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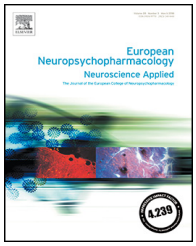

# Impaired heme metabolism in schizophrenia-derived cell lines and in a rat model of the disorder: Possible involvement of mitochondrial complex I

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**Abstract**
Accumulating data point to heme involvement in neuropsychiatric disorders. Heme plays a role in major cellular processes such as signal transduction, protein complex assembly and regulation of transcription and translation. Its synthesis involves the mitochondria, which dysfunction, specifically that of the complex I (Co-I) of the electron transport chain is involved in the pathophysiology of schizophrenia (SZ). Here we aimed to demonstrate that deficits in Co-I affect heme metabolism. We show a significant decrease in heme levels in Co-I deficient SZ-derived EBV transformed lymphocytes (lymphoblastoid cell lines - LCLs) as compared to healthy subjects-derived cells ($n = 9$/cohort). Moreover, protein levels assessed by immunoblotting and mRNA levels assessed by qRT-PCR of heme catabolic enzyme, heme Oxygenase 1 (HO-1), and protein levels of heme downstream target phosphorylated eukaryotic initiation factor 2-alpha (Peif2a/eif2a) were significantly elevated in SZ-derived cells. In contrast, protein and mRNA levels of heme synthesis rate limiting enzyme aminolevulinic acid synthase-1 (ALAS1) were unchanged in SZ derived LCLs. In addition, inhibition of Co-I by rotenone in healthy subjects-derived LCLs ($n = 4$/cohort) exhibited an initial increase followed by a later decrease in heme levels. These findings were associated with opposite changes in heme's downstream target and

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HO-1 level, similar to our findings in SZ-derived cells. We also show a brain region specific pattern of impairment in Co-I subunits and in HO-1 and PeIF2$\alpha$/eIF2$\alpha$ in the Poly-IC rat model of SZ ($n = 6$/cohort). Our results provide evidence for a link between CoI and heme metabolism both *in-vitro* and *in-vivo* suggesting its contribution to SZ pathophysiology.


## 1. Introduction

Schizophrenia (SZ), a chronic brain disorder which affects 1% of the population, is one of the top 15 leading causes of disability worldwide (Vos et al., 2017). Clinical manifestations include positive symptoms (agitation, paranoia, delusions and hallucinations) and negative symptoms (apathy, social withdrawal and anhedonia) as well as deficits in various neurocognitive functions (Javitt and Sweet, 2015). Previous studies by our group and others have shown that mitochondrial dysfunction play a major role in the pathophysiology of the disease (Ben-Shachar, 2016; Chouinard et al., 2017; Manji et al., 2012; Prabakaran et al., 2004; Rajasekaran et al., 2015a). Imaging studies demonstrated reduction in mitochondrial originated high energy phosphates, such as ATP and phosphocreatine as well as in other cellular factors whose metabolism is strongly suggested to be linked to mitochondrial ATP production, in SZ-relevant brain structures (Fujimoto et al., 1992; Jayakumar et al., 2006; Volz et al., 2000). Genetic transcriptomic, proteomic, metabolomic molecular and biochemical studies, point to abnormalities in mitochondria in both the periphery and the brain in this disorder (Bergman and Ben-Shachar, 2016a, 2016b; Föcking et al., 2011; Rajasekaran et al., 2015b; Washizuka et al., 2006). Investigation of the mitochondrial oxidative phosphorylation system (OXPHOS) by our group and others, revealed alterations of the enzymatic activities of several complexes but specifically that of the first and largest complex, complex I (Co-I) in post-mortem brain specimens and in peripheral blood cells of SZ patients(Ben-Shachar and Karry, 2008; Haghighatfard et al., 2018; Karry et al., 2004; Rollins et al., 2018).

The biosynthesis of heme (iron protoporphyrin IX), an essential iron-containing molecule, is an important function of the mitochondria (Ponka, 1999). The first and rate limiting step in heme biosynthesis pathway, as well as the last three steps, all take place in the mitochondria. Alterations in heme have been shown to affect the mitochondria. Both increase and decrease in heme levels were found to corrupt mitochondrial membrane potential (Higdon et al., 2012; Homedan et al., 2015). Though not fully understood, association between heme and Co-I, generating ~40% of the proton-motive force needed for ATP production, has been suggested by two studies. A 52% decrease in Co-I activity was reported in a heme deficient mouse model (Homedan et al., 2014), and inhibition of Co-I was associated with decreased heme biosynthesis in HeLa cells (Gielisch and Meierhofer, 2015).

Traditionally, heme was believed to merely control oxygen transfer. In the past few decades, however, heme was shown to take part in intricate processes such as signal transduction, assembly of protein complexes and regulation of transcription and translation, all of which are necessary for neuronal survival (Smith et al., 2011; Yang and Wang, 2010). One such example is regulation of protein synthesis by phosphorylation of eukaryotic initiation factor 2-alpha (eIF2$\alpha$) via the mediator enzyme heme regulated inhibitor (HRI). HRI can bind four heme molecules; two structural irreversibly bound to the N-terminus domain, and two that reversibly bind at the C-terminus kinase insertion domain (Chefalo et al., 1998; Chen and London, 1995). When heme levels are low, the C-terminus domain is unoccupied resulting in auto-phosphorylation of HRI, which in turn phosphorylates eIF2$\alpha$ $\alpha$ subunit at Ser-51. Phosphorylated eIF2$\alpha$ (PeIFf2$\alpha$) is inactive, hindering global translation and therefore an indicator of cellular heme levels (Chefalo et al., 1998). Heme has also been implicated in additional intricate processes such as mitochondrial proteolysis (Tian et al., 2011), acceleration of mRNA degradation (Cable et al., 1996; Hamilton et al., 1991), control of ion channels such as $Ca^{2+}$-activated potassium channels (Tang et al., 2003) and binding nuclear receptor transcription factors such as Rev-erb $\alpha$ and $\beta$ (Raghuram et al., 2007), nuclear receptors that function as transcription repressors.

Inappropriate heme metabolism has been linked to several neuropsychiatric disorders. In the hippocampi of Alzheimer's disease (AD) patients, for example, heme was found to binds intracellular amyloid $\beta$ (A$\beta$), resulting in reduced heme bioavailability and its functional deficiency (Atamna and Frey, 2004). Heme Oxygenase-1 (HO-1), the heme catabolic rate limiting enzyme, was found to be overexpressed in the cerebral cortex and hippocampus of individuals with AD (Barone et al., 2012; Smith et al., 1994), the substantia nigra of Parkinson's disease (PD) patients (Schipper et al., 1998) and its mRNA was overexpressed in the prefrontal cortex of patients with SZ (Prabakaran et al., 2004). These data suggest an increase in HO-1 activity in the pathophysiology of several neuropsychiatric disorders. Furthermore, neuroporphyria, a condition in which heme levels are scarce, is characterized by psychiatric manifestations several are similar to SZ such as psychosis, hallucinations and paranoia as well as anxiety, insomnia and depression (Anderson et al., 2001). As heme is associated with both neuropsychiatric pathology and the mitochondria, we hypothesize that alterations in Co-I may affect heme metabolism in SZ.

## 2. Experimental procedures

### 2.1. Cell lines

Epstein-Barr Virus (EBV) transformed lymphocyte cell lines (LCLs) were established as previously described (Bernacki et al., 2003). Lymphocytes were obtained from DSM-IV diagnosed SZ patients and healthy subjects with no history of psychiatric illness, verified

by a structured review interview. SZ hospitalized patients (eight men, seven women; average age 40.1 ± 2.5 [±SEM], range 22-61 years) and control subjects (eight men, nine women; average age 38.2 ± 2.2 [±SEM], range 22-50 years) staff and students who had no connection with the experimenters, were recruited from the Beer-Sheva Mental Health Center. Out of theses cohorts nine SZ derived LCLs (four men, five women; average age; 37.5 ± 2.4 [±SEM], range 22-58 years) with significant mitochondria dysfunction and nine healthy subject derived LCLs (four men, five women; average age; 36.0 ± 2.1 [±SEM], range 22-50 years) were assessed in this study. All subjects provided a written informed consent. The study was approved by the Beer-Sheva Hospital Helsinki Committee Internal Review Board. The EBV transformed lymphocytes were obtained as a gift from the R. Ebstein group, Jerusalem Israel.

Transformed cells from 9 patient and 9 controls were grown for a week before starting the experiments at 37 °C, with 5% $CO_2$ in RPMI-1640 growth medium (Biological industries, Kibbutz Bet-Haemek, Israel), supplemented with 16% European-grade fetal bovine serum (FBS) (Biological industries, Kibbutz Bet-Haemek, Israel), 5% penicillin-streptomycin and 5% L-glutamine (Gibco, Invitrogen, CA, USA). The Co-I inhibitor rotenone (Sigma-Aldrich, St Louis, MO, USA) dissolved in Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Israel) was applied at 10, 25, 50 and 100 nM final concentrations to four different lines of healthy subject derived LCLs. DMSO concentration in all samples was 0.5%. Equal amount of DMSO was added to control samples. Medium was replaced every 24 h for up to 72 h. Since rotenone is known to retain in serum (Newhouse et al., 2004), 1% FBS level was used after optimization of culturing conditions. Viability of cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and was conducted with four different dosages of rotenone (10 nM, 25 nM, 50 nM, 100 nM), in the presence of four FBS concentrations (0%, 1%, 5% and 16%).

## 2.2. Poly I:C treated rat brains

Right brain hemispheres of adult rats ($n = 6$/group), offspring of mothers treated with polyinosinic:polycytidylic acid (poly I:C), which activates their immune system, or with saline on gestational day 15 were provided by the laboratory of I. Weiner, School of Psychological Sciences, Tel Aviv University, Israel. Four SZ-related brain areas: medial pre-frontal cortex [mPFC, coordinates from bregma in mm: 3.7 - 2.2 anterior/posterior (AP); 0-1 lateral (L); 3.5-5 ventral (V)], striatum [(STR; 1.7-(−0.4) AP; 1.0-3.7 L; 3.6-6.5 V)], ventral hippocampus [(VH; (−4.4)-(−5.5) AP; 3.5-5.8 L; (−6)-(−8.5) V)], dorsal hippocampus [(DH; (−2.3)-(−3.3) AP; 0.2-2 L; 2.2-3.5 V)] were incised with a sterile blade from frozen brains, sectioned to reveal the brain region of interest by cryostat (Leica, CM1850) and kept at −80 °C until use.

## 2.3. Total heme levels

Free Heme is readily oxidized, therefore oxidized heme levels in the form of hemin ($Fe^{3+}$) are used to assess intracellular heme levels. Heme/hemin levels were assessed using a fluorescence assay, as previously described (Sinclair et al., 2001). Briefly, cells were collected and protein concentration was measured by Bradford protein assay (Ernst and Zor, 2010) (BIO-RAD, Munchen, Germany). Cells (5 μg/μl protein) were re-suspended in 300 μl 2 M oxalic acid, half of which was heated at 100 °C for 30 min (sample), while the remaining half was left at room temperature and used as a normalizing blank. To avoid crystallization of oxalic acid at room temperature an equal volume of PBS was added to the blank immediately and to the sample following heating. All samples were centrifuged at 16,000 × *g* for 2 min at room temperature and absorbance was measured at 662 nm in the supernatant containing protoporphyrin following excitation at 405 nm using Infinite® 200 PRO plate reader (TECAN, Männedorf, Switzerland).

## 2.4. Isolation of mitochondria

Mitochondria were isolated from healthy and SZ derived LCLs as previously described (Brenner-Lavie et al., 2009). Briefly, cells ($2\text{-}5 \times 10^8$) were centrifuged at room temperature and re-suspended in ice cold Tris-sucrose-ethylenediaminetetraacetic (EDTA) buffer (2.5 ml, pH 7.4) containing Tris-HCl (10 mM), sucrose (250 mM), K-EDTA (1 mM), and protease-phosphatase inhibitor cocktails, (Complete™ protease inhibitor, Roche Diagnostics, Mannheim, Germany and Phosphatase inhibitor cocktail I & II, Sigma Aldrich, St. Louis, MO, USA). The following procedure was performed at 4 °C: cells were homogenized in cooled Dounce glass-glass homogenizer (Kimble Kontes, Vineland, NJ, USA). Following centrifugation of homogenized cells, the upper (1000 × *g*) mitochondria-containing supernatant was transferred to a new tube and re-centrifuged at 12,000 × *g*. The pellet was suspended in Tris-sucrose buffer supplemented with percoll (Sigma aldrich, Israel) (23% v:v) and centrifuged at 10,000 × *g*. Lastly, the pellet was washed with Tris-sucrose buffer by centrifugation at 12,000 × *g*. Protein concentration was determined and the mitochondria were kept at −80 °C until use.

## 2.5. Basal cellular respiration

To confirm that Co-I activity is inhibited by rotenone, Co-I driven respiration was measured by the rate of $O_2$ consumption ($\Delta O_2$ μM/h) in intact cells. Cellular respiration was polarographically determined by the rate of cellular oxygen consumption using a thermostatically controlled (37 °C) Clark oxygen electrode (Strath-Kelvin Instrument, North Lanarkshire, Scotland) (Brenner-Lavie et al., 2009). Cells ($3\text{-}5 \times 10^6$) were washed and suspended in potassium-phosphate mannitol buffer (1 ml, pH 7.4) containing mannitol (0.3 M), KCl (10 mM), $MgCl_2$ (5 mM), $KH_2PO_4$ (10 mM), ATP (0.4 mM) and bovine serum albumin (BSA) (0.25 mg). Cell membrane was permeabilized with digitonin (0.001%) to avoid the interference of uptake mechanisms for each of the substrates. Co-I induced respiration rates were assessed in the presence of Co-I substrates glutamate (5 mM) and malate (1 mM).

## 2.6. Western blot

Samples were homogenized in Radio Immuno-Precipitation Assay (RIPA) lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1% NP-40, 2 mM EDTA, 0.1% SDS including protease and phosphatase inhibitors described above, by a 1.5 ml Dounce glass-glass homogenizer (Kimble Kontes, Vineland, NJ, USA) as previously described (Yaniv et al., 2008). Protein concentration was measured by a modified Bradford protein assay (BIO-RAD, Munchen, Germany). 60-100ug total protein was diluted 1:1 in SeeBlue electrophoresis sample buffer (Invitrogen, Carlsbad, CA, USA). Protein samples were separated on a 7.5, 10 or 14% SDS acrylamide gels and transferred to a PVDF membrane using wet transfer system (Amersham Biosciences, Piscataway, NJ, USA). Quality of transfer was assayed by Ponceau staining (Sigma Aldrich, St. Louis, MO, USA). Following blocking of nonspecific binding sites in 2-5% BSA, membranes were incubated with primary antibodies and then with corresponding secondary antibodies (Santa Cruz Biotechnology, CA, USA) (Supplementary Table A). Membranes were developed with ECL reagent (Amersham Biosciences, Buckinghamshire, UK) and exposed to XLS Kodak film. Films were analyzed using a densitometer (Vilber-Lourmat, France). β-actin, was used for normalization as

it was not affected by treatments. In addition, a single batch of protein was used as a positive internal control.

### 2.7. RNA extraction and qRT-PCR analysis

Total RNA was extracted with Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Yaniv et al., 2008) and spectrophotometrically analyzed for quantity and quality at 280, 230 and 260 nm. RNA was reverse transcribed into first-strand cDNA by using the verso cDNA synthesis kit (ABgene, Epsom, UK). cDNA was amplified by real-time PCR (Stratgene Mx30000P), in the presence of gene-specific primers designed according to sequence obtained from Gene-Bank using Primer 3 software (http://primer3.sourceforge.net/) and SYBR green ROX mix (Thermo Scientific, USA. (Supplementary Table 2). Each experimental set included a control for cross comparison. Three reference genes, GAPDH, 18S and $\beta$-Actin were studied. $\beta$-Actin showed the lowest variance across treatments and therefore used for normalization.

### 2.8. Statistical analysis

Results were analyzed for normal distribution using the Kolmogorov-Smirnov test. As all data showed normal distribution, analysis was done by a two-sample *T*-test, two-way and one-way ANOVA followed by LSD post-hoc test. Differences between groups were considered significant if $P \leq 0.05$. IBM SPSS software (version 23.0) was used for statistical analyses.

## 3. Results

### 3.1. Cellular heme levels are decreased and eIF2$\alpha$ phosphorylation increased in SZ-derived cells

All SZ-derived cell lines used in the present study showed multifaceted impairments in their mitochondria including in Co-I activity and its subunits' expression (data not shown). Cellular heme levels were significantly decreased in SZ derived cell line as compared to controls (C: $159.3 \pm 14.4$, SZ: $128.0 \pm 22.8$ pmol/mg protein) (Fig 1A). Levels of its downstream target peIF2$\alpha$/ eIF2$\alpha$, were significantly increased in SZ- as compared to control-derived cell line (C: 0.91 $\pm$0.09, SZ: 2.25 $\pm$0.84 OD), with no change in basal eIF2$\alpha$ levels, suggesting a higher phosphorylation activity of the heme dependent enzyme HRI, which phosphorylates eIF2$\alpha$ at low heme levels and thereby inhibits its activity (Fig 1B).

### 3.2. HO-1 levels are elevated, while mitochondrial heme and ALAS1 levels show no change in SZ-derived cells

To identify a possible cause for decreased heme levels in SZ, levels of the two main rate-limiting enzymes in heme metabolism were studies. Protein levels of HO-1, the cytosolic heme catabolic rate-limiting enzyme, were significantly higher in SZ compared to control cell line (C: 0.62 $\pm$0.045, SZ: 0.98 $\pm$0.18 OD) (Fig 1C). HO-1 is highly inducible and its regulation heavily relies on RNA transcription (Choi and Alam, 1996). Concomitantly, HO-1 mRNA levels were 1.8-fold higher in SZ-derived LCLs compared to control cell line (C: $6.7 \times 10^{-5} \pm 1.9 \times 10^{-5}$, SZ: $1.2 \times 10^{-4} \pm 3.1 \times 10^{-5}$) (Fig. 1D). Protein and mRNA levels of ALAS1, the mitochondrial heme biosynthesis rate-limiting enzyme, exhibited no change between SZ derived LCLs compared to controls (Fig 1E and F). In line with the latter, no change was observed in heme levels of isolated mitochondria between the two cohorts (Fig. 1A), suggesting intact synthesis and increased catabolism of heme in SZ cells.

### 3.3. Co-I inhibition is associated with altered heme metabolism in healthy subjects-derived LCLs

Given that Co-I deficit is a major cause for mitochondrial dysfunction in SZ (Ben-Shachar, 2016), we studied the effect of Co-I on heme metabolism by inhibiting Co-I activity with rotenone in healthy subjects-derived LCLs. Following optimization of rotenone treatment, the 100 nM rotenone was omitted due to toxicity. The lowest FBS concentration with the highest viability was achieved at 1% FBS and used in further experiments (Supplementary data and Fig. 1S).

#### 3.3.1. Validation of Co-I inhibition by cellular respiration

Analysis revealed a significant effect of both rotenone dosage [$F_{(3,36)} = 77.5$, $P < 0.001$] and duration of treatment [$F_{(2,36)} = 7.9$, $P < 0.001$] on $O_2$ consumption rates. Our data showed a dosage-dependent declined in respiration at all dosages. For example, following 24 h of treatment control: $6.6 \pm 0.87$, 10 nM: $2.5 \pm 0.47$, 25 nM: $1.9 \pm 0.35$, 50 nM: $1.22 \pm 0.19$ $\Delta O_2$ $\mu$M/h. A time dependent decrease in respiration following rotenone administration between 24 h and 72 h, and between 48 h and 72 h following treatment was also observed. For example, following 25 nM of rotenone (24 h: $2.3 \pm 0.57$, 48 h: $2.18 \pm 0.45$, 72 h: $1.18 \pm 0.07$ $\Delta O_2$ $\mu$M/h). No statistically significant difference was observed in the respiration rates between the three time points in the control groups (Fig. 2).

#### 3.3.2. Total heme levels are altered following Co-I inhibition

Cellular heme levels were affected by rotenone treatment in a dosage and treatment duration dependent manner. A significant main effect of dosage [$F_{(3,28)} = 4.5$, $P < 0.001$] duration [$F_{(2,28)} = 18.5$, $P < 0.001$] and dosage*duration interaction [$F_{(6,28)} = 12.9$, $P < 0.001$] was observed by two way ANOVA . Heme levels significantly increased 24 h following 50 nM rotenone treatment as compared to both control and 10 nM (control: $136 \pm 2.0$, 10 nM:$145 \pm 0.6$, 50 nM:$177 \pm 14.6$ pmol/mg protein). Forty-eight hours following rotenone treatment heme levels decreased in a dosage-dependent manner in virtually all rotenone dosages (control: $181 \pm 12.0$, 10 nM:$155 \pm 11.0$, 25 nM: $111 \pm 9.7$, 50 nM:$78 \pm 2.4$ pmol/mg protein). Although no significant dosage dependent difference was witnessed at 72 h following treatment, levels significantly decreased in a time-dependent manner compared to 48 h and 24 h following treatment (Fig. 3A).

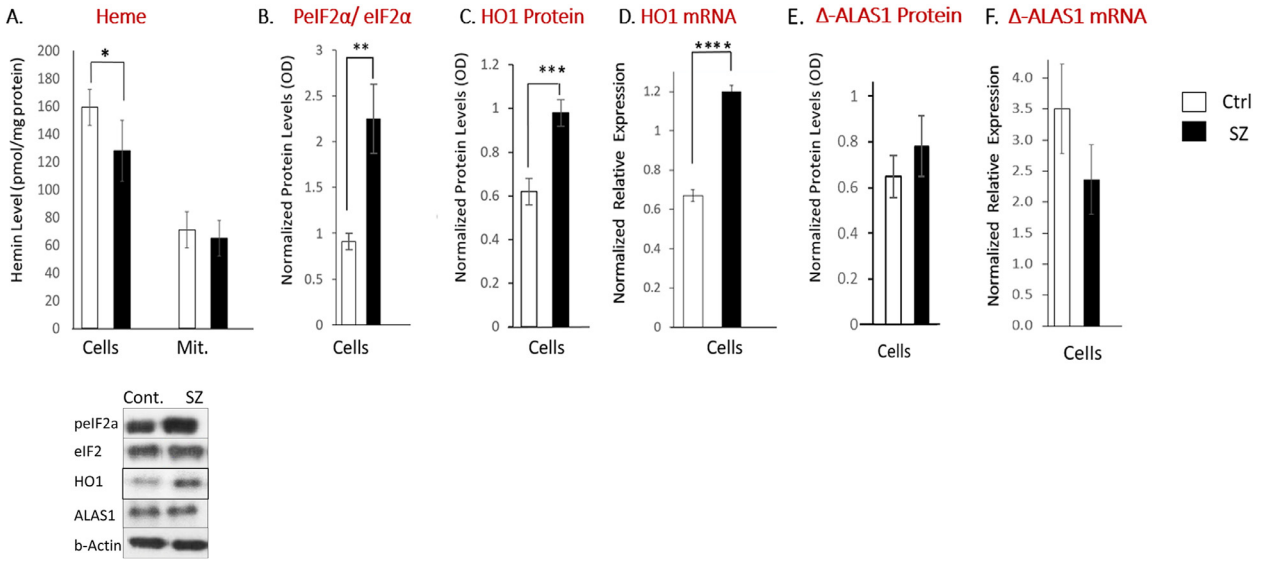


**Fig. 1** Heme metabolism in SZ and control derived LCLs (quantification and representative SDS-PAGE gels). **A.** Cellular Heme levels were lower in whole cell lysate of SZ cell line, while no change was observed in mitochondrial heme levels. **B.** Increased PeIF2$\alpha$/eIF2$\alpha$ was observed in SZ cell line. **C.** Increased HO-1 protein levels were observed in SZ cell line. **D.** HO-1 mRNA expression was 1.8-fold higher in SZ cell line. **E, F.** ALAS1 protein and mRNA levels did not differ between the two groups. Values are means $\pm$ SEM of 2 experiments, each done in duplicates. $n = 9$ cell lines/group. Data were normalized for housekeeping genes or proteins as detailed in experimental procedures section. $^{*}P < 0.032$, $^{**}P < 0.004$, $^{***}P < 0.002$. $^{****}P < 0.043$.

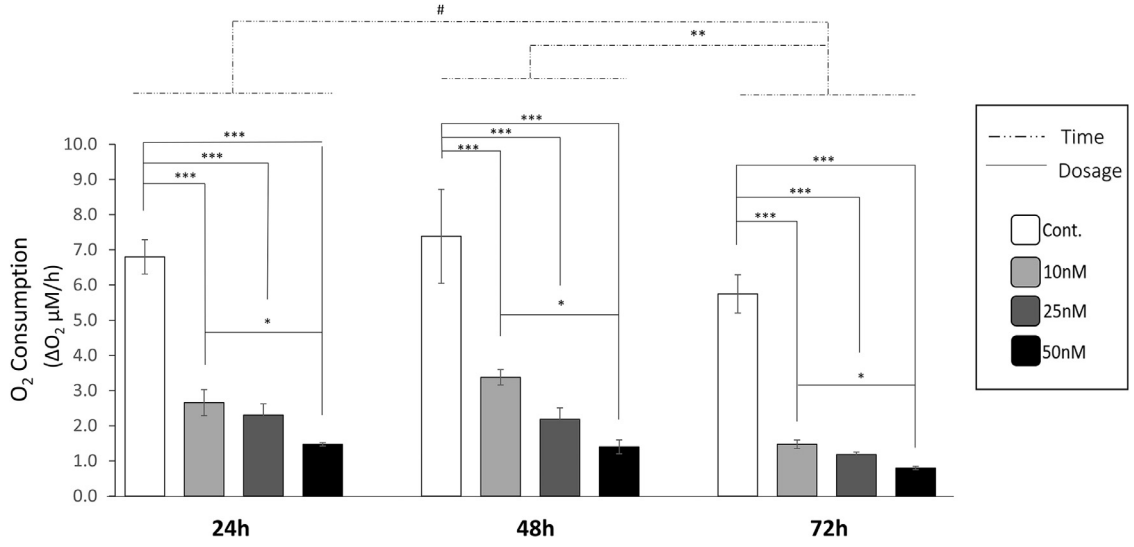


**Fig. 2** Cellular oxygen consumption (respiration) in healthy-subject derived lymphoblasts treated with rotenone. Respiration declined in a dose dependent manner following rotenone treatment. Decline in respiration is also treatment duration-dependent. Values are means $\pm$ SEM of 4 different experiments. $^{*}P < 0.012$, $^{**}P < 0.014$. $^{***}$ $P < 0.001$, $\#P < 0.002$.

### 3.3.3. HO-1 and eIFf2$\alpha$ phosphorylation are altered following Co-I inhibition

HO-1 protein levels showed an overall increase, opposite to the trend of change in heme levels, in rotenone treated cells. Two way ANOVA revealed a significant effect of dosage [$F_{(3,24)} = 2.99$, $P < 0.005$] and treatment duration [$F_{(2,24)} = 12.8$, $P < 0.001$]. Levels of HO-1 protein were significantly increased following 50 nM rotenone treatment at all time-points. For example following 48 h an increase in HO1 protein level was observed in the 50 nM rotenone as compared to control and 10 nM (C: $0.618 \pm 0.135$, 10 nM: $0.680 \pm 0.153$, 50 nM: $0.960 \pm 0.209$ OD). Forty-eight hours following treatment, levels of HO-1 were not significantly affected by the duration of treatment. However, 72 h following treatment, levels of HO-1 exhibited a significant increase compared to 24 h and 48 h following treatment (24 h: $0.662 \pm 0.076$, 48 h: $0.960 \pm 0.209$, 72 h: $1.325 \pm 0.157$ OD) (Fig. 3B). The increase in HO-1 protein level was accompanied by elevation in its transcript levels. Two-way ANOVA revealed a significant effect of dosage [$F_{(3,12)} = 51$, $P < 0.001$], treatment duration [$F_{(2,12)} = 192$, $P < 0.001$], and dosage*treatment duration interaction [$F_{(6,12)} = 17$,

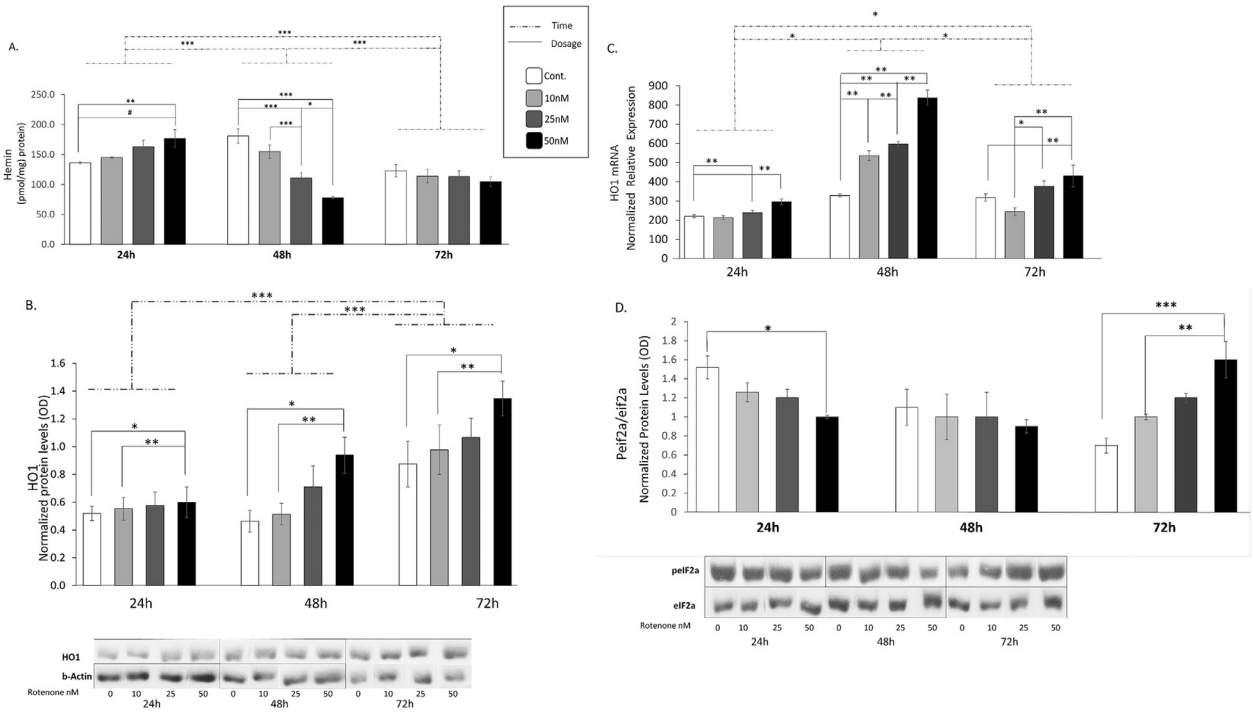


**Fig. 3** Alterations in heme metabolism in healthy-subject derived lymphoblasts treated with rotenone (quantification and representative SDS-PAGE gels). **A.** Heme levels increased 24 h following 50 nM rotenone treatment in control lymphoblast ($n = 4$ cell lines/group), while a decrease was observed 48 h following 25 and 50 nM rotenone treatment. Treatment duration-dependent decrease in heme was witnessed 48 h and 72 h following treatment as compared to 24 h. Values are means ± SEM of 4 different experiments, each done in duplicates. $^{*}P < 0.009$, $^{**}P < 0.005$, $\#P < 0.026$, $^{***}P < 0.001$. **B.** HO-1 protein levels were significantly increased in a dose dependent manner at the 50 nM condition. Treatment duration-dependent increase was witnessed at 72 h compared to 24 h and 48 h following treatment. Values are means ± SEM of 2 experiments, each done in duplicates. $^{*}P < 0.01$, $^{**}P < 0.03$, $^{***}P < 0.001$. **C.** HO-1 mRNA levels were significantly increased in a dose dependent manner, especially at the 50 nM condition. Significant treatment duration-dependent increase was witnessed 24 h following treatment compared to 48 h and 72 h, while at 72 h compared to 48 h a decrease was witnessed. Values are means ± SEM of 2 different experiments, each done in duplicates $^{*}P < 0.05$, $^{**}P < 0.03$. $^{***}P < 0.001$. **D.** eIF2$\alpha$ phosphorylation levels (PeIF2$\alpha$/eIF2$\alpha$) decreased upon short-term treatment with 50 nM rotenone and significantly increased 72 h following treatment. Values are means ± SEM of 2 experiments, each done in duplicates. $^{*}P < 0.042$, $^{**}P < 0.05$, $^{***}P < 0.02$.

$P < 0.001$]. Twenty-four hours following rotenone treatment HO-1 mRNA levels increased in the 50 nM rotenone treated cells. Forty-eight hours following treatment a significant increase in HO-1 mRNA level was observed in all rotenone treated cells compared to controls. Seventy-two hours following treatment, HO-1 mRNA levels increased in the 50 nM rotenone treated cells compared to controls (24 h - C: 220.5 ± 7.4, 10 nM: 213 ± 15.2, 25 nM: 239 ± 22, 50 nM: 262 ± 10.9 OD; 48 h - C: 325.9 ± 3.5, 10 nM: 531 ± 62, 25 nM: 596 ± 35, 50 nM: 835 ± 10.9 OD; 72 h - C: 314 ± 15, 10 nM: 241 ± 15.5, 25 nM: 372 ± 14.4, 50 nM: 430 ± 167 OD). Initial time dependent elevation in HO-1 mRNA levels was witnessed 48 h following rotenone administration compared to 24 h ($P < 0.001$), and a significant decrease was witnessed at 72 h as compared to 48 h following treatment. Nevertheless, HO-1 mRNA levels were still 29% elevated at 72 h as compared to 24 h. For example, following 25 nM treatment 24 h: 239 ± 22, 48 h: 596 ± 68, 72 h: 376 ± 40 OD (Fig. 3C).

The alterations in heme level due to Co-I inhibition by rotenone were associated with opposite alterations in eIF2$\alpha$ phosphorylation. Duration of rotenone treatment had no effect on eIF2$\alpha$ phosphorylation. Therefore, one-way ANOVA analysis was applied which revealed that 24 h following treatment, eIF2$\alpha$ phosphorylation was significantly decreased in the 50 nM rotenone treated cells compared to controls (24 h - C: 1.12 ± 0.12, 50 nM: 0.90 ± 0.11 OD). No significant difference was observed 48 h following rotenone administration. Seventy-two hours following rotenone treatment, however, a significant increase in eIF2$\alpha$ phosphorylation was observed in the 50 nM condition compared to controls, 10 nM and 25 nM (C: 0.7 ± 0.08, 10 nM: 1 ± 0.03, 50 nM: 1.6 ± 0.19 OD) (Fig. 3D).

## 3.4. Mitochondrial proteins are altered in Poly I:C-induced model of SZ

The Poly I:C induced maternal immunization activation (MIA) model is widely used to study SZ and related brain disorders (Bauman et al., 2014; Oh-Nishi et al., 2016; Piontkewitz et al., 2012). In order to extend our findings beyond cell culture, mitochondrial dysfunction and heme metabolism were assessed in the brains of Poly I:C treated rats. First we validated that mitochondria is altered in brain

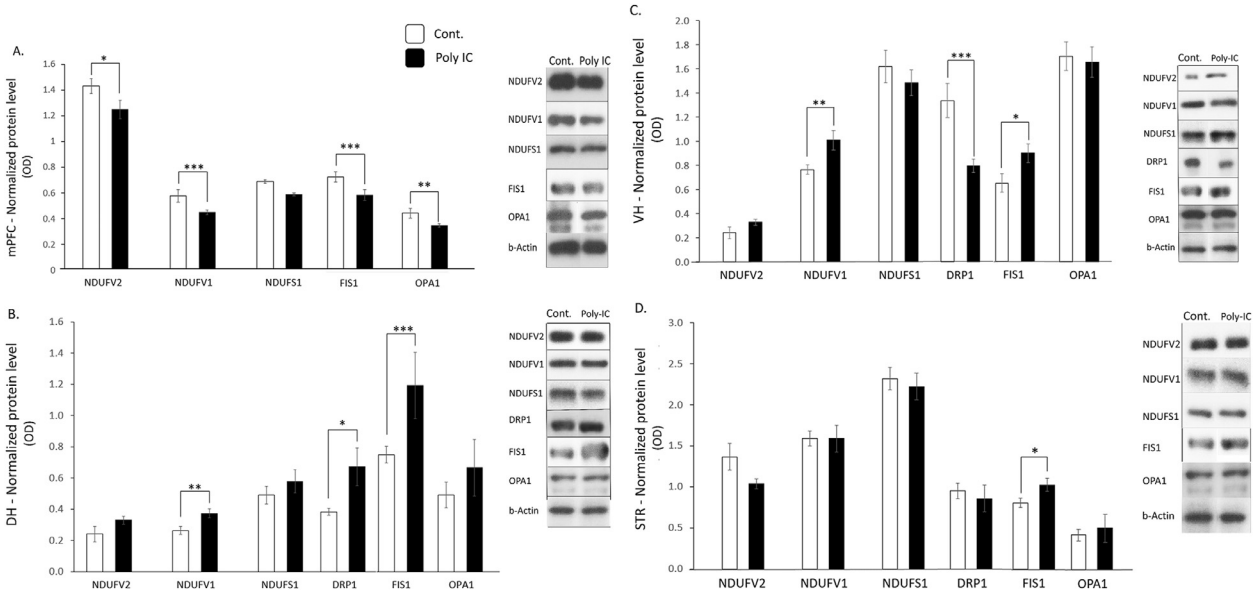


**Fig. 4** Alterations in mitochondrial related proteins in different brain regions of Poly I:C prenatally exposed offspring (quantification and representative SDS-PAGE gels) **A.** mPFC - A significant reduction in protein levels of mitochondrial Co-I subunits NDUFV2 as well as mitochondrial pro-fission protein FIS1 and pro-fusion protein OPA1 was observed in poly-IC exposed offspring. $^{*}P < 0.048$, $^{**}P < 0.05$, $^{***}P < 0.03$. **B.** DH - A significant elevation in protein levels of NDUFV1, mitochondrial pro-fission proteins FIS1 and Drp1. $^{*}P < 0.018$, $^{**}P < 0.024$, $^{***}P < 0.05$. **C.** VH - A significant elevation in protein levels of NDUFV1 and FIS1 as well as a reduction in Drp1. $^{*}P < 0.05$, $^{**}P < 0.017$, $^{***}P < 0.007$. **D.** STR - A significant elevation in FIS1 was witnessed in Poly I:C exposed offspring. $^{*}P < 0.05$. Values are means ± SEM, all done in duplicates.

by analyzing the mPFC, DH, VH, and STR, which have been repeatedly reported to be involved in SZ (Harrison, 2004; Meyer-Lindenberg et al., 2002; Pantelis et al., 1997). Immunoblotting analyses of these areas in the adult brains of control and prenatally Poly I:C-exposed rats revealed that the mPFC of Poly I:C-exposed offspring exhibits a significant reduction in: (1) mitochondrial Co-I subunits NDUFV1 (C: 0.58 ± 0.048, Poly I:C: 0.45 ± 0.017 OD) and NDUFV2 (Control: 1.43 ± 0.057, Poly I:C: 1.25 ± 0.07 OD) (2) mitochondrial pro-fission protein FIS1 protein (C: 0.73 ± 0.04, Poly I:C: 0.59 ± 0.04 OD) and (3) mitochondrial profusion protein OPA1 (C: 0.45 ± 0.038, Poly I:C: 0.35 ± 0.013 OD) (Fig. 4A). Levels of NDUFV1 and FIS1 were significantly elevated in both dorsal and ventral hippocampus (DH and VH, respectively) of Poly-IC exposed offspring [DH - (NDUFV1 - C: 0.26 ± 0.03, Poly I:C: 0.37 ± 0.03 OD and FIS1 - C: 0.75 ± 0.017, Poly I:C: 1.19 ± 0.21 OD); VH - (NDUFV1 - C: 0.76 ± 0.04, Poly I:C: 1.01 ± 0.082 OD and FIS1 - C: 0.65 ± 0.079, Poly I:C: 0.90 ± 0.077 OD). Levels of the pro-fission protein DRP1 were significantly increased in the DH (C: 0.38 ± 0.02, Poly I:C: 0.67 ± 0.12 OD), but decreased in the VH (C: 1.34 ± 0.14, Poly I:C: 0.79 ± 0.05 OD) of Poly-IC exposed offspring (Fig. 4B and C). In the striatum only FIS1 was significantly decreased in the Poly I:C-exposed offspring (C: 0.80 ± 0.058, Poly I:C: 1.02 ± 0.081 OD) (Fig. 4D).

### 3.5. HO-1 levels and eIF2α activation are altered in Poly I:C brains

Similar to our findings in the SZ derived LCLs and rotenone treated cells cellular protein levels of HO-1 were significantly increased in the mPFC and the DH of prenatally Poly I:C exposed offspring compared to saline controls, while levels of HO-1 in the VH and STR were unchanged [mPFC - (C: 0.55 ± 0.14, Poly I:C: 0.95 ± 0.07 OD) and DH - (C: 0.18 ± 0.02, Poly I:C: 0.38 ± 0.05 OD)] (Fig. 5A). Levels of PeIF2α /eIF2α were significantly increased in the DH and VH as expected, while surprisingly decreased in the mPFC of Poly I:C treated offspring compared to controls [DH- C: 0.16 ± 0.02, Poly I:C: 0.21 ± 0.02 and VH- (C: 0.26 ± 0.03, Poly I:C: 0.33 ± 0.04 OD)] (Fig. 5B).

## 4. Discussion

Mitochondrial dysfunction has been repeatedly reported in SZ, and genetic, molecular and biochemical impairments in Co-I are consistently reported in various brain and somatic cells of SZ patients (Ben-Shachar et al., 1999; Bergman and Ben-Shachar, 2016a, 2016b; Cavelier et al., 1995; Dror et al., 2002; Gubert et al., 2013; Maurer et al., 2001; Prince et al., 1999). A major obstacle in SZ research is the limited accessibility of the brain. LCLs have been shown to retain many SZ-related abnormalities including those of the mitochondria and unlike fresh lymphocytes have been suggested as a medication-free peripheral cell model for SZ and other disorders (Gangadhar et al., 2004; Sei et al., 2007; Washizuka et al., 2009). Therefore, SZ- derived LCLs, which have repeatedly shown to have impaired Co-I activity and reduced oxygen consumption rates (Robicsek et al., 2018; Rosenfeld et al., 2011) were used in this study. It was previously shown that EBV viral load and ATP levels in LCLs used as covariates in gene expression analyses,

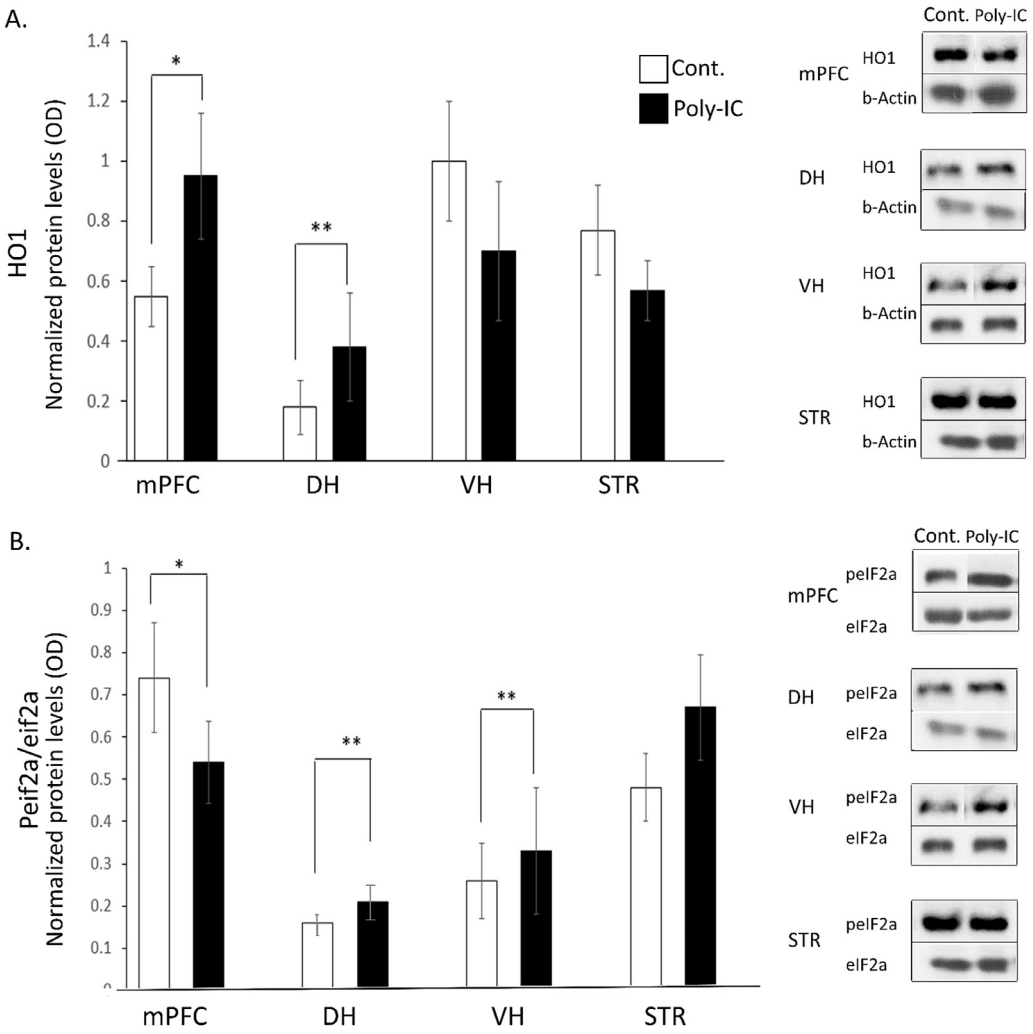


**Fig. 5** Alterations in HO-1 and eIF2$\alpha$ phosphorylation (PeIF2$\alpha$/ eIF2$\alpha$) in different brain regions of Poly I:C prenatally exposed offspring (quantification and representative SDS-PAGE gels) **A**. HO-1 protein levels were significantly elevated in the and DH of Poly I:C treated offspring. HO-1 protein levels in the VH and STR did not differ and. Values are means $\pm$ SEM, with all samples done in duplicates. $^{*}$ $P < 0.04$, $^{**}$ $P < 0.001$. **B.** Levels of PeIF2$\alpha$/ eIF2$\alpha$ were significantly elevated in the DH and VH of Poly I:C offspring. PeIF2$\alpha$/ eIF2$\alpha$ levels in the mPFC have significantly declined in Poly I:C offspring. Values are means $\pm$ SEM, with all samples done in duplicates. $^{*}P < 0.006$, $^{**}P < 0.03$.

can affect gene expression (Choy et al., 2008). We have previously shown that there was no significant difference in EBV viral load between our SZ and healthy subjects derived LCLs (Rosenfeld et al., 2011). In addition, in contrast to gene experiments in which every LCL is used once, we have repeated each experiment several times and used different LCLs, minimizing the effects of EBV viral load and ATP levels on our outcomes. Here we show that the CoI deficiency is associated with alterations in heme metabolism and signaling. In the SZ-derived cell lines a 20% reduction in cellular heme levels were observed, while its levels in SZ-derived isolated mitochondria was similar to the control. Reduced cellular heme is in complement with previous reports indicating reduced heme levels in disorders exhibiting SZ-like symptomology such as porphyria (Homedan et al., 2014) and Co-I deficiencies (Gielisch and Meierhofer, 2015). Unchanged mitochondrial heme is in line with unaltered protein and mRNA levels of the mitochondrial heme synthesis rate-limiting enzyme $\Delta$-ALAS1 in SZ-derived cells and unaltered levels and activity of complex IV (Ben-Shachar et al., 1999), which contains two hemes, cytochrome a and $a_3$. These data suggest that heme biosynthesis pathway is unaffected, while cytosolic heme metabolism is abnormal in SZ derived cells. Indeed, elevated HO-1 protein and mRNA were witnessed, suggesting increased heme degradation in these cells. It was previously shown that selective overexpression of HO-1 in astrocytes of GFAP HMOX1 transgenic mice resulted in subcortical mitochondrial autophagy and SZ-like behaviors (Song et al., 2012). In contrast, it was reported that overexpression of HO1 protects the heart from oxidative injury by regulating mitochondrial quality control, upregulating the expression of genes involved in mitochondrial biogenesis and fusion and downregulating those involved in fission (Hull et al., 2016). Other showed that in cells, inhibition of HO1 prevents mitochondria associated protection by an antioxidant (Duarte et al., 2018). In addition, a growing number of

studies report alteration of HO-1 expression and activity in neuropsychiatric disorders (Barone et al., 2012; Prabakaran et al., 2004; Schipper et al., 1998; Smith et al., 1994). Taken together, these findings suggest an intricate mechanism underlying the reciprocal interaction between mitochondria or CoI and HO1 in general and in SZ in particular.

A decrease in heme levels enables the phosphorylation of eIF2$\alpha$ by HRI and thereby inhibition of eIF2$\alpha$ activity. Indeed, the relatively small change in cytosolic heme levels in SZ cells showed downstream functional consequences manifested by almost 50% elevation in the phosphorylation of eIF2$\alpha$ in SZ-derived LCLs as compared to control, complementing prior reports of reduction in global protein translation in SZ (English et al., 2015). Nevertheless, the exact mechanism responsible for eIF2a phosphorylation is yet to be determined. In mammals, four protein kinases are known to phosphorylate eIF2's $\alpha$ subunit at Ser-51, one of which is HRI (Bauer et al., 2001). At least dozen other agents such as polyamines can also affect physphorylation of eIF2a (Landau et al., 2010). Unraveling the role of HRI in eIF2alpha phosphorylation in SZ cells, by direct measures of HRI levels and phosphorylation and its inhibition or silencing in CoI deficient cells as well as the mechanism linking HRI kinase and CoI activities is currently under investigation.

In order to study whether there is a link between Co-I deficits and alterations in cytosolic heme metabolism we inhibited Co-I activity by rotenone. An initial increase (24 h) with a later decrease (48 h) in heme levels following Co-I inhibition was observed, suggesting that impaired Co-I activity affects heme metabolism, inducing an initial compensatory mechanism that eventually fails. The decrease in heme levels is in line with two previous studies indicating a positive correlation between decrease in Co-I activity and levels of heme (Gielisch and Meierhofer, 2015; Homedan et al., 2015). At the longest duration examined, 72 h, and after repeated rotenone treatments, heme levels have stabilized, and rotenone did not seem to elicit a further effect on heme levels. This could be the result of the induction of cell compensatory mechanism when heme level reaches a certain threshold or a direct effect of rotenone on heme metabolism, as rotenone is known to cause secondary impacts on cells such as apoptosis and ER stress, both activating a number of different pathways (Han et al., 2014; Newhouse et al., 2004). Alterations in heme levels due to Co-I inhibition were associated with a significant increase in HO-1 protein levels at all-time durations at the highest rotenone dosage. HO-1 mRNA levels also increased at highest rotenone dosage, but with a moderate increase at the longest duration examined, 72 h. This lag between HO-1 protein and mRNA could suggests that mRNA may not necessarily correlate with protein levels of this enzyme following long-term exposure to rotenone, in line with reports of an overall ~40% correlation between mRNA and protein expression (Vogel and Marcotte, 2012). The mechanism by which Co-I affects cytosolic heme metabolism is still an open question. One possible mechanism is elevated oxidative stress state due to impairment in Co-I, which is a major site for superoxide production and if impaired, as observed in SZ cells and in repeated exposure to rotenone, can lead to reactive oxygen species (ROS) production and lipid peroxidation (Barrientos and Moraes, 1999). HO-1 is highly induced by elevate oxidative stress (Barone et al., 2012; Choi and Alam, 1996) which can then lead to increased degradation of heme. The reduction in heme due to Co-I inhibition by rotenone was functionally manifested by the opposite, yet with a time shift pattern of change in eIF2$\alpha$ phosphorylation similarly to our findings in SZ cells.

The reduction in heme levels in SZ cells, probably due to increased HO-1 and its possible link to Co-I impairment, is supported by our findings in brains of Poly I:C offspring of mitochondrial impairments including that of Co-I, increased protein levels of HO-1 and alterations of eIF2$\alpha$ phosphorylation in a brain-area specific pattern. Mitochondrial impairments were most severe in the mPFC, where virtually all examined proteins, Co-I subunits NDUFV1, NDUFV2 and the fission/fusion proteins FIS1 and OPA1, were decreased. This is in line with previous reports of decreased NDUFV1, NDUFV2 and OPA1 in the prefrontal cortex postmortem specimens and in cells of SZ patients (Dror et al., 2002; Rosenfeld et al., 2011). Changes in mitochondrial proteins were also witnessed in the hippocampus, and to a lesser extent in the striatum of the Poly I:C offspring. These changes in mitochondria add to previously reported reduction in mPFC neurons of mitochondrial membrane potential ($\Delta\psi_m$), to which Co-I is a major contributor, of ATP in mPFC neurons and splenocytes of Poly-I:C offspring (Giulivi et al., 2013; Robicsek et al., 2018) and in Poly-I:C transfected cell lines (Chen et al., 2016). HO-1 protein levels were elevated in the mPFC and DH of Poly I:C offspring, but not in the VH and the striatum, while phosphorylation of eIF2$\alpha$ was enhanced in both VH and DH. The increase in HO-1 and PeIF2$\alpha$/eIF2$\alpha$ in the DH suggests a decrease in heme levels in this brain area, since HO-1 catabolizes heme and deficiency in heme is a known cause for eIF2$\alpha$ phosphorylation. In the VH, levels of HO-1 were not elevated but PeIF2$\alpha$/eIF2$\alpha$ was still increased. In the mPFC a decrease in PeIF2$\alpha$/eIF2$\alpha$ was witnessed in the of Poly I:C treated offspring despite elevated levels of HO-1. A plausible explanation is that additional factors that are unrelated to heme/HRI pathway such as oxidative stress or other kinases affect phosphorylation of eIF2$\alpha$ in these brain areas (Holcik and Sonenberg, 2005). Different susceptibility to an insult between brain area are a common phenomenon and examples include differences in VH and DH responsiveness to oxidative stress specific (Steullet et al., 2010), eIF2$\alpha$ phosphorylation in the frontal cortex but not in the cerebellum in AD rat model (Ma et al., 2013) and brain-area dependent pattern of impairments in Co-I in SZ patients (Ben-Shachar and Karry, 2007). Interestingly, the only brain area that did not exhibit altered heme metabolism is the striatum, the brain area in which we did not find altered levels of Co-I subunits.

The current study demonstrates a link between heme metabolism and mitochondrial Co-I, which deficits instigate abnormalities in the metabolism of heme and its downstream targets both *in-vitro* in patients and healthy subjects derived LCLs and *in-vivo* in brains of SZ-rat model. A further study of the link between heme metabolism and mitochondrial Co-I, in postmortem brain specimens in comparison with peripheral cells of patients, as well as in Poly I:C rat model will further substantiate this link. The mechanism by which Co-I abnormal activity induces deficit in heme metabolism whether direct or indirect and the prevalence and functional importance of this process in the pathophysiology of SZ, is still an open question. Regardless,

heme was shown to plays an important role in neuronal functioning. It was shown that a decrease in neuronal heme, suppresses NMDA receptor expression and instigates neurite damage (Chernova et al., 2007, 2006) and that HO-1 enhances the expression of BDNF in dopaminergic neurons and GDNF in glia cells both eminent to neuronal survival (Hung et al., 2008; Mendoza et al., 2011). In addition, heme depletion can induce apoptosis via the pro-apoptotic JNK signaling pathway and suppressing of pro-survival Ras-ERK1/2 signaling pathway (Sengupta et al., 2005). These findings together with our data calls for a further study of heme metabolism impairments and its consequence in SZ.

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## Contributors

Authors LSI and DBS designed the study and wrote the protocol. LSI and HME performed experiments and statistical analyses. LSI and DBS wrote the first draft of the manuscript and discussed it with KME. DBS wrote the final version of the MS. All authors contributed to and have approved the final manuscript.

## Conflict of interest

None for all authors.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.03.011.

## References

Atamna, H., Frey, W.H., 2004. A role for heme in Alzheimer's disease: heme binds amyloid beta and has altered metabolism. Proc. Natl. Acad. Sci. USA 101, 11153–11158. doi:10.1073/pnas.0404349101.

Barone, E., Di Domenico, F., Sultana, R., Coccia, R., Mancuso, C., Perluigi, M., Butterfield, D.A., 2012. Heme oxygenase-1 posttranslational modifications in the brain of subjects with Alzheimer disease and mild cognitive impairment. Free Radic. Biol. Med. 52, 2292–2301. doi:10.1016/j.freeradbiomed.2012.03.020.

Barrientos, A., Moraes, C.T., 1999. Titrating the effects of mitochondrial complex I impairment in the cell physiology. J. Biol. Chem. 274, 16188–16197.

Bauer, B.N., Rafie-Kolpin, M., Lu, L., Han, A., Chen, J.J., 2001. Multiple autophosphorylation is essential for the formation of the active and stable homodimer of heme-regulated eIF2$\alpha$ kinase. Biochemistry 40, 11543–11551. doi:10.1021/bi010983s.

Bauman, M.D., Iosif, A.M., Smith, S.E.P., Bregere, C., Amaral, D.G., Patterson, P.H., 2014. Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. Biol. Psychiatry doi:10.1016/j.biopsych.2013.06.025.

Ben-Shachar, D., 2016. Mitochondrial multifaceted dysfunction in schizophrenia; complex I as a possible pathological target. Schizophr. Res. doi:10.1016/j.schres.2016.10.022.

Ben-Shachar, D., Karry, R., 2008. Neuroanatomical pattern of mitochondrial complex I pathology varies between schizophrenia, bipolar disorder and major depression. PLoS One 3, e3676. doi:10.1371/journal.pone.0003676.

Ben-Shachar, D., Karry, R., 2007. Sp1 expression is disrupted in schizophrenia; a possible mechanism for the abnormal expression of mitochondrial complex I genes, NDUFV1 and NDUFV2. PLoS One 2, e817.

Ben-Shachar, D., Zuk, R., Gazawi, H., Reshef, A., Sheinkman, A., Klein, E., 1999. Increased mitochondrial complex I activity in platelets of schizophrenic patients. Int. J. Neuropsychopharmacol. 2, 245–253. doi:10.1017/S1461145799001649.

Bergman, O., Ben-Shachar, D., 2016a. Mitochondrial Oxidative Phosphorylation System (OXPHOS) deficits in schizophrenia: possible interactions with cellular processes. Can. J. Psychiatry 61, 457–469. doi:10.1177/0706743716648290.

Bergman, O., Ben-Shachar, D., 2016b. Mitochondrial oxidative phosphorylation system (OXPHOS) deficits in schizophrenia: possible interactions with cellular processes. Can. J. Psychiatry 61. doi:10.1177/0706743716648290.

Bernacki, S.H., Stankovic, A.K., Williams, L.O., Beck, J.C., Herndon, J.E., Snow-Bailey, K., Prior, T.W., Matteson, K.J., Wasserman, L.M., Cole, E.C., Stenzel, T.T., 2003. Establishment of stably EBV-transformed cell lines from residual clinical blood samples for use in performance evaluation and quality assurance in molecular genetic testing. J. Mol. Diagn. 5, 227–230. doi:10.1016/S1525-1578(10)60478-3.

Brenner-Lavie, H., Klein, E., Ben-Shachar, D., 2009. Mitochondrial complex I as a novel target for intraneuronal DA: modulation of respiration in intact cells. Biochem. Pharmacol. 78, 85–95. doi:10.1016/j.bcp.2009.03.024.

Cable, E.E., Gildemeister, O.S., Pepe, J.A., Donohue, S.E., Lambrecht, R.W., Bonkovsky, H.L., 1996. Hepatic 5-aminolevulinic acid synthase mRNA stability is modulated by inhibitors of heme biosynthesis and by metalloporphyrins. Eur. J. Biochem. 240, 112–117.

Cavelier, L., Jazin, E.E., Eriksson, I., Prince, J., Båve, U., Oreland, L., Gyllensten, U., 1995. Decreased cytochrome-c oxidase activity and lack of age-related accumulation of mitochondrial DNA deletions in the brains of schizophrenics. Genomics 29, 217–224. doi:10.1006/geno.1995.1234.

Chefalo, P.J., Oh, J., Rafie-Kolpin, M., Kan, B., Chen, J.J., 1998. Heme-regulated eIF-2$\alpha$ kinase purifies as a hemoprotein. Eur. J. Biochem. 258, 820–830. doi:10.1046/j.1432-1327.1998.2580820.x.

Chen, H., Wang, D.L., Liu, Y.L., 2016. Poly (I:C) transfection induces mitochondrial-mediated apoptosis in cervical cancer. Mol. Med. Rep. doi:10.3892/mmr.2016.4848.

Chen, J.J., London, I.M., 1995. Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase. Trends Biochem. Sci. 20, 105–108 S0968000400889756 [pii].

Chernova, T., Nicotera, P., Smith, A.G., 2006. Heme deficiency is associated with senescence and causes suppression of

N-Methyl-D-aspartate receptor subunits expression in primary cortical neurons. Mol. Pharmacol. 69, 697–705. doi:10.1124/mol.105.016675.uct.

Chernova, T., Steinert, J.R., Guerin, C.J., Nicotera, P., Forsythe, I.D., Smith, A.G., 2007. Neurite degeneration induced by heme deficiency mediated via inhibition of NMDA receptor-dependent extracellular signal-regulated kinase 1/2 activation. J. Neurosci. 27, 8475–8485. doi:10.1523/JNEUROSCI.0792-07.2007.

Choi, A.M., Alam, J., 1996. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am. J. Respir. Cell Mol. Biol. 15, 9–19. doi:10.1165/ajrcmb.15.1.8679227.

Chouinard, V.-A., Kim, S.-Y., Valeri, L., Yuksel, C., Ryan, K.P., Chouinard, G., Cohen, B.M., Du, F., Öngür, D., 2017. Brain bioenergetics and redox state measured by 31 P magnetic resonance spectroscopy in unaffected siblings of patients with psychotic disorders. Schizophr. Res. 187, 11–16. doi:10.1016/j.schres.2017.02.024.

Choy, E., Yelensky, R., Bonakdar, S., Plenge, R.M., Saxena, R., De Jager, P.L., Shaw, S.Y., Wolfish, C.S., Slavik, J.M., Cotsapas, C., Rivas, M., Dermitzakis, E.T., Cahir-McFarland, E., Kieff, E., Hafler, D., Daly, M.J., Altshuler, D., 2008. Genetic analysis of human traits in vitro: drug response and gene expression in lymphoblastoid cell lines. PLoS Genet. 4, e1000287. doi:10.1371/journal.pgen.1000287.

Dror, N., Klein, E., Karry, R., Sheinkman, A., Kirsh, Z., Mazor, M., Tzukerman, M., Ben-Shachar, D., 2002. State-dependent alterations in mitochondrial complex I activity in platelets: a potential peripheral marker for schizophrenia. Mol. Psychiatry 7. doi:10.1038/sj.mp.4001116.

Duarte, A.R., Chenet, A.L., Souza de Almeida, F.J., Balbinotti Andrade, C.M., Roberto de Oliveira, M., 2018. The inhibition of heme oxigenase-1 (HO-1) abolishes the mitochondrial protection induced by sesamol in LPS-treated RAW 264.7 cells. Chem. Biol. Interact. 296, 171–178. doi:10.1016/j.cbi.2018.09.012.

English, J.A., Fan, Y., Föcking, M., Lopez, L.M., Hryniewiecka, M., Wynne, K., Dicker, P., Matigian, N., Cagney, G., Mackay-Sim, A., Cotter, D.R., 2015. Reduced protein synthesis in schizophrenia patient-derived olfactory cells. Transl. Psychiatry 5, e663. doi:10.1038/tp.2015.119.

Ernst, O., Zor, T., 2010. Linearization of the Bradford protein assay. J. Vis. Exp. doi:10.3791/1918.

Föcking, M., Dicker, P., English, J.A., Schubert, K.O., Dunn, M.J., Cotter, D.R., 2011. Common proteomic changes in the hippocampus in schizophrenia and bipolar disorder and particular evidence for involvement of cornu ammonis regions 2 and 3. Arch. Gen. Psychiatry doi:10.1001/archgenpsychiatry.2011.43.

Fujimoto, T., Nakano, T., Takano, T., Hokazono, Y., Asakura, T., Tsuji, T., 1992. Study of chronic schizophrenics using 31P magnetic resonance chemical shift imaging. Acta Psychiatr. Scand. 86, 455–462.

Gangadhar, B.N., Jayakumar, P.N., Subbakrishna, D.K., Janakiramaiah, N., Keshavan, M.S., 2004. Basal ganglia high-energy phosphate metabolism in neuroleptic-naive patients with schizophrenia: a 31-phosphorus magnetic resonance spectroscopic study. Am. J. Psychiatry 161, 1304–1306. doi:10.1176/appi.ajp.161.7.1304.

Gielisch, I., Meierhofer, D., 2015. Metabolome and proteome profiling of complex I deficiency induced by rotenone. J. Proteome Res. 14, 224–235. doi:10.1021/pr500894v.

Giulivi, C., Napoli, E., Schwartzer, J., Careaga, M., Ashwood, P., 2013. Gestational exposure to a viral mimetic poly(I:C) Results in long-lasting changes in mitochondrial function by leucocytes in the adult offspring. Mediat. Inflamm. 2013. doi:10.1155/2013/609602.

Gubert, C., Stertz, L., Pfaffenseller, B., Panizzutti, B.S., Rezin, G.T., Massuda, R., Streck, E.L., Gama, C.S., Kapczinski, F., Kunz, M., 2013. Mitochondrial activity and oxidative stress markers in peripheral blood mononuclear cells of patients with bipolar disorder, schizophrenia, and healthy subjects. J. Psychiatr. Res. 47, 1396–1402. doi:10.1016/j.jpsychires.2013.06.018.

Haghighatfard, A., Andalib, S., Amini Faskhodi, M., Sadeghi, S., Ghaderi, A.H., Moradkhani, S., Rostampour, J., Tabrizi, Z., Mahmoodi, A., Karimi, T., Ghadimi, Z., 2018. Gene expression study of mitochondrial complex I in schizophrenia and paranoid personality disorder. World J. Biol. Psychiatry 19, S133–S146. doi:10.1080/15622975.2017.1282171.

Hamilton, J.W., Bement, W.J., Sinclair, P.R., Sinclair, J.F., Alcedo, J.A., Wetterhahn, K.E., 1991. Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. Arch. Biochem. Biophys. 289, 387–392.

Han, G., Casson, R.J., Chidlow, G., Wood, J.P.M., 2014. The mitochondrial complex I inhibitor rotenone induces endoplasmic reticulum stress and activation of GSK-3?? in cultured rat retinal cells. Investig. Ophthalmol. Vis. Sci. 55, 5616–5628. doi:10.1167/iovs.14-14371.

Harrison, P.J., 2004. The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications. Psychopharmacology (Berl) 174, 151–162. doi:10.1007/s00213-003-1761-y.

Higdon, A.N., Benavides, G.A., Chacko, B.K., Ouyang, X., Johnson, M.S., Landar, A., Zhang, J., Darley-Usmar, V.M., 2012. Hemin causes mitochondrial dysfunction in endothelial cells through promoting lipid peroxidation: the protective role of autophagy. AJP Hear. Circ. Physiol. 302, H1394–H1409. doi:10.1152/ajpheart.00584.2011.

Holcik, M., Sonenberg, N., 2005. Translational control in stress and apoptosis. Nat. Rev. Mol. Cell Biol. 6, 318–327. doi:10.1038/nrm1618.

Homedan, C., Laafi, J., Schmitt, C., Gueguen, N., Lefebvre, T., Karim, Z., Desquiret-Dumas, V., Wetterwald, C., Deybach, J.C., Gouya, L., Puy, H., Reynier, P., Malthièry, Y., 2014. Acute intermittent porphyria causes hepatic mitochondrial energetic failure in a mouse model. Int. J. Biochem. Cell Biol. 51, 93–101. doi:10.1016/j.biocel.2014.03.032.

Homedan, C., Schmitt, C., Laafi, J., Gueguen, N., Desquiret-Dumas, V., Lenglet, H., Karim, Z., Gouya, L., Deybach, J.-C., Simard, G., Puy, H., Malthièry, Y., Reynier, P., 2015. Mitochondrial energetic defects in muscle and brain of a Hmbs-/- mouse model of acute intermittent porphyria. Hum. Mol. Genet. 24, 5015–5023. doi:10.1093/hmg/ddv222.

Hull, T.D., Boddu, R., Guo, L., Tisher, C.C., Traylor, A.M., Patel, B., Joseph, R., Prabhu, S.D., Suliman, H.B., Piantadosi, C.A., Agarwal, A., George, J.F., 2016. Heme oxygenase-1 regulates mitochondrial quality control in the heart. JCI Insight. 1, e85817. doi:10.1172/jci.insight.85817.

Hung, S., Liou, H., Kang, K., Wu, R.-M., Wen, C., Fu, W.-M., 2008. Overexpression of heme oxygenase-1 protects dopaminergic neurons against 1-methyl-4-phenylpyridinium-induced neurotoxicity. Mol. Pharmacol. 74, 1564–1575. doi:10.1124/mol.108.048611, mol.108.048611 [pii]\n.

Javitt, D.C., Sweet, R.A., 2015. Auditory dysfunction in schizophrenia: integrating clinical and basic features. Nat. Rev. Neurosci. doi:10.1038/nrn4002.

Jayakumar, P.N., Venkatasubramanian, G., Keshavan, M.S., Srinivas, J.S., Gangadhar, B.N., 2006. MRI volumetric and 31P MRS metabolic correlates of caudate nucleus in antipsychotic-naïve schizophrenia. Acta Psychiatr. Scand. doi:10.1111/j.1600-0447.2006.00836.x.

Anderson, K.E., Sassa, S., Bishop, D.F., D., R.J., et al., 2001. Disorders of heme biosynthesis: x-linked sideroblastic anemia and the porphyrias. In: Scriver, C.R., Beaudt, A.L., Sly, W.S., Valle, D., Barton, C., Kinzler, K.W., et al. (Eds.), The Metabolic and

Molecular Bases of Inherited Disease. The McGraw-Hill Companies, Inc, New York, pp. 2991–3062.

Karry, R., Klein, E., Shachar, D.Ben, 2004. Mitochondrial complex I subunits expression is altered in schizophrenia: a postmortem study. Biol. Psychiatry 55, 676–684.

Landau, G., Bercovich, Z., Park, M.H., Kahana, C., 2010. The role of polyamines in supporting growth of mammalian cells is mediated through their requirement for translation initiation and elongation. J. Biol. Chem. doi:10.1074/jbc.M110.106419.

Ma, T., Trinh, M.A., Wexler, A.J., Bourbon, C., Gatti, E., Pierre, P., Cavener, D.R., Klann, E., 2013. Suppression of eIF2$\alpha$ kinases alleviates Alzheimer's disease-related plasticity and memory deficits. Nat. Neurosci. 16, 1299–1305. doi:10.1038/nn.3486.

Manji, H., Kato, T., Di Prospero, N.A., Ness, S., Beal, M.F., Krams, M., Chen, G., 2012. Impaired mitochondrial function in psychiatric disorders. Nat. Rev. Neurosci. 13, 293–307 10.1038/nrn3229nrn3229 [pii].

Maurer, I., Zierz, S., Moller, H., 2001. Evidence for a mitochondrial oxidative phosphorylation defect in brains from patients with schizophrenia. Schizophr. Res. 48, 125–136.

Mendoza, M.C., Er, E.E., Blenis, J., 2011. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. Trends Biochem. Sci. doi:10.1016/j.tibs.2011.03.006.

Meyer-Lindenberg, A., Miletich, R.S., Kohn, P.D., Esposito, G., Carson, R.E., Quarantelli, M., Weinberger, D.R., Berman, K.F., 2002. Reduced prefrontal activity predicts exaggerated striatal dopaminergic function in schizophrenia. Nat. Neurosci. 5, 267–271. doi:10.1038/nn804.

Newhouse, K., Hsuan, S.L., Chang, S.H., Cai, B., Wang, Y., Xia, Z., 2004. Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic SH-SY5Y cells. Toxicol. Sci. 79, 137–146. doi:10.1093/toxsci/kfh089.

Oh-Nishi, A., Koga, K., Maeda, T., Suhara, T., 2016. A possible serologic biomarker for maternal immune activation-associated neurodevelopmental disorders found in the rat models. Neurosci. Res. doi:10.1016/j.neures.2016.07.003.

Pantelis, C., Barnes, T.R., Nelson, H.E., Tanner, S., Weatherley, L., Owen, A.M., Robbins, T.W., 1997. Frontal-striatal cognitive deficits in patients with chronic schizophrenia. Brain 120, 1823–1843. doi:10.1093/brain/120.10.1823.

Piontkewitz, Y., Arad, M., Weiner, I., 2012. Tracing the development of psychosis and its prevention: what can be learned from animal models. Neuropharmacology 62, 1273–1289 10.1016/j.neuropharm.2011.04.019S0028-3908(11)00164-X [pii].

Ponka, P., 1999. Cell biology of heme. Am. J. Med. Sci. 318, 241–256.

Prabakaran, S., Swatton, J.E., Ryan, M.M., Huffaker, S.J., Huang, J.-J.T., Griffin, J.L., Wayland, M., Freeman, T., Dudbridge, F., Lilley, K.S., Karp, N.A., Hester, S., Tkachev, D., Mimmack, M.L., Yolken, R.H., Webster, M.J., Torrey, E.F., Bahn, S., 2004. Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. Mol. Psychiatry 9, 684–697. doi:10.1038/sj.mp.4001511.

Prince, J.A., Blennow, K., Gottfries, C.G., Karlsson, I., Oreland, L., 1999. Mitochondrial function is differentially altered in the basal ganglia of chronic schizophrenics. Neuropsychopharmacology 21, 372–379. doi:10.1016/S0893-133X(99)00016-0.

Raghuram, S., Stayrook, K.R., Huang, P., Rogers, P.M., Nosie, A.K., McClure, D.B., Burris, L.L., Khorasanizadeh, S., Burris, T.P., Rastinejad, F., 2007. Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta. Nat. Struct. Mol. Biol. doi:10.1038/nsmb1344.

Rajasekaran, A., Venkatasubramanian, G., Berk, M., Debnath, M., 2015a. Mitochondrial dysfunction in schizophrenia: pathways, mechanisms and implications. Neurosci. Biobehav. Rev. 48, 10–21. doi:10.1016/j.neubiorev.2014.11.005.

Rajasekaran, A., Venkatasubramanian, G., Berk, M., Debnath, M., 2015b. Mitochondrial dysfunction in schizophrenia: pathways, mechanisms and implications. Neurosci. Biobehav. Rev. 48, 10–21. doi:10.1016/j.neubiorev.2014.11.005.

Robicsek, O., Ene, H.M., Karry, R., Ytzhaki, O., Asor, E., McPhie, D., Cohen, B.M., Ben-Yehuda, R., Weiner, I., Ben-Shachar, D., 2018. Isolated mitochondria transfer improves neuronal differentiation of schizophrenia-derived induced pluripotent stem cells and rescues deficits in a rat model of the disorder. Schizophr. Bull. 44, 432–442. doi:10.1093/schbul/sbx077.

Rollins, B.L., Morgan, L., Hjelm, B.E., Sequeira, A., Schatzberg, A.F., Barchas, J.D., Lee, F.S., Myers, R.M., Watson, S.J., Akil, H., Potkin, S.G., Bunney, W.E., Vawter, M.P., 2018. Mitochondrial Complex I Deficiency in Schizophrenia and Bipolar Disorder and Medication Influence. Mol. Neuropsychiatry 3, 157–169. doi:10.1159/000484348.

Rosenfeld, M., Brenner-Lavie, H., Ari, S.G.-B., Kavushansky, A., Ben-Shachar, D., 2011. Perturbation in mitochondrial network dynamics and in complex I dependent cellular respiration in schizophrenia. Biol. Psychiatry 69. doi:10.1016/j.biopsych.2011.01.010.

Schipper, H.M., Liberman, A., Stopa, E.G., 1998. Neural heme oxygenase-1 expression in idiopathic Parkinson's disease. Exp. Neurol. 150, 60–68. doi:10.1006/exnr.1997.6752.

Sei, Y., Ren-Patterson, R., Li, Z., Tunbridge, E.M., Egan, M.F., Kolachana, B.S., Weinberger, D.R., 2007. Neuregulin1-induced cell migration is impaired in schizophrenia: association with neuregulin1 and catechol-o-methyltransferase gene polymorphisms. Mol. Psychiatry 12, 946–957. doi:10.1038/sj.mp.4001994.

Sengupta, A., Hon, T., Zhang, L., 2005. Heme deficiency suppresses the expression of key neuronal genes and causes neuronal cell death. Brain Res. Mol. Brain Res. 137, 23–30. doi:10.1016/j.molbrainres.2005.02.007.

Sinclair, P.R., Gorman, N., Jacobs, J.M., 2001. Measurement of heme concentration. Curr. Protoc. Toxicol. doi:10.1002/0471140856.tx0803s00, Chapter 8, Unit 8.3.

Smith, A.G., Raven, E.L., Chernova, T., 2011. The regulatory role of heme in neurons. Metallomics 3, 955–962. doi:10.1039/c1mt00085c.

Smith, M.a, Kutty, R.K., Richey, P.L., Yan, S.D., Stern, D., Chader, G.J., Wiggert, B., Petersen, R.B., Perry, G., 1994. Heme oxygenase-1 is associated with the neurofibrillary pathology of Alzheimer's disease. Am. J. Pathol. 145, 42–47.

Song, W., Zukor, H., Lin, S.-H., Hascalovici, J., Liberman, A., Tavitian, A., Mui, J., Vali, H., Tong, X.-K., Bhardwaj, S.K., Srivastava, L.K., Hamel, E., Schipper, H.M., 2012. Schizophrenia-like features in transgenic mice overexpressing human HO-1 in the astrocytic compartment. J. Neurosci. 32, 10841–10853. doi:10.1523/JNEUROSCI.6469-11.2012.

Steullet, P., Cabungcal, J.-H., Kulak, A., Kraftsik, R., Chen, Y., Dalton, T.P., Cuenod, M., Do, K.Q., 2010. Redox dysregulation affects the ventral but not dorsal hippocampus: impairment of parvalbumin neurons, gamma oscillations, and related behaviors. J. Neurosci. 30, 2547–2558. doi:10.1523/JNEUROSCI.3857-09.2010.

Tang, X.D., Xu, R., Reynolds, M.F., Garcia, M.L., Heinemann, S.H., Hoshi, T., 2003. Haem can bind to and inhibit mammalian calcium-dependent Slo1 BK channels. Nature doi:10.1038/nature02003.

Tian, Q., Li, T., Hou, W., Zheng, J., Schrum, L.W., Bonkovsky, H.L., 2011. Lon peptidase 1 (LONP1)-dependent breakdown of mitochondrial 5-aminolevulinic acid synthase protein by heme in human liver cells. J. Biol. Chem. doi:10.1074/jbc.M110.215772.

Vogel, C., Marcotte, E.M., 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet. 13, 227–232. doi:10.1038/nrg3185.

Volz, H.P., Riehemann, S., Maurer, I., Smesny, S., Sommer, M., Rzanny, R., Holstein, W., Czekalla, J., Sauer, H., 2000. Reduced

phosphodiesters and high-energy phosphates in the frontal lobe of schizophrenic patients: A31P chemical shift spectroscopic-imaging study. Biol. Psychiatry doi:10.1016/S0006-3223(00)00235-3.

Vos, T., Abajobir, A.A., Abbafati, C., et al., 2017. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet (17) doi:10.1016/S0140-6736, 32154-2.

Washizuka, S., Iwamoto, K., Kakiuchi, C., Bundo, M., Kato, T., 2009. Expression of mitochondrial complex I subunit gene NDUFV2 in the lymphoblastoid cells derived from patients with bipolar disorder and schizophrenia. Neurosci. Res. 63, 199–204. doi:10.1016/j.neures.2008.12.004.

Washizuka, S., Kametani, M., Sasaki, T., Tochigi, M., Umekage, T., Kohda, K., Kato, T., 2006. Association of mitochondrial complex I subunit gene NDUFV2 at 18p11 with schizophrenia in the Japanese population. Am. J. Med. Genet. Neuropsychiatr. Genet. 141 (B), 301–304. doi:10.1002/ajmg.b.30285.

Yang, F., Wang, E.-D., 2010. Heme regulates protein homeostasis at transcription, protein translation, and degradation levels. Front. Biol. (Beijing). 5, 516–523. doi:10.1007/s11515-010-7700-5.

Yaniv, S.P., Ben-Shachar, D., Klein, E., 2008. Norepinephrine-glucocorticoids interaction does not annul the opposite effects of the individual treatments on cellular plasticity in neuroblastoma cells. Eur. J. Pharmacol. 596. doi:10.1016/j.ejphar.2008.08.006.