Neuropharmacology xxx (2017) xxx-xxx

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com

# Stimulation of the brain serotonin receptor 7 rescues mitochondrial dysfunction in female mice from two models of Rett syndrome

Daniela Valenti[a, **], Lidia de Bari[a], Daniele Vigli[b], Enza Lacivita[c], Marcello Leopoldo[c], Giovanni Laviola[b], Rosa Anna Vacca[a, 1], Bianca De Filippis[b, *, 1]

[a] Institute of Biomembranes and Bioenergetics, National Council of Research, Bari, Italy
[b] Dept. Cell Biology and Neuroscience, Istituto Superiore di Sanità, Roma, Italy
[c] Dept. Pharmacy, University of Bari "A. Moro", via Orabona 4, 70125 Bari, Italy

ARTICLE INFO

*Article history:*
Received 27 December 2016
Received in revised form 21 March 2017
Accepted 14 April 2017
Available online xxx

*Keywords:*
Neurodevelopmental disorders
Intellectual disability
Transgenic mice
Rho GTPases
Energy metabolism
Oxidative stress

*Chemical compound:*
LP-211(PubChem CID:25107716)

ABSTRACT

Rett syndrome (RTT) is a rare neurodevelopmental disorder, characterized by severe behavioral and physiological symptoms. Mutations in the methyl CpG binding protein 2 gene (*MECP2*) cause more than 95% of classic cases, and currently there is no cure for this devastating disorder. Recently we have demonstrated that neurobehavioral and brain molecular alterations can be rescued in a RTT mouse model, by pharmacological stimulation of the brain serotonin receptor 7 (5-HT7R). This member of the serotonin receptor family, crucially involved in the regulation of brain structural plasticity and cognitive processes, can be stimulated by systemic repeated treatment with LP-211, a brain-penetrant selective agonist. The present study extends previous findings by demonstrating that LP-211 treatment (0.25 mg/kg, once per day for 7 days) rescues mitochondrial respiratory chain impairment, oxidative phosphorylation deficiency and the reduced energy status in the brain of heterozygous female mice from two highly validated mouse models of RTT (MeCP2-308 and MeCP2-Bird mice). Moreover, LP-211 treatment completely restored the radical species overproduction by brain mitochondria in the MeCP2-308 model and partially recovered the oxidative imbalance in the more severely affected MeCP2-Bird model. These results provide the first evidence that RTT brain mitochondrial dysfunction can be rescued targeting the brain serotonin receptor 7 and add compelling preclinical evidence of the potential therapeutic value of LP-211 as a pharmacological approach for this devastating neurodevelopmental disorder.



## 1. Introduction

Rett syndrome (RTT) is a rare neurodevelopmental disorder, characterized by severe behavioral and physiological symptoms (Hagberg, 2002; Rett, 1966). One essential feature of RTT is the apparently normal perinatal development until about 6–18 months of age, when RTT patients start losing their acquired cognitive, social, and motor skills and develop a wide variety of symptoms, including autistic-like behaviors, anxiety, motor disturbances, stereotypic hand movements and severe cognitive dysfunction (Hagberg, 2002). Mutations in the methyl CpG binding protein 2 gene (*MECP2*) cause more than 95% of classic cases (Amir et al., 1999; Chahrour and Zoghbi, 2007; Guy et al., 2001). *MeCP2* encodes a multifunctional protein that binds to methylated DNA and mainly acts as a key transcriptional regulator (Guy et al., 2011). How mutations in the *MeCP2* gene lead to the neurobehavioral features of RTT is still unknown and there is no cure for this devastating disorder.

We recently demonstrated that stimulation of central serotonin receptor 7 (5-HT7R) with LP-211, a brain penetrant selective agonist which binds with high affinity at the human cloned 5-HT7R (Hedlund et al., 2010; Leopoldo et al., 2008, 2011), substantially rescues the neurobehavioral phenotype in a mouse model of RTT (De Filippis et al., 2014b, 2015a). 5-HT7R is the most recently discovered serotonin receptor and is involved in a number of neuro-physiological phenomena relevant for RTT, including regulation of the circadian rhythm, sleep, mood and cognitive processes, and in the regulation of structural plasticity in brain circuits (Canese et al., 2014; Gasbarri and Pompili, 2014; Meneses, 2014; Volpicelli et al., 2014). Consistent with these observations, 5-HT7R activation stimulates signaling cascades known to play a prominent role in synaptic plasticity and cognition, such as the extracellular-signal regulated kinases (ERKs), the cyclic AMP protein kinase (PKA) and the Cyclin-dependent kinase 5 (Cdk5) (Guseva et al., 2014; Volpicelli et al., 2014).

**Abbreviations:** 5-HT7R, 5-hydroxytryptamine, serotonin 7; ASC, ascorbate; $H_2O_2$, hydrogen peroxide; GLU, glutamate; MAL, malate; MeCP2, methyl CpG binding protein 2 gene; MRC, mitochondrial respiratory chain; OXPHOS, oxidative phosphorylation; SUCC, succinate; TMPD, *N,N,N′,N′*-tetrametil-*p*-fenilendiammina; ROS, reactive oxygen species; RTT, Rett syndrome; wt, wild-type

* Corresponding author. Dept. Cell Biology and Neuroscience, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Roma, Italy.
** Corresponding author. Institute of Biomembranes and Bioenergetics, National Council of Research, Via Amendola 165/A, 70126 Bari, Italy.
*Email addresses:* d.valenti@ibbe.cnr.it (D. Valenti); bianca.defilippis@iss.it (B. De Filippis)

[1] Equally contributed to the study.

http://dx.doi.org/10.1016/j.neuropharm.2017.04.024
0028-3908/

Notably, stimulation of the central 5-HT7R with LP211 can also activate in mouse brain the Rho GTPases, which are low-molecular-weight guanine nucleotide binding proteins critically involved in different forms of intellectual disabilities (De Filippis et al., 2014a; Etienne-Manneville and Hall, 2002), and can rescue the abnormal activation of Rho GTPases effectors in RTT mouse brain (De Filippis et al., 2012). This family of proteins regulates a variety of important processes, including vesicle transport, microtubule dynamics, cell-cycle progression and gene expression (Feltri et al., 2008; Hall, 2005; Luo, 2000; Nakayama et al., 2000; Tashiro et al., 2000). From a molecular point of view, Rho GTPases have been historically linked to signaling pathways related to cytoskeletal remodeling (Ramakers, 2002); aberrant Rho GTPases signaling is in fact critically associated to cognitive dysfunction and to the accompanying alterations in dendritic spine morphology (Bassani et al., 2013; Ramakers, 2002). Recent evidence also suggests a role for Rho GTPases in the regulation of a signaling pathway critically involved in the regulation of local protein synthesis (De Filippis et al., 2014a; De Rubeis et al., 2013).

Recently, we have provided innovative evidence that Rho GTPases may be critically involved in the regulation of brain mitochondria (De Filippis et al., 2015b, 2015c), whose dysfunction has been indicated as a central player in several pathological conditions associated with intellectual disabilities (Valenti et al., 2014a). This was achieved by intracerebroventricular (icv) administration in mouse brain of CNF1, a bacterial protein produced by several strains of *Escherichia coli* (De Filippis et al., 2012; Loizzo et al., 2013). The CNF1 bacterial protein specifically activates the Rho GTPases through its C-terminal catalytic domain (Fabbri et al., 2013). We demonstrated that specific activation of Rho GTPases by CNF1 in a RTT mouse model improves the neurobehavioral phenotype and rescues in the brain the defective oxidative phosphorylation (OXPHOS) apparatus, the mitochondrial molecular machinery responsible for the majority of cell energy production. The mitochondrial overproduction of $H_2O_2$ associated with the decrease in brain energy status was also contrasted by CNF1 in RTT mouse brain (De Filippis et al., 2015b, 2015c). Importantly, the use as a control treatment of a mutant CNF1 protein whose Rho enzymatic activity was abrogated (Fabbri et al., 2013), allowed us to unequivocally confirm the pivotal role of Rho GTPases in the rescue of mitochondrial dysfunction in CNF1-treated RTT mouse brains. This also provided compelling evidence that modulation of brain Rho GTPases affects brain mitochondrial functionality.

Based on this evidence, we argued that modulation of Rho GTPases signaling by 5-HT7R stimulation might have similar beneficial effects on brain mitochondrial defects for RTT. This has important implication from a translational point of view, because the LP-211 treatment can be systematically administered, thus bearing a higher clinical relevance compared to the CNF1 icv administration.

The present study thus verified whether repeated systemic treatment with LP-211 rescues mitochondrial dysfunction and the subsequent redox imbalance in RTT mouse brain. To this aim, we applied the same LP-211 treatment schedule we have previously reported to exert long-term beneficial effects on behavioral and molecular alterations in a mouse model of RTT (De Filippis et al., 2015a). To substantiate our results, the study was carried out in symptomatic heterozygous female mice, the genetic and hormonal milieus that more closely resemble those of RTT patients. To strengthen our results, the study was carried out using two highly validated mouse models of RTT: (i) the MeCP2-308 model, that bears a truncating mutation, leading to the expression of a truncated protein (De Filippis et al., 2010; Shahbazian et al., 2002); (ii) the MeCP2-Bird model that bears a null mutation (Guy et al., 2011). In agreement with clinical data from RTT patients carrying C-terminal deletions of the MeCP2 gene (Díaz de León-Guerrero et al., 2011), MeCP2-308 hemizygous male mice present a delayed onset of symptoms and a prolonged life-span in comparison with knockout male mice from the MeCP2-Bird model (Ricceri et al., 2008, 2013).

## 2. Materials and methods

### *2.1. Subjects*

The experimental subjects were 8–10 months old heterozygous female mice and wild-type (wt) littermates from two strains: the MeCP2-308 strain [B6.129S-MeCP2tm1Heto/J, stock number: 005439] or the MeCP2-Bird strain [B6.129P2(C)-Mecp2 tm1.1Bird/J, Stock No: 003890] from the Jackson Laboratories (USA), backcrossed to C57BL/6J mice for at least 12 generations.

Mice were housed in groups of 2–3 in polycarbonate transparent cages (33 × 13 × 14 cm) with sawdust bedding and kept on a 12-h light-dark schedule (lights off at 8:00am). Temperature was maintained at 21 ± 1 °C and relative humidity at 60± 10%. Animals were provided *ad libitum* with tap water and a complete pellet diet (Altromin, Germany). All procedures were carried out in accordance with the European Communities Council Directive (2010/63/EU) as well as Italian law, and formally approved by Italian Ministry of Health.

### *2.2. Drug and treatment*

LP-211 was prepared following the same synthetic procedure described in (Leopoldo et al., 2008). The compound was dissolved in a vehicle solution of 1% dimethyl sulfoxide (DMSO) in saline (0.9% NaCl). MeCP2-mutated mice and wt littermate controls were randomly assigned to be daily intra-peritoneally (ip) injected (between 9.00 and 11.00 a.m.) for 7 consecutive days with either LP-211 (0.25 mg/kg) or vehicle (1% of DMSO in saline). The dose was chosen based on previous studies (Adriani et al., 2012; De Filippis et al., 2015a).

### *2.3. Mitochondrial analysis*

One month after the last ip injection of LP-211 or control, MeCP2-mutated heterozygous females and their wt littermates were sacrified and the brains were explanted. The estrous status of the experimental mice at the time of the sacrifice was not controlled, based on recent evidence demonstrating that brain mitochondrial function is not affected by hormonal fluctuations during the estrous cycle (Gaignard et al., 2015).

Immediately after their explantation, the brains were added to an ice-cold cryopreservation solution consisting of 50 mM K-MES (pH 7.1), 3 mM $K_2HPO_4$, 9.5 mM $MgCl_2$, 3 mM ATP plus 20% glycerol and 10 mg/ml BSA, and stored at −80 °C until assayed. Previous data demonstrates that cryopreserved brain tissues show mitochondrial membrane potential, outer and inner membrane integrity and mitochondrial ATP production capacity comparable to mitochondria isolated from fresh brains (Valenti et al., 2014a).

#### *2.3.1. Measurement of mitochondrial ATP production rate*

The rate of ATP production by OXPHOS was determined in isolated mitochondria, essentially as previously described in (Valenti et al., 2010). Briefly, mitochondria isolated from total brain (0.5 mg protein) were incubated at 37 °C in 2 ml of respiratory medium consisting of 210 mM mannitol, 70 mM sucrose, 20 mM Tris/HCl, 5 mM $KH_2PO_4/K_2HPO_4$, (pH 7.4) plus 5 mg/ml BSA, 3 mM $MgCl_2$, in the

presence of the ATP detecting system consisting of glucose (2.5 mM), hexokinase (HK, 2 e.u.), glucose 6-phosphate dehydrogenase (G6P-DH, 1 e.u.) and $NADP^+$ (0.25 mM) in the presence of glutamate (GLU) plus malate (MAL) (5 mM each) or succinate (SUCC, 5 mM) plus rotenone (ROT, 3 μM), or ascorbate (ASC, 0.5 mM) plus *N,N,N′,N′*-tetrametil-*p*-fenilendiammina (TMPD, 0.25 mM), as energy sources. The reduction of $NADP^+$ in the extramitochondrial phase, which reveals ATP formation from externally added ADP (0.5 mM), was monitored as an increase in absorbance at 340 nm. Care was taken to use enough HK/G6P-DH coupled enzymes to ensure a non-limiting ADP-regenerating system for the measurement of ATP production.

*2.3.2. Measurement of mouse brain ATP levels*

Total brain was weighted (approx. 20–40 mg) and subjected to perchloric acid extraction as described in (Khan, 2003). In brief, tissues were homogenized in 600 μl of pre-cooled 10% perchloric acid and then centrifuged at 14,000 *g* for 10 min, 4 °C. The amount of tissue ATP was determined enzymatically in KOH neutralized extracts, as described in (Valenti et al., 2010).

*2.3.3. Measurement of mitochondrial respiratory chain complex (MRC) activities*

Measurements of mitochondrial respiratory chain (MRC) complex activities were carried out in mitochondrial membrane-enriched fractions obtained from isolated mitochondria. For isolation of mitochondrial membrane-enriched fractions, mitochondrial pellets were first frozen at −80 °C, then thawed at 2–4 °C, suspended in 1 ml of 10 mM Tris-HCl (pH 7.5) plus 1 mg/ml BSA and exposed to ultrasound energy for 8 s at 0 °C (11 pulse 0.7 s on, 0.7 s off) at 20 kHz, intensity 2. The ultrasound-treated mitochondria were centrifuged at 600 g for 10 min, 4 °C. The supernatant was centrifuged again at 14000*g* for 10 min, 4 °C and the resulting pellet was kept at −80 °C until use. Measurement of MRC complex activities were performed essentially as in (Valenti et al., 2013), by three assays which rely on the sequential addition of reagents to measure the activities of: i) NADH:ubiquinone oxidoreductase (complex I) followed by ATP synthase (complex V), ii) succinate:ubiquinone oxidoreductase (complex II) and iii) cytochrome *c* oxidase (complex IV) followed by cytochrome *c* oxidoreductase (complex III).

*2.3.4. Detection of mitochondrial superoxide anion/hydrogen peroxide production*

Production of superoxide anion and $H_2O_2$ by mitochondria was measured (without discriminating between them) as $H_2O_2$ production rate in the presence of endogenous and exogenous (70 e.u) superoxide dismutase (SOD). $H_2O_2$ production rate was measured using homovanillic acid (HOVA, 200 μM) and horseradish peroxidase (POX, 8 e.u.) forming a fluorescent dimer monitored at excitation/emission wavelengths of 312/420 nm (Barja, 2002). In each experiment the arbitrary fluorescence units were converted to amounts of $H_2O_2$ by measuring the increase in fluorescence after the addition of known amounts of $H_2O_2$ in the presence of POX, HOVA and SOD ($H_2O_2$-detecting system, $H_2O_2$-ds).

Soon after isolation, mitochondria from mouse brain hemispheres (0.5 mg of mitochondrial protein) were incubated at 37 °C in a final volume of 2 ml of assay medium consisting of 145 mM KCl, 30 mM Hepes-Tris, 5 mM $KH_2PO_4$, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.1% fatty-acid-free albumin (pH 7.4). The mitochondrial production of $H_2O_2$ was detected after the addition of the respiratory substrates glutamate/malate (GLU/MAL, 5 mM each) or succinate (SUCC, 5 mM). In this latter case, the complex I inhibitor rotenone (ROT, 5 μg/10 μL) was added to the mitochondrial suspension before the substrate in order to block the reverse electron flow from SUCC to complex I, this preventing reactive oxygen species production by complex I. The rate of $H_2O_2$ generation was obtained from the tangent to the progress curve and expressed as pmol of $H_2O_2$ formed/min x mg of mitochondrial proteins.

*2.4. Statistical analysis*

The values are given as means ± standard deviation (SD). The brains of at least three mice per experimental group were analyzed. Statistical evaluation of the differential analysis between groups was performed by one-way ANOVA and Tukey's post hoc test. $p < 0.05$ was considered to be statistically significant.

## 3. Results

*3.1. Stimulation of 5-HT7R by LP-211 rescues mitochondrial dysfunction and the reduced energy status in the brain of RTT mouse models*

*3.1.1. MeCP2-308 female mice*

To assess whether LP-211 treatment is able to improve the defective mitochondrial energy function in MeCP2-308 mouse brain (De Filippis et al., 2015b, 2015c), we first evaluated the effects of the LP-211 treatment on the activity of the MRC complexes. A focus was made on complexes II and V, as they were previously proven to be selectively impaired in whole brain of MeCP2-308 mice (De Filippis et al., 2015b, 2015c) (Fig. 1A). In addition, the activity of complex I, previously found not to be affected in whole brain of MeCP-308 mice (De Filippis et al., 2015b), was also measured (Fig. 1A). As expected, veh-injected MeCP2-308 mice showed a significant reduction in the activity of complex II and V compared to veh-injected wt mice (complex II: $p < 0.01$ after post hoc comparison on the Gen*Treat interaction: $F(1,8) = 24,300$, $p = 0,001$; complex V: $p < 0,01$ after post hoc comparison on the Gen*Treat interaction: $F(1,8) = 181,818$, $p < 0,0001$). A complete restoration in the activity of both the defective MRC complexes in LP-211-treated RTT mice was found (Fig. 1A; $p < 0,01$ compared to vehicle-treated RTT mice after post hoc comparisons on the Gen*Treat interactions). By contrast, no significant changes in complex I activity were found in MeCP2-308 mice respect to wt (Fig. 1A).

To ascertain whether the full recovery in the MRC defective complex activities found in LP-211-treated MeCP2-308 mice was accompanied by normalization in their bioenergetics efficiency, the mitochondrial ATP synthesis and whole brain ATP levels were measured. As expected, veh-injected MeCP2-308 mice showed a significant reduction in both mitochondrial ATP synthesis, using succinate, the respiratory substrates of complex II, as energy source (Fig. 1B; $p < 0,01$ after post hoc comparison on the Gen*Treat interaction: $F(1,8) = 44,100$, $p = 0,002$), and whole brain ATP levels in comparison to veh-injected wt mice (Fig. 1C; $p < 0,01$ after post hoc comparison on the Gen*Treat interaction: $F(1,8) = 315,875$, $p < 0,001$). A complete rescue to wt values of both parameters was found in the brain of LP-211- injected MeCP2-308 mouse brain ($p < 0,01$ after post hoc comparison on the Gen*Treat interactions; Fig. 1B and C).

*3.1.2. MeCP2-bird female mice*

To investigate whether and how mitochondrial bioenergetics is similarly compromised in a second mouse model of RTT and to evaluate the effect of LP-211 treatment thereon, we monitored the ATP production via the oxidative phosphorylation (OXPHOS) apparatus in mitochondria isolated from the whole brain of MeCP2-Bird female

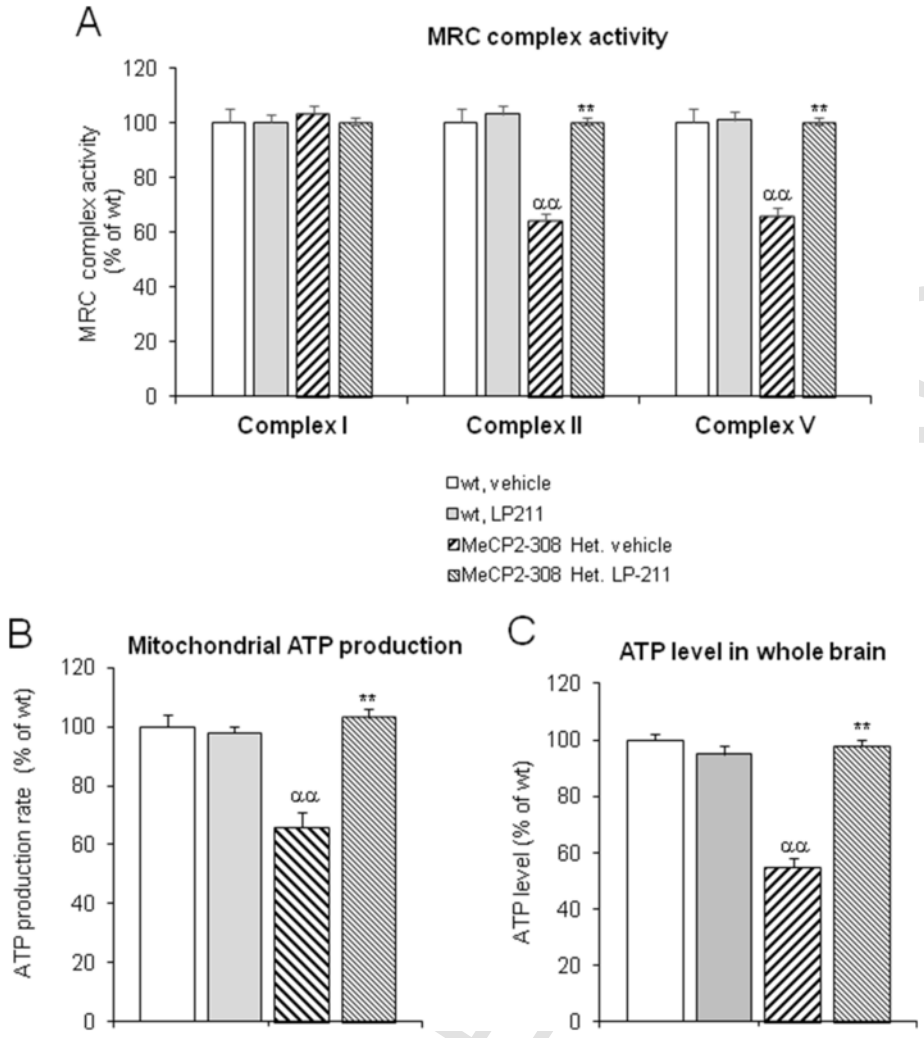


**Fig. 1. LP-211 treatment restored MRC complex II and V activities, mitochondrial ATP production and ATP levels in the brain of MeCP2-308 female mice.** (A) The activities of the complex I, complex II and complex V were measured spectrophotometrically in mitochondrial membrane enriched fractions from cryopreserved brain hemispheres of vehicle-injected MeCP2-308 female mice (MeCP2-308 Het, vehicle), LP-211-injected MeCP2-308 female mice (MeCP2-308 Het, LP-211), vehicle-injected wt littermates controls (wt, vehicle) and LP-211- injected wt mice (wt, L-211). Complex activities are expressed as percentage of activity measured in wt. Data are mean rates ± SD obtained from three independent experiments. (B) The rate of mitochondrial ATP production was measured in mitochondria isolated from cryopreserved brain hemispheres in the presence of the complex II respiratory substrate succinate plus rotenone (SUCC). Values are mean rates ± SD obtained from three independent experiments and expressed as percent of wt. (C) The ATP level was measured as described in Material and Methods.

mice with respect to wt littermates, administered with either LP-211 or vehicle. The relative contribution of the individual MRC complexes of the OXPHOS apparatus in the mitochondrial ATP production was also addressed by adding the respiratory substrates of either complex I (GLU/MAL), complex II (SUCC) or complex IV (ASC/TMPD), as energy sources (Fig. 2A). The rate of mitochondrial ATP synthesis appeared strongly reduced in mitochondria from MeCP2-Bird mice compared to wt controls, when either GLU/MAL or SUCC were used as energy substrates ($p < 0,01$ after post hoc comparison on the Gen*Treat interactions: GLU/MAL: $F(1,8) = 33,379$, $p = 0,0004$, 34± 5%; SUCC: $F(1,8) = 9,394$, $p < 0,015$, 30 ± 4%). Conversely, no significant differences were found in the complex IV-dependent rate of mitochondrial ATP production from both mutant and wt mouse brains (Fig. 2A).

Interestingly, the levels of ATP, assayed in the brain of MeCP2-Bird mice, were strongly lower compared to wt littermates (Fig. 3B, $p < 0,01$ after post hoc comparison on the Gen*Treat interaction: $F(1,8) = 118,564$, $p < 0,001$), thus suggesting that alterations in ATP production by mitochondria affect the whole brain energy status in MeCP2-Bird mice.

Notably, a complete normalization in the rate of complex I- and II-dependent mitochondrial ATP synthesis (Fig. 2A), and a full restoration to wt values of the brain ATP levels (Fig. 2B), were found in the brain of LP-211-treated MeCP2-Bird mice. In line with the results obtained by measuring mitochondrial ATP synthesis by OXPHOS, functional analysis in MeCP2-Bird mouse brain of the five MRC complexes revealed a significant reduction in the activities of both complex I and complex II ($p < 0,01$ after post hoc comparison on the Gen*Treat interactions: complex I: $F(1,8) = 171,125$, $p < 0,001$; 31± 4%; complex II: $F(1,8) = 20,167$, $p = 0,002$; 37± 3%) in comparison to wt littermates (Fig. 3A and B). No significant difference between MeCP2-Bird and wt samples was detected in the activity of complex III and complex IV (Fig. 3C and D). Interestingly, ATP synthase (complex V) activity was also found significantly impaired in mitochondria from MeCP2-Bird brains compared to veh-injected wt mice (Fig. 3E; $p < 0,01$ after post hoc comparison on the

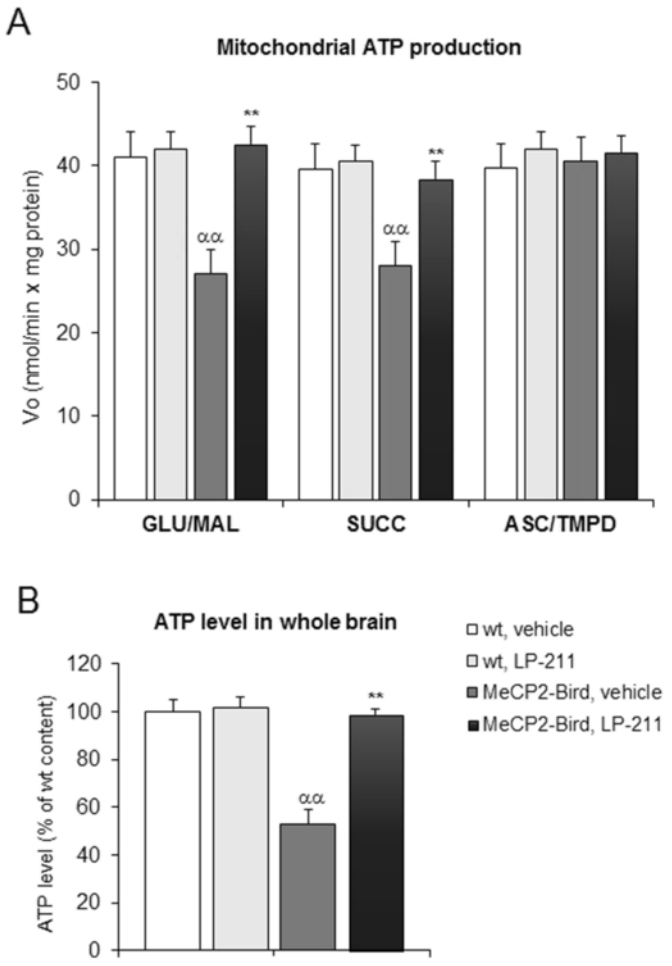


**Fig. 2. Mitochondrial ATP synthesis impairment in MeCP2-Bird female mice: beneficial effects by LP-211 treatment.** (A) Rate of ATP production in mitochondria isolated from cryopreserved brain hemispheres in the presence of the respiratory substrates glutamate plus malate (GLU/MAL) or succinate plus rotenone (SUCC), or ascorbate plus TMPD (ASC/TMPD). Values are mean rates ± SD obtained from three independent experiments for each experimental group: vehicle-injected MeCP2-Bird female mice (MeCP2- Bird Het, vehicle), LP-211-injected MeCP2- Bird female mice (MeCP2-Bird Het, LP-211), vehicle-injected wt littermates controls (wt, vehicle) and LP-211-injected wt mice (wt, L-211). (B) The ATP level in mitochondria isolated from cryopreserved brain hemispheres. Data are mean rates ±SD obtained from three independent experiments and expressed as percent of ATP amount measured in wt. Significant differences were calculated with one-way ANOVA and Tukey's test. αα = $p < 0{,}01$: wt vehicle *vs*. MeCP2-Bird vehicle; ** = $p < 0{,}01$: MeCP2-Bird vehicle *vs*. MeCP2-Bird LP-211.

Gen*Treat interaction: $F(1,8) = 841{,}000$, $p < 0{,}001$; 47± 2%). LP-211 treatment increased the activity of all the defective MRC complexes in MeCP2-Bird brains, thus restoring wt-like activity values (Fig. 3).

It should be noted that no changes into OXPHOS protein levels were found in RTT whole brains compared to wt mice (Supplementary Fig. 1).

### *3.2. LP-211 treatment affects ROS overproduction in RTT mouse brain*

We next asked whether the beneficial effects exerted by the LP-211 treatment extended beyond the defective energy function and affected reactive oxygen species (ROS) production in RTT mouse brain. Aberrant MRC functionality can in fact increase ROS production (Dröse and Brandt, 2012), contributing to oxidative stress (Raha and Robinson, 2000). ROS generation was thus detected by monitoring $H_2O_2$ production due to the addition of either complex I (GLU/MAL) or complex II (SUCC) respiratory substrates (Fig. 4).

In line with our previous findings (De Filippis et al., 2015a) and with the absence of inhibition in complex I activity in the brain of RTT MeCP2-308 mice (Fig. 1), we found no significant differences in the rate of complex I-dependent ROS production by mitochondria isolated from RTT MeCP2-308 and wt brain samples (Fig. 4A). By contrast, aberrant complex II MRC functionality is accompanied by succinate-dependent ROS overproduction in brain mitochondria from the RTT MeCP2-308 mouse model compared to wt controls (1,5- fold higher than wt controls; $p < 0{,}01$ after post hoc comparison on the Gen*Treat interaction: $F(1,18) = 40{,}171$; $p < 0{,}001$; Fig. 4B). Importantly, a complete prevention of ROS overproduction was found as a result of the LP-211 treatment in the brain of MeCP2-308 female mice ($p < 0{,}01$ after post hoc comparison on the Gen*Treat interaction; Fig. 4B).

We extended our investigation to mitochondria isolated from MeCP2-Bird mouse brains. We found that the deficit in complex I and II activities was accompanied by a significant increase in ROS production from both complexes I (Fig. 4C) and II (Fig. 4D) in the brain of MeCP2- Bird mice with respect to wt controls ($p < 0.01$ after post hoc comparison on the Gen*Treat interactions: complex I: $F(1,14) = 13{,}351$, $p = 0{,}003$; complex II: $F(1,14) = 4{,}092$, $p = 0{,}063$). The LP211 treatment partially but significantly prevented ROS overproduction by complex I in the brain of MeCP2-Bird LP211 treated mice in comparison to veh-treated MeCP2-Bird mice (Fig. 4C, $p < 0{,}05$ after post hoc comparison on the Gen*Treat interaction); however, ROS production by complex II was not affected by the LP-211 treatment in this mouse model (Fig. 4D).

## 4. Discussion

The present study demonstrates that a systemic treatment with LP-211, a selective agonist of the 5-HT7 receptor, significantly improves RTT-related impairments in mitochondrial functionality in mouse brain. Reactivation of respiratory chain complexes by LP-211 completely rescued the reduced brain energy status. Importantly, the LP-211 treatment was found to be effective in MeCP2-mutated heterozygous female mice from two highly validated RTT models, MeCP2 Bird and MeCP2-308 mice. A restoration of the mitochondrial overproduction of ROS in RTT mouse brains is also reported.

We have previously demonstrated that repeated systemic treatment with LP-211 restores RTT-related gross phenotypic alterations, the anxiety-related profile, motor abilities, exploratory behavior, as well as memory performance and synaptic plasticity in symptomatic MeCP2-308 male and female mice (De Filippis et al., 2014b, 2015a). In the brain of RTT mice, LP-211 treatment also reversed the abnormal activation of key regulators of both actin cytoskeleton dynamics and protein translational control (De Filippis et al., 2014b). The present study extends our previous findings by demonstrating that the widespread beneficial effects of the LP-211 treatment on the neurobehavioral phenotype of RTT mouse models is associated with the rescue of mitochondrial abnormalities in RTT mouse brain. These results provide compelling preclinical evidence of the potential therapeutic value of a pharmacological approach targeting the brain 5HT7R for RTT, a devastating disorder for which no cure is currently available.

This is, to the best of our knowledge, the first report suggesting a direct link between mitochondria functionality and the 5-HT7R. Interestingly, some lines of evidence are already available suggesting a relationship between the neurotransmitter serotonin and mitochondria

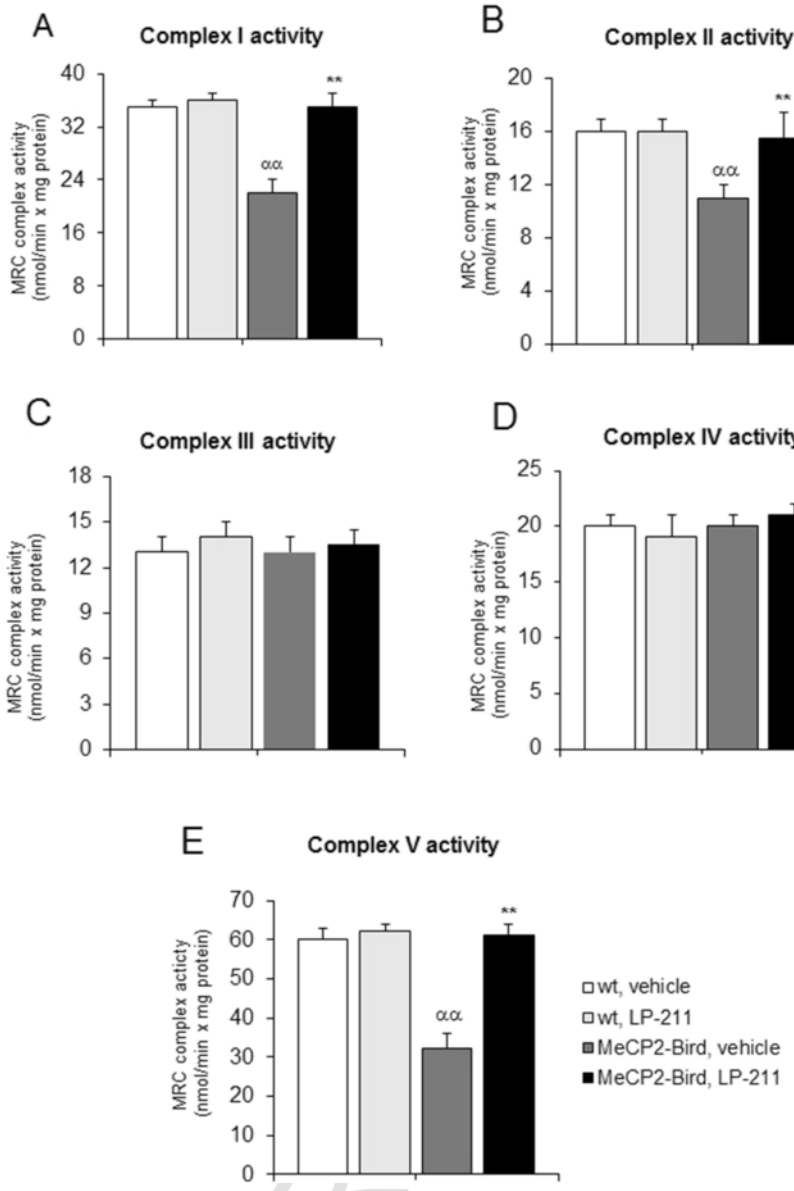


**Fig. 3. The reduced functionality of the mitochondrial respiratory chain complexes in the brain of MeCP2-Bird female mice is counteracted by LP-211.** The activities of complex I (NADH:ubiquinone oxidoreductase) (A), complex II (succinate:ubiquinone oxidoreductase) (B), complex III (cytochrome *c* reductase) (C), complex IV (cytochrome *c* oxidase) (D) and complex V (ATP synthase) (E) were measured spectrophotometrically in mitochondrial membrane-enriched fractions from cryopreserved brain hemispheres of vehicle-injected MeCP2-Bird female mice (MeCP2- Bird Het, vehicle), LP-211-injected MeCP2- Bird female mice (MeCP2- Bird Het, LP-211), vehicle-injected wt littermates controls (wt, vehicle) and LP-211-injected wt mice (wt, L-211). Data are means ± SD of three independent experiments. Significant differences, calculated with one-way ANOVA and Tukey's test, are indicated as follow: wt vehicle *vs*. MeCP2-Bird vehicle, αα = $p < 0{,}01$; MeCP2-Bird vehicle *vs.* MeCP2-Bird LP-211,** = $p < 0{,}01$.

(Chen et al., 2007; de Oliveira, 2016). Previous studies have in fact demonstrated that the enhanced availability of serotonin in the synaptic cleft, provided by acute and chronic treatment with SSRI, affects mitochondria functionality in the nervous system (Braz et al., 2016; da Silva et al., 2015). Moreover, serotonin has been found to be critical for neuroendocrine coordination of mitochondrial stress signaling and proteostasis (Berendzen et al., 2016). Taken together, these studies are in favor of a role for the serotonergic system in the regulation of mitochondria homeostasis. However, the type of serotonin receptor responsible for such effects has not been established yet. Present results suggest that the effects of serotonin on mitochondria functionality may be, at least in part, mediated by the 5-HT7R. Consistently, systemic treatment with 8-OH-DPAT (a mixed 5-HT1A/5-HT7 agonist) in a mouse model of age-related oxidative stress in the retina was found to induce anti-oxidant protection and to preserve the retina from mitochondrial oxidative stress (Biswal et al., 2015). Although the authors of this study did not address the relative contribution of 5-HT1A/5-HT7 receptors in the reported effects, these results provide support to our hypothesis.

Even though further studies are needed to dissect the underlying molecular mechanisms, we argue here that the observed rescue of mitochondrial functional alterations in RTT mouse brain might be mediated by LP-211 effects on the activation of Rho GTPases (De Filippis et al., 2014b). This hypothesis stems from our previous data demonstrating that modulation of brain Rho GTPases by a selective activator provides similar beneficial effects on mitochondrial alterations in a RTT mouse model (De Filippis et al., 2015c). Although we cannot exclude at the moment that other signaling pathways known to be

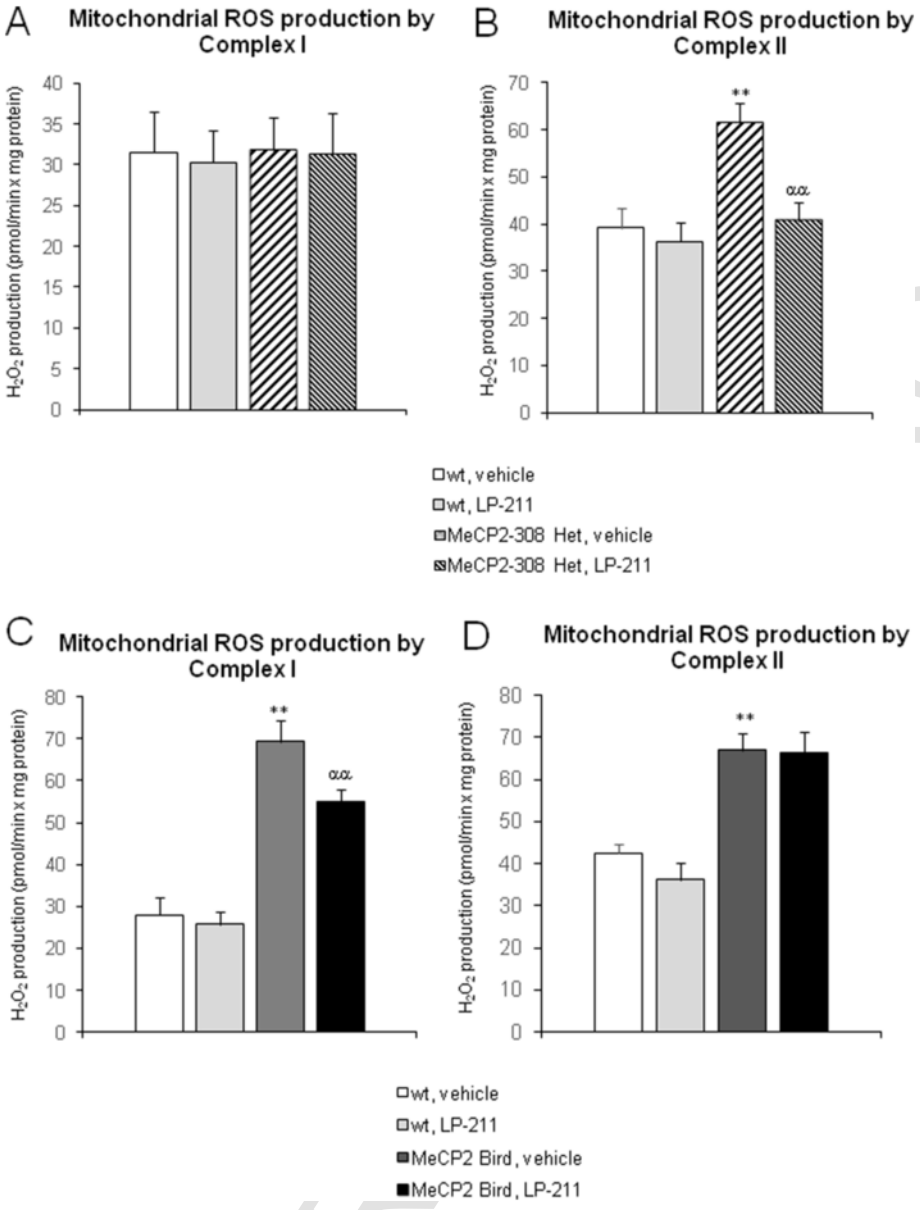


**Fig. 4. Complex I- and complex II-dependent mitochondrial H2O2 production in MeCP2-308 Het and MeCP2-Bird female mouse brain.** $H_2O_2$ production rate was measured in mitochondria isolated from whole brain in the presence of the complex I respiratory substrates glutamate plus malate (GLU/MAL) (A, C) or complex II substrate succinate (SUCC) (B, D). Data are means ± SD of at least three independent experiments. Significant differences, calculated with one-way ANOVA and Tukey's test, are indicated as follow: wt vehicle *vs*. MeCP2-308 Het vehicle, αα = $p < 0,01$; MeCP2-308 Het vehicle *vs*. MeCP2-308 Het LP-211,** = $p < 0,01$; wt vehicle *vs*. MeCP2-Bird vehicle, αα = $p < 0,01$; MeCP2-Bird vehicle *vs*. MeCP2-Bird LP-211,* = $p < 0,05$.

controlled by 5-HT7R (Nikiforuk, 2015) may be in part responsible for the therapeutic efficacy of the proposed treatment, present data stress the relevance of the Rho GTPases family of proteins as therapeutic targets for RTT.

Another important result of this study concerns the long-lasting duration of the effects exerted by the treatment with LP-211 on mitochondria functionality in RTT mouse brain. We report here a complete restoration of mitochondria functionality on brain samples collected one month after the last administration of the 7-day long LP-211 treatment. These results are consistent with our previous data suggesting long-lasting effects of the LP- 211 treatment on RTT symptomatology. In particular, we have demonstrated that the beneficial effects on neurobehavioral and molecular parameters were evident up to two months after the last injection of LP-211 (De Filippis et al., 2015a). Similar results were previously observed in RTT mice treated with the selective activator of Rho GTPases CNF1 (De Filippis et al., 2015c), suggesting a role for this family of proteins in mediating the enduring sequence of beneficial effects exerted by LP-211 in RTT mice. Consistently, long-lasting effects of the treatment were also evidenced over the activation status of the Rho-GTPases effector rpS6 (De Filippis et al., 2014b), the downstream target of mTOR and S6 kinase responsible for the altered protein translational control in RTT mouse brain (Ricciardi et al., 2011). Taken together, these results suggest that plastic remodeling of the brain circuitries mediated by RhoGTPases in RTT mouse brain might account for the long-lasting effects of treatment with LP-211 (Cerri et al., 2011; De Filippis et al., 2012; Diana et al., 2007; Loizzo et al., 2013).

It is worth noting that, to test the robustness and generalization of the outcomes of the LP-211 treatment, two different mouse models have been adopted in the present study, a needed requirement for RTT preclinical research (Katz et al., 2012). This approach allowed us to confirm that the LP-211 treatment is effective in both RTT mouse models, thus representing a highly reliable preclinical approach. Interestingly, however, we uncovered a complex picture of mitochondrial dysfunction in RTT mouse brains, with different mouse models displaying different patterns of mitochondrial alterations. Consistent with previous data from our group (De Filippis et al., 2015b), we demonstrate that complexes II and V are profoundly affected in whole brain of the MeCP2-308 model. Thanks to the analysis of mitochondria in different brain regions, we have previously demonstrated that in this RTT mouse model these complexes are compromised in several brain regions as well as in the whole brain, whereas deficient complex I activity is evident only in mitochondria isolated from cerebellum and striatum. In this line, in the whole brain of MeCP2-308 mice, a selective defect in complex II-mediated ATP production is evident and mitochondrial ROS overproduction is mainly ascribable to complex II.

We demonstrate here that a more severe profile is evident in the MeCP2-Bird model, in which the same alterations characterizing the MeCP2-308 model are found to be accompanied by a more generalized defect in complex I activity, leading to abnormalities in ATP and ROS production mediated by both complex I and II. These results are in line with a previous study reporting an important reduction of brain ATP level in the MeCP2-Bird model (Saywell et al., 2006). However, other studies, especially those looking at more defined brain regions, have reported contrasting data (Fischer et al., 2009; Toloe et al., 2014), suggesting that different brain areas may behave differently at the mitochondrial level. This is in fact in agreement with our previous results demonstrating that in MeCP2-308 female mice mitochondrial respiratory chain dysfunctions differ in different brain areas (De Filippis et al., 2015b). Importantly, this is, to our knowledge, the first time that brain mitochondrial functionality is addressed in MeCP2-Bird heterozygous female mice, as previous studies addressed this topic in MeCP2-Bird hemizygous males. This has high translational relevance, given that sex-dependent differences have been described in brain mitochondrial function (Gaignard et al., 2015).

Taken together, present results confirm our previous findings and demonstrate that brain mitochondrial functionality is more affected in the MeCP2-Bird model than in MeCP2-308 mouse brain. This is consistent with the more severe behavioral phenotype presented by the MeCP2-Bird mice (Guy et al., 2001). Indeed, the two models adopted in the present study present profound differences in gene mutations and the consequent protein integrity (Ricceri et al., 2008). This comes also with profound differences in the gravity of symptoms and in the age of their onset. In this line, LP-211 treatment completely restored ROS overproduction in the MeCP2-308 model, whereas a partial recovery in ROS overproduction was observed in the MeCP2-Bird model. We argue here that the differential responsiveness to the treatment of the two mouse models may be ascribed to a worst plasticity in the more affected MeCP2-Bird model, which may prevent these mice from properly responding to this Rho GTPases modulating treatment. A more systematic comparison between the two models, at the behavioral, molecular and neurobiological level, also keeping into account possible interactions with the sex of the subjects, is however needed to understand the actual differences and to shed light on the underlying molecular mechanisms.

As a whole, these results confirm and extend our previous findings and provide further evidence that the brain serotonin receptor 7 represents a potential target for the treatment of RTT. Moreover, this study demonstrates that LP-211 treatment is effective in MeCP2-mutated heterozygous female mice from two highly validated RTT models, MeCP2-Bird and MeCP2-308 mice, thus increasing the translational value of the study.

An important aspect to be addressed in the next future concerns the potential effects exerted by the systemic LP211 treatment on mitochondrial functionality on peripheral tissues. Given the widespread distribution of the 5-HT7 receptor in the periphery, especially in the gastrointestinal tract (Bard et al., 1993; Liu et al., 2001), it is plausible that stimulation of the peripheral receptors may uncover potentially interesting results.

Given the involvement of the 5-HT7R in a number of physiological processes relevant for neuropsychiatric disorders (Nikiforuk, 2015), it is conceivable that the effects mediated by 5-HT7R on mitochondrial functionality we uncovered in the present study may be relevant also for other disorders, particularly for those associated with cognitive disturbances and reduced synaptic plasticity, two processes which strictly depend on brain mitochondrial energy metabolism and oxygen supply (Erecinska et al., 2004; Valenti et al., 2014b). In this line, stimulation of 5-HT7R is emerging as an innovative potential treatment for different intellectual disabilities [Fragile X and Rett: (Costa et al., 2012; De Filippis et al., 2014b)]. Future studies aimed at evaluating the role played by mitochondria in mediating the therapeutic efficacy of this promising pharmacological approach in other disorders are needed to shed light on the translational relevance of the suggested link between brain mitochondria functionality and the 5-HT7R.

## Authors disclosures

None of the authors declare financial interests or potential conflict of interests.

## Acknowledgements

The authors are grateful to Livia Cosentino for technical assistance, Luigia Cancemi and Valerio Tomassini for animal care, Nadia Francia and Stella Falsini for administrative assistance. This research was supported by Jerome Lejeune Foundation (France) and AIRETT (Italy) to G.L., and by IRSF HeART Award (#3107) to B.D.F.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2017.04.024.

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