AEF0117 blocked as well this behavior. Furthermore, both pregnenolone and AEF0117 prevented the THC-induced signaling cascade (see Fig 5 and 6 in the article and Fig. 33d for a schematic representation). Altogether those results represent a significant step forward in understanding both the CB1 intracellular signaling pathway that may lead to hyperlocomotion and possibly towards a better understanding of THC-induced psychosis and addiction. Although it is difficult to draw parallels between THC doses in humans and mice, our experiments provide a solid basis for further work, such as THC's chronic treatment, which is more consistent with THC effects related to psychosis and addiction. By contrast, a typically nuclear phosphoprotein, CREB, was detected only within the cell core. Akt that is known to be mostly cytosolic has been found mostly (~95%) within the cytosol and in little amounts in the core and microsomes. Altogether the data collected and disserted in this article demonstrate that AlphaLISA is a perfectly suited technique in mouse brain signaling

pathway research, allowing the rapid, semiquantitative, reproducible detection of phosphoproteins concomitantly from several signaling pathways.

My contribution to this work has been the concept and design of the experiments, the performance of the experiments, the data acquisition, analysis, interpretation, article writing, and the pictures drawing with Marion Zanese under the supervision of Monique Vallée and Jean-Michel Revest.

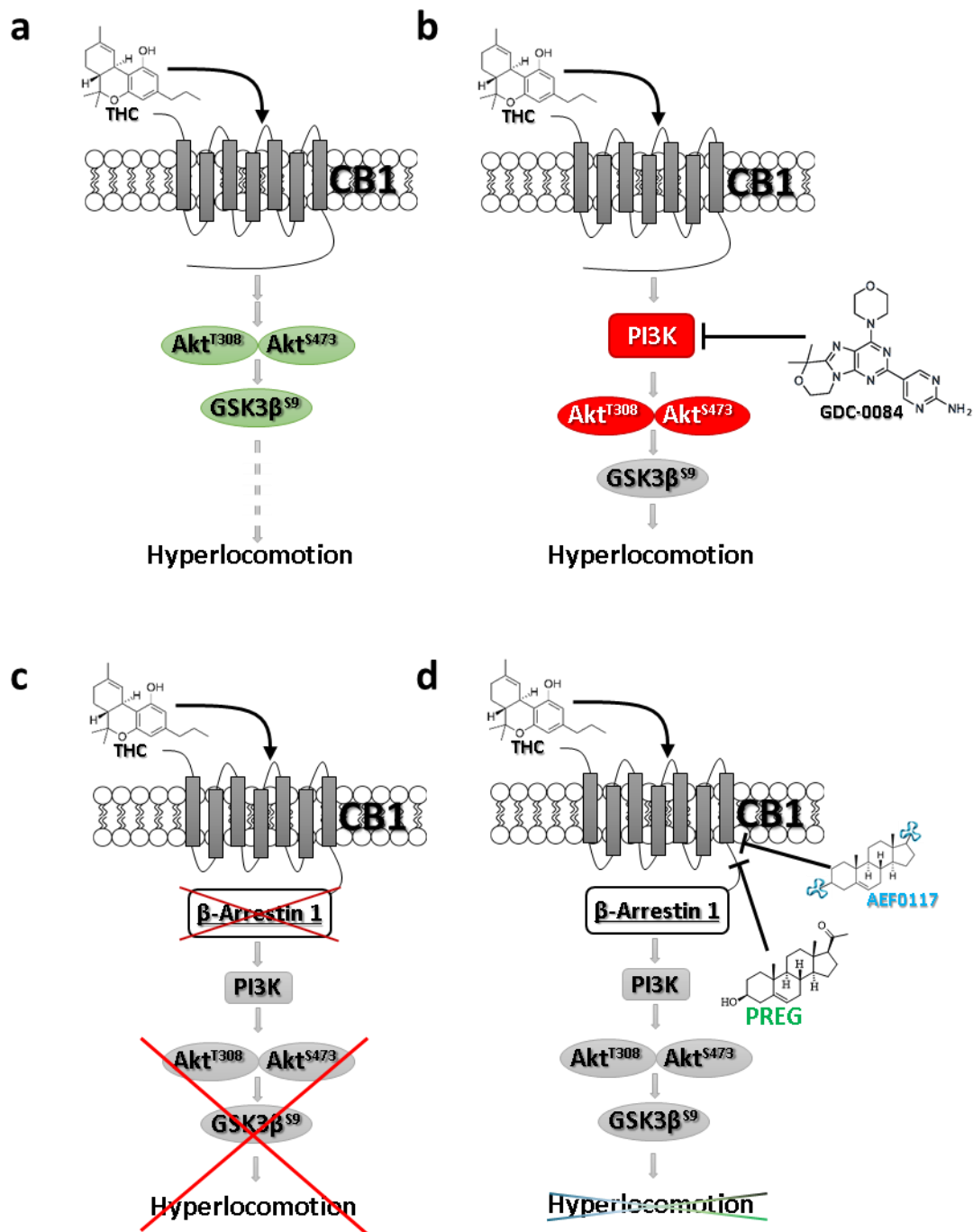


Figure 33: Schematic representation of the results in Tomaselli et al. 2020 (to be submitted): a) 30 minutes after THC injection, Akt^{T308/S473}, and GSK3β^{S9} resulted overphosphorylated in NAC, and CB .45 minutes later, THC caused hyperlocomotion in mice. **b)** GDC-0084 caused a sharp decrease in phosphorylation of Akt^{T308/S473} and rescued GSK3β^{S9} to control levels. GDC-0084 did not create any per se effect on the locomotor activity but rescued THC-treated mice's behavior. **c)** β-Arrestin 1 KO mice did not show any THC-induced hyperlocomotor behavior, while their wild type littermate did. Furthermore, β-Arrestin 1 KO mice did not show the Akt-GSK3β increase phosphorylation at AlphaLISA analyses. **d)** AEF0117 or PREG administration blocked the THC-induced hyperlocomotion 45 minutes post-injection. Furthermore, both AEF0117 and PREG rescued the Akt-GSK3β signaling in THC treated mice.

**Pregnenolone-like AEF0117 blocks the THC-induced GSK3 β -dependent
hyperlocomotion in mice**

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Abstract

Background: THC, the main active component of cannabis Sativa, is one of the most abused illicit drugs around the world, and it is correlated to several mental pathologies such as cannabis use disorder (CUD), depression, bipolar disorder, and psychosis. In animal model of addiction and psychosis, the hyperlocomotion induced by THC is one of the typical altered behavior. Behavioral and cellular outputs of THC are mediated via the type-1 cannabinoid receptor (CB1), a G-protein coupled-receptor that presents biased signaling. However, the intracellular events that THC triggers to produce hyperlocomotion are mostly unknown. A better knowledge of those events would significantly improve our understanding on the activity of CB1 receptor, since this receptor presents a high degree of complexity due to its biased signaling. Recently, it has been discover that the endogenous steroid pregnenolone (PREG) being a CB1 biased negative allosteric modulator, was able to rescue, among other behaviors, THC-induced hyperlocomotion in mice. Accordingly, synthetic analogues of PREG, and in particular the lead compound AEF0117, show similar activity on the CB1 receptor, and are able to rescue THC-induced hyperlocomotion but with a higher efficacy and a better therapeutic profile than PREG.

Aim: Our main goal was to thoroughly analyze the intracellular CB1 signaling pathways in several areas of the mouse brain that can mediate the effect of THC. The objectives of the current work were to study the CB1 signaling pathway that is involved in THC-induced hyperlocomotion at 0.3 mg/kg in mice, to reveal the signaling transducer that is recruited into this signaling pathway, and to further elucidate the mechanism of action of AEF0117.

Results: Our findings, through *in vitro* and *in vivo* pharmacological and genetic studies, demonstrate a correlative and causal link between the activation of β -Arrestin1- mediated PI3K-Akt-GSK3 β signaling pathway in the nucleus accumbens, striatum and cerebellum and THC-induced hyperlocomotion in mice. In addition, PREG and AEF0117 were able to reverse THC-induced hyperlocomotion and PI3K-Akt-GSK3 β signaling modulation.

Introduction

Cannabis Sativa is one of the most common illicit drugs of abuse around the world [1]. Δ^9 -tetrahydrocannabinol (THC) is the principal cannabis psychoactive component responsible for both its pleasant and aversive effects by acting on the central cannabinoid type-one (CB1) receptor. The long-term consequences of THC abuse include cannabis use disorder (CUD), which leads to some of the worst symptoms of cognitive deficits, heavy dependence and paranoia [2–4], and cannabinoid-induced psychosis [3,5,6]. In animal models, THC implies biphasic behavioral effects: to name some, low doses of THC result in reward [7], enhance memory and cognition [8], and increase locomotion behavior [7,9], while high doses of THC generate aversion [7], impair memory and cognition (Calabrese and Rubio-Casillas, 2018), and decrease locomotion behavior [7,10] in rodents. In line with the opposite effect of THC on reward and aversion in animals, it was reported that usual THC consumers adjust the puffs intensity of cannabis cigarettes to intake small amounts of THC [11]. Low doses of THC are indeed worthy of study, representing the most common condition of THC abuse in human patients, since these are mainly low doses of THC that are addictive in humans. Hyperlocomotor activity elicited by psychostimulant drugs is considered to be correlated to addiction [12–15] and positive symptoms of psychosis [9,16,17], and is commonly observed in rodents treated with low doses of CB1 agonists.

Locomotion is a complex function that is carried on both centrally and peripherally. Centrally, two main systems carry on motor functions: the pyramidal system, deeply involved in voluntary movement, and is constituted of motor fibers, formed of pyramidal neurons (located in some regions such as primary motor cortex, supplementary motor area, premotor cortex, somatosensory cortex, parietal lobe, and cingulate gyrus), and extrapyramidal system, that fulfills an indirect control of movements. Several brain areas are part of the extrapyramidal system, of which the basal ganglia (of which are part of the striatum, the nucleus accumbens,

substantia nigra, globus pallidus, ventral pallidum, and subthalamic nucleus) and cerebellum are among the most important [18]. Interestingly, the basal ganglia and the cerebellum are also rich in CB1 receptors [19]. This work aims to study and define which intracellular signaling pathway and area of the mouse brain is responsible for THC-induced hyperlocomotor behavior. As the signaling of the CB1 receptor is biased [20,21], implying that the activation of distinct signaling cascades may differ between CB1 agonists, cellular contexts or the expression of signaling transducers [22], we used a validated high-throughput technique to detect intracellular signaling events in cells and mouse brain tissues with a rapid, high sensitive, and reproducible Alpha (Amplified Luminescent Proximity Homogeneous Assay) method [23].

We studied several signaling cascades that have been detected in response to CB1 activation, and are also correlated to locomotion, the MAPK and Akt-GSK3 β pathways [24–28]. To unravel the THC-induced signaling that leads to hyperlocomotion in mice, we studied at first the kinetic effects of THC on CB1 signaling in individual mouse brain areas (nucleus accumbens, striatum, and cerebellum) that are among the most rich in CB1 receptors and involved in locomotion. We found that the Akt-GSK3 β pathway was amplified in those brain areas in correlation with the hyperlocomotor behavior. To further establish a causal link between signaling and behavioral events, we pharmacologically blocked upstream signaling and evaluated its effects on both. Then, in order to determine which transducer was involved in the signaling modulation we performed complementary *in vitro* and *in vivo* experiments with a genetic approach using β -Arrestin1 KO (arrb1 KO) mice. Finally, to prove that THC modulation of CB1 signaling is physiologically involved in the behavioral outcome, we tested the effect of the steroid pregnenolone, which is an endogenous negative allosteric modulator of the CB1 receptor and can shut down the toxic effects of THC in rodents [29], as well as psychotic-like behaviors, particularly those on hyperlocomotion in mice [9]. We have also tested a synthetic analogue of PREG that is part of a new class of CB1-signaling specific

inhibitors (CB1-SSi), which is highly effective in inhibiting several THC-induced behaviors [30]. This PREG analogue also demonstrated great clinical potential, being a non-metabolized steroid-like compound with good ADMET properties [30]. Our study will therefore constitute a breakthrough in both the understanding of the CB1 signaling pathway and the pharmacology of this new class of CB1-SSi compounds by revealing, for the first time, an *in vivo* mechanism of action.

Results

THC-induced Akt-GSK3 β signaling over-phosphorylation 30 minutes post-injection in mice

In order to investigate the role of CB1 signaling in THC-induced hyperlocomotor behavior, we first performed the locomotor activity to a novel environment in THC-treated mice, as described previously [9]. THC, administrated at the dose of 0.3 mg/kg, caused hyperlocomotor behavior in mice 45 minutes after injection (Fig 1b). In order to understand which was the intracellular signaling pathway that leads to this behavior, we investigated the phosphorylation of targets from MAPK and Akt-GSK3 β pathways, two of the most studied signaling linked to locomotion [24,28], also known to be altered by THC [25,27]. We performed analyses in the NAc and CB, two structures strongly implicated in locomotion, and part of the extrapyramidal system, also rich in CB1, and in the Hpc, as a negative control, being a CB1-rich brain area, but not implicated in locomotor activity. We tested those targets at two main time-points antecedent to the hyperlocomotion: 15 and 30 minutes post-injection. Thirty minutes post-THC injection Akt^{T308}, Akt^{S473} and GSK3 β ^{S9} showed increased levels of phosphorylation in the NAc (Fig 1c) and CB (Fig 1d), while no effect was detected in the Hpc (Fig. 1e). Nor in NAc nor CB it was observed a modification of total Akt expression (Fig 1e). No significant alteration was observed in Akt-GSK3 β signaling 15 minutes post-injection (Fig S1a, b, c), nor in targets from Erk1/2 pathway 15, and 30 minutes after injection (Fig S1d, e, f).

GDC-0084 blocked the THC-induced hyperlocomotion and Akt pathway over-phosphorylation

The first experiment showed a correlation between the THC-induced Akt-GSK3 β signaling and the hyperlocomotion. However, it was not possible to claim any causal relationship. For this reason, we used a pharmacologic approach in order to prove the causality between signaling and behavior. We used an inhibitor of PI3K, an upstream target of the Akt-GSK3 β signaling, to prevent the over-phosphorylation of the pathway. First, we started by studying a GDC-0084 dose-response curve to choose the highest sub-effective dose of the compound on locomotion (Fig. S2b). Among all the GDC-0084 doses we used in mice, 6 mg/kg was the one that did not show any *per se* behavioral effect. Pretreatment with this sub-effective dose of GDC-0084 prevented the THC-induced hyperlocomotion while it did not cause any *per se* effect (Fig. 2b). THC increased Akt^{T308}, Akt^{S473}, and GSK3 β ^{S9} phosphorylation in the NAc (Fig. 2c), Str (Fig. 2d), and CB (Fig. 2e). Since GDC-0084 is a mTORC1 inhibitor [31], we investigated the phosphorylation of mTORC1, too, by checking the phosphorylation state mTORC1 downstream targets. No THC-induced altered phosphorylation of mTOR downstream targets was observed in the NAc, Str, and CB (Fig. S2c). Interestingly, GDC-0084 caused a significant drop in phosphorylation of Akt^{T308}, Akt^{S473}, and RPS6^{S240/S244}, while it rescued GSK3 β phosphorylation in the NAc and Str (Fig. 2c, d), in the NAc and Str (Fig. 2c,d; Fig S2c). In the CB, GDC-0084 caused a severe drop in phosphorylation of Akt^{T308}, Akt^{S473}, GSK3 β ^{S9}, and 4EBP1^{T37/T46} (Fig. 2e; Fig S2c).

STHdh^{Q7/Q7} cells transfected with *ARRB1* showed a THC-induced Akt-GSK signaling alteration

We investigated the signaling transducer in an *in vitro* model. For this task, we chose a model of murine striatal medium spiny neurons that endogenously express CB1 receptor: the STHdh^{Q7/Q7} cell line. However, we were not able to detect any alteration of the Akt-GSK3 β signaling pathway in those cells, despite it was described in the literature [32]. We then investigated the expression of the most common signaling transducer genes through the ddPCR

(digital droplet polymerase chain reaction). Our analyses showed in our *STHdh*^{Q7/Q7} cells a very low gene expression of β -Arrestin 1 compared to the other signaling transducers (Fig. 3a; Fig. S3a). By transfecting transfected *STHdh*^{Q7/Q7} cells with the *ARRB1* gene (encoding for the human β -Arrestin 1), we were able to reveal significant increase in Akt^{T308}, Akt^{S473}, GSK3 β ^{S9}, and Erk^{T202/Y204} phosphorylation (Fig. 3d, Fig. S3c, respectively). A similar increase in Erk1/2 phosphorylation was found in cells transfected or not with the *ARRB1* gene (Fig. 3d, Fig. S3b and S3c).

β -Arrestin 1 KO mice do not show THC-induced hyperlocomotor behavior

To confirm the previous *in vitro* data showing that β -Arrestin 1 was necessary for the alteration of Akt-GSK3 β pathway induced by THC, we tested the effects of THC at the dose of 0.3 mg/kg in β -Arrestin1 KO (*arrb1* KO) mice. Wild type mice treated with THC showed increased locomotor activity, while *arrb1* KO mice did not, compared to their respective vehicle (Fig. 4b). To exclude that this lack of effect of THC in increasing locomotion in *arrb1* KO mice, was not related to a behavioral phenotype of the transgenic mice, we tested whether amphetamine was able to induce hyperlocomotion in these mice. Since, *Arrb1* KO mice showed a hyperlocomotor response to amphetamine, even more robust than in their wild type littermates (Fig.S4b), we could confirm that β -Arrestin 1 is indeed required for THC-induced hyperlocomotion. Moreover, the phosphorylation of the protein GSK3 β was increased by THC in the NAc, Str, and CB in wild-type mice, but not in *arrb1* KO mice (Fig. 4c), further confirming the need of β -Arrestin 1 in the modulation of the Akt-GSK3 β pathway by THC.

PREG rescued the THC-induced hyperlocomotion and increase in Akt-GSK3 β phosphorylation

We tested whether PREG, an endogenous CB1 negative allosteric modulator, could inhibit the effect of THC on locomotion and on the CB1-mediated Akt-GSK3 β pathway as well. Indeed, the pre-treatment with PREG at the dose of 6 mg/kg could block the THC-induced

hyperlocomotion (Fig. 5b), and it was able to prevent the increase in Akt^{T308}, Akt^{S473}, and GSK3β^{S9} phosphorylation in the NAc, Str, and CB (Fig. 5c) without showing any per se effect.

AEF0117 rescues the THC-induced hyperlocomotion and increase in Akt-GSK3β pathway

We tested the effect of AEF0117, a synthetic analog of pregnenolone, being part of a new class of CB1 signaling specific inhibitors (CB1-SSi) compounds [30]. The pre-treatment with AEF017 at the dose of 0.015 mg/kg was able to block THC-induced hyperlocomotion in mice (Fig. 6b). Moreover, AEF0117 was able to rescue the effect of THC on the Akt-GSK3β pathway. Indeed, AEF0117 prevented the phosphorylation of the Akt^{T308}, Akt^{S473}, and GSK3β^{S9} proteins within the NAc (Fig. 6c), Str (Fig. 6c), and CB (Fig. 6d). Furthermore, mice pretreated with AEF0117 did not show any per se effect on both behavior and CB1 signaling.

Discussion

Although it is well known that cannabinoids induce hyperlocomotor activity in rodents [7,9,33], the mechanism by which this occurs has never been fully understood, and the intracellular signaling pathway underlying this event has never been extensively studied. In this work, we discovered that the Akt-GSK3 β signaling resulted hyperphosphorylated in the nucleus accumbens, striatum, and cerebellum when mice were treated with THC at 0.3 mg/kg, while no effect was detected within the hippocampus. Interestingly, the action of THC was observed in specific cerebral zones involved in locomotion, while the hippocampus is not involved [34], strongly suggesting a role of this pathway in mice locomotor activity. Those studies were correlative, and in order to clearly define the role of the Akt-GSK3 β pathway in THC-induced locomotor activity, we decided to inhibit this signaling with the GDC-0084, a PI3K and mTORC1 inhibitor. Accordingly, we found a drop in the phosphorylation of all the targets downstream to PI3K, and so of Akt^{T308/S473}, of RPS6^{S240/S244}, and 4EBP1^{T37/T46} (targets downstream to mTORC1) following GDC-0084 treatment. Sub-effective doses of GDC-0084 were sufficient to block the THC-induced hyperlocomotion. Interestingly, GDC-0084 rescued GSK3 β in the nucleus accumbens and striatum when altered by THC; otherwise, it was not affected. Since most of the targets were altered by sub-effective doses of GDC-0084, with the only exception of GSK3 β within the nucleus accumbens and the striatum, we concluded that GSK3 β phosphorylation in those structures plays an essential role in THC-induced hyperlocomotion.

Investigating the CB1 signaling transducer that underlies the Akt-GSK3 β signaling, we started by testing the effect of THC in the *STHdh*^{Q7/Q7} cell line. The choice of this cell line was supported by the fact that they are mouse striatal derived cell line, having CB1 receptor and presented an overphosphorylation of Akt on both the epitopes Thr308 and Ser473 following THC treatment [32]. However, we could not detect any alteration of the Akt-GSK3 β signaling

following THC treatment on those cells. By analyzing the gene expression of several signaling transducers through ddPCR we observed that *arrb1* was considerably less expressed, compared with the majority of the other transducers, which suggested that *STHdh*^{Q7/Q7} cells present a system bias, as already described [32]. A system that presents such a difference between the signaling transducers may induce a considerable bias towards the most abundant [22]. Our data may appear in discord with the work of Laprairie and coworkers showing through qPCR analysis a higher expression of *arrb1* compared to *arrb2*, although the other transducer were not studied [32]. This genetic expression discrepancy observed in *STHdh*^{Q7/Q7} could be explained by the fact that cell lines express a certain variability, from mild to high, within the same line [35] or even the same batch [36], as well as with the difference in the PCR analyses used, since we preferred the ddPCR approach, which is more sensitive than q-PCR, to analyze the low expression of *arrb1* and *arrb2* in *STHdh*^{Q7/Q7} cells. We therefore tested and confirmed our hypothesis of biased system by transfecting *STHdh*^{Q7/Q7} cell line the *ARRB1* gene (the human gene for β -Arrestin 1). Interestingly, THC induced an increase in the phosphorylation of Akt^{T308/S473} and GSK3 β ^{S9} signaling in cells transfected with the *ARRB1* gene, while no alteration was found in non-transfected cells, showing the significance of β -Arrestin 1 in the THC-induced Akt-GSK3 β signaling modulation. Furthermore, THC induced an increase as well of Erk phosphorylation, however both in cells non-transfected and transfected with *ARRB1*, showing that Erk is not recruited by β -Arrestin 1 in the *STHdh*^{Q7/Q7} cells. The study in the *arrb1* KO mice that constitutively do not express the protein β -Arrestin 1, further confirms the involvement of the β -Arrestin 1-PI3K-Akt-GSK3 β pathway in the effect of THC, since THC at the dose of 0.3 mg/kg did not increased locomotion of the *arrb1* KO mice, while it did in wild type littermates. According to the behavioral data, we detected an increase in the phosphorylation of GSK3 β in the nucleus accumbens, striatum, and cerebellum of wild type mice, but not of *arrb1* KO mice.

Lastly, we revealed the physiologic and therapeutic significance of the uncovered THC-induced GSK3 β -dependent hyperlocomotion by using the endogenous CB1-SSi, pregnenolone, and the high-efficacy CB1-SSi, its synthetic analog AEF0117. Indeed, both compounds could reverse THC-induced hyperlocomotion in mice, as previously found [9,30], but as well rescue the Akt-GSK3 β signalization within the nucleus accumbens, striatum, and cerebellum of mice. Such evidence would be helpful in understanding the molecular mechanism underlying the preclinical and potentially clinical action of AEF0117.

Altogether, this work shows, for the first time, that the CB1-induced intracellular signaling pathway that leads to THC-induced hyperlocomotion is the β -Arrestin 1-Akt-GSK3 β . This signaling modulation could be mistaken for dopaminergic D2 receptor modulation, which may be involved in locomotion behavior, but main considerations rule out this possibility. Firstly, the D2 receptor recruits and activates the phosphatase 2A (PP2A) to alter the Akt phosphorylation. However, PP2A can only dephosphorylate Akt on the epitope Thr308, but we observed a clear effect on the epitope Ser473 as well. Secondly, the canonical dopaminergic pathway leading to hyperlocomotion is the Akt^{T308}-GSK3 β ^{S473} dephosphorylation [37], while we observed the Akt^{T308/S473}-GSK3 β ^{S9} hyperphosphorylation. Thirdly, D1 and D2 receptors inhibitors did not block the Akt-GSK3 β phosphorylation in CD1 mice [25]. Altogether, although the dopaminergic signaling in the nucleus accumbens and striatum is canonically one the most described cause of hyperlocomotion [38], the above compiled data disagree with a dopamine implication in THC-induced hyperlocomotor behavior in mice. Finally, the D2 receptor signaling involves the β -Arrestin 2 protein, while we revealed that the CB1 signaling analyzed in the present work implicates the β -Arrestin 1 protein. We can therefore conclude that the CB1-mediated Akt-GSK3 β pathway that has been identified is responsible for cannabinoid-induced dopamine-independent locomotion in mice.

Star Methods

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

Animals

C57BL6N

Arrb1KO

Drugs

THC

Amphetamine

GDC-0084

PREG

AEF0117

Cell cultures

STHdhQ7/Q7

PCR technique

Behavioral tests

Locomotor activity test

Behavioral experimental design

THC kinetics of action in mouse brain

GDC-0084 per se effect

GDC-0084 effect on THC-induced hyperlocomotion and signaling

Responsiveness of β -Arrestin 1 KO mice to THC (0.3 mg/kg) induced hyperlocomotion

Responsiveness of β -Arrestin 1 KO mice to amphetamine (5 mg/kg) induced hyperlocomotion

PREG effect on THC-induced behavior and signaling

AEF0117 effect on THC-induced behavior and signaling

Protein extraction from brain tissues and protein quantitation

AlphaLISA analyses

Data analyses

Author contribution

GT, GM, PVP, JMR, and MV designed the project; GT, ABG, MZ, PVP, JMR, and MV designed the experiments; GT, ABG, VL, MZ, VRL, GD, MMo, MMe, AG, and MV performed the experiments; GT, ABG, VL, MZ, and GD analyzed data; GT, JMR and MV interpreted data and wrote the manuscript.

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Figures:

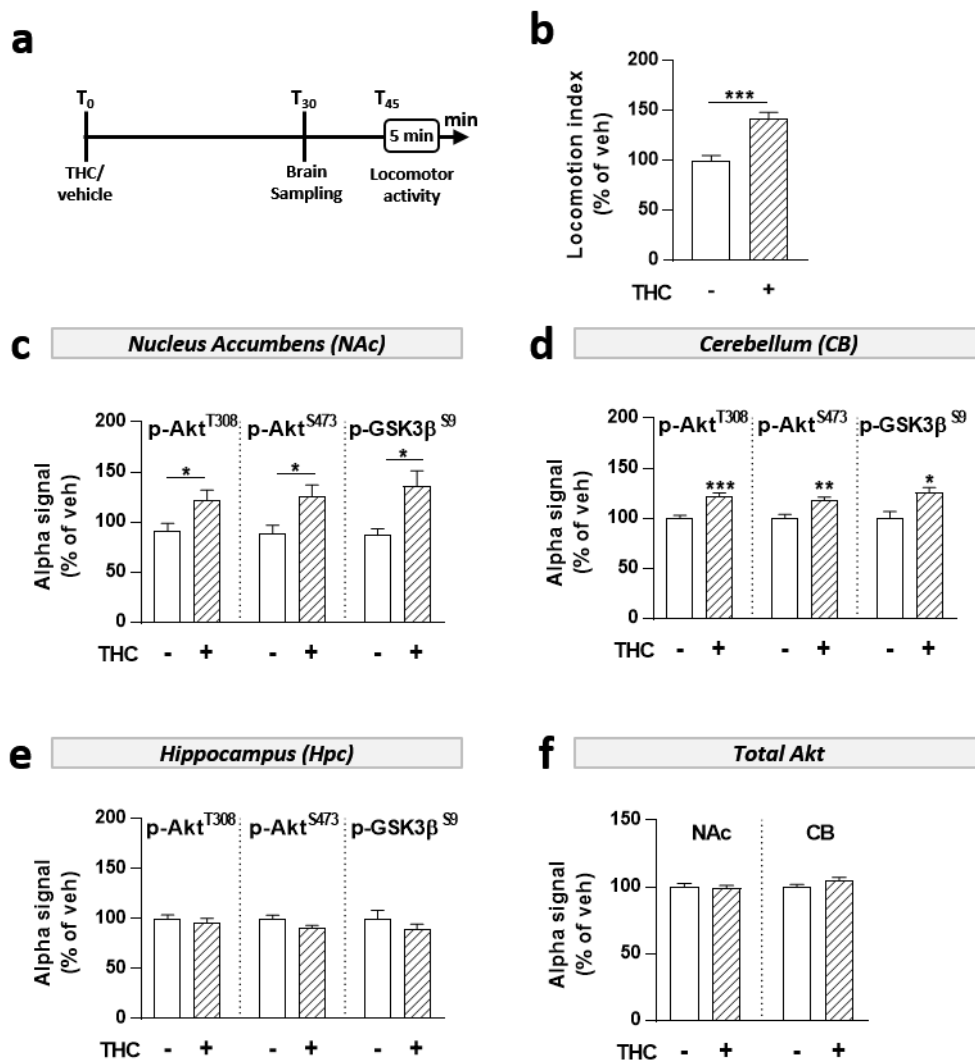


Figure 1: The treatment with THC at the dose of 0,3 mg/kg alters the locomotor activity and the Akt-GSK3 β intracellular pathway. a) Two groups of mice were treated with THC or its vehicle. Brain sampling was performed in one group 30 minutes post-treatment, and the mice of the second group were subjected 45 minutes post-treatment to locomotor activity in a novel environment for five minutes. THC treatment increased the locomotion in mice (b) and the phosphorylation of Akt and GSK3 β proteins in the c) nucleus accumbens and d) cerebellum but not e) in the hippocampus. f) No significant alteration of Akt's total amount was detected in the nucleus accumbens and cerebellum. Data are expressed as percentage of the vehicle groups and are represented as mean +SEM. Student's t-test; THC vs Vehicle, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

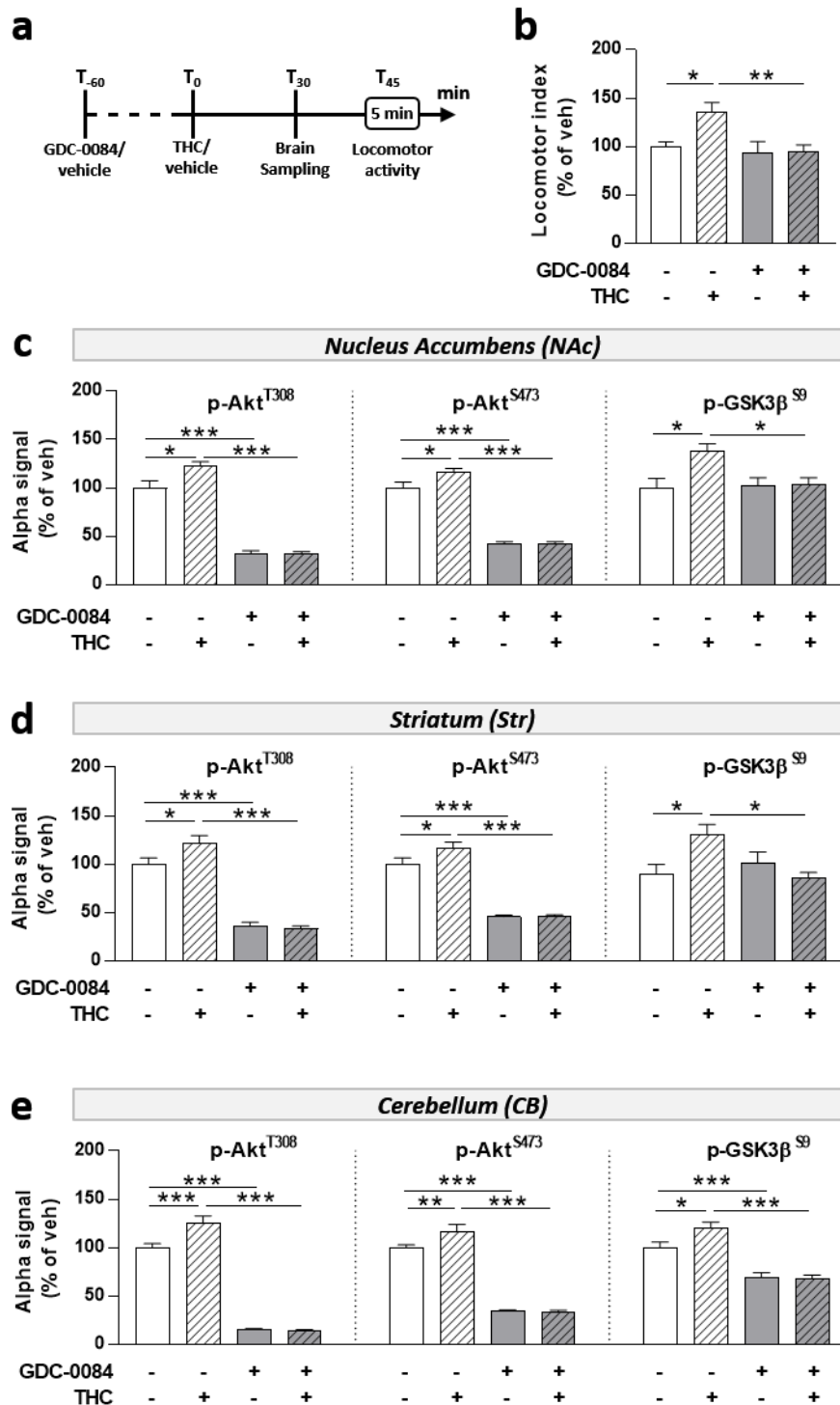


Figure 2: GDC-0084 blocks both THC-induced hyperlocomotion and CB1 signaling modulation. *a)* Mice were pre-treated with GDC-0084 or its vehicle 60 minutes before THC or its vehicle injection. 30 minutes post-injection mice were sacrificed for further analyses. 45 minutes post-THC injection, mice were subjected to locomotor activity in a novel environment test. *b)* GDC-0084 rescued normal locomotion in THC-treated mice, without showing any per se effect. GDC-0084 caused a sharp drop of phosphorylation for Akt in the *c)* nucleus accumbens, *d)* striatum, and *e)* cerebellum, and GSK3β on the epitope Ser9 in the cerebellum. Data are expressed as percentage of the Veh^{GDC-0084}-Veh^{THC} groups and are represented as mean +SEM. Tukey's multiple comparison test; **p*<0.05, ***p*<0.01, ****p*<0.001.

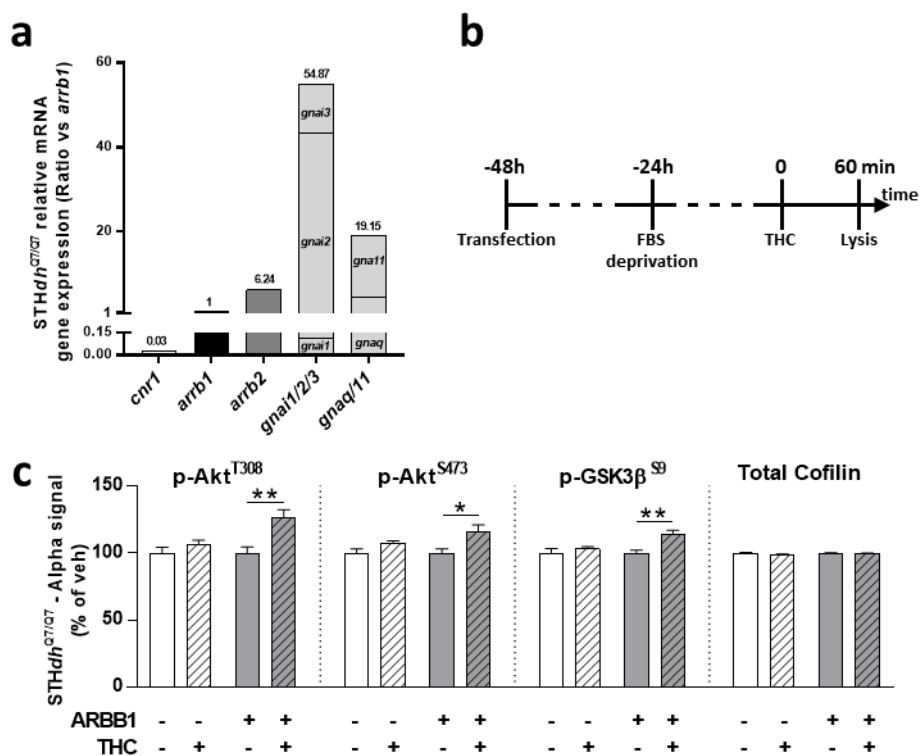


Figure 3: In vitro analyses of β -Arrestin 1. a) ddPCR analysis of the expression of several signaling transducers and CNR1 receptor normalized with the arrb1 gene in STHdhQ7/Q7 cells. b) Experimental plan of transfection and treatment. c) Only STHdhQ7/Q7 cells transfected with the ARRB1 gene showed THC-induced overphosphorylation of the Akt-GSK3 β signaling. Data are expressed as a ratio between the gene expression and arrb1 expression (a) or the percentage of the Vehicle groups (c). Data are represented as mean (a), or mean +SEM (c). Student's t-test; * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$.

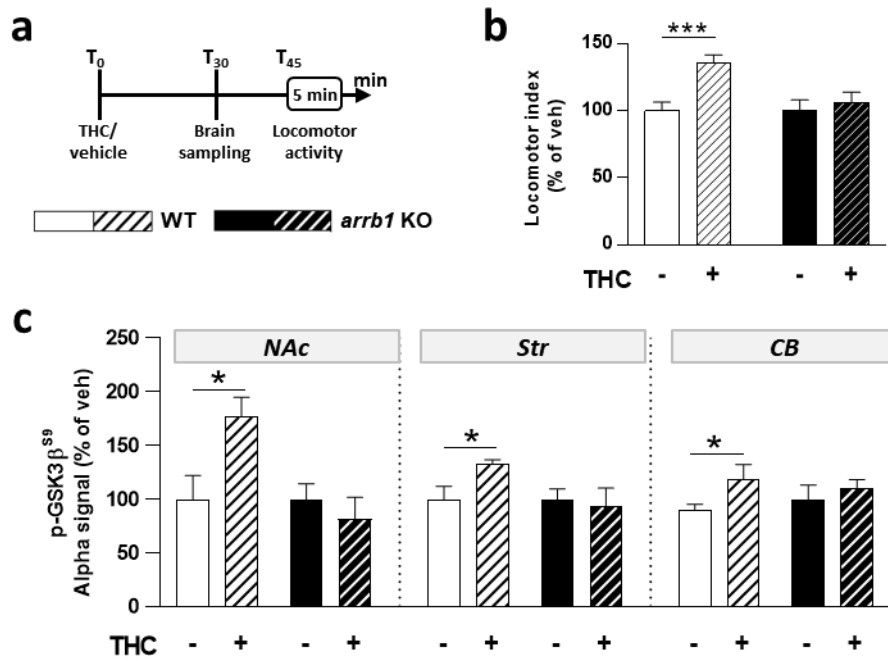


Figure 4: The treatment with THC at the dose of 0,3 mg/kg did not alter the locomotor activity and the Akt-GSK3 β intracellular pathway in β -Arrestin 1 (*arrb1*) KO mice. a) Two groups of *arrb1* KO mice and wild-type (WT) littermates were treated with THC or its vehicle. Brain sampling was performed in one group 30 minutes post-treatment, and the mice of the second group were subjected 45 minutes post-treatment to locomotor activity in a novel environment for five minutes. Wild-type, but not *arrb1* KO mice, treated with THC showed b) hyperlocomotor activity, and c) significantly increased phosphorylation of the protein GSK3 β in the nucleus accumbens (NAc), striatum (Str), and cerebellum (CB). Data are expressed as percentage of WT-VehTHC and *arrb1* KO-VehTHC groups and are represented as mean \pm SEM. Student's *t*-test; ** $p < 0.01$, *** $p < 0.001$.

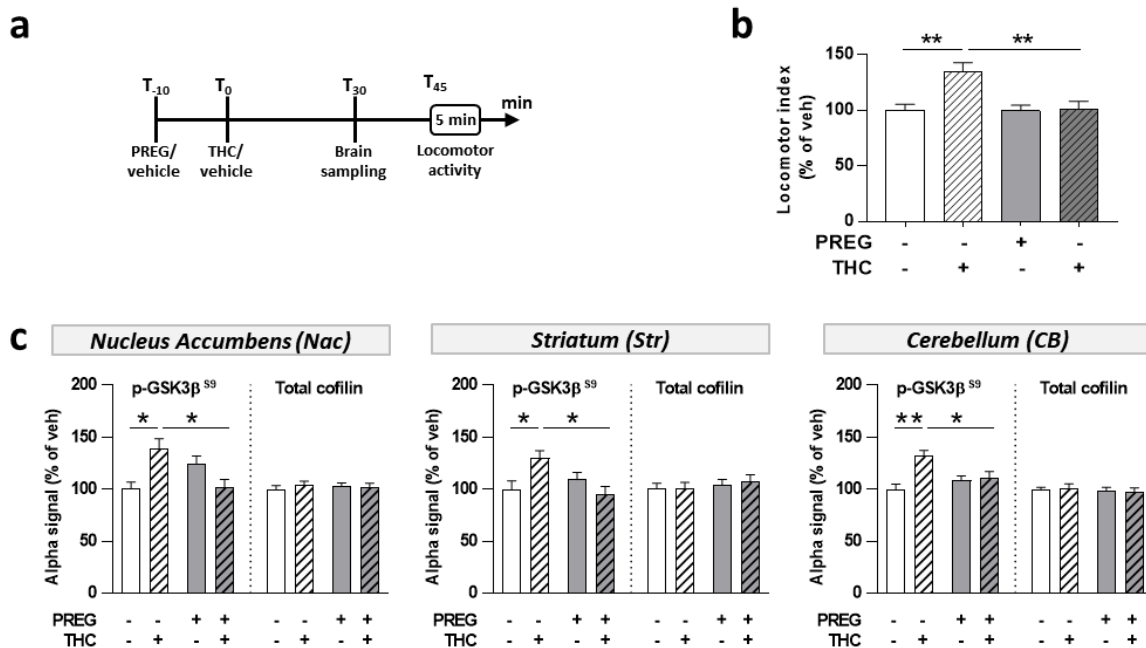


Figure 5: Pregnenolone (PREG) rescued both THC-induced hyperlocomotion and CB1 signaling modulation. a) Two groups of mice were treated with PREG or its vehicle 30 minutes before THC or its vehicle. Brain sampling was performed in one group 30 minutes post-treatment, and the mice of the second group were subjected 45 minutes post-treatment to locomotor activity in a novel environment for five minutes. b) Pregnenolone did not show any per se effect, while it was able to rescue normal locomotor behavior in THC-treated mice. c) and d) Pregnenolone blocked the THC-induced signaling alteration in the nucleus accumbens (Nac), striatum (Str), and cerebellum (CB), respectively, without showing any per se effect. Data are expressed as percentage of the Veh^{PREG}-Veh^{THC} group, and are represented as mean +SEM. Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$.

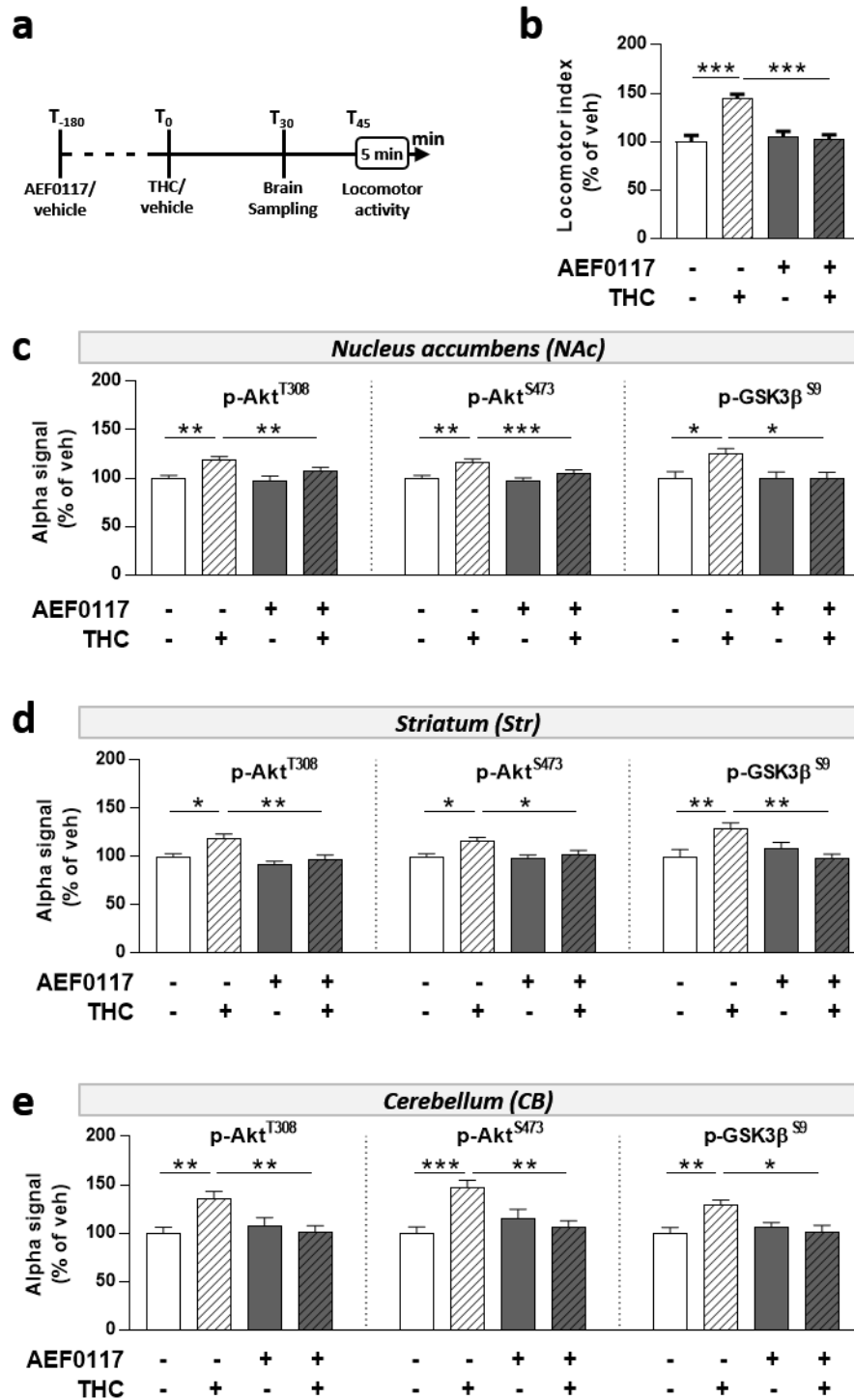


Figure 6: AEF0117 rescued both THC-induced hyperlocomotion and CB1 signaling modulation. a) Two groups of mice were treated with AEF0117 or its vehicle 180 minutes before THC or its vehicle. Brain sampling was performed in one group 30 minutes post-treatment, and the mice of the second group were subjected 45 minutes post-treatment to locomotor activity in a novel environment for five minutes. AEF0117 rescued b) locomotor behavior and Akt-GSK3 β signaling in the c) NAc, d) Str, and e) CB of THC-treated mice without showing any per se effect nor in behavior nor signaling. Data are expressed as percentage of the Veh^{AEF0117}-Veh^{THC} groups and are represented as mean +SEM. Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplementary material:

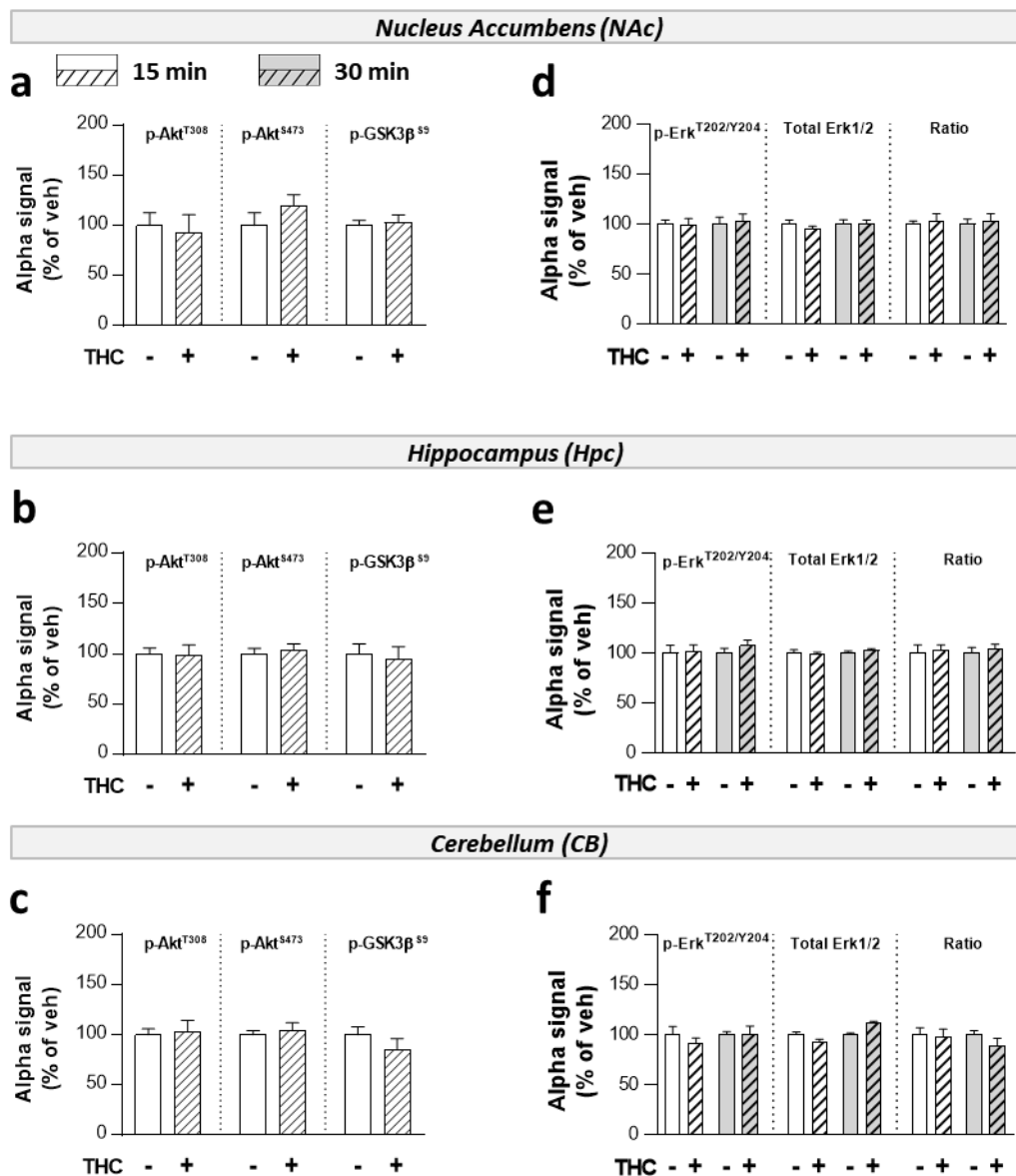


Figure S1: THC did not show any significant effect in the Akt-GSK3 β pathway 30 minutes post-injection in the a) NAc, b) Hpc, and c) CB. The Erk pathway was not affected by THC nor at 15 or 30 minutes post THC in the d) NAc, e) Hpc, and f) CB. Data are expressed as percentage of the Veh^{THC} groups respectively, and are represented as mean +SEM. Statistical analyses used Tukey's multiple comparison test.

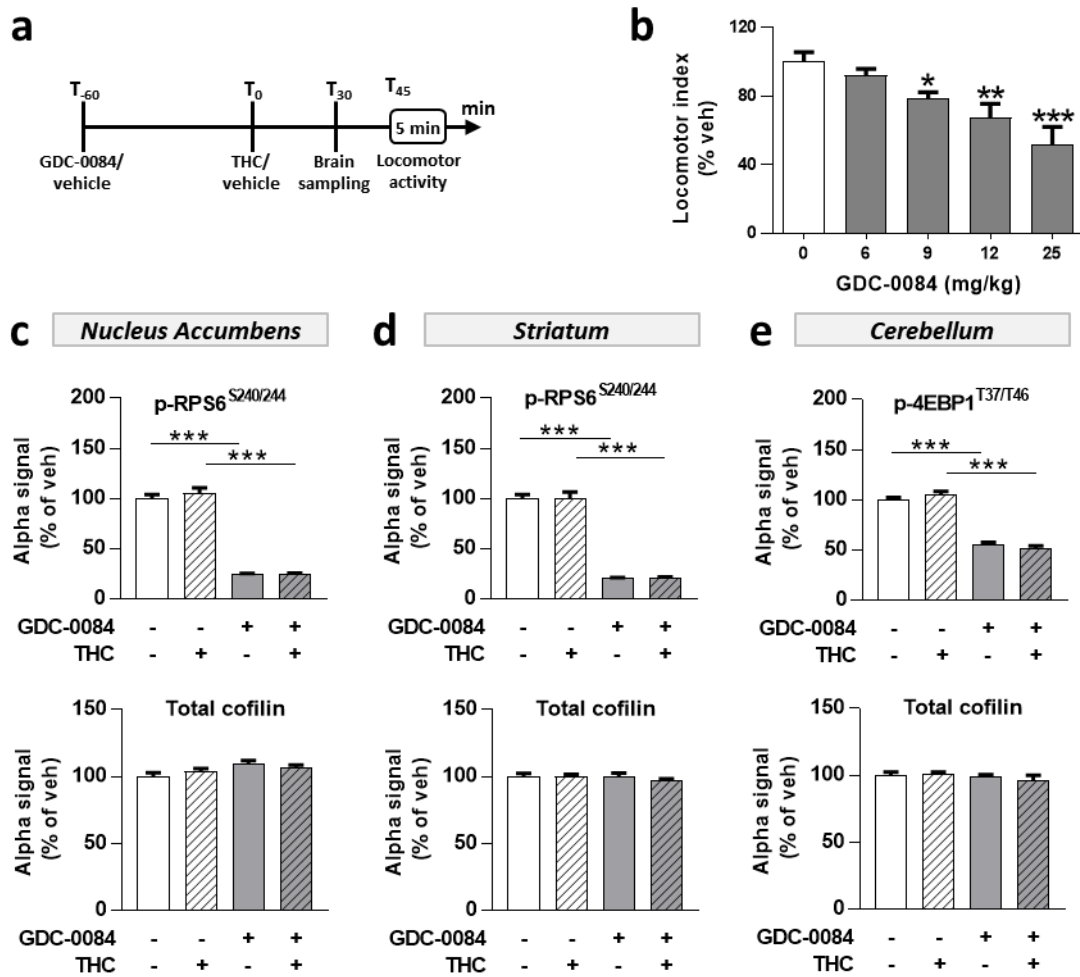


Figure S2: Effect of the PI3K inhibitor, GDC-0084 on locomotion and CB1 signaling. a) Two groups of mice were treated with GDC-0084 or its vehicle 60 minutes before THC or its vehicle. Brain sampling was performed in one group 30 minutes post-treatment, and the mice of the second group were subjected 45 minutes post-treatment to locomotor activity in a novel environment for five minutes. b) GDC-0084 induced a decrease in locomotion starting from the dose of 9 mg/kg, but not at 6 mg/kg. c), d) and e) THC did not show any significant effect in the mTORC1 pathway in any studied brain area (Nucleus accumbens, striatum and cerebellum, respectively). However GDC-0084 was able to decrease the mTORC1 pathway phosphorylation, without affecting total cofilin in all brain areas. Data are, expressed as the percentage of the Veh^{GDC-0084} - Veh^{THC} groups, and are represented as mean +SEM. Tukey's multiple comparison test; *p<0.05, **p<0.01.

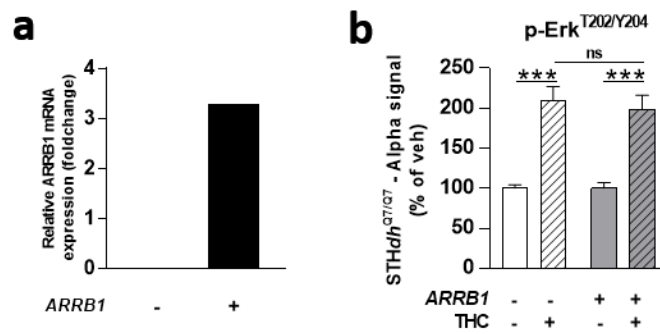


Figure S3: a) It was detected the presence of the human gene of β -Arrestin 1 only in the STHdh^{Q7/Q7} cells transfected with ARRB1 gene, b) in both groups (transfected and non transfected with ARRB1) THC sharply increased Erk phosphorylation compared to vehicle. No significant difference was observed between the two groups treated with THC. Data are expressed as a) the fold change of mRNA expression, and b) as percentage of the Vector-Veh^{THC} and ARRB1-Veh^{THC} groups, respectively. Data are represented as a) mean and b) mean +SEM. Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$.

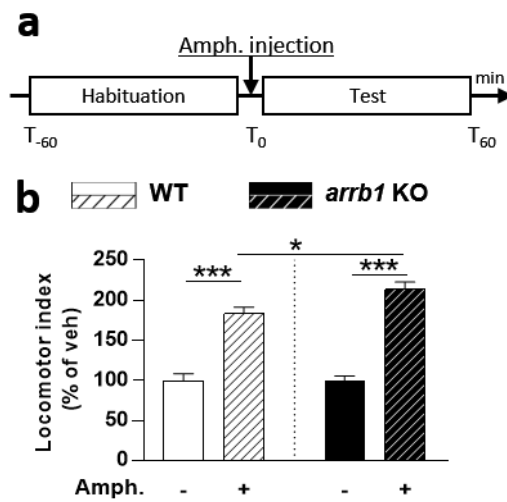


Figure S4: Locomotor activity in wild-type (WT) *bArr1* KO mice. a) After a period of 1h habituation in the actimetry chambers, the mice were treated with amphetamine and tested for locomotion in the same actimetry chambers. b) Amphetamine induced an increase in the locomotor activity in both the WT and *bArr1* KO mice. *bArr1* KO mice showed a more intense increase of locomotor activity compared to the WT mice. Data are expressed as percentage of the WT-Veh^{THC}, and *arrb1* KO-Veh^{THC} groups, respectively. Data are represented as mean +SEM. Tukey's multiple comparison test; **p*<0.05, ***p*<0.01.

Gene name	GenBank ID	Primer sequence	
		Forward (5'-3')	Reverse (5'-3')
Cnr1	NM_007726	TCAGTTGCTGTGGAATCTTTAAAAA	CTGATCGCAGGACCCCTAGA
Arrb1	NM_177231	CCCCATGTGTGAAGGGCTAGTA	TCACCCTCATATCCCTTTCAGTAGT
Arrb2	BC016642	CCGCTATGGCCGAGAAGAC	CTGGTAGGTGGCGATGAACA
Gnai1	NM_010305	TCGTGCCATTGAAACAAAATCA	TGGGAAGGCGAGTCAGCTT
Gnai2	NM_008138	GCCGGGAGTAGCCATGGTA	CCCCAGAACAAGACAACGGTTA
Gnai3	NM_010306	GCTGTGGCTCGGATCCATT	CAGTTCTGACCATCAACCTTCTTTC
Gnaq	NM_008139	CTTCTTAGGAGCCTGCGTATATTGT	GTTCCAGGTGTTCTTTAAATATCGC
Gna11	NM_010301	CCACCTTCCCACACTAGGCTC	TGTAGTGTATGGGATGGCACAGAT

Table 3: List of the primers used for the ddPCR

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STAR methods

- Animals

Mice were housed individually in a temperature- (22°C) and humidity- (60 %) controlled animal facility under a constant light-dark cycle (light on: 7 am – 7 pm). Food and water were available ad libitum. When needed, mice were quickly sacrificed by decapitation. In order to conserve protein phosphorylation, brain areas were quickly dissected on ice and placed in dry ice-cold Precellys tubes (Bertin Technologies, Montignyle- Bretonneux, France) stored at -80°C before protein extraction [23]. Nine-weeks-old male C57BL/6N (weighing 25-30 g) were purchased from Janvier Laboratories (France), and six-twelve-weeks-old male β -Arrestin1 constitutive KO (B6.129X1(Cg)-Arrb1^{tm1Jse}/J – Arrb1) mice (weighing 25-35 g) were purchased from The Jaxon Laboratory [39]. All experiments involving mice were conducted according to the protocols approved by the local ethical committee (authorization number APAFIS#10936-201708091043277 v5) in strict agreement with the French Ministry of Agriculture and Fisheries (authorization number A33-063-98) and European Communities Council Directive (2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of mice used while maintaining reliable statistics.

- Drugs

THC (#THC-1295S-250; 10 ml/kg, 0.3 mg/kg) was purchased from THC Pharm GmbH (Germany), and was dissolved in a solution of 4% ethanol, 4% cremophor, 92% saline (0.9% NaCl) to be administrated through intraperitoneal injection (*i.p.*), in volume 10 ml/kg, at the dose of 3 mg/kg, while the 10 μ M solution for cell treatment was dissolved in DMSO. GDC-0084 (#S8163) was purchased from selleckchem.com (UE) and was dissolved in a solution of 0.2% methylcellulose, 0.2% tween80, to be administrated orally, via gavage (*p.o.*), in volume of 5 ml/kg, at the doses of 6, 9, 12, 25 mg/kg; AEF0117 was obtained from Aelis Farma biotech

(France) and was dissolved in corn oil to be administered orally via gavage (*p.o.*), in volume of 5 mg/kg, at the dose of 0.015 mg/kg. PREG was dissolved in a solution of 1% tween80, 2% DMSO, 97% saline (0.9% NaCl) as described in previous works [9,29] to be administered subcutaneously, in volume of 10 ml/kg, at the dose of 6 mg/kg (*s.c.*); amphetamine sulfate (A5880) was purchased from Sigma and dissolved in saline solution, to be administered through intraperitoneal injection (*i.p.*), in volume 10 ml/kg, at the dose of 5 mg/kg.

- Cell cultures

One day before the transfection, the *STHdh*^{Q7/Q7} cells (CHDI-90000073, Coriell Ref # CH00097) were plated in a 96 well plate (Nunc Thermo Scientific: ref #167008) at 2x10⁴ cells per well in 100µL of complete medium (DMEM– Gibco: ref #61965; 10% FBS not inactivated – Gibco: ref #10270; 1% penicillin/streptomycin – Gibco ref: 15140; 1% G418– Gibco: ref #11811 stock 40 mg/mL). Cells were incubated in a humidified atmosphere at 33°C of 5% CO₂. Then *STHdh*^{Q7/Q7} cells were transfected with Lipofectamine 2000 reagent (Invitrogen-: ref #11668) in OptiMEM medium (GIBCO: ref #51985) according to the manufacturer's instruction at a final ratio of 120 µg cDNA / 0.4 µl Lipofectamine per well with *ARRB1* gene (Origen: ref #SC303424) or pcDNA3.1(+). 24h post-transfection, cells were deprived of FBS, penicillin, streptomycin, and G418 and maintained in 100µl of DMEM (Gibco: ref #61965) per well. After 24 h of deprivation, the culture medium was removed and changed with fresh DMEM (50 µL). Then cells were treated, adding 150 µL of THC solution (40 µM in DMEM, final concentration 10 µM) or its vehicle (DMEM, DMSO 0.2%; final concentration of DMSO, 1.05%). After 30 minutes of incubation at 33°C, the culture medium was removed, and the cells were lysed with 100µL of AlphaLISA lysis buffer (Perkin Elmer: ref# AL003) supplemented with phosphatase and protease inhibitors 1% and 0.5% v/v respectively (#P8340 and #P0044, Sigma, USA). The plate was agitated on a plate shaker (350rpm) for 10 min at room temperature, and samples were stored at -80°C for further Alpha analyses [23]

- **Quantitative real-time PCR (q-PCR)**

Total RNA was extracted using a standard chloroform/isopropanol protocol and purified by incubation with Ambion™ DNase I – RNase free (Thermo Scientific) [40]. The purity and concentration of RNA samples were determined from OD_{260/280} readings using ND1000 UV spectrophotometer (Fisher Scientific) and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit run on the Bioanalyzer 2100 (Agilent Technologies). cDNA was synthesized from 2 µg of RNA with RevertAid Premium Reverse Transcriptase (Fermentas) using a mix of random primers (Fermentas) and oligo(dT)₁₈ primers (Fermentas). Transcript-specific primers were generated with Primer Express software (Applied Biosystems) based on GenBank sequence information, verified by NCBI BLAST search, and custom synthesized (Eurogentec). The sequences of all primer pairs can be found in Table 1. Each primers set was tested by melting curve analysis and gel electrophoresis for the absence of primer-dimer artifacts and multiple products. Amplification efficiency of each set was determined using repetitive dilution series of a mixture of cDNA. Only primers with an efficiency of ~2 were used.

PCR were performed in a final volume of 20 µl containing the required QX200 ddPCR EvaGreen Supermix (Bio-Rad) with a final concentration of 150 nM of each primer sets and 2 µl cDNA equivalent to 4 ng total RNA input. Each ddPCR assay mixture (20 µl) was loaded into a disposable droplet generator cartridge (Bio-Rad). Then, 70 µl of droplet generation oil (Bio-Rad) was distributed into each of the eight oil well of the cartridge. The cartridge was then placed inside the QX200 droplet generator (Bio-Rad). When droplet generation was completed, the droplets were transferred to a 96-well PCR plate (xxxx) heatsealed with foil in a PX1 PCR Plate Sealer (Bio-Rad) and amplified with a Mastercycler Nexus Gradient Thermal Cycler (Eppendorf). Thermal cycling conditions for EvaGreen assays were as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec and 61°C for 1 min, followed by 4°C 5min and a final

inactivation step at 90°C for 5min. A no template control and a negative control for each reverse transcription reaction were included in the assay. After thermal cycling the sealed plate was placed in the QX200 Droplet Reader (Bio-Rad) for data acquisition. The resulting data was analyzed using QuantaSoft Software (version 1.7; Bio-Rad).

- **Behavioral tests**

Mice were subjected to locomotor activity to novel environment test 45 minutes post THC or its vehicle injection mice. Mice were put in the center of a transparent, open field (45 x 35 x 30 cm) with a gridline drawn on the bottom (each square measured 10 x 10 cm) for five minutes under controlled light (110 lux). Line-crosses and rearings were recorded with a mechanical counter by hand, and the results expressed as locomotion index of horizontal and vertical locomotion (crosses + rearings) [9].

Six- to twelve-weeks old β -Arrestin1 KO mice were subjected to spontaneous locomotor activity test. Mice were put one hour in the actimetry cages (Imetronics), and their basal activity was registered. Amphetamine was administrated in Arrb1 KO mice and immediately put in the actimetry cages for another hour, and their horizontal activity was registered as the number of cell breaks during the period of the experiment.

- **Experimental design**

THC 0.3 mg/kg kinetics of action in mouse brain: C57BL/6N mice were sacrificed 15 minutes, and 30 minutes post-injection (n=5-6 per group), and the third group have been subjected to the locomotor activity in novel environment test 45 minutes post-injection (n=10 per group) (Fig. 1a). Nucleus accumbens (NAc), hippocampus (Hpc), and cerebellum (CB) were withdrawn for Alpha analyses.

GDC-0084 effects on THC-induced hyperlocomotion and signaling: To find the subeffective dose of GDC-0084, C57BL/6N mice were treated with four increasing doses of GDC-0084: 0 (n=21), 6 (n=17), 9 (N=10), 12 (N=7), and 25 (N=5) mg/kg. Sixty minutes after GDC-0084 administration *p.o.* (gavage), mice were injected with THC vehicle, and 45 minutes later, mice were subjected to locomotor activity in novel environment (Fig. S2a). Then C57BL/6N mice were pretreated with the GDC-0084 at the sub-effective dose (6 mg/kg, *p.o.*) or its vehicle and treated with THC or its vehicle (n=9-12 per group). Forty-five minutes after injection, mice were subjected to locomotor activity in novel environment, while thirty minutes after injection, mice were sacrificed. NAc, Striatum (Str), Hpc, and CB were withdrawn for Alpha analyses (Fig. 2a).

Responsiveness of β -Arrestin 1 KO mice to THC (0.3 mg/kg) induced hyperlocomotion: β -Arrestin1 KO mice were treated with THC, or its vehicle (n=9-12 per group). Forty-five minutes after injection, mice were subjected to locomotor activity in novel environment (Fig. 4a). Mice were submitted to locomotor activity in a novel environment test forty-five minutes after THC-injection. Then, to control that the locomotor behavior of this breed of mice did not show any locomotor impairment, we tested locomotor behavior following amphetamines. β -Arrestin1 KO (n=15 per group) mice were put in the actimetry test cage for habituation. One hour later, all mice were treated with amphetamine sulphate and put again in the actimetry test cage for one hour (Fig. S4a).

PREG effect on THC-induced behavior and signaling: C57BL/6N mice were pretreated with PREG or its vehicle and treated with THC (0.3 mg/kg) or its vehicle (n=8-12 per group). Forty-five minutes after injection, mice were subjected to locomotor activity in novel environment, while 30 minutes after injection, mice were sacrificed. Nucleus accumbens (NAc), striatum (Str), and cerebellum (CB) were withdrawn for Alpha analyses (Fig 5a).

AEF0117 effect on THC-induced behavior and signaling: C57BL/6N mice were pretreated with AEF0117 or its vehicle and treated with THC (0.3 mg/kg) or its vehicle (n=12 per group). Forty-five minutes after injection, mice were subjected to locomotor activity in novel environment, while 30 minutes after injection, mice were sacrificed. Nucleus accumbens, striatum, and cerebellum (CB) were withdrawn for Alpha analyses (Fig 6a).

- **Protein extraction from brain tissues and protein quantitation**

Extraction of total proteins from mice brain structures was performed in AlphaLISA SureFire Ultra lysis buffer supplemented with protease and phosphatase inhibitors (#P8340 and #P0044, Sigma, USA) using the Precellys 24 homogenizer (a benchtop device for biological samples grinding, lysis, and homogenization; Bertin Technologies, Montigny-le Bretonneux, France) [23]. A two cycles homogenizing protocol (30 sec at 5000 rpm) with a 10-sec pause between the two cycles using ceramic CK14 beads (#03961-1-0032, Bertin Technologies) have been used. Then the samples were centrifuged three times at 10.000 rpm, 10 min at 4°C. Supernatants were kept for quantitation. Total proteins quantitation was done using a Direct Detect X spectrometer that analyzes the number of amide bonds (Merck Millipore) by mid-infrared spectroscopy. Then the protein samples were stored at -80°C until use [23].

- **AlphaLISA Analysis**

p-Erk1/2^{T202/Y204} (ALSU-PERK-A10K, and ALSU-PERK-A500), total Erk1/2 (ALSU-TERK-A10K, and ALSU-TERK-A500), Akt1/2/3 total (ALSU-TAKT-A500), p-Akt1/2/3^{T308} (ALSU-PAKT-A10K, and ALSU-PAKT-A500), p-Akt1/2/3^{S473} (ALSU-PAKT-B500), p-GSK3β^{S9} (ALSU-PGS3B-A500), p-RPS6^{S240/244} (ALSU-PS6R-A500), p-4EBP1^{T37/46} (ALSU-P4EBP-A500), and total Cofilin (ALSU-TCOF-A10K, and ALSU-TCOF-A500) levels were quantified using AlphaLISA SureFire Ultra kits, following PerkinElmer's instructions. In an OptiPlate-384 white opaque microplate (PerkinElmer, USA) were poured 10 µl/well of diluted protein

samples and 5 μ l of acceptor mix (containing both antibodies and Acceptor beads) [23]. The plate was sealed with a transparent adhesive film and put at room temperature for one or two hours of incubation for AlphaLISA SureFire Ultra and AlphaScreen SureFire. Then five μ l of donor mix (containing Donor beads) were poured into the wells under subdued light (<100 lux). The plate was sealed again, covered with foil, and put at room temperature in the dark for the second incubation of the same duration of the first. Then the plate was read with an Alpha technology-compatible plate reader (EnSpire Alpha plate reader, PerkinElmer) using default AlphaLISA/Screen settings. Alpha measurements were performed in duplicates: each sample was systematically transferred in two independent wells, and the mean Alpha signal value of both wells was used [23]. The samples were diluted in lysis buffer at the desired concentration: 0.625 μ g of total proteins in 10 μ l of lysis buffer was used to measure p-Erk1/2^{T202/Y204}, total Erk1/2, and p-4EBP1^{T37/46}; 1.25 μ g of total proteins in 10 μ l of lysis buffer were used to measure p-GSK3 β ^{S9}, and p-RPS6^{S240/244}; 2.5 μ g of total proteins in 10 μ l of lysis buffer were used to measure total Cofilin, total Akt1/2/3, p-Akt1/2/3^{T308}, and p-Akt1/2/3^{S473}.

- **Data analysis**

The Student's t-test was performed for pairwise comparisons. When the total interaction was significant, Tukey's test was performed for multiple comparisons. Behavioral analyses are expressed as percentage of the control group, AlphaLISA analyses are expressed as percentage of the control group.

Exploration of THC effects at middle and high doses (3 and 10 mg/kg) on behavior and CB1 signaling in mice

Introduction

THC, and more in general cannabinoids, when it reaches a toxic concentration in the brain, stimulate through a CB1-dependent process the production of pregnenolone (PREG), that can reach and surpass the 1500% of its average production in the brain when the doses of cannabinoids are elevated. In Vallée's and colleagues' work (2014), it was shown that C57BL6N mice started to significantly produce pregnenolone with doses of THC superior to 1.5 mg/kg. Since it is known that pretreatment with pregnenolone abolishes the majority of known THC-dependent effect in mice, it is possible to speculate that the endogenously produced pregnenolone dulls the effect of THC, making the outcome observed a resultant between the THC agonism and the PREG modulation of CB1 (Fig. 34).

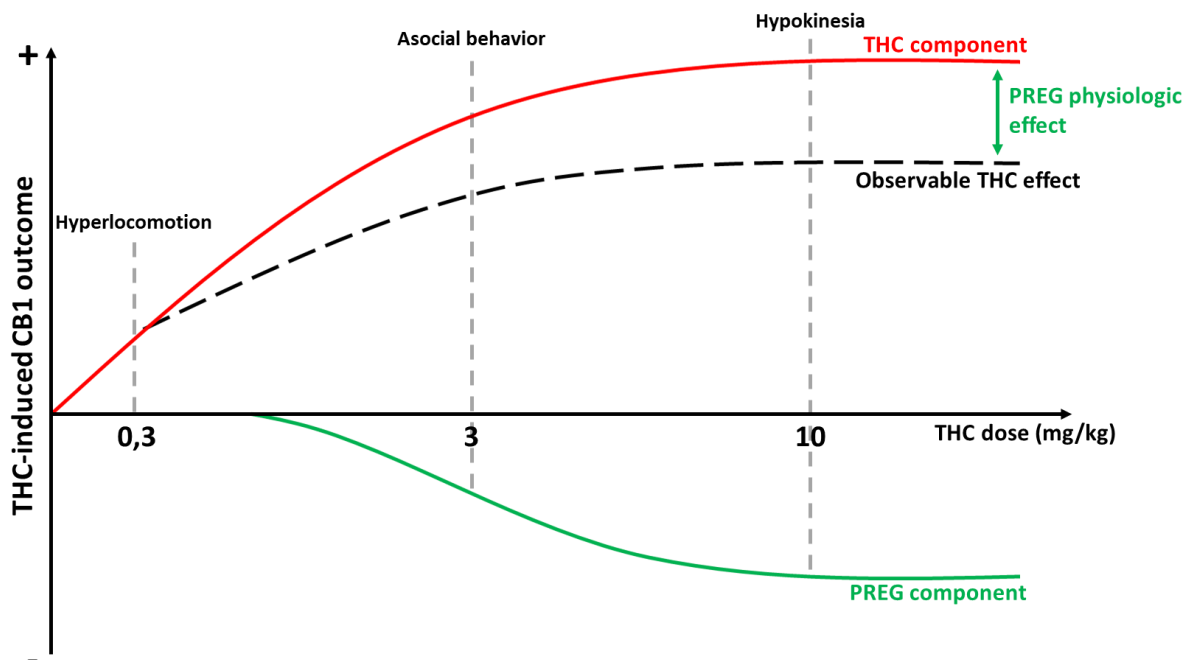


Figure 34: The observed effect of THC on CB1-mediated behavior and signaling may result from THC and PREG's action on the CB1 receptor. At the dose of 3 and 10 mg/kg, THC causes PREG production in the mouse brain, submaximally and maximally, respectively, while at the dose of 0.3 mg/kg, THC does not affect PREG production. When THC activates CB1 and induces PREG synthesis, PREG inhibits THC-induced CB1 activation acting as negative feedback. It is possible to speculate that the observed THC-dependent effects result from THC and PREG's opposite effects.

To better understand PREG's physiologic role and the mechanism of action of the CB1-SSi, it is imperative to study the THC-induced signaling pathway at higher doses of THC. To this end, in this section of the thesis, are provided the studies on the CB1 signaling following THC at the doses of 3 and 10 mg/kg, doses that are known to trigger PREG production in the brain, to collect pieces of information for the understanding of the pregnenolone' endogenous role, and better to understand the mechanism of action of PREG and AEF0117.

The two THC doses of choice, 3 and 10 mg/kg, respectively, represent a THC dose at which it is possible to detect a significant production of PREG and the THC dose at which the maximal PREG production is detected. THC is known at these doses to cause respectively asocial behavior (Busquets-Garcia et al., 2017b) and hypolocomotion (Katsidoni et al., 2013; Metna-Laurent et al., 2017). The fact that THC modifies sociability in humans is well-known for a long time (Foltin and Fischman, 1988; Tart, 1970), and it has been investigated in rodents as well (Jan M. van Ree et al., 1984). Although in humans, several works show that low doses of THC may improve sociability in rodents (Hall and Degenhardt, 2009; Tart, 1970), and it remains unclear if it positively or negatively affects aggressive behavior (Ostrowsky, 2011). It has been shown that in mice, quite consistently, THC reduces the interaction between mice (Busquets-Garcia et al., 2017b; Dorr and Steinberg, 1976). This effect has been proposed as a possible model of negative symptoms of cannabinoid-induced psychotic-like behaviors in mice (Busquets-Garcia et al., 2017b; C. Arnold et al., 2012). For what concerns the THC-induced hypolocomotion (or hypokinesia), it is one of the most studied effects of THC in mice. Several works point out a dopaminergic contribution to this effect. Navarro and colleagues (1993) demonstrated that Wistar rats, following acute oral administration of high doses of THC (5 mg/kg), showed impaired locomotor activity and that within the striatum, the dopamine receptor D1 was significantly diminished. Those data were further confirmed in another work (Romero et al., 1995). Other works pointed out that after high doses of THC can induce CB1 activity in

the striatum that could affect the glutamatergic release (Gerdeman and Lovinger, 2001), an event known to lead to hypolocomotion (Miller et al., 1998).

Furthermore, it has been proposed that THC's hypokinetic effect may be due to the increase of GABA release, a common effect of cannabinoids (Miller and Walker, 1998). It was observed that anandamide was able to reduce dopamine release in the striatum of rats significantly. This effect seemed to be due not only to the CB1 receptor but to the vanilloid-like receptors as well (de Lago et al., 2004). In a more recent work, Polissidis and colleagues (2013) proposed that WIN55,212-2, at high doses would sharply increase the release of dopamine, and at the same time, strongly decrease the release of glutamate in both the striatum and nucleus accumbens of rats, concluding that its action on the glutamatergic transmission caused the hypolocomotor effect of WIN55,212-2 and not the dopaminergic signal. This seems to be in contrast with the previous works. However, the agonist WIN55,212-2 has a very high affinity for CB1 (low nanomolar range), while THC has a lower affinity for CB1, which may indeed be a reason for this discrepancy. The author also proposed that the WIN55,212-2 affinity to CB2, and though a possible involvement of this receptor activity should not be excluded. However, even if WIN55,212-2 still shows high affinity for both CB1 and CB2, being CB2 very low expressed in the brain, and CB1 one of the most abundant GPCRs in the brain, it seems unlikely that the observed effect should come from CB2. Although several works showed the THC-induced biphasic effect on rodents' locomotor activity, none attempted to explore the intracellular signaling pathway underlying it.

To study the signaling pathway responsible for the THC-induced asocial behavior and hypokinesia in mice, our methodological strategy consisted of starting to investigate the kinetic effects of THC on the CB1 intracellular pathway. Then, we studied the effect of AEF0117 at one chosen time-point, corresponding to THC's maximal effect. Lastly, we started to investigate the signaling transducer carrying on the pathways.

Materials and methods

- Animals

Mice were housed individually in a temperature- (22°C) and humidity- (60 %) controlled animal facility under a constant light-dark cycle (light on 7 am – 7 pm) during one week before the test. Food and water were available ad libitum. When needed, animals were quickly sacrificed by decapitation. To preserve phosphorylation, brain areas were quickly dissected on ice, placed in dry ice-cold Precellys tubes (Bertin Technologies, Montignyle- Bretonneux, France) and stored at -80°C before protein extraction (Revest et al., 2010; Zanese*, Tomaselli* et al., 2020).

In experiment 1 (sociability), nine-weeks-old male C57BL/6N (weighing 25-30 g) were purchased from Janvier Laboratories (France), or eleven-twelve-weeks-old male β -Arrestin1 constitutive KO (B6.129X1(Cg)-Arrb1tm1Jse/J – Arrb1) mice (weighing 25-35 g) were purchased from The Jaxon Laboratory (Conner et al., 1997).

In experiment 2 (locomotion), nine-weeks-old male C57BL/6J (weighing 25-30 g) were purchased from Janvier Laboratories (France).

All experiments involving mice were performed according to the protocols approved by the Aquitaine-Poitou Charentes local ethical committee (authorization number APAFIS#10936-201708091043277 v5) in strict compliance with the French Ministry of Agriculture and Fisheries (authorization number A33-063-98) and European Communities Council Directive (2010/63/EU).

- Drugs

THC (#THC-1295S-250) was purchased from THC Pharm GmbH The Health Concept (Germany) and was dissolved in a solution of 4% ethanol, 4% cremophor, 92% saline (0.9% NaCl). It was administrated intraperitoneally at the doses of 3 or 10 mg/kg at a volume of 10 ml/kg. AEF0117 was obtained from Aelis Farma biotech (France) and was synthesized by

Atlanchim (France). It was dissolved in corn oil and was administrated via gavage at the dose of 0.015 mg/kg with a volume of 5 ml/kg, corresponding to the most observed dose in mice of ED100 for the inhibition of THC behavioral effects (Piazza et al., 2013). The timing of administration of AEF0117 has been chosen based on the pharmacokinetics of AEF0117 in mice (Piazza et al., 2013).

- **Behavioral tests**

Sociability: The sociability chamber was composed of an open field (35 cm x 35 cm) in which were put two cages on opposite corners, made of Plexiglas with little holes, to allow mice to see, smell, and hear each other, but not to touch each other (Fig. 35). One cage was left empty, while within a separate cage was put a mouse (social mouse) purchased in the same batch, with the same age, strain, and sex as the tested mice.

The area around the empty cage was named “non-social zone,” while the area around the cage with the social mouse was named "social zone." At the beginning of each session, the mouse to be tested was put at the cage's center and then left in the cage for five minutes (Fig 35). The light was set at 50 lux, and the mouse spent interacting with the other mouse or with the empty cage while it was in the social and non-social zone, respectively, was handly recorded offline. The social index was calculated as:

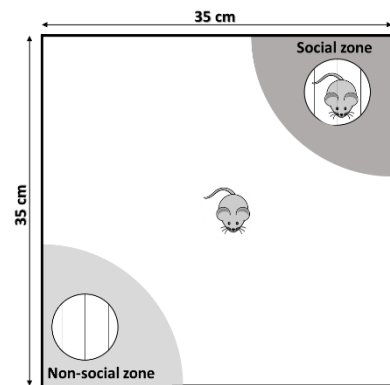


Figure 35: Sociability test in an open-field

$$\frac{\text{time spent in social zone}}{\text{total interaction time}} = \text{social index}$$

As control of activity, the total interaction was calculated by the time spent in both social and non-social zones (Busquets-Garcia et al., 2017b).

Locomotor activity: The automatic activity test (Imetronic) was composed of eight identical cages (22 x 12 x 18 cm), detecting eight mice's locomotion concurrently. Within each cage, there were infrared beam cells 3 cm above the floor. The experiment was conducted at fixed light (20 lux), and the number of cell breaks was recorded automatically for each mouse.

- **Experimental design**

Experiment 1: When used, AEF0117 or its vehicle was administrated via gavage 180 minutes before THC or its vehicle injection. Four groups of animals were used and received the same drug solutions. Three groups of mice were sacrificed 15, 30, or 90 minutes after THC or its vehicle injection, and the mice of the fourth group were subjected to sociability test for five minutes 120 minutes post-injection, and then sacrificed 130 minutes after injection (Fig 36). Sacrifices were performed by quick decapitation, and the brains were dissected at ice-cold temperature. Frontal cortex, striatum, nucleus accumbens, hippocampus, and cerebellum were collected. Then the structures were and stored at -80°C until extraction.

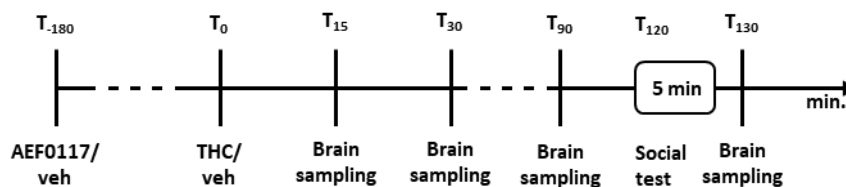


Figure 36: Experimental design of experiment 1

Experiment 2: Two groups of mice were used and received identical drug solutions. 30 minutes after THC or its vehicle injection, the mice of one group were subjected to the locomotor activity test for 15 minutes, while the mice of the other group were sacrificed, using

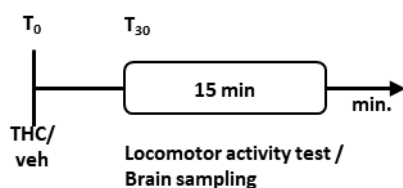


Figure 37: Experimental design of experiment 2

the same protocol that in experiment 1 (Fig. 37). Frontal cortex, striatum, nucleus accumbens, and hippocampus were collected. Then the structures were and stored at -80°C until extraction.

- **Protein extraction from brain tissues and protein quantitation**

Extraction of total proteins from mice brain structures was performed in AlphaLISA SureFire Ultra lysis buffer supplemented with protease and phosphatase inhibitors (#P8340 and #P0044, Sigma, USA) using the Precellys 24 homogenizer (a benchtop device for biological samples grinding, lysis, and homogenization; Bertin Technologies, Montignyle - Bretonneux, France) (Revest et al., 2010). A two cycles homogenizing protocol (30 sec at 5000 rpm) with a 10-sec pause between the two cycles using ceramic CK14 beads (#03961-1-0032, Bertin Technologies) were used. Then the samples were centrifuged three times at 10.000 rpm, 10 min at 4°C. Supernatants were kept for quantitation. Total proteins quantitation was done using a Direct Detect X spectrometer that analyzes the number of amide bonds (Merck Millipore) by mid-infrared spectroscopy. The protein samples were then stored at -80°C until use (Revest et al., 2010; Zanese, Tomaselli et al., 2020).

- **AlphaLISA Analysis**

p-Erk1/2^{T202/Y204} (ALSU-PERK-A10K), total Erk1/2 (ALSU-TERK-A10K), JNK1/2/3^{T183/Y185} (ALSU-PJNK-A500), Akt1/2/3 total (ALSU-TAKT-A500), p-Akt1/2/3^{T308} (ALSU-PAKT-A500 and ALSU-PAKT-A10K), p-Akt1/2/3^{S473} (ALSU-PAKT-B500), p-GSK3 β ^{S9} (ALSU-PGS3B-A500), p-mTOR^{S2448} (ALSU-PMTOR-A500), p-RPS6^{S240/244} (ALSU-PS6R-A500), p-4EBP1^{T37/46} (ALSU-P4EBP-A500), p-eIF4E^{S209} (ALSU-PEIF4-A500), p-CREB^{S133} (ALSU-PCREB-A500), and p-PDK1 (TGRPS500) levels were quantified using AlphaLISA SureFire Ultra kits, while p-PDK1^{S241} (TGRPS500) levels were quantified using AlphaScreen Surefire kit, accordingly to PerkinElmer's instructions. In an OptiPlate-384 white opaque microplate (PerkinElmer, USA) were poured 10 μ l/well of diluted protein samples and 5 μ l of acceptor mix (containing both antibodies and Acceptor beads) (Zanese, Tomaselli, et al., 2020). The

plate was sealed with a transparent adhesive film and put at room temperature for one or two hours of incubation for AlphaLISA SureFire Ultra and AlphaScreen SureFire, respectively. Then five μl of donor mix (containing Donor beads) were added to wells under subdued light (<100 lux). The plate was sealed again, covered with foil, and put at room temperature in the dark for the second incubation of the same duration as the first. The plate was then read with an Alpha technology-compatible plate reader (EnSpire Alpha plate reader, PerkinElmer) using default AlphaLISA/Screen settings. Alpha measurements were performed in duplicates: each sample was systematically transferred in two independent wells, and the mean Alpha signal value of both wells was used (Zanese, Tomaselli, et al., 2020). The samples were diluted in lysis buffer at the desired concentration: 0.625 μg of total proteins in 10 μl of lysis buffer was used to measure p-Erk1/2^{T202/Y204}, total Erk1/2, and p-4EBP1^{T37/46}; 1.25 μg of total proteins in 10 μl of lysis buffer were used to measure p-PDK1^{S241}, p-GSK3 β ^{S9}, p-RPS6^{S240/244}, and p-eIF4E; 2.5 μg of total proteins in 10 μl of lysis buffer were used to measure p-MEK^{S218/222} total Cofilin, total Akt1/2/3, p-Akt1/2/3T308, and p-Akt1/2/3S473; and 5 μg of total protein in 10 μl of lysis buffer was used to measure p-JNK1/2/3, p-mTOR²⁴⁴⁸ and p-CREB^{S133}.

- **Data analysis**

All efforts were made to minimize animal suffering and to reduce the number of mice used while maintaining reliable statistics. Student's t-test was used for pairwise comparisons of the groups in the kinetic experiment. For the THC and AEF0117 experiment, Two-Way ANOVA was performed, and Tukey's test was performed for multiple comparisons. Values were expressed as mean + SEM or mean \pm SEM.

THC-induced un-social behavior: main data, discussion, and perspectives

THC (3 mg/kg) alters CB1 signaling related to sociability in the frontal cortex, nucleus accumbens, and cerebellum.

The first step of this study was to confirm the THC-induced asocial behavior in mice and to study the THC-induced kinetics of intracellular signaling cascades. The brain areas were chosen among the most CB1-rich, while the CB1-dependent pathways have been analyzed through AlphaLISA. THC-treated mice 120 minutes after injection showed significantly decreased sociability, with no variation in total exploration (Fig. 38a). Since there was no significant alteration of the total amount of time spent for exploration, it was possible to exclude a reduced activity due to a hypothetic THC depressor effect (Busquets-Garcia et al., 2017). Given the AlphaLISA considerable throughput, we obtained a vast amount of data, analyzing several targets within different brain structures and time-points (Table 3), so we decided to consider a signaling pathway altered only when more than half of the tested targets from the same signaling pathway resulted coherently altered. Using this rule, we identified as the most fitting candidates for the THC-induced asocial behavior in the **frontal cortex**, 30 minutes post THC injection, where it was observed a decreased phosphorylation of PDK1 (on the epitope Ser241), Akt1/2/3 (on both the epitopes Thr308 and Ser473), mTOR (on the epitope Ser2448), RPS6 (on the epitopes Ser240/244), Erk1/2 (on the epitope Thr202/Tyr204), and CREB (on the epitope S133) (Fig. 38b). We also found in the **nucleus accumbens**, 130 minutes post-injection, a decrease of mTOR (epitope Ser2448), RPS6 (epitopes Ser240 and Ser244), Erk (epitopes Thr202 and Tyr204), and of JNK (epitopes Thr183 and Tyr 185). Plus, there was detected in the **cerebellum** 30 minutes post-THC injection increased phosphorylation of Akt1/2/3 (epitopes Thr308 and Ser473), and a long-lasting decrease in Erk phosphorylation starting 30 minutes and lasting 130 minutes after the injection of THC (Fig. 38d).

It is well known and established that the **frontal cortex** has quite a relevant role in sociability (Bicks et al., 2015; Bruin, 1991; Kolb, 1974) and that THC may alter the frontal cortex transmission in rats, disrupting their sociability (Zamberletti et al., 2014). Our data further support mice's frontal cortex, a decrease in the PDK1-Akt-mTORC1-p70S6K-RPS6 signaling pathway 30 minutes following THC administration, which may be related to the un-social effect of THC (Fig. 38b). Although we did not analytically detect p70S6K phosphorylation through our studies, we can deduce its involvement through two main other observations: RPS6 and mTORC1 phosphorylations. RPS6 presents five epitopes that can be phosphorylated to cause its activation (Ser235, Ser236, Ser240, Ser244, and Ser247). Still, the ones we have observed, Ser240 and Ser244, can be phosphorylated only by p70S6K, a direct target of mTORC1 (Hutchinson et al., 2011). The second substantial proof of p70S6K involvement in this pathway is mTOR's phosphorylation on the epitope Ser2448. mTOR present seven epitopes that can be phosphorylated (Ser1264, Ser1415, Ser2159, Ser2164, Thr2446, Ser2448, and Ser2481), and the Ser2448 is phosphorylated only by p70S6K through a feedback loop (Figueiredo et al., 2017). As previously disserted, mTOR is indirectly activated by Akt, which phosphorylates and inactivates PRAS40 and TSC1/2, leading to mTOR activation. The phosphorylation on Ser2448 seems to lead to a minor activation of mTOR itself that is negatively regulated by its downstream target loop, even if it is still under discussion (Figueiredo et al., 2017). Controversially, several works point out that mTOR, when hyperactive, has a central role in social deficit (Xing et al., 2019), while our observation would lead to the opposite conclusion. However, while we observed CB1 dependent mTORC1 inactivation, the canonical mTOR hyperactivity in mice frontal cortex leading to asocial and autistic-like behaviors was reported to be NMDA dependent (Burket et al., 2015).

Furthermore, the possibility of an inverted "U-shaped" curve for the activity of mTORC1 on social behavior cannot be excluded. In this case, both low and high activity of mTORC1 may

lead to social impairment. However, being the current experiment, a correlative and not causal study, mTOR may not cause the social deficit observed in mice but a collateral event. On the other hand, low phosphorylation of Erk1/2 within mouse frontal cortex have been already correlated with social impairment (Faridar et al., 2014), making of the MAPK in the frontal cortex another candidate for causal studies between THC and social impairment, as well as a candidate target for PREG and AEF0117 mechanism of action.

In the **nucleus accumbens** 130 minutes after THC injection, five minutes after the behavior, mice showed a decreased phosphorylation of mTORC1 and Erk pathways. Since there was no modification of the GSK3 β target, but there are decreases of both mTORC1 and RPS6 phosphorylation, it is possible to presume that this effect is indeed a mTORC1 specific pathway, even without direct information about Akt. However, the role of a decrease in the phosphorylation of mTORC1 in disrupted sociability may indeed raise more questions than answers since no other work seems to find a relationship between the reduced mTORC1 activity and disrupted sociability. On the other hand, similarly to the frontal cortex, it was detected in the nucleus accumbens a decrease in the phosphorylation of the MAPK, this time for both Erk and JNK proteins. Several works point out the centrality of the nucleus accumbens in social behaviors, suggesting that the pathway observed may be implicated in THC-induced asocial behavior (Francis and Lobo, 2017; Wallace et al., 2009).

Interestingly, this effect has been observed 130 minutes post-injection, meaning that the time observed is the closest to the behavior itself. Arguably, it could be speculated that socializing with the other mouse could have triggered in mice a pathway modification, and since the THC-treated mice did not socialize with other mice, showed a lack of increase, that compared to the vehicle appeared as a decrease, representing an effect and not the cause of the asocial behavior. There is a correlation between social behavior and the mTOR and MAPK pathway within the

nucleus accumbens, but it is still needed to understand the causality relation between the behavior and the pathway.

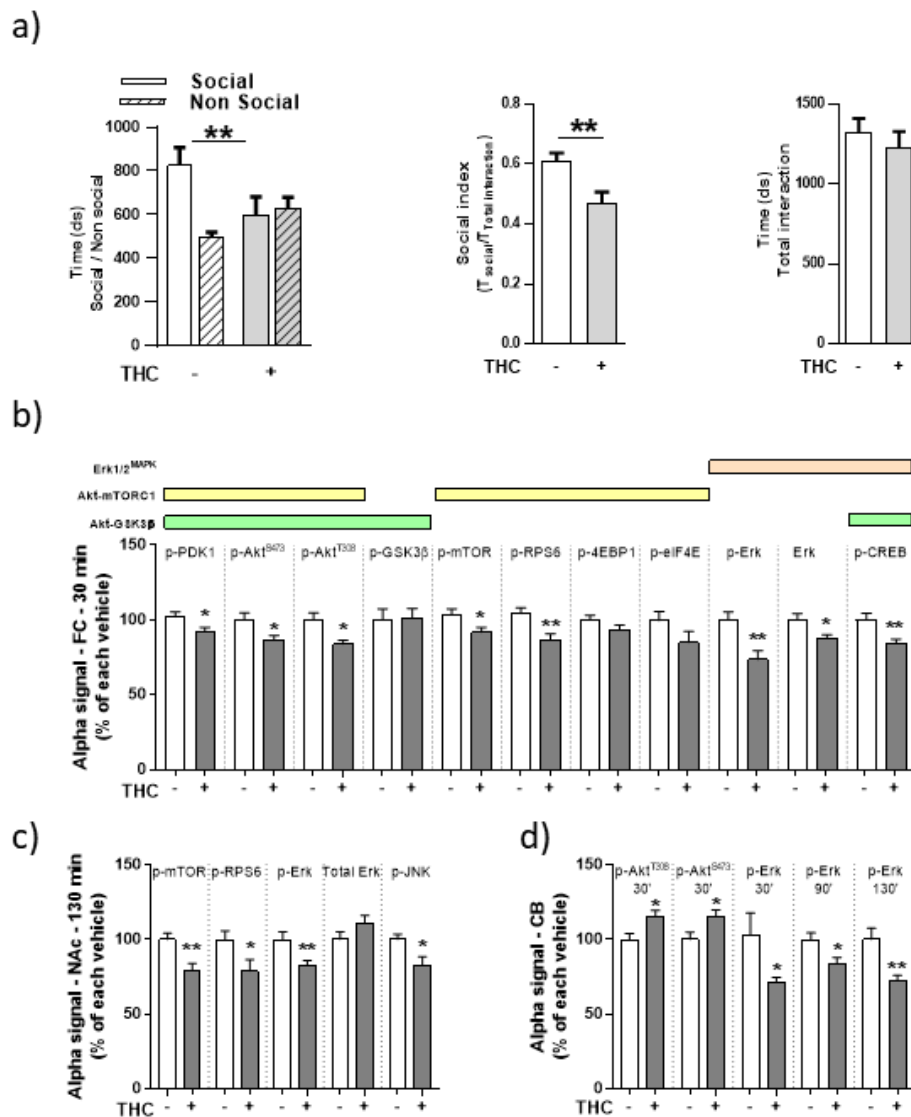


Figure 38: THC-induced asocial behavior and altered brain signaling pathways. **a)** THC (3 mg/kg) induced a social impairment in mice but did not decrease the time of total exploration (time spent socializing + time spent exploring the empty cage). **b)** In the frontal cortex, 30 minutes after THC injection, a significant decrease of phosphorylation was observed for all the targets of the Akt-mTOR pathway (designated by the yellow boxes) and the Erk1/2MAPK pathway (designated by the pink box), and almost all the targets of the Akt-GSK3 β pathway (designated by the green boxes) were decreased as well. **c)** It was detected a decrease of the mTORC1 pathway and the MAPKs Erk and JNK in the Nucleus Accumbens. **d)** In the cerebellum, a contextual increase of Akt (30 and 90 minutes post THC injection) and a long-lasting decrease of Erk phosphorylation were observed. Data are shown as mean +SEM. Comparison between THC vs vehicle groups, Student's t-test; *p<0.05; **p<0.01.

For what concerns the cerebellum, this structure's role in several cognitive and emotional brain functions is now well-established, and its role in autism spectrum disorder (Hampson and Blatt, 2015; Reeber et al., 2013). Several studies point out to the cerebellar functions in sociability (D'Angelo, 2019) and reward (Carta et al., 2019).

A - Frontal Cortex	15 minutes	30 minutes	90 minutes	130 minutes
p-PDK ^{S241}	105,9 ± 5,3	90,3 ± 2,9 *	99,2 ± 2,4	96,0 ± 1,4
p-Akt ^{T308}	114,7 ± 11,3	83,9 ± 2,7 *	104,8 ± 5,9	103,1 ± 5,7
p-Akt ^{S473}	105,3 ± 6,8	88,1 ± 2,8 *	98,0 ± 3,8	100,9 ± 3,9
p-GSK3β ^{S9}	102,8 ± 8,0	101,3 ± 6,4	93,4 ± 6,3	110,2 ± 6,5
p-mTOR ^{S2448}	99,6 ± 3,8	88,4 ± 3,2 *	96,5 ± 5,0	94,7 ± 3,7
p-RPS6 ^{S2402/4}	95,1 ± 4,3	82,9 ± 4,3 **	97,0 ± 4,4	77,5 ± 6,3 **
p-4EBP1 ^{T37-46}	100,1 ± 4,6	93,8 ± 2,9	100,0 ± 3,9	93,4 ± 2,2
p-eIF4E ^{S209}	104,8 ± 9,5	85,4 ± 7,3	91,9 ± 10,6	87,7 ± 6,9
p-CREB ^{S133}	91,2 ± 4,8	84,4 ± 2,8 **	104,7 ± 7,5	91,7 ± 3,2
p-Erk ^{T202/Y204}	104,5 ± 8,3	73,7 ± 5,7 **	96,8 ± 9,7	71,0 ± 4,1 **
Erk total	104,2 ± 4,5	87,6 ± 2,6 *	99,3 ± 3,6	100,8 ± 3,1
p-Erk ^{T202/Y204} / Erk total	100,1 ± 4,7	80,2 ± 6,8 *	95,0 ± 7,5	75,0 ± 5,0 **

B - Nucleus Accumbens	15 minutes	30 minutes	90 minutes	130 minutes
p-GSK3β ^{S9}	114,6 ± 18,7	129,2 ± 10,3 *	103,1 ± 8,4	121,1 ± 11,0
p-mTOR ^{S2448}	102,4 ± 4,6	101,8 ± 2,7	103,7 ± 2,3	79,9 ± 4,3 **
p-RPS6 ^{S2402/4}	130,6 ± 10,6 *	105,8 ± 5,8	98,1 ± 5,9	78,9 ± 7,6 *
p-Erk ^{T202/Y204}	118,4 ± 5,8 *	105,5 ± 6,4	91,9 ± 5,4	89,8 ± 4,7 *
Erk total	103,1 ± 3,0	105,9 ± 2,8	106,8 ± 1,8 *	108,1 ± 2,6 *
p-Erk ^{T202/Y204} / Erk total	115,0 ± 6,2 *	98,5 ± 4,6	85,3 ± 5,9	82,8 ± 5,6 *
JNK ^{T183/Y185}	94,8 ± 2,0	99,9 ± 2,9	108,8 ± 2,1 **	82,7 ± 5,9 *

C - Striatum	15 minutes	30 minutes	90 minutes	130 minutes
p-Akt ^{T308}	107,7 ± 11,4	107,9 ± 5,7	108,5 ± 7,1	106,6 ± 6,9
p-Akt ^{S473}	107,6 ± 7,7	108,0 ± 5,2	111,5 ± 6,2	107,3 ± 4,6
p-GSK3β ^{S9}	126,7 ± 25,1	128,3 ± 12,0 *	105,5 ± 8,9	103,7 ± 11,1
p-mTOR ^{S2448}	97,4 ± 3,2	106,7 ± 3,0	97,2 ± 2,8	98,3 ± 2,6
p-RPS6 ^{S2402/4}	105,1 ± 11,0	103,5 ± 8,9	139,5 ± 19,7 *	107,6 ± 13,3
p-4EBP1 ^{T37-46}	98,0 ± 2,3	99,1 ± 2,9	102,3 ± 3,7	87,5 ± 2,2 *
p-eIF4E ^{S209}	116,4 ± 9,8	116,8 ± 9,2	118,3 ± 9,7	134,1 ± 11,4 *
p-CREB ^{S133}	89,8 ± 9,8	94,8 ± 4,5	97,6 ± 4,5	92,3 ± 17,7
p-Erk ^{T202/Y204}	108,6 ± 4,9	95,2 ± 6,5	111,4 ± 6,8	79,7 ± 5,3 *
Erk total	104,3 ± 2,1	99,8 ± 2,7	106,2 ± 4,1	98,2 ± 3,5
p-Erk ^{T202/Y204} / Erk total	103,7 ± 4,4	96,5 ± 7,4	104,7 ± 5,2	81,2 ± 3,9 **

D - Hippocampus	15 minutes	30 minutes	90 minutes	130 minutes
p-Akt ^{T308}	101,7 ± 4,5	102,4 ± 5,3	119,7 ± 4,5 **	108,9 ± 4,3
p-Akt ^{S473}	100,8 ± 3,5	101,9 ± 5,7	114,5 ± 4,2 *	103,4 ± 6,7
p-GSK3β ^{S9}	98,5 ± 6,6	107,2 ± 6,9	110,9 ± 6,6	124,7 ± 6,9 *
p-mTOR ^{S2448}	96,4 ± 3,0	98,6 ± 2,1	103,9 ± 3,5	105,1 ± 2,8
p-RPS6 ^{S2402/4}	88,4 ± 3,6 **	105,2 ± 6,3	109,5 ± 5,0	93,0 ± 2,9
p-4EBP1 ^{T37-46}	93,7 ± 2,5	98,0 ± 3,2	103,3 ± 3,8	100,1 ± 2,6
p-eIF4E ^{S209}	85,2 ± 3,9 *	104,0 ± 7,7	94,6 ± 5,0	111,1 ± 6,1
p-CREB ^{S133}	101,0 ± 5,6	96,1 ± 4,4	119,1 ± 7,2 *	113,8 ± 4,5
p-Erk ^{T202/Y204}	96,5 ± 5,9	109,9 ± 7,5	104,9 ± 6,2	99,3 ± 5,4
Erk total	92,2 ± 2,6	103,4 ± 3,1	107,5 ± 3,8	106,7 ± 2,2
p-Erk ^{T202/Y204} / Erk total	104,6 ± 3,7	106,2 ± 6,3	97,3 ± 3,8	107,4 ± 3,8
JNK ^{T183/Y185}	96,9 ± 3,5	102,8 ± 6,2	98,5 ± 2,8	104,2 ± 4,3

E - Cerebellum	15 minutes	30 minutes	90 minutes	130 minutes
p-Akt ^{T308}	98,0 ± 5,0	115,1 ± 4,3 *	99,5 ± 2,9	119,5 ± 5,2 **
p-Akt ^{S473}	95,9 ± 4,5	115,2 ± 4,6 *	98,9 ± 3,5	121,4 ± 5,7 **
p-GSK3β ^{S9}	94,8 ± 7,1	102,4 ± 5,6	89,7 ± 3,9	110,8 ± 5,3
p-mTOR ^{S2448}	91,4 ± 3,3	99,9 ± 2,9	96,9 ± 2,0	100,6 ± 3,0
p-RPS6 ^{S2402/4}	91,8 ± 3,0	99,2 ± 6,1	97,0 ± 3,8	88,9 ± 5,4
p-4EBP1 ^{T37-46}	94,1 ± 2,5	97,8 ± 2,1	91,1 ± 2,9	101,0 ± 2,1
p-eIF4E ^{S209}	88,1 ± 5,2	109,3 ± 10,8	82,5 ± 2,7 **	121,0 ± 9,1 *
p-CREB ^{S133}	96,0 ± 8,8	96,6 ± 8,5	97,1 ± 5,0	120,0 ± 12,0
p-Erk ^{T202/Y204}	83,1 ± 2,7 *	71,4 ± 3,1 *	84,1 ± 3,8 *	72,8 ± 3,2 **
Erk total	88,9 ± 1,5 *	93,9 ± 2,9	94,7 ± 2,7	106,3 ± 2,3
p-Erk ^{T202/Y204} / Erk total	93,0 ± 2,6	80,7 ± 3,8 *	89,3 ± 1,7 *	70,4 ± 3,3 ***

Table 3: THC (3mg/kg) kinetics on intracellular signaling pathways. Analyses of protein phosphorylation following the THC or its vehicle treatment. All the analytes at all times in the frontal cortex, striatum, nucleus accumbens, hippocampus, and cerebellum. Data are expressed as mean (percentage) ± sem, *p<0.05; **p<0.01; ***p<0.001

Within the **cerebellum**, we have observed two distinct signalings. The increase of phosphorylation of Akt1/2/3 on both the epitopes Ser473 and Thr308 (Fig. 38d) seemed to happen in waves. The first and second waves were detected 30 and 130 minutes after THC injection, respectively, while no effect on Akt has been detected 15 and 90 minutes after THC injection (Table 3). Erk1/2 phosphorylation decrease was a long-lasting effect (Fig. 38d; Table 3). Interestingly both the effects on Akt1/2/3 and Erk1/2 were more intense after the behavior (130 minutes) (Fig. 39). mTORC1 signaling was not involved in this signaling pathway since either mTOR or its downstream targets' phosphorylation resulted altered at AlphaLISA analyses. In the hippocampus, according to our rule, no significant effect was detected in any pathway.

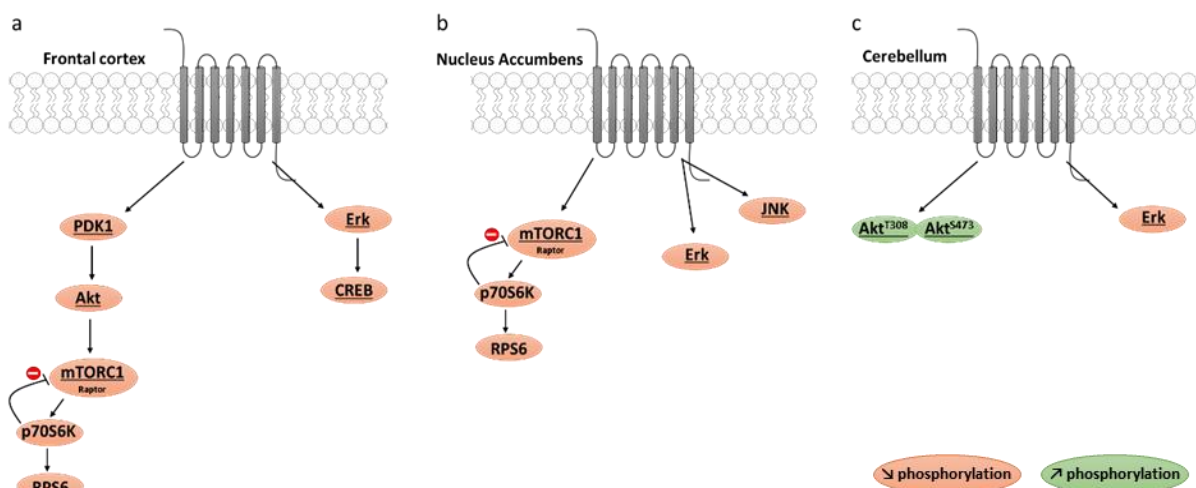


Figure 39: Signaling pathway alterations observed after THC (3 mg/kg) injection: a) in the frontal cortex, 30 minutes after injection; b) in the nucleus accumbens 130 minutes after injection; c) in the cerebellum the increase of Akt on both the epitopes and the long-lasting Erk decrease, from 30 to 130 minutes after injection.

To pinpoint the THC induced signaling pathway that leads to asocial behavior with accuracy was still not possible in these conditions. For this reason, causal studies are needed to investigate further the THC-induced signaling that impairs social behavior. Due to the observed alteration's complexity, finding a clear method to test causality may be indeed a long and challenging task. An indirect but still effective strategy to study which targets are more relevant

for THC-induced asocial behavior could be to study which of the THC-induced signaling pathway are affected and rescued by AEF0117. In this way, it could be possible to reduce the number of candidate targets and better understand which time-point is more relevant in this study.

- β -Arrestin 1 deletion enhances the THC-induced asocial behavior

The following step has been to study the signaling transducer involved in the THC-induced signaling pathway that leads to asocial behavior in mice by administrating THC to β -Arrestin 1 KO mice. In this exploratory experiment, we used the same individuals used for THC-induced locomotor hyperactivity after a drug-free period of inactivity of two weeks. Interestingly, the deletion of β -Arrestin 1 drastically increased the asocial behavior in mice (Fig. 40a and 40c), while it was not observed any significant difference in the total exploration of the animals (Fig. 40b). Possibly, this preliminary information only suggests that β -Arrestin 1 exerts a role in the CB1 signalization that leads to the cannabinoid regulation of sociability. However, instead of recruiting the signaling, β -Arrestin 1 may actively cause the receptor's internalization, and the

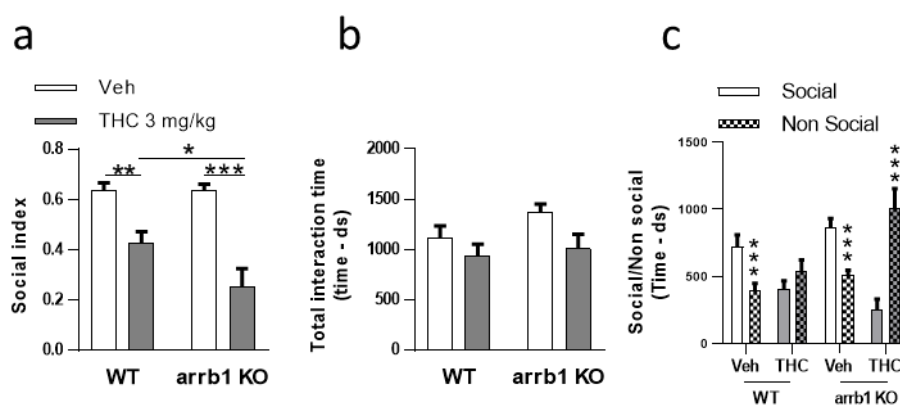


Figure 40: β -Arrestin 1 KO mice show enhanced THC-induced asocial behavior a) the social index was decreased in WT and arrb1 KO mice treated with THC compared to vehicle-treated mice. b) The total interaction time, expressed in deciseconds, was similar in all groups. c) The time spent in the social zone and the unsocial zone is represented for each group, expressed in deciseconds. Data are expressed as mean + SEM; Student's t-test was used for pairwise comparisons (social/non-social) of the groups, while Tukey's test has been used for multiple comparisons (social index and social interaction). * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

cease of signal (Fig. 41). As discussed in the introductory section, β -Arrestin 1 is part of a family of proteins named Arrestins. These proteins have been originally discovered to cease the GPCR signaling through the receptor's internalization and desensitization (Jones and Hinkle, 2005). Most likely, β -Arrestin 1, in this specific case, allows internalization and desensitization of the CB1 receptor, and not its trafficking, and its absence would enhance the CB1 signaling, explaining why β -Arrestin 1 KO mice show an increased THC-induced asocial behavior.

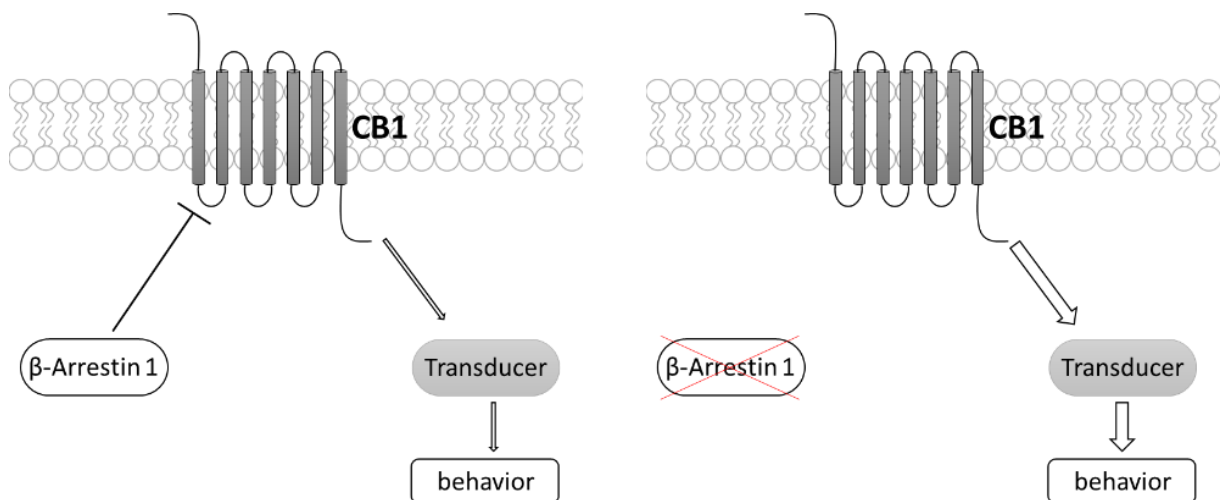


Figure 41: Schematic representation of the suggested role of β -Arrestin 1 in THC-induced asocial behavior. On the left, is represented the signaling in the WT mouse brain, on the right, is represented the signaling in the β -Arrestin 1 KO mice after exposure to THC 3 mg/kg. β -Arrestin 1 would not represent the signaling transducer responsible for recruiting the THC-induced signaling leading to the behavior, but an element that, through receptor internalization, may help to cease the signaling. In this context, the *arrb1* KO mice would enhance THC-induced output since the signalization blockade element is lacking.

- AEF0117 blocks the THC (3 mg/kg)-induced asocial behavior

Once identified the kinetics of the intracellular cascades, the following step has been to test the efficacy of AEF0117 on THC-induced asocial behavior. We confirmed that AEF0117 reversed THC's effect, as previously found (Piazza et al., 2013), and as it was also demonstrated with pregnenolone (Busquets-Garcia et al., 2017b).

Mice treated with THC, as expected, showed reduced sociability, with a significant decrease in the social index, and AEF0117 rescued normal sociability in mice, while it did not show any *per se* effect (Fig. 42). Also, THC did not affect total exploration nor locomotion.

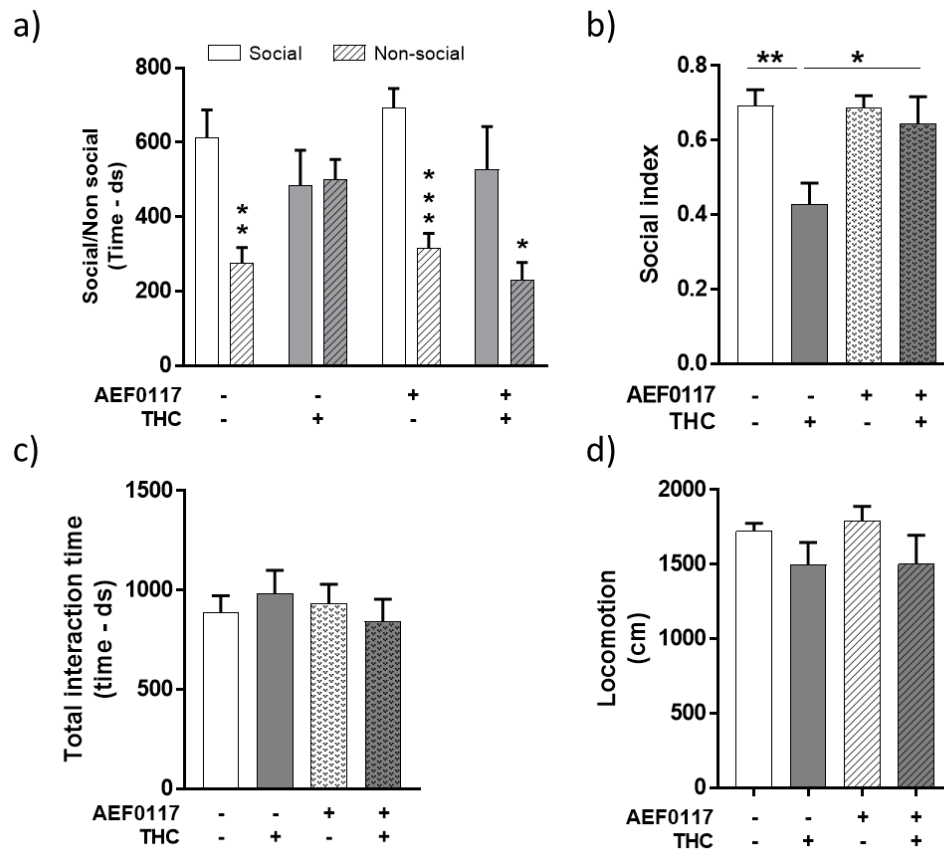


Figure 42: AEF0117 rescues THC-induced asocial behavior a) and b) THC treated mice showed an impairment of sociability, while in c) and d) did not show any significant difference total interaction time locomotion. Data are expressed as mean \pm SEM. The student's t-test was used for pairwise comparisons of the groups, while Tukey's test was used for multiple comparisons. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

THC-induced hypolocomotion: main data, discussion, and perspectives

As already described in the literature, mice showed a severe drop of locomotion following treatment with a high dose of THC (10 mg/kg) (Canazza et al., 2016; Katsidoni et al., 2013, 2013; Marshall et al., 2014; Metna-Laurent et al., 2017; Wiebelhaus et al., 2012; Wiley et al., 2005) (Fig. 43a). Since we have already proven that the signaling cascade causing hyperlocomotor behavior is detectable 30 minutes after injection, we decided to study the THC signaling pathway causing hypolocomotion at the same time-point.

The frontal cortex, nucleus accumbens, and striatum presented the same signaling pattern: Akt phosphorylated on the epitope Thr308, but not on Ser473 resulted in overphosphorylated, and Erk phosphorylated on the epitopes Thr202/Tyr204 phosphorylation resulted decreased, while GSK3 β phosphorylation on the epitope Ser9, tested only in nucleus accumbens, resulted increased (Fig. 43b,c,d; Table 4). Within the hippocampus, it has been found hyperphosphorylated Akt on the epitope Thr308, but not Ser473, GSK3 β on the epitope Ser9, eIF4E on the epitope Ser209, and as well Erk, phosphorylated on the epitope Thr202/Tyr204. Interestingly the signaling pathway observed in the striatum, nucleus accumbens, and frontal cortex resulted in different from the one observed in the hippocampus. Within the hippocampus, it has been observed increased phosphorylation of both the Akt-GSK3 β and Akt-mTOR and the Erk pathways, while in the three other structures, it has been detected an increase of the Akt-GSK3 β and a decrease of the Erk pathway. Among the results shown, however, the most interesting are surely the ones observed in the striatum, nucleus accumbens, and frontal cortex, being structures responsive to THC, CB1-rich, and deeply involved locomotor activity.

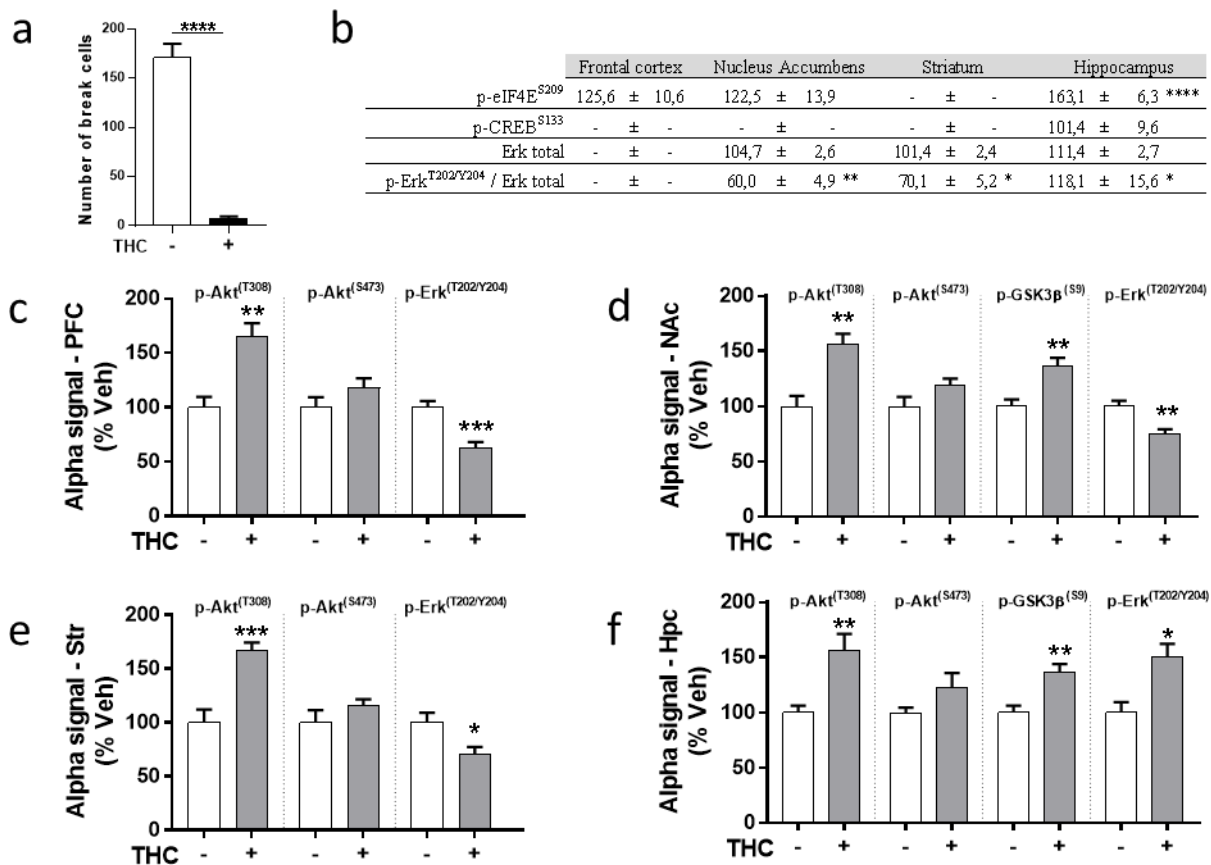


Figure 43: THC-induced hypolocomotion and its related signaling pathway a) THC (10 mg/kg) treated mice showed a hypolocomotion. Alpha analyses of protein phosphorylation from b) eIF4E, CREB, and total Erk; c) Akt-GSK3 β and Erk signaling in c) prefrontal cortex, d) nucleus accumbens, e) striatum and f) hippocampus. Data are expressed as mean + SEM. Student's t-test was used for pairwise comparisons of the groups. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The signaling observed in the THC treated mice seem to be the exact opposite of the D2R signaling after activation through dopamine. As shown in the introduction, after activation, the D2R recruits β -Arrestin 2 as a signaling transducer, which activated the phosphatase 2A (PP2A), which, in turn, acts on Akt unphosphorylating the epitope Thr308 only, without having any effect on the epitope Ser473. Akt, unphosphorylated, cannot phosphorylate and inactivate GSK3 β , which in turn is in its unphosphorylated form, and thus activated state (Beaulieu et al., 2005, 2004; Beurel et al., 2015), and contextually the Erk phosphorylation increase (Valjent et al., 2000). In our experiments, we have observed increased phosphorylation of Akt on the epitope Thr308 and not in Ser473, and in GSK3 β , with a contextual decrease of Erk phosphorylation on the epitopes Thr202/Tyr204. Compared to the results obtained from THC

0.3 mg/kg, the profile seemed to be quite different, even if, in both cases, Akt on the epitope Thr308 and GSK3 β on the epitope Ser9 resulted overphosphorylated. In fact, for the THC-induced hyperlocomotion, we demonstrated that the CB1 dependent process is recruited by β -Arrestin 1, and pass through PI3K, leading to active phosphorylation of Akt and GSK3 β ; furthermore, Akt was overphosphorylated in both the epitopes (Thr308 and Ser473), and no effect was observed in Erk phosphorylation. On the other side, the evidences showed about THC-induced hypolocomotion would point to an inhibition of the intracellular D2 pathway, where a reduced PP2A activity would lead to the absence of dephosphorylation of Akt and thus increased phosphorylation of GSK3 β . However, it has been demonstrated that high doses of THC (and more in general of cannabinoids) promote dopamine release, and at such a high dose it should be expected an intense dopamine release in the striatum and the nucleus accumbens (Polissidis et al., 2010; Vallée et al., 2014). It is also a fact that high doses of cannabinoids impair locomotor activity (Katsidoni et al., 2013; Metna-Laurent et al., 2017; Polissidis et al., 2010; Vallée et al., 2014). It is known that amphetamine, the prototypical dopaminergic drug, has an "inverted U" shaped effect on locomotion (Dunnett and Robbins, 1992), and that dopamine may have a self-inhibitory activity (Benoit-Marand et al., 2001; Groves et al., 1975), and so one possibility could be that high doses of THC stimulate a massive release of dopamine, that lead to hypolocomotion due to dopaminergic self-inhibition. The fact that GSK3 β could be overphosphorylated in both the cases, leading to two opposite effects, could be explained through its unique characteristics: as disserted before, GSK3 β alone exerts no activity on its downstream targets, unless those latter are not pre-phosphorylated four amino acids before the epitope target of GSK3 β (Doble, 2003) (Fig. 23). In this regard, it is possible to suppose that GSK3 β activity and outcomes profoundly depend on other kinases that pre-phosphorylate different targets, allowing GSK3 β to act on different pathways. Indeed, this would mean that the hypolocomotion and the hyperlocomotion induced by different doses of THC would not be

carried on by the same cells, nor by the same cellular process, demonstrating that CB1 exerts its behavioral effects by regulating other systems or acting on its own. Further studies proving the causality between the Akt-GSK3 β /Erk signaling pathway and the hypolocomotion are needed and the understanding of the signaling transducer involved. Indeed, it will be essential to approach studies on the THC-induced neurotransmission, and lastly, to test the AEF0117 on both behavior and signaling pathways.

Discussion

The endocannabinoid system is a complex regulatory system in the brain, mainly composed of endocannabinoids, lipid-based molecules that are active on the cannabinoid receptors, CB1, CB2, and the GPR55, and all the enzymes that produce and degrade the endocannabinoids. The endocannabinoid system modulates a plethora of different functions in the central nervous system and periphery. The CB1 receptor, one of the most abundant GPCRs in the brain, and the GPR55 are mainly central, although CB1 can be found in the periphery as well, while the CB2 receptor is mainly peripheral (Busquets-Garcia et al., 2017a, 2016). THC, the main active component of the *Cannabis sativa* plant, is one of the most commonly abused drugs around the world (Hardwick and King, 2008). THC acts on the CB1 receptor, one of the most abundant G-protein-coupled receptors in the brain, expressed in a large number of brain areas, including the basal ganglia (Julian et al., 2003), frontal cortex (Flores et al., 2013; Lin et al., 2009), and cerebellum (Marcaggi, 2015), and several cell types, mainly GABAergic and glutamatergic neurons, but also in astrocytes (Busquets-Garcia et al., 2017a) and mitochondria (Bérnard et al., 2012). THC forces the activation of the circuitry of reward and addiction acting on the CB1 receptor present in the GABAergic neurons of the ventral tegmental area (VTA), causing the release of dopamine in the nucleus accumbens (Lupica et al., 2004). THC is now known as also being strongly correlated to several mental pathologies: addiction (Curran et al., 2016), bipolar disorder (Khan and Akella, 2009), and cognitive impairment (Calabrese and Rubio-Casillas, 2018) are considered relatively mild THC-related effects, while the worst cases present cannabis use disorder (Hayley et al., 2017; O'Brien, 2011) and cannabinoid-induced psychosis (S. Andréasson et al., 1987; D'Souza et al., 2016). Since the CB1 receptor is involved in many physiologic functions, THC, depending on its dose, can cause several different outcomes, so in order to study cannabinoid-related pathologies more effectively, it is important to study appropriate doses and their related behavior in animal models. For this work, we have chosen to focus on three THC doses in mice: 0.3 mg/kg, leading to hyperlocomotion (Busquets-

Garcia et al., 2017b); 3 mg/kg leading to asocial behavior (Busquets-Garcia et al., 2017b); and 10 mg/kg leading to hypolocomotion (Metna-Laurent et al., 2017). It is known that cannabinoids exert a biphasic effect on locomotion (Katsidoni et al., 2013): low doses of cannabinoid agonists cause hyperlocomotion, while high doses cause hypolocomotion. In order to study the biphasic effects of THC in mice, the dose of THC of 0.3 mg/kg was used to reproduce hyperlocomotion (Busquets-Garcia et al., 2017), while the dose of 10 mg/kg was used to reproduce hypolocomotion (Metna-Laurent et al., 2017). All the psychostimulant drugs that are known to enhance locomotor activity cause a significant release of dopamine in the nucleus accumbens (Di Chiara and Imperato, 1988), an event that is closely related to reward (Nicola et al., 2005) and addiction (Adinoff, 2004), while it is known that a blockade of dopaminergic signaling leads to catalepsy and hypolocomotion (Casti et al., 2004). Furthermore, the CB1 receptor is extremely present in several brain areas involved in locomotion, such as the frontal cortex, cerebellum, and almost all the basal ganglia structures (Flores et al., 2013). The biphasic effect of THC on locomotion could involve dopaminergic transmission. Poddar and Dewey (1980) showed that THC at low doses promoted dopamine release in the hypothalamus and striatum, while high doses of THC tended to block it. However, although the effect on locomotion is biphasic, cannabinoid-induced dopamine release has been shown to be linear in several other works, and WIN55-212,2 at doses at which rats show hypolocomotion also induces a strong release of dopamine in the striatum (Polissidis et al., 2013; Vallée et al., 2014). Polissidis et al., (2013) proposed that cannabinoid-induced hyperlocomotion could be due to the increase in dopamine release, while cannabinoid-induced hypolocomotion due to the cannabinoid-induced blockade of glutamatergic transmission is observed only at high doses of cannabinoids. However, an auto-inhibitory role of such a massive release of dopamine cannot be ruled out (Benoit-Marand et al., 2001; Moquin and Michael, 2009). The dopamine D2 receptor is located both pre- and post-synaptically. The

postsynaptic functions of D2R are mainly excitatory, but the presynaptic role is mainly inhibitory, and it is usually activated after sustained firing (De Mei et al., 2009). Furthermore, it has been observed that the dose at which THC causes hyperlocomotion is not sufficient to also cause dopamine release in the striatum (Busquets-Garcia et al., 2017b; Vallée et al., 2014). We have therefore chosen to focus on a third dose as well, representing the medium dose of THC (3 mg/kg), which is known to cause sociability in mice (Busquets-Garcia et al., 2017). THC can alter sociability in humans and animals. It has been shown that it could modulate sociability in a biphasic way. One of the first works on the social effect of cannabis abuse showed that people who used cannabis felt well disposed towards other people (Tart, 1970). However, it was observed that people who used cannabis diminished social conversation (Higgins and Stitzer, 1986) but increased coaction in the absence of verbal communication (Foltin and Fischman, 1988). Nevertheless, people addicted to cannabis showed social withdrawal and lower interpersonal communications, which was especially true during withdrawal symptoms (Haney et al., 1999). Animal models have also shown that THC acts on sociability in a biphasic manner: low doses of THC improve sociability, while high doses disrupt it (Jan M. van Ree et al., 1984).

In reviewing CB1 signaling, we realized that it has been only partially investigated and that no in-depth studies have examined the intracellular CB1 signaling pathways leading to specific THC-induced behaviors. CB1 is known to be one of the most abundant GPCR and this cannabinoid receptor was initially identified as an inhibitory receptor coupled to protein G_i (Devane et al., 1988; Matsuda et al., 1990). However, during the last decades, the level of complexity of the signaling of this receptor has been found to be remarkable, with a biased signaling pathway (Ibsen et al., 2017). For a receptor, having a signaling bias means that under different conditions, it can rely on different signaling transducers for different signaling pathways (Smith et al., 2018). The CB1 receptor can trigger different signaling pathways, and

several pieces of evidence have shown that it can bind to and use the three families of G proteins (i, q, and s) (Turu and Hunyady, 2010), as well as β -Arrestins (Daigle et al., 2008b; Nguyen et al., 2012; Nogueras-Ortiz and Yudowski, 2016). Among the several signaling pathways that CB1 can trigger, the ones that have been chosen for this work are those known to play a role in locomotion (Beaulieu et al., 2007; Valjent et al., 2000) and sociability (Sato et al., 2011; Xing et al., 2019) and pathologies related to them (Doble, 2003, 2003; Engel et al., 2009; Gourley et al., 2008; Muneer, 2017): the MAPK pathway (Asimaki and Mangoura, 2011), Akt-GSK3 β (Ozaita et al., 2007), and Akt-mTORC1 (Raptor) (Puighermanal et al., 2009).

Understanding the signaling pathway underlying THC-induced pathological behaviors may boost the discovery of new molecules that are active on pathological signaling only, reducing the potential risks of an orthosteric inhibition of the receptor. In fact, to counteract the negative effect of CB1 activation, rimonabant was initially used as a CB1 antagonist (Poncelet et al., 1999). Although this drug was effective in preclinical studies to inhibit CB1-mediated THC effects (Carai et al., 2006, 2006; Patel and Pathak, 2007), clinical studies revealed its limitations. It has proved to be effective in treating obesity (Curioni and André, 2006), but such a drastic blockade of CB1 signaling ended up causing significant side-effects (such as anxiety and depression), leading to its withdrawal from the market (Sam et al., 2011). The importance of the CB1 receptor in the brain as a suitable target for many pathologies, and the risks of using orthosteric inhibitors of the receptor, shifted attention to positive and negative allosteric modulators of CB1 (PAM and NAM, respectively), namely molecules that interact with allosteric sites that are different from those the agonist or antagonist engage with. Since allosteric modulators regulate signaling without affecting its basal signaling, it is strongly believed that they should not present the same drawback issues as rimonabant. To date, some of the most widely-studied CB1 allosteric modulators have been the synthetic ORG-compounds, of which ORG27569 is considered the prototypical NAM of CB1 (Osborne et al.,

2019), and the steroid precursor pregnenolone, that is an endogenous negative allosteric modulator of the CB1 receptor (Vallée et al., 2014). THC induced a CB1 and dose-dependent increase in pregnenolone levels in the brains of mice and rats, by over 1500% for the highest doses, and pregnenolone can abolish several THC-induced behaviors and toxic effects in mice and rats (Busquets-Garcia et al., 2017; Vallée et al., 2014), strongly suggesting an endogenous negative feedback of pregnenolone on CB1 over-activation (Vallée, 2016; Piazza et al., 2013). Furthermore, as it is able to block Erk phosphorylation, but not cAMP inhibition in CHO cells transfected with *hCB1*, pregnenolone has been identified as a biased negative allosteric modulator (Vallée et al., 2014). However, since pregnenolone is a steroid precursor (and can be metabolized into downstream steroids), and shows very low bioavailability *per os* and half-life (Vallée, 2016), a therapeutic strategy has led to the synthesis of new non-metabolized pregnenolone-like compounds, thereby leading to a new pharmacological class, the CB1-Signaling Specific Inhibitors (CB1-SSi) with AEF0117 as the lead compound (Piazza et al., 2012). This compound presents the advantage of not being metabolized by any endogenous metabolic enzyme into a downstream steroid, of showing high bioavailability *per os* and high half-life, and blocking several THC-induced behaviors in animal models (Piazza et al., 2013). Not only is this molecule an optimal candidate for human treatment of cannabinoid-related diseases, but is also an excellent tool for studying the mechanism of action of pregnenolone, modulating CB1 in a similar manner but being impossible to convert into any downstream steroid.

The current work aims to identify the CB1 intracellular signaling pathway triggered by the three doses of THC mentioned above (0.3, 3, or 10 mg/kg) leading respectively to hyperlocomotion, asociability, and hypolocomotion, and to unravel the mechanism of action of AEF0117 on these signaling pathways. The three doses of THC used in this work induced a progressive increase in pregnenolone synthesis, with no production at 0.3, mild production at 3, and maximal

production at 10; suggesting a parallel between CB1 activation by THC and CB1 inhibitory modulation by pregnenolone. It follows that the mechanism of action of AEF0117 on the CB1 intracellular pathway can be achieved more easily through a THC dose that does not cause pregnenolone production in the brain, currently the dose of THC that induces hyperlocomotion in a mouse model.

As this work is mainly focused on signaling pathway research and as signaling pathways are mainly composed of kinases that exert their activity by phosphorylating their targets, it was necessary to develop a technique that would allow rapid, semiquantitative, sensitive, and reproducible screening of phosphoproteins, with a considerable throughput. We decided to turn to Perkin Elmer's AlphaLISA technique. However, this technique has been reported to be effective only on cell lysates and never on brain tissue samples. The first step in this work was therefore to fine-tune this technique for our purposes. In this respect, we have published a methodological paper, in which we have shown that AlphaLISA is a reliable, highly reproducible technique which, when compared to the western blot that is the gold standard technique in this field, requires less than 1/10th of the amount of sample to work, shows less variability due to its easy-to-use protocol, and boasts a throughput that is between 12 and 24 times higher. We also showed that this technique was perfectly suited for cell fractionation and synaptosomal preparation. Altogether, we demonstrated that for brain intracellular signaling research, AlphaLISA is perfectly suited. Then we investigated the THC-induced CB1 signaling that leads to hyperlocomotion in mice. This work was carried out in the form required by Current Biology, the journal to which it was to be sent. In this work, we worked in parallel to perform a study of THC-induced hyperlocomotion and an investigation of the kinetics of activation of the CB1 signaling pathway. The brain areas we analyzed were selected from among those that correspond to two characteristics at the same time: being involved in locomotor activity and being CB1-rich (nucleus accumbens, striatum, and cerebellum), plus we

decided to consider a structure (the hippocampus) with a high level of CB1 receptors but not involved in the locomotor activity, as a negative control. Through this first experiment, we were able to find a correlation between hyperlocomotion (observable 45 minutes after THC injection) and overphosphorylation of the Akt^{T308}, Akt^{S473}, and GSK3 β ^{S9} targets, while no effect was detected among the MAPK and mTOR signaling pathways thirty minutes post-injection in the nucleus accumbens and cerebellum. In contrast, no effect was detected in the hippocampus. To understand whether the observed pathway and the behavior were causally related or if there were only contextual outcomes, we decided to use a PI3K (a target upstream of Akt and GSK3 β) inhibitor: GDC-0084. As GSK3 β is constitutively active, it was not possible to use a GSK3 β inhibitor, since its function was already inhibited through phosphorylation. We pretreated mice with a sub-effective dose of GDC-0084 that showed no *per se* effect on their locomotion and treated them with THC. Interestingly, GDC-0084 was able to abolish the THC-induced hyperlocomotion and contextually, it rescued GSK3 β signaling in the nucleus accumbens and striatum. Furthermore, GDC-0084 caused a drastic decrease in phosphorylation in all the other targets downstream from PI3K (Akt on both the epitopes, RPS6). Since the disruption of the phosphorylation of Akt on Ser473, Thr308 and the mTOR pathway was not sufficient to cause any effect on locomotor activity in mice, but rescuing GSK3 β also rescued locomotion, we supposed that the latter has a hyper-specific role in THC-induced locomotor activity. Then we decided to investigate the intracellular signaling transducer that the CB1 receptor recruits following THC. We started a treatment with THC of a model of the murine striatal medium spiny neuron cell line, STHdh^{Q7/Q7}. However, we were not able to find a THC-dependent increase in phosphorylation of Akt or GSK3 β . Then we investigated the expression of the genes encoding for several signaling transducers. Since the ratio between *arrb1* (the gene encoding for β -Arrestin 1) and *gnai* (the gene encoding for the α subunit of Gi protein) was ~1:50, we hypothesized that there was a system bias within the STHdh^{Q7/Q7}, meaning that the relative

abundance of one transducer over the other biased the system towards the latter (Smith et al., 2018). To prove this concept, we transfected the cells with the *ARRB1* gene, encoding for human β -Arrestin 1. The cells transfected with the *ARRB1* gene, when treated with THC, showed an increase in the Akt^{Thr308/Ser473}-GSK3 β pathway. To further prove that the PI3K-Akt-GSK3 β pathway was carried by β -Arrestin 1, we tested THC in β -Arrestin 1 KO mice. While wild type mice showed increased locomotion due to THC, the KO mice did not, demonstrating once and for all that this signaling pathway is recruited by β -Arrestin 1, and is necessary for THC-induced locomotor activity. Once that we discovered the THC-induced CB1 signaling pathway leading to hyperlocomotion, we tested AEF0117 in THC-treated mice. AEF0117 rescued normal locomotion in mice, without showing any *per se* effect on behavior. Furthermore, it rescued phosphorylation of the Akt^{Thr308/Ser473}-GSK3 β pathway. Altogether this work shows, for the first time, a CB1 signaling pathway leading to a specific behavior. At first sight, the Akt^{Thr308}-GSK3 β ^{S9} signaling could be confused with a dopaminergic response, since it has been well described that the D2 receptor, when activated, causes the activation of GSK3 β and hyperlocomotion. However, a few major details strongly suggest that there is no involvement of the dopaminergic pathway. The first is that such low doses of THC do not cause dopamine release in the striatum (Vallée et al., 2014), and so such stronger dopaminergic signaling would be highly improbable. Secondly, D2 receptor signaling needs to activate GSK3 β to cause hyperlocomotion, and since the latter is constitutively activated, its phosphorylation blocks its activity, it does not enhance it. If there was a dopamine-dependent hyperlocomotion, the observed effect would have been diminished phosphorylation of Akt on epitope Thr308 and GSK3 β on epitope Ser9. The fact that Akt has been found phosphorylated on Ser473 is another important element to consider. In fact, according to the known literature about dopaminergic D2 signaling, the receptor recruits β -Arrestin 2, which in turn activates the phosphatase 2A (Beaulieu et al., 2007). The latter can recognize Akt only on epitope Thr308

and not on epitope Ser473, showing that as there is a significant effect on this latter Akt epitope, the signaling cannot be attributed to the dopamine D2 receptor. On the other hand, it has been shown that the D1 receptor can cause phosphorylation of the whole Akt^{Thr308/Ser473}-GSK3 β pathway, through interaction with the RTK receptor (Beaulieu et al., 2011). If the signaling had been “mediated” by the dopaminergic neurons, in the *STHdh*^{Q7/Q7} cells that are medium spiny neurons, and so GABAergic neurons, it would have been impossible to reach the Akt^{Thr308/Ser473}-GSK3 β signaling pathway, regardless of the presence or not of *ARRB1* transfection. Furthermore, the GABAergic neurons represent the vast majority of the neuronal population in the nucleus accumbens and striatum, as well as in the cerebellum.

This work showed the mechanism of action of AEF0117 on the CB1 intracellular pathway that leads to hyperlocomotion in mice. Such signaling can be achieved only through a THC dose that does not cause pregnenolone production in the brain. In this model, we have explored the pharmacological role of AEF0117 and CB1-SSi, but not the physiologic role of pregnenolone. In order to better understand the physiologic role of pregnenolone in the cannabinoid system and CB1 signaling, we decided also to investigate the CB1 signaling following two other doses of THC: one at which it is known that pregnenolone is produced, and one at which the maximal production of pregnenolone is reached (Vallée et al., 2014). This part of the project is still ongoing, but we have been able to collect essential information about the biased signaling of CB1 that leads to other behaviors induced by medium and high doses of THC. The medium dose of THC disrupts social behavior a relatively long time after THC administration (120 minutes post-injection); for this reason, we decided to analyze the THC-induced signaling pathway at different time-points. As discussed before, in the frontal cortex we found decreased Akt-mTOR signaling and decreased Erk phosphorylation 30 minutes after injection; in the nucleus accumbens, we found a decreased mTOR pathway, and decreased phosphorylation of Erk and JNK 130 minutes post-injection; in the cerebellum, we detected increased Akt

phosphorylation 30 minutes post-injection, and a long-lasting decrease of Erk phosphorylation (from 30 to 130 minutes post-injection). Then we tested THC on β -Arrestin 1 KO mice and observed that they showed greater asocial behavior. One interpretation of this data may be that β -Arrestin 1, in this case, does not recruit the signaling pathway, the role for which there should be another signaling transducer, but instead stops it, and in mice lacking β -Arrestin 1, this effect could be enhanced for the diminished control over receptor desensitization. Lastly, we showed that AEF0117 was able to rescue sociability in THC-treated mice, without showing any *per se* effect.

The high dose of THC that leads to hypolocomotion has also been explored. Interestingly, in this case, 30 minutes after THC injection we observed an increase in Akt phosphorylation on Thr308, no effect on Ser473 and a decrease in Erk1/2 phosphorylation in the frontal cortex and striatum (while GSK3 β was not tested in those two structures), and in the nucleus accumbens an increase in GSK3 β phosphorylation was also detected. In the hippocampus, an increase in Akt phosphorylation was also observed on Thr308 and a GSK3 β phosphorylation increase, while an increase of eIF4E and Erk1/2 was observed (unlike the nucleus accumbens). In this case, the profile of the signaling pathway seemed to be more related to the dopaminergic signal, since it seemed to be the actual opposite signaling of the D2 receptor on both the Akt^{T308}-GSK3 β ^{S9} increased (while no effect was detected on the Akt^{Ser473}), and a decrease of Erk1/2. The fact that high doses of THC promote dopamine release in the nucleus accumbens and striatum does not contradict the fact that we may observe inhibition of the D2 signaling. It is possible that as THC causes a massive release of dopamine, which is known for yielding an "inverse U" shaped function of locomotor activity (Dunnett and Robbins, 1992), that high dopamine release may cause autoinhibition of dopaminergic neurons (Benoit-Marand et al., 2001). More causal experiments should be carried out to prove further this theory, i.e., the use of GDC-0084 at its sub-effective dose could prove effective once again, preventing GSK3 β

overphosphorylation and inactivation. The fact that GSK3 β phosphorylation under different conditions may lead to different behavioral outcomes it not to be discarded. As previously stated, GSK3 β possesses more than 100 downstream targets, which must previously be phosphorylated in position n-4 (with n=amino acid position recognized by GSK3 β). Without such preventive activity, GSK3 β does not phosphorylate the target, meaning that the cellular context may profoundly influence GSK3 β activity, even resulting in opposite outcomes (Beaulieu et al., 2007, 2005, 2004; Beurel et al., 2015; Cole, 2012; Doble, 2003; McCubrey et al., 2014; Medunjanin et al., 2016).

Altogether, the three main experiments confirm that CB1 signaling is biased. In fact, at the different doses, it triggered different intracellular pathways: at the low dose, it triggered its own signaling pathway, leading to cannabinoid-dependent hyperlocomotion, with the signaling pathway being carried on by β -Arrestin 1. At the medium dose, we observed several signaling pathways involved, but the signaling transducer that led to asocial behavior was surely not β -Arrestin 1. Lastly, what information we obtained from the high dose of THC allows us only to speculate that the CB1 receptor blocked the D2 dopamine receptor signaling through an indirect mechanism.

AEF0117 was also able to rescue both the hyperlocomotor and the asocial behavior safely, as it has already been shown for its lead compound, pregnenolone (Busquets-Garcia et al., 2017). Most importantly, we have been able to identify the mechanism of action of AEF0117 on the THC-induced signaling pathway that leads to hyperlocomotion, abolishing the THC-induced signaling pathway altogether, but without showing any *per se* effect on the CB1 intracellular signaling pathway. Undoubtedly, this work represents a significant step towards a better understanding of CB1-SSi in general and the physiologic role of pregnenolone in the organism. This compound has been extensively studied from a preclinical, behavioral and toxicological point of view, and it has been shown that its therapeutic index is superior to 7000. It has been

proven to be valid for blocking almost all the THC-induced behaviors it was tested with (Piazza et al., 2013), and it is currently in phase two of human experimentation for CB1-related addiction and CUD (ClinicalTrials.gov Identifier: NCT03717272). However, the effect of AEF0117 on intracellular brain signaling in mice has never been described before. AEF0117 is a safe drug that did not show any side-effects comparable to those of rimonabant. Although it was quite effective, rimonabant needed to be withdrawn from the market for its side effects, often leading to depression and other psychiatric side effects (Sam et al., 2011).

For this reason, the discovery of biased negative allosteric modulators such as pregnenolone and its derivatives gives hope of a promising future for cannabinoid-related pharmacology, modulating the receptor and causing a "reboot" of its signaling, but without causing any harmful alteration of cannabinoid system signaling. Since the cannabinoid system regulates a vast amount of brain functions, drastic inhibition of this receptor leads to dangerous physiological and behavioral outcomes. On the other side of the coin, however, cannabinoid abuse is growing worldwide, with more and more cases of cannabis use disorder and cannabinoid-induced psychiatric disorders. The new cannabis strains, that are so abundant in THC and deficient in cannabidiol (CBD), such as the skunk or super skunk, and the diffusion of synthetic cannabinoids with even more addictive power, such as spice, are mowing down new victims every day (Every-Palmer, 2011; Freeman et al., 2013; Johnson et al., 2011; Kolliakou et al., 2012). However, the only suitable drug to address this worldwide emergency, rimonabant, was withdrawn from the market for its dangerous side-effects. Avoiding the side effects derived from the full blockade of the CB1 receptor is imperative to develop new molecules with therapeutic potential to win the war against cannabinoid abuse around the world. In this context, CB1-SSi represent a milestone in cannabinoid pharmacology, being able to work physiologically to restore receptor signaling and to abolish the behavioral outputs that derive from it.

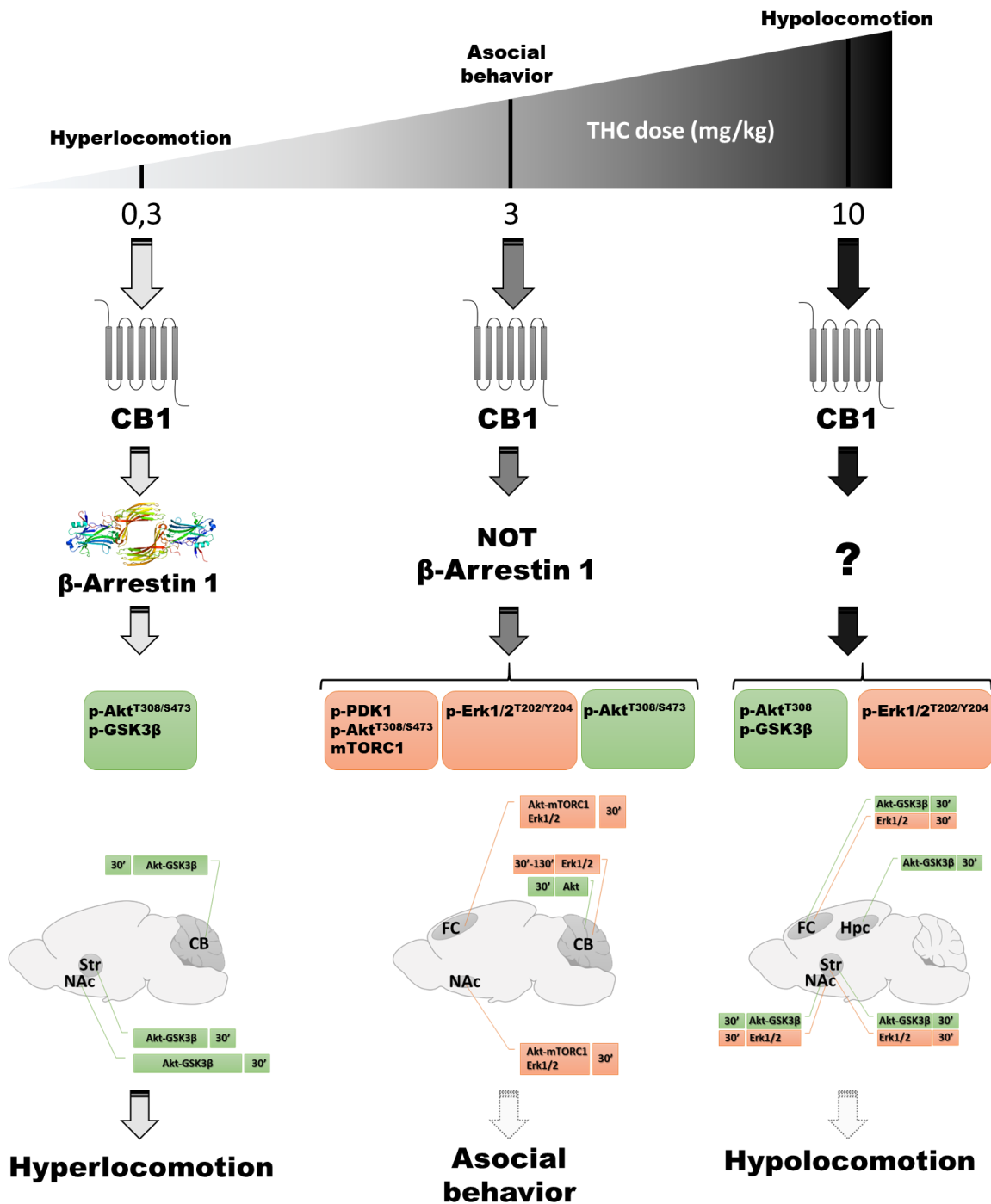


Figure 44: Schematic representation summary of the data of the current thesis work. In this work, three THC doses have been analyzed (0.3, 3, 10 mg/kg). For the dose of 0.3 mg/kg, we have identified that the signaling pathway analyzed was directly recruited by CB1 and that the signaling transducer was the β-Arrestin 1. The signaling leading to the hyperlocomotor behavior was Akt^{T308}/Akt^{S473}, and GSK3β, observable within the nucleus accumbens, striatum, and cerebellum, 30 minutes after THC injection. Furthermore, both the behavior and the signaling were abolished by pretreatment with PREG or AEF0117. For the dose of 3 mg/kg, we identified that the signaling transducer involved β-Arrestin 1, and that several signaling could be involved in the asocial behavior. In the frontal cortex and nucleus accumbens, 30 and 130 minutes after THC injection respectively, it was observed a decrease of phosphorylation of the Akt-mTORC1 signaling pathway, as well as a decrease of the Erk1/2MAPK pathway. In contrast, in the cerebellum, it was observed an increase of Akt^{T308}/Akt^{S473} phosphorylation, and a long lasting decrease, starting 30 minutes after THC injection, of the Erk1/2MAPK signaling. Lastly, mice treated with THC at the dose of 10 mg/kg showed a contextual increase of phosphorylation of Akt^{T308}, but no effect was observed on Akt^{S473}. Moreover, there was a significant decrease in Erk1/2MAPK in the frontal cortex, striatum, and nucleus accumbens. A causal link between the Akt-GSK3β pathway's alterations observed after the treatment with THC at the dose of 0.3 mg/kg was demonstrated, but more studies are needed for assessing the causality at 3 and 10 mg/kg.

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