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MITODREADDs: a tool to study G-protein signaling in mitochondrial trafficking

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Contents

List of abbreviations

2-AG	2-arachidonoylglycerol
AEA	Anandamide
AMPK	AMP-activated protein kinase
cAMP	cyclic adenosine monophosphate
CB1	Cannabinoid receptor type-1
CMV	Cytomegalovirus
CNO	Clozapine N-oxide
CoxVIII	Cytochrome c oxidase subunit VIII
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DHC	Dynein heavy chain
DIV	Days *in vitro*
DMSO	Dimethyl sulfoxide
DREADD	Designer receptors exclusively activated by a designer drug
Drp1	Dynamin related protein 1
ECS	Endocannabinoid system
ERK ½	Extracellular regulated kinase 1 and 2
FAAH	Fatty acid amide hydrolase
FBS	Fetal bovine serum
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GPCR	G-protein-coupled receptor

HA	Hemagglutinin
IMM	Inner mitochondrial membrane
IRES	Internal ribosome entry site
JNK	c-Jun N-terminal kinase
KIF5	Kinesin-1 family
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinases
MCS	Multiple cloning site
Mfn1/2	Mitofusin 1 and 2
mitoDREADD	mitochondrial designer receptors exclusively activated by a designer drug
MLS	Mitochondrial leader sequences
mtCB1R	mitochondrial cannabinoid receptor type-1
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy factor 1
PKA	Protein Kinase A
PLC	Phospholipase C
RASSLs	Receptors activated solely by synthetic ligands
TOM20	Translocase of the outer mitochondrial membrane subunit 20
TRAK	Trafficking kinesin protein

Abstract

G-protein coupled receptors (GPCRs) are widely expressed throughout the brain, regulating a vast array of functions such as cognition, pain and synaptic transmission. The cannabinoid type-1 receptor (CB1R), the most abundant GPCR in the brain, is a powerful regulator of metabolic activity and synaptic plasticity by coupling to multiple Gα protein subunits. Interestingly, several of those Gα proteins have been located to the mitochondria. Moreover, CB1R was shown to be present at the membranes of neuronal mitochondria (mitochondrial cannabinoid receptor type 1, or mtCB1R), where it directly regulates cellular respiration and energy production. More recently, mtCB1R has been linked to the modulation of mitochondrial trafficking, which is essential for synaptic transmission and neuronal survival. To dissect the pathways involved in this process, we developed a series of new chemogenetic receptors to control Gs and Gi protein signaling in mitochondria. We dubbed it as mitochondrial Designer Receptors Exclusively Activated by a Designer Drug (mitoGi-DREADD and mitoGs-DREADD), by comparison to already existent Gi-DREADD and Gs-DREADD. Previous results demonstrated that mitoGi and mitoGs-DREADD are targeted to the mitochondria through the addition of four mitochondrial leader sequences (MLS); here, we showed that mitoGs and mitoGi-DREADD display a unique signaling profile in Hela and HEK cells as a consequence of mitochondrial location. Both receptors were unable to activate the canonical GPCR-dependent extracellular regulated kinase (ERK) pathway. Furthermore, the receptors exerted a bi-directional modulation of mitochondrial respiration, with mitoGi-DREADD causing a mtCB1R-like depressing effect in respiration and mitoGs-DREADD promoting an increase. We then focused on the effect of Gi/mitoGi-DREADD on mitochondrial trafficking in neurons. However, none of the receptors was detected in hippocampal neurons. To improve this, a pIRES bicistronic vector was created to co-express the receptors with the fluorescent protein MitoDsRed, but this approach was also unsuccessful. Nevertheless, mitochondrial trafficking was assessed and we found first that mitoGi-DREADD appears to abolish mitochondrial motility *per se* in Hela cells, which could indicate a toxic effect on mitochondrial function. On the other hand, mitochondrial motility and velocity was within the normal parameters for neurons transfected with mitoGi and Gi-DREADD. In this regard, we showed that CNO causes a tendency for decreased mitochondrial motility in hippocampal neurons transfected with mitoGi-DREADD, but not with Gi-DREADD. Overall, we conclude that mitoDREADDs are a highly promising chemogenetic tool to control mitochondrial activity via G-protein signaling, but they still require considerable optimization to minimize deleterious effects.

Keywords: GPCR, mtCB1R, mitochondrial trafficking, mitoDREADD

Resumo

Os recetores acoplados a proteínas G (GPCRs) encontram-se amplamente distribuídos no cérebro, onde regulam diversas funções como a cognição, algesia e transmissão sináptica. O principal representante desta classe no cérebro, o recetor de canabinóides tipo 1 (CB1R), é um elemento chave na regulação da atividade metabólica e plasticidade sináptica, através do acoplamento a diversas subunidades de proteínas G. Paralelamente, algumas destas proteínas G exibem localização mitocondrial. A juntar a isso, já foi demonstrado que o CB1R se encontra presente nas membranas de mitocôndrias neuronais (recetor mitocondrial de canabinóides tipo 1, ou mtCB1R), onde regula diretamente a respiração celular e a produção de energia. Recentemente, o mtCB1R foi associado à modulação do tráfego mitocondrial, um processo essencial à transmissão sináptica e sobrevivência dos neurónios. De modo a descobrir quais as vias de sinalização envolvidas neste processo, desenvolvemos um conjunto de recetores quimiogenéticos capazes de controlar a sinalização por proteínas G na mitocôndria. A estes demos o nome de recetores mitocondriais de desenhador exclusivamente ativados por fármacos de desenhador, ou mitoDREADDs . Previamente, foi revelado que os mitoDREADDs são direcionados para a mitocôndria ao promover a sua fusão com quatro sequências repetitivas de localização mitocondrial (MLS). Neste trabalho, demonstrámos que tanto o mitoGi-DREADD como o mitoGs-DREADD apresentam um perfil sinalizador único em células HEK e Hela como consequência da localização mitocondrial. Ambos os recetores foram incapazes de promover a fosforilação de ERK 1/2, uma via canónica associada aos GPCRs. Além disso, os recetores exerceram um controlo bidirecional da respiração mitocondrial, com mitoGi-DREADD a causar um decréscimo da mesma, semelhante ao obtido com mtCB1, ao passo que mitoGs-DREADD estimulou a respiração. Com base nestes resultados, focámo-nos no efeito de Gi e mitoGi-DREADD no tráfego mitocondrial em neurónios do hipocampo. No entanto, não foi possível detetar nenhum dos recetores em neurónios. De forma a resolver isto, foi criado um vetor bicistrónico pIRES com o intuito de co-expressar os recetores com a proteína fluorescente MitoDsRed, mas esta estratégia falhou. Mesmo assim, procedemos à análise do transporte mitocondrial e descobrimos que mitoGi-DREADD parece eliminar por si só a mobilidade mitocondrial em células Hela, o que indica um possível efeito tóxico do recetor. Por outro lado, a mobilidade e velocidade mitocondrial encontravam-se dentro dos parâmetros normais em neurónios transfectados com mitoGi e Gi-DREADD. De um modo geral, foi possível concluir que os mitoDREADDs representam uma aplicação quimiogenética extremamente promissora para controlar sinalização por proteínas G na mitocôndria, mas é necessário otimizar consideravelmente a técnica de modo a minimizar os seus efeitos prejudiciais.

Palavras-chave: GPCR, mtCB1R, tráfego mitocondrial, mitoDREADD

Chapter 1 - Introduction

Chapter 1 - Introduction

G-protein coupled receptors: from structure to function

With about 850 members, G-protein coupled-receptors (GPCRs) form the largest and most diverse superfamily of human proteins involved in signal transduction across cell membranes. [1] GPCRs regulate physiological responses to a variety of stimuli that include endogenous ligands such as biogenic amines, peptides, hormones and glycoproteins, as well as various exogenous ligands for sensory perception. As a consequence, these receptors are involved in multiple physiological functions such as vision, olfaction, cellular metabolism, growth, inflammatory and immune responses [2 3]. More than 90% of these GPCRs are expressed in the brain, where they play important roles in cognition, mood, appetite, pain, and synaptic transmission through presynaptic and postsynaptic modulation of neurotransmitter release. Despite their vast diversity, all GPCRs share a common structural signature of a seven transmembrane(TM) domain composed of α-helices spanning the biological membrane, with an extracellular amino terminus and an intracellular carboxyl terminus (**Fig.1**).

As the name indicates, GPCRs are molecular receptors coupled to heterotrimeric G-proteins (composed by Gα, Gβ and Gγ subunits). For most GPCRs, binding of the endogenous hormone or neurotransmitter promotes a conformational change that results in the activation of receptor-associated heterotrimeric G-proteins and consequent modulation of downstream effector proteins (**Fig. 1**). Heterotrimeric G-proteins act as molecular switches alternating between the "off" state when bound to guanosine diphosphate (GDP) and the "on" state (activated) when bound to guanosine triphosphate (GTP). The Gα subunit is at the core of the molecular switch because signaling is tied to its GTP binding and intrinsic GTPase activity. [4,5] Upon ligand binding, the activated GPCR acts as a guanine nucleotide exchange factor (GEF), catalyzing the exchange of bound GDP on the Gα subunit for GTP. The exchange in the guanine nucleotides leads to a reduction in the affinity of the Gα subunit for the Gβγ dimer and functional dissociation of the heterotrimer (**Fig. 1**). The activated Gα and Gβγ proteins can then transmit signals to effector proteins, such as enzymes and ion channels, resulting in rapid changes in the concentration of intracellular signaling molecules, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), inositol phosphates, diacylglycerol, arachidonic acid, and cytosolic ions, which affect a variety of cellular functions(**Fig. 1**).

However, it is now appreciated that GPCRs also mediate cell signaling by recruiting GPCR interacting G-proteins, which modulate GPCR function and signal transduction. GPCR regulation can be carried out by steric exclusion, receptor internalization/recycling or transcriptional control, and involves several protein families such as the G-protein-coupled receptor kinases (GRKs) protein kinase A or C and β-arrestins. The three proteins are integrated in the process of receptor desensitization, characterized by the loss of response to prolonged or repeated administration of an agonist.

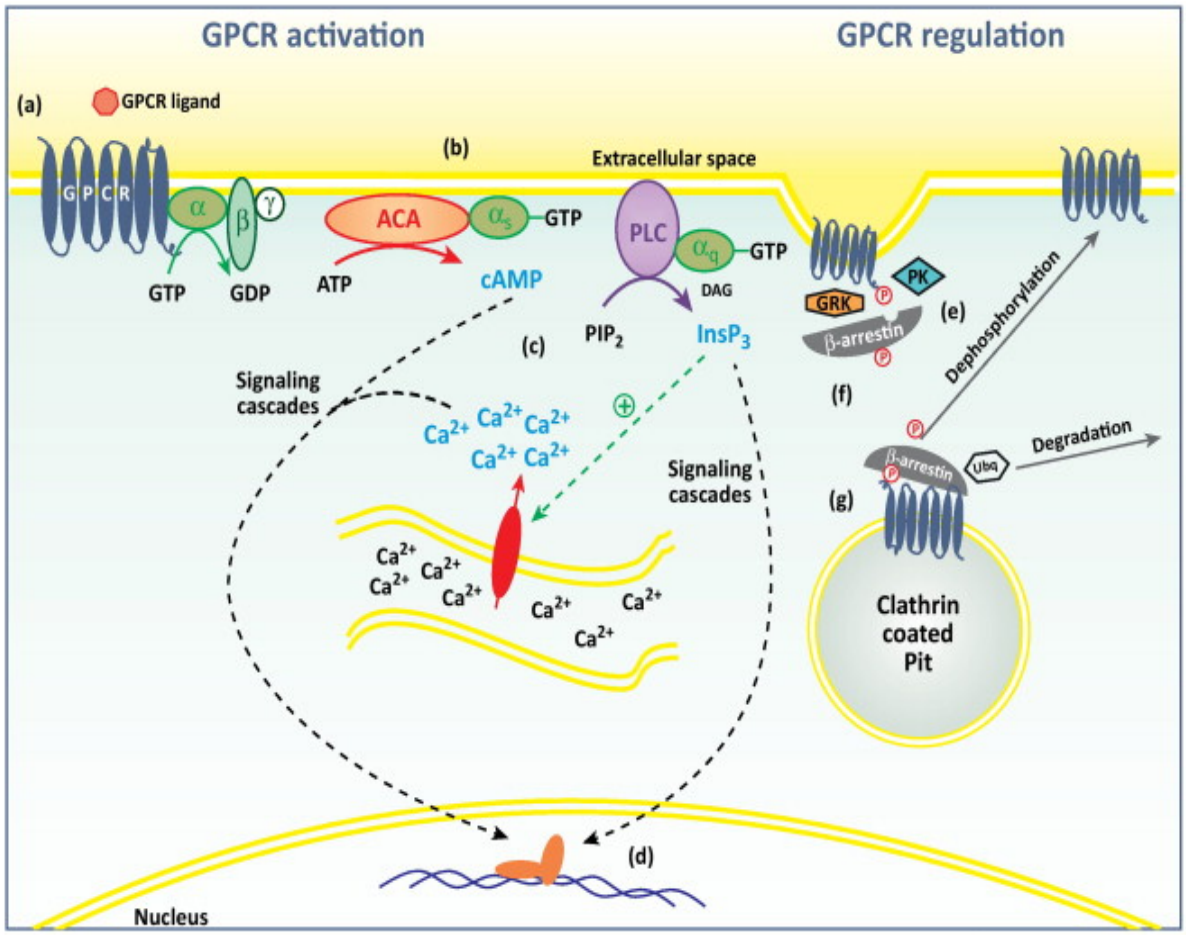

Figure 1. G-protein-coupled receptor (GPCR) activation and regulation. (a) Binding of a GPCR ligand to the extracellular region of the receptor induces a conformational change in the GPCR and the exchange of GDP to GTP by the subunit of the G-protein. **(b)** The GTP-bound α subunit dissociates from βγ and acts on a primary effector such as adenylate cyclase (ACA) (Gαs) or phospholipase C (PLC) (Gαq), leading to their activation **(c)** This leads to the release of second messenger molecules like cAMP and inositol-1,4,5-triphosphate ($InsP_3$), which are direct products of enzymatic conversion of ATP and phosphatidylinositol-4,5-bisphosphate (PIP_2) respectively, whereas cytosolic Ca^{2+} is released upon activation of reticular calcium channels. **(d)** Second messenger molecules can trigger cascade reactions that will lead to a downstream biological event, such as gene transcription or modulation of synaptic plasticity **(e)** GPCR responsive elements such as protein kinases (PKs) or G-protein-coupled receptor kinases (GRKs) phosphorylate the intracellular side of the receptor and promote β-Arrestin binding, leading to G-protein uncoupling and desensitization **(f)** β-arrestins also trigger the internalization process by interacting with adaptor proteins associated to clathrin complexes. **(g)** Modifications on the β-arrestin molecule such as dephosphorylation or ubiquitination define the fate of the internalized molecule either to recycling or degradation respectively. Figure adapted from Martins et. al, 2012 [6].

G alpha subunit is a crucial mediator of GPCR signaling

Among the several players involved in GPCR activity, the Gα subunit plays a crucial in the mediation of GPCR signaling. A wide array of Gα subunits are involved in the GPCR-dependent regulation of neuronal communication exerted by various neurotransmitters, such as glutamate, GABA or serotonin[7]. Gα subunits are typically grouped into four main classes, Gαs, Gαi/o, Gαq/11 and Gα12/13, which regulate distinct sets of effector proteins and downstream second messengers. Briefly, Gs proteins activate adenylyl cyclase to stimulate the production of cAMP; Gq proteins activate phospholipase Cβ(PLCβ) to produce diacylglycerol and inositol 1,4,5-trisphosphate (IP3), which elicits Ca^{2+} signaling; Gi proteins inhibit adenylyl cyclase, regulate ion channels, and activate some isoforms of PLCβvia release of Gβγ subunits; last but not least, Gα12/13 proteins activate a small family of Rho GEFs termed RhoGEF RGS proteins, which control cell motility [8]. A more detailed description of G-protein signaling can be found in **Table 1**, including alternative effectors. Taking the human adenosine A1 receptors as an example, the binding of adenosine to presynaptic and postsynaptic terminals for neuromodulation leads to the activation of the inhibitory subunit of heterotrimeric G-protein ($G\alpha_{i/o}$), which negatively regulates adenylyl cyclase activity, cAMP accumulation and activation of protein kinase A (PKA), leading to the induction of long-term depression. Concurrently, the released Gβγ dimers inhibit P/Q-type and N-type Ca^{2+}channels and reduce presynaptic Ca^{2+} influx [7]. Moreover, G-proteins are further modulated by proteins known as regulators of G-protein signaling (RGS) family. RGS proteins contain a canonical RGS domain of ~120 amino acids which binds activated G-proteins and act as GTPase activating proteins (GAPs). These GAPs, in turn, catalyze GTP hydrolysis and accelerate the G-protein cycle[9].

Now, growing evidence is showing that many GPCRs have much more complex signaling behavior. As the matter of fact, several GPCRs can stimulate multiple signaling systems, and specific ligands can have different relative efficacies to different pathways, a behavior known as “biased agonism”. In the extreme case, even opposite activities for different signaling pathways are observed (for example, β2 adrenaline receptor, agonists for the arrestin/MAP kinase pathway are also inverse agonists for the classical Gαs), activities that are further complicated by mechanisms such as GPCR oligomerization.

Table 1: Overview of Gα signaling and interacting partners			
G alpha subunit	**Binding effectors**	**Downstream signaling**	**GPCRs in the brain**
Gαi/o	Adenylyl Cyclase (decrease in cAMP levels)	Activation of ERK 1/2 and JNK (the latter via Gαo)	CB1R, P2Y1, D2R
	c-Src kinase	C3G and Rho stimulation	
	Rap1 GAPII	Rap inhibition and ERK 1/2 activation	
Gαs	Adenylyl Cyclase (increase in cAMP levels)	Activation of PKA, leading to Raf-1 inhibition, ERK 1/2 differential regulation and p38 MAPK stimulation Activation of EPAC, resulting in Rap1 stimulation	D1R, B1AR
Gαq/11	Phospholipase C-β	Intracellular calcium mobilization by Ca^{2+} release from IP3-regulated intracellular stores and DAG-dependent protein kinase C (PKC) activation. Ca^{2+} - dependent CAMKII stimulation Activation of ERK1/2, p38 mAPK and JNK	mGluRs, P2Y12, M3R
	PI3K	Inhibition of Akt pathway	
	p63-RhoGEF (RhoA activation)	Control of the dynamics of actin cytoskeleton, cell rounding, SRF and NFκB - dependent gene expression	
Gα12/13	RH-RhoGEF (RhoA activation) Other Ras proteins (CDC42, Rac)	Control of the dynamics of actin cytoskeleton, cell rounding, SRF and NFκB - dependent gene expression Attenuation of ERK 1/2 , JNK activation and differential regulation of p38 MAPK (Gα12 inhibition, Gα13 activation	a1AR, P2Y6

The CB1 receptor

CB1 receptor in the CNS

The cannabinoid type-1 receptor (CB1R), the main molecular target of endocannabinoids and cannabis active components, is the most abundant GPCR in the mammalian brain. CB1R is widely distributed in areas such as the cortex, hippocampus, cerebellum, and basal ganglia. The molecular cloning and characterization of CB1R, followed by the CB2 receptor, led to the discovery of an important endogenous system known as the endocannabinoid system (ECS)[10]. Further research in the last 15 years has consolidated the ECS as a key player in mediating a broad range of physiological functions such as the control of food intake, cardiovascular regulation, cognition, energy metabolism, immune response and reproduction.

The ECS as whole comprises (1:) at least two G-protein coupled receptors (GPCRs), known as the CB1 and CB2 receptors (CB2R) (2:) the endogenous ligands, known as endocannabinoids, of which anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized; and (3:) synthetic and degradative proteins that regulate endocannabinoid levels, as well as the receptors through which they signal [11].

AEA and 2-AG are produced, often with their congeners, from cell membrane phospholipids after cell stimulation and are immediately released to target CB1R and CB2R However, there are differences concerning the regulation of endocannabinoid levels, which is essential to define the "ECS" tone in biological systems. 2-AG is synthesized in the brain by the enzyme diacylglycerol lipase (DAGL) alpha, whereas AEA formation is thought to occur through different pathways, often involving N-acylphosphatidylethanolamine (NAPE). The degradation process is also different for both molecules; while 2-AG is hydrolyzed by the enzyme monoacylglycerol lipase (MAGL), the degradation of AEA is primarily catalyzed by fatty acid amide hydrolase (FAAH) [10,11].

In the CNS, CB1R is the main responsible for mediating endocannabinoid activity through the modulation of synaptic strength[12,13]. Indeed, CB1R is expressed in the presynaptic and/or postsynaptic terminals of neurons that regulate feeding, energy expenditure and reward. Modulation of synaptic strength occurs through retrograde signaling via the CB1R, although there is also evidence suggesting that endocannabinoids signal in a non-retrograde or autocrine manner[14]. Furthermore, CB1-dependent regulation of synaptic activity is also involved in the reduction of excitatory activity and consequent modulation of learning and memory. Finally, also it has been shown that the dysregulation of CB1-endocannabinoid signaling is implicated in neuropsychiatric conditions such as depression, anxiety and schizophrenia[15 16].

On the other hand, CB2R is mainly found within cells of the immune system, in line with its role as a major modulator of immune function [11]. Still, CB2R is also expressed in the cortex, hippocampus and cerebellum, and recent studies support a role for CB2R as a modulator of neuronal excitability in the CNS[13]. However, further investigation is required to unveil precise cellular mechanisms and contributions of CB2Rs to brain function.

G-protein signaling at the CB1 receptor

CB1Rs and CB2Rs primarily couple to Gi/o proteins, which leads to the inhibition of adenylyl cyclase and a reduction in cAMP levels, and to the activation of mitogen-activated protein kinase (MAPK) pathways (**Fig. 2A**). However, activation of CB1Rs, but not CB2Rs, results in the inhibition of various voltage-gated Ca^{2+} channels and activation of G-protein-gated inwardly rectifying potassium (GIRK) channels [17,18]. Both inhibition of adenylyl cyclase and modulation of ion channels are the two main mechanisms by which CB1R modulates synaptic strength.

For short-term plasticity, in which CB1Rs are activated for a few seconds, the mechanism involves direct G-protein-dependent (likely via the beta/gamma subunits) inhibition of presynaptic Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs). Conversely, long-term plasticity requires inhibition of adenylyl cyclase and downregulation of the cAMP/PKA pathway via Gi/o coupling[13]. As a result, neurotransmitter release is inhibited, an effect already observed in GABAergic, glutamatergic and dopaminergic synapses[7]. These lasting changes in synaptic strength are also dependent on the recruitment of complex intracellular protein kinase networks, usually involving extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK) cascades[18]. In line with this last point, CB1R is known to stimulate MAPK pathways in a Gi/o-dependent manner, not only involving ERK but also JNK and p38 MAPK(**Fig. 2**). However, this is highly dependent on the cell type and nature of the agonist. Δ9-tetrahydrocannabinol promoted JNK activation, but this was not the case after treatment with HU-210. Moreover, HU-210 administration to Neuro2a cells was shown to activate ERK1/2 pathways, but not JNK or p38 MAPK.

Interestingly, CB1R displays an alternative coupling to other G-proteins (**Fig. 2B**). Indeed, CB1R is able to stimulate adenylyl cyclase upon inactivation of Gi/o with pertussis toxin (Ptx), thus increasing cAMP levels[19]. Moreover, CB1R may also promote Ca^{2+} signaling through a VGCC-independent mechanism, by means of Gq protein activation. Indeed, an increase in intracellular Ca^{2+} was observed in insulinoma cells after stimulation with CB1R agonist arachidonoyl-chloro-ethanolamide, a response which was found to be Gq/PLC-dependent[20]. However, the mechanisms involved in this response are not clear and seem to differ between cell types[21].

Multiple receptor G-protein coupling could result from the artificial activation of non-preferred G-proteins due to their overexpression or Gi/o blocking via Ptx. However, both Gs and Gq/11 coupling has also been observed in models where cannabinoid receptors are endogenously expressed[19]. Furthermore, successive activation of Gs and Gi/o by increasing concentrations of cannabinoid agonists suggests a dual control of neurotransmitter release upon modulation of synaptic plasticity. This may have a protective role against excessive excitability and might be in the origin of the neuroprotective effect displayed by a restricted population of CB1Rs recently identified in glutamatergic terminals[22]. Overall, the multiple coupling displayed byCB1R may represent a powerful advantage towards the adaptation of diverse functional responses

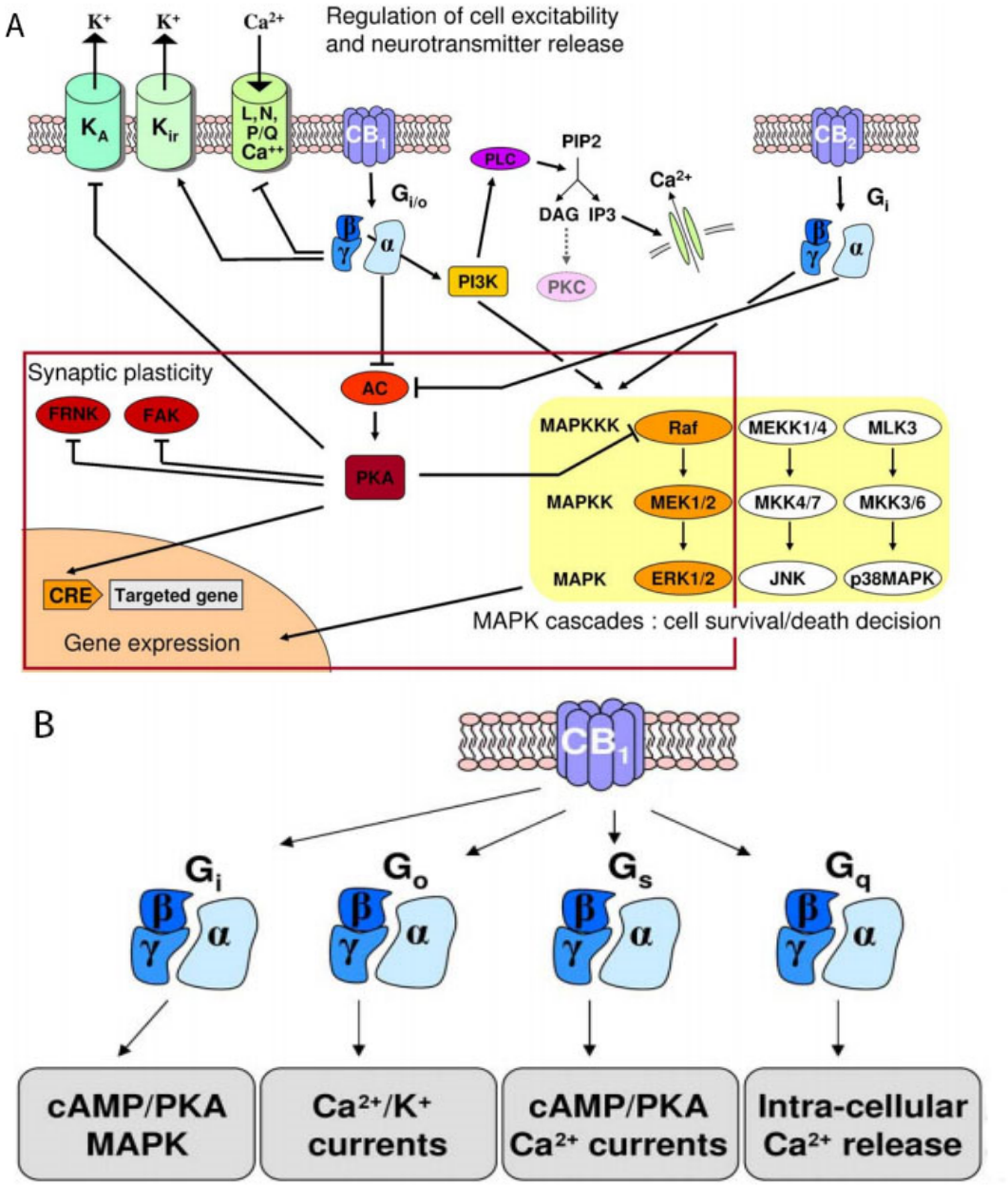

Figure 2: Complexity at cannabinoid receptor signaling, highlighting the multifunctional G-protein coupling displayed by CB1R. (A) Both CB1R and CB2R are associated with Gαi/o-dependent inhibition of adenylyl cyclase activity and Gβγ-dependent activation of the different MAPK cascades. In addition, the CB1R negatively regulates voltage-gated Ca^{2+} channels and positively regulates inwardly rectifying K+ channels, both through Gβγ coupling, which regulates neurotransmission. Finally, CB1R promotes Ca^{2+} signaling through Gβγ-/Gq - dependent activation of PLC (A and β). Cross-talks between signaling pathways are illustrated by the variety of responses requiring cannabinoid-mediated inhibition of PKA. This will lead to modifications in gene expression, activation of MAPK pathways and modulation of synaptic. Besides, it is now demonstrated that activation of CB1Rs also leads to activation or Gs and Gq proteins (B). Figure adapted from Bosier et al, 2010 [19]

GPCR mitochondrial signaling: the special case of CB1 receptor

G-protein non-canonical signaling

Although the traditional view on G-protein-coupled signaling implies that heterotrimeric G-proteins performed their functions while bound to the cytoplasmic surface of the plasma membrane, recent evidence indicates that G-protein behavior is highly dynamic. Indeed, G-protein subunits can translocate reversibly from the PM to endomembranes, such as endosomes and Golgi [23], where they are coupled to GPCRs. This paradigm was also challenged by the existence of GPCRs on the nuclear membrane, where they mediate signaling by multiple ligands[24]. Furthermore, there is now consistent evidence that mitochondria contain G-proteins. This had already been suggested after observing that P2Y-like purine receptors, which normally signal through G-proteins, modulate calcium flux across mitochondrial membranes in liver cells[25]. The first discovery concerning mitochondrial G-proteins happened in 2007, with the localization of Gαi subunit to the mitochondria of HEK293T fibroblasts[24]. More recent findings have identified the mitochondria as a non-canonical localization for G-proteins, including Gα12[26] Gβ2[27], and Gαq/Gα11[28], The latter supports the mitochondrial PLCβ signaling previously associated to P2Y receptors. The aforementioned subunits are involved in several mitochondrial functions, such as motility, morphology or dynamic behavior, as explained in **Table 2**.

Table 2: Characterization of the four mitochondrial G-proteins that have been identified so far.

Subunit	Location (mitochondria)	Binding partners/ effectors	Function
Gαi	Surface/inner membrane	Unknown	Unknown
Gα12	Outer membrane (mostly)	RhoGEF, Hsp90	Regulation of mitochondrial morphology and motility; Gα12 depletion increases mitochondrial motility
Gβ2	Outer membrane	Mitofusin 1	Regulation of mitochondrial fusion by modulation of Mfn1 mobility; Depletion of Gβ2 increases mitochondrial fragmentation
Gαq/11	Inner membrane (Gβγ at outer membrane)	Drp1, OPA1	Regulation of mitochondrial dynamics and bioenergetics; Gαq/11 promotes mitochondrial fusion and protects OPA1

Moreover, several reports have shown the intramitochondrial localization of potential downstream effectors of G-protein signaling, such as PLCβ, phosphodiesterase [29] and protein kinase A (PKA)[30]. Thus, cAMP production can occur in the mitochondria, leading to the activation of PKA signaling and phosphorylation of mitochondrial proteins, which optimizes oxidative phosphorylation and energy output. In addition, Acin-Perez et al demonstrated the existence of a mitochondrial cAMP-PKA signaling cascade dependent on soluble adenylyl cyclase, which serves as a metabolic sensor modulating ATP generation and reactive oxygen species (ROS) production in response to nutrients[31]. Overall, the upstream mechanisms regulating the intramitochondrial cAMP–PKA signaling cascade in neurons remain poorly understood. Interestingly, also a regulator protein of GPCR desensitization, GRK2, has been found in mitochondria, where it is involved in mitochondrial biogenesis and ATP production[32]. All together, these evidence also supports the existence of G-protein signaling and GPCRs in the mitochondria.

CB1 receptors are the first mitochondrial GPCRs

Early studies revealed that exogenous cannabinoids could impact mitochondrial functions, long before the discovery of cannabinoid receptors. Later, CB1Rs were found to maintain a positive energy balance by modulating, among others, mitochondrial activity. However, the mitochondrial effects of cannabinoids were traditionally interpreted either as indirect consequences of plasma membrane CB1Rs activation, or as unspecific alterations of mitochondrial membranes by these lipid compounds[33]. Still, the lipophilic nature of most cannabinoids kept alive the possibility that receptor–ligand interactions might occur not only at plasma membranes, but also inside cells, as verified in other well-described systems, such as glucocorticoid-mediated signaling. Strikingly, glucocorticoid receptors (GRs) also translocate into mitochondria and modulate mitochondrial gene expression[34]. Standing on that hypothesis, the connection between CB1R signaling and mitochondria was progressively strengthened across the years. For instance, CB1R stimulation decreases mitochondrial biogenesis in white adipocytes through eNOS downregulation and p38 MAPK activation and impairs mitochondrial function in metabolically active tissues of dietary obese mice[35]. Moreover, different intracellular compartments were shown to contribute for the regulation of endocannabinoid metabolism, including mitochondria, where an endocannabinoid-hydrolyzing enzyme was found[36]. Finally, CB1Rs were shown to functionally signal in lysosomal or endosomal intracellular membranes.[37]

Despite the strong correlation between CB1R and mitochondrial activity, the existence of mitochondrial cannabinoid receptors remained a matter of speculation. Although CB1Rs were often observed on neuronal mitochondria in electron microscopy experiments, they were generally considered nonspecific background labeling. This paradigm changed when Bénard et al demonstrated that CB1R was in fact present at the membranes of brain neuronal mitochondria (mtCB1R), where it directly regulated cellular respiration and energy production[5]. . Activation of CB1R by synthetic agonist WIN55,212-2 (WIN) decreased mitochondrial PKA activity, cAMP content, oxygen consumption and complex I activity in an mtCB1R-dependent manner, probably due to the reduction in cAMP-dependent phosphorylation of COX subunit I and

consequent decrease of OXPHOS efficiency. Notably, a highly significant inverse correlation was observed between 2-AG levels and mitochondrial respiration in wild-type brain mitochondria, suggesting that the organelle is equipped with endocannabinoids able to activate mtCB1R *in situ*[36]. In addition to the regulation of mitochondrial bioenergetics, mtCB1R was also found to be involved in depolarization-induced suppression of inhibition (DSI), a short-term form of synaptic plasticity related to endocannabinoid retrograde modulation of GABAergic transmission[38]. Briefly, mtCB1R-induced depression of mitochondrial respiration might contribute to strong DSI by altering energy supply required for neurotransmitter release. Overall, mtCB1R identification and characterization was a major breakthrough in the field of G-protein intracellular signaling, as it represented the first description of a mitochondrial GPCR.

Given the risks of false positive results associated to immunostaining, the functional presence of mtCB1Rs was rapidly challenged, claiming that the original observation was likely due to artifact results. A recent report further confirmed the existence and functionality of mtCB1R, while highlighting the importance of adequate immunostaining procedures and mitochondrial fractionation in the whole process[39].

In conclusion, mtCB1Rs seem to play an important role in the control of mitochondrial respiration and bioenergetics, which in turn may have a significant impact in synaptic plasticity. Moreover, mitochondria work as an intracellular Ca^{2+} buffer and regulate and redox potential, which can affect neuronal activity and neurotransmitter release. Rapid trafficking of mitochondria has also been recently proposed to modulate synaptic plasticity, raising the possibility that mtCB1R might interfere with these processes. Furthermore, G-proteins have been shown to regulate mitochondrial motility, dynamics and morphology (Table2). Thus, mtCB1R may pose as a multifunctional GPCR capable of modulating both mitochondrial activity and trafficking. This would further increasing the importance of CB1R in neuronal energy modulation, which is highly dependent on mitochondrial regulation.

Overview of mitochondrial transport

Main functions

Despite its relatively small size (2% in body weight), the human brain accounts for about 20% of the body's resting energy production[40]. Indeed, a resting cortical neuron in human brain consumes up to 4.7 billion ATP molecules per second to power various biological functions [41] and more than 90% of this ATP is produced by mitochondria. The energy demand is especially high at the synapses, due to the release of neurotransmitters and maintenance of ion gradients[42], where, mitochondria also ensure neurotransmission by buffering presynaptic Ca^{2+}. Notably, mitochondria induce certain forms of short-term synaptic plasticity by modulating Ca^{2+} dynamics at presynaptic terminals[43].

Constant mitochondrial ATP production is also essential for axonal growth and branching, synapse assembly and generation of action potentials. Therefore, ensuring the correct mitochondrial distribution is crucial for the normal functioning of neurons. However, neurons pose as a considerable challenge for proper mitochondrial distribution due to their

extremely complex morphology. Neurons are polarized cells with highly ramified dendrites and axons that can extend up to a meter long in the case of human peripheral nerves or corticospinal tracts[42,44]. Therefore, they require specialized mechanisms to efficiently distribute mitochondria to far-flung cellular regions where energy demands are high, such as synaptic terminals, active growth cones, and axonal branches. However, the polarity and distance are not the only issues to overcome. More than recognizing the polarity of the cell, mitochondrial transport must promote an adequate energy supply according to the local demand for ATP, Ca^{2+} and several other functions. Axonal branches and synapses are highly plastic and undergo spontaneous as well as activity-dependent remodeling, thereby changing mitochondrial trafficking and distribution. This constant adaptation to a dynamic context can be visualized, for instance, by the arresting or enhancement of mitochondrial movement when electrical activity is stimulated or blocked, respectively[45].

Furthermore, mitochondrial function itself is highly reliant on trafficking mechanisms, especially at distal terminals. Neurons are postmitotic cells surviving for the lifetime of the organism, whereas mitochondrial proteins exist for a shorter time span. As a consequence, mitochondrial function becomes compromised, with dysfunctional mitochondria producing less ATP, displaying a lower Ca^{2+} buffering capacity and posing as a source of ROS, which leads to the initiation of apoptotic cascades[46]. Therefore, mitochondrial transport is essential to remove aged of dysfunctional mitochondria and replenish them by healthy ones at distal terminals. Continuous turnover of mitochondria and maintenance of peripheral populations are also supported by mitochondrial fusion and fission. These processes, which will be later explained, contribute for the viability of stationary and distal mitochondrial by allowing the exchange of materials with the motile fraction[42].

In conclusion, mitochondrial trafficking is essential to correctly distribute mitochondria throughout the neuron according to metabolic and synaptic demands, as well as to ensure the renewal of mitochondrial population. A growing body of evidence clearly suggest that impaired mitochondrial transport and turnover, with consequent mitochondrial pathology, is associated to multiple neurodegenerative disorders, including Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease[44,47].

Mitochondrial movement is driven by motor-adaptor complexes

In axons, some mitochondria move persistently over long distances (tens to hundreds of micrometers), whereas other appear anchored or are otherwise stationary. Mitochondrial transport is characterized by continuous bidirectional runes, frequently intermingled by brief pauses and direction changes[44]. This transport between the soma and distal processes is mainly dependent on microtubule-based motor proteins, which drive their cargo through mechanisms relying on ATP hydrolysis. Microtubules are polarized tubulin polymers uniformly arranged in axons, with the fast growing plus ends oriented towards synaptic terminals, while more stable minus ends are directed to the soma[48]. So far, motor proteins driving microtubule-based transport are classified into two main categories: kinesins and dyneins. Given the axonal uniform polarity of microtubules, kinesin motors drive plus end-directed mitochondrial anterograde transport towards the synaptic terminals, while dynein motors mediate minus end-

oriented retrograde movement toward the soma (**Fig. 3A**). The balance between anterograde and retrograde transport is continuously shifted in either direction, depending on several aspects such as axonal growth and specific stimuli.

Among the several members of the kinesin superfamily, the kinesin-1 branch (collectively known as KIF5) is the key motor driving anterograde mitochondrial transport in neurons. KIF5 heavy chain (KHC) contains a motor domain with ATPase at the N terminus and a C-terminal tail for binding cargo directly or indirectly via adaptor proteins. KIF5 motors associate with mitochondria through adaptor proteins, forming motor/adaptor complexes that are essential to target mitochondria and distribute them according to local needs. Disruption of this coupling in hippocampal neurons impairs mitochondrial transport, thus reducing mitochondrial density in distal axons[49].

On the other hand, cytoplasmic dynein is the major motor driving microtubule-based retrograde transport in axons. As a multifunctional protein complex, dynein is composed by two catalytic heavy chains (DHC) with ATPase activity and several intermediate (DIC), light intermediate (DLIC) and light chains (DLC), which mediate cargo binding or regulate motor activity. Dynein heavy chain 1 (Dync1h1) is involved in retrograde transport in axons, while the C-terminus of DHC is the motor domain required for dynein movement. Furthermore, dynein has an important associated protein complex called dynactin, which binds directly to dynein and microtubules. Dynactin regulates dynein activity and the binding capacity of dynein for its cargos.

Recently, a motor/adaptor complex involving KIF5 was described as a mediator of mitochondrial transport in neurons and possibly most animal cells (**Fig. 3B**) [42]. KIF5 heavy chain lies at the core of this complex, as well as other two proteins: Miro, a protein of the RhoGTPase family which is anchored to the outer surface of the mitochondria, and Milton, a protein that links Miro (as a receptor) to the KIF5 cargo-binding domain [50]. Milton was originally identified in a Drosophila where its mutation reduces mitochondrial trafficking to synapses. The protein appears specific for mitochondrial trafficking because the trafficking of other cargoes such as synaptic vesicles is not affected [51] [52,53]. Milton has two mammalian orthologues, trafficking kinesin-bindinG-protein 1 and 2 (TRAK1 and TRAK2), both required for mitochondrial trafficking. Indeed, depleting TRAK1 or expressing its dominant negative mutants in hippocampal neurons leads to the impairment of mitochondrial motility[54], whereas overexpression of TRAK2 has the opposite effect[54].

Furthermore, TRAK1 and TRAK2 may have different roles in regulating mitochondrial motility in axons versus dendrites. A recent study showed that TRAK1 binds to both kinesin-1/KIF5 and dynein/dynactin and steers mitochondria into axons, whereas TRAK2 predominantly interacts with dynein/dynactin and mediates dendritic targeting[54]

On the other extremity of this complex, lies the Miro protein, a RhoGTPase composed by two GTPase domains that flank a central Ca^{2+} - binding region containing two canonical EF-hands, and a C-terminal transmembrane domain tethering the protein to the outer mitochondrial membrane (OMM)[55]. Miro functions as a mitochondrial receptor by binding the motor adaptor Milton, thereby recruiting KIF5 motors to the mitochondrial surface. Moreover, the EF hands allow Miro to function as a Ca^{2+} dependent switch for mitochondrial movement, whereas the GTP domains influence mitochondrial morphology. Together, EF hand and GTPase domains regulate ER-mitochondrial connections[55]. Similarly to Milton, Miro mutations

impair mitochondrial anterograde transport, thus depleting mitochondria at distal synaptic terminals[56]. In addition, Miro also possesses two mammalian isoforms, Miro1 and Miro2. Overexpression of Miro1 increases mitochondrial transport, probably via enhancement of TRAK2 recruitment, while the loss of Miro1 causes depletion of mitochondria from corticospinal tract axons and progressive neurological deficits[57]. Interestingly, this may result from the combined impairment of anterograde and retrograde transport, as loss of Drosophila Miro affects both kinesin and dynein-driven transport[58]. As a matter of fact, it has been proposed that Miro serves as an adaptor for both KIF5 and dynein motors in Drosophila, thus enabling a model in which opposite motors can be coordinated by the adaptor/receptor complex. The dual role of TRAK1/2 also supports this, given the mutually exclusion between kinesin dynein and binding. The multifunctional role of adaptor proteins might also imply that different motor proteins are simultaneously bound to the outer mitochondrial membrane[59]. Moreover, dynein can colocalize with mitochondria moving in either direction[60], suggesting that kinesin and dynein coordinate the transport of individual mitochondria instead of opposing each other.

Although the Miro/Milton complex is widely regarded as the main system behind the interaction between motor proteins and mitochondria, there are several other proteins which have been proposed as alternative adaptors, such as syntabulin (**Fig. 3B**)[44], a protein that is targeted to the mitochondria through a C-terminal transmembrane domain and directly interacts with the cargo-binding domain of KIF5.

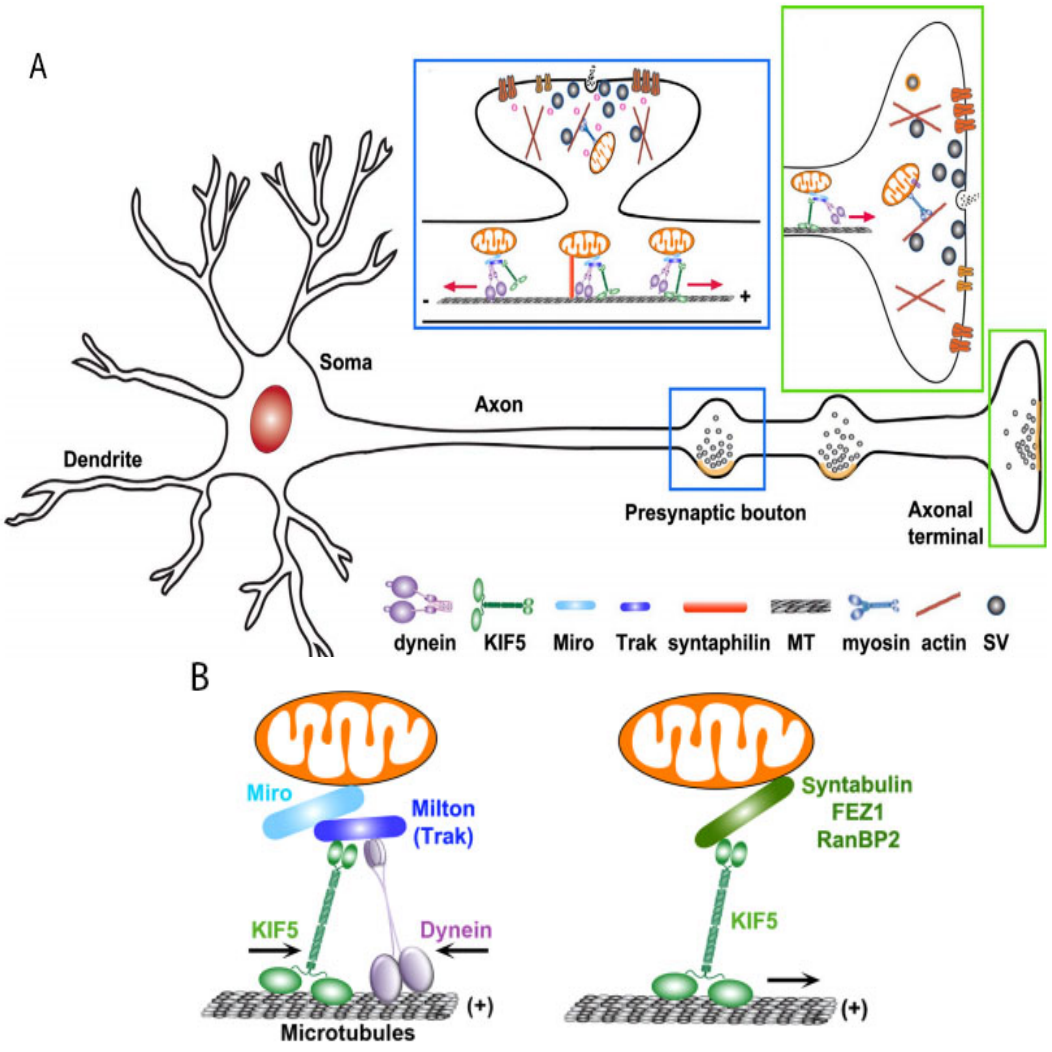

Figure 3. Mitochondrial trafficking and anchoring in neurons. Due to their highly polarized and complex morphology, neurons require highly specialized mechanisms for mitochondrial transport, which is driven by motor/adaptor complexes. A) Mitochondrial transport relies on microtubule (MT)-based motor proteins and is related to MT polarity. In axons, MT plus ends are oriented towards synaptic terminals, whereas the minus ends are directed towards the soma. Kinesins drive anterograde transport towards the synaptic terminals, while dyneins mediate retrograde transport back to the soma. In the highlighted regions, namely the presynaptic bouton (blue) and the axonal terminal (green), myosin drives short-range mitochondrial transport. Formation of stationary mitochondrial pools from the recruitment of motile organelles is crucial to ensure local ATP supply and Ca^{2+} buffering. B) Motor-adaptor complexes are crucial to regulate mitochondrial movement; the main complex involves KIF5, mitochondrial receptor Miro and adaptor protein Milton, which links Miro to KIF5. Dynein can also bind Milton, thus ensuring bidirectional control of mitochondrial trafficking. Alternative complexes have been proposed involving alternative motors such as syntabulin, FEZ1 and RanBP2. Figure was modified from (Sheng, 2014) and (Lin and Sheng, 2015)[44,50]

Regulation of mitochondrial transport

Regulation by cytosolic Ca^{2+}

Mitochondrial trafficking and distribution is closely tied to the levels of neuronal activity and synaptic function. Efficient regulation of mitochondrial motility is crucial to ensure that metabolically active areas are adequately supplied with ATP. Thus, stationary mitochondria ideally serve as local power plants for stable and continuous ATP supply, essential for the functioning of Na+/K+ ATPase and synaptic transmission (**Fig. 3A**). Anchored mitochondria also participate in the regulation of calcium homeostasis at synapses, as well as in axonal branching and maintenance. The pool of stationary mitochondria can be re-mobilized and re-distributed, whereas motile mitochondria can also be arrested. The balance between these two processes depends on axonal physiology and synaptic activity. However, since two thirds of mitochondria are stationary for a wider period of time within the axons, neurons require mechanisms to efficiently recruit motile mitochondria into stationary pools and anchoring them to the cytoskeleton.

One of the most interesting mechanisms involves the protein synthaphilin, (**Fig. 3A**). Syntaphilin locates to the OMM through a C-terminal mitochondrial targeting domain and binds specifically to axonal microtubules via an N-terminal axon-sorting sequence. Overall, synthaphilin acts as a "static anchor", arresting motile mitochondria by attaching them to the microtubules. Deleting synthaphilin results in a robust increase of axonal mitochondria in motile pools, whereas the over-expression of synthaphilin abolishes axonal mitochondrial transport. The actions of synthaphilin and other anchoring systems are essential to maintain a pool of stationary mitochondria at energy-demanding sites, such as synapses. Indeed, mitochondria are recruited to synapses in response to elevated intracellular Ca^{2+} arising from sustained synaptic activity, either by the activation of voltage-dependent calcium channels at presynaptic terminals or NMDA receptors at postsynaptic sites. It is widely believed that this regulation has the purpose of providing Ca^{2+} buffering capacity and ATP for Ca^{2+} active transport from the cell[42]. Several mechanisms have been proposed to explain Ca^{2+} - dependent arrest of motile mitochondria, mostly based on the KIF5-Milton-Miro transporter complex. As the matter of fact, several studies have identified the mitochondrial "receptor" Miro as a Ca^{2+} sensor. In short, rising of Ca^{2+} levels causes Miro to change conformation upon Ca^{2+} binding

to EF-hands, either inactivating or disassembling the transporter complex, which immobilizes mitochondria at active synapses.

A possible mechanism for Miro-Ca^{2+} sensing was proposed by Macaskill et al. 2009 [61], suggesting that Ca^{2+} binding to Miro detaches KIF5 motors from mitochondria (**Fig. 4A**). This could either be caused by a disruption of the KHC/Milton interaction or the milton/Miro interaction. However, there is substantial evidence against this model. One of the most striking is that the three proteins continue to co-precipitate as a complex even in the presence of very high Ca^{2+}, suggesting that Miro conformational change does not imply KIF5 detachment. Moreover, equivalent amounts of KHC are present on mitochondria before and after Ca^{2+} - induced arrestment. Thus, it appears that motor dissociation from mitochondria is not a decisive factor in the formation of stationary pools.

Still, KIF5 appears to be involved in mitochondrial arrest, but in a different manner (**Fig. 4B**). Indeed, an alternative model for Ca^{2+} - dependent arrest has been proposed, [62] in which Ca^{2+} binding allows Miro to interact directly with the motor domain of kinesin-1, preventing motor/microtubule interactions. Miro and KHC were shown to co-precipitate in elevated Ca^{2+}, even in the absence of cotransfected Milton. However, Milton was still required in Ca^{2+} free conditions for Miro-KHC association. Therefore, KIF5 switches from a low Ca^{2+} active state where it is bound to Miro only via Milton, to an elevated Ca^{2+} inhibited state in which direct binding to Miro prevents KIF5 from interacting with the microtubules.

However, knockdown or depletion of Miro1 in cell lines or primary cortical neurons does not abolish Ca^{2+} -induced mitochondrial immobilization. Moreover, and despite the bi-directional role ascribed to the transporting complex, the Ca+2-Miro-induced disruption of KIF5 does not necessarily mean the switching towards dynein-driven transport. This raised the possibility of additional pathways mediating the mitochondrial arrest, with syntaphilin assuming an emergent role. Indeed, activation of Miro-Ca^{2+} pathway fails to specifically arrest axonal mitochondria in syntaphilin-null hippocampal neurons[57]. This has expanded the role of syntaphilin towards an activity-related mediator of mitochondrial arrest and recruitment to stationary pools. The syntaphilin-based mechanism, proposed in the same study, was described as “engine-switch and brake” model (Fig. 4C). In response to a “stop” sign, such as the elevated Ca^{2+} at active synapses, the Miro-Ca^{2+} sensor releases the C-terminal tail of KIF5 to bind syntaphilin, resulting in the inhibition of the motor ATPase. Thus, syntaphilin switches off the kinesin motor and acts as a “brake” in mitochondrial movement, hence anchoring them to microtubules.

Syntaphilin-based model has allowed to solve the controversy surrounding Ca^{2+}-dependent regulation of mitochondrial transport in axons and dendrites. More precisely, Ca^{2+} binding to Miro disconnects KIF5 motors from dendritic mitochondria, whereas axonal mitochondria remain associated to KIF5 in the presence of high Ca^{2+} levels. The specificity of syntaphilin targeting allows for an axonal-unique mechanism in which the static anchor is recruited upon Ca^{2+} rising. Thus, while supporting the Miro-Ca^{2+} - sensing pathway, syntaphilin “engine-switch and brake” model implies that motor loading is insufficient for regulation of mitochondrial axonal motility, which also requires the modulation of anchoring mechanisms[50,57].

Despite the controversy surrounding the previous mechanisms, it is evident that Miro/Milton complex plays a crucial role in this process, which reflects its importance in

driving mitochondrial transport. Moreover, calcium binding to Miro inhibits both anterograde and retrograde transport, once again highlighting the bidirectional role of the complex and association to different motor proteins.

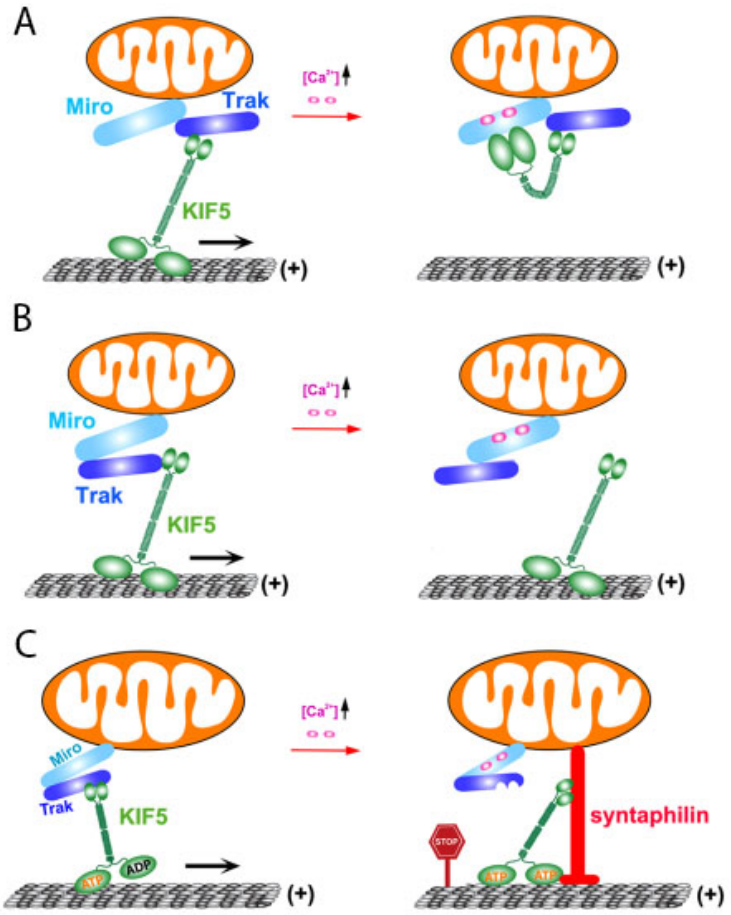

Figure 4. Regulation of mitochondrial transport by cytosolic Ca^{2+} can involve three different mechanisms. (A and B) Miro–Ca^{2+} sensing models for activity-dependent regulation of mitochondrial motility. The C-terminal cargo-binding domain of KIF5 motors binds to the Miro–TRAK adaptor complex. Ca^{2+} binding to Miro EF-hands favors direct association between Miro and KIF5, causing the dissociation of the motor from microtubule tracks, arresting mitochondria (A). Alternatively (B), Ca^{2+} binding releases KIF5 motors from mitochondria, but without detaching them from the tracks. Thus, Ca^{2+} influx upon synaptic activity arrests motile mitochondria at activated synapses. (C) Syntaphilin-mediated "engine-switch and brake "model. A Miro–Ca^{2+} sensing pathway triggers the binding switch of KIF5 motors from the Miro–TRAK adaptor complex to docking receptor syntaphilin, which immobilizes axonal mitochondria via inhibiting motor ATPase activity. Thus, syntaphilin turns off the "Engine" (KIF5 motor) by sensing a "Stop Sign" (elevated Ca^{2+}) and putting a brake on mitochondria. Figure adapted from Lin and Sheng, 2015 [50]

Alternative mechanisms

Recent evidence has allowed to expand the mechanisms by which mitochondrial transport is regulated, in different means from the readily reversible arrest induced by cytosolic Ca^{2+}. In addition, it had already been demonstrated that mitochondrial transport in cortical

neurons was independent of intracellular calcium elevations. This was observed for a wide variety of conditions, from spontaneous firing to sustained glutamate stimulation, always with the same effect. Thus, alternative mechanisms pose as an important contribution for understanding Ca^{2+} - independent regulation of mitochondrial transport.

One of the most promising hypothesis is focused on the protein kinase PINK1 (PTEN-induced putative kinase 1) and the ubiquitin E3 ligase Parkin, which are involved in a mitophagy-initiating pathway. . Interestingly, this pathway might be coordinated with mitochondrial movement and transport, a hypothesis that stemmed from the observation of a biochemical interaction between PINK1/Parkin and Miro/Milton. In this regard, depolarized or dysfunctional mitochondria may enhance PINK1-Miro association, thereby recruiting Parkin. This, in turn, will lead to Parkin-mediated Miro degradation, causing the release of kinesin or dynein from the mitochondrial surface and movement arrest. PINK1/Parkin mechanism is a less conventional, although fully functional argument to demonstrate the role of mitochondrial transport in the renewal of the organelle pool; in a certain way, Miro serves as an intermediate capable of promoting the elimination of damaged mitochondria.

Moreover, other proteins have been involved in the regulation of mitochondrial transport, either through the association with Miro/Milton complex or by modulating the actin cytoskeleton. Interestingly, it has also been proposed that regulatory proteins can serve as the intermediates in the control of mitochondrial transport by environmental stimulus. The strongest candidate is hypoxia up-regulated mitochondrial movement regulator (HUMMR), which is markedly induced by hypoxia-inducible factor 1 α (HIF-1α) and interacts with Miro 1/2, enhancing kinesin-1 recruitment[63]. Loss of HUMMR function decreases mitochondrial axonal content and reduces anterograde transport, an effect which is particularly noticeable during hypoxia. Interestingly, it also enhances retrograde mitochondrial transport, hence reflecting the bidirectional and coordinated control of mitochondrial transport. HUMMR may work as a switch, inhibiting kinesin-1 binding to Miro/Milton and turning “on” dynein-driven transport[58].

As previously mentioned, CB1R might also play an important role in the control of mitochondrial trafficking. Boesmans et al demonstrated that CB1R signaling modulates transport of mitochondria in enteric neurons, as well as network activity and synaptic vesicle recycling[64]. The team showed that activation of CB1Rs decreases the number of transported mitochondria in both directions, while receptor blockade had precisely the opposite effect. CB1R activation also turned down spontaneous network Ca^{2+} spiking, thus suggesting that CB1R mediates tonic inhibition in enteric nervous system. According to the previous results, it is not clear whether CB1R-mediated arrest of mitochondrial motility is due to receptor signaling or modulation of network activity. However, there are two interesting aspects pointing towards CB1R signaling. First of all, CB1R blocking and consequent increase of network activity should have resulted in a decrease of axonal mitochondrial motility, which was not verified. The increase of network activity, especially if sustained throughout time, should have promoted the arrest of mitochondrial movement in the axons via syntaphilin translocation. Moreover, it was proven that ENS possesses an endogenous cannabinoid ligand, probably AEA, since FAAH blockade shuts down Ca^{2+} spiking.

With the discovery of mtCB1R, a new link emerged between the endocannabinoid system and the regulation of mitochondrial motility. Following this hypothesis, preliminary results from our team demonstrated that mtCB1R is involved in the regulation of mitochondrial transport. In a first stance, it was shown that CB1R agonist HU-210 causes a specific reduction of mitochondrial axonal mobility in CB1R expressing neurons (CB1 +/+) by comparison to non-expressing ones, thus supporting the role of CB1R in regulation of mitochondrial trafficking (**Fig. 5A**). Next, to discriminate between the effects of plasma membrane CB1R and mtCB1R, HU-210 was replaced by a biotinylated version (HU-biot), which is unable to penetrate the cell due to the hydrophilic biotin bulk. Interestingly, HU-biot had no effect in mitochondrial mobility, indicating that activation of plasma membrane CB1R alone is not enough to reproduce the reduction obtained with HU-210 (**Fig. 5B**). Therefore, mtCB1R could be the responsible for the decrease in motility, thus suggesting that the receptor is able to regulate mitochondrial motility. This gives a whole new meaning to endogenous cannabinoid production in neuron networks as an intracellular control system and a complete mechanism for the regulation of mitochondrial G-proteins involved in motility. Thus, mtCB1R might in fact be one of the key players behind mitochondrial arrest by coupling to several G-proteins located in the mitochondria. To further elucidate that, the next section will focus on several G-protein-associated mechanisms which may regulate mitochondrial transport and thereby constitute possible mtCB1R-mediated pathways associated to this function.

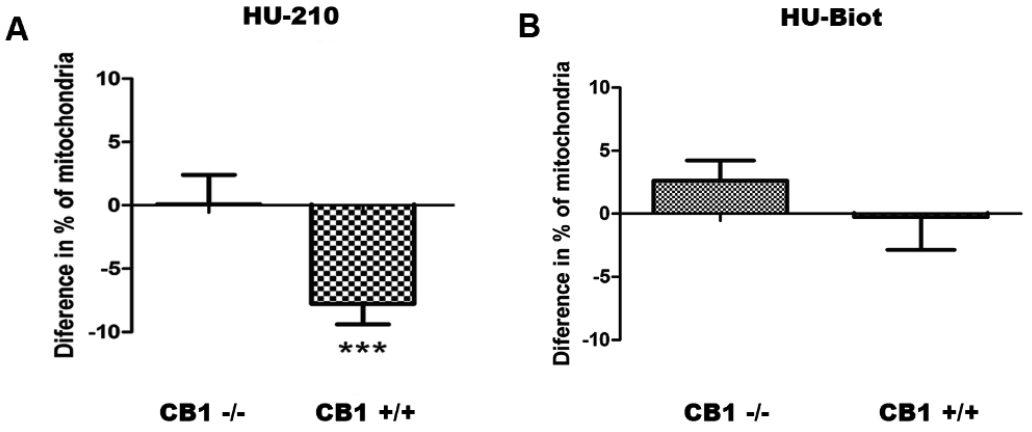

Figure 5: Activation of plasma membrane CB1R **alone is not sufficient to decrease mitochondrial motility.** A) Effect of CB1R agonist HU-210 in the total percentage of motile mitochondria before and after the treatment with HU-210, in CB1R knockout neurons (CB1-/-) and CB1R-expressing neurons (CB1 +/+). B) Effect of the non-permeable HU-Biot in in the total percentage of motile mitochondria before and after the treatment, in CB1R knockout neurons (CB1-/-) and CB1R-expressing neurons (CB1 +/+). ***P < 0.001 as compared to CB1 -/-

G-protein signaling pathways in the regulation of mitochondrial transport

AMPK modulation and ROS production

Stationary or anchored mitochondrial pools are essential as local power sources and are thought to be highly correlated with axonal growth and branching. Capturing of mitochondria at branching points is crucial to support cytoskeleton reorganization, localized protein synthesis and axonal transport. Recent studies suggest that this mechanism is probably mediated by AMPK, an AMP-activated protein kinase[50]. AMPK is a metabolic sensor which is activated in stress conditions upon ATP depletion, hence playing a pivotal role in whole body energy balance. Indeed, activation of AMPK increases anterograde flux of mitochondria into axons and promotes axonal branching in regions where mitochondria are docked in an ATP-dependent manner. Moreover, a recent study revealed that the serine/threonine liver kinase B1 (LKB1) regulates terminal axon branching of cortical neurons in both in vitro and in vivo systems through activating downstream kinase NUAK1, an AMPK-like kinase[65]. Conditional deletion after axon specification LKB1 or NUAK1 knockdown decreases the pool of stationary mitochondria and drastically impairs axon branching, whereas overexpressing LKB1 or NUAK1 increases the proportion of immobilized mitochondria along axons, as well as axon branching [65]. Although the mechanisms for capturing mitochondria are still unclear, it is very likely that LKB1-NUAK1 axis mediates axonal branching and stabilization by recruiting mitochondria to branch points. Syntaphilin is thought to play a role in this process, but the mechanisms are still unclear.

Since there is cumulative evidence indicating that GPCRs modulate AMPK activity, G-protein signaling might promote mitochondrial arrest through AMPK or LKB1/NUAK1-mediated pathways. The cAMP-PKA pathway is particularly important in this context, since it exerts a bidirectional control of AMPK activation[66]. Indeed, cAMP-PKA can either decrease AMPK activity by PKA-dependent CAMKK inhibition/phosphorylation at Ser485/491 or increase AMPK activity through the activation of LKB1[66]. The latter point suggests that GPCR-mediated signaling may in fact promote mitochondrial arrest and enhance axonal branching, more precisely via Gs coupling, cAMP elevation and LKB1 activation. It is important to mention that the association of G-proteins to AMPK regulation is still relatively unknown. Moreover, as cAMP-PKA also displays the opposite effect, AMPK-dependent G-protein-dependent regulation of mitochondrial motility will likely be cell and context-specific. Interestingly, LKB1 was recently found to mediate microtubule-dependent trafficking of the canalicular bile acid transporter, [67] , which may indicate that the protein has also a direct effect in microtubules, independent of AMPK.

The cAMP-PKA-LKB1-AMPK model is highly promising in the field of GPCR-dependent control of mitochondrial transport. However, there are some aspects which can limit the potential of this hypothesis at the level of mitochondrial G-protein signaling and, especially, mtCB1R. First of all, it is still unclear if mitochondrial G-protein signaling can modulate the activity of AMPK. Second, and most important, mtCB1R activation was reported to decrease cAMP levels and PKA activity[66]. Thus, the cAMP-PKA – dependent activation of LKB1 would be prevented, which in turn would promote mitochondrial movement. CB1R activation is known to impair mitochondrial motility, hence contradicting this possibility. Still, one could

propose that CB1R might regulate the opposite pathway, namely the inhibition of AMPK. That way, CB1R-dependent reduction of PKA activity would prevent AMPK inhibition, thus leading to mitochondrial arrest. However, AEA is known to reduce the levels of AMPK mRNA, which argues against the increase of mitochondrial motility promoted by CB1R-linked activation of AMPK[66]. Moreover, the context and cell type will likely be determinant in these mechanisms, as well as the nature of the agonists. Indeed, THC is able to activate AMPK in the brain, but only at high doses. Although this might implicate a possible stimulation of AMPK by CB1R, it is crucial to highlight that exogenous and endogenous cannabinoids agonists often exhibit opposite effects.

Metabolic activity of mitochondria is also an endogenous source of ROS, which were recently demonstrated to suppress mitochondrial motility (unpublished data, with access to poster). This rising connection might also be modulated by mitochondrial G-protein signaling, as mitochondrial cAMP-PKA cascade minimizes ROS production[31]. Although the upstream effector sAC is not linked to G-protein signaling, mtCB1R decreases cAMP levels, thereby blocking the protective effect of cAMP-PKA cascade in ROS levels and causing a ROS-dependent suppression of mitochondrial motility.

Control of fusion/fission balance

Mitochondria are dynamic organelles which are continuously changing their morphology through the combined actions of fission and fusion. These processes, originally thought to interfere only with mitochondrial shape, are now widely regarded as crucial events for the maintenance of mitochondrial function and integrity. Fusion allows the exchange of contents, DNA, and metabolites between neighboring mitochondria, while fission is necessary for proper mitochondrial transport and regulation of apoptosis. Furthermore, dysregulation of mitochondrial fusion and fission has been linked to several neuromuscular disorders. Thus, the mitochondrial protection exerted by fusion-fission cycles has a strong impact in cell physiology and survival.

These two opposing processes are regulated by members of the dynamin GTPase superfamily, with GTP hydrolysis being an integral step of fusion and fission pathways. The OMM proteins Mitofusin 1 and 2 (Mfn1 and Mfn2), together with the inner mitochondrial membrane (IMM) protein optic atrophy factor 1 (OPA1), regulate mitochondrial fusion, whereas fission is mediated by dynamin related protein 1 (Drp1)[68]. During mitochondrial fusion, Mfn1 and Mfn2 mediate OMM association through the formation of Mfn heterodimers or homodimers, while OPA1 is involved in IMM fusion. OPA1, in turn, is closely regulated by nuclease and proteolytic cleavage, and mutations in OPA1 were found to be associated with the human neurodegenerative condition, autosomal dominant optic atrophy[68].

On the other hand, mitochondrial fission can be divided into two main steps: the first consists in the inhibition of fusion proteins, and the second is the recruitment of Drp1 from the cytosol to OMM, where it mediates mitochondrial scission by interacting with other partners, such as human fission factor-1 (Fis1). Binding to its numerous receptors leads to the oligomerization of Drp 1 into a helical structure which, upon GTP hydrolysis, constricts the

mitochondrial membrane until the lipid bilayers are sufficiently destabilized to break apart from each other.

In line with the central role of fusion and fission in mitochondrial function and integrity, many of these dynamin-related proteins are linked to other processes, such as mitochondrial transport. In a functional perspective, it is expected that mitochondrial morphology has a direct impact on motility, but the connections go beyond that. For instance, hippocampal neurons expressing defective Drp1, a mitochondrial fission protein, display accumulated mitochondria within the soma and reduced mitochondrial density in dendrites[69]. Moreover, Drp1 is required for the delivery of mitochondria to neuromuscular junctions in Drosophila[70]. Therefore, regulation of Drp1 activation and recruitment is also important for controlling mitochondrial motility. Although the pathways subjacent to this are still unclear, it is suggested that other mitochondrial fission proteins mediate Drp1 recruitment and assembly, such as Mff and mitochondrial elongation factors. Recently, mitochondrial G-proteins have been introduced into this equation, due to their location and apparent role in controlling morphology and motility. More precisely, the Gq/11 subunit, targeted to the mitochondria through its N-terminal, is able to decrease mitochondrial fragmentation induced either by Drp1 expression or CCCP, a mitochondrial uncoupler. Therefore, Gαq/11 acts as a powerful inhibitor of mitochondrial fission, probably by interfering with the action of Drp1. Since the loss of Drp1 function in Drosophila leads to clustering in the cell body and depletion of axonal mitochondria, it is likely that Gq/11-mediated inhibition of Drp1 via GPCR activation will lead to mitochondrial arrest. The activation of Gq/11, in turn, is probably linked to a mitochondrial GPCR, although this is not approached in original study. This possibility is raised because the Gαby heterodimer is located at the OMM. As a matter of fact, the interaction between Gα and Gβγ appears to be important for targeting the heterodimer to the OMM, although the Gα subunit is mainly located inside the mitochondria. If Gαq/11 subunit was targeted alone to the mitochondria, it could be the result of a GPCR activation at the plasma membrane, followed by the dissociation of Gαq/11 and posterior mitochondrial targeting. As the whole heterotrimer is located to the mitochondria, Gαq/11 activation is likely coupled to a mitochondrial GPCR. Furthermore, given that CB1R can also signal via Gq/11 proteins[19,21] we suggest that mtCB1R activation will lead to mitochondrial Gαq/11 activation and arrest of mitochondrial motility via Drp1 inhibition (Fig. 5).

Similar to Drp1, mitofusins are also linked to mitochondrial transport. Indeed, Mfn2 mutations are the most commonly identified cause of the axonal Charcot-Marie-Tooth disease type 2A (CMT2A), since they produce a marked decrease in overall motility of axonal mitochondria in sensory neurons [71-73].This, in turn, may lead to the degeneration of peripheral sensory and motor axons observed in CMT2A[73]. Given the role of Mfn2 in mitochondrial fusion, defective axonal transport in CMT2A could arise from an impairment in the fusion process. However, disruption of mitochondrial fusion via Opa1 knockdown had no effect on mitochondrial motility, suggesting that loss of fusion does not inherently alter mitochondrial transport[72]. Interestingly, both Mfn2 and Mfn1 interact with mammalian Miro (Miro1/Miro2) and Milton (OIP106/GRIF1) proteins[72]. Thus, the interaction between mitofusins and the transporting complex may be essential to correctly mediate mitochondrial transport. Although Mfn2 is thought to have a more important role in mitochondrial trafficking than Mfn1, given the correlation with CMT2A, recent evidence places Mfn1 as a promisor contestant. As a matter

of fact, exogenous expression of Mfn1 was able to compensate for loss of Mfn2 in DRG neurons, raising the possibility that Mfn1 may in fact play an important albeit secondary role in mitochondrial trafficking[72]. Strikingly, recent studies suggest that Mfn1 activity might be dependent on G-protein mediated signaling, since the mitochondrial Gβ2 subunit physically interacts with Mfn1 and regulates its mobility on the mitochondrial surface[27]. This interaction is important for inducing mitochondrial aggregation, with Gβ2 depletion decreasing mitochondrial fusion. This effect is specific for mitochondria and Mfn1: downregulation of Gβ2 had little effect in Mfn2 surface mobility[27]. Therefore, by regulating Mfn1 mobility at the mitochondria, Gβ2 might be essential to ensure the interaction between Mfn1 and Miro/Milton, thereby contributing to the correct distribution of mitochondria. This suggests that mitochondrial GPCR signaling via Gβ2 subunit might promote mitochondrial motility in both directions by enhancing Mfn1 interaction with the transporting complex, thus exerting the opposite effect of Gαq/11 (Fig. 5).

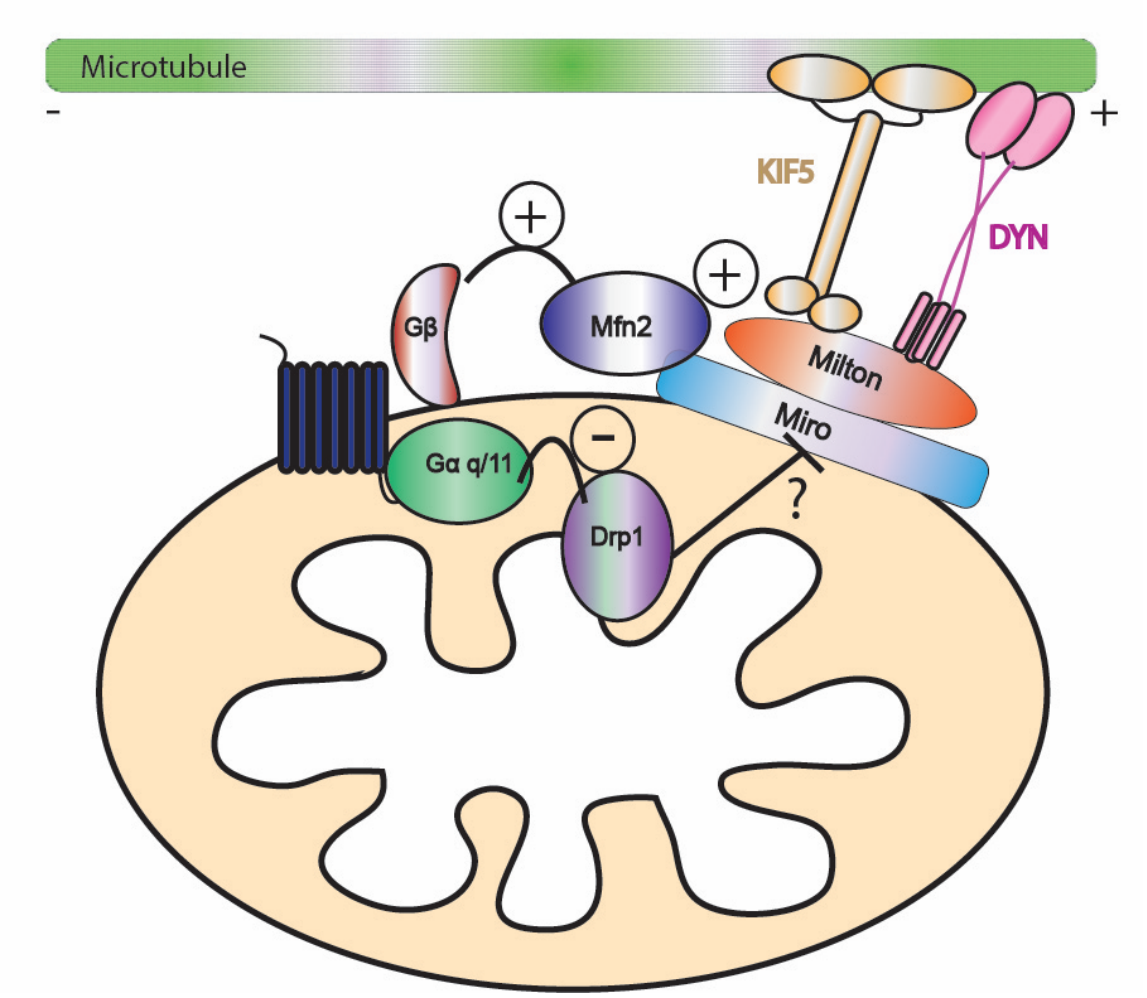

Figure 6. Putative modulation of fusion-fission balance by G-protein signaling can regulate mitochondrial trafficking. By controlling Mfn2 mobility on the mitochondrial surface, the OMM-located protein Gβ can enhance Mfn2 interaction with Miro/Milton complex, thus promoting mitochondrial motility in both directions. On the other hand, Gαq/11 signaling might lead to the inhibition of fission protein Drp1, which in turn causes a destabilization of Miro/Milton complex, although the subjacent mechanism is currently unknown. Original figure.

p38 MAPK and JNK pathways

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with a wide range of cellular activities. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK). JNK and p38 MAPK pathways are activated by pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β environmental and genotoxic stresses. Moreover, both proteins act in a cell context-specific and cell-type specific manner to control proliferation, differentiation, survival and migration[74,75]. Interestingly, GPCRs and G-protein signaling are critically involved in the regulation of different mitogen-activated protein kinase (MAPK) networks, including JNK and p38 MAPK (**Table 1**).

Recent reports associate p38 MAPK and JNK to mitochondrial transport regulation, which in turn might be dependent on G-protein signaling. As a matter of fact, the depletion of endogenous Gα12 in HUVEC cells was shown to increase the percentage of motile mitochondria, probably through a JNK-related effect[26]. A mutant version of Gα12, Gα12p115, which is unable to bind RhoGEF and activate JNK, caused the complete fragmentation of mitochondrial network and lead to the decrease of mitochondrial membrane potential[26]. Thus, Gα12 is probably signaling through JNK, although there is no direct connection with the regulation of mitochondrial transport. In addition, having into account that 40% of Gα12 is targeted to the mitochondria in these same cells, it is likely that mitochondrial Gα12 is contributing to this effect.

The decrease of motility observed upon Gα12 depletion might be related to a previously described activity of JNK, since this protein was shown to phosphorylate kinesin-1 heavy chains, inhibiting its microtubule-binding activity[76]. Therefore, Gα12 activation might lead to JNK stimulation and consequent phosphorylation of KIF5 heavy chain, displacing mitochondria from microtubule tracks and decreasing the overall motility of the organelle (**Fig. 7**). In addition, and although CB1R is not coupled to Gα12/13, it still can activate JNK via Gαq or Gαi. The latter is particularly important in this situation, since mtCB1R activation decreased cAMP levels and PKA activity, probably via Gαi coupling (**Table 1**)[77]. Thus, mtCB1R activation may lead to a decrease in mitochondrial motility through JNK stimulation in a Gαi depending manner, thereby explaining the results obtained in enteric neurons upon CB1R activation (Fig 6). However, having into account the intramitochondrial location of Gαi (**Table 2**), it is still unclear whether activated mitochondrial JNK (as described in[78]) is translocated to the cytosol to phosphorylate KIF5 (Fig 6).

Furthermore, a recent study demonstrated that p38 MAPK-dependent phosphorylation of kinesin-1 heavy chain at Serine 175-176 also leads to an inhibition of kinesin translocation along axonal microtubules[79]. Therefore, activation of p38 MAPK by Gαs or Gαq might lead to mitochondrial arrest through the uncoupling between kinesin-1 and microtubules (**Fig. 7**). The coupling of Gαs and Gαq to CB1R suggests that the receptor can mediate mitochondrial transport through any one of these proteins.

Finally, a last possibility for the previous effects elicited by G-protein signaling is that they might be also dependent on the regulation of actin polymerization through the Rho pathway, which, in this case, is activated by Gαq or Gα12/13 (**Table 1**)

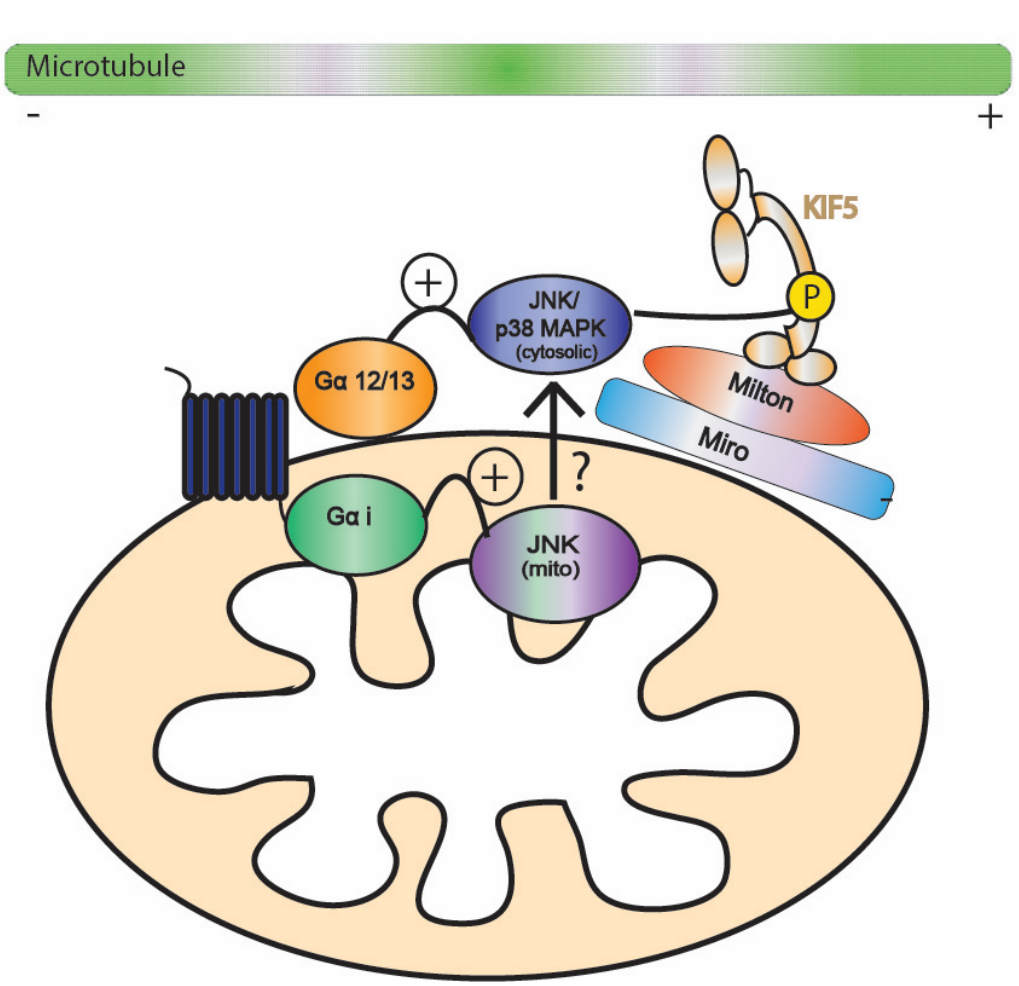

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Figure 7. Possible mechanisms for G-protein-dependent regulation of mitochondrial motility through MAPK pathways. Gα12/13 subunit, located in the OMM, can promote JNK/p38 MAPK recruitment, activating both proteins, which in turn will phosphorylate KIF5 heavy chains, causing its displacement from microtubule tracks. The same effect can happen for intramitochondrial Gαi activation and JNK signaling although this requires a so far unknown mechanism in which mitochondrial JNK translocates to the cytosol, exerting the same effect on KIF5 binding to microtubules. Original figure.

Overall, there are several G-protein linked pathways through which mtCB1R might regulate mitochondrial trafficking. Therefore, the physiological roles played by mtCB1R in the brain will likely result from the integrated outcome of different pathways on multiple cell types and tissues, a trace shared by the vast majority of the GPCR superfamily. In order to identify and characterize specific pathways, an experimental system allowing a selective activation of GPCRs in a cell-type or tissue-specific manner would be the ideal approach.

DREADDs: A tool to control GPCR signaling

As previously mentioned, GPCRs are widely distributed along the brain, where they are able to modulate a vast array of functions in different cell types, making almost impossible to regulate a specific GPCR-signaling pathway in a selective cell population with conventional methods. Genetic studies are normally restricted to loss-of-function phenotypes, while selective drugs often exhibit "off-target" effects, which in turn interferes with pharmacological studies. This is further hindered by the presence of endogenous receptor ligands.[80,81].

In this context, the development of chemogenetic tools to control GPCR activity has become a powerful alternative to study the in vivo relevance of GPCR signaling pathways. Chemogenetics refers to the process by which macromolecules (such as G-protein coupled receptors) can be engineered to be selectively activated by small molecules that have otherwise no effect on cellular signaling[82]. Initially, these customized receptors consisted in engineered GPCRs that could be efficiently activated by preexistent synthetic drugs but displayed low sensitivity to its endogenous ligands. Such mutant receptors were classified as receptors activated solely by synthetic ligands (RASSLs)[81]. Despite their usefulness, first-generation RASSLs displayed high levels of constitutive activation, which is a major concern for chemogenetic technologies. Moreover, the most commonly used RASSL synthetic ligands retained high affinity and/or potency at the endogenous receptors, limiting the *in vivo* applications, especially in tissues with a wild-type receptor present[80,81]. To overcome these issues, Bryan Roth and colleagues recently developed a series of mutated muscarinic receptors that can be activated by the pharmacologically inert compound clozapine-N-oxide (CNO) with high potency and efficacy, while being insensitive to the native ligand acetylcholine. This third-generation of chemogenetic tools became known as designer receptors exclusively activated by designer drugs (DREADDs)[80,83]. These customized receptors show the same G-protein coupling preference as their parent receptors while displaying minimal constitutive activity *in vitro* and *in vivo*, even at high levels of neuronal expression. Since their inception, DREADDs have become an essential chemogenetic tool used for neuroscientists to map neuronal circuits underlying several CNS functions, including behavior, perception, regulation of food intake and motor functions species ranging from flies to nonhuman primates[84]. This is largely due to their ability to control neuronal activity through G-protein mediated pathways. Nowadays, Gq, Gi and Gs-coupled DREADDs have been largely used in neurobiology and their essential properties are described in Table 3. Therefore, from a neuroscientific point of view, DREADDs evolved from a GPCR-oriented tool to a versatile technology able to switch on and off specific neurons in a time and space-controlled manner. Currently, there are four main classes of DREADDs, listed in **Table 3**.

Table 3: Classes of DREADDs and respective properties

Designer receptor	Coupling preference	Application in neurobiology
Gq-DREADD	Gq/11	Induces neuronal firing
Gi-DREADD	Gi/o	Mediates neuronal and synaptic silencing
Gs-DREADD	Gs/off	Modulates neuronal activity
Darr-DREADD	Arrestin-2, -3	

Although DREADD technology has been mostly used with the intent of exerting remote control of neural activity, we are more interested in its core features. Indeed, several DREADD-based experiments have provided key information to dissect physiological and pathophysiological roles of G-protein pathways. For instance, extensive characterization of transgenic mice expressing Gq-DREADD has allowed to establish B-cell Gq signaling as a crucial pathway to improve B-cell function and glucose homeostasis in vivo[85]. On the other hand, a recent study involving Gi-DREADD allowed to highlight the role of Ga13/RhoA axis in suppressing tumor growth in vivo[86]. Several mechanistic insights have also been obtained from in vitro studies in cultured cells, such as the GPCR-dependent modulation of B-catenin signaling [87]

As a versatile and finely-tuned technology, DREADDs pose as a highly promising approach to dissect the mechanisms by which mtCB1R can regulate mitochondrial transport. However, it would be highly desirable to modulate G-protein signaling via DREADDs in a mitochondrial-specific fashion, thus allowing an even more precise characterization of the different mtCB1R-elicited pathways.

mitoDREADDs: a promising tool to study mitochondrial activity (preliminary studies)

Our lab has recently started to develop two variants of Gi-DREADD and Gs-DREADD which are specifically targeted to the mitochondria. These receptors, created by Luigi Bellochio and colleagues, were dubbed as mitoDREADDs and were inspired by the process of mitochondrial protein import. Indeed, more than 99% of mitochondrial proteins are encoded by nuclear genes and later imported across the mitochondrial membranes. The majority of these proteins are imported via the general entry gate, the translocase of the outer membrane or TOM complex. To ensure this, mitochondrial proteins contain targeting signals within their primary or secondary structure that direct them to the organelle with the assistance of elaborate protein translocating and folding machine. These signals exist either as a cleavable sequence in the N-terminal region, termed as mitochondrial leader sequences (MLS), or as noncleavable internal signals, located in mature regions of mitochondrial proteins[88]. Although both signals are present in several mitochondrial proteins, MLS-based protein import is better characterized. In classical MLS targeting, TOM 22 and TOM 20 subunits of the TOM complex interact with the MLS and direct the preproteins across the OMM. Further interaction with translocase of the inner

membrane (TIM complex) ensures the import across the inner membrane and into the matrix, where mitochondrial processing peptidase (MPP) releases the N-terminal sequence[88]. Therefore, it was likely that MLSs could be used to engineer DREADDs targeted specifically to the mitochondria. We chose the MLS cytochrome c oxidase subunit VIII (CoxVIII) a transmembrane enzyme of the mitochondrial respiratory chain, and fused this MLS to the N-terminal region of Gi and Gs-DREADD. There is strong evidence showing that CoxVIII MLS can be used to achieve mitochondrial localization in diverse proteins such as Green Fluorescent Protein (GFP) or mCherry[89,90]. To ensure the detection and mitochondrial localization of the modified DREADDs, a HA-Tag was also added to the N-terminal region. However, inserting a single CoxVIII MLS to the N-terminal of DREADD-Gs and DREADD-Gi was unable to promote mitochondrial targeting in HeLa cells (data not shown). To overcome this issue, we increased to four the number of identical Cox VIII MLS fused to the N-terminal of Gi-DREADD and Gs-DREADD, thereby creating a 4xMLS-HA Gi-DREADD (mitoGi-DREADD) and a 4xMLS-HA-Gs-DREADD (mitoGs-DREADD), as shown in **Fig. 8A**. Strikingly, the presence of four MLS tandem repeats in Gs-DREADD and Gi-DREADD resulted in a significant increase of mitochondrial localization in HeLa cells (**Fig. 8B-E**).

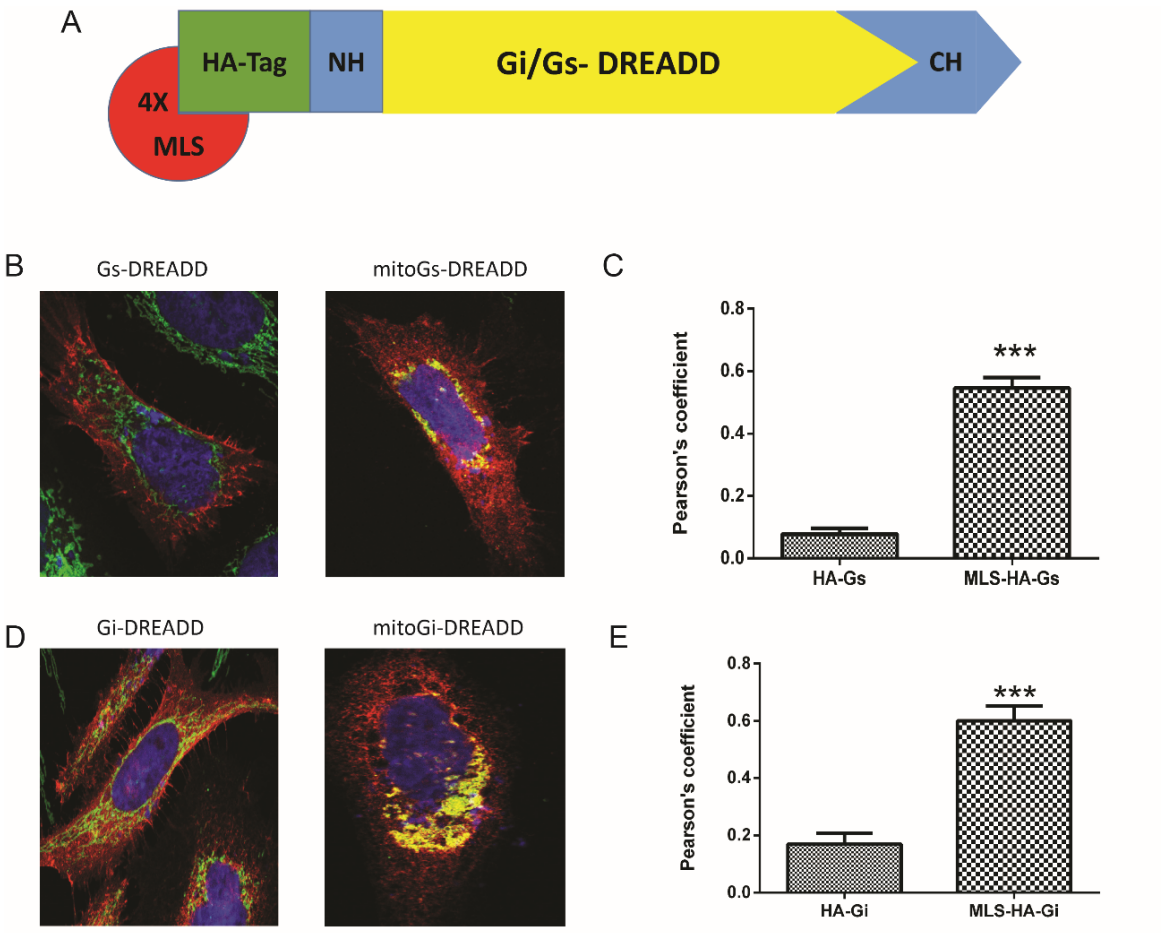

Figure 8: Attachment of four MLS identical sequences to Gs and Gi-DREADD creates mitochondrially-targeted mitoDREADDs. A) Schematic representation of the mito Gs/Gi-DREADD, with the four MLS tandem repeats attached to the N-terminal of Gs and Gi-DREADD, followed by a HA-Tag. B-E mitoGs-DREADD (B-C) and mitoGi-DREADD (E-D) exhibit a higher mitochondrial co-localization when compared to Gs and Gi-DREADD, as shown by the significant increase in Pearson's coefficient. Mitochondria are represented in green and HA-tag in red. *** P>0.0001 as compared to Gs and Gi-DREADD. Data are expressed as mean + S.E.M from 5-6 independent experiments. Figure adapted from Bellochio, L. et al. DREADDs a tool to manipulate mitochondrial activity, poster communication, Neurocentre Magendie Seminar, November 2015.

Main objectives

Targeting of DREADDs to the mitochondria represents an important step forward to achieve control of G-protein signaling and activity in this specific organelle. However, the change of location is not enough to consider mitoDREADDs as an appropriate pharmacogenetic tool. Therefore, the objectives of this work were twofold: 1) to functionally characterize the mitoDREADD signaling and effects on mitochondrial activity; 2)to apply newly characterized mitoDREADDs for the unveiling of G-protein pathways involved in the regulation of mitochondrial trafficking. For the first part, cell lines were used to evaluate the effects of mitoGi and Gs-DREADD in the activation of a GPCR canonical ERK pathway and mitochondrial respiration. During this section, a constant comparison to non-targeted Gs and Gi-DREADDs was always performed to determine the emergent signaling properties of mitoDREADDs. Only after this it was possible to change to a neuronal context and, by consequence, focus on mitochondrial transport. Here, a preliminary objective consisted in developing several strategies for optimizing mitoDREADD expression in hippocampal neurons, which is crucial for the reliability of mitochondrial trafficking studies. After this, mitoDREADD impact in mitochondrial transport was evaluated through live recordings of hippocampal neurons.

Chapter 2 – Materials and Methods

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Animals

Experiments were performed according to guidelines of the French Ministry of Agriculture and Forestry (authorization number 3306369). Timed pregnant mice were generated by crossing homozygote C57BL/6-N males and females (Neurocentre Magendie, INSERM U1215). Mating day was considered embryonic day (E0) and the day of birth postnatal day 0 (P0). Postnatal pups for primary cultures were then obtained at P0-P1 stage.

Drugs

CNO was obtained from Tocris Bioscience (Bristol, UK). For in vitro experiments, CNO was dissolved in a mixture of water with 20% DMSO.

Plasmids and Cloning

A bicistronic expression vector encoding both MitDsRed and plasma membrane/mitochondria-targeted DREADDs was generated through a combination of PCR and double blunt-based cloning. Initially, MitDsRed was subcloned into an Internal Ribosome Entry Site (IRES)-based vector. MitDsRed was amplified by high-fidelity PCR with primers containing a 5'XbaI or NotI site (Forward primer for XbaI 5'-CATGTCTAGAATGTCCGTCCTGACGCCGCTGCTGC-3' and
Reverse primer for NotI 5' CATGGCGGCCGCCTAAGACAGGAACAGGTGGTGGCGG-3'), cleaved with XbaI and NotI and ligated into a XbaI/NotI -cleaved pIRES vector. Insertion of MitDsRed was first verified by PCR amplification with internal primers for CMV pIRES promoter (Forward 5'-CGCAAATGGGCGGTAGGCGTG-3') and MitDsRed (Reverse 5'-GCGCACACTAGACTTCCCCTCC-3'), and by specific cleavage with NcoI and NotI/XbaI. Aftewards, pIRES-MitDsRed (P30M) construct was used as a template to generate MitDsRed-Gi-DREADD(P30M-55) and MitDsRed-mitoGi-DREADD (P30M-82) First, pIRES-MitDsRed was cleaved with NheI/MluI and re-polymerized with T4 Pol to create a blunt-end vector, which was later de-phosphorlyated to prevent spontaneous ligation. Next, pcDNA5-Gi-DREADD and –pcDNA3.1-mitoGi-DREADD plasmids were digested with PmeI and ligated into the NheI/MluI-cleaved pIRES-MitDsRed vector. This modification allowed the co-expression of Gi-DREADD/mitoGi-DREADD with the fluorescent protein MitoDsRed. Again, ligation of Gi-DREADD/mitoGi-DREADD was confirmed through PCR amplification with the CMV-directed primer (Forward 5'-CGCAAATGGGCGGTAGGCGTG-3') and a reverse primer for Gi-DREADD (Reverse 5'- GTGGACTCGGCTGCGACTGG -3'), followed by restriction analysis with SacI or NheI. Finally, the construct was sequenced (Mix2Seq, Eurofins Genomics) to confirm Gi-DREADD/mitoGi-DREADD insertion.

For the generation of Myc-tagged mitoGi-DREADD, HA-tag was replaced by Myc-Tag in the pcDNA3.1-mitoGi-DREADD expression vector. First, HA-tagged GiDREADD was amplified by high fidelity PCR with a forward primer encoding the Myc tag

(5'ATGCATGGAATTCATGGAGCAGAAACTCATCTCAGAAGAGGATCTGGCCAACT TCACACCTGTCAATGGCAGCTCGGGC-3'), replacing HA-Tag, and preceded by a restriction site for EcoRI. A restriction site for XbaI was inserted in the other extremity of the HA-GiDREADD with the appropriate reverse primer (5'-ATCGTCTAGACTCGACCTACCTGGCAGTGCC-3'). Next, the GiDREADD fragment was digested with EcoRI and XbaI and inserted into an EcoRI/XbaI-cleaved HA-4MLS-Gi-DREADDpc3.1DNA vector. Insertion of the Myc-tag was confirmed by sequencing the construct (Mix2Seq, Eurofins Genomics).

Cell culture and transfection

HeLa and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium GlutaMAX™ High Glucose supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids, 120 ugml-1 Penicillin/Streptomycin and 0,1% Gentamicin, and maintained at 37ºC in a 5% CO_2 controlled environment. Upon reaching confluence, cells were trypsinized and seeded at the desired density onto 6-well multiwells (ERK phosphorylation), glass coverslips (immunocytochemistry) or 35-mm glass bottom dishes (Mattek Corporation), for live imaging. After one day, cells were transfected using Polyethylenamine (PEI), according to the manufacturer's instructions and with a 1:3 DNA to PEI ratio.

For the preparation of primary hippocampal cultures, neonatal (P0-P1) pups were decapitated and their brains were extracted in ice-cold dissection medium (0.5% BSA and 0.6% D-Glucose in sterile PBS, pH 7.4). Hippocampi were dissociated with a specific kit for postnatal neurons, according to manufacturer's instructions (Miltenyl Biotech). Next, cells were seeded onto 0.5 mgml-1 poly-l-lysine (Sigma-Aldrich)-coated coverslips (for immunocytochemistry, at a density of $5x10^5$ cells/well) or 35-mm glass bottom dishes(Mattek corporation, for live imaging, at a density of $6x10^{5/}$ml), in Neurobasal medium supplemented with B-27 (1X), 120 ugml-1 Penicillin/Streptomycin and 1 mM glutamine. Cells were maintained at 37ºC in a 5% CO_2 controlled environment until the day of the experiment (DIV 7-8). Neuron transfection was carried out at 3-5 DIV for both live imaging and immunocytochemistry, using a standard calcium phosphate transfection protocol.

Oxygen consumption measurements

The oxygen consumption of HeLa cells was monitored at 37ºC in a glass chamber equipped with a Clark oxygen electrode (Hansatech, U.K.) Intact cells were trypsinized and transferred directly into the chamber. After recording basal respiration during 2-3 minutes (or until rate time difference became constant), CNO was added to the chamber at the final concentration of 50 µM and oxygen consumption was measured for another 5 minutes.

ERK phosphorylation

HEK cells were seeded onto 6-well plates at the density of $3x10^5$ cells/well and transfected in the following day with Gs-DREADD/mitoGs-DREADD and Gi-DREADD/mitoGi-DREADD. After one day of recovery in serum-containing medium, cells were starved overnight in serum-free DMEM. Next, cells were treated for 30 minutes with CNO (50 mM), FBS (10%) or vehicle (DMSO), at 37°C in a 5% CO_2 controlled atmosphere. Medium was then aspired and the samples were snap-frozen in liquid nitrogen and stored at -80°C.

Protein extraction and Western Blot

HeLa cells were placed in ice-cold lysis buffer (1mM EGTA, 50mMTris pH 7.5, 0.1% Triton X-100, 0.2% B-mercaptoetanol, supplemented with phosphatase and protease inhibitors), collected by scraping and pelleted by centrifugation for 5 minutes at 12000 rpm and 4°C, to remove cell debris. After recovery of the supernatant, which contained the fraction of cytoplasmic proteins, total protein content was measured through Bradford assay in a POLARstar Omega (BMG Labtech), at 570 nm. Samples were loaded with Laemli buffer, heated for 5 minutes at 100°C and stored at -20°C.

For Western Blotting, 30 ug of each protein extract was loaded into Tris-Glycine 10% acrylamide gels (Bio-Rad laboratories, Inc) and separated by SDS-PAGE electrophoresis, in Tris-Glycine-SDS Buffer (25 mM Tris-HCI, 192 mM glycine, 0.1% SDS, pH 8.3). Next, proteins were electroblotted onto PVDF membranes for 1 hour at 100 V, in Tris-Glycine Buffer (25 mM Tris-HCI, 192 mM glycine, 20% methanol, pH 8.3). Next, membranes were blocked in TBS-Tween20 0.05% with 5% BSA for 1 hour at room temperature. Specific proteins were detected upon incubation with primary antibodies (**Table 1**) against p-ERK (Cell Signaling, 1:2000, overnight, 4°C°) and ERK (Cell Signaling, 1:1000, 1 h, room temperature). Membranes were then washed with TBS-T 0.05% and incubated with appropriate secondary HRP-conjugated antibodies for 1h. HRP signal was revealed with Clarity Western ECL Substrate (Bio-Rad) and visualized in a Bio-Rad ChemiDoc MP System. Image processing and protein quantification was performed in ImageLab software.

Live imaging of HeLa cells and axonal transport of mitochondria

HeLa cells and hippocampal neurons were seeded onto Poly-L-Lysine 35 mm glass plates (Mattek corporation), co-transfected with Gi-DREADD/MLS-Gi-DREADD and MitoDsRed in a 10:1 ratio and recorded 24h to 48h (HeLa cells) or 72h to 96h after (for neurons) using an inverted Leica DMI 6000 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a confocal head Yokogawa CSU-X1 (Yokogawa Electric Corporation, Tokyo, Japan) and a sensitive Quantem camera (Photometrics, Tucson, USA). The diode lasers used were at 491 nm and 561 nm and the objective was HCX PL APO CS 63X oil 1.32 NA. The z stacks were done with a piezo P721.LLQ (Physik Instrumente (PI), Karlsruhe, Germany), the 37°C atmosphere was created with an incubator box and an air heating system (Life Imaging Services, Basel, Switzerland) in the presence of 5% CO_2. This system was controlled by MetaMorph software (Molecular Devices, Sunnyvale, USA).

For the imaging of mitochondrial transport, axonal processes were identified according to morphological criteria and directionally determined for each axon. Time lapse series of image stacks composed of 18 images each (0.5 μm step size) were taken every 1.7 s during 10 min. This was followed by a 15 min treatment with CNO 50 μM. Next, a new time lapse series was taken during 10 min, exactly in the same conditions. For Hela live recordings, isolated cells were localized and time lapse series of image stacks composed of 18 images each (0.5 mm step size) were taken every 30s during 90 min. All stacks were processed with MetaMorph software for video compilation and mounted as TIF files for video compilation. Further image edition and analysis was done with ImageJ software, (version 1.5b, NIH, USA). In order to quantify mitochondrial transport, kymographs were generated with KymoToolBox[91]. In the presented kymographs, vertical axis represents the time, while the horizontal axis represents the distance along an axonal process. Stationary mitochondria are represented as vertical white lines, while motile mitochondria appear as diagonal lines, with the slopes providing for the velocity. Distances and speeds of retrograde and anterograde transport were measured separately from the corresponding kymographs as previously described[92]. A specific region of interest (ROI) was assigned to each mitochondrion, which was considered motile only when travelling more than 5 um in at least one of the two directions (Anterograde/Retrograde) during the 10 min of recording. Mitochondria moving in both directions were included in retrograde and anterograde moving groups.

Immunostaining

For immunocytochemistry, HeLa cells and hippocampal neurons were seeded onto glass coverslips in 12-welll plates and transfected with several combinations of plasmids, which are represented in Table 4:

Table 4: Plasmids used to transfect hippocampal neurons (DIV 3-5) and HeLa cells (24h post-seeding)

Hippocampal Neurons	Hela
Gi-DREADD/mitoGi-DREADD	P30M
GsDREADD/mitoGs-DREADD	P30M-55
P30M	P30M-82
P30M-55	mitoGi-mycDREADD
P30M-82	
mitoGi-mycDREADD	

Cells were fixed in warm 4% paraformaldehyde for 20 minutes, permeabilized in phosphate buffer saline with Triton X100 0.15% (PBS-T) and blocked for 1 hour in PBS-T 0.15% with 10% Donkey Serum. Coverslips were then incubated with primary antibodies against HA (1 hour at room temperature, 1:2000, Thermo Scientific), TOM-20 (1 h, room temperature, 1:1000, Santa Cruz) and Myc (2h, room temperature, 1:500). This was followed by 1 h incubation with the correspondent A488, A545 and A647 Alexa Fluor antibodies (1:1000, Thermo Scientific), in combination with DAPI nuclear stain (1:20000). All the antibody incubations were performed in PBS-T 0.15%% supplemented with 3% Donkey Serum. Confocal fluorescence images were acquired in an inverted Leica DMI 6000 microscope (Leica

Microsystems, Wetzlar, Germany) equipped with equipped with a confocal head Yokogawa CSU-X1 (Yokogawa Electric Corporation, Tokyo, Japan) and a sensitive Quantem camera (Photometrics, Tucson, USA). The diode lasers used were at 405 nm, 491 nm, 561 nm and 635 nm and the objective was HCX PL APO CS 63X oil 1.32 NA. The z stacks were done with a piezo P721.LLQ (Physik Instrumente (PI), Karlsruhe, Germany). This system was controlled by MetaMorph software (Molecular Devices, Sunnyvale, USA). Pixel quantification and colocalization analysis were perfomed with ImageJ software (version 1.5b, NIH, USA).

Statistical analysis

Statistical analysis of the results was performed using the GraphPad Prism software, version 5.04 (GraphPad Software Inc., San Diego, CA, USA). All the results for graphs are expressed as the mean ± standard error of the mean (SEM. Data was analyzed with the one-sample t-test, unpaired t-test with Welch's correction, one-way ANOVA (followed by Tukey's *post-hoc* test), two-way ANOVA (followed by a Sidak's *post hoc* test), when appropriate. A *p*-value below 0.05 was considered statistically significant.

Chapter 3 – Results

Chapter 3 - Results

Mitochondrial Gs and Gi DREADDs are unable to activate MAPK/ERK pathway.

In order to characterize newly obtained mitoGs and Gi-DREADD, we set out a series of functional assays based on GPCR signaling and mitochondrial activity. This characterization was performed in two distinct cell lines: HEK293 and HeLa, since the protocols for cell transfection, protein extraction and oxygen consumption assays are well optimized for the two models[93].

One of the main properties of DREADDs is the ability to activate downstream pathways in a similar manner to their parent GPCRs. A common downstream signaling event is the phosphorylation of MAPK/ERK pathway proteins, ERK 1/2, which is known to be activated through all G-protein α subunits[80,94]. Indeed, preliminary results obtained in our lab revealed that Gs and Gi-DREADD are able to recapitulate this pathway *in* vitro. However, as the nature of G-protein signaling in mitochondria is still unclear, regulation of ERK 1/2 pathway could be changed upon activation of mitoGs and mitoGi-DREADD. Therefore, we evaluated the effect of CNO on ERK 1/2 phosphorylation in HEK cells transfected with Gs/mitoGs and Gi/mitoGi-DREADD. As expected, treatment with CNO 50 μM for 30 minutes enhanced the levels of ERK 1/2 phosphorlyation (p-ERK 1/2) in HEK cells expressing Gs-DREADD (**Fig. 9A**) and Gi-DREADD (**Fig. 9B**), but this effect was lost in HEK cells expressing mitoGs-DREADD (**Fig. 9A**) or mitoGi-DREADD (**Fig. 9B**). Fetal bovine serum (FBS) 10%, used as positive control, produced a similar increase in ERK 1/2 phosphorylation for both groups, as expected[80]. These results imply that the mitochondrial targeting of DREADDs causes a loss of activation of the ERK pathway, a canonical GPCR feature.

Mitochondrial Gs and Gi-DREADDs modulate mitochondrial respiration.

Benard et al. demonstrated that mtCB1R activation decreases oxygen consumption and complex I activity in mitochondria, likely through PKA inhibition[77]. Therefore, we sought to evaluate how mitoGs and mitoGi-DREADD could modulate mitochondrial respiration in HeLa cells. HeLa cells were chosen to study mitochondrial respiratory chain activity because they generate around 80% of cellular ATP through oxidative phosphorylation. [95,96]. This ensured that the impact of mitoDREADDs was evaluated in highly active mitochondria, thus providing a realistic measure of their influence on mitochondrial respiration. To assess these effects, we measured O_2 consumption in cells following treatment with CNO 50 μM for 5 minutes. The treatment significantly increased oxygen consumption in HeLa transfected with mitoGs-DREADD (**Fig. 9C**), but not in Gs-DREADD or mitochondria-targeted GFP (MLS-GFP)-transfected cells **(Fig. 9C**), Conversely, CNO treatment promoted a significant decrease of oxygen consumption in cells transfected with mitoGi-DREADD cells (**Fig. 9D**), but not in Gi-

DREADD or MLS-GFP-transfected cells (**Fig. 9D)**. Therefore, it appears that mitoDREADD signaling modulates mitochondrial respiration in a G-protein-specific fashion.

Together with the ERK 1/2 phosphorylation assay, these two experiments demonstrate that mitoGs and Gi-DREADD are able to differentially modulate mitochondrial activity and display a unique G-protein signaling profile. Both effects are likely connected to the prominent mitochondrial targeting of the receptors

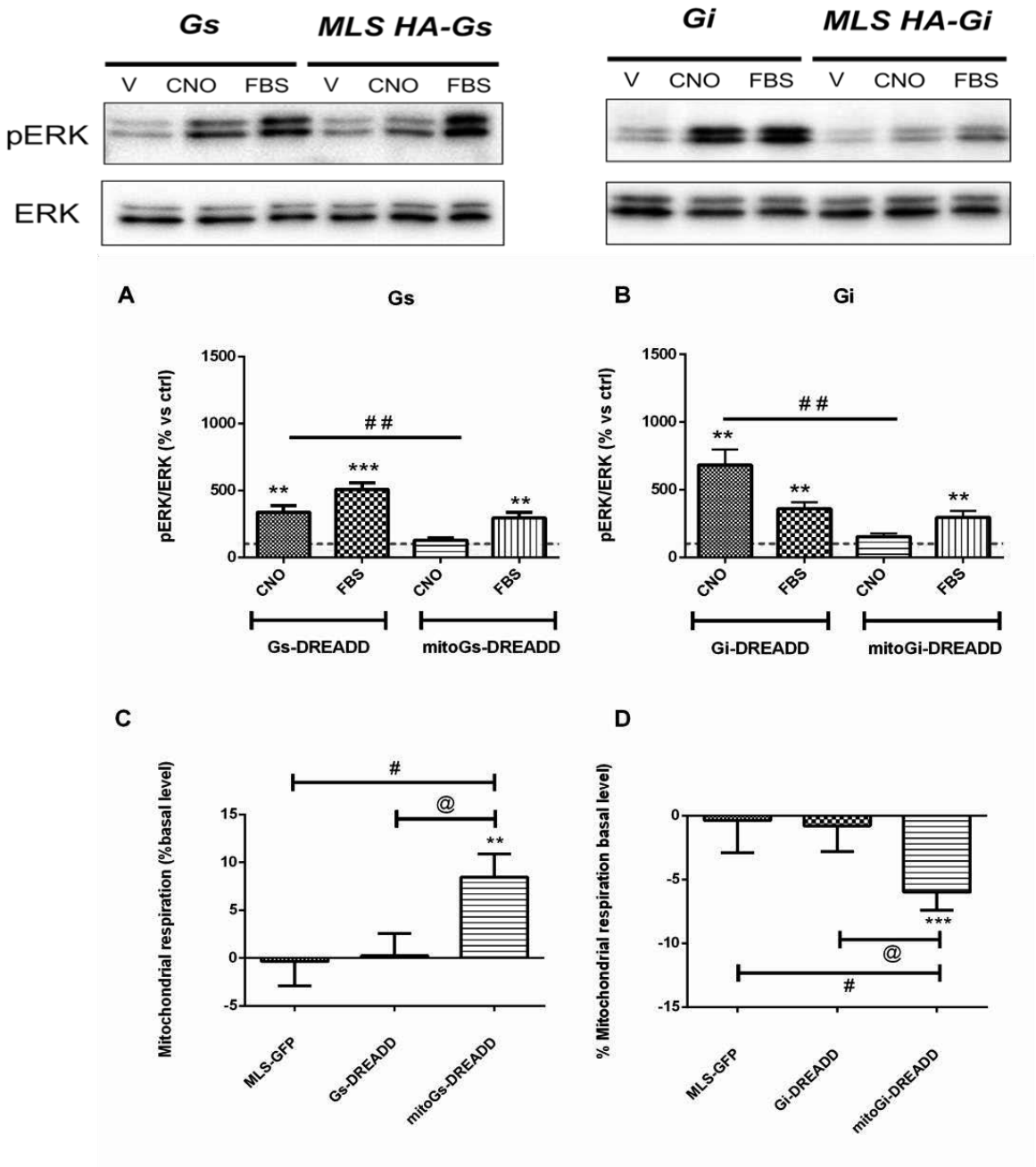

Figure 9: mitoGs and mitoGi-DREADD are unable to activate canonical MAPK pathway in HEK cells. A and B) (Upper) Representative immunoblot for the assessment of ERK phosphorylation in HEK cells expressing either Gs/mitoGs-DREADD (A) or Gi/mitoGi-DREADD(B), after a 30 min incubation with 50 μM CNO, using 10% FBS as positive control and DMSO as vehicle (Lower) Quantification of the ratio between p-ERK 1/2 and total ERK 1/2 for Gs-DREADD/mitoGs-DREADD (A) and Gi-DREADD/mitoGi-DREADD (B), expressed as the percentage of vehicle (DMSO), which was considered 100% and is represented by the red dashed line. Data are expressed as mean+s.e.m of 8 independent experiments; One-sample t-test (** P< 0.01, *** P< 0.001) was used to compare the mean of each group against 100%. Unpaired Welch's t-test (## P< 0.01) was used to compare the effect of CNO for Gs-DREADD vs mitoGs-DREADD and Gi-DREADD vs mitoGi-DREADD.
C and D) Effect of 5 min treatment with CNO 50 μM in cellular oxygen consumption of HeLa cells transfected with Gs/mitoGs-DREADD (C) or Gi/mitoGi-DREADD (D), expressed as the percentage of basal level. Value represents the mean of oxygen consumption at t=3min and t=4min. Data represent the mean+s.e.m. of 13-25 independent experiments. One-sample t-test (** P< 0.01, *** P< 0.001) was used to compare the mean of each group against zero. Unpaired Welch's t-test was used to compare mitoGs-DREADD vs Gs-DREADD (@ P< 0.05), mitoGi-DREADD vs Gi-DREADD (@ P< 0.05), mitoGs-DREADD vs MLS-GFP (# P< 0.05) and mitoGi-DREADD vs MLS-GFP (# P< 0.05).

mitoGi-DREADD is not detected in hippocampal primary neurons

Functional characterization of mitoDREADDs put us one step closer to use them as a tool to study G-protein regulation of mitochondrial transport. Next, it was necessary to check whether mitoDREADDs were still targeted to mitochondria in neurons. As a model for this and subsequent mitochondrial trafficking studies, primary hippocampal cultures were chosen, since they are more likely to recapitulate the properties of neuronal cells in vivo than any other cell lines. For this particular set of experiments, we focused on mitoGi-DREADD due to its mtCB1R-like effects in mitochondrial respiration and to the possible induction of mitochondrial arrest through JNK activation (**Fig. 7**).

Similar to what was performed in HeLa cells, primary hippocampal neurons were transfected with mitoGi-DREADD and Gi-DREADD in order to compare the mitochondrial location. Mitochondria were visualized by TOM-20 staining. However, it was impossible to evaluate the localization of mitoDREADDs, since there was no detection of the HA-Tag in any of the experimental groups. (**Fig 10 A-C**). In order to find out whether this was a technical issue with the immunostaining procedure, another primary antibody against HA was used, but the outcome was the same (not shown).

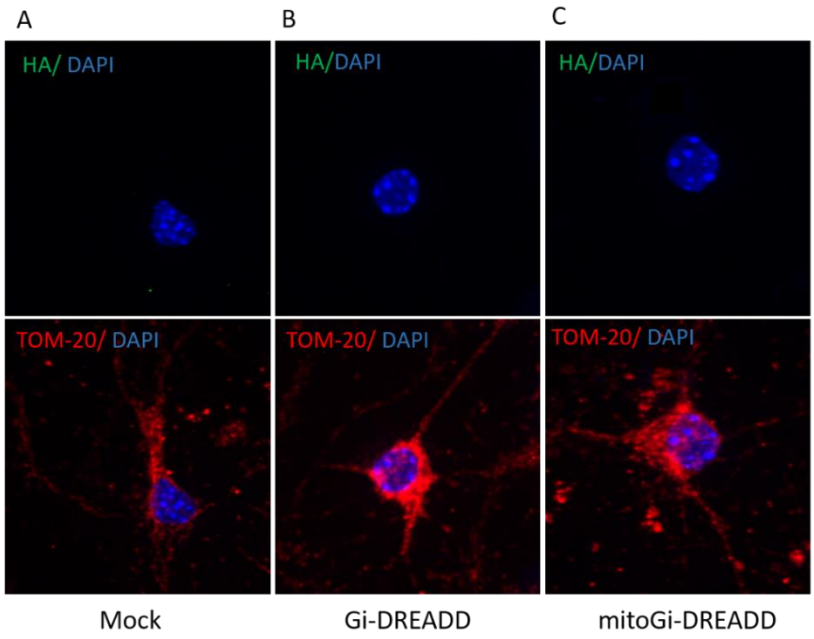

Figure 10: HA-tagged Gi-DREADD and mitoGi-DREADD are not detected in primary hippocampal neurons and thus cannot be co-localized with mitochondria. A-C) Representative confocal fluorescence images of hippocampal neurons transfected with Mock control (A), Gi-DREADD (B) and mitoGi-DREADD (C). Mitochondria were visualized with TOM-20 staining (red), nuclei were stained with DAPI (blue) and DREADDs were visualized using anti-HA antibody. Images were taken at a 63X immersion oil objective, using diode lasers of 405 nm, 491 nm and 635 nm. Images were processed with ImageJ and adequate filters were applied based on the Mock group in order to remove unspecific background.

A bicistronic pIRES allows the co-expression of Gi/mitoGi-DREADD and MitoDsRed in Hela cells, but not in primary hippocampal neurons

The previous results cast strong doubts over the ensuing functional studies on mitochondrial trafficking. First of all, as we cannot detect the receptors, there is no assurance that neurons are in fact expressing them, preventing us from studying the different impact of mitoDREADD/DREADD signaling on mitochondrial trafficking. Moreover, mitochondrial targeting cannot be evaluated, making impossible to assess the specific effects of mitoDREADDs. To overcome these issues, we developed a series of new Gi and mitoGi-DREADD constructs, either focused on ensurinG-protein expression or improving receptor detection.

To solve the problem of uncertainty in mitoDREADD expression, we designed a bicistronic Internal Ribosome Entry Site (IRES)-based expression vector encoding simultaneously mitoGi-DREADD/Gi-DREADD and the mitochondrially targeted red fluorescent protein MitoDsRed (**Fig. 11A**). As a matter of fact, this strategy has already been used to express Gi-DREADD together with fluorescent protein mCitrene in hippocampal neurons *in vivo* [97]. We chose the mammalian expression vector pIRES (Clontech, US), which allows high level expression of two genes of interest from the same bicistronic mRNA

transcript. The vector contains the encephalomyocarditis virus (ECMV) IRES, flanked by two multiple cloning sites (MCS A and B), an arrangement that allows cap-independent translation of the gene cloned into MCS B[98]. Hence, translation of the gene cloned into MCS B ensures automatically that the first protein is also expressed. By inserting MitoDsRed into MCS B, detection of this fluorescent protein at 561 nm implies necessarily that DREADD is being expressed, even in the absence of HA signal. This also poses as an advantage for mitochondrial trafficking studies, since MitoDsRed is a commonly used probe in this field [99].Additionally, pIRES utilizes a partially disabled IRES sequence that reduces the rate at which the gene cloned into MCS B is translated relative to that of MCS A, meaning that DREADD expression is always expected to be higher than MitoDsRed[98,100]. After obtaining and sequencing the new constructs, we checked their functionality by transfecting them into HeLa cells. We observed both MitoDsRed and DREADD expression in HeLa cells transfected with Gi-DREADD-MitoDsRed (P30M-55, **Fig. 11D**) and mitoGi-DREADD-MitoDsRed (P30M-82, **Fig. 11F**). As expected, control pIRES plasmid encoding only MitoDsRed (P30M, **Fig. 11B**) displayed no HA-signal. Overall, it appears that P30M-55 and 82 vectors are functional in HeLa cells, ensuring DREADD expression in an easily detectable manner.

We subsequently addressed the functionality of P30M-55 and P30M-82 in primary hippocampal neurons. By contrast to HeLa cells, neurons transfected with P30M-55 and P30M-82 showed no expression of MitoDsRed(**Fig. 11E and 11G**, respectively), which was only verified when using the P30M vector (**Fig. 11C**), although in a sparse amount of neurons. This suggested that the new construct was unable to promote neuronal DREADD expression. Interestingly, there was always a short number of astrocytes successfully transfected with P30M-55 and 82. Nevertheless, the results clearly indicate that pIRES-based strategy failed to solve the issue of DREADD detection in neurons. Moreover, we started to consider that neurons were unable to cope with high levels of mitoGi/Gi-DREADD expression, but this required further experiments.

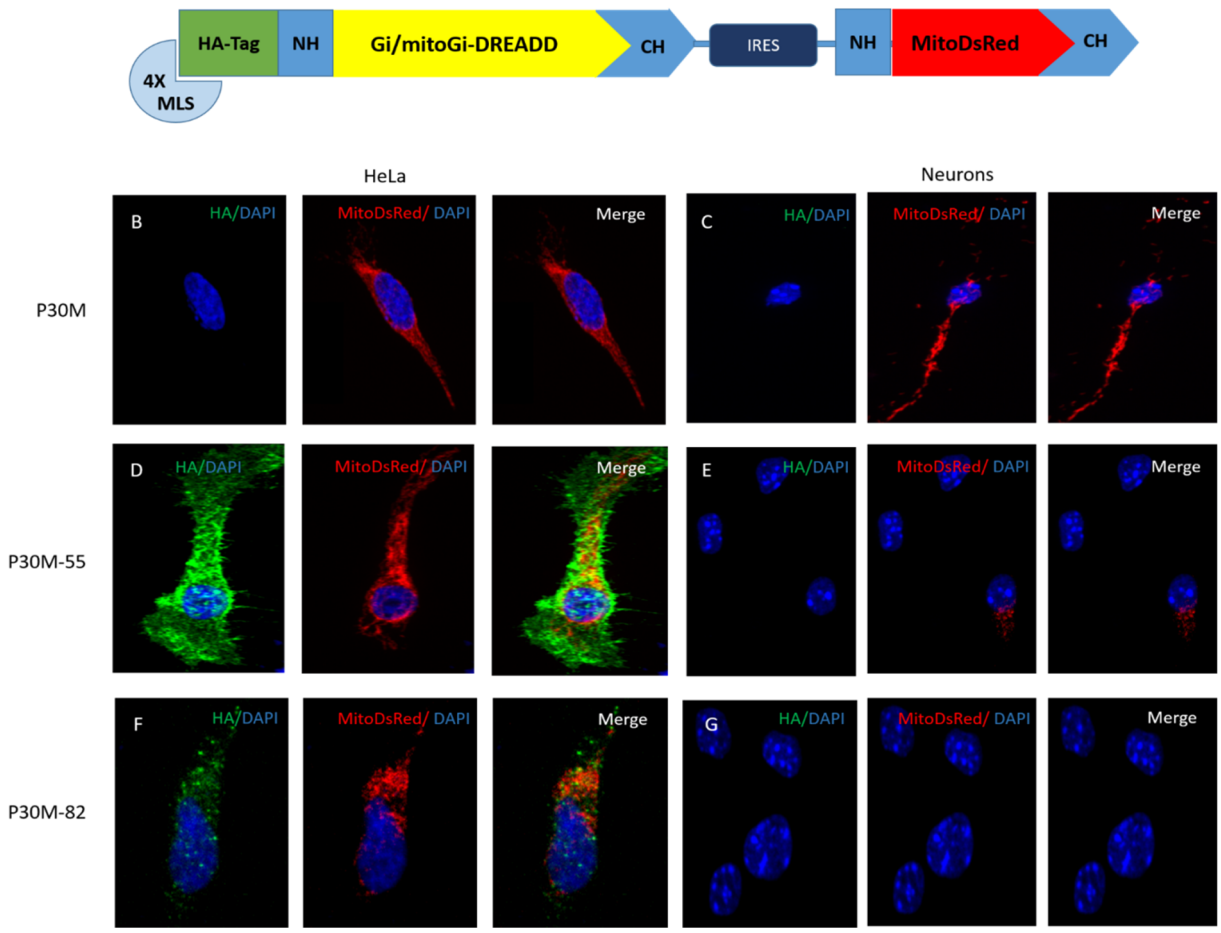

Figure 11: A bicistronic pIRES vector successfully promoted co-expression of Gi-DREADD/mitoGi-DREADD and MitoDsRed in Hela cells, but not in primary hippocampal neurons. A) Schematic representation of the bicistronic pIRES vector. Gi-DREADD or mitoGi-DREADD were inserted in MCS A, while MitoDsRed was placed in MCS B. IRES sequence allows for cap-independent initiation of translation in the middle of the transcript, thus allowing expression of both proteins. (B-F) Representative fluorescent confocal images of HeLa and primary hippocampal neurons transfected with P30M (B-C), P30M-55 (D-E) and P30M-82 (F-G). Nuclei were stained with DAPI (blue) and DREADDs were visualized using an anti-HA antibody. Images were taken at a 63X immersion oil objective, using diode lasers of 405 nm, 491 nm and 635 nm. Images were processed and adequate filters were applied based on the P30M group, in order to remove unspecific background.

Introduction of a Myc-epitope in the N-terminal of mitoGi-DREADD

In our hands, primary antibody used for HA-Tag displayed a strong background in neurons, even in mock-transfected cells. Changing of the primary antibody was also unsuccessful, since the alternative antibody displayed a non-specific nuclear signal in mock-transfected cells. Hence, we sought to replace the N-terminal HA-Tag in Gi and mitoGi-DREADD for a similar-sized Myc-Tag, in order to improve receptor detection at a fluorescence level. Myc-Tag contains a 10 amino acid segment of human protooncogene Myc (EQKLISEEDL) and it is a widely used detection system, due to the availability of highly

specific anti-Myc monoclonal antibodies[101]. So far, only a Myc-tagged mitoGi-DREADD was developed (**Fig.12 A-B**). Unfortunately, analysis of this construct was limited due to time constraints.

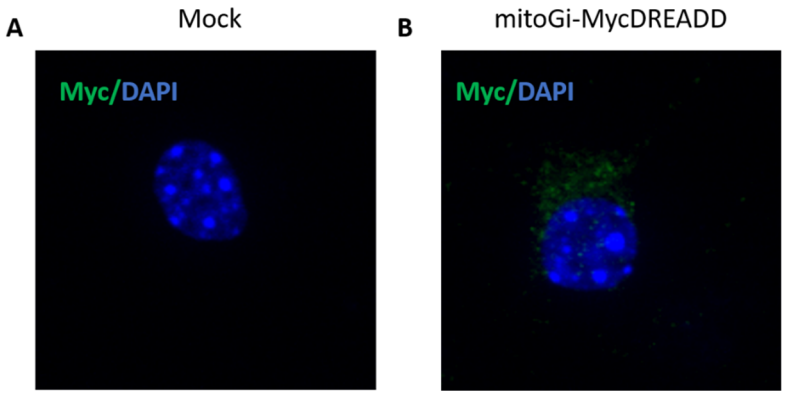

Figure 12: Preliminary results on the replacement of HA-Tag by a Myc-Tag in mitoGi-DREADD
A) Representative confocal fluorescence images of hippocampal neurons transfected with Mock control and Myc-tagged mitoGi-DREADD. Nuclei were stained with DAPI (blue) and DREADDs were visualized using anti-Myc antibody. Images were taken at a 63X immersion oil objective, using diode lasers of 405 nm and 491 nm. Images were processed and adequate filters were applied based on the Mock group, in order to remove unspecific background.

Mitochondrial motility appears to be highly affected by mitoGi-DREADD in HeLa cells

Unsuccessful outcome of the previous strategies lead to the hypothesis that neurons might be more vulnerable to the expression of the receptors, which at normal/high expression levels may lead to apoptosis. This is further supported by the observation of altered mitochondrial networks in HeLa cells transfected with mitoDREADDs (**Fig. 8 B-E**). Hence, it could be possible that mitoDREADD expression at high levels would affect per se mitochondrial function and health. To address this issue, we performed live recordings of HeLa cells co-transfected with MitoDsRed and either Gi-DREADD or mitoGi-DREADD, without any CNO treatment. First of all, we observed again an abnormal morphology of mitochondrial network in mitoGi-DREADD-transfected cells. Strikingly, altered-shape mitochondria appear to be motionless in HeLa cells transfected with mitoGi-DREADD. By contrast, Gi-DREADD appears to have no effect on mitochondrial motility, which is further supported by the normal aspect of mitochondrial network. These results, although still preliminary and lacking proper quantification, clearly suggest that expression of mitoGi-DREADD is enough to impair mitochondrial movement in HeLa cells.

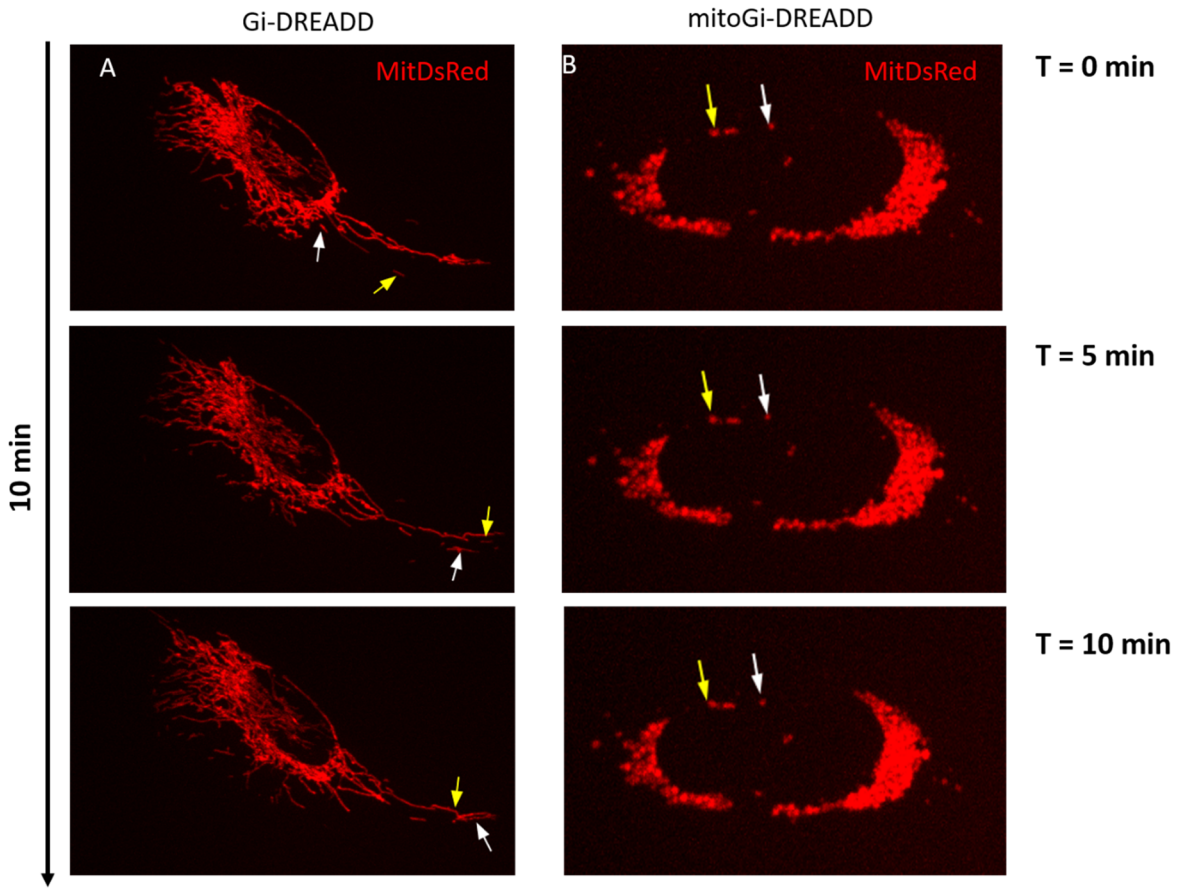

Figure 13 Expression of mitoGi-DREADD impairs mitochondrial motility in HeLa cells. (A-B). Series of three representative projections taken at every 5 minutes, of live HeLa cells expressing either Gi-DREADD (A) or mitoGi-DREADD (B), together with the mitochondrially-targeted protein MitoDsRed (coloured in red). Coloured arrows identify the same mitochondria at different time points.

CNO treatment produces a tendency for the decrease of mitochondrial motility in neurons transfected with mitoGi-DREADD

Despite the problems involving DREADD detection and expression, we noticed that MitoDsRed was normally expressed in neurons when co-transfected with either DREADD-Gi or mitoGi-DREADD. Therefore, it was still possible that the receptors were being expressed at a sub-detection level. Assuming that mitoGi-DREADD could be anyway targeted to neuronal mitochondria, we decided to assess the functional impact of Gi-coupled pathways on mitochondrial trafficking. For that, we recorded in live-imaging experiments MitoDsRed-tagged mitochondria in the axons of hippocampal neurons transfected either Gi-DREADD or mitoGi-DREADD. This system allows for an unambiguous distinction between anterograde and retrograde transport, because axons exhibit a uniform microtubule polarity (**Fig.3**). Mitochondrial transport was quantified before and after a 15 min treatment with 50 µM CNO, a dose which matches the one used in mitoDREADD functional studies. We analyzed several trafficking parameters before and after the treatment with CNO, using DMSO as a vehicle.

In control (pre-treatment) conditions, Gi-DREADD and mitoGi-DREADD-transfected neurons exhibited similar and prominent mitochondrial trafficking, with an average of 66% and 65% motile mitochondria, respectively (Fig. 15A and 15B). The distance covered by mitochondria (Gi-DREADD - anterograde: 40.396 ± 3.591 µm , retrograde: 38.832± 3.594 µm; mitoGi-DREADD – anterograde: 35.959 ± 3.295 µm, retrograde: 34.289 ± 3.267 µm) and the total speed at which mitochondria were transported (Gi-DREADD - anterograde: 0.450 ± 0.036 µm s^{-1} , retrograde: 0.428 ± 0.028 µm s^{-1} ; mitoGi-DREADD – anterograde: 0.427 ± 0.042 µm s^{-1}, retrograde: 0.385 ± 0.034 µm s^{-1}) were similar in both directions for the two groups (Fig. 15C and 15D). Overall, the parameters are within the reported in previous studies[99,100]. Thus, mitochondrial motility in neurons is apparently unaffected by the transfection of Gi/mitoGi-DREADD.

Surprisingly, CNO treatment produced a strong tendency towards the reduction of the fraction of motile mitochondria in mitoGi-DREADD transfected neurons (**Fig. 16E**). By contrast, CNO had no effect in mitochondrial motility for neurons transfected with Gi-DREADD (**Fig. 16E**). Moreover, in neurons transfected with mitoGi-DREADD, CNO induced a significant and selective decrease of the fraction of mitochondria moving in retrograde direction, without altering anterograde transport, which may explain the tendency for mitochondrial arrest (Δv= -11.30 µm s^{-1}, 2-way ANOVA, $p < 0.05$, **Fig. 16F and 16G**). However, this effect is likely misleading, since DMSO unexpectedly interfered with the fraction of motile mitochondria in both directions (Fig 16F and 16G). Neither total velocity nor distance covered by mitochondria were affected upon CNO exposure in both Gi-DREADD and mitoGi-DREADD-transfected neurons (**Fig. 16H-I and K-M**). Dwelling time, defined as the time spent paused by motile mitochondria, also remained unaltered (**Fig. 16J**).

Taken together, these experiments suggest that selective activation of mitochondrial Gi-coupled pathways may decrease mitochondrial motility by impairing retrograde transport, but not other parameters. At the same, the results indicate that mitoGi-DREADD may be expressed in neurons and is targeted to neuronal mitochondria. We further conclude that is crucial to increase the number of independent experiments, in order to consolidate this effect and to reduce the high variability in DMSO-related parameters.

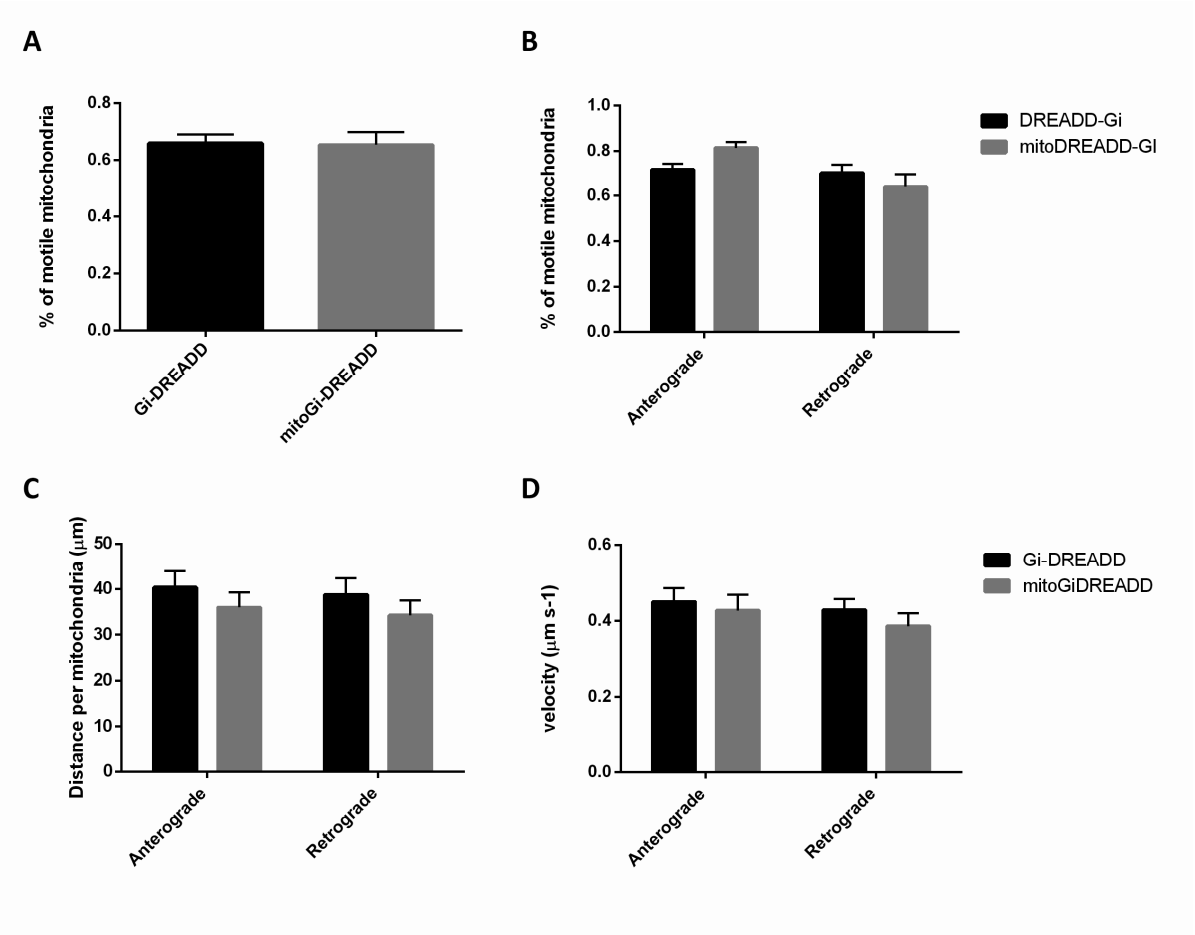

Figure 15: Control neurons (pre-CNO or pre-DMSO treatment) co-transfected with Gi-DREADD/mitoGi-DREADD and MitoDsRed display similar and prominent motility rates, velocity and distance travelled by mitochondria, in both directions. (A-B) Graphic representation of the total fraction of motile mitochondria (A) or specific retrograde and anterograde fractions (B) in Gi-DREADD and mitoGi-DREADD transfected (C-D) Graphic representation of the distance covered by individual mitochondria(C) and their total velocity (D) in anterograde and retrograde direction. All data were obtained from pre-treatment recordings and are expressed as mean+s.e.m from 15-16 independent experiments.

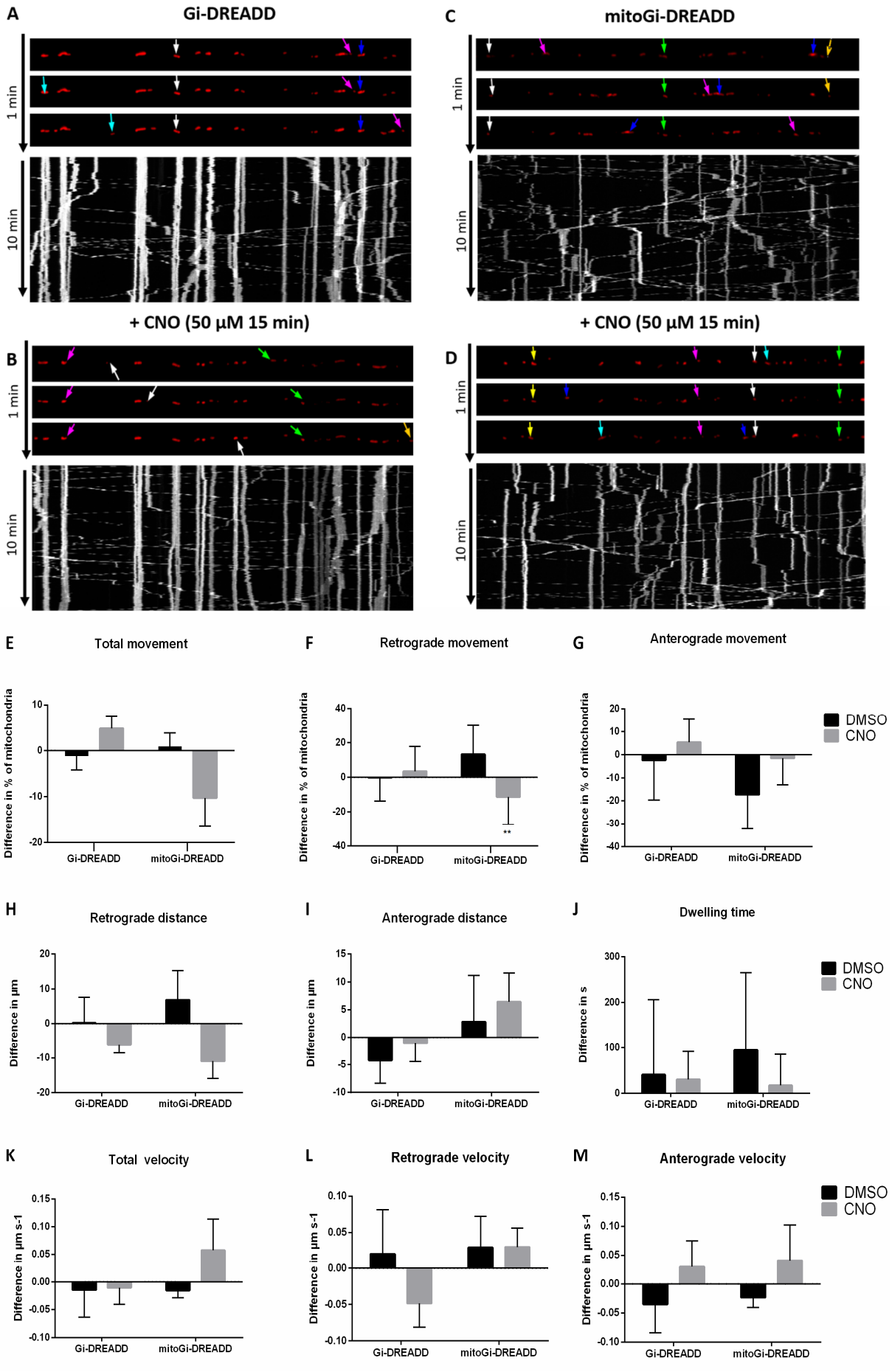
A
Gi-DREADD
1 min
10 min
C
mitoGi-DREADD
1 min
10 min
+ CNO (50 µM 15 min)
B
1 min
10 min
+ CNO (50 µM 15 min)
D
1 min
10 min
E
Total movement
Difference in % of mitochondria
Gi-DREADD
mitoGi-DREADD
F
Retrograde movement
Difference in % of mitochondria
**
Gi-DREADD
mitoGi-DREADD
G
Anterograde movement
Difference in % of mitochondria
DMSO
CNO
Gi-DREADD
mitoGi-DREADD
H
Retrograde distance
Difference in µm
Gi-DREADD
mitoGi-DREADD
I
Anterograde distance
Difference in µm
Gi-DREADD
mitoGi-DREADD
J
Dwelling time
Difference in s
DMSO
CNO
Gi-DREADD
mitoGi-DREADD
K
Total velocity
Difference in µm s-1
Gi-DREADD
mitoGi-DREADD
L
Retrograde velocity
Difference in µm s-1
Gi-DREADD
mitoGi-DREADD
M
Anterograde velocity
Difference in µm s-1
DMSO
CNO
Gi-DREADD
mitoGi-DREADD

Figure 16: CNO produces a tendency for decreasing mitochondrial motility in mitoGi-DREADD-transfected neurons, while having no effect in neurons transfected with Gi-DREADD A-D) Series of four representative confocal images, taken every 30 s, of live axons expressing either Gi-DREADD (A-B) or mitoGi-DREADD (C-D) together with mitochondrially-targeted protein MitoDsRed (in red) before (A,C) and after treatment with 50 μM CNO for 15 minutes (B,D). Below, representative kymographs (1 per condition) showing the full time 10 min acquisition before and after the treatment. Vertical lines represent stationary mitochondria. Coloured arrows identify single mitochondria during the 1 minute representative projections. E-M) Graphical representation of several mitochondrial transport parameters in Gi-DREADD and mito Gi-DREADD transfected neurons treated with CNO or DMSO, always expressed as the difference in comparison to pre-treatment situation. E-G) Graphical representation of the changes in the percentage of motile mitochondria, as a total, retrograde or anterograde-moving fraction H-I) Graphical representation of the changes in the distance covered by individual mitochondria in retrograde or anterograde direction J)Graphical representation of the changes in dwelling time, defined as the ratio between the total pause time and total number of motile mitochondria K-M) Graphical representation of changes in mitochondrial total, retrograde and anterograde velocity. Data are expressed as mean+s.e.m from 7-8 independent experiments, each corresponding to one axon. Data were analyzed with two-way ANOVA followed by Sidak's *post*-hoc test, with ** $p<0.05$ for mitoGi-DREADD CNO vs mitoGi-DREADD DMSO

Chapter 4 - Discussion

Chapter 4 - Discussion

The recent discovery of mtCB1R, together with the unveiling of its possible role in mitochondrial transport, has protracted a new link between mitochondrial motility and G-protein mediated signaling. Third-generation chemogenetic tools, known as DREADDs, represent the ideal strategy to further explore this connection through their specific targeting to mitochondria.

In the first part of this study, we report that mitochondrial Gs and Gi-DREADDs have a distinct functional profile, which makes them a suitable chemogenetic tool to control mitochondrial G-protein signaling. First of all, our data shows that both mitoDREADDs are unable to recapitulate the canonical GPCR-ERK pathway, by opposition to Gi-DREADD and Gs-DREADD. Therefore, mitochondrial targeting of DREADDs will likely prevent them from inducing the phosphorylation of ERK proteins, since these are anchored in cytoplasm together with Raf protein and MAPK kinase, hence being inaccessible to mitochondrial signaling [94]. Consequently, only DREADDs located in the plasma membrane are able to activate ERK signaling cascade, thus mimicking their "parental" GPCRs. Contrary to this hypothesis, recent studies suggest that a subset of ERK 1/2 is targeted to mitochondria in several cell types such as cardiomyocytes and macrophages as part of anti-apoptotic and antioxidant mechanisms [102],[103],[104]. Although there are no reports linking this specific ERK pathways to mitochondrial GPCR signaling, this pool is thought to be constitutively expressed in the matrix and inner membrane of the organelle, where it can be activated by MEK, hence supporting a possible connection with GPCR signaling[103]. Thus, our data can be interpreted in a different manner, in which the modification of DREADDs renders them dysfunctional and unable to promote mitochondrial ERK phosphorylation. However, to first validate this hypothesis, we would need first to assess p-ERK levels in a mitochondrial preparation after CNO treatment, since the fraction of mitochondrial ERK is usually much lower than the cytoplasmic one[105]. Moreover, further characterization of the mechanisms involved in mitochondrial ERK regulation is also necessary, since the process can be independent of GPCR signaling. Indeed, it has already been proven that mitochondrial activity and redox signaling are necessary to activate the inner ERK pool, favoring a GPCR-independent modulation[103]. Nevertheless, all interpretations point towards an alteration of the signaling profile of mitoDREADDs being unable to increase phosphorylation of total ERK, with the issue lying on whether they can still activate G-protein pathways at the mitochondria.

In line with the previous questions, we showed that mitoGi and mitoGs DREADDs are able to differentially modulate mitochondrial respiration in HeLa cells, as opposite to non-targeted DREADDs. Hence, both receptors appear to be activating specific G-protein pathways regulating mitochondrial activity. In addition, the results not only support a possible and yet unknown mitochondrial location for Gαs but also a role for this subunit in promoting respiration. Intriguingly, another Gα subunit, Gαq, is necessary for the regulation of mitochondrial respiration, ATP production and membrane potential [28].Thus, developing a mitoGq-DREADD could be an interesting strategy to further establish the role of mitochondrial Gαq subunits in regulating respiration and support the effects obtained with mitoGi and Gs-

DREADD. Finally, the reduction in respiration produced by mitoGi-DREADD resembles that produced by mtCB1R, indicating this effect on oxygen consumption may occur due to activation of Gi-coupled pathways and consequent decrease of mitochondrial PKA activity.

To further expand the characterization of mitoDREADDs in a more physiological system and evaluate its impact on mitochondrial trafficking, we used hippocampal neurons. However, when transfecting the previously characterized Gi-DREADD/mitoGi-DREADD in neurons, we were unable to detect the receptor. This could either happen because the receptor was not being expressed at detectable levels or due to a specific problem of HA immunostaining in neurons. Initially, to overcome these problems, we designed a pIRES-based strategy for expressing mitoGi-DREADD and Gi-DREADD in a visually detectable manner, but again this was unsuccessful in neurons, despite the promising results in HeLa cells. The latter observation helped rule out any problems with the vector, together with the extensive reports of using pIRES to simultaneously express two proteins in neurons, including DREADDs[97,106]. As the problem could also arise from a low efficiency of calcium phosphate-based transfection, we tried two alternative high-efficiency methods, Lipofectamine 3000 (Thermofisher Scientific) and Amaxa 4D nucleofection (Lonza). However, both approaches were unfit to enable DREADD expression in P30M-55 and P30M-82 transfected neurons (data not shown).

Considering the failure of all previous strategies, we postulated that hippocampal neurons are much more vulnerable to the expression of Gi-DREADD or mitoGi-DREADD, even at normal levels, which in turn may lead to cell death. Previous observations already suggested that mitoGi-DREADD expression could be harmful for mitochondria, since HeLa cells transfected with the receptor exhibited abnormal, aggregated-like mitochondrial networks (**Fig. 8B and 8D**). Strikingly, this phenotype closely resembles the one observed in situations of excessive mitochondrial fragmentation, as well as the mitochondrial clustering which occurs during apoptosis (**Fig. 17 A-B**)[107,108]. In accordance to that, live recordings of Hela cells performed in this work demonstrated that mitochondrial dynamics is highly affected when expressing mitoGi-DREADD, by opposition to normal DREADDs. This absence of movement, in combination with the clustered-like network, clearly suggests that mitoGi-DREADD expression interferes with mitochondrial dynamics and, consequently, may lead to apoptosis. As neurons rely on mitochondrial trafficking to ensure axonal growth, synapse assembly and energy production at distal terminals, they are much more susceptible to changes in mitochondrial function, thereby aggravating the consequences of mitoDREADD expression. We postulate that these effects arise from forcing the expression of a rather large transmembrane protein (DREADD) into the inner membrane of mitochondria. This may lead to profound alterations in fission/fusion balance, which is crucial to regulate mitochondrial transport and ensure cell survival[107,109]. As the aberrant morphology can either result from hyperfusion (**Fig. 17A**) or excessive fragmentation, measurements of Mfn1/2, Opa1 and Drp1 are required, followed by evaluation of apoptosis through caspase-3 assays, always comparing Gi-DREADD and mitoGi-DREADD-expressing cells[107]. Additionally, this could be combined with experiments on the activation of autophagy survival pathways, given their tight connection with mitochondrial morphology, which may dictate survival of the organelle[110]. All together, these experiments would allow to decipher if really exists a correlation between mitoGi-DREADD-induced mitochondrial clustering and apoptosis.

As for Gi-DREADD, the results are completely unexpected, given its widespread use in the control of neuronal activity *in vivo* and *in vitro* without any expression or toxicity issues. Furthermore, Gi-DREADD appears to have no impact in mitochondrial dynamics or morphology in Hela cells. Here, a possible improvement could be achieved by using embryonic neuronal cultures that display better survival, although the original report on Gi-DREADD development and characterization used postnatal hippocampal neurons to assess the receptor effects on neuronal firing, without any cytotoxic effects [80].

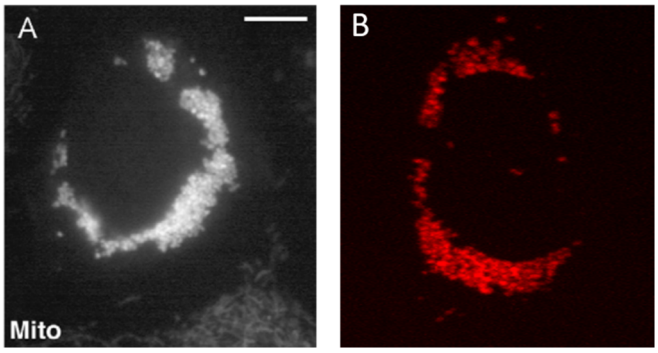

Fig. 17 mitoGi-DREADD may cause mitochondrial aggregation combined with excessive fragmentation. A) Aggregates of small, fragmented mitochondria are visible after Mfn2 overexpression in rat liver cell line clone 9. B) One of the timepoint projections obtained in live recordings of Hela cells, where MitoGi-DREADD seems to cause a similar effect in mitochondrial aggregation and fragmentation. Mitochondria were visualized with Mito-GFP in A) and MitoDsRed in B). Figure 16A) was adapted from Huang, P. et al. (2007).

At the same time, and having into account the novelty of mitoDREADD as a chemogenetic tool, we aim to develop strategies able to minimize its deleterious effects. A possible approach consists in replacing CoxVII-MLS by another targeting sequence directed to outer mitochondrial membrane (OMM), avoiding an excessive protein packing into the inner membrane and transmembrane space. This poses as a risky challenge, since outer membrane proteins contain the targeting sequences within mature regions of the protein[88]. Therefore, DREADD structure would have to be modified, which may change receptor function. To minimize the risks, the ideal candidate would be outer membrane proteins with C-tail anchors as targeting signals, such as Omp25 or Bcl-2, since previous studies show that fusion of these anchors with other proteins resulted in targeting and insertion of the hybrid protein into the OMM[111,112].

Overexpression of both Gi-DREADD and mitoGi-DREADD might be also contributing to their negative impact, because the cytomegalovirus (CMV) promoter present in both vectors is known for producing high, possibly supra-physiological expression levels[113]. Therefore, strong constitutive CMV promoter could be replaced by a series of weaker promoters, such as synthetic mutations of CMV, in order to modulate DREADD expression[113].

Besides the development of the pIRES-based strategy, we also replaced HA-Tag by a Myc-tag in mitoGi-DREADD receptor. Although HA tag is widely used to detect proteins because of its strong immunoreactivity, Myc-tag could enhance the detection in this particular case. However, it is so far unclear whether this has produced any improvements, since our

preliminary observations lack mitochondrial co-localization assays. Moreover, Myc-tagged Gi-DREADD is also being developed. Simultaneously, we intend to fuse the CoxVII MLS to the already existent and well-characterized mCherry-Gi-DREADD (UNC Chapel Hill, Roth Lab), thus creating a mitochondria-targeted fluorescent Gi-DREADD. However, this strategy is considered troublesome, since it would produce an even larger-sized DREADD targeted to mitochondria, probably aggravating its deleterious effect. Nonetheless, it would allow us to study mitochondrial trafficking in live cell imaging without any uncertainty regarding receptor expression and eliminating the need for co-transfection with a fluorescent protein. .

Finally, we showed that mitoGi-DREADD activation may decrease mitochondrial motility in axons of primary hippocampal neurons, by opposition to Gi-DREADD. For now, these are preliminary results, since we need to increase the number of experiments and eliminate the high variability of DMSO-associated effects, especially regarding retrograde transport. Moreover, as there is no possibility so far of assessing DREADD expression in neurons, we cannot state for sure that any possible effect results either from receptor expression or from its specific targeting. Nevertheless, the tendency for decreasing motility supports the idea that a population of neurons is expressing mitoDREADD-Gi at sub-optimal levels, but who are nonetheless functional and capable of modulating mitochondrial trafficking. Indeed, this putative low expression appears to have no effect *per se,* since the data also revealed that mitochondrial motility in a control state is normal for both mitoGi-DREADD and Gi-DREADD. Therefore, the tendency for decrease is in fact caused by CNO treatment and, consequently, by mitoGi-DREADD activation.

Chapter 5- Conclusion and future perspectives

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In this work we describe the first attempt to develop chemogenetic mitoGs and Gi-DREADDs capable of controlling specific G-protein pathways in mitochondria. We initially demonstrated that mitoGs and Gi-DREADDs exhibit a distinct signaling profile than non-modified counterparts as a consequence of their mitochondrial targeting. This is manifested as a loss of ERK pathway activation and a bi-directional modulation of respiration, which may have unveiled a new role for Gs-dependent signaling in mitochondria. As a future perspective, we intend to develop a mitoGq-DREADD to compare with the previous effects and widen our possibilities. These observations represent a considerable advance in establishing mitoDREADDs as an exciting albeit unpolished tool to expand the yet unknown field of G-protein mitochondrial signaling.

Despite the promising insights on functional characterization, we were unable to detect mitoGi-DREADD and Gi-DREADD in neurons. The latter, which contrasts with numerous reports, is more likely caused by technical issues, such as misadjusted expression levels or a higher vulnerability of our cultures. On the other hand, we found that mitoGi-DREADD causes a dysfunction in organelle dynamics and motility by itself, an effect which we attribute to an unnatural transmembrane protein expression in the IMM. In line with that, future assays will probably reveal deregulated levels of fusion/fission proteins and an increased apoptosis in mitoGi-DREADD transfected cells, as well as changes in autophagic pathways. Overall, this constitutes a strong handicap for the validation of mitoDREADDs as a chemogenetic tool to control G-protein mitochondrial signaling. Nonetheless, we consider that the several strategies previously proposed will be able to minimize the negative impact of mitoGi-DREADD. In this regard, combination of an OMM-targeted MLS with weaker CMV promoters will likely reduce the overload on the inner mitochondrial space. However, detection issues are expected after applying these strategies, since they will reduce the expression of the receptor. Fortunately, pIRES-MitoDsRed- based strategy or double-promoter strategies can be adapted to the new mitoGi/Gi-DREADD constructs. Moreover, the process of inserting a Myc tag is not yet completed and may help improving the detection of any new constructs.

Despite the constraints imposed by mitoGi-DREADD in neurons, this tool has still a great potential in the context of mitochondrial G-protein signaling, which is partially confirmed by our observations on mitochondrial trafficking. In particular, the selective tendency for decreased motility in mitoGi-DREADD transfected neurons indicates that a sub-detectable expression of the receptor may still be able to modulate trafficking. After increasing the number of individual experiments, we expect a significant decrease on mitochondrial motility upon mitoGi-DREADD activation. This will allow establish a firm connection between mtCB1R-induced mitochondrial arrest and activation of Gi-coupled pathways at the mitochondria. If this is the case, the following step would consist in evaluating the role of JNK especially regarding anterograde transport (**Fig,7**).Together with the effect of mitoGi-DREADD in mitochondrial respiration, this work would allow us to establish two new Gi-dependent pathways modulating mitochondrial activity. As a future perspective, and depending on the successful optimization of mitoGi-DREADD, it would be extremely interesting to compare its effects on mitochondrial trafficking with mitoGs-DREADD and a yet hypothetic mitoGq-DREADD, which may also

decrease mitochondrial motility (**Fig. 6**). This way, we could explore several other pathways by which mtCB1R exerts its regulation of mitochondrial trafficking. In this regard, newmitoDREADDs may also provide new insights on emergent mtCB1R properties and novel mitochondrial G-protein pathways.

Chapter 6 - References

Chapter 6 - References

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