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Metyrapone effects on systemic and cerebral energy metabolism

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ABSTRACT

Metyrapone is a cytochrome P_{450} inhibitor that protects against ischemia- and excitotoxicity-induced brain damages in rodents. This study examines whether metyrapone would act on energy metabolism in a manner congruent with its neuroprotective effect. In a first investigation, the rats instrumented with telemetric devices measuring abdominal temperature, received i.p. injection of either metyrapone or saline. One hour after injection, their blood and hippocampus were sampled. Hippocampus metabolite concentrations were measured using ^{1}H high-resolution magic angle spinning-magnetic resonance spectroscopy (^{1}H HRMAS-MRS). The hippocampus levels in phosphorylated mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK) were measured by Western Blot analysis and those of c-fos and HSP70-2 mRNA were quantified by RT-PCR. In a second investigation, the rats received the same treatment and were sacrificed 1 h after. The functioning of mitochondria was immediately studied on their whole brain. Metyrapone provoked a slight hypothermia which was correlated to the increase in blood glucose concentration. Metyrapone also increased blood lactate concentrations without modifying hippocampus lactate content. In the hippocampus, metyrapone decreased γ-aminobutyric acid (GABA) and glutamate levels but increased glutamine and N-acetyl-aspartate contents (NAA). Phosphorylated mTOR and AMPK and the c-fos and HSP70-2 mRNA levels were similar between treatment groups. Metyrapone did not modify blood corticosterone levels. Mitochondrial oxygen consumption was similar in both groups whatever the substrate used. These metabolic modifications, which take place without modifying blood glucocorticoid levels, are consistent with the neuroprotective properties of metyrapone as demonstrated in animal models.

1. Introduction

Metyrapone is a cytochrome P_{450} inhibitor that exhibits neuroprotective effects in animal models. It reduces ischemia-induced damages in the hippocampus of rats (Krugers et al., 1998, 2000; Smith-Swintosky et al., 1996) and gerbils (Adachi et al., 1999). Metyrapone also reduces kainic acid-induced brain injury (Smith-Swintosky et al., 1996; Stein and Sapolsky, 1988) and HSP70 expression (Czyrak et al., 2000). The mechanisms involved in the metyrapone-induced neuroprotection deserve discussion.

Since metyrapone blocks glucocorticoid synthesis (Jenkins et al., 1958), it has been suggested that the protective effect of metyrapone could be related to the limitation of glucocorticoid production. This was supported by the deleterious effect of corticosterone supplementation together with the protective effect of metyrapone on damages induced by hypoxia, ischemia or kainate (Krugers et al., 2000; Smith-Swintosky et al., 1996) and alterations in CA1 field potentials (Krugers et al., 2000). However, the corticosterone co-treatment, does not (Krugers et al., 1998) or not completely (Smith-Swintosky et al., 1996) reverse the neuroprotective effect of metyrapone, suggesting that a part of this effect might be independent from glucocorticoid synthesis inhibition. Consequently, other factors addressing to brain metabolism should be evaluated. Firstly, metyrapone may act through inducing hypothermia since it has been shown to occur in guinea-pig (Werner, 1988) and rats (Drouet et al., 2010; Michel et al., 2007b). In turn, hypothermia would be protective by acting on several pathways, including the decrease in extracellular glutamate levels and the improvement of ATP recovery (Zhao et al., 2007). Secondly, metyrapone may act through cell energy metabolism modifications. Hyperglycemia is known to enhance ischemia-induced brain damages (Li et al., 1999; Payne et al., 2003) at least by favoring high extracellular glutamate levels (Li et al., 1999). Metyrapone which induces hyperglycemia (Rotllant et al., 2002;

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Werner, 1988) also decreases cerebral glucose uptake (Bruno et al., 1972), and blocks the hyperglycemia-induced aggravation of ischemic damages (Payne et al., 2003). However, this leads to the following paradox: metyrapone reduces brain glucose metabolism and limits ischemia-induced brain damages. As a consequence, metyrapone might act on other brain metabolism pathways to produce energy for cells.

The aim of this study was therefore to determine the ways by which metyrapone may act to explain its neuroprotective effects in animals. Metabolic pathways were studied in the hippocampus through the simultaneous assessment of the concentration of 18 metabolites in intact biopsies including glutamate, γ-aminobutyric acid (GABA) glutamine and N-acetylaspartate (NAA) using ^{1}H high-resolution magic angle spinning magnetic resonance spectroscopy (^{1}H HRMAS MRS). NAA is mainly synthesized in neuron and its exact function remains unknown, even if several hypotheses have been proposed. However, NAA is frequently quoted as a marker of mitochondrial function since NAA synthesis and energy availability would be biochemically linked in mitochondria (Demougeot et al., 2004; Moffett et al., 2007; Patel and Clark, 1979) and (ii) the mitochondrial oxygen consumption in the forebrain. Hippocampus energy status was determined by the assessment of (i) adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways activities as metabolic sensors (Dennis et al., 2001; Hardie, 2004) and (ii) HSP70-2 mRNA levels as its transcription is induced by energy deprivation (Imuta et al., 1998).

2. Materials and methods

2.1. Animals

The investigation was conducted in 53 male OFA Sprague–Dawley rats (Janvier, Le Genet-St-Isle, France) weighing 175–200 g upon arrival at the laboratory. The rats were housed three per cage (26×40×15 cm) in a room at constant ambient temperature (23±2 °C), constant relative humidity (50±10%), and under a 12 h light–12 h dark cycle (light-on at 08h00). Ten days were allowed to the rats to be accustomed to laboratory environment. All experimental procedures were reviewed and approved by the institutional ethics committee for animal care and performed in accordance with the principles of animal care (NIH publication no. 86-23, revised 1985) and the 24 November 1986 European Community Council Directive (86/609 EEC).

2.2. Drugs

Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was purchased from Sigma-Aldrich (St-Quentin Fallavier, France). It was used at the dose of 150 mg/kg. The individual dose was suspended in 1 ml sterile saline and immediately injected i.p. The control rats received the same volume of sterile saline.

2.3. Experimental design

Two separate experiments were carried out. The first experiment was dedicated to energy metabolism analysis. Thirty-three rats were equipped with TA10TA-F40 telemetric devices allowing abdominal temperature assessment. After recovery, the rats were randomly distributed into 2 groups according to the saline (n=16) or metyrapone (n=17) treatment. On the experimental morning, the rats received their treatment i.p. and were left undisturbed in their home cage during 1 h to record abdominal temperature. They were then quickly decapitated and trunk blood was sampled into tubes containing lithium heparinate (Sarstedt, Marnay, France). Blood was centrifuged (4 °C, 4500 *g* for 5 min) and plasma was aliquoted and stored at −80 °C until analysis. The hippocampus was dissected and cut transversally: the ventral part was immediately frozen in liquid nitrogen for HRMAS MRS and Western Blot analysis. Samples were kept at −80 °C until analysis. The dorsal part was put into RNAlater® (Ambion, Courtaboeuf, France) for mRNA transcription analysis. Samples were then placed at 4 °C for 24 h before being stored at −20 °C until analysis.

The second experiment aimed at studying mitochondrial functioning. On the experimental morning, the 20 rats received randomly either metyrapone (n=10) or saline (n=10) and were left undisturbed during 1 h. They were then decapitated and their brain was quickly removed. The brain mitochondria were prepared according to Rosenthal et al.'s (1987) method in ice-cold buffer MSHE containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA pH 7.4. Mitochondrial protein concentration was determined by a bicinchoninic acid assay (Pierce® BCA proteins assay).

2.4. Physiological variables

Implantable TA10TA-F40 radio transmitters were used to record abdominal temperature. The telemetric signal was acquired with a DataQuest system running on ART-gold software 4.1 (Data Sciences, Saint-Paul, Mn, USA). The TA10TA-F40 sensor was surgically inserted in the abdominal cavity under deep anesthesia (pentobarbital sodium, 60 mg/kg, i.p.) according to a procedure described elsewhere (Michel et al., 2007a). Briefly, a sagittal abdominal incision was made and the telemetric device placed in the peritoneal cavity. Muscles and skin were then stitched up. Immediately after surgery, the rats received antibiotic (Extencilline®, Sanofi-Aventis, 60,000 IU per rat, i.p.) and analgesic treatments (Finadyne®, Schering-Plough, Segre, France, 1 mg/kg, s.c.). The rats were allowed 10 days to recover from surgery.

Abdominal temperature was measured during 10 s every 2 min. Abdominal temperature values were then averaged into 15-min time periods during the hour preceding and following the injection. The 15-min epoch preceding sacrifice was used for correlation analysis.

2.5. Biological variables

2.5.1. Blood variables assessment

Plasma corticosterone concentration was analyzed using specific radioimmunoassay kits ([^{125}I] RIA kit, DPC France, La Garenne Colombes, France). Plasma glucose, lactate, triglycerides and cholesterol were assayed on a Hitachi 912 Analyzer (Roche Diagnostics, Meylan, France) with the colorimetric method using Roche™ reagents (Roche Diagnostics). All analyses were performed according to manufacturer's instructions.

2.5.2. mRNA quantification by RT-PCR

The mRNA quantification of HSP70-2 (NM_212504.1), c-fos (NM_022197.2), CycA (NM_017101.1), HPRT (NM_012583) and ARBP (NM_022402.1) was done using the following primers: HSP70-2 (FW: ACCATCGAGGAGGTGGATTAGAGG; RW: ACCAGCAGCCATCAAGAGTCT GTC), c-fos (FW: CGGAGAATCCGAAGGGAAAG; RW: TGGCAATCTCG GTCTGCAAC), Cyca (FW: GGCAAATGCTGGACCAAACAC; RW: CTTCCCA AAGACCACATGCTTG), HPRT (FW: CTCATGGACTGATTATGGACAGGAC, RW: GCAGGTCAGCAAAGAACTTATAGCC), ARBP (FW: CCTGCACACTCG CTTCCTAGAG; RW: CAACAGTCGGGTAGCCAATCTG).

The mRNA extraction was carried out on a MagNA Pure LC mRNA Isolation Kit II (Roche Applied Science, Mannheim, Germany). Reverse transcription was performed using oligo-dT (Reverse Transcription Core Kit, Eurogentec, Seraing, Belgium). Real-time PCR was carried out with the Light Cycler Fast Start DNA Master SYBR Green kit (Roche Applied Science) using LightCycler (Roche Applied Science). Quantification cycles were assessed using the second derivative maximum method. The specificities of the PCR amplification were documented with the LightCycler melting curve analysis. The quantification was achieved using a pool of cDNA samples as

calibrator according to the comparative threshold cycle method. The normalization was done using geometric average of the three internal validated control gens, Cyca, HPRT and ARPB.

2.5.3. Protein quantification by Western Blot

Frozen samples of the hippocampus were homogenized in 15 volumes of ice cold RIPA buffer (Tebu-Bio, Le Perray-en-Yvelines, France). Protein concentration was determined by the Bradford dye-binding method. The protein extract obtained from each sample was separated by SDS-PAGE (5% stacking gel, 8% resolving gel) and transferred to nitrocellulose membrane (GE Healthcare Amersham, Orsay, France). Membranes were incubated overnight at 4 °C with the appropriate primary antibody: (i) the mTOR pathway: phospho-mTOR (Ser2448), mTOR, phospho-S6 Ribosomal Protein (Ser 240/244)(phospho-S6R), S6R, phospho-p70 S6 kinase (Thr389), p70 S6 kinase and (ii) the AMPK pathway: phospho-AMPKα (Thr172), AMPKα, phospho-Acetyl-CoA Carboxylase (Ser79) (phospho-ACC), ACC and β-actin (Cell Signalling Technology, Danvers, MA, antibodies nos. 2971, 2972, 4838, 2217, 9205, 9202, 2535, 2603, 3661, 3676 and 4970, respectively). The protein bands were detected using Enhanced Chemiluminescence (ECL) Western Blot System and visualized by exposure to Hyperfilm ECL film (GE Healthcare Amersham, Orsay, France). Specific bands were quantified using GS-800 Calibrated Densitometer and Quantity one 1-D Analysis software version 4.6.1 (BioRad, Hercules, CA, USA). Membranes were stripped out of the phosphorylated antibody before reprobing with the corresponding total antibody, as recommended by the manufacturer. For quantization analysis, the mean density of bands under study was calculated and normalized by the amount of β-actin for total protein. The results are presented as ratio of phosphorylated protein to total protein.

2.5.4. ^{1}H HRMAS MRS

2.5.4.1. Sample preparation. Approximately 15 mg of the frozen biopsies was rapidly introduced in a 4 mm ZrO_2 rotor and a cold 1 mM D_2O solution of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP) was added as chemical shift internal standard ($\delta = 0$ ppm). The rotor was fitted with a 50 µl spherical insert and then transferred in the NMR probe, which had been previously cooled at +4 °C. The whole HRMAS NMR study was performed at +4 °C. The acquisition was started immediately after 7 min at equilibrium.

2.5.4.2. HRMAS data acquisition. Spectra were recorded on a Bruker Avance 400 spectrometer (proton frequency 400.13 MHz), equipped with a 4 mm ^{1}H-^{13}C-^{31}P HRMAS probe-head. Samples were spun at 4000 Hz. 1D spectra were acquired with a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence to attenuate macromolecule and lipid resonances, synchronized with the spinning rate (interpulse delay 250 µs, total spin echo time 30 ms). Residual water signal was presaturated during the 2-s relaxation delay time. Total acquisition of one spectrum with 256 scans lasted 16 min. Resonance assignment was performed as previously described (Rabeson et al., 2008).

2.5.4.3. Data processing. Metabolite quantification was performed with the software package java-based magnetic resonance user interface (jMRUI) using the "subtract-QUEST" procedure. This procedure uses a simulated metabolite database set including: acetate (Ace), alanine (Ala), aspartate (Asp), creatine and phosphocreatine (Pcr), choline (Cho), γ-aminobutyric acid (GABA), glutamate (Glu), glutamine (Gln), glutathione (Gsh), glycerophosphocholine (Gpc), glycine (Gly), lactate (Lac), myo-inositol (M-ins), N-acetylaspartate (NAA), phosphoethanolamine (PE), phosphocholine (PC), and taurine (Tau). The first 16 data points were used to model the background signal of macromolecules and lipids, which were not totally removed by the 30-ms CPMG. The metabolite-only signal can thus be simulated. The amplitude of metabolites calculated by QUEST was normalized to the total spectrum signal and then only relative concentrations were produced. Reliability of quantification was evaluated using the Cramer Rao lower bounds (CRLB) determined by the jMRUI algorithm, which are estimates of the Standard Deviations of the fit for each metabolite.

2.5.5. Mitochondrial respiration

Mitochondrial oxygen consumption was measured polarographically at 30 °C using a Clark type oxygen electrode in a Mitocell S200® (Strathkelvin Instruments, Glasgow, United Kingdom).

Mitochondria (1 mg) were suspended in 1 ml "KET" medium containing 125 mM KCl, 1 mM EGTA, 20 mM Tris pH 7.2 with 0.1% BSA and 5 mM Pi. Oxygen consumption rate was measured after addition of 300 µM ADP (respiration state 3) or 75 µM DNP (uncoupling state) with 5 mM glutamate plus 2.5 mM malate (complex I) or 5 mM succinate (complex II) or 2 mM ascorbate plus 50 µM tetramethyl-p-phenylenediamine (TMPD) (complex IV) as substrates.

2.6. Statistical analysis

Statistical analysis was performed with Statistica software v7.1 (StaSoft-France, Maisons-Alfort, France). Time courses of abdominal temperature were analyzed using analysis of variance (ANOVA) for repeated measures. Comparisons between treatments were done by one factor ANOVA. Correlations between variables were assessed using linear regression analysis. The significance level was set at $P<0.05$. Values were presented as means ± standard error of the mean (S.E.M.).

3. Results

3.1. Physiological and biochemical effects of metyrapone

After injection, saline rats exhibited a stress-induced hyperthermia after injection. Metyrapone blocked this reaction (Fig. 1A; metyrapone, $P<0.001$; and metyrapone × repetition, $P<0.001$) and induced a hypothermia 45–60 min after the injection (saline: 37.3 ± 0.1 °C; metyrapone: 36.2 ± 0.2 °C, $P<0.001$).

Metyrapone did not affect blood corticosterone levels (Table 1). Compared to saline, metyrapone increased plasma glucose ($P<0.001$), lactate ($P<0.001$), creatinine ($P<0.001$) and urea ($P<0.01$) concentrations. Metyrapone did not modify blood triglycerides, cholesterol ($P=0.066$) and protein levels. Plasma glucose concentration was negatively correlated to abdominal temperature in Metyrapone rats (Fig. 1B; $r^2=0.58$; $P<0.001$), but not in saline rats (Fig. 1B; $r^2=0.03$; $P=0.53$). Lactate was not correlated to abdominal temperature.

3.2. Effect of metyrapone on the hippocampus

A typical ^{1}H HRMAS NMR spectrum of rat hippocampus 1 h after metyrapone administration is represented in Fig. 2. Metyrapone did not modify lactate, alanine (Table 2), acetate, aspartate, creatine, choline, glutathione, glycerophosphocholine, glycine, myo-inositol, phosphocreatine, phosphoethanolamine, phosphocholine, and taurine levels (data not shown). Compared to saline, metyrapone decreased GABA ($P<0.01$) and glutamate ($P<0.05$) concentrations in the hippocampus (Table 2). Conversely, metyrapone induced a significant increase in glutamine ($P<0.01$) and NAA ($P<0.05$) levels as compared to saline-treated rats (Table 2). No correlation was observed between glutamine, glutamate, GABA and blood glucose, abdominal temperature.

Metyrapone did not change c-fos and HSP70-2 mRNA levels in the hippocampus (Table 2). No difference in hippocampus protein content was observed between treatment groups for mTOR (saline: 0.98 ± 0.10; metyrapone: 1.16 ± 0.08, ns.), p70 S6K (saline: 0.98 ± 0.05;

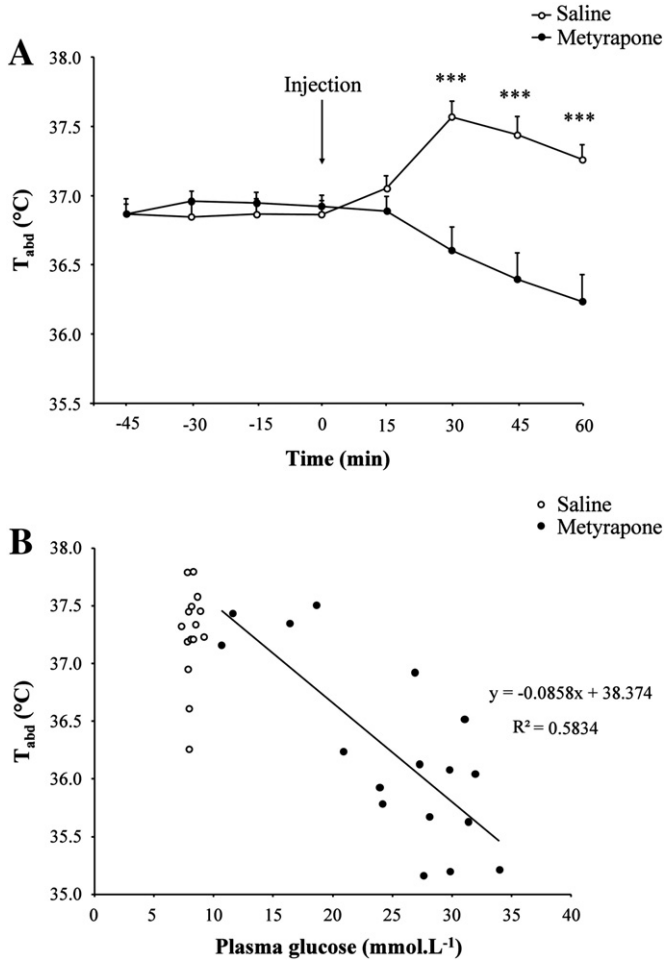

Fig. 1. Effect of metyrapone administration (150 mg/kg i.p.) on abdominal temperature. A. Time course of abdominal temperature (A, °C) in saline (saline: ○, n = 16) and metyrapone (metyrapone: ●, n = 17) rat groups. Comparisons between groups were performed with factorial ANOVA and significant differences are indicated as follows: *: P<0.05 and ***: P<0.001. Data are expressed as means ± S.E.M. B. Correlations between plasma glucose levels (mmol/l) and final (45–60 min) abdominal temperature (B, °C) in saline (○; n = 16) and metyrapone (●; n = 17) rats.

metyrapone: 1.12 ± 0.08, ns.), S6R (saline: 0.94 ± 0.09; metyrapone: 1.02 ± 0.09, ns.), AMPk (saline: 1.04 ± 0.07; metyrapone: 1.07 ± 0.06, ns.) and ACC (saline: 1.17 ± 0.14; metyrapone: 1.21 ± 0.13, ns.). Metyrapone treatment did not affect the ratio of phosphorylated protein/total protein of mTOR, p70 S6K, S6R, AMPKα and ACC (Fig. 3).

Table 1
Effects of metyrapone (150 mg/kg i.p., n = 17) or saline injection (i.p., n = 16) on glucose (mmol/l), lactate (mmol/l), triglycerides (mmol/l), cholesterol (mmol/l), creatinine (μmol/l), urea (mmol/l), protein (g/ml), and plasma corticosterone (ng/ml). Comparisons between groups were performed with factorial ANOVA and significant differences are indicated as follows: 'a': P<0.01; 'b': P<0.001. Data are expressed as means ± S.E.M.

	Saline	Metyrapone
Corticosterone	111.21 ± 13.17	93.16 ± 6.14
Glucose	8.16 ± 0.12	24.94 ± 1.71 [b]
Lactate	2.46 ± 0.12	4.68 ± 0.21 [b]
Triglycerides	1.65 ± 0.1	1.7 ± 0.08
Cholesterol	2.28 ± 0.05	2.14 ± 0.05
Creatinine	24.19 ± 0.59	33.59 ± 1.12 [b]
Urea	5.41 ± 0.16	6.29 ± 0.20 [a]
Protein	58.63 ± 0.62	58.51 ± 0.54

3.3. Effect of metyrapone on brain mitochondria

The maximum functioning of mitochondria was not altered after acute treatment with metyrapone: the oxygen consumption measurements of brain mitochondria were similar between treatment groups whatever the substrates employed (Table 3).

4. Discussion

In this study, we show that metyrapone may modify several pathways known to be involved in neuroprotection, such as glutamine–glutamate–GABA cycle, and glucose-lactate metabolic shift.

We observed hypothermia in the metyrapone-treated rats. The decrease in body temperature had been previously reported in rats (Drouet et al., 2010; Michel et al., 2007b) and guinea pigs (Werner, 1988), but not in mice in which metyrapone only potentiates the cannabinoid-induced hypothermia (Pryce et al., 2003). In spite of the neuroprotective properties of hypothermia (Zhao et al., 2007), it is unlikely that hypothermia supports the neuroprotective activity of metyrapone as the latter was observed in anesthetized animals kept warm (Adachi et al., 1999; Krugers et al., 1998, 2000; Smith-Swintosky et al., 1996). Alternatively, both hypothermia and neuroprotection may be the consequence of a depression in glucose metabolism since 2-deoxy-D-glucose treatment produces the same effects (Niwa et al., 1999; Wei et al., 2003). In that perspective, the decrease in body core temperature would be the consequence of a global inhibition of energy metabolism. This hypothesis is supported by the fact that metyrapone decreases both oxygen consumption (Werner, 1988) and glucose utilization (Bruno et al., 1972) and increases blood glucose concentration (Drouet et al., 2010; Rotllant et al., 2002; Werner, 1988). This effect is consistent with correlation we observed between the decrease in body temperature and the extent of hyperglycemia.

Such a decrease in glucose metabolism does not occur in a context of decreased cerebral energy expenditure. The waking effect of metyrapone immediately upon injection (Drouet et al., 2011) is likely to be accompanied by an increase in energy consumption (Cespuglio et al., 2005). In addition, we did not observe any decrease in cell activation since c-fos mRNA expression in hippocampus did not change 1 h after metyrapone injection. This observation is in agreement with the lack of difference in Fos-like immunostaining in the hippocampus 2 and 3 h after metyrapone injection in spite of the increase of the Fos-like immunostaining in other brain areas (Rotllant et al., 2002). Furthermore, the metyrapone-induced inhibition of glucose utilization was not associated to any energy impairment. Indeed, we did not observe any difference between treatment groups in phosphorylated protein/total protein ratio for AMPK (Hardie, 2004) and mTOR (Dennis et al., 2001) and HSP70-2 mRNA levels (Imuta et al., 1998).

The paradoxical maintenance in energy status in spite of a decreased glucose metabolism and some neuroprotective effects in animal model may suggest changes in mitochondrial functioning. Metyrapone did not alter the maximum functioning of mitochondria in conditions of oxygen and substrates saturation. Metyrapone did not change the transfer rate of electrons along the electron transport chain, suggesting that either metyrapone had no direct effect on the electron transport chain, or if the effect exists, it did not change fundamentally and irreversibly the electron transport chain.

The analysis of brain samples using ^{1}H HRMAS MRS produces highly resolved spectra that are efficiently quantified with the QUEST procedure of jMRUI (Rabeson et al., 2008), allowing the comparison between metyrapone-treated animals and saline one. Interestingly, metyrapone-treated rats exhibited an increase in hippocampus NAA levels as compared to saline animals. NAA is produced by neuronal mitochondria according to acetylCoA availability (Demougeot et al., 2004; Moffett et al., 2007; Patel and Clark, 1979).

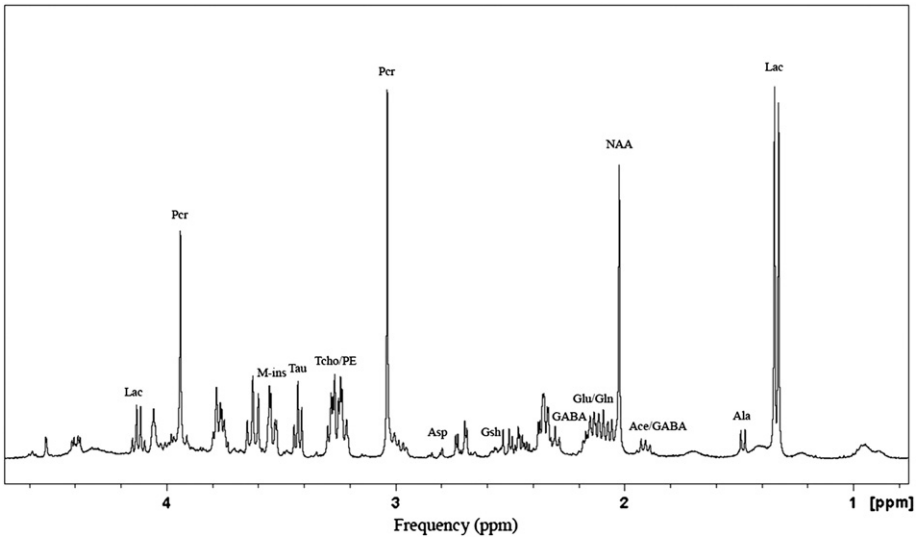

Fig. 2. Typical proton HRMAS NMR spectrum in one rat hippocampus sample 1 h after metyrapone treatment. Abbreviations are given in the Materials and methods section.

Table 2
Effects of metyrapone (150 mg/kg i.p., n = 17) or saline (i.p., n = 16) on hippocampus levels of c-fos and HSP70-2 mRNA measured by RT-PCR (a.u.), and neurochemicals assessed by ^{1}H HRMAS MRS (10^{3} au; NAA, phosphoCre, GABA, lactate, alanine, glutamate and glutamine). Comparisons between groups were performed with factorial ANOVA and significant differences are indicated as follows: 'a': $P<0.05$; 'b': $P<0.01$. Data are expressed as means ± S.E.M.

	Saline	Metyrapone
c-fos mRNA	1.18 ± 0.18	1.50 ± 0.14
HSP70-2 mRNA	1.04 ± 0.06	1.10 ± 0.05
Alanine	4.93 ± 0.12	4.81 ± 0.13
GABA	10.80 ± 0.30	9.64 ± 0.28[b]
Glutamate	33.76 ± 0.74	31.62 ± 0.50[a]
Glutamine	26.53 ± 0.63	28.62 ± 0.36[b]
Lactate	56.02 ± 1.15	56.49 ± 1.38
NAA	34.33 ± 0.45	35.50 ± 0.30[a]

Taken together, the absence of energy impairment, the normal cellular activation despite the decrease in glucose utilization and the increase in hippocampus NAA strongly suggest that another substrate than glucose is used by the brain during metyrapone impregnation.

Firstly, metyrapone may favour a shift toward a preferential consumption of lipid as suggested by the decrease in the respiratory quotient (Werner, 1988). Secondly, lactate, by conversion to pyruvate, represents also an alternative source of substrate for mitochondria. Although we observed a large increase in plasma lactate concentration, lactate level remained unaffected in the hippocampus. This discrepancy might be explained by the substantial cerebral utilization of blood lactate described in case of hyperlactatemia (Bouzier et al., 2000). The consumption of blood lactate by the brain may underpin the neuroprotective properties of metyrapone observed in animals. *In vitro*, lactate is preferentially used by brain tissue to recover from

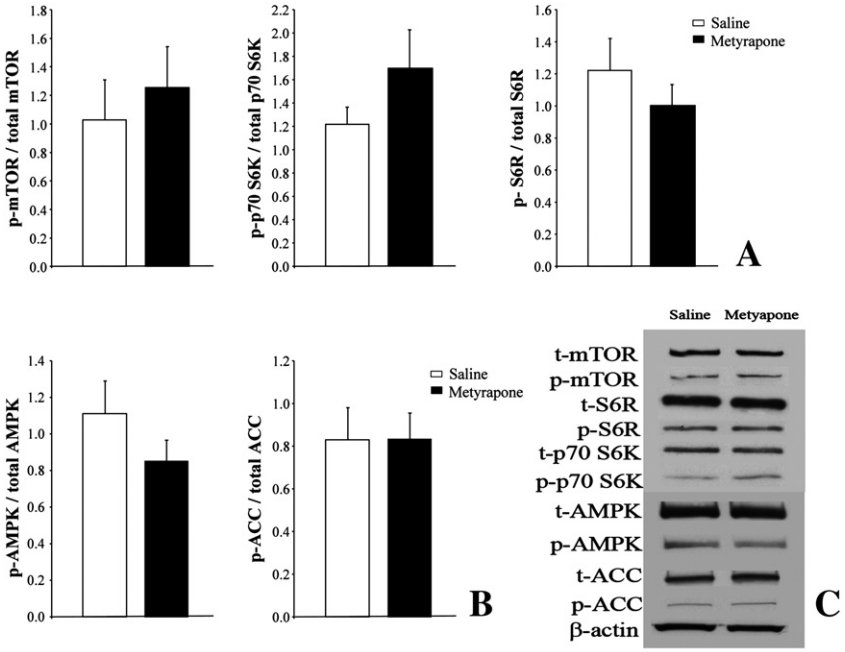

Fig. 3. Effect of metyrapone on mTOR and AMPk pathway activities in the hippocampus. A: analysis of mTOR, S6R and P70 S6 kinase phosphorylation status. B: analysis of AMPk and ACC phosphorylation status. C: representative protein bands from Western Blots of total mTOR, phospho-mTOR (Ser2448), total p70 S6 kinase, phospho-p70 S6 kinase (Thr389), total S6R, phospho-S6R (Ser 240/244), total AMPKα,, phospho-AMPKα (Thr172), total ACC, phospho-ACC (Ser79) and β-actin. Bands given come from the same animal. Values are expressed as means ± S.E.M.

Table 3
Effects of metyrapone (150 mg/kg i.p., n = 10) or saline (i.p., n = 10) on oxygen consumption (nM O_2/min/mg prot). Glutamate/malate and succinate referred to state 3 and ascorbate/TMPD to uncoupling state. Data are expressed as means ± S.E.M.

	Saline	Metyrapone
Glutamate/malate	69.3 ± 2.3	70.0 ± 3.2
Succinate	89.9 ± 2.5	92.2 ± 2.4
Ascorbate/TMPD	118.6 ± 3.4	121.5 ± 2.6

hypoxia during reoxygenation (Schurr et al., 1997). Furthermore lactate helps brain to recover during reperfusion (Berthet et al., 2009). This may be also pertinent for humans since blood lactate is an important source of energy for human brain (van Hall et al., 2009) and protects it during hypoglycaemia (Maran et al., 1994). Thirdly, the transfer of glutamine between astrocytes and neurones may be enhanced. Metyrapone injection was followed by a decrease in glutamate and GABA together with an increase in glutamine content, as measured by HRMAS MRS. This can be explained by an accelerated conversion of glutamate into glutamine within astrocytes or a slowing conversion of glutamine into glutamate in neurons or both (Albrecht et al., 2007). In one hand, metyrapone may have enhanced the glutamate uptake in astrocytes. This could be achieve through the decrease in body core temperature since glutamate re-uptake is impaired by hyperthermia and favored by hypothermia (Asai et al., 2000; Zhao et al., 1997). In the other hand, metyrapone may have also enhanced glutamine production through the stimulation of glutamine synthetase. This cannot be ruled out as the deleterious effects of methionine sulfoximine, a glutamine synthetase inhibitor, are counteracted by metyrapone (Berel et al., 1977). Hyperammonemia that stimulates glutamine synthetase (Suarez et al., 2002), is not relevant for metyrapone as we observed only a slight increase in blood urea concentration and we did not observed any increase in brain lactate and alanine as described in case of hyperammonemia (Chatauret et al., 2003; Kala and Hertz, 2005). Whatever the mechanism considered, the effects of metyrapone on the glutamate–glutamine cycle may participate to its neuroprotective activity in animal models.

Although metyrapone inhibits glucocorticoid synthesis (Jenkins et al., 1958), previous reports suggest that glucocorticoid synthesis inhibition does not fully explain the neuroprotective properties of metyrapone and that other mechanism should be evoked (Krugers et al., 1998; Smith-Swintosky et al., 1996). Our results support this view as the physiological and biochemical modifications induced by metyrapone we observed take place without difference in blood corticosterone levels. The latter is not surprising since other reports show that metyrapone (100 to 200 mg/kg) does not affect circulating corticosterone levels in resting conditions (Czyrak et al., 2000; Drouet et al., 2010; Herman et al., 1992; Rotllant et al., 2002) although it blocks the stress-induced rise in corticosterone (Baez and Volosin, 1994; Baez et al., 1996; Drouet et al., 2010; Kennet et al., 1985).

Besides the direct action of glucocorticoid synthesis inhibition, metyrapone may act through other related mechanisms. Firstly, metyrapone reduces the intracellular corticosterone signalling by inhibiting the brain 11β-hydroxysteroid dehydrogenase type 1 (Raven et al., 1995) which converts 11-dehydrocorticosterone into corticosterone (Yau and Seckl, 2001). This mechanism is unlikely to explain the metyrapone-induced neuroprotection: (i) It does not explain the decrease in abdominal temperature since both adrenalectomy and glucocorticoid receptor antagonist RU38486 potentiate stress-induced hyperthermia (McClellan et al., 1994; Morrow et al., 1993) and a supplementation with high dose of corticosterone does not reverse the metyrapone-induced hypothermia (Drouet et al., 2010). (ii) It is not congruent with the increased in glutamine observed in metyrapone-treated rats since dexamethasone enhances glutamine synthetase activity (Rao et al., 2003). Secondly, metyrapone activates the hypothalamic–pituitary–adrenocortical (HPA) axis as evidenced by the increase in blood ACTH (Rotllant et al., 2002). ACTH or ACTH metabolites which can cross the blood–brain barrier (Banks and Kastin, 1994) may have borne the neuroprotective effect of metyrapone as the $ACTH_{4-9}$ analogue [H-Met(O_2)-Glu-His-Phe-$_D$-Lys-Phe-OH] (ORG2766) protects the hippocampus from ischemic damages (Antonawich et al., 1999; Hwang et al., 2004). This hypothesis is unlikely since the chronic treatment with ORG2766 increases the hippocampus glucose utilization (McCulloch et al., 1982), an effect in contradiction with the decrease in glucose utilization induced by metyrapone (Bruno et al., 1972). In addition, ORG2766 neither affects blood glucose concentration nor body temperature (Antonawich et al., 1999). Therefore, changes in ACTH concentration cannot explain the changes we observed in metabolism after metyrapone administration.

5. Conclusions

In conclusion, metyrapone profoundly modifies brain metabolism, favoring lactate utilization and glutamate–glutamine cycling, without altering mitochondrial functioning. The contrast between brain activation and lack of energy depletion suggests that all these pathways are susceptible to underpin the neuroprotective activity of metyrapone without modifying blood glucocorticoid concentration.

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