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Validation of the long-term assessment of hypothalamic-pituitary-adrenal activity in rats using hair corticosterone as a biomarker

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Running title: Chronic stress and hair corticosterone in rats

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List of abbreviations

ADX: adrenalectomy

AUC: area under the curve

CUS: chronic unpredictable stress

HC: hair corticosterone

IMO: immobilization

IMOch: chronic immobilization

RESTch: chronic restraint

Abstract

The evaluation of chronic activity of the hypothalamic-pituitary-adrenal (HPA) axis is critical for determining the impact of chronic stressful situations. However, current methods have important limitations. The potential use of hair glucocorticoids as a non-invasive, retrospective biomarker of long term HPA activity is gaining acceptance in humans and wild animals. However, there is no study examining hair corticosterone (HC) in laboratory animals. The present study validates a method for measuring HC in rats and demonstrates that it properly reflects chronic HPA activity. The HC concentration was similar in male and female rats, despite higher total plasma corticosterone levels in females, tentatively suggesting that it reflects free rather than total plasma corticosterone. Exposure of male rats to two different chronic stress protocols (chronic immobilization and chronic unpredictable stress) resulted in similarly higher HC levels compared to controls (1.8-fold). HC also increased after a mild chronic stressor (30 min daily restraint). Chronic administration of two different doses of a long-acting ACTH preparation dramatically increased HC (3.1-fold and 21.5-fold, respectively), demonstrating that a ceiling effect in HC accumulation is unlikely under other more natural conditions. Finally, adrenalectomy significantly reduced HC. In conclusion, HC measurement in rats appears appropriate to evaluate integrated chronic changes in circulating corticosterone.

Keywords: Chronic Immobilization, Chronic Unpredictable Stress, ACTH, Glucocorticoids.

INTRODUCTION

Exposure to systemic and emotional stressors involves a rapid activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, resulting in the stimulation of adrenocortical secretion of glucocorticoids. The primary glucocorticoid in rodents is corticosterone, while in humans, non-human primates and most mammals it is cortisol. Corticosterone and cortisol regulate numerous biological processes and play a critical role in the proper adaptation of the organism to stressors (1). One particularly relevant function of glucocorticoids is to inhibit the activation of the HPA axis caused by stressors through negative feedback-loops exerted at different levels, including the pituitary gland, the hypothalamus and some extra-hypothalamic areas, such as the hippocampal formation and the medial prefrontal cortex (2). If exposure to the stressor is prolonged and the stress response continues, this can lead to a syndrome of distress and to deleterious effects on health (3). The activation of the HPA axis is considered a gold standard biomarker of acute stress (4), but the ability of glucocorticoids to reflect long term HPA axis activity is limited. Thus, establishing biomarkers of chronic stress is a great challenge for investigators.

To date, glucocorticoid concentrations have been analyzed mostly in blood serum, saliva, urine and feces, and each of these approaches has clear limitations. Glucocorticoid levels in blood and saliva reflect transient changes in the HPA axis, show high intra-individual variation and are strongly affected by environmental disturbances (5) and circadian and ultradian rhythms (6). Currently, urine and fecal samples are widely accepted as a non-invasive technique (7,8) and they reflect a somewhat longer period of HPA activity (up to 24-48 hours). However, they require repeated sampling from the same individual and are subjected to cross-contamination. More importantly, none of these matrices is able to reflect the long-term accumulative impact of chronic stress exposure.

Therefore, the potential use of hair as a non-invasive, retrospective biomarker of long term HPA activity is of great interest. Hair has been recognized as a biomaterial that accumulates glucocorticoids over weeks to months and that could eventually reflect chronic stress (9). Compared to other matrices, hair does not require special storage or shipping condition prior to analysis and it is a matrix stable for years to centuries (10,11). Several studies in humans have investigated the association between long-term stress exposure and hair cortisol levels, covering a wide range of physical and emotional stressors (12-17). In contrast, only two papers have examined the chronic hair cortisol response to chronic stress in rhesus macaques and vervets monkeys (9,18).

It has been assumed that hair glucocorticoids reflect free rather than total plasma glucocorticoids (19,20), but evidence is scarce. In rats, total plasma corticosterone levels are clearly higher in female compared to male rats (21,22), but plasma levels of corticosteroid-binding globulin are also higher in females (23). Hence, it appears

that the free fraction of circulating corticosterone is similar in male and female rats as supported by similar brain levels of corticosterone (24,25). Similar levels of hair corticosterone in both sexes could provide evidence for the latter possibility.

To our knowledge, there is no report on the technical validation of the measurement of glucocorticoids (corticosterone) in the hair of laboratory rodents and its utility to evaluate chronic stress. This is critical because prior studies in humans and non-human primates, although of great interest, offer few opportunities to introduce manipulations that could more appropriately validate the use of hair as a measure of chronic HPA activation. For instance, it is not known whether hair glucocorticoids reflect the intensity of the chronic stressful situation and the amount of glucocorticoid released. Moreover, this evaluation is needed considering the extensive use of rodents in animal models of diseases. Thus, the aims of the present work were a) to develop and validate a reliable non-invasive method for measuring hair corticosterone concentration in rats, b) to provide evidence that supports the hypothesis that hair glucocorticoids is representative of the free rather than the total fraction of circulating glucocorticoids, and c) to demonstrate that hair corticosterone accurately reflects long term HPA axis activity.

MATERIALS AND METHODS

Animals and general procedures

Male and female Sprague–Dawley rats obtained from the breeding center of the Universitat Autònoma de Barcelona were used. Animals were approximately 60 days old at the beginning of the experiments and were assigned randomly to the experimental groups. Animals were housed individually (experiments 1 and 2) or in pairs (experiment 3) in polypropylene opaque wire-topped cages with a solid-bottom (21.5 × 46.5 × 14.5 cm; Type "1000 cm2", Panlab S.L.U., Barcelona, Spain) containing wood shavings bedding (Lignocel 3/4, Harlan Interfauna Ibérica, Barcelona, Spain); standard temperature conditions (21 ± 1 °C) and a 12-12 h light/dark schedule (lights on at 08:00 h) were used. Food (Global Diet 2014, Harlan Laboratories, Madison, WI) and water were available ad libitum. Animals were individually housed in some experiments because they took part of a bigger experiment studying the influence of stress on saccharine intake (26), this requiring individual housing. We have previously reported on no major differences in general parameters and HPA function in individually versus group housed male Sprague-Dawley rats (27). The animals were allowed to acclimate to the housing conditions for at least one week prior to the beginning of the experimental treatments. If not otherwise stated, experiments were carried out in the morning. The experimental protocols were approved by the Committee of Ethics of the Universitat Autònoma de Barcelona, following the "Principles of laboratory animal care" and were carried out in accordance to the European Communities Council Directive (86/609/EEC).

Starting at least three days after their arrival, all animals were handled three times (2 min each) over a period of one week. The experiments started 48-72 hours after the third handling session. On the last day of handling, animals were subjected to a tail-nick procedure to habituate them to the blood sampling procedure. The tail-nick consisted of gently wrapping the animals with a cloth, making a 2-mm incision at the end of one of the tail veins and then massaging the tail while collecting, within two minutes, 300 µl of blood into ice-cold EDTA capillary tubes (Sarstedt, Granollers, Spain). The tail-nick procedure is extensively used in our laboratory because low resting levels of hormones are obtained (28). After centrifugation (4960 x g, 15 min at 4ºC), plasma was stored at -20ºC. In all experiments, cage-mates were processed simultaneously, including blood sampling. Animals were always stressed in a room different from the animal room and the sampling room.

Only a few seconds of wrapping the animals were necessary to collect a large amount of hair. Given its lipophilic nature, corticosterone is assumed to be incorporated into follicles during the anagen phase of the hair through passive diffusion from the blood (29-31). Thus, hair was shaved in the morning with an electric grooming clipper as close to the skin as possible. Approximately 50 cm^2 of old hair was removed from the region delimited cranially by the 8th lumbar vertebra, caudally by the ischial tuberosity and laterally by the

posterior limbs. This area corresponds approximately to the ischiatic region of the animal. If a second sample of new hair was required by the experimental design, the sample was collected within the same region, being careful not to cut any old hair. Samples were placed into aluminum foil for protection, then into small plastic pouches, numbered, and kept at 4ºC until the time of assay.

Experiment 1: comparison of male and female rats

Sixteen rats (eight males, eight females) were housed individually and blood samples were taken under basal conditions at different time points on a single day (10:00 h; 13:00 h; 17:00 h; 20:00 h; 23:00 h; 10:00 h) to assess total plasma corticosterone circadian rhythm. Hair samples were collected at the end of the experiment.

Experiment 2: effect of two chronic stress procedures on hair corticosterone.

This experiment was conducted to assess if two relatively severe chronic stress models increase hair corticosterone. Eighteen male rats housed individually were randomly assigned to the following three groups: control (n=6), chronic immobilization (IMOch) (n=6) and chronic unpredictable stress (CUS) (n=6). On day one prior to starting the treatments, hair samples were taken from all animals. Body weight and food intake were measured several times throughout the experimental period. Hair was sampled at day 12 instead of on the day after the last stressor (day 10) because hair growth was not sufficient for removal. Animals were then sacrificed, and their adrenal gland and thymus were removed.

Two chronic stress procedures were IMOch and CUS. The chronic treatment lasted from experimental days one to nine. Control animals remained undisturbed. The first model consisted of daily exposure to 1 h of immobilization (IMO) at 09:30 h. IMO rats were stressed by taping their four limbs to metal mounts attached to a board (32). Head movements were restricted with two plastic pieces (7x6 cm) placed in each side of the head and the body was subjected to the board by means of a piece of plastic cloth (10 cm wide) attached with *Velcro* that surrounded the trunk. The CUS procedure (26) consisted of exposing rats to different types of stressors: restraint, electric foot-shocks and forced swimming. For restraint, animals were placed into PVC cylindrical tubes measuring 6-cm diameter and 21.5-cm length for 30, 60 or 90 minutes. The rear top of the apparatus was closed by a cork letting the tail of the rat hang off. Several holes (0.5 cm in diameter) in the walls of the cylinder provided fresh air. For shock, rats received a six second shock (1.5 mA) each minute for 30, 60 or 90 minutes. Rats were put into individual clear Plexiglas boxes with a metal grid floor connected to a shocker (Cibertec, Madrid, Spain). For the swimming condition, animals were placed in transparent cylindrical plastic tanks (height= 40 cm, internal diameter = 19 cm) containing water (25ºC) to a level of 24 cm, as described previously (33). Animals remained in the tank for 20 min. The rats were then withdrawn from the water, returned to their home cages and exposed to swim for an additional 10 min period 1 h later. Similar to others

well-established chronic variable stress procedures (i.e. 34), our CUS model involved changing the stressor and the time every day: day 1, 30 min restraint at 11:00 h; day 2, 30 min of shocks at 12:30 h; day 3, swim at 15:00 h; day 4, 60 min of shocks at 10:30 h; day 5, swim at 08:00 h; day 6, 60 min of restraint at 15:30 h; day 7, swim at 16:00 h; day 8, 90 min of restraint at 10:00 h; and day 9, 90 min of shocks at 14:30 h.

Experiment 3: effects of an intermediate intensity chronic stressor (restraint)

This experiment was conducted to assess if daily repeated exposure to an intermediate intensity, extensively used, stressor (restraint) could be able to increase hair corticosterone. We have previously observed that restraint is of lower intensity than IMO (35). Eighteen male rats housed individually were randomly assigned to control (n=8) or chronic restraint stress (RESTch) (n=8) groups. The latter animals were exposed daily to 30 min restraint for 14 days. The restraint was carried out in PVC cylindrical tubes measuring 6-cm diameter and 21.5-cm length. The rear top of the apparatus was closed by a cork letting the tail of the rat hang off. Several holes (0.5 cm in diameter) in the walls of the cylinder provided fresh air. On the day before starting stress exposure, hair samples were taken from all animals and again on day 15. Body weight and food intake were measured several times throughout the experimental period. Animals were then sacrificed, and their adrenal gland and thymus were removed.

Experiment 4: effect of chronic ACTH administration

The aim of this experiment was two-fold, (a) to demonstrate that hair corticosterone quantitatively reflects the magnitude of chronic corticosterone production and (b) to rule out a ceiling effect in hair accumulation that limits its validity to reflect actual corticosterone secretion under certain conditions. To this end, we administered tetracosactide (Nuvacthén® Depot 1 mg/ml; Defiante farmacêutica, Funchal, Portugal), a synthetic analogue of ACTH consisting of the first 24 amino acids of the naturally occurring hormone. This treatment maintains high levels of ACTH over the 24 h period, thus resulting in a marked and prolonged activation of adrenocortical secretion (36), while maintaining physiological levels of glucocorticoids.

Twenty-four male rats housed in pairs were randomly assigned to three groups (eight rats per group) and received daily saline solution (control) or tetracosactide (20 µg/kg or 100 µg/kg) for 15 days, to allow appropriate hair growth. Injections were administered s.c. from days one to fifteen at 10:00 h. Hair samples were collected on day one (prior to starting the treatments) and again on day 16. Body weight and food intake were monitored throughout the experimental period. The values reported are the food consumption per day/rat, and the statistical unit was the cage. At the end of the experiment, animals were sacrificed and the adrenal gland removed.

Experiment 5: effect of adrenalectomy

This experiment was conducted to assess if hair corticosterone quantitatively reflects the strong reduction of circulating corticosterone after adrenalectomy (ADX). Eight male rats housed individually were randomly assigned to ADX and SHAM groups (n=4 per group). All animals were weighed and shaved on the day prior to surgery, and again on days 15 and 30 post-surgery. Two post-ADX periods were studied because a prior pilot study demonstrated only partial decrease of HC in the two weeks following ADX. Blood samples were collected at days 14 and 29 on two times point on a single day (10:00 h, 19:00 h) as described previously. ADX was performed in rats anesthetized with 2-3% Isofluorane (IsoFlo® Fl. 250 ml, Esteve Spa, Barcelona, Spain) and placed in ventral recumbency. A dorsal midline incision was made with its midpoint centered over the 13th rib and the underlying muscle wall on either side of the spinal column was pierced by scissors. The adrenal glands were located and the forceps were used to tear away the gland and its fat pad. The abdominal muscle was sutured with single interrupted absorbable suture and the skin incision closed with 1-2 simple interrupted non-absorbable sutures. SHAM group was treated the same way as ADX animals but the adrenal glands were not removed. Buprenorphine (Buprex® 0,3 mg/ml, RB Pharmaceuticals Limited, Barcelona, Spain) was injected subcutaneously 30 minutes before surgery and after 24 hours to all animals. ADX rats received 0.9% saline as drinking solution after surgery until the end of the experiment. Animals were then sacrificed and necropsy was performed to ensure the absence of adrenal tissue.

Corticosterone radioimmunoassay and validation of the hair corticosterone assay method

Plasma and hair corticosterone levels were assayed by double antibody radioimmunoassay (RIA). In brief, corticosterone RIA used 125I-corticosterone–carboximethyloxime–tyrosine–methylester (ICN-Laboratorios Leti, Barcelona, Spain) and synthetic corticosterone (Sigma, Barcelona, Spain) as the standard and an antibody raised in rabbits against corticosterone–carboximethyloxime-BSA that was kindly provided by Dr. G. Makara (Inst. Exp. Med., Budapest, Hungary). The characteristics of the antibody and the basic RIA procedure have been described previously (37). The range of the standard curve was between 6.25 pg and 1600 pg of corticosterone per tube. The antibody used has a cross-reactive of 2.3% with progesterone, 1.5% with desoxycorticosterone and less than 0.1% with any other steroid tested. All samples that were statistically compared were run in the same assay to avoid inter-assay variability.

For hair corticosterone extraction we developed a washing protocol based on that reported by Davenport et al. (9) in rhesus macaques and subsequently applied successfully in other species (38,39). For corticosterone extraction, hair samples were washed twice by mixing with 15 ml of undiluted 2-propanol (Sigma, Barcelona, Spain) per ~200 mg of hair using a tube mixer that provides 360º rotation at 20 rpm/min (Minilabroller; Sigma,

Barcelona, Spain); each wash was followed by centrifugation at 1500 ×g (1 min, 4°C) and removal of the supernatant. Hair samples were put on aluminum foil inside an incubator at 37 °C for approximately 30 min to completely dry the samples. Dried hair was frozen with liquid nitrogen and minced in a mortar while it was frozen. Five ml of methanol HPLC grade (Scharlau, Barcelona, Spain) were added to 100 mg of powdered hair and mixed at room temperature by rotation at 20 rpm/min for 21 h. After a centrifugation at 10,000 ×g (10 min, 4°C), 4 ml of supernatant were recovered in a glass tube and dried at 38 °C under a gentle stream of nitrogen gas. Dried extracts were reconstituted with 200 µl of 0.2 M sodium phosphate buffer, pH 7.6, and stored at -20ºC. On the day of the analysis, samples were centrifuged at 16,000 x g (30 min, 4ºC) and the supernatant was recovered and assayed. In the final validated protocol, 10 µl of reconstituted hair extract were used per tube.

Statistical analysis

Data were analyzed with the Statistical Program for Social Sciences (SPSS), version 17. A two-way ANOVA with sex as the between-subjects factor (two levels), and time of day as the within-subjects factor (six levels) was used for the study of the circadian rhythm of plasma corticosterone in Exp 1. When appropriate, further comparisons were performed. In other cases, a t-test was used to compare two independent means (e.g., sex differences in hair corticosterone). The one-way ANOVA was followed by a post-hoc Student-Newman-Keuls (SNK) test when more than two independent means were compared (the criterion for significance was set at $p<0.05$ in the SNK test). When variances were not homogenous between groups, logarithmic transformation of data was used. The area under the curve (AUC) for each animal was calculated from the circadian rhythm data with GraphPad Prism 5. To calculate AUC, corticosterone concentration (ng/ml) was plotted in the Y axis versus time (hours) in the X axis. The area is computed connecting a straight line between every set of adjacent points defining the curve, and sums up the area beneath these lines.

RESULTS

Technical validation

To validate the described methods, the linearity, accuracy, precision and reproducibility of the assay were studied. The validation summary is shown in table 1. Assay linearity was evaluated by assaying three different dilutions of three independent samples. A good parallelism between sample dilution and standard curve was found. To evaluate assay accuracy, each of five reconstituted hair extracts were divided in three independent aliquots and spiked with three different known corticosterone concentrations, mixed and assayed. The percentage of recovery were determined as follows ((measured corticosterone in spiked sample / measured corticosterone in non-spiked sample + corticosterone added)*100). Recovery was 97.9 ± 10.3 % (mean ± SD) indicating a good selectivity of the assay. Taken together, parallelism and selectivity results demonstrate the proportionality between hair corticosterone and the reference standard, and the absence of significant matrix interferences, at least in the range of hair extracts assayed. Precision was estimated by repeatedly extracting and assaying samples in the same assay (6 times). Reproducibility was estimated by assaying three samples in 8 independent assays. The mean intra-assay and inter-assay CV were 4.3% and 12.2%, respectively. The lowest corticosterone standard used in the assay was 6.25 pg/tube; using 8 mg of hair per tube, hair corticosterone can be assayed with reasonable accuracy and precision with a sensitivity of 0.78 pg/mg of hair.

Biological validation

Comparison of male and female rats

Figure 1 shows comparison of corticosterone concentration in male and female rats. The two-way ANOVA of the circadian pattern of plasma corticosterone revealed significant effects of sampling time ($F(5,70)= 4.6$, $p<0.001$), sex ($F(1,14)= 7.1$, $p=0.019$) and the interaction sampling time x sex ($F(5,70)= 3.9$, $p=0.021$). The decomposition of the interaction showed that plasma corticosterone levels were higher in females at 10:00 h. The t-test analysis revealed that the corticosterone AUC was significantly higher in females than males ($t(14)= 2.68$, $p=0.018$). However, no differences were found in the hair corticosterone concentration between sexes ($t(14)= -0.22$, $p=0.826$).

Effects of two chronic severe stressors

Table 2 shows the results for body weight gain, food intake and adrenal and thymus weights. Statistical analysis revealed significant differences between groups in body weight gain ($F(2,17)=26.2$, $p<0.001$) and food intake ($F(2,17)=14.3$, $p<0.001$). Post-hoc comparisons with the SNK test showed that CUS decreased body weight gain compared with controls, but the effect of IMOch was more pronounced and differed from both control and

CUS rats. Although CUS tended to decrease food intake, the effect only reached significance in IMOch compared to controls. Neither adrenal nor thymus weight were significantly affected by CUS or IMOch.

The amount of hair obtained after chronic exposure to the stressors was similar in all groups, suggesting no effect of stress on hair growth (not shown). Hair corticosterone concentrations after the chronic stress procedures are shown in Figure 2. We separately analyzed hair corticosterone before and after chronic stress because they represent completely different time domains for corticosterone accumulation and hair growth is not uniform across its cycle (40). Samples taken after chronic stress represents only the period of exposure to chronic stress. As expected, the one-way ANOVA revealed no group differences on day one samples (before chronic stress)(Control: 16.5±1.6 pg/mg, IMOch: 16.2±1.8 pg/mg and CUS: 17.1±1.6 pg/mg). The one-way ANOVA revealed significant group differences on day 12 ($F(2,17)=10.2$, $p=0.002$). Further post-hoc comparisons showed that both IMOch and CUS significantly increased hair corticosterone concentration (approximately 80%) compared to controls ($p<0.05$), with no differences between both chronic stress paradigms.

Effects of chronic restraint stress

RESTch did not alter food intake or adrenal and thymus weights (not shown), but significantly reduced body weight gain (90.8 g vs 69.0 g, $t(14)=4.73$, $p<0.001$). Regarding hair corticosterone, no differences between groups were observed on day 1 (before chronic restraint stress)(Control: 31.0±4.1 pg/mg, RESTch: 35.9±2.7 pg/mg). As we can predict that chronic stress should increase hair corticosterone, one-tail t test was used that revealed significant increase after the chronic stress procedure ($t(14)=-1.82$, $p=0.045$) (Figure 3).

Effects of chronic ACTH administration

Table 3 shows the results for body weight gain, food intake and adrenal weight. The one-way ANOVA revealed significant differences between groups in absolute body weight ($F(2,23)=17.42$, $p<0.001$), body weight gain ($F(2,23)= 70.4$, $p<0.001$), both absolute and relative adrenal weights ($F(2,23)=93.7$ and $F(2,23)=110.0$, respectively, $p<0.001$ in both cases) and food intake ($F(2,11)= 6.3$, $p=0.019$). Post-hoc SNK comparisons showed that only the higher dose of ACTH significantly decreased absolute body weight, but both doses of ACTH caused a statistically significant reduction in body weight gain, with a significantly greater effect in the high dose group. These results illustrate that greater sensitivity of body weight gain instead of absolute body weight to detect the impact of stress. Changes in absolute and relative adrenal weight followed the same pattern as body weight gain. ACTH treatment caused a reduction in food intake that was statistically significant only for the high dose.

Regarding hair corticosterone, the one-way ANOVA did not reveal significant differences between groups on day one (before drug administration)(Control: 25.6±1.3 pg/mg, 20 µg/kg: 27.5±1.8 pg/mg and 100 µg/kg 27.0±1.8 pg/mg) but did on day 16 ($F(2,23)= 182.7$, $p<0.001$) (Figure 4). A post-hoc SNK test revealed that both doses dramatically increased hair corticosterone concentration ($p<0.05$), with a much greater increase in the 100 µg/kg group. In the latter case, ACTH administration caused more than a 20-fold increase in hair corticosterone levels.

Effect of adrenalectomy

ADX drastically decreased the circulating corticosterone levels to less than 10ng/ml, even in the peak of the circadian cycle. There were not differences between groups on hair corticosterone on day one (SHAM: 17.8±1.5 pg/mg; ADX: 17.4±1.8 pg/mg). However, a progressive decrease in hair corticosterone compared with SHAM group was found on days 15 and 30 after surgery (Figure 5). The t-test revealed statistically significant differences between ADX and SHAM rats on both on day 15 ($t(6)=3.32$, $p=0.016$) and day 30 ($t(6)=10.08$, $p<0.001$).

DISCUSSION

The present results represent the first technical and biological validation of hair corticosterone measurement in rats. We have validated a specific washing protocol and extraction assay method that has good sensitivity. More importantly, the present results give tentative support to the hypothesis that hair corticosterone reflects plasma free corticosterone fraction and that it is a good marker of chronic HPA activation in typical chronic stress procedures lasting several weeks.

The results of the first experiment showed the expected and well-characterized circadian pattern of plasma corticosterone with lower levels in the morning and the highest levels occurring around the onset of the dark cycle. As expected (21,22), females showed higher levels of corticosterone throughout the 24 h period than males. However, hair corticosterone concentration was similar in both sexes. As it has been reported that brain corticosterone concentration, which reflects free plasma corticosterone, is similar in male and female rats (24,25), our results support the hypothesis (19,20) that hair glucocorticoids are a reflection of free rather than total circulating glucocorticoid levels. The present results are supported by prior studies in humans and other species failing to find sex differences in hair cortisol or higher levels in men under non-stressful conditions (14,15,29,41-43). Small and inconsistent differences have been reported in salivary cortisol, which reflects free cortisol, between men and women under resting conditions (44-46). However, women consistently respond less to acute stressors (47). Nevertheless, alternative possibilities cannot be completely rule out, including sex differences in the rate of hair growth or hair corticosterone accumulation/degradation that may compensate for differences in circulating corticosterone. Moreover, sex differences could appear after exposure to chronic stress rather than under basal conditions.

Hair cortisol has attracted great interest as a means to evaluate chronic stress in humans (see 20,43,48) and has potential as a fundamental tool in human stress research. However, studies in experimental animals are needed to validate the procedure. To our knowledge, there are only two studies that aimed to characterize hair cortisol changes after chronic stress in non-human primates. Chronic stress was induced by moving animals from one facility to another or to a new social environment; in both cases, higher hair cortisol levels were observed 14 and 29 weeks after the chronic stress (9,18).

In the present experiment, we directly assessed the influence of two chronic stress models: chronic repeated IMO and CUS. By measuring not only hair corticosterone but also other well-known stress-related parameters (food intake, body weight gain, adrenal and thymus weight), we can better characterize the overall intensity of the stressors and compare the above parameters with the changes in hair corticosterone concentration. IMOch and CUS both significantly reduced body weight gain, and the former also reduced food intake, suggesting that

a greater overall physiological impact of IMOch than CUS. However, both stressors increased hair corticosterone concentration to the same extent, demonstrating for the first time that hair corticosterone is appropriate to evaluate chronic stress in laboratory rodents. A contribution of chronic-stress induced changes in hair growth was rule out as no group differences have been observed. Although food intake and body weight gain are rather good markers of the severity of stressors, a partial dissociation between different stress markers is not unlikely. Interestingly, in the present experiment, changes in relative adrenal weight of the adrenals and thymus were not significant. Thus, it appears that the measurement of hair corticosterone appropriately reflects chronic stress states lasting only nine days and that hair corticosterone is perhaps more sensitive than changes in adrenal or thymus weight.

Chronic IMO and the present CUS procedure are severe stressful situations. We then wanted to study a chronic stressful situation of lower intensity than the previous one. Restraint is clearly of lower intensity than IMO (35), and an extensively used model. We found that 30 min restraint for 14 days increased hair corticosterone, although the increase was, as expected, of lower magnitude than that observed after IMO and CUS.

The preceding results clearly demonstrated that hair corticosterone is a sensitive index of chronic stress. However, it is unclear the extent to which accumulation of corticosterone in hair saturates, thus diminishing its validity as a marker when using severe stressors and/or more prolonged periods of exposure. In fact, a saturation effect could explain the lack of differences between IMOch and CUS. To answer this question, we did an experiment administering two doses of a long-acting ACTH preparation (tetracosactide), which slowly release ACTH and can stimulate the adrenal for hours after its administration. This procedure has been previously used in the literature and results in strong increases in adrenal weight (49-51). Our results demonstrated dose-dependent reductions in body weight and food intake, as well as dose-dependent increases in adrenal weight and hair corticosterone. The decreases in food intake and body weight gain observed with the highest dose of ACTH are in accordance with one previous report (51). In contrast, the lowest dose did not affect food intake but nevertheless decreased body weight, likely because of the well-known catabolic effects of glucocorticoids. Dramatic dose-dependent increases in hair corticosterone were observed after ACTH administration. These data not only confirm that hair corticosterone reflects the magnitude of the chronic activation of the HPA axis but also showed no evidence for saturation of corticosterone incorporation into the hair matrix. Therefore, it is clear that a ceiling effect in hair corticosterone accumulation under more natural conditions of chronic stress is unlikely.

The preceding experiments indicate that hair corticosterone is able to reflect increased in circulating corticosterone, but how this variable responds to reduced levels is unknown. Then we studied hair corticosterone after ADX. In a pilot study we observed only a partial decrease two weeks after ADX.

Therefore, we repeated the experiment taking hair at 15 and 30 days after surgery. Again, we observed a moderate decrease after the first hair sampling, but after the second sampling a much stronger decrease was found. It thus appears that hair shaved two weeks after ADX only partially reflects the strong reduction of circulating corticosterone. One possible explanation is that the portion of hair under the skin, which accumulated corticosterone under regular conditions, erupted later to be sampled two-weeks after ADX, thus partially masking the effect of ADX. However, it appears that this part of the hair takes at least 3 days to emerge (52) and it is unable to explain why the decrease in hair corticosterone was so modest. Although the precise mechanisms of glucocorticoid incorporation to hair are unknown (53), it is possible that concentration of corticosterone is much higher in follicular cells that are generating the shaft than in the shaft itself. Therefore it takes more days for the follicular cells born in absence of glucocorticoids to reflect the effect of ADX.

Conclusions and methodological concerns

In conclusion, the washing and extraction procedure used was validated for rats and could also be potentially applied to other laboratory animals, such as mice. The present data indicates that hair corticosterone concentration is similar in male and female rats and could reflect plasma free corticosterone levels. Hair corticosterone was elevated in response to various chronic stressful procedures and chronic ACTH administration, demonstrating that it provides a good direct index of HPA activity over long periods of time that better compares with indirect parameters, such as adrenal or thymus weight. Although hair corticosterone was eventually able to reflect the decrease in circulating corticosterone after ADX, this was clearly found only 3-4 weeks after the intervention. Considering the irregular hair growth of rats and the latter concern, it appears that this procedure could be most appropriate to evaluate chronic changes (i.e. one month) in circulating corticosterone. With much shorter periods of time, classical urine and fecal samples taking several times are still valuable tools. Finally, this technique enables the use of rats to better characterize the impact of genetically or environmentally generated individual differences on the HPA axis and the impact on environmental conditions and chronic stressors, particularly those involving complex designs and stimuli.

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REFERENCES

1. Sapolsky, R.M., Romero, L.M., and Munck, A.U. (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.***21**, 55-89.

2. Armario, A.(2006) The hypothalamic-pituitary-adrenal axis: what can it tell us about stressors? *CNS Neurol. Disord. Drug Targets***5**, 485-501.

3. McEwen, B.S.1(1998) Stress, adaptation, and disease. Allostatis and allostatic load. *Ann. N. Y. Acad. Sci.***840**, 33-44.

4. Armario, A., Daviu, N., Muñoz-Abellan, C., Rabasa, C., Fuentes, S., Belda, X., Gagliano, H., and Nadal, R. (2012) What can we know from pituitary-adrenal hormones about the nature and consequences of exposure to emotional stressors? *Cell. Mol. Neurobiol.***32**, 749-58.

5. Hellhammer, J., Fries, E., Schweisthal, O.W., Schlotz, W., Stone, A.A., and Hagemann, D. (2007) Several daily measurements are necessary to reliably assess the cortisol rise after awakening: state- and trait components. *Psychoneuroendocrinology***32**, 80-86.

6. Lightman, S.L., Wiles, C.C., Atkinson, H.C., Henley, D.E., Russell, G.M., Leendertz, J.A., McKenna, M.A., Spiga, F., Wood, S.A., and Conway-Campbell, B.L. (2008). The significance of glucocorticoid pulsatility. *Eur. J. Pharmacol.***583**, 255-262.

7. Rooney, N.J., Gaines, S.A., and Bradshaw, J.W.S. (2004) Behavioural and glucocorticoid responses of dogs (*Canis familiaris*) to kenneling: Investigating mitigation of stress by prior habituation. *Physiol. Behav.***92,** 847-854.

8. Buchanan, K.L., and Goldsmith, A.R. (2007). Noninvasive endocrine data for behavioral studies: The importance of validation. *Anim. Behav.***67**, 183-185.

9. Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., and Meyer, J.S. (2006) Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *Gen. Comp. Endocrinol.***147**, 255-261.

10. Kintz, P. 2004. Value of hair analysis in postmortem toxicology. *Forensic Sci. Int.***142**, 127-134.

11. Webb, E., Thomson, S., Nelson, A., White, C., Koren, G., Rieder, M., and Van Uum, S.H.M. (2010) Assessing individual systemic stress through cortisol analysis of archaeological hair. *J. Archaeol. Sci.***37**, 807-812.

12. Yamada, J., Stevens, B., de Silva, N., Gibbins, S., Beyene, J., Taddio, A., Newman, C., and Koren, G. (2007) Hair cortisol as a potential biologic marker of chronic stress in hospitalized neonates. *Neonatology***92**, 42-49.

13. Van Uum, S.H.M., Sauve, B., Fraser, L.A., Morely-Forster, P., Paul, T.L., and Koren, G. (2008) Elevated content of cortisol in hair of patientes with severe chronic pain: a novel biomarker for stress. *Stress***11**, 483-488.

14. Dettenborn, L., Tietze, A., Bruckner, F., and Kirschbaum, C. (2010) Higher cortisol content in hair among long-term unemployed individuals compared to controls. *Psychoneuroendocrinology***35**, 1404-1409.

15. Karlén, J., Ludvigsson, J.,Frostell, A., Theodorsson, E., and Faresjo, T. (2011) Cortisol in hair measured in young adults – a biomarker of major life stressors? *BMC Clin. Pathol.***25**, 11-12.

16. Grassi-Oliveira, R., Pezzi, J.C., Daruy-Filho, L., Wendt Viola, T., D'Avila Francke, I., Leite C.E., and Brietzke, E. (2012). Hair cortisol and stressful life events retrospective assessment in crack cocaine users. *Am. J. Drug Alcohol Abuse***38**, 535-538.

17. Skoluda, N., Dettenborn, L., Stalder, T., and Kirschbaum, C. (2012) Elevated hair cortisol concentrations in endurance athletes. *Psychoneuroendocrinology***37**, 611-617.

18. Fairbanks, L.A., Jorgensen, M.J., Bailey, J.N., Breidenthal, S.E., Grzywa, R., and Laudenslager, M.L. (2011). Heritability and genetic correlation of hair cortisol in vervet monkeys in low and higher stress environments. *Psychoneuroendocrinology***36**, 1201-1208.

19. Meyer, J.S., and Novak, M.A. (2012) Minireview: Hair cortisol. A novel biomarker of hypothalamic-pituitary-adrenocortical activity. *Endocrinology***153**, 4120-4127.

20. Russell, E., Koren, G., Rieder, M., and Van Uum, S.H.M. (2012). Hair cortisol as a bilogical marker of chronic stress: current status, future directions and unanswered questions. *Psychoneuroendocrinology***37**, 589-601.

21. Hiroshige, T., Abe, K., Wada, S., and Kaneko, M. (1973). Sex difference in circadian periodicity of CRF activity in the rat hypothalamus. *Neuroendocrinology***11**, 306-320.

22. Atkinson, H.C., and Waddell, B.J. (1997) Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. *Endocrinology***138**, 3842-3848.

23. Gala, R.R., and Westphal, U. (1965) Corticosteroid-binding globulin in the rat: studies on the sex difference. *Endocrinology***77**, 841-851.

24. Droste, S.K., de Groote, L., Atkinson, H.C., Lightman, S.L., Reul, J.M.H.M., and Linthorst, A.C.E. (2008) Corticosterone levels in the brain show a distinct ultradian rhytm but a delayed response to forced swim stress. *Endocrinology***149**, 3244-3253.

25. Droste, S.K., de Groote, L., Lightman, S.L., Reul, J.M.H.M., and Linthorst, A.C.E. (2009) The ultradian

and circadian rhytms of free corticosterone in the brain are not affected by gender: an in vivo microdialysis study in wistar rats. *J. Neuroendocrinol.***21**, 132-140.

26. Pastor-Ciruana, J., Rabasa, C., Ortega-Sánchez, JA., Sanchís-Ollè, M., Gabriel-Salazar, M., Ginesta, M., Belda, X., Daviu, N., Nadal, R., and Armario, A. (2014) Prior exposure to repeated immobilization or chronic unpredictable stress protects from some negative sequels of an acute immobilization. *Behav Brain Res***265**, 155-162.

27. Giralt, M., and Armario, A. (1989) Individual housing does not influence the adaptation of the pituitary-adrenal axis and other physiological variables to chronic stress in adult male rats. *Physiol. Behav*.**45**, 477-481.

28. Belda, X., Márquez, C., and Armario, A. (2004) Long-term effects of a single exposure to stress in adult rats on behavior and hypothalamic-pituitary-adrenal responsiveness: comparison of two outbred rat strains. *Behav. Brain Res.154*, 399-408.

29. Cone, E.J., and Joseph, R.E. (1996) The potential for bias in hair testing for drugs of abuse. In *Drug testing hair* (Kintz, P., Ed.) pp. 69-93, CRC Press, Boca Raton, Fla, U.S.A.

30. Raul, J.S., Cirimele, V., Ludes, B., and Kintz, P. (2004) Detection of physiological concentrations of cortisol and cortisone in human hair. *Clin. Biochem.***37**, 1105-1111

31. Pragst, F., and Balikova, M.A. (2006) State of the art in hair analysis for detection of drug and alcohol abuse. *Clin. Chim. Acta***370**, 17-49.

32. García, A., Martí, O., Vallès, A., Dal-Zotto, S., and Armario, A. (2000) Recovery of the hypothalamic-pituitary-adrenal response to stress. Effect of stress intensity, stress duration and previous stress exposure. *Neuroendocrinology***72**, 114-125.

33. Dal-Zotto, S., Martí, O., and Armario, A. (2000) Influence of single or repeated experience of rats with forced swimming on behavioural and physiological responses to the stressor. *Behav. Brain Res.***114**, 175-181.

34. Ulrich-Lai, Y.M., Figueiredo, H.F., Ostrander, M.M., Choi, D.C., Engeland, W.C., and Herman, J.P. (2006) Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *Am. J. Physiol. Endocrinol. Metab.* 291, E965-E973.

35. Campmany, L., Pol, O., and Armario, A. (1996) The effects of two chronic intermittent stressors on brain monoamines. *Pharmacol. Biochem. Behav.***53**, 517-523.

36 . Riondel, A.M., Rebuffat, P., Mazzocchi, G., Nussdorfer, G.G., Gaillard, R.C., Bockhorn, L., Nussberfer, J., Vallotton, M.B., and Muller, A.F. (1987). Long-term effects of ACTH combined with angiotensin II on steroidogenesis and adrenal zona glomerulosa morphology in the rat. *Acta Endocrinol (Copenh)***114**, 47-54.

37. Zelena, D., Mergl, Z., Foldes, A., Kovács, K.J., Tóth, Z., and Makara, G.B. (2003) Role of hypothalamic inputs in maintaining pituitary-adrenal responsiveness in repeated restraint. *Am. J. Physiol. Endocrinol. Metab* **285**, E1110-E1117.

38. Macbeth, B.J., Cattet, M.R.L., Stenhouse, G.B., Gibeau, M.L., and Janz, D.M. (2010) Hair cortisol concentration as a nonivasive measure of long-term stress in free-ranging grizzly bears (*Ursus arctos*): considerations with implications for other wildlife. *Can. J. Zool.***88**, 935-949.

39. Bennett, A., and Hayssen, V. (2010) Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. *Domest. Anim. Endocrinol.***39**, 171-180.

40. Priestley, G.C. (1966) Rates and duration of hair growth in the albino rat. *J. Anat.***100,** 147-157.

41. Manenschijn, L., Spijker, A.T., Koper, J.W., Jetten, A. M., Giltay, E.J., Haffmans, J., Hoencamp, E., and van Rossum, E.F.C. (2012) Long-term cortisol in bipolar disorder: Associations with age of onset and psychiatric co-morbidity. *Psychoneuroendocrinology***37**, 1960-1968.

42. O'Brien, K.M., Tronick, E.Z., and Moore, C.L. (2012) Relationship between Hair Cortisol and Perceived Chronic Stress in a Diverse Sample. *Stress Health.***6**. doi: 10.1002/smi.2475

43. Stalder, T., and Kirschbaum, C. (2012) Analysis of cortisol in hair – state of the art and future directions. *Brain Behav. Immun.***26**, 1019-1029.

44. King, J.A., Rosal, M.C., Ma, Y., Reed, G., Kelly, T.A., Stanek, E.J., and Ockene, I.S. (2000) Sequence and seasonal effects of salivary cortisol. *Behav. Med.***26**, 67-73.

45. Larsson, C.A., Gullberg, B., Råstam, L., and Lindblad, U. (2009) Salivary cortisol differs with age and sex and shows inverse associations with WHR in Swedish women: a cross-sectional study. *BMC Endocr. Disord*.**21**, 9-16.

46. Lederbogen, F., Kühner, C., Kirschbaum, C., Meisinger, C., Lammich, J., Holle, R., Krumm, B., von Lengerke, T., Wichmann, H.E., Deuschle, M., and Ladwig, K.H. (2010) Salivary cortisol in a middle-aged community sample: results from 990 men and women of the KORA-F3 Augsburg study. *Eur. J. Endocrinol.***63**, 443-51.

47. Kudielka, B.M., Hellhammer, D.H., and Wüst, S. (2009) Why do we respond so differently? Reviewing determinants of human salivary cortisol responses to challenge. *Psychoneuroendocrinology***34**, 2-18.

48. Staufenbiel, S.M., Penninx, B.W.J.H., Spijker, A.T., Elzinga, B.M., and Van Rossum, E.F.C. (2013) Hair cortisol, stress exposure, and mental health in humans: A systematic review. *Psychoneuroendocrinology***38**, 1220-1235.

49. Stark, E., Ács, E., Makara, G.B., and Mihály, K. (1968) The hypophyseal-adrenocortical response to various different stressing procedures in ACTH-treated rats. *Can. J. Physiol. Pharmacol*.**46**, 567-571.

50. Alario, P., Gamallo, A., Beato, M.J., and Trancho, G. (1987) Body weight gain, food intake and adrenal development in chronic noise stressed rats. *Physiol. Behav*.**40**, 29-32.

51. Giralt, M., Garcia-Marquez, C., and Armario, A. (1987) Previous chronic ACTH administration does not protect against the effects of acute or chronic stress in male rats. *Physiol. Behav*.**40**, 165-170.

52. Liu, L., Guo, D., Xin, X., and Fang, J. (2008) Observation of a system of linear loops formed by re-growing hairs on rat skin. *The anatomical recor*.**291,** 858-868.

53. Meyer, J.S., and Novak, M.A. (2012) Minireview: hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. *Endocrinology***153**, 4120-4127.

Figures Legends

Figure 1. Plasma and hair corticosterone levels in male and female rats. The upper panel shows the circadian rhythm of total plasma corticosterone. Samples were taken at different time points during the lights on period (10:00 h, 13:00 h, and 17:00 h) and the nocturnal period (20:00 h and 23:00 h, marked in shadow). Means ± SEM (n=8 per group) are represented. Circadian rhythm data were analyzed by two-way ANOVA with repeated measures for the factor time. The area under the curve (AUC) of plasma corticosterone was calculated from the circadian rhythm data for each animal (left bottom panel). Hair corticosterone levels are shown in the right bottom panel; * $p<0.05$ between sexes.

Figure 2. Effects of two chronic stress procedures on hair corticosterone. Chronic exposure to IMO (IMOch) or to various different stressors (CUS) started on day one and was completed on day nine. Hair samples were taken before the experiment (day 1) and at day 12. Only the results for day 12 are shown. Means ± SEM (n=6 per group) are represented. Groups were statistically compared with a one-way ANOVA followed by a SNK post-hoc test when appropriate; * $p<0.05$ *vs.* control group.

Figure. 3. Effects of chronic restraint (RESTch) on hair corticosterone. Animals were exposed to RESTch for 14 days. Hair samples were taken before the experiment (day 1) and at day 15. Only the results for day 15 are shown. Means ± SEM (n=8 per group) are represented. Groups were statistically compared with a t-test; * $p<0.05$ vs control group.

Figure. 4. Effects of chronic ACTH (tetracosactide) administration on hair corticosterone. Vehicle or two doses of tetracosactide (20 µg/Kg and 100 µg/Kg) were injected for 15 days. Hair samples were taken before (day 1) and after drug administration (day 16). Only the results for day 16 are shown. Means ± SEM (n=8 per group) are represented. Groups were statistically compared with a one-way ANOVA followed by a SNK post-hoc test when appropriate; * $p<0.05$ *vs.* control group; $ $p<0.05$ *vs.* 20 µg/Kg group.

Figure. 5. Effect of adrenalectomy (ADX) on hair corticosterone. Hair samples were collected at day 1 (before surgery), 15 and 30, but only the results for day 15 and 30 are shown. Means ± SEM (n=4 per group) are represented. Groups were statistically compared with a t-test; * $p<0.05$ vs SHAM group.

Table 1. Linearity and recovery

1.1 Linearity

Sample	Hair used (mg/tube)	Corticosterone (pg/mg)	%
1	8	19.9±0.3	100
	4	20.5±0.1	103
	2	20.6±0.5	104
2	1.4	14.8±0.2	100
	0.7	14.4±0.5	97
	0.35	15.1±0.3	102
3	8	16.6±0.6	100
	4	20.4±0.5	123
	2	22.8±0.8	137

1.2 Recovery

Sample	Corticosterone		
	Added (pg/tube)	Observed (pg/tube)	%
4	0	74.1±4.8	
	50	133.4±15.8	107
	100	168.4±8.0	97
	200	273.0±0.4	100
5	0	39.2±3.5	
	50	97.8±0.8	110
	100	152.7±4.6	110
	200	222.9±18.0	93
6	0	13.8±1.7	
	50	52.5±4.6	81
	100	114.0±19.8	100
	200	188.4±24.0	88
7	0	40.8±4.9	
	50	73.0±0.2	80
	100	143.5±2.9	102
	200	235.8±26.0	98
8	0	14.9±0.2	
	50	73.6±2.3	114
	100	101.5±0.2	89
	200	216.5±8.2	100

To assay linearity, three independent samples were diluted with a ratio of ½ and ¼. The average concentration (mean±SEM) and the percent of the expected value for each sample is shown. To assay recovery, three known concentrations of corticosterone were added (+50 pg; +100 pg and + 200 pg per tube) to five hair extracts. The average concentration (mean±SEM) is shown. Percent recovery was calculated on the observed *vs* expected.

Table 2. Effects of two chronic stress procedures on body weight gain, food intake, and adrenal and thymus weight (Experiment 2).

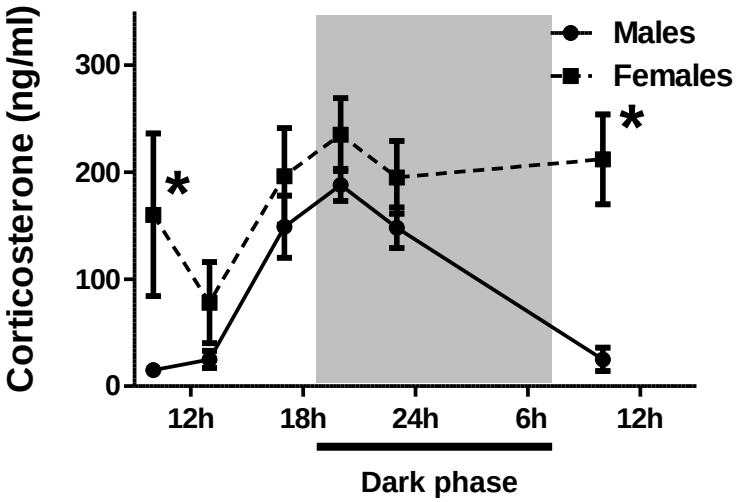
					Adrenal Weight		Thymus Weight	
	Body Weight at day 9 (g)	Body Weight Gain (g)	Food Intake (g/rat/day)	Body Weight at day 12 (g)	Absolute (mg)	Relative mg of (tissue/100 g body weight)	Absolute (mg)	Relative mg of (tissue/100 g body weight)
Control	375.7±17.8	60.5±2.5	28.2±1.0	441.4±54.6	57.2±3.1	13.0±0.7	576.9±65.1	129.3±10.6
IMOch	329.0±14.9	12.2±5.7*	22.2±0.8*	395.0±29.0	53.8±2.5	13.6±0.6	450.3±52.7	115.9±16.0
CUS	352.9±12.1	29.9±5.4*$	26.2±0.5	389.6±29.8	59.6±3.1	15.3±0.7	451.8±33.1	117.2±10.7

Table 3. Effects of two doses of chronic tetracosactide administration on body weight gain, food intake and adrenal weight (Experiment 3).

				Adrenal weight	
	Body Weight (g)	Body Weight Gain (g)	Food Intake (g/rat/day)	Absolute (mg)	Relative (mg of tissue/100g body weight)
Vehicle	344,9±9.0	84.3±4.3	47.0±1.7	41.5±2.3	12.0±0.5
20 µg/Kg	324,4±8.9	63.6±3.5 *	42.4±1.0	56.5±4.0 *	17.5±1.3 *
100 µg/Kg	273,7±8.3*$	12.8±5.2 *$	39.1±1.9*	113.9±5.0 *$	42.0±2.4 *$

Total body weight gain (n=8 per group) and average food intake per day and cage (n=4 per group) during the chronic administration period (between day 1 and 15). Absolute body weight and adrenal weight was measured on day 16. In this experiment animals were housed in pairs so food consumption refers to the home cage intake and not to the individual animals. Means ± SEM are represented. Groups differences were analyzed by one-way ANOVA followed by SNK post-hoc comparisons; * $p<0.05$ *vs* vehicle group; $^{\$}$ $p<0.05$ *vs* the lower dose of the drug.

Figure 1


Corticosterone (ng/ml)
300
200
100
0
Males
Females
*
*
12h
18h
24h
6h
12h
Dark phase

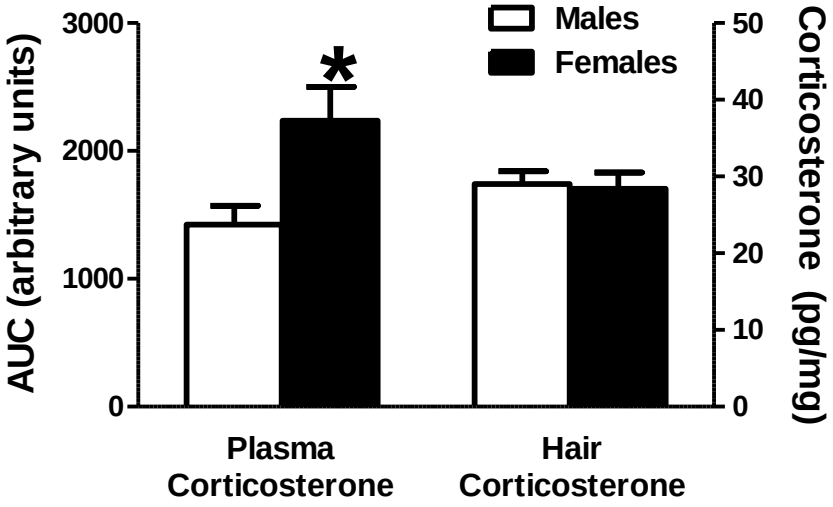

AUC (arbitrary units)
3000
2000
1000
0
Males
Females
*
50
40
30
20
10
0
Corticosterone (pg/mg)
Plasma Corticosterone
Hair Corticosterone

Figure 2

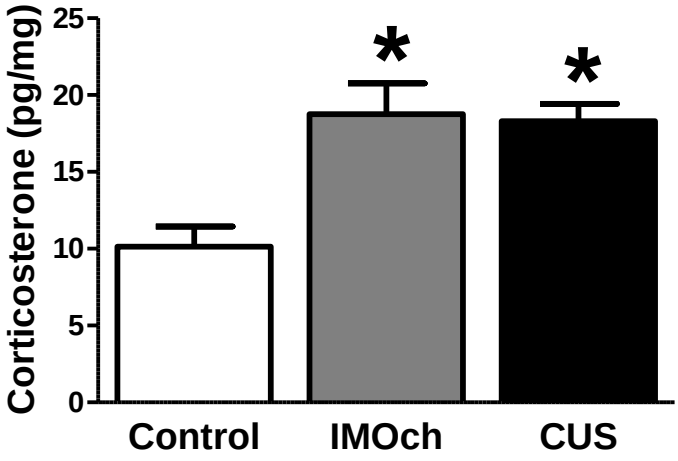


Figure 3

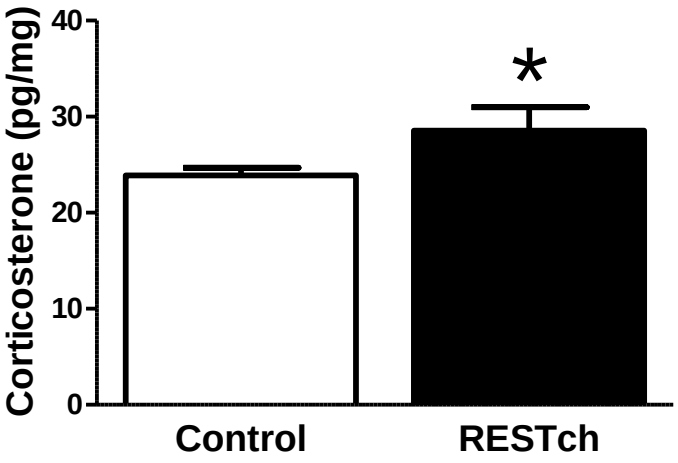


Figure 4

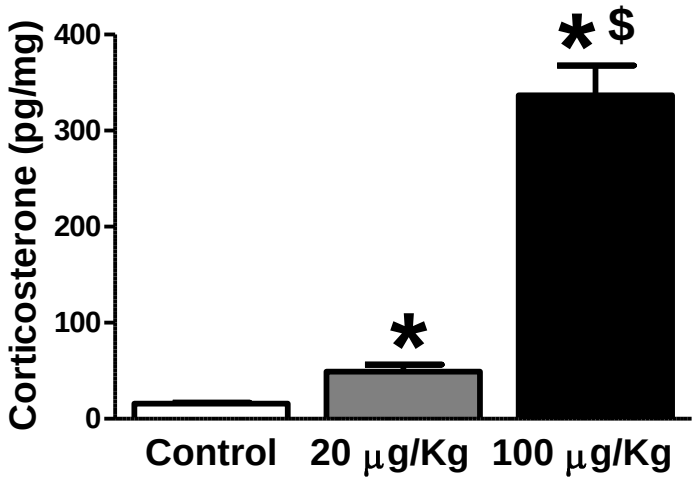

Corticosterone (pg/mg)
400
300
200
100
0
*
* $
Control
20 μg/Kg
100 μg/Kg

Figure 5