Energization by multiple substrates and calcium challenge reveal dysfunctions in brain mitochondria in a model related to acute psychosis

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Abstract

Schizophrenia etiology is unknown, nevertheless imbalances occurring in an acute psychotic episode are important to its development, such as alterations in cellular energetic state, REDOX homeostasis and intracellular Ca^{2+} management, all of which are controlled primarily by mitochondria. However, mitochondrial function was always evaluated singularly, in the presence of specific respiratory substrates, without considering the plurality of the electron transport system. In this study, mitochondrial function was analyzed under conditions of isolated or multiple respiratory substrates using brain mitochondria isolated from MK-801-exposed mice. Results showed a high H_2O_2 production in the presence of pyruvate/malate, with no change in oxygen consumption. In the condition of multiple substrates, however, this effect is lost. The analysis of Ca^{2+} retention capacity revealed a significant change in the uptake kinetics of this ion by mitochondria in MK-801-exposed animals. Futhermore, when mitochondria were exposed to calcium, a total loss of oxidative phosphorylation and an impressive increase in H_2O_2 production were observed in the condition of multiple substrates. There was no alteration in the activity of the antioxidant enzymes analyzed. The data demonstrate for the first time, in an animal model of psychosis, two important aspects (1) mitochondria may compensate deficiencies in a single mitochondrial complex when they oxidize several substrates simultaneously, (2) Ca^{2+} handling is compromised in MK-801-exposed mice, resulting in a loss of phosphorylative capacity and an increase in H_2O_2 production. These data favor the hypothesis that disruption of key physiological roles of mitochondria may be a trigger in acute psychosis and, consequently, schizophrenia.

Keywords Calcium handling · Mitochondria · Brain · Schizophrenia · Metabolism · Redox homeostasis

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Introduction

Schizophrenia is a neuropsychiatric disorder that affects about 21 million people around the world (World Health Organization 2018) Its complex etiology includes genetic, environmental, psychological and social factors (Picchioni and Murray 2007). This is currently recognized as a neurodevelopmental disorder, including a prodromal phase characterized by attenuated symptoms, followed by an acute exacerbation of psychotic symptoms that usually lead to the chronic disturbance (McCutcheon et al. 2019). The first psychotic episode generally occurs in late adolescence and early adulthood, including mainly positive symptoms, such as delusions and hallucinations, but also negative symptoms, such as blunted affect and social withdrawal and cognitive deficits (Insel 2010; Lewis and Levitt 2002). Duration of untreated psychosis is a key factor for the prognostic (Harrigan et al. 2003), thus, understanding the changes occurring during the

first psychotic episode may contribute to the understanding of schizophrenia development.

It is well established that there is an imbalance in dopamine and glutamate neurotransmitter systems related to the pathology (Insel 2010). This led to the emergence of neurochemical hypotheses to explain the signs and symptoms of schizophrenia. N-methyl-D-aspartate glutamate receptors (NMDAR) were first implicated in schizophrenia pathophysiology after the observation that NMDAR antagonists, such as phencyclidine (PCP) (Allen and Young 1978) and ketamine (Lahti et al. 1995), are able to induce delusional states, thought disorder as well as negative symptoms and cognitive deficits in healthy individuals, and exacerbate these symptoms in schizophrenia patients (Adler et al. 1998; Malhotra et al. 1997). In rodents, these antagonists (including MK-801) consistently induce behavioral and neurochemical endophenotypes analogous to acute psychosis manifestations, reproducing positive, negative and cognitive symptoms (Andine et al. 1999; Lodge and Mercier 2015; Manahan-Vaughan et al. 2008; Sams-Dodd 1996). For example, the impairment in sensory gating manifested by deficits in the prepulse inhibition of the startle reflex (PPI) is clearly observed in animals exposed to NMDAR antagonists (Jones et al. 2011; Mansbach 1991). Pharmacologically-induced hypofunction of NMDAR also leads to an acute increase in glutamate release and, consequently, disinhibition of brain regions such as the hippocampus and frontal cortex (Greene 2001), resembling the changes detected in schizophrenia patients by neuroimaging studies (Chiu et al. 2018; Theberge et al. 2002; Tregellas et al. 2014; Uhlhaas and Singer 2010). Moreover, acute challenges with NMDA antagonists induce metabolic changes similar to those described in first episode unmedicated schizophrenia patients (Soyka et al. 2005; Eyjolfsson et al. 2006). For these reasons, animals acutely exposed to NMDAR antagonists are used as models of NMDA hypofunction related to acute psychosis (Greene 2001; Howes et al. 2015; Jones et al. 2011). Such acute models are useful tools for the identification of initial changes related to NMDA receptors hypofunction that might occur during an acute psychotic episode and, consequently, contribute to the chronic state of the disorder.

The pathophysiology of psychotic episodes and schizophrenia is also correlated to changes in fundamental cellular processes which regulate the energetic and REDOX status. This is supported by several evidence found in patients brains: (i) reduction in glucose metabolism, related to several alterations in glycolytic enzymes [for review see (Martins-De-Souza et al. 2010)], impaired glucose tolerance and insulin resistance are already manifested in acutely psychotic first episode patients (Perry et al. 2016); (ii) decreased expression of the E1 subunit of the pyruvate dehydrogenase complex, which directly reflects in mitochondrial acetyl-CoA levels (Prabakaran et al. 2004); (iii) changes in expression and activity of the Krebs cycle enzymes (Bubber et al. 2011); (iv)

detachment of hexokinase 1 from the external mitochondrial membrane, the main link between glucose metabolism and ATP production in brain (Regenold et al. 2012). In addition, several studies with patients or animal models show both an increase in the generation of reactive oxygen species (ROS) as well as a reduction in the efficiency of antioxidant systems, which leads to oxidative stress [for review see (Koga et al. 2016)]. Moreover, high baseline oxidative stress is present in first episode unmedicated patients and seems to be related to gray matter lost, cognitive outcomes and a worst disorder prognostic (Fraguas et al. 2017).

There is a lack of studies on mitochondrial function in acutely psychotic patients and the available data reflects activity of peripheral samples only. A decrease in mitochondrial DNA copy number was detected in blood samples of unmedicated patients (Hjelm et al. 2015; Li et al. 2015). However, many groups showed brain mitochondria changes in chronic schizophrenia (Rajasekaran et al. 2015). These changes are both quantitative and morphological, such as alterations in mitochondrial density and the structure of the cristae (Uranova et al. 2007). In addition, further evidence associates genetic polymorphisms in mitochondrial DNA to schizophrenia. Some of these polymorphisms result in structural and functional modifications in mitochondrial proteins such as OPA1 and components of the electron transport system (Ben-Shachar and Karry 2007; Bergman and Ben-Shachar 2016; Iwamoto et al. 2005; Marchbanks et al. 2003; Maurer et al. 2001; Prince et al. 1999; Rosenfeld et al. 2011). Taken together, these data point to mitochondrial imbalance as a hallmark in the pathophysiology of schizophrenia and understanding the early changes that might precede these chronicle alterations is a necessary to a further understanding of the disorder.

Mitochondrion is the organelle to which all cellular energy metabolism converges and the main source of ATP and ROS generation in brain cells. In addition, it plays a key role in synaptic plasticity and modulation of neuronal activity (Li et al. 2004) and changes in these processes may impact neural communication and development. Although mitochondrial complexes have been investigated due to its importance in the pathophysiology of psychosis (Bergman and Ben-Shachar 2016), they have always been evaluated in isolation, using enzymatic activity or oxygen consumption assays in the presence of specific substrates for each of them, without considering the plurality of the electron transport system and the mitochondrial ability to oxidize different substrates. Physiologically, mitochondria use several energetic substrates simultaneously and pathological conditions or stressors could modify this pattern, as previously shown in skeletal muscle (Martins et al. 2018). These changes may directly reflect on mitochondrial ATP synthesis and ROS generation, which are both altered in the disorder (Martins-de-Souza et al. 2011). In addition, brain mitochondria represent an important

compartment for calcium storage and plays a central role in calcium homeostasis and modulation of neurotransmitter release (Jonas et al. 1999; Mnatsakanyan et al. 2017), which is also disrupted in schizophrenia (Lidow 2003; Lisek et al. 2016). Despite these facts, few studies have directly investigated mitochondrial calcium handling on experimental models of schizophrenia (Lisek et al. 2016; Bustamante et al. 2016).

Therefore, in this work, mitochondrial electron transport system, ROS generation and calcium uptake were evaluated in the presence of different respiratory substrates, used in isolation and together, in an animal model of NMDA hypofunction related to acute psychosis. Our results indicate that the disruption of NMDA activity may lead to disturbances in mitochondrial calcium handling in brain that follows a severe mitochondrial dysfunction under stress conditions which could be implicated in psychosis and, consequently, schizophrenia pathology.

Materials and methods

Animals

Adult male Swiss mice (PND60–90) were used. Animals were kept in groups of 4 or 5 in plastic cages with free access to filtered water and standard diet. Mice were kept under a 12 h light-dark cycle (lights off at 6:00 pm) with controlled temperature (23 ± 2 °C) and humidity (65%). All experimental protocols were developed according to the guidelines of the National Research Ethical Committee (published by National Heath Council-MS, 1998) and were approved by the local Ethical Committee (CEUA-UFRJ n. DFBCICB045).

MK-801 exposure

All the biochemical experiments were preceded by the pre-pulse inhibition of the startle reflex (PPI) test. Mice were transferred to the experimental room and remained for 1 h on-site for habituation. Subsequently, they were randomly divided into four groups: saline (NaCl 0.9%), MK-801 0.1 mg/kg, MK-801 0.3 mg/kg and MK-801 1.0 mg/kg. MK-801 hydrogen maleate (Sigma-Aldrich, Brazil) was dissolved in saline. All animals were injected intraperitoneally at 1 mL/100 g body weight and doses are expressed as free base. After injection, animals were kept undisturbed for 30 min and submitted to the PPI test.

Pre-pulse inhibition of startle reflex (PPI)

The experimental protocol was performed according to (Neves et al. 2013) with minor modifications. PPI test was performed in an isolated chamber containing a movement-sensitive platform and a loudspeaker to produce the acoustic stimulus (Panlab, Harvard Instruments, Barcelona, Spain). The experimental protocol consisted of 5 min habituation (background white noise 65 dB) followed by 5 exposures to the pulse (115 dB, 50 ms) for startle stabilization. Subsequently, animals were exposed to five different blocks of stimulus (10 times each, with 10 to 30 s intertrial interval): (1) control (background noise); (2) pulse (115 dB); (3) pulse preceded by pre-pulse of 72 dB; (4) pulse preceded by 80 dB pre-pulse; (5) pulse preceded by 90 dB pre-pulse. Interstimulus interval (prepulse-pulse) was fixed at 100 ms. Startle magnitude in each trial was digitalized and the percentual inhibition of the startle reflex for each pre-pulse intensity was calculated as: 100 - [(average startle from pre-pulse plus pulse trials / average startle from pulse alone trials) × 100].

Mitochondrial isolation

Soon after the PPI test, the animals were euthanized by decapitation and the forebrains were removed for mitochondrial isolation by digitonin according to the protocol described by Rosenthal et al. with small modifications (Rosenthal et al. 1987). To test if PPI alone would affect mitochondrial function, we compared mitochondrial respiration from animals that passed through PPI test or not. No difference was found in mitochondrial function between animals submitted or not to PPI test, thus no specific effect of PPI was detected (data not shown). Briefly, the forebrains were immersed in mitochondrial isolation buffer containing: 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 1 mM BSA, 10 mM HEPES pH 7.2 and kept on ice. This solution was repeatedly changed for excess blood removal. Subsequently, the brains were minced and softly homogenized 10x in glass *potter* with Teflon piston in isolation buffer. The produced homogenate was centrifuged at 1330 g for 3 min. After centrifugation, the pellet was discarded and the supernatant was separated and centrifuged again at 21200 g for 8 min. Thereafter, the resulting pellet was resuspended in 5 mL of isolation buffer and gently homogenized 3x in the glass *potter* in the presence of 0.1% (*w/v*) digitonin. This homogenate was centrifuged again at 21200 g for 8 min to remove the detergent. At the end of the process, pure mitochondria were obtained, since digitonin disrupts the cell membranes to promote an enriched mitochondrial preparation. The amount of mitochondrial protein was determined by the Lowry method using bovine serum albumin BSA as standard (Lowry et al. 1951).

High resolution respirometry

Measurements of mitochondrial oxygen consumption were performed with high-resolution oxygraphy (Oxygraph-2 k Oroboros Instruments, Innsbruck,

Austria) at 37 °C with constant stirring. It was used 0.1 mg/mL of mitochondrial protein in MIR05 medium containing: 110 mM sucrose, 20 mM HEPES, 20 mM taurine, 60 mM K-lactobionate, 3 mM $MgCl_2$, 10 mM KH_2PO_4, 0.5 mM EGTA, BSA 1 g/L, pH 7.1 (Gnaiger et al. 2000). Substrates and inhibitors of mitochondrial function were added sequentially, always after signal stabilization, using two different protocols. The following additions were made for experiments with isolated substrates: 2/2 mM Pyruvate/Malate or 5 mM Succinate; 1 mM ADP; 0.1 µg/mL Oligomycin; 2.5 µM Antimycin A. The following additions were made to the experiments in the presence of multiple substrates 2/2/10 mM Pyruvate/Malate/glutamate; 5 mM Succinate; 5 mM Glycerol Phosphate; 1 mM ADP; 0.1 µg/mL Oligomycin; 2.5 µM Antimycin A. Multiple substrate runs were performed in the absence or presence of 200 µM $CaCl_2$. Data were collected through a specific software that shows, in real time, the concentration of oxygen and the specific oxygen flow, which means the negative time derivative of oxygen concentration (DatLab software 5.0, Oroboros Instruments, Innsbruck, Austria).

H_2O_2 production

The H_2O_2 generation was measured by two protocols. In the first part of the work (Fig. 2), when mitochondria were evaluated with isolated respiratory substrates, mitochondrial H_2O_2 release was measured by the fluorescence of Amplex Red probe using a spectrofluorometer (Varian Cary Eclipse; Agilent Technologies, Santa Clara, CA). Fluorescence was detected at an excitation wavelength of 563 nm (slit 5 nm) and an emission wavelength of 587 nm (slit 5 nm) in MIR05 medium in the presence of 0.1 mg/mL mitochondrial protein and 2 U/mL horseradish peroxidase. The titration sequence was identical to the respirometry experiments: 2/2 mM Pyruvate/Malate - or 5 mM Succinate; 1 mM ADP; 0.1 µg/mL Oligomycin; 2.5 µM Antimycin A. The rate of H_2O_2 production was determined using linear regression curve fitting with Origin 8.0.

In the second part of the work (Figs. 3, 6 and 7), the measurements of H_2O_2 production in the presence of multiple substrates were made simultaneously to the oxygen consumption measurements, through the coupling of an optical sensor to the oxygraph chambers (O2k-Fluo LED2-Module). This module, associated with the software (DatLab software 5.0, Oroboros Instruments, Innsbruck, Austria) is able to record the fluorescence emitted by the Amplex-Red-Peroxidase system. This protocol was performed in the absence and presence of 200 µM $CaCl_2$. Equipment calibration was done by addition of a known concentration of H_2O_2 at the beginning of the run.

Enzymatic activities

Glutathione reductase (GR) The activity of Glutathione reductase was measured in a reaction medium containing 0.1 M K^+ phosphate buffer, pH 7 and 0.1 mM NADPH by following the consumption of NADPH, represented by the reduction of its light absorbance at 340 nm. The reaction was started by adding 1 mM of oxidized glutathione (GSSG) (adapted from (Flohe and Gunzler 1984)).

Glutathione peroxidase (GPx) The activity of Glutathione peroxidase was measured indirectly through exogenous glutathione reductase activity coupling. The exogenous GR reduces the GSSG formed by the GPx coupled to the consumption of NADPH. The reaction medium contained 50 mM KH_2PO_4 pH 7, 1 mM NaN_3, 0.2 mM NADPH, 1 U/mL exogenous GR, 1 mM GSH and the reaction was started with 0.25 mM H_2O_2. Reduction of light absorbance of NADPH was monitored at 340 nm (adapted from (Mills 1957)).

Thioredoxin reductase (TR) The activity of Thioredoxin reductase was measured in medium containing 100 mM KH_2PO_4 pH 7, 2.5 mM DTNB, 10 mM EDTA. The reaction was started by adding 0.3 mM NADPH and the method is based on the ability of this enzyme to use 5,5 ¢ -dithiobis (2-nitrobenzoic acid) (DTNB) as the substrate to generate 5-thio-2-nitrobenzoic acid (TNB), which produces a strong yellow color that is measured at 412 nm (Holmgren and Bjornstedt 1995).

Superoxide dismutase (mnSOD and CuZnSOD isoforms) The activity of mnSOD was monitored using superoxide radicals from the xanthine/xanthine oxidase system coupled to the reaction. The rate of cytochrome C reduction by the superoxide radical was monitored at 480 nm in medium containing 5–20 µg mitochondrial protein, 50 µM xanthine, 20 µM potassium cyanide (inhibitor of the CuZnSOD isoform), 20 µM cytochrome C and 2 U/mL xanthine oxidase (McCord and Fridovich 1969). The experiments were repeated in buffer without potassium cyanide to obtain the total SOD activity. The activity of the CuZnSOD isoform was determined by the difference between the total activity by the activity in the presence of potassium cyanide.

All enzymatic assays were performed on a Victor Perkin-Elmer plate reader at 37 °C with constant stirring.

Mitochondrial Ca^{2+} uptake

To evaluate the mitochondrial calcium retention capacity, mitochondria were submitted to $CaCl_2$ titration, with additions of 100 µM in fixed 4 min intervals. The medium contained 10 mM HEPES, 2 mM MgCl2, 150 mM KCl, 2 mM KH2PO4, 1 mM ADP and 2 mM pyruvate and malate or

5 mM succinate and 0.1 μM of CaGreen, a probe that emits fluorescence when bound to Calcium (λ_{ex} 506 nm and λ_{em} 532 nm) (Facundo et al. 2005; Rajdev and Reynolds 1993). Each CaCl$_2$ addition resulted in fluorescence peak representing the binding of the ion to the fluorescent probe (Fig. 4a, b and 5a). Over time, a reduction in fluorescence is observed, indicating calcium detachment of the probe and its entry into the mitochondria. Titrations were performed until there was no more mitochondrial calcium uptake and the calculations were made after the subtraction of the baselines at the end of each calcium addition by the initial baseline. The assays were performed at 37 °C, with constant stirring in a spectrofluorometer (Varian Cary Eclipse; Agilent Technologies, Santa Clara, CA).

Statistics

Data were plotted on GraphPad Prism 6® software. PPI data was analyzed by a two-way repeated measure ANOVA, considering pre-pulse intensity as the first factor and treatment as the second, followed by Bonferroni post-test. For all other experiments, it was used One-way ANOVA with Bonferroni post-test for multiple comparisons. Differences were considered statistically significant when $p < 0.05$.

Results

Impairment of PPI by MK-801

PPI is the measure of startle reflex reduction induced by a pre-stimulus (Powell et al. 2009) and is considered an indicative of sensory-motor filter integrity. This filter allows the discrimination of external physiological or cognitive relevant from irrelevant stimulus and is extremely conserved among several species of mammals, including humans and rodents (Weiss and Feldon 2001). Pre-pulse inhibition is impaired in both acutely psychotic patients and animal models and reflects an important aspect of the disorder: the deficit in pre-attentional gating and information processing (Braff and Geyer 1990; Powell et al. 2009; Weiss and Feldon 2001).

Higher prepulse intensities induce a progressive startle inhibition, as shown in Fig. 1 (two-way RM ANOVA, $F_{intensity(2,94)} = 43.98$, $p < 0.001$). MK-801 treatment impaired PPI at all doses (two-way RM ANOVA, $F_{treatment(3,94)} = 23.65$, $p < 0.001$, Bonferroni post-test $p < 0.003$) showing that NMDAR blockade had the expected effect. PPI disruption was slightly more prominent for the smaller doses of MK-801 (Bonferroni post-test $p < 0.024$ comparing 1.0 mg/kg effect with the other doses). There was no difference in startle amplitude between groups (ANOVA, $F_{(3,47)} = 1.73$, $p = 0.175$). Taking together, these data validates the acute NMDAR hypofunction model in our experimental conditions.
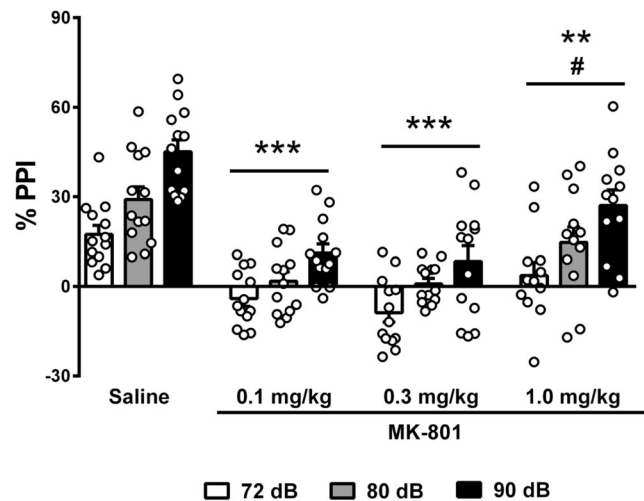

Fig. 1 NMDAR blockade by MK-801 impairs pre-pulse inhibition of the startle reflex. Mice were treated i.p. with saline or MK-801 30 min before test. Data are expressed as mean ± S.E.M. ($n = 12$–13). Data was analyzed by a two-way repeated measure ANOVA followed by Bonferroni post-test. ***$p < 0.001$ **$p < 0.01$ vs. saline group; #$p < 0.05$ vs. other MK-801 groups

Mitochondrial O$_2$ and H$_2$O$_2$ fluxes with complex i, ii or multiple substrates

In order to evaluate the mitochondrial function in MK-801-treated animals we measured the fluxes of oxygen consumption and H$_2$O$_2$ generation in the presence of isolated substrates that feed the electron transport system via complex I (pyruvate/malate) or complex II (succinate). In Fig. 2a, d typical traces of the flux of oxygen consumption (solid black line) and H$_2$O$_2$ levels (solid gray line) are shown. As expected, both oxygen and H$_2$O$_2$ fluxes respond to sequential addition of mitochondrial substrates and inhibitors in accordance with the variation of the mitochondrial membrane potential ($\Delta\Psi_M$) variation (Korshunov et al. 1997). More importantly, H$_2$O$_2$ fluxes are higher with succinate than with pyruvate/malate (Fig. 2a, d, gray line). The O$_2$ fluxes follow this same tendency for CII compared to CI -linked substrates (Fig. 2a, d, black lines). We detected a significant increase in H$_2$O$_2$ production in the MK-801 0.3 mg/kg group compared to controls in the presence of pyruvate/malate and ATP (Fig. 2c). However, there was no change in oxygen consumption (Fig. 2b). This parameter did not change in the presence of succinate alone (Fig. 2e, f).

Under physiological conditions, cells from the central nervous system (CNS) metabolize multiple substrates from NAD and FAD- enzymes simultaneously and converge the electrons to coenzyme Q (Q-junction) and finally to oxygen (Gnaiger 2014). We evaluated the O$_2$ consumption and H$_2$O$_2$ production in the presence of pyruvate/malate/glutamate/succinate and glycerol 3-phosphate, simultaneously as a mimetic of in vivo system complexity. Figure 3a shows typical traces of
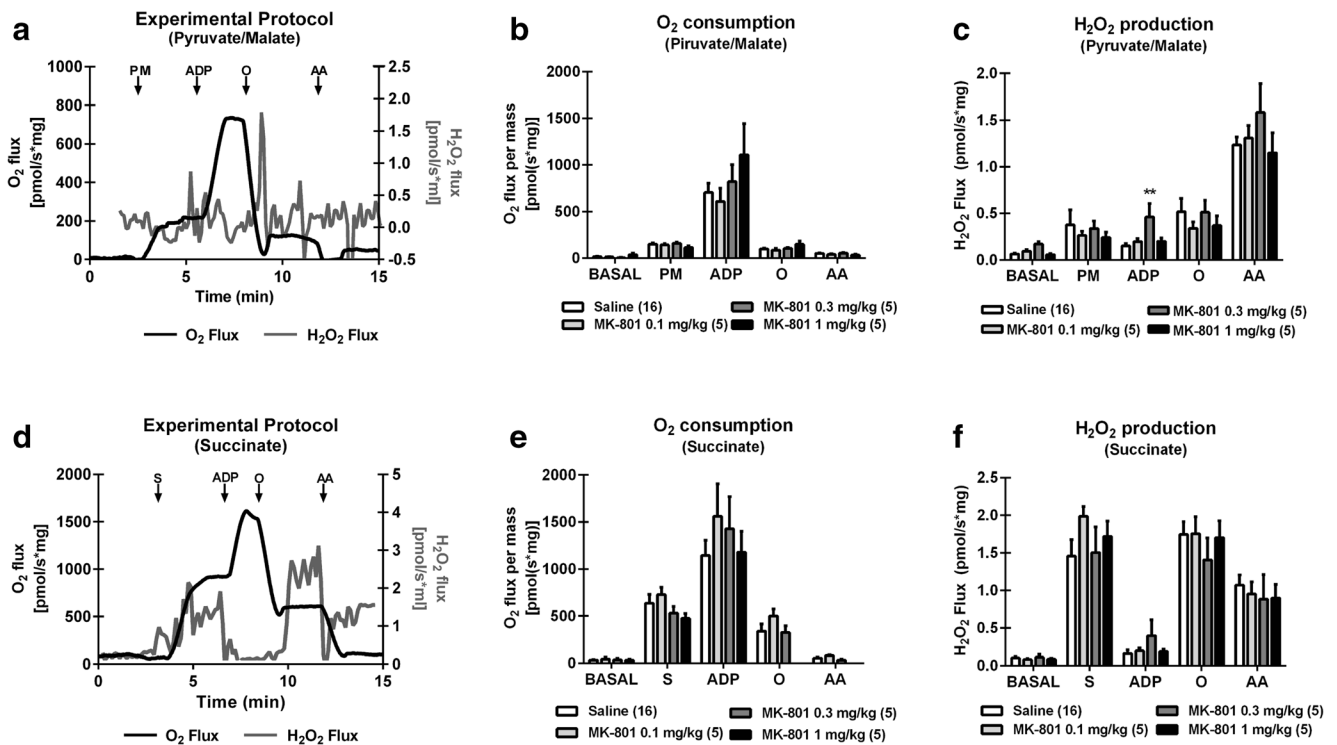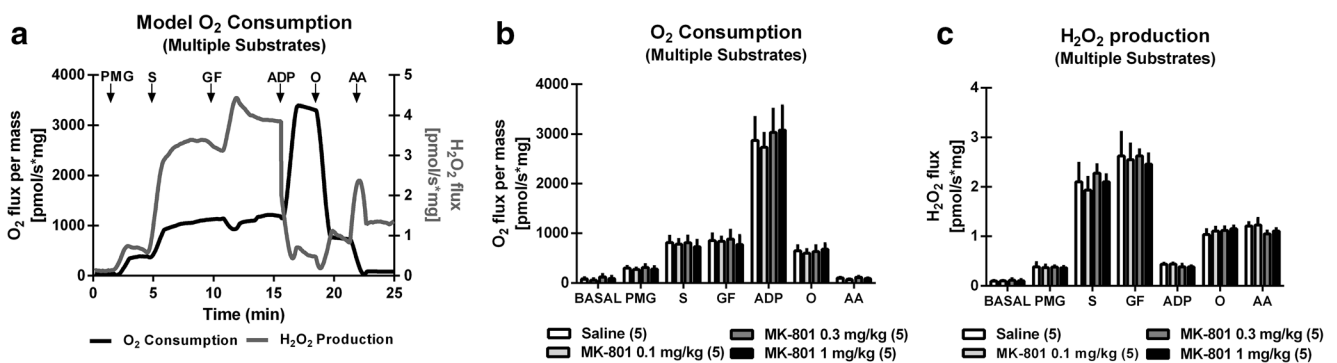
Fig. 2 Effect of MK-801 treatment on oxygen consumption and H_2O_2 generation in isolated mitochondria using substrates linked to complexes I or II. **a** and **d** Representative experiments. The arrows represent the moments of the additions. PM - Pyruvate/Malate (2/2 mM); S - Succinate (5 mM); ADP (1 mM); O - Oligomycin (0.1 μg / mL); AA - Antimycin A (2.5 μM). The sequence of additions and substrates concentrations were identical in oxygen consumption and

H_2O_2 generation experiments. **b** and **e** High resolution respirometry in the presence of pyruvate/malate (**b**) or succinate (**e**). **c** and **f** Production of H_2O_2 measured by fluorescence of Amplex Red in the presence of pyruvate/malate (**c**) or succinate (**f**). Data were analyzed by One-way ANOVA with Bonferroni post-test for multiple comparisons and are expressed as the mean ± standard error. ** $p = 0.0093$ compared to saline group. The numbers of individual experiments are in parentheses

O_2 and H_2O_2 fluxes. There was no change between groups either in oxygen consumption (Fig. 3b) or H_2O_2 production (Fig. 3c) in the presence of multiple substrates. These results indicate that with the multiple substrate approach the observed effect of higher ROS generation via complex I (Fig. 2c, MK-801 0.3 mg/kg) is abolished.

Effect of MK-801 in the antioxidant systems

In order to investigate whether the observed increase in H_2O_2 generation linked to complex I substrates was due to a reduction in enzymatic antioxidant capacity, the activities of important enzymes responsible for the clearance and control of ROS

Fig. 3 Effect of MK-801 treatment on oxygen consumption and H_2O_2 generation in isolated mitochondria in the presence of multiple substrates. **a** Representative experiment. The arrows represent the moments of the additions. PMG - Pyruvate/Malate/Glutamate (2/2/ 10 mM); S - Succinate (5 mM); GF - Glycerol Phosphate (5 mM); ADP (1 mM); O - Oligomycin (0.1 μg / mL); AA - Antimycin A

(2.5 μM). **b** High resolution respirometry and (**c**) H_2O_2 production in the presence of multiple substrates. Data were analyzed by One-way ANOVA with Bonferroni post-test for multiple comparisons and are expressed as the mean ± standard error. MK-801 groups were not significantly different from saline group. The numbers of individual experiments are in parentheses

Table 1 - Antioxidant enzyme activities of MK-801-treated mice isolated brain mitochondria

Enzyme	Saline	MK-801 0.1 mg/mg	MK-801 0.3 mg/kg	MK-801 1 mg/kg	Statistics
GPx (μMol/min.mg)	0.0049 ± 0.0014	0.0045 ± 0.0012	0.0053 ± 0.0021	0.0039 ± 0.0006	NS
GR (μMol/min.mg)	0.0337 ± 0.0118	0.0324 ± 0.0063	0.0297 ± 0.0156	0.0288 ± 0.0109	NS
TR (μMol/min.mg)	0.3651 ± 0.1058	0.3564 ± 0.0992	0.2838 ± 0.1123	0.2649 ± 0.0883	NS
MnSOD (U/mg ptn)	64.6827 ± 38.3535	56.7802 ± 25.7867	66.7013 ± 11.5198	51.4727 ± 17.5449	NS
CuZnSOD (U/mg ptn)	24.9734 ± 2.9349	27.6239 ± 16.4766	15.9334 ± 2.7275	10.9718 ± 6.1337	NS

GPx, Glutathione Peroxidase; GR, Glutathione Reductase; TR, Thioredoxin Reductase; MnSOD, Mn Superoxide Dismutase; CuZnSOD, CuZn Superoxide Dismutase. The number of experiments is equals to 5 for all tested enzymes. Data are expressed as the mean ± standard error. NS: not statistically significant

levels in neural cells were measured. As shown in Table 1, no changes were observed in the activities of the tested enzymes. This result suggests that the higher H_2O_2 levels in the presence of pyruvate/malate was due to an increase in H_2O_2 production instead of a decrease in the antioxidant enzymatic capacity.

Effects of MK-801 on mitochondrial Ca^{2+}-uptake

In addition to oxygen consumption and H_2O_2 generation, calcium retention capacity is another important aspect of mitochondrial physiology, especially in the CNS where a precise balance in mitochondrial calcium has a key role in mechanisms of neuroplasticity, such as long-term potentiation (LTP) (Jonas et al. 1999). Intracellular calcium variations occur for a variety of reasons, including activation of NMDA receptors, and mitochondria play a key role in the uptake of this ion in order to control intracellular calcium levels. To evaluate this ability, the mitochondria were exposed to a titration of calcium until their maximum absorption capacity, in the presence of different substrates (Figs. 4 and 5). In Figs. 4a, d we show typical traces of mitochondrial Ca^{2+} uptake in the presence of pyruvate/malate or succinate, respectively. Although the maximum calcium retention capacity is the same for all MK-801 treated groups (Fig. 4c, f), the uptake kinetics is different in all drug treated groups relative to saline group (Fig. 4b, e, closed circles, dotted lines), starting from 400 μM in the presence of pyruvate/malate and from 300 μM succinate. In the presence of both pyruvate/malate or succinate, NMDA blockage led to a slowdown in calcium uptake into the mitochondria, observed in the graphs by a shift to the left of the titration curves, which implies a Ca^{2+} retention deficit (Fig. 4b, e). This displacement indicates a higher concentration of calcium outside the mitochondria and bound to the fluorescent probe CaGreen after the time set for titrations (increased residual baselines). This result suggests that the kinetic of calcium uptake is altered in the MK-801 treated groups.

In the data presented in Fig. 5 the same experiment was conducted, but in the presence of multiple substrates feeding the electron transport system. Under this condition, the effect of NMDA blockade was exacerbated. Figure 5a shows a schematic representation of the Ca^{2+} titration. There is a significant shift to the left for all treated groups compared to the control group (Fig. 5b). Furthermore, the maximal Ca^{2+} retention capacity is reduced in the MK-801 treated groups in the presence of multiple substrates (Fig. 5c). Together, these results indicate that not only the kinetic of calcium uptake is affected by NMDA blockage but also the more physiological condition provided by the presence of multiple substrates exacerbates this effect.

## Effect of calcium in O$_2$ consumption and in H$_2$O$_2$ production

Since calcium handling is altered in the mitochondria of MK-801-exposed animals, we wondered whether, in the face of a stressful situation, such as an increase in calcium concentration, differences in oxygen consumption and H_2O_2 generation would be detected. To address this issue, we subjected the mitochondria to a condition of excess calcium (200 μM) as a stressor and performed the measurements in the presence of multiple substrates.

Figures 6 shows representative experiments of oxygen consumption (Fig. 6a) or H_2O_2 production (Fig. 6c). The stressful condition unmasked a drastic reduction in oxygen consumption by mitochondria from animals exposed to MK-801 in the highest doses (0.3 and 1.0 mg/kg) compared to saline and MK-801 0.1 mg/kg groups (Fig. 6b). The presence of calcium completely abolished oxidative phosphorylation in mitochondria from animals exposed to the highest doses of MK-801 (Fig. 6b). In addition, these doses of 0.3 and 1 mg/kg of MK-801 led to an impressive increase in H_2O_2 production in the presence of calcium (Fig. 6d). In this condition, the basal
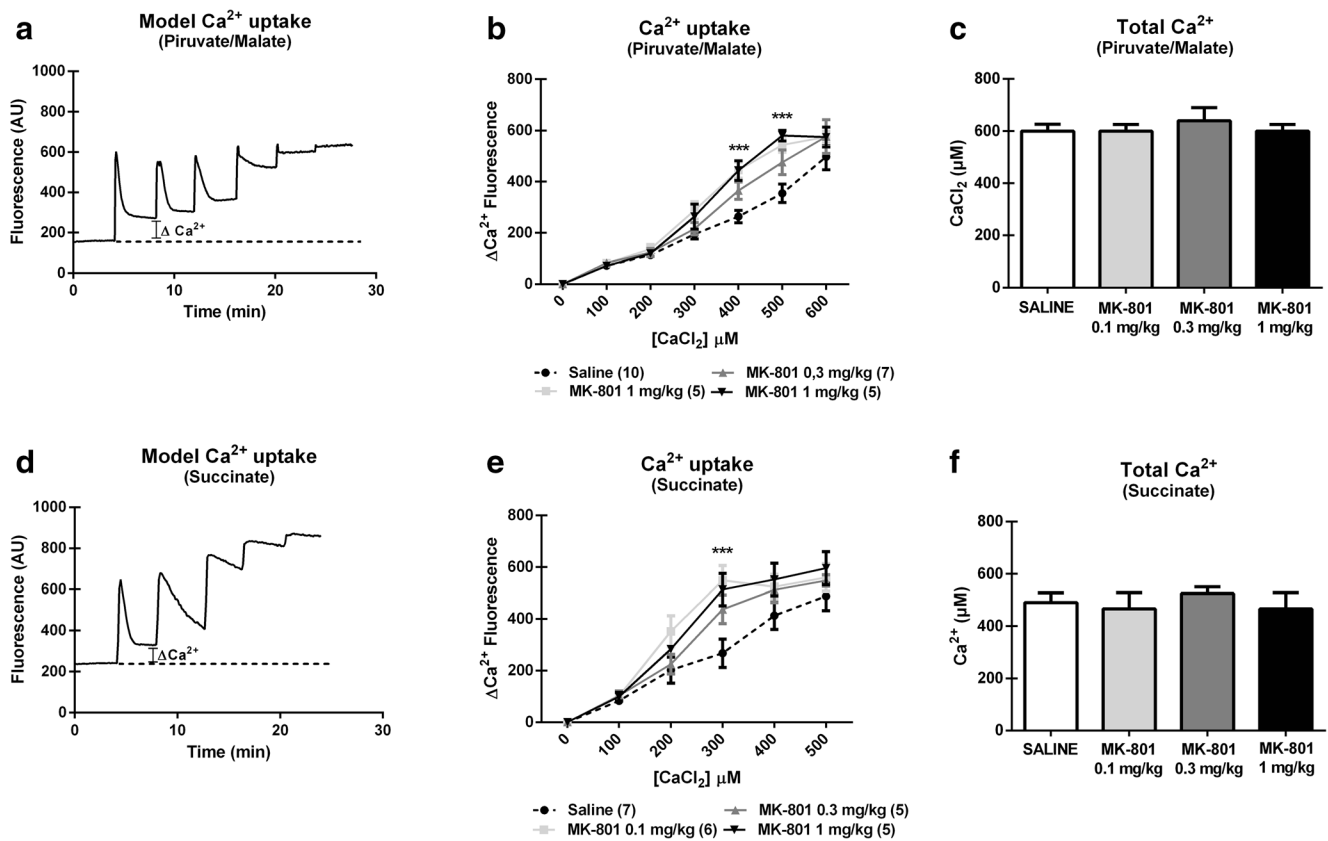
Fig. 4 Mitochondrial Ca²⁺ uptake in MK-801 treated mice mitochondria in the presence of isolated substrates. a and d Representative experiments of calcium uptake curves in the presence of (**a**) pyruvate/malate or (**d**) succinate. Each peak represents the addition of 100 μM $CaCl_2$. Additions were made every 4 min until maximal Ca²⁺ uptake. The baselines after each addition were used to calculate the ΔCa^{2+} fluorescence, after subtraction of the initial baseline. **b** and **e** Calcium uptake curves in the presence of (**b**) Pyruvate/Malate or (**e**) Succinate. **c** and **f** Maximal calcium absorption in the presence of (**c**) Pyruvate/Malate or (**f**) Succinate. Data were analyzed by One-way ANOVA with Bonferroni post-test for multiple comparisons and are expressed as the mean ± standard error. *** $p < 0.0001$ vs. Saline group. The numbers of individual experiments are in parentheses
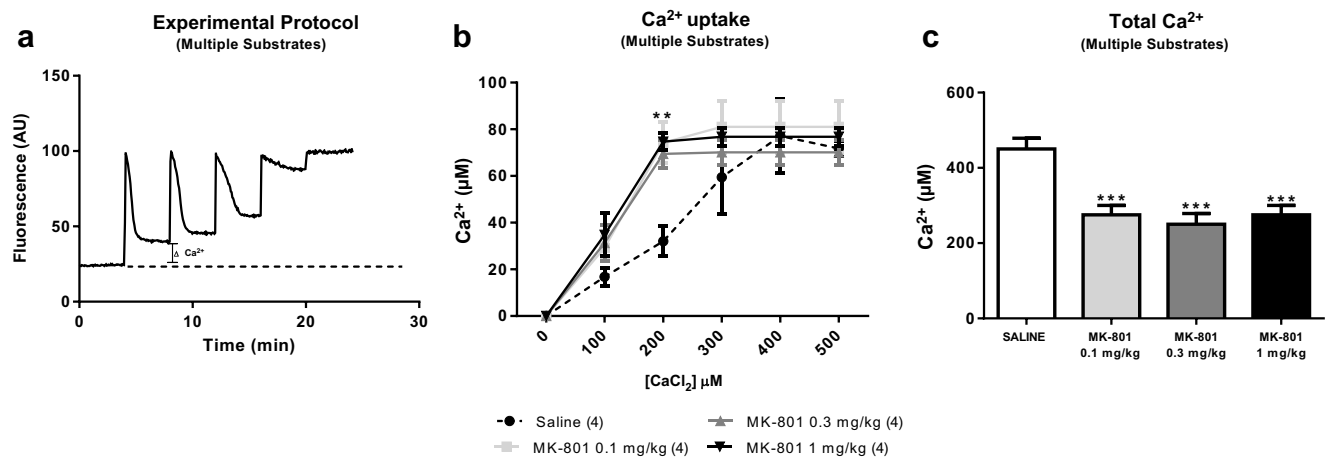
Fig. 5 Mitochondrial Ca²⁺ uptake in MK-801 treated mice mitochondria in the presence in the multiple substrates. a Representative experiments of calcium uptake curves in the presence of pyruvate, malate, glutamate, succinate and glycerol phosphate as substrates. Each peak represents the addition of 100 μM $CaCl_2$. Additions were made every 4 min until maximal Ca²⁺ uptake. The baselines after each addition were used to calculate the ΔCa^{2+} fluorescence, after subtraction of the initial baseline. **b** Calcium uptake curves in the presence of multiple substrates. **c** Maximal calcium absorption in the presence of multiple substrates. Data were analyzed by One-way ANOVA with Bonferroni post-test for multiple comparisons and are expressed as the mean ± standard error. ** $p = 0.0014$ vs. saline group. *** $p = 0.0007$ vs. saline group. The number of individual experiments is in parentheses
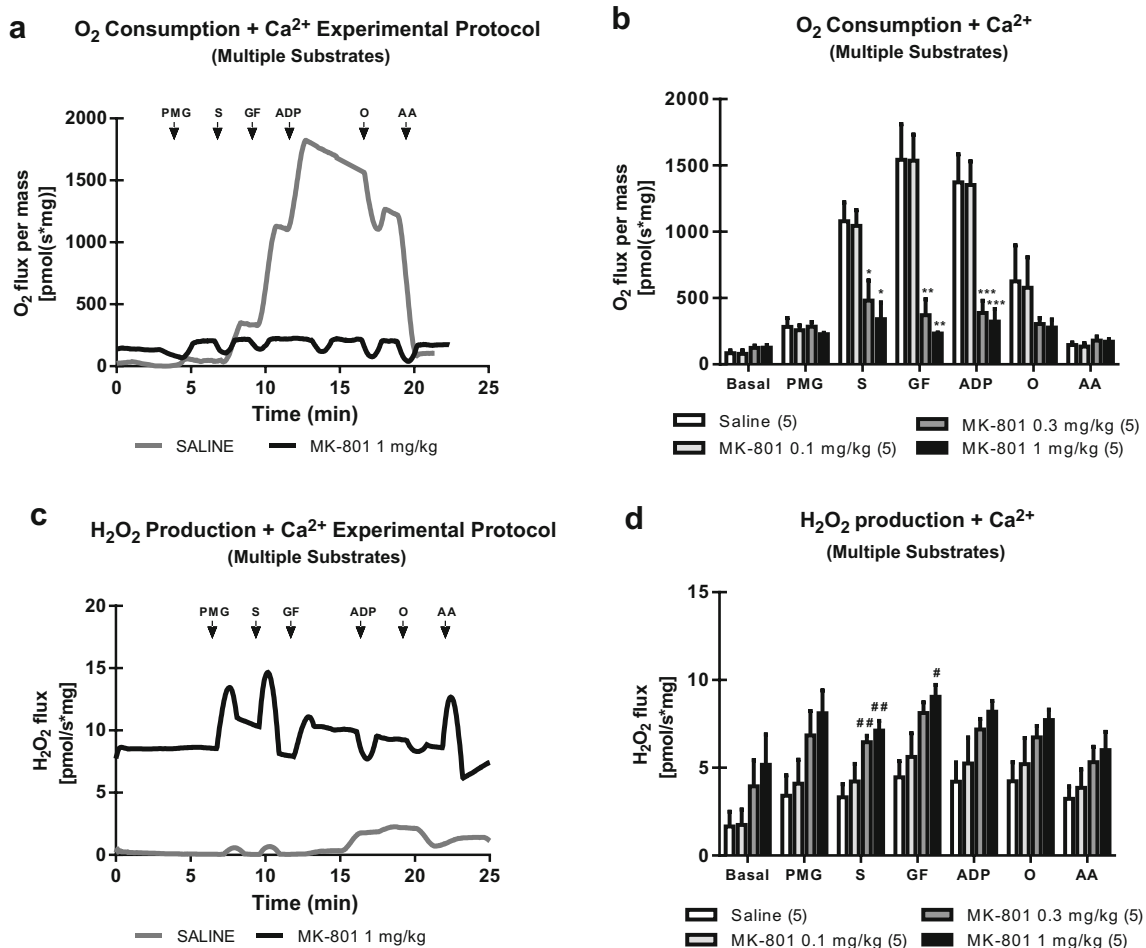
Fig. 6 **Effect of calcium on oxygen consumption and H_2O_2 generation in isolated brain mitochondria of treated mice in the presence of multiple substrates.** **a** and **c** Representative experiments of oxygen consumption (**a**) and H_2O_2 generation (**b**) in the presence of multiple substrates. The arrows represent the moments of the additions. PMG - Pyruvate/Malate/Glutamate (2/2/10 mM); S - Succinate (5 mM); GF - Glycerol Phosphate (5 mM); ADP (1 mM); O - Oligomycin (0.1 μg / mL); AA - Antimycin A (2.5 μM). Experiments were already initiated in the presence of 200 μM $CaCl_2$. **b** High resolution respirometry in the presence of 200 μM $CaCl_2$ and multiple substrates. **d** Production of H_2O_2 in the presence of 200 μM $CaCl_2$ and multiple substrates. Data were analyzed by One-way ANOVA with Bonferroni post-test for multiple comparisons and are expressed as the mean ± standard error. * $p = 0.0018$ vs saline and MK-801 0.1 mg/mL groups; ** $p = 0.0002$ vs saline and MK-801 0.1 mg/mL groups; *** $p = 0.0001$ vs saline and MK-801 0.1 mg/mL groups; # $p = 0.0207$ vs saline group; ## $p = 0.0044$ vs saline group. The numbers of individual experiments are in parentheses

levels of H_2O_2 raised in the groups exposed to the highest doses of MK-801 in relation to the control group and this difference remains until the end of the experiment. It was also observed that the addition of ADP was not able to decrease H_2O_2 levels, in accordance with the loss of phosphorylating capacity (Fig. 6d). Together, these data show that treatment with MK-801 greatly alters oxygen uptake capacity and H_2O_2 generation in mitochondria exposed to high calcium concentrations.

Discussion

The present study shows for the first time that NMDAR blockade by acute MK-801 administration drastically alters calcium handling in brain mitochondria in the presence of multiple substrates, which results in reduction of oxygen consumption, loss of the capacity of ATP production and an increase in H_2O_2 generation.

The acute administration of MK-801 at different doses significantly impaired PPI response (Fig. 1), as expected from other studies (Andine et al. 1999; Varty and Higgins 1995). Importantly, this result relates the MK-801 doses used to impairments found in acutely psychotic patients, validating the model for the analysis of putative psychosis-related mitochondrial alterations in our experimental conditions. The relationship between changes in mitochondrial function and chronic schizophrenia is well established. Various studies described changes in mitochondrial DNA (Iwamoto et al. 2005; Marchbanks et al. 2003), in the expression and function of proteins related to mitochondrial structure (Rosenfeld et al. 2011) and ETS (Electron Transport System) (Ben-Shachar

and Karry 2007; Bergman and Ben-Shachar 2016; Maurer et al. 2001; Prince et al. 1999) as well as functional losses of these complexes in patients (Bergman and Ben-Shachar 2016; Cavelier et al. 1995; Maurer et al. 2001; Prince et al. 1999). However, few studies have focused in unmedicated first episode patients or used animal models of acute NMDA hypofunction to thoroughly assess early acute changes in mitochondrial function that may be important for the disturbance. Most importantly, none of the previous studies have investigated the role of Ca^{2+} handling by brain mitochondria in a condition of multiple substrates. This situation mimics a physiologic stressful state of the mitochondria, which are exposed to large variations in calcium concentrations.

Acute NMDA blockade with MK-801 did not result in alteration in oxygen consumption when mitochondria were exposed to substrates of complex I or complex II in isolation (Fig. 2b, e). These data contrast with previous results published by Millo and Chari-Bitron (1973) where brain mitochondria from rats acutely treated with 10 mg/kg PCP showed a decrease in oxygen consumption in the presence of pyruvate/malate and in the presence of succinate, with a pronounced effect in complex I (Millo and Chari-Bitron 1973). Venancio et al. (2015) also showed a decrease in the activity of complex I with glutamate/malate after acute treatment with high doses of ketamine (Venancio et al. 2015). Differences between the experimental models, antagonists and doses used in each study may explain the divergence, since there is no study that relates the effects of these drugs on different species of rodents nor the equivalence among doses. In addition, it should be noted that the activity of the isolated complex I should be analyzed differently from its activity in an integrated and complex system.

In the same study, Venancio et al. (2015) also verified that mitochondria stimulated with glutamate/malate or with succinate alone showed a high rate of H_2O_2 production, which partially agrees with the present work, that showed higher H_2O_2 production in the presence of ADP only when mitochondria were exposed to substrates for complex I (Fig. 2c, f). In fact, several studies point to an increase in the production of ROS related to psychosis (for review, see (Koga et al. 2016)). Animal models based on repeated exposure to NMDA antagonists also show data consistent with our findings (de Oliveira et al. 2009, 2011; Faizi et al. 2014). For example, Faizi et al. (2014) observed in adult rats treated with 30 mg/kg ketamine for 5 consecutive days the collapse in mitochondrial membrane potential, increase in ROS production, mitochondrial swelling and cytochrome C release (Faizi et al. 2014). de Oliveira et al. (2011) treated adult rats with 25 mg/kg ketamine for one week and observed changes in the enzymatic activity of all mitochondrial complexes in different regions of the brain (de Oliveira et al. 2011). Zhou et al. (2012) observed changes in complexes I, II and III after seven days of treatment with 0.7 mg/kg MK-801(Zhou et al. 2012). Thus,

the changes described in our work might represent an initial acute step that may lead to a more complex mitochondrial disfunction after a longer NMDA hypofunction. However, both changes in oxygen consumption as well as production of ROS seen in all these studies do not consider that, under physiological conditions, mitochondria feeds simultaneously NAD and FAD molecules with electrons derived from different metabolic sources. Moreover, the type and amount of ROS produced depends on the substrate used (Yin et al. 2014). In none of these animal models the oxygen consumption capacity or ROS production were measured in the presence of NAD and FAD electron donors.

In Fig. 3 we observed that, when substrates (NAD and FAD electron donors) other than pyruvate/malate (NAD electron donors) were introduced, the profile of higher H_2O_2 production via complex I was lost. In general, animal models related to psychosis and schizophrenia that evaluated mitochondrial function with isolated substrates found changes related to complex I. In addition, studies with tissue from schizophrenia patients show predominantly deficiencies in the same complex (Bergman and Ben-Shachar 2016). If there is a change in complex I, it is expected a decrease in O_2 consumption and increase in ROS, especially if the experimental condition allows the entry of electrons into the system exclusively via complex I, as in most studies with isolated substrates. It is possible that physiologically, even if there is a defect in a certain mitochondrial complex, the simultaneous oxidation of several substrates may compensate for this deficiency, in order to reduce the impact of this defect on mitochondrial function. Thus, a compensatory effect could justify the results obtained with multiple substrates and reinforces the importance of a more detailed mitochondrial analysis.

As important as the evaluation of ROS production is the analysis of antioxidant systems, since the level of these species in the cells is a consequence of the balance between its production and purification. A huge range of studies shows a correlation between alteration in antioxidant enzyme systems and first episode psychosis or schizophrenia, as recently reviewed by Koga et al. (2016). Despite this, there is still considerable divergence among results for the same enzymes. For example, SOD has decreased activity in approximately half of the studies analyzed and increased activity in the other half, with a few showing no difference (Koga et al. 2016). Glutathione peroxidase presents reduced activity in most studies, but an increase in a few of them (Koga et al. 2016). A metanalysis including four studies in early onset unmedicated psychotic patients showed no differences in the activity of SOD, catalase and glutathione peroxidase when compared to blood samples of control individuals (Fraguas et al. 2017), in accordance with our findings. However, analysis of antioxidant enzymes activity also presents controversial results in other studies. While Venancio et al. (2015) showed an increase in the activity of SOD in acute high dose ketamine model, de

Oliveira et al. (2009) evidenced a reduction in the activity of the enzyme in the same model with smaller doses. In the present study, no significant differences were observed in any of the antioxidant enzymes analyzed (Table 1), suggesting that, in our model, the most plausible explanation for the increase in ROS associated with acute psychosis is a higher production of ROS, and not a decrease in the clearance capacity.

Mitochondria have functions that go beyond the generation of ATP. In addition to act as a metabolic control center, it is associated with various cellular signaling pathways, which include not only the control of ROS generation but also the maintenance of intracellular calcium levels (Brini et al. 2014; Murphy 2009). For this reason, the analysis of mitochondrial function should consider oxygen consumption, ROS production and calcium handling. The analysis of calcium retention capacity in the presence of isolated substrates showed that, although total retention capacity was not altered (Fig. 4c, f) there was a drastic change in the kinetics of the uptake of this ion by mitochondria from animals treated with MK- 801 (Fig. 4b, e). When Ca^{2+} titration was performed in the presence of multiple substrates, a condition considered as more physiological, it was observed that both maximal Ca^{2+} retention capacity and the uptake kinetic were significantly modified by MK-801 treatment (Fig. 5). These data highlight the importance of the approach with multiple substrates when mitochondria function is analyzed since there are differences that can be abolished or revealed only in this scenario.

Although there is no similar data in the literature related to Ca^{2+} handling with acute treatment with MK-801 in mice, there are two recent studies with chronically treated animals with ketamine showing changes in calcium management. Bustamante et al. (2016) treated adult rats with ketamine 40 mg/kg for three consecutive days and observed a decrease in calcium concentration in the mitochondria from the hippocampus of these animals, as well as a reduction in the intracellular levels of Ca^{2+} induced by KCl (Bustamante et al. 2016). Lisek et al. (2016) injected 30 mg/kg ketamine in adult rats for five consecutive days and analyzed cells from various brain regions at the end of treatment, which led to a relevant increase in intracellular $[Ca^{2+}]$ in the cortex, cerebellum, hippocampus as well as altered expression of proteins related to the control of cellular calcium levels (Lisek et al. 2016). Although the experimental models are different and the acute and chronic treatments lead to distinct metabolic consequences, our results and previous evidences are consistent with the relevance of calcium management in psychosis.

The alterations in calcium handling (Figs. 4 and 5) raised a concern on the integrity of the mitochondria when it is exposed to high calcium concentrations. In the presence of calcium, we observed a complete loss of mitochondrial function in the groups treated with the highest doses of MK-801 in the presence of multiple substrates (Fig. 6). In addition, there was

an impressive increase in H_2O_2 production in this condition (Fig. 6d). It is noteworthy that, in the presence of multiple substrates but without the calcium challenge, MK801-treated mitochondria presented exactly the same profile of oxygen consumption and H_2O_2 production as the control group (Fig. 3). Only under a stressful situation the difference emerges.

Ca^{2+} is an important second messenger that controls various metabolic pathways and is involved in fundamental processes, such as synaptic transmission, learning and memory (Brini et al. 2014; Friel 2000). The activation of voltage-gated channels, transmembrane receptors may lead to an increase of cytoplasmic calcium, which rapidly changes from nanomolar to micromolar concentrations (LaFerla 2002). The endoplasmic reticulum and the mitochondria are the main organelles involved in storage and release of intracellular calcium after the increase observed during depolarization, for example. In addition to Ca^{2+} uptake, mitochondria are also responsible for providing ATP to plasma membrane ATPases that transport Ca^{2+}. The entrance of Ca^{2+} in mitochondria via Mitochondrial Calcium Uniporter (MCU) leads to an increase in the activity of several mitochondrial enzymes that will ultimately rise ATP generation (Brini et al. 2014). For this reason, mitochondria need to be able to tolerate large variations in Ca^{2+} concentration without losing the phosphorylative capacity or the control of ROS generation. However, as seen in Fig. 6, both processes become extremely affected by MK-801 acute treatment. The role of synaptic and non-synaptic mitochondria in neuroplasticity through appropriate calcium buffering may involve MCU (Kirichok et al. 2004; Penna et al. 2018), the interaction of endoplasmic reticulum with mitochondria (Park et al. 2017), and/or a fine regulation of permeability transition pore opening (Jonas et al. 1999; Mnatsakanyan et al. 2017). Our data give support for the proposal that alterations in one or more of these crucial steps may result in important cellular dysfunctions (Figs. 4, 5 and 6). However, at present, we do not know which of these targets are involved.

Schematic representations of control mitochondria (Fig. 7a) and MK-801-exposed mitochondria (Fig. 7b) highlight alterations in the mitochondrial function caused by high calcium concentrations in both cases. Data show that mitochondria derived from MK-801 treated animals present two negative characteristics concerning Ca^{2+} handling: (1) they have a reduced capacity of Ca^{2+} uptake and (2) they are extremely sensitive to incoming Ca^{2+}, which abolishes oxidative phosphorylation and increases the generation of H_2O_2 in the condition of multiple substrates. On a real situation, where these mitochondria are in an active neuron, the result would be catastrophic, leading to a longer time for the clearance of Ca^{2+} from the cytosol, a drastic decrease in ATP production and an increase in H_2O_2 production. Altogether, this could impact several other cellular processes. In long term, the result of the impairment in Ca^{2+} management by mitochondria could

Fig. 7 Schematic
representation of mitochondria
fed by multiple substrates in the
presence of calcium. In the
scheme, mitochondrial
complexes attached to the
membrane are represented as
indicated. OMM – Outer
Mitochondrial Membrane; IMM
– Inner Mitochondrial
Membrane; CI – NADH
Dehydrogenase; CII – Succinate
Dehydrogenase; CIII –
Ubiquinone Cytochrome C
Oxidoreductase; CIV –
Cytochrome C Oxidase; CV –
ATP Synthase. Solid black lines
represent the electron pathway
through the ETS and the passing
of protons through complex V
resulting in ATP formation.
Dotted black lines indicate the
formation of superoxide as a
result of the electron transport.
Thick light blue arrows represent
the proton pumping from the
mitochondrial matrix to the
intramembrane space through the
indicated complexes. Red solid
arrow indicates the entering of
Ca^{2+} through Mitochondrial
Calcium Uniporter (MCU).
Mitochondria representations of
saline (**a**) or MK-801 treated (**b**)
groups show the consequences of
calcium entrance in the matrix
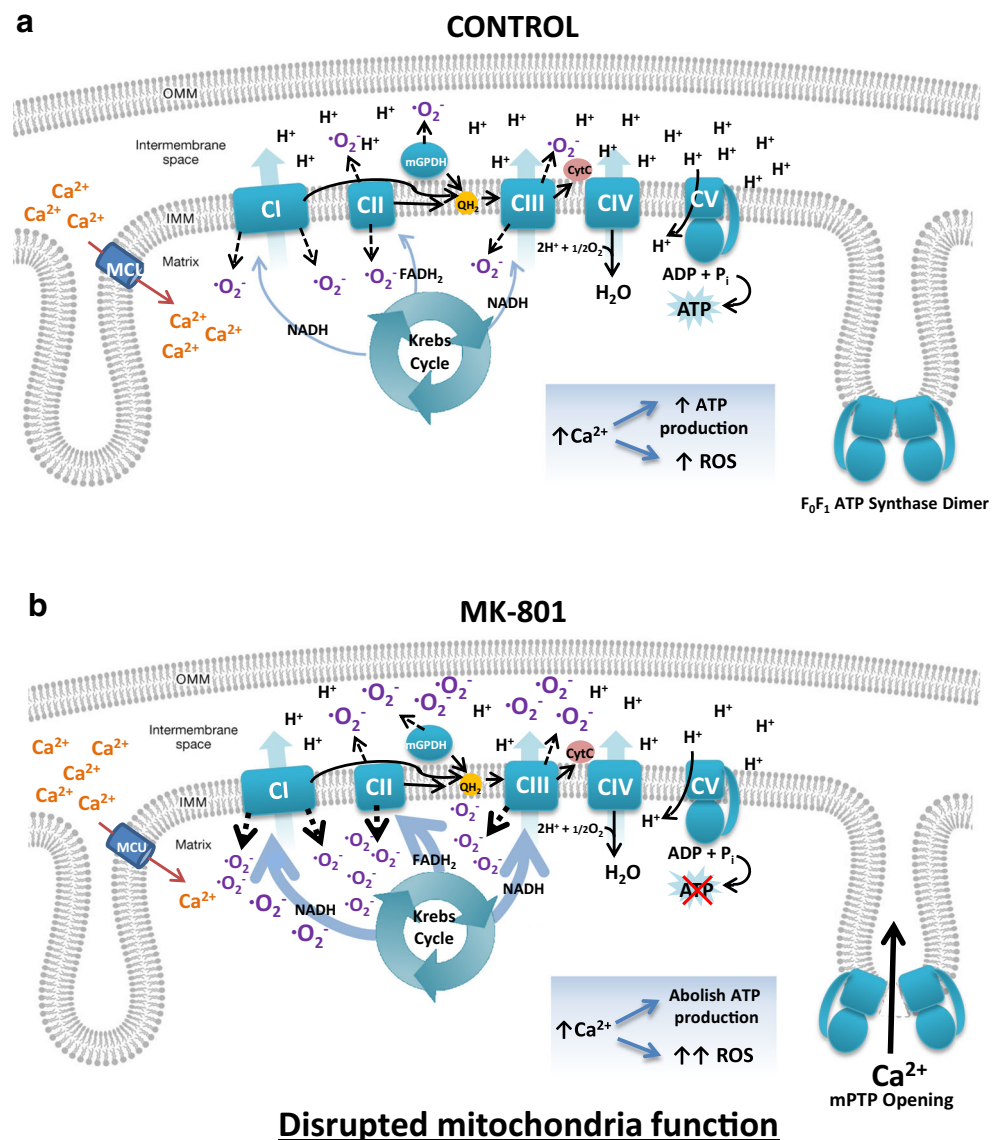
lead to a pathological context with dysregulation of neural
function.

Changes in critical cellular pathways, such as calcium sig-
naling, are in focus and are proposed to be the main basis for
pathologies with multiple potential targets (Sawa and Snyder
2002). Since early 80's this proposal has been based on sev-
eral studies that point to a dysregulation in intracellular calci-
um signaling in schizophrenia (Lidow 2003). Acute blockade
of NMDAR is related to an increase in glutamate levels in key
brain regions (Moghaddam et al. 1997). The excess of this
neurotransmitter may activate all glutamate receptors, such
as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
receptor (AMPAR), kainic acid receptors (KAR) and metab-
otropic glutamate receptors (mGluR) that, ultimately, raises
the intracellular calcium levels in these cells in a process that
may lead to glutamate excitotoxicity (for review see (Kritis
et al. 2015)). Thus, the deficit in mitochondrial calcium

buffering could be explained by a previous rise in this ion
due to an overflow of glutamate promoted by MK-801 treat-
ment. Another possibility could be the rising in intracellular
calcium concentration promoted by dopamine, since it is well
established that NMDAR antagonists, such as MK-801, lead
to dopamine release in the striatum and that dopamine recep-
tor activation also result in increasing intracellular calcium
concentrations (Breier et al. 1998; Hasbi et al. 2010;
Whitton et al. 1992). It is important to highlight that, in the
presence of calcium, mitochondria derived from treated
groups are no longer able to generate ATP aerobically,
resulting in a drastic energy imbalance. In this scenario, there
is also a severe REDOX deregulation, caused by the signifi-
cant increase in H_2O_2 production that could culminate in ab-
normal cellular signaling. Changes in glutamate signaling are
related with the signs and symptoms of psychosis and the
observed imbalance in energy production, REDOX

equilibrium and calcium handling disruption might represent initial changes occurring in acute psychotic episodes that will possibly contribute to changes in neural circuits that can underline the emergence of the syndrome.

In summary, our data show that the analysis of mitochondrial function in the condition of isolated substrates should be carefully evaluated, since the mitochondria is able to oxidize several substrates simultaneously and to compensate for the deficiencies that may exist linked to a single mitochondrial complex. Moreover, the changes demonstrated in this study regarding the management of calcium in the presence of multiple substrates may have several implications in the context of acute psychosis and, possibly, schizophrenia and contribute to the hypothesis that the management of calcium may be a trigger for the pathophysiological changes seen in this complex pathology.

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