Role(s) of the mitochondrial type-1 cannabinoid receptor in the brain

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université de BORDEAUX

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RÔLE(S) DU RÉCEPTEUR AUX CANNABINOÏDES MITOCHONDRIAL DE TYPE 1 DANS LE CERVEAU

Sous la direction de Giovanni MARSICANO

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Rôle(s) du récepteur aux cannabinoïdes mitochondrial de type 1 dans le cerveau

Le récepteur aux cannabinoïdes de type 1 (CB_1) est un récepteur couplé aux protéines G, abondamment exprimé dans le cerveau et régulant plusieurs processus physiologiques. Cependant, les mécanismes cellulaires par lesquels les CB_1 régulent ces processus n'ont été que peu analysés. Bien que les CB_1 localisés dans les membranes plasmiques sont connus pour induire la transduction de signal; une partie de ces récepteurs sont aussi fonctionnels au niveau des mitochondries ($mtCB_1$), où leur stimulation réduit la respiration mitochondriale. L'objectif de cette thèse fut d'évaluer l'impact de l'activation des récepteurs $mtCB_1$ du cerveau sur les effets connus des cannabinoïdes. Afin de distinguer la fonction des $mtCB_1$ de celle des autres populations de récepteurs, nous avons développé des outils basés sur la signalisation induite par les $mtCB_1$. Dans les mitochondries isolées de cerveau, l'activation des protéines $G_{\alpha i/o}$, dépendante des $mtCB_1$ diminue l'activité de l'adénylyl cyclase soluble (sAC). L'inhibition locale de l'activité de sAC prévient l'amnésie, la catalepsie et partiellement l'hypolocomotion induite par les cannabinoïdes. De plus, nous avons généré une protéine fonctionnelle mutante CB_1 (DN22-CB_1) dépourvue des 22 premiers acides aminés des CB_1 ainsi que de sa localisation mitochondriale. Contrairement aux CB_1, l'activation des DN22-CB_1 n'affecte pas l'activité mitochondriale. Enfin, l'expression des DN22-CB_1 dans l'hippocampe bloque à la fois la diminution de la transmission synaptique et l'amnésie induites par les cannabinoïdes. Ces travaux démontrent l'implication des $mtCB_1$ dans certains effets des cannabinoïdes et le rôle clé des processus bioénergétiques contrôlant les fonctions cérébrales.

Mots clés: *récepteurs $mtCB_1$, mitochondrie, adénylyl cyclase soluble, phosphorylation oxydative, bioénergétique, mémoire de reconnaissance d'objet, contrôle moteur, hippocampe, Substance Noire réticulée.*

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Résumé substantiel

Introduction générale

Il y a des millénaires, le peuple chinois cultivait le Cannabis à des fins nutritives, médicinales et/ou psychoactives (Russo et al., 2008). Ce n'est qu'au 19ème siècle que le cannabis fut importé en Europe par le docteur irlandais William Brooke O'Shaughnessy. Il étudia les effets aigues du Cannabis de façon expérimentale sur des animaux et humains et publia ces résultats (O'Shaughnessy, 1843). Peu après, le Cannabis entra dans la pharmacopée occidentale où il était recommandé pour ses propriétés anticonvulsivantes, antiémétiques, antinociceptives et hyperphagiques. Ces effets thérapeutiques étaient souvent accompagnés par des effets indésirables tels que des troubles psychotiques, troubles mnésiques et une perturbation dans les performances motrices.

L'intérêt scientifique se porta alors sur la caractérisation pharmacologique des molécules contenues dans le cannabis, les cannabinoïdes. La compréhension de leur mode d'action fut le fruit d'une recherche laborieuse. Il faudra attendre les années 1940 pour que les études de Roger Adams et celles de Raphaël Mechoulam en 1964 identifient et établissent définitivement la structure chimique du principal composant psychoactif du cannabis, nommé le ∆9-tetrahydrocannabinol (THC ; Adams, 1942 ; Gaoni et Mechoulam, 1964).

Malgré la nature lipidique du THC et sa faculté de perturber la fluidité des membranes, l'hypothèse d'un mode d'action par l'activation de récepteurs fut avancée. En 1988, Allyn Howlett et ses collaborateurs montrèrent que la plupart des effets attribués aux cannabinoïdes étaient dus à leur capacité d'activer des récepteurs spécifiques (Howlett et al., 1988). Deux types de récepteurs appartenant à la famille des récepteurs couplés aux protéines G (noté GPCR) furent clonés; le récepteur aux cannabinoïdes de type 1 (CB_1 ; Matsuda et al., 1990) et de type 2 (CB_2 ; Munro et al., 1993). Les récepteurs CB_1 sont abondamment exprimés dans le cerveau et avec des niveaux plus faibles dans beaucoup de tissus périphériques (Matsuda et al., 1990). Quant aux récepteurs CB_2, ils sont principalement localisés

en périphérie dans des cellules immunitaires et hématopoïétiques et avec en faible densité dans le système central. De par cette distribution différentielle et par des outils génétiques et pharmacologiques, il a été établit que la plupart des effets centraux des cannabinoïdes impliquent les récepteurs CB_1 et leur effets immunomodulateurs sont dus, au contraire, principalement à l'activation des CB_2 (Munro et al., 1993).

Les deux principaux ligands endogènes des récepteurs aux cannabinoïdes, appelés endocannabinoïdes (eCBs), ont été identifiés dans les années 1990 et connus sous le nom d'anandamide et du 2-arachidonoylglycerol (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). A l'inverse de neurotransmetteurs classiques, les aCBS sont des composés lipidiques synthétisés *de novo* à partir de précurseurs lipidiques membranaires et se comportent comme des messagers neuronaux rétrogrades. Les eCBs sont synthétisés à la demande suite à une augmentation de calcium intracellulaire de l'élément post-synaptique induite par une dépolarisation. Les eCBS vont ensuite aller activer les récepteurs CB_1 pré-synaptique localisés sur les terminaisons axonales (Kim et Alger, 2004). Une fois stimulé par leur ligands, les récepteurs CB_1 déclenchent de nombreuses cascades intracellulaires, telles que la phosphorylation de protéines (par ex : la protéine ERK, extracellular signal-regulated kinase), la synthèse protéique et la modulation de certains canaux ioniques. Ces changements rapides induits par la stimulation des CB_1 hyperpolarisent la présynapse et conduisent à une inhibition transitoire ou persistante de la libération des neurotransmetteurs, un des mécanismes synaptiques les mieux caractérisé des cannabinoïdes (Mackie et al., 1993; Deadwyler et al., 1995; Hampson et al., 1995; Mu et al., 1999).

De par leur large distribution en terme de structures et de cellules cérébrales, les récepteurs CB_1 régulent un vaste éventail de fonctions cognitives et somatiques. Ainsi, la compréhension des rôles complexes des CB_1 requiert une cartographie précise de leurs sites d'action à toutes les échelles (régional, cellulaire et subcellulaire). Les récepteurs CB_1 sont les GPCRs les plus fortement exprimés dans le cerveau, avec des densités différentes d'une région à l'autre (Herkenham et al., 1990). On les retrouve fortement exprimés dans la substance noire réticulée, le globus pallidus, le cervelet et l'hippocampe (Tsou et al., 1998). Ces récepteurs sont localisés sur différents types de populations cellulaires. Majoritairement présents sur

les neurones GABAergiques, ils se retrouvent en plus faible densité dans les neurones glutamatergiques corticaux (Mailleux et al., 1992; Marsicano et al., 2003; Marsicano et Kuner, 2008; Marsicano et Lutz, 1999). Malgré la minorité de récepteurs CB_1 sur des cellules glutamatergiques, ces récepteurs jouent des rôles importants dans effets physiologiques et pharmacologiques comme la protection contre la excitotoxicitée dans l'hippocampe, l'anxiété et la prise alimentaire (Bellocchio et al., 2010; Dubreucq et al., 2012; Metna-Laurent et al., 2012). De plus, les récepteurs CB_1 sont fonctionnellement exprimés sur d'autres types de cellules non-neuronales. C'est le cas des récepteurs CB_1 localisés dans les cellules astrocytaires où ils jouent un rôle dans la transmission synaptique et participent aux processus mnésiques et métaboliques (Navarrete et Araque, 2010; Navarrete et Araque 2008 ; Bosier et al., 2013 ; Han et al., 2012).

La localisation subcellulaire semble également tenir un rôle déterminant dans la signalisation et la fonction des récepteurs CB_1. Au niveau subcellulaire, les CB_1 se retrouvent exprimés en majorité à la surface des cellules insérés dans la membrane plasmique mais la présence des CB_1 sur les compartiments intracellulaires et en particulier sur mitochondries avait été suggéré (Ong et 1999 Mackie; Rodriguez et d'autres., 2001). Cependant, jusqu'à présent les effets dépendants des CB_1 ont été exclusivement attribués aux CB_1 de la membrane plasmique ($pmCB_1$) et la possibilité que les récepteurs CB_1 sur les mitochondries puissent participer à la transduction du signal induit par les cannabinoïdes n'a jamais été prise en considération et leur présence était contestée. Cela pour deux raisons :

- Les immuno-marquages anti-CB_1 étaient considérés comme aspécifiques à cause du manque de contrôle négatif approprié tel que les animaux génétiquement modifiés et n'exprimant pas les récepteurs CB_1 (CB_1 knock-out, noté $CB_1^{-/-}$).

- Les GPCRs sont traditionnellement considérés comme des médiateurs de la communication intercellulaire et sont pour cela recrutés à la surface cellulaire où ils transfèrent les signaux extracellulaires aux effecteurs intracellulaires via la stimulation des protéines G.

Défiant ce prédicat logique, des études ont démontrés la présence fonctionnelle des GPCRs sur les membranes intracellulaires, y compris, les endosomes, les noyaux et les mitochondries (Belous et al., 2004; Irannejad et von Zastrow, 2014). De façon similaire, l'équipe de Rozenfeld a observé l'activité des récepteurs CB_1

localisés dans les compartiments endosomal/lysosomal, dont la stimulation déclenche la signalisation dépendante de protéine G et contribuent ainsi aux cascades intracellulaires induites par les cannabinoïdes (Rozenfeld et Devi, 2008).

Dans ce contexte, en 2012, une étude de notre laboratoire a démontré qu'une portion de récepteurs CB_1 était fonctionnellement présente sur les membranes mitochondriales et nommé $mtCB_1$ (Benard et al., 2012). Par microscopie électronique et marquage immunohistochimie, les auteurs ont montré qu'approximativement 10 à 15 % de récepteurs CB_1 totaux dans la région hippocampique CA1 sont situés dans les mitochondries de souris sauvages-types, en grande partie au-dessus des niveaux de bruit quantifiés dans les tissus de souris mutantes $CB_1^{-/-}$.

Les mitochondries sont des organelles à doubles membranes essentielles pour la survie de nos cellules et remplissent diverses fonctions. La plus connue est la production d'adénosine triphosphate (ATP) au cours de la phosphorylation oxydative (OXPHOS). Dans la matrice mitochondriale, les intermédiaires métaboliques issues du cycle de Krebs vont subir des séries de réactions d'oxydo-réduction au niveau d'un ensemble de quatre complexes enzymatiques localisés sur la membrane interne de l'organelle. Ce processus libère les électrons qui sont transférés le long de cette chaîne respiratoire, l'oxygène, étant l'accepteur final d'électrons sera transformé en eau. Le flux d'électron s'accompagne d'une accumulation de protons dans l'espace intermembranaire à l'origine d'un gradient électrochimique de part et d'autre de la membrane mitochondriale interne. La dissipation de ce gradient s'opère par le retour de protons dans la matrice via l'ATP synthétase, permettant la synthèse d'ATP (Brown, 2004).

L'approvisionnement constant en ATP est fondamental pour le cerveau et représente le pilier de toutes les fonctions cérébrales. Chez les mammifères, le cerveau est un des les organes les plus exigeants en ATP. Au repos, alors qu'il représente seulement 2 % de la masse corporelle totale, le cerveau consomment 25 % de l'énergie de corps totale et 20 % de l'oxygène total (Erecinska et Argent, 2001; Kety, 1957; Rolfe et Brun, 1997; Sokoloff, 1960). L'ATP cellulaire est principalement produit par les mitochondries et utilisée pour mobiliser les vésicules synaptiques, maintenir les flux d'ions et l'excitabilité neuronale, les réarrangements du cytosquelette des cellules du cerveau, et la régulation l'activité synaptique (Attwell et Laughlin, 2001).

Les travaux menés par Benard et ses collègues ont démontrés que l'activation des mtCB_1, exprimés sur la membrane externe mitochondriale, régulent la fonction respiratoire des mitochondries (Benard et al., 2012). En effet sur des mitochondries isolées de cerveau de souris sauvage, l'application exogène d'agonistes aux CB_1 tel que le THC, l'anandamide et le HU210 diminue significative la consommation d'oxygène mitochondrial, alors qu'aucun changement n'est observé sur des mitochondries de cerveau de souris $CB_1^{-/-}$, indiquant une inhibition directe de la respiration mitochondriale par cannabinoïdes via les récepteurs CB_1 (Benard et al., 2012).

Objectif de la thèse

Alors que le dysfonctionnement à long terme de l'activité mitochondriale peut avoir des conséquences dramatiques sur la neurotransmission (Cai et al., 2011) et conduire à de nombreuses maladies neurologiques et psychiatriques (Mattson et al., 2008), on en sait peu sur les rôles physiologiques de la régulation de l'activité mitochondriale dans le cerveau et quant à leur rôle dans la régulation aiguë du comportement. L'activation des CB_1 représente un potentiel thérapeutique important, tels que des effets analgésiques, mais cause également des effets indésirables.

L'objectif de ma thèse était de déterminer la potentielle participation des mtCB_1 dans le cerveau dans ces effets pharmacologiques induit par les cannabinoïdes et dépendants des CB_1. En particulier, j'ai étudié le potentiel rôle des mtCB_1 dans les effets secondaires des cannabinoïdes, y compris l'altération de la mémoire, la réduction d'activité locomotrice spontanée (hypolocomotion) et l'incapacité d'initier le mouvement (la catalepsie; Holtzman et d'autres., 1969).

Mise en place d'outils génétiques et pharmacologiques

Le premier défi de ce projet consistait à développer des outils expérimentaux pharmacologiques et génétiques capables de distinguer la fonction des mtCB_1 de celle des autres populations de récepteurs. L'approche pharmacologique a été basée sur la signalisation induite par les mtCB_1 impliquée dans la réduction de l'activité

respiratoire mitochondriale. Dans les mitochondries isolées de cerveau, l'activation des protéines $G\alpha_{i/o}$, dépendante des $mtCB_1$ diminue l'activité OXPHOS par l'inhibition de la cascade intra-mitochondriale soluble adénylyl cyclase/ adénosine monophosphate cyclique/ phosphate kinase A, noté sAC/cAMP/PKA. L'inhibition de l'activité de sAC, par un inhibiteur pharmacologique spécifique, le KH7, bloque les effets de l'activation des $mtCB_1$ sur la fonction respiratoire mitochondrial dans des cultures cellulaires et les mitochondries isolées de cerveau. En combinaison, nous avons utilisé l'Hemopressin, un agoniste inverse des récepteurs CB_1 qui de par sa nature peptidique est non-perméable aux membranes cellulaires, ce qui permet de distinguer le rôle des $pmCB_1$ par rapport a celui des CB_1 intracellulaires (Heimann et al., 2007, Dodd 2013). Pour élargir la palette d'outils pour étudier les fonctions des $mtCB_1$, nous avons généré une protéine mutante CB_1 (DN22-CB_1) dépourvue des 22 premiers acides aminés des CB_1 ainsi que de sa localisation mitochondriale. Contrairement aux CB_1, l'activation des DN22-CB_1 n'affecte pas l'activité respiratoire mitochondriale. Néanmoins, cette protéine est fonctionnellement couplée aux protéines G et son activation déclenche une voie de signalisations bien connue des CB_1 qui est la phosphorylation de la protéine ERK.

Résultats :

Par ces approches innovantes, nous avons utilisé ces outils pharmacologiques et génétiques, afin d'évaluer le rôle potentiel des récepteurs $mtCB_1$ *in vivo* dans la régulation de mémoire de reconnaissance d'objets et le contrôle moteur. Les données actuelles suggèrent fortement que le contrôle direct de l'activité mitochondriale par les récepteurs $mtCB_1$ dans différentes régions du cerveau participe aux effets comportementaux induits cannabinoïdes y compris troubles de la mémoire et de l'altération de l'activité motrice. Plusieurs éléments convergent pour soutenir cette conclusion:

1. Des enregistrements électrophysiologiques sur des tranches d'hippocampes ont révélé que le KH7 bloque la diminution de la libération glutamatergique dépendante des récepteurs CB_1. Cet effet est rétablit chez les souris $CB_1^{-/-}$ par la réexpression virale de CB_1, mais pas par la réexpression fonctionnelle des protéines DN22-CB_1.

2. Dans l'effet amnésique des cannabinoïdes sur la mémoire de la reconnaissance d'objets (ORM) des précédentes études ont mis en évidence la participation des CB_1 dans l'hippocampe, une région cérébrale responsable de processus mnésiques. L'injection locale de KH7 prévient l'effet amnésiant des cannabinoïdes chez les souris sauvages. De plus, ce test est adéquat pour nos études car les souris $CB_1^{-/-}$ ne répondent pas à l'administration de cannabinoïdes et ne montrent aucune altération spontanée des performances mnésiques (Puighermanal et al ., 2009), permettant ainsi la réexpression virales dans l'hippocampe des protéines CB_1 sauvages ou des DN22-CB_1. La réexpression de CB_1 viral intra-hippocampal chez les souris $CB_1^{-/-}$, est suffisante pour rétablir l'altération des performances mnésiques induite par les cannabinoïdes. Au contraire, la réexpression de DN22 -CB_1 chez les souris $CB_1^{-/-}$ ne restaure pas l'effet du WIN sur l'ORM, indiquant l'implication de mtCB_1 dans cet effet.

3. En utilisant les outils pharmacologiques, nous avons identifié le SNr comme une région du cerveau où les cannabinoïdes exercent les effets cataleptiques et hypolocomoteurs. En effet, l'administration intra-SNr de l'agoniste inverse lipidique des récepteurs CB_1 perméable aux membranes (AM251) bloque l'hypolocomotion et la catalepsie. Au contraire, l'hemopressin, un agoniste inverse peptidique et non-perméable, injecté localement ne prévient partiellement l'hypolocomotion mais pas l'effet cataleptique du THC, suggérant la participation de récepteurs CB_1 intracellulaires.

4. Après identification par microscopie électronique de l'expression de mtCB_1 au sein de la SNr, l'infusion locale du KH7, inhibiteur de la sAC, bloque l'effet cataleptique du THC et réduit fortement sont effet hypolocomoteur.

Conclusion

Les résultats obtenus durant ma thèse démontrent l'implication des mtCB_1 dans certains effets secondaires des cannabinoïdes (catalepsie et troubles de la mémoire) et le rôle clé des processus bioénergétiques contrôlant les fonctions cérébrales.

L'identification des partenaires de signalisation des CB_1 intramitochondriaux tels que la protéine $G_{i/o}$ s'accompagne d'un couplage étroit entre le récepteur et la sAC. Cette cascade cause l'inhibition de la fonction respiratoire mitochondriale et

contribue aux effets comportementaux des cannabinoïdes. Au delà de leur fonction respiratoire, les mitochondries exercent une myriade de fonctions essentielles à l'activité synaptique et aux fonctions cérébrale, tel que la production d'espèces réactives à l'oxygène (ROS), le métabolisme des neurotransmetteur, l'homéostasie du calcium et beaucoup d'autres processus physiologiques. Il est difficile de connaître précisément par quel mécanisme "post-mitochondrial" l'activation de récepteur $mtCB_1$ est couplé aux les effets cognitifs ou moteur observés puisque les fonctions mitochondriales différentes sont interdépendantes (Gunter et Sheu, 2009, Vos et d'autres., 2010). Des travaux de recherche sont en cours pour identifier d'autres possibles régulations des $mtCB_1$ sur les fonctions mitochondriales tel que l'homéostasie du calcium ou la dynamique mitochondriale.

Ces données rassemblées pendant mon doctorat démontrent de façon causale que la régulation des fonctions mitochondriales dans le cerveau par l'activation des $mtCB_1$ participe aux effets comportementaux caractéristiques des cannabinoïdes comme la catalepsie et l'amnésie. Ainsi, la régulation l'activité mitochondriale représente un mécanisme central qui sous-tend les fonctions cérébrales complexes, ce qui jusqu'à présent était sous-estimé. De par l'utilisation de cannabinoïdes pour des indications thérapeutiques, la mise en évidence des $mtCB_1$ dans les effets secondaires des cannabinoïdes chez la souris, offre potentiellement de nouvelles alternatives dans le développement d'agonistes CB_1 qui ciblerai exclusivement les $pmCB_1$ pour induire des effets thérapeutiques en limitant les effets secondaires. C'est pourquoi, il serait nécessaire de disséquer les effets des cannabinoïdes dépendants des fonctions mitochondriales de ceux qui sont indépendants.

université de BORDEAUX

THESIS FOR THE

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Tifany DESPREZ

ROLE(S) OF THE MITOCHONDRIAL TYPE-1 CANNABINOID RECEPTOR IN THE BRAIN

Under the supervision of Giovanni MARSICANO

Defended on May 13th 2015

Members of the jury:

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Role(s) of the mitochondrial type-1 cannabinoid receptor in the brain

Type-1 cannabinoid receptor CB_1 is a G protein-coupled receptor (GPCR), widely expressed in the brain, which regulates numerous physiological processes. However, the cellular mechanisms of CB_1-mediated control of these functions are poorly understood. Although CB_1 are known to signal at the plasma membrane, a portion of these receptors are also present in mitochondria ($mtCB_1$), where $mtCB_1$ activation decreases mitochondrial activity. The goal of this thesis was to dissect the impact of brain $mtCB_1$ signaling in known behavioral effects induced by cannabinoids. To distinguish the functions of $mtCB_1$ from other receptor pools, we developed tools based on the characterization of the intra-mitochondrial molecular cascade induced by $mtCB_1$ receptors. In isolated brain mitochondria, we found that intra-mitochondrial decrease of soluble-adenylyl cyclase (sAC) activity links $mtCB_1$-dependent activation of $G_{\alpha i/o}$ proteins to decrease cellular respiration. Local brain inhibition of sAC activity blocks cannabinoid-induced amnesia, catalepsy and contributes to the hypolocomotor effect of cannabinoids. In addition, we generated a functional mutant CB_1 protein (DN22-CB_1) lacking the first 22 amino acid of CB_1 and its mitochondrial localization. Differently from CB_1, activation of DN22-CB_1 does not affect mitochondrial activity. Hippocampal *in vivo* expression of DN22-CB_1 abolished both cannabinoid-induced impairment of synaptic transmission and amnesia in mice. Together, these studies couple mitochondrial activity to behavioral performances. The involvement of $mtCB_1$ in the effects of cannabinoids on memory and motor control highlights the key role of bioenergetic processes as regulators of brain functions.

Key words: *$mtCB_1$ receptors, mitochondria, soluble adenylyl cyclase, oxidative phosphorylation, bioenergetic, object recognition memory, motor control, hippocampus, and Substantia Nigra pars reticulata.*

A mon grand-père, Jean-Jacques Dubois

« Il convient de préparer d'abord le cerveau de l'homme avant que d'y rien imprimer. Il faut premièrement apprendre à douter avant d'apprendre à croire tout. » de Ludvig Holberg. Extrait des Pensées morales.

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16.07.11: 8th World Congress of IBRO, Florence, Italy

LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
ABHD6	α-β hydrolase 6
AC	Adenylyl cyclase
AD	Alzheimer disease
AKAP	A-kinase anchoring protein
AEA	Arachidonoylethanolamide, Anandamide
ANLS	Astrocyte-to-neuron lactate shuttle
ALS	Amyotrophic Lateral Sclerosis
Aralar	Aspartate-glutamate carrier
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
$CB_1^{-/-}$	*CB_1 null* mice
$CB_1^{+/+}$	*CB_1* wild type
CB_1 receptor	Type-1-cannabinoid receptor
CB_2 receptor	Type-2 cannabinoid receptor
CNR1	*CB1* gene
CCK	Cholecystokinin
CNS	Central nervous system
CoQ	Coenzyme Q10
COX	Cytochrome c oxidase
D_1/D_2	Dopamine receptor type-1/2
DAG	Diacylglycerol
DAGL/DGL	DAG lipase
DNA	Deoxyribonucleic acid
DRP1	Dynamin related protein 1
DSE/DSI	Depolarization-induced suppression of excitation/ inhibition
eCB	Endocannabinoid
eCB-LTD	Endocannabinoid long-term depression
ECS	Endocannabinoid system
EPN	*Entopeduncular nucleus*
EPSC/IPSC	Excitatory / inhibitory post-synaptic currents
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide
FAAH	Fatty acid amide hydrolase
FCCP	4-(trifluoromethoxyl)s-phenyl-hydrazone
FLAT	FAAH-like protein complex
GABA	Gamma-Aminobutyric acid
GAD65	Glutamic acid decarboxylase 65
GDP	Guanosine diphosphate

GFP	Green fluorescent protein
GLUT	Glucose transporter
GTP	Guanosine triphosphate
GFAP	Glial fibrillary acidic protein
GPCR	G protein-coupled receptor
H_2O	Water
H_2O_2	Hydrogen peroxide
h*CB_1*	Human *CB_1*
HEK	Human embryonal kidney cell
HD	Huntington disease
IM/OM	Inner/outer membrane
IMS	Intermembrane space
IP3/IP3R	Inositol triphosphate/receptor
JNK	c-Jun N-terminal kinase
K^+	Potassium
KIF	Kinesin superfamily
LTD/LTP	Long-term depression/ Long-term potentiation
mAchR	Metabotropic acetylcholine receptor
MAGL	Monoacylglycerol lipase
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MAS	Malate–aspartate shuttle
MCU	Mitochondrial Ca^{2+} uniporter
MF	Mouse fibroblast
mGluR	Metabotropic glutamate receptor
Miro	Mitochondrial Rho-GTPase
MLS	Mitochondrial leading sequence
MS	Multiple sclerosis
MSN	Medium spiny neuron
MT	Microtubule
mtCB_1	Mitochondrial CB_1 receptors
mTOR	Mammalian target of rapamycin
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
NAPE	N-Acylphosphatidylethanolamine
NAPE-PLD	NAPE-phospholipase D
NMJ	Neuromuscular junction
NO	Nitric oxide
NOS	Nitric oxide synthase
NMDA	N-methyl-D-aspartate receptor
O_2	Oxygen
ORM	Object recognition memory
OXPHOS	Oxidative phosphorylation
PD	Parkinson disease
Pi	Inorganic phosphate
PI3K	Phosphatidylinositol 3-kinase
PKA, B, C	Protein kinases A, B, C
PLC	Phospholipase C

$pmCB_1$	Plasma membrane CB_1
PTX	Pertussis toxin
rCB_1	Rodent CB_1 receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sAC	Soluble adenylyl cyclase
SCs	Schaffer collaterals
Slp2	Stomatin-like protein 2
SNr	*Substantia Nigra pars reticulata*
STD/STP	Short-term depression/Short-term potentiation
STN	*Subthalamic nucleus*
THC	Delta-9-tetrahydrocannabinol
tmAC	Transmembrane adenylyl cyclase
TRAK	Trafficking protein kinesin
VDCC/VGCC	Voltage-dependent Ca^{2+} channel/ Voltage-gated Ca^{2+} channel
VDAC	Voltage-dependent anion channel

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GENERAL INTRODUCTION

I. THE ENDOCANNABINOID SYSTEM

I.1 Cannabis and humans: a long history

The transmission of knowledge between generations is a determinant factor for the well-being and human survival. For example, awareness of the medicinal proprieties of plants has been used to cure diseases and alleviate discomfort. Already about 2700 years ago, in the ancient China, the Cannabis was cultivated for providing food from comestible seeds, oil and textiles but also probably employed as a medicinal or psychoactive agent (Russo et al., 2008). In the Middle East, historical traces reveal that Cannabis was recommended for its benefits against malaria, constipation and rheumatic pains and taken with wine, as a surgical analgesic (Mechoulam, 1986).It was not until the 19th century that Dr. William Brooke O'Shaughnessy, an Irish scientist and physician working for the British Army, observed the use of Cannabis in India and brought it to Western medicine (O'Shaughnessy, 1843). He experimentally tested the seeds from the plant on animals and humans and concluded about their utility in the treatment of pain, convulsions, spams, and as an appetite stimulant. Thereafter, studies in Europe and North America on the plant emerged and private laboratories developed a market of Cannabis extracts in response to scientific and medical demand (*e.g.* Merck, Eli Lilly or Parke-Davis; Mathre, 1997).

Despite the potential therapeutic effects of Cannabis, its administration was often hampered by undesired effects (Miller and Branconnier, 1983; Hall and Solowij, 1998). In all history of Cannabis use, consumption of this plant was known to produce a range of adverse effects depending on the dose, including hallucinations, psychosis, motor disorders and memory impairments (Hall and Solowij, 1998; Miller and Branconnier, 1983; O'Shaughnessy, 1843). Because of the adverse effects of Cannabis and a parallel development of new compounds effective for pain relief and migraine such as the aspirin or opioids, the consumption of Cannabis-based compounds as potential therapeutics was progressively discarded and they were eventually banned for their psychotropic effects and their abuse liability (Sznitman et al., 2008). Thereby, the early 20th century was marked by the decrease of legal use of cannabinoids and the progressive application of a stringent control of Cannabis market at the international level. Then, following the ratification of Geneva Convention (1925), cannabis use was outlawed in Europe and US and removed from Europe and North America pharmacopeia. However, biological and clinical cannabinoid research was almost abandoned since the active component(s) of the plant were not identified yet and oral administration of Cannabis extracts was leading to variable and unpredictable responses, making difficult scientific interpretations (Sznitman et al., 2008).

From the late 1940s until 1964, scientific studies performed by Adams and Mechoulam, respectively, led to the identification of the chemical identity of specific Cannabis molecules, called the cannabinoids, including Δ^9-tetrahydrocannabinol (THC; Gaoni and Mechoulam, 1964). Numerous reports on the pharmacology of THC showed that this compound mediates the major psychoactive properties of the plant including sedative, hyperphagic and antinociceptive effects (Carlini et al., 1974; Kiplinger and Manno, 1971). Prof. Raphael Mechoulam and colleagues then achieved the complete synthesis of the pure compounds, established their molecular structures, and began to study their structure-activity relationships (Mechoulam et al., 1972; Razdan, 1986). The consequences of cannabis consumption were thus rediscovered and precisely characterized by systemic administration of various doses of THC in animals (Kiplinger and Manno, 1971).

Cannabis is, nowadays, the most widely illegal consumed drug plant and probably one of the most accessible illegal drugs on the market. There are between 119 and 224 million cannabis users worldwide (World Drug Report 2012 United Nations publication, Sales No. E.12. XI.1). However, due to the progress in the understanding of cannabis' effect on physiological processes, cannabis-based compounds have been authorized in several countries of Europe (UK, Netherlands, Spain, Italy), in Canada, Australia, Israel, Switzerland and in 23 American States. Clinical trials have demonstrated their efficacy on: spasticity, nausea and vomiting, loss of appetite and chronic pain (Borgelt et al., 2013; Grotenhermen and Muller-Vahl, 2012). They are now being used such as Marinol® (dronabinol) to stimulate appetite or Sativex® (THC and cannabidiol) for the symptomatic relief of neuropathic pain in adults with multiple sclerosis (MS) or in chemotherapy (Pertwee, 2009; Russo, 2007). At the end of 2013, Uruguay became the first country to fully legalize this drug (http://www.elpais.com.uy/informacion/marihuana-ley-senado-uruguay-parlamento.html). In France, cannabis medications will be released in the market in 2015, under medical supervision, for relief of spasticity and neuropathic pain in the context of MS pathology (http://www.metronews.fr/info/sativex-le-medicament-au-cannabis-arrive-en-pharmacie/moaC!1aaCQ3iPDjUyE/). Nevertheless, the use of cannabis components for medical purposes remains a controversial issue. Therefore, it is crucial to unmask by which mechanisms Cannabis components mediated beneficial and adverse effects.

I.2 From cannabinoids to the endocannabinoid system

Intense research has been conducted during the last decades to identify the chemical components of Cannabis and their biological effects, the so-called cannabinoids. To date, the plant is known to contain a mixture of more than 100 cannabinoids (Mechoulam et al., 2014). One of the pioneer studies in cannabinoid research was the isolation of cannabinol and cannabidiol in the 1940's (Adams, 1940; Jacob and Todd, 1940). Then, in 1964, Gaoni and Mechoulam succeeded in identifying the main psychoactive compound present in Cannabis, THC (Gaoni and Mechoulam, 1964). After this discovery, extensive works on the plant constituent

THC were carried on and several studies revisited the large range of proprieties mediated by Cannabis as well as their dose and time-dependent responses (Holtzman et al., 1969; Kiplinger and Manno, 1971). Despite this regain of interest, the mode of action of THC and other cannabinoids was unsolved and two possible mechanisms were postulated to mediate their known effects. The first hypothesis was that cannabinoids might act *via* a chemical interaction with biological membranes due to their highly lipophilic nature able to modify membrane properties (Hillard et al., 1985). The second one suggested that cannabinoids might interact through still undiscovered receptors, thereby inducing or inhibiting cellular signaling. This second hypothesis was based on the observation that THC promotes the decrease of adenylyl cyclase (AC) activity in particular cell types but not in others, indicating that this effect would not be a ubiquitous phenomenon, as it would be expected of cannabinoid-induced membrane fluidity changes, but rather specific (Howlett et al., 1988).

The advances in the chemistry of the plant led to the development of potent synthetic cannabinoids, such as CP-55,940 (Johnson, 1986). The high-affinity-binding sites of CP-55,940 in biological tissues sections resulted in a specific labeling profile. Indeed, the absence of labeling in the white matter and the low non-specific binding supported the view of a specific receptor on both brain membranes and brain sections (Devane et al., 1988; Herkenham et al., 1991c; Herkenham et al., 1990; Howlett et al., 1988). Moreover, the central localization of the potential cannabinoid receptor was found to be in accordance with the cognitive and psychotropic effects of cannabinoids (Herkenham et al., 1990). In 1990, the first cannabinoid receptor was cloned, named CB_1 receptor and found to be highly expressed in the brain (Matsuda et al., 1990). Later on, a second receptor, CB_2, was identified, whose distribution is mainly found in peripheral cells and the immune system (Munro et al., 1993). These major discoveries, suggested the potential existence of endogenous molecules that target these receptors in order to regulate similar functions as the exogenous cannabinoids. Because of the lipophilic proprieties of exogenous cannabinoids, it was assumed that possible endogenous cannabinoid molecules would also be lipids. In 1992, anandamide (AEA), composed by ethanolamide and arachidonic acid, was the first endocannabinoid (eCB) discovered (Devane et al., 1992) followed by 2-

arachidonoylglycerol (2-AG; Mechoulam et al., 1995; Sugiura et al., 1995). Later on, their specific synthesis and degradation pathways were uncovered (Marzo et al., 1994). The identification of endogenous receptors (CB_1 and CB_2) and ligands (eCBs) promoted the concept that an "endocannabinoid system" (ECS) exists in the body, participating in the regulation of physiological processes (Piomelli, 2003). Notably, the ECS was identified not only in mammals but it was also found in non-mammalian vertebrates and invertebrates (Elphick et al., 2003; Soderstrom et al., 2000). The importance of this system is also underlined by the finding of a high degree of evolutionary conservation across species, which highlights the fundamental physiological role in animal adaptation and survival (De Petrocellis et al., 1999).

I.3 Composition and expression of the endocannabinoid system

I.3.1 Cannabinoid receptors: focus on CB_1 receptors

CB_1 and CB_2 receptors belong to the superfamily of G protein–coupled receptors (GPCRs), which represent the largest group of membrane receptors, in eukaryotes (Fredriksson et al., 2003). Cannabinoid receptors, like other GPCRs, structurally consist of an extracellular N-terminal domain, seven transmembrane domains and a C-terminal intracellular tail (Matsuda et al., 1990; Shim et al., 2011). However, CB_1 and CB_2 receptors only share 44% amino acid sequence similarity and have a different pattern of expression. As mentioned above, CB_1 receptors are likely the most highly expressed GPCRs in the brain, with densities similar to GABA and glutamate receptors (Herkenham et al., 1991c), and they are also expressed, at lower levels, in many peripheral tissues (Pagotto et al., 2006). On the other hand, CB_2 receptors are found primarily in peripheral immune and hematopoietic cells (Munro et al., 1993) and in microglial brain cells (Nunez et al., 2004), whereas their presence in neurons is still under scrutiny, due to the lack of suitable tools (Onaivi et al., 2006; Onaivi et al., 2012).

In addition to CB_1 and CB_2 receptors, pharmacological studies have revealed numerous other receptors that mediate the effects of cannabinoids, including

vanilloid receptors, peroxisome proliferator-activated receptors and the formerly "orphan" designated receptors GPR55 and GPR119 (Pertwee, 2010).

On the other hand, there is some evidence that cannabinoids, in particular cannabidiol, can act through cannabinoid receptor-independent mechanisms, possibly *via* an interaction with cholesterol-rich microdomains of the cell membrane, also known as lipid rafts (DeMorrow et al., 2007).

However, it is now clearly established that most of central effects of cannabinoids in the brain are due to CB_1 receptor activation (Ledent et al., 1999; Zimmer et al., 1999). CB_1 receptor cDNA was originally cloned from rat (Matsuda et al. 1990) and subsequently in human (Gérard et al., 1991) and in mouse (Chakrabarti et al., 1995). Two splice variants of the human CB_1 gene (CNR1) noted h*CB$_1$*A and h*CB$_1$*B have been described (Ryberg et al., 2005; Shire et al., 1995). Few studies have attempted to compare the pharmacology of the human CB_1 receptors and splice variants. Interestingly, they have reported a differential binding proprieties and pharmacology (Straiker et al., 2012; Ryberg et al. 2005). Although both h*CB$_1$* receptor variants are found in low quantity, their physiological relevance remains to be understood.

I.3.1.1 Brain localization of CB_1 receptors

Cannabinoids exert a multitude of effects that depend on CB_1 receptor activation. The comprehension of the complex roles of the CB_1 protein in the physiological-behavioral effects of cannabinoids requires a precise mapping of their sites of action at the regional, cellular and subcellular level. As mentioned above, CB_1 receptors are likely the most abundant GPCRs in the mammalian brain (Herkenham et al., 1990). After their cloning, in situ hybridization (ISH) studies were designed to detect CB_1 mRNA in rodents (Matsuda et al., 1993; Marsicano and Lutz, 1999) and in humans (Mailleux et al., 1992; Westlake et al., 1994). Later on, immunohistochemistry (IHC) was developed using antisera to reveal CB_1 protein (Katona et al., 1999; Pettit et al., 1998; Tsou et al., 1998). IHC revealed the highest density of CB_1 protein in the *cerebellum* and *basal ganglia* including the *Substantia Nigra pars reticulata* (SNr), *Globus Pallidus*, *Entopeduncular nucleus* [EP, the rodent

analog of the *internal Globus Pallidus* (GPi) in humans] and dorsolateral caudate putamen. CB_1 receptor immunoreactivity is also dense in the *hippocampus*, the cerebral *cortex* and olfactory system, and moderate in the in the *amygdala*, *hypothalamus*, *thalamus* and *habenula* (Moldrich and Wenger, 2000; **Figure 1)**. In-situ hybridization studies revealed that CB_1 mRNA expression in some areas does not correspond to CB_1 protein expression. A striking example is the *Substantia Nigra pars reticulata*. This brain region contains no or very low CB_1 mRNA, but is one of the most densely stained regions in immunohistochemistry and ligand binding experiments (Matsuda et al., 1993; Herkenham et al., 1990). This discrepancy resides in the fact that CB_1 receptor protein is mainly expressed at pre-synaptic axonal terminals when observed at high magnification using electron microscopy (Puente et al., 2010; Reguero et al., 2011; Katona et al., 1999) that the strong CB_1 receptor expression in this region corresponds to axonal terminals of projecting cells towards the SNr.

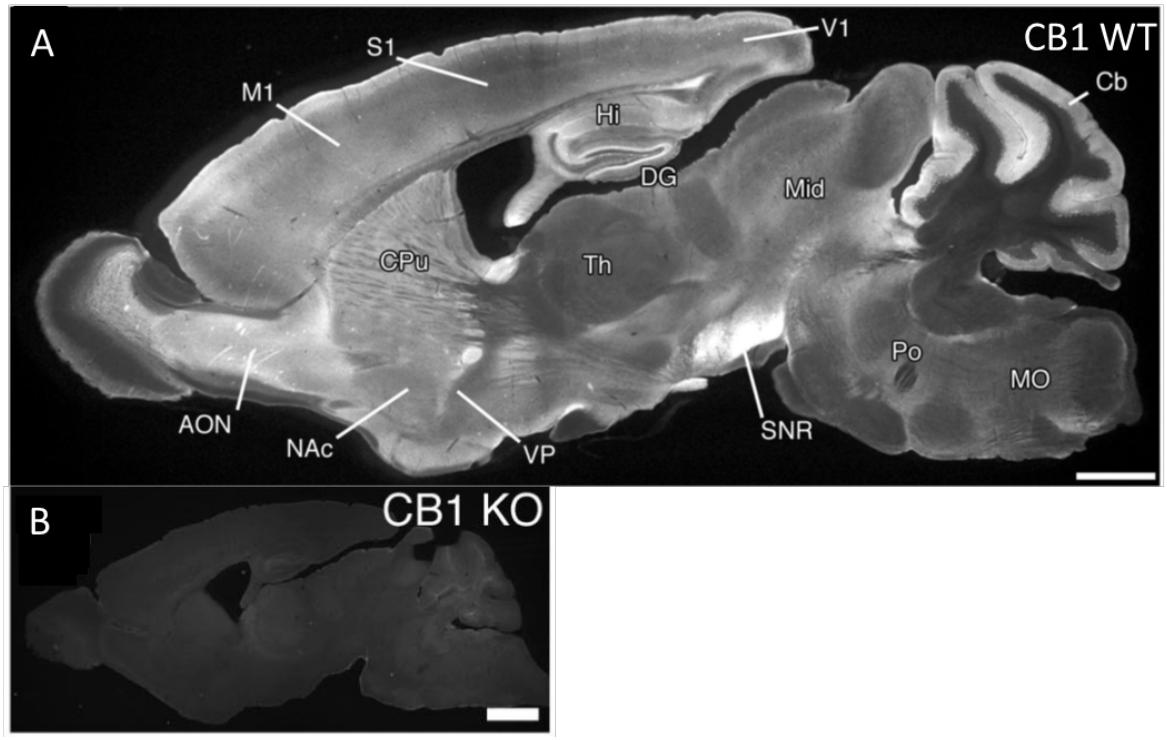

Figure 1.Brain distribution of the mouse CB_1receptor protein. Immunolabelling of the CB_1 receptor protein in parasagittal brain slices of wild-type **(A)** and *CB_1*-KO **(B)** mice. Note the high levels ofCB1expression in the anterior olfactory nucleus (AON), *neocortex* (M1, primary motor cortex; S1, primary somatosensory cortex; V1, primary visual cortex), caudate putamen (CPu), substantia Nigra pars reticulata (SNR), hippocampus (Hi; DG, dentate gyrus), thalamus (Th) and cerebellum (Cb). CB_1 protein is absent in the *CB_1*-KO mouse brain **(B)**. NAc, nucleus accumbens, VP, ventral pallidum; Mid, midbrain; PO, pons; MO, medulla oblongata; EP, entopedoncular nucleus. Scale bars: 1 mm (A), 200 µm (B) (from Kano et al., 2009).

I.3.1.2 CB_1 receptors: cellular expression profile

Despite the wide expression of CB_1 receptors in the CNS, their cellular distribution appears to be restricted to particular cell types. Within cortical regions, CB_1 mRNA is mainly found in GABAergic interneurons co-expressing glutamic acid decarboxylase (GAD65) and cholecystokinin (CCK), and are virtually absent in inhibitory interneurons expressing parvalbumin (Marsicano and Lutz, 1999). Glutamatergic neurons also contain CB_1 but in considerable lower amount as compared to GABAergic interneurons (Mailleux et al., 1992; Marsicano et al., 2003; Marsicano and Kuner, 2008; Marsicano and Lutz, 1999). The progress in genetics greatly contributed to highlight the relevance of CB_1 receptors according to their

cellular localization. Genetic deletion of CB_1 receptors in cortical glutamatergic neurons revealed their presence in pyramidal neurons in the *hippocampus*, *neocortex* and *amygdala* (Bellocchio et al., 2010; Domenici et al., 2006; Monory et al., 2006). Despite the minority of CB_1 receptors on glutamatergic cells, these receptors play important roles in functions modulated by cannabinoids such as resistance to excitotoxic insults in the *hippocampus* (Monory et al., 2006), fear coping, stress and anxiety (Dubreucq et al., 2012; Metna-Laurent et al., 2012) and food intake (Bellocchio et al., 2010). These findings might be due to cell-specific CB_1 signaling proprieties as CB_1 receptors on glutamatergic neurons are surprisingly more efficiently coupled to G protein signaling than 'GABAergic' CB_1 receptors (Steindel et al., 2013). In addition, serotonin-releasing neurons projecting from the raphe nuclei to the basolateral *amygdala* and the hippocampal CA3 region also express CB_1 receptors (Haring et al., 2007). Given the major cellular role of CB_1 receptors in decreasing neurotransmitter release, the presence of CB_1 receptors within different neuronal types suggest a regulation of CB_1 receptors upon several major neurotransmitter systems.

Recently, using pharmacological tools and conditional mutants, several data demonstrated the low but functionally important presence of CB_1 receptors on astrocytes (Bosier et al., 2013; Han et al., 2012; Navarrete and Araque, 2008). Indeed, astroglial CB_1 receptors were shown to regulate synaptic transmission (Navarrete and Araque, 2010; Navarrete and Araque 2008), astrocyte leptin signaling (Bosier et al., 2013), and to be responsible for the impairment in working memory performance induced by cannabinoids (Han et al., 2012).

Together, the anatomical and functional data shortly summarized above show that CB_1 receptors expressed in specific cell types play important physiological and pharmacological roles independently of the relative levels of expression. Thus, cellular localization is likely a better predictor of the functions of CB_1 receptors than their levels of expression (Marsicano and Kuner 2008). In addition, recent data suggest that also the differential subcellular localization of CB_1 receptors within the cell determines the functional outcome of their signaling.

I.3.1.3 Subcellular localization of CB_1 receptors

Immuno-electron microscopy and accurate cell fractioning have been two indispensable techniques to investigate the subcellular localization of CB_1 proteins. CB_1 receptors are preferentially targeted to plasma membranes of presynaptic terminals and axons, where they likely mediate the well-known inhibitory actions on neurotransmitter release (Chevaleyre and Castillo, 2003; Wilson and Nicoll, 2001; Katona et al., 1999). Additionally, CB_1 receptors were also observed in somatodendritic neuronal compartments in several brain regions, where they appear to be rather intracellular than located at plasma membranes (Ong and Mackie, 1999; Pickel et al., 2004; Sierra et al., 2014; Wilson-Poe et al., 2012). Due to this absence from plasma membranes, somatodendritic CB_1 receptors are generally considered as inactive proteins being transported to or from their functional localization at presynaptic membranes (Katona et al., 1999; Freund and Hajos, 2003). However, electrophysiological studies strongly suggested that somatodendritic CB_1 receptors participate in the autocrine regulation of neuronal excitability, although the signaling mechanisms of these functions are not fully clarified yet (Bacci et al., 2004; Marinelli et al., 2009).

The classical paradigm of GPCR functioning postulates that GPCRs localize on the cell surface and once activated by their agonists they activate G proteins signaling and initiate various intracellular signaling pathways. In contrast, their presence in intracellular compartments such as the endoplasmic reticulum (ER), where they are synthesized and trafficked to cell surface, or on endosomes, where the receptors are internalized after activation, was assimilated to a non-functional state (Ferguson et al., 2001). However, this idea was recently challenged by emerging evidence showing the functionality of different GPCRs at intracellular membranes, including ER, endosomes, nuclei and mitochondria (Belous et al., 2004; Irannejad and von Zastrow, 2014). CB_1 receptors were recently shown to share such unconventional intracellular localization. Rozenfeld and colleagues showed that CB_1 receptors present in endosomal/lysosomal compartments are able to activate G protein-dependent signaling (Rozenfeld and Devi, 2008). Early studies *in vitro* showed the accumulation of cannabinoid-binding probes in subcellular fractions,

including mitochondria (Colburn et al., 1974). Interestingly, more recent studies reported also anatomical evidence pointing to the presence of CB_1 immunolabeling in intracellular compartments, and in particular on mitochondria (Ong and Mackie 1999; Rodriguez et al., 2001). Indeed, the mitochondrion was described as one of the most commonly labeled organelles by CB_1 immunoparticles (Rodriguez et al., 2001). However, the possibility that mitochondrial CB_1 receptors could be functional was never taken into consideration: mitochondrial effects of cannabinoids were ascribed to the lipophilic nature of these compounds **(see Chapter III for more details)** and mitochondrial staining with CB_1 receptor antisera was considered as unspecific background and sometimes even used to "normalize" specific plasma membrane staining in semi-quantitative anatomical experiments (Pedro Grandes, personal communication). More recently, our group performed a rigorous analysis of mitochondrial CB_1 receptor expression in brain mitochondria, quantifying CB_1 immunogold labeling in brain tissues from wild-type and *CB_1*-KO mice (Benard et al., 2012). The results clearly showed low, but specific CB_1 receptor localization at mitochondria **(Figure 2)**. This pool of CB_1 receptors was named $mtCB_1$ to distinguish it from other subcellular localization (Benard et al., 2012). Further experiments indicated that $mtCB_1$ receptors are specifically present at mitochondrial outer membranes (OMs) and they have a topological orientation with a cytoplasmic protein N-terminus and the C terminus facing the interior of the organelle (Benard et al., 2012). There, $mtCB_1$ receptors impact negatively on mitochondrial respiratory chain activity (Benard et al., 2012; **see below Chapter III for further details)**.

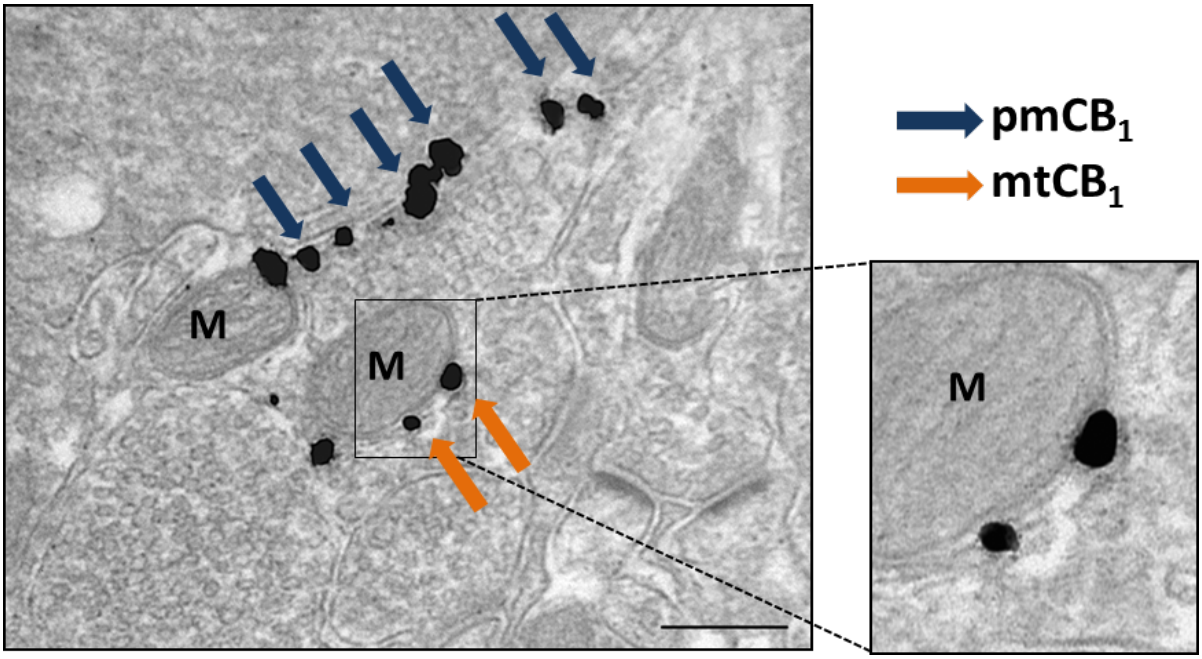

Figure 2.Electron microscopy image of immunogold staining of CB_1 proteins in the hippocampus.CB_1 receptors are present on both plasma membrane ($pmCB_1$, blue arrows) and mitochondrial membrane ($mtCB_1$, orange arrows). M: mitochondria.Scale bar, 500 nm.

Taken together, the data shortly summarized above indicate that the anatomical localization of CB_1 receptors is an extremely important determinant of their functions. This is evident at cellular level, with the differential roles of CB_1 receptors expressed in different cell types (excitatory, inhibitory or modulatory neurons or astroglial cells), but also at subcellular level. In neurons, the localization of CB_1 receptors at pre- or postsynaptic sites determines different functions. The presence of CB_1 receptors in intracellular organelles such as mitochondria adds complexity to the repertoire of potential effects of cannabinoid signaling. This Thesis work specifically addresses the functional characterization of the mitochondrial localization of CB_1 receptors ($mtCB_1$). However, before proceeding with these aspects, the other elements of the ECS and the known functions and mechanisms of action of CB_1 receptors in the brain will be described.

I.4 Endocannabinoids

The eCBs are defined as the endogenous ligands for cannabinoid receptors (Di Marzo et al., 1998b). They are mainly lipids derived from amides or esters of long chain fatty acids (arachidonic acid). The first eCB identified was arachidonoyl-ethanolamide, named anandamide (AEA; Devane et al., 1992), from the Sanskrit word “ananda”, which means “bliss”. The second discovered e*CB*, 2-arachidonoyl-glycerol (2-AG) was isolated from canine intestines (Stella et al., 1997; Mechoulam et al., 1995; Sugiura et al., 1995). Recently, a new family of endogenous ligands of CB_1 receptors was discovered and, surprisingly these new eCBs were revealed to be peptides, such as Hemopressin and Pepcans (Heimann et al., 2007). In the paragraphs below, I will first briefly describe the molecular actors implicated in the synthesis, degradation and transport of the ‘classical’ lipid eCB. Later, I will present the newly discovered (and less characterized) peptide eCBs.

I.4.1 Synthesis of lipid endocannabinoids

A feature that distinguishes classical eCBs from many other neurotransmitters is that they are lipids and, therefore, they are not stored in the aqueous medium of synaptic vesicles (Di Marzo et al., 1998). Rather, lipid eCBs are synthesized directly from cell membranes (Piomelli 2003). The main described biosynthetic pathways of eCBs start from the hydrolysis of membrane phospholipid precursors by specific enzymes. Briefly, AEA is produced from the cleavage of N-acyl-phosphatidyl-ethanolamine (NAPE;Di Marzo et al., 1994; Piomelli 2003), catalyzed by a NAPE-selective phospholipase D (NAPE-PLD) enzyme (Okamoto et al., 2004). In contrast, 2-AG is derived from other arachidonic acid-containing membrane phospholipids such as phosphatidylinositol through the action of phospholipase C β (PLCβ) leading to the formation of diacylglycerol (DAG; Piomelli 2003). DAG is further hydrolyzed by DAG lipase α or β, eventually forming 2-AG (Bisogno et al., 2003). Interestingly, these enzymes are often detected at the postsynaptic sites of brain synapses, providing anatomical support to the role of eCBs as retrograde synaptic modulators **(see below for more details;** Uchigashima et al., 2007; Yoshida et al., 2006). The

biosynthesis of the eCBs is induced by various stimuli causing intracellular Ca^{2+} elevation, such as depolarization and/or activation of metabotropic $G_{q/11}$-coupled receptors like group I metabotropic glutamate receptors (mGluR) or muscarinic acetylcholine receptors (mAChR; Kim et al., 2002; Varma et al., 2001). Thus, eCB de novo production in the brain likely depends on synaptic activity (Piomelli, 2003; Cadas et al., 1996).

I.4.2 Transport of lipid endocannabinoids

As better detailed below, eCBs are thought to act as retrograde signaling molecules at brain synapses (Piomelli, 2003). This function implies that postsynaptically-produced eCBs travel the aqueous synaptic cleft to activate presynaptic CB_1 receptors. But, how do these hydrophobic molecules diffuse through aqueous media across synaptic cleft or within the cytosol? Different hypotheses have been proposed to explain their trafficking in and out of the cell but it seems rather a complex process that remains to be elucidated. To date, several intracellular AEA binding proteins have been reported, including fatty acid binding proteins (FABPs; Elmes et al., 2015) and FAAH-like anandamide transporter (FLAT), a truncated and catalytically-silent variant of the degrading enzyme fatty acid amide hydrolase (FAAH, see below; Fu et al., 2012). In addition to their binding, FLAT facilitates the transport of AEA from cell membranes to FAAH for a rapid clearance and inactivation (Fu et al., 2012; Leung et al., 2013). More recently, it was shown that neosynthesized AEA could be also released into secreted extracellular membrane vesicles from microglial cells, from which AEA activates CB_1 receptors and inhibits presynaptic transmission (Gabrielli et al., 2015). If little is known concerning the transport of AEA, even less has been clarified on the mechanisms of 2-AG transport (Di Marzo et al., 2015). Hence, the mechanisms by which eCBs are trafficking represent nowadays a hot-topic in this research field.

I.4.3 Degradation of endocannabinoids

Once their molecular target is activated, the eCBs are rapidly removed and subsequently broken down inside the cell, thereby limiting the temporal window of

CB_1 receptor activation. AEA is mainly degraded by the fatty acid amide hydrolase (FAAH) mainly located in postsynaptic neurons (Gulyas et al., 2004). FAAH-dependent degradation of AEA generates arachidonic acid and ethanolamine, which can then be re-used to generate new AEA (Cravatt et al., 1996). On other hand, 2-AG is processed at both sites of the synapse. 2-AG is hydrolyzed trough the action of the monoacylglycerol lipase (MAGL), resulting in the production of arachidonic acid and glycerol. At lower extent, but potentially important in specific cells and cell locations, 2-AG can also be hydrolyzed by the α/β hydrolase domain 6 (ABHD6) enzymes or oxidized by the cytochrome c oxidase subunit II (COX2), a mitochondrial enzyme involved in the respiratory chain (Feledziak et al., 2012). Given the pharmacological and anatomical evidence of the presynaptic localization of MAGL (making it well-sited to break down 2-AG), 2-AG is considered as the main effector of CB_1 receptor-mediated retrograde signaling (Hashimotodani et al., 2007; Kim and Alger, 2004; Straiker et al., 2009). However, 2-AG can also act on postsynaptic CB_1 receptors to modulate neuronal excitability (Bacci et al., 2004; Marinelli et al., 2009).

Synthesis, transport and degradation of eCBs often occur in a short period of time and only when cells are under particular conditions. Thus, eCBs actions are often referred to as “on demand”, because they are synthetized de novo where and when they are required, and rapid degradation interrupts their activity (Piomelli 2003). Overall, the qualitative and spatial expression mitochof the enzymes involved in synthesis and degradation of AEA and 2-AG may allow the two eCBs to exert different functions in the brain (Di Marzo and De Petrocellis, 2012).

I.4.4 Other endogenous molecules binding CB_1 receptors

Over the past years, the CB_1 receptor has been the focus on a number of studies identifying putative endogenous orthosteric and allosteric ligands. A α-hemoglobin-derived peptide composed by the amino acid sequence PVNFKFLSH (called hemopressin) was found to serve as an inverse agonist of CB_1 receptors (Heimann et al., 2007). Hemopressin blocks several *in vitro* CB_1-mediated signaling pathways similarly as rimonabant. *In vivo*, hemopressin acts through CB_1 receptors to reduce food intake in rodents (Dodd et al., 2010). Since then, additional

endogenous peptide derivatives of hemopressin, called Pepcans were shown to act as negative allosteric modulators of CB_1 receptors (Bauer et al., 2012). These novel endogenous modulators of CB_1 receptor signaling are still under close investigation, as they likely participate in the diversity of large panel of effects exerted by CB_1 receptors.

Other endogenous modulators have been found including the lipids oleamide (Leggett et al., 2004), lipoxin A4 (Pamplona et al., 2012) and pregnenolone (Vallee et al., 2014). Lipoxins and the neurosteroid pregnenolone also act as allosteric modulators, with lipoxin A4 increasing and pregnenolone inhibiting CB_1 receptor signaling, respectively. Pregnenolone is particularly interesting, because its production in the brain is induced by excessive activation of CB_1 receptors. By acting as an allosteric inhibitor, therefore, this neurosteroid represents a clear endogenous molecular inhibitory loop to limit the excessive activation of CB_1 receptors (Vallee et al., 2014).

In conclusion, the physiological activity of CB_1 receptors is assured by a complex machinery for the synthesis, transport and degradation of several types of different endogenous ligands. The molecular and physiological details of these mechanisms, as well as the discovery of new ligands, are and will be the subject of many studies in the field.

I.5 Central CB_1 receptor signaling and modulation of synaptic transmission and plasticity

Animals have the capacity to learn and adjust their behavior in response to changes in the environment. It is now widely accepted that this ability relies on brain functional and morphological re-organizations of synaptic connections between neurons, which are commonly referred to as “plasticity” (Milner et al., 1998). Plasticity is a dynamic phenomenon, characterized by local changes of synaptic strength of neuronal connections in response to the use or disuse of specific synaptic pathways (Tsukahara, 1981). As a consequence, local structural rearrangements occur to rewire synaptic connections (Bernardinelli et al., 2014). Importantly, CB_1 receptors

have a key role in regulating synaptic transmission and plasticity (Chevaleyre and Castillo, 2003; Han et al., 2012; Marsicano et al., 2002). Many forms of synaptic plasticity are induced by the regulation of diffusible second messengers such as cAMP or Ca^{2+} (Calabresi et al., 2000; Kandel et al., 2014; Chevaleyre et al., 2007). Synaptic plasticity can be also temporally and spatially defined. Synaptic modifications can last for only some seconds (short-term plasticity), or for longer periods of time (long-term plasticity). They can also occur at given synapses independently from their neighbors (Kim et al., 2011; Soler-Llavina and Sabatini, 2006). The spatiality of plastic events is likely to be underlined by molecular signals confined in microdomains, rather than diffused in the whole cell (Kim et al., 2011; Soler-Llavina and Sabatini, 2006). Two basic mechanisms have been introduced for the compartmentalization of molecular transduction pathways: 1) diffusional physical barriers such as dendritic spines or organelles (Korkotian and Segal, 2006), 2) colocalization of enzymes that interact together, which is mediated *via* structural proteins that contain binding sites for various synaptic elements (anchoring proteins; Kim et al., 2011). In the paragraphs below, I will mention the participation of CB_1 receptors in numerous molecular cascades regulating neuronal excitability and plasticity.

I.6 Classical CB_1 dependent signaling pathways

I.6.1 cAMP/PKA pathway

The first characterized pathway for agonist-stimulated CB_1 receptor activation was the inhibition of the cAMP-protein kinase A pathway (cAMP/PKA) *via* the decrease of adenylyl cyclase (AC) activity (Cadas et al., 1996; Derkinderen et al., 1996). Inhibition of AC and subsequently a reduction in cAMP level, the second messenger produced by AC was observed following the addition of cannabinoids on neuroblastoma cells (Howlett and Fleming, 1984). The major role of cAMP is to activate the cAMP-dependent protein kinase (PKA; Brandon et al., 1997). PKA is a serine/threonine kinase, composed of two regulatory and two catalytic subunits. Once they bind to cAMP, the catalytic subunits are activated and diffuse through the

cytosol to further phosphorylate various substrates (Rababa'h et al., 2015). In contrast, the decrease of cAMP/PKA signaling by CB_1 receptor activation induces a concomitant decrease of PKA-dependent phosphorylation (Zhuang et al., 2005). The cAMP/PKA pathway has been implicated in learning and memory (Alberini, 1999; Heyser et al., 2000) and synaptic plasticity (Calabresi et al., 2000; Chevaleyre et al., 2007). Therefore, the CB_1-dependent decrease of PKA activity can explain many of the pharmacological effects of cannabinoids, such as memory impairment (Hampson and Deadwyler, 1998). Importantly, decrease of cAMP/PKA pathway is mediated through CB_1 receptor activation coupled to G inhibitory/off ($G_{i/o}$) proteins (Di Marzo et al., 1998a; Howlett, 2005; Howlett et al., 1986; Piomelli, 2003) *via* a pertussis toxin (PTX)-sensitive mechanism (Howlett et al., 1986). Pertussis toxin is able to block the dissociation of α and β/γ subunits of $G_{i/o}$, thereby abolishing the G protein-mediated inhibition of AC (Howlett et al., 1986). In addition, the coupling of CB_1 receptors to G-proteins on isolated membranes can be measured by receptor-stimulated [^{35}S]GTPyS binding. The technic is based on the exchange of bound GDP for GTP (or [35S]GTPyS) when G proteins are activated in the presence of excess GDP (Strange, 2010). In purified brain membranes, cannabinoids stimulate [^{35}S]GTPyS binding in a CB_1-depedent manner because this activation is fully blocked by CB_1 receptor antagonists (Selley et al., 1996).

The cAMP/PKA is present at different subcellular locations. Whereas it was originally established that the only known source of cAMP in mammals was transmembrane AC (tmAC), a truncated and soluble isoform of AC (sAC) was purified from rat testis cytosol (Braun and Dods, 1975) and later found in the cytoplasm, in mitochondria, in the centriole, and within the nucleus (Feng et al., 2006; Zippin et al., 2003; Zippin et al., 2004). Moreover, cAMP is also localized in intracellular compartments together with sAC such as in the nucleus and mitochondria (Lefkimmiatis et al., 2013; Zippin et al., 2010). In addition, PKA was reported to signal in compartmentalized microdomains within the cytosol of cardiomyocytes (Buxton and Brunton, 1983) as well as in the brain (Dell'Acqua et al., 2006; Kim et al., 2011). This organization is mediated by anchoring proteins, which are structural proteins that contain binding sites for various enzymes. A-kinase anchoring proteins (AKAPs) have been shown to sequester PKA at given locations

such as in cytosol and in intracellular organelles, which facilitate local phosphorylation of PKA substrates (Wong and Scott, 2004). In particular, AKAP 121 and SPHKAP/SKIP were identified to mediate PKA targeting to the outer mitochondrial membrane (OM) and intermembrane space (IMS) in proximity of local targets (Feliciello et al., 2005; Kovanich et al., 2010; Lieberman et al., 1988). Importantly, such a spatially limited cAMP/PKA signaling has been shown to regulate synapse plasticity in the striatum (Oliveira et al., 2012). Therefore, CB_1 receptor stimulation could result in a compartmentalized down-regulation of cAMP/PKA signaling in order to locally modulate plasticity. The differential pharmacology of sAC *versus* tmAC and the impact of CB_1 receptor activation on sAC signaling will be discussed in the 2nd and 3rd chapter.

I.6.2 Other CB_1 receptor-dependent signaling pathways

CB_1 receptors are able to stimulate different cellular pathways including (among others): the mitogen-activated protein kinases / extracellular signal-regulated kinase (MAPK/ERK), c-Jun N-terminal kinases (JNK), mammalian target of rapamycin (mTOR) and phosphatidylinositol 3-kinases (PI3K) pathways both *in vitro* and *in vivo* (Andre and Gonthier, 2010; Bouaboula et al., 1995; Puighermanal et al., 2009; Valjent et al., 2001**; Figure 3)**. Among these, I will discuss more in detail the ERK and the mTOR pathways, which have been shown to play important roles in the cannabinoid-dependent regulation of behavior and synaptic plasticity (Andre and Gonthier, 2010; Bouaboula et al., 1995; Puighermanal et al., 2009; Valjent et al., 2001).

I.6.2.1 MAPK/ERK pathway

The MAPK/ERK pathway is a key signaling in cell development, differentiation and apoptosis, and is a well-established CB_1 receptor-dependent signaling (Galve-Roperh et al., 2002). In the brain, this pathway also controls the shape of synaptic terminals promotes spine formation, and has been strongly associated with synaptic plasticity (Wiegert and Bading, 2011). ERK is a family of two, highly homologous proteins denoted as ERK1 (also known as p44) and ERK2 (p42). By phosphorylating

their serine/threonine sites (Dalton and Howlett, 2012), activation of CB_1 receptors stimulates both of the extracellular signal-regulated kinases in the striatum as well as the *hippocampus* and *cerebellum* (Daigle et al., 2011; Derkinderen et al., 2003; Valjent et al., 2001). These effects are prevented by SR141716A or PTX treatments and absent in $CB_1^{-/-}$ mice (Bouaboula et al., 1995; Derkinderen et al., 2003), indicating the involvement of CB_1 receptors through $G_{i/o}$ signaling. ERK-dependent signaling activates one of the final outcomes of this signaling takes place in the nucleus, where several transcription factors such as c-Fos, c-Jun and CREB. ERK pathway has been involved in synaptic plasticity (English and Sweatt, 1997; Wiegert and Bading, 2011) and learning and memory (Atkins et al., 1998; Selcher et al., 2002). Thus, protein phosphorylation and gene regulation through MAP kinase activation by CB_1 receptors could account for some behavioral effects of cannabinoids.

I.6.2.2 mTOR pathway

More recently, attention has focused on the modulation of mTOR signaling pathway by CB_1 receptors. mTOR is a protein kinase that regulates various processes including protein synthesis, mitochondrial activity, cell proliferation, synaptic plasticity, food intake and learning and memory (Costa-Mattioli et al., 2009; Cota et al., 2006; Diaz-Alonso et al., 2014; Haissaguerre et al., 2014; Parsons et al., 2006; Santini et al., 2014). mTOR is the catalytic subunit of two structurally distinct complexes: mTOR complex 1 (C1) and mTOR complex 2 (C2). Both complexes localize to different subcellular compartments and phosphorylate a different set of substrates, which determines their respective functions (Betz and Hall, 2013; Wullschleger et al., 2006). In particular, mTORC1 phosphorylates and modulates the activity of the ribosomal protein S6 kinase 1 (S6K1 or P70-S6 Kinase; Wullschleger et al., 2006). The mTOR/S6K1-signaling pathway is modulated by cannabinoids for controlling food intake and memory consolidation, in a CB_1 receptor-dependent manner (Puighermanal et al., 2009; Senin et al., 2013). Indeed, gastric CB_1 receptors modulate ghrelin production through the mTOR/S6K1 pathway to regulate food intake (Senin et al., 2013). In the *hippocampus*, activation of the mTOR/p70S6K-

signaling pathway couples CB_1 receptors stimulation to impairment of memory consolidation (Puighermanal et al., 2009). More recently, the implication of mTOR signaling in other CB_1-dependent effects such as anxiolytic effect was shown (Puighermanal et al., 2013), indicating that the mTOR activation is a key molecular player of CB_1 receptor-dependent regulation of different behaviors.

I.7 CB_1 receptor signaling at central synapses

I.7.1 Modulation of ion channels

Synaptic activity can be modulated at three different levels: 1) neuronal excitability, 2) presynaptic release probability and 3) activity of postsynaptic receptors and their intracellular effectors. A major effect of CB_1 activation is the regulation of both neuronal excitability and neurotransmitter release, through the modulation of voltage-dependent ion channels conductance (Azad et al., 2008; Chevaleyre et al., 2007). Therefore, it is necessary to understand how CB_1 receptors are coupled to ion channels **(Figure 3)**.

I.7.2 CB_1 receptors control neuronal excitability

CB_1 receptors modulate potassium channel activity. Potassium (K^+) currents, specifically A-type outward currents (K^+_A) contain a potential site for PKA phosphorylation (Deadwyler et al., 1995). This ion channel permits the movement of positively charged K^+ ions from the intracellular to the extracellular space at resting potential. Activation of PKA down-regulates these channels by shifting the activation curve to more positive potentials (Hoffman and Johnston, 1998). In this line, early studies found that, in hippocampal cell culture, cAMP/PKA inhibition mediated by cannabinoids, reduces the PKA-dependent inhibition of this ion channel. Therefore, the ion channels open and produce efflux of potassium ions leading to hyperpolarization of the cell (Deadwyler et al., 1995; Hampson et al., 1995; Mu et al., 1999).

A more direct way for cannabinoids to hyperpolarize cell membranes through CB_1 receptors is the direct modulation of K^+ ion channels. Indeed, CB_1 receptor activation leads to the stimulation specific subunits of inwardly rectifying potassium channels (K^+_{ir}) called GIRK and it is mediated by $G_{\alpha i/o}$ protein signaling (Henry and Chavkin, 1995; Mackie et al., 1995). This effect allows the ions to flow outwardly, making CB_1 signaling to promote the hyperpolarization of the cell.

I.7.2.1 CB_1 receptors control the neurotransmitter release

At presynaptic active zones, voltage-dependent calcium (Ca^{2+})-channels activation rapidly increases intracellular Ca^{2+}, which drives the exocytosis of vesicles, leading to the spread of neurotransmitters into the synaptic cleft (Schneggenburger and Neher, 2005). CB_1 receptor activation acts negatively on these channels. Indeed, cannabinoids and their analogues have been found to inhibit N- and P/Q-type Ca^{2+} channels in hippocampal cell culture (Mackie et al., 1993). The addition of non-hydrolysable analogues of cAMP or inhibitors of phosphodiesterase, the enzyme that hydrolyzes cAMP, did not alter the inhibitory effect of CB_1 receptors onto Ca^{2+} channels, indicating an adenylyl cyclase-independent mechanism (Mackie et al., 1993). Hence, the modulation of voltage-dependent ion channels by CB_1 receptors results in a decrease in intracellular Ca^{2+} concentration and a subsequent reduction of synaptic transmission.

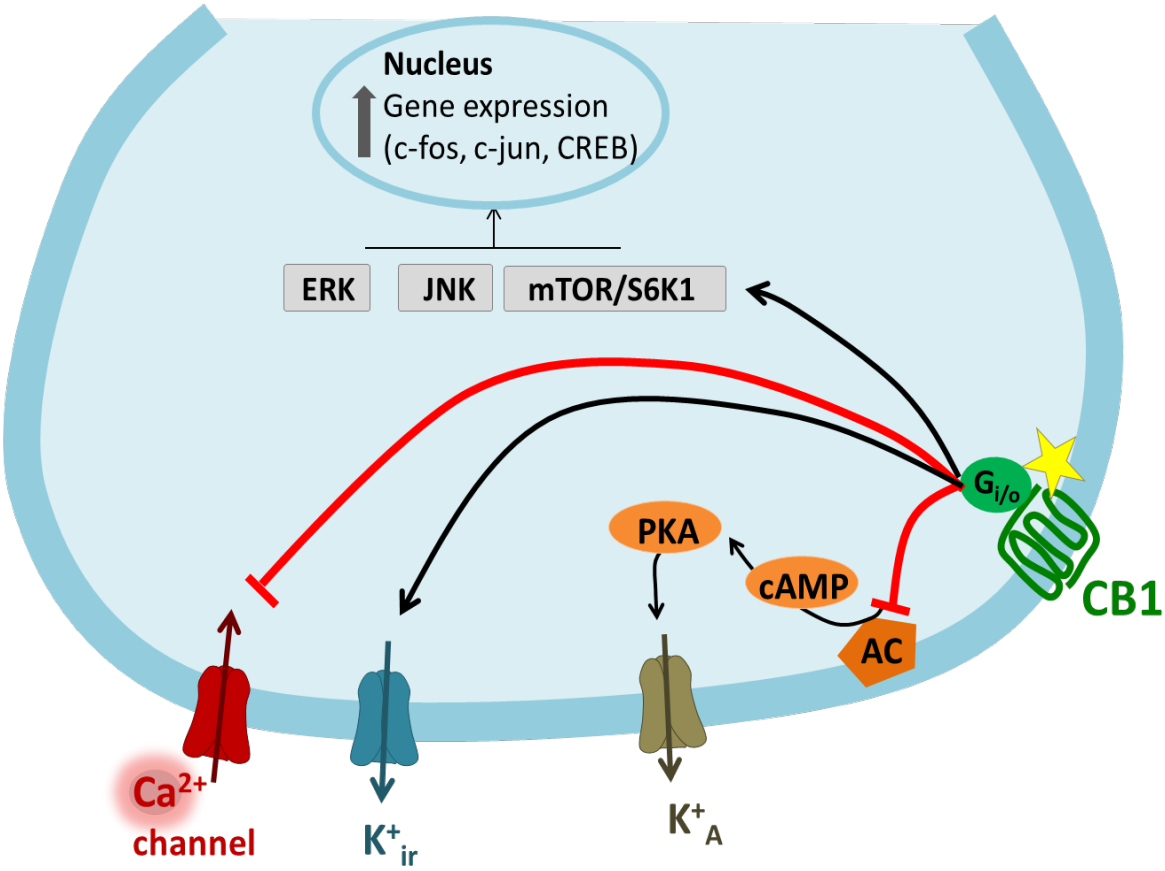

Figure 3.Main intracellular CB_1 receptor signaling pathways. Stimulation of CB_1 receptors lead to 3 main cascades of events. A direct modulation of ion channels conductance including an inhibition of Ca^{2+} channel and activation of K^+_{ir} channels. Activation of A-type K^+ channels (K^+_A) can be induced through the inhibition of adenylyl cyclase (AC). Activation of several protein kinases including ERK, JNK and mTOR leads to *de novo* gene expression. Adapted from Pagotto et al., 2006.

I.7.2.2 CB_1 receptor-mediated modulation of synaptic transmission and plasticity

CB_1 receptor activation decreases basal neurotransmission

Once the neurotransmitters are released, they translocate and bind postsynaptic receptors to generate inhibitory or excitatory inputs within the dendrites, named inhibitory postsynaptic potentials (IPSPs) and excitatory postsynaptic potentials (EPSPs), respectively (Ameri et al., 1999).The *hippocampus* is a suitable structure for extracellular recording techniques *in vitro* and *in vivo*, because of its

laminar organization in which neuronal cells are tightly packed and they all receive synaptic inputs in the same area. According to this, it is well established that CA1 pyramidal cells receive excitatory inputs from the pyramidal cells in the CA3 region *via* excitatory afferents called Schaffer collaterals (SCs). These SC-CA1 connections can be monitored by electrical stimulation and extracellular recording of different cells, called fields, in the well-defined stratum radiatum layer of the *hippocampus*; where the main dendritic arborization of pyramidal CA1 cells is present. Notably, it was shown that cannabinoids decrease field EPSPs (fEPSP) at SC-CA1 synapses in hippocampal slices (Ameri et al., 1999). This effect on basal neurotransmission is a broad and long-lasting mechanism, which reduces the strength of excitatory synaptic connections of a larger number of CA1 neurons (Marder and Thirumalai, 2002). Importantly, this effect is fully blocked by CB_1 receptor antagonists and absent in $CB_1^{-/-}$ mice (Bajo et al., 2009; Takahashi and Castillo, 2006). It is important to note that this response is not defined as plasticity because it is fully abolished by application of CB_1 receptor antagonists 10 minutes after cannabinoid application (Chevaleyre et al., 2006; Hajos et al., 2001; Kawamura et al., 2006). In contrast, it rather suggests the necessity of a continuous activation of CB_1 receptors for cannabinoid depression of transmission at SC-CA1 synapses, a characteristic of transient synaptic depression but not of synaptic plasticity (Chevaleyre et al., 2006).

CB_1 receptor signaling modulates short-term synaptic plasticity

In addition to decreasing basal synaptic transmission, CB_1 receptors play a key role in neuroplasticity. Plasticity is defined as the change in synaptic strength by either making it more efficient or weaker, known as potentiation/facilitation or depression, respectively, in the absence of the inducer, either chemical or electrical (Hebb, 1949). This process can be classified as short- or long-term, depending on its duration. Short-term potentiation (STP) and depression (STD) last from few seconds up to a minute. Conversely, long-term potentiation (LTP) and depression (LTD) have a longer time-scale, from minutes to hours (Blitzer, 2005). LTP is characterized by an increase of synaptic strength, whereas LTD is characterized by a decrease of synaptic strength and both long-term forms of synaptic plasticity rely on production of new proteins (Hoeffer and Klann, 2010).

CB_1 receptors mediate depolarization-induced suppression of neurotransmitter release

Various forms of eCB-STD have been reported and the most studied ones are the depolarization-induced suppression of transmission mediated by CB_1 receptors (Kano et al., 2009; Wilson and Nicoll, 2001). This form of eCB-STD has been widely reported at hippocampal and cerebellar synapses (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001). A brief depolarization (up to few seconds) of the post-synaptic neurons, for instance the pyramidal neurons of the *hippocampus*, induces a transient suppression (< 90 s) of either GABAergic (depolarization-induced suppression of inhibition, DSI) or glutamatergic synaptic inputs (depolarization-induced suppression of excitation, DSE), as measured by the amplitudes of evoked inhibitory or excitatory postsynaptic currents (eIPSC and eEPSC, respectively; Kreitzer and Regehr, 2001; Pitler and Alger, 1992). DSI and DSE can be induced *via* increase of Ca^{2+} influx in the post-synaptic neuron *via* voltage-dependent Ca^{2+} channels (VDCCs) after depolarization, triggering eCBs synthesis and retrograde signaling. Both DSI and DSE are prevented by Ca^{2+} chelators, such as BAPTA and EGTA (Kreitzer and Regehr, 2001; Pitler and Alger, 1992; Wilson and Nicoll, 2001). The eCB 2-AG is likely to be the main mediator of these processes since specific blockade of 2-AG degradation (JZL184) but not AEA (URB597) prolongs DSE in Purkinje neurons and DSI in CA1 pyramidal neurons (Pan et al., 2009**; Figure 4)**. Moreover, these short-term synaptic change resulting in the decrease of postsynaptic currents are a consequence of presynaptic transmission changes trough CB_1 receptors as the postsynaptic responses of GABA-A receptors induced by locally-applied GABA are not altered by DSI induction (Pitler and Alger, 1992).

Additionally, it is possible to induce a postsynaptic production of eCBs and to inhibit afferent synaptic transmission by activating postsynaptic Gq-linked receptors including the Group I metabotropic glutamate receptors (I-mGluRs) and the metabotropic muscarinic receptors (mAChRs; Maejima et al., 2001; Straiker and Mackie, 2005). These receptors are likely to engage phospholipase C (PLCβ) and diacylglycerol lipase (DGL), which synthesis 2-AG.

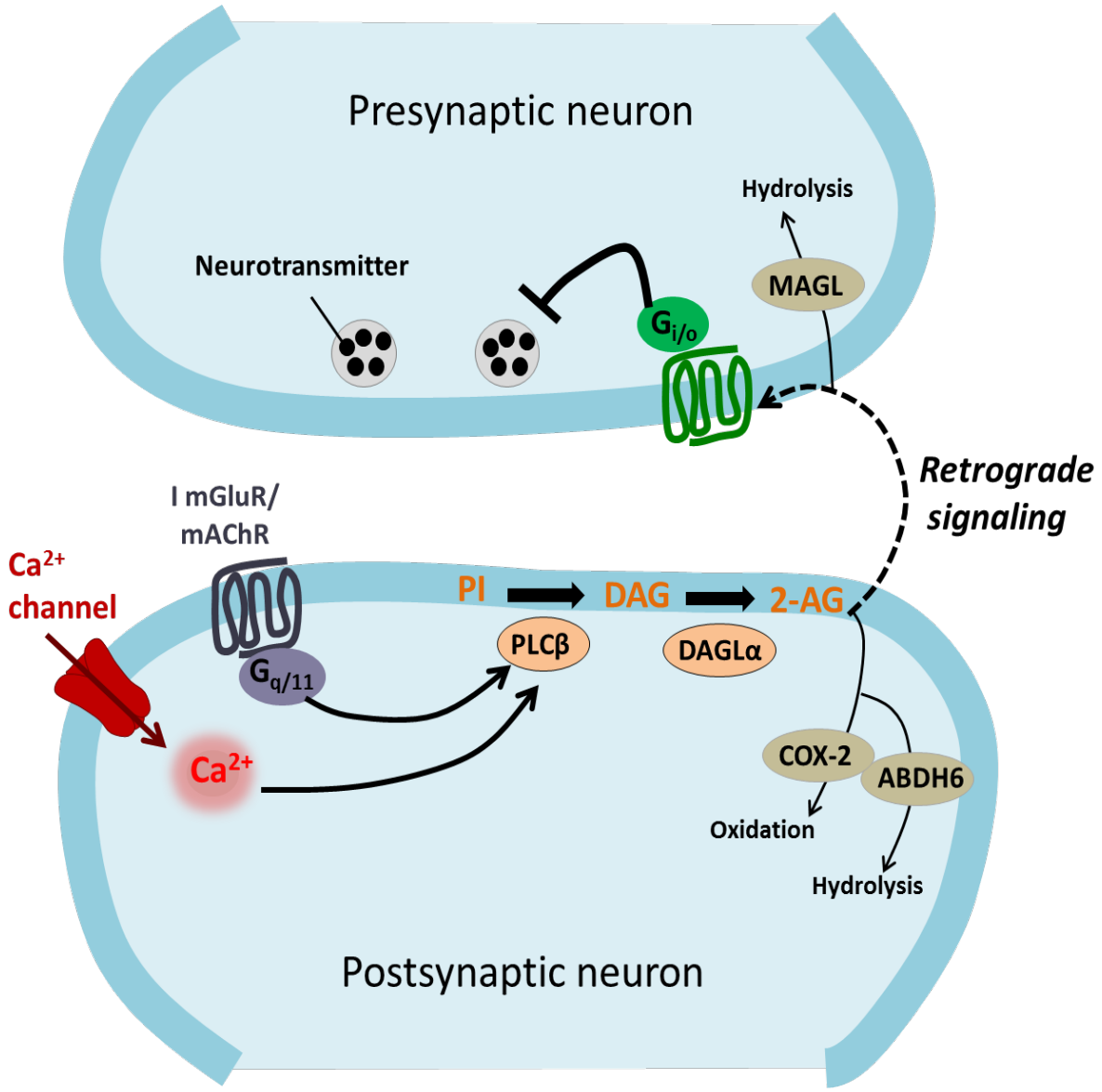

Figure 4.Molecular mechanisms of eCB-STD. When a large Ca^{2+} elevation is caused by activation of voltage-gated Ca^{2+} channels or $G_{q/11}$-coupled receptors (I-GluR and mAChR), 2-AG is generated in a DAGL-dependent manner. Synthesis of 2-AG from postsynaptic neurons, activates presynaptic *CB_1*receptors, and induces transient suppression of transmitter release. 2-AG is degraded by presynaptic MAGL. In the postsynaptic neuron, 2-AG is degraded by ABHD6 or oxidized by COX-2.Reproduced with modification from Kano 2014.

CB_1 receptor signaling modulates long-term synaptic plasticity

CB_1 receptors can also take part in long-term synaptic plasticity, potentially the cellular mechanism responsible for CB_1-dependent effects on memory and

behavioral adaptation (Castillo et al., 2012; Huang et al., 2008; Marsicano et al., 2002). In particular, their role in LTD has been highly documented in the *neocortex* and *hippocampus* (Chevaleyre et al., 2006; Rasooli-Nejad et al., 2014). Unlike eCB-induced basal neurotransmission depression, eCB-LTDs maintenance does not require continuous CB_1 receptor stimulation making it a form of plasticity (Chevaleyre et al., 2006).

While classical LTD requires NMDA receptors activation and Ca^{2+} increase (Malenka, 2003), eCB-LTD can be triggered by activation of postsynaptic I-mGluRs to induce eCB synthesis *via* phospholipase C (PLC) activation (Heifets and Castillo, 2009). The expression of eCB-LTD depends on the activation of presynaptic CB_1 receptors coupled to $G_{i/o}$ protein, leading to reduction in neurotransmitter release (Chevaleyre et al., 2006; Huang et al., 2008). What makes eCBs mediating short-term *versus* long-term plasticity? It has been put in evidence that the type of synaptic plasticity (short *versus* long) may depend on how long the CB_1 receptor is occupied by eCBs or exogenous cannabinoids. For example, DSE/DSI are presumably induced by a brief synthesis of eCBs associated to a transient CB_1 receptor activation, while eCB-LTD requires cannabinoid activation for several minutes (Chevaleyre and Castillo, 2003; Younts and Castillo, 2014).

Typically, the modulation of synaptic activity consists in cellular events occurring in pre- and postsynaptic neurons. However, over the last years, astrocytes were identified as a third partner hosting cellular signaling cascades involved in the direct modulation of synaptic activity, beyond their well-recognized role in brain metabolism (Araque et al., 2014; Dallerac et al., 2013). In this sense, astrocytes could act as sensors of neuronal activity and consequently control back neuronal activity trough synthesis of gliotransmitters, such as glutamate, ATP or D-serine (Hamilton and Attwell, 2010; Navarrete and Araque, 2010; Panatier et al., 2011). In this context, it was found that activation of astrocytic CB_1 receptors leads to modulation of synaptic function. For instance, *in vivo* exogenous application of cannabinoids induces a form of LTD of hippocampal synaptic transmission dependent on N-methyl-D-aspartate (NMDA) receptor activation (Han et al., 2012). This *in vivo* LTD was not inducible in conditional mutant mice, lacking CB_1 receptor

expression in GFAP-positive astrocytes (Han et al., 2012). Interestingly, this form of plasticity was associated to impairment in working memory induced by astrocytic CB_1 receptors (Han et al., 2012). In contrast to neurons, CB_1 receptors in astrocytes can be coupled to PLC *via* $G_{q/11}$-proteins and thereby can increase the intracellular Ca^{2+} levels and, plausibly, trigger an exocytotic release of gliotransmitters (Navarrete and Araque, 2010). However, more studies are needed to determine the precise mechanisms of such neuron-astrocyte regulation *via* astrocytic CB_1 receptors.

I.8 Effect of CB_1 receptors on memory and motor function

I.8.1 CB_1 receptors and memory

The research on the pharmacological proprieties of plant *Cannabis Sativa* has shown that cannabis intake can induce memory loss in humans and animals (Bolla et al., 2002; Castellano et al., 2003; Davies et al., 2002; Miller et al., 1977; Puighermanal et al., 2009; Ranganathan and D'Souza, 2006). Additionally, ECS signaling has been shown to be a key regulator of memory processes (Marsicano and Lafenetre, 2009; Marsicano et al., 2002; Rueda-Orozco et al., 2008). Large part of my Thesis work focuses, in particular, on the potential involvement of $mtCB_1$ receptors in the pharmacological effects of cannabinoids on memory. In the following paragraphs, I will shortly describe the current definition of memory, I will summarize known effects of cannabinoids on different types of memory, and I will introduce the memory task that I used, underlying its experimental advantages for the specific aims of my Thesis work.

I.8.1.1 The memory systems

Short and long –lasting memories

Memory refers to the faculty of retaining and recalling past experiences based on the processes of learning, retention and retrieval (Milner et al., 1998). Information can be retained for a short period of time (*e.g.* seconds to minute range), for a longer time period (*e.g.* days, months) or remote period (*e.g.* years; Milner et al., 1998). The

functional dissociation of time-dependent memory systems comes from studies of memory performances in brain-lesioned patients and of the cellular mechanisms associated to learning-induced changes in synaptic properties in invertebrates (Milner et al., 1998). For instance, long-term memories were shown to depend on *de novo* protein synthesis (Kandel, 2009; Milner et al., 1998). The observation that long-term changes in synaptic plasticity require new protein synthesis represents one of the most important clues leading to the hypothesis that memory processes are indeed mediated by changes in synaptic plasticity (Kandel, 2009; Milner et al., 1998). Moreover, experiments in rodents led to the theory that a shift from *hippocampus*-dependent to neocortical-dependent memory might delineate ancient memory storage (Bontempi et al., 1999; Frankland et al., 2004).

Long-term memory systems

The existence of multiple memory systems was already proposed at the early beginning of the 19th century, notably based on the distinction of memory for facts, or representative memory, and memory for skills, or habits (Kandel, 2009; Milner et al., 1998). Again, the specific memory impairments described in amnesic patients across tasks led to neuropsychological dissociations between memory types, which were further identified in animal lesion studies **(figure 5;** Kandel, 2009; Kandel et al., 2014). Indeed, human memory can be declarative, as defined by the capacity of explicit recollection about fact and events, or "*knowing that*" (Squire et al., 1993). Human declarative memory can be further dissociated in semantic memory, defined by general knowledge unrelated to specific experience ("*Santiago is the capital of Chile*"), and episodic memory, which is related to specific, contextualized learning episodes or experiences ("*I went to Santiago last year*"). Declarative memory allows referring to a remembered event so as to infer its relationships, similarities and contrasts with other events or items (Squire et al., 1993). In animals, this form of memory is often called reference or relational memory, in which the medial temporal lobes are critically involved (Jaffard et al., 2000; Slangen et al., 1990; Squire, 2004). Memory can be also non declarative, implicit, referring to several additional memory subtypes, requiring specialized performances such as skills and habits ("*knowing how*"), sensory learning, priming (influence of a presented stimulus on later

responses), classical or Pavlovian conditioning and non-associative learning (sensitization / habituation;(Squire, 2004; Squire and Zola-Morgan, 1988). It is important to note that these distinctions are not strict. Adapted behaviors often require several memory processes that interact in a given situation (Levens and Phelps, 2010; Phelps, 2004; Squire, 2004).

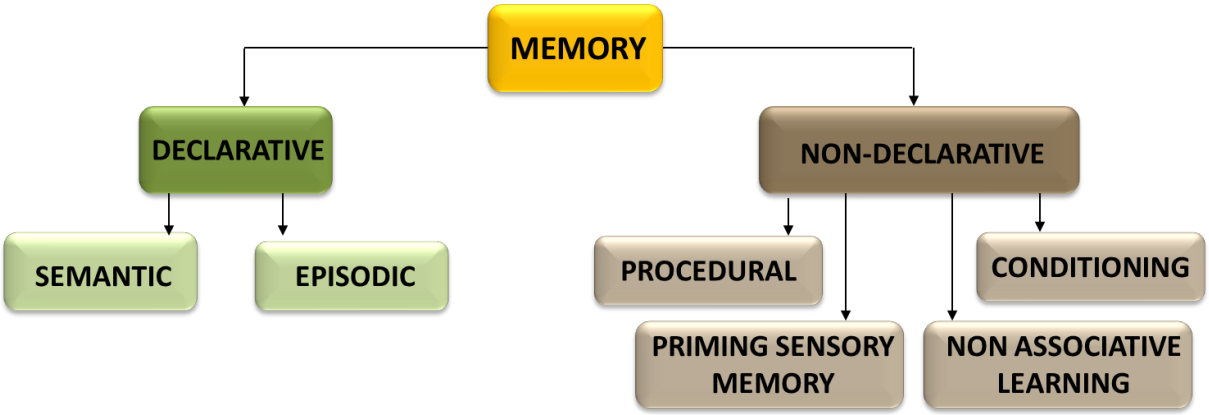

Figure 5.Long-term memory systems. Taxonomy of the mammalian long-term memory systems. Adapted from Squire 2004.

I.8.1.2 Hippocampal CB_1 receptor activation induces memory impairment

The amnesic effect of cannabinoids has been widely documented. In humans, chronic exposure to cannabis is associated with dose-related cognitive impairments, most consistently in attention, working memory and verbal learning (Bolla et al., 2002). Acutely, cannabinoids impair both encoding and recall of verbal and non-verbal information, in a dose-dependent manner (Ranganathan and D'Souza, 2006). In animal studies, cannabinoids alter long-term memory in a variety of experimental conditions evaluating both declarative (*e.g.* spatial and recognition memory) and non-declarative processes (*e.g.* non-associative learning, classical conditioning and procedural memory) (Castellano et al., 2003; Lichtman et al., 1995; Wise et al., 2009). The pharmacological effects of cannabinoids on memory performances fully depend on CB_1 receptors as they are absent in $CB_1^{-/-}$animals (Galanopoulos et al., 2014; Puighermanal et al., 2009; Reibaud et al., 1999; Varvel and Lichtman, 2002).

The *hippocampus* is a key brain structure involved in memory formation (Squire, 2004). Given the well-established role of the *hippocampus* in synaptic plasticity and memory processes and the high expression of CB_1 receptors in this structure (Herkenham et al., 1990; Marsicano and Lutz, 1999), it is likely that the negative effects of cannabinoids on at least certain forms of memory are ascribed to their actions within this brain region. Indeed, several studies have demonstrated the negative impact of cannabinoid agonists infused intra-hippocampally in different memory tasks such as object-location recognition memory and spatial memory (Abush and Akirav, 2010; Clarke et al., 2008; Lichtman et al., 1995; Suenaga and Ichitani, 2008). Many of these cannabinoid on memory effects are reversed by intra-hippocampal blockade of CB_1 receptors, revealing the necessary role of hippocampal CB_1 receptors (Clarke et al., 2008; Wise et al., 2009).

I.8.1.3 Object Recognition memory to study the potential role of $mtCB_1$ receptors

Large part of the present Thesis work aims at dissecting the potential involvement of mitochondrially-expressed CB_1 receptors in the amnesic effects of cannabinoids. To this aim, I chose to use object recognition memory (ORM). This behavioral task evaluates episodic-like memory, which is a type of declarative memory (Dere et al., 2005). Additionally, the *hippocampus* is essential for this type of memory (Zola-Morgan and Squire, 1985). Medial temporal lesions in monkeys impair memory on a variety of tasks sensitive to human amnesia). Below, I will shortly describe the principles of this task and explain why, considering the available experimental tools, it is the best suited memory test to study the potential role of $mtCB_1$ receptors.

Principles of object recognition memory (ORM)

Behavioral tests that evaluate the ability of recognizing a previously presented stimulus constitute the core of animal models of human amnesia (Baxter, 2010).Animals have the tendency to show an innate preference for novelty. This preference is displayed by spending more time exploring novel than familiar stimuli (recognition memory; Ennaceur and Delacour, 1988). Experimentally, in the version

of the task that we used, a mouse is placed in an L maze (L-M/OR) containing two identical objects at the end of each arm and then freely allowed to explore these objects for several minutes ("sample" phase, which represents the memory acquisition; Busquets-Garcia et al., 2011; Puighermanal et al., 2013; Puighermanal et al., 2009). After a delay the mouse is placed back in the maze, which now contains one familiar object (a copy of the sample phase objects) and a novel object ("test" phase, which represents the memory retrieval; Winters et al., 2008). Recognition memory is indicated by greater exploration of the novel object (Puighermanal et al., 2009). Thus, this memory consists in evaluating the memory performance for unique episodes or events (one-trial learning), which is adequately suited for studying the effects of cannabinoids on different stages of memory: acquisition, consolidation and retrieval. Moreover, a short delay (*e.g.* minutes) between the sample phase and the test phase will allow assessing the short-term memory whereas a longer delay (*e.g.* hours to days) will permit to test long-term memory. CB_1 receptor activation mediates both short-term (personal communications) and long-term memory impairment (Clarke et al., 2008; Puighermanal et al., 2009).

ORM is well-suited for the aims of this Thesis

As compared to other hippocampal-dependent memory tasks, this test presents several advantages for studying the impact of $mtCB_1$ receptor signaling on cannabinoid-induced amnesia: (i) the acquisition of L-M/OR occurs in one step and, previous studies revealed that the consolidation of this type of memory depends on hippocampal CB_1 receptors and is deeply altered by acute immediate post-training administration of cannabinoids (Puighermanal et al., 2013; Puighermanal et al., 2009); (ii) this test allows repeated independent measurements of memory performance in individual animals (Puighermanal et al., 2013), thereby allowing within-subject comparisons, eventually excluding potential individual differences in viral infection and/or expression of proteins **(see below)**; (iii) importantly, $CB_1^{-/-}$ mice do not respond to the administration of cannabinoids, but they do not show any spontaneous impairment of performance in L-M/OR (Puighermanal et al., 2009), thereby allowing the use of re-expression approaches to study the role of

hippocampal mtCB_1 receptors in the cannabinoid-induced blockade of memory consolidation **(see below)**.

I.8.2 CB_1 receptors mediate the hypolocomotor and cataleptic effects of cannabinoids

Typically, cannabinoids at high doses produce four characteristic behavioral and autonomic effects in mice, known as the “tetrad” effects of cannabinoids, whose study has been standardized by the team of Martin (Compton et al., 1993; Little et al., 1988). The “tetrad” is characterized by a reduction of spontaneous locomotor activity (hypolocomotion), body temperature (hypothermia), sensory pain perception (antinociception) and the inability to initiate the movement (catalepsy; Holtzman et al., 1969; Little et al., 1988). This assay represented an early screening to isolate novel cannabinoids and emphasize the impact of cannabinoids on motor activity and reactivity. As previously seen, CB_1 proteins are highly dense and widespread within the *basal ganglia* circuit (Herkenham et al., 1990; Matsuda et al., 1990). The *basal ganglia* are a cluster of brain nuclei participating in the planning and control of movements **(Figure 6)**. As the primary input of the *basal ganglia,* the striatum receives convergent glutamatergic inputs from the *cortex* and *thalamus* onto the dendrites of striatal medium spiny neurons (MSNs), the principal output neurons of the striatum (Calabresi et al., 1996; Graybiel, 1990). In turn the GABAergic MSNs project onto the *Entopeduncular nucleus* (EPN), the rodent homologue of the *internal Globus Pallidus* (GPi) in primates and the SNr (Bolam et al., 2000). These MSNs can be divided into two classes, based on the expression of either the dopamine receptor D_1 (D_1R) or the dopamine receptor D_2 (D_2R; Gerfen et al., 1990; Le Moine and Bloch, 1995). CB_1 receptors mRNA is highly expressed in the dorsolateral part of the striatum where it is co-expressed with GAD65 and D_1R or D_2R mRNA (Hermann et al., 2002; Martin et al., 2008). In a simplistic view, the D_1R population sends direct inhibitory projections to the GPi and SNr (the direct pathway; Zhou and Lee, 2011), facilitating movement. There, CB_1 protein is found at the synaptic terminals of striatal neurons projecting to the SNr and GPi (Herkenham et al., 1991a; Mansour et al., 1992). In contrast, the D_2R population projects indirectly to the GPi/SNr complex

(indirect pathway), *via* the *external Globus Pallidus* and the s*ubthalamic nucleus* (STN; Gerfen and Surmeier, 2011). Activation of the indirect pathway results in the inhibition of movement (Gerfen, 1992; Gerfen and Surmeier, 2011; Kawaguchi, 1997). In this context, deletion of CB_1 receptor gene restricted to D_1R- positive neurons in mice (D_1-$CB_1^{-/-}$) abolishes the induction of catalepsy by cannabinoids, whereas the hypolocomotion is still present in these mice (Monory et al., 2007). Given that most of the double CB_1- and D_1-positive cells are MSNs, it is likely that the direct striatonigral pathway is involved in this effect. Accordingly, activation of CB_1 cannabinoid receptors inhibits GABAergic neurotransmission in the SNr, where most of the CB_1 receptors are found on GABAergic terminal (Wallmichrath and Szabo, 2002), suggesting that SNr could be a well-sited structure to modulate the hypolocomotor and cataleptic effects of cannabinoids. Part of the present Thesis work is dedicated to the identification of the neuronal circuits mediating hypolocomotor and cataleptic effects of cannabinoids, with a specific focus on the SNr and the potential role played by mitochondrial CB_1 receptors.

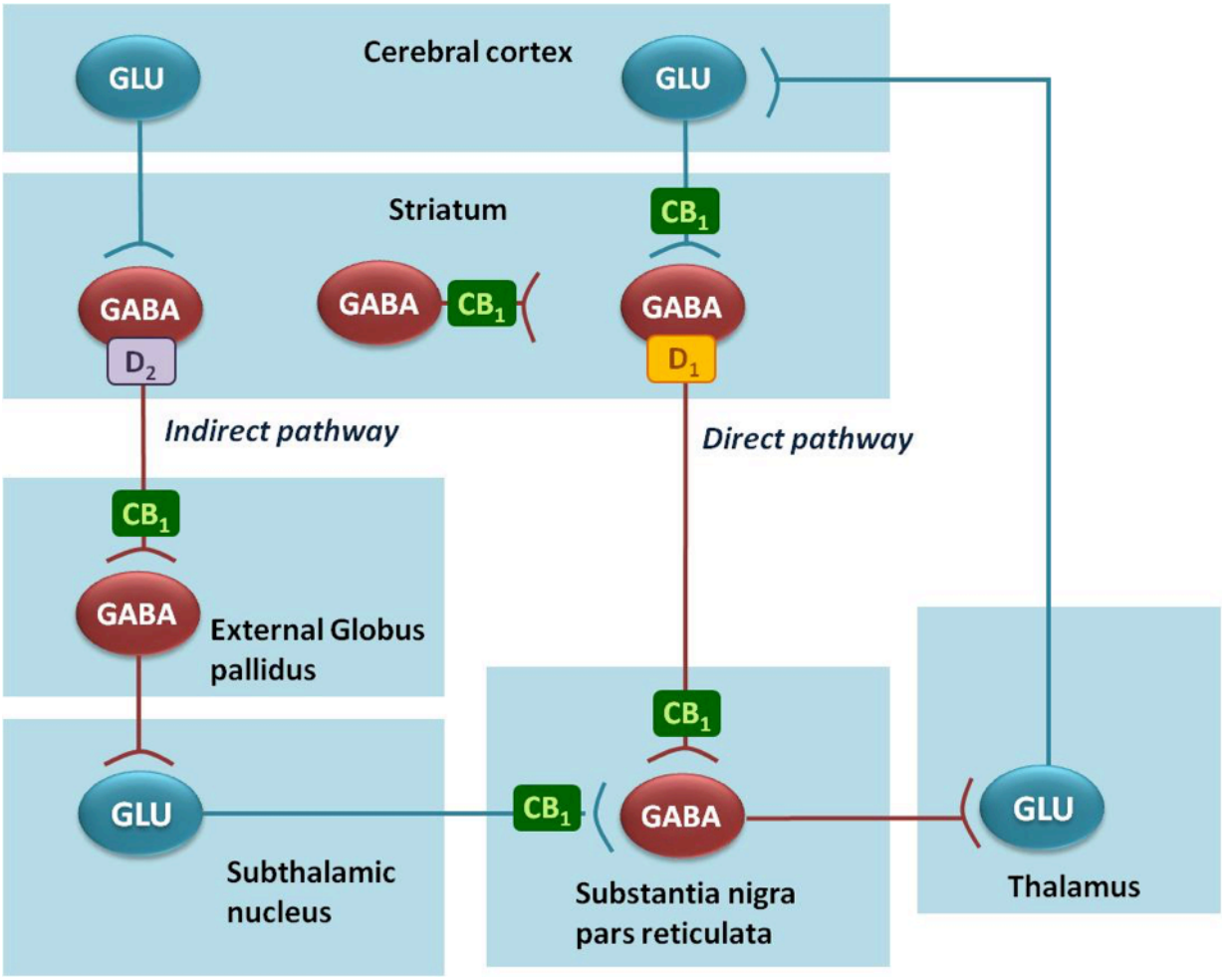

Figure 6. **Location of CB_1 receptors in specific neuronal subpopulations within basal ganglia circuit.** Excitatory inputs are indicated in blue, whereas inhibitory are indicated in red. Unknown neurons are shown in black. GABA, g-aminobutiric acid; GLU, glutamate; D_1 and D_2 Dopamine receptors type 1 and type 2. Adapted from Fernández-Ruiz, 2009.

II. MITOCHONDRIA

The brain is one of the most energy demanding organs of the mammalian body. In adults, whereas the brain represents only about 2% of the total body mass, the brain consumes ~25 % of the total body energy and it spends ~20 % of the total oxygen (O_2; Erecinska and Silver, 2001; Kety, 1957; Rolfe and Brown, 1997; Sokoloff, 1960). These values illustrate that brain functioning is tightly associated with high-energy expenditure and, therefore, requires high and constant levels of energy supply (Attwell et al., 2010; Mintun et al., 2001). Regarding energy production, mitochondria are the most important organelles able to transform energy supplies (*e.g.* from food) into ATP, which is the main source of energy used by cells for their survival and functioning. Indeed, one of the major roles of mitochondria is the generation of ATP, mainly through oxidative phosphorylation. Beyond their central role in energy metabolism, brain mitochondria participate to other key cellular functions including Ca^{2+} homeostasis, production of reactive oxygen species (ROS), synthesis and metabolism of neurotransmitters and other signaling molecules, and apoptotic processes (Turrens, 2003).

Synaptic plasticity and behavior largely depend on the capacity of neurons to meet the energy demands imposed by neuronal activity (Attwell and Laughlin, 2001). Emerging evidence indicates that events associated with cellular energy balance can impact synaptic and cognitive function (Suzuki et al., 2011; Wu et al., 2004). Importantly, mitochondria were shown to serve as a node for neurotransmission, synaptic plasticity, network activity and behavioral processes (Benard et al., 2012;

Guo et al., 2005; Kann and Kovacs, 2007; Li-Byarlay et al., 2014; Sun et al., 2013). Consequently, mitochondrial malfunctions are associated to the onset and progression of several neurodegenerative diseases (Manji et al., 2012; McInnes, 2013). Thus, understanding the functional role and consequences of metabolic energetic processes is a critical issue in neuroscience that needs to be further investigated. Altogether, these studies have opened new doors for the investigation of the biological basis of these diseases. Nevertheless, basic questions on synaptic metabolism persist: what are the regulatory mechanisms that control mitochondrial activity in the brain? Which features of synaptic functions require mitochondrial activity? How disruption of mitochondrial-dependent functions can impact on synaptic activity and physiological processes?

Before addressing these issues and discussing the role of mitochondria in brain functions, I will shortly describe the structure, properties and function of these organelles in the cell.

II.1 Origins of mitochondria

According to electron microscopy studies (Palade 1953), mitochondria are grain-like shaped organelles resembling to bacteria, composed by two highly specialized membranes and containing their own DNA (Palade, 1953; Taanman, 1999). Because of these characteristics, during the Sixties of the last century, Lynn Margulis proposed an endosymbiotic theory, postulating that eukaryotic cells have evolved from the acquisition of an aerobic prokaryotic bacterium (Margulis, 2001). Nowadays, this theory, supported by additional evidence, is commonly accepted (Richards and Archibald, 2011). From the evolutionary point of view, this symbiotic process provided eukaryotic cells with the great advantage of giving access to OXPHOS, a metabolic process that produces more than 15 times as much ATP as cytosolic glycolysis and the capacity to produce ATP from diverse substrates (Alberts, 2002). Throughout evolution, symbiotic bacteria likely became intracellular organelles (mitochondria) *via* a progressive transfer of genetic material to the host nucleus, causing a reduction of the mitochondrial genome (Dyall et al., 2004; Mossmann et al., 2012). Many endosymbiont genes were lost because they became

dispensable (such as those required for cell-wall building or motility) and many of them migrated to the nuclear genome (Lang et al., 1999). The transfer of genes to the nuclear DNA (nDNA) was accompanied by the development of protein targeting and transport machinery back to the organelle (Lang et al., 1999; Lucattini et al., 2004). However, mitochondria still contain their own genome and translation machinery. In mammalian cells, 37 genes are encoded by the mtDNA including 13 that encode proteins involved in cellular respiration and the rest for the transcription of mitochondrial DNA into protein (2 ribosomal RNA genes and 22 transfer RNAs; Mishra and Chan, 2014). Yet, the question why mitochondria have retained part of their own genome remains to be answered. Several hypotheses are debated on this issue, largely falling into two groups: “hydrophobicity” (Adams, 2003) and “redox control” (Allen, 2003). The first view holds that hydrophobic proteins are poorly imported by organelles; therefore, they have to be encoded by organelle’s genes (Adams, 2003). The second proposes that each mitochondria needs to have a proper control on the expression of genes that participate in the respiratory chain because the cellular ATP needs to fluctuate and a dysregulation of these processes could promote a deleterious effects (Allen, 2003).

II.2 Mitochondrial structure and dynamics

II.2.1 Mitochondrial ultrastructure

To obtain a comprehensive view of mitochondrial functions in the brain, one might first consider the spatial organization of mitochondrial compartments. As we mentioned previously, mitochondria contain an outer (OM) and an inner membrane (IM) with differential properties and functions. These membranes create two different mitochondrial compartments: the internal lumen called the matrix, and a much narrow intermembrane space (IMS; Alberts, 2002). The OM expresses transport proteins called porins. The porins create large channels in the lipid bilayer and render it largely permeable to molecules of 5000 Daltons or less, including ATP (Shoshan-Barmatz and Gincel, 2003). On the contrary, the IM is largely impermeable, but it contains a variety of carrier proteins that makes it selectively permeable. In addition,

the IM is highly folded, thereby increasing the total membrane surface area and the available “working space” (Alberts, 2002). Hence, the IM forms two domains that are structurally and functionally distinct: 1- the inner-boundary membrane closely apposed to the OM, and 2- the invaginations, called *cristae* (Herrmann and Riemer, 2010). The complexes forming the respiratory chain are mainly located in the *cristae* (Stroud and Ryan, 2013). In contrast, the control of ionic exchange and the import of metabolites and proteins between the cytosol and the matrix take mainly place in inner-boundary membranes (Davies et al., 2011; Vogel et al., 2006; Wurm and Jakobs, 2006). This structural description represents mitochondria as rather static and isolated organelles, shaped as "bean-like" within the cells' cytoplasm. However, mitochondria are not discrete or autonomous but form highly dynamic, interconnected networks whose structures and shapes are highly influenced by the needs of the cell (Lackner, 2014), as shortly described below **(Figure 7)**.

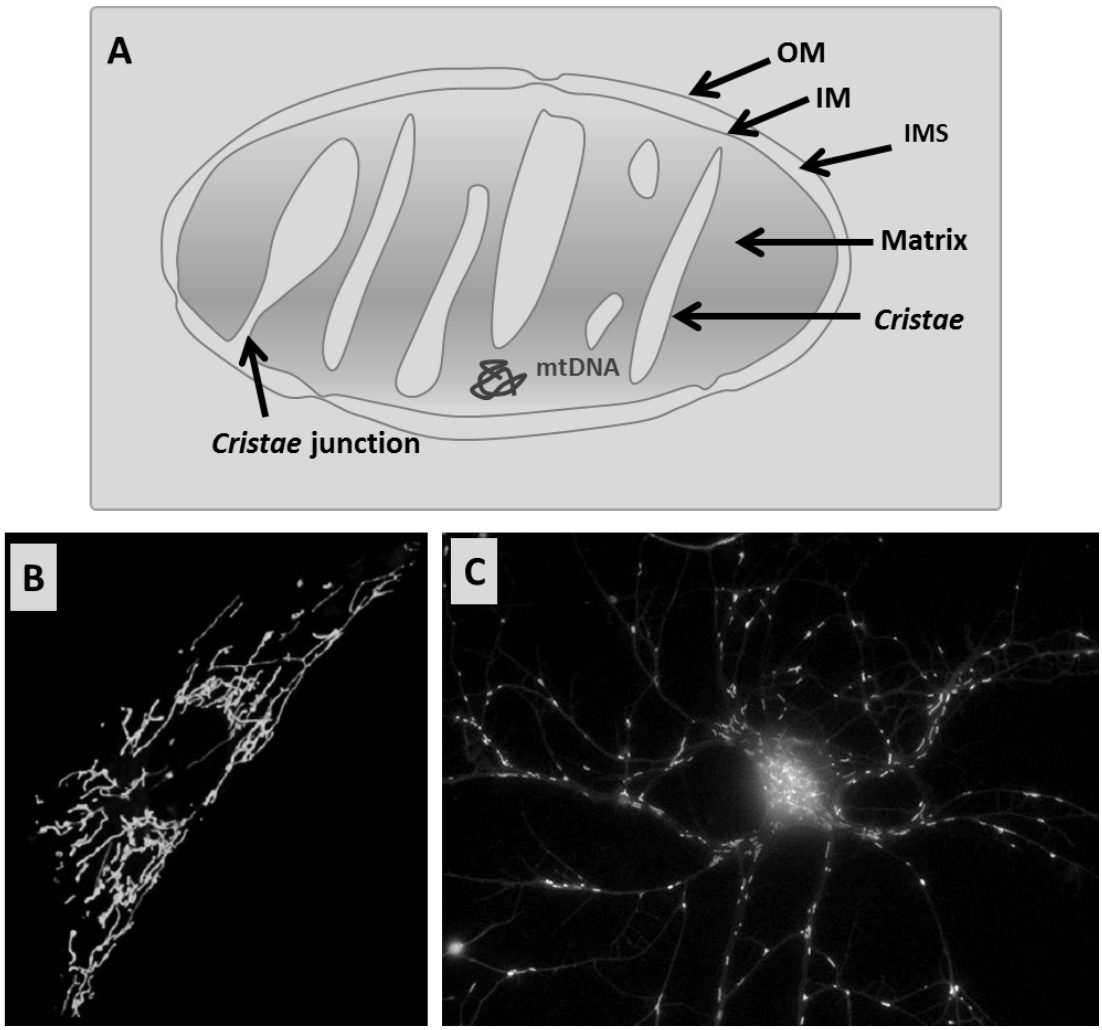

Figure 7.Mitochondrial structure and newtwork. (A) Schema of mitochondrial structure; mitochondrial outer membrane (OM), mitochondrial inner membrane (IM), intermembrane space (IMS). **(B)** Tomographic slice from Perkins et al., 2001, where mitochondria (M) localized nearby synaptic vesicle (SV) Scale bar = 100 nm. **(C)** Labeled mitochondrial network in a neuron, the organelles are positioned throughout the axon, cell body, and dendrites, taken from Brent T. Harris Lab site.

II.2.2 Mitochondria: a dynamic organelle

The traditional view of mitochondria as static organelles has evolved and it is nowadays recognized that they are highly mobile and they are organized in dynamic networks. Mitochondria undergo repetitive cycles of fusion and fission, and they are rapidly distributed towards active sites of the cell. These processes are necessary to

maintain mitochondrial viability and, in brain cells, they participate in the regulation of synaptic functions (Frazier et al., 2006). Here below, we will see briefly the mechanisms governing mitochondrial dynamics (fusion/fission) and their functional impact onto cell physiology.

Depending on environmental conditions, mitochondria appear in different shapes ranging from numerous small individual organelles to a single large interconnected membrane-bound tubular network (Picard et al., 2013; Rafelski, 2013). These morphological transitions are determined by fission and fusion events that constantly remodel the mitochondrial network. In neurons, mitochondria are widely distributed in the cell, including the soma and synapse (Sheng, 2014), and the balance between mitochondrial fission and fusion events varies among subcellular compartments (Popov et al., 2005). Indeed, 3D reconstructions using electron microscopy revealed that, in hippocampal neurons, dendrites display elongated mitochondria, forming a reticular network of interconnected "tubes", whereas axonal processes display smaller discrete mitochondrial bodies (Popov et al., 2005). However, additional studies are required to uncover the functional relevance of such neurite-specific morphological properties of mitochondria. The key proteins that control mitochondrial dynamic processes are Dynamin related proteins (DRPs), which belong to the family of GTP-hydrolyzing enzymes (Labbe et al., 2014). Briefly, sequential steps process mitochondrial fusion that results in lipid content mixing. First, the OM membranes tether and fuse together followed by IMs association and fusion. The first step is mediated through mitofusin 1 and 2 (Mfn 1 and 2), whereas IM fusion is operated by optic atrophy 1 (OPA 1; **Figure 8A)**. The opposing process of mitochondrial fission relies on the constriction of mitochondria mainly operated by dynamin-related protein 1 (DRP1; Picard et al., 2013;Smirnova et al., 2001; **Figure 8B)**.

Mitochondrial functions are highly dependent on their dynamics, and alterations of these processes can lead to dramatic consequences (Itoh et al., 2013). Several proteins regulating mitochondrial morphology have been identified as determinants of OXPHOS function. Defect in mitochondrial dynamics is strongly coupled to severe defects in respiratory activity. Silencing of OPA1 or DRP1 in cellular cultures results

in the blockade of mitochondrial fusion and fission, respectively (Benard et al., 2007; Chen et al., 2005). In addition, mutant mice lacking mitofusins or DRP1 die before birth, suggesting that mitochondrial dynamics represent vital processes (Chen et al., 2003a; Wakabayashi et al., 2009). In humans, strong evidence shows that disturbed mitochondrial fusion and fission are central pathological components of neurodegenerative disorders (Burte et al., 2015). A striking example is that genetic mutations of the mitofusin Mfn2 cause axonal Charcot–Marie–Tooth disease type 2A, a neuropathy affecting both motor and sensory nerves (Burte et al., 2015). Notably, this disease predominantly affects specialized neurons that require precise transport of mitochondria over long axonal distances (Burte et al., 2015).

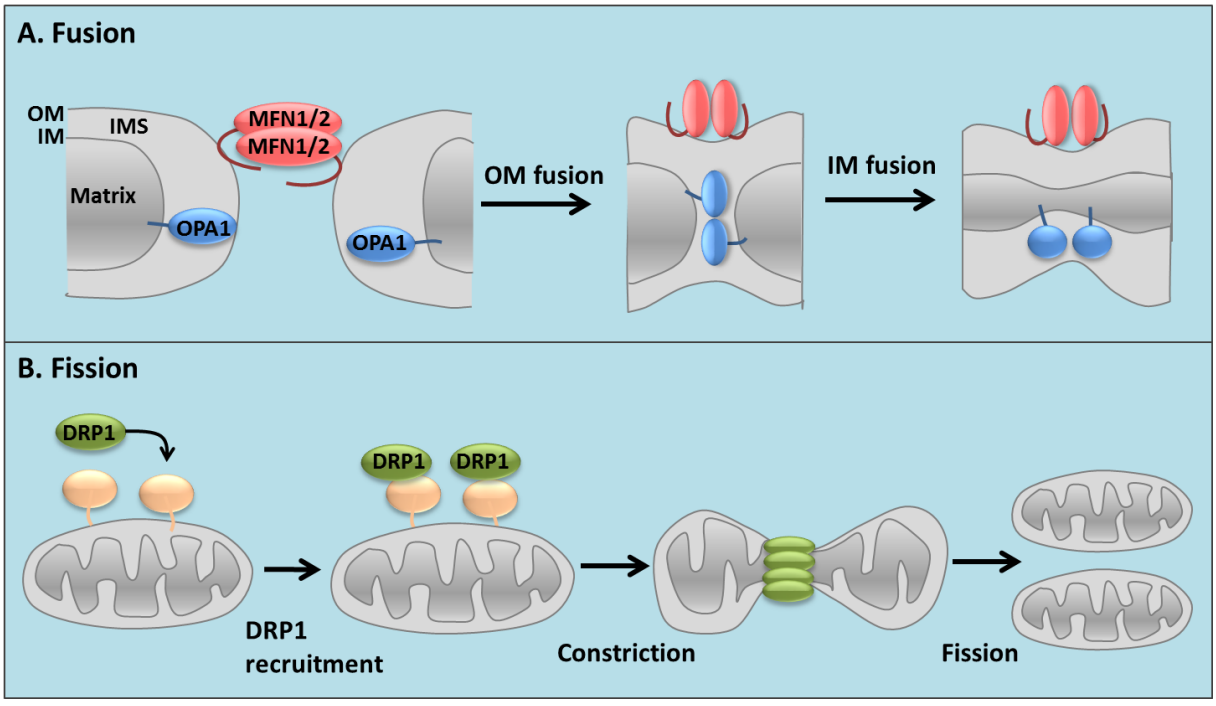

Figure 8.Mitochondrial fusion and fission in mammalian cells. The key factors in both fusion and fission are large GTP-hydrolyzing enzymes of the dynamin superfamily. **(A)** Mfn1 and 2 mediate OMM fusion whereas OPA1 mediates inner mitochondrial membrane fusion. **(B)** Mitochondrial fission machinery. Drp1 (Dynamin related protein 1) is found soluble in the cytosol of cells from where it shuttles onto and bind receptors at the surface of mitochondria. Drp1 assembles into spirals at division sites around the outer mitochondrial membrane to drive the fission process. Adapted from Mishra 2015.

II.3 Mitochondrial protein import

One feature of mitochondria is their ability to import proteins and metabolites that are essential to their function. As previously mentioned, the ancestral organelle that became the mitochondrion has likely undergone an evolutionary reduction of its genomic content with a concomitant gene transfer to the nucleus. Based on proteomic analyses, mammalian mitochondria are estimated to contain ~1500 different proteins (Giorgianni et al., 2014; Opalinska and Meisinger, 2014). Hence, the vast majority of mitochondrial proteins (≈98%) are synthesized in cytosolic ribosomes in their precursor forms. To acquire their mature and functional state these precursor proteins need to be imported into mitochondria and correctly distributed to one or more of the four mitochondrial subcompartments in order to ensure the correct mitochondrial biogenesis and functional maintenance of mitochondria (Lucattini et al., 2004; Stojanovski et al., 2012).

Molecular mechanisms underlying mitochondrial proteins targeting, import and sorting

The import of precursor proteins into mitochondria generally occurs in a post-translational manner. Cytosolic chaperones help fully translated proteins to reach their docking sites in the mitochondria. The chaperones shield the hydrophobic segments of mitochondrial precursor proteins to protect them from the aqueous cytosolic environment and to further prevent misfolding and aggregation (Dudek et al., 2013; Mossmann et al., 2012). Once they reach the organelle surface, they are recognized, docked and transported by mitochondrial membrane-embedded translocation machineries. These machineries are present at both OM and IM and consist of groups of associated proteins forming several different complexes. The first step of mitochondrial docking of proteins is their transport across the OM *via* the translocase of the outer mitochondrial membrane complex (TOM; Chacinska et al., 2009; Dukanovic and Rapaport, 2011; Kutik et al., 2007). After import, different transport routes exist depending on the final destination of the precursor (OM or IMS or IM), which is determined by their targeting information. The translocase of the inner membrane (TIM) is a complex of proteins found in the IM of which facilitates

translocation of matrix-targeted proteins into the mitochondrial matrix. The inner membrane potential (Δψ) drives this transport (Dudek et al., 2013).

The import of mitochondrial proteins is thought to occur mainly *via* post-translational processes (as seen above). However, messenger RNAs of mitochondrial proteins are enriched at the mitochondrial surface, where they are recruited into mitochondria by specific RNA-binding proteins (Gadir et al., 2011; Saint-Georges et al., 2008). Thus, recent studies have proposed an additional model in which some proteins are imported by a co-translational mechanism (Weis et al., 2013). The model involves interactions of ribosome with mitochondria OM proteins. Recently, an outer membrane protein (OM14) was identified as a receptor for cytosolic ribosome (Lesnik et al., 2014). This protein was shown to facilitate the import of proteins newly synthetized by ribosomes located at the mitochondrial surface, supporting the view of co-translational import into mitochondria (Lesnik et al., 2014). Further investigations need to be conducted in order to reveal the identity of mitochondrial mRNAs, their mode of transport and the function of this local translation of specific transcripts.

Interestingly, different types of G proteins and associated signaling partners are nowadays known to be present within mitochondria, such as Gαi (Kuyznierewicz and Thomson, 2002; Lyssand and Bajjalieh, 2007), Gα12 (Andreeva et al., 2008), Gβ2 (Zhang et al., 2010), and Gαq (Beninca et al., 2014). This fact suggests that G protein signaling participate in mitochondrial functions. However, the specific roles of G proteins and the mechanisms regulating their functions are poorly explored so far. For instance, the presence of G protein-coupled receptors (one of the most important regulators of G proteins) on mitochondrial membranes have been suggested by indirect pharmacological studies (*e.g.* P2Y purinergic receptors, Krzeminski et al., 2007), but no direct evidence for this has been presented until some years ago. The demonstration that CB_1 receptors are functionally present on brain mitochondrial membranes (Benard et al., 2012; Hebert-Chatelain et al., 2014; Koch et al., 2015) represents, therefore, a novel field of research.

These data, together with the previously described effects of cannabinoids on mitochondrial activity, raised the possibility that CB_1 receptors could also be functionally present on mitochondrial membranes, in direct contact with the organelle.

II.4 Mitochondrial functions

Within the cell, mitochondria play key roles in a number of important functions, including essential pathways of amino acid biosynthesis, steroid metabolism and apoptosis. However, the main role of mitochondria consists in providing oxidative energy metabolism, in the form of ATP and Ca^{2+} handling, which will be detailed below.

II.4.1 ATP synthesis

ATP synthesis in the cytosol

Energy metabolism is a highly coordinated cellular process, in which enzymes are organized into discrete metabolic pathways that cooperate to produce energy substrates from nutrients **(Figure 9)**. In the brain, nerve cells require continuous fuel supply from different metabolic pathways present in the cytosol (i.e. glycolysis) and in the mitochondria (i.e. Krebs cycle; Alberts, 2002). The energy used by nerve cells mainly derives from the breakdown of glucose that is permeable through the blood–brain barrier (Upadhyay, 2014). Once the glucose reaches the brain and is transported through cerebral blood flow, it is metabolized in both astrocytes and neurons through glucose transporters (GLUT) present at the plasma membrane (Leybaert, 2005).

The first step of glucose catabolism is glycolysis, taking place in the cytosol under anaerobic conditions (Alberts, 2002). Glycolysis is the process by which one molecule of glucose is converted into two molecules of pyruvate. In turn, pyruvate can be converted to lactate and vice versa, by lactate dehydrogenase (LDH). In particular, it has been proposed that glycolysis from astrocytes serves at supporting neuronal energy demands by providing lactate to neurons, where it is being

reconverted in pyruvate. This process is called astrocyte to neurons lactate shuttle (ANLS; Belanger et al., 2011; Pellerin and Magistretti, 2012; Sotelo-Hitschfeld 2015).

ATP synthesis in mitochondria

Once pyruvate has been produced by glycolysis in the cytosol, it translocates into mitochondria where it undergoes enzymatic degradation to produce acetyl-CoA. In turn, each acetyl-CoA enters into the Krebs cycle where, after eight sequential enzymatic reactions, it will produce 3 NADH (nicotinamide adenine dinucleotide), 1 $FADH_2$ (flavin adenine dinucleotide), 1 GTP (Guanosine-5'-triphosphate) and 2 CO_2. GTP is a molecule involved in the energy transfer within the cell and can be subsequently be used to produce ATP. The reducing products NADH and $FADH_2$ will be used as substrates to fuel the electron transport chain (ETC) in the OXPHOS pathway (Nelson and Rajagopalan, 2004). In addition to glycolysis, glucose can be stored by astrocytes in the form of glycogen. Glycogen can be rapidly metabolized without the need for ATP hydrolysis to provide energy substrates during periods of increased tissue energy demand (Brown, 2004).

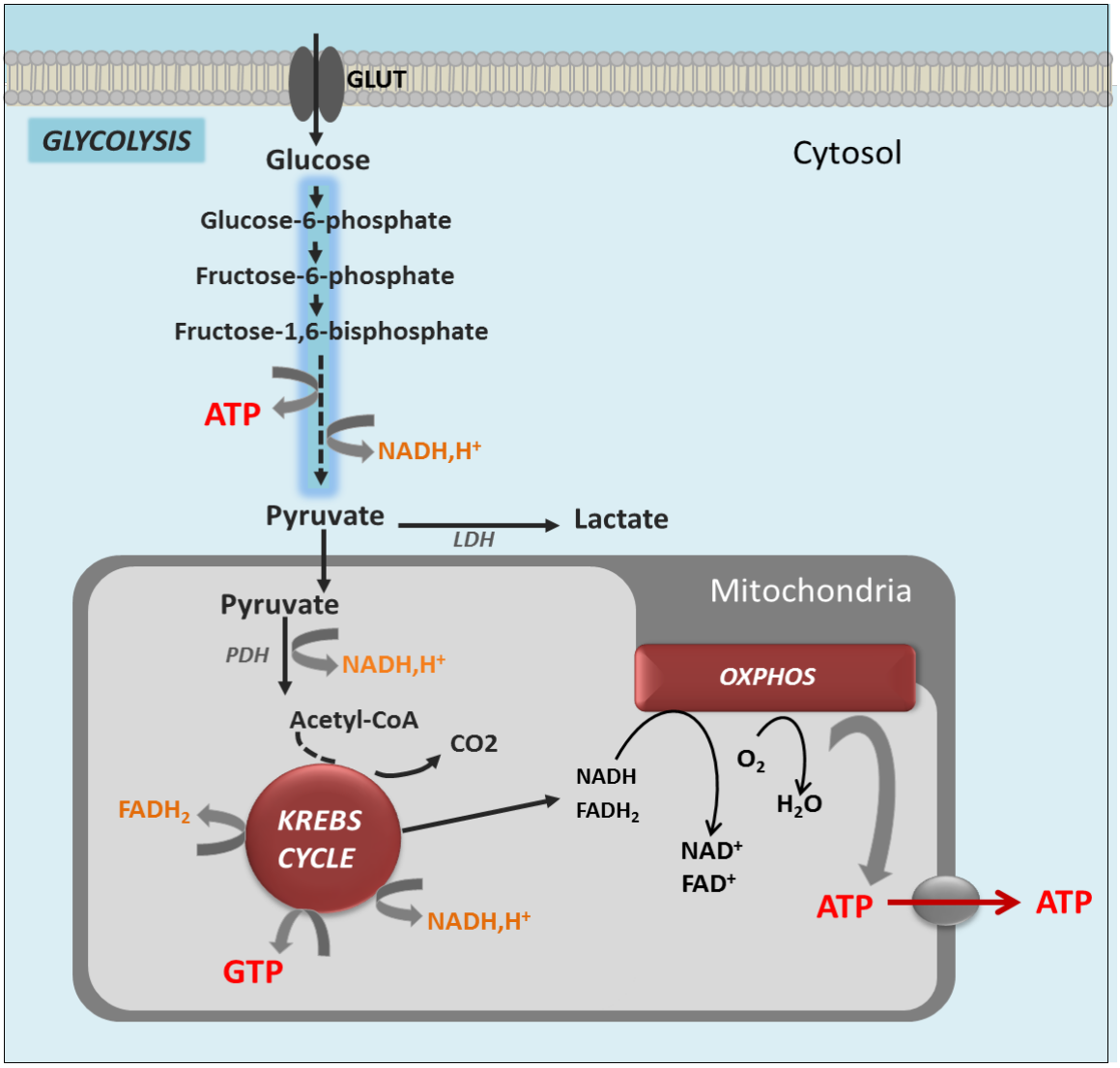

Figure 9. Main pathways of cellular and mitochondrial energy metabolism. The two main metabolic pathways, i.e. glycolysis and oxidative phosphorylation (OXPHOS) are linked by the enzyme complex pyruvate dehydrogenase (PDH). Briefly, glucose is transported inside the cell and oxidized to pyruvate. Under aerobic conditions, the complete oxidation of pyruvate occurs through the Kreb's cycle to produce NADH and/or FADH2. These reduced equivalents are further oxidized by the mitochondrial respiratory chain. Adapted from Bellance 2008.

II.4.1.1 Oxidative phosphorylation (OXPHOS)

The brain is a highly oxidative organ and, as a consequence, most of the energy is derived from oxidative reactions (Cai et al., 2011). Neuronal communication depends on energetically demanding processes such as reversing the ion influxes involved in action potential generation and synaptic transmission, which require

mitochondrial ATP production (Harris et al., 2012). Oxidative metabolism of pyruvate provides 32 ATP/glucose whereas glycolysis and glycogenolysis produce 2 ATP or 3 ATP/glucose, respectively. During OXPHOS, redox reactions and the associated proton pumping are coupled to ATP synthesis and are mediated by 5 enzymatic complexes **(Figure 10;** Garret, 2013). Complexes I-IV are large multi-subunit enzymatic complexes that form the ETC whereas Complex V does not transfer electrons but rather produces ATP. The electrons that are moving along the ETC are provided by a series of oxidation-reduction (redox) reactions, realized between an electron donor (such as NADH and $FADH_2$) and an electron acceptor (such as O_2;Garret, 2013). As the electrons get transferred from one molecule to another, they go into lower energy state and they release energy. The free energy extracted from the movement of electrons drives the pumping of H^+ ions (protons) from the matrix to the IMS by complexes I, III, and IV. In more details, complex I, known as NADH ubiquinone oxidoreductase, is responsible for the oxidation of NADH while Complex II, succinate ubiquinone reductase, oxidizes $FADH_2$. From these two complexes, electrons converge to the coenzyme Q10 (CoQ; Garret, 2013). CoQ is a lipid-soluble electron carrier that transfers electrons from complexes I/II to complex III. By receiving the electrons coming from complex I and II, CoQ is reduced and takes the name of ubiquinol. In this form, it will carry electrons to Complex III, also called cytochrome bc1 complex, and it will be converted back to its oxidized state (ubiquinone). The oxidation of CoQ will allow the Complex III to catalyse the reduction of cytochrome c, another electron carrier. The electrons transferred to the cytochrome c are in turn delivered to the complex IV, cytochrome oxidase (Garret, 2013). This is the last enzyme in the respiratory chain in mitochondria where the electrons are transferred to an O2 molecule, forming two molecules of water. The translocation of protons during these processes generates an electrochemical proton gradient (ΔμH+) across the IM. This gradient is approximately 180 - 220 mV and results from the mitochondrial membrane potential (ΔΨm) and pH gradient (ΔpHm; Mitchell and Moyle, 1969; Nicholls, 1974). In turn, protons will be transported back to the matrix through the ATP synthase (complex V), which uses the energy produced by this transport to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). The ATP is then translocated out of

mitochondria, through the adenine nucleotide transporter (ANT) at the IM and through porins at the OM level, and is used to ensure neural cellular processes.

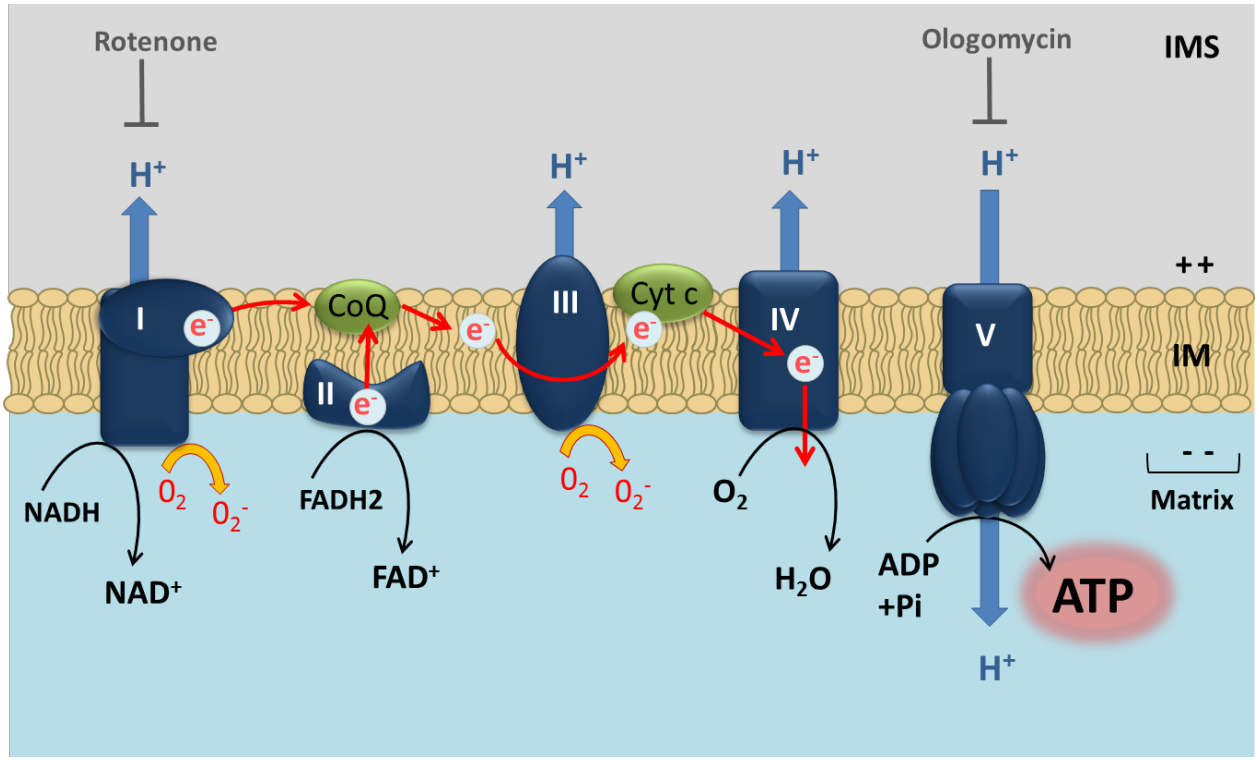

Figure 10. Mitochondrial respiratory chain. For mammals, the respiratory chain consists of four enzyme complexes (complexes I - IV) and two intermediary substrates (coenzyme Q and cytochrome c). The NADH and $FADH_2$ produced by the intermediate metabolism are oxidized further by the mitochondrial respiratory chain to establish an electrochemical gradient of protons, which is finally used by the F1F0-ATP synthase (complex V) to produce ATP, the only form of energy used by the cell, adapted from Bellance 2008.

II.4.1.2 Reactive oxygen species (ROS): by-products of the mitochondrial energy metabolism

During mitochondrial respiration, significant amounts of reactive oxygen species (ROS) are formed, resulting from the incomplete reduction of the O_2 molecule into water. Any free radical involving O_2 can be referred to as ROS (Datta et al., 2000). Free radicals such as the hydroxyl radical (OH^-) and the superoxide anion (O_2^-) are transient and chemically unstable due to unpaired electrons in their atomic orbitals. Hence, they tend to rapidly react with other compounds, trying to capture the needed electron to gain stability (Balaban et al., 2005). Hydrogen peroxide (H_2O_2) is not a

free radical (no unpaired electrons) but it is included in ROS because it can be easily converted into OH− and could rapidly damage molecules. Mitochondria are considered as the major sites of ROS production in the cell (Cadenas and Davies, 2000). When electrons are transferred along the ETC, a small quantity of electrons can leak out from the classical redox reactions pathway and be transferred directly to O_2. Complex I and III are thought to be the main ROS generation sites as compared to Complex II (Balaban et al., 2005). In contrast, complex IV, due to its high catalytic capacity of O_2 reduction to water, does not normally produce ROS, but it rather prevents their production by keeping the cellular O_2 concentration low (Papa and Skulachev, 1997). ROS could have either deleterious effects by promoting cellular damages or and physiologic roles through a signaling activity (Popa-Wagner et al., 2013). On one hand, excessive levels of ROS can be toxic causing protein damage and mtDNA and nDNA mutagenesis (Marchi et al., 2012). Indeed, increased cellular ROS levels production is a common feature of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (Hroudova and Fisar, 2013). Importantly, beyond their damaging effects on cells, ROS have been proposed to act as signal molecules modulating a myriad of cellular processes including apoptosis, inflammation, synaptic plasticity and memory formation (Hu et al., 2006; Massaad and Klann, 2011). However, under normal conditions, ROS production only accounts for 2% of the overall cellular O_2 consumption (Chance et al., 1979). Hence, most of the O_2 consumption is due to the respiratory activity of mitochondria in order to produce ATP, which is needed for survival, function and activity of the cell.

II.4.1.3 Cellular respiration is governed primarily by ATP needs: focus on OXPHOS regulation

In the brain, energy requirements are very high, and vary in time and space according to levels of activity. Therefore, it is very likely that brain-specific regulatory mechanisms exist to ensure an adequate delivery of energy by mitochondria. Mitochondrial functions can adapt to meet cellular energetic needs by modulating OXPHOS performance. This regulation can occur at different levels, including the control of:

- Changes in OXPHOS capacity in response to cellular and mitochondrial microenvironment (*e.g.* energy substrate availability)
- Changes in OXPHOS efficiency through kinetic parameters (*e.g.* supercomplexes)
- Changes in OXPHOS chain activity (*e.g.* post-translational modifications)

Changes in OXPHOS capacity in response to cellular and mitochondrial microenvironment (*e.g.* energy substrate availability)

Mitochondria are in constant communication with the cytosol to coordinate the balance between the energy demands of the cell and energy production by OXPHOS. OXPHOS requires a supply of NADH, $FADH_2$ (or other source of electrons at high potential), O_2, ADP, and Pi. A well-known mechanism regulating OXPHOS is mediated by the availability of ADP as substrates and is called “respiratory control” (Brand and Nicholls, 2011). This effect was observed in isolated mitochondria where the respiratory activity (measured by O_2 -sensitive electrode) is reversibly modulated by the availability of energy substrates ADP (Lardy and Wellman, 1952). The rate of O_2 consumption by mitochondria increases markedly when ADP is added and then goes back to its initial value when the added ADP has been converted into ATP. Because of the tight coupling between electrons transport and ATP synthesis, electrons do not usually flow through the electron transport chain toward O_2, unless ADP is simultaneously phosphorylated to ATP (Garret, 2013). Thus, the physiological significance of this regulatory mechanism is that when ATP is consumed for cellular mechanisms, ADP levels increase. The further elevation of ADP will exert a positive feedback control on respiration. Hence, the positive regulation of OXPHOS activity by ADP levels indicates the strong dependence of physiological respiration on energy demand (Zharova and Vinogradov, 2012).

Changes in OXPHOS efficiency through kinetic parameters (*e.g.* supercomplexes)

The classical view of ETC as discrete enzymes diffused in the IM has been replaced by the “solid state model”, postulating that respiratory complexes are associated with each other to form supramolecular complexes, called respiratory supercomplexes or respirasomes (Genova and Lenaz, 2014). Using blue native

polyacrylamide gel electrophoresis (BN PAGE), studies in mammalian mitochondria showed that Complex I, III and IV were organized in supercomplexes in various combinations (Dudkina et al., 2011; Schagger et al., 2004). Almost all the complexes I are assembled into supercomplexes whereas most of complexes II were found in a free, non-associated form and only a small proportion is associated with supercomplex I/III/IV (Acin-Perez et al., 2008). Hence, remodeling of supercomplexes was hypothesized to facilitate OXPHOS specialization and to directly control OXPHOS bioenergetic functions (Acin-Perez et al., 2008; Huttemann et al., 2008). Consistent with this view, destabilization of supercomplexes is associated with a decrease in respiration (Lenaz and Genova, 2012). Thus, it is proposed that supercomplexes confer a kinetic advantage for substrate channeling. Indeed, to the spatially close arrangement of the complexes that makes the diffusion of the mobile electron carriers faster as the later will only travel short distances (Althoff et al., 2011).

Post-translational processes regulating OXPHOS chain activity: the case of the mitochondrial cAMP/PKA signaling pathway mediated by sAC

It has been shown that Complex I undergoes a multitude of post-translational modifications in several of its subunits (De Rasmo et al., 2010). As an important point of my Thesis, I will discuss about post-translational modifications of complex I subunits by phosphorylation induced by the cAMP/PKA pathway. The majority of the complex I subunits are encoded by nDNA, whereas 7 are encoded by mtDNA (Schon et al., 2012). *In vitro*, there is strong evidence demonstrating that mitochondrial cAMP/PKA-dependent phosphorylation regulates complex I activity (Papa et al., 2012).

It was first found that pharmacological up-regulation of cAMP levels positively impacts on complex I activity and endogenous respiration sensitive to the Complex I inhibitor rotenone in mouse embryonic fibroblasts (MEFs; Scacco et al., 2000). Interestingly, this increase of mitochondrial activity was accompanied by serine phosphorylation in the 18-kDa subunit of complex I (Scacco et al., 2000) In addition, dibutyryl cAMP, an analog of cyclic AMP that stimulates cAMP-dependent protein kinases, resulted in a similar stimulation of mitochondrial respiration and subunit

complex I phosphorylation. Overall, these data indicate that cAMP-dependent phosphorylation directly regulate cellular respiration likely trough the modulation of complex I activity (Scacco et al., 2000). Furthermore, NDUFS4 subunit of complex I was identified as the target that undergoes post-translational phosphorylation, mediating the increase of Complex I activity (De Rasmo et al., 2010; Scacco et al., 2003). Indeed, the serine-173 in C terminus of the NDUFS4 protein is a site phosphorylated by PKA (Scacco et al., 2000).

The following question was whether cAMP and PKA could act directly within mitochondria. On one hand, PKA can be localized in defined microdomains such as the mitochondria (Kapiloff et al., 2014; Means et al., 2011). A-kinase anchoring proteins (AKAPs) mediate the transport of PKA into mitochondria, allowing PKA to phosphorylate target substrates preferentially located in the matrix Means, 2011 (Means et al., 2011). Together, these data support the view that PKA likely signals within the mitochondria (Sardanelli et al., 2006) and phosphorylates Complex I subunit NDUFS4. As seen above, cAMP-mediated phosphorylation of mitochondrial enzymes plays a role in OXPHOS regulation. However, cAMP generated in the cytosol does not enter mitochondria (Di Benedetto et al., 2013). Thus, it was postulated that a source of this second messenger might reside inside mitochondria (Papa et al., 1999). A soluble form of adenylyl cyclase called sAC was found expressed in mitochondrial matrix, where it generates cAMP (Zippin et al., 2003). This enzyme has specific molecular characteristics, as it seems to be insensitive to heterotrimeric G protein regulation and forskolin stimulation and it is modulated by bicarbonate ions, Ca^{2+} and ATP (Litvin et al., 2003; Zippin et al., 2003).

Interestingly, Acin-Perez demonstrated that the pharmacological inhibition of sAC, using a sAC inhibitor named KH7, decreases rotenone-sensitive dependent respiration. Conversely, sAC activation by bicarbonate increases mitochondrial respiratory function (Acin-Perez et al., 2009b). More recently, De Rasmo and colleagues showed that KH7 treatment causes a reduction in respiratory activity and a decrease of the expression levels of several complex I subunits (NDUFS4, NDUFV2 and NDUFA9; De Rasmo et al., 2015). Notably, inhibition of proteasomes blocked the effects of KH7 on both subunit expression levels and on complex I

activity, suggesting that the drug regulates post-translational events. It was then proposed that intramitochondrial cAMP exerts this positive effect on complex I by preventing digestion of nDNA-encoded complex I subunits by mitochondrial protease. Importantly, both effects were abolished by a non-hydrolysable cell permeant cAMP analog (8-Br-cAMP), but not by the increase of cytosolic cAMP induced by Beta-adrenoreceptor agonist (De Rasmo et al., 2011). Altogether, these findings clearly suggest that sAC-dependent production of mitochondrial cAMP promotes respiratory functions *via* modifications of complex I activity.

II.4.2 Mitochondrial role in calcium homeostasis

II.4.2.1 Mitochondrial use of calcium

Beyond their role in generating ATP, neuronal mitochondria contribute to Ca^{2+} homeostasis. Many characteristics of synaptic transmission and plasticity depend on changes in presynaptic and post-synaptic Ca^{2+} levels (Zucker, 1999). Under physiological resting conditions, the cytosolic Ca^{2+} concentration is remarkably low (about 50–100 nM), but it can transiently rise during electrical activity to levels that are ten to 100 times higher (Berridge et al., 2000). As compartmentalization is one of the key features of cellular signaling, the regulation of neural processes by intracellular Ca^{2+} signals depends on its spatial and temporal limitations. Indeed, Ca^{2+} signaling is highly versatile and can operate over a large time range, from neurotransmitter release at the microsecond scale to gene transcription, which lasts for minutes and hours. Similarly, the organization of discrete cellular microdomains, in which the Ca^{2+}-sensitive enzymes such as AC resides, plays a key role in neuronal processes (Berridge et al., 2003; Willoughby et al., 2010). Together with the endoplasmic reticulum (ER), mitochondria play key roles in modulating the concentration of cytosolic Ca^{2+} (Grimm, 2012). Pulses in Ca^{2+} influx, *e.g.* during synaptic activity, is matched by Ca^{2+} efflux during periods of rest to avoid either Ca^{2+} buildup or depletion (Balaban, 2002). The ability of mitochondria to uptake Ca^{2+} allows modifying the shape of cytosolic Ca^{2+} pulses, but it also permits the stimulation the OXPHOS activity (Gunter et al., 2004). In excitable cells, elevation of Ca^{2+} increases OXPHOS indirectly by increasing ATP consumption, *via* active Ca^{2+}

transport, and by directly stimulating ATP production *via* activating complex V and Krebs' cycle enzyme activity (Bender and Kadenbach, 2000; Carafoli and Crompton, 1978; Gunter et al., 2004; Lehninger et al., 1978; Vale et al., 1983). Indeed, Ca^{2+} ions have been reported to activate the rate of NADH production, one of the substrate for OXPHOS, by up-regulating the isocitrate dehydrogenase and α-ketoglutarate dehydrogenase present in the Kreb's cycle (Denton, 2009; Gunter et al., 2004). Furthermore, large evidence highlights a role of Ca^{2+} in modulating the activity of Complex V and cytochrome oxidase *via* post-translational modifications (Balaban, 2009; Bender and Kadenbach, 2000). More recently, it was shown that Ca^{2+} entry into the mitochondrion induces the up-regulation of mitochondrial cAMP/PKA signaling trough sAC, which results in the increase in ATP synthesis (Di Benedetto et al., 2013). Thus, it was suggested that Ca^{2+} could be one of the positive regulators of mitochondrial energy metabolism at nerve terminals by potentially acting as a feed-forward mechanism to boost ATP synthesis in order to prevent energy drop during synaptic activity (Rangaraju et al., 2014).

II.4.2.2 Mitochondria buffer cellular calcium

Mitochondrial Ca^{2+} uptake depends on the strong driving force ensured by the membrane potential across the inner membrane (-180 mV, negative inside the matrix) built by the respiratory chain and inducing Ca^{2+} to penetrate into mitochondria (Bianchi et al., 2004). The use of chemical uncouplers that perturbs the proton gradient across the IMM and depolarizes the mitochondrial matrix, such as carbonyl cyanide 4-(trifluoromethoxyl)s-phenyl-hydrazone (FCCP), strongly inhibits mitochondrial Ca^{2+} uptake (Graier et al., 2007). Ca^{2+} import across the OM occurs through the voltage dependent anion channel (VDAC; Hajnoczky et al., 2006; Simamura et al., 2008; Szabadkai et al., 2006). However, the identification of ion channels responsible for Ca^{2+} entering the mitochondrial matrix has been a difficult task. Because of specific kinetic characteristics of mitochondrial Ca^{2+} uptake, it was proposed that Ca^{2+} is likely transported through a gated channel rather than a classical carrier (Kirichok et al., 2004). Only recently, De Stephani and colleagues revealed the molecular nature of a mitochondrial Ca^{2+} uniporter (MCU) embedded in

the IM. Thereby, using short interfering RNA (siRNA) silencing of MCU in HeLa cells, they observed a markedly reduced mitochondrial Ca^{2+} (De Stefani et al., 2011). Since then, additional proteins have been described that are able to modulate the function of MCU-dependent transport of Ca^{2+} and its assembly in the mitochondrial IM, such as mitochondrial calcium uniporter regulator 1 (MCUR1) and essential MCU regulator (EMRE), respectively (Mallilankaraman et al., 2012; Sancak et al., 2013). Thus, MCU has been characterized as a complex of molecules, but future research needs to characterize the exact composition and stoichiometry of MCU complexes as well their functional impact on synaptic activity. In this context, the recent molecular identification of the MCU (De Stefani et al., 2011) allowed investigating its impact on neuronal excitotoxicity induced by NMDA receptor activation (Qiu et al., 2013). While Ca^{2+} uptake can stimulate the respiratory chain, excessive amounts of mitochondrial Ca^{2+} can be detrimental for the cell and lead to excitotoxicity and apoptosis (Benedict et al., 2012; Hajnoczky et al., 2006). Knockdown of MCU expression, using shRNA-targeting MCU in hippocampal and cortical neurons, decreases NMDA-induced accumulation of mitochondrial Ca^{2+} (Qiu et al., 2013). This effect largely prevents mitochondrial depolarization and confers a resistance to NMDA-induce excitotoxic cell death (Qiu et al., 2013), suggesting a role of mitochondrial Ca^{2+} uptake capacity in coupling excessive Ca^{2+} entrance to apoptosis. However, the functional role of MCU remains under controversy. A recent study using $MCU^{-/-}$ mice has reported that the Ca^{2+} content in mitochondria of mutant cells was only partially reduced as compared to wild type animals (Pan et al., 2013). While the lower levels of mitochondrial Ca^{2+} levels can be relevant for neuronal toxicity (Qiu et al., 2013), MCU does not appear to be the only route for mitochondrial Ca^{2+} uptake across the IM, indicating the existence of alternative Ca^{2+} uptake pathways or that Ca^{2+} target other mitochondrial proteins without penetrating in the matrix. Interestingly, the identification of Ca^{2+}-regulated mitochondrial carriers (CaMCs) revealed an additional target of cytosolic Ca^{2+} signals in neuronal mitochondria for regulation OXPHOS (del Arco and Satrustegui, 2004). The critical difference between these pathways is that Ca^{2+}-dependent regulation of mitochondrial respiration through the carriers operates by the action of Ca^{2+} at the surface of the IM, rather than in the matrix and therefore does not require mitochondrial Ca^{2+} uptake (Llorente-Folch et al., 2015). Cytosolic

Ca^{2+} controls OXPHOS by facilitating pyruvate supply to mitochondria through its action on the Ca^{2+}-regulated mitochondrial carriers, the aspartate-glutamate carrier (Aralar), a component of the malate–aspartate shuttle (MAS; Llorente-Folch et al., 2013). Indeed, the formation of pyruvate from its precursors (*e.g.* glucose, lactate) is coupled to NAD+ regeneration. NAD+ levels are promoted by cytosolic Ca^{2+}*via* MAS activity, thereby increasing pyruvate formation (Gellerich et al., 2013). In turn, pyruvate mitochondrial import will boost substrates production from Kreb's cycle resulting in increased energy production from OXPHOS, a process called "the mitochondrial gas pedal" (Llorente-Folch et al., 2013; Rueda et al., 2014). Thus, the mechanisms by which Ca^{2+} activates mitochondrial function remain a hot research spot and future experiments will detail the actors of such system and their physiological relevance **(Figure 11)**.

Another relevant issue for intracellular Ca^{2+} dynamics is the physiological function of the ER and mitochondrial physical apposition (Csordas et al., 2006). Electron tomography experiments revealed that ER and mitochondria can be tightly connected at specific membrane contact sites, called the mitochondria-associated membranes (MAMs; Csordas et al., 2006). Ca^{2+} is mobilized from the ER and subsequently transferred to mitochondria *via* inositol trisphosphate receptor (IP3R; Cardenas et al., 2010; Esterberg et al., 2014). Remarkably, IP3Rs within the ER are juxtaposed to the mitochondrial VDAC, by which Ca^{2+} enter mitochondria, supporting the idea of Ca^{2+} transfer in MAMs (Szabadkai et al., 2006). In particular, IP3R Ca^{2+} release and mitochondrial uptake likely provide an essential functional regulation of mitochondrial bioenergetics autophagy (Mendes et al., 2005). According to this, genetic deletion or pharmacological blockade using siRNA or inhibitors of IP3R decrease O_2 consumption and lead to a reduction in mitochondrial ATP production (Cardenas et al., 2010). In turn, the ATP depletion causes an increase of cell death by autophagy (Cardenas et al., 2010). Thus, these data suggest that mitochondrial Ca^{2+} intake *via* IP3R has a protective role against autophagy by stimulating mitochondrial OXPHOS.

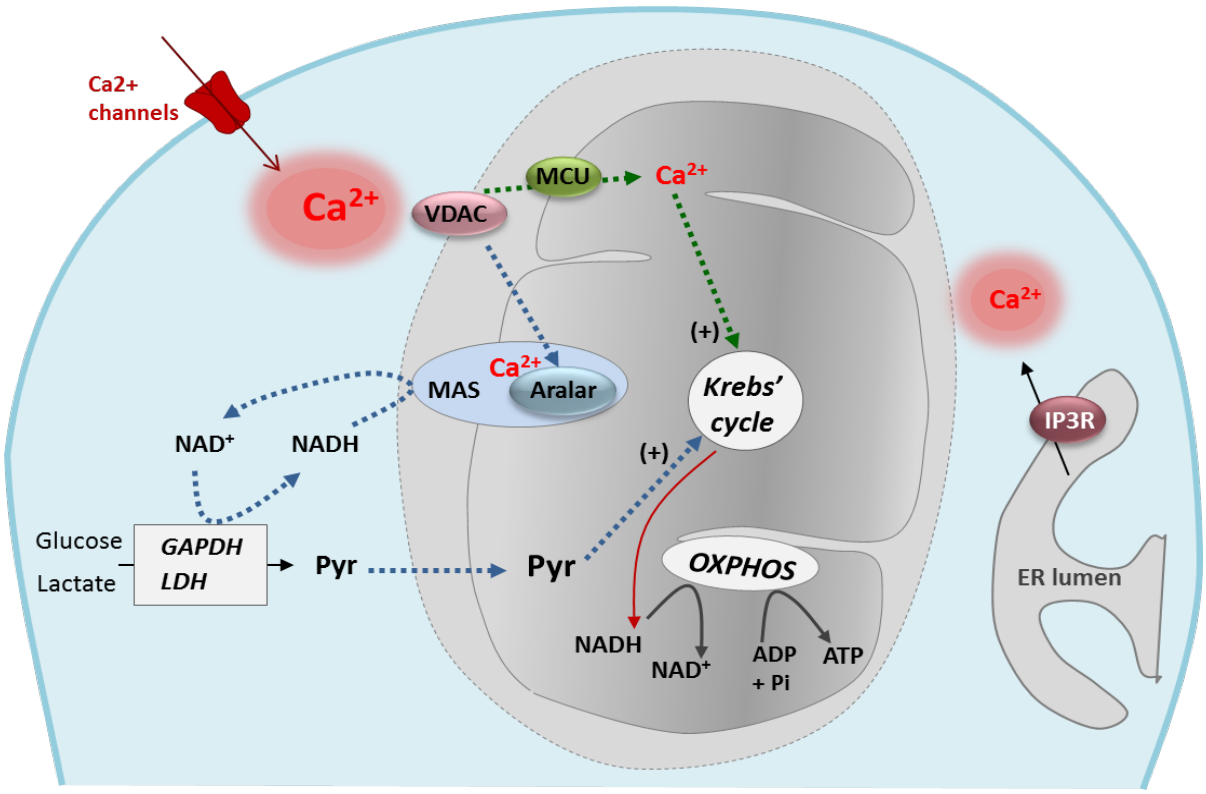

Figure 11. Ca^{2+} modulates OXPHOS by increase NADH production. Mitochondrial Ca^{2+} directly modulates Kreb's cycle dehydrogenase (green dotted line). Cytosolic Ca^{2+} regulates pyruvate supply to mitochondria (glue dotted line). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), pyruvate (Pyr) the malate–aspartate shuttle (MAS), mitochondrial Ca^{2+} uniporter (MCU), aspartate-glutamate carrier (Aralar), inositol triphosphate receptor (IP3R), voltage dependent anion channel (VDAC). Adapted from Gellerich 2013.

II.5 Mitochondrial functions during synaptic activity

The brain greatly depends on the cellular and mitochondrial metabolic pathways to fuel its activity, making it vulnerable to metabolic dysfunctions. For instance, the

mitochondria are essential components of synaptic transmission, since they are a major source of energy and participate to calcium buffering (Chen et al., 2010; MacAskill and Kittler, 2010). Long-term disruption of mitochondrial activity, including impairment of OXPHOS and especially of complex I activity, results in dramatic effects and is involved in several neurological disorders (Moran et al., 2012; Papa et al., 2012; Zsurka and Kunz, 2013). However, little is known about the impact of acute disruption of mitochondrial function in the brain. The paragraphs below will review about the importance of the mitochondrial energy metabolism for the neurotransmission and the implication for the behavior.

II.5.1 Neuronal activity regulates mitochondrial mobility

Mitochondria are mobile organelles using motor proteins and microtubules network to ensure their transport. Mitochondrial trafficking is essential for neuronal functions (Kang et al., 2008; Sun et al., 2013).In neurons, mitochondrial trafficking is a complex process divided into two phases: a bidirectional mobility phase and a stationary phase. Mitochondria move both anterogradely (from soma to cell extremities) to distribute towards active sites, and retrogradely (from cell extremities to the soma), a process involved in removing dysfunctional mitochondria by autophagy (Scherz-Shouval and Elazar, 2007). During their mobility phase, their mean velocity is approximately 0.6 µm/s (Jackson et al., 2014). In hippocampal neurons, labeled axonal mitochondria display different motion profiles and, at any given time, approximately 55% of mitochondria are stationary out of synapses site, around 15% are docking at synapses, 15% are moving passing through synapses, 15 % are pausing at synapses start moving again (Sun et al., 2013). Neuronal mitochondria are retained at sites of high activity and Ca^{2+} -buffering need, including postsynaptic and presynaptic terminals, in active growth cones and in axonal branches (Perkins et al., 2010; Ruthel and Hollenbeck, 2003). At these sites, the organelles stop moving and become stationary in order to provide local ATP synthesis and Ca^{2+} sequestration depending on the demand (MacAskill et al., 2010). Knowledge of mitochondrial mobility is technically provided by kymographs from time-lapse live-imaging movies, which provide a spatio-temporal map of

mitochondrial transport activity in the neural process of interest (Wang and Schwarz, 2009).

Due to this complex mobility pattern of axonal mitochondria, it is likely that each mitochondrion is associated to machinery complexes to ensure mitochondrial trafficking and arrest at precise locations (Cai et al., 2011). These machineries consist in motor/adaptor/receptor proteins that are coupled together and are regulated in response to changes in neuronal activity **(Figure 12;** Sheng, 2014). Mitochondria are carried along the cytoskeleton through the activity of microtubule (MT)-based motor proteins, such as proteins of the kinesin superfamily (KIFs) and dynein (Alberts, 2002; Molecular Biology of the Cell. 4th edition). Motor proteins mediate long-distance transport of mitochondria (up to 1 meter in humans) as well as other organelles or vesicles. The direction of transport exerted by these motor proteins is determined by the polarity of the MTs: one end, termed the “plus end”, points toward axon terminal and distal dendrites, while the polarity is mixed in proximal dendrite microtubules (Alberts, 2002). KIF5 proteins are the main motor effectors of plus end-directed anterograde transport of mitochondria, whereas dyneins drive mitochondrial retrogradely in axons. The motor proteins contain catalytic domains (ATPase) mediating the motor activity, and cargo-binding domains, that are associated with mitochondria trough adaptor proteins. The two main adaptor proteins for mitochondrial transport in neurons are the mitochondrial Rho-GTPase Miro, anchored to the mitochondrial outer surface, and the trafficking protein kinesin-binding (TRAK; mammalian Milton orthologue), recruiting KIF5 and associated to Miro (see reviews of Lin and Sheng, 2015; MacAskill and Kittler, 2010; Sheng, 2014).

Mitochondrial mobility and subcellular distribution are tightly regulated by neuronal activity to meet energy demands. Increase in neuronal signaling reduces mitochondrial movements (MacAskill and Kittler, 2010; Sheng, 2014). Pharmacological blockade of synaptic activity by the voltage sensitive Na+ channels blocker TTX increases mitochondrial mobility and reduces axonal mitochondrial occupancy (Sheng, 2014). Conversely, increase of synaptic activity induced by electrical field stimulation leads to decreased axonal mitochondrial mobility in hippocampal cultures (Sheng, 2014), suggesting that synaptic activity arrests mobile

mitochondrial at active sites. Neuronal activity is characterized by an increase of intracellular Ca^{2+} observed upon arrival of an action potential at the nerve terminal. In turn, increase of cytosolic Ca^{2+} down-regulates mitochondrial movement along the MT, suggesting that Ca^{2+} might mediate mitochondrial arresting in response to synaptic activity (Sheng, 2014). Further studies revealed that, when mobile mitochondria pass by active synaptic terminals, the local increase in Ca^{2+} levels disrupts the Miro–TRAK–KIF5 complex and arrests the mitochondria at the synapse (Macaskill et al., 2009; Wang and Schwarz, 2009). Furthermore, the group of Zu-Hang Sheng identified Syntaphilin (SNPH) as the protein mediating anchoring of KIF5-bound mitochondria to the MT (Chen and Sheng, 2013). Synaptic activity and elevated Ca^{2+} levels promote the interaction between SNPH and KIF5 by disrupting the Miro–TRAK–kinesin complex. In neurons, the SNPH–KIF5 interaction maintains KIF5 attachment to mitochondria, but inhibits the motor ATPase activity, indicating that SNPH prevents KIF5 from moving along MTs at pre-synaptic boutons (Chen and Sheng, 2013). Overall, these data indicate a clear down-regulation of mitochondrial mobility during synaptic activity possibly to fulfill bioenergetic and Ca^{2+} buffering requirements.

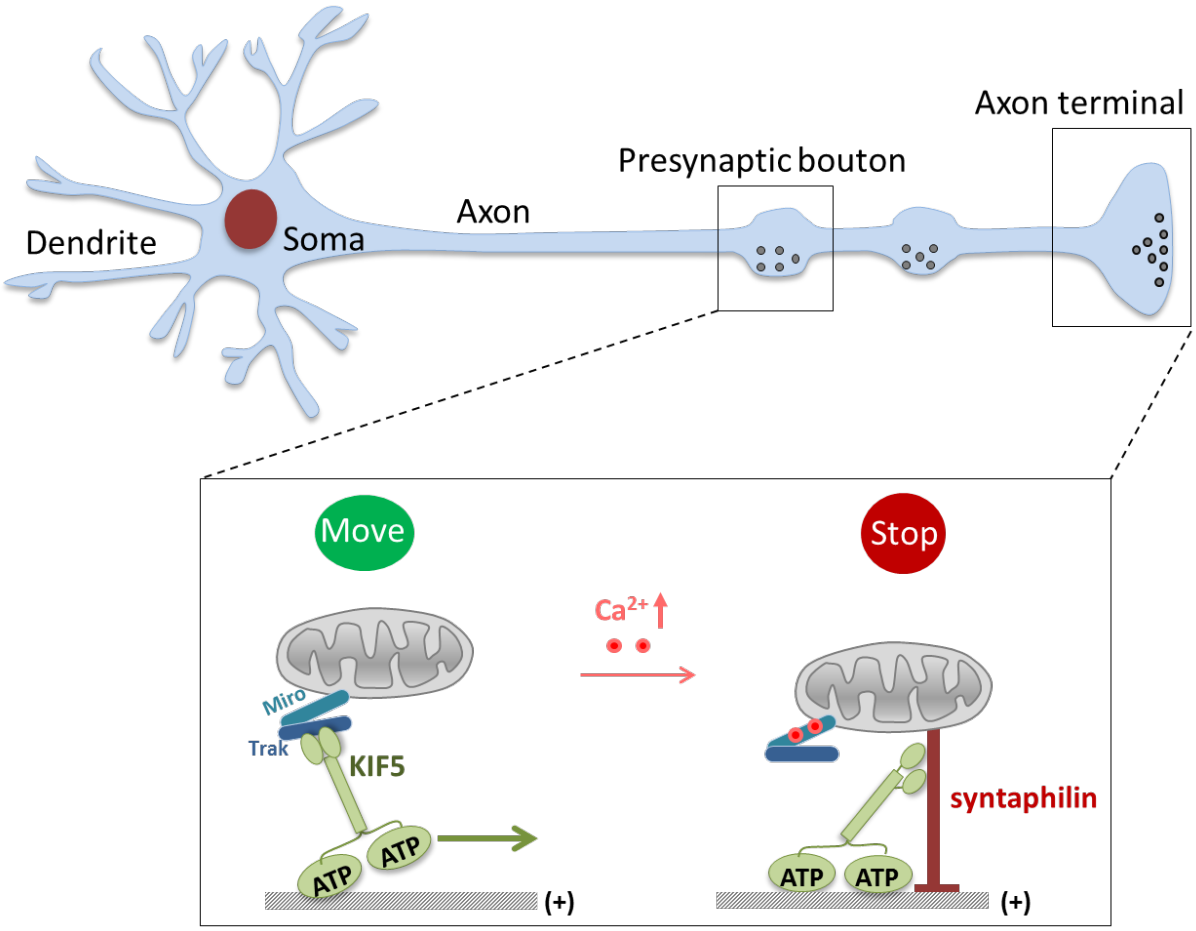

Figure 12.Activity-dependent regulation of mitochondrial transport. The Miro–Milton (or Miro–TRAK) adaptor complex mediates KIF5-driven mitochondrial transport. Ca^{2+} binding to Miro causes the release of KIF5 motors from mitochondria. Thus, Ca^{2+} influx after synaptic activity arrests motile mitochondria at activated synapses. Additionally, a Miro-Ca^{2+}–sensing pathway triggers the binding switch of KIF5 motors from the Miro–TRAK adaptor complex to anchoring protein syntaphilin, which immobilizes axonal mitochondria *via* inhibiting motor ATPase activity. Adapted from Sheng 2014.

In astrocytes, it was recently revealed that mitochondria are also distributed along the fine processes of astrocytes (Jackson et al., 2014). Their trafficking is bidirectional, depends on MT assembly and appears to be regulated by glutamate and neuronal activity (Jackson et al., 2014). However, mitochondria moved more slowly in astrocytes compared to the ones present in neurons (Jackson 2014). These results suggest that different motor proteins might govern astrocyte mitochondrial trafficking (Stephen et al., 2014). Interestingly, it has been shown that astrocytes

express kinesin motor proteins (KIF11 and KIF22; Cahoy et al., 2008); however, their involvement in regulating astrocyte mitochondrial mobility has yet to be determined.

II.5.2 The necessary role of presynaptic mitochondria for maintenance of synaptic activity

Presynaptic terminals have a high energetic demand and require a fine-tuning of Ca^{2+} buffering. Interestingly, these areas are often enriched in mitochondria (Perkins et al., 2010; **Figure 13)**.

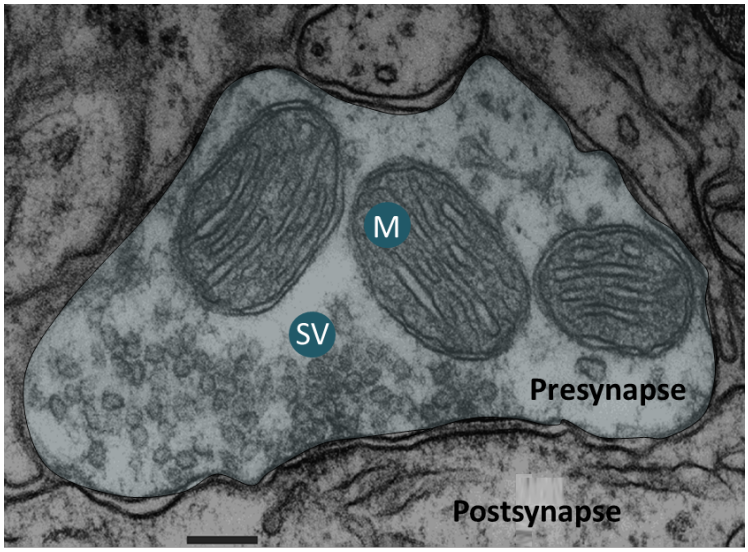

Figure 13. Mitochondrial presynaptic localization in neurons. Electron microscopy from mouse cerebellar section where mitochondria (M) (transversally sectioned) in the presynaptic axonal bouton localized nearby synaptic vesicle (SV).Scale = 250 nm (http://synapses.clm.utexas.edu/atlas/1_1_4_2.stm)

The functional relevance of presynaptic mitochondria was first reported at the neuromuscular junctions (NMJs) of *Drosophila melanogaster* by two different laboratories (Guo et al., 2005; Verstreken et al., 2005). NMJ is the type of synapse connecting spinal motor neurons (presynaptic elements) to skeletal muscle cells (postsynaptic elements). In Drp1 or Miro (mitochondrial Rho-GTPase) loss-of-function mutant flies, mitochondrial fission or axonal mitochondrial transport is inhibited, respectively. Both mutations induce lack of mitochondria at synapses accompanied by organelles accumulation in the cell body (Guo et al., 2005; Verstreken et al., 2005). Mutant Drp1 or Miro proteins lead to an impairment of neurotransmission during high stimulation frequencies but not during weak stimulations (Guo et al., 2005; Verstreken et al., 2005), suggesting that intense

synaptic activity likely rely on mitochondrial function (Guo 2005). Accordingly, supplementing synapses with exogenous ATP largely rescued this decrease of neurotransmission (Verstreken et al., 2005). Failure of the Drp1 mutants to maintain neuronal activity upon intense stimulations was associated with the inability to mobilize reserve pool (RP) vesicles and this effect is mimicked by pharmacological inhibition of mitochondrial respiration in an ATP-dependent manner (Verstreken et al., 2005). In addition to play critical roles in the maintenance of synaptic activity, mitochondria may also be required for neuroplasticity. Disruption of Syntabulin, a KIF5 motor adaptor in sympathetic neurons, is associated with a decrease of presynaptic mitochondria and causes an impairment of synaptic transmission at high-frequency firing as well as an alteration of presynaptic short-term plasticity (Ma et al., 2009). Here again, these effects were reversed by the application of exogenous ATP, indicating a key role of presynaptic mitochondria in fueling short-term neuronal plasticity (Ma et al., 2009). Altogether, these data indirectly suggested the critical role of presynaptic mitochondria in providing ATP supply to sustain synaptic activity evoked by intense stimulation and plasticity processes. However, a recent study provided direct evidence of the need of presynaptic ATP for supporting synaptic activity (Rangaraju et al., 2014). In this study, a new quantitative optical presynaptic ATP reporter, called syn-ATP, was used to precisely measure pre-synaptic ATP levels. Surprisingly, the concentration of presynaptic ATP was shown to be rather constant during electrical activity or TTX application in hippocampal neuronal cultures (Rangaraju et al., 2014). Considering that neuronal activity is a major brain processes consuming brain ATP, the authors hypothesized that fast ATP turnover might explain this lack of activity-dependent regulation of ATP levels. Indeed, blockade of glycolysis and/or OXPHOS decreased ATP levels at active synapses and dramatically impaired synaptic transmission, suggesting that rapid *de novo* ATP synthesis is required to maintain neuronal activity (Rangaraju et al., 2014). Thus, neuronal activity is directly linked to presynaptic ATP consumption derived from both glycolytic and respiratory processes.

Beyond the bioenergetics need for synaptic recycling (Vos et al., 2010), other processes require neuronal synthesis of ATP. For instance, ion pumps activity such as the Na+/K+-ATPases, which ensure the maintenance of ion gradients across the

plasma membrane, have been suggested to be mainly fueled by mitochondrial ATP (Fernandez-Moncada and Barros 2014). In addition, cytoskeleton reorganization or phosphorylation reactions and neurotransmitter metabolism are dependent of ATP supply (Attwell and Laughlin, 2001), but experimental data are needed for determining whether mitochondrial OXPHOS activity could directly impact on these processes.

One of the prominent features of brain functioning is the synchronous oscillatory activity of neuronal networks (Duzel et al., 2010; Gloveli, 2010), and mitochondria have been involved in these processes (Kann et al., 2011). For instance, fast neuronal network oscillations in the gamma range have been involved in complex behavioral task such as memory formation (Nyhus and Curran, 2010). Gamma oscillations in the *hippocampus* are associated to oxidative processes and O_2 consumption (Kann et al., 2011). Pharmacological inhibition of complex I, using rotenone, impairs O_2 consumption and provokes a rapid and complete loss in the power of gamma oscillations (Kann et al., 2011), indicating the necessity of mitochondrial respiration to sustain network synchronization. From these results, it is tempting to hypothesize that disruption of mitochondrial function could also have an impact on memory performances associated with gamma oscillations.

Altogether, the data shortly summarized above indicate that synaptic activity and network oscillations require mitochondrial activity at synapses. However, would such a modulatory role of mitochondrial function be sufficient to impact on animal behavior?

II.5.3 Mitochondrial function/dysfunction and behavior

Many studies pointed out the role of mitochondria in different psychiatric and neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), Amyotrophic Lateral Sclerosis (ALS) and Schizophrenia (Demetrius and Driver, 2013; Lezi and Swerdlow, 2012; Manji et al., 2012; Mattson et al., 2008). These disorders result from synaptic dysfunction in specific brain areas. For example, PD results from a loss of the dopamine-secreting cells in the *Substantia Nigra* (SN; Damier et al., 1999), while schizophrenia likely

results from compromised function of a population of interconnected cells in the prefrontal *cortex*, temporal *cortex* and *thalamus* (Goldman-Rakic and Selemon, 1997). Notably, these diseases are all accompanied by changes in mitochondrial-dependent functions including respiratory activity, ROS production, Ca^{2+} buffering and dynamics (Lezi and Swerdlow, 2012; Mattson et al., 2008). For instance, in patients suffering from schizophrenia, it was observed a decrease of mitochondrial respiratory function (Rozenfeld and Devi, 2011), which is likely due to impairment in complex I activity and decrease expression levels of several subunits (Mattson et al., 2008).

While it is well established that mitochondrial dysfunctions are often present in neuropathologies and psychiatric disorders, it is still unclear whether these impairments contribute to the onset and progression or whether they are consequences of brain disorders. Recent findings suggest that mitochondria may serve as key mediators of the onset and progression of some types of neurodegeneration (McInnes, 2013). For instance, a causal relationship between organelle alterations and synaptic dysfunctions was reported in a genetic AD mouse model *via* excessive mitochondrial ROS production (Lee et al., 2012). In addition, pharmacological chronic inhibition of mitochondrial respiratory function by rotenone is one of the best established PD models (Betarbet et al., 2000), reproducing most features of the disease, including degeneration of dopaminergic neurons, bradykinesia, rigidity (Betarbet et al., 2002), delayed initiation of movement (Fleming et al., 2004), reduced motor activity (Sherer et al., 2003b) and prolong latency in the catalepsy test (Sharma and Nehru, 2013). Interestingly, recent computational studies revealed the likely causal link between bioenergetic processes and motor neuron degeneration, a key feature of ALS (Le Masson et al., 2014).

Thus, mitochondrial integrity is gaining wide attention in neuropathology and could represent a therapeutic target (Le Masson et al., 2014; Moreira et al., 2010). However, which mitochondrial dependent functions predominantly participate in the onset of specific features of different neurodegenerative diseases is just starting to be addressed. A parallel action of subcellular ATP insufficiency, ROS production and mitochondrial DNA mutations has been proposed (Zsurka and Kunz, 2013). In

particular, it was shown that antioxidant therapy in preclinical stages could delay or prevent the onset of several neurodegenerative diseases (Lee et al., 2012; Moreira et al., 2010).

Beyond the implication of mitochondria in neurodegenerative and psychiatric diseases, very little is known about how acute impairment of mitochondrial activity could be responsible for changes in animal behavior. The team of Gene Robinson has revealed that increased aggression in insects is associated with whole-brain down-regulation of several OXPHOS enzymes (Alaux et al., 2009) and has later found a causal relationship between mitochondrial bioenergetics and behavior (Li-Byarlay et al., 2014). In this study, pharmacological manipulation of mitochondrial respiratory activity led to an increase in aggressive behavior in honey bees (Li-Byarlay et al., 2014). Furthermore, in Drosophila, neuronal RNAi silencing of the complex I subunit CG2014 (NADH ubiquinone oxidoreductase-like) induces an elevation of aggressive performance (Li-Byarlay et al., 2014). Thus modification on OXPHOS pathway and in particular complex I activity, appears to modulate specific behavioral states. Additional studies will have to address the potential causal link between mitochondrial OXPHOS activity and the modulation of behavior in the mammalian brain. This Thesis work represents one of the first experimental attempts in this direction.

III. THE LINK BETWEEN THE ENDOCANNABINOID SYSTEM AND MITOCHONDRIA

In the early Seventies of last century, few years after the identification of the chemical structures of Cannabis compounds (the cannabinoids, Gaoni and Mechoulam, 1964), CB_1 receptors were not discovered yet and a great interest rose to identify the biological mechanisms underlying the effects of this class of plant-derived molecules, particularly of the main psychoactive component THC. Among other effects, some authors showed a significant impact of cannabinoids on mitochondrial functions. In particular, cannabinoid compounds were shown to alter ATPase activity and morphology of rat liver mitochondria (Chari-Bitron and Bino, 1971; Bino et al., 1972), to decrease Complex I activity in rat brain and heart mitochondria (Bartova and Birmingham, 1976), and to inhibit monoamine oxidase (MAO) in brain human and porcine mitochondria (Schurr and Livne, 1975, 1976; Schurr et al., 1978). At that time, THC was thought to exert non-receptor-mediated effects due to the lipophilic nature of cannabinoids, which could explain cellular and mitochondrial effects *via* alteration of membrane properties (Bartova and Birmingham, 1976; Howlett, 2002). After the discovery of cannabinoid receptors as typical plasma membrane GPCRs, an indirect downstream mechanism of $pmCB_1$ receptor signaling on mitochondrial activity was suggested for CB_1-induced apoptosis (Campbell, 2001). The potential implication of CB_1 receptors in cannabinoid effects

on OXPHOS was refuted at that time, as these effects were similar in both cellular and isolated mitochondrial extracts (Athanasiou et al., 2007). This was due to the fact that CB_1 receptors, as all GPCRs, could not be possibly considered to be present on mitochondria at that time. For instance, concluding the abstract of their paper on cannabinoid impact on mitochondrial activity, Athanasiou and colleagues wrote, "These data demonstrate that AEA, THC, and HU 210 are all able to cause changes in integrated mitochondrial function, directly, in the absence of cannabinoid receptors" (Athanasiou et al., 2007). Interestingly, the evidence of the effects of cannabinoids occurred "in the absence of cannabinoid receptors" was exclusively based on the common knowledge assuming that GPCRs cannot possibly be present at mitochondrial membranes and, as such, this possibility was not discussed (Athanasiou et al., 2007). Therefore, until few years ago, cannabinoids were known to directly alter mitochondrial functions, but these effects were fully ascribed to unspecific membrane disturbance induced by these lipid molecules. However, recent results challenged this idea, indicating that CB_1 receptors are present also at mitochondrial membranes in the periphery, such as in spermatozoa (Aquila et al., 2010) or skeletal muscles (Pedro Grandes, personal communication), and in the brain, where they directly regulate mitochondrial OXPHOS activity (Benard et al., 2012; Hebert-Chatelain et al., 2014; Vallee et al., 2014). Therefore, mitochondrial effects of cannabinoids can be nowadays ascribed to two potential molecular mechanisms: unspecific alterations of membrane properties and specific receptor-mediated intramitochondrial signaling pathways (Fisar et al., 2014). In this Chapter, I will mention old and new studies addressing the interplay between cannabinoids and mitochondrial functions.

III.1 The interplay between cannabinoids and mitochondrial functions

In the attempt to decipher the biological effects of cannabinoids, it was proposed that THC could potentially alter monoamine release in the brain by acting on the enzyme MAO, an oxidoreductase located in the mitochondrial OM and responsible for metabolism of monoamine neurotransmitters (Shih et al., 1999). After

release into the synaptic cleft, monoamines' action is terminated by their reuptake into the presynaptic terminal, where they can be recycled into synaptic vesicle or degraded by MAO (Mukherjee and Yang, 1999). Brain monoamines such as dopamine, serotonin and norepinephrine play a key role in the regulation of brain functions in animals and humans, including motor control, mood or cognitive functions (Koob and Le Moal, 2001). Interestingly, both MAO inhibitors and CB_1 receptor agonists exert antidepressant-like effect (Fiedorowicz and Swartz, 2004; Hill and Gorzalka, 2005). In this context, early studies found that cannabinoid compounds inhibit the activity of MAO in isolated brain mitochondria (Schurr and Livne, 1975, 1976; Schurr et al., 1978). The inhibitory effect of CB_1 receptor agonists (THC, WIN and AEA) on MAO activity with serotonin as a substrate was confirmed in a crude mitochondrial fraction isolated from mammalian brain cortex (Fisar, 2010).

Besides the regulation of mitochondrial MAO, numerous studies reported also effects of endogenous and exogenous cannabinoids on respiratory functions (Bartova and Birmingham, 1976; Bino et al., 1972; Chari-Bitron and Bino, 1971). Bartova and colleagues showed that acute THC treatment of mitochondria isolated from different brain regions strongly impairs NADH-oxidase activity (Complex I) (Bartova and Birmingham, 1976). Furthermore, exogenous and endogenous CB_1 receptor agonists (THC, HU210 and AEA) inhibit mitochondrial O_2 consumption and disrupt mitochondrial membrane potential from rat heart purified mitochondria (Athanasiou et al., 2007; Silva et al., 2013; Zaccagnino et al., 2011). The impairment of mitochondrial membrane potential by THC was also found to be associated with a following decline in the bioavailability of cellular ATP in lung cells cultures, indicating a potential decrease of mitochondrial respiratory activity contributing to the cellular ATP depletion (Sarafian et al., 2003).

By releasing cytochrome c in the cytosol, mitochondria are the main actors of programmed cell death or apoptosis (Gordman 2000). In the nervous system, apoptotic mechanisms have been associated with various cellular processes such synaptic plasticity and neurodegenerative disorders (Mattson 2000, Li 2012). The specific action of CB_1 receptors on apoptotic processes was broadly examined. Prolonged THC applications cause reduction of cell viability in a dose- and time-

dependent manner in cultured hippocampal or cortical neurons, (Campbell, 2001). Exposure of cultured cortical neurons to THC (30–360 min) reproduced the morphological and biochemical features of apoptosis including nuclear DNA fragmentation, release of mitochondrial cytochrome c and activation of caspase-3 (Campbell, 2001). Importantly, the THC-induced apoptosis was blocked by the CB_1 receptor antagonist AM251 and pertussis toxin (PTX), suggesting that CB_1-dependent apoptosis *via* mitochondria involves receptor-mediated activation of the G-protein subtypes $G_{i/o}$ (Campbell, 2001).

CB_1 receptors also participate in the regulation of mitochondrial transport along processes (Boesmans et al., 2009). As described above, mitochondrial trafficking towards active sites of neurons is an essential mechanism to support ATP supply during synaptic activity (Rangaraju et al., 2014; Verstreken et al., 2005). Interestingly, the number of mitochondria transported in enteric neuronal fibers is decreased by CB_1 receptor agonists and conversely enhanced by CB_1 receptor inhibition, indicating a role of CB_1 receptors in slowing down mitochondrial trafficking (Boesmans et al., 2009).

Overall, these results emphasized the effects exerted by cannabinoids on mitochondrial functions and regulation. Some of these effects were considered to be CB_1 receptor-independent and due to membrane-perturbing properties of lipophilic cannabinoids (*e.g.* modulation of OXPHOS or of MAO activity in isolated mitochondria; (Athanasiou et al., 2007; Fisar, 2010). Conversely, other effects, such as induction of apoptosis and regulation of mitochondrial mobility, were shown to involve CB_1 receptors (Campbell, 2001). However, given the predominant idea that functional GPCRs are exclusively present at cellular plasma membranes, the involvement of CB_1 receptors in mitochondrial effects of cannabinoids has been long considered to be indirect, through cytoplasmic signaling pathways. Recent data challenged this idea.

III.2 The ECS directly regulate brain mitochondrial respiration *via* mtCB$_1$ receptors

In 2012, a study from our laboratory demonstrated the functional presence of CB_1 receptors on mitochondrial membranes, identifying mitochondrial CB_1 receptors ($mtCB_1$) as a direct modulator of bioenergetics cellular processes in the brain (Benard et al., 2012). Pharmacological activation of CB_1 receptors accompanied by rigorous controls using $CB_1^{-/-}$ mutant mice showed that $mtCB_1$ receptors directly impact on endogenous respiration of brain mitochondria.

In more details, electron immunohistochemistry showed that approximately 10 to 15% of total CB_1 receptors in the CA1 hippocampal region are located in mitochondria of wild-type mice, largely above background levels quantified in tissues from $CB_1^{-/-}$ mutants (Benard et al., 2012). $MtCB_1$ receptors are densely present in GABAergic interneurons and to a lesser extent on glutamatergic neurons, which goes in parallel with the well-known higher densities of 'GABAergic' CB_1 receptors as compared to 'glutamatergic' ones (Marsicano and Lutz 1999; Marsicano and Kuner 2008). At the subcellular level, $mtCB_1$ receptors are equally distributed to both dendrites and axon terminals, which differ from the pool localized at plasma membranes ($pmCB_1$), largely found at the presynaptic terminals (Benard et al., 2012). The direct impact of $mtCB_1$ receptors on respiratory functions of mitochondria was tested in purified brain mitochondria from wild-type and $CB_1^{-/-}$ mice**(Figure 14)**, in CB_1-transfected primary mouse fibroblast (MFs) from $CB_1^{-/-}$ mice and in transfected HEK293 (Benard et al., 2012; Hebert-Chatelain et al., 2014; Vallee et al., 2014). Exogenous application of the CB_1 receptor agonists THC, WIN and HU210 decreases endogenous mitochondrial respiration, whereas no changes were observed in control-transfected cells or brain mitochondria from $CB_1^{-/-}$ mice, indicating a direct regulation of respiration by cannabinoids trough CB_1 receptors (Benard et al., 2012; Hebert-Chatelain et al., 2014; Vallee et al., 2014).

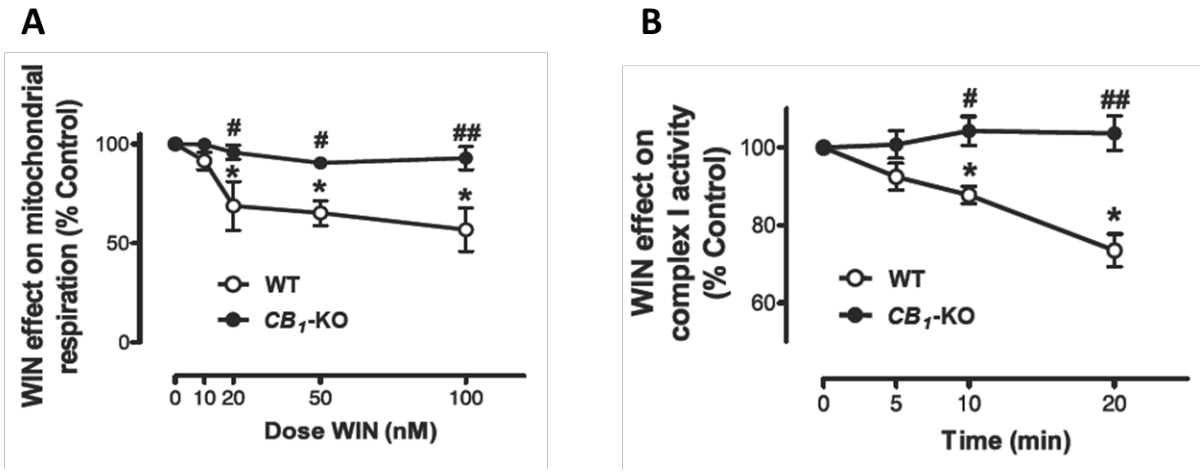

Figure 14.Direct regulation of mitochondrial activity by mtCB$_1$ receptors in the brain.(A-B) Direct dose-response effect of the CB$_1$receptor agonist WIN55,212-2 (WIN) on respiration and Complex I activity of purified mitochondria from WT and *CB$_1$*-KO brains. From Benard et al., 2012.

In purified brain mitochondrial preparation, the cannabinoid-induced decrease of mitochondrial respiration is associated with a decrease of complex I activity as well as a reduction of mitochondrial cAMP levels and PKA activity (Benard et al., 2012). To discriminate the respective influence of pmCB$_1$ *versus* intracellular CB$_1$ receptors on these effects, indirect pharmacological tools were developed. A biotinylated version of the lipophilic CB$_1$ receptor agonist HU210 (HU210-biotin, hereafter HU-biot), in which the presence of the hydrophilic biotin extension prevents the cell penetration, was synthesized and used *in vitro*. Interestingly, HU-biot treatment does not alter O_2 consumption in intact CB$_1$-expressing cells, whereas the effect appears when CB$_1$-MFs are permeabilized (Benard et al., 2012), indicating that reduction of the respiratory activity in living cells is limited to the action of intracellular CB$_1$ receptors and most likely trough mtCB$_1$ receptors. Given the major coupling of CB$_1$ with protein $G_{i/o}$ resulting in the decrease of cAMP and previous evidence reporting the presence of intra-mitochondrial cAMP/PKA signaling regulation on OXPHOS, it is probable that mtCB$_1$ receptors could modulate respiration through inhibition of the cAMP/PKA cascade (Acin-Perez et al., 2009a; De Rasmo et al., 2010; Howlett, 2005; Piomelli, 2003).

The detailed characterization of the precise molecular mechanisms by which $mtCB_1$ receptors induce decrease of O_2 consumption is one of the subjects of the present thesis **(see below)**.

CB_1 receptor signaling is at the cross of the pharmacological effects of cannabinoids and the physiological roles of the ECS. Thus, an important question to address was whether the ECS physiologically modulates mitochondrial activity *via* $mtCB_1$ receptors. FAAH, the primary degradative enzyme for AEA, is densely present in the mitochondria (Benard et al., 2012) and Benard et al, (2012) found that purified mitochondria contain AEA- and 2-AG–degrading activity that is ascribed to FAAH and MAGL, representing approximately 18% and 12% from the total cellular enzyme activity, respectively (Benard et al., 2012). Pharmacological inhibition of MAGL in purified mitochondria increases 2-AG levels and decreases respiration. Interestingly, a strong inverse correlation was found between the levels of endogenous 2AG in mitochondria and O_2 consumption (Benard et al., 2012), suggesting that endogenous $mtCB_1$ receptor signaling within mitochondria control the respiratory activity of the organelles.

The idea that $mtCB_1$ receptors might participate in eCB-dependent modulation of synaptic plasticity was further tested. As mentioned above, CB_1 receptors mediate DSI, a form of short-term plasticity of inhibitory neurotransmission **(see Chapter 1)**. To remind briefly, the depolarization of a postsynaptic cell leads to eCBs mobilization, which retrogradely activate presynaptic CB_1 receptors and transiently decrease GABAergic inhibitory neurotransmission (Alger, 2002; Kano et al., 2009). DSI levels are proportional to the intensity and/or duration of the depolarization step, with stronger or longer depolarization inducing higher levels of presynaptic inhibition (Alger, 2002; Kano et al., 2009). DSI is blocked by CB_1 receptor antagonists, but it is also prevented (occluded) by CB_1 receptor agonists, which occupy CB_1 receptors and impede further actions of endogenously mobilized eCBS (Alger, 2002; Kano et al., 2009). In hippocampal slices, HU210 completely occluded “strong DSI” induced by 5-sec depolarization steps, whereas HU-biot at saturating doses had only a partial effect (approximately 50% occlusion). Similarly, the cell-impermeant CB_1 receptor antagonist, the peptide Hemopressin (Heimann et al., 2007) partially reduced “strong

DSI", whereas the membrane-permeant CB_1 receptor antagonist AM251 abolished it. Finally, rotenone, a mitochondrial complex I inhibitor, was ineffective on "strong DSI", but potentiated "weak DSI", suggesting that mitochondrial processes participate in the expression of "strong DSI". Interestingly, the participation of intracellular CB_1 receptors was limited to DSI: HU-biot and HU 201 equally decreased hippocampal basal inhibitory transmission, suggesting that intracellular CB_1 receptors do not participate in pharmacological effects of cannabinoids on inhibitory synaptic transmission. Thus, CB_1 receptors are present on brain mitochondria, where they regulate cellular bioenergetics processes and possibly participate in eCB-dependent synaptic plasticity.

However, further studies and more powerful tools than indirect pharmacology are needed to investigate the impact of $mtCB_1$ on synaptic transmission, brain functions and behavior. This Thesis work, based on the solid anatomical and functional data described above, aimed at providing first answers to these questions by generating and using novel tools to dissect the functions of $mtCB_1$ receptors in the brain.

AIMS OF THE THESIS

As detailed in the introduction, the brain is one of the most energy-demanding organs in our body. Brain functions critically depend on an adequate energy supply, in the form of oxygen and nutrients provided by the blood. This energy is largely used to mobilize synaptic vesicles, maintain ion fluxes underlying neuronal excitability, to induce action potentials, to provide morphological rearrangements of brain cells, and to regulate synaptic activity (Attwell and Laughlin, 2001). The correct functioning of mitochondria is fundamental for the generation of most cellular ATP and its impairment may have dramatic consequences (Cai et al., 2011). Accordingly, mitochondrial dysfunction has been implicated in many neurological and psychiatric diseases (Mattson et al., 2008). It is well established that brain energy metabolism, including mitochondrial respiration, supports brain activity (Attwell and Laughlin, 2001; MacAskill and Kittler, 2010; Rangaraju et al., 2014). However, little is known about the physiological roles of mitochondrial activity in the acute regulation of behavior.

Progress in understanding the mechanisms of action of cannabinoids was made after discovery of the ECS that consists in cannabinoid receptors, their endogenous ligands endocannabinoids and the enzymatic machineries for the synthesis, degradation and transport of endocannabinoids (Di Marzo, 2006). Endogenous and pharmacological activation of CB_1 receptors at synaptic terminals results in the regulation of neurotransmitter release and synaptic plasticity, which in turn affects physiological and behavioral processes (Kano et al., 2009). We recently discovered the presence of CB_1 receptors on brain mitochondrial membranes ($mtCB_1$) and its direct control of cellular respiration and energy production suggesting a potential role of mitochondria in regulating animal behavior (Bénard et al., 2012; Hebert-Chatelain et al., 2014). Thus, the contribution of brain $mtCB_1$ receptor activation in behavioral processes deserves further investigation.

The general aim of this thesis work is to unravel the role(s) played by brain $mtCB_1$ receptors in behavioral effects of cannabinoids. In order to do that, we used new experimental approaches, combining pharmacological and genetic tools (objective 1), and to investigate the role of $mtCB_1$ receptor

signaling in motor and memory effects of cannabinoid agonists (objective 2).

Objective 1- Development of pharmacological and genetic tools to study $mtCB_1$ receptors in the brain.

To study the involvement of $mtCB_1$ receptors in cell, network and behavioral functions, the first challenge resides in the functional discrimination between effects mediated by $mtCB_1$ and others dependent on CB_1 receptors at other cellular locations. This challenge implies the development, the characterization and the validation of tools aimed at stimulating or to inhibiting these two receptor pools, independently.

First, we focused on the identification of the molecular mechanisms by which $mtCB_1$ receptors modulate mitochondrial function. By exploiting the specific signaling cascades of $mtCB_1$ receptors, (characterized in collaboration with Etienne Hebert-Chatelain), we used pharmacological compounds to block $mtCB_1$ receptor-dependent signaling *in vivo*.

Second, we adopted a genetic approach consisting in the generation and functional characterization of a mutant CB_1 protein (DN22-CB_1) that does not localize at mitochondrial membranes (developed in collaboration with Luigi Bellocchio and Anna Delamarre).

Objective 2- Role of $mtCB_1$ receptor signaling on motor and memory performance.

Motor alterations and deficits in memory performance are amongst the best characterized behavioral effects of cannabinoids (Carter and Weydt, 2002; Solowij and Pesa, 2010). Moreover, mitochondrial impairment and oxidative stress has been associated with pathologies where these functions are altered (Demetrius and Driver, 2013; Lezi and Swerdlow, 2012; Manji et al., 2012; Mattson et al., 2008).Thus, I assessed the potential dependency of memory and motor alterations induced by cannabinoids on $mtCB_1$ receptor activation.

Objective 2.1:

To evaluate the role of $mtCB_1$ receptor signaling in the modulation of memory consolidation: Long-term memory is a process that has high metabolic

demands within the underlying active neuronal network (Suzuki et al., 2011). Post-training injection of cannabinoids impair long-term object recognition memory (ORM), a subcategory of declarative memory, that is mediated by hippocampal CB_1 receptors (Clarke et al., 2008; Puighermanal et al., 2009). The goal of this first study was to assess the contribution of $mtCB_1$ receptor signaling to ORM consolidation. *In vitro* electrophysiological recordings in mouse *hippocampus* allowed us to describe the implication of $mtCB_1$ receptors on hippocampal basal excitatory neurotransmission (in collaboration with Federico Massa and Geoffrey Terral). Once established this role, I addressed the following questions:

→ Does pharmacological inhibition of intra-hippocampal $mtCB_1$-dependent signaling prevent cannabinoid effects of ORM consolidation?

→ Is viral re-expression of CB_1 or of DN22-CB_1 receptors in the *hippocampus* of $CB_1^{-/-}$ mice sufficient to rescue the cannabinoid-dependent alteration of ORM consolidation?

Objective 2.2:

To determine the role of $mtCB_1$ receptor signaling in the modulation of motor control: It is well established that high doses of cannabinoids induce decreased locomotor activity and catalepsy, *via* activation of CB_1 receptors (Zimmer 1999, Monory 2007). The *Substantia Nigra* (SNr) is a structure integrated in the *basal ganglia* circuit, controlling motor functions and expressing considerably high level of CB_1 receptors (Herkerham 1991). Thus, I addressed the following questions:

→ Is the SNr a brain site where cannabinoids alter locomotor performance in mice?

→ Are intracellular CB_1 receptors in the SNr necessary for locomotor and cataleptic effects of cannabinoids?

→ Do mitochondrial effects of cannabinoids *via* $mtCB_1$ receptors in the SNr contribute to cannabinoid-induced regulation of motor control?

The work described below is under consideration for publication in these two articles **(see annex for manuscripts)**:

1- Etienne Hebert-Chatelain[*], Tifany Desprez[*], Edgar Soria-Gomez, Luigi Bellocchio, Anna Delamarre, Geoffrey Terral, Peggy Vincent, Arnau Busquets-Garcia, Laurie M. Robin, Michelangelo Colavita, Nagore Puente, Leire Reguero, Uzaskun Elezgarai, Gabriel Barreda-Gómez, Maria-Luz Lopez-Rodriguez, Federico Massa, Pedro Grandes, Giovanni Bénard[*], Giovanni Marsicano[*]. **Mitochondrial CB_1 receptors are required for amnesic effects of cannabinoids.** Submitted.

2- Tifany Desprez, Edgar Soria-Gomez[*], Etienne Hebert-Chatelain[*], Julia Goncalves, Lea Moreau, Luigi Bellocchio, Nagore Puente, Pedro Grandes, Giovanni Benard, Giovanni Marsicano. **The subcellular localization of CB_1 receptors shapes its effect on motor control.** In preparation

[*]Share authorship.

MATERIALS AND METHODS

Mice

All experimental procedures were approved by the Committee on Animal Health and Care of INSERM and the French Ministry of Agriculture and Forestry (authorization number 3306369). Mice were maintained under standard conditions (food and water *ad libitum*; 12h/12h light/dark cycle, light on 7 a.m.; experiments were performed between 9 a.m. and 5 p.m.). Wild-type ($CB_1^{+/+}$) and KO ($CB_1^{-/-}$) female and male mice (2–4 months old) were obtained, bred and genotyped as described (Marsicano et al., 2002). Only male mice were used for behavioral experiments. For most experiments $CB_1^{+/+}$ and $CB_1^{-/-}$were littermates. For primary cell cultures, pups were obtained from homozygote pairs. C57BL/6-N mice were purchased from Janvier (France).

Drugs

THC was obtained from THC Pharm GmbH (Frankfurt, Germany). HU210 and HU210-Biotin were synthesized as described (Martin-Couce et al., 2012). WIN55-212-2, KH7, PTX, Bicarbonate (HCO_3^-), Forskolin, FCCP, Oligomycin, Picrotoxin and the rest of chemicals used in this study were purchased from Sigma Aldrich (St-Louis, IL, USA) were obtained from Sigma Aldrich (France).

For behavioral experiments, THC (10mg/kg) was dissolved in 5% ethanol, 4% cremophor and saline. WIN55,212-2, (10mg/kg) was dissolved in a mixture of saline (0.9% NaCl) with 2% DMSO and 2% cremophor. KH7 (2 µg/0.5 µl/injection) was dissolved in 10% cremophor, 2.5% DMSO and saline. Hemopressin (5 µg/0.5 µl/injection) was dissolved in saline. AM251 (4 µg/0.5µl/injection) was dissolved in 10% dimethyl sulfoxide (DMSO) and 10% cremophor. Rotenone (1 µg/2 µl/injection) was dissolved in a mixture of saline (0.9% NaCl) with 2% DMSO and 2% cremophor. All vehicles solutions contained the same amounts of solvents. All drugs were prepared freshly before the experiments.

For biochemical experiments, PTX (1 µg/ml), HCO_3^- (5 mM) and Forskolin (10 µM) were dissolved in water. KH7 (5 µM) and WIN (100 nM and 1 µM) were dissolved in DMSO. THC (800 nM and 1 µM), Oligomycin (2 µg/ml) and FCCP (1 µM)

were dissolved in ethanol. Corresponding vehicle solutions were used in control experiments.

Cell culture and transfection

Mouse 3T3 cells (3T3 F442A) and HEK293 cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose, 2 mM glutamine, 1 mM pyruvate. HEK293 cells were transfected with control plasmid, CB_1 or DN22-CB_1 cDNA coupled with mCherry cloned in pcDNA 3.1(+), respectively. 3T3 cells were transfected with sAC-HA kindly offered by Dr. G. Manfredi from Cornell University, [see (Acin-Perez et al., 2009b)]. The transfections were done using FugeneHD (Roche, France) according to the manufacturer's protocol.

Primary hippocampal cultures were prepared from $CB_1^{+/+}$ and $CB_1^{-/-}$P0-P1 mice. Briefly, after sacrifice by decapitation, hippocampi were extracted in dissection medium (10 mM Hepes, 0.3% glucose in Hank's salt balanced solution, pH 7.4) and dissociated in 0.25% trypsin for 30 min. Where indicated, dissociated cells were transfected with sAC-HA using Amaxa P3 primary cell 4D-nucleofector kit (Lonza, France), according to the manufacturer's protocol. Cells were plated on poly-L-lysine-coated 96-wells dishes using neurobasal/B27 medium (supplemented with 5% FBS, 2mM glutamine, 1 mM pyruvate, 1mM sodium lactate, 0.3% glucose and 37.5 mM NaCl) at a density of 50,000 cells/well. One hour after plating, the serum was removed. Primary hippocampal cultures contained both neurons and astrocytes, and were used at 3 DIV.

Mouse fibroblasts (MFs) were generated from P0-P1 $CB_1^{-/-}$ pups. After sacrifice by decapitation, the dorsal skin was excised and minced in 1x phosphate buffer (PBS). Cells were then separated by incubation in 0.25% trypsin solution in PBS, collected by centrifugation and resuspended in DMEM with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin solution (Invitrogen, France). Cells were seeded in 25 cm^2 flasks and then expanded in 75 cm^2 flasks until reaching 90% confluence. Transfections were carried out by using a BTX electroporator ECM 830 (Harvard Apparatus, France) (175 V, 1-ms pulse, five pulses, 0.5-s interval between pulses). Cells were electroporated in Optimem medium (Invitrogen, France) at

$2x10^7$cells/ml (fibroblasts from two 75 cm^2 flasks at 90% confluence in 300 µl) in a 2-mm-gap cuvette using 30 µg of either control plasmid, CB_1 or DN22-CB_1 cDNA coupled with mCherry. After electroporation, cells were resuspended in DMEM with 10% fetal bovine serum, 1% l-glutamine and 2% penicillin/streptomycin solution (Invitrogen, France) and seeded in three 100 cm^2 petri dishes. All cells were maintained at 37°C and 5% CO_2 and harvested 48h after transfection for respiration experiments.

Isolation of mitochondria and cell fractionation

The brains of $CB_1^{+/+}$ and $CB_1^{-/-}$ littermates were dissected and mitochondria were purified using Ficoll gradient as previously described (Benard et al., 2012; Hebert-Chatelain et al., 2014). Briefly, mice brains were extracted in ice-cold isolation buffer (sucrose 250 mM, Tris 10 mM, EDTA 1 mM, pH 7.6) containing protease inhibitors (Roche, France) and 2 mM NaF and homogenized with a Teflon potter. Homogenates were centrifuged at 1,500 *g* for 5 min (4°C). The supernatant was then centrifuged at 12,500 *g* (4°C). The pellet was collected and the cycle of centrifugation was repeated. To purify mitochondria, the final pellet was resuspended in 400 µl of isolation buffer, layered on top of a discontinuous Ficoll gradient (10% and 7% fractions) and centrifuged at 100,000 *g* for 1 h (4°C). All experiments using freshly isolated brain mitochondria were performed within 3 h following purification.

The 3T3 cells were harvested, resuspended in isolation buffer and disrupted with 25 strokes using a 25G needle. The total cell lysate was centrifuged at 500 *g* (4°C) to remove cells debris and nuclei. The supernatant was kept and centrifuged at 12,500 *g* for 10 min (4°C). Then, the supernatant was kept (cytosolic fraction) and the pellet was resuspended and the centrifugation cycle was repeated. Finally, the mitochondrial fractions were obtained from the last pellet.

Oxygen consumption measurements

The oxygen consumption of isolated mitochondria and cell lines was monitored at 37 °C in a glass chamber equipped with a Clark oxygen electrode (Hansatech, U.K.). Purified mitochondria (75-100 µg) were suspended in 500 µl of respiration buffer (75 mM mannitol, 25 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, pH 7.4, 50 mM EDTA) in the chamber. Respiratory substrates were added directly to the

chamber. Pyruvate (5 mM), malate (2 mM) and ADP (5 mM) were successively added to follow mitochondrial respiration. The experiments using 3T3 cells were performed on 2 × 10^6 cells ml^{-1} in growth medium. Intact cells were transferred directly into the chamber and basal respiration was recorded. Drugs were added directly into the chambers. Mitochondria were incubated with PTX, KH7 and H89 during 5 min before addition of CB_1 receptor agonists. HCO_3^- and 8Br-cAMP were added 5 min after the addition of CB_1 receptor agonists.

Oxygen consumption of primary hippocampal cultures was monitored using XF96 Seahorse Bioscience analyzer (Seahorse Bioscience, Denmark), according to the manufacturer's protocol. When indicated, oligomycin (2 µg/ml) and Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 µM) were injected directly into the wells. Other drugs were directly added into the medium 1h before measurements.

ATP content measurements

The intracellular ATP content was measured by using the bioluminescent ATP kit HS II (Roche, France). $CB_1^{+/+}$ and $CB_1^{-/-}$primary hippocampal cultures (50,000 cells/well in a 96 wells dish) were treated with THC (1 µM), WIN (1 µM) or vehicle (EtOH 1:30.000 for THC and DMSO 1:500 for WIN, respectively) in the presence or absence of rotenone (0.1 µM) during one hour. Then, ATP measurements were done as described (Jose et al., 2011). Briefly, cells were lysed to release the intracellular ATP by using the lysis buffer provided with the kit (equal volume) for 20 min. The lysate was then analyzed in a 96-well plate luminometer (Luminoskan, Thermo Scientific, France) using the luciferin/luciferase reaction system provided with the kit. 100 µl of luciferin/luciferase was injected in the wells and after 10 s of incubation; bioluminescence was read (1 s integration time). Standardizations were performed with known quantities of standard ATP provided with the kit. The ATP content derived from mitochondria was determined by subtracting $ATP_{rotenone}$ values from ATP_{total} ($ATP_{mito} = ATP_{total} – ATP_{rotenone}$).

Trypsin sensitivity assays

100 µg of mitochondria were suspended in isolation buffer, untreated or incubated with 0.01% trypsin in the presence or absence of 0.05% Triton X-100

during 15 min at 37°C. Proteins were then processed for Western immunoblot analyses.

Immunoprecipitation assays

Freshly purified brain mitochondria were resuspended in PBS (5 mg/ml) supplemented with protease inhibitors cocktail (Roche, France) and 2 mM NaF, and solubilized with 1% lauryl maltoside for 30 min (4°C). For co-immunoprecipitation of sAC and Gα, mitochondria were incubated with THC (800 nM) or vehicle for 5 min at 37°C. Proteins were incubated with a C-terminal anti-CB_1antibody (Cayman, USA) or sAC R21 antibody (CEP Biotech, USA) overnight (4°C). Protein A/G PLUS-Agarose beads (Santa Cruz, USA) were then added and the incubation continued for 4h (4°C). The elution was performed using glycine buffer (0.2M glycine, 0.05% lauryl maltoside, pH 2.5) and samples were processed for Western immunoblotting.

ERK phosphorylation assays

Following transfection (control, CB_1 or DN22-CB_1, respectively), cells were allowed to recover in serum containing medium for 24 h. Cells were then starved overnight in serum-free DMEM before treatment and lysis. The cells then were treated at 37°C with HU210 (100 nM) or vehicle for 10 min. The media were rapidly aspirated and the samples were snap-frozen in liquid nitrogen and stored at −80°C before preparation for western blotting.

Protein Extraction and Western immunoblotting

Lysis buffer (1 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 50 mM Tris pH 7.5 , 1 % Triton X-100, protease inhibitors, 30 mM 2-mercaptoethanol) was added and the cells were then collected by scraping and pelleted by centrifugation at 12,500 *g* (4°C) for 5 min to remove cell debris. Protein concentrations were measured using the Pierce BCA protein assay kit (Thermo Scientific), loaded with Laemmli buffer and kept at −80 °C.

For Western Immunoblotting, the proteins were separated on Tris-glycine 7, 10 or 12% acrylamide gels and transferred to PVDF membranes. Membranes were soaked in 5% milk (or 5% BSA for phosphorylation immunoblots) in TBS-Tween20

(0.05%). Mitochondrial proteins were immunodetected using antibodies against complex III core 2 (Abcam, 1:1,000,

1 h, room temperature), SDHA (Abcam, 1:10,000, 1 h, room temperature) and TOM20 (Santa Cruz, 1:1,000, 1 h, 4 °C). Cytosolic proteins were probed with LDHa (Santa Cruz, 1:500, overnight, 4 °C). Samples were also probed with antibodies against Gα proteins (Enzo Life Science, 1:1,000, 1 h, room temperature), sAC (sAC R21, CEP Biotech, 1:500, overnight, 4°C), PKA (cAMP protein kinase catalytic subunit, Abcam, 1:1,000, 1 h, room temperature), an antiserum directed against the C terminus of CB_1receptor (Cayman, 1:200, overnight, 4°C), HA (Abcam, 1:500, overnight, 4°C), P-ERK (Phospho-p44/42 MAPK) corresponding to residues around Thr202/Tyr204 (Cell signaling, 1:1000, overnight, 4°C) and ERK (p44/p42 MAPK; Cell signaling, 1:2000, 1 h, room temperature).

Then, membranes were washed and incubated with appropriate secondary HRP-coupled antibodies (1 h, room temperature). Finally, HRP signal was revealed using the ECL plus reagent (Amersham) and detected by the Bio-Rad Quantity One system. Labelings were quantified by densitometric analysis using Image J (NIH) software.

PKA activity and cAMP content assays

Cyclic AMP levels and PKA activity were assayed on brain-isolated mitochondria using the Direct Correlate-EIA cAMP kit (Assay Designs Inc., USA) and an ELISA kit (Enzo Life Science), respectively, according to the manufacturers' instructions. The different treatments described in the main text were performed at 37 °C for 1 h.

Cloning of DN22-CB_1 receptors

The deletion of the first 22 aminoacids (66 base pairs) of the N-terminal in the mouse CB_1 receptor coding sequence was generated by polymerase chain reaction (PCR). Briefly a forward primer hybridizing from the 67^{th} base starting from ATG was coupled to a reverse primer hybridizing to the end of the coding sequence, including TGA stop codon. In order to guarantee proper translation of the construct the forward primer included an ATG codon upstream the hybridizing sequence. The cDNA for

ΔN22-CB_1 was amplified using HF Platinum DNA polymerase (Invitrogen) and inserted into PCRII-Topo vector (Invitrogen) according to manufacturer instruction. The absence of amplification mismatches was then verified by DNA sequencing. Then the cDNA sequence for ΔN22-CB_1 was inserted into pcDNA3.1 mammalian expression vector using BamHI-EcoRV according to standard cloning procedures. Primers used were: forward 5'-**ATG**gtgggctcaaatgacattcag-3' (in bold the inserted ATG), reverse 5'-**TCA**cagagcctcggcagacgtg-3' (in bold the stop codon).

Preparation of adeno-associated viruses (AAV)

The cDNAs coding for mouse CB_1, ΔN22-CB_1 and hrGFP were subcloned into pAM-*CB*A vector using standard molecular cloning techniques (Soria-Gomez et al., 2014). The resulting vectors were transfected by Calcium Phosphate precipitation in HEK 293 cells together with rAAV helper plasmid pFd6 and AAV1/2 serotype packaging plasmids pRV1 and pH21 (McClure et al., 2011). The viruses were then purified and tittered as previously described (Benard et al., 2012; Chiarlone et al., 2014; Soria-Gomez et al., 2014). Virus titers expressed as genomic copies/ml were $2.9*10^{10}$ for rAAV-GFP, $3.5*10^{10}$ for rAAV-CB_1 and $8.3*10^{10}$ for rAAV-ΔN22-CB_1.

[35S]GTPγS binding studies

[35S]GTPγS binding studies were carried out according to the patented methodology for the screening of molecules that act through G protein-coupled receptors using Cell Membrane Microarrays (Rodriguez and Gutierrez-de-Teran, 2013). Briefly, Cell Membrane Microarrays were dried 20 min at room temperature (RT), then they were incubated in assay buffer (50 mM Tris-Cl; 1 mM EGTA; 3 mM MgCl2; 100 mM NaCl; 0,5% BSA; pH 7,4) for 15 min at RT. Microarrays were transferred into assay buffer containing 50 µM GDP and 0.1 nM [35S]GTPγS, with the cannabinoid agonist, WIN55.212-2, at increasing concentrations (0, 10 nM, 100 nM, 1 µM and 10 µM), and incubated at 30ºC for 30 min. Non-specific binding was determined with GTPγS (10 µM). Finally, microarrays, together with ARC [14C]-standards, were exposed to films, developed, scanned and quantified. Protein concentration in each spot was measured by the Bradford method and used to normalize the [35S]GTPγS binding results to nCi/ng protein. Data from the dose–response curves (5 replicates in triplicate) were analyzed by using the program

PRISM (Graph Pad Software Inc., San Diego, CA) to yield EC50 (Effective concentration 50%) and Emax (maximal effect) for WIN55,212-2.

Surgery and drug/virus administration

C57BL/6-N, $CB_1^{+/+}$ and $CB_1^{-/-}$mice (7-9 weeks of age) were anesthetized by intraperitoneal injection of a mixture of ketamine (100mg/kg, Imalgene 500®, Merial) and Xylazine (10mg/kg, Rompun, Bayer) and placed into a stereotaxic apparatus (David Kopf Instruments) with mouse adapter and lateral ear bars. Mice were allowed to recover for at least one week in individual cages before the beginning of the experiments. Mice were weighed daily and individuals that failed to regain the pre-surgery body weight were excluded from the following experiments.

For intra-HPC injections of drugs, C57BL/6-N mice were bilaterally implanted with 1.0 mm stainless steel guide cannulae targeting the *hippocampus* (HPC) with the following coordinates: anterior/posterior (AP) −3.1; medial/lateral(ML) ±1.3; dorsal/ventral (DV) -0.5. Guide cannulae were secured with cement anchored to the skull by screws. The drug injections intra-HPC was performed by using injectors protruding 1 mm from the tip of the cannula.

For viral intra-HPC AAV delivery, $CB_1^{+/+}$ and $CB_1^{-/-}$mice were submitted to stereotaxic surgery (as above) and AAV vectors were injected with the help of a microsyringe (10 µl Hamilton syringe with a 30-gauge beveled needle) attached to a pump (UMP3-1, World Precision Instruments). Mice were injected directly into the HPC (0.5 µl per injection site at a rate of 0.5 µl per min), with the following coordinates: dorsal HPC, AP -1.8; ML ±1; DV -2.0 and -1.5 ; ventral HPC: AP -3.5; ML ±2.7; DV -4 and -3. Following virus delivery, the syringe was left in place for 1 minute before being slowly withdrawn from the brain. $CB_1^{+/+}$ mice were injected with AAV-GFP to generate $CB_1^{+/+}$(GFP) mice; $CB_1^{-/-}$ mice were injected with AAV-GFP, AAV-CB_1 or AAV-DN22-CB_1, to obtain $CB_1^{-/-}$(GFP), $CB_1^{-/-}$(CB_1) and $CB_1^{-/-}$(DN22-CB_1) mice, respectively. Animals were used for experiments 4-5 weeks after injections. . CB_1 receptor expression was verified by immunofluorescence or electron microscopy (see below).

For intra-SNr injections of drugs, C57BL/6-N mice were bilaterally implanted with 3.5 mm stainless steel guide cannulae (Plastics One, USA) targeting the SNr with the

following coordinates: AP -3.1, L ± 1.3, DV –3.5, according to Paxinos and Franklin (Paxinos, 2004) . Guide cannulae were secured with cement anchored to the skull by screws. The drug injections intra-HPC and intra-SNr were performed by using injectors protruding 1 mm from the tip of the cannula.

Fluorescent immunohistochemistry

Mice were anesthetized with chloral hydrate (400 mg/kg body weight), transcardially perfused with Ringer solution [NaCl (135 mM), KCl (5.4 mM), $MgCl_2$ $6H_2O$ (1 mM), $CaCl_2$ $2H_20$ (1.8 mM), HEPES (5 mM)]. Heparin choay (25000 UI/5 mL) was added extemporarily and tissues were then fixed with 500 ml of 4% formaldehyde dissolved in PO4 buffer (0.1M, pH 7.4) and prepared at 4°C. After perfusion, the brains were removed and incubated several additional hours in the same fixative. Serial vibrosections were cut at 50 µm and collected in PO4 buffer at room temperature (RT). Sections were pre-incubated in a blocking solution of 10% donkey serum, 0.1% sodium azide and 0.3% Triton X-100 prepared in PO4 buffer for 30 minutes at RT. Free-floating sections were incubated for 48h (4^oC) with goat anti-CB_1 polyclonal antibodies raised against a 31 aminoacid C-terminal sequence (NM007726) of the mouse CB_1 receptor (CB_1-Go-Af450-1; 2 µg/ml; Frontier Science Co. Ltd). The antibody was prepared in 10% donkey serum/PB containing 0.1% sodium azide and 0.5% Triton X-100. Then, the sections were washed in PO4 buffer for 30 minutes at RT. After, the tissue was incubated with fluorescent anti-goat Alexa 488 (1:200, Jackson ImmunoResearch) for 4 hours and then washed in PO4 at RT, before being incubated with DAPI (1:20000) during 10 min for nuclear counterstaining. Finally, sections were washed, mounted, dried, and coverslipped with DPX (Fluka Chemie AG). The slides were analyzed with an epifluorescence Leica DM6000 microscope (Leica).

Immuno-electron microscopy

For the characterization of DN22-CB_1 protein, $CB_1^{+/+}$(GFP), $CB_1^{-/-}$(GFP), $CB_1^{-/-}$ (CB_1) and $CB_1^{-/-}$(DN22-CB_1) mice (n = 3 each) were processed for electron microscope pre-embedding immunogold labeling as recently described (Benard et al., 2012; Hebert-Chatelain et al., 2014). Immunodetection was performed in 50-µm-thick sections of *hippocampus* with goat anti-CB_1 polyclonal antibodies raised against

a 31 aminoacid C-terminal sequence (NM007726) of the mouse CB_1 receptor (CB_1-Go-Af450-1; 2 µg/ml; Frontier Science Co. Ltd).

For intra-SNr electron microscopy, C57BL/6-N mice were processed for electron microscope pre-embedding immunogold labeling as recently described (Benard et al., 2012; Hebert-Chatelain et al., 2014). Immunodetection was performed in 50-µm-thick sections of *hippocampus* with goat anti-CB_1 polyclonal antibodies raised against a 31 aminoacid C-terminal sequence (NM007726) of the mouse CB_1 receptor (CB_1-Go-Af450-1; 2 µg/ml; Frontier Science Co. Ltd).

Semi-quantification of mtCB_1 receptors

Immunogold particles were identified and counted. To exclude the risk of counting possible false positive mitochondrial labeling, we used a strict semi-quantification method of mtCB_1 receptors as recently described, excluding immunogold particles that were located on mitochondrial membranes but at a distance ≤ 80 nm from other cellular structures (Hebert-Chatelain et al., 2014). Normalized mtCB_1 receptor labeling *versus* total CB_1 showed mtCB_1 proportion *versus* total CB_1.

Field excitatory post-synaptic potentials (fEPSP) recording

Mice were anesthetized with isoflurane and killed by decapitation. Brains were rapidly removed and chilled in an ice-cold, carbogenated (bubbled with 95% O_2-5% CO_2) cutting solution containing 180 mM Sucrose, 2.5 mM KCl, 1.25 mM $CaCl_2$, 12 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 11 mM glucose (pH 7.4). Sagittal hippocampal slices (350 µm thick) were cut using a Leica VT1200S vibratome and incubated with artificial cerebrospinal fluid (ACSF) containing 123 mM NaCl, 1.25 mM NaH_2PO_4, 11 mM glucose, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 26 mM $NaHCO_3$ (osmolarity of 298±7, pH 7.4) for 30 min at 34°. The slices were subsequently transferred to a holding chamber where they were maintained at room temperature until experiments. Slices were individually transferred to a submerged chamber for recording and continuously perfused with oxygenated (95% O_2-5% CO_2) bathing medium (3–5 mL/min). All experiments were performed at room temperature. Extracellular evoked field potentials (fEPSPs) were recorded using glass micropipettes (2–4 mOhm) filled with normal ACSF bathing

medium. Slices from the dorsal *hippocampus* were used preferentially. fEPSPs responses were evoked by stimulation (0.1 ms duration, 10–30 V amplitude) delivered to stratum radiatum to stimulate the Schaffer collateral fibers using similar glass electrodes used for the recordings, in the presence of picrotoxin 100uM. Recordings were obtained using an Axon Multiclamp 700B amplifier (Molecular Devices). Signals were filtered at 2 kHz, digitized, sampled, and analyzed using Axon Clampfit software (Molecular Devices).

Novel object-recognition memory task

To study the impact of mtCB$_1$ receptor signaling on cannabinoid-induced amnesia, we used the hippocampal-dependent novel object recognition memory task in a L-maze [L-M/OR; (Busquets-Garcia et al., 2011; Puighermanal et al., 2013; Puighermanal et al., 2009)]. This task was performed with a L-maze made out of dark gray Plexiglas with two corridors (35 cm and 30 cm long respectively for external and internal V walls, 4.5cm wide and 15 cm high walls) set at a 90° angle and under a weak light intensity (50 Lux).

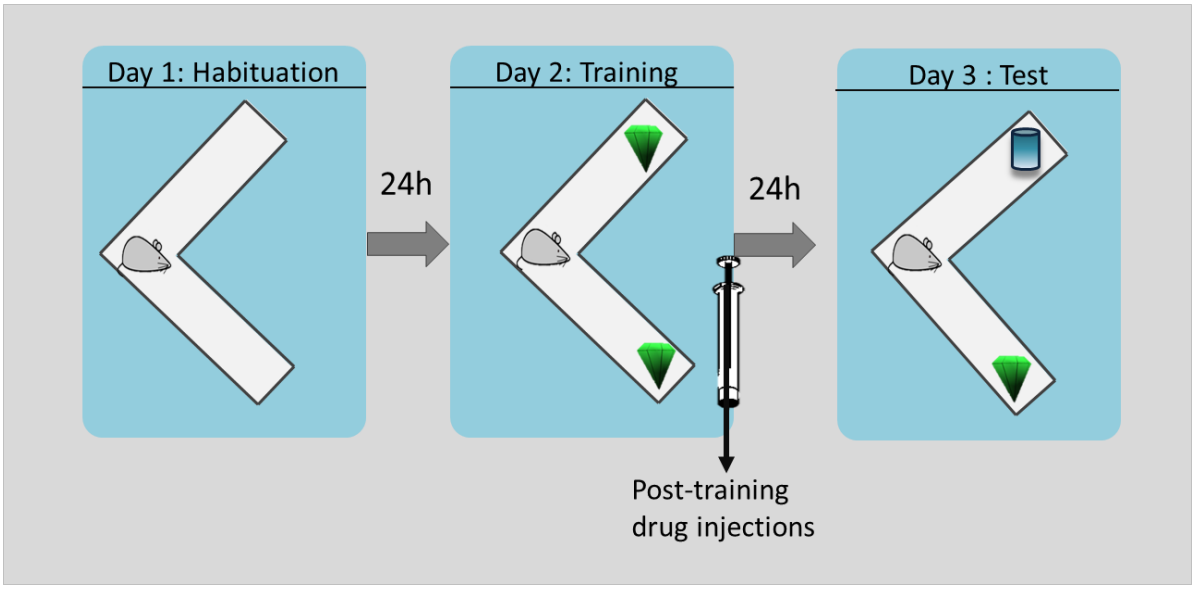

Figure 15.Novel object recognition memory task in a L-maze (L-M/OR).

The task consisted of 3 sequential daily trials of 9 minutes. Day 1 (habituation): mice were placed at the intersection of the two arms and were let free to explore the maze. Day 2 (training): two identical objects were disposed at the end of each arm.

After 9 min of exploration, mice were removed from the maze, injected and placed them in their home cages. Day 3 (test): A novel object different in shape, color and texture was placed at the end of one of the arm, whereas the familiar object remained at the other arm's end. The position of the novel object and the associations of novel and familiar were randomized. Memory performance was assessed by the discrimination index (DI). The D.I. was calculated as the difference between the time spent exploring the novel (TN) and the familiar object (TF) divided by the total exploration time (TN+TF): DI=[TN-TF]/[TN+TF]. Mice receiving the acute intra-hippocampal infusion of KH7 (10mM) and WIN (5mg/kg) i.p. were submitted to a single L-M/OR session, whereas virally injected mice were tested twice with one week interval using different pairs of objects and treated the first time with vehicle and the following with WIN. Every pairs of objects were screened to determine whether the animals exhibited significant preferences for any specific item.

Open field and horizontal bar test

AM251, Hemopressin or KH7 were bilaterally injected into the SNr in a volume of 0.5 µl per side. Immediately after, THC (10 mg/kg) was injected i.p. Mice were tested, 30 min post-injections, successively for: locomotor activity and catalepsy. To evaluate locomotor activity, mice were placed in a plastic transparent box [45cm (length) X 34cm (width) X 20cm (height)] with a floor divided into squares. Number of crossed squares was measured during five minutes. Then, catalepsy was determined by the time of immobility on a bar. To this aim, mice were positioned in a new plastic cage (identical to their home cage) without bedding with on a horizontal cylindrical bar (0.7cm Diameter) placed at 4.5 cm high with their forepaws gripping the bar and its hind paws in the plastic box. The time spent in its initial position on the bar was scored for two minutes. All equipment was cleaned with ethanol 25% and dried with paper towels between all the trials.

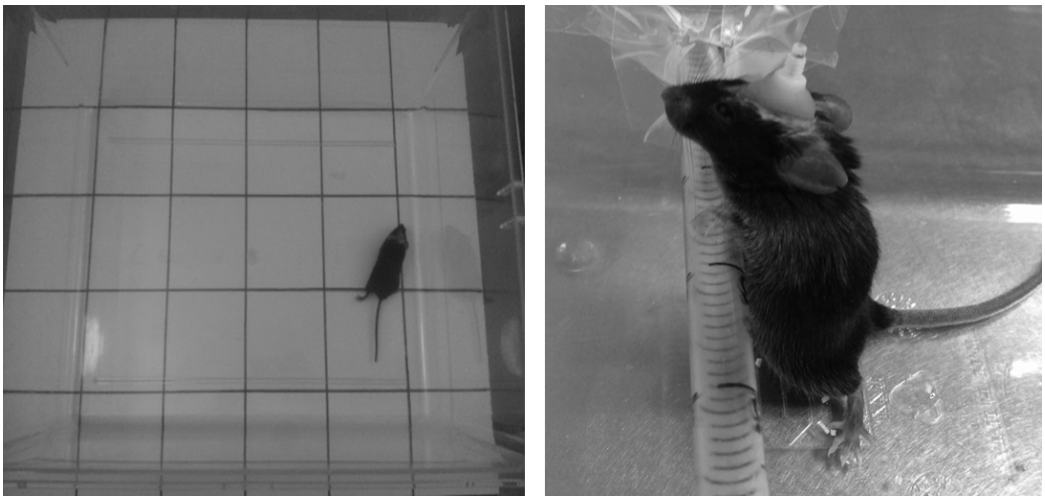

Figure 16. Open field and horizontal bar test.

RESULTS

Cannabinoid administration in humans induces several therapeutic-relevant effects accompanied with undesired side effects, particularly a decline in memory performance (Howlett, 2002; Koppel et al., 2014; Pacher et al., 2006). Genetic and pharmacological approaches demonstrated that most of these effects are mediated by CB_1 receptors in the brain (Monory et al., 2007; Pacher et al., 2006; Puighermanal et al., 2009; Wise et al., 2009). Nevertheless, the molecular mechanisms involved have been only partially explored so far.

Cannabinoid receptors type-1 are G protein coupled receptor (GPCRs) highly expressed in the brain and particularly enriched in the *hippocampus*, *cortex* and basal ganglia structures (Herkenham et al., 1990; Marsicano and Kuner, 2008). These receptors are expressed in different cell types and, at cellular level, they are found at both plasma membrane and intracellular compartments (Benard et al., 2012; Hebert-Chatelain et al., 2014; Rozenfeld and Devi, 2008). However, to what extent the sub-cellular localization of CB_1 receptors shapes their functions is virtually unknown.

The fact that the brain, weighing 2% of the body, consumes up to 20% of body energy, indicates that bioenergetics processes play peculiar roles in this organ, beyond mere “housekeeping” metabolic processes (MacAskill and Kittler, 2010; Mattson et al., 2008; Rangaraju et al., 2014). Mitochondria, the main organelles devoted to the processing of energy sources and their transformation into ATP, play a central role in the modulation of synaptic plasticity and associated physiological and pathological brain processes (Kann and Kovacs, 2007; Mattson et al., 2008). By providing local ATP supply, buffering of intracellular calcium and other ions, modulation of reactive oxygen species, metabolism of steroid synthesis, production of several neurotransmitters and many other key cellular functions, mitochondria are involved in the large majority of brain functions (MacAskill and Kittler, 2010; Mattson et al., 2008; Rangaraju et al., 2014). Accordingly, mitochondrial dysfunctions can contribute to the development of neurodegenerative diseases and memory deficits (Gibson and Shi, 2010; Mattson et al., 2008; Papa et al., 2012). However, whereas the impact of long-term mitochondrial dysfunctions on the brain is the subject of

intense research, these studies cannot establish a direct link between mitochondrial functions and behavioral outcomes. In fact, long-term mitochondrial dysfunctions lead to altered cellular processes, including cell damage and death, which can produce behavioral impairments as secondary effects. Conversely, the acute involvement of mitochondria in the regulation of animal behavior might establish a more solid link between brain bioenergetics processes and functions. However, likely due to the lack of specific pharmacological and molecular targets, the acute impact of mitochondrial function on memory processes has not been deeply explored so far.

Our previous studies demonstrated that CB_1 receptors are functionally present at mitochondrial membranes of brain cells (Benard et al., 2012; Vallee et al., 2014), for methodological discussions see (Hebert-Chatelain et al., 2014; Morozov et al., 2013). Despite the fact that $mtCB_1$ receptor activation by cannabinoids decreases mitochondrial respiration and consequently leads to decrease of ATP cell production in brain cells (Benard et al., 2012; Hebert-Chatelain et al., 2014; Vallee et al., 2014), the impact of these processes in the central effects of cannabinoids is still unknown. Addressing this issue is a difficult task, because it implies the functional discrimination, at single cell level in intact animals and tissues, between $mtCB_1$ receptors located at mitochondrial membranes and CB_1 receptors located at other subcellular sites. Therefore, the development of new tools is necessary to specifically investigate the functional roles of $mtCB_1$ receptors both *in vitro* and *in vivo*.

The following results have been submitted for publication (Tools and amnesic effects of cannabinoids) or are in preparation (involvement of sAC signaling in cannabinoid-induced locomotor effects). The manuscripts are presented in Annexes. In sake of brevity, some data panels are presented only in the Annexes and specific references to these panels is made in the text.

I. DEVELOPING THE TOOLS

Since no tool is currently available to specifically target mtCB$_1$ receptor activity, we decided to deeply investigate the molecular mechanisms linking mtCB$_1$ receptor activation to mitochondrial respiration, aiming at identifying specific signaling elements, which might be then experimentally targeted *in vivo*.

I.1 MtCB$_1$ receptors decrease brain mitochondrial respiration through G$_{\alpha i/o}$ proteins

Activation of mtCB$_1$ receptors decreases respiration of purified brain mitochondria, but this effect was not investigated in living brain cells (Benard et al., 2012; Hebert-Chatelain et al., 2014; Vallee et al., 2014). The prototypical plant-derived and synthetic CB$_1$ receptor agonist THC, decreased basal, oligomycin-insensitive and uncoupled respiration states (using FCCP, see methods) by approximately 25 % in primary hippocampal cultures derived from wild-type mice, but bore no effect in cultures from *CB$_1^{-/-}$* mice **(Figure 17A-B)**. These effects were associated with reduced cellular and mitochondrial ATP levels **(Figures 17C)**.

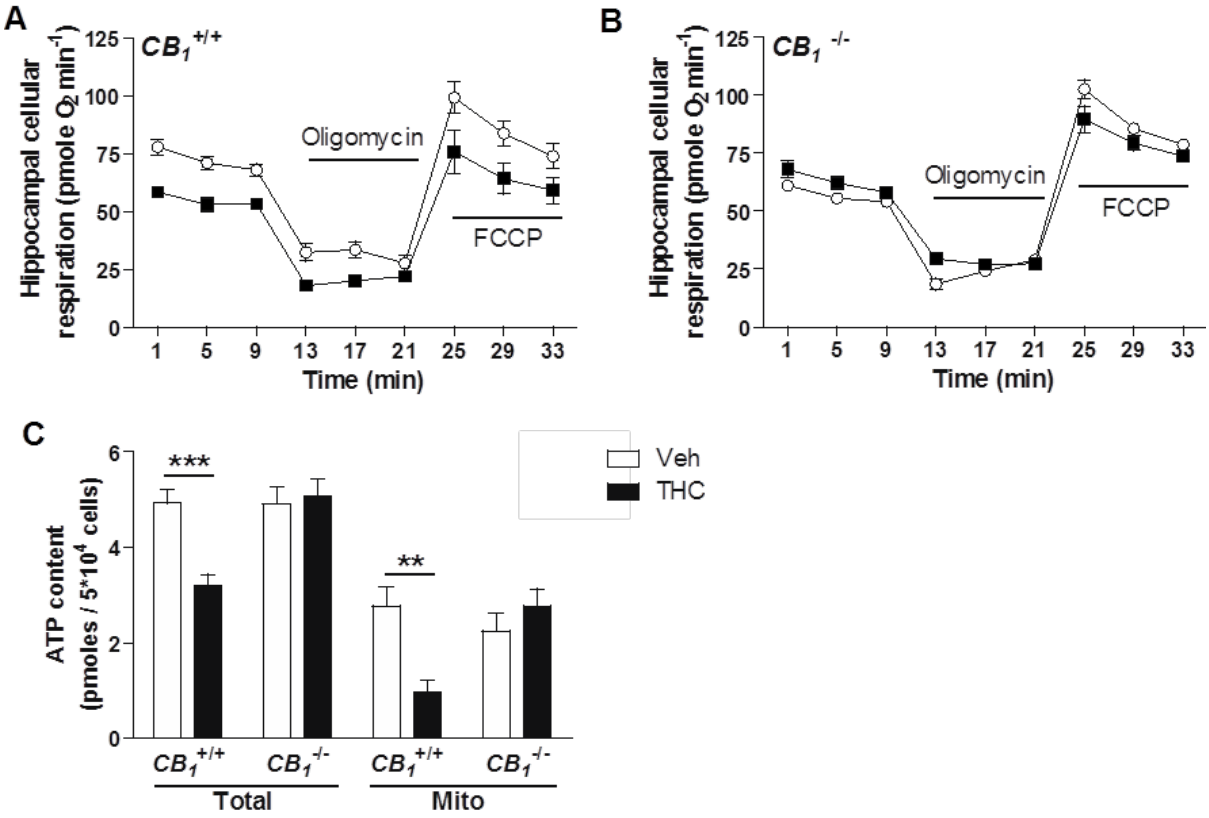

Figure 17. CB_1 receptor agonist, THC, impacts on hippocampal cellular respiration and ATP levels. (A-B) THC (1 µM) decreases cellular respiration of primary hippocampal cultures derived from $CB^{1+/+}$ but not from CB_1-/- mice (n=15). **(C)** THC (1 µM) reduces both total and mitochondrial (Mito) ATP levels of primary hippocampal cultures derived from $CB_1^{+/+}$ but not from $CB_1^{-/-}$ mice (n=7-8). FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (mitochondrial oxidative uncoupler). Data, mean ± s.e.m. * p<0.05, ** p<0.01, *** p<0.001.

The presence of G protein signaling has been identified in mitochondria (Andreeva et al., 2008; Beninca et al., 2014; Kuyznierewicz and Thomson, 2002; Lyssand and Bajjalieh, 2007), together with many downstream molecular cascades, such as adenylyl cyclase and protein kinase A (PKA) activities (Acin-Perez et al., 2009b; Valsecchi et al., 2013). Thus, we tested whether the GPCR $mtCB_1$ regulates brain mitochondrial activity *via* this signaling cascade. Trypsin sensitivity and co-immunoprecipitation assays revealed that Gα proteins are present inside isolated brain mitochondria **(Figure 18A)**, where they physically interact with $mtCB_1$ receptors **(Figure 18B)**. Activation of $mtCB_1$ receptors by THC induced the release of Gα proteins from these receptors **(Figure 18C-D)**.

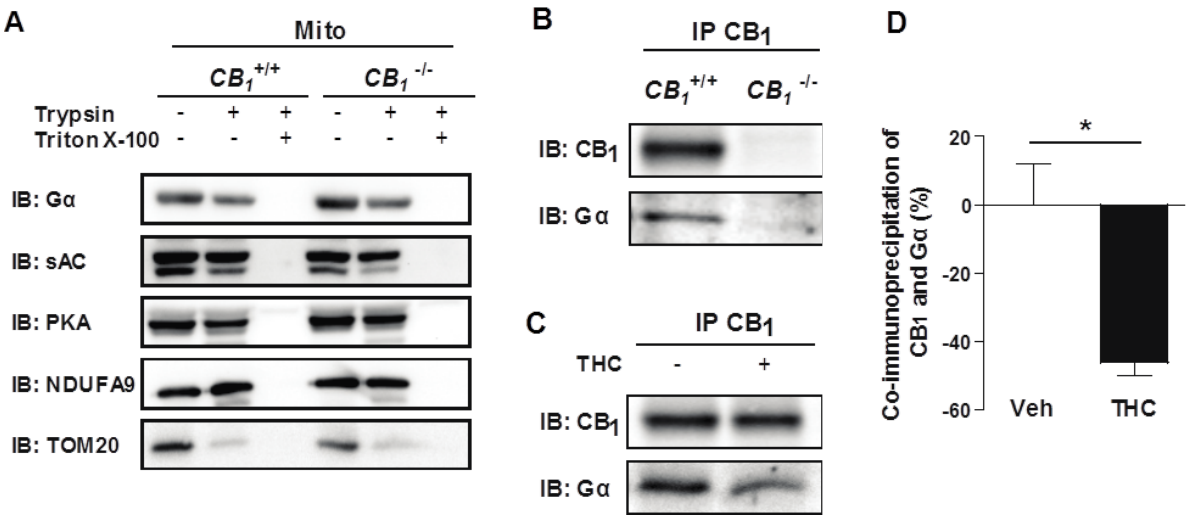

Figure 18. Intramitochondrial signaling. **(A)** Representative trypsin sensitivity assay (3 independent experiments) showing the intramitochondrial localization of G_{α}, sAC and PKA. IB, immunoblotting; NDUFA9, subunit of complex I; TOM20, translocase of outer mitochondrial membrane. **(B)** Representative immunoblotting (3 independent experiments) showing that Gα proteins co-immunoprecipitate with $mtCB_1$ receptors in purified brain mitochondria. **(C)** Representative immunoblotting (3 independent experiments) showing that co-immunoprecipitation of Gα proteins with $mtCB_1$ receptors decrease after THC treatment (1 µM) in purified brain mitochondria, indicating release of the G protein upon activation of $mtCB_1$ receptors. **(D)** Quantification of data presented in C (n=3). Data are mean ± s.e.m. * $p<0.05$.

The inhibitor of Gαi/o proteins pertussis toxin (PTX) abolished the effects of cannabinoids on mitochondrial respiration, Complex I activity, cAMP levels and PKA activity (**Figure 19A-D)**, but it did not alter mitochondrial cAMP levels and PKA activity in brain mitochondria from *$CB_1^{-/-}$* mice **(Figure 19E-F)**. PTX treatment also blocked the effect of THC on respiration of primary hippocampal cultures **(Figure 19G)**. Thus, cannabinoids regulate brain mitochondrial respiration through $mtCB_1$ receptor-dependent activation of $G_{\alpha i/o}$ proteins.

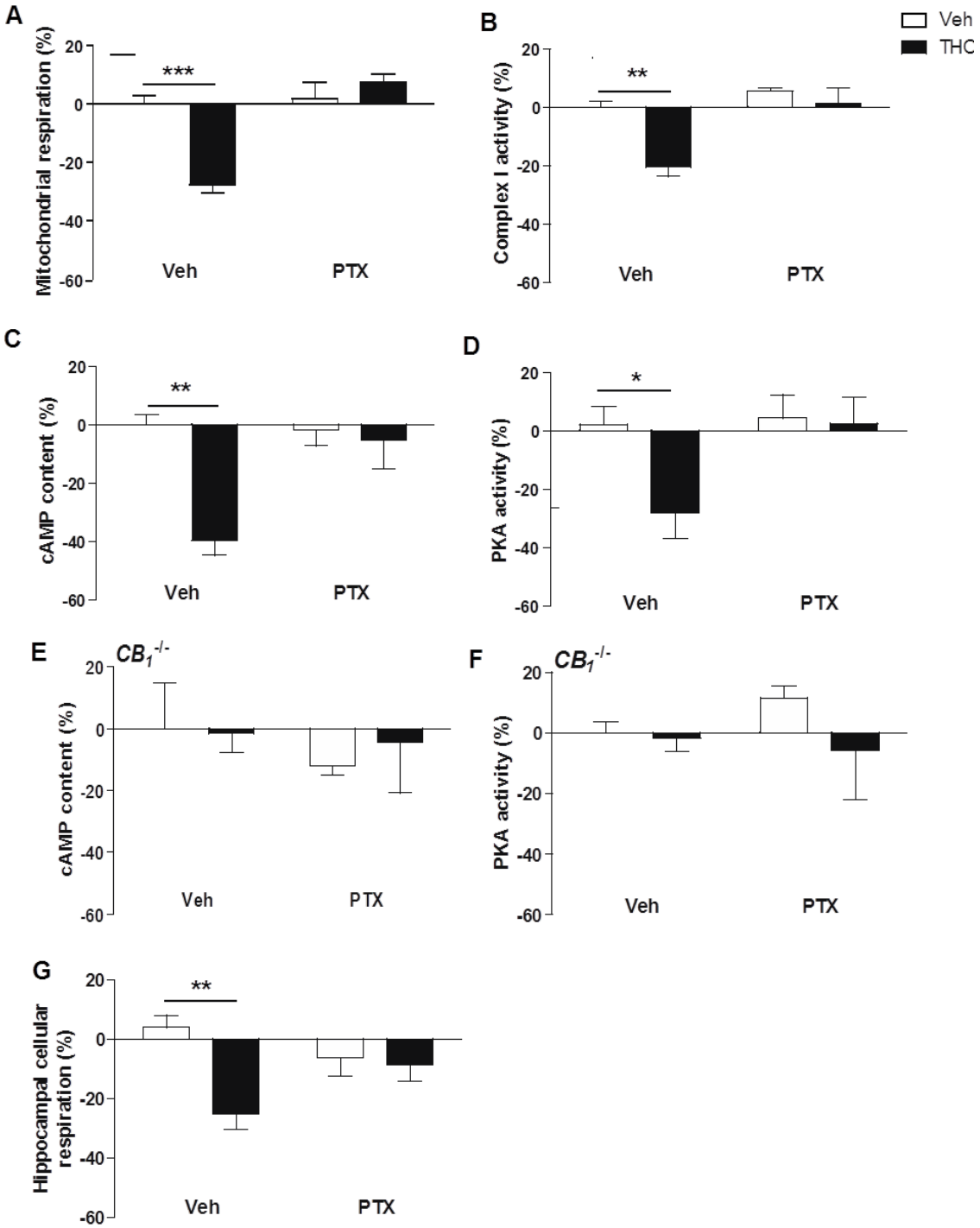

Figure 19. Activation of mtCB$_1$ receptor impacts OXPHOS through Gαi/o. (A-D) Pertussis toxin (PTX: 1 µg/ml) blocks the effect of THC (800nM) on (A) mitochondrial respiration (n=4), (B) complex I activity (n=3), (C) cAMP levels (n=4), and (D) PKA activity (n=6-7) of purified brain mitochondria from CB1+/+ mice. (E, F) PTX (1 µg/ml) and THC (800nM) do not impact on (E) cAMP levels (n=4) and (F) PKA activity (n=5) in $CB_1^{-/-}$ purified brain mitochondria. (G) Pertussis toxin blocks the effect of THC (1 µM) on basal cellular respiration in primary hippocampal cultures prepared from $CB_1^{+/+}$ mice (n=8-9). Data are mean ± s.e.m. * p<0.05, ** p<0.01, *** p<0.001.

I.2 Mitochondrial soluble adenylyl cyclase (sAC) is necessary for mtCB$_1$ receptor signaling

In mitochondria, cAMP is mainly produced by soluble adenylyl cyclase (sAC), regulating mitochondrial activity through PKA-mediated phosphorylation of OXPHOS components (Acin-Perez et al., 2009b). Trypsin sensitivity assays showed that sAC and PKA are present within purified brain mitochondria **(Figure 18A)**. The activator of the non-mitochondrial transmembrane adenylyl cyclase (tmAC) forskolin had no effect *per se* and did not alter the effect of THC on mitochondrial cAMP levels **(Figure 20A)**, indicating that tmACs are not involved in the regulation of OXPHOS by mtCB$_1$ receptors. Conversely, the activator of sAC bicarbonate [HCO_3^-,(Chen et al., 2000) fully reversed the effects of THC on cAMP in brain mitochondria **(Figure 20B)**.

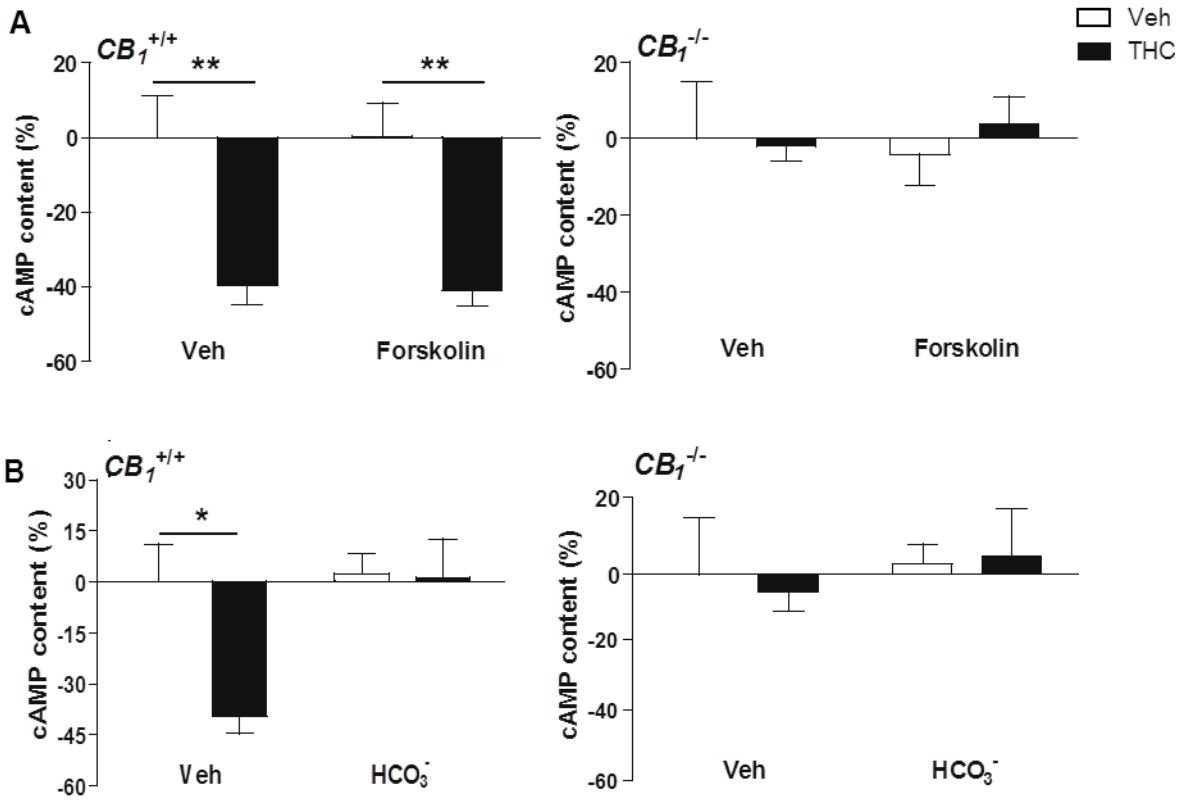

Figure 20. Soluble adenylyl cyclase (sAC) and not transmembrane adenylyl cyclase (tmAC) mediates the effects of mtCB$_1$ activation. (A) Forskolin (10 µM) does not reverse the effect of THC (800 nM) on cAMP content of *CB$_1^{+/+}$*brain mitochondria (n=4) a-nd has no effect on basal cAMP levels of *CB$_1^{-/-}$* brain mitochondria (n=3-4). **(B)** The sAC activator bicarbonate (HCO_3^-, 5mM) reverses the effect of THC (800 nM) on cAMP content of *CB$_1^{+/+}$*brain mitochondria (n=4) and has no effect on basal cAMP levels of *CB$_1^{-/-}$* brain mitochondria (n=3-4). Data are mean ± s.e.m. * p<0.05, ** p<0.01.

In addition, HCO_3^- abolished cannabinoid effects on respiration in isolated brain mitochondria **(Figure 21A)** and in cultured hippocampal cells **(Figure 21B)**. Consistently, KH7, a sAC inhibitor that does not affect the activity of membrane-bound adenylyl cyclase(Hess et al., 2005)completely occluded the THC-induced decrease of mitochondrial respiration, complex I activity and PKA activity in purified brain mitochondria in *CB$_1^{+/+}$***(Figure 21C-E)**. Neither KH7 nor THC had any effect on mitochondria derived from *CB$_1^{-/-}$*mice **(Figure 21F)**.

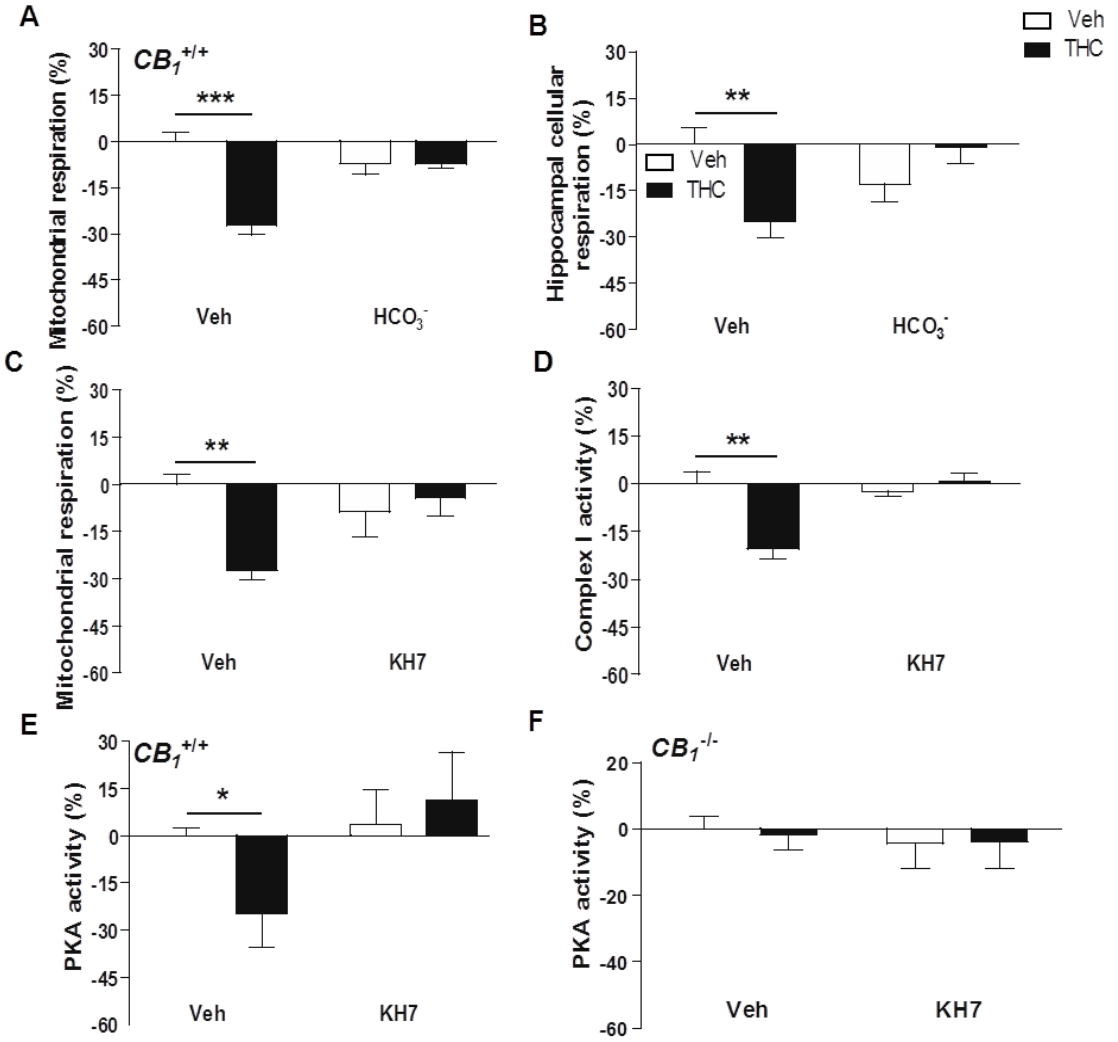

Figure 21. Soluble adenylyl cyclase (sAC) mediates the effects of mtCB$_1$ receptor activation. **(A)** The sAC activator bicarbonate (HCO_3^-, 5mM) reverses the effect of THC (800 nM) on mitochondrial respiration (n=4-6) in purified brain mitochondria from $CB_1^{+/+}$mice. **(B)** Bicarbonate reverses the effect of THC (1 µM) on basal cellular respiration of primary hippocampal cultures derived from CB1+/+ mice (n = 8-11). **(C)** The sAC inhibitor KH7 (5 µM) blocks the effect of THC (800 nM) on mitochondrial respiration (n=3-4) and **(D)** complex I activity (n=3) of purified brain mitochondria from $CB_1^{+/+}$mice. **(E-F)**KH7 (5 µM) blocks the effect of THC (800 nM) on PKA activity (n=6) of purified brain mitochondria from $CB_1^{+/+}$but not from$CB_1^{-/-}$mice (n=5). Data are mean ± s.e.m. * p<0.05, ** p<0.01, *** p<0.001.

To further confirm the importance of sAC in mtCB$_1$ receptor-dependent regulation of OXPHOS, the expression of mitochondrial sAC was modified in cultured 3T3 cells using HA-tagged sAC protein (sAC-HA), present both in cytosolic and mitochondrial fractions, and a mitochondria-targeting form of HA-tagged sAC,

[mtsAC-HA, (Acin-Perez et al., 2009b); **Figure 22A**]. Trypsin sensitivity assays showed that CB_1 receptors, G_α, endogenous sAC and PKA proteins **(Figure 22B)**, and sAC-HA and mtsAC-HA proteins were present inside mitochondria of transfected 3T3 cells **(Figure 22C)**. The decrease of cellular respiration induced by THC in mock-transfected cells was completely blocked in cells transfected with sAC-HA or mtsAC-HA, showing the necessity of mitochondrial sAC in $mtCB_1$ receptor-dependent signaling **(Figure 22D)**. Accordingly, the decrease of complex I activity measured on mitochondrial fractions treated with THC was completely blocked in 3T3 cells transfected with sAC-HA **(Figure 22E)**. In addition, sAC-HA expression also blocked the effect of THC on cellular respiration of primary hippocampal cultures **(Figure 22F)**. Notably, G_α proteins co-immunoprecipitated with mitochondrial sAC in brain mitochondrial extracts, and THC treatment increased this interaction **(Figure 22G-H)**. Thus, the regulation of OXPHOS by $mtCB_1$ receptors depends on mitochondrial sAC activity. Importantly in the present context, the sAC blocker KH7 occludes these effects, providing a reasonable pharmacological tool to study mitochondrial-related effects of cannabinoids *in vivo*.

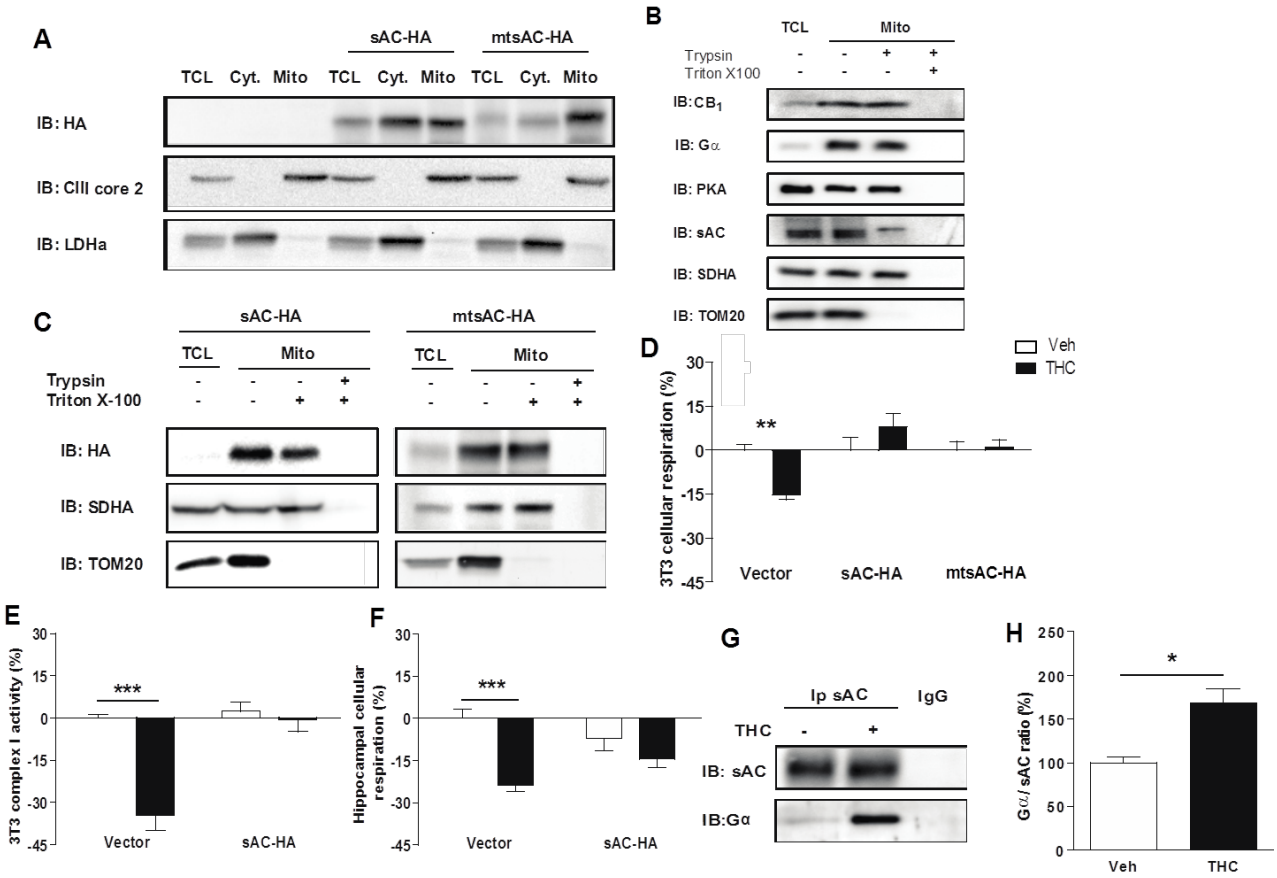

Figure 22. Soluble adenylyl cyclase (sAC) mediates the effects of mtCB$_1$ receptor activation. **(A)** Representative immunoblot (3 independent experiments) showing that sAC-HA is present in cytosolic and mitochondrial fractions whereas mtsAC-HA is specifically targeted to mitochondrial fractions of transfected 3T3 cells. **(B)** Representative trypsin sensitivity assay (from 3 independent experiments) showing intramitochondrial localization of CB$_1$ receptors, G$_\alpha$ proteins, PKA and sAC in 3T3 cells. **(C)** Representative trypsin sensitivity assay (3 independent experiments) showing that sAC-HA and mtsAC-HA are localized inside mitochondria in transfected 3T3 cells. **(D)** Over-expression of sAC-HA or mtsAC-HA in 3T3 cells blocks the effect of THC (800 nM) on basal cellular respiration (n=4). **(E)** Over-expression of sAC-HA in 3T3 cells blocks the effect of THC (1 µM) on complex I activity measured in mitochondrial fractions (n=3-4). **(F)** Expression of sAC-HA in CB$_1^{+/+}$ primary hippocampal cultures blocks the effect of THC (1 µM) on basal cellular respiration (n = 8-10). **(G)** Representative immunoblotting showing co-immunoprecipitation of G$_\alpha$ proteins with sAC from purified brain mitochondria, which is increased after THC (800 nM) treatment. **(H)** Quantification of data presented in G (n=3). Data are mean ± s.e.m. * p<0.05, ** p<0.01, *** p<0.001.

I.3 *In silico* identification of DN22-CB_1, a CB_1 mutant protein theoretically lacking mitochondrial localization

The results obtained so far strongly indicate that the mitochondrial effects of CB_1 receptor agonists mediate specific behavioral effects of cannabinoids. However, these data were obtained using pharmacological tools that are intrinsically exposed to uncontrollable potential caveats such as unspecific or off-target effects of drugs. Therefore, in order to extend our findings and to provide further tools to study the roles of mtCB_1 receptors, we investigated the possibility to exclude, by genetic mutagenesis, CB_1 receptors from mitochondrial membranes. The freely accessible softwares MitoProt (http://ihg.gsf.de/ihg/mitoprot.htm) and PSORT (http://psort.hgc.jp/) allow identifying the theoretical probability of mitochondrial localization of any given protein sequence (Claros, 1995). Interestingly, using these tools, the CB_1 receptor displays an approximate 40-45% probability to be imported to mitochondria (40.6% for MitoProt and 46.7% for PSORT, respectively **(Figure 23)**. These values are comparable to the ones of typical mitochondrial proteins and are more than one order of magnitude higher than the ones of many other G protein coupled receptors (Benard et al., 2012). By *in silico* "mutagenesis" tests, we found that the deletion of the first 22 N-terminal amino acids of the CB_1 protein sequence decreased its theoretical probability to be imported to mitochondria to approximately 1% for MitoProt and to 3% for PSORT, respectively **(Figure 23)**. Interestingly, as calculated by PSORT, the percentage of probability of plasma membrane localization was unchanged by the mutation (60% for both wild type and mutant CB_1 proteins **(Figure 23)**. Thus, the deletion of the first 22 amino acids of the CB_1 protein was predicted to exclude the CB_1 receptor from mitochondrial membranes, but to maintain its presence at cellular plasma membranes. To test this *in silico* prediction in "real life", we generated a mutant CB_1 protein by deleting the first 22 amino acids of its sequence and we called it DN22-CB_1 (for Delta N-terminus 22 amino acids deletion).

		Probability of Targeting		
		MITOCHONDRIA		PLASMA MEMBRANE
Protein	**Amino acid sequence**	**MitoProt** Prediction (Claros et al., 1995)	**PSORT** Prediction (Nakai et al., 1999)	**PSORT** Prediction (Nakai et al., 1999)
CB_1	MKSILDGLADTTFRTITTDLLYVGSNDIQYEDIKGDMASKLGYFPQKFPLTSFR GSPFQEKMTAGDNSPLVPAGDTTNITEFYNKSLSSFKENEDNIQCGENFMD MECFMILNPSQQLAIAVLSLTLGTFTVLENLLVLCVILHSRSLRCRPSYHFIGSLA VADLLGSVIFVYSFVDFHVFHRKDSPNVFLFKLGGVTASFTASVGSLFLTAIDRY ISIHRPLAYKRIVTRPKAVVAFCLMWTIAIVIAVLPLLGWNCKKLQSVCSDIFPLI DETYLMFWIGVTSVLLLFIVYAYMYILWKAHSHAVRMIQRGTQKSIIIHTSEDG KVQVTRPDQARMDIRLAKTLVLILVVLIICWGPLLAIMVYDVFGKMNKLIKTVF AFCSMLCLLNSTVNPIIYALRSKDLRHAFRSMFPSCEGTAQPLDNSMGDSDCL HKHANNTASMHRAAESCIKSTVKIAKVTMSVSTDTSAEL	**41%**	**47%**	**60%**
DN22-CB_1	MGSNDIQYEDIKGDMASKLGYFPQKFPLTSFRGSPFQEKMTAGDNSPLVPA GDTTNITEFYNKSLSSFKENEDNIQCGENFMDMECFMILNPSQQLAIAVLSLT LGTFTVLENLLVLCVILHSRSLRCRPSYHFIGSLAVADLLGSVIFVYSFVDFHVFH RKDSPNVFLFKLGGVTASFTASVGSLFLTAIDRYISIHRPLAYKRIVTRPKAVVAF CLMWTIAIVIAVLPLLGWNCKKLQSVCSDIFPLIDETYLMFWIGVTSVLLLFIVY AYMYILWKAHSHAVRMIQRGTQKSIIIHTSEDGKVQVTRPDQARMDIRLAKT LVLILVVLIICWGPLLAIMVYDVFGKMNKLIKTVFAFCSMLCLLNSTVNPIIYALR SKDLRHAFRSMFPSCEGTAQPLDNSMGDSDCLHKHANNTASMHRAAESCIK STVKIAKVTMSVSTDTSAEL	**1%**	**3%**	**60%**

Figure 23. Table of the *in silico* "mutagenesis" tests. The deletion of the first 22 N-terminal amino acids of the CB_1 protein sequence decreased its theoretical probability to be imported to mitochondria.

I.4 DN22-CB_1 receptor is functional but it does not impact on mitochondrial respiratory activity

To determine whether the deletion of the first 22 amino acids impacts on the ability of the CB_1 receptor to mediate the cannabinoid effects on cellular respiration, we electroporated newborn mouse fibroblasts (MFs) derived from $CB_1^{-/-}$ mice with plasmids expressing either wild-type CB_1 or mutant DN22-CB_1 proteins to obtain MF-CB_1 and MF-DN22-CB_1, respectively. The synthetic CB_1receptor agonists HU 210 or WIN did not alter respiration in mock-transfected MFs (mCherry), but they inhibited oxygen consumption by approximately 20% in MFs-CB_1, respectively **(Figure 24A)**. Interestingly, the same treatments in MF-DN22-CB_1 did not alter respiration as

compared to vehicle conditions **(Figure 24A)**. In addition, to further confirm the differential participation of the intracellular *versus* extracellular CB_1 receptor population, we used HU 210-biotin, a synthesized biotinylated version of the CB_1 receptor agonist HU 210, which does not penetrate plasma membranes (Benard et al., 2012; Martin-Couce et al., 2012).

These data indicate that the DN22-CB_1 mutant protein lost the ability to mediate the intracellular cannabinoid effects on respiration, suggesting that the 22 amino acids from the N-terminal tail of the CB_1 receptor are necessary for localization and/or function of the receptor at mitochondrial membranes. However, this lack of cannabinoid effects on cellular oxygen consumption in cells expressing the DN22-CB_1 protein might be merely due to unspecific alterations of the general functionality of the CB_1 receptor. Thus, to test the ability of DN22-CB_1 to mediate CB_1 receptor-dependent cellular pathways different from the control of mitochondrial respiration, we transfected the HEK293 cell line with mCherry (mock), CB_1 or DN22-CB_1, respectively. On these cells, we studied the cannabinoid-induced effects on cellular respiration and on extracellular-regulated kinases (ERKs) signaling, which is one of the most important intracellular effects of CB_1 receptor activation (Howlett, 2002). HU 210 decreased oxygen consumption in HEK293 cells transfected with a plasmid expressing the wild-type CB_1 receptor, but not in cells expressing a mock protein (mCherry) or DN22-CB_1 **(Figure 24B)**, confirming the lack of impact of DN22-CB_1 receptors on cannabinoid-dependent mitochondrial effects in different cell types. Notably, however, the administration of the CB_1 receptor agonist did not alter the ERK pathway in mock-transfected HEK293 cells (mCherry), but it clearly stimulated ERKs phosphorylation in both CB_1- and DN22-CB_1-transfected cells to similar extent **(Figure 24C-D)**, showing that the mutant CB_1 protein is still able to activate CB_1 receptor-dependent pathways not directly related to control of mitochondrial activity. Importantly, the cannabinoid agonist WIN55,212-2 stimulated [^{35}S]-GTP-gS binding in both CB_1- and DN22-CB_1-transfected HEK293 cells, with similar potency (214 nM vs 260 nM, respectively) and efficacy (74.7% vs 74.3%, respectively), demonstrating that the DN22-CB_1 mutant receptors conserved the same ability to activate G protein signaling as the wild-type CB_1 receptors **(Figure 24E)**, and consequently that the mutation did not alter the general functionality of the receptor.

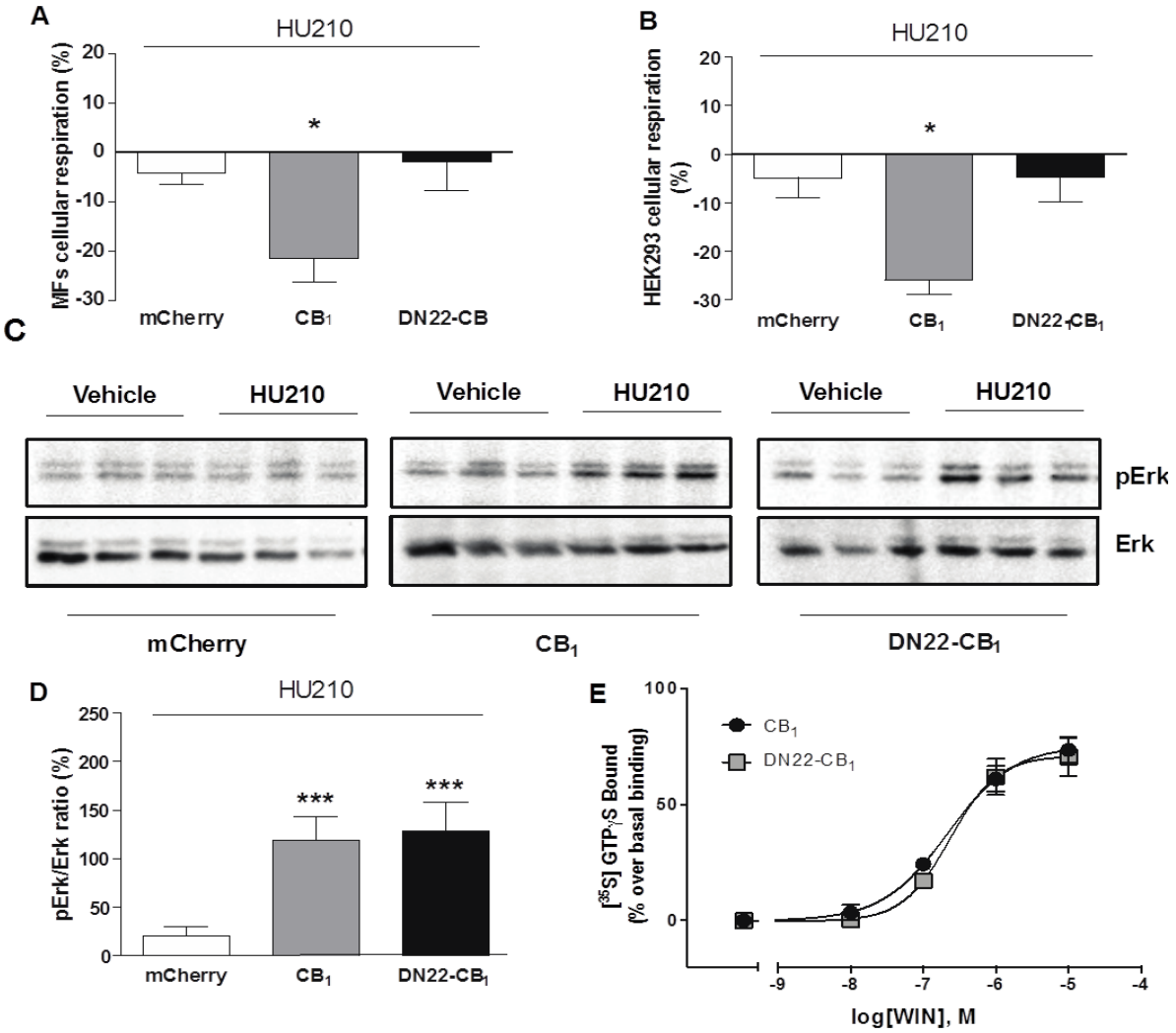

Figure 24. Mutant DN22-CB_1 receptor does not mediate cannabinoid-induced alterations of mitochondrial activity, but it can efficiently activate ERK and G protein signaling. (A) Mouse fibroblasts (MF) were obtained from *$CB_1^{-/-}$* pups (P0-P1) and electroporated either with a vector expressing mCherry (mock) or the CB_1 or DN22-CB_1 receptors. Acute treatment with HU 210 (100nm) reduced cellular oxygen consumption in CB1-expressing intact *$CB_1^{-/-}$*MF, whereas no alteration was observed in DN22-CB_1-expressing *$CB_1^{-/-}$*MF (n=3-10). **(B)** HEK293 cells were transfected with plasmids expressing mCherry or CB_1 or DN22-CB_1 receptor. HU 210 (200 nM) treatment decreases HEK293 cellular respiration in cells expressing CB1 but not DN22-CB_1 receptors (n=4). **(C**) Representative Immunoblotting showing the effect of HU 210 (100 nM) on pERK/ERK ratios in HEK293 transfected with plasmids expressing mCherry, CB_1 or DN22-CB_1, respectively. **(D)** Quantification of pERK/ERK ratios induced by HU 210, as in D (n=7-13). **(E)** Concentration response-curves of WIN55,212-2-stimulation of [35S]GTPγS binding in membranes isolated from HEK293 cells transfected with wild-type CB_1 or mutant DN22-CB_1, respectively. Data

Altogether, these results show that the mutant DN22-CB_1 protein does not functionally impact on mitochondrial cellular respiration, but it still maintains other CB_1 receptor-like signaling properties, including the ability to stimulate G protein signaling and ERK phosphorylation.

I.5 DN22-CB_1 is excluded from mitochondrial membranes

To assess the subcellular localization and function of the DN22-CB_1 mutant protein *in vivo*, we generated adeno-associated viruses carrying the expression of wild-type CB_1 or DN22-CB_1 proteins (AAV-CB_1 and AAV-DN22-CB_1, respectively). Either of these AAVs was infused into the *hippocampus* of $CB_1^{-/-}$ mice **(Figure 25A)** to obtain mice carrying exclusive hippocampal expression of CB_1 [called $CB_1^{-/-}$(CB_1) mice] or DN22-CB_1 proteins [called $CB_1^{-/-}$(DN22-CB_1) mice], respectively. Immunofluorescence analysis revealed that the efficiency of infection and expression of the two viruses were comparable **(Figure 25B)**.

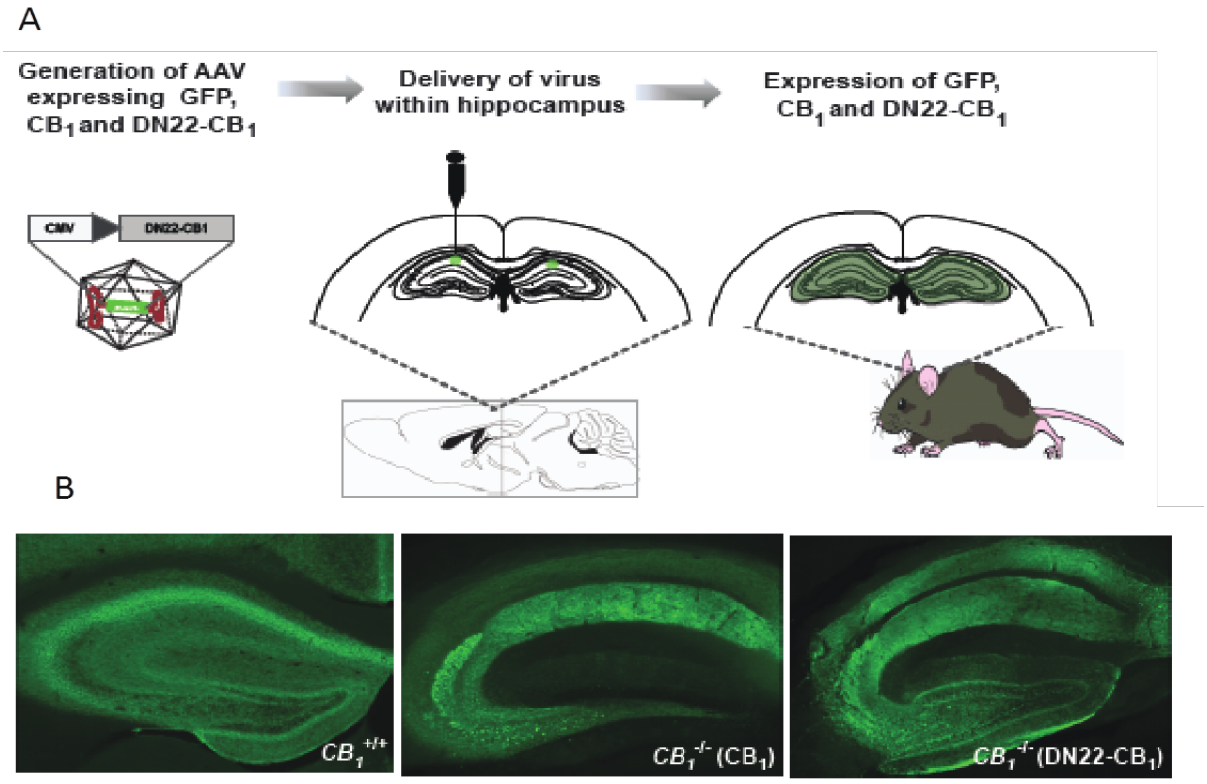

Figure 25.Selective expression of adeno-associated virus into the hippocampus. (A) Schematic representation of the AAV vectors delivery used in this study (control AAV-GFP and AAV- CB_1 and AAV-DN22-CB_1). Expression was driven by the CMV promoter. High titer, purified virus was prepared for stereotaxic injection into the hippocampus of 7-9 weeks $CB_1^{+/+}$and $CB_1^{-/-}$ mice. Mice were tested at least 4 weeks after gene delivery. **(B)** Immunofluorescence for CB_1 receptors in hippocampal brain sections from $CB_1^{-/-}$ mice bilaterally injected into the hippocampi with AAV-DN22-CB_1 or CB_1 or GFP (control), and their respective wild-type $CB_1^{+/+}$ littermates injected with GFP.

To determine the subcellular localization of the two proteins, we examined CB_1 receptor immunoreactivity in the hippocampal CA1 region by immunogold electron microscopy. As expected (Benard et al., 2012; Hebert-Chatelain et al., 2014), CB_1 receptor gold particles were observed at both plasma and mitochondrial membranes of tissues derived from wild-type mice locally treated with a control AAV [$CB_1^{+/+}$(GFP) mice, **Figure 26A**], but not in tissues from $CB_1^{-/-}$ mice treated with the same control virus [$CB_1^{-/-}$(GFP) mice, **Figure 26A**). Interestingly, the hippocampal infection of $CB_1^{-/-}$ mice with AAV-CB_1 [$CB_1^{-/-}$(CB_1) mice] led to the expression of CB_1 receptor protein

both at plasma and mitochondrial membranes (Figure 26A), whereas the DN22-CB_1 protein appeared to be excluded from mitochondrial membranes in *$CB_1^{-/-}$*(DN22-CB_1) mice **(Figure 26A)**. Semi-quantitative counting of gold particles using strict parameters to define mitochondrially-located CB_1 receptor immunoreactivity (Hebert-Chatelain et al., 2014) confirmed these observations. Approximately 10% of the total CB_1 receptor-positive gold particles were identified on CA1 mitochondria in wild-type *$CB_1^{+/+}$*(GFP), whereas less than 2% of background gold particles were observed at mitochondrial membranes in hippocampi of negative control *$CB_1^{-/-}$*(GFP) mice **(Figure 26B)**. The viral re-expression of the entire CB_1 protein in *$CB_1^{-/-}$*(CB_1) mice led to a similar proportion of mtCB_1 gold particles as in wild-type controls **(Figure 26B)**. Notably, *$CB_1^{-/-}$*(DN22-CB_1) mice displayed a very similar proportion of mitochondrial staining as *$CB_1^{-/-}$*(GFP) littermates **(Figure 26B)**, indicating that the mtCB_1 proportion in these mice did not overcome background levels.

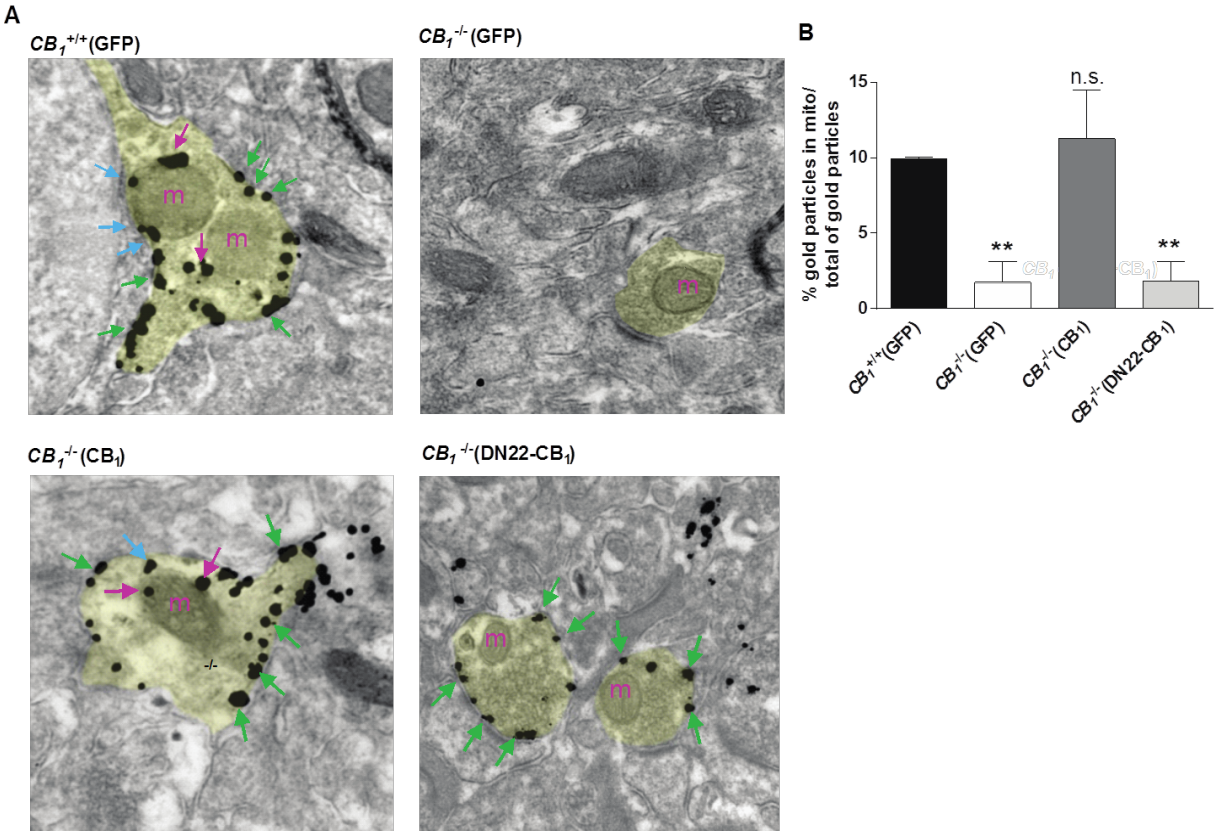

Figure 26. DN22-CB_1 receptors are excluded from mitochondrial membranes in hippocampus. (A) Electron microscopy of immunogold staining in the CA1 hippocampal region showing the expression of CB_1 in different mutant mice. The presence of mtCB_1receptors is observed in $CB_1^{+/+}$ (GFP) and $CB_1^{-/-}$(CB_1), but not in $CB_1^{-/-}$(GFP) or $CB_1^{-/-}$(DN22-CB1). **(B)** Semi-quantification of the percentage of gold particles in mitochondria normalized by the total gold particles (n=3). $CB_1^{+/+}$(GFP) and $CB_1^{-/-}$(CB_1) exhibit a significant higher proportion of mtCB_1 proteins as compared to$CB_1^{-/-}$(DN22-CB_1) and $CB_1^{-/-}$(GFP). Data are mean ± s.e.m. ** p<0.01 as compared to the percentage of the $CB_1^{+/+}$ (GFP). See main text for definition of the different groups of mutant mice.

Thus, the mutant DN22-CB_1 protein is excluded from brain mitochondria and is functionally unable to modulate mitochondrial functions. The mutant protein, however, can still localize to other subcellular sites (*e.g.* plasma membrane) and regulate CB_1 receptor-dependent activation of G proteins and the ERK pathway. Thus, DN22-CB_1 is an optimal tool to study the role of mtCB_1 receptor activation in brain functions and behavior.

Conclusion

In order to address the potential role of intracellular $mtCB_1$ receptors, we identified the precise intramitochondrial biochemical cascade linking $mtCB_1$ receptor activation to decrease of cellular respiration and ATP production. We found that the soluble adenylyl cyclase (sAC), the only AC known to be present in mitochondria (Valsecchi et al., 2013), is necessary for these effects.

To enlarge the tool panel to study $mtCB_1$ receptor functions and to overcome potential caveats of pharmacological tools, such unspecific or off-target effects of drugs like KH7, we identified and generated a mutant protein, DN22-CB_1, which lacks the first 22 amino acids of the CB_1 receptor sequence. Agonist stimulation of DN22-CB_1 receptors in transfected cells does not alter mitochondrial respiration, but still induces other CB_1 receptor-dependent signaling, such as activation of P-ERK and activation of G proteins.

II. ROLE OF HIPPOCAMPAL MTCB_1 RECEPTOR ACTIVATION IN SYNAPTIC ACTIVITY AND MEMORY

Next, we aimed at determining the specific impact of mtCB_1 receptor signaling on memory-disrupting effects of cannabinoids. Thus, we took advantage of the identification of the molecular pathways induced by mtCB_1 receptor activation leading to decreased mitochondrial activity in brain cells, and used drugs able to block these pathways to investigate the specific roles of mtCB_1 receptor signaling in typical cannabinoid effects such as reduction of neurotransmitter release and amnesia. Then, we strengthened these pharmacological studies by using the genetic tool described above (DN22-CB_1) specifically designed to exclude the mitochondrial localization of CB_1 receptors and, thereby, to lack mtCB_1 receptor signaling. Our data demonstrate the specific roles of mtCB_1 receptor signaling in cannabinoid amnesic effects and, thereby, establish a causal link between bioenergetics and behavioral memory processes.

II.1 sAC activity is required for cannabinoid-induced decrease of hippocampal excitatory neurotransmission

Cannabinoids are well known to decrease neurotransmitter release in the *hippocampus* (Kano et al., 2009). Despite some initial studies on inhibitory neurotransmission using indirect tools (Benard et al., 2012), no data are so-far available concerning the potential role of mtCB_1 receptors in the cannabinoid-induced decrease of excitatory hippocampal neurotransmission. We examined the effect of sAC inhibition on the cannabinoid-induced decrease of field excitatory postsynaptic potentials (fEPSP) at hippocampal CA3-CA1 synapses. Applications of the CB_1 receptor agonist HU 210 reduced the slope of fEPSP by almost 50% of the baseline

levels **(Figure 27A)**. The application of the sAC inhibitor KH7 did not exert any measurable effect on baseline fEPSPs **(Figure 27B)**, but it abolished the HU 210-induced decrease of fEPSP slopes **(Figure 27A)**.

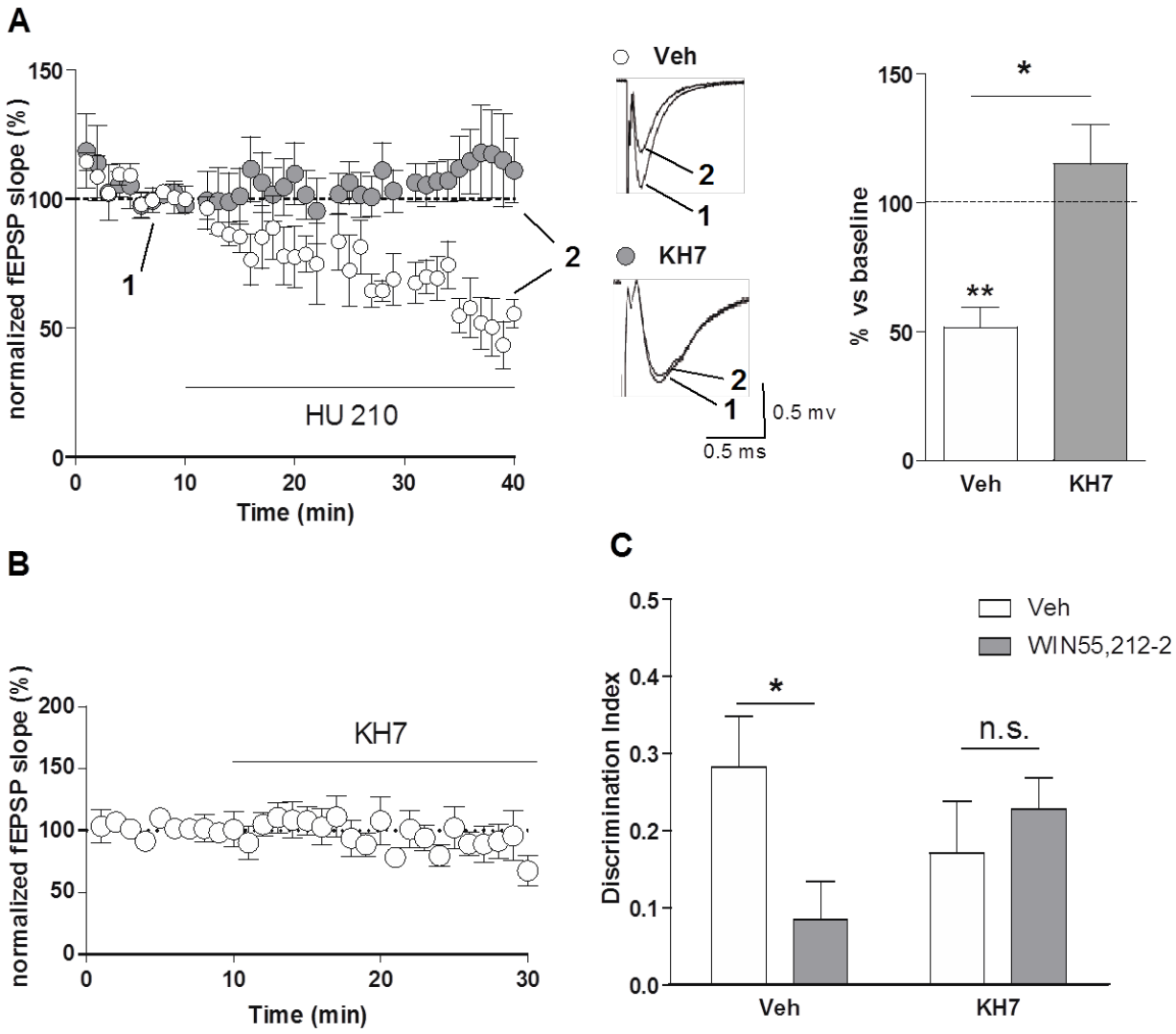

Figure 27. Blockade of mtCB$_1$ receptor signaling in the hippocampus impairs the effects of cannabinoid on glutamatergic transmission and object recognition memory.(A) Effects of KH7 (10 µM) on HU 210 (2.5 µM)-induced *in vitro* decrease of fEPSP at CA3-CA1 synapses from C57BL/6-N mice. (Left) Plots of normalized fEPSP slopes *in vitro* with representative fEPSP traces before (1) and after (2) vehicle or HU 210 incubation. Vehicle (n=4) or KH7 (n=5) were pre-incubated 10 min before cannabinoid application. (Right) Histogram summarizing the average changes in percentage of fEPSP slope before (100% baseline, dotted line) and after HU 210 treatment in presence or absence of KH7. **(B)** KH7 does not affect the basal fEPSP. **(C)** Cannabinoid-induced inhibition of object recognition memory performance is blocked by KH7 injection in the hippocampus. Discrimination index values in the object-recognition test showing the effect of WIN55,212-2 (i.p., 5 mg/kg) in C57BL/6-N miceintra-hippocampally injected with vehicle or KH7 (2 µg in 0.5 µl).N=7-14 per group. Data are mean ± s.e.m. * p<0.05, ** p<0.01.

II.2 sAC activity is required for cannabinoid-induced impairment of object recognition memory

Cannabinoids strongly impair hippocampal-dependent novel object recognition memory tested in a L-maze [L-M/OR; (Busquets-Garcia et al., 2011; Puighermanal et al., 2013; Puighermanal et al., 2009)]. As expected, the systemic administration of the CB_1 receptor agonist WIN immediately after task acquisition impaired L-M/OR performance in C57BL6-N mice **(Figure 27C)** without altering total objects exploration (not shown). Interestingly, however, the local intra-hippocampal administration of KH7 abolished this effect **(Figure 27C)**, indicating the involvement of sAC activity in this cannabinoid-induced alteration of memory performance.

Taken together, these results show that KH7 occludes the $mtCB_1$-dependent reduction of mitochondrial respiration and inhibits specific effects of cannabinoids, such as reduction of excitatory synaptic transmission and consolidation of memory in the *hippocampus*.

II.3 Hippocampal $mtCB_1$ receptor signaling mediates the cannabinoid effects on glutamatergic transmission and object recognition memory

The effects of cannabinoid agonists on fEPSPs in the CA3-CA1 pathway are known to depend on CB_1 receptors and to be absent in hippocampal slices from $CB_1^{-/-}$ mice (Kano et al., 2009). Thus, to study the role of $mtCB_1$ receptors in this electrophysiological effect, we used hippocampal slices derived from $CB_1^{-/-}(CB_1)$ and $CB_1^{-/-}(DN22\text{-}CB_1)$ mutant mice, respectively. Viral re-expression of CB_1 receptor was able to rescue the effect of HU 210 in hippocampal slices derived from $CB_1^{-/-}(CB_1)$ mice **(Figure 28A)**. However, the CB_1 receptor agonist was not able to reduce fEPSP slopes in hippocampal slices derived from $CB_1^{-/-}(DN22\text{-}CB_1)$ mice **(Figure 28A)**, indicating that mitochondrial localization of the CB_1 receptor is required for this electrophysiological effect of cannabinoids.

To study the impact of hippocampal mtCB$_1$ receptor signaling on cannabinoid-induced impairment of memory, *CB$_1^{+/+}$*(GFP), *CB$_1^{-/-}$*(GFP), *CB$_1^{-/-}$*(CB$_1$) and *CB$_1^{-/-}$* (DN22-CB$_1$) mice were tested in the L-M/OR task. As expected (Puighermanal et al., 2009), all mice treated after acquisition with vehicle displayed a normal discrimination index during testing 24 hours later, independently of genotype and viral treatment **(Figure 28B)**. One week later, the same animals were trained with a different pair of objects (Puighermanal et al., 2013) and challenged immediately post-acquisition with the cannabinoid agonist WIN (i.p.). The drug did not alter total object exploration in any group (not shown). However, WIN strongly reduced the object discrimination index in positive controls *CB$_1^{+/+}$*(GFP) mice, but not in negative controls *CB$_1^{-/-}$* (GFP; **Figure 28B)**, confirming that the amnesic effect of cannabinoids in the L-M/OR task depends on CB$_1$ receptors (Puighermanal et al., 2009). Notably, the effect of WIN was fully restored by hippocampal viral re-expression of wild-type CB1 receptors in *CB$_1^{-/-}$*(CB$_1$; **Figure 28B)**. However, *CB$_1^{-/-}$*(DN22-CB$_1$) carrying re-expression of the mutant CB$_1$ protein lacking mitochondrial localization and function remained insensitive to WIN **(Figure 28B)**.

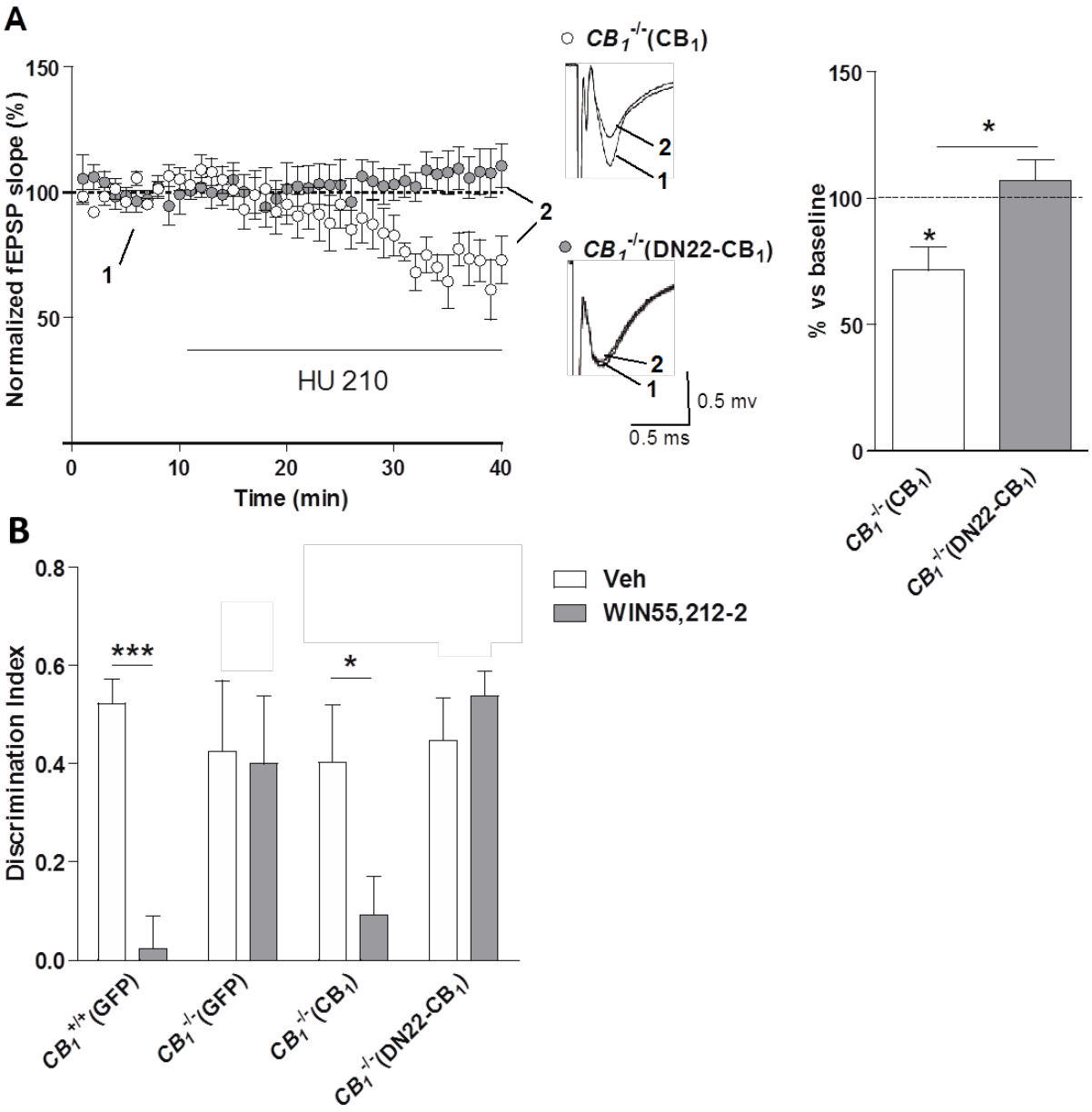

Figure 28. DN22-CB_1 receptors in the hippocampus impair the effects of cannabinoid on glutamatergic transmission and object recognition memory.(A) Effect of HU 210 (2.5 µM) of CA3-CA1 fEPSP slopes in hippocampal slices derived from $CB_1^{-/-}$(CB_1) and $CB_1^{-/-}$(DN22-CB_1) mice. **(Left)** Plots of normalized fEPSP slopes in vitro with representative fEPSP traces before (1) and after HU 210 treatment (2). **(Right)** Histogram summarizing the average change in percentage of fEPSP slopes. CB_1 but not DN22-CB_1 receptors activation decreases fEPSP slope in the hippocampus. **(B)** Cannabinoid-induced alteration of object recognition memory performance is dependent of mtCB_1 activation in the hippocampus. Discrimination index values in the object-recognition test of $CB_1^{+/+}$ (GFP) (n=7), $CB_1^{-/-}$(GFP) (n=16), $CB_1^{-/-}$(CB_1) (n=12) and $CB_1^{-/-}$(DN22-CB_1) (n=11) mice treated with Vehicle (white bars) and the CB1 agonist WIN55,212-2 (5 mg/kg, i.p., grey bars). Data, mean ± s.e.m. * p<0.05, *** p<0.001.

Altogether, these data indicate that hippocampal $mtCB_1$ receptors are necessary for the cannabinoid-induced reduction of excitatory synaptic transmission and for the amnesic effects of cannabinoid drugs.

Conclusion

The present study shows that the direct control of brain mitochondrial activity by hippocampal $mtCB_1$ receptors mediates well-known cannabinoid-induced synaptic and behavioral effects. Several pieces of evidence converge to support this conclusion **(See Graphical abstract Figure 35)**.

Cannabinoids are well known to reduce hippocampal synaptic transmission and to impair object recognition memory (Kano et al., 2009; Puighermanal et al., 2012). Notably, the sAC inhibitor KH7 is able to block these *in vitro* and *in vivo* hippocampal effects of cannabinoids, strongly suggesting that $mtCB_1$ receptor signaling is necessary for these effects. Viral expression of DN22-CB_1 or wild-type CB_1 proteins in the *hippocampus* of $CB_1^{-/-}$ mice revealed that the mutant protein is not addressed to mitochondrial membranes. *In vitro* electrophysiological recordings and *in vivo* object recognition memory assays revealed that CB_1 receptor-dependent decrease of fEPSPs in hippocampal slices and the amnesic effect of cannabinoids on object recognition memory are rescued in $CB_1^{-/-}$ mice bearing viral re-expression of wild-type CB_1 receptor in the *hippocampus*, but not by viral re-expression of the DN22-CB_1 mutant, respectively. Thus, by using several different and independent experimental approaches, we present converging evidence that $mtCB_1$ receptor signaling in the brain is necessary for amnesic effects of cannabinoid drugs, demonstrating an acute role of mitochondrial activity in mnemonic processes.

III. SOLUBLE ADENYLYL CYCLASE (SAC) MEDIATES THE EFFECTS OF INTRACELLULAR CB1 RECEPTORS ON MOTOR CONTROL

It is well established that cannabinoid administration induces decrease of spontaneous locomotor activity and impairment of movement's initiation, also known as catalepsy (Daigle 2011, Monory et al., 2007, Sulcova 1998). These responses are mediated by CB_1 receptors as they are blocked by specific CB_1 receptor antagonists and are absent in *null* $CB_1^{-/-}$ mice (Howlett, 2002; Zimmer et al., 1999; Monory et al., 2007). CB_1 receptor is the main target of cannabinoids in the central nervous system and it is considered as one of the most abundant G protein coupled receptor (GPCRs) in the brain (Herkenham et al., 1990; Marsicano and Kuner, 2008)(Marsicano and Kuner 2008). The basal ganglia circuit, which consists in several nuclei participating in the planning and the control of motor behaviors, is particularly enriched of CB_1 receptors, (Herkenham et al., 1990). Within this circuit, the *Substantia Nigra pars reticulata* (SNr) contains one of the highest densities of brain CB_1 receptors, mainly located at axonal terminals (Herkenham et al., 1990; Wallmichrath and Szabo, 2002a Marsicano, 2008). The use of CB_1 mutant mice helped to reveal the cell specificity of the cataleptic effect produced by cannabinoids. Particularly, D_1-$CB_1^{-/-}$ mice, carrying a CB_1 receptor deletion in dopamine receptor 1 (D_1)-positive cells do not present catalepsy under cannabinoid treatment (Monory 2007).

Additionally, CB_1 receptors are found in several cell populations and different subcellular compartments (Freund and Hajos, 2003; Marsicano and Kuner, 2008; Rozenfeld and Devi, 2011). Our previous studies demonstrated that CB_1 receptors are functionally present at mitochondrial membranes of brain cells [mtCB_1, (Benard et al., 2012; Vallee et al., 2014), and data above in this Thesis)]. Shortly, we have shown that mtCB_1 receptors decrease mitochondrial respiration and complex I

enzymatic activity *via* soluble adenylyl cyclase (sAC)-dependent signaling. Mitochondrion in the brain is a key organelle for maintaining cellular energy homeostasis, but it is also involved in others processes such as calcium buffering, free radicals production and regulation of apoptosis (Mattson et al., 2008)(MacAskill and Kittler, 2010). In addition, impaired mitochondrial function negatively affects synaptic plasticity and leads to cognitive deficits and behavioral abnormalities (Kann and Kovacs, 2007; Rangaraju et al., 2014). Accordingly, mitochondrial dysfunctions are associated with locomotor neurodegenerative diseases, such as Parkinson's disease and Huntington's disease (Chaturvedi and Flint Beal, 2013). However, the direct effect of acute mitochondrial impairment on motor behavior has not been investigated yet.

In this part of the work, I first investigated whether the SNr is the brain region where THC-dependent effects on locomotor performance are exerted. Then, I addressed whether THC acts at plasma membrane or intracellular CB_1 receptors and whether sAC activity is necessary to exert these effects. Finally, as suggested by the anatomical identification of $mtCB_1$ receptors in the SNr, we asked whether acute alteration of brain mitochondrial activity might participate in these effects of THC.

III.1 CB_1 receptors in the SNr mediate THC-induced hypolocomotion and catalepsy

Interestingly, the SNr is one of the brain regions containing the highest levels of CB_1 receptor protein (Herkenham et al., 1991a; Marsicano and Kuner, 2008; Wallmichrath and Szabo, 2002). Immunoflurorescence experiments revealed, indeed, an intense CB_1 receptor in the SNr of $CB_1^{+/+}$ mice, but not in $CB_1^{-/-}$mice **(Figure 29A-B)**. Therefore, I decided to test whether a blockade of CB_1 receptors in this brain region is able to inhibit the motor effects induced by THC. The systemic administration of a high dose of THC (10 mg/kg) induced strong hypolocomotion and catalepsy in C57BL/6-N mice. The local infusion of the specific CB_1 receptor antagonist AM251 into the SNr fully blocked both the cataleptic and the

hypolocomotor effects of THC **(Figure 29C-D)**. This indicates that CB_1 receptor activation within the SNr is necessary for the THC effects on motor performance.

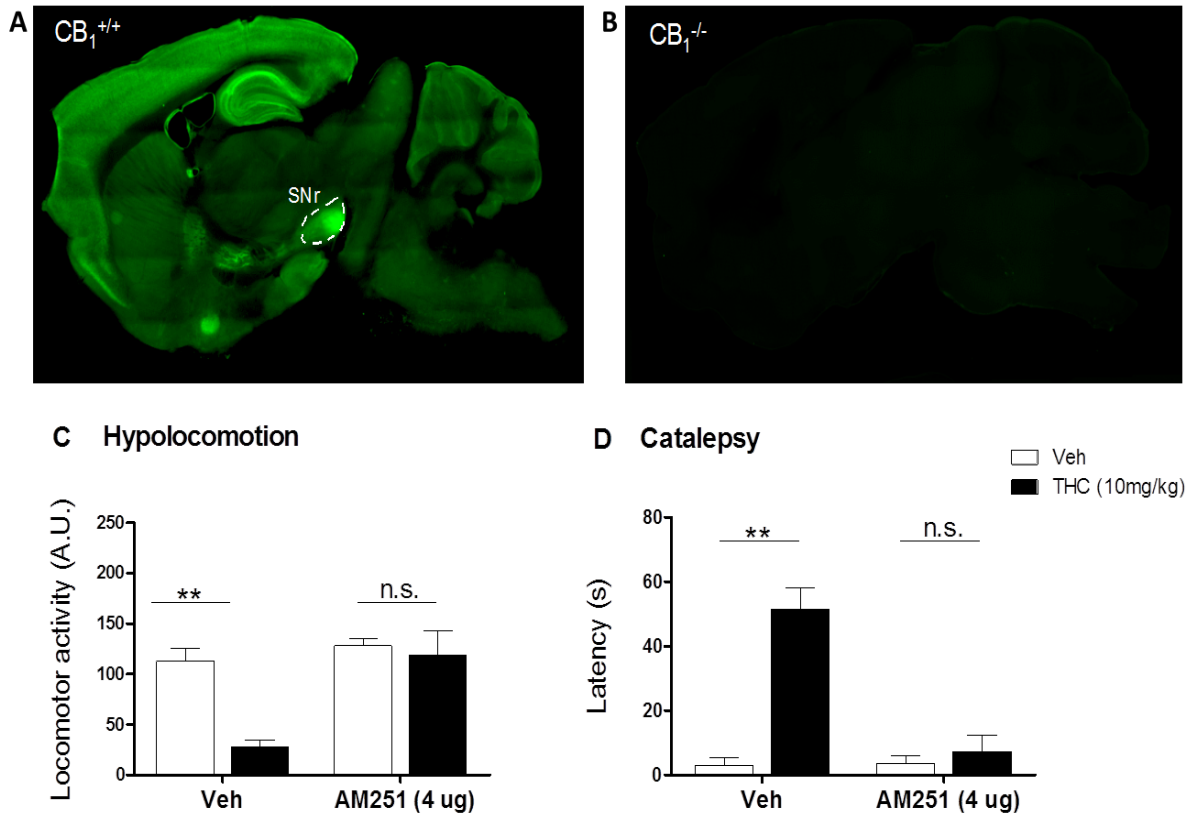

Figure 29. CB_1 receptor activation into the SNr mediates the hypolocomotor and cataleptic effects of THC. Immunofluorescence for CB_1 protein on sagittal sections of $CB_1^{+/+}$ and $CB_1^{-/-}$ mice confirmed the strong expression of CB_1 receptor in the SNr (**A** and **B**, dotted line). **(C-D)** Intra-SNr bilateral injection of AM251 (4µg) blocks the hypolocomotor and cataleptic effects induced by THC (10mg/kg, i.p.) on C57BL/6-N mice (n=5-11). Data, mean ± s.e.m. *p<0.05, ** p<0.01, *** p<0.001

III.2 Subcellular localization of CB_1 receptors determines THC-induced motor effects

III.2.1.1 Effect of Hemopressin, a cell-impermeant CB_1 receptor antagonist, on hypolocomotion and catalepsy induced THC

THC and AM251 are lipophilic compounds, which can easily pass through lipid membranes and, thereby, can activate or antagonize both plasma membrane and

intracellular CB_1 receptors, respectively. To distinguish between the relative impacts of these two subcellular pools of CB_1 receptors on the THC-induced hypolocomotion effects in the SNr, we locally administered the CB_1 receptor antagonist Hemopressin into this brain area. Hemopressin is a peptide compound, which is unable to penetrate intracellular compartments and, thereby, can antagonize only $pmCB_1$ receptors, but not intracellular ones (Benard et al., 2012; Heimann et al., 2007; Rozenfeld and Devi, 2008). Intra-SNr Hemopressin, without any effect by itself, partially blocked the hypolocomotor effect induced by THC (10 mg/kg; **Figure 30A)**. In contrast, Hemopressin administered into the SNr did not reverse the THC-induced catalepsy **(Figure 30B)**.

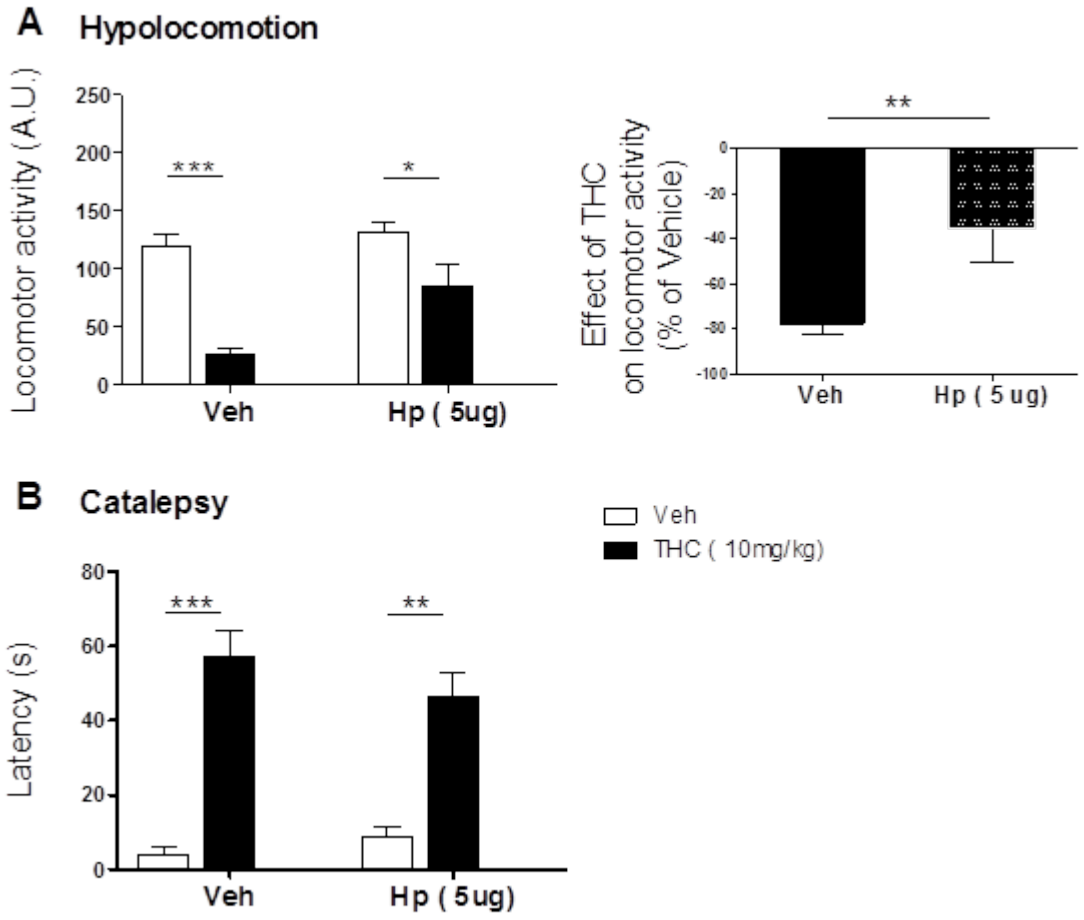

Figure 30. Plasma membrane CB_1 receptors in the SNr partially mediate cannabinoid-induced hypolocomotion. (A; Left) Hemopressin local injections cause a significant, but partial, blockade of THC-induced decrease of locomotor activity. **(Right)** Intra-SNr Hemopressin partially reversed the hypolocomotion induced by THC (10mg/kg, i.p.). (B) Intra-SNr Hemopressin injections do not block the cataleptic effect of THC (10mg/kg, i.p.) on C57BL/6-N mice. Mean ± s.e.m. *p<0.05, *** p<0.001 as compared to vehicle.

III.2.1.2 Presence of mtCB_1 receptors in the SNr

We have previously shown the functional presence of CB_1 receptors on mitochondrial membranes of the *hippocampus* (Benard et al., 2012; Hebert-Chatelain et al., 2014). Thus, we examined the distribution of CB_1 receptors in the SNr using the immunogold electron microscopy. Like in many other brain regions, the majority of CB_1 protein is located at the plasma membrane (pmCB_1) of synaptic terminals of the SNr **(Figure 31A-B)**. However, semi-quantifications of gold particles using strict

parameters to identify mitochondrial-localized immunoreactivity (Hebert-Chatelain et al., 2014) revealed that approximately 10-15% of SNr mitochondria contain CB_1 receptor immunoreactivity ($mtCB_1$) whereas less than 2% of mitochondrial sections showed non-specific immunoreactivity in $CB_1^{-/-}$mice **(Figure 31C)**. Thus, $mtCB_1$ receptors are present also in the SNr and might participate in the hypolocomotor and/or cataleptic effects of THC.

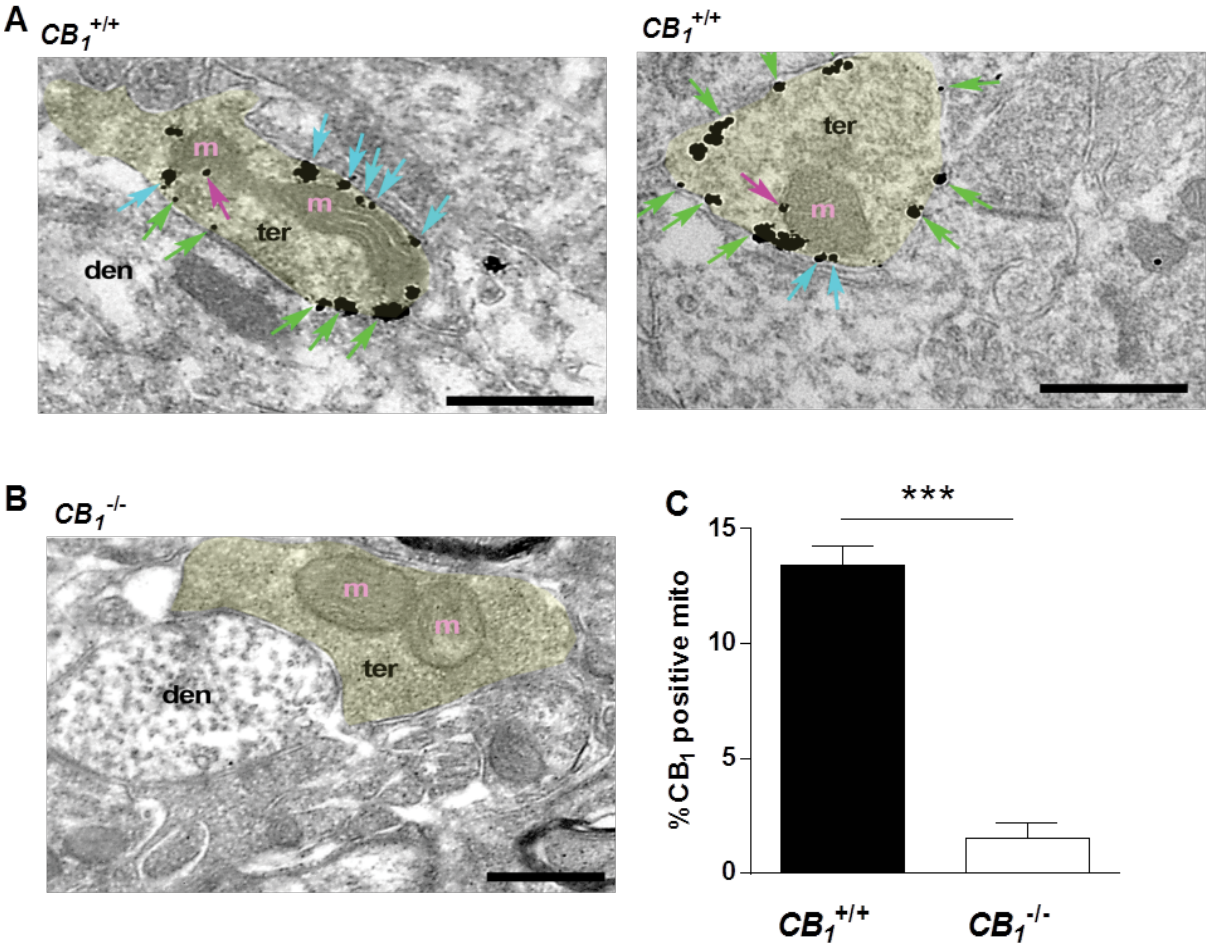

Figure 31.Subcellular distributions of CB_1 receptors in the SNr. (A, B) Immuno electron detection of CB_1receptors in neuronal terminals of the SNr. Pre-embedding silver-intensified immunogold method. **(A)** In $CB_1^{+/+}$ mice, CB_1 immunoparticles are localized on presynaptic terminal (ter) membranes (green arrows) and on mitochondrial (m) membrane segments close to (distance ≤ 80 nm; blue arrows) or far away from other membranes (distance ≥ 80 nm; pink arrows). **(B)** The CB_1 expression pattern is abolished in the SNr from $CB_1^{-/-}$ mice. den: dendrite. Scale bars: 0.5 µm. **(C)** Semi-quantitative analysis of the proportion of CB_1 immunolabeled mitochondria in the SNR from $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. Only mitochondria with particles distant from other membranes (≥ 80 nm) were considered (see panel A). Data are obtained from at least 15 images of each mouse (n=3 per genotype). These data were provided by Pedro Grandes (Bilbao, Spain).

III.2.1.3 Locomotor effects of THC depend on sAC activity in the SNr

As described above, we found that $mtCB_1$ receptor signaling within mitochondria involves the soluble adenylyl cyclase (sAC), and this pathway regulates in vivo amnesic effects induced by cannabinoids *via* $mtCB_1$ receptors. Thus, we

decided to use the sAC inhibitor KH7 to evaluate the potential role of intramitochondrial $mtCB_1$ receptor signaling in THC locomotor and cataleptic effects.

Hypolocomotion

Intra-SNr infusions of KH7 alone (2µg/site) did not affect locomotor activity **(Figure 32A)**, whereas, as expected, THC alone (10 mg/kg, i.p.) induced strong hypolocomotion **(Figure 32A)**. However, the co-administration of systemic THC and intra-SNr KH7 resulted in a significant reduction (albeit incomplete) of this effect, similarly to hemopressin **(Figure 32A)**.

Catalepsy

Intra-SNr infusions of KH7 alone did not induce any observable cataleptic effect, whereas, the systemic injection of THC profoundly reduced the ability of mice to initiate movements **(Figure 32B)**. The combination of the two treatments, however, strongly reduced the cataleptic effect of THC **(Figure 32B)**. These data show that CB_1 receptor-dependent sAC activity within the SNr is necessary for THC-induced catalepsy.

Altogether, these results suggest that activation of both $pmCB_1$ receptors and $mtCB_1$-dependent signaling in the SNr participates in the regulation of hypolocomotor effects of THC. However, only CB_1 receptor-dependent sAC activity within the SNr mediates the THC-induced catalepsy.

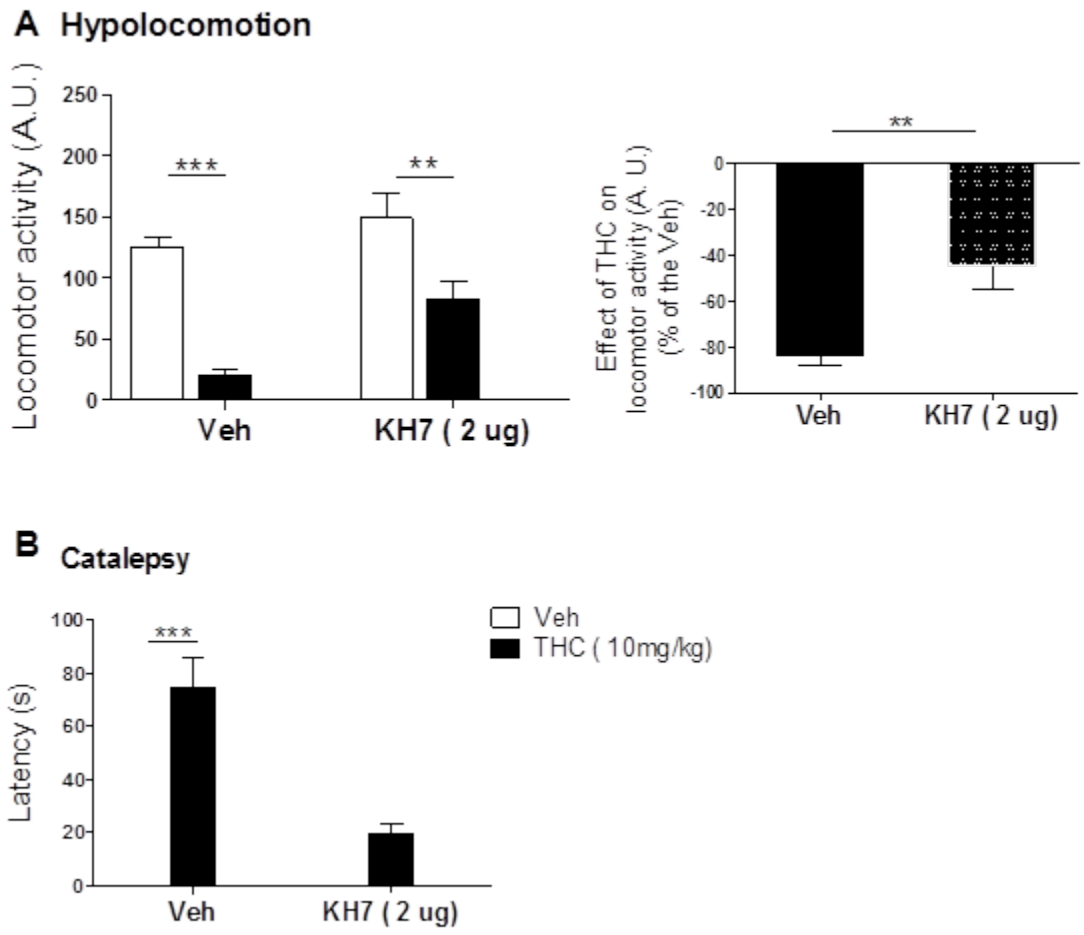

Figure 32.sAC activity in the SNr mediates cannabinoid-induced hypolocomotion and catalepsy. (A; Left) Intra-SNr injection of KH7 causes a significant reduction but partial of cannabinoid-induced decrease of locomotion. **(Right)**Intra-SNr KH7 injections partially reversed the hypolocomotion induced by THC (10mg/kg, i.p.).**(B)** Intra-SNr KH7 infusion fully reverses the cataleptic effect of THC (10mg/kg) Data, mean ± s.e.m. *p<0.05, *** p<0.001 as compared to vehicle.

III.3 Reduction of brain mitochondrial activity facilitates motor effects of THC

Why not using rescue approaches: technical considerations

The results presented so far indicate that intracellular CB_1receptor-dependent modulation of sAC activity in the SNr partially mediates cannabinoid-induced hypolocomotion, whereas it fully accounts for the cataleptic effect of THC.

Considering that sAC is necessary for the effects of mtCB$_1$ receptor activation and that mtCB$_1$ receptors are present at synaptic terminals in the SNr, these data suggest an involvement of mitochondrial processes in these effects. In this specific case, the use of the DN22-CB$_1$ mutant receptor is more difficult than in the case of memory processes in the *hippocampus***(see above)**, because CB$_1$ receptors in the SNr are expressed at neuronal terminals, whose cell bodies are likely located very distally, mainly in the striatum, but possibly also in other brain regions. The striatum is a large region and targeting all neurons projecting to the SNr by local viral injections is an extremely difficult task. Indeed, I made several attempts to re-express CB$_1$ receptors in the dorsal striatum (the striatal part containing higher levels of CB$_1$ transcripts, (Marsicano and Lutz, 1999) of *CB$_1^{-/-}$* mice, but I was never able to rescue the locomotor effects of THC in these mice (data not shown). This could be due to several reasons: (i) Viral vectors can infect only a limited number of neurons in a given brain region. As there is no clear topographical pattern in the distribution of specific nigral-projecting neurons in the striatum, it is virtually impossible to know which terminals of striatonigral neurons expressing CB$_1$ receptors are the ones involved in the locomotor effects of THC. Therefore, the viral CB$_1$ receptor re-expression might simply target the “wrong” neurons in the dorsal striatum. (ii) Previous data suggest that D$_1$R-expressing neurons should be the ones responsible of the cataleptic effects of THC (Monory et al., 2007). Therefore, we might need more specific expression vectors to obtain rescue of THC effects in global *CB$_1^{-/-}$* mice only in certain neuronal subpopulations. (iii) Besides striatal neurons, other neuronal populations projecting to the SNr could be the ones responsible for the locomotor effects of THC. For instance, local interneurons might contain CB$_1$ receptors and participate in the locomotor effects of THC. Moreover, in these re-expression experiments, we focused our attention to the dorsal striatum, because this portion contains the highest amounts of CB$_1$ receptors (Marsicano and Lutz, 1999). However, also neurons located in the ventral striatum (nucleus accumbens) contain moderate, but significant levels of CB$_1$ mRNA (Hermann et al., 2002; Marsicano and Lutz, 1999; Marsicano and Kuner, 2008). A reduction of CB$_1$ receptor expression in the ventral striatum has been described in conditional mutant mice lacking CB$_1$ receptors in D$_1$R-positive striatal neurons has been observed, together with their loss

of THC-induced cataleptic effect (Monory et al., 2007). Considering that accumbal neurons can also directly project to the SNr (Heimer et al., 1991), it is possible that ventral striatal neurons might be the ones to target for viral CB_1 re-expression experiments.

These considerations will be taken into account for future experiments. However, independently of its methodological causes, the lack of rescue of THC locomotor effects in $CB_1^{-/-}$ mice carrying local expression of wild-type CB_1 in the dorsal striatum (not shown) impeded so far the comparison with DN22-CB_1 local expression in the same area.

Effects of central rotenone on THC-induced hypolocomotion and catalepsy

Thus, to test the potential link between the hypolocomotor and cataleptic effects of activation of intracellular SNr CB_1 receptors and mitochondrial processes, we adopted an indirect pharmacological approach. We evaluated whether central mitochondrial alteration by the classical complex I inhibitor rotenone, could impact THC effects on motor performance.

As expected, the systemic administration of 10 mg/kg THC induced strong hypolocomotion and catalepsy in mice **(Figure 33A-B)**. However, a lower dose of THC (2 mg/kg i.p.) was not sufficient to produce any observable effect on locomotion or catalepsy **(Figure 33A-B)**. Similarly, intra-cerebro-ventricular (i.c.v.) injections of a low dose of rotenone (1 µg) did not induce any significant effect **(Figure 33C-D)**. However, the co-administration of rotenone (1 µg i.c.v.) and the low dose of THC (2 mg/Kg i.p.) produced very similar hypolocomotion and catalepsy as the higher dose of THC **(Figure 33C-D**, compared with **Figure 33A-B)**, indicating that alterations of brain mitochondrial activity are able to potentiate these cannabinoid effects. Together with the data presented above, these results strongly suggest a role of mitochondria in the cannabinoid regulation of motor performance, reinforcing the idea that mtCB_1 receptors in the SNr might be responsible of these effects.

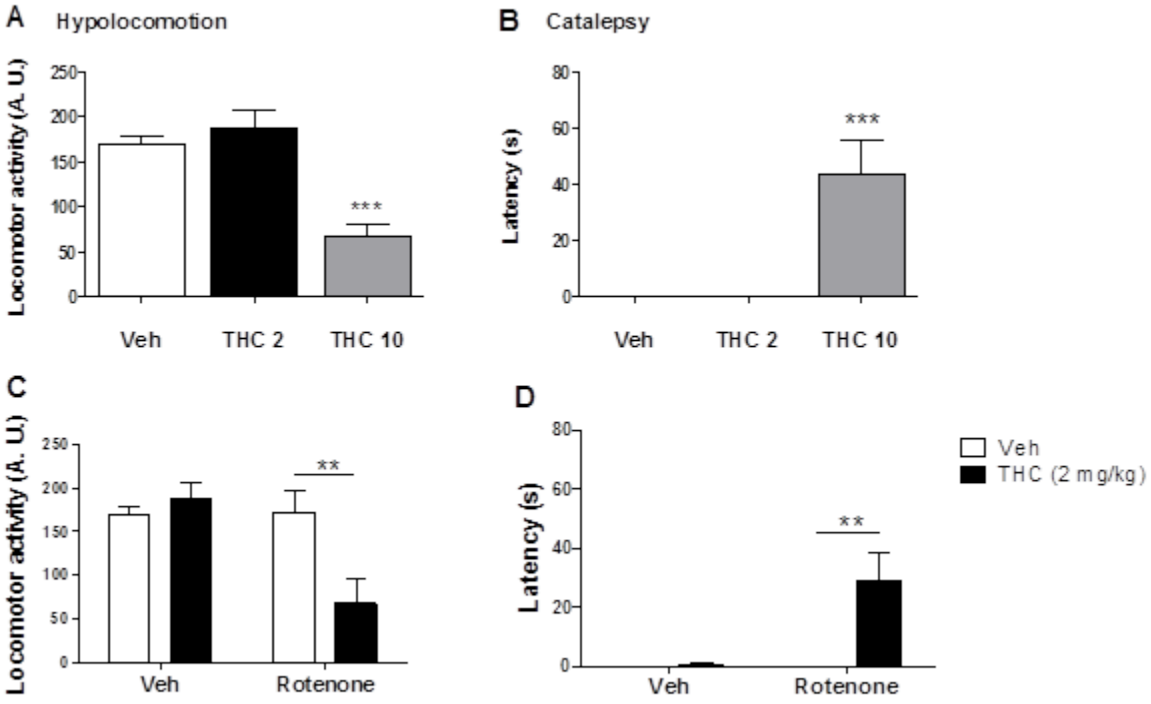

Figure 33.Inhibition of mitochondrial activity potentiates CB_1 dependent hypolocomotion and catalepsy. Effect of THC (2 and 10 mg/kg) on C57BL/6N mice on (A) locomotor activity in an open field, and (B) on immobility in the bar test for catalepsy. Co-administration of rotenone (1 µg icv) and the subthreshold dose of THC (2 mg/kg) produce hypolocomotion (C) and catalepsy (D). Veh, vehicle. Values, mean ± s.e.m. **P < 0.01 as compared to control.

Conclusions

Using systemic and local pharmacology and neuroanatomical approaches, we identified the SNr as a brain region where THC exerts hypolocomotor and cataleptic behavioral effects. Immunogold electron microscopy revealed that CB_1 receptors are present both at plasma and mitochondrial membranes of neuronal terminals in the SNr. Local injections of the cell permeable or impermeable CB_1 receptor antagonists AM251 or Hemopressin, respectively, indicated that intracellular SNr CB_1 receptors are necessary for hypolocomotor and cataleptic effects of THC. Intra-SNr administration of KH7 indicated that sAC signaling (previously shown to depend on

$mtCB_1$ receptor activation) is involved in THC-induced hypolocomotion (partial blockade) and catalepsy (complete blockade). Finally, i.c.v. injections of a *per se* ineffective dose of the mitochondrial inhibitor rotenone were able to potentiate both hypolocomotor and cataleptic effects of THC.

Thus, these data present anatomical and pharmacological compelling evidence suggesting that $mtCB_1$ receptor signaling in the SNr is responsible, at least in part, of locomotor effects of cannabinoids in mice.

DISCUSSION

IV. SUMMARY OF MAIN RESULTS

Cannabinoids affect a variety of functions including appetite, pain, mood, motor control, memory and perception through CB_1 receptors. These receptors are widely distributed throughout the brain and localize at both plasma and intracellular membranes. Since their discovery as classical GPCRs, the impact of CB_1 receptors on behavior is commonly assigned to the receptor pool located at plasma membranes, whereas CB_1 receptor immunolabeling observed within the cell is generally considered evidence of trafficking of the protein (Jong et al., 2014). Recently, our laboratory has demonstrated that a portion of intracellular CB_1 receptors is functionally located at mitochondrial membranes ($mtCB_1$), where they can depress brain mitochondrial activity (Benard et al., 2012). My work aimed at determining to which extent $mtCB_1$ receptors participate to mnemonic and locomotor CB_1-dependent behavioral effects of cannabinoids.

The first challenge of this project was to tease apart the roles of $mtCB_1$ *versus* CB_1 receptors at different cellular location. To this aim, we adopted novel pharmacological and genetic tools. The pharmacological approach was based on the characterization of the intra-mitochondrial signaling cascade linking $mtCB_1$ receptor activation to decrease of respiration. We found that $mtCB_1$ receptor-dependent decrease of OXPHOS depends on the inhibition of the mitochondrial sAC/cAMP/PKA pathway. Specific pharmacological targeting of sAC, using the inhibitor KH7, blocked the effect of $mtCB_1$ receptor activation in cell cultures and in brain isolated mitochondria. In combination, we used Hemopressin, a cell-impermeant CB_1 receptors antagonist, which permits to discriminate the role of $pmCB_1$ *versus*

intracellular CB_1 receptors (Heimann et al., 2007). To further enlarge the tool panel to study $mtCB_1$ receptor functions, we designed a functional mutant protein, DN22-CB_1, which lacks the first 22 amino acids of the CB_1 sequence. Using electron microscopy approach, we confirmed that viral hippocampal expression of DN22-CB_1 receptor is not addressed at the mitochondrial compartment as compared to wild-type CB_1 receptor. Furthermore, cannabinoid treatments of cells expressing DN22-CB_1 did not alter mitochondrial respiration, but they still induced other CB_1 receptor-dependent signaling, such as phosphorylation of ERKs and, importantly, activation of G protein. We further used these pharmacological and genetic tools, to evaluate the potential role of $mtCB_1$ receptors *in vivo* in regulating long-term object recognition memory and motor control. The current data strongly suggest that the direct control of brain mitochondrial activity by $mtCB_1$ receptors in different brain regions mediates well-known cannabinoid-induced behavioral effects including memory impairment and alteration of locomotion. Several pieces of evidence converge to support this conclusion:

1. Electrophysiological recordings in hippocampal slices revealed that KH7 blocks the CB_1 receptor-dependent decrease of fEPSPs. This effect is rescued in $CB_1^{-/-}$ mice by viral re-expression of wild-type CB_1, but not of DN22-CB_1 protein.
2. Intra-hippocampal infusion of KH7 prevents cannabinoid-induced ORM impairment and hippocampal re-expression of wild-type CB_1 rescues this effect of cannabinoids in $CB_1^{-/-}$ mice, whereas DN22-CB_1 lost this ability.
3. Using pharmacology, we identified the SNr as a brain region where cannabinoids exert both cataleptic and hypolocomotor effects. Both these effects are indeed blocked by a membrane permeable lipid CB_1 receptor antagonist (AM251) locally administered into the SNr. However, a membrane impermeable peptide antagonist (Hemopressin) blocks only partially the hypolocomotor but not the cataleptic effect of THC, suggesting the participation of intracellular CB_1 receptors.
4. The sAC inhibitor KH7 administered intra-SNr fully blocks THC-induced catalepsy and partially reduces hypolocomotion, strongly indicating the

participation of $mtCB_1$ receptor signaling in the sedative effects of cannabinoids.

In summary, the data collected during my PhD work causally link the direct impact of cannabinoids on brain mitochondrial functions to some of their most important behavioral effects, such as catalepsy and amnesia. This study establishes that the activation of a G protein coupled receptor can interfere with behavior *via* direct alteration of mitochondrial functions. The importance of bioenergetic processes is emerging as a key and so far rather underestimated element of the complex regulation of brain processes. The dichotomy between mitochondrial-dependent and -independent effects of cannabinoids opens new hopes for a targeted and specific exploitation of the large therapeutic potentials of this class of drugs, possibly avoiding undesired and limiting side effects. In the following, I will discuss more in detail the implications of these results.

V. GENERATION AND VALIDATION OF TOOLS TO STUDY MTCB_1 RECEPTORS

V.1 The presence of mtCB_1 receptors in the brain

Contrary to previous assumptions, functional GPCRs do not permanently reside on the plasma membrane, but signal within the cell on endomembranes (Beninca et al., 2014). GPCRs have a critical involvement in nearly all physiological processes (Rozenfeld and Devi, 2011), but, until recently, the functional presence of GPCRs on mitochondrial membranes was considered a remote and unlikely possibility. Indeed,

GPCRs were described as one of the most important means for cells to convert external stimuli to intracellular signaling, and their localization at plasma membranes represented a logic prerequisite to exert this function (Devi et al., 2005). However, recent data challenged the idea that GPCRs can signal exclusively when located at plasma membranes. To date, GPCRs were discovered on endosomal membranes (β2-adrenergic receptor; Irannejad et al., 2013), ER membranes (Metabotropic glutamate receptor 5 (mGluR5; Purgert et al., 2014), lysosomes (CB_1 receptors; Rozenfeld 2008), nuclei (mGluR5; Purgert et al., 2014) and within mitochondrial membranes (purinergic receptors; Krzeminski et al., 2007). At endosomal locations these receptors can contribute to different molecular cascades and cellular processes, among them ERK1/2 activation (Rozenfeld and Devi, 2008) and cellular cAMP level (Irannejad et al., 2013), synaptic plasticity (Purgert et al., 2014).

Thus, the discovery that metabotropic CB_1 receptors are functionally present in mitochondria (Benard et al., 2012; Hebert-Chatelain et al., 2014; Koch et al., 2015) is a new but not completely unexpected finding. Indeed, other studies have previously observed labeling of mitochondria labeled by CB_1 receptor antisera within the brain (Rodriguez et al., 2001; Sierra et al., 2014) and in peripheral cells, such as sperm and heart cells (Aquila et al., 2010), but the functional implications of these observations were only recently addressed (Benard et al., 2012).

From the anatomical point of view, this study reveals that, in addition to *hippocampus* and *hypothalamus* (Aquila et al., 2010) $mtCB_1$ receptors are present also at synaptic terminals of the SNr. Due to the low levels of $mtCB_1$ receptors, the danger of false-positive results is always present in such immunogold electron microscopic experiments (Morozov et al., 2013) see also below). Therefore, an important aspect to consider for the rigorous identification of $mtCB_1$ receptors is the use of negative controls and strict standard quantitative methods to avoid misidentification. Because several brain mitochondria are found close to other cell membranes, to avoid that CB_1 immunogold particles were wrongly interpreted as $mtCB_1$, restrictive criteria were applied for semi-quantitative analyses (Morozov et al., 2013). By this way, only the particles located on mitochondrial membranes that are at least 80nm (the average dimension of gold particles) far from other membranes were

considered as mtCB$_1$ receptors in the present study. According to this and similarly to other brain regions, approximately 13% of mitochondria of the SNr contained CB$_1$ particles whereas less than 3% were found in tissues from *CB$_1^{-/-}$* mice, clearly confirming the presence of mtCB$_1$ also in this brain region.

V.2 For CB1 receptors, quantity is not always quality

The approximately 10-15% of total CB$_1$ receptors located on the mitochondrial outer membrane of brain cells might seem a limited amount. However, this expression accounts for up to 25% of mtCB$_1$-dependent reduction of respiration in purified brain mitochondria, suggesting a strong impact of cannabinoid signaling onto the specific organelles expressing mtCB$_1$. Moreover, a general rule of CB$_1$ receptor signaling is that even low amounts of the protein can bear crucial functional significance. For instance, less than 1% of total brain CB$_1$ protein is expressed in glutamatergic neurons (Bellocchio et al., 2010; Soria-Gomez et al., 2014), but many of the functions of the ECS are indeed exerted by this limited percentage of receptors. Examples of this "paradoxical" way of functioning of the ECS can be found in the protection against excitotoxic seizure or in the orexigenic properties of endocannabinoid signaling, which are mainly exerted by "glutamatergic" CB$_1$ receptors (Bellocchio et al., 2010; Soria-Gomez et al., 2014). Therefore, 15% of brain total CB$_1$ protein located in the mitochondrion cannot be considered a negligible amount from a functional point of view.

V.3 New discoveries generate controversy

Considering that GPCRs are generally considered to be functionally present predominantly at plasma membranes, the discovery of mtCB$_1$ receptors attracted the interest of the scientific community. Some authors performed functional studies in isolated mitochondria suggesting the existence of both CB$_1$ receptor-dependent and –independent effects of cannabinoids on OXPHOS activity (Fisar 2014). Another group performed a study directly challenging the discovery, claiming that CB$_1$ receptors labeled on mitochondria are likely due to the unspecific binding of CB$_1$

antisera to the mitochondrial stomatin-like protein 2 (Slp2), instead of CB_1 protein (Morozov et al., 2013). An essential missing part in this study was the use and quantification of negative controls for determining antisera specificity, especially when highly sensitive methods are used. As described above, $CB_1^{-/-}$ brain tissues display a dramatic reduction in $mtCB_1$ protein labeling (Benard et al., 2012; Hebert-Chatelain et al., 2014), clearly indicating the specificity of the antisera used in the experiments. Nevertheless, the possibility that the decrease in the immunolabeling in $CB_1^{-/-}$ might reflect a reduction or a loss of Slp2 proteins due to off-target binding of CB_1 antisera cannot be neglected. For this purpose, Western immunoblotting experiments using an antiserum against Slp2 was performed and revealed no differences in the amount of Slp2 protein in brain extracts from wild type and $CB_1^{-/-}$ mice (Hebert-Chatelain et al., 2014). Thus, these results clearly indicate that the deletion of the *CB1* gene does not alter the expression levels of a potential off-target of anti-CB_1 antisera and confirm the finding of $mtCB_1$ receptors. Nowadays, the presence of $mtCB_1$ receptors seems to be accepted by the scientific community, as the same researchers who raised the controversy recently confirmed the presence of $mtCB_1$ receptors in the *hypothalamus* (Koch et al., 2015).

V.4 $mtCB_1$ receptor activation inhibits OXPHOS and ATP production

The negative relationship between cannabinoids (eCBs and synthetic cannabinoids) and mitochondrial functions is well documented (Badawy et al., 2009; Bartova and Birmingham, 1976; Silva et al., 2012; Zaccagnino et al., 2011). These results can now be explained, at least in part, by the impact of $mtCB_1$ receptors on endogenous respiration (Benard et al., 2012; Hebert-Chatelain et al., 2014; Vallee et al., 2014). In addition, high doses of cannabinoids also depress mitochondrial OXPHOS by a non-receptors mechanism (Fisar, 2010). Indeed, cannabinoids are lipophilic compounds that accumulate within cell membranes and they can negatively impact on mitochondrial activity by altering membrane fluidity (Fisar, 2010; Velenovska and Fisar, 2007). Moreover, unpublished results from our laboratory showed that higher doses of cannabinoids start having effects also on brain

mitochondrial fraction from *$CB_1^{-/-}$*, indicating a nonspecific effect. Therefore, both receptor- and non- receptor-mediated mechanisms of cannabinoid action on mitochondrial respiration are possible (Fisar, 2010). Thus, rigorous experimental approaches are needed to differentiate receptor-dependent and –independent effects of exogenous cannabinoids.

Previous reports have found a reduction of *ex vivo* hypothalamic and brain mitochondrial respiration after systemic injections of cannabinoids in mice (Benard et al., 2012; Koch et al., 2015), supporting the view that cannabinoids decrease *in vivo* brain mitochondrial OXPHOS activity. However, the effect observed in isolated mitochondria might be different from that taking place in intact cells, where cellular networks and environment are undisturbed, representing a more physiologically relevant model for experiments on metabolism (Althoff et al., 2011). Here, we demonstrated that cannabinoid treatments decrease, in a CB_1-dependent manner, rotenone-sensitive endogenous respiration by approximately 25 % in living hippocampal cells. Consistently, cellular and mitochondrial ATP levels were decreased in the cells after cannabinoid treatments. Cellular ATP levels mostly result from the concomitant actions of glycolysis, Kreb's cycle activity and OXPHOS. The inhibition of OXPHOS, likely causing a decrease in mitochondrial ATP production, also impacts on the total cytosolic ATP levels. Thus, other metabolic sources of ATP such as glycolysis are not sufficient to compensate the $mtCB_1$ receptor-dependent reduction of cellular ATP levels (approximately 35%), indicating that a decrease of mitochondrial respiration *via* $mtCB_1$ receptor signaling is associated with an important reduction of cellular ATP levels that could significantly impact on the cell physiology.

V.5 G protein signaling in the mitochondria

The alteration of rotenone-sensitive endogenous respiration induced by $mtCB_1$ receptors on isolated brain mitochondria is associated with a decrease in cAMP levels and in PKA and complex I activities (Benard et al., 2012). Whether these effects were mediated by $G\alpha_{i/o}$-dependent signaling and through which molecular partner was still unknown.

Our present data established that $mtCB_1$ receptor-dependent inhibition of mitochondrial $G\alpha_{i/o}$ proteins mediates a decrease of mitochondrial cAMP levels and PKA activity, resulting in the inhibition of Complex I activity and respiration. Thus, seven transmembrane receptors located at brain mitochondria can stimulate intra organelle G protein signaling. It is well established that $pmCB_1$ receptors can be coupled and signal through different types of Gα, including $G\alpha_i$, $G\alpha_o$, $G\alpha_q$ and $G\alpha_s$ (Turu and Hunyady, 2010). Our data show that the decrease of mitochondrial bioenergetic processes induced by $mtCB_1$ receptor activation depends on their coupling with $G\alpha_{i/o}$ proteins. The functional coupling of $mtCB_1$ receptors to $G\alpha_{i/o}$ is further supported by the finding that Gα proteins co-immunoprecipitate with $mtCB_1$ receptors in purified brain mitochondria and by the ability of THC to decrease this interaction. As pointed out in the Chapter 3 of this work, this molecular mechanism is in agreement with recent data showing the presence of G protein in mitochondria and other intracellular membranes (Andreeva et al., 2008; Beninca et al., 2014; Kuyznierewicz and Thomson, 2002; Lyssand and Bajjalieh, 2007; Purgert et al., 2014). Thus, G protein-mediated signaling, previously considered an exclusive inter-cellular communication mechanism, is emerging as a universal mechanism used to induce a chain of cellular cascades at different cellular membranes. In this context, the intracellular functional localization of CB_1 receptors is particularly interesting, because the main endogenous and exogenous ligands of these receptors are lipid molecules, which can easily penetrate cells and move along intracellular membranes. Thus, (endo)cannabinoids reaching cells from outside or generated within cell membranes might directly alter mitochondrial activity, thereby directly linking extracellular or intracellular signals to cellular bioenergetic status (Benard 2012). Whether CB_1 receptors can be functionally present at other intracellular membranes such as the ER or the nucleus is still an open question, and new tools and approaches will be required to study intracellular CB_1 receptors. Indeed, the potential of intracellular and mitochondrial GPCRs signaling as regulators of cellular functions has been neglected for a long time (Belous et al., 2006), likely because of the technical difficulties in isolating intracellular GPCRs from total GPCRs signaling. This limitation mainly originates from the lack of methods to exert spatial control over GPCRs inside a living cell (O'Neill, 2014). In this work, we introduced innovative

pharmacological (KH7) and genetic tools (DN22-CB_1 protein) in order to directly study mtCB_1 receptor functions.

V.6 Downstream effectors of G proteins coupled to mtCB_1 receptors: the role of sAC

CB_1 receptors modulate brain functions through different signaling pathways including, among others, inhibition of transmembrane adenylyl cyclase, modulation of ion channels and activation of mitogen-activated and focal adhesion kinases (Howlett, 2005; Piomelli, 2003). Our results indicate a novel signaling pathway within mitochondria: activation of mtCB_1 receptors decreases mitochondrial respiration and complex I activity through inhibition of the soluble form of AC and PKA activity. Indeed, we showed that mitochondrial respiration was blocked by the sAC activator bicarbonate and occluded by the sAC inhibitor KH7. In this line, co-immunoprecipitation of Gα proteins with sAC from purified brain mitochondria revealed a physical interaction between mitochondrial sAC and Gα proteins in the brain, which was even promoted by mtCB_1 receptor activation. Additionally, targeting of sAC into mitochondria rescues the decrease of O_2 consumption induced by cannabinoids. Overall, these findings clearly established the causal role of sAC activity in mediating the effect of mtCB_1 receptor signaling on OXPHOS within mitochondria. To date, this study is the first to report a direct modulation of sAC activity *via* heterotrimeric G-protein alpha subunits present in the mitochondria. This is actually surprising, because sAC was shown to be independent from G protein signaling in certain tissues, such as testis (Buck et al., 1999). This apparent discrepancy might underlie tissue-specific differential regulatory pathways of sAC activity.

We showed that sAC inhibition induced by cannabinoids down-regulates cAMP level and leads to the decrease of PKA activity in the mitochondria. Indeed, both changes in cAMP levels and decrease of PKA activity were blocked by PTX, bicarbonate or KH7. Thus, the mitochondrial cAMP/PKA pathway appears to be involved in the decrease of complex I activity and respiratory activity produced by cannabinoids **(Figure 34)**. The presence of the different components of this pathway

in the mitochondria has been demonstrated in numerous papers (Acin-Perez et al., 2009b; Means et al., 2011; Sardanelli et al., 2006; Zippin et al., 2003) and their regulatory role on the complex I activity and on respiration has been clearly established (Acin-Perez et al., 2009b; Papa et al., 2012).

The identification of which respiratory chain complex is targeted by mitochondrial sAC signaling is still under investigation. While cAMP/PKA pathway has been showed to participate in the phosphorylation and consequently in the maintenance of the complex I subunit NDUFS4 (De Rasmo et al., 2010; De Rasmo et al., 2015; Scacco et al., 2003), others have suggested that the complex IV, cytochrome c oxidase (COX), is an additional direct target of PKA phosphorylation (Acin-Perez et al., 2011). These proteins contain a PKA serine consensus site and genetic defects in the phosphorylation site of NDUFS4 or protein mutagenesis at Ser-85 of COX subunit IV-1 impair OXPHOS efficiency (De Rasmo 2010, Acin-Perez 2011a). Preliminary data from our laboratory indicate that $mtCB_1$ activation reduces mitochondrial PKA-dependent phosphorylation of the complex I subunit NDUFS2, suggesting that this downstream mechanism might ultimately mediate the direct cannabinoid effects on OXPHOS (Hebert Chatelain et al., *in preparation*).

Overall, the identification of a mitochondrial sAC signaling pathway clearly provides a mechanism for phosphorylation of mitochondrial enzymes implicated in OXPHOS. Further research is needed to explore whether the mitochondrial sAC pathway regulates additional intra-organelle processes and to complete the characterization of $mtCB_1$ receptor signaling. These studies will likely reveal additional mitochondrial signaling pathways and will extend our knowledge on how GPCRs can modulate mitochondrial bioenergetics processes.

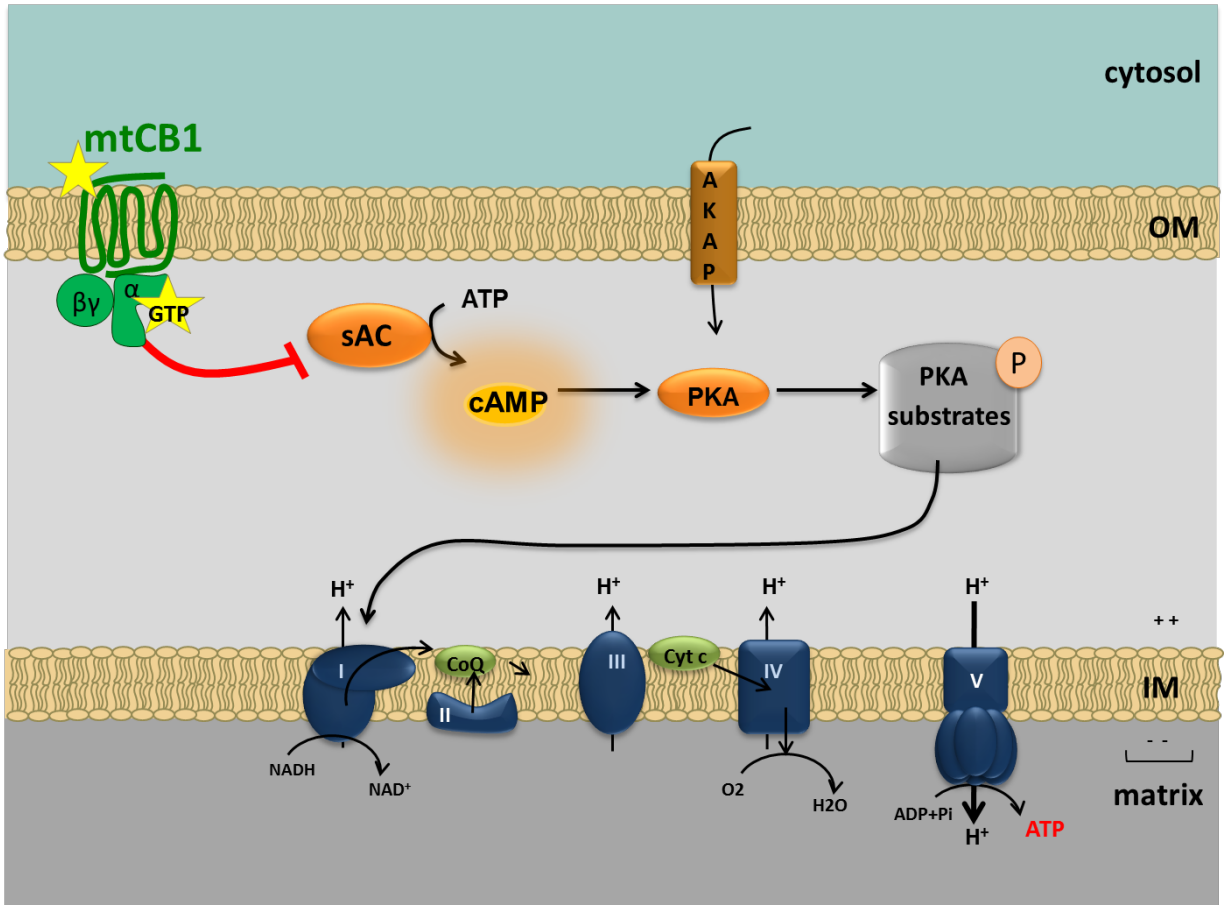

Figure 34.Soluble adenylyl cyclase (sAC) mediates the effects of mtCB$_1$ receptor activation.

V.7 mtCB$_1$ receptor-dependent function could rely on its cellular distribution

As mentioned earlier, ex vivo brain mitochondrial respiration is modulated by CB$_1$ receptor agonists in wild type (Koch et al., 2015), but not in *CB$_1^{-/-}$* mice (Benard et al., 2012). Interestingly, it was found that high doses of the CB$_1$ receptor agonist, ACEA, negatively impact on mitochondrial respiratory activity whereas low doses induce an opposite effect, enhancing respiration (Koch et al., 2015). Such biphasic effects of cannabinoids have been already reported *in vivo* and were attributed to CB$_1$ receptor activation on different cell types or brain regions (Bellocchio et al., 2010; Metna-Laurent et al., 2012; Soria-Gomez et al., 2014). As previously mentioned, CB$_1$

receptors are expressed on many cell types (Domenici et al., 2006; Haring et al., 2007; Hermann et al., 2002; Marsicano and Kuner, 2008; Marsicano and Lutz, 1999). The use of conditional mutant mice allows controlling for the cell-type specificity of CB_1 receptor activation or repression. These genetic tools allowed revealing that several cannabinoid-dependent behavioral, cellular or molecular effects are differentially mediated by CB_1 receptors expressed on GABAergic or glutamatergic neurons or others. As an example, a bimodal control of food intake by CB_1 receptors was recently shown. Whereas CB_1 receptor activation in glutamatergic neurons promotes food intake, a contrary hypophagic function is exerted by CB_1 receptor signaling within GABAergic neurons (Bellocchio et al., 2010). Interestingly, "glutamatergic" CB_1 receptors are pharmacologically activated by low doses of exogenous cannabinoids to increase food intake, whereas higher doses exert hypophagic effects *via* "GABAergic" CB_1 receptors. Accordingly, analysis of cannabinoid-stimulated [35S]GTPγS binding in hippocampal extracts showed that "glutamatergic" CB_1 receptors are more efficiently coupled to G protein signaling than GABAergic CB_1 receptors (Steindel et al., 2013), providing a potential molecular and cellular mechanism for the biphasic effects of cannabinoids (Bellocchio et al., 2010; Metna-Laurent et al., 2012). $MtCB_1$ receptors are present in both GABAergic and glutamatergic neurons (Benard et al., 2012). Moreover, both terminal and somatodendritic neuronal compartments contain $mtCB_1$ receptors (Benard et al., 2012). Hence, $mtCB_1$ receptor signaling could vary in function of its subcellular and cellular expression, thereby increasing the physiological repertoire of possible CB_1 receptor responses to single molecules. Thus, it is possible that $mtCB_1$ receptors could exert a different role in synaptic and behavioral functions modulated by cannabinoids according to their cell-type localization.

As a following of the present work, the question of whether $mtCB_1$ receptors can have potentially a cell-type differential effect on OXPHOS was addressed. As detailed in the first chapter, CB_1 receptors are functionally present in many brain cell types, including GABAergic and glutamatergic neurons, but also in astrocytes (Bosier et al., 2013; Han et al., 2012). Thus, unpublished experiments from Etienne Hebert-Chatelain in the laboratory have investigated the role of astrocytic $mtCB_1$ receptors. Indeed, preliminary results indicate that the impairment of O_2 consumption following

cannabinoid applications is absent in brain mitochondria derived from mice lacking CB_1 receptors from glial fibrillary acidic protein (GFAP)-expressing cells (including brain astroglial). Although cannabinoid effects through neuronal $mtCB_1$ cannot be discarded at this stage, this interesting finding suggests that astroglial mitochondria might respond differently to $mtCB_1$ receptor activation.

Interestingly, it appears that mitochondria have cell-type-specific functional and morphological characteristics. For example, the *cristae* of astrocytic and neuronal mitochondria are different, presenting a parallel arrangement in neurons and a random arrangement in astrocytes (Kristian et al., 2006). Functionally, a higher respiration rate and complex I activity is found in astrocytic mitochondria as compared to neuronal mitochondria (Bolanos et al., 1995; Kristian et al., 2006). Interestingly, during short-term bioenergetic inhibition, astrocytic but not neuronal mitochondria exhibit characteristic morphological changes such as swelling (Gerencser et al., 2008), suggesting that astrocytes might be more sensible to oxidative stress as compared to neurons. Moreover, the $mtCB_1$ receptor effect on respiration is mediated by the reduction of sAC activity, which has been found to be 5 fold higher in glial cells as compared to neurons in cerebellum tissue (Chen et al., 2013). Hence, the intrinsic difference of mitochondria among the cell type needs further attention. The understanding of the respective role of astrocytic *versus* neuronal $mtCB_1$ receptors in cannabinoid impairment of OXPHOS will require the use of advanced tools in future studies. For instance, the generation of conditional knock-in mice, expressing DN22-CB_1 instead of wild-type CB_1 in specific cell type will allow directly tackling many important questions in this new field **(see below Perspectives)**.

V.8 KH7, a tool to investigate the *in vivo* $mtCB_1$ receptor dependent function

So far, sAC is the only AC known to be present in mitochondria (Valsecchi et al., 2013) and the present data showed that silencing of cellular sAC activity by KH7 fully abolished the decrease of respiration induced by cannabinoids. Therefore, KH7 was used to study the role of $mtCB_1$ receptors *in vitro* and *in vivo* on different

cannabinoid-induced effects. However, it is important to keep in mind that while numerous studies have found sAC within mitochondria (Acin-Perez et al., 2009b; Kumar et al., 2009; Zippin et al., 2003), its presence was also observed in other subcellular compartments such as the cytoplasm (Zippin et al., 2003) and the nucleus (Zippin et al., 2003). As all pharmacological tools, the only commonly used sAC inhibitor KH7 might exert non-specific effects. Thus, careful interpretation has to be drawn when using KH7, and other pharmacological or genetic tools should be employed to confirm the implication of mtCB_1 receptors. Other structurally different sAC inhibitors start being generated by several industrial or academic laboratories, and their use will be instrumental in future experiments. Cell permeable or impermeable CB_1 receptor ligands can provide indications on the involvement of plasma membrane or intracellular CB_1 receptors (Benard et al., 2012; Heimann et al., 2007), present work). However, the most direct evidence for a role of CB_1 receptors at mitochondrial membranes can be achieved by specifically excluding this location. This was obtained by generating the DN22-CB_1 mutant protein.

V.9 Generation of a truncated form of CB_1, the DN22-CB_1

To overcome potential caveats of pharmacological tools and enlarge the tool panel to study mtCB_1 receptor functions, we identified and generated a mutant protein, DN22-CB_1, which lacks the first 22 aminoacids of the CB_1 receptor sequence. Viral expression of DN22-CB_1 or wild-type CB_1 protein in the *hippocampus* of $CB_1^{-/-}$ mice demonstrated that DN22-CB_1 is virtually absent from mitochondrial membranes, thereby providing a genetic tool to dissect the functions of mtCB_1 from other localizations of the receptor.

Our data indicate that the first 22 aminoacid of the CB_1 protein are necessary for mitochondrial localization of the receptor. The reasons of this function are not clear at the moment, but two possibilities can be identified: (i) These 22 amino-acids (N22) might serve as a mitochondrial leading sequence for CB_1 receptors to be transported to the mitochondria. (ii) The N-terminal truncation might help the stabilization of the receptor at the plasma membrane, as proposed by other authors (Andersson et al., 2003; Fay and Farrens, 2013). Specific studies will be necessary

to test whether either or both these hypotheses are correct. On one hand, preliminary data from our laboratory suggest that N22 might be not sufficient to lead an exogenous protein (GFP) to mitochondria (data not shown). However, this does not exclude that the N22, which plays a necessary role for mitochondrial CB_1 receptor localization, might co-operate with other CB_1-specific sequences to favor such trafficking process. On the other hand, some studies suggested that deletion of different portions of the N-terminal part of the CB_1 protein does not impair signaling, but “stabilize” the protein at the plasma membrane (Andersson et al., 2003; Fay and Farrens, 2013). In this sense, N22 might inhibit plasma membrane stability of the protein, rather than addressing it to other locations. However, the interpretation of those data was embedded by the concept that the “natural” localization of GPCRs is the plasma membrane. In this sense, those authors used the word “stabilization”. However, if we hypothesize that CB_1 receptors, after translation can be addressed to different cellular locations, the data described above can be re-interpreted as “proportional increase” of plasma membrane localization, due to the lack of mitochondrial targeting, rather than "stabilization". For sure, the mechanisms through which CB_1 receptors are targeted to mitochondria, plasma membranes or other cellular location is a very difficult and challenging issue, which will require specific and detailed future studies.

However, in the present study, we could show that DN22-CB_1 is functional (P-ERK and G protein activation), but it lacks mitochondrial localization. Consistently, it is not able to mediate cannabinoid-induced alteration of OXPHOS. Thus, these data represent a strong genetic indication that the effect of cannabinoids on mitochondrial respiration can be fully attributed to specific activation of mtCB_1 receptors. Importantly, the lack of respiratory effects and of mitochondrial localization makes of the DN22-CB_1 mutant protein an optimal tool to study the specific impact of mtCB_1 receptors on cellular and behavioral functions.

V.10 Effect of mtCB$_1$ receptors activation on neurotransmission and behavior

V.10.1 Activation of mtCB1 receptors impairs excitatory synaptic activity

V.10.1.1 SYNAPTIC TRANSMISSION: A HIGH ENERGY DEMAND PROCESS

Cannabinoids are well known to reduce hippocampal synaptic transmission and to impair ORM (Kano et al., 2009; Puighermanal et al., 2012; Puighermanal et al., 2009). Neurotransmitter release is a very energy-demanding process, and mitochondria can modulate its efficiency by different means (Mattson et al., 2008). Accordingly, mitochondrial dysfunctions generate electrophysiological and behavioral abnormalities. For example, conditional deletion of Cytochrome c oxidase restricted to cortical fast-spiking parvalbumine interneurons, a type of neuron which has particularly high energy utilization, induces a decrease in inhibitory synaptic transmission (Kann et al., 2014). Moreover, in these mutants mice, the impairment of synaptic activity correlated with behavioral dysfunctions including lack of sensory gating, increased anxiety and impaired sociability, indicating that the bioenergetic impairment greatly influence behavior (Inan et al., 2015).In addition, as seen in the introduction, presynaptic mitochondrial activity and ATP synthesis are crucial for the maintenance of synaptic activity and plasticity (Guo et al., 2005; Ma and Blenis, 2009; Verstreken et al., 2005). ATP participates in various synaptic processes such as restoration of the membrane resting potential following depolarization, mobilization of synaptic vesicles, neurotransmitter recycling and axonal and dendritic protein trafficking (Attwell and Laughlin, 2001; Wong-Riley, 2010). The use of cell-impermeant CB$_1$ receptor ligands and mitochondria-interfering drugs previously provided evidence that inhibition of mitochondrial activity *via* mt*CB$_1$* receptor stimulation are partially involved in the eCBs-dependent control of short-term synaptic plasticity at inhibitory hippocampal GABAergic synapses (Benard et al., 2012). Notably, however, those experiments also revealed that cell permeant and impermeant CB$_1$ receptor agonists (HU210 and HU 210-biotin, respectively) were equally able to pharmacologically reduce inhibitory basal synaptic transmission,

suggesting that intracellular CB_1 receptors (including mitochondrial ones) are possibly not necessary for the exogenous cannabinoid-dependent negative regulation of GABAergic synaptic release (Benard et al., 2012). In contrast, the present data show that cannabinoids cause an $mtCB_1$-dependent inhibition of field excitatory transmission at CA3–CA1 synapses. Taken together, these data clearly suggest that the regulation of mitochondrial activity by $mtCB_1$ receptors participate in the decrease of excitatory synaptic activity and in the eCB-dependent inhibitory plasticity in the *hippocampus* (Benard et al., 2012). A recent study on energy demand of hippocampal synaptic transmission has revealed a considerable contribution of presynaptic processes, which consume approximately 50% of the O_2 (Liotta et al., 2012). Considering that CB_1 receptors are highly dense at presynaptic level (Marsicano and Kuner, 2008) both at plasma and mitochondrial membranes (Benard et al., 2012), our demonstration that $mtCB_1$ receptor activation decreases ATP production allows speculating that this mechanism might be responsible for the decrease of excitatory synaptic activity induced by cannabinoids. Accordingly, recent studies clearly demonstrated that even temporary interruptions of local ATP supply profoundly alter synaptic functions in neurons (Rangaraju et al., 2014).

V.10.1.2 PRE OR POST-SYNAPTIC MTCB1 RECEPTORS

The portions of hippocampal mitochondria carrying $mtCB_1$ receptors are similar in axonal terminals and somatodendritic compartments (Benard et al., 2012). Interestingly, somatodendritic CB_1 receptors have been proposed to play a necessary role in autocrine slow self-inhibition (SSI) induced by depolarization of GABAergic and glutamatergic cortical neurons (Bacci et al., 2004; Marinelli et al., 2009). Considering that many authors agree that somatodendritic CB_1 receptors are virtually never localized at plasma membrane, these functions are likely to ascribe to intracellular CB_1 receptors (Sierra et al., 2014). Thus, the interesting possibility emerges that somatodendritic $mtCB_1$ receptors might be involved in the CB_1-dependent autocrine regulation of neuronal excitability (Bacci et al., 2004; Marinelli et al., 2009).

V.10.1.3 ROLE OF MTCB_1 RECEPTORS IN EXCITATORY *VERSUS* INHIBITORY BASAL TRANSMISSION

The fact that mtCB_1 receptor activation is possibly not required for cannabinoid control of inhibitory basal transmission (Benard et al., 2012), but it is necessary for cannabinoid-induced decrease of excitatory transmission is quite surprising. What can explain this differential impact of mtCB_1 signaling on basal excitatory *versus* inhibitory transmission? Assuming that mtCB_1 receptors effects on neurotransmission are due to a depletion of mitochondrial ATP synthesis, a possibility to take into account would be that these two different synaptic processes require a differential ATP budget, making them more or less sensible to the shortage of energy production. Indeed, the energy budget can vary depending on the subcellular brain compartment (*e.g.* somatic/synaptic, pre-/postsynaptic), cell type, cell activity, synaptic processes (*e.g.* network oscillation, basal transmission, plasticity) and environmental conditions (Attwell and Laughlin, 2001; Kann et al., 2011; Wong-Riley, 2010). For example, excitatory synapses are predicted to consume more energy as compared to inhibitory ones. Indeed, in the mature brain the chloride reversal potential is close to the resting potential so restoring postsynaptic Chlore (Cl-) gradients uses less energy than for Na+ gradients (Howarth 2010). It is therefore possible that, due to unequal energy budgets of these processes, mtCB_1 receptor signaling might differentially affect glutamatergic and GABAergic neuronal transmission. However, more powerful and precise tools (*e.g.* conditional cell type-specific DN22-CB_1 knock-in mutant mice) will be necessary to gather detailed information on the differential involvement of mtCB_1 receptors in distinct synaptic processes.

V.10.1.4 POTENTIAL POST-MITOCHONDRIAL EVENTS LINKING MTCB_1 RECEPTORS TO DECREASE OF SYNAPTIC ACTIVITY AND BEHAVIOR

Unraveling the link between a specific mitochondrial process and its consequences on neuronal function is very challenging, mostly because different mitochondrial functions are interdependent (Graier et al., 2007; Gunter and Sheu, 2009). Thus, experimental results obtained using drugs or mutations that interfere with only one aspect of mitochondrial physiology need to be interpreted with care

(Vos et al., 2010). Indeed, by providing ATP, regulating RedOx potentials, metabolizing neurotransmitters, providing Ca^{2+} clearance and many other mechanisms, mitochondria exert myriads of functions that are potentially directly linked to synaptic activity and brain functions (Mattson et al., 2008). Thus, we still do not know which "post-mitochondrial" effects link $mtCB_1$ receptor activation to the observed alterations of synaptic and behavioral functions. Because ATP synthesis, one of the major roles of mitochondria, is altered by $mtCB_1$ receptor activation, it is tempting to hypothesize that this mechanism might be implicated in cataleptic, hypolocomotor and amnesic effects of cannabinoids.

As mentioned above, however, mitochondria exert many different functions, which are all potentially suitable to modulate brain processes, and are directly or indirectly linked to OXPHOS activity and ATP production. As $mtCB_1$ receptor physiology is a recent field of research, very little is known concerning the possible "post-mitochondrial" events involved in its functions. Our present data indicate that the modulation of sAC activity by $mtCB_1$ receptors is responsible for cannabinoid-induced decrease of excitatory hippocampal transmission, impairment of ORM and alteration of motor performance. However, other complementary or alternative molecular mechanisms previously described to depend on CB_1 receptor activation might be linked to mitochondrial activity and, thus, might involve $mtCB_1$ receptors. Among them, I will discuss the interplay between CB_1 receptor signaling and mTOR, ROS and nitric oxide (NO) that are modulated by brain CB_1 receptors (Donadelli et al., 2011; Nunn et al., 2012; Puighermanal et al., 2009). Importantly, mTOR, ROS and NO signaling have been shown to modulate both mitochondrial functions and synaptic activity and behavior (Morita et al., 2015).

mTOR forms two distinct complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2). In particular, mTORC1-dependent signaling regulates different processes such as protein synthesis, cell growth, autophagy and mitochondrial activity (Betz and Hall, 2013; Morita et al., 2013). Activation of CB_1 receptors in the brain was recently shown to up regulate the mTORC1 pathway, underlying the amnesic effect of cannabinoids in hippocampal dependent tasks (Puighermanal et al., 2009). Considering that a strong link exists between mTORC1 and mitochondrial

respiration (Morita et al., 2013; Ramanathan and Schreiber, 2009) the possible interaction between mitochondrial effects of cannabinoids and the mTORC1 signaling cascades appears as a rational and intriguing hypothesis worth to investigate in future studies.

ROS are highly reactive molecules that are known to produce cellular damage but also to participate in cellular physiology (Popa-Wagner et al., 2013). ROS can modulate hippocampal synaptic plasticity and memory (Hu et al., 2006; Kamsler and Segal, 2003). It was recently shown that CB_1 receptor activation leads to an increase of ROS production *in vitro* (Donadelli et al., 2011; Rajesh et al., 2010) and *in vivo* (Koch et al., 2015). Mitochondria are producing the largest amount of reactive oxygen species (ROS) in the cell (Massaad and Klann, 2011). Mitochondrial ROS generation can occur through the leaking of electrons at the mitochondrial complex I of the respiratory chain (Chen et al., 2003b; Paradies et al., 2004). In particular, blockade of Complex I activity trough sAC inhibition causes ROS elevation (De Rasmo et al., 2015). Given that $mtCB_1$ receptors inhibit mitochondrial O_2 consumption through decrease of complex I activity, it is possible that cannabinoids generate ROS elevation *via* $mtCB_1$ receptors, which could in turn affect neurotransmission and behavior. Supporting this hypothesis, it was shown that inhibition of respiratory activity by cannabinoids is coupled to an increase in mitochondrial hydrogen peroxide (H_2O_2) production (Athanasiou et al., 2007). Interestingly, recent unpublished data from the laboratory indicate that ROS inhibitors can block several acute effects of cannabinoids *in vivo* (Busquets-Garcia et al., *in preparation*), supporting the hypothesis that CB_1 receptors (and possibly specific $mtCB_1$ receptors) might act *via* modulation of these processes.

The endocannabinoid system could modulate mitochondrial function *via* nitric oxide (Lipina et al., 2014; Nunn et al., 2012).Activation of CB_1 receptors has been shown to increase NO production by inhibiting the expression and/or the activity of neuronal NO synthase, the enzyme responsible for catalyzing the synthesis of NO (Esposito et al., 2001; Waksman et al., 1999), in different cell types including monocytes and neuroblastoma cells and in brain slice preparations and (Carney et al., 2009; Prevot et al., 1998; Stefano et al., 1996). NO regulates mitochondrial

function by inhibiting cytochrome c oxidase, the terminal acceptor in the mitochondrial electron transport chain (ETC), thereby inhibiting energy production and increasing H_2O_2(Brown, 2001). Thus, $mtCB_1$ receptors could additionally mediate the decrease of ATP production trough inhibition of NO. Future experiments are needed to explore this possibility. Interestingly, THC was shown to exert normal analgesic effects in mice lacking the neuronal form of NOS, but locomotor effects of the drug were impaired by the mutation (Azad et al., 2001). These results suggest that NO signaling might indeed participate in some behavioral effects of cannabinoids involving $mtCB_1$ receptor activation.

Overall, besides direct regulation of ATP levels, the data described above suggest that mTOR, ROS and NO signaling might represent mitochondrial processes linking the activity of $mtCB_1$ receptors to brain functions. $MtCB_1$ receptors signaling might contribute to the modulation of synaptic activity or plasticity (Benard et al., 2012) by different means and systematic studies will be needed to clarify this aspect.

V.10.1.5 DO $MTCB_1$ RECEPTORS IMPACT ON THE MOBILITY OF MITOCHONDRIA IN NEURONS?

The precise positioning of mitochondria at their functional locations in dendrites, spines, axons or synaptic terminals is of key importance in powering synapses (Chang et al., 2006; MacAskill et al., 2010). Principal neurons and motor neurons project their process far from their cell body and mitochondria are formed at the soma. If mitochondria were not transported to distal cellular locations, ATP synthesized in the soma would take over 2 min to diffuse to the end of a 200 µm-long dendrite, and ~10 years to diffuse to the end of a 1 meter-long axon, preventing rapid adaptation of the ATP supply in response to changing pre- and postsynaptic activity. Instead, mitochondria are transported long distances around neurons by kinesin and dynein motors, moving on microtubule tracks (MacAskill et al., 2010) playing a fundamental role in the modulation of synaptic transmission and plasticity (Guo et al., 2005; Kang et al., 2008; Sun et al., 2013; Verstreken et al., 2005). Interestingly, CB_1 receptor agonists reduce the fraction of transported mitochondria in enteric nerve fibers (Boesman 2009) and preliminary results from our laboratory indicate that this effect in hippocampal neurons directly involves $mtCB_1$ receptor signaling (Serrat et

al., *in preparation*). Thus, mtCB1 receptors could impact synaptic and behavioral functions also by regulating mitochondrial motility and their subcellular distribution.

V.10.1.6 DOES sAC INHIBITION MEDIATED BY MTCB_1 RECEPTORS IMPACT ON THE ASTROCYTE-NEURON COUPLING?

From where the metabolic substrate(s) needed for OXPHOS activity and ATP production are provided to synaptic mitochondria? The morphology and location of astrocytes, with an extensive endfoot around blood vessels, is well suited to uptake glucose from the blood, transform it into pyruvate or lactate and distribute them to the neighboring neurons *via* their processes closely unsheathing synapses (Allaman et al., 2011). Furthermore, while most brain energy is used by synapses, the unique brain energy store, glycogen, which can sustain neuronal functions for a few minutes when the glucose and oxygen supply is compromised (Choi et al., 2012), is mainly located in astrocytes (Gruetter, 2003), supporting their role in metabolite transfer to neurons. Moreover, neuronal activity elevates lactate levels in the brain and activation of astrocytes by glutamate increases lactate production subsequently secreted in the extracellular medium (Pellerin and Magistretti, 1994). Such processes could regulate the energy supplied to neurons in response to their activity, since glutamate released by active neurons could promote lactate production in astrocytes (Pellerin and Magistretti, 1994). Thus, an astrocyte-neuron lactate shuttle (ANLS) mechanism was proposed, whereby astrocytes generate lactate, eventually exporting it to neurons where it would be converted to pyruvate for ATP generation in mitochondria (Allaman et al., 2011; Pellerin and Magistretti, 2012). In this context, metabolic coupling between astrocytes and neurons was shown to be dependent on sAC signaling within astrocytes (Choi et al., 2012). Physiological increases in extracellular K^+ concentration cause astrocyte depolarization and permit the entry of bicarbonate into astrocyte, resulting in the activation of sAC in astrocytes (Choi et al., 2012). In turn, sAC activation causes glycogen breakdown, enhanced glycolysis, and the release of lactate into the extracellular space, which is subsequently taken up by neurons for use as an energy substrate (Choi et al., 2012). Soluble AC is mainly localized around and inside mitochondria (Bundey and Insel, 2004; Zippin et al.,

2003). Hence, it is tempting to hypothesize that $mtCB_1$ receptors could participate in the modulation of the metabolic coupling that occurs during transmission and thereby altering the maintenance of synaptic activity. In addition, recent evidence has revealed the role of astrocytic CB_1 receptors and their strong participation in the (endo)cannabinoid-dependent modulation of synaptic plasticity and memory processes (Han et al., 2012; Navarrete and Araque, 2008, 2010). Thus, assuming that $mtCB_1$ receptors are functional in astrocytes, we cannot discard the possibility that blockade of sAC activity by $mtCB_1$ receptors activation could affect, in addition to the decrease of mitochondrial respiration, the metabolic coupling between astrocyte and neurons during synaptic activity. Despite the fact that only preliminary data show that $mtCB_1$ receptors might be present also in astroglial cells, it is intriguing to speculate that bioenergetics control of astrocyte functions by (endo)cannabinoids might participate in their pharmacological or physiological effects. Also, it has been well established that astrocytes are endogenous regulators of synaptic transmission and plasticity (Araque et al., 2014; Panatier et al., 2011; Papouin et al., 2012). Future experiments are needed to explore the potential effect of $mtCB_1$ receptors in astrocytes. Given that astrocytes have higher complex I activity and sAC expression than neurons, astrocytic $mtCB_1$ receptors are potential good candidates to mediate some of the effects of cannabinoids on synaptic transmission and behavior. Our present experiments did not address this issue, as ubiquitous promoters were used to express CB_1 and DN22-CB_1 in the *hippocampus*. More specific approaches are currently being performed in the laboratory to address these possibilities.

V.11 Hippocampal mitochondrial inhibition mediated by $mtCB_1$ receptors impairs ORM performances

Cannabinoids are well known to impair ORM performance and this pharmacological effect was assigned to hippocampal CB_1 receptors (Puighermanal et al., 2009). In the present study, we showed that post-training inhibition of mitochondrial activity trough $mtCB_1$ receptor activation impairs ORM performance. This finding, linking mitochondrial activity with ORM, is consistent with the high energy requirement for recurrent firing that mediates memory processes (Rojas et al.,

2012). Interestingly, a recent study conducted by Hara and colleagues suggested a key role of mitochondria in modulating synaptic transmission, brain function and cognition, revealing the relationship between abundance and morphology of presynaptic mitochondria and memory performance in monkeys (Hara et al., 2014). However, future studies are needed to further investigate the crucial role of mitochondrial activity in memory processes.

V.12 CB_1 receptors in the SNr mediate cataleptic and hypolocomotor effects of THC

Cannabinoid drugs display potential therapeutic applications in several diseases (Pacher et al., 2006). However, these therapeutic effects are often accompanied with undesired effects such as memory, motor impairments and psychotic-like states, mostly related to alterations of brain processes (Pacher et al., 2006). For instance, CB_1 receptor agonists are powerful analgesic drugs, but their use in clinics is limited by important side effects, including alterations in movement initiation and coordination (Howlett, 2002). In mice, cannabinoids impair spontaneous locomotor activity and induce catalepsy in a CB_1-dependent manner (Monory et al., 2007; Zimmer et al., 1999). My Thesis work identified the SNr as a crucial brain structure where CB_1 receptor signaling is necessary for the induction of catalepsy and hypolocomotion by cannabinoids. Interestingly, the SNr has a central role in the basal ganglia circuits and local cannabinoid injections in this brain region affect motor performance (Fernandez-Ruiz, 2009; Sanudo-Pena et al., 1996). The SNr contains CB_1 receptors on terminals of striatal projecting neurons co-expressing CB_1 and D_1 receptors (the so-called 'direct' striatal efferent pathway), which use GABA as a neurotransmitter (Hohmann and Herkenham, 2000). Indeed, it was reported that the activation of CB_1 cannabinoid receptors inhibits GABAergic neurotransmission in the SNr (Wallmichrath and Szabo, 2002). Interestingly, inhibition of GABAA receptors in the SNr is associated with the induction of catalepsy (Kolasiewicz et al., 1988) whereas elevation in intra-SNr GABA level promotes hyperactivity (Matsui and Kamioka, 1978). Moreover, deletion of the *CB_1* gene in D_1-positive neurons in mouse (D_1-*$CB_1^{-/-}$*) abolishes the induction of catalepsy by cannabinoids (Monory et al.,

2007). This work shows that antagonizing CB_1 receptors in the SNr prevents both hypolocomotor and cataleptic effects of THC. Thus, the present data show that pharmacological activation of CB_1 receptors in the SNr mediates these effects of THC, likely *via* a modulation of striatonigral neurotransmission.

V.13 Local inhibition of mitochondrial activity mediates cannabinoid-induced catalepsy and hypolocomotion

Neurodegenerative diseases are tightly linked to mitochondrial dysfunctions. Among them, Parkinson's and Huntington's diseases primarily compromise the basal ganglia circuit, thereby affecting motor behavior. Rotenone is a highly lipophilic compound and a classic complex I inhibitor that ultimately leads to energy deficiency. Interestingly, chronic rotenone injection is widely used as an animal model of Parkinson's disease because it progressively induces motor abnormalities, including delayed initiation of movement (Fleming et al., 2004), reduced motor activity and prolonged latency in the catalepsy test (Sharma and Nehru, 2013). Thus, mitochondrial inhibition is clearly linked to motor disabilities (Klein et al., 2011). In the current study, the combination of subthreshold doses of rotenone and cannabinoids, which both directly exert effects on complex I activity, results in the induction of hypolocomotion and catalepsy. Therefore, it is rational to suggest that these drugs have synergist effects due to the dual inhibition of Complex I activity. Importantly, intra-SNr inhibition of $mtCB_1$ receptor signaling using the sAC inhibitor KH7 prevents catalepsy and decreases hypolocomotion induced by high doses of THC. Altogether, these results support the view that acute local inhibition of mitochondrial activity by $mtCB_1$ receptors mediates the motor effects induced by cannabinoids.

Motor impairment is generally considered as an important undesired side-effect of cannabinoids, profoundly limiting the use of these drugs for potential therapeutic effects (Pacher et al., 2006). In addition, CB_1 receptor modulation have been proposed to offer a novel type of therapy for HD and PD, delaying, arresting or repairing the striatal and nigral damage and the associated symptoms (Lastres-Becker et al., 2005; Sagredo et al., 2012). Thus, these data provide mechanistic theoretical ground for the future development of cannabinoid drugs inducing lower

side effects. For instance, recent studies revealed that the neurosteroid pregnenolone acts as an allosteric negative modulator of CB_1 receptors in the brain, and this effect is at least partially exerted through $mtCB_1$ receptors at the level of mitochondrial respiration (Vallee et al., 2014). Thus, our data and this recent study suggest that the combination of pregnenolone with cannabinoids might ameliorate side effects of therapeutic cannabinoids.

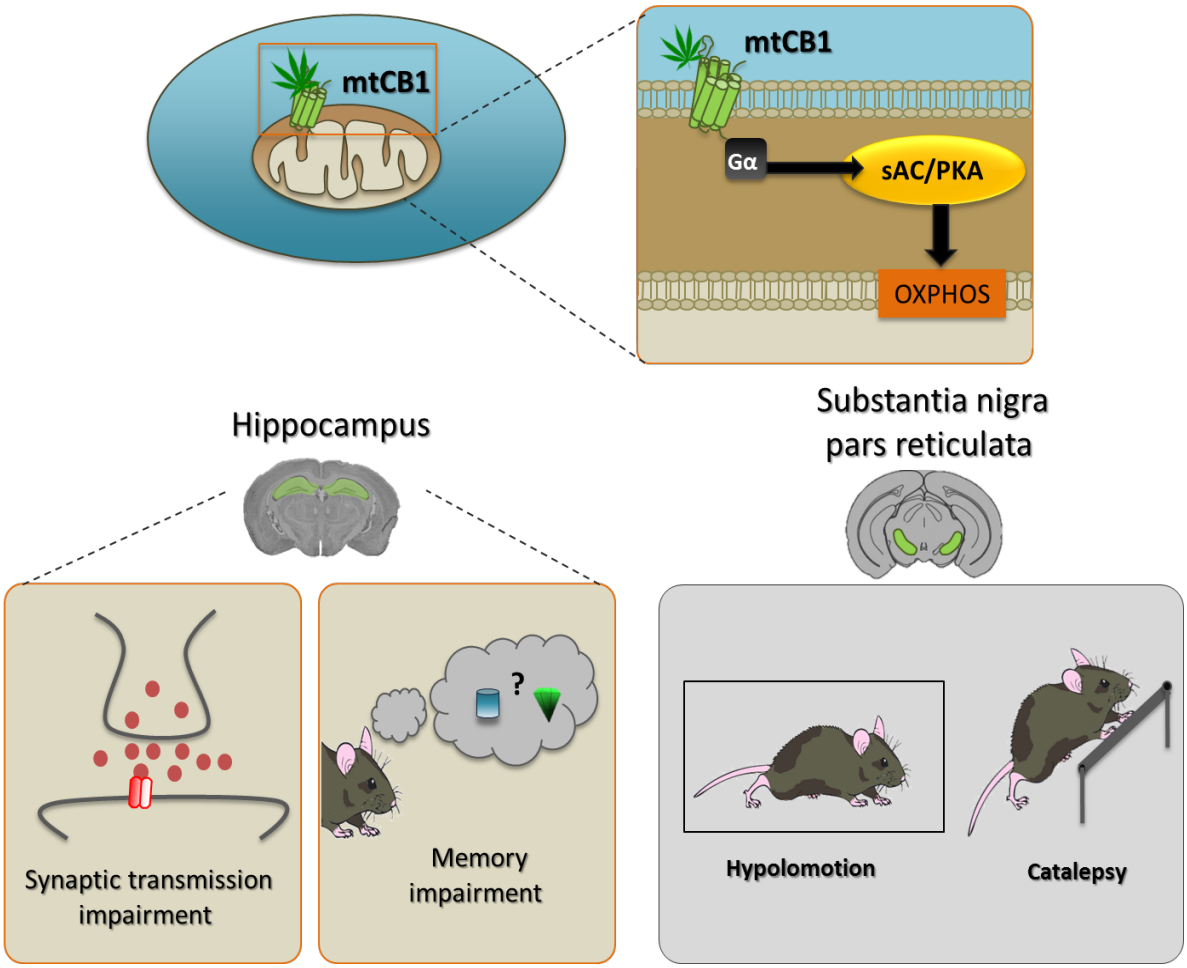

Figure 35.$mtCB_1$receptors and/or sAC signaling activation mediate behavioral effects of cannabinoids.

Conclusion

In summary, I believe that my Thesis work has contributed to a better understanding of the underlying mechanisms of cannabinoid effects on behavior. We have approached the role of $mtCB_1$ receptors in different brain regions trough different and complementary methods. In isolated mitochondria, $mtCB_1$ receptors

target complex I-dependent respiration through $G\alpha_{i/o}$ proteins. We then identified mitochondrial sAC as the target of $mtCB_1$ receptor signaling. In addition, we generated DN22-CB_1 mutant protein as a good strategy to study $mtCB_1$ receptor function *in vivo*. By taking advantage of these novel approaches, we revealed the necessary role of sAC signaling and $mtCB_1$ receptors in mediating specific central effects of cannabinoids including amnesia, catalepsy and hypolocomotion **(Figure 34)**. *In vitro* hippocampal electrophysiological recordings allowed us describing that $mtCB_1$ receptor activation mediates the cannabinoid-induced decrease of excitatory transmission in the *hippocampus*. Overall, these functional analyses may serve as a representative example of how impairment in bioenergetic processes could affect neurotransmission, memory and motor control

V.14 Perspectives

The recent discovery of the presence of $mtCB_1$ receptors in the brain generated several important scientific questions; dealing with the “upstream” and “downstream” mechanisms linking cannabinoid signaling, cell bioenergetics processes and brain functions. To address these novel questions, specific innovative tools are needed. The present Thesis work, by generating some experimental tools and using them to investigate some specific effects of exogenous cannabinoids, represents just a first step in the quest of understanding the implications of that discovery. Obviously, much work is still to do. In the following, I propose some feasible directions for future experiments.

- **Development of precise genetic tools to study *in vivo* effect of $mtCB_1$ receptors**

More powerful and precise tools will be necessary to gather detailed information on the differential involvement of $mtCB_1$ receptors in distinct synaptic processes.

- Based on the present data, our lab is currently generating a conditional cell type-specific DN22-CB_1 knock-in mutant mouse, which will express the mutant protein at the place of wild-type CB_1. These mutant mice will be instrumental to dissect the specific roles of $mtCB_1$ receptors.

- DN22-CB_1 receptors are excluded from mitochondria and, thereby, can provide information on the necessary role played by $mtCB_1$ receptors in brain functions. To address its potential sufficient role, we are in the process to generate additional genetic tools allowing an increase of CB_1 receptor mitochondrial location. Mitochondrial leading sequences (MLS) will be fused to the CB_1 protein to "force" mitochondrial localization (MLS-CB_1). These constructs, if functional, will allow obtaining "exclusive $mtCB_1$" mutant mice, which will provide highly valuable information on the sufficient roles played by $mtCB_1$ receptors in brain functions.

- **Understanding the mechanisms of mitochondrial targeting of CB_1 receptors**

The cellular mechanisms promoting the mitochondrial localization of CB_1 receptors are not known. Specific cell biological studies are needed to disentangle these mechanisms, which, moreover, could lead to the generation of more precise tools to study the functions of $mtCB_1$ receptors.

- **Test the role of mitochondrial sAC pathway in cannabinoid-dependent effects**

Down regulation or overexpression of mitochondrial sAC using viral or transgenic approaches will allow definitely establishing the role played by this enzyme in cannabinoid-induced effects, likely mediated by $mtCB_1$ receptors.

- **Assess the role of $mtCB_1$ receptors in other cannabinoid-mediated effects**

In particular, as cannabinoids are well-known food intake regulators, it will be very interesting to characterize the metabolic response of DN22-*CB_1* knock-in mutant mice after cannabinoids. Secondly, given that cannabinoids exert therapeutically relevant effects such as anti-convulsant and anti-nociceptive effects in animal models and patients, it will be interesting to test the whether these effects require the activation of $mtCB_1$ receptors.

- **Physiological stimulation of mitochondrial activity**

In the brain it is well established that, in the absence of glucose, ketone bodies are used as an alternative source of energy. The ketone bodies are oxidized, releasing acetyl-CoA, which enters directly to the Kreb's cycle, thereby shunting glycolysis. In particular, ketogenic diet appears to stimulate mitochondrial biogenesis and activity. Thus, ketogenic diet could permit highlighting the functions of $mtCB_1$ receptors in body physiology.

- **Testing "post-mitochondrial" mechanisms of action of $mtCB_1$ receptors**

As mentioned above, several "post-mitochondrial" pathways can be involved in the brain functions of $mtCB_1$ receptors. Pharmacological and genetic tools will be established to dissect the processes linking $mtCB_1$ receptor activation to modulation of brain functions.

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