Cosentino *et al. Molecular Autism* (2024) 15:39
https://doi.org/10.1186/s13229-024-00617-1

Molecular Autism

RESEARCH Open Access

Pharmacological inhibition of the CB1 cannabinoid receptor restores abnormal brain mitochondrial CB1 receptor expression and rescues bioenergetic and cognitive defects in a female mouse model of Rett syndrome

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Abstract

Background Defective mitochondria and aberrant brain mitochondrial bioenergetics are consistent features in syndromic intellectual disability disorders, such as Rett syndrome (RTT), a rare neurologic disorder that severely affects mainly females carrying mutations in the X-linked *MECP2* gene. A pool of CB1 cannabinoid receptors (CB1R), the primary receptor subtype of the endocannabinoid system in the brain, is located on brain mitochondrial membranes (mtCB1R), where it can locally regulate energy production, synaptic transmission and memory abilities through the inhibition of the intra-mitochondrial protein kinase A (mtPKA). In the present study, we asked whether an overactive mtCB1R-mtPKA signaling might underlie the brain mitochondrial alterations in RTT and whether its modulation by systemic administration of the CB1R inverse agonist rimonabant might improve bioenergetics and cognitive defects in mice modeling RTT.

Methods Rimonabant (0.3 mg/kg/day, intraperitoneal injections) was administered daily to symptomatic female mice carrying a truncating mutation of the *Mecp2* gene and its effects on brain mitochondria functionality, systemic oxidative status, and memory function were assessed.

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Results mtCB1R is overexpressed in the RTT mouse brain. Subchronic treatment with rimonabant normalizes mtCB1R expression in RTT mouse brains, boosts mtPKA signaling, and restores the defective brain mitochondrial bioenergetics, abnormal peripheral redox homeostasis, and impaired cognitive abilities in RTT mice.

Limitations The lack of selectivity of the rimonabant treatment towards mtCB1R does not allow us to exclude that the beneficial effects exerted by the treatment in the RTT mouse model may be ascribed more broadly to the modulation of CB1R activity and distribution among intracellular compartments, rather than to a selective effect on mtCB1R-mediated signaling. The low sample size of few experiments is a further limitation that has been addressed replicating the main findings under different experimental conditions.

Conclusions The present data identify mtCB1R overexpression as a novel molecular alteration in the RTT mouse brain that may underlie defective brain mitochondrial bioenergetics and cognitive dysfunction.

Keywords Rett syndrome, Mouse model, Intellectual disability, Brain mitochondria, Energy metabolism, CB1 cannabinoid receptor, PKA

Background

The endocannabinoid system (ECS) is a widespread neuromodulatory network that controls neuronal activity, neurotransmission, and synaptic plasticity [1, 2]. Its broad expression in the central nervous system and its involvement in cell-cell communication make the influence of ECS pivotal in the regulation of cognitive, motor, sensory, and emotional processes both during early time windows of development and in adulthood [3].

The effects of cannabinoid binding in the brain are largely mediated by the CB1 cannabinoid receptor (CB1R), a G protein-coupled receptor densely expressed at pre-synaptic sites, where its influence extends over various neurobiological processes, including synaptic transmission, plasticity, and the regulation of cognitive abilities [4, 5]. In particular, the critical role of CB1R in learning and memory processes [6] is increasingly highlighted by the emerging relevance of this receptor in the pathophysiology of syndromic intellectual disability disorders, a broad class of pathological conditions that arises early in life and entails defective intellectual functioning and adaptive behavior. Indeed, pharmacological inhibition of CB1R was found to improve synaptic function and cognitive performance in murine models of syndromic intellectual disability disorders, such as fragile X syndrome [4, 7] and Down syndrome [8] for which a phase 1/2 clinical trial (NCT05748405) is currently underway to assess the safety and tolerability of novel CB1R inhibitors in patients with Down syndrome.

Groundbreaking immunogold electron microscopy experiments led to the discovery that a proportion of brain CB1R is present at the mitochondria-associated membrane (mtCB1R) [9]; follow-up studies revealed that these subcellularly localized receptors are key for promoting cannabinoid effects on memory functions. In fact, mitochondria-specific knockout of CB1R prevented the deficits in synaptic and memory functions induced by cannabinoid administration in mice [10, 11]. Interestingly, the effects of mtCB1R stimulation by cannabinoids are mediated by the inhibition of intra-mitochondrial protein kinase A (mtPKA) activity, which leads to altered phosphorylation of the mitochondrial respiratory chain (MRC) complexes subunits and decreased energy production [9, 12]. These studies uncover the role of mtCB1R-mtPKA signaling in the control of cellular bioenergetics and confirm that brain mitochondria processes intimately control cognitive functions [10, 13, 14].

Interestingly, defective mitochondrial respiratory capacity resulting in reduced ATP synthesis and reactive oxidizing species (ROS) overproduction has been observed in the brain of mouse models and in tissues from patients with different syndromic intellectual disability disorders [15–17], including Rett syndrome (RTT, OMIM #312750), the primary genetic cause of severe intellectual disability in females (1:10,000 live births) [18, 19]. Most RTT patients carry *de novo* loss-of-function mutations in the X-linked gene methyl-CpG-binding protein 2 (*MECP2*) which codes for the homonym transcriptional and epigenetic regulator. A peculiar course characterizes the clinical presentation of RTT, where an apparently typical neurobehavioral development is followed by a growth arrest at around 1 year of age, followed by a progressive deterioration of acquired cognitive and motor skills [19]. At the fully symptomatic stage, patients with RTT display cognitive impairments, abnormal fine and gross motor skills, communicative difficulties, distinctive repetitive hand motions, and a significant decline in overall functioning.

Mitochondrial dysfunction and oxidative stress have been suggested to play a contributing role in the pathogenic process of RTT [20]. In RTT murine models, changes in brain bioenergetics are noticeable during the neonatal period [21] and worsen concomitantly with symptom progression. At the symptomatic stage, a decline in mitochondrial membrane potential alters the physiological function of the MRC and leads to lower ATP synthesis [22, 23]. Additionally, the impairments in specific MRC complexes induce ROS overproduction by

RTT brain mitochondria [24–26]. Although accumulating evidence suggests that targeting mitochondrial function may be beneficial to contrast RTT symptomatology [27–29], the mechanisms underlying mitochondrial impairments are still unclear.

In the present study, we asked whether an overactive mtCB1R-mtPKA signaling might underlie the brain mitochondrial alterations in RTT and whether its modulation by a systemic administration of the CB1R inverse agonist rimonabant might improve bioenergetics and cognitive defects in mice modeling RTT. To this aim, rimonabant was administered subchronically to symptomatic female mice carrying a truncating mutation of the *Mecp2* gene (Mecp2-308) [30] as previously described [31], and its effects on brain mitochondria functionality, systemic oxidative status, and memory function were assessed.

Materials and methods

Additional details are described in the supplementary materials and methods.

Subjects

The experimental subjects were fully symptomatic 8-12-month-old, experimentally naïve Mecp2-308 heterozygous female mice (RTT) and wild-type (Wt) littermates (B6.129 S-*Mecp2*^{tm1Hzo}/J from Jackson Laboratories, USA, stock number: 005439) bred in our facility on a C57/BL6J background [32]. Female mice were used in the present study since RTT almost exclusively affects girls and mutant female mice more closely phenocopy the typical temporal features of RTT, which is characterized by a delayed onset of overt symptoms [33]. Since diagnosis currently occurs when symptoms are already present, we tested treatment efficacy on fully symptomatic females under conditions mirroring those in which patients might actually receive treatment. All procedures were carried out during the dark phase.

Drug

Rimonabant (0.3 mg/kg/day) and vehicle (Cremophor® EL: ethanol: saline in a ratio of 1:1:18) emulsions were freshly prepared every day 15 min before the first injection of the day [31]. Based on a previous study highlighting the need for continuous treatment to maintain therapeutic efficacy [31], rimonabant was administered daily for either 4 or 7 consecutive days.

Experiment 1

To unravel the effects of CB1R inhibition on RTT-related bioenergetics alterations, RTT mice and Wt controls were injected intraperitoneally (i.p.) once a day for 4 consecutive days with rimonabant or vehicle. Four hours after the 4th injection, when the drug was eliminated from the brain [34], animals were sacrificed, and blood and brain tissues were collected for biochemical analyses (Supplementary Fig. 1A).

Mitochondrial bioenergetics

Brain dissection and cryopreservation. The brains were dissected in 2 hemispheres, cryopreserved as described previously [35], and stored at −80 °C until assay.

Brain ATP levels. Cryopreserved tissues were subjected to perchloric acid extraction as described previously [36]. The amount of tissue ATP was determined enzymatically in KOH-neutralized extracts, as described previously [37].

Mitochondrial isolation. Mitochondria were isolated from cryopreserved tissues by differential centrifugation of brain homogenate, and controls were made for checking mitochondrial integrity and function, as previously reported [35].

Mitochondrial ATP production rate. The rate of ATP production by oxidative phosphorylation (OXPHOS) was determined in mitochondria isolated from cryopreserved brain tissues essentially as previously described [37].

MRC complex I and V activities. The activities of MRC complex I and V (ATP synthase) were measured spectrophotometrically in mitochondrial membrane-enriched fractions obtained from mitochondria isolated from cryopreserved brain tissues essentially as described previously [23].

CB1R and PKA signaling

CB1R expression. Proteins of brain homogenate and mitochondrial fractions were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% fatty-acid-free dry milk in 500 mM NaCl, 20 mM Tris, 0.05% Tween-20 (pH 7.4; TTBS) for 3 h at 4 °C and probed with antibodies against CB1R (1:200, Cayman, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and cytochrome oxidase subunit 1 (COX1). After being washed in TTBS, the membrane was incubated for 60 min with an anti-rabbit IgG peroxidase-conjugated antibody. Immunodetection was then performed, after further TTBS washes, with enhanced chemiluminescence (ECL) (Euroclone, UK). Image acquisition was performed using the ChemiDoc imaging system (Bio-Rad, Italy), and densitometric analysis was performed with the Image Lab software (Bio-Rad).

Western blot analyses were replicated in mitochondria isolated using Ficoll gradient ultracentrifugation to control for the purity of mitochondrial fractions [38].

PKA activity. The PKA kinase activity was measured in brain homogenate and mitochondrial fractions using

a solid phase enzyme-linked immuno-absorbent assay (ELISA, ENZO Life Sciences, USA), as recommended by the manufacturer. This assay utilizes a specific synthetic peptide precoated on the wells that once phosphorylated by PKA is recognized by a phosphospecific antibody. Then, the phosphospecific antibody is recognized by a peroxidase-conjugated secondary antibody. Subsequently, the addition of tetramethylbenzidine develops color in proportion to PKA activity (450 nm). Colorimetric detection and quantitative analysis were performed by VICTORX Multilabel Plate Reader.

Whole blood oxidative stress

Trunk whole blood of the experimental subjects was collected into heparinized tubes at sacrifice to evaluate ROS levels. The oxidation of the spin probe 1-hydroxy-3-carboxypyrrolidine (CPH) to the correspondent 3-carboxy-peroxyl radical (CP•) [29] was monitored by Electron Paramagnetic Resonance (EPR). The oxidation of CPH in the CP• is not specific to a singular oxidant, but it is suitable to screen the totality of ROS (among which O2•, •OH, peroxynitrite, transition metal-catalyzed reactions) produced in biological samples. If the intensity of CP• is significantly increased, the presence of a pro-oxidant status is suggested.

Experiment 2

To assess whether CB1R inhibition was able to rescue RTT-related cognitive deficits, experimental animals were injected daily for 7 consecutive days with rimonabant or vehicle. Behavioral testing was carried out from day 5 to day 7 of the treatment schedule, between 10:00 am and 6:00 pm. Four hours after the 7th injection animals were sacrificed, and blood and brain tissues were collected for biochemical analyses (Supplementary Fig. 1B).

Object location task. The effects of treatment with rimonabant on episodic memory were assessed with the object location test, which is devoted to assess memory of spatial configurations based on the spontaneous tendency of rodents to explore novel stimuli [39]. On day 5, mice were *habituated* for 30 min to an empty open field. On day 6, each subject returned to the arena for a 10-minute re-*habituation* phase; then, two identical clean objects were placed in different quadrants of the arena for a 5-minute *training* session. One hour later, one of the objects was moved into the opposite quadrant (novel location) and mice were allowed to explore freely for 5 min for the *test* phase. The time spent in the object-containing quadrants was used to compute the preference index [displaced/(non-displaced + displaced), chance level 50%], which was considered a measure of spatial memory retention. Drug administration was performed 30 min before *habituation* and immediately after *training* on the 5th and the 6th experimental days, respectively, to exploit the maximal inhibitory effect of rimonabant in the brain [34].

Y-maze test. The rimonabant treatment effects on spatial short-term memory were assessed using a spontaneous spatial novelty preference task performed using the Y-maze apparatus, as previously described [40]. On day 7, 30 min after the drug injection, each mouse was exposed to two arms (start and familiar), while the entrance to the third, *novel*, arm was prevented by a block of white Perspex, for a 5-minute *familiarization* phase. Then, the entrance to the novel arm was opened for the *test* phase, where mice were free to explore the entire apparatus for 2 min. The preference index (time spent in novel arm/total test time, chance level 33%) was calculated as a measure of novelty discrimination.

General health status evaluation. After the Y-maze test, the general health status of the experimental mice was scored by a trained observer blind to mouse genotype and treatment, following a protocol that is widely accepted and applied in RTT preclinical studies [41, 42]. Body weight was also recorded at each scoring session.

Mitochondrial isolation. Mitochondria were isolated from cryopreserved brain hemispheres by differential centrifugation of brain homogenate, as reported above.

Mitocondrial bioenergetics. The rate of ATP production by OXPHOS was measured in mitochondria isolated from cryopreserved brain tissues as reported above.

CB1R expression. The total protein concentration from the whole homogenate, as well as the mitochondrial-enriched fractions, was determined using the BCA method (Pierce, USA). Fifteen μg of proteins from brain homogenate and mitochondrial fractions were separated by 4–15% gradient SDS-PAGE, using Criterion TGX (Tris-Glycine extended) Stain-Free precast gels (Bio-Rad) in Tris/Glycine/SDS (TGS) Running Buffer (Bio-Rad). Following electrophoresis and gel imaging, the proteins were transferred via the TransBlot Turbo semi-dry blotting apparatus (Bio-Rad) onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with 3% of bovine serum albumin (SERVA Electrophoresis GmbH, Germany) in TBS solution containing 0.01% Tween 20 and incubated overnight at 4 °C with the following primary antibodies: CB1R (Cayman) and COX1 (Novus Biological, USA). Subsequently, membranes were incubated at room temperature with the respective horseradish peroxidase-conjugated secondary antibody (Sigma–Aldrich, USA). Membranes were developed with Clarity ECL substrate (Bio-Rad) and then acquired with ChemiDoc MP (Bio-

Rad) and analyzed using Image Lab 6.1 software (Bio-Rad) that allows the normalization of a specific protein signal by the total protein load.

Statistical analyses

Statistical analyses were performed using SPSS Statistics for Windows (Version 28.0, IBM Corp., USA). Data distribution among experimental groups was compared using parametric tests, i.e. Student's t and analysis of variance (ANOVA). The ANOVA included genotype and treatment as between-subject fixed factors and *post-hoc* comparisons were performed using Tukey's test. Animals were excluded from the analyses when identified as outliers with the Grubb's test. When the normality and homoscedasticity criteria, assessed by Shapiro-Wilk's and Levene's tests, were not met, the non-parametric Kruskal-Wallis test was used, followed by Dunn's *post-hoc* testing. Outliers in non-parametric tests were identified using the interquartile range rule. The alpha level was set at 5%. Plots were created with GraphPad Prism for Windows (Version 9.0, GraphPad Software, USA).

Results

Treatment with rimonabant normalized mtCB1R overexpression and boosted mtPKA activity in the brain of RTT mice

To test the hypothesis that mtCB1R-mtPKA signaling is altered in RTT mice and evaluate the effects of rimonabant, we measured the protein levels of mtCB1R as well as the activity of mtPKA in the brains of experimental animals. As hypothesized, mtCB1R was found overexpressed in the brain mitochondria of RTT mice compared to Wt controls (genotype*treatment interaction: $F_{1,8}$=4.28, p=0.072; p<0.05 after *post-hoc* comparisons between RTT-vehicle and Wt-vehicle; Fig. 1A). A 4-day-long treatment with rimonabant decreased mtCB1R overexpression in brain mitochondria of RTT mice to control values (p<0.05 after *post-hoc* comparisons between RTT-rimonabant and RTT-vehicle on the genotype*treatment interaction, Fig. 1A). To exclude the possibility that the observed overexpression of mtCB1R in RTT mouse brains was due to contamination of non-mitochondrial membranes in the mitochondrial fraction, mitochondria were purified through Ficoll gradient ultracentrifugation in a separate cohort of mice. Results confirmed that mtCB1R is overexpressed in RTT mice (t_8 = -2.88; p=0.021; see Supplementary Methods and Supplementary Fig. 2A, C). The effects were also replicated in subjects undergoing a longer 7-day treatment schedule (main effect of experimental group: H_3=15.62, p=0.001; p<0.05 after *post-hoc* comparisons between Wt-vehicle versus RTT-vehicle and p<0.01 after *post-hoc* comparisons between RTT-vehicle versus Wt-vehicle; Fig. 1B and Supplementary Fig. 3).

A 20% reduction in the activity of the mtCB1R target mtPKA was also found in the RTT mouse brain compared to Wt controls (Fig. 1C). Treatment with rimonabant increased the activation of mtPKA selectively in the RTT mouse brain (genotype*treatment interaction: $F_{1,11}$=7.09, p=0.022; p<0.05 after *post-hoc* comparisons between RTT-rimonabant and RTT-vehicle; Fig. 1C).

Importantly, no differences were evident in CB1R expression and PKA activation in brain homogenates of RTT and Wt mice (Supplementary Fig. 2B, C, 3 and 4A-C). Treatment with rimonabant, however, provided a transient reduction of CB1R levels in brain homogenates of RTT mice after 4 days of treatment (genotype*treatment interaction: $F_{1,8}$=12.69, p=0.007; p<0.01 after *post-hoc* comparisons between RTT-rimonabant and RTT-vehicle; Supplementary Fig. 4A), without affecting overall PKA activity (Supplementary Fig. 4C). Of note, no differences were found in CB1R expression in the hippocampus or striatum of mice from different genotype/treatment groups (Supplementary Fig. 4D, E). Consistent with this, the levels of anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG), the major endocannabinoids, did not significantly differ in the hippocampus or the striatum of Wt and RTT mice and were unaltered by rimonabant treatment (Supplementary Table 1).

Rimonabant treatment restores mitochondrial bioenergetics in the brain of RTT mice

To explore whether the normalization of mtCB1R signaling in rimonabant-treated RTT mouse brain is accompanied by a rescue of cerebral mitochondrial dysfunctions, ATP synthesis, as well as MRC activity were analyzed in the whole brain of experimental animals. In line with our previous studies [23, 27, 29, 43], mitochondrial ATP synthesis tended to be reduced in RTT mouse brains when succinate, the respiratory substrate of mitochondrial complex II, was provided as energy source (genotype*treatment interaction: $F_{1,8}$=4.80, p=0.060; p<0.1 after *post-hoc* comparisons between RTT-vehicle and Wt-vehicle; Fig. 2A; genotype*treatment interaction: $F_{1,20}$=116.80, p<0.001; p<0.001 after *post-hoc* comparisons between Wt-vehicle versus RTT-vehicle; Fig. 2B). Treatment with rimonabant increased mitochondrial ATP production in the brain of RTT mice (4-day treatment: p<0.05 after *post-hoc* comparisons between RTT-rimonabant and RTT-vehicle on the genotype*treatment interaction; Fig. 2A, and 7-day treatment: p<0.001 after *post-hoc* comparisons between RTT-rimonabant and RTT-vehicle on the genotype*treatment interaction; Fig. 2B), restoring the levels observed in Wt brains.

In line with previous results [23–25], a significant reduction in the activity of the MRC complex V was observed in the RTT mouse brain compared to Wt

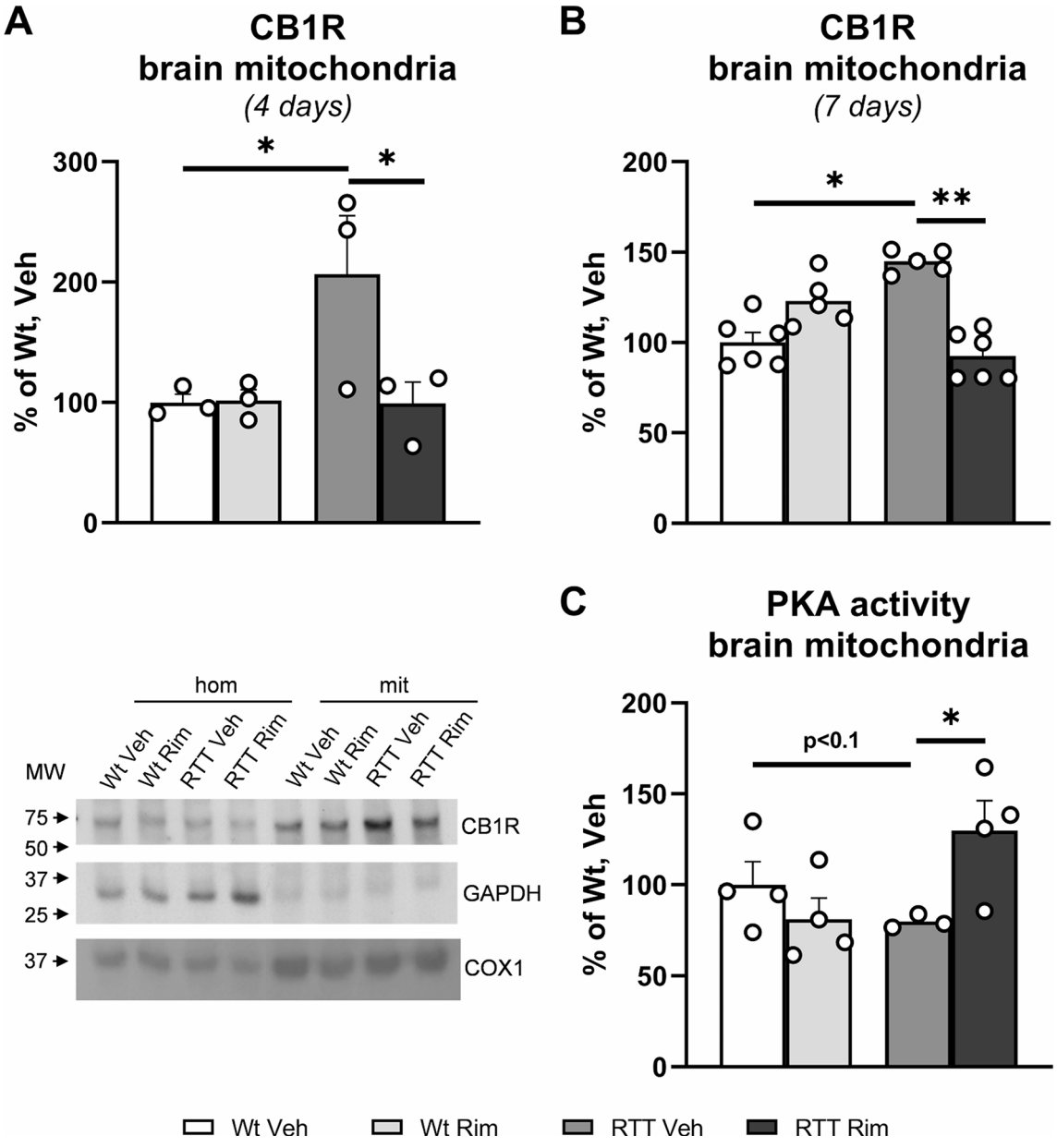

Fig. 1 Rimonabant treatment normalizes mtCB1R overexpression and boosts mtPKA signaling in the RTT mouse brain. After 4 or 7 days of systemic treatment with the CB1 cannabinoid receptor (CB1R) inverse agonist rimonabant (Rim, 0.3 mg/kg) or vehicle (Veh), and precisely 4 h after the last injection, Mecp2-308 heterozygous female mice (RTT) and wild-type (Wt) controls were sacrificed and the brain tissue was sampled to assess the CB1R and protein kinase A (PKA) activity in isolated brain mitochondria. **(A)** Treatment with Rim normalizes the levels of CB1R expressed at the mitochondrial membranes (mtCB1R) that are increased in the brain of RTT mice compared to Wt controls. Immunoblots were assembled to show examples from 1 animal from each experimental group. Protein loading was assessed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody for homogenate (hom) and cytochrome oxidase subunit 1 (COX1) for mitochondrial fractions (mit). (**B**) The effects of Rim on mtCB1R expression were replicated after a longer 7-day treatment schedule. Two observations were removed from the mtCB1R data after 7 days of treatment as they significantly exceeded the group's distribution. (**C**) Rim boosts the activation of the mtCB1R-target PKA in the RTT mouse brain mitochondria (mtPKA). A single observation was removed from the mtPKA data as it was significantly outside the group average. $N = 3–6$. The histograms are mean ± SEM and represent the arbitrary densitometric units expressed as a percentage of the values of Wt and Veh. * $p < 0.05$, ** $p < 0.01$, two-way ANOVA followed by Tukey's *post-hoc* test or Kruskal-wallist test followed by Dunn's *post-hoc* test

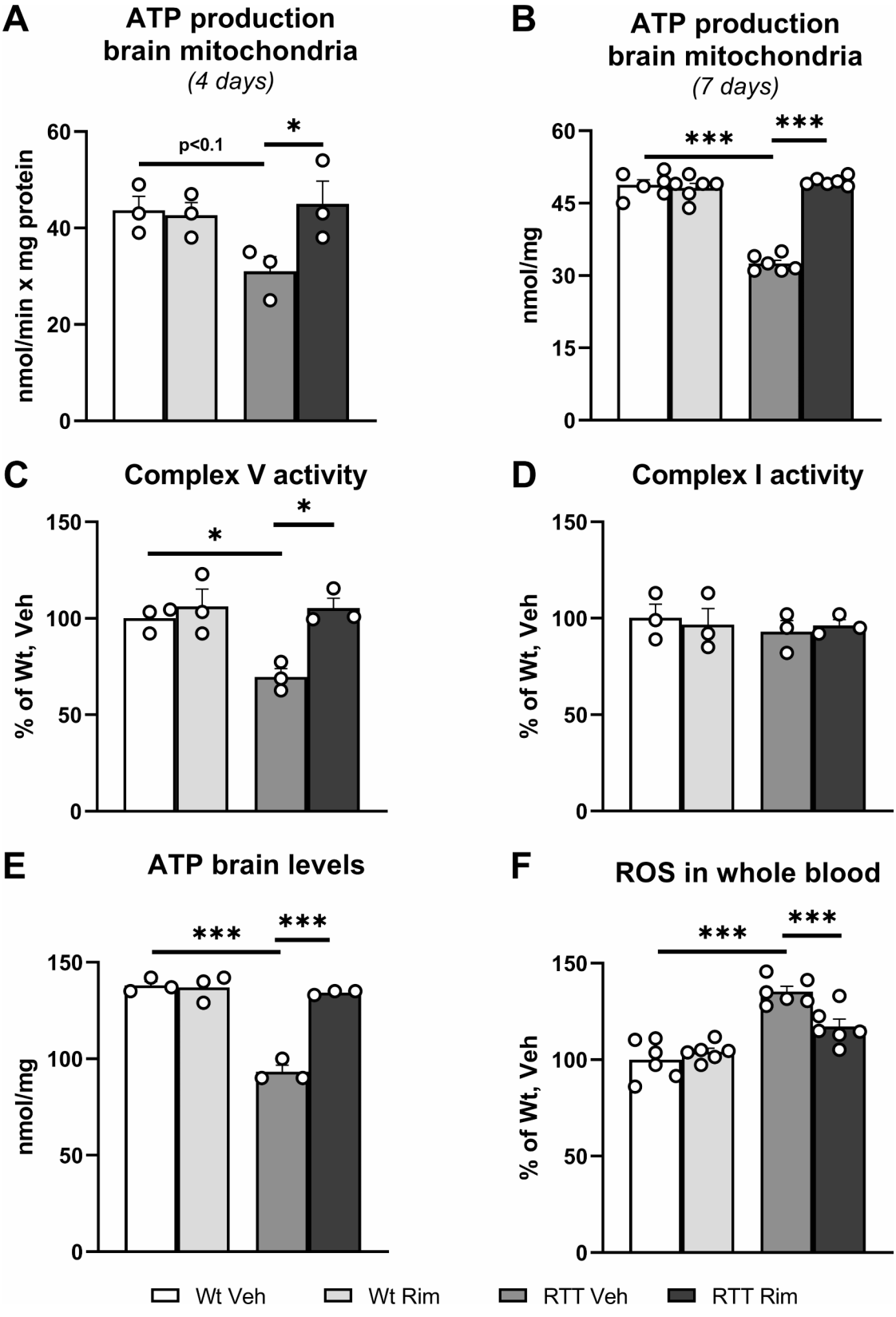

Fig. 2 (See legend on next page.)

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Fig. 2 Rimonabant treatment rescues the defective brain bioenergetics and increased peripheral oxidative stress in RTT mice. Mecp2-308 heterozygous female (RTT) mice and wild-type (Wt) controls received intraperitoneal daily injections with the CB1 cannabinoid receptor inverse agonist rimonabant (Rim, 0.3 mg/kg) or vehicle (Veh) for 4 or 7 days; 4 h after the last injection mice were sacrificed and the brains were collected and cryopreserved for analyses of mitochondrial bioenergetics. (**A-B**) Mitochondria were isolated from cryopreserved tissues and the rate of ATP production by oxidative phosphorylation was determined when succinate was provided as an energy source. RTT brain mitochondria generated lower levels of ATP, and treatment with Rim restored Wt-like ATP production rate. (**C-D**) The activity of the mitochondrial respiratory chain (MRC) complexes I and V was measured spectrophotometrically in mitochondrial membrane-enriched fractions of cryopreserved brain tissues and is expressed as a percentage of the activity measured in Wt and Veh. Treatment with Rim normalized the reduced complex V activity in the brain of RTT mice, restoring Wt-like levels. The activity of complex I did not differ between the two genotypes and rimonabant did not affect it. (**E**) The levels of ATP were measured in brain extracts. RTT mice treated with Veh displayed reduced ATP levels in brain homogenates, which were normalized by treatment with Rim. (**F**) At sacrifice, whole blood was collected to assess treatment effects on peripheral oxidative stress status. RTT mice displayed elevated blood levels of reactive oxidizing species (ROS), indicative of excessive oxidative stress; a 4-day treatment with Rim decreased blood ROS levels in RTT mice. $N = 3–6$. Data are mean ± SEM. * $p < 0.05$, *** $p < 0.001$, two-way ANOVA followed by Tukey's *post-hoc* test

controls (genotype*treatment interaction: $F_{1,8}=6.19$, $p=0.038$; $p<0.05$ after *post-hoc* comparisons between RTT-vehicle and Wt-vehicle; Fig. 2C), which was rescued by treatment with rimonabant ($p<0.05$ after *post-hoc* comparisons between RTT-rimonabant and RTT-vehicle on the genotype*treatment interaction; Fig. 2C). Of note, the activity of MRC complex I was unaltered in the brain of RTT mice and unaffected by treatment with rimonabant (Fig. 2D).

Consistent with an extensive improvement in the functionality of brain mitochondria, treatment with rimonabant normalized the impaired ATP levels in the brain of RTT mice, restoring a normal brain bioenergetic status (genotype*treatment interaction: $F_{1,8}=54.75$, $p<0.001$; $p<0.001$ after *post-hoc* comparisons on Wt-vehicle versus RTT-vehicle and RTT-vehicle versus RTT-rimonabant; Fig. 2E).

Treatment with rimonabant rescues the increased oxidative stress in RTT mouse blood

We next asked whether the treatment with rimonabant can restore normal redox homeostasis in RTT mice. In fact, as described previously [29, 43], a pro-oxidant status characterizes the whole blood of RTT mice, which display significantly higher levels of ROS (measured as the rate of CP• formation) compared to Wt controls (genotype*treatment interaction: $F_{1,20}=11.15$, $p=0.003$; $p<0.001$ after *post-hoc* comparisons between RTT-vehicle and Wt-vehicle; Fig. 2F). Importantly, treatment with rimonabant reduced ROS overproduction restoring Wt-like levels in RTT mouse blood ($p<0.001$ after *post-hoc* comparisons between RTT-rimonabant and RTT-vehicle on the genotype*treatment interaction; Fig. 2F).

Treatment with rimonabant rescues the spatial memory deficits in RTT mice

To determine if treatment with rimonabant can rescue cognitive deficits in RTT mice, treatment effects on their spatial memory capacity were tested. Spatial memory and discrimination, a cognitive process severely impaired in RTT [44, 45], was assessed via the object location test. As expected, while Wt mice exhibited a clear preference for the novel object location ($t_4=5.76$, $p=0.005$ versus a 50% chance level; Fig. 3A), RTT mice displayed spatial memory deficits, as demonstrated by a lack of preference for the displaced over the non-displaced object (Fig. 3A). Sub-chronic treatment with rimonabant increased the preference for the displaced object in RTT mice thus restoring Wt levels ($t_7=-2.51$, $p=0.040$ versus a 50% chance level; Fig. 3A).

Spatial working memory was assessed by evaluating spatial novelty preference in the Y-maze test. Consistent with the defective spatial discrimination previously described in this RTT mouse model [40], RTT mice injected with vehicle did not show a preference for the novel arm over the familiar one (Fig. 3B). Notably, treatment with rimonabant restored the spatial novelty preference of RTT mice ($t_7=3.92$; $p=0.006$ versus a 33% chance level; Fig. 3B) thus returning the discrimination capacity to the level of Wt controls ($t_5=2.81$; $p=0.038$ versus a 33% chance level; Fig. 3B).

Strikingly, rimonabant exerted negative effects on the memory abilities of Wt animals, as Wt mice treated with rimonabant failed to exhibit spatial memory discrimination in both tasks (Fig. 3A, B).

Of note, rimonabant did not affect total object/arm exploration or total distance traveled by experimental animals during neither of the two tests [Mean ± standard deviation (SD) – object location: Wt-vehicle = 151.76 ± 51.90 s and 11.69 ± 2.10 m, Wt-rimonabant = 151.23 ± 70.05 s and 13.44 ± 5.12 m, RTT-vehicle = 145.10 ± 47.66 s and 14.00 ± 5.44 m, RTT-rimonabant = 109.63 ± 48.04 s and 10.96 ± 4.70 m; Y-maze: Wt-vehicle = 85.98 ± 7.44 s and 5.41 ± 1.08 m, Wt-rimonabant = 75.14 ± 12.32 s and 4.61 ± 0.76 m, RTT-vehicle = 81.20 ± 13.23 s and 4.73 ± 0.46 m, RTT-rimonabant = 87.63 ± 9.97 s and 5.91 ± 2.42 m].

The effects of CB1R inhibition on the general health conditions of RTT mice at a fully symptomatic stage and Wt controls were evaluated by an experienced observer. As expected, RTT mice suffered from an impaired health status, measured by the overall evaluation of symptoms like abnormal gait, reduced mobility, breathing issues, kyphosis, poor fur condition, hindlimb

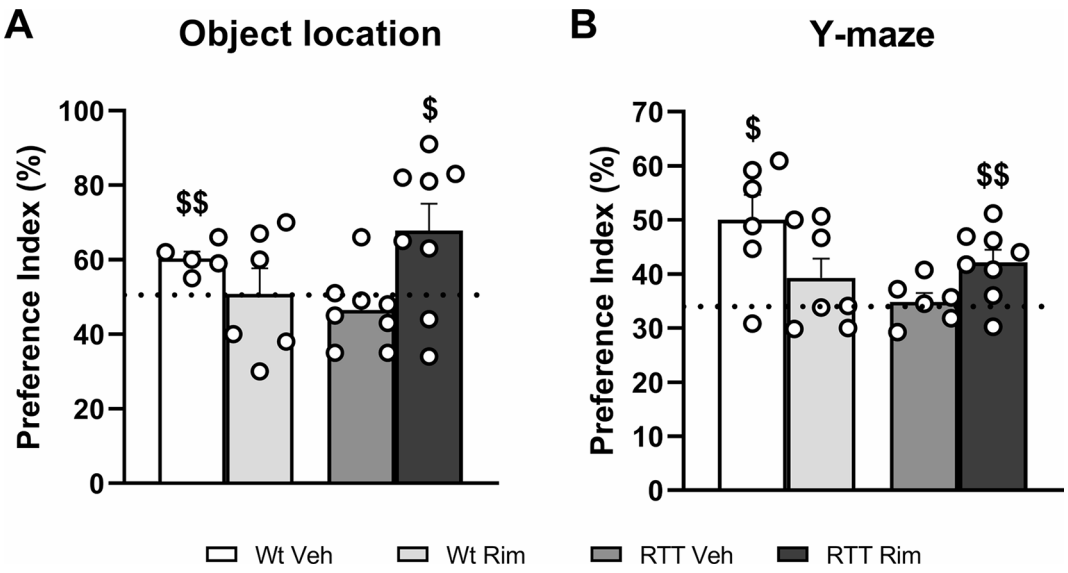

Fig. 3 Treatment with rimonabant rescues the spatial memory deficits in RTT mice. Mecp2-308 heterozygous female (RTT) mice and wild-type (Wt) controls received intraperitoneal daily injections with the CB1 cannabinoid receptor inverse agonist rimonabant (Rim, 0.3 mg/kg) or vehicle for 7 days. Behavioral testing was performed to assess drug effects on the cognitive impairments typically displayed by RTT mice at a symptomatic stage: on the 6th day of treatment mice underwent the object location test to assess memory of spatial configurations; on the 7th day spatial short-term memory was assessed using the Y-maze test. Both tests are based on the spontaneous tendency of rodents to explore novel stimuli (displaced object or unexplored arm of the maze). The preference index (time exploring the novel/(familiar + novel) objects/arms) was calculated and considered a measure of spatial memory retention and novelty discrimination. (**A**) Treatment with Rim restores the ability of RTT mice to discriminate spatial novelty in both the object location and (**B**) Y-maze tests. The dashed line represents the chance levels (50% in A; 33% in B). $N = 5–8$. Data are mean ± SEM. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ (one sample Student's t-test versus chance level)

clasping, tremors, and seizures, compared to control animals (main effect of genotype: $F_{1,23}$=4.13, p=0.054) while no difference in body weight was detected compared to Wt controls. A subchronic treatment regimen with rimonabant in RTT mice did not significantly affect the general health status (Wt-vehicle = 1.02 ± 0.37 average units (AU), Wt-rimonabant = 1.01 ± 0.38 AU, RTT-vehicle = 1.31 ± 0.48 AU, RTT-rimonabant = 1.29 ± 0.23 AU) and body weight (Wt-vehicle = 24.53 ± 2.27 g, Wt-rimonabant = 24.84 ± 2.25 g, RTT-vehicle = 24.47 ± 1.86 g, RTT-rimonabant = 24.35 ± 0.94 g).

Discussion

In the present study, we demonstrate that mtCB1R expression is selectively altered in the brain mitochondria of a mouse model of RTT, considered the leading cause of severe intellectual disability in females. Treatment with the CB1R inverse agonist rimonabant normalized mtCB1R overexpression, boosted mtPKA signaling and concomitantly restored brain mitochondrial bioenergetics, peripheral redox homeostasis, and cognitive abilities in RTT mice. The present data identify the brain mtCB1R overexpression as a novel druggable molecular alteration in RTT.

Impaired mitochondrial functionality and quality control have been largely described in RTT patients and mouse models [24, 27, 46, 47]. These alterations lead to decreased brain energy availability and have been suggested to contribute to RTT-related impairments in higher brain functions, such as learning and memory [27, 29, 48, 49]. Yet, in spite of the increasing evidence supporting the relevance of these brain alterations for RTT pathophysiology, the underlying molecular mechanisms have not been uncovered. We here provide innovative evidence that intracellular compartmentalization of CB1R is altered in the RTT mouse brain, with CB1R localized at mitochondrial membranes, the pool of receptors that critically controls mitochondrial respiration and energy production in neurons [9, 50] being selectively overexpressed. Interestingly a similar profile has been previously reported in models of traumatic brain injury [50], in which trauma-induced mtCB1R overexpression was found to promote metabolic abnormalities coupled with a deficit in ATP production. Collectively, these findings underscore the pathological relevance of this molecular alteration.

An upregulation of brain CB1R was previously reported in the more compromised male mice carrying a *Mecp2*-null mutation [51], suggesting that Mecp2 might be involved in the regulation of CB1R expression levels. Present results extend these findings by demonstrating that the trafficking of CB1R among intracellular compartments is altered in the RTT mouse brain. Of note, treatment with rimonabant reduced total CB1R levels and

normalized mtCB1R overexpression in the brain of RTT mice, confirming that the expression and distribution of CB1R are intimately intertwined with its activity [52]. Taken together, these results provide a complex picture in which CB1R signaling appears severely compromised in the RTT mouse brain, stressing the need to explore the relevance of this molecular signature for the pathogenesis and the treatment of this disorder.

Of utmost importance, pharmacological inhibition of CB1R rescued mitochondrial dysfunction in symptomatic RTT mouse brains, restoring normal levels of ATP production. Furthermore, the normalization of brain mitochondrial functionality by rimonabant treatment was accompanied by the restoration of memory impairments. These results are consistent with compelling evidence demonstrating that mtCB1R signaling pathways, by directly controlling brain mitochondria functionality, play a pivotal role in the regulation of synaptic transmission and memory formation and support the role of these receptors as promising therapeutic targets for RTT. Indeed, several studies have already suggested that targeting the ECS may represent a promising strategy to treat RTT [53]. We complement this evidence by suggesting that the beneficial effects exerted by ECS modulation may involve the restoration of mitochondria functionality in the RTT brain. Interestingly, we report negative effects of subchronic treatment with a low dose of rimonabant on Wt animals at 1 year of age. This was unexpected given that multiple studies show beneficial effects of rimonabant in cognitive processes [54, 55]. However, the function of CB1R in learning and memory seems to be heavily influenced by various factors [54], with variations in drug administration protocols affecting rimonabant effects on cognitive performance [56]. Consistent with our findings, an age-dependent effect was also reported, showing nootropic function in young mice and memory-impairing effects later on [57, 58]. Notably, mitochondria-related dynamics are suggested to be involved in the age-dependent memory decline sustained by CB1R [59].

Importantly, we found that the rimonabant treatment boosted mtPKA activation in the RTT mouse brain. Since previous studies have demonstrated that stimulation of mtPKA increases mitochondrial respiration and ATP synthesis by targeting subunits of mitochondrial complexes I, IV, and V [60, 61], it is conceivable that the rimonabant-induced mtPKA activation, observed in the brain of RTT mice, may account for the rescue of mitochondrial dysfunction. In fact, previous studies reported that PKA signaling modulates several mitochondrial processes such as mitochondrial respiratory chain activity and organization [62–64], and mitochondrial biogenesis [65]. Also, many data point to a role of PKA in the regulation of mitochondrial dynamic and mitophagy [66, 67], two key mechanisms in the maintenance of mitochondrial homeostasis that have been reported to be altered in RTT [47]. It is however worth noting that while rimonabant treatment rescued Complex V hypofunctionality in the RTT mouse brain mitochondria, it had no effect on the unaltered activity of Complex I, providing support to a selective impact of the pharmacological treatment on specific abnormalities observed in the RTT mouse brain.

Of note, in a pioneering study [11], it was also shown that the same cascade of events that involves intra-mitochondrial activation of PKA and controls mitochondrial respiration and ATP production in neurons mediates mtCB1R effects on memory function. Based on this evidence, it can be speculated that the boost in mtPKA activity induced by treatment with rimonabant might have played a role also in the rescue of proper memory functions, possibly by restoring the dendritic spine dysgenesis and impaired synaptic plasticity in RTT mouse brains. In fact, activation of mtPKA signaling was found to promote dendritogenesis and enhance dendrite outgrowth [66, 67], suggesting that rimonabant may have exerted similar beneficial effects in the RTT mouse model [7, 68]. However, we cannot exclude that other mechanisms may have contributed to mtCB1R effects on cognitive function since recent evidence has demonstrated that mtCB1R-dependent effects on mitochondrial calcium in neurons can also affect learning and memory in mice [69]. Further studies will have to explore this possibility. Another intriguing explanation for the current findings concerns the possibility that the identified alterations may be at least partly mediated by astrocytic mtCB1R-mtPKA signaling. In fact, astrocytic mtCB1R has been found in the vicinity of hippocampal synapses [70] and has been recognized as a key determinant of synaptic plasticity [71]. Of particular relevance to our study, the activation of the astrocytic mtCB1R has been demonstrated to influence neuronal signaling and redox status through the PKA-mediated downregulation of OXPHOS and lactate production [72]. Although the specific effects of astrocytic mtCB1R on memory function remain unexplored, astrocytic-derived lactate is known to sustain memory consolidation [73]. Based on this evidence, and on the literature pointing to astrocytes playing a non-cell-autonomous role in the neuropathology of RTT [74, 75] and RTT-related impairment in mitochondrial bioenergetics [32, 76, 77], it will be interesting in future studies to clarify the cellular specificity of the abnormal mtCB1R-mtPKA signaling in the RTT mouse brain.

Of note, rimonabant rescued the aberrant pro-oxidant status of RTT mice by normalizing systemic ROS production. This is in line with previous studies showing a significant boost of the antioxidant defense mechanisms and reduced ROS accumulation after chronic

administration of rimonabant at a low dose in aging rats [78]. Interestingly, PKA was also formerly identified as a regulator of the antioxidant defense, through the modulation of the nuclear factor erythroid 2–related factor 2 (Nrf2) signaling pathway [79], which is known to be impaired in RTT [43], suggesting that the mtPKA boost following rimonabant in RTT mice contributes, at least in part, to the reduction in ROS production. Given the involvement of the neuron-astrocyte crosstalk in oxidative mechanisms in RTT [80], it may be interesting in future studies to address the relative contribution of different cell types to the reported rescue of the aberrant pro-oxidant status of RTT mice.

Taken together, the present results identify the overexpression of mtCB1R as a molecular signature to be pharmacologically modulated to improve brain mitochondrial alterations and cognitive deficits in RTT. The translational relevance of this observation is stressed by the fact that CB1R antagonism is under active scrutiny at the clinical level as a promising target to rescue cognitive defects in syndromic intellectual disability disorders (NCT05748405), based on the promising results obtained at the preclinical level [7, 8]. Our results complement previous knowledge by demonstrating that the mechanism underlying the beneficial effects of these drugs may reside in the specific targeting of CB1R present at mitochondrial membranes. This is fully supported by the increasing evidence demonstrating that mtCB1R is specifically responsible for the control of learning and memory processes [11, 69]. However, to confirm that mtCB1R may represent a common druggable therapeutic target to counteract cognitive dysfunctions, further studies are needed to verify whether abnormal mtCB1R-mtPKA signaling is present in the brain of other syndromic intellectual disability disorders characterized by brain mitochondrial alterations. This could further raise the interest of pharmaceutical companies, thus speeding up the process towards the development of novel drugs to treat cognitive dysfunction in syndromic intellectual disability disorders.

Limitations

Although the present work contributes valuable insights to the field of syndromic intellectual disability disorders, it is essential to acknowledge certain limitations that warrant cautious interpretation of the findings. Firstly, further investigations will be critical to uncover whether an approach that selectively targets the brain mtCB1R achieves the same efficacy as rimonabant treatment for RTT. We cannot exclude that the beneficial effects exerted by the treatment in the RTT mouse model may be ascribed to the modulation of CB1R activity and distribution among intracellular compartments, rather than to a selective effect on mtCB1R-mediated signaling.

Despite the low sample size being a main limitation, the alterations under study demonstrate extensive replicability across distinct experimental conditions [23, 28, 29, 43, 81], and supplementary materials). Additionally, the main findings were replicated in different cohorts of experimental animals, strongly advocating for their strength and reliability.

Furthermore, the observation that the reported biochemical alterations are evident in the whole RTT brain makes the present results compelling and therapeutically relevant, since they provide evidence that the treatment has a broad and robust impact. However, we previously reported that different brain regions in the RTT mouse brain present specific defects [27, 81]. Follow-up studies are thus needed to pinpoint whether area-dependent effects of rimonabant may occur in the RTT mouse brain, as these may influence the extent of treatment efficacy to specific functional domains [29].

Conclusions

In conclusion, our study identifies mtCB1R overexpression as a novel molecular signature in the brain of RTT mice, which may underlie defective brain mitochondrial bioenergetics and cognitive dysfunction; additional investigation is necessary to validate this subcellular pool of CB1R as novel druggable targets with therapeutic potential for RTT and confirm the generalizability of our findings to other syndromic intellectual disability disorders characterized by mitochondrial dysfunctions [16, 82]. Even though further studies are needed, these findings put mitochondria in the spotlight as a valuable source of unexplored therapeutic targets for the treatment of RTT and other syndromic intellectual disability disorders with a metabolic component, and open to the implementation of innovative targeted interventions.

Abbreviations

2-AG	2-arachidonoyl-glycerol
AEA	Anandamide
ANOVA	Analysis of variance
AU	Average units
CB1R	Type-1 cannabinoid receptor
COX1	Cytochrome oxidase subunit 1
CP•	3-carboxy-peroxyl radical
CPH	1-hydroxy-3-carboxypyrrolidine
ECL	Enhanced chemiluminescence
ECS	Endocannabinoid system
EPR	Electron Paramagnetic Resonance
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
i.p.	Intraperitoneal
MECP2	Methyl-CpG-binding protein 2
Mecp2-308	Female mice carrying a truncating mutation of the Mecp2 gene
MRC	Mitochondrial respiratory chain
mtCB1R	Brain CB1R present at the mitochondria-associated membrane
mtPKA	Intra-mitochondrial protein kinase A
Nrf2	Nuclear factor erythroid 2–related factor 2
OXPHOS	Oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
Rim	Rimonabant

ROS	Reactive oxidizing species
RTT	Rett syndrome
SD	Standard deviation
Veh	Vehicle
Wt	Wild-type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13229-024-00617-1.

Supplementary Material 1

Acknowledgements
The authors are grateful to Alessandra Monaco, Daniele Vigli, Nicole Pavoncello, and Maribel Evoli for assistance with mouse behavioral phenotyping; Enrico Caldarelli, Andrea Giovannelli, Yvan Gilardi and Antonio Di Virgilio for animal care; Federica Fratini for assistance with ultracentrifugation; Antonio Maione and Sabrina Alviti for technical/administrative assistance.

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Conceptualization: B.D.F.; Methodology: L.C., C.U., C.L., D.D.R., D.V., F.D.D., D.P., T.B., A.S., R.A.V., B.D.F.; Investigation: L.C., C.U., C.L., D.D.R., D.V., M.P., M.C.Q., F.P., M.K., F.D.D., D.P., A.S.; Visualization: L.C., C.U., B.D.F.; Funding acquisition: B.D.F.; Project administration: B.D.F.; Supervision: D.P., T.B., A, S., R.A.V., B.D.F.; Writing – original draft: L.C., C. U., B.D.F; Writing – review & editing: all authors; Validation: D.D.R., D.P., T.B., A.S., R.A.V., B.D.F.; Formal Analysis: L.C., C.U.; Resources: D.P., T.B., A.S., R.A.V., B.D.F.; Data Curation: L.C., C.U., C.L., D.D.R, D.V., M.P., M.C.Q, F.P., M.K., F.D.D., D.P., A.S.

Funding
This study was supported by the Italian Ministry of Health (#GR-2018-12366210) to B.D.F. and by the Italian Ministry of Education, University and Research, FOE 2022 (#DSB.AD006.371 "*InvAt-Active Aging and Health*") to T.B.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Experiments were approved by the Italian Ministry of Health and are in accordance with the European Communities Council Directive (10/63/EU) as well as the Italian law (26/2014).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 2 December 2023 / Accepted: 16 August 2024
Published online: 19 September 2024

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