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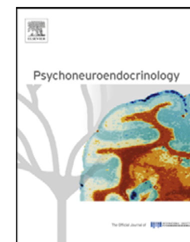

Metyrapone blunts stress-induced hyperthermia and increased locomotor activity independently of glucocorticoids and neurosteroids

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EEG;
Arousal

Summary Metyrapone, a cytochrome P_{450} 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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1. Introduction

Metyrapone is a cytochrome P_{450} 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 foot-shock 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-adrenocorticotrope (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 (anxiolysis 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_{abd}) 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_S) was recorded because it increases after social conflict (Sgoifo et al., 2002) and saline injection (Marinelli et al., 1997). Brain activation was assessed by recording the electroencephalogram (EEG). Arousal is reflected by fast β2 (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_{abd} and A_S. 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 5-α reductase using finasteride (Lephart et al., 1996). In fact, the transformation of DOC into tetrahydro-DOC (THDOC) by 3- and 5-α reductases (Raven et al., 1996; Rupprecht et al., 1998) acts as a positive modulator of $GABA_A$ 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^{-1}, 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^{-1}) or at a pharmacological dosage (40 mg kg^{-1}). The latter dosages were chosen as they mimic the stress-induced blood corticosterone concentrations observed respectively in naive (Baez et al., 1996; Calvo and Volosin, 2001) and metyrapone-treated rats (Krugers et al., 1998). Finasteride was used at 50 mg kg^{-1}, a dosage that blocks completely the 5-α reductase (Lephart et al., 1996).

2.3. Variables

2.3.1. T_{abd} and A_S

T_{abd} and A_S 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

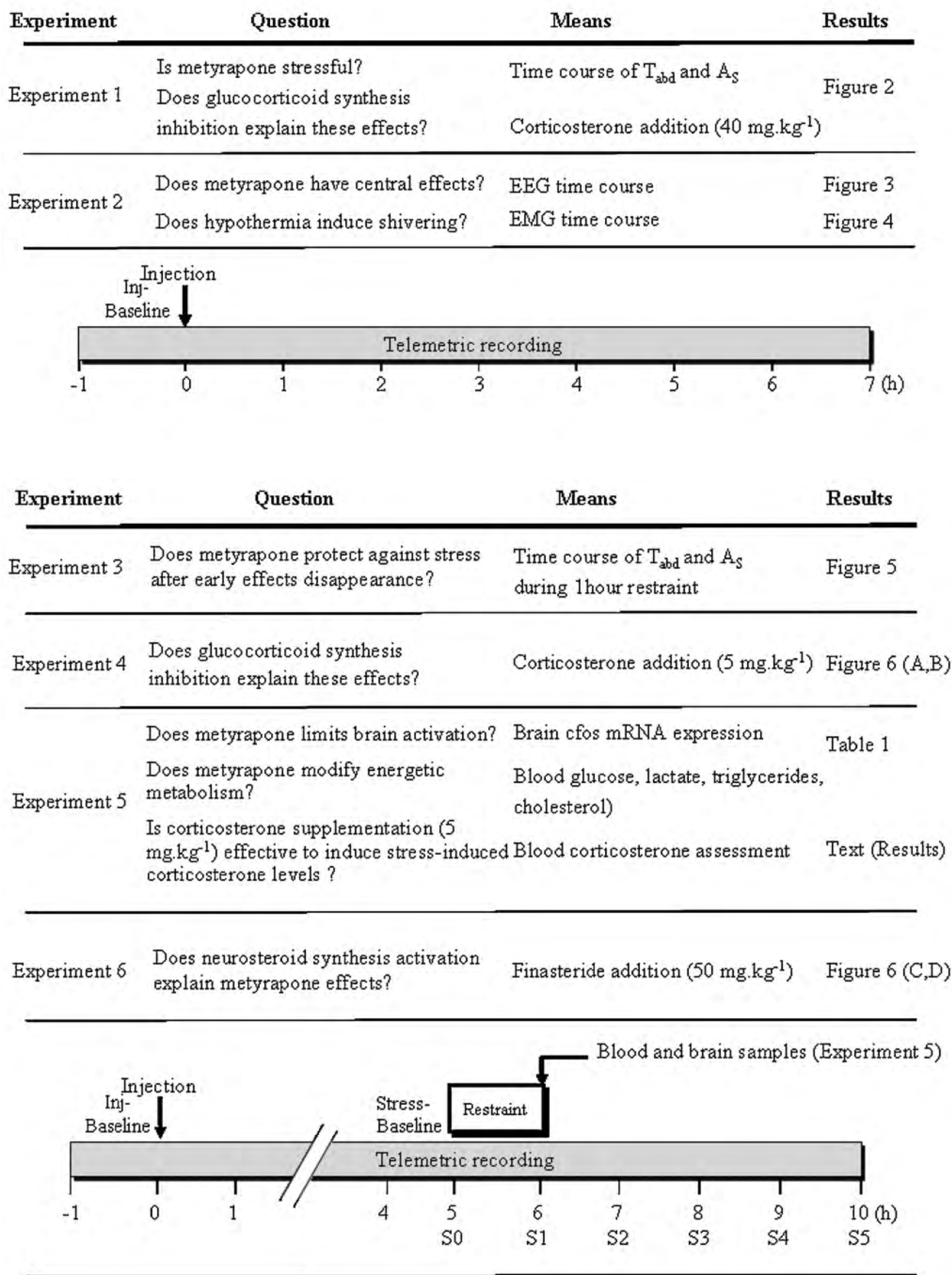

Figure 1 Questions to be answered by each experiment, means used to obtain an answer, related figure or table, and schematic view of experimental procedures. In Experiments 1 and 2, T_{abd}, A_S, EEG and EMG were measured before and after treatment injection. T_{abd} and A_S were both measured before and after drug injection and restraint in Experiments 3—6. Only data obtained after restraint are shown. Blood and brain samples were collected after stress and related data are presented in Table 1.

(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_{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 (x) were normalized using z-scores: z-score $(x) = (x - \mu)/\sigma$, standard deviation (σ) and mean (μ) 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_{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

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}, IM, Merial, 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 (δ: 0.5—4 Hz; θ: 4—8 Hz; α: 8—11 Hz; σ: 11—15 Hz; β1: 15—19 Hz; β2: 19—30 Hz; γ1: 30—35 Hz and γ2: 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 (^{125}I 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 RNAlater (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 (Peinnequin 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 (Peinnequin et al., 2004). The validity of internal control genes (Cyca, β-actin and ARPB) 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 T_{abd} and A_S 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 T_{abd} and A_S 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 T_{abd} and A_S during and after a 1-h restraint exposure carried out after normalization of metyrapone-induced T_{abd} and A_S 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.

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_{abd} and A_S 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 TA10TA-F40 telemetric devices, received the assigned pharmacological treatment. They were left undisturbed during 5 h, then restrained during 1 h, and lastly 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 RNAlater (Ambion, Courtaboeuf, France). These samples were maintained at 4 °C during 24 h then stored at −20 °C until mRNA quantification by RT-PCR.

3.6. Experiment 6

Experiment 6 evaluated the role of THDOC in the metyrapone-induced T_{abd} and A_S changes using finasteride co-treatment. The 45 rats were randomly assigned to saline—vehicle (SAL—VEH, n = 12), saline—finasteride (SAL—FIN, n = 11), metyrapone—vehicle (MET—VEH, n = 11) and metyrapone—finasteride (MET—FIN, n = 11). The experimental protocol was strictly similar to that of Experiment 4.

3.7. Statistical analysis

Statistical analysis was performed with Statistica v7.1 (StaSoft-France, Maisons-Alfort, France). Comparisons were done by two-factor analysis of variance (ANOVA). The T_{abd} and A_S time courses were analyzed using ANOVA for repeated measures with factor effects. If necessary, *post hoc* tests were done using Bonferroni tests. Values are presented as means ± standard error of the mean (SEM).

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_{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_S (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_{abd} time course with or without metyrapone co-treatment. Saline injection induced also an immediate increase in A_S (Fig. 2B), which was not observed after metyrapone. However, A_s was enhanced 2 h after metyrapone injection (MET, ns; MET × repetition, $p < 0.001$). Co-administration

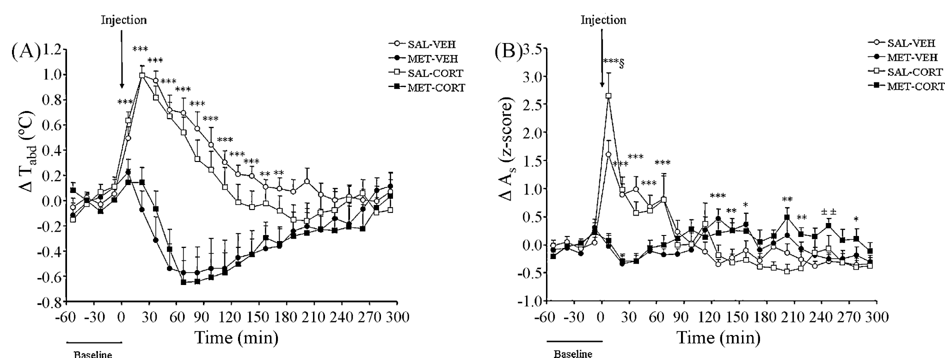

Figure 2 Effect of acute administration of 150 mg kg^{-1} metyrapone IP and 40 mg kg^{-1} corticosterone SC on relative variations of abdominal temperature (A, ΔT_{abd} expressed in °C) and locomotor activity (B, ΔA_S, z-score). According to their experimental group, the rats received saline and sesame 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: $^{\pm\pm}p < 0.01$ and interaction between MET and CORT effects: $^{\S}p < 0.05$. Data are expressed as means ± SEM.

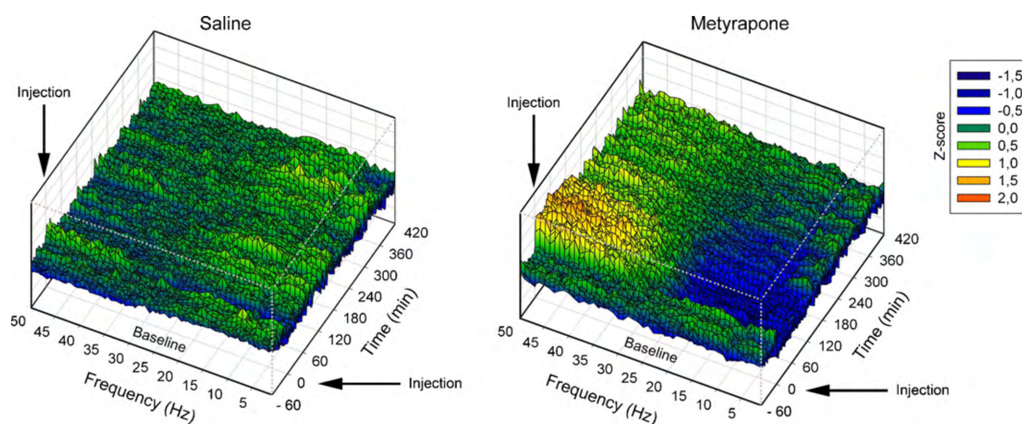

Figure 3 Change in EEG spectral profile after metyrapone administration. Eight animals received saline injection first (saline IP) and metyrapone 2 days later (150 mg kg^{-1} metyrapone IP). Spectral powers between 0.5 and 50 Hz were analyzed with a 1-Hz resolution. The EEG spectral data were transformed into z-scores and averaged by 1-min epochs. Then, z-score data were averaged over 8 animals and color-coded.

of corticosterone did not affect A_S time course after saline or metyrapone, although it amplified the increase in A_S during the first 15 min after saline injection ($p < 0.05$). During the 5th hour post-injection, T_{abd} and A_S 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 α band relative power but did not affect the relative power of the δ, θ, σ and $\beta 1$ bands. The main effect resided in the large increase that occurred in the $\gamma 1$, $\gamma 2$ bands and in the $\beta 2$ band. The effects of metyrapone lasted 2 h after injection for α, $\beta 2$ 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_S 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 ± 0.1 °C; MET—R: 36.7 ± 0.2 °C; SAL—S: 37.0 ± 0.1 °C and MET—S: 36.8 ± 0.2 °C) and A_S (SAL—R: -0.2 ± 0.1; MET—R: 0.0 ± 0.1; SAL—S: -0.3 ± 0.1 and MET—S: -0.3 ± 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_S (Fig. 5B; stress, ns; and stress $\times$ repetition, $p < 0.001$). Metyrapone limited the stress-induced increase in A_S (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_S were observed after saline injection, while hypothermia and a reduction in A_S followed metyrapone injection. Corticosterone administration did not modify the concomitant T_{abd} or A_S time courses in saline and metyrapone rats (data not shown).

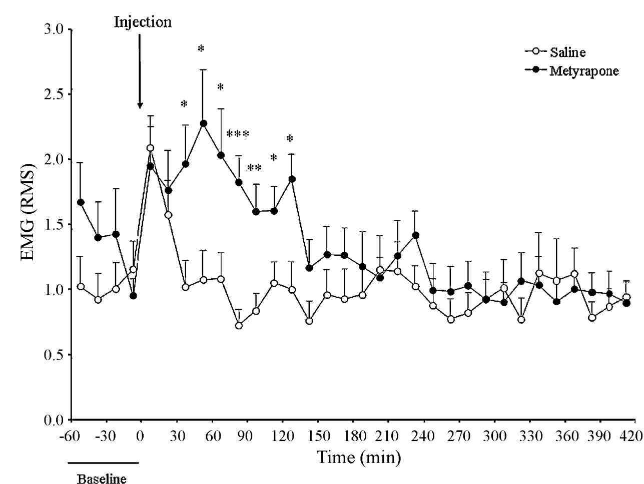

Figure 4 Changes in EMG after metyrapone administration. Eight animals received saline injection first (saline IP) and metyrapone 2 days later (150 mg kg^{-1} metyrapone IP). The EMG was expressed as the 10^5 root mean square of the activity (RMS). Comparisons between groups were made through a two-way factorial ANOVA and significant differences are indicated as follows: MET effect: *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$. Data are expressed as means $\pm$ SEM.

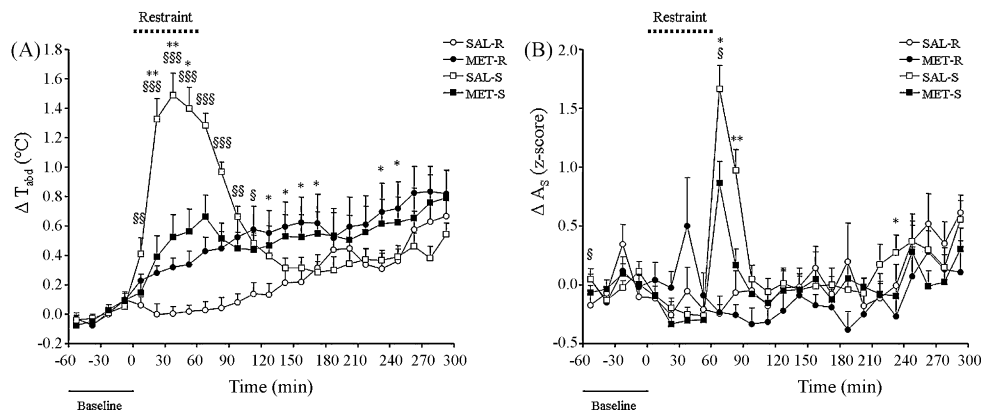

Figure 5 Effect of a 1-h restraint on abdominal temperature (A, ΔT_{abd}, °C) and locomotor activity (B, ΔA_S, *z*-score) in rats treated 5 h earlier with either 150 mg kg^{-1} metyrapone IP or saline IP. The rats were conditioned as follows: saline and rest (SAL—R, $n = 12$), metyrapone and rest (MET—R, $n = 13$), saline and stress (SAL—S, $n = 13$) or metyrapone and stress (MET—S, $n = 13$). Restraint duration is represented by the dotted line. Comparisons between groups used two ways factorial ANOVA and significant differences are indicated as follows: MET effect: $^{*}p < 0.05$ and $^{**}p < 0.01$; interactions between MET and stress effects: $^{\S}p < 0.05$; $^{\S\S}p < 0.01$ and $^{\S\S\S}p < 0.001$. Data are expressed as means ± SEM.

During stress—baseline, compared to saline rats, metyrapone rats exhibited higher T_{abd} (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_S (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_S (Fig. 6B; MET, $p < 0.01$; and MET × repetition, $p < 0.001$). Corticosterone supplementation did not change T_{abd} and A_S 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^{-1}, MET—R: 123.1 ± 16.0 ng ml^{-1}). Restraint induced an increase in corticosterone levels that was blunted by metyrapone (SAL—S: 476.2 ± 15.3 ng ml^{-1}, MET—S: 186.4 ± 8.0 ng ml^{-1}, 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^{-1}, SAL + CORT—S: 358.3 ± 39.8 ng ml^{-1}; SAL—S vs. SAL + CORT—S: $p < 0.01$). Corticosterone addition had the opposite effect in metyrapone-treated rats (MET—S: 186.4 ± 8.0 ng ml^{-1}, MET + CORT—S: 284.6 ± 19.9 ng ml^{-1}; 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 Effect of metyrapone and stress on glucose (mMol L^{-1}), lactate (mMol L^{-1}) triglycerides (mMol L^{-1}) and cholesterol (mMol L^{-1}), and frontal cortex and hippocampus c-fos mRNA (a.u.). The animals received either saline and were stressed (SAL—S, $n = 10$) or rested (SAL—R, $n = 10$) or metyrapone and were stressed (MET—S, $n = 10$) or rested (MET—R, $n = 10$. The 1-h restraint was applied 5 h after treatment injection. Blood and brain samples were collected immediately at the end of restraint. The effects of metyrapone and stress were analyzed using a two-way ANOVA. Statistical differences are expressed in the first column with MET effect: $^{*}p < 0.05$; $^{***}p < 0.001$ and stress effect: $^{\pm\pm}p < 0.01$; $^{\pm\pm\pm}p < 0.001$. Data are expressed as means ± SEM.

	p	SAL—R	MET—R	SAL—S	MET—S
Frontal cortex c-fos	±±±	0.4 ± 0.1	0.4 ± 0.1	1.3 ± 0.3	1.0 ± 0.1
Hippocampus c-fos	±±	0.6 ± 0.1	0.6 ± 0.2	1.1 ± 0.1	0.9 ± 0.1
Glucose	*** ±±±	10.9 ± 0.3	12.0 ± 0.3	12.1 ± 0.3	13.1 ± 0.3
Lactate		2.3 ± 0.2	2.3 ± 0.2	2.4 ± 0.4	2.4 ± 0.2
Triglycerides	*	1.2 ± 0.1	1.6 ± 0.2	1.1 ± 0.1	1.6 ± 0.2
Cholesterol		1.8 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1

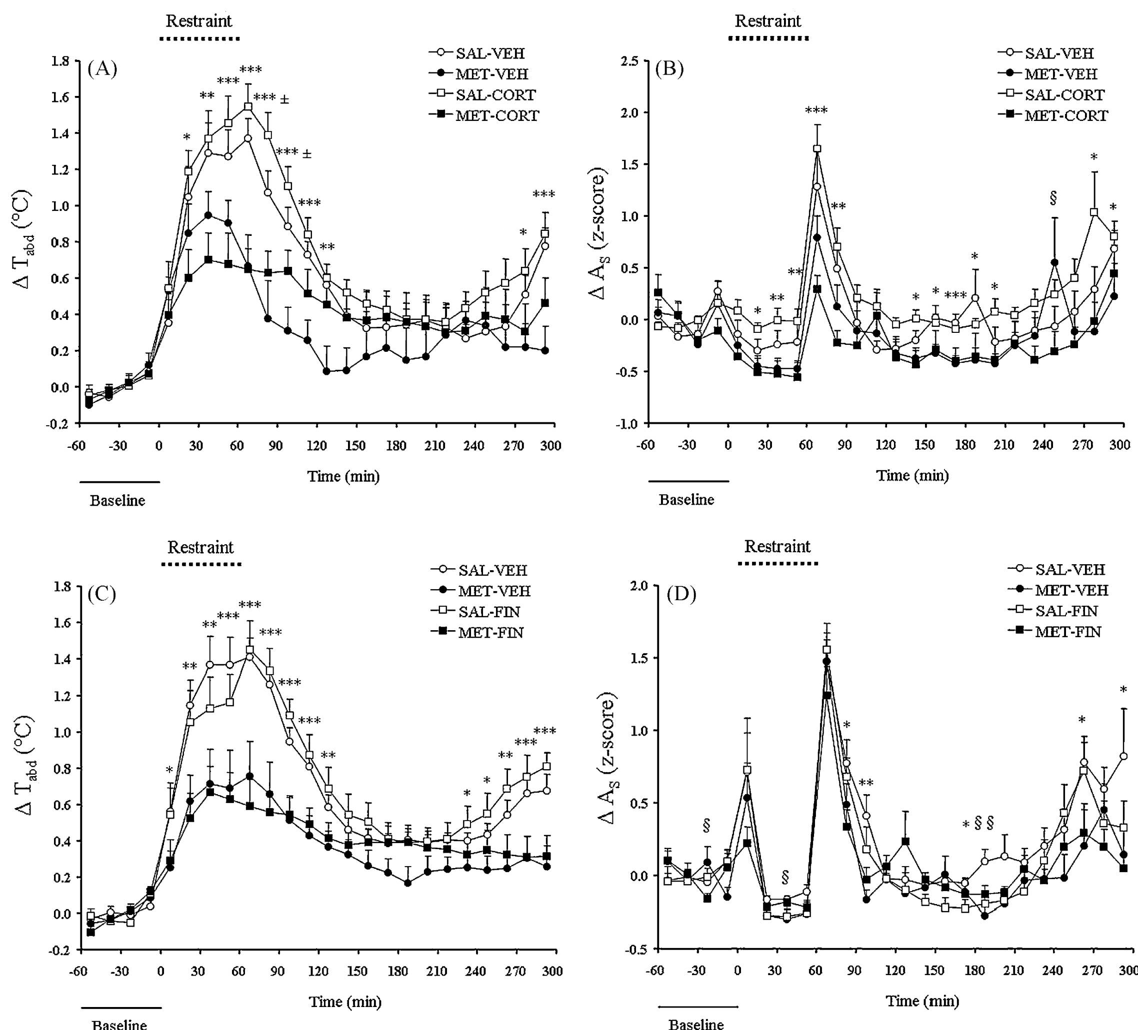

Figure 6 (A and B) Effect of a 1-h restraint on abdominal temperature (A, ΔT_{abd}, °C) and locomotor activity (B, ΔA_S, z-score) in rats treated 5 h earlier with 150 mg kg^{-1} metyrapone IP and 5 mg kg^{-1} corticosterone SC. According to their experimental group, the rats received saline and sesame oil (SAL—VEH, n = 11), saline and corticosterone (SAL—CORT, n = 11), metyrapone and sesame oil (MET—VEH, n = 10) or both metyrapone and corticosterone (MET—CORT, n = 11). (C and D) Effect of a 1-h restraint on body temperature (C, ΔT_{abd}, °C) and locomotor activity (D, ΔA_S, z-score) in rats treated 5 h earlier with 150 mg kg^{-1} metyrapone IP and/or 50 mg kg^{-1} finasteride SC. According to their experimental group, the rats received saline and sesame oil (SAL—VEH, n = 12), saline and finasteride (SAL—FIN, n = 11), metyrapone and sesame oil (MET—VEH, n = 11) or both metyrapone and finasteride (MET—FIN, n = 11). All the rats were restrained. Restraint duration is represented by the dotted line. Comparisons between treatment groups were made through a two-way factorial ANOVA and significant differences are indicated as MET effect: $^*p < 0.05$; $^{**}p < 0.01$ $^{***}p < 0.001$; CORT effect: $^{\pm}p < 0.05$; interactions between MET and CORT or FIN: $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$. Data are expressed as means ± SEM.

p = 0.09). Stress was ineffective on these variables (stress, ns; MET × stress, ns).

4.6. Experiment 6

Similarly to previous observations on immediate effects, metyrapone induced hypothermia and blocked the increase in A_S observed immediately after injection (data not shown). Finasteride co-treatment did not alter the concomitant T_{abd} and A_S 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 ± 0.1 °C; MET—VEH: 37.4 ± 0.1 °C; SAL—FIN: 36.9 ± 0.1 °C and MET—FIN: 37.3 ± 0.1 °C, MET, $p < 0.001$). However, A_S remained similar (SAL—VEH: −0.4 ± 0.1; MET—VEH: −0.3 ± 0.1; SAL—FIN: −0.3 ± 0.1; MET—FIN: −0.3 ± 0.1, ns). During restraint, metyrapone-treated rats exhibited a reduced hyperthermia compared to saline-treated rats (Fig. 6C; MET, $p < 0.001$; MET × 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 × repetition, $p < 0.001$). Finasteride did not modify A_S 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_{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 (Pryce et al., 2003). Hypothermia was not related to the depressed motor activity as both events ran under different time courses. Moreover, hypothermia 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 θ 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 γ 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_{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 modified environmental appraisal as indicated by the decrease in α power which has been related to cognitive performance in mammals (Klimesch, 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; Roozendaal 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 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_{abd}, A_S, EEG power spectra. Moreover, cerebral c-fos mRNA level and plasma corticosterone concentration were similar in both resting treatment groups 1 h after. As expected, T_{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 (Selye, 1950; Nonogaki and Iguchi, 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_{abd} and A_S. Although metyrapone had increased basal blood glycemia, 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 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 $5\text{-}HT_{1A}$ receptor through its effect on intracellular level of corticosteroid (Lopez et al., 1998). A $5\text{-}HT_{1A}$-related mechanism may explain both its hypothermic 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-induce-anxiety disorders and brain ischemia.

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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.

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