

# Mitochondrial CB<sub>1</sub> receptors regulate neuronal energy metabolism

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The mammalian brain is one of the organs with the highest energy demands, and mitochondria are key determinants of its functions. Here we show that the type-1 cannabinoid receptor (CB<sub>1</sub>) is present at the membranes of mouse neuronal mitochondria (mtCB<sub>1</sub>), where it directly controls cellular respiration and energy production. Through activation of mtCB<sub>1</sub> receptors, exogenous cannabinoids and *in situ* endocannabinoids decreased cyclic AMP concentration, protein kinase A activity, complex I enzymatic activity and respiration in neuronal mitochondria. In addition, intracellular CB<sub>1</sub> receptors and mitochondrial mechanisms contributed to endocannabinoid-dependent depolarization-induced suppression of inhibition in the hippocampus. Thus, mtCB<sub>1</sub> receptors directly modulate neuronal energy metabolism, revealing a new mechanism of action of G protein-coupled receptor signaling in the brain.

The brain represents only 2% of the total body weight in mammals, but it consumes up to 20% of the body's resting energy production<sup>1,2</sup>. Ensuring and regulating cellular energy supplies, mitochondria are key elements of eukaryotic cell functions<sup>1,3</sup> that are crucial for the regulation of brain functions<sup>1,4</sup>. The involvement of neuronal energetics in brain physiology and pathology is the focus of intensive research<sup>1,4,5</sup>, but the molecular mechanisms linking mitochondrial activity to brain functions are still poorly documented.

G protein-coupled receptors (GPCRs) represent one of the largest protein families controlling neuronal activity. The presence of GPCRs on neuronal mitochondria and their potential ability to modulate neuronal energetics and function is still a matter of speculation<sup>6</sup>. However, there is now consistent evidence that mitochondria contain G proteins<sup>7,8</sup>. Moreover, several reports have shown the intramitochondrial localization of potential downstream effectors of G protein signaling, such as soluble adenylyl cyclase<sup>9</sup>, phosphodiesterase<sup>10,11</sup> and protein kinase A (PKA)<sup>12</sup>. Thus, cAMP can be produced in mitochondria, leading to activation of PKA and phosphorylation of mitochondrial proteins, eventually regulating mitochondrial respiration and energy production<sup>10,11,13,14</sup>. However, the upstream mechanisms regulating the intramitochondrial cAMP-PKA signaling cascade in neurons, and the mechanisms coupling mitochondrial activity and neuronal physiology, remain poorly understood<sup>1,4</sup>.

CB<sub>1</sub> receptors are GPCRs highly enriched in neuronal plasma membranes, where they tightly control neuronal activity, metabolism and functions<sup>15–17</sup>. Early studies suggested that the *Cannabis sativa* (marijuana)-derivative cannabinoid Δ<sup>9</sup>-tetrahydrocannabinol (THC) could affect mitochondrial functions<sup>18</sup>. However, with the identification of cannabinoid receptors as typical plasma membrane GPCRs<sup>15,19</sup>, mitochondrial effects of lipophilic cannabinoids on neurons were ascribed to nonspecific alterations of membrane properties<sup>20</sup>. Recent evidence points also to the ability of CB<sub>1</sub> receptor signaling to regulate mitochondrial biogenesis in peripheral non-neural tissues<sup>21,22</sup>. The lipophilic nature of most cannabinoids<sup>15</sup> implies that receptor-ligand interactions might occur not only at plasma membranes, but also inside cells. Indeed, different intracellular compartments contribute to the regulation of endocannabinoid metabolism<sup>23,24</sup>, and CB<sub>1</sub> receptors have been shown to functionally signal in lysosomal or endosomal intracellular membranes<sup>25</sup>.

The present study stemmed from the neuroanatomical observation that CB<sub>1</sub> receptor immunogold particles are often detected on neuronal mitochondria in electron microscopy experiments, but they are generally considered nonspecific background labeling. This observation prompted us to investigate the specificity of this signal by using mutant mice constitutively lacking the CB<sub>1</sub> receptor<sup>26</sup>. The results showed that CB<sub>1</sub> receptors are present on neuronal mitochondrial

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membranes, and their direct activation regulates mitochondrial energetics. Furthermore, stimulation of intracellular CB<sub>1</sub> receptors and inhibition of presynaptic mitochondrial activity participate in endocannabinoid-dependent regulation of short-term synaptic plasticity in the hippocampus, providing a possible link between neuronal energy metabolism and specific neuronal functions.

## RESULTS

### CB<sub>1</sub> receptor protein is localized on neuronal mitochondria

CB<sub>1</sub> receptor immunoreactivity was detected by immunogold electron microscopy on mitochondrial membranes of CA1 hippocampal neurons of wild-type mice (mtCB<sub>1</sub>; Fig. 1a and Supplementary Fig. 1a), but not *Cnr1*<sup>-/-</sup> (here called CB<sub>1</sub><sup>-/-</sup>) littermates (Fig. 1b and Supplementary Fig. 1b). Approximately 30% of wild-type CA1 mitochondria displayed CB<sub>1</sub> immunolabeling, whereas only 2.9% ± 0.7% of CA1 mitochondria in CB<sub>1</sub><sup>-/-</sup> mice showed nonspecific labeling (Fig. 1c). MtCB<sub>1</sub> receptors were comparably distributed between axon terminals and somato-dendritic compartments in wild-type mice (Supplementary Fig. 2a). The density of mtCB<sub>1</sub> immunogold labeling was 18.3 ± 2.0 particles per μm<sup>2</sup> of mitochondria in wild type and dropped to 0.4 ± 0.1 particles per μm<sup>2</sup> in CB<sub>1</sub><sup>-/-</sup> mice (Fig. 1d). Further quantifications revealed that 15.5% ± 4.2% of CA1 neuronal total CB<sub>1</sub> protein was localized to mitochondria. Western immunoblotting confirmed the specific presence of CB<sub>1</sub> immunoreactivity in purified wild-type brain mitochondria (Fig. 1e and Supplementary Fig. 3). Semiquantification of immunogold images revealed that approximately 95% of mtCB<sub>1</sub> was localized to the outer membrane of mitochondria (Fig. 1f). This was confirmed by Western immunoblotting of purified mitochondria treated with trypsin with or without digitonin presolubilization, suggesting that the N-terminal

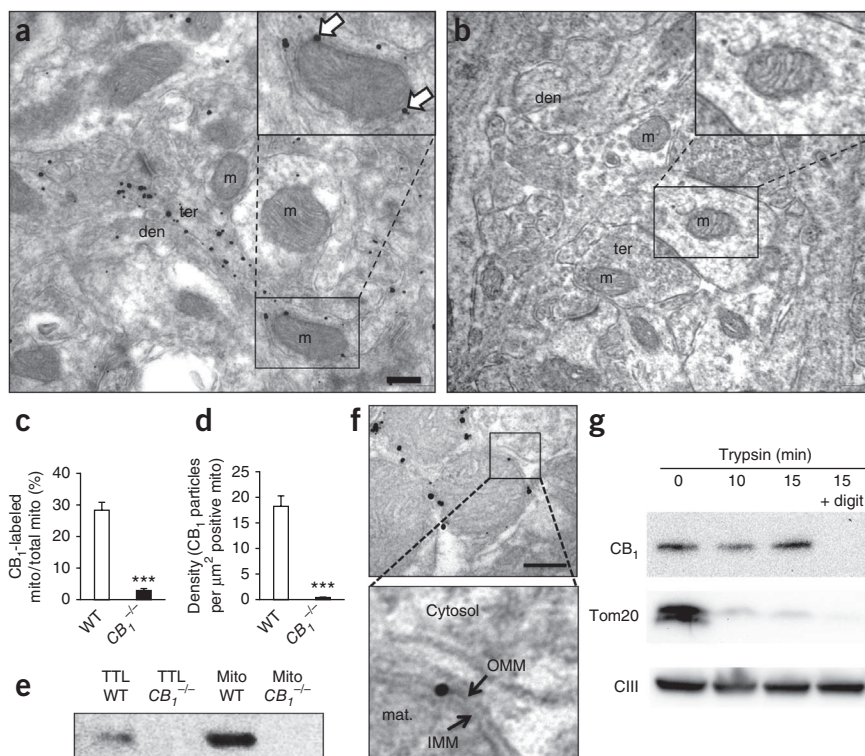
portion of mtCB<sub>1</sub> is exposed to the cytosol, whereas the C terminus is likely located in the mitochondrial intermembrane space (Fig. 1g and Supplementary Fig. 4). Thus, a substantial portion of CB<sub>1</sub> receptor protein is present at the surface of neuronal mitochondria.

CB<sub>1</sub> receptors are expressed in different neuronal populations, where they negatively regulate excitability and neurotransmission<sup>15–17</sup>. These include inhibitory neurons containing GABA and excitatory glutamatergic neurons<sup>24</sup>, where activation of CB<sub>1</sub> receptors differentially affects brain functions and behavior<sup>16,17,27,28</sup>. We analyzed the anatomical distribution of mtCB<sub>1</sub> receptors in the hippocampus of conditional mutant mice lacking CB<sub>1</sub> either in cortical glutamatergic (*Glu-CB<sub>1</sub>*<sup>-/-</sup>) or in GABAergic (*GABA-CB<sub>1</sub>*<sup>-/-</sup>) neurons, respectively<sup>27,28</sup>. MtCB<sub>1</sub> was still present in CA1 hippocampal neurons of both mutant strains (Fig. 2a,b and Supplementary Fig. 1c,d), with similar reductions in the percentage of labeled mitochondria relative to that in wild-type mice (Fig. 2c). In contrast, the density of mtCB<sub>1</sub> was reduced by 50.4% in *GABA-CB<sub>1</sub>*<sup>-/-</sup>, but not in *Glu-CB<sub>1</sub>*<sup>-/-</sup>, mutants (Fig. 2d). No difference between these two genotypes was observed concerning the proportion of mtCB<sub>1</sub> versus total CB<sub>1</sub> expression (Fig. 2e). As expected, most mtCB<sub>1</sub> was detected in terminals bearing asymmetric synapses (presumably excitatory) in *GABA-CB<sub>1</sub>*<sup>-/-</sup> mice, whereas it was almost exclusively present on mitochondria of presumably inhibitory symmetric synapses in *Glu-CB<sub>1</sub>*<sup>-/-</sup> mice (Supplementary Fig. 2b). Thus, both neuronal populations contain mtCB<sub>1</sub> receptors, which are, however, more densely expressed in hippocampal GABAergic neurons than in glutamatergic ones.

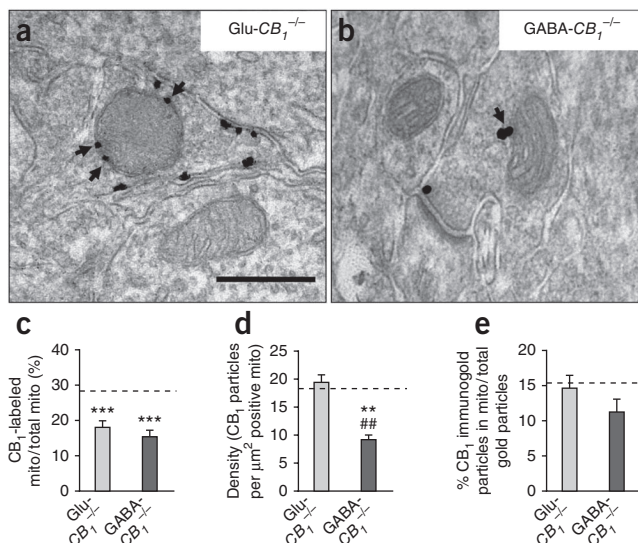
### MtCB<sub>1</sub> directly regulates brain mitochondrial activity

The acute administration of THC to C57BL/6/N mice (intraperitoneally, 5 mg per kilogram body weight) reduced mitochondrial

**Figure 1** CB<sub>1</sub> receptors in neuronal mitochondria. (a,b) Electron immunogold detection of CB<sub>1</sub> receptors on mitochondrial membranes of neurons in the CA1 hippocampal region of wild-type (a) and CB<sub>1</sub><sup>-/-</sup> mice (b). Insets, details of single mitochondria. Arrows, specific CB<sub>1</sub> receptor localization. Scale bar, 200 nm. (c) CB<sub>1</sub>-immunopositive mitochondria (mito) as a proportion of total mitochondria (WT, *n* = 463 mitochondria from 3 mice; CB<sub>1</sub><sup>-/-</sup>, *n* = 458 mitochondria from 3 mice). (d) Density of CB<sub>1</sub> receptor immunoparticles per area of the immunopositive subset of mitochondria (WT, total mitochondrial area, 35.7 μm<sup>2</sup>; CB<sub>1</sub><sup>-/-</sup>, total mitochondrial area, 29.4 μm<sup>2</sup>). (e) Immunoblotting of CB<sub>1</sub> receptor protein in whole brain total tissue lysate (TTL) from wild-type (WT) and CB<sub>1</sub><sup>-/-</sup> mice and purified mitochondria from WT and CB<sub>1</sub><sup>-/-</sup> mice. Full-length blots are shown in Supplementary Figure 3c. (f) Detail of CB<sub>1</sub> receptor gold particles on the outer mitochondrial membrane. Scale bar, 200 nm. (g) Trypsin and digitonin assay of CB<sub>1</sub> receptor expression in mitochondria. Resistance of mtCB<sub>1</sub> C-terminal domain to trypsin digestion but not to mild membrane permeabilization by digitonin (digit) indicates that the receptor is localized on the outer membrane with the C-terminal portion of the protein facing the mitochondrial intermembrane space. Full-length blots are shown in Supplementary Figure 4. Den, dendrites; ter, terminals; m, mitochondria; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; mat., matrix; Tom20, translocase of outer mitochondrial membranes 20 kDa; CIII, core 2 protein of mitochondrial complex III. Values, mean ± s.e.m. \*\*\**P* < 0.001 as compared to WT.



**Figure 2** Mt-CB<sub>1</sub> is present in both GABAergic and glutamatergic CA1 hippocampal neurons. (a,b) Immunogold localization of mtCB<sub>1</sub> (arrows) in the CA1 hippocampal region of Glu-CB<sub>1</sub><sup>-/-</sup> (a) and GABA-CB<sub>1</sub><sup>-/-</sup> mice (b). (c) Proportion of CB<sub>1</sub>-immunopositive mitochondria normalized to the total number of mitochondria in the CA1 hippocampal region of Glu-CB<sub>1</sub><sup>-/-</sup> (*n* = 473 mitochondria from 3 mice) and GABA-CB<sub>1</sub><sup>-/-</sup> mice (*n* = 320 mitochondria from 3 mice). (d) Density of mtCB<sub>1</sub> in Glu-CB<sub>1</sub><sup>-/-</sup> and GABA-CB<sub>1</sub><sup>-/-</sup> mice. (e) Proportion of mtCB<sub>1</sub> receptor normalized to the total CB<sub>1</sub> signal in the CA1 hippocampal region of Glu-CB<sub>1</sub><sup>-/-</sup> (*n* = 31 fields) and GABA-CB<sub>1</sub><sup>-/-</sup> (*n* = 27 fields) mice. Scale bar, 500 nm. Values, mean ± s.e.m. \*\**P* < 0.01; \*\*\**P* < 0.001 as compared to WT; ##*P* < 0.01 as compared to Glu-CB<sub>1</sub><sup>-/-</sup> mice. Dashed horizontal lines, wild-type values.



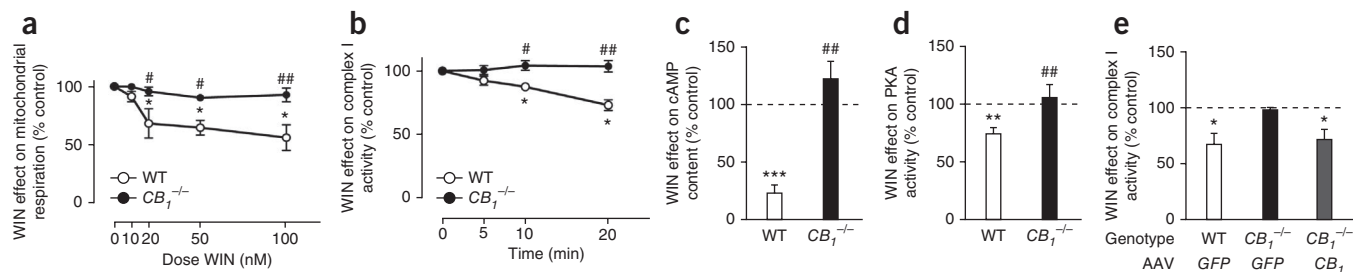
respiratory chain complex I activity (particularly involved in neuronal bioenergetics)<sup>4</sup> in purified hippocampal mitochondria (vehicle, 94.7 ± 6.2; THC, 65.9 ± 8.1 nmol oxidized NADH per minute per milligram protein; *n* = 6, *P* < 0.05). CB<sub>1</sub> receptor signaling typically involves reduction of cAMP<sup>15,19,29</sup>, and mitochondrial forms of adenylyl cyclase and protein kinase A (PKA) have been suggested to regulate complex I activity and mitochondrial respiration<sup>7,8,11,13,14,30</sup>. Acute THC treatment decreased mitochondrial cAMP by approximately 30% (vehicle, 0.60 ± 0.06; THC, 0.41 ± 0.06 nmol per mg protein; *n* = 6, *P* < 0.05). To test whether the effects of CB<sub>1</sub> receptor activation were due to a direct activation of mtCB<sub>1</sub> or to an indirect stimulation of CB<sub>1</sub> receptors located on the plasma membrane, brain mitochondria were purified from wild-type and CB<sub>1</sub><sup>-/-</sup> littermates (Supplementary Fig. 3a) and treated *in vitro* with the synthetic CB<sub>1</sub> agonist WIN55,212-2 (WIN). The drug dose-dependently decreased oxygen consumption in brain mitochondria from wild-type, but not CB<sub>1</sub><sup>-/-</sup>, mice (Fig. 3a). WIN specifically decreased mitochondrial complex I activity (Fig. 3b) and cAMP content (Fig. 3c). Hippocampal neuronal mitochondria contained the A-kinase anchor protein 121 (AKAP121; Supplementary Fig. 1e), confirming that PKA activity occurs in these organelles<sup>12</sup>. WIN decreased mitochondrial PKA activity in an mtCB<sub>1</sub>-dependent manner (Fig. 3d). To establish whether hippocampal CB<sub>1</sub> expression is sufficient for the mitochondrial effects of WIN, we injected the hippocampi of CB<sub>1</sub><sup>-/-</sup> mice with neurotropic adeno-associated viruses (AAV) expressing either a control protein (green fluorescent protein, AAV-GFP) or CB<sub>1</sub> (AAV-CB<sub>1</sub>)<sup>31</sup>. The effect of WIN on complex I activity of purified hippocampal mitochondria was fully rescued by *in vivo* viral re-expression of the CB<sub>1</sub> receptor in CB<sub>1</sub><sup>-/-</sup> hippocampal mitochondria (Fig. 3e).

To investigate whether mtCB<sub>1</sub> is responsive to natural plant-derived cannabinoids, we measured the effects of THC on respiration, cAMP

levels and PKA activity in neuronal mitochondria from wild-type and CB<sub>1</sub><sup>-/-</sup> littermates. THC induced effects similar to those of WIN (Fig. 4a–c), indicating that the functions of mtCB<sub>1</sub> are not dependent on the type of ligand.

Brain mitochondria have substantial levels of endocannabinoid degrading activity (Supplementary Table 1). To test whether endocannabinoids regulate mitochondrial activity *in situ*, we treated purified brain mitochondria with JZL195 (JZL), a dual inhibitor of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), the two main endocannabinoid degrading enzymes<sup>15,32</sup>, and measured mitochondrial endocannabinoid levels and respiration. The endocannabinoid anandamide was detected in purified neuronal mitochondria, but its levels were below the limits of reliable quantification (data not shown). However, JZL dose-dependently increased mitochondrial 2-arachidonoylglycerol (Fig. 4d) and reduced respiration in brain mitochondria from wild-type, but not CB<sub>1</sub><sup>-/-</sup>, mice (Fig. 4e). Notably, a highly significant inverse correlation was observed between 2-arachidonoylglycerol levels and mitochondrial respiration in wild-type brain mitochondria (*r* = 0.82, *P* < 0.0001; Fig. 4f).

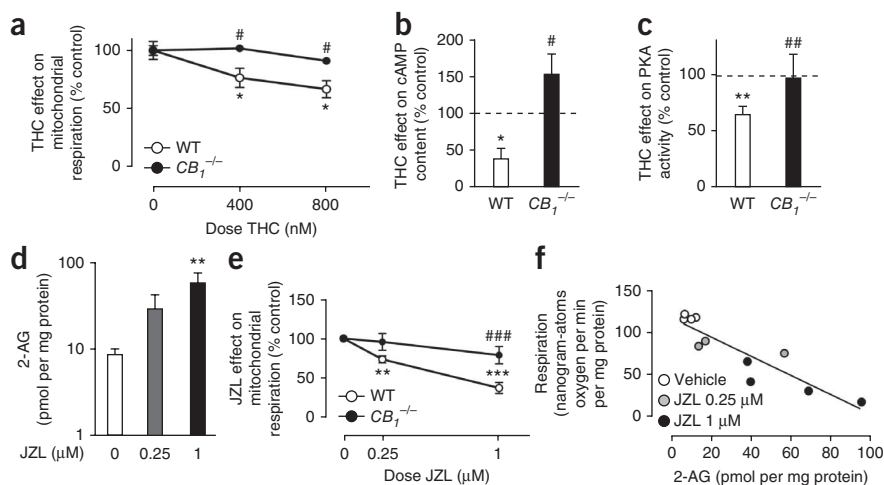
Together, these data show that (i) mtCB<sub>1</sub> signaling directly affects mitochondrial cAMP accumulation, PKA activity, complex I activity and respiration in the brain, and (ii) mitochondria are equipped with endocannabinoids able to activate mtCB<sub>1</sub> *in situ*.



**Figure 3** Direct regulation of mitochondrial activity by mtCB<sub>1</sub> receptors in the brain. (a) Dose–response curve for the effect of the CB<sub>1</sub> receptor agonist WIN on respiration of purified mitochondria from wild-type (WT) and CB<sub>1</sub><sup>-/-</sup> brains (*n* = 4 independent experiments). (b) Time course of enzymatic activity of complex I in the presence of 100 nM WIN in purified mitochondria from WT and CB<sub>1</sub><sup>-/-</sup> brains (*n* = 3). (c) cAMP content after 5 min treatment of purified mitochondria with 100 nM WIN (*n* = 4). (d) Effect of 100 nM WIN on mitochondrial PKA activity (*n* = 3). (e) Effect of 100 nM WIN on complex I activity in purified mitochondria from WT and CB<sub>1</sub><sup>-/-</sup> hippocampi locally injected with AAV-GFP, and from CB<sub>1</sub><sup>-/-</sup> hippocampi locally injected with AAV-CB<sub>1</sub> (*n* = 6 per group). Values, mean ± s.e.m. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 as compared to control (vehicle or time 0); #*P* < 0.05, ##*P* < 0.01 as compared to WT. Dashed horizontal lines, values for vehicle-treated controls.

**Figure 4** MtCB<sub>1</sub> receptors are activated by THC, and mitochondria contain biologically active endocannabinoids. (a) Dose–response curve for the effect of THC on respiration of purified mitochondria from wild-type (WT) and *CB<sub>1</sub><sup>-/-</sup>* brains. (b) Effect of THC on mitochondrial cAMP content in WT and *CB<sub>1</sub><sup>-/-</sup>* littermates. (c) Effect of THC on PKA activity in WT and *CB<sub>1</sub><sup>-/-</sup>* littermates.

(d) Levels of the endocannabinoid 2-arachidonoylglycerol (2-AG) in purified brain mitochondria after treatment with the double MAGL and FAAH inhibitor JZL195 (JZL). Data are presented on a logarithmic scale ( $n = 3–5$  pools of mitochondria purified each time from 10–15 mice). (e) Effect of JZL on respiration of purified brain mitochondria ( $n = 3–5$  pools of mitochondria purified each time from 10–15 mice). (f) Correlation between 2-AG and respiration in purified brain mitochondria from WT mice (values are from d,e). Values, mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  as compared to vehicle control; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  as compared to WT. Dashed lines, vehicle values.



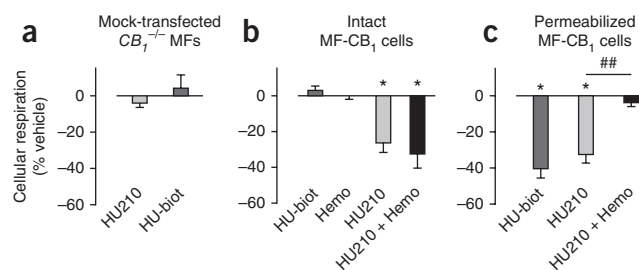
### Intracellular CB<sub>1</sub> receptors participate in DSI

If mtCB<sub>1</sub> can control brain mitochondrial activity, are mitochondria involved in endocannabinoid-dependent neuronal functions? Retrograde control of hippocampal GABAergic transmission is an important endocannabinoid-dependent form of synaptic plasticity<sup>15–17</sup>. Depolarization of CA1 postsynaptic pyramidal neurons mobilizes endocannabinoids, which retrogradely activate presynaptic CB<sub>1</sub> receptors, transiently decreasing GABAergic inhibitory neurotransmission in a process known as depolarization-induced suppression of inhibition (DSI)<sup>15–17</sup>. DSI is blocked by CB<sub>1</sub> receptor antagonists and is occluded by CB<sub>1</sub> receptor agonists, which prevent the binding and the action of endogenously mobilized endocannabinoids<sup>16</sup>. If intracellular mtCB<sub>1</sub> receptors participate in the expression of DSI, cell-impermeant CB<sub>1</sub> receptor antagonists and agonists should not be able to fully block and occlude DSI, respectively. The peptide CB<sub>1</sub> receptor antagonist hemopressin<sup>25,33,34</sup> provided a first tool to test this hypothesis. As a peptide, hemopressin should not be able to penetrate plasma membranes<sup>25</sup>. This was confirmed both by measurements of intracellular fluorescence of cells treated with a fluorescent derivative of hemopressin (Supplementary Fig. 5a–c) and by spectrometric analysis of extracts from hippocampal slices incubated with hemopressin and the lipophilic CB<sub>1</sub> antagonist AM251 (Supplementary Fig. 5d–f). Thus, hemopressin can be used as a cell-impermeant CB<sub>1</sub> receptor antagonist.

To investigate the occluding effects of cell-impermeant CB<sub>1</sub> receptor agonists on DSI and to discriminate the relative influence of plasma membrane and intracellular CB<sub>1</sub> receptors on cellular respiration, we synthesized a biotinylated version of the lipophilic CB<sub>1</sub> receptor agonist HU210 (HU210-biotin, hereafter HU-biot). The presence of the hydrophilic biotin bulk should impede the cell penetration of this compound, and the presence of a spacer between the HU210 scaffold and the biotin moiety should preserve the affinity for CB<sub>1</sub> receptors. Radioligand competition experiments revealed that HU-biot displayed a comparable affinity for recombinant CB<sub>1</sub> receptors to that of HU210 (HU210,  $K_i = 0.2 \pm 0.07$  nM; HU-biot,  $K_i = 1.5 \pm 0.4$  nM). To characterize the ability of HU210 and HU-biot to penetrate plasma membranes and stimulate mtCB<sub>1</sub> receptors, we transfected primary mouse fibroblast cultures derived from *CB<sub>1</sub><sup>-/-</sup>* pups with a plasmid expressing the CB<sub>1</sub> receptor (creating MF-CB<sub>1</sub> cells). Neither HU210 nor HU-biot had any effect on cellular respiration of

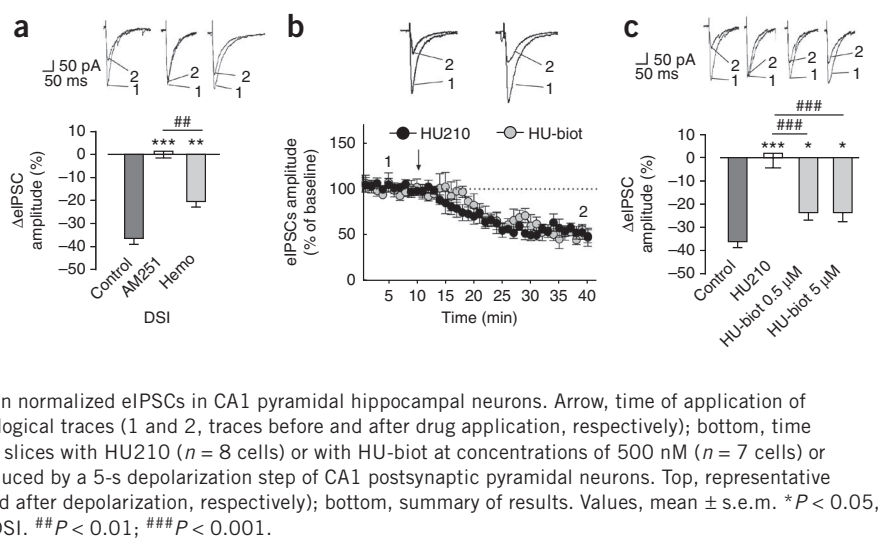
mock-transfected *CB<sub>1</sub><sup>-/-</sup>* mouse fibroblasts (Fig. 5a). Conversely, only HU210, but not HU-biot, decreased cellular respiration in MF-CB<sub>1</sub> cells (Fig. 5b), suggesting that cell penetration is a necessary step for this effect. Accordingly, HU-biot became as efficient as HU210 in depressing cell respiration in permeabilized MF-CB<sub>1</sub> cells (Fig. 5c), indicating that both drugs activate mtCB<sub>1</sub> receptors, but the difference relies on their cell permeance. Notably, hemopressin was not able to alter the effect of HU210 on intact MF-CB<sub>1</sub> cells (Fig. 5b), but it fully blocked this effect in permeabilized cells (Fig. 5c). These data indicate that (i) the lipophilic CB<sub>1</sub> agonist HU210 can penetrate cells and alter cellular respiration, (ii) HU-biot and hemopressin do not penetrate intact cells and do not alter cellular respiration, and, notably, (iii) only the activation of intracellular CB<sub>1</sub> receptors affects mitochondrial respiration of living cells.

We next analyzed the impact of the cell-permeant CB<sub>1</sub> antagonist AM251 and agonist HU210 on inhibitory synaptic transmission and DSI, and compared their effects to those of the cell-impermeant antagonist



**Figure 5** Hemopressin and HU-biot do not penetrate cells and cannot alter cellular respiration in intact cells. Mouse fibroblasts (MFs) were obtained from *CB<sub>1</sub><sup>-/-</sup>* pups and transfected with a vector expressing either the fluorescent protein mCherry (mock) or the CB<sub>1</sub> receptor (MF-CB<sub>1</sub>). (a) Neither HU210 nor HU-biot altered cellular respiration in mock-transfected *CB<sub>1</sub><sup>-/-</sup>* MFs ( $n = 6$  independent experiments). (b) HU-biot and hemopressin (Hemo) did not alter cellular respiration in MF-CB<sub>1</sub> cells, whereas the lipophilic agonist HU210 significantly decreased oxygen consumption. Hemo was not able to antagonize this effect of HU210 in intact MF-CB<sub>1</sub> cells ( $n = 4–7$  independent experiments). (c) In permeabilized MF-CB<sub>1</sub> cells, HU-biot reduced cellular respiration and Hemo became able to fully block the depression by HU210 ( $n = 3–5$  independent experiments). Values, mean  $\pm$  s.e.m. \* $P < 0.05$  as compared to vehicle control. ## $P < 0.01$ .

**Figure 6** Intracellular CB<sub>1</sub> receptors contribute to depolarization-induced DSI. **(a)** Effects of the lipophilic CB<sub>1</sub> receptor antagonist AM251 (4 μM, *n* = 5 cells) and of the cell membrane-impermeant CB<sub>1</sub> receptor antagonist peptide hemopressin (Hemo, 10 μM; *n* = 8 cells) on the amplitude of strong DSI induced by a 5-s depolarization step from -70 to 0 mV of CA1 postsynaptic pyramidal neurons (Control, *n* = 9 cells). Top, representative electrophysiological traces (1 and 2, traces before and after depolarization, respectively); bottom, summary of results. **(b)** Effects of the lipophilic synthetic CB<sub>1</sub> receptor agonist HU210 (500 nM; *n* = 7 cells) and of the cell membrane-impermeant CB<sub>1</sub> receptor agonist HU-biot (500 nM; *n* = 6 cells) on normalized eIPSCs in CA1 pyramidal hippocampal neurons. Arrow, time of application of HU210 or HU-biot. Top, representative electrophysiological traces (1 and 2, traces before and after drug application, respectively); bottom, time course. **(c)** Occlusion effect of 30-min incubations of slices with HU210 (*n* = 8 cells) or with HU-biot at concentrations of 500 nM (*n* = 7 cells) or 5 μM (*n* = 5 cells) on the amplitude of strong DSI induced by a 5-s depolarization step of CA1 postsynaptic pyramidal neurons. Top, representative electrophysiological traces (1 and 2, traces before and after depolarization, respectively); bottom, summary of results. Values, mean ± s.e.m. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.0001 as compared to control DSI. ##*P* < 0.01; ###*P* < 0.001.

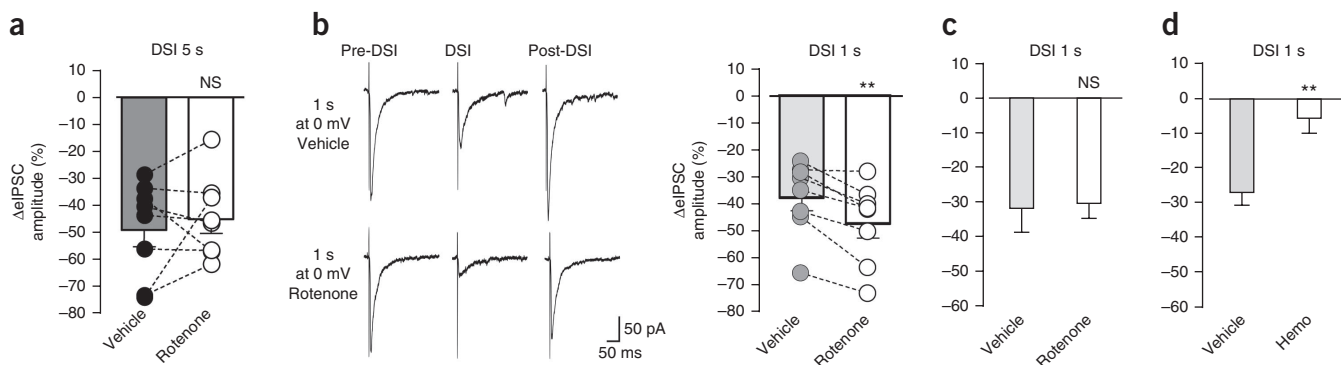


hemopressin and agonist HU-biot, respectively. As expected<sup>16</sup>, a 'strong' DSI induced by a 5-s depolarization of CA1 pyramidal neurons was fully blocked by AM251 (Fig. 6a). The application of hemopressin at a high dose (10 μM) did not alter evoked inhibitory postsynaptic currents (eIPSCs) when applied to hippocampal slices (Supplementary Fig. 6a,b). However, the peptide antagonist only partially and reversibly reduced DSI amplitude (Fig. 6a and Supplementary Fig. 6c,d). High concentrations of HU210 and HU-biot (500 nM) displayed a very similar ability to reduce eIPSCs (Fig. 6b), independently of the frequency of stimulation of afferent fibers (Supplementary Fig. 6a,b). Notably, the extracellular antagonist hemopressin fully blocked the depression of eIPSCs by both HU210 and HU-biot (Supplementary Fig. 6a,b), suggesting that intracellular CB<sub>1</sub> receptor signaling is not required for this pharmacological cannabinoid effect and that the biotin moiety does not alter the effect of HU210 on hippocampal GABAergic transmission. The maximal eIPSCs-depressing effect of both HU210 and HU-biot was observed after 25–30 min of incubation (Fig. 6b and Supplementary Fig. 6a). As expected, a 30-min incubation with HU210 fully occluded DSI induction in hippocampal slices (Fig. 6c and Supplementary Fig. 6d). In contrast, HU-biot only partially occluded DSI at concentrations

of both 500 nM and 5 μM (Fig. 6c and Supplementary Fig. 6d). Thus, cell penetration of CB<sub>1</sub> antagonists and agonists is necessary for the respective full blockade and occlusion of DSI induced by 5-s depolarization of postsynaptic pyramidal neurons.

#### Inhibition of mitochondrial activity contributes to DSI

Rotenone is a well known specific and potent mitochondrial complex I inhibitor. The application of rotenone to hippocampal slices (2.5 μM) had no effect on strong DSI induced by a long depolarization period (5 s; Fig. 7a). However, if a weaker DSI was induced by a 1-s depolarization step, rotenone reliably enhanced DSI amplitude (Fig. 7b). Rotenone was unable to affect eIPSCs in the absence of depolarization (Supplementary Fig. 7a), to induce DSI in slices from CB<sub>1</sub><sup>-/-</sup> mice in the presence of the depolarization (Supplementary Fig. 7b), to alter the inhibitory effects of HU-biot on eIPSCs (Supplementary Fig. 7c), or to modify the levels of mitochondrial endocannabinoids (Supplementary Fig. 7d). These results indicate that the effect of rotenone on DSI was not due to a direct alteration of inhibitory neurotransmission, that it was dependent on CB<sub>1</sub> receptors, and that it was not due to increase in local endocannabinoid levels. DSI is mediated by presynaptic CB<sub>1</sub> receptors<sup>16</sup>. Rotenone did not



**Figure 7** Inhibition of mitochondrial activity potentiates DSI. **(a)** Amplitudes of strong DSI induced by a 5-s depolarization step from -70 to 0 mV of CA1 postsynaptic pyramidal neurons before and after bath application of the mitochondrial complex I inhibitor rotenone (2.5 μM). **(b)** Amplitudes of weak DSI induced by a 1-s depolarization step of CA1 postsynaptic pyramidal neurons before and after bath application of rotenone (2.5 μM). Left, representative electrophysiological traces. Right, summary data plot. **(c)** Amplitude of DSI induced by a 1-s depolarization step of CA1 postsynaptic pyramidal neurons with postsynaptic intracellular application of vehicle (*n* = 6 cells) or rotenone (2.5 μM; *n* = 6 cells). **(d)** Effect of hemopressin (Hemo, 10 μM; *n* = 7 cells) on the amplitude of DSI induced by a 1-s depolarization step of CA1 postsynaptic pyramidal neurons (gray bar, *n* = 7 cells). Values, mean ± s.e.m. \*\**P* < 0.01 as compared to control. NS, not significant.

produce any effect when infused postsynaptically (Fig. 7c), suggesting the involvement of presynaptic mitochondria in the expression of strong DSI. Consistent with these data, hemopressin was able to fully abolish a weak DSI induced by a 1-s depolarization (Fig. 7d), further confirming that intracellular CB<sub>1</sub> receptors contribute to strong, but not weak, forms of DSI.

## DISCUSSION

The present results show that the CB<sub>1</sub> GPCR is present in a portion (approximately 30% in the hippocampus) of brain neuronal mitochondria, where it directly regulates mitochondrial respiration. This function may be exerted through the modulation of complex I activity, by regulating cAMP levels and PKA activity, which have been recently proposed to occur in mitochondria and to modulate the organelle's respiration and energy production<sup>7,8,11,13,14,30</sup>. Intracellular CB<sub>1</sub> receptors are involved in DSI, an endocannabinoid-dependent form of short-term plasticity of inhibitory neurotransmission in the hippocampus, and this function is mimicked by direct inhibition of complex I activity. Thus, these data establish the existence of mtCB<sub>1</sub> receptors and their role in the regulation of neuronal bioenergetics, which might be involved in endocannabinoid-dependent synaptic plasticity.

The hypothesis that GPCRs may directly regulate mitochondrial activity has been recently proposed<sup>6,30</sup>. The open source program MitoProt II (ref. 35) predicted the CB<sub>1</sub> receptor protein to bear a 39.3% probability of mitochondrial localization (Supplementary Table 2). This theoretical value is much higher than that of many GPCRs and is comparable to the values of several known or recently identified mitochondrial proteins (Supplementary Table 2). Our experimental data demonstrate that the metabotropic CB<sub>1</sub> receptor is present on mitochondrial membranes of neurons, where it acutely regulates energy metabolism and likely contributes to the expression of DSI.

The proportion of CB<sub>1</sub> receptors located on the mitochondrial membrane (approximately 15% of total cell CB<sub>1</sub> receptors) might seem low. However, this expression accounts for up to 30% of mtCB<sub>1</sub>-dependent reduction of respiration in purified brain mitochondria. Notably, approximately 30% of neuronal mitochondria do contain CB<sub>1</sub> receptors, suggesting a strong impact of cannabinoid signaling on the subset of organelles expressing mtCB<sub>1</sub>. Moreover, a low abundance of CB<sub>1</sub> receptors can bear crucial functional significance in specific locations. Although a minimal amount (less than 10%) of total cortical CB<sub>1</sub> receptor proteins are expressed in glutamatergic neurons<sup>24,27</sup>, many of the functions of the endocannabinoid system are exerted by this limited percentage of receptors. For instance, protection against excitotoxic seizures or the orexigenic properties of endocannabinoid signaling are mainly exerted by glutamatergic-neuron CB<sub>1</sub> receptors<sup>27,28</sup>. Therefore, the 15% of hippocampal CB<sub>1</sub> protein located on mitochondrial membranes cannot be considered a negligible amount from a functional point of view.

Neuronal bioenergetics is emerging as a key player in brain functions<sup>1,2,4,5</sup>. This holds true for neurodegenerative disorders, in which mitochondrial alterations are thought to be of etiopathological relevance<sup>4</sup>. However, mitochondria also influence the normal functioning of the brain, regulating synaptic plasticity and possibly behavior<sup>1,2,4,5</sup>. The identification and functional characterization of brain mtCB<sub>1</sub> receptors provides a new mechanism of modulation of neuronal activity, which might have key roles in both physiological and pathological brain processes.

CB<sub>1</sub>-dependent forms of synaptic plasticity such as DSI are likely to underlie many functions of endocannabinoid signaling in the brain<sup>15-17</sup>. Neurotransmitter release is a very energy-demanding

process<sup>5</sup>, and mitochondria can modulate its efficiency by different means<sup>4</sup>. The membrane-impermeant CB<sub>1</sub> receptor agonist HU-biot and the antagonist hemopressin only partially occluded and blocked DSI, respectively. In addition, inhibition of presynaptic mitochondrial activity by rotenone potentiated this form of synaptic plasticity, suggesting the involvement of mitochondrial activity in DSI. The latter effect could also be due to indirect effects of rotenone, which might potentiate endocannabinoid signaling by stimulation of neurotransmitter (for example, glutamate)<sup>36</sup> release, which can in turn increase endocannabinoid mobilization<sup>16</sup>. This is, however, unlikely because the application of rotenone postsynaptically or in the absence of postsynaptic depolarization did not alter inhibitory transmission. If rotenone were stimulating indirectly endocannabinoid mobilization *per se*, its application to slices should result into alterations of eIPSCs. Indeed, previous studies have shown that much higher concentrations of rotenone and much longer application periods are needed to alter synaptic transmission in the hippocampus<sup>37</sup>. Reduction of eIPSCs by exogenous application of cannabinoids does not seem to involve intracellular CB<sub>1</sub>. Conversely, our data clearly show that intracellular CB<sub>1</sub> receptors (likely mtCB<sub>1</sub>) participate in strong DSI. These apparently contradictory results suggest a mechanistic difference between the decrease of eIPSCs induced by exogenously applied cannabinoids or endogenously mobilized endocannabinoids. As yet, the mechanistic bases of this paradoxical difference are unknown, but they might rely on temporal and/or spatial aspects<sup>17</sup>. DSI is short-lasting (a few tens of seconds), likely due to efficient endogenous degradation of endocannabinoids<sup>15,17</sup>. Conversely, drug applications (especially of lipophilic cannabinoids) are generally long-lasting, their duration depending only on pharmacokinetics properties of the drug, and might induce extra effects (for example, tolerance)<sup>29</sup>. From the spatial point of view, the mobilization of endocannabinoids by depolarization and/or synaptic stimulation might be limited to specific cellular subdomains. Conversely, exogenous cannabinoids might display preference for other subdomains, thereby triggering different mechanisms. Finally, depolarization steps able to endogenously mobilize endocannabinoids might trigger other, so far unknown cellular events to favor additional means of endocannabinoid signaling, which would otherwise remain silent.

Taken together, our results show that intracellular CB<sub>1</sub> receptors participate in DSI and suggest that mtCB<sub>1</sub> might be the mediator of this intracellular endocannabinoid signaling. Endocannabinoids acting at presynaptic mtCB<sub>1</sub> receptors might contribute to DSI by temporarily decreasing mitochondrial respiration and altering the energy supply in the form of ATP needed for the ongoing release of neurotransmitters<sup>4,5</sup>. Moreover, mitochondria regulate intracellular calcium levels and redox potential, which can also modulate neuronal activity and neurotransmitter release<sup>4</sup>. Rapid trafficking of mitochondria has also been recently proposed to modulate synaptic plasticity<sup>1</sup>, raising the possibility that mtCB<sub>1</sub> might interfere with these processes. Thus, mtCB<sub>1</sub> receptors might contribute to DSI and possibly to other forms of synaptic regulation and plasticity by different means.

The discovery that intracellular CB<sub>1</sub> receptor signaling and mitochondrial regulation are involved in DSI further extends the range of mechanisms through which endocannabinoid signaling influences brain functions and will possibly add new pharmacological targets for the therapeutic exploitation of the endocannabinoid system. The demonstration that CB<sub>1</sub> receptors are functionally expressed on neuronal mitochondria will pave the way for new studies investigating in deeper detail the relationship between GPCR signaling in the brain and cellular energy regulation, with unforeseeable effect on the development of new theoretical frameworks to better understand brain functioning in health and disease.

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Note: Supplementary information is available on the Nature Neuroscience website.

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## AUTHOR CONTRIBUTIONS

N.P., J.L. and L.B. equally contributed to experiments. P.G. and R.R. equally supervised different parts of this work. G.B., P.G., R.R. and G.M. designed the study. G.B. performed the biochemical experiments. F.M., J.L. and C.M. performed the electrophysiological studies. N.P. performed the anatomical studies. L.B. and E.S.-G. performed *in vivo* studies and stereotactic injections of viruses. A.D., M.M.-L., E.H.-C. and A.C. participated in biochemical experiments. I.M. measured endocannabinoids and endocannabinoid-degrading enzymatic activities. S.O.-G., M.M.-F. and M.L.L.-R. synthesized and biochemically characterized HU-biot and measured intracellular hemopressin and AM251. J.G. generated fluorescent hemopressin and performed *in vitro* studies on cell penetration. M.K., S.G. and B.L. provided the viruses for local re-expression of CB<sub>1</sub> receptors. F.C. supervised part of the work. P.G. supervised the anatomical studies. R.R. supervised biochemical experiments. G.M. conceived and supervised the whole study and wrote the manuscript. All authors edited the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Mice.** Experiments were approved by the Committee on Animal Health and Care of INSERM and the French Ministry of Agriculture and Forestry (authorization number 3306369). Mice were maintained under standard conditions. Female mice (2–4 months old) were used for anatomy and biochemistry. Mice 20–30 d old of both sexes were used for electrophysiology.  $CB_1^{-/-}$ ,  $Glu-CB_1^{-/-}$  and  $GABA-CB_1^{-/-}$  mice (elsewhere called  $CB_1$ -KO,  $Glu-CB_1$ -KO and  $GABA-CB_1$ -KO, respectively) were as described<sup>26–28,38,39</sup>.  $C57BL/6N$  were from Janvier (France). Experimenters were blind to genotypes and treatments. A total of 312 female and 22 male mice were used.

**Drugs.** Drugs were from Sigma, Tocris, Abbott Laboratories Inc. or Ascent Scientific. THC was dissolved in ethanol; WIN55,252-2 (WIN), HU210 and HU-biot (see below) in DMSO; hemopressins in PBS. Vehicles contained the same amounts of solvents (0.1–0.2% ethanol; 0.1% DMSO).

**Synthesis of and  $CB_1$  binding assay for HU-biot.** HU-biot was prepared by condensation between HU210 (ref. 40) and *N*-(+)-biotinyl-6-aminohexanoic acid in the presence of dicyclohexylcarbodiimide, *N,N*-dimethylaminopyridine and *N*-hydroxybenzotriazole as coupling reagents (yield: 32%) and purified by column chromatography. Spectroscopic data were consistent with the structure. Purity (elemental analysis) > 95%. The  $CB_1$  binding assay was performed as previously described<sup>41</sup>.

**Synthesis of fluorescent hemopressin.** Rat hemopressin was synthesized using standard Fmoc solid-phase synthesis chemistry on a CS336S Peptide Synthesizer (CS Bio Co.). The introduction of fluorescein to the peptide was done by a covalent coupling of a 6.25 mg ml<sup>-1</sup> solution of fluorescein-5-maleimide (Pierce) to the cysteine of 5 mg of hemopressin in dimethylformamide for 2 h at room temperature in the dark. The product was reconstituted in DMSO and purified and analyzed with preparative HPLC and ESI-MS.

**$CB_1$  binding assay with fluorescent hemopressin.** The  $K_d$  value of the fluorescent hemopressin was determined using CHO-K1 cells expressing human  $CB_1$  (h $CB_1$ ). The peptide was incubated with  $1 \times 10^5$  cells suspended in 200  $\mu$ l binding buffer (50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.5% BSA, pH 7.4) in silanized plastic tubes (30 min, 30 °C). After centrifugation and three washes with ice-cold binding buffer, fluorescence intensities were measured in a 96-well plate reader (Tecan Farcyte, 485-nm excitation and 535-nm emission). Specific binding was determined after washing and subtracting of signals from untransfected control cells. Concentration response curves were analyzed by nonlinear curve fitting (one-site binding). The apparent  $K_d$  value for the  $CB_1$  receptor was  $119 \pm 11$  nM.

**Immunocytochemistry for electron microscopy.** Wild-type,  $Glu-CB_1^{-/-}$ ,  $GABA-CB_1^{-/-}$  and  $CB_1^{-/-}$  mice ( $n = 3$  each) were processed for electron microscope pre-embedding immunogold labeling as recently described<sup>42</sup>. Antibodies used in 50- $\mu$ m-thick hippocampal sections: goat  $CB_1$  antiserum (2  $\mu$ g ml<sup>-1</sup>; CB1-Go-Af450-1, Frontier Science Co. Ltd); goat AKAP 121 antiserum (2  $\mu$ g ml<sup>-1</sup>; AKAP 149/121, Santa Cruz Biotechnology).

**Semiquantification of mtCB1.** The detailed procedure has been described elsewhere<sup>42</sup>. The number of labeled mitochondria normalized to total mitochondria determined the proportion of hippocampal CA1  $CB_1$ -immunopositive mitochondria. MtCB1 labeling density was calculated on the area of labeled mitochondria. Normalized mtCB1 labeling versus total  $CB_1$  showed mtCB1 proportion versus total  $CB_1$ . Axon terminals identification: abundant synaptic vesicles, mitochondria, synapses with dendritic elements. No difference in percentage of labeled mitochondria, in density of mtCB1 or proportion of mtCB1 versus total  $CB_1$  was observed between the wild-type littermates of the different mutant mice ( $P > 0.6$ , for each parameter, one-way ANOVA), allowing the pooling of respective wild-type littermate controls. Normalized CA1 asymmetric or symmetric terminals with mtCB1 versus total asymmetric or symmetric synaptic boutons containing mitochondria revealed the percentages of axon terminals containing mtCB1 in  $Glu-CB_1^{-/-}$  and  $GABA-CB_1^{-/-}$  mice.

**Cell culture and transfection.** Mouse primary fibroblasts (MFs) were generated from P0–P1  $CB_1^{-/-}$  mice. Dorsal skin was excised and minced in PBS and

then incubated with in 0.25% trypsin solution. Cells were collected by centrifugation and resuspended in DMEM with 10% FBS, 1% L-glutamine and 2% penicillin/streptomycin solution (Invitrogen). Cells were seeded onto 25-cm<sup>2</sup> flasks. Transfections were carried out by using a BTX electroporator ECM 830 (Harvard apparatus, 175V, 1-ms pulse, five pulses, 0.5-s interval between pulses). Cell were electroporated in Optimem medium (Invitrogen) at  $2 \times 10^7$  cells ml<sup>-1</sup> in a 2-mm-gap cuvette using 30  $\mu$ g of DNA. After electroporation, cells were resuspended in culture medium and experiments were performed 3 d later.

**Mitochondrial purification from mouse hippocampi or whole brain.** After death, the brains or hippocampi of  $CB_1^{-/-}$  and wild-type littermates were dissected and mitochondria were purified as previously described<sup>43</sup>.

**Trypsin and digitonin assays.** Mitochondria diluted in isolation buffer (100  $\mu$ g) were treated or not with digitonin (0.25%, w/v, room temperature, 15 min). Trypsin digestion (0.1% w/v) was performed for 0, 5, 10 or 15 min (room temperature). The reaction was stopped by adding protease inhibitor cocktail (Roche). All samples were centrifuged (12,000g, 4 °C, 10 min). Each pellet was suspended in sample buffer and analyzed by western blot.

**Oxygen consumption assays.** Oxygen consumption was monitored at 30 °C in a glass chamber equipped with a Clark oxygen electrode (Hansatech). Purified mitochondria (1 mg) were suspended in 1 ml of the respiration buffer (75 mM mannitol, 25 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, pH 7.4, 50 mM EDTA) in the chamber. Respiratory substrates (10 mM pyruvate and malate) were added directly to the chamber. A coupled respiratory state was obtained by adding 2 mM ADP.

The experiments using MFs were performed on  $2 \times 10^6$  cells ml<sup>-1</sup> in growth medium. Intact cells were transferred directly in the chamber and respiration was recorded. For experiments with permeabilized cells, MFs were supplemented with respiratory substrates (10 mM pyruvate and malate) and 2 mM ADP and permeabilized with digitonin (20  $\mu$ g ml<sup>-1</sup>).

The effects of hemopressin, WIN, JZL195, HU210 and HU-biot were analyzed by adding the drugs directly to the chamber.

**NADH-ubiquinone oxidoreductase (complex I) activity assay.** We followed the reaction of NADH oxidation into NAD<sup>+</sup> by complex I as previously described<sup>43</sup>.

**Immunoblotting.** All samples were supplemented with proteases inhibitors (Roche) and diluted in sample buffer. Samples were supplemented with 2% beta-mercaptoethanol and 50 mM DTT and boiled for ~5 min. Nonreducing conditions (beta-mercaptoethanol and DTT omitted) were also used in some experiments (Supplementary Fig. 3b). Proteins were separated on Tris-glycine 4–16% acrylamide gels and transferred to PVDF membranes. Membranes were soaked in 5% milk in PBS-Tween20 (0.05%). Mitochondrial proteins were immunodetected using antibodies against complex III (Mitosciences, 2 h, room temperature) and Tom-20 (Santa Cruz, overnight, 4 °C). Cytosol, endoplasmic reticulum (ER), synaptosomes and plasma membrane contaminations were investigated using antibodies respectively against GAPDH (Santa Cruz, overnight, 4 °C), calreticulin (Santa Cruz, overnight, 4 °C), KDEL (ER; Enzo Life Sciences, 1 h, room temperature), synaptophysin (Millipore, 2 h, room temperature) and cadherin (Santa Cruz, overnight, 4 °C). The presence of the  $CB_1$  in mitochondrial fractions was analyzed using an antiserum directed against the C terminus of the receptor (Cayman, overnight, 4 °C). Then membranes were washed and incubated with secondary HRP-coupled antibodies (1 h, room temperature). Finally, HRP signal was revealed using the ECL plus reagent (Amersham) and detected by the Bio-Rad Quantity One system.

**Cyclic AMP and PKA activity assays.** Cyclic AMP activity and PKA activities were assayed on isolated mitochondria using the Direct Correlate-EIA cAMP kit (Assay Designs Inc) and an ELISA kit (Enzo Life Science), respectively, according to the manufacturers' instructions. Reactions in presence or absence of WIN or THC were performed at 30 °C for 1 h.

**Purification and quantification of endocannabinoids and *N*-acylethanolamides.** Purification and quantification of anandamide, 2-arachidonoylglycerol (2-AG), palmitoylethanolamide and oleoylethanolamide from the brain total membrane fraction and purified mitochondria were performed as described



previously<sup>44</sup>. The amounts of anandamide and 2-AG were determined using a calibration curve and were expressed as nmol or pmol per mg of protein, respectively. Anandamide was detected in the samples, but at levels below the limit of reliable quantification. Therefore, only data relating to 2-AG are presented.

**Fatty acid amide hydrolase and monoacylglycerol lipase assays.** FAAH or MAGL activities were detected by using brain total membrane fraction or purified mitochondria (50 µg protein), and [<sup>14</sup>C]anandamide (for FAAH, 5.0 mCi mmol<sup>-1</sup>, 1.8 µM, ARC Inc.) or 2-oleoyl-[<sup>3</sup>H]glycerol (for MAGL, 1.0 mCi mmol<sup>-1</sup>, 25 µM, ARC Inc.) as substrates. Reactions were carried out in 50 mM Tris-HCl, pH 9 (30 min, 37 °C) for FAAH and in Tris-HCl buffer, pH 7 (20 min, 37 °C) for FAAH and MAGL, respectively. The [<sup>14</sup>C]ethanolamine (for FAAH) or [<sup>3</sup>H]glycerol (for MAGL) produced were measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of CHCl<sub>3</sub>/CH<sub>3</sub>OH (1:1 v/v). Enzymatic activities were expressed as pmol of hydrolyzed anandamide or 2-AG per min per mg protein.

**Viral rescue of CB1 expression in the hippocampus.** Cloning of hemagglutinin (HA)-tagged rat CB<sub>1</sub> receptor (AAV-CB<sub>1</sub>) or green fluorescent protein (AAV-GFP) into an AAV expression cassette, packaging of AAV1/2 chimeric vectors and determination of genomic titers were as described<sup>31,45</sup>. One µl of either AAV-GFP or AAV-CB<sub>1</sub> (6 × 10<sup>11</sup> viral genomes ml<sup>-1</sup>) was injected bilaterally at 150 nl min<sup>-1</sup> into the dorsal and ventral hippocampus (-2.0 mm AP; ± 2.0 mm ML, -2.0 mm DV; -3.5 mm AP; ± 3.4 mm ML, -3.0 mm DV from bregma, respectively). Animals were used for experiments five weeks after viral injection<sup>31</sup>.

**Cellular localization of fluorescent hemopressin.** 1 × 10<sup>6</sup> hCB<sub>1</sub>-expressing CHO-K1 cells were incubated with 10 µM fluorescent hemopressin in 50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA and 5 mM MgCl<sub>2</sub> (30 min, 30 °C), washed different times (Supplementary Fig. 4a–c) in incubation buffer plus 0.5% BSA, detached with trypsin, and fixed with 0.01% formaldehyde (10 min). Fluorescence was measured by flow cytometry (FACScan, Cytex). Some cells were permeabilized with 0.5% NP-40 in PBS before incubation. Images were acquired with a Nikon TS100 fluorescence microscope and video camera using the NIS Elements D software.

**Measurement of hemopressin and AM251 by liquid chromatography/mass spectrometry.** Slices previously incubated with 10 µM hemopressin or AM251 (same conditions as in electrophysiology experiments) were rinsed with water, centrifuged, Dounce-homogenized in chloroform:methanol:water (2:1:1; 2 ml), and the organic and aqueous layers separated by centrifugation. For hemopressin, the aqueous phase was dried under a stream of argon and reconstituted in 100 µL H<sub>2</sub>O, and 60 µL were analyzed by LC/MS. For AM251, the organic phase was dried under a stream of argon and reconstituted in 100 µL of acetonitrile, and 50 µL were analyzed by LC/MS. LC/MS was carried out in an Agilent 1200LC-MSD VL instrument with an Eclipse XDB-C18 column coupled to an ESI source in SIM mode. The mobile phases were A (95:5 water:methanol) and B (95:5 methanol:water) with 0.1% ammonium hydroxide and 0.1% formic acid. Fractions were quantified by measuring the area under the peak and compared to corresponding standards.

**Electrophysiology.** Preparation of hippocampal slices and recording of eIPSCs were as previously described<sup>28,46</sup>. After reaching stable baseline (~10 min after

reaching whole-cell configuration for infusion of intracellular solution), IPSCs were evoked using a patch pipette filled with a HEPES-based extracellular solution positioned in the stratum radiatum, 100 µm from the CA1 pyramidal cell layer. DSI expression was checked before starting each experiment with a 5-s depolarization protocol<sup>46</sup>. Control experiments showed that this 'initial' DSI had no effect on following recorded DSI (not shown).

To determine the effects of HU210 (500nM), HU-biot (500nM) and rotenone (2.5 µM) on basal inhibitory synaptic transmission, IPSCs were evoked every 10–15 s. To determine the effects of HU210, HU-biot, hemopressin (10 µM) and rotenone on basal inhibitory synaptic transmission using the same conditions of afferent stimulation as during DSI protocols (see below), IPSCs were evoked every 3 s and a stable baseline was recorded (time 0). Then, drugs (or vehicle) were applied and patched cells were left undisturbed. The same stimulation protocol was then applied every 10 min after drug applications up to 30 min.

For DSI, IPSCs were evoked every 3 s, and DSI was induced by a 1-s or a 5-s voltage step from -70 to 0 mV applied to the postsynaptic pyramidal neuron and calculated as described<sup>46</sup>.

Two different treatment schemes were used to evaluate the effect of the different drugs on DSI. In some experiments (Fig. 6a,c and 7d), slices were first incubated with drugs (or vehicle) for 30 min. A CA1 pyramidal cell was then patched and, after a stable baseline was obtained, a DSI protocol was applied. In other experiments (Fig. 7a–c and Supplementary Figs. 6a–c and 7b,c), a pairwise schedule was used: CA1 pyramidal neurons were patched and, after we obtained a stable baseline, a DSI protocol was applied. Then, drugs were applied and a new DSI protocol was applied after 15 min of baseline recording. The effect of postsynaptic rotenone on DSI was studied by adding the drug (2.5 µM) to the intracellular solution in the patch pipette.

**Statistical analyses.** All graphs and statistical analyses were performed using GraphPad software (version 5.0). Results were expressed as means of independent data points ± s.e.m. Data were analyzed using a paired or unpaired Student's *t*-test, one-way ANOVA (followed by Newman-Keuls *post hoc* test), or two-way ANOVA (followed by Bonferroni's *post hoc* test), as appropriate.

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## Corrigendum: Mitochondrial CB<sub>1</sub> receptors regulate neuronal energy metabolism

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In the version of this article initially published online, the description of the procedure given in the Online Methods for synthesis of fluorescent hemopressin, taken from different protocol not used in this work, was incorrect. The entire paragraph has been replaced. The error has been corrected in the PDF and HTML versions of this article.