Acute exposure of rats to a severe stressor alters the circadian pattern of corticosterone and sensitizes to a novel stressor: relationship to pre-stress individual differences in resting corticosterone levels

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Short title: Stress alters daily corticosterone rhythm

Abstract

Traumatic events have been proposed to be associated with hypo-activity of the hypothalamic-pituitary-adrenal (HPA) axis, but data in animal models exposed to severe stressors are controversial and have important methodological concerns. Individual differences in resting or stress levels of corticosterone might explain some of the inconsistencies. We then studied this issue in male rats exposed to 2 h immobilization on boards (IMO), a severe stressor.

Thirty-six rats were blood sampled under resting conditions four times a day on three non-consecutive days. Then, they were assigned to control (n=14) or IMO (n=22) to study the HPA response to IMO, the stressor-induced alterations in the circadian pattern of corticosterone (CPCORT), and the behavioral and HPA responsiveness to an open-field.

Individual differences in pre-IMO resting corticosterone were inconsistent, but averaging data markedly improved consistency. The CPCORT was markedly altered on day 1 post-IMO (higher trough and lower peak levels), less altered on day 3 and apparently normal on day 7. Importantly, when rats were classified in low and high resting corticosterone groups (LCORT and HCORT, respectively), on the basis of the area under the curve (AUC) of the averaged pre-IMO data, AUC differences between LCORT and HCORT groups were maintained in controls but disappeared in IMO rats during the post-IMO week. Open-field hypo-activity and corticosterone sensitization were similar in LCORT and HCORT groups nine days after IMO. A single IMO exposure causes long-lasting HPA alterations, some of them dependent on pre-stress resting corticosterone levels, with no evidence for post-IMO resting corticosterone hypo-activity.

Key words: Hypothalamic-pituitary-adrenal axis, PTSD, Immobilization Stress, Individual Differences, Aggregated data, Circadian Rhythm, Hypercorticosteronemia, Hypocorticosteronemia

1. Introduction

The evaluation of glucocorticoid release has been the main focus of stress research for decades since Hans Selye coined the concept of stress in the first half of the XX century. Both physical and emotional stressors are processed by the brain (Ulrich-Lai and Herman, 2009), with stimulatory signals eventually conveying at the paraventricular nucleus of the hypothalamus (PVN), the key brain area in the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The PVN releases the corticotrophin releasing hormone/factor (CRH or CRF) and other secretagogues to the pituitary portal blood to stimulate the synthesis and release of adrenocorticotropic hormone (ACTH) by anterior pituitary corticotrope cells, which in turns controls the secretion of glucocorticoids (corticosterone in rats and mice, cortisol in humans and most mammals) by the zona fasciculata of the adrenal cortex.

Glucocorticoids exert a wide range of physiological and behavioral effects that have a critical adaptive value to cope with stress (Sapolsky et al., 2000), acting peripherally and within the brain through genomic and non-genomic receptors (De Kloet et al., 1998; Haller et al., 2008). Moreover, stress-induced glucocorticoid release contributes to return HPA activity to pre-stress (resting) conditions through a glucocorticoid negative feedback exerted at multiple levels, including the anterior pituitary, the hypothalamus and supra-hypothalamic areas such as the hippocampal formation and the medial prefrontal cortex (Armario, 2006). Given the important role of glucocorticoids, a considerable effort has been dedicated to characterize, both in animals and humans, how individual differences in resting and stress levels of glucocorticoids might be related to particular psychological/behavioral characteristics (Chida and Hamer, 2008) and to pathophysiology and psychiatric diseases (Chrousos, 2009; Zorn et al., 2017). In addition to the inherent complexity of characterizing individual differences and studying certain pathologies, particularly psychiatric diseases, it is methodologically difficult to obtain representative individual values of glucocorticoid secretion. This is mainly because of the particular sensitivity of glucocorticoids to environmental conditions and minor stressors and the marked pulsatile and circadian changes in circulating glucocorticoid levels (Spiga et al., 2014). This precludes that a single sample could be representative of true glucocorticoid secretion even if the time of day is strictly controlled. In human studies, there is evidence using salivary cortisol that taking samples on different days under the same conditions and using the aggregated (averaged) data from all these days can clearly improve the relationship between glucocorticoid secretion and certain individual characteristics (Garcia et al., 2017; Lai et al., 2010; Li et al., 2007; Pruessner et al., 1997). However, to our knowledge, have not been attempts to evaluate the possible usefulness of averaged data in laboratory animals.

Whereas the activation of the HPA axis in response to acute and chronic stressors in adult laboratory animals has been extensively characterized for decades, most studies dealing with acute stressors initially focused on the first 24 h after the stressor. However, the growing interest in post-traumatic stress disorder (PTSD) in humans has greatly encouraged the study of the long-lasting behavioral and endocrine effects of a single exposure to certain severe stressors, considered as putative animal models of PTSD. There are two dominant theories about the putative relationship between the HPA axis and PTSD (DePierro et al., 2019; Olff and van Zuiden, 2017). One theory postulates that a defective glucocorticoid response to traumatic stressors might be relevant for, or at least be a marker of, the future development of PTSD after trauma. The other postulates that basal hypo-activity of the HPA axis is a consequence of the development of PTSD. It is however of note that there are discrepancies in the literature about the changes in the HPA axis in PTSD patients (Meewisse et al., 2007), and that traumatic stressors can results in the development of PTSD but also of depression (Breslau et al., 2000), which is associated to different alterations in the HPA axis (Staufenbiel et al., 2013).

Given the importance attributed to the HPA axis in PTSD, various laboratories have used a single exposure to severe stressors (at least some of them considered as putative animal models of PTSD) to explore these hypotheses. Comparing individual or strain differences, there is some support for the theory that low resting levels of corticosterone and/or a defective response to severe stressors can favor the development of PTSD-like behavioral changes (Cohen et al., 2006; Danan et al., 2018; Milde et al., 2003; Reznikov et al., 2015; Rod et al., 2012), although the relationship between resting and stress levels is still poorly known. Moreover, the results are markedly controversial regarding the long-term impact of acute exposure to severe stressors on the activity of the HPA axis. For instance, normal resting corticosterone levels have been reported one week after exposure to immobilization on boards (IMO) (Belda et al., 2008), inescapable tail-shock session (Fleshner et al., 1995) or the single prolonged stress (SPS) model (Ganon-Elazar and Akirav, 2012; Kohda et al., 2007), whereas other authors reported high levels after SPS (Laukova et al., 2014; Serova et al., 2013) or cat urine exposure (e.g. Kovlovsky et al., 2009a, 2009b).

In addition to the possibility that exposure to severe stressors could result in PTSD-like or depression-like behavior in animals, there are considerable methodological concerns regarding these studies. First, circulating levels of glucocorticoids show a marked circadian pattern (CP) in mammals, with peak levels just around the start of the activity period (lights off in rats and mice) and low levels during most of the inactive period. Therefore, to study the consequences of a severe stressor on resting corticosterone levels we need to study how the CP is affected over the days following exposure. Second, in male rats and mice, truly resting levels of corticosterone are on average 10-30 ng/ml or less in the initial lights on period (morning) and 100-200 ng/ml at the

lights off peak (Armario, 2006; Spencer and Deak, 2017). Unfortunately, most laboratories are unable to obtain actual resting levels and values of 50-100 ng/ml are frequently reported in the morning. There are several reasons to explain these high values: a) the increasingly use of ELISA instead of radioimmunoassay (RIA) techniques to measure corticosterone; b) the inclusion of some brief anesthesia procedure before sampling, as almost all anesthetic drugs strongly activates the HPA axis (e.g. Arnold and Laghans, 2011); and c) the minor stress associated with taking animals from their cages and the blood sampling procedure itself. Considering that exposure to severe stressors can induce sensitization of the HPA response to novel mild stressors (Belda et al., 2015, 2016), the interpretation of corticosterone data in terms of altered resting activity of the HPA axis is problematic in most studies.

In the present work we used in adult male rats immobilization on boards (IMO) as a severe stressor model (Armario et al., 2008). IMO is more severe than restraint, forced swim, odor exposure or electric-shocks when they have been compared using classical biological markers of stress intensity, including the initial activation of the HPA axis, the post-stress recovery of the HPA axis and its impact on food intake (Marquez et al., 2002; Martí et al., 2001; Muñoz-Abellán et al., 2008; Rabasa et al., 2015). Importantly, a single exposure to IMO has been found to enhance acoustic startle response (Fuentes et al., 2014) and cause spatial memory deficit in the Morris water maze (Andero et al., 2011), behavioral sensitization to further brief stressors (Belda et al., 2008) and impaired fear extinction (Andero et al., 2010), when studied even one week after the stressor. Another method of IMO (plastic bags) has also reported to increase anxiety-like behavior in the long-term (Mitra et al., 2005). We then characterized the CP of corticosterone before and after IMO as well as the behavioral and HPA response to a brief and mild superimposed stressor. Our two main purposes were: (i) to demonstrate persistent IMO-induced alterations in the circadian pattern of corticosterone and its responsiveness to further stressors; and (ii) to explore whether individual differences (low versus high resting or stress corticosterone levels) can affect the endocrine and behavioral consequences of IMO. The experimental design can be seen in Fig. 1.

2. Methods

2.1. Animals and general procedure

Male Sprague–Dawley rats obtained from the breeding centre of the Universitat Autònoma de Barcelona were used. They were 3-months-old at the beginning of the experiments. The animals were housed in pairs in polypropylene opaque wire-topped cages with solid-bottom (21.5 × 46.5 × 14.5 cm; Type "1000 cm^2", Panlab S.L.U., Barcelona, Spain) containing wood

shavings bedding (Lignocel 3/4, Harlan Interfauna Ibérica, Barcelona, Spain). They were maintained under standard conditions of temperature (21 ± 1 ºC) and in a 12:12 h light/dark schedule (lights on at 07:00 h), with food (SAFE-diet A04, Panlab S.L.U., Barcelona, Spain) and water available *ad libitum*. The experimental protocol was approved by the Committee of Ethics of the Universitat Autònoma de Barcelona and by the Generalitat de Catalunya and was carried out in accordance to the European Communities Council Directive (2010/63/EU) and Spanish legislation (BOE53-2013). A maximal effort was done to minimize the number and suffering of animals.

The experimental treatments were always carried out in the morning (between 1 and 5 h after light on), except when otherwise stated. Starting two days after being placed in the housing room, all animals were handled at least three times on different days for approximately 2 min a day. In addition, one blood sample (200-250 µl) was taken under basal conditions to habituate animals to the procedure. Blood samples were taken by tail-nick as described previously (Belda et al., 2004). This procedure is extensively used in our lab and others because very low resting levels of hormones are obtained under appropriate conditions (Belda et al., 2004; Vahl et al., 2005). Cage-mates were sampled simultaneously (two experimenters were sampling at the same time and a third was gently holding the two rats). Blood was centrifuged at 4930 x g (15 min, 4º C), and plasma was frozen (-20º C) until assay. Animals were assigned at random to the different groups in function of their date of birth and body weight. The two animals of a cage were assigned to the same group.

2.2. Experimental design

The overall picture of the experimental procedures can be seen in Fig. 1. The consistence of resting corticosterone levels during various times of the day and the impact of a single exposure to IMO on behavior and HPA activity were studied. For this purpose, animals were initially divided into control (n=14) and IMO (n=22) groups. On days 1, 4 and 7, all animals were blood sampled at 9:00 AM, 3:30 PM, 7:30 PM and 11:30 PM hours of the day under resting conditions. On day 12, all animals were individually introduced into an open field (OF) for 5 min. Just after OF exposure, control rats were returned in their respective home-cages to the vivarium, whereas IMO rats were exposed to 2 h of IMO in another room. Blood samples were taken in both groups following the same schedule: immediately after stressor (END) and at 1 h after its termination (R1h). On days 13, 15 and 19 (days 1, 3 and 7 post-IMO, respectively), all animals were blood sampled at 9:00 AM, 3:30 PM, 7:30 PM and 11:30 PM hours of the day under resting conditions. Finally, on day 21, all rats were again exposed to the OF for 5 min and a blood sample was taken just after the test.

IMO rats were immobilized on boards as previously described (Belda et al., 2012). Briefly, rats were restrained in a prone position by attaching their four limbs to metal mounts with adhesive tape.

2.3. Open Field (OF)

Exposure to the OF was done to have some behavioral outcome of the long-term consequences of IMO exposure. The OF consisted in a plastic gray rectangular box (56 x 36.5 x 31 cm) opened at the top, where each animal was initially placed facing a corner. The apparatus was cleaned carefully between animals with a tap water solution containing ethanol (5% v/v). OF behavior was recorded with a video camera (Sony SSC-M388 CE, BW) situated 150 cm above the center of the cage. A blind experimenter to the treatment estimated the distanced travelled (using video tracking analysis; Smart version 2.5.21, Panlab-Harvard, Barcelona, Spain) and the number of rearings (manually) as measures of activity.

2.4. Biochemical analysis

Plasma ACTH and corticosterone levels were determined by double antibody radioimmunoassay (RIA) following our general procedures (Muñoz-Abellán et al., 2011). In brief, ACTH RIA used ^{125}I-ACTH (PerkinElmer Life Science, Boston, USA) as the tracer, rat synthetic $ACTH_{1–39}$ (Sigma, Barcelona, Spain) as the standard and an antibody raised against rat ACTH (rb7) kindly provided by Dr. W.C. Engeland (Department of Surgery, University of Minnesota, Minneapolis, USA). The characteristics of the antibody have been described previously (Engeland et al., 1989) and we followed a non-equilibrium procedure. Corticosterone RIA used ^{125}I-corticosterone-carboximethyloxime-tyrosine-methylester (ICN-Biolink 2000, Barcelona, Spain), synthetic corticosterone (Sigma, Barcelona, Spain) as the standard and an antibody raised in rabbits against corticosterone–carboximethyloxime-BSA kindly provided by Dr. G. Makara (Institute of Experimental Medicine, Budapest, Hungary). The characteristics of the antibody and the basic RIA procedure have been described previously (Zelena et al., 2003) and we followed an equilibrium procedure. All samples to be statistically compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 5.1% for ACTH and 7.6% for corticosterone. The sensitivity of the assays was 25 pg/ml for ACTH and 2 ng/ml for corticosterone.

2.5. Statistical analysis

Data were analyzed by means of the Statistical Program for Social Sciences (SPSS) version 24.0 (IBM Corp., Armonk, N.Y., USA). To study hormonal data, two different types of analysis were done using General Linear Model (GLM). For circadian levels of corticosterone on the days before exposure to IMO, a GLM analysis with IMO as between-subjects factor (two levels) and DAY (three levels) and SAMPLING TIME (four levels) as within-subjects factors. For the HPA response to IMO and the circadian levels of corticosterone on each of the days after IMO, GLM analysis were carried out with IMO as between-subjects factor and SAMPLING TIME (two levels for the response to IMO and four levels for circadian levels) as within-subjects factor. To study behavioral differences in the OF, the Student t-tests were used. Hormonal data were log-transformed to achieve homogeneity of variances. In order to study the relationship between pre-stress resting corticosterone levels and the response to IMO, control and IMO rats were assigned to low and high corticosterone groups on the basis of the median of the pre-IMO averaged AUCs (low and high groups: LCORT, HCORT). Then, the possible changes in the AUCs between LCORT and HCORT throughout the next week was separately assessed in control and IMO groups using GLM analysis, with corticosterone levels and days as between-subjects and within-subjects factors, respectively. If an interaction between factors was found, a decomposition of the interaction was performed examining the simple effect of one factor at each of the different levels of the other factor. The effect size was calculated with the partial eta square coefficient (η^2).Pearson correlations (two-tailed) were also calculated. The criterion for significance was set at $p < 0.05$.

3. Results

3.1. Pre-IMO corticosterone levels

Fig. 2 shows the CP of plasma corticosterone levels on three non-consecutive days before exposure to IMO. The GLM analysis revealed no effect of GROUP (those assigned to be controls or exposed to IMO, not shown), but significant effects of DAY ($F(2, 204) = 6.7$; $p = 0.002$; $\eta^2 = 0.164$), SAMPLING TIME ($F(3, 204) = 327.6$; $p < 0.001$; $\eta^2 = 0.906$) and the interaction DAY x SAMPLING TIME ($F(6,204) = 4.0$; $p = 0.001$; $\eta^2 = 0.105$). Further comparisons showed higher plasma corticosterone levels both at 9:00 AM and at 11:30 PM on the seventh experimental day.

Since studies in humans show that aggregating data from several days improve the consistency of the results (Garcia et al., 2017; Lai et al., 2010; Li et al., 2007; Pruessner et al., 1997), we first

studied correlations between individual time points and also between the AUCs in all rats in the three days prior to IMO. Correlations were low and inconsistent in all cases either considering particular time points or AUCs (not shown). However, when data from the three days were averaged, consistent correlations were observed between these averaged data and the three days values considering both individual time points and AUCs (Table 1). Importantly, averaged data of controls were compared with the values obtained in the next three sampling days (corresponding to the post-IMO phase): correlations were significant for the AUCs (Table 2), but not for individual time points (not shown). Such correlations in the AUCs were not found in the IMO group (Table 2).

3.2. HPA response to IMO

The HPA response to IMO is shown in Fig. 3. As expected, the GLM analysis of plasma ACTH levels in response to IMO revealed significant effects of SAMPLING TIME ($F(1,34) = 91.3$; $p < 0.00$; $\eta^2 = 0.729$), IMO ($F(1,34) = 323.1$; $p < 0.001$; $\eta^2 = 0.905$) and SAMPLING TIME x IMO ($F(1,34) = 92.3$; $p < 0.001$; $\eta^2 = 0.729$). Decomposition of the interaction showed that ACTH levels were higher in IMO than control group at the two time points ($p < 0.001$ in both cases). Regarding plasma corticosterone, we observed a marginally significant effect of SAMPLING TIME ($F(1,34) = 3.9$, $p = 0.07$; $\eta^2 = 0.103$), and significant effects of IMO ($F(1,34) = 165.4$; $p < 0.001$; $\eta^2 = 0.829$) and SAMPLING TIME x IMO ($F(1,34) = 18.9$; $p < 0.001$; $\eta^2 = 0.358$), with higher plasma corticosterone levels in IMO than control group at the two times ($p < 0.001$ in both cases). A significant correlation was found between corticosterone levels obtained 1 h after the termination of IMO (R1h) and resting levels on the morning on the day after (Fig. 3C).

3.3. Impact of IMO on the circadian pattern of corticosterone

After exposure to IMO, control and IMO groups were compared at each particular post-IMO day and time of day (Fig. 4). On Day 1 post-IMO, the GLM analysis showed significant effects of SAMPLING TIME ($F(3,102) = 52.7$; $p < 0.001$; $\eta^2 = 0.608$) and the interaction SAMPLING TIME x IMO ($F(3,102) = 6.3$; $p = 0.001$; $\eta^2 = 0.156$). Further decomposition revealed an altered pattern of plasma corticosterone levels across the day in the group previously exposed to IMO, with higher levels in the morning (9:00 AM) and lower levels just after lights off (07:30 PM). On Day 3 post-IMO, the GLM analysis showed significant effects of SAMPLING TIME ($F(3,102) = 136.9$; $p < 0.001$; $\eta^2 = 0.801$) and SAMPLING TIME x IMO ($F(3,102) = 3.9$; $p = 0.011$; $\eta^2 = 0.103$). Further comparisons showed that plasma corticosterone levels were lower in IMO than control group only at 11:30 PM. Finally, on Day 7 post-IMO, only statistically significant effect of SAMPLING TIME was found ($F(3,102) = 90.4$; $p < 0.001$; $\eta^2 = 0.727$).

To explore whether pre-IMO levels of corticosterone were related to the consequences of exposure to IMO we classified rats by the median on the basis of the averaged AUCs of the pre-IMO resting levels into low and high corticosterone (LCORT, HCORT) and studied these two groups during the post-IMO period. We classified controls in the same way (Fig. 5). The range of AUC values were 3632-6552 in controls and 3028-7307 in IMO rats, the cut-off values being 5069 and 4345 respectively. In controls, the GLM analysis revealed significant effects of GROUP ($F(1,12) = 7.8$; $p = 0.016$; $\eta^2 = 0.394$) and DAY ($F(3, 36) = 3.9$; $p = 0.017$; $\eta^2 = 0.243$), without significant interaction. Further comparisons showed higher AUCs in controls on day 1 post-IMO versus pre-stress levels ($p = 0.012$). Regarding IMO rats, the GLM analysis revealed no significant effect of GROUP, but significant effects of DAY ($F(3, 60) = 4.8$; $p = 0.005$; $\eta^2 = 0.194$) and the interaction GROUP x DAY ($F(3, 60) = 2.8$; $p = 0.047$; $\eta^2 = 0.123$). Decomposition of the interaction showed that LCORT-HCORT pre-stress differences were not observed at any day post-IMO. In fact, within the LCORT group, differences versus pre-stress levels were significant on day 1 and 3 post-IMO ($p < 0.001$ in the two cases), and marginally significant on day 7 ($p = 0.07$). In contrast, IMO had no impact on HCORT group at any time.

3.4. Behavioral and endocrine response to the open-field

To assess the long-term impact of IMO on the behavioral response to the OF, the changes in the distance travelled and the number of rearings between the two OF exposures were calculated for each group (Fig. 6). One rat of the IMO group had to be excluded to the analysis because it was not videotaped during the first day of OF. The t-test showed an inhibitory effect of IMO on the two behavioral parameters ($t(33) = 2.8$; $p = 0.008$ for distance travelled; $t(33) = 3.5$; $p = 0.002$ for rearings). Regarding the endocrine response to this second OF exposure (Fig. 6), the t-test revealed no differences in ACTH but did greater corticosterone response in IMO than in controls rats ($t(34) = 2.2$; $p = 0.037$). We calculated correlations between hormonal data and OF behavior. No significant correlation was observed (not shown) between: (i) averaged pre-IMO AUCs or time points from all the animals (n=35) and their behavior during the first OF exposure; (ii) averaged pre-IMO AUCs or time points of the IMO group rats (n=21) and their behavior during the second OF exposure or the magnitude of the change between the two exposures; (iii) the response of ACTH and the impact of IMO (change between first and second OF exposure). However, a significant correlation was found between the corticosterone levels after the second OF exposure and the change (decrease) in horizontal activity ($r = 0.65$, $p = 0.002$).

4. Discussion

The present results demonstrate that a single exposure to a severe stressor caused alterations in the CP of corticosterone over the next week after the stressor. Such alterations were partially dependent on individual differences in the pre-IMO resting levels of corticosterone calculated using the averaged data from the three blood sampling days prior to stress. Moreover, IMO-exposed rats showed behavioral hypo-activity and enhanced corticosterone response to an open-field when measured 9 days after the stressor, regardless of pre-stress resting corticosterone levels. These results did not support the hypothesis that a single exposure of adult rats to an acute severe stressor can induce hypo-activity of the HPA axis.

4.1. The difficulty of consistently assessing individual differences in HPA activity: the value of using averaged data

Thirty-six male rats were blood sampled under resting conditions four times through the 24 h period in three non-consecutive days. A typical CP was observed with very low corticosterone levels during the morning, higher levels in the afternoon and peak levels just after lights off and a decline thereafter. On the 3 sampling days prior to IMO, plasma corticosterone levels were found to be similar in those rats assigned to controls and those assigned to IMO, but small but significant differences were observed at some time points between the 3 days, suggesting some minor influences of situational factors. In fact, correlations between values obtained at each particular time point were inconsistent and the same occurred with the AUCs. However, when averaged data over the 3 days were considered, consistent and good correlations were observed with the values corresponding to each day (either AUCs or time points). Interestingly, for control rats only, the averaged AUC still showed a good correlation with the individual AUCs obtained on the days corresponding to the post-IMO period, although this consistency was not observed with particular time points.

To our knowledge, the validity of using averaged data has not been previously examined in laboratory animals, but our results give support to previous studies in humans that has shown increased consistency with averaged (aggregated) data for salivary cortisol when trying to relate cortisol levels with personality traits (Garcia et al., 2017; Lai et al., 2010; Li et al., 2007; Pruessner et al., 1997). The rationale for this improvement is clear. Even controlling for the pronounced CP of corticosterone in laboratory animals and cortisol in humans, values corresponding to a particular sampling in particular individual are affected by transient situational changes and the marked pulsatile secretion of cortisol in humans (Krieger et al., 1971; Weitzman et al., 1971) or

corticosterone in rats (Jasper and Engeland, 1991; Windle et al., 1998a, 1998b). This means that a particular sample is unlikely to be representative of the average cortisol (or corticosterone) secretion of an individual at a particular time of day. This problem is more critical when we try to correlate this particular cortisol (corticosterone) value with relatively stable individual differences; for instance, personality factors in humans or behavioral traits in animals. Our present data strongly encourage the use of averaged data when trying to establish a relationship between resting corticosterone activity and any behavioral or physiological characteristic of animals.

4.2. Long-lasting effects of IMO on resting corticosterone levels

A main purpose of this experiment was to know whether or not a single exposure to a traumatic stressor can affect in the long-term the CP of corticosterone beyond the first 24 h after the stressor. More particularly, it aimed at directly testing the hypothesis that severe stressors can induce hypo-corticosteronemia. Our results demonstrated that a single IMO exposure increased resting levels of corticosterone in the next morning but reduced it at the lights off peak. In two previous reports that evaluated plasma corticosterone at both the trough (lights on) and the peak (lights off) of the CP using tail-shock as the stressor, only the increase at lights on was detected (Brennan et al., 2000; Fleshner et al., 1995; Ottenweller et al., 1994). Similarly, chronic IMO exposure also increased plasma corticosterone in the morning, and differences were not significant at lights off (Martí et al., 1993). This partial discrepancy may be explained by the high sample size used in the present as compared with the other previous studies or by the exact time points studied.

The activation of the HPA in response to predominantly emotional stressors is transient, with a return of corticosterone to normal resting levels in less than 1 h after the termination of the stressor. However, exposure to a severe stressor such as IMO has been repeatedly demonstrated to maintain high levels of corticosterone for at least a few hours after the stressor, in contrast to less severe stressors (e.g. García et al., 2000; Márquez et al., 2002; Martí et al., 2001). In addition, increased resting levels of corticosterone has been reported in the next morning following exposure to various severe stressors, some of them considered as putative animal models of PTSD (Deslauriers et al., 2018): IMO (Belda et al., 2012, 2008; Martí et al., 1996), tail-shocks (Fleshner et al., 1995; Ottenweller et al., 1994; Servatius et al., 1995) and SPS (Ganon-Elazar and Akirav. 2012; Kohda et al., 2007; Sun et al., 2017). It is of note that the effect of IMO on resting levels of ACTH and corticosterone were not found in female rats that already showed higher levels of the two hormones in non-IMO rats (Gagliano et al., 2014). Sex differences in the HPA axis, particularly higher resting and stress levels of plasma corticosterone, has been repeatedly reported in female versus male rats (Goel et al., 2014) and further studies in females are needed.

To know whether high corticosterone levels in the next morning were related to the HPA response to IMO, we correlated these measures. Interestingly, we observed a significant positive correlation between plasma corticosterone levels during the post-IMO recovery period and next morning levels, with any other significant correlation. These results indicated that delayed recovery of corticosterone is someway related to high morning post-stress levels, this phenomenon apparently being independent of ACTH levels. Although more detailed studies are needed, these data emphasized the importance of extra-ACTH regulation of adrenocortical function (see later).

In the present study the altered corticosterone CP was relatively short-lasting, as 3 days after IMO the only difference was high levels of corticosterone in the middle of the dark period. No alterations were detected 7 days after IMO. Therefore, our results do not appear to support the hypothesis that an acute exposure to severe stressors could induce long-term alteration of resting levels of corticosterone. How severe stressors alter resting levels of corticosterone beyond the first 24 h post-stress is a very controversial topic in the literature. In some studies, using IMO or tail-shocks, the initial high morning levels progressively vanished over the next week (Belda et al., 2008; Brennan et al., 2000; Fleshner et al., 1995; Ottenweller et al., 1994; Servatius et al., 1995). High corticosterone levels have been reported one week after cat urine odor exposure, but levels are typically very high as compared with normal resting levels in other studies (e.g. Kozlovsky et al., 2009a, 2009b). Particularly inconsistent is the case of the SPS model as resting corticosterone levels have been found to increase one week after SPS (Laukova et al., 2014; Serova et al., 2013), not to change (Ganon-Elazar and Akirav, 2012; Kohda et al., 2007) or to be lower than controls (Lin et al., 2016; Zhang et al., 2012, 2015). But, again, some methodological problems can contribute to the results. In the first two papers, low resting corticosterone levels were observed 3 or 4 weeks after SPS, but levels reported were markedly above actual resting levels and SPS rats were singly housed whereas controls were group-housed. Sampling rats maintained in groups involves much more cage disturbance than sampling singly housed rats, thus spuriously resulting in apparent hypo-corticosteronemia in SPS rats. In the second, rats were exposed to cued fear conditioning, extinction and retrieval for several days before blood sampling were obtained and it is unclear whether the post-SPS procedure could have affected corticosterone levels. Models more closely mimicking PTSD (e.g. SPS) should be carefully tested in further studies. Moreover, in view of the possibility that blood-sampling might sensitize the HPA system, it would be better not to introduce any experimental procedure such as repeated blood sampling before testing changes in resting HPA function.

4.3. Long-lasting IMO-induced changes in the response to an open-field

Exposure to IMO significantly reduced activity in the OF when assessed 7 days later, suggesting a longer lasting impact of the stressor. Long-lasting behavioral effects of a single exposure to stressors considered as putative animal models of PTSD have been described, reflecting hyperarousal, anxiety-like behavior and potentiated fear, although the results are not always consistent (Armario et al., 2008; Deslauriers et al., 2018; Richter-Levin et al., 2019). In this regard, a single IMO exposure has been found to impair spatial memory in rats and fear extinction in mice (Andero et al., 2010, 2011) and to enhance acoustic startle response (Fuentes et al., 2014).

In addition to inhibit activity in the OF, prior IMO resulted in normal ACTH response to the 5 min exposure to the OF, but higher corticosterone response. Previous studies have consistently found that a single exposure to IMO and other severe stressors causes sensitization of the ACTH and corticosterone responses to further novel (heterotypic) stressors that typically lasted for several days, although occasionally longer-lasting effects have been reported (Belda et al., 2008, 2016; Johnson et al., 2002; O'Connor et al., 2003). Interestingly, sensitization of the response to the OF was observed with corticosterone but not ACTH. Although differences in the time-course of the response of the two hormones is a likely explanation, we cannot rule out that sensitization affects the adrenal cortex independently of ACTH, this specific adrenal effect being longer-lasting. Although the precise mechanisms are not known, there is evidence that the brain can modulate the sensitivity of the adrenal cortex to ACTH through sympathetic innervation of the gland (Bornstein et al., 2008).

Behavior in the OF did not correlate with the endocrine measures of the present study, except for corticosterone levels after the second OF exposure, which positively correlated with the magnitude of IMO-induced hypoactivity. Consequently, the higher the corticosterone response the greater the decrease in activity. Importantly, this correlation was not observed with the ACTH response of IMO rats to the OF and was not found in control rats. Although the results are difficult to explain, it is possible that ACTH-independent corticosterone sensitization might be a peripheral marker of vulnerability to severe stressors. Unfortunately, this is a novel aspect of the HPA axis that has never been studied.

4.4. Individual differences in resting and stress levels of corticosterone

Taking advantage of the number of rats included and the use of averaged data, we wanted to explore the influence of pre-stress resting levels on the consequences of IMO. More particularly, we examined whether corticosterone secretion over the day might be related to the response to IMO and the consequences of such an exposure regarding dysregulation of the CP of

corticosterone and sensitization to the OF exposure. We divided both control and IMO groups by the median on the basis of the averaged AUCs into LCORT and HCORT groups and studied the influence of IMO or parallel procedures in controls to alter the LCORT-HCORT differences over the next week. In controls, the differences between LCORT and HCORT were maintained over the week, although in both groups the AUCs were significantly higher on day 1 post-IMO versus pre-IMO values, suggesting some mild sensitization of the HPA axis caused by blood sampling the day before. In striking contrast, prior IMO did alter the LCORT-HCORT differences in that such differences disappeared during all the post-IMO phase studied. This reflects that HCORT rats showed no post-IMO versus pre-IMO differences, whereas a significant increase was found in LCORT rats. Importantly, LCORT-HCORT groups did not differ in the ACTH and corticosterone response to IMO. This suggests that resting and stress levels of HPA hormones are typically dissociated and that the differential impact of the stressor is not directly related to a distinct HPA response.

These above results indicate that the protracted impact of IMO on resting corticosterone levels is partially dependent on the pre-stress resting levels, affecting more to those animals showing lower levels. It is difficult to know the actual functional meaning of these results since there is no precedent in the literature. Nevertheless, the results suggest that characterization of resting pre-stress levels could be of great value to study the long-term impact of a single exposure to severe stressors. In this regard, a recent paper shows that decreased amplitude of corticosterone pulses in individual rats under resting conditions predicted a lower response to cat urine odor and greater behavioral alterations in the long-term (Danan et al., 2018). However, the consistency of such individual differences in samples taken on different days was not assessed.

4.5. Methodological concerns and limitations

It is unlikely that differences observed between animals across the different days and between groups are due to methodological problems. Controls and IMO rats were alternated for sampling and the same order of blood sampling was followed each day. The inconsistency between days are likely to be due to pulsatile secretion, but we cannot rule out a minor contribution of minor stress despite our corticosterone levels are under the lowest range of published data. Moreover, repeated blood sampling, particularly if done on two consecutive days as was the case in the last pre-IMO and the first post-IMO days, could sensitize the HPA axis and interfere with the effects of IMO per se. The alternative of using cannulated animals, which can reduce to a minimum stress associated with blood sampling, involves surgery and catheter maintenance, precluding the simultaneous handling of an elevated number of animals in order to characterize individual differences.

5. Conclusion

The present study suggest that repeated blood sampling on different days, if done appropriately, could not be necessary if we are interested in the average impact of any particular factor on plasma corticosterone. However, we strongly encourages the use of averaged data from various days if we are specifically interested in characterizing individual (trait) differences in resting corticosterone levels in a population of animals and their relationship with other physiological variables, particular behavioral traits or the susceptibility/resilience to stressors. Our data indicate that exposure to a severe stressor induced protracted changes in the CP of corticosterone that are partially influenced by differences in pre-stress resting levels. These differences were not related to the HPA response to the stressor, suggesting the involvement of different regulatory mechanisms. It is clear that more studies are needed on resting levels of glucocorticoids in animal models to increase the translational value of these models regarding human pathologies that mainly rely on the characterization of resting cortisol levels.

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Declaration of competing interest

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TABLES

Table 1. Pearson correlations of plasma corticosterone levels on the days before IMO

	D1-preIMO	D4-preIMO	D7-preIMO
$\bar{X}$ 9:00 AM preIMO	9:00 AM		
	0.72**	0.61**	0.88**
$\bar{X}$ 3:30 PM preIMO	3:30 PM		
	0.71**	0.79**	0.72**
$\bar{X}$ 7:30 PM preIMO	7:30 PM		
	0.76**	0.78**	0.66**
$\bar{X}$ 11:30 PM preIMO	11:30 PM		
	0.78**	0.72**	0.69**
$\bar{X}$ AUCs preIMO	AUC		
	0.74**	0.72**	0.78**

Abbreviations: AUC, area under curve; D, day. $\bar{X}$ represents the averaged values of the three non-consecutive days before exposure to IMO taking into account each particular time of day or the daily AUC. Correlations were calculated between these averaged values and the corresponding values in each of the three days. All animals (n=36) were included in the analysis.
**p<0.01

Table 2. Pearson correlations of AUCs of plasma corticosterone both before and after IMO.

		AUC					
		D1 preIMO	**D4 preIMO**	**D7 preIMO**	**D1 postIMO**	**D3 postIMO**	**D7 postIMO**
$\bar{X}$ **AUCs preIMO**	**Control**	**0.63***	**0.81****	**0.66***	**0.63***	**0.72****	**0.60***
	IMO	**0.81****	**0.66****	**0.82****	**-0.07**	**0.48***	**0.36**

Abbreviations: AUC, area under curve; D, day. $\bar{X}$ represents the averaged AUC of the three non-consecutive days before exposure to IMO, separately for Control (n=14) and IMO (n=22) groups. Correlations were calculated between these averaged values and the corresponding AUCs values in each of the pre-IMO and post-IMO days. *p<0.05, **p<0.01.

LEGENDS TO FIGURES

Fig. 1. Scheme of the experimental design. BS-CP: blood sampling (BS) four times a day to study the circadian corticosterone pattern (CP); D0 was the day of exposure to IMO. Note that this day all rats were exposed to the open-field (OF, 5 min) and immediately after that to 2 h IMO (IMO group) or returned to their home cages (Control group). BS: blood sampling to all rats on D0 and D21. On D0 BS was done immediately after 2 h IMO and again at 1 h after the termination of IMO (R1h in the text and Figures).

Fig. 2. Plasma corticosterone at different day time points on three non-consecutive days before exposure to IMO. Means and SEM (n=36) are represented. Panel A represents bars corresponding to values at 9:00 am to better see the differences. * p< 0.05, ** p< 0.01 vs corresponding time on D1; + p< 0.05, ++ p< 0.01 vs corresponding time on D4.

Fig. 3. Plasma ACTH and corticosterone in response to IMO. Means and individual values of plasma levels obtained immediately after 2 h IMO or 1 h after the termination of IMO (R1h) are represented (Panel A and B). Control rats (n=14) were only sampled in parallel with IMO rats (n=22). *** p< 0.001 vs corresponding control values. Panel C represents the Pearson correlation between corticosterone (ng/ml) levels of IMO exposed rats at R1 and the levels at the 9:00 am on the day after.

Fig. 4. Plasma corticosterone at different day time on three non-consecutive post-IMO days in control (n=14) and IMO-exposed (n=22) rats (Panels A-C). Means and SEM are represented. * $p< 0.05$ vs corresponding control values at the same day time.

Fig. 5. Influence of pre-stress resting levels of corticosterone on the post-stress levels. Both control (n=14) and IMO (n=22) rats were divided by the median of the averaged AUC of daily plasma corticosterone during the three sampling days before IMO (low and high corticosterone groups, LCORT and HCORT). After exposing the IMO group to the stressor, the AUCs of control and IMO groups were followed throughout the next week. In control rats the LCORT-HCORT pattern was maintained over time (* $p< 0.05$: significance of the main factor GROUP; the LCORT-HCORT differences in pre-IMO values, *** $p< 0.001$, are indicated separately only to parallel corresponding data of IMO rats), although both groups showed higher levels on D1 vs pre-IMO. In IMO rats, differences between LCORT and HCORT in the pre-IMO period (*** $p< 0.001$) disappeared during the post-IMO period; # $p=0.07$; +++ $p< 0.001$ in the LCORT group between post-IMO and pre-IMO values.

Fig. 6. Behavioral and endocrine response to a 5 min open-field (OF) in control (n=14) and IMO (n=21-22) rats. All rats were exposed to the OF before IMO and again 9 days after IMO and the mean of the differences in horizontal activity and rearings between the two days were calculated (delta). Hormonal data were obtained only on the last day. *< 0.05; ** < 0.01 vs corresponding control values.

Figure 1

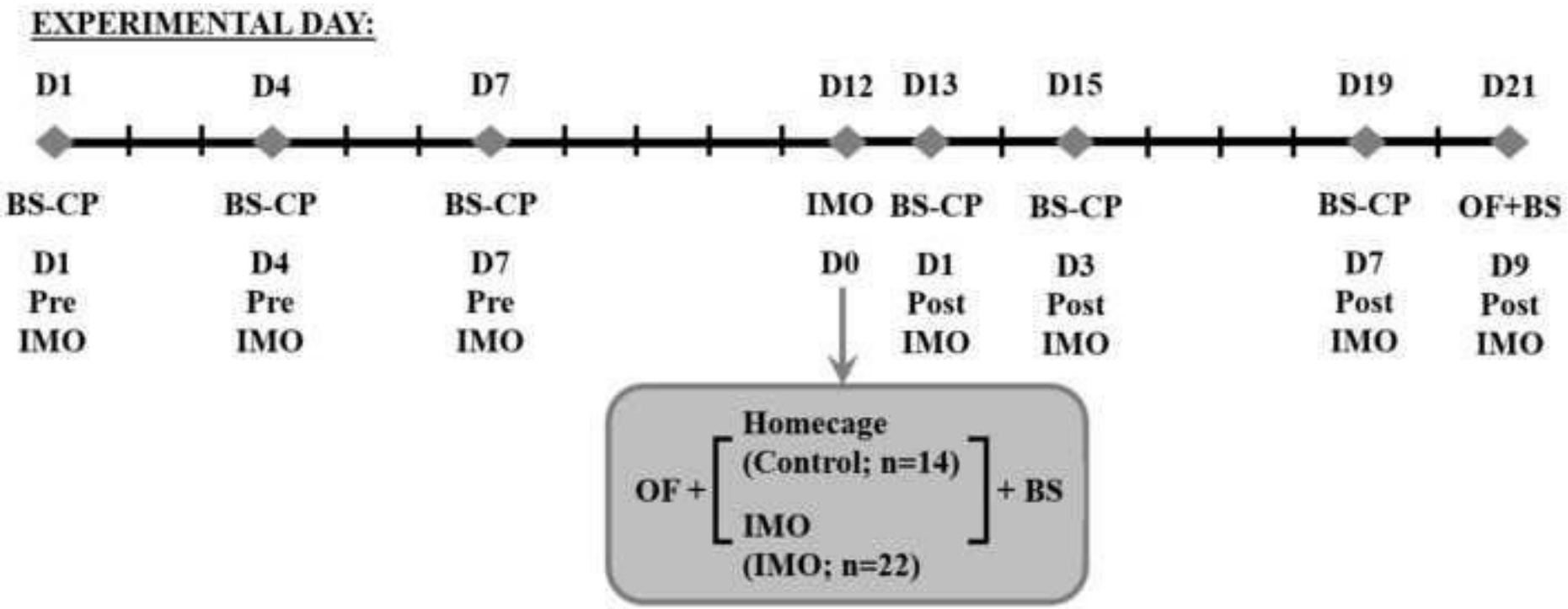

EXPERIMENTAL DAY:
D1
D4
D7
D12
D13
D15
D19
D21
BS-CP
BS-CP
BS-CP
IMO
BS-CP
BS-CP
BS-CP
OF+BS
D1 Pre IMO
D4 Pre IMO
D7 Pre IMO
D0
D1 Post IMO
D3 Post IMO
D7 Post IMO
D9 Post IMO
OF +
Homecage (Control; n=14)
IMO (IMO; n=22)
+ BS

Figure 2

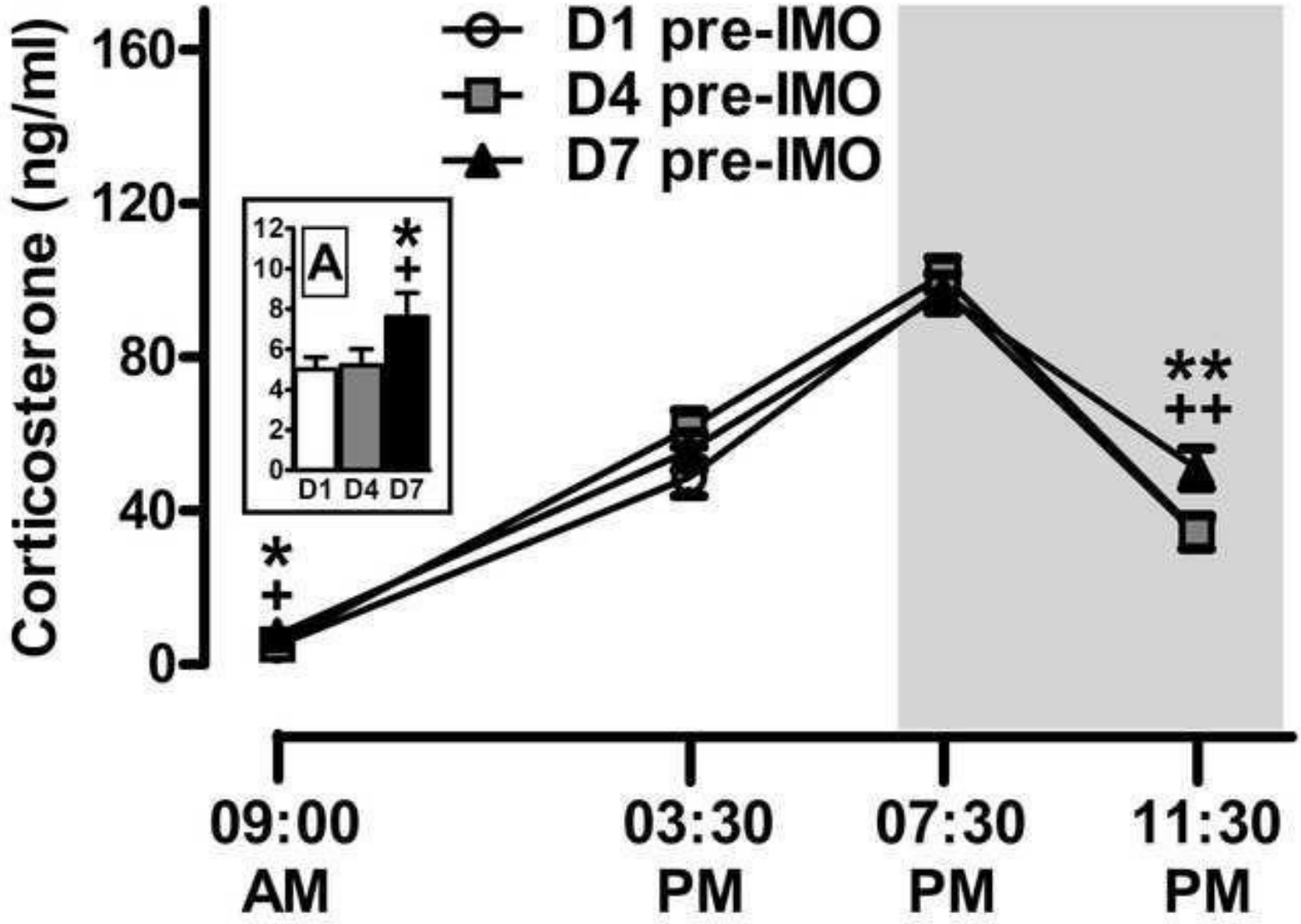

D1 pre-IMO
D4 pre-IMO
D7 pre-IMO
Corticosterone (ng/ml)
160
120
80
40
0
A
12
10
8
6
4
2
0
D1 D4 D7
*
+
*
+
**
++
09:00
AM
03:30
PM
07:30
PM
11:30
PM

Figure 3

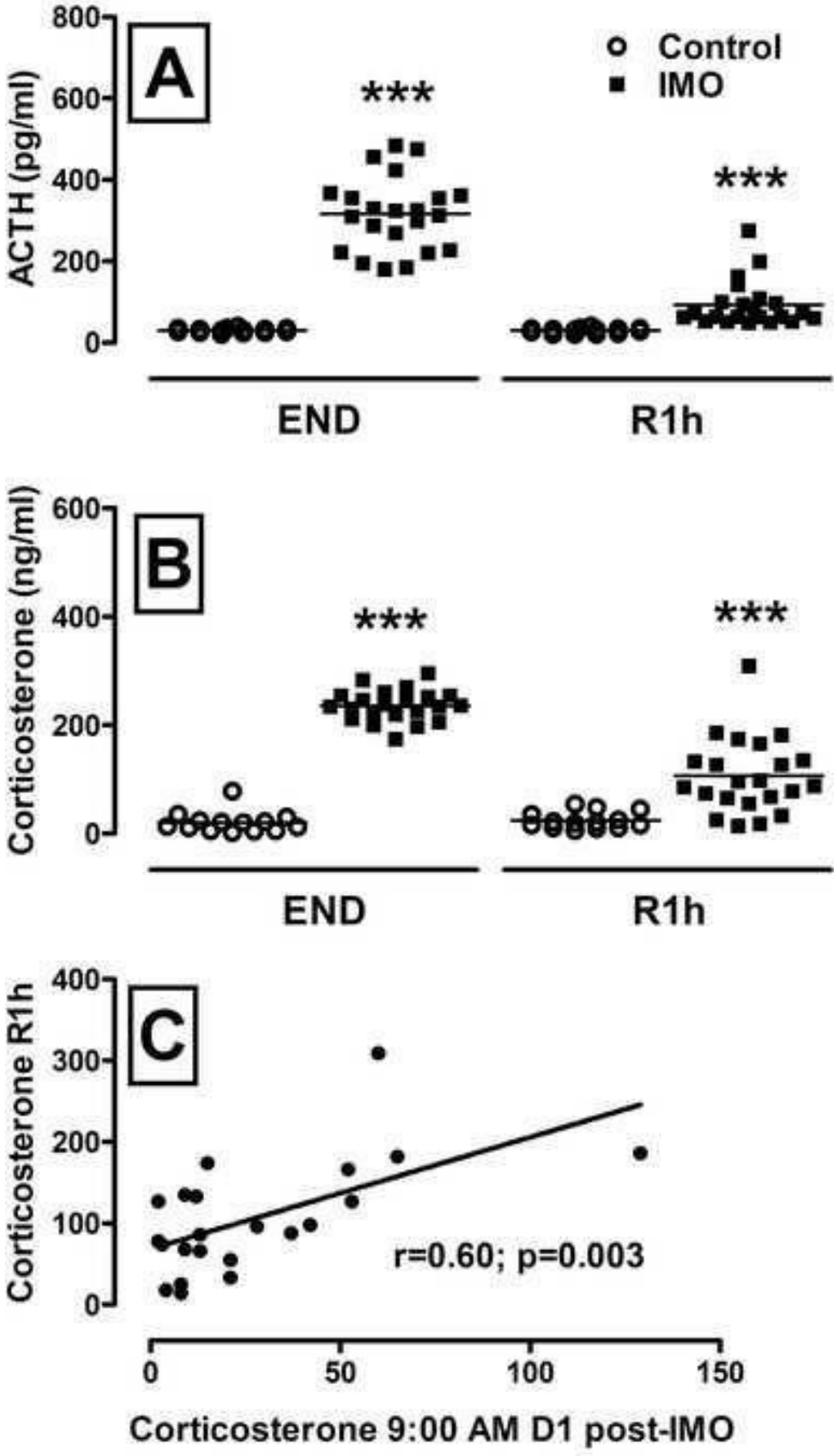

A
Control
IMO
ACTH (pg/ml)
800
600
400
200
0
***
***
END
R1h
B
Corticosterone (ng/ml)
600
400
200
0
***
***
END
R1h
C
Corticosterone R1h
400
300
200
100
0
r=0.60; p=0.003
0
50
100
150
Corticosterone 9:00 AM D1 post-IMO

Figure 4

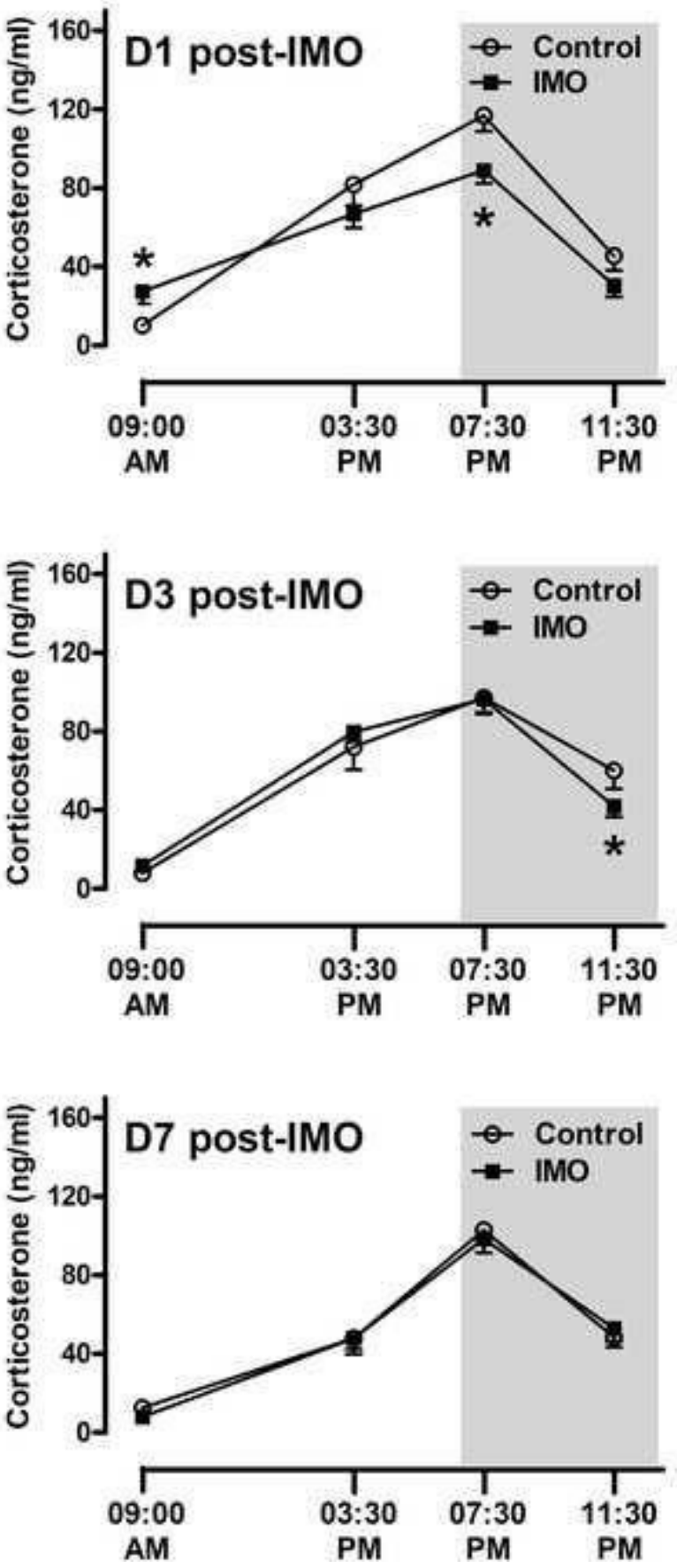

D1 post-IMO
Control
IMO
Corticosterone (ng/ml)
160
120
80
40
0
*
*
09:00 AM
03:30 PM
07:30 PM
11:30 PM
D3 post-IMO
Control
IMO
Corticosterone (ng/ml)
160
120
80
40
0
*
09:00 AM
03:30 PM
07:30 PM
11:30 PM
D7 post-IMO
Control
IMO
Corticosterone (ng/ml)
160
120
80
40
0
09:00 AM
03:30 PM
07:30 PM
11:30 PM

Figure 5

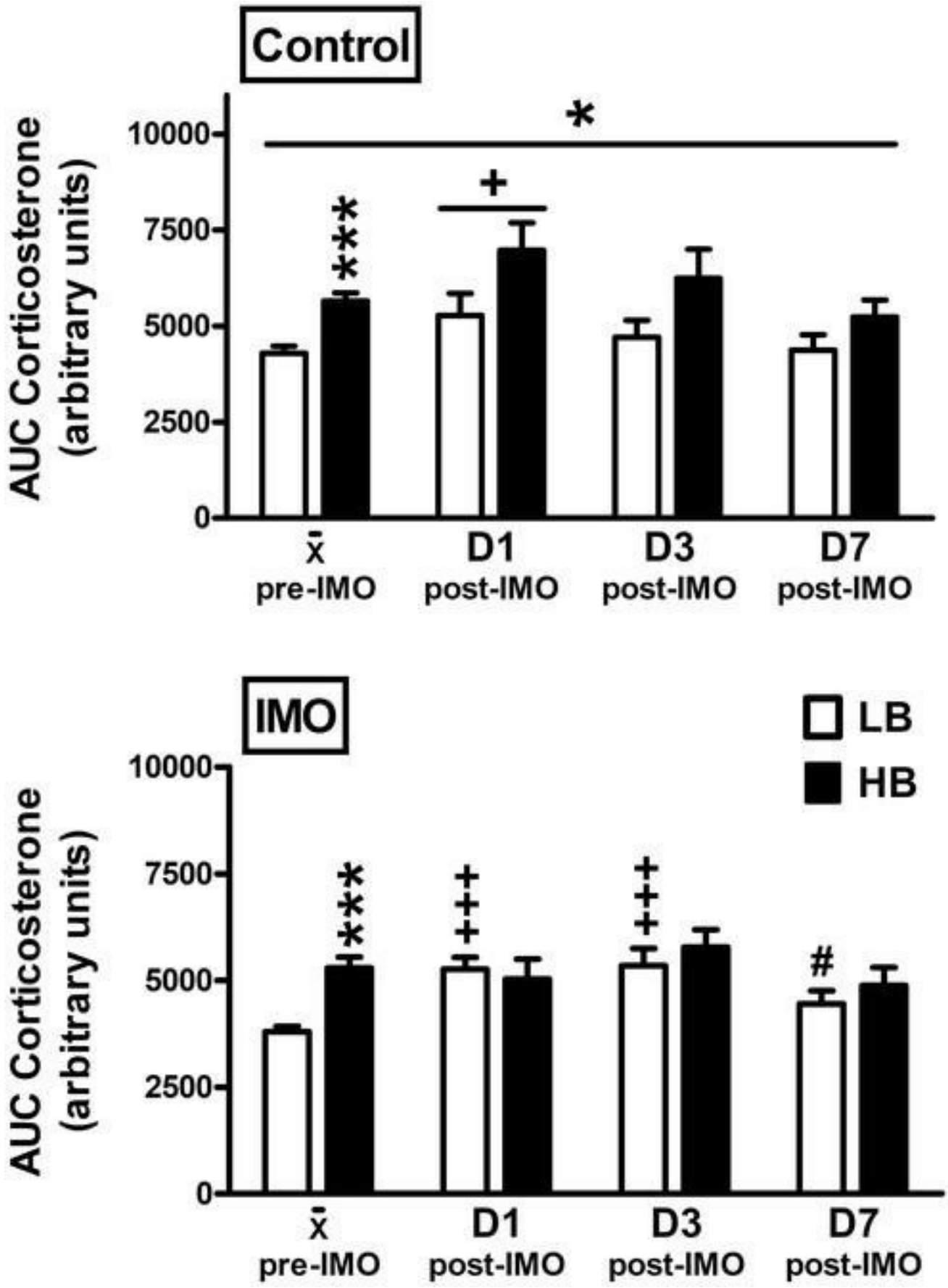
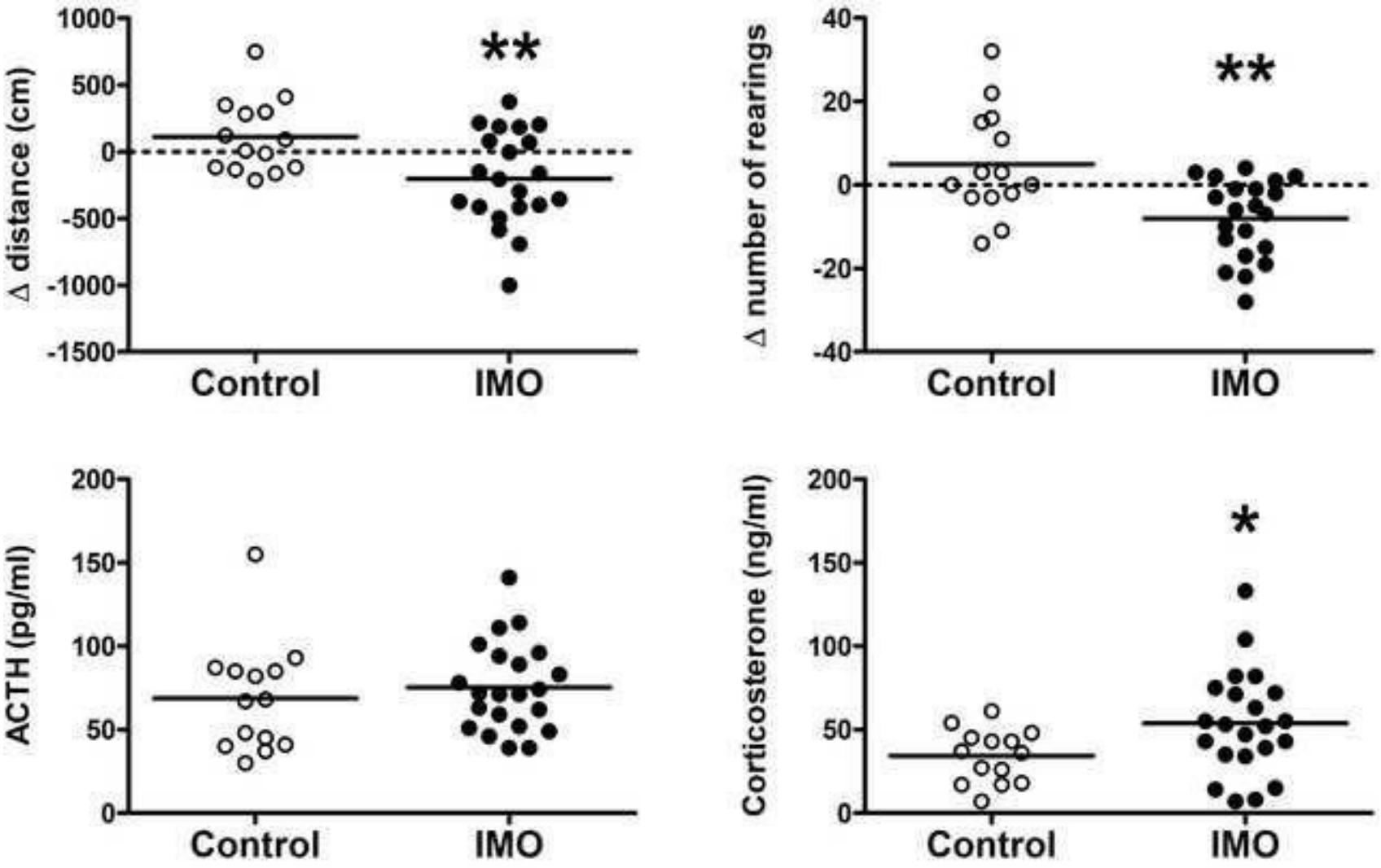

Control
AUC Corticosterone (arbitrary units)
10000
7500
5000
2500
0
*
+
***
x̄
pre-IMO
D1
post-IMO
D3
post-IMO
D7
post-IMO
IMO
LB
HB
****
+++
+++
#

Figure 6


Δ distance (cm)
1000
500
0
-500
-1000
-1500
**
Control
IMO
Δ number of rearings
40
20
0
-20
-40
**
Control
IMO
ACTH (pg/ml)
200
150
100
50
0
Control
IMO
Corticosterone (ng/ml)
200
150
100
50
0
*
Control
IMO