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To cite this version:

HAL Id: inserm-00593914
http://www.hal.inserm.fr/inserm-00593914
Submitted on 27 Sep 2011
Metyrapone blunts stress-induced hyperthermia and increased locomotor activity independently of glucocorticoids and neurosteroids

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Received 29 September 2009; received in revised form 1 March 2010; accepted 1 March 2010

KEYWORDS
Locomotor activity; Stress-induced hyperthermia; Corticosterone; Neuroactive steroids; EEG; Arousal

Summary Metyrapone, a cytochrome P450 inhibitor used to inhibit corticosterone synthesis, triggers biological markers of stress and also reduces stress-induced anxiety-like behaviors. To address these controversial effects, 6 separate investigations were carried out. In a first set of investigations, abdominal temperature ($T_{abd}$), spontaneous locomotor activity ($A_S$) and electroencephalogram (EEG) were recorded in freely moving rats treated with either saline or 150 mg kg$^{-1}$ metyrapone. An increase in $T_{abd}$ and $A_S$ occurred in saline rats, while, metyrapone rats exhibited an immediate decrease, both variables returning to basal values 5 h later. Concomitantly, the EEG spectral power increased in the gamma and beta 2 bands and decreased in the alpha frequency band, and the EMG spectral power increased. This finding suggests that metyrapone depressed stress-induced physiological response while arousing the animal. In a second step, restraint stress was applied 5 h after injection. Metyrapone significantly blunted the stress-induced $T_{abd}$ and $A_S$ rise, without affecting the brain c-fos mRNA increase. Corticosterone (5 and 40 mg kg$^{-1}$) injected concomitantly to metyrapone failed to reverse the observed metyrapone-induced effects in $T_{abd}$ and $A_S$. Finasteride (50 mg kg$^{-1}$), which blocks neurosteroid production, was also unable to block these effects. In conclusion, metyrapone acutely reduced stress-induced physiological response in freely behaving rats independently from glucocorticoids and neurosteroids.

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0306-4530/S — see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.psyneuen.2010.03.001
1. Introduction

Metyrapone is a cytochrome P<sub>450</sub> inhibitor (Williamson and O’Donnell, 1969) that blocks the 11β-hydroxylation of deoxycorticosterone (DOC) into corticosterone in the adrenal cortex (Jenkins et al., 1958). It has been extensively used in rodents to study the role of glucocorticoids in stress processes (Mousa et al., 1981; Haleem et al., 1988; Calvo et al., 1998; Mikics et al., 2005). However, metyrapone administration leads to apparently contradictory findings.

Metyrapone limits stress-induced behaviors. Metyrapone reduces immobility time when administrated one (Healy et al., 1999) or 3 h (Baez and Volosin, 1994) prior to forced swim test. Its administration 3 h before inescapable footshock exposure also decreases the percentage of inactive trials during the test (Baez et al., 1996). These behavioral changes occurring during stress exposure are likely to reflect a decrease in stress-induced anxiety. The subsequent anxiety-like behaviors are also reduced 24 h after stressor exposure. Metyrapone administration 3 h prior restraint exposure increases the time spent in open arms during an elevated-plus-maze test carried out 1 day after (Calvo et al., 1998; Calvo and Volosin, 2001).

Conversely, as stated by Rotllant et al. (2002), “metyrapone can act as a stressor”. Metyrapone increases c-fos mRNA expression in the hypothalamic paraventricular nucleus (PVN) (Herman et al., 1992) and Fos-like immunostaining throughout the brain (Rotllant et al., 2002). Metyrapone also activates the hypothalamo-pituitary-adrenocortico (HPA) axis. The heteronuclear corticotropin-releasing factor (CRF) mRNA transcription is enhanced in the PVN (Herman et al., 1992) and vasopressin and CRF concentrations are increased in the pituitary portal blood (Conte-Devolx et al., 1992). Plasma ACTH concentration rises (Conte-Devolx et al., 1992; Herman et al., 1992; Rotllant and Armario, 2005) as well as that of 11-desoxycortisol (Conte-Devolx et al., 1992) and deoxycorticosterone (DOC, Krugers et al., 2000).

In order to examine the apparent controversial effects of metyrapone administration (anxiety concomitant to brain activation), a set of 6 investigations was carried out in freely moving rats. The first experimental step aimed at analyzing the immediate and delayed reactions to the injection of metyrapone through measuring two physiological variables. Abdominal temperature (T<sub>abd</sub>) was taken to approach anxiolytic properties of metyrapone through the stress-induced hyperthermia paradigm (Bouwknecht et al., 2007; Vinkers et al., 2009). Spontaneous locomotor activity (A<sub>S</sub>) was recorded because it increases after social conflict (Sgoffo et al., 2002) and saline injection (Marinelli et al., 1997). Brain activation was assessed by recording the electroencephalogram (EEG). Arousal is reflected by fast β (19–30 Hz) and γ (γ1: 30–35 Hz and γ2: 35–50 Hz) frequency bands (Maloney et al., 1997). Variations in locomotor activity are associated with variations in the θ (4–8 Hz) band (Oddie and Bland, 1998). It increases with locomotion speed (Slawinska and Kasicki, 1998), but disappears when the animal is immobile (Whishaw and Vanderwolf, 1971). The second experimental step aimed at analyzing the effects of stress by applying a 60-min restraint after the extinction of the immediate response to metyrapone administration. The effects of metyrapone on stress-induced physiological activation were addressed using the time course of T<sub>abd</sub> and A<sub>S</sub>. The cerebral effects were assessed using brain c-fos mRNA expression (Chan et al., 1993). Metabolic effects of metyrapone administration were quantified in the blood using glycaemia, which increases after metyrapone (Werner, 1988; Rotllant et al., 2002) and stress (Armario et al., 1990), triglyceride concentration, which decreases after stress (Ricart-Jané et al., 2002), and lactate concentration, a marker of anaerobic metabolism. The role of the inhibition of glucocorticoid synthesis in the effects observed after metyrapone administration was evaluated through corticosterone supplementation. The place of the increased DOC production (Krugers et al., 2000) was analyzed by blocking the 5α-reductase using finasteride (Lephart et al., 1996). In fact, the transformation of DOC into tetrahydro-DOC (THDOC) by 3- and 5α-reductases (Raven et al., 1996; Rupprecht et al., 1998) acts as a positive modulator of GABA<sub>A</sub> receptor (Reddy, 2006).

2. Methods

2.1. Animals

The investigation was conducted in 250 male OFA Sprague–Dawley rats (Charles River Laboratories, L’arbresle, France) weighing 175–200 g upon arrival at the laboratory. Animals were housed at constant temperature (23 ± 2°C) and relative humidity (50 ± 10%), and in a 12 h–12 h light-dark cycle (light on at 0800 h). The rats were accustomed to laboratory conditions during 10 days before surgery and were allowed 10 days to recover from the surgical operation. They were weighed 5 days a week to reduce handling stress (Briese and de Quijada, 1970). Experimental procedures were 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 European Community Council Directive (86/609 EEC).

2.2. Drugs

Metyrapone was purchased from Interchim (Montluçon, France) for investigations no. 1, 3 and 6 (Fig. 1) and from Sigma–Aldrich (St-Quentin Fallavier, France) for investigations no. 2, 4 and 5 (Fig. 1). The substance was dissolved in 1 ml sterile saline (SAL) and injected IP. The chosen dosage was 150 mg kg<sup>−1</sup>, in order to block the stress-induced increase in blood corticosterone (Haleem et al., 1988). Corticosterone (CORT, Sigma–Aldrich) and finasteride (FIN, Interchim) were dissolved in 300 μL sesame oil (VEH) and injected SC. CORT was used either at a physiological (5 mg kg<sup>−1</sup>) or at a pharmacological dosage (40 mg kg<sup>−1</sup>). The latter dosages were chosen as they mimic the stress-induced blood corticosterone concentrations observed respectively in naïve (Baez et al., 1996; Calvo and Volosin, 2001) and metyrapone-treated rats (Krugers et al., 1998). Finasteride was used at 50 mg kg<sup>−1</sup>, a dosage that blocks completely the 5α-reductase (Lephart et al., 1996).

2.3. Variables

2.3.1. T<sub>abd</sub> and A<sub>S</sub>

T<sub>abd</sub> and A<sub>S</sub> were assessed with a TA10TA-F40 implantable radiotransmitter and the telemetric signal was acquired using a DataQuest system running on ART-gold software 3.1

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The TA10TA-F40 sensor was implanted into the abdominal cavity under deep anaesthesia (pentobarbital sodium, 60 mg kg\(^{-1}\), IP) according to a procedure described elsewhere (Michel et al., 2007a). As stated previously, the rats were allowed 10 days to recover from surgery. The \(T_{\text{abd}}\) and \(A_S\) values were measured every 2 min. \(A_S\) was calculated from variations in the transmitted power signal due to the rat position changes in its home cage. The \(A_S\) values were normalized using z-scores: 

\[
z\text{-score}(x) = \frac{(x - \mu)}{\sigma},
\]

where standard deviation \(\sigma\) and mean \(\mu\) being calculated for each rat during the weekend preceding the experimental day (Chevrier et al., 2006). Weekends measures of \(A_S\) were thought to represent natural circadian variations, due to the absence of handling. Values in \(T_{\text{abd}}\) and \(A_S\) were averaged in 15-min and 1-h time periods. Baseline was assumed to be the mean value of the hour preceding injection or restraint exposure. Final data were

(Data Sciences, Saint-Paul, MN, USA). The TA10TA-F40 sensor was implanted into the abdominal cavity under deep anaesthesia (pentobarbital sodium, 60 mg kg\(^{-1}\), IP) according to a procedure described elsewhere (Michel et al., 2007a). As stated previously, the rats were allowed 10 days to recover from surgery. The \(T_{\text{abd}}\) and \(A_S\) values were measured every 2 min. \(A_S\) was calculated from variations in the transmitted power signal due to the rat position changes in its home cage. The \(A_S\) values were normalized using z-scores: 

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calculated as a difference to the relevant baseline (Δ values).

2.4. Electroencephalogram and electromyogram

To assess EEG and EMG, the TL10M3-F50-EEE transmitter (Data Sciences) was inserted into a pouch in the interscapular subcutaneous tissue under deep anaesthesia (pentobarbital sodium, 60 mg kg⁻¹, IP). The electrodes maintained by screws were placed in 5 holes drilled in the calvarium: one pair at 2 mm anterior and ±3 mm lateral to the bregma; and one pair at 4 mm posterior and ±2 mm lateral to the central suture. The reference electrode was placed 10 mm posterior to the bregma. Electrodes were anchored with dental cement (Dentalon Plus, Heraeus Kulzer, Dormagen, Germany). The EMG electrodes were placed inside the dorsal neck muscle. The rats received antibiotic (Extencilline™, Sanofi-Aventis, 60,000 IU per rat, IP) and anti-inflammatory treatments (Ketofen 3 mg kg⁻¹, Sanofi-Aventis, Lyon, France) and were allowed 10 days to recover. The signal was recorded at a sampling rate of 500 Hz (EEG) and 100 Hz (EMG). Data were imported to the Neuroscore software (v1.1.1, Data Science) for spectral analysis of the EEG by Fast-Fourier Transform (FFT) between 0.5 and 50 Hz with a 1 Hz resolution (hamming window, 10-s epochs). After artefact removal, data were analyzed according to two methods: (i) values were normalized by z-scores using parameters calculated for each rat on the signal assessed during the 2 h preceding injection. The calculated values were then averaged by 1-min epochs and plotted using Sigmaplot (Systat Software Inc., San Jose, CA, USA); (ii) values were divided into 8 frequency bands: 1: 0.5—4 Hz; 2: 30—111 Hz; 3: 11—15 Hz; 4: 15—19 Hz; 5: 19—30 Hz; 6: 30—50 Hz; 7: 35—50 Hz and expressed as percent of total spectral power (0—50 Hz). The EMG was averaged in 15-min epochs and expressed as root mean square of the activity (RMS).

2.5. Blood variables assessment

Plasma corticosterone concentration was analyzed using specific radioimmunoassay kits (¹²⁵I RIA kit, DPC France, La Garenne Colombes, France). Plasma glucose, lactate, triglycerides and cholesterol were assayed on a Hitachi 912 Analyser (Roche Diagnostics, Meylan, France) with the colorimetric method using Roche™ reagents (Roche Diagnostics). Analyses were performed according to the manufacturers’ instructions.

2.6. mRNA quantification by RT-PCR

The mRNA quantification of c-fos (NM_022197.2), CycA (NM_017101.1), β-actin (NM_031144.2) and ARBP (NM_022402.1) was done according to previously described methods (Michel et al., 2007a), using the same primers except for ARBP (Barbier et al., 2009). Briefly, the samples were conditioned in RINaPrep (Qiagen, Courtaboeuf, France) until RNA extraction using MagNA Pure LC mRNA Isolation Kit II (Roche Applied Science, Mannheim, Germany). Reverse transcription was performed with oligo-dT (Reverse Transcription Core Kit, Eurogentec, Seraing, Belgium). Real-time PCR was carried out with the LightCycler Fast Start DNA Master SYBR Green kit (Roche Applied Science) with LightCycler (Roche Applied Science). Quantification cycles were assessed using the second derivative maximum method from a pool of cDNA samples as calibrator (Penninquin et al., 2004), according to the comparative threshold cycle method (Livak and Schmittgen, 2001). Specificity of PCR amplification was verified with the LightCycler melting curve analysis (Penninquin et al., 2004). The validity of internal control genes (CycA, β-actin and ARBP) was checked using geNorm (Vandesompele et al., 2002).

3. Experimental designs

Fig. 1 recapitulates the questions to be answered by each experiment, the experimental means that were used and the corresponding figures and tables presenting the results obtained.

3.1. Experiment 1

Experiment 1 analyzed (i) whether metyrapone induces stress using Tabd and As time courses and (ii) whether metyrapone effects are related to glucocorticoid synthesis inhibition using a 40 mg kg⁻¹ corticosterone supplementation. The 43 rats were randomly distributed into 4 groups: saline—vehicle (SAL—VEH, n = 11), saline—corticosterone (SAL—CORT, n = 10), metyrapone—vehicle (MET—VEH, n = 11), and metyrapone—corticosterone (MET—CORT, n = 11). The rats, previously instrumented with TA10TA-F40 telemetric devices, received the assigned treatment and were left undisturbed for Tabd and As recordings.

3.2. Experiment 2

Experiment 2 investigated (i) whether metyrapone elicits cerebral (central) effects and (ii) whether metyrapone-induced hypothermia triggers shivering through EEG and EMG recordings, respectively. The experiment was performed in 8 rats previously instrumented with TL10M3-F50-EEE telemetric devices. After recovery from surgery, each rat received an injection of saline and, 2 days later, another injection of metyrapone. Substances were given 120 min after lights were turned on and all recordings were made during the light period.

3.3. Experiment 3

Experiment 3 aimed at assessing the protective effect of metyrapone against stressor exposure by analyzing the time course of Tabd and As during and after a 1-h restraint exposure carried out after normalization of metyrapone-induced Tabd and As alterations. The 51 rats were randomly distributed into saline/rest (SAL—R, n = 12), saline/stress (SAL—S, n = 13), metyrapone/rest (MET—R, n = 13) and metyrapone/stress (MET—S, n = 13). The rats, previously instrumented with TA10TA-F40 telemetric devices, received the assigned pharmacological treatment and were then left undisturbed during 5 h. At this time, the rats belonging to stress groups were restrained during 1 h, then released and left again undisturbed. The rats belonging to rest groups remained undisturbed in their home cage throughout.
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3.4. Experiment 4

Experiment 4 evaluated the involvement of glucocorticoid synthesis inhibition in the reduction of stress-induced physiological response after metyrapone. The time course of $T_{\text{abd}}$ and $A_8$ was studied in the same way as in Experiment 3. However, a 5 mg kg$^{-1}$ corticosterone supplementation was administered concomitantly to metyrapone to obtain a corticosterone-clamp condition. The 43 rats were randomly affected to saline–vehicle (SAL–VEH, $n = 11$), saline–corticosterone (SAL–CORT, $n = 11$), metyrapone–vehicle (MET–VEH, $n = 10$) and metyrapone–corticosterone (MET–CORT, $n = 11$). The rats, previously instrumented with TAI10TA-F40 telemetric devices, were left undisturbed during 5 h, then restrained during 1 h, and received the assigned pharmacological treatment. They were previously instrumented with TA10TA-F40 telemetric devices, and then restrained during 1 h, and received the assigned pharmacological treatment. They were left undisturbed during 5 h, then restrained during 1 h, and finally released to remain undisturbed again.

3.5. Experiment 5

Experiment 5 analyzed (i) whether metyrapone was capable of limiting stress-induced biological responses in the same experimental paradigm as in Experiment 4 and (ii) whether a 5 mg kg$^{-1}$ corticosterone supplementation was sufficient to obtain stress-like levels of glucocorticoid blood concentration. The 60 rats were randomly distributed into saline/rest (SAL–R, $n = 10$), metyrapone/rest (MET–R, $n = 10$), saline/stress (SAL–S, $n = 10$), metyrapone/stress (MET–S, $n = 10$), metyrapone–corticosterone/stress (MET + CORT–S, $n = 10$) and saline–corticosterone/stress (CORT–S, $n = 10$). Five hours after treatment, the rats were exposed to a 1-h restraint (stress groups) or were left undisturbed in their home cage (rest groups). Immediately after restraint, the rats were quickly anaesthetized with 100% O$_2$ ventilation containing 4% halothane (Laboratoire Belmont, Paris, France). Blood samples were placed in tubes containing lithium heparinate (Sarstedt, Marnay, France). Plasma was sampled after centrifugation and stored at −80 °C until assessment of blood variables. Prefrontal cortex and hippocampus were dissected and placed into RNA later (Ambion, Courtaboeuf, France). These samples were maintained at 4 °C during 24 h then stored at −20 °C until mRNA quantification by RT-PCR.

4. Results

4.1. Experiment 1

During the baseline before injection, slight statistical differences (MET, ns; CORT, ns; interaction $p < 0.01$) were observed for $T_{\text{abd}}$ (SAL–VEH: 37.0 ± 0.1 °C; MET–VEH: 37.1 ± 0.1 °C; SAL–CORT: 37.2 ± 0.1 °C and MET–CORT: 36.9 ± 0.1 °C), but not for $A_8$ (SAL–VEH: −0.2 ± 0.1; MET–VEH: −0.1 ± 0.1; SAL–CORT: −0.1 ± 0.1 and MET–CORT: −0.2 ± 0.1).

Saline injection induced a strong hyperthermia (Fig. 2A). In rats receiving metyrapone, the hyperthermia was blocked and was followed by a 3-h hypothermia (Fig. 2A; MET, $p < 0.001$; and MET × repetition, $p < 0.001$). Corticosterone treatment did not modify $T_{\text{abd}}$ time course with or without metyrapone co-treatment. Saline injection induced also an immediate increase in $A_8$ (Fig. 2B), which was not observed after metyrapone. However, $A_8$ was enhanced 2 h after metyrapone injection (MET, ns; MET × repetition, $p < 0.001$). Co-administration

Figure 2  Effect of acute administration of 150 mg kg$^{-1}$ metyrapone IP and 40 mg kg$^{-1}$ corticosterone SC on relative variations of abdominal temperature (A, $\Delta T_{\text{abd}}$ expressed in °C) and locomotor activity (B, $\Delta A_8$, z-score). According to their experimental group, the rats received saline and sesame oil (SAL–VEH, $n = 11$), metyrapone and sesame oil (MET–VEH, $n = 11$), saline and corticosterone (SAL–CORT, $n = 11$) or metyrapone and corticosterone (MET–CORT, $n = 10$). Comparisons between groups used two ways factorial ANOVA and significant differences are indicated as follows: MET effect: $^{**}p < 0.01$ and $^{***}p < 0.001$; CORT effect: $^{±±}p < 0.01$ and interaction between MET and CORT effects: $^5p < 0.05$. Data are expressed as means ± SEM.
of corticosterone did not affect A5 time course after saline or metyrapone, although it amplified the increase in A5 during the first 15 min after saline injection ($p < 0.05$). During the 5th hour post-injection, $T_{abd}$ and $A_5$ resumed pre-injection values and SAL—VEH and MET—VEH groups did not differ. This time period was thus selected as the reference period (stress—baseline) for the evaluation of metyrapone effects on stress reaction.

4.2. Experiment 2

Saline injection induced a 5-min decrease in the EEG spectral power in frequency bands between 0.5 and 25 Hz and a slight increase between 35 and 50 Hz (Fig. 3). No change was detected thereafter. Compared to saline, metyrapone modified the time course of EEG spectral power profoundly. It decreased $\alpha$ band relative power but did not affect the relative power of the $\theta$, $\delta$, and $\gamma$ bands. The main effect resided in the large increase that occurred in the $\gamma_1$ and $\gamma_2$ bands and in the $\beta_2$ band. The effects of metyrapone lasted 2 h after injection for $\alpha$, $\beta_2$ and $\gamma_1$ frequency bands and 3 h for the $\gamma_2$ frequency band (data not shown). Concomitantly, metyrapone induced an increase in EMG activity, especially during the second hour following injection (Fig. 4; MET, ns and MET $\times$ repetition, $p < 0.05$).

4.3. Experiment 3

Before restraint stress, as in Experiment 1, saline injection triggered hyperthermia. Metyrapone administration blocked this stress-induced hyperthermia, and induced a subsequent hypothermia. The stress-induced increase in $A_5$ observed in saline rats was also blocked by metyrapone (data not shown).

During stress—baseline, no difference was observed between groups for $T_{abd}$ (SAL—R: $37.0 \pm 0.1 ^\circ C$; MET—R: $36.7 \pm 0.2 ^\circ C$; SAL—S: $37.0 \pm 0.1 ^\circ C$ and MET—S: $36.8 \pm 0.2 ^\circ C$) and $A_5$ (SAL—R: $-0.2 \pm 0.1$; MET—R: $0.0 \pm 0.1$; SAL—S: $-0.3 \pm 0.1$ and MET—S: $-0.3 \pm 0.1$). During restraint, $T_{abd}$ increased sharply in saline rats (Fig. 5A; stress, ns; and stress $\times$ repetition, $p < 0.001$) and the stress-induced hyperthermia was blunted by metyrapone. In the resting condition, $T_{abd}$ levels remained higher in metyrapone than in saline rats (MET, ns; MET $\times$ repetition, $p < 0.001$ and MET $\times$ stress $\times$ repetition, $p < 0.001$).

After restraint release, saline rats exhibited a sharp increase in $A_5$ (Fig. 5B; stress, ns; and stress $\times$ repetition, $p < 0.001$). Metyrapone limited the stress-induced increase in $A_5$ (MET, ns; MET $\times$ repetition, $p < 0.001$ and MET $\times$ stress $\times$ repetition, $p < 0.05$).

4.4. Experiment 4

Similarly to observations made in Experiment 1, hyperthermia and increased $A_5$ were observed after saline injection, while hypothermia and a reduction in $A_5$ followed metyrapone injection. Corticosterone administration did not modify the concomitant $T_{abd}$ or $A_5$ time courses in saline and metyrapone rats (data not shown).
supplementation did not change T < C0/C0/VEH: MET—CORT: 37.2 < C6/C6/MET—VEH: 37.2 < C6/C6/123.1 saline and metyrapone rats. p < 476.2 < p MET/C2/C2/Restraint induced an increase in cor-
ticosterone supplementation limited the stress-induced increase in plasma corticosterone in saline rats (SAL—S: 476.2 ± 15.3 ng ml < C0/C0/C0/SAL + CORT—S: 358.3 ± 39.8 ng ml < C0/C0/C0/SAL—S vs. SAL + CORT—S: p < 0.01). Corticosterone addition had the opposite effect in metyrapone-treated rats (MET—S—CORT: 186.4 ± 8.0 ng ml < C0/C0/C0/MET + CORT—S: 284.6 ± 19.9 ng ml < C0/C0/C0/MET, p < 0.001; CORT, ns; CORT × MET, p < 0.001; MET—S vs. MET + CORT—S: p < 0.05).

Metyrapone did not alter resting levels of cortical and hippocampal c-fos mRNA expressions. c-fos mRNA expression increased after restraint in the frontal cortex (Table 1; stress, p < 0.001) and hippocampus (stress, p < 0.01). This reaction was not affected by prior metyrapone treatment (MET, ns; MET × stress, ns).

Plasma glucose level was increased by stress and metyrapone, without any interaction between the two conditions (Table 1; MET, p < 0.001; stress, p < 0.001; MET × stress, ns). Metyrapone modified energetic metabolism as shown by the increase in plasma triglycerides (MET, p < 0.05) and the slight decrease in plasma cholesterol concentrations (MET,

During stress—baseline, compared to saline rats, metyrapone rats exhibited higher T<sub>abd</sub> (SAL—VEH: 37.0 ± 0.1 °C; MET—VEH: 37.2 ± 0.1 °C; SAL—CORT: 36.8 ± 0.1 °C and MET—CORT: 37.2 ± 0.1 °C, MET, p < 0.01) and A<sub>5</sub> (SAL—VEH: −0.3 ± 0.1; MET—VEH: −0.2 ± 0.1; SAL—CORT: −0.4 ± 0.1 and MET—CORT: 0.0 ± 0.1, MET, p < 0.05; MET × CORT, p < 0.05). Metyrapone reduced stress-induced hyperthermia (Fig. 6A; MET, p < 0.01; and MET × repetition, p < 0.001) and blunted the increase in A<sub>5</sub> (Fig. 6B; MET, p < 0.01; and MET × repetition, p < 0.001). Corticosterone supplementation did not change T<sub>abd</sub> and A<sub>5</sub> time courses in saline and metyrapone rats.

4.5. Experiment 5

Metyrapone did not modify blood corticosterone levels in resting condition (SAL—R: 125.9 ± 26.7 ng ml < C0/C0/C0/MET—R: 123.1 ± 16.0 ng ml < C0/C0/C0/1). Restraint induced an increase in corticosterone levels that was blunted by metyrapone (SAL—S: 476.2 ± 15.3 ng ml < C0/C0/C0/MET—S: 186.4 ± 8.0 ng ml < C0/C0/C0/MET, p < 0.001; stress, p < 0.001; MET × stress, p < 0.001). In stressed rats, corticosterone supplementation limited the stress-induced increase in plasma corticosterone in saline rats (SAL—S: 476.2 ± 15.3 ng ml < C0/C0/C0/SAL + CORT—S: 358.3 ± 39.8 ng ml < C0/C0/C0/SAL—S vs. SAL + CORT—S: p < 0.01). Corticosterone addition had the opposite effect in metyrapone-treated rats (MET—S—CORT: 186.4 ± 8.0 ng ml < C0/C0/C0/MET + CORT—S: 284.6 ± 19.9 ng ml < C0/C0/C0/MET, p < 0.001; CORT, ns; CORT × MET, p < 0.001; MET—S vs. MET + CORT—S: p < 0.05).

Metyrapone did not alter resting levels of cortical and hippocampal c-fos mRNA expressions. c-fos mRNA expression increased after restraint in the frontal cortex (Table 1; stress, p < 0.001) and hippocampus (stress, p < 0.01). This reaction was not affected by prior metyrapone treatment (MET, ns; MET × stress, ns).

Plasma glucose level was increased by stress and metyrapone, without any interaction between the two conditions (Table 1; MET, p < 0.001; stress, p < 0.001; MET × stress, ns). Metyrapone modified energetic metabolism as shown by the increase in plasma triglycerides (MET, p < 0.05) and the slight decrease in plasma cholesterol concentrations (MET,

### Table 1

<table>
<thead>
<tr>
<th>p</th>
<th>SAL—R</th>
<th>MET—R</th>
<th>SAL—S</th>
<th>MET—S</th>
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</thead>
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<tr>
<td>Frontal cortex c-fos</td>
<td>±±±</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Hippocampus c-fos</td>
<td>±±</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.2</td>
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</tr>
<tr>
<td>Glucose</td>
<td>***</td>
<td>10.9 ± 0.3</td>
<td>12.0 ± 0.3</td>
<td>12.1 ± 0.3</td>
</tr>
<tr>
<td>Lactate</td>
<td>±±</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>*</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.2</td>
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</tr>
<tr>
<td>Cholesterol</td>
<td>±±</td>
<td>1.8 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>
4.6. Experiment 6

Similarly to previous observations on immediate effects, metyrapone induced hypothermia and blocked the increase in $A_o$ observed immediately after injection (data not shown). Finasteride co-treatment did not alter the concomitant $T_{abd}$ and $A_o$ time courses in saline- and metyrapone-treated rats (data not shown).

During stress—baseline, metyrapone rats showed higher $T_{abd}$ than saline rats (SAL—VEH: $37.0 \pm 0.1$ °C; MET—VEH: $37.4 \pm 0.1$ °C; SAL—FIN: $36.9 \pm 0.1$ °C and MET—FIN: $37.3 \pm 0.1$ °C, MET, $p < 0.001$). However, $A_o$ remained similar (SAL—VEH: $-0.4 \pm 0.1$; MET—VEH: $-0.3 \pm 0.1$; SAL—FIN: $-0.3 \pm 0.1$; MET—FIN: $-0.3 \pm 0.1$, ns). During restraint, metyrapone-treated rats exhibited a reduced hyperthermia compared to saline-treated rats (Fig. 6C; MET, $p < 0.001$; MET x repetition, $p < 0.001$). Finasteride did not change $T_{abd}$ time course in both saline and metyrapone rats. The increased locomotion observed after restraint in saline rats was reduced by metyrapone (Fig. 6D; MET, $p < 0.05$; MET x repetition, $p < 0.001$). Finasteride did not modify $A_o$ time course in both treatment groups.

5. Discussion

The present investigations demonstrated that metyrapone rapidly induced hypothermia and depressed locomotion.
while triggering cerebral arousal. After dissipation of these immediate effects, metyrapone also reduced the stress-induced hyperthermia and enhanced motor activity. The effects of metyrapone on body core temperature and locomotion were independent from glucocorticoid synthesis inhibition and THDOC synthesis.

5.1. Immediate effects of metyrapone

Immediately after saline injection, $T_{\text{abd}}$ and $A_S$ increased in relation to the injection-induced stress reaction. Hyperthermia occurs after handling (Briese and de Quijada, 1970; Vinkers et al., 2009), restraint (De Paula et al., 2000), and social (Sgoifo et al., 2002) and predator (Briese and de Quijada, 1970) confrontations. Increased motor activity is also observed after handling, saline injection (Vinkers et al., 2009) and social confrontation (Sgoifo et al., 2002). Both hyperthermia (Monda et al., 1998; Kiyatkin and Wise, 2001) and increased locomotion (Slawinska and Kasicki, 1998) are tightly linked to cerebral activation. These responses are evoked by cerebral injection of CRF (Morimoto et al., 1993) and reduced by anxiolytic substances such as GABA$_A$ and 5-HT$_{1A}$ receptor agonists (Bouwknecht et al., 2007; Conley and Hutson, 2007; Vinkers et al., 2009). The close relation between stress-induced hyperthermia and brain activation accounts for the opinion that stress-induced hyperthermia represents a model of anxiety (Bouwknecht et al., 2007).

Metyrapone did not allow hyperthermia and increased spontaneous locomotion to occur immediately after injection. It did not only block the stress-induced hyperthermia but also induced a slight hypothermia. This is in agreement with other descriptions reported in rats (Michel et al., 2007b) and guinea pigs (Werner, 1988), but not in mice (Pyce et al., 2003). Hypothermia was not related to the depressed motor activity as both events ran under different time courses. Moreover, hyperthermia occurred despite the increased dorsal neck muscle activity that was strictly limited to the duration of hypothermia. It suggests that hypothermia would be limited by shivering (Gordon, 1990). The neuroprotective properties of metyrapone observed in warmed and anesthetized rats (Smith-Swintosky et al., 1996; Krugers et al., 1998, 2000) might be enhanced by its effect on body temperature (Zhao et al., 2007).

Metyrapone also depressed locomotion acutely in agreement with other studies (Halmy et al., 1970; Canini et al., 2009). Metyrapone may have acted at a peripheral level as (i) its effect on locomotion was concomitant to hypothermia and (ii) the decrease in locomotion occurred without any modification in the $\delta$ frequency band, which is closely related to locomotion speed (Whishaw and Vanderwolf, 1971; Oddie and Bland, 1998; Slawinska and Kasicki, 1998). The decreased locomotion was also concomitant to a large increase in relative EEG spectral power in the rapid $\beta_2$, $\gamma_1$ and $\gamma_2$ frequency bands. Since elevated $\gamma$ activities have been linked to cortical arousal and attentive behavior (Maloney et al., 1997), it may be that metyrapone had an arousing effect. This effect is congruent to other observations reporting that metyrapone administration is followed by an increase in c-fos mRNA expression in the PVN (Herman et al., 1992) and a rise in cerebral Fos-like immunostaining (Rotllant et al., 2002). All these observations exclude that the effect of metyrapone on $T_{\text{abd}}$ and $A_S$ were a consequence of a sedative effect (Mikics et al., 2004). Arousal is unlikely to be related to hypothermia (Deboer, 1998) since the increase in rapid frequencies occurred quickly after injection, at a time devoid of any hypothermia. This immediate arousal was more probably due to a direct action of metyrapone on the brain. Metyrapone may have modest environmental appraisal as indicated by the decrease in $\alpha$ power which has been related to cognitive performance in mammals (Klimples, 1999; Basar et al., 2000). Such a hypothesis may explain that, in our animals, metyrapone induced immobility in a safe environment and reduced immobility during exposure to stressor (Baez and Volosin, 1994; Baez et al., 1996; Rozendaal et al., 1996). This hypothesis is also supported by the reduction of subsequent behavioral anxiety (Calvo et al., 1998; Calvo and Volosin, 2001). Further studies are therefore needed to better delineate the original anxiolytic properties of metyrapone.

5.2. Subsequent effects of metyrapone

To evaluate whether metyrapone may have modified appraisal to stressor, the rats were submitted to restraint $5 \text{ h}$ after injection. At this time, the immediate effects of metyrapone were extinct. During stress—baseline, no differences were observed between saline- and metyrapone-treated rats regarding $T_{\text{abd}}$, $A_S$, EEG power spectra. Moreover, cerebral c-fos mRNA level and plasma corticosterone concentration were similar in both resting treatment groups $1 \text{ h}$ after. As expected, $T_{\text{abd}}$ increased in saline rats immediately after the beginning of restraint, and locomotion was enhanced just after restraint release. Concomitant cerebral activation was suggested by the increase in c-fos mRNA level in the hippocampus and frontal cortex (Senba and Ueyama, 1997; Chowdhury et al., 2000; Herman et al., 2003). The rats were also hyperglycaemic, such as usually observed in stressed fed rats (Selvey, 1950; Nonogaki and Iuchi, 1997). Altogether, the above reactions suggest a strong stress response in saline-treated animals. Compared to saline, metyrapone administration induced a lower increase in $T_{\text{abd}}$ and $A_S$. Although metyrapone had increased basal blood glycaemia, the latter was further increased after stressor exposure. Such an additive effect suggests that metyrapone and stress acted by different pathways and that metyrapone-treated rats were able to mobilize glucose when exposed to a stressor. Moreover, the c-fos mRNA level increase was statistically similar in both treatment groups after restraint. Metyrapone rats may have perceived their challenging environment, although the physiological consequences were different from those observed in saline-treated rats.

5.3. Mechanisms of action of metyrapone

The biochemical mechanisms by which metyrapone may have produced these effects deserve discussion. Although metyrapone is known to block glucocorticoid synthesis (Jenkins et al., 1958), a major role of blood glucocorticoids in the blockade of the stress-induced hyperthermia and increased locomotion triggered by metyrapone is unlikely to occur. The following reasons may be proposed: (i) metyrapone did not induce glucocorticoid depletion because the resting blood corticosterone concentration was similar in metyrapone and saline rats $6 \text{ h}$ after injec-
tion (Experiment 5), at a time at which stress reactivity was blunted (Experiments 3, 4, 6); (ii) corticosterone supplementation at a dosage that mimics stress levels of blood corticosterone failed to modify the time course of $T_{abd}$ and $A_S$ during both the early and the late phases; (iii) a high dose of corticosterone supplementation in metyrapone rats was also unable to modify the $T_{abd}$ and $A_S$ time course (Experiment 1), whereas it increases locomotion in saline rats suggesting treatment efficiency (Sandi et al., 1996). However, the fact that blood glucocorticoid concentration was not related to the previous effects does not rule out a role of glucocorticoid at the cellular level. Metyrapone can modify tissue glucocorticoid concentration by inhibiting specifically 11β-hydroxysteroid dehydrogenase type 1 (11 β-HSD1) (Raven et al., 1995). This enzyme, which is widely expressed by neurons, converts inactive 11-dehydrocorticosterone into active corticosterone (Yau and Seckl, 2001).

Metyrapone may also act through the HPA axis activation since it increases brain CRF mRNA, arginine vasopressin and ACTH levels (Herman et al., 1992; Rotllant and Armario, 2005). Although CRF may have been implicated in arousal, it does not explain the blunt in $T_{abd}$ and $A_S$ since it produces the opposite effects (Morimoto et al., 1993). The accumulation of DOC (Conte-Devolx et al., 1992; Raven et al., 1996) further converted into the positive GABA A receptor modulator THDOC (Paul and Purdy, 1992) is also to be considered. Under GABA stimulation, metyrapone normalized the maximal chloride uptake previously reduced by restraint (Calvo et al., 1998). Such a positive GABA A modulation could account for metyrapone-induced hypothermia and diminished locomotion. However, the blockade of 5α-reductase by finasteride, at a dose reducing brain concentration of neurosteroids (Verleye et al., 2005), failed to reverse the effects of metyrapone. Moreover, the metyrapone-induced brain activation suggested by EEG activation and cerebral c-fos induction (Herman et al., 1992) is not in accordance with such a mechanism. Therefore, metyrapone did not act through neurosteroid production.

Alternatively, metyrapone may act by modulating brain neurotransmission. Metyrapone is known to inhibit monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT) (Parvez and Parvez, 1973). It may therefore enhance noradrenaline and dopamine neurotransmission. However, COMT and MAO inhibitors are known to rather trigger hyperthermia and enhanced motor activity (Feldberg and Lang, 1970; Ashkenazi et al., 1983; Rivas et al., 1999). Metyrapone can also modify serotonin neurotransmission by upregulating serotonin 5-HT1A receptor through its effect on intracellular level of corticosteroid (Lopez et al., 1998). A 5-HT1A-related mechanism may explain both its thermogenic and anxiolytic properties (De Vry et al., 2004; Hedlund et al., 2004).

Metyrapone may also simply act through metabolic modifications. Metyrapone may have reduced energetic metabolism as suggested by hypothermia. Actually, metyrapone induces metabolic depression in guinea pigs (Werner, 1988), although not in rats (Ohno and Kuroshima, 1986). The metabolic depression may be explained by an inhibition in glucose utilization (Bruno et al., 1972) and a shift towards lipid utilization, as supported by the decrease in respiratory quotient (Werner, 1988). This hypothesis is congruent with the development of hyperglycemia (Werner, 1988; Rotllant et al., 2002) and the observed increase in plasma triglyceride concentration.

6. Concluding remarks

Administration of metyrapone in rats triggered an immediate cerebral activation and a concomitant blockade of the injection-induced hyperthermia and enhanced locomotion. Subsequently, long after these early effects, metyrapone limited the consequences of restraint on body core temperature and locomotion. These effects were not mediated by blood corticosteroid concentration or neurosteroid production. These results shed light on the effect of $P_{450}$ inhibition on energy metabolism, brain activation and behavior, opening new potential pharmacological opportunities in the fields of stress-induced-anxiety disorders and brain ischemia.

Role of funding source

This work was supported by the grant no. 06co016 from the DGA. The DGA had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Conflicts of interest

The authors declare that over the past three years André Peinnequin has received compensation from ROCHE DIAGNOSTICS, the manufacturer of the qPCR reagents and devices used in this work. The authors have not other financial or competing interest to declare.

Acknowledgements

We are indebted to J. Denis, L. Vachez-Colomb and V. Leroux for the corticosterone dosages and C. Mouret for her advice.

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