Adaptation of the hypothalamus-pituitary-adrenal axis to daily repeated stress does not follow the rules of habituation: a new perspective.

Cristina Rabasa[1,3], Humberto Gagliano[1], Jordi Pastor-Ciurana[1], Silvia Fuentes[2], Xavier Belda[1], Roser Nadal[2] and Antonio Armario[1]

[1]Institut de Neurociències and Unitat de Fisiologia Animal (Facultat de Biociències), Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona; Red Temática de Investigación Cooperativa en Salud (RETICS-Trastornos Adictivos), Spain

[2]Institut de Neurociències and Unitat Psicobiologia (Facultat de Psicologia), Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona; Red Temática de Investigación Cooperativa en Salud (RETICS-Trastornos Adictivos), Spain

[3]Present address: Department of Physiology/Endocrinology. Institute of Neuroscience and Physiology. The Sahlgrenska Academy at University of Gothenburg. Gothenburg, Sweden.

+Corresponding author: Antonio Armario, Animal Physiology Unit (School of Biosciences), Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. Tel.: +34 93 5811664, fax: +34 93 5812390, antonio.armario@uab.es

Running title: HPA adaptation to stress is not a habituation process.

Abstract

Repeated exposure to a wide range of stressors differing in nature and intensity results in a reduced response of prototypical stress markers (i.e. plasma levels of ACTH and adrenaline) after an acute challenge with the same (homotypic) stressor. This reduction has been considered to be a habituation-like phenomenon. However, direct experimental evidence for this assumption is scarce. In the present work we demonstrate in adult male rats that adaptation of the hypothalamus-pituitary-adrenal (HPA) axis to repeated stress does not follow some of the critical rules of habituation. Briefly, adaptation was stronger and faster with more severe stressors, maximally observed even with a single exposure to severe stressors, extremely long-lasting, negatively related to the interval between the exposures and positively related to the length of daily exposure. We offer a new theoretical view to explain adaptation to daily repeated stress.

Keywords: Habituation, Repeated Stress, Immobilization, Restraint, water stress, ACTH, corticosterone, c-*fos*.

1. Adaptation to repeated stress as a habituation process, evidences and contradictions

Exposure to different types of stressors results in a wide range of physiological and behavioral responses, some of them related to the particular nature of the stressor. Activation of the hypothalamus-pituitary-adrenal (HPA) axis constitutes one of the prototypical responses to all kind of stressors. The HPA axis, along with a few set of other physiological responses (i.e. plasma levels of prolactin, adrenaline and glucose) appear to be good markers of the intensity of emotional or predominantly emotional stressors (Armario et al, 2012; Kvetnansky et al, 2009; Martí and Armario, 1998). When animals are daily exposed to the same stressor for several days or a few weeks, reduction of the response of the HPA axis and other physiological variables, mainly plasma levels of adrenaline and hyperglycemia, has been very often observed (Martí and Armario, 1998), suggesting that those variables that are sensitive to the intensity of stressors are also sensitive to repeated experience with the stressors. The progressive reduction of the HPA and adrenaline response to repeated exposure to the same stressor was initially termed adaptation, but later on, the term habituation has been more widely accepted on the assumption that adaptation to the same (homotypic) daily repeated stressor appears to follow the rules of habituation (i.e. De Boer et al, 1990; Ma and Lightman, 1998; Natelson et al, 1988).

Reduction of the HPA response to daily repeated stress is not always achieved and is likely to involve different processes depending on the nature of the stressors. We can broadly distinguish between physical (systemic) stressors that represent a direct challenge to homeostasis and survival (i.e. hypovolemia, infection), and psychological (emotional) stressors that represent potential, not actual, danger (i.e. an unknown unprotected environment, predator odor). Although most laboratory stressors have some physical component (exercise and hypothermia after forced swim, minor tissue damage/inflammation after footshock, altered temperature and intense struggle after restraint or immobilization), under typical laboratory conditions the physical component do not represent any challenge for survival. In addition, the pattern of brain c-fos expression strongly suggests that they are more alike to emotional than physical stressors and therefore we call them predominantly emotional stressors. Adaptation to physical and emotional stressors probably encompasses markedly different processes (see Armario, 2015) and the present work will focus on emotional or predominantly emotional stressors.

Habituation has been defined as a response decrement that results from repeated exposure to a stimulus and does not involve either sensorial adaptation or sensorial/motor fatigue. Although it was originally considered as a primitive, non-associative type of learning, more recent views about habituation emphasize that it represents a wide range of phenomena, distinguish between short-term (STH) and long-term habituation (LTH) and considers the possibility that some of these phenomena may also involve associative processes (Cristofferssen, 1997; Grissom and Bhatnagar, 2009; Rankin et al, 2009; Thompson, 2009). It is obvious that factors involved in adaptation to daily repeated stress are likely to be closer to LTH than STH. There is a consensus among researchers about the main characteristics of habituation (Rankin et al, 2009):

1) Repeated application of a stimulus results in a progressive decrease in some parameters of the response to an asymptotic level.

2) If the stimulus is withheld after response decrement, the response recovers at least partially (spontaneous recovery).

3) After multiples series of stimulus repetitions and spontaneous recovery, the response decrement becomes successively more rapid and/or more pronounced.

4) Other things being equally, more frequent stimulation results in more rapid and/or pronounced response decrement, and more rapid spontaneous recovery.

5) Within a stimulus modality, the less intense the stimulus, the more rapid and/or more pronounced the behavioural decrement. Very intense stimuli may yield no significant observable decrement.

6) The effects of repeated stimulation may continue to accumulate even after the response has reached an asymptotic level. This effect of stimulation beyond asymptotic level can alter subsequent behaviour, for example, delaying the onset of spontaneous recovery.

7) Within the same stimulus modality, the response decrement shows some stimulus specificity.

8) Presentation of a different stimulus results in an increase of the decremented response to the original stimulus. This phenomenon is termed dishabituation.

9) Upon repeated application of the dishabituating stimulus, the amount of dishabituation is reduced (habituation of dishabituation).

10) Some stimulus repetition protocols may result in properties of the response decrement that last hours, days or weeks. This persistence of aspects of habituation is termed long-term habituation.

The hypothesis that adaptation to repeated stress is an habituation-like phenomenon neither has been theoretically developed nor is strongly supported by experimental evidence (Grissom and Bhatnagar, 2009). To our knowledge, only a few papers have generated information directly concerning to the hypothesis of habituation to repeated stress in rats. In a first paper comparing stressors differing in intensity (handling, restraint prone and restraint supine) the authors concluded that the stronger the stressors the lower the magnitude of corticosterone reduction after repeated stress (Natelson et al, 1988). However, there is strong evidence to suggest that plasma corticosterone neither appropriately reflect plasma ACTH nor adaptation to daily repeated stress for two main reasons (Armario et al, 2006; 2012): (i) saturation of adrenal cortex secretion of glucocorticoids with intermediate levels of ACTH; and (ii) increase in maximum adrenal cortex glucocorticoid secretion after a history of chronic exposure to severe stressors.

In another report, De Boer et al (1990) studied how the interval between stressor exposure (24 versus 72 h) could affect adaptation of corticosterone, noradrenaline, adrenaline and glucose to five repeated exposure to a novel environment (cylinder) containing a low level of water. They found a progressive reduction of the response of all these variables after repeated stress, which was more pronounced with the 24 h than the 72 h interval. This is in contrast to the lower LTH observed after massed (six 30 min sessions of noise on one day) as compared with spaced (six daily 30 min sessions) stress exposure (Massini et al, 2008), although it is of note that repeated exposure to a stressor within the same day are likely to involve different processes that repeated exposure with intervals of 24 h or more. In another paper, Ma and Lightman (1998) studied the corticosterone response to a final restraint in function of the different schedules of previous exposure to the stressor: once a week, twice a week or daily exposure. They observed a progressive reduction of the corticosterone response to a final restraint in function of the number of previous experiences with the situation, although in this case the interval between exposures also differed. Negative evidence for dishabituation of the corticosterone response to daily repeated restraint stress has been found after presentation of a different (heterotypic) stressor (Pace et al, 2001).

Finally, a single exposure to some stressors, including immobilization (IMO), was found to induce a long-lasting reduction of the HPA response to the homotypic stressor that was positively related to the intensity of the stressors (Dal-Zotto et al, 2004; 2003; 2002; Martí et al, 2001; Vallès et al, 2003; 2006). IMO is a severe stressor on the basis of all physiological markers of stressor intensity (Márquez et al, 2002; Martí et al, 2001; Vallès et al, 2000) and therefore the above results were opposite to those predicted by the rules

of habituation. We then hypothesized that long-lasting effects of IMO and adaptation after daily repeated exposure to the stressor could reflect two different phenomena (Armario et al, 2004). However, an alternative explanation is that adaptation does not actually follow the rules of habituation in that even a single exposure to a severe stressor may be enough to induce nearly maximum HPA adaptation.

In summary, strong experimental evidence supporting that the reduced HPA axis response after daily repeated exposure to stress is a habituation-like phenomenon is lacking or controversial. In view of the above concerns, the aim of the present work was to systematically test in adult male rats whether HPA adaptation actually follows the main rules of habituation.

2. Methods

2.1. Animals and general procedures

Adult male Sprague–Dawley rats obtained from the breeding centre of the Universitat Autònoma de Barcelona were used. Rats were maintained under standard animal housing conditions (21°C, 55% to 65% humidity, and a 12-hour light/dark cycle: 8 am-8 pm). Rats were housed in pairs and food and water were available at libitum. The experimental protocols were approved by the Committee of Ethics of the Universitat Autònoma de Barcelona and Generalitat de Catalunya, and were carried out in accordance to the European Communities Council Directive (1010-63-UE) and Spanish legislation (RD 53/2013).

In all experiments blood samples were taken by tail-nick, a procedure extensively used in our lab and by others because very low resting levels of hormones are obtained under appropriate conditions (i.e. Belda et al, 2004; Vahl et al, 2005). Blood was centrifuged at 4930 x g (15 min, 4º C), and plasma was frozen (-20º C) until assay. In all experiments, rats were sampled immediately after stress and at different times post-stress (30, 45, 60 or 90 min after the termination of stressor exposure, R30, R45, R60 and R90

respectively), depending on the particular experiment.

2.1.1: Stressors

Immobilization (IMO): Rats were immobilized by taping their four limbs to metal mounts attached to a board (i.e. Gagliano et al, 2008). Head movements were restricted with two plastic pieces (7 × 6 cm) and the body was subjected to the board by means of a piece of plastic cloth (10 cm wide) attached with Velcro® which surrounded all the trunk.

Restraint (REST): Animals were placed in open-ended transparent Plexiglas cylindrical restrainers (WPI, UK, Ref. STR554) measuring 6 cm in diameter and 21.5 cm in length (Rabasa et al, 2011). Several holes in the walls of the cylinder provided fresh air.

Water stress (WS): Animals were placed in transparent Plexiglas cylindrical tanks (height: 40 cm, internal diameter: 19 cm), containing water (36°C) to a level of 5 cm. Tanks were separated by opaque screens.

Open-field (OF): Animals were exposed for 15 min to a rectangular gray plastic box opened at the top (56 × 36.5 × 31 cm) with dim illumination provided by a white 25 W bulb placed 1.20 m above the centre of the surface of the box. Animals were initially placed in a corner of the open-field facing the wall. The box was cleaned between animals with ethanol solution (5%, v/v in tap water).

2.2. Techniques

Radioimmunoassay: Plasma ACTH and corticosterone levels were determined by double-antibody radioimmunoassay (RIA). 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). All samples to be statistically compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 3.8 % for ACTH and 7.8 % for corticosterone. The sensitivity of the assays was 12.5 pg / ml for ACTH and 0.1 µg / dl for corticosterone.

In situ hybridization hystochemistry: Animals were deeply anesthetized by inhalation with isofluorane (Laboratorios Esteve, Barcelona), with oxygen flow of 0.8L/min. Then, they were transcardially perfused as previously described (Ons et al, 2010), and coronal sections, including medial prefrontal cortex (mPFC), paraventricular nucleus of the hypothalamus (PVN), medial and basolateral amygdala (MeA) and the posterior paraventricular thalamic nucleus (pPVTh), were obtained with a cryostat at 20 µm. In-situ hybridization was performed as previously described (Ons et al, 2010) without modifications. Thereafter, the slides were exposed to a XAR-5 Kodak Biomax MR auto-radiography film (Kodak, Madrid). Exposition time varied in function of the intensity of the signal. After autoradiography film exposure, slides from PVN and MeA, were defatted in xylenes, and dipped in LM-1 nuclear emulsion (GE Healthcare). Slides were exposed for some days and developed in D19 developer (Kodak, Madrid) for 4 min at 14ºC, counter-stained with thionine 0.25%, dehydrated with ethanol series and cover slipped with DPX (Sigma, Barcelona).

The c-fos RNA levels were semi-quantitatively determined in three-four sections per brain area (including both hemispheres) and animal, according with the stereotaxic atlas of Paxinos and Watson (1998). Digitalized images (NIKON, DMX-1200 - Eclipse-E400 system) were quantified using Image software (Scion Corporation, Frederick, MD) by grey level thresholding. Measures were obtained in arbitrary units (pixel area × average sum grey). Slides exposed to emulsion, were analyzed in the same way but using dark field instead of light field to make photographs.

2.3. Statistical Analysis

Data are presented as means ± SEM. The 'statistical package for social science' (SPSS) was used. Data were analyzed by a generalized linear model (GzLM), usually with repeated measures (generalized estimating equations model or GEE (see Hardin and Hilbe, 2003). As a method of estimation, the maximum likelihood (ML) was used in all cases. Normality distribution and identity as a link function was chosen to be the one that better fit the data. The significance of the effects was determined by the Wald chi-square statistic. Day and sampling time were used as within-subject factors to study the hormonal response changes along days in the same groups. When different groups were compared, group was used as a between-subject factor, and sampling time as a within-subject factor. When necessary, data was corrected using post-hoc sequential Bonferroni comparisons. In some cases, the area under the curve (AUC) of plasma hormone levels in response to stressors was calculated with Graph Pad Prism (version 4.01) that computes the AUC using the trapezoid rule. Differences were considered significant if $p<0.05$.

3. Testing some critical characteristics of habituation

3.1. Does the response progressively decrease with the number of exposures to the stimulus? (Characteristic number 1)

This criterion predicts that the HPA axis response will decrease with daily repeated exposure to the same stressor to an asymptotic level. To directly test this hypothesis, animals were assigned to either chronic or acute IMO groups. Those assigned to the chronic IMO group were daily exposed to 1 h of IMO for 7 days and the HPA response was followed in the same animals on days 1, 2 and 7. Those assigned to the acute IMO group were only blood sampled on days 1 and 2, but only exposed to 1 h of IMO on day 7. This group was introduced to demonstrate that prior blood sampling did not alter the response to an acute IMO. As compared to day 1, a significant reduction of the ACTH response was already observed on day 2 (Fig 1A), with no further significant reduction after repeated exposure to IMO for 5 additional days (7^{th} IMO). In accordance with previous reports (i.e. Márquez et al, 2004; Martí et al, 2001), the reduction of corticosterone levels after prior single or repeated experience with IMO was only observed during the post-IMO period, and higher corticosterone levels immediately after IMO were achieved just after the 7^{th} day

(see Fig 1B). A minor, but significant, further reduction of plasma levels of corticosterone during the post-IMO period was found after the 7th as compared to the 2nd IMO, suggesting that factors other than ACTH could control plasma corticosterone after repeated exposure to stress by reducing adrenocortical responsiveness to circulating ACTH. It is likely that this could be achieved by neural innervation of the adrenal gland (Bornstein et al, 2008; Herman et al, 2003).

It is of note that after chronic IMO, plasma corticosterone just after the stressor increased despite the decrease in ACTH. We have repeatedly found and discussed the same phenomenon (i.e. Armario et al, 1988; Márquez et al, 2004), which can be explained in two ways: (i) first, chronic IMO usually increased adrenal weight and maximum capability of the adrenal cortex to secrete glucocorticoids (Armario et al,1988), the two variables showing good correlation (Márquez et al.,2004); (ii) the reduction of plasma ACTH levels observed after chronic IMO still maintain levels above those needed to maximally activate the adrenal due to the saturation of adrenocortical synthesis with relatively low levels of ACTH (probably around 300 pg/ml). In contrast, when ACTH levels are decreasing during the post-IMO period below those maximally activating the adrenal, the lower ACTH response in chronic IMO rats was reflected in lower plasma corticosterone.

The above results indicate, in contrast to the criterion for habituation, that a single exposure to 1 h of IMO was enough to induce a reduction of the response of the HPA axis to the same stressor that was almost unaffected by further exposures to the stressor. It is unlikely that additional days of exposure to IMO could have eventually result in much lower response since in a previous report in which response to repeated IMO was followed on days 1, 4, 7, 10 and 13, the most important reduction of the HPA response was already observed on day 4, with only minor differences thereafter (Márquez et al, 2004). Therefore, it appears that adaptation to IMO rapidly develops and reaches a ceiling effect with only a single exposure to IMO. These data are in striking contrast to the criterion for habituation and indicate that the homotypic long-term reduction of the HPA axis after a single stress exposure (we initially termed homotypic desensitization) and adaptation after daily repeated exposure are the same phenomenon.

In order to know whether stress-induced brain activation followed a pattern similar to that of HPA hormones, different groups of animals were exposed to a single 1 h IMO on day 1 or on day 11, whereas

other rats were left undisturbed or daily exposed to the stressor for 11 days. On day 12, all groups were exposed to 1 h of IMO and their brain processed for c-fos induction by in situ hybridization. C-fos is considered a good marker of neuronal activation (Hoffman and Lyo, 2002). We selected the medial dorsal parvocellular subdivision of the paraventricular nucleus of the hypothalamus (mpdPVN), the key area in the control of the HPA axis where ACTH secretagogues, mainly the corticotropin-releasing hormone (CRH), are located (Herman et al, 2003). In addition, we measured c-fos expression in other brain areas known to be important in the processing of emotional or predominantly emotional stressors and their adaptation to repeated stress (Dayas et al, 1999; Herman et al. 2003, 2013): the prelimbic (PrL) and infralimbic (IL) areas of the medial prefrontal cortex (mPFC), the posterior part of the paraventricular thalamic nucleus (pPVTh), the medial (MeA) and basolateral (BLA) amygdala.

The pattern of c-fos expression after prior experience with IMO was not homogeneous among the different areas. The results showed a significant reduction of c-fos expression in the mpdPVN (Fig 2A) after a single experience with IMO (regardless of time elapsed between the first and the final exposure), with an additional reduction after chronic IMO. In the mPFC (Fig 2B and 2C), the response to an acute IMO was only significantly reduced after repeated but not after single previous single experience with IMO, whereas in the pPVTh (Fig 2D) the results showed a significant reduction of c-fos expression after chronic IMO and also in the rats exposed to the second IMO on the day after the first MO, but not in those exposed to the second IMO 11 days after the first exposure. In the MeA and the BLA (Fig 2E and 2F respectively) the reduction of the response was independent of the number of previous exposures and the time elapsed between them. Interestingly, c-fos expression in the MeA, an area that appears to be important for the control of the response to emotional stressors (Dayas et al, 1999), followed the same pattern as the HPA hormones. A similar pattern was observed in the BLA, supporting the possibility of an important role of this area in adaptation to repeated stress mediated by activation of β-adrenergic receptors (Grissom and Bhatnagar, 2011). In contrast, the PVN itself was sensitive to a single experience, yet repeated IMO exposure caused a stronger reduction of c-fos expression than a single experience. Regarding the differential impact of the time elapsed between the two exposures to IMO in the pPVTh, we cannot rule out at present that prior exposure to a severe stressor might transiently impair the c-fos response to any further stressor on the day after. A previous study using different days of exposure to ferret odor showed a more homogenous pattern of reduction in c-fos expression among the different brain areas, but the rats

had at least three experiences with the stressor (Weinberg et al, 2009). It thus appears that heterogeneity is particularly evident with only one single experience.

The regional heterogeneity in the consequences of single versus repeated experience with IMO respect to c-fos expression suggests that not all stress-induced behavioral and physiological responses would follow a similar pattern of adaptation, with adaptation of the HPA axis being faster and/or stronger than that of other physiological systems (i.e. Chen and Herbert, 1995; Dumont et al, 2000; Bhatnagar et al, 2006; Schmidt et al, 2010). In our hands, stress-induced hyperglycemia is very sensitive to repeated exposure to the same stressor (Armario et al, 1990; Márquez et al, 2004), but the impact of a single IMO experience is not consistent (unpublished data). It would be important to study the sensitivity of plasma levels of noradrenaline and adrenaline to single versus repeated experience with the same stressor. Unfortunately, this requires using chronically catheterized animals and individual housing, making it difficult to simultaneously handle an elevated number of animals.

3.2. Is spontaneous recovery influenced by the number of prior experiences with the stressor? (Characteristic number 2)

The theory of habituation predicts a progressive spontaneous recovery of the HPA response over time. There is some evidence showing that HPA adaptation to repeated restraint (REST) or noise stress is long-lasting, being at least partially maintained after 3-4 weeks (Bhatnagar et al, 2002; Nyhuis et al, 2010). In contrast, a recent paper suggests that discontinuation of exposure to REST for 2 days is enough to attenuate both HPA and behavioral responses (Zhang et al, 2014). We then decided to study this phenomenon using IMO as the stressor. Although single and repeated exposure to IMO caused the same reduction of the HPA response to the homotypic stressor, it is still possible that consolidation of memory about the situation was stronger after repeated exposure and such a difference in consolidation could be reflected in a greater spontaneous recovery after a single experience as observed with other paradigms of habituation (i.e. Sanderson and Bannerman, 2011).

Animals were assigned to three experimental groups: (a) acute IMO group, only exposed to acute IMO on day 72; (b) prior single IMO group, exposed to IMO on day 12 and again on day 72; and (c) chronic IMO

group, daily exposed to IMO from day 1 to 12 and again on day 72. IMO always lasted for 1h. We firstly demonstrated a strong reduction of the HPA response to IMO in those animals daily exposed to the stressor for 12 days (Fig 3A-B). Two months later we still observed reduced HPA response to IMO in the two groups of animals having prior experience with the stressor when compared with stress naïve animals (Fig 3C-D). Importantly, the reduction was similar after single or repeated experience with the stressor. Therefore, the data confirm that adaptation of the HPA axis to severe stressors is extremely long-lasting and of similar characteristics regardless of the number of exposures to the stressor. Thus, evidence for complete dishabituation was not found more than two months later. In conclusion, one single experience with a severe stressor is enough to fully consolidate long-term memory about the situation, with only minor improvement after repeated experience.

3.3. The weaker the stimulus, the more rapid and/or more pronounced is habituation? (Characteristic number 5)

Although the previous results demonstrated that one single previous exposure to 1 h of IMO was enough to induce nearly maximum and long-lasting HPA adaptation, we reported that the reduction of the HPA response after a single exposure was greater with more severe stressors (i.e. IMO) than with lower intensity ones (i.e. restraint) (Martí et al, 2001). This is opposite to the criterion for habituation that the weaker the stimulus the higher the reduction of the response after repeated exposure. Moreover, if long-lasting reduction of the HPA axis caused by a single exposure to stress and adaptation are the same phenomenon, the positive relationship between the intensity of stressors and the magnitude of the long-term homotypic reduction of the HPA response may be explained in two ways. First, more days of exposure are needed to achieve maximum adaptation (for this particular stressor) with less severe stressors so that one single exposure would result in submaximum adaptation. Second, maximum absolute HPA adaptation would be stronger with more severe stressors. To test the two hypotheses we chose to compare two stressors differing in intensity: REST versus IMO (Armario et al, 1990; Campmany et al, 1996; Rabasa et al, 2011). Two groups of animals were daily exposed to either 1 h of IMO or 1 h of REST for 12 days (Fig 4).

Similar to the previous experiment (see Fig 1), a strong reduction of the HPA response to IMO was already observed on day 2, with only a slight additional decline on day 3 (Fig 4A). In contrast, after REST the decrease in ACTH on day 2 was only significant during the post-REST period, whereas a significant reduction of the initial ACTH response to the stressor was only observed on day 12 (Fig 4C). Plasma corticosterone followed a similar pattern, although some differences were observed when using severe stressors such as IMO for the reason already described. Thus, the reduction of corticosterone response on day 2 was greater in absolute terms with IMO (noted only in the post-stress period) than REST, and the reduction was not consistent with the latter stressor until day 6 (Fig 4B and 4D). These data support the hypothesis that lower intensity stressors need more days to achieve maximum adaptation. Importantly, the two stressors not only differed in the number of days needed for maximum adaptation, they strongly differed in the magnitude of the absolute decrease of the ACTH responses to repeated exposure, clearly greater after IMO than after REST as better observed with the AUCs (Fig 4 A'-D'). Comparison of AUCs on day 12 versus day 1 showed that the absolute decrease of ACTH was greater after IMO than REST ($p < 0.01$), and the same trend was found regarding corticosterone, but differences were not significant, supporting the relative insensitivity of plasma corticosterone to reflect ACTH release with severe stressors. Therefore, more severe stressors slightly enhance the speed of adaptation of the HPA axis, but strongly potentiate the maximum adaptation achieved as evaluated with plasma ACTH. The results not only demonstrate that adaptation of the HPA axis to daily repeated stress does not follow the criterion for habituation, but they confirm that that long-term reduction of the HPA response after a single experience and adaptation to daily repeated stress are the same phenomenon.

3.4. The higher the frequency of stimulation, the more rapid and/or more pronounced is habituation? (Characteristic number 4)

This criterion has an important degree of ambiguity in that it does not specify whether the number of exposures is maintained constant, changing only the inter-stressor interval (ISI). One of the strongest support to the consideration of adaptation to stress as an habituation-like phenomenon are the results by de Boer et al. (1990), showing that adaptation of corticosterone, adrenaline and glucose to repeated exposure (15 min) to a cylinder containing a small amount of water (herein water stress, WS) was markedly dependent on the interval between successive exposures. More specifically, with equal number

of exposures to the stressor, the decline over the days of all responses was greater with the 24 h than with the 72 h interval, in accordance with the rule number 4 of habituation that higher frequency of exposure results in stronger reduction of the response.

However, we have previously found that a single 20 min exposure to IMO was enough to induce reduction of the response of the HPA axis to the same stressor when tested one week later (Dal-Zotto et al, 2003; 2004). This is certainly in contrast to the results by De Boer et al. in which a 72 h interval clearly impaired adaptation to water stress. Moreover, there have not been attempts to replicate De Boer et al's findings. We then wanted to confirm them in a new experiment testing the influence of the ISI in the process of adaptation of the HPA axis using IMO and WS. The former stressor was also included for two other reasons: (i) to have a more complete picture of the influence of ISI; and (ii) because in prior experiments adaptation was maximum with a single exposure to 1 h of IMO and it remains possible that the ISI may be more important for lower intensity stressors (WS) than for the severe stressors (IMO). We followed as close as possible the design by De Boer et al. (1990) and used 20 min exposure to both stressors and ISIs of 24 or 72 h. We observed that adaptation of ACTH was better with the 72 than the 24 h interval both after IMO and WS (Fig 5A, 5B, 5E, 5F). Nevertheless, with IMO, a reduction of the ACTH response (restricted to the post-IMO period) was already observed on day 2 with both 24 and 72 h ISIs, although such a reduction progressively accentuated over the sessions with the 72 h interval (Fig 5B), whereas only a slight reduction over the sessions was found with the 24 h interval (Fig 5A). Similarly, with the 24 h interval of WS (Fig 5E), a significant reduction of the ACTH was not observed until the 3rd exposure, with a maximum reduction during the 5th exposure, whereas with the 72 h interval a marked (nearly maximum) reduction was already observed during the 2nd exposure (Fig 5F).

The above experiment was designed to replicate De Boer et al's design and include four groups, making it difficult to include more than two sampling times. Therefore, sampling times were not particularly appropriate to detect repeated stress-induced changes in plasma corticosterone, particularly after IMO. Maximum corticosterone levels are usually achieved far beyond the 20 min IMO period so that the R30 period probably detected changes in ACTH just after the stressor (García et al, 2000). Nevertheless, in animals exposed to WS, a lower intensity stressor, corticosterone was sensitive to prior stress experience and again the effect was faster with the longer ISI (Fig 5G and 5H).

As the results were in striking contrast to those by De Boer et al. (1990), we repeated the experiment using only WS, with the same results (not shown). We have carefully revised the protocols to find possible reasons for the discrepancies between our results and those by De Boer et al. (1990), but we have been unable to find them. Moreover, the results of this experiment argue against spontaneous recovery of the HPA response after interruption of stress exposure for 2 days as reported by Zhang et al. (2014).

Direct comparison of the ACTH response to the first exposure demonstrated a much greater ACTH response to IMO than to WS stress, supporting that IMO was a stronger stressor. With the 24 h interval (Fig 5A and 5E), which allowed us to compare the present experiment with our previous results, a significant reduction of the response was found on day 2 with IMO (at R30) and only on day 3 with WS (immediately after stress), supporting again a positive relationship between the intensity of the stressor and the number of exposures needed to achieve a significant degree of adaptation.

To more precisely know whether the magnitude of adaptation was affected by the intensity of the stressor, we calculated the AUC of the response to compare the last day with the initial day of exposure in all groups. The absolute decrease in ACTH was greater with IMO than WS (regardless of ISI), and greater with the 72 h ISI (regardless of the stressor), whereas the percent decrease was similar with both stressors and greater with the 72 h ISI (Table 1). Therefore, the results confirm that the higher the stressor intensity the greater the absolute reduction of the ACTH response after daily repeated exposure in stress. Once again, the pattern observed with corticosterone differed from that of ACTH in that the reduction respect to day 1 was greater with WS, for the reasons already discussed.

The unexpected results regarding the influence of the ISI require a theoretical interpretation. One possible explanation is that the kind of memory about the situation that determines the reduction of the HPA response would require some days to fully consolidate after relatively short exposure to stressors (20 min), this consolidation being faster with more severe stressors. Evidence for such a consolidation exists in fear conditioning paradigms (i.e. Houston et al, 1999; Pickens et al, 2009; Siegmund and Wotjak, 2006). Alternatively, exposure to stressors could induce a sensitization of the HPA response to any further stressor that would last for some days and partially counteract the adaptation process. Disappearance of

this transient sensitization 2 days after initial exposure would unmask adaptation. The latter explanation is unlikely as HPA sensitization is consistently observed after a single exposure to severe stressors such as IMO and tail-shock (Belda et al, 2008; 2012; Johnson et al, 2002; O'Connor et al, 2003), but not after less severe stressors such as REST (Wong et al, 2000). If sensitization affected the response to the homotypic stressor this would mask more HPA adaptation to IMO than to WS, in contrast to the empirical evidence.

3.5. The length of the daily exposure as a critical factor to explain the reduced response

The previous results strongly argue against the consideration of adaptation of the HPA axis to repeated, predominantly emotional, stressors as a habituation-like process. We then suggest a new hypothesis to explain adaptation to daily repeated stress. The physiological changes elicited by exposure to emotional or predominantly emotional stressors constitute an anticipatory, evolutionary developed, response so far as exposure to such stressors has a high probability to be followed by an actual challenge to homeostasis (i.e. intense physical activity, hemorrhage, wounds and infections). This anticipatory response involves both behavioral and physiological changes, the activation of the HPA axis being one paramount example of a physiological response that is proportional to the intensity of stressors. If such exposure is not followed, under laboratory or natural conditions by any actual challenge, a safety signal would develop that is proportional to the magnitude of the initial activation caused by the stressor, but also to the time being exposed to the stressor without any additional evidence for actual homeostatic challenge. Upon a second exposure to the same stressor, two opposite processes are triggered by the sensory information generated by the stressor: one stimulatory that would remain constant over the days and another inhibitory (safety signal), which developed as a consequence of the previous experience with the same situation. This inhibitory safety signal would oppose to the stimulatory inputs and eventually reduce the magnitude of the HPA response to the present situation. This putative inhibitory signal does not appear to block the response to a novel stressor when both the repeated and the novel stressor are applied simultaneously (Masini et al, 2012), suggesting independent processing of the two stimuli. It is of note that we have obtained evidence for a lack of interference between cat odor exposure and IMO regarding long-term context fear conditioning when animals were exposed to the odor while immobilized (Muñoz-Abellán et al, 2011), supporting the possibility of at least partially independent brain processing of two different stressors.

This hypothesis assumes that the length of the first exposure to the stressor without any sign of injury may be critical to determine the magnitude of adaptation of the HPA axis. To test such a hypothesis rats were daily exposed to IMO for 1 or 3 h and sampled on days 1, 2 and 7 (Fig 6). Repeated exposure to 1 h of IMO resulted in a lower ACTH response on day 2 that was restricted to the post-IMO period (R45) and only weakly declined from day 2 to day 7 in the post-IMO period (Fig 6A). In contrast, repeated exposure to 3 h of IMO resulted in a marked reduction of the initial ACTH response on day 2 that was again only weakly affected by additional experiences with the situation (Fig 6B). To directly compared animals exposed to 1 versus 3 h of IMO, we performed an additional statistical analysis comparing the common sampling time (just after 1 h of IMO). The analysis showed lower ACTH response in those animals daily exposed to 3 h of IMO than in those daily exposed for 1h on days 2 and 7 ($p < 0.001$ in the two cases). These results not only confirm that one single exposure to IMO is enough to induce similar adaptation of the HPA axis as repeated exposure, but give support to our hypothesis that nearly maximum adaptation is strongly dependent on the length of exposure to the stressor.

In the present experiment, rats repeatedly exposed to 1 h IMO did not show reduction of ACTH response just after the stressor, in contrast to the results observed in previous experiments using the same length of IMO. Nevertheless, a clear reduction was observed during the post-IMO period. It appears that the balance between presumably stimulatory and inhibitory inputs differ among the different cohorts of animals. When stronger inhibitory inputs develop, the initial response is already reduced, whereas in cases when such inhibitory inputs are lower only the post-IMO period is affected, likely because stimulatory inputs are declining at this time.

The greater effect of the 3 h IMO exposure are unlikely to be explained by greater negative glucocorticoid feedback in the 3 h than 1 h IMO group when animals were again exposed to the stressor on the next day so far as we demonstrated in another experiment that prior exposure to 30 min or 4 h IMO on the day before, not only did not reduce the HPA response to a novel (heterotypic) stressor, but such response was enhanced, revealing HPA sensitization (**Fig 7**) and also confirming previous reports (Belda et al, 2008; 2012; Johnson et al, 2002; O'Connor et al, 2003). Importantly, similar HPA sensitization was observed after 30 min and 4 h IMO, thus ruling out a differential contribution of negative glucocorticoid feedback to explain the differential homotypic adaptation after 1 versus 3 h of IMO.

It is noteworthy that these results were unexpected in lights of the accepted view in the learning field on the greater efficacy of spaced as compared to massive learning (i.e. Lattal, 1999; Rescorla, 1988). Applied to our procedure, this should have been reflected in greater HPA adaptation after 6 daily exposures to 1 h of IMO than after a single exposure to 3 h of IMO. However, the opposite results were found.

4. General discussion

The present work presents evidence strongly arguing against the consideration of the reduced HPA response to a daily repeated stressor as a habituation-like phenomenon. We propose that the denomination of the process of adaptation to daily repeated stress should change and one possibility is to define it as tolerance. This name can apply to both physical and psychological stressors and does not require to perfectly fit to very specific rules.

The present results suggest that exposure to an emotional (or mainly emotional) stressor initially triggers HPA activation that is positively related to the intensity of the stressor (as independently evaluated by other different physiological systems). During a first prolonged exposure to stressors there is a progressive decline of ACTH response despite the persistence of exposure that can be explained by the contribution of several mechanisms, including negative glucocorticoid feedback (Rivier and Vale, 1987), but also by some kind of safety signal that is proportional to the intensity of the stressor and gets stronger with the period of exposure to the stressor provided that no actual danger follows. This safety signal is a learning-like process that are likely to need more than one day to fully consolidate if the length of exposure to the stressors is very short. The absolute magnitude of the reduction of the response when the animals are again exposed to the same stressor is directly proportional to the intensity of the stressor and the length of prior exposure. Re-exposure to the same stressor activates the memory about the safety signal, what generates an inhibitory signal that opposes to the ongoing stimulatory one. The integration of these two opposite signals would determine the magnitude of the reduction of the response.

The concept of safety signals has been taken from classical studies of shock-induced fear conditioning where certain stimuli predict the occurrence of a period without shocks and they are called safety signals (Christianson et al, 2012). Importantly, as stated by the latter authors, safety signals are learned only if subjects expect danger that does not occur. Here we use this term to indicate that exposure to emotional stressors generate an emotional state and trigger an anticipatory physiological response aiming to better cope with a possible biological insult. As maintenance of such response represents a high cost for the organism, this response is progressively reduced during exposure to the stressor when no actual biological insult is detected. We call this process stress-in safety signal, to differentiate it from another safety, stress-out, signal generated by the release from the situation. In contrast to the stress-in safety signal, which is mainly generated by internal biological inputs, the stress-out signal is generated by external cues associated with the termination of stress exposure: i.e. manipulations needed to release the animals from the stressful situation, taking them from the stress context, moving them to the animal living room. Since there is evidence using the tail-shock paradigm of learned helplessness that the insula is critical for the beneficial influence of safety signal to reduce the negative consequences of shocks (Christianson et al, 2008; 2011), we propose that this region might be similarly involved in the elaboration of the safety signals leading to adaptation to repeated stress.

An intriguing question in stress research is why only a very restricted number of physiological variables have been found to be sensitive to the intensity of stressors (Armario et al, 2012). In general, it appears that those that are sensitive to the intensity of stressors (plasma levels of ACTH, corticosterone, noradrenaline, adrenaline and glucose) are also reduced after daily repeated exposure to the same stressor, although results regarding prolactin are inconsistent (Martí and Armario, 1998). This makes sense as the inhibitory processes associated to previous experience with the stressor would reduce the stimulatory effect caused by the stressor. Those variables that are not sensitive to the intensity of stimulatory inputs should not be sensitive to the reduction of such inputs. Unfortunately, there are no parametric studies either in animals or humans aiming at characterizing whether or not some stress-sensitive variables such as heart rate, blood pressure or skin conductance are sensitive to stressor intensity. On the basis of available data in human, plasma levels of catecholamine could be less sensitive than cortisol to one single prior experience with stressors (Schommer et al, 2003; von Kanel et al, 2006; Jonsson et al, 2010). Other physiological variables such as heart rate, systolic and diastolic blood pressure

are inconsistent or not sensitive (von Kanel et al, 2006; Jonsson et al, 2010; Elfering and Grebner, 2012). In animals, when both plasma ACTH and/or corticosterone and other stress-sensitive variables have been studied, a similar trend emerges, with clear reduction of the HPA response after repeated experience and milder, slower or null changes in stress-induced changes in cardiovascular measures and body temperature (Chen and Herbert, 1995; Bhatnagar et al, 2006; Barnum et al, 2007; Schmidt et al, 2010). Reduction of tachycardia and hyperthermia after repeated stress is better observed with a lower intensity stressor such as noise (Masini et al.,2008), strongly suggesting that variables other than those related to the HPA axis are less sensitive to repeated stress and therefore are insufficient to consistently reduce certain responses when facing with relatively severe stressors. Thus, translation of putative safety-associated inhibitory signals to each particular system is different, as also illustrated in the present work with the differential sensitivity of c-fos expression to prior experience with the stressor, depending on each particular brain area.

The extent to which adaptation of the HPA axis is dependent on the context has been explored in different laboratories. Whereas Grissom et al. (2007) showed evidence for a partial contribution of the context to the reduced HPA response to repeated REST, Nyhuis et al. (2010) did not observe any contribution of context to repeated noise stress. In accordance with the latter report, our group has not found such evidence after repeated REST or IMO (Rabasa et al, 2011). Although we cannot rule out at present a contribution of contextual signals to adaptation, the present evidence is weak.

It is important to remark the heterogeneous pattern of reduction of c-fos expression after single versus repeated exposure (present results) and the different pattern obtained when different physiological or behavioral systems has been studied (Cullinan et al, 1995; Ons et al, 2004; Pacak and Palkovits, 2001). These data suggest that the sensitivity of each system to daily repeated stress markedly differ, at least in its temporal pattern. Unfortunately, there is scarce theoretical elaboration about the fact that among the myriad of physiological and behavioral changes associated with exposure to stress, only a few appears to be clearly sensitive to the repeated experience with the situation (Martí and Armario, 1998). Although in general there is a good correlation between the sensitivity of certain physiological variables to the intensity of the stressors and their sensitivity to adaptation, this topic merits to be explored in depth, Moreover, future studies should clearly focus on systems other than the HPA axis (i.e. plasma noradrenaline and

adrenaline response) to know whether or not they share similar characteristics even with different degrees of sensitivity.

The neurobiological processes underlying adaptation to daily repeated stress are poorly known. Stress-induced glucocorticoid release is not a necessary requisite for the induction of adaptation as it is still observed in adrenalectomized rats maintained with low levels of corticosterone (Dal-Zotto et al, 2002; Jafery and Bhatnagar, 2006), but these hormones may play a partial role acting through both mineralocorticoid (MR) and glucocorticoid (GR) receptors in the pPVTth to induce adaptation (Jafery and Bhatnagar, 2006). Interestingly, blockade of mineralocorticoid, but not glucocorticoid, receptors using peripheral administration of the drugs impedes the expression of adaptation (Cole et al, 2000). It is thus clear that MR and GR are likely to partially contribute to adaptation, but mechanisms other than those associated to daily glucocorticoid release are important for proper adaptation of the HPA axis. Recently, it has been reported that brain blockade of vasopressin V1a receptors impair adaptation of the HPA axis to repeated REST without affecting the response to the first exposure (Gray et al, 2012), suggesting a role of vasopressin via V1a receptors to induce HPA adaptation. The precise locus of action of vasopressin remains to be characterized. Finally, there is also some evidence about a possible role of endogenous cannabinoids in HPA adaptation (Hill et al, 2010), although we have obtained negative evidence on this regard (Rabasa et al, 2015).

Evaluation of neuronal activation with the immediate early gene c-fos after repeated exposure to the same stressor revealed reduction of the response in telencephalic and diencephalic regions, but not brainstem nuclei involved in early sensorial processing (Girotti et al, 2006). Therefore, we have no clues about those brain regions primarily involved in the reduction of the response, likely because multiple areas participate in the process. Functional blockade of the medial geniculate nucleus has been found to impair adaptation of the HPA axis to daily repeated noise stress (Day et al, 2009). In contrast, auditory cortex is not needed for HPA adaptation to repeated noise exposure (Masini et al, 2012). These results indicate that the processing of information in sensorial specific thalamic nuclei, but not in cortical areas, may play an important role in the induction of HPA adaptation to specific stressors. Some evidence points to a role of the pPVTh in HPA adaptation to repeated stress (Bhatnagar et al, 2002). The role of the pPVTh appears to be more important for the induction than the expression of adaptation and to involve local action of

glucocorticoids (Jaferi and Bhatnagar, 2006), but how this is compatible with the typical reduction of c-fos expression after chronic stress in this area is unclear. Moreover, our present results showed dissociation between reduced c-fos expression and HPA adaptation to IMO in the group exposed to a single IMO 11 days before. The possible role of the anterior PVTh is not conclusive (Fernandes et al, 2002). The BLA may play an important role in the induction of adaptation as daily local administration of the beta blocker propranolol impaired HPA adaptation to repeated restraint (Grissom and Bhatnagar, 2011). A role of the BLA is compatible with our c-fos data showing similar reduction after single and repeated exposure to IMO and with the well-accepted role of the BLA in consolidation of different types of associative learning (McGaugh, 2004). On the basis of inactivation studies, a role of the mPFC in the expression of adaptation has been proposed (Weinberg et al, 2010), although the precise role is unclear.

The available experimental data indicate that adaptation of the HPA axis to repeated stress appears to be a phenomenon very resistant to disruption. It is likely that this could be due to the existence of multiple redundant mechanisms to assure a reduction of such costly response.

5. Conclusions

In conclusion, the present set of experiments indicate that the rules defining adaptation of the HPA axis after previous experience with the homotypic stressor do not fit with the concept of habituation. Adaptation is likely to be a learning-like process with particular characteristics not conforming previously accepted types of learning. The present results, by demonstrating that a single exposure to a severe stressor is, under certain conditions, enough to induce maximum HPA adaptation will allow to use more simple experimental designs to study this phenomenon. It remains to be established whether or not the rules governing adaptation of the HPA axis also apply to other adaptation-sensitive stress system such as plasma catecholamines and associated physiological responses. Both theoretical approaches and efforts to better characterize the neurobiological substrate of adaptation are needed.

Acknowledgements

The laboratory was supported by Spanish grants to AA and/or RN from Ministerio de Economía y Competitividad (SAF2011-28313), Plan Nacional sobre Drogas (2011/021), Instituto de Salud Carlos III (RD12/0028/0014, Redes Temáticas de Investigación Cooperativa en Salud, Ministerio de Sanidad y Consumo), and Generalitat de Catalunya (SGR2014-1020). Silvia Fuentes was a recipient of a PTA-MICINN fellowship (PTA 2010-3472-I). Roser Nadal is a recipient of an ICREA Academia fellowship (Generalitat de Catalunya). We thank Dr. Serge Campeau and Dr. Bruce McEwen for their comments on an earlier version of the manuscript.

BIBLIOGRAPHIC REFERENCES:

Armario, A.,2015. Studying chronic stress in animals: purposes, models and consequences, In: Neuroendocrinology of Stress, Masterclass in Neuroendocrinlogy Series. Editors J A Russell & M J Shipston. Wiley, New York.

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

Armario, A., Daviu, N., Muñoz-Abellán, C., Rabasa, C., Fuentes, S., Belda, X., Gagliano, H., 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, 749-758.

Armario, A., Hidalgo, J., Giralt, M., 1988. Evidence that the pituitary-adrenal axis does not cross-adapt to stressors: comparison to other physiological variables. Neuroendocrinology. 47, 263-267.

Armario, A., Martí, J., Gil, M., 1990. The serum glucose response to acute stress is sensitive to the intensity of the stressor and to habituation. Psychoneuroendocrinology. 15, 341-347.

Armario, A., Vallès, A., Dal-Zotto, S., Márquez, C; Belda, X., 2004. A single exposure to severe stressors causes long-term desensitisation of the physiological response to the homotypic stressor. Stress. 7, 157-172.

Barnum, C.J., Blandino, P., Jr Deak, T., 2007. Adaptation in the corticosterone and hyperthermic responses to stress following repeated stressor exposure. J. Neuroendocrinol. 19, 632-642.

Belda, X., Daviu, N., Nadal, R., Armario, A., 2012. Acute stress-induced sensitization of the pituitary-adrenal response to heterotypic stressors: independence of glucocorticoid release and activation of CRH1 receptors. Horm Beh. 62, 515-524.

Belda, X., Fuentes, S., Nadal, R., Armario, A., 2008. A single exposure to immobilization causes long-lasting pituitary-adrenal and behavioral sensitization to mild stressors. Horm Behav. 54, 654-661.

Belda, X., Márquez, C., 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.

Bhatnagar, S., Huber, R., Nowak, N., Trotter, P., 2002. Lesions of the posterior paraventricular thalamus block habituation of hypothalamic-pituitary-adrenal responses to repeated restraint. J. Neuroendocrinol. 14, 403-410.

Bhatnagar, S., Vining, C., Iyer, V., Kinni, V., 2006. Changes in hypothalamic-pituitary-adrenal function, body temperature, body weight and food intake with repeated social stress exposure in rats. J. Neuroendocrinol. 18, 13-24.

Bornstein, S.R., Engeland, W.C., Ehrhart-Bornstein, M., Herman, J.P., 2008. Dissociation of ACTH and glucocorticoids. Trends Endocrinol Metab. 19, 175-180.

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

Chen, X., Herbert, J., 1995. Regional changes in c-fos expression in the basal forebrain and brainstem during adaptation to repeated stress: correlations with cardiovascular, hypothermic and endocrine responses. Neuroscience. 64, 675-685.

Christianson, J.P., Benison, A.M., Jennings, J., Sandsmark, E.K., Amat, J., Kaufman, R.D., Baratta, M.V., Paul, E.D., Campeau, S., Watkins, L.R., Barth, D.S., Maier, S.F., 2008. The sensory insular cortex mediates the stress-buffering effects of safety signals but not behavioral control. J. Neurosci. 28, 13703-13711.

Christianson, J.P., Fernando, A.B., Kazama, A.M., Jovanovic, T., Ostroff, L.E., Sangha, S., 2012. Inhibition of fear by learned safety signals: a mini-symposium review. J. Neurosci. 32, 14118-14124.

Christianson, J.P., Jennings, J.H., Ragole, T., Flyer, J.G., Benison, A.M., Barth, D.S., Watkins, L.R., Maier, S.F., 2011. Safety signals mitigate the consequences of uncontrollable stress via a circuit involving the sensory insular cortex and bed nucleus of the stria terminalis. Biol Psychiatry. 70, 458-64.

Cole, M.A., Kalman, B.A., Pace, T.W., Topczewski, F., Lowrey, M.J., Spencer, R.L., 2000. Selective blockade of the mineralocorticoid receptor impairs hypothalamic-pituitary-adrenal axis expression of habituation. J. Neuroendocrinol. 12, 1034-1042.

Cristofferssen, G.R.J., 1997. Habituation: events in the history of its characterization and linkage to synaptic depression. A new proposed kinetic criterion for its identification. Prog. Neurobiol. 53, 45-66.

Cullinan, W.E., Herman, J.P., Battaglia, D.F., Akil, H., Watson, S.J., 1995. Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience. 64, 477-505

Dal-Zotto, S., Martí, O., Armario, A., 2002. Is repeated exposure to immobilization needed to induce adaptation of the hypothalamic-pituitary-adrenal axis? Influence of adrenal factors. Behav Brain Res. 129, 187-195.

Dal-Zotto, S., Martí, O., Armario, A., 2003. Glucocorticoids are involved in the long-term effects of a single immobilization stress on the hypothalamic-pituitary-adrenal axis. Psychoneuroendocrinology. 28, 992-1009.

Dal-Zotto, S., Martí, O., Delgado, R., Armario, A., 2004. Potentiation of glucocorticoid release does not modify the long-term effects of a single exposure to immobilization stress. Psychopharmacology (Berl). 177, 230-237.

Day, H.E., Masini, C.V., Campeau, S., 2009. Reversible inactivation of the auditory thalamus disrupts HPA axis habituation to repeated loud noise stress exposures. Brain Res. 1276, 123-130.

Dayas, C.V., Buller, K.M., Day, T.A., 1999. Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. Eur J Neurosci. 11, 2312-2322.

De Boer, S.F., Koopmans, S.J., Slangen, J.L., Van der Gugten, J., 1990. Plasma catecholamine, corticosterone and glucose responses to repeated stress in rats: effect of interstressor interval length. Physiol Behav. 47, 1117-1124.

Dumont, E. C., Kinkead, R., Trottier, J. F., Gosselin, I., Drolet, G., 2000. Effect of chronic psychogenic stress exposure on enkephalin neuronal activity and expression in the rat hypothalamic paraventricular nucleus. J. Neurochem. 75, 2200-2211.

Engeland, W.C., Miller, P., Gann, D.S., 1989. Dissociation between changes in plasma bioactive and immunoreactive adrenocorticotropin after hemorrhage in awake dogs. Endocrinology. 124, 2978-2985.

Elfering, A., Grebner, S., 2012. Getting used to academic public speaking: global self-esteem predicts habituation in blood pressure response to repeated thesis presentations. Appl Psychophysiol Biofeedback. 37, 109-120.

Fernandes, G.A., Perks, P., Cox, N.K., Lightman, S.L., Ingram, C.D., Shanks, N., 2002. Habituation and cross-sensitization of stress-induced hypothalamic-pituitary-adrenal activity: effect of lesions in the paraventricular nucleus of the thalamus or bed nuclei of the stria terminalis. J. Neuroendocrinol 14, 593-602.

Gagliano, H., Fuentes, S., Nadal, R., Armario, A., 2008. Previous exposure to immobilization and repeated exposure to a novel environment demonstrate a marked dissociation between behavioral and pituitary-adrenal responses. Behav Brain Res. 187, 239-245.

García, A., Martí, O., Vallès, A., Dal-Zotto, S., 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.

Girotti, M., Pace, T.W., Gaylord, R.I., Rubin, B.A., Herman, J.P., Spencer, R.L., 2006. Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. Neuroscience. 138, 1067-1081.

Gray, M., Innala, L., Viau, V., 2012. Central vasopressin V1A receptor blockade impedes hypothalamic-pituitary-adrenal habituation to repeated restraint stress exposure in adult male rats. Neuropsychopharmacology. 37, 2712-2719.

Grissom N., Bhatnagar S., 2009. Habituation to repeated stress: get used to it. Neurobiol Learn Mem. 92, 215-234.

Grissom, N.M., Bhatnagar, S., 2011. The basolateral amygdala regulates adaptation to stress via beta-adrenergic receptor-mediated reductions in phosphorylated extracellular signal-regulated kinase. Neuroscience. 178, 108-122.

Grissom, N., Iyer, V., Vining, C., Bhatnagar, S., 2007. The physical context of previous stress exposure modifies hypothalamic-pituitary-adrenal responses to a subsequent homotypic stress. Horm Behav. 51, 95-103.

Hardin, J.W., Hilbe, J.M., 2003. Generalized Estimating Equations. Chapman & Hall/CRC, Boca Raton.

Herman, J.P., 2013. Neural control of chronic stress adaptation. Front Behav Neurosci. 7, 61.

Herman, J.P., Figueiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C., Cullinan, W.E., 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol. 24, 151-180.

Hill, M.N., McLaughlin, R.J., Bingham, B., Shrestha, L., Lee, T.T., Gray, J.M., Hillard, C.J., Gorzalka, B.B., Viau, V., 2010. Endogenous cannabinoid signaling is essential for stress adaptation. Proc Natl Acad Sci U S A. 107, 9406-9411.

Hoffman, G.E., Lyo, D., 2002. Anatomical markers of activity in neuroendocrine systems: are we all 'fos-ed out'? J. Neuroendocrinol. 14, 259-268.

Houston, F.P., Stevenson, G.D., McNaughton, B.L., Barnes, C.A., 1999. Effects of age on the generalization and incubation of memory in the F344 rat. Learn Mem. 6, 111-119.

Jaferi, A., Bhatnagar, S., 2006. Corticosterone can act at the posterior paraventricular thalamus to inhibit hypothalamic-pituitary-adrenal activity in animals that habituate to repeated stress. Endocrinology. 147, 4917-4930.

Johnson, J.D., O'Connor, K.A., Deak, T., Stark, M., Watkins, L.R., Maier, S.F., 2002. Prior stressor exposure sensitizes LPS-induced cytokine production. Brain Behav Immun. 16, 461-476.

Jönsson, P., Wallergård, M., Osterberg, K., Hansen, A.M., Johansson, G., Karlson, B., 2010. Cardiovascular and cortisol reactivity and habituation to a virtual reality version of the Trier Social Stress Test: a pilot study. Psychoneuroendocrinology. 35, 1397-1403.

Kvetnansky, R., Sabban, E.L., Palkovits, M., 2009. Catecholaminergic systems in stress: structural and molecular genetic approaches. Physiol Rev. 89, 535-606.

Kwon, M-S., Seo, Y-J., Shim, E-J., Lee, J-K., Jang, J-E., Park, S-H., Jung, J-S., Suh, H-W., 2008. The differential effects of emotional or physical stress on pain behaviors or on c-Fos immunoreactivity in paraventricular nucleus or arcuate nucleus. Brain Res. 1190, 122-131.

Lattal, K.M., 1999. Trial and intertrial durations in Pavlovian conditioning: issues of learning and performance. J. Exp. Psychol.-Anim. Behav. Process. 25, 433-450.

Ma, X.M., Lightman, S.L., 1998. The arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats. J. Physiol. 510, 605-614.

Márquez, C., Belda, X., Armario, A., 2002. Post-stress recovery of pituitary-adrenal hormones and glucose, but not the response during exposure to the stressor, is a marker of stress intensity in highly stressful situations. Brain Res. 926, 181-185.

Márquez, C., Nadal, R., Armario, A., 2004. The hypothalamic-pituitary-adrenal and glucose responses to daily repeated immobilization stress in rats: individual differences. Neuroscience. 123, 601-612.

Martí, O., Armario, A., 1998. Anterior pituitary response to stress: time-related changes and adaptation. Int J. Dev Neurosci. 16, 241-260.

Martí, O., Garcia, A., Vallès, A., Harbuz, M.S., Armario, A., 2001. Evidence that a single exposure to aversive stimuli triggers long-lasting effects in the hypothalamus-pituitary-adrenal axis that consolidate with time. Eur. J. Neurosci. 13, 129-136.

Masini, C.V., Babb, J.A., Nyhuis, T.J., Day, H.E., Campeau, S., 2012. Auditory cortex lesions do not disrupt habituation of HPA axis responses to repeated noise stress. Brain Res. 1443, 18-26.

Masini, C.V., Day, H.E., Gray, T., Crema, L.M., Nyhuis, T.J., Babb, J.A., Campeau, S., 2012. Evidence for a lack of phasic inhibitory properties of habituated stressors on HPA axis responses in rats. Physiol Behav. 105, 568-575

Masini, C.V., Day, H.E.W., Campeau,S., 2008. Long-term habituation to repeated loud noise is impaired by relatively short interstressor intervals in rats. Behav Neurosci. 122, 210-223.

McGaugh, J.L., 2004. The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annu Rev Neurosci. 27,1-28.

Muñoz-Abellán, C., Rabasa, C., Daviu, N., Nadal, R., Armario, A., 2011 Behavioral and endocrine consequences of simultaneous exposure to two different stressors in rats: interaction or independence? PloS one. 6, e21426

Natelson, B. H., Ottenweller, J. E., Cook, J. A., Pitman, D., McCarty, R., Tapp, W. N., 1988 Effect of stressor intensity on habituation of the adrenocortical stress response. Physiol Behav. 43, 41-46.

Nyhuis, T.J., Sasse, S.K., Masini, C.V., Day, H.E., Campeau, S., 2010. Lack of contextual modulation of habituated neuroendocrine responses to repeated audiogenic stress. Behav Neurosci. 124, 810-820.

O'Connor, K.A., Johnson, J.D., Hammack, S.E., Brooks, L.M., Spencer, R.L., Watkins, L.R., Maier, S.F., 2003. Inescapable shock induces resistance to the effects of dexamethasone. Psychoneuroendocrinology. 28, 481-500.

Ons, S., Martí, O., Armario, A., 2004. Stress-induced activation of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) is restricted to telencephalic areas in the rat brain: relationship to c-fos mRNA. J. Neurochem 89, 1111-1118.

Ons, S., Rotllant, D., Marín-Blasco, I.J., Armario, A., 2010. Immediate early gene response to repeated immobilization: Fos protein and arc mRNA levels appears to be less sensitive than c-fos mRNA to adaptation. Eur. J. Neurosci. 31, 2043-2052.

Pacak, K., Palkovits, M., 2001. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. Endocr Rev. 22, 502-548.

Pace, T.W., Cole, M.A., Ward, G., Kalman, B.A., Spencer, R.L., 2001. Acute exposure to a novel stressor further reduces the habituated corticosterone response to restraint in rats. Stress. 4, 319-331.

Paxinos, G., Watson, C., 1998. The rat brain in stereotaxic coordinates. Academic Press, INC.

Pickens, C.L., Golden, S.A., Adams-Deutsch, T., Nair, S.G., Shaham, Y., 2009. Long-lasting incubation of conditioned fear in rats. Biol Psychiatry. 65, 881-886.

Rabasa, C., Delgado-Morales, R., Muñoz-Abellán, C., Nadal, R., Armario, A., 2011. Adaptation of the hypothalamic-pituitary-adrenal axis and glucose to repeated immobilization or restraint stress is not influenced by associative signals. Behav Brain Res. 217, 232-239.

Rabasa, C., Pastor-Ciurana, J., Delgado-Morales, R., Gómez-Román, A., Carrasco, J., Gagliano, H., García-Gutiérrez, M.S., Manzanares, J., Armario, C., 2015. Evidence against a critical role of CB1 receptors in adaptation of the hypothalamic-pituitary-adrenal axis and other consequences of daily repeated stress. Eur. Neuropsychopharmacol. *In press.*

Rankin, C.H., Abrams, T., Barry, R.J., Bhatnagar, S., Clayton, D.F., Colombo, J., Coppola, G., Geyer, M.A., Glanzman, D.L., Marsland S., McSweeney, .FK., Wilson, D.A., Wu, C.F., Thompson, R.F., 2009. Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. Neurobiol Learn Mem. 92, 135-138.

Rescorla, R.A., 1988. Behavioral studies of Pavlovian conditioning. Annu Rev Neurosci. 11, 329-352.

Rivier, C., Vale, W., 1987. Diminished responsiveness of the hypothalamic-pituitary-adrenal axis of the rat during exposure to prolonged stress: a pituitary-mediated mechanism. Endocrinology. 121, 1320-1328.

Sanderson, D.J., Bannerman, D.M., 2011. Competitive short-term and long-term memory processes in spatial habituation. J. Exp Psychol Anim B. 37, 189-199.

Schmidt, A., Hödl, S., Möstl, E., Aurich, J., Müller, J., Aurich, C., 2010. Cortisol release, heart rate, and heart rate variability in transport-naive horses during repeated road transport. Domest Anim Endocrinol. 39, 205-213.

Schommer, N.C., Hellhammer, D.H., Kirschbaum, C., 2003. Dissociation between reactivity of the hypothalamus-pituitary-adrenal axis and the sympathetic-adrenal-medullary system to repeated psychosocial stress. Psychosom Med. 65, 450-460

Siegmund, A., Wotjak, C.T., 2006. Toward an animal model of posttraumatic stress disorder. Ann N Y Acad Sci. 1071, 324-334.

Thompson, R.F., 2009. Habituation: a history. Neurobiol Learn Mem. 92, 127-134.

Vahl, T.P., Ulrich-Lai, Y.M., Ostrander, M.M., Dolgas, C.M., Elfers, E.E., Seeley, R.J., D'Alessio, D.A., Herman, J.P., 2005. Comparative analysis of ACTH and corticosterone sampling methods in rats. Am J. Physiol Endocrinol Metab. 289, 823-8.

Vallès, A., Martí, O., García, A., Armario, A., 2000. Single exposure to stressors causes long-lasting, stress-dependent reduction of food intake in rats. Am J. Physiol Regul Integr Comp Physiol. 279, 1138-1144.

Vallès, A., Martí, O., Armario, A., 2003. Long-term effects of a single exposure to immobilization stress on the hypothalamic-pituitary-adrenal axis: transcriptional evidence for a progressive desensitization process. Eur. J. Neurosci. 18, 1353-1361.

Vallès, A., Martí, O., Armario, A., 2006. Long-term effects of a single exposure to immobilization: a c-fos mRNA study of the response to the homotypic stressor in the rat brain. J. Neurobiol. 66, 591-602.

Von Känel, R., Kudielka, B.M., Preckel, D., Hanebuth, D., Fischer, J.E., 2006. Delayed response and lack of habituation in plasma interleukin-6 to acute mental stress in men. Brain Behav Immun. 20, 40-48.

Weinberg, M.S., Bhatt, A.P., Girotti, M., Masini, C.V., Day, H.E., Campeau, S., Spencer, R.L., 2009. Repeated ferret odor exposure induces different temporal patterns of same-stressor habituation and novel-stressor sensitization in both hypothalamic-pituitary-adrenal axis activity and forebrain c-fos expression in the rat. Endocrinology. 150, 749-761.

Weinberg, M.S., Johnson, D.C., Bhatt, A.P., Spencer, R.L., 2010. Medial prefrontal cortex activity can disrupt the expression of stress response habituation. Neuroscience 168, 744-756.
Wong, Y.N., Cassano, W.J.,Jr, D'mello, A.P., 2000. Acute-stress-induced facilitation of the hypothalamic-pituitary-adrenal axis. Neuroendocrinology. 71, 354-365.

Zelena, D., Mergl, Z., Foldes, A., Kovacs, K.J., Toth, Z., Makara, G.B., 2003. Role of hypothalamic inputs in maintaining pituitary-adrenal responsiveness in repeated restraint. Am J. Physiol Endocrinol Metab. 285, 1110-7.

Zhang, W., Hetzel, A., Shah, B., Atchley, D., Blume, S.R., Padival, M.A., Rosenkranz, J.A., 2014. Greater Physiological and Behavioral Effects of Interrupted Stress Pattern Compared to Daily Restraint Stress in Rats. PLoS One 9, e102247.

Caption for figures:

Fig 1: Plasma levels of ACTH (A) and corticosterone (B) in rats daily exposed to chronic IMO for 1 h (black bars) and stress-naïve rats exposed to IMO for the first time on day 7 (white bars). Mean and S.E.M are represented. Animals (n=12 for each group) were sampled on days 1, 2 and 7, immediately after 1h of IMO and again at 45 and 90 min after its termination (R45 and R90). Chronic IMO group on day 1 did not differ from the acute IMO group. The analysis of the response over the days in the chronic IMO group revealed significant effects for day [Wald X2 (2) = 37. 4], sampling time [Wald X2 (2) = 185.1] and the interaction day x sampling time [Wald X2 (4) = 44.1] regarding ACTH, and day [Wald X2 (2) = 44.4], sampling time [Wald X2 (2) = 559.1] and the interaction day x sampling time [Wald X2 (4) = 575.7] regarding corticosterone (always $p<0.001$). Further appropriate comparisons are seen in the Figure. The magnitude of adaptation of the ACTH response was similar on days 2 and 7, suggesting that one single IMO is enough to induce an almost maximum adaptation. *Significance versus the same group at the same sampling time on day 1; + significance versus the same group at the same sampling time on day 2. One symbol $p<0.05$, 2 symbols $p<0.01$ and 3 symbols $p<0.001$.

Fig 2: C-fos mRNA levels in the medial dorsal parvocellular subdivision of the paraventricular hypothalamic nucleus (mpdPVN), prelimbic (PrL) and infralimbic (IL) regions of the medial prefrontal cortex, posterior part of the paraventricular thalamic nucleus (pPVTh), medial amygdala (MeA), and basolateral amygdala (BLA). Mean and S.E.M are represented (n=8 for each group) of the semi-quantitative measurements expressed in arbitrary units (AU). The groups are as follows: CONTROL-IMO, only exposed to IMO on day 12; IMO d1-IMO, exposed to IMO on days 1 and 12; IMO d11, exposed to IMO on days 11 and 12; Chronic IMO-IMO, exposed to IMO daily for 12 days. Differences between groups were observed in all areas analyzed (at least $p<0.05$): mpdPVN $X^2(3) = 33.8$, PrL $X^2(3) = 10.5$, IL $X^2(3) = 15.3$, pPVTh $X^2(3) = 28.1$, MeA $X^2(3) = 89.4$, and BLA $X^2(3) = 47.8$. Groups labelled with different letters are statistically different (at least $p <0.05$ after sequential Bonferroni post-hoc comparisons). These comparisons revealed a marked reduction of c-fos expression in all brain areas analyzed after chronic IMO. Animals previously exposed to a single IMO 1 or 11 days before the last exposure did not reach a significant adaptation in the medial prefrontal cortex (2B and 2C), whereas they showed a partial reduction in the mpdPVN (2A), and a maximum reduction in the MeA and BLA (2E and 2F, respectively). The c-fos response in the pPVTh (2D)

was reduced in the rats exposed to IMO on two consecutive days and chronically, but the effect was not observed in rats exposed to IMO 11 days before the last exposure.

Fig 3: Previous experience with IMO reduced HPA response to an acute challenge with the same stressor, shortly or two months later. Means and S.E.M are represented (n=8 for each group). Acute IMO indicates exposure to the stressor the last day; prior single IMO, exposure to the stressor on day 12 and again on day 72; chronic IMO, daily exposure to IMO from day 1 to 12 and again on day 72. Animals were sampled immediately after 1h of IMO and again at 30, 60 and 90 min after its termination on days 12 and 72 (IMO, R30, R60, R90). On day 12, an additional sample before stress was taken. The analysis of day 12 data (A, B) revealed significant effects for group [Wald $X^2(1) = 38.4$ for ACTH, and Wald $X^2(1) = 48.6$ for corticosterone, both $p<0.001$], sampling time [Wald $X^2(3) = 120.3$ for ACTH and Wald $X^2(3) = 53.4$ for corticosterone, both $p<0.001$], and the interaction group x sampling time [Wald $X^2(3) = 67.5$ for ACTH and Wald $X^2(3) = 31.3$ for corticosterone, both $p<0.001$]. Chronic IMO rats clearly adapted to the stressor as observed in the post-IMO period. The analysis of HPA response to IMO on day 72 (C,D) in animals having single or repeated (chronic) experience with IMO two months before revealed effect for group [Wald $X^2(2) = 8.1$ for ACTH and Wald $X^2(2) = 7.5$ for corticosterone, both $p<0.05$], sampling time [Wald $X^2(3) = 114.9$ for ACTH and Wald $X^2(3) = 35.5$ for corticosterone, both $p<0,001$], and the interaction group x sampling time [Wald $X^2(6) = 31.0$, $p<0.001$ for ACTH and Wald $X^2(6) = 20.7$, $p< 0.01$, for corticosterone]. Significant adaptation was observed in single and chronic IMO groups, demonstrating an extremely long-lasting adaptation. * Significance versus acute IMO group; & Significance versus prior single IMO group (always at the same sampling time). One symbol $p<0.05$, 2 symbols $p<0.01$ and 3 symbols $p<0.001$.

Fig 4: Plasma levels of ACTH (A,C) and corticosterone (B,D) in rats daily exposed to two different stressors: IMO (black bars) or REST (grey bars). Samples were taken on days 1, 2, 3, 6 and 12, immediately after 1 h of stress and at 30 and 60 min after the termination of IMO/REST (R30 and R60). The inserts on the right represent the area under the curve (AUC) for plasma levels of ACTH (A',C') and corticosterone (B',D'). Means and S.E.M are represented (n=8-10 for each group). The analysis of ACTH revealed effects for stressor [Wald $X^2(1) = 41.7$], day [Wald $X^2(4) = 83.3$], sampling time [Wald $X^2(2) = 252.5$], and the interaction stressor x day x sampling time [Wald $X^2(8) = 89.1$]; in all cases $p<0.001$. The analysis of corticosterone revealed significant effect for stressor [Wald $X^2(1) = 28.56$], day [Wald $X^2(4) =$

48.84] and sampling time [Wald $X^2(2) = 320.8$], as well as for the interaction stressor x day x sampling time [Wald $X^2(8) = 140.1$]; in all cases $p<0.001$. Animals exposed to IMO adapted faster and stronger than those exposed to REST. The same pattern was observed with AUCs with significant stressor x day interaction for ACTH and corticosterone [Wald $X^2(4) = 69.7$, $p<0.001$ and Wald $X^2(4) = 26.3$, $p<0.001$ respectively]. Significance always refers to the corresponding sampling time, where appropriate.* versus the corresponding sampling time on day 1; + versus the corresponding sampling time on day 2. One symbol $p<0.05$, 2 symbols $p<0.01$ and 3 symbols $p<0.001$.

Fig 5: Plasma levels of ACTH in animals exposed to IMO or water stress (WS) with different inter-stress intervals (ISI): 24 h (A, C, E and G) or 72 h (B, D, F and H). Means and S.E.M are represented (n=10 for each group). Samples were obtained on days 1, 2, 3, 4 and 5 (24 h ISI) or on days 1, 4, 7, 10 and 13 (72 h ISI), immediately after 20 min of stress and at 30 min after its termination. Analysis of ACTH from IMO groups (A and B) showed significant effects for day [Wald $X^2(4) = 109.4$, $p<0.001$], sampling time [Wald $X^2(1) = 289.7$, $p<0.001$], and the interaction ISI x day x sampling time [Wald $X^2(4) = 11.5$; $p<0.05$]. Analysis of corticosterone response of IMO groups (C and D) showed significant effect for sampling time [Wald $X^2(1) = 14.6$, $p<0.001$] and the interaction day x sampling time [Wald $X^2(4) = 26.9$, $p<0.001$]. Analysis of ACTH response to WS (E,F) showed results similar to those observed with IMO: significant effect for day [Wald $X^2(4) = 48.6$], sampling time [Wald $X^2(1) = 84.3$], and the interaction ISI x day x sampling time [Wald $X^2(4) = 37.8$]; in all cases $p<0.001$. Regarding corticosterone response to WS (G,H), rats exposed to WS showed significant effects for day [Wald $X^2(4) = 106.8$; $p<0.001$], sampling time [Wald $X^2(1) = 62.5$; $p<0.001$], ISI [Wald $X^2(1) = 9.4$; $p<0.01$] and the interaction ISI x day x sampling time [Wald $X^2(4) = 24.5$; $p<0.05$]. The results suggest that longer ISI is associated with better ACTH adaptation to both repeated IMO and repeated WS. Animals exposed to the 72 h ISI achieved faster a significant decline of the ACTH response to the stressors, particularly evident just after the stressors. Repeated exposure to IMO did not affect corticosterone response, but WS did in the post-stress period, with the WS-72h groups showing a faster reduction of the response after repeated exposure than the WS-24h group. * Significance versus the same group and sampling time on day 1; + Significance versus the same group and sampling time on day 2. One symbol: $p<0.05$, two symbols: $p<0.01$ and three symbols $p<0.001$.

Fig 6: Plasma levels of ACTH (A, C) and corticosterone (B, D) in animals repeatedly exposed to 1 h or 3 h of IMO for seven days. Means and S.E.M are represented (n=8-10 for each group). Samples were obtained on days 1, 2 and 7. In those animals exposed to IMO for 1 h, samples were obtained immediately after 1 h of IMO (1H) and at 45 min after the termination of stress (R45), while in those exposed to IMO for 3 h, samples were obtained at 1 h from the beginning of IMO (1H), at the end of IMO (3H) and at R45. The analysis of the response over the days of the Chronic IMO-1H group showed, for ACTH (A), significant effects for day [Wald $X^2(2) = 14.1$], sampling time [Wald $X^2(1) = 524.7$] and the interaction day x sampling time [Wald $X^2(2) = 22.3$], and for corticosterone (B), significant effect for day [Wald $X^2(2) = 50.2$], sampling time [Wald $X^2(1) = 169.1$] and the interaction day x sampling time [Wald $X^2(2) = 47.9$]. The analysis of the response over the days of the Chronic IMO-3H group revealed, for ACTH (C) significant effect for day [Wald $X^2(2) = 398.6$], sampling time [Wald $X^2(2) = 978.0$] and the interaction day x sampling time [Wald $X^2(4) = 13.2$], and for corticosterone (D), significant effect for day [Wald $X^2(2) = 59.4$], sampling time [Wald $X^2(2) = 317.7$] and the interaction day x sampling time [Wald $X^2(4) = 300.7$]. In all above effects p was always < 0.001. Further comparisons showed that repeated exposure to 1 h IMO only affected ACTH and corticosterone during the post-IMO period, whereas after repeated exposure to 3 h IMO, the ACTH response was already reduced during exposure to the stressor. * Significance versus the same group at the corresponding sampling time on D1; + Significance versus the same group at the corresponding sampling time on D2. One symbol $p<0.05$, 2 symbols $p<0.01$ and 3 symbols $p<0.001$.

Fig 7: Effects of prior exposure to 30 min or 4 h of IMO on the day before on plasma ACTH and corticosterone response to 15 min open-field (OF) exposure. Control rats were not stressed on the preceding day. Means and S.E.M. are represented (n=10 for each group). Statistical analysis of hormonal responses to the OF showed significant group effect for both ACTH [Wald $X^2(2) = 15.95$; $p<0.001$] and corticosterone [Wald $X^2(2) = 30.80$; $p<0.001$]. Further comparisons showed similarly enhanced hormonal response to the OF in the two IMO groups as compared to controls. **$p<0.01$ and ***$p<0.001$ versus controls.

Table 1: Reduction of the area under the curve (AUC) of ACTH and corticosterone response to repeated stress.

Means and SEM (n= 10) of the absolute and relative (percent) reduction of the ACTH and corticosterone response after repeated exposure to 20 min of immobilization (IMO) or water stress (WS) with inter-stress intervals (ISI) of 24 or 72 h. Differences between day 1 (D1) and day 5 (D5) were calculated. The results were analyzed by GLzM including two between-subjects factors: type of stressor and ISI. * Refers to differences between ISI 24h vs ISI 72h, and Δ refers to differences between rats exposed to WS and IMO. One symbol $p<0.05$, 2 symbols $p<0.01$ and 3 symbols $p<0.001$.

Table(s)

	ACTH		Corticosterone	
	Absolute reduction	Relative reduction (%)	Absolute reduction	Relative reduction (%)
	** /Δ Δ Δ	***	Δ Δ Δ	* / Δ Δ Δ
IMO 24 h	6833 ± 1511	26.0 ± 6.7	-209 ± 532	-5.8 ± 7.6
IMO 72 h	12695 ± 1442	49.3 ± 3.6	827 ± 431	10.5 ± 6.0
WS 24 h	3813 ± 1131	28.4 ± 7.5	1518 ± 323	26.8 ± 7.7
Ws 72 h	5694 ± 922	47.5 ± 9.0	1779 ± 216	40.3 ± 6,0

Figure 1

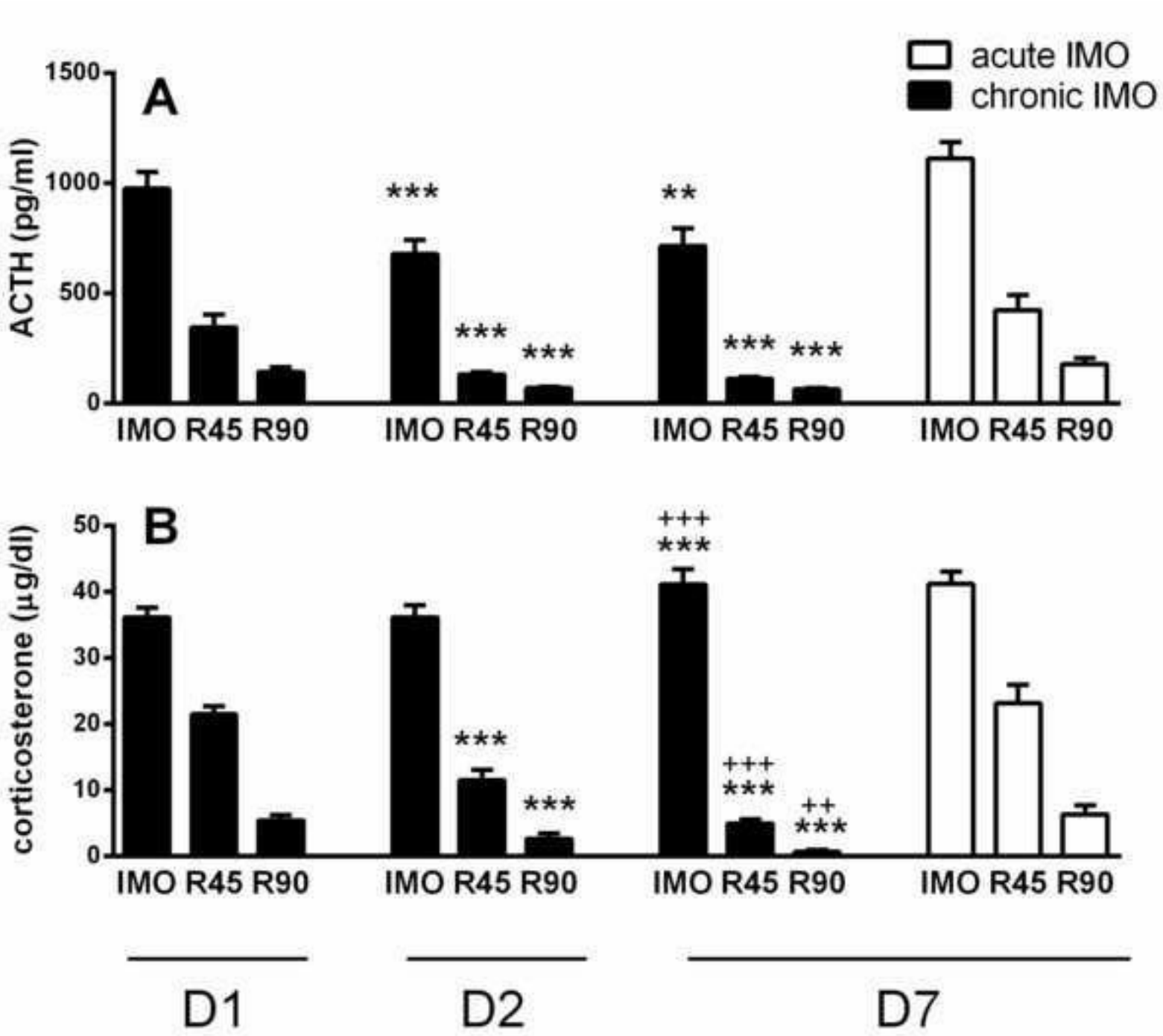

acute IMO
chronic IMO
A
ACTH (pg/ml)
1500
1000
500
0
***
***
***
**
***
***
IMO R45 R90
IMO R45 R90
IMO R45 R90
IMO R45 R90
B
corticosterone (µg/dl)
50
40
30
20
10
0
***
***
+++
***
+++
***
++
***
IMO R45 R90
IMO R45 R90
IMO R45 R90
IMO R45 R90
D1
D2
D7

Figure 2

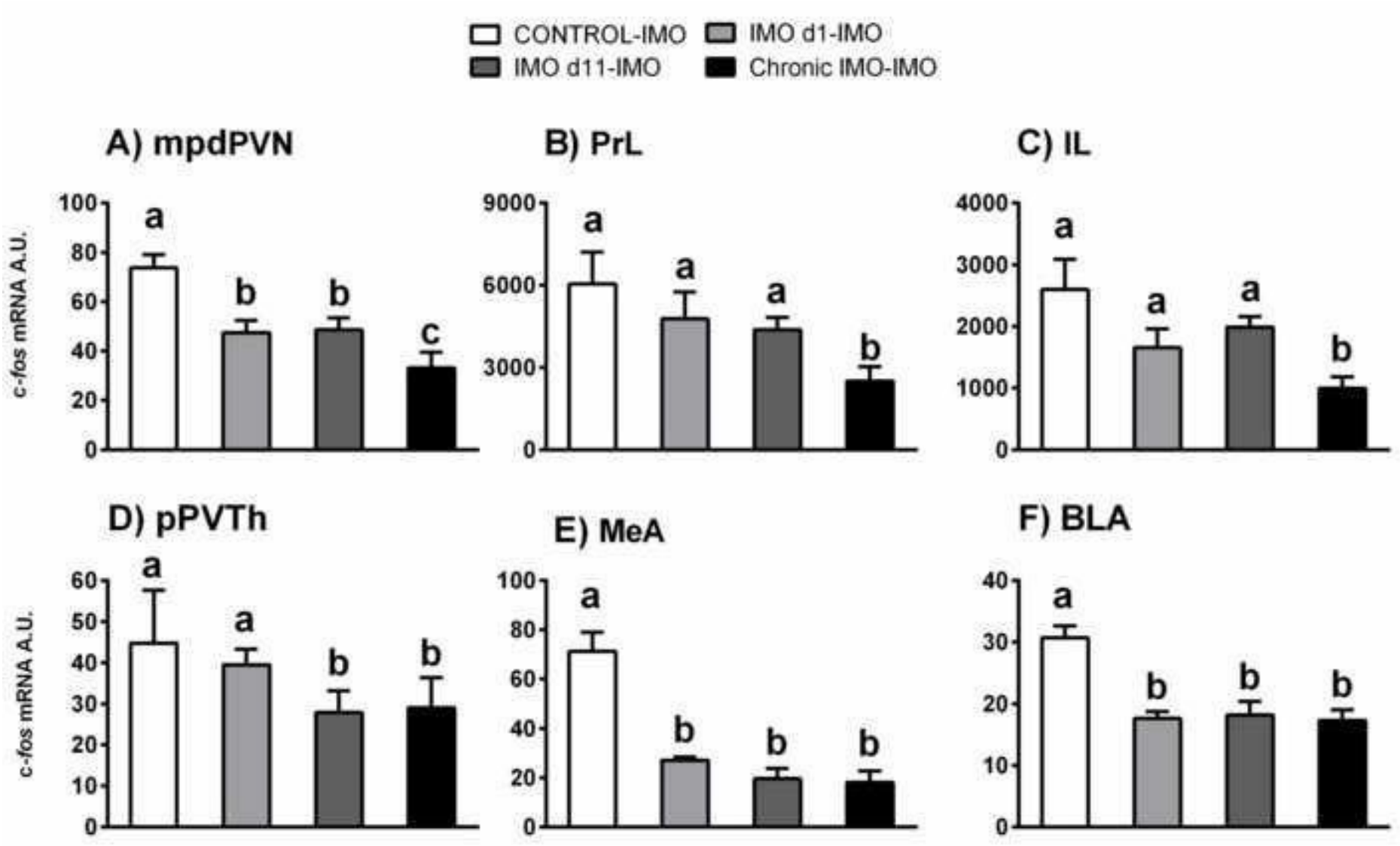

CONTROL-IMO
IMO d1-IMO
IMO d11-IMO
Chronic IMO-IMO
A) mpdPVN
B) PrL
C) IL
D) pPVTh
E) MeA
F) BLA
c-fos mRNA A.U.
c-fos mRNA A.U.
a
b
b
c
a
a
a
b
a
a
a
b
a
a
b
b
a
b
b
b
a
b
b
b

Figure 3

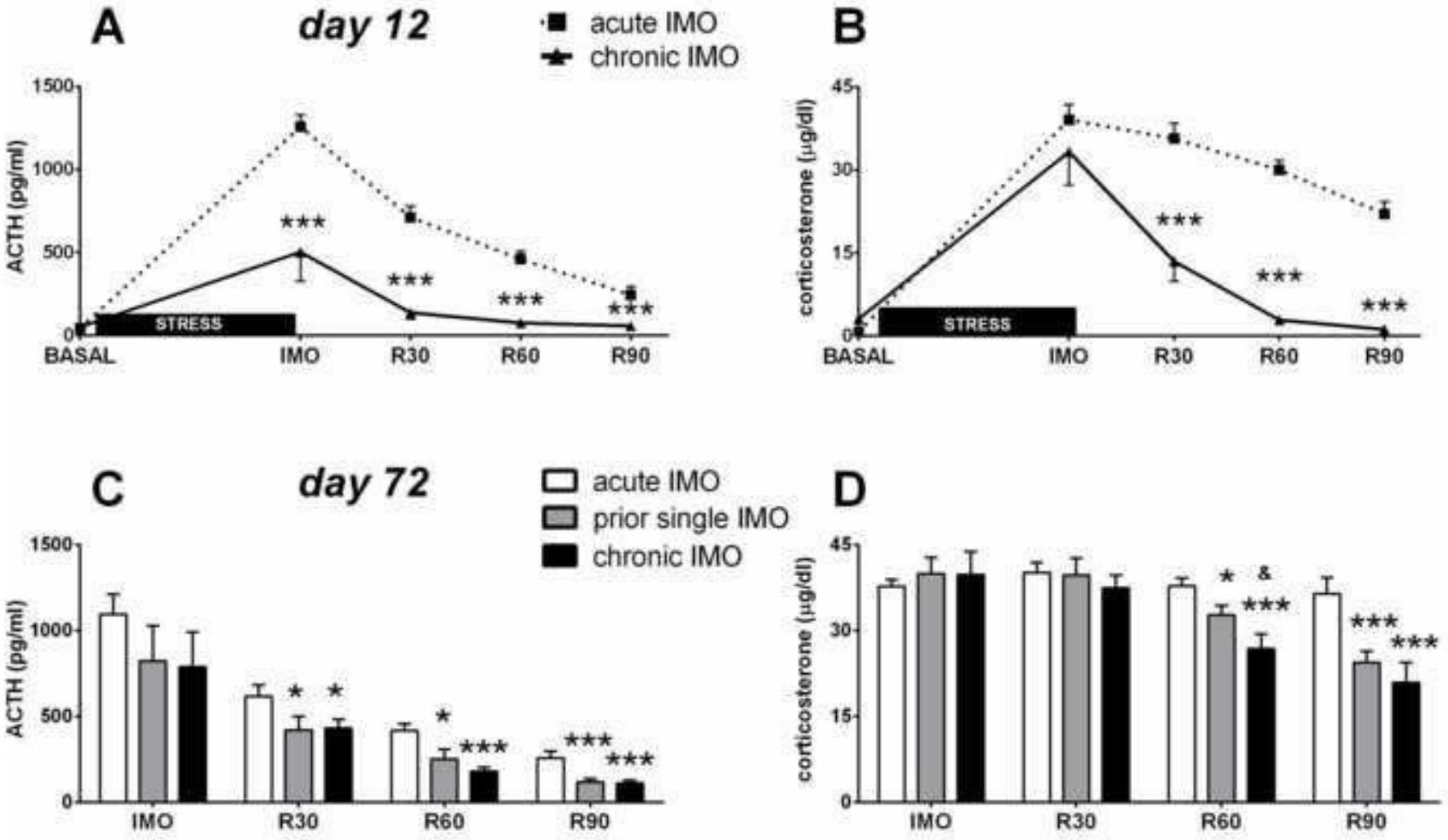

A
day 12
acute IMO
chronic IMO
ACTH (pg/ml)
1500
1000
500
0
STRESS
***
BASAL
IMO
R30
R60
R90
B
corticosterone (µg/dl)
45
30
15
0
C
day 72
acute IMO
prior single IMO
chronic IMO
D
&

Figure 4

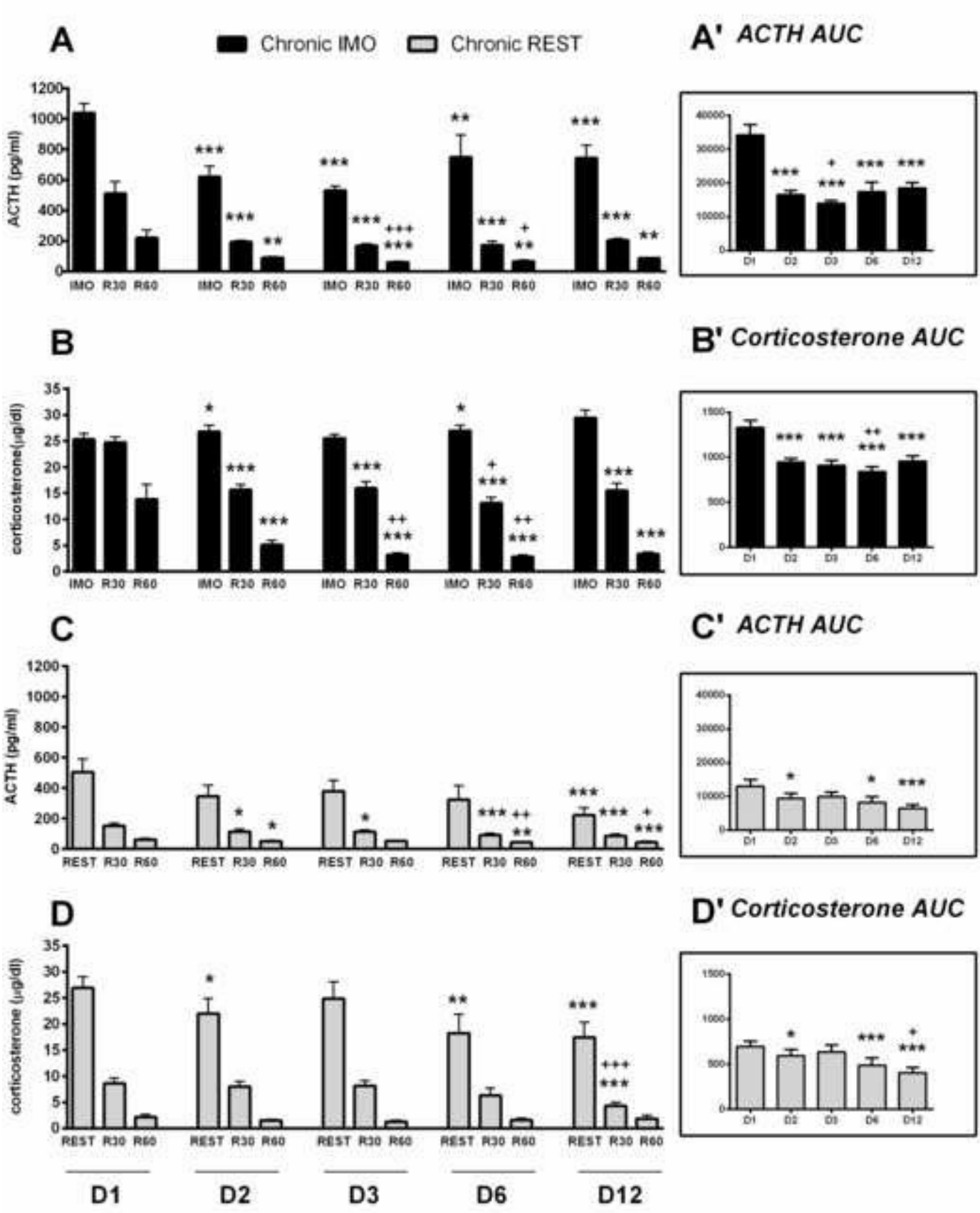

A
Chronic IMO
Chronic REST
A' ACTH AUC
ACTH (pg/ml)
IMO R30 R60
B
B' Corticosterone AUC
corticosterone(µg/dl)
C
C' ACTH AUC
REST R30 R60
D
D' Corticosterone AUC
corticosterone (µg/dl)
D1
D2
D3
D6
D12

Figure 5

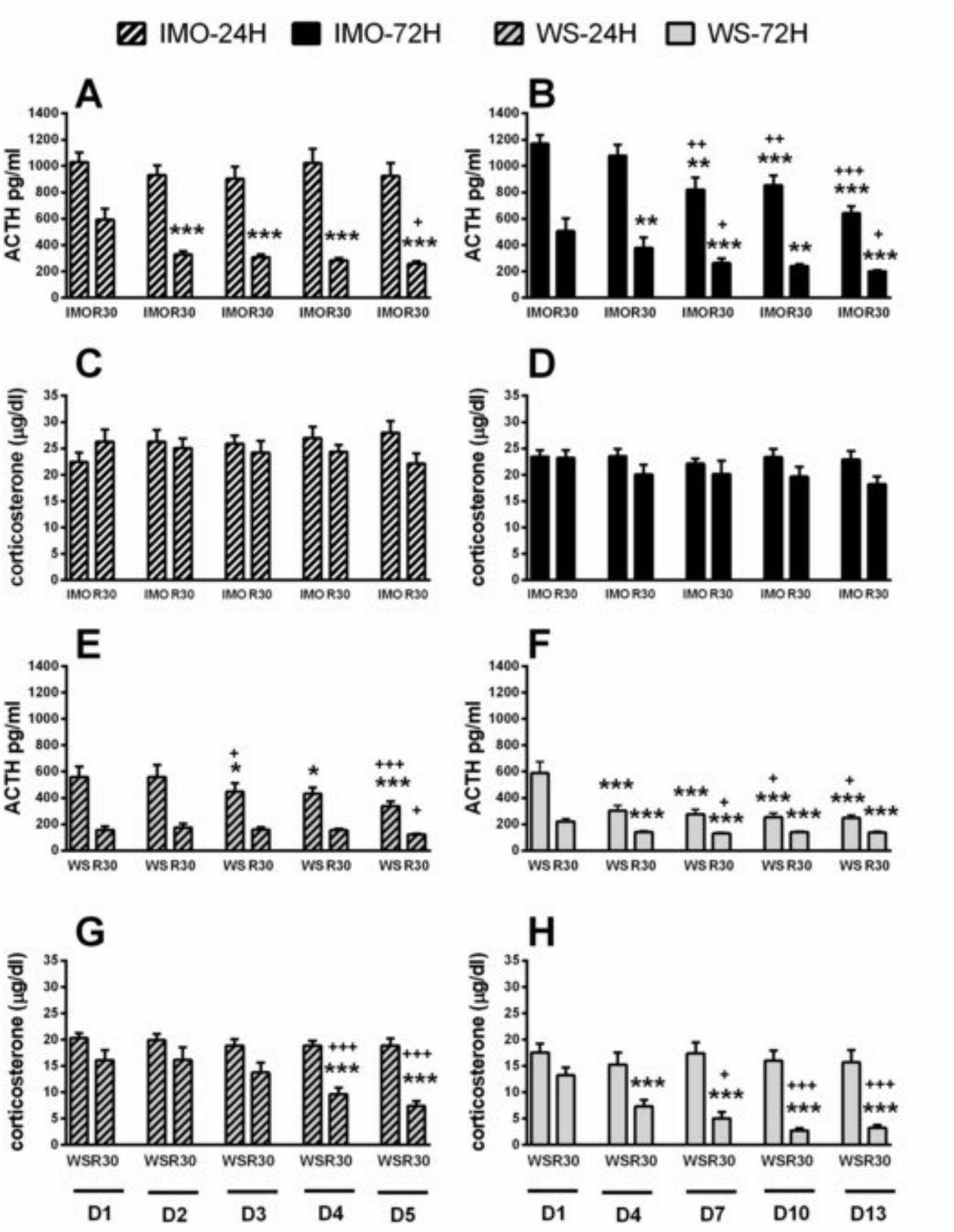

IMO-24H
IMO-72H
WS-24H
WS-72H
A
B
C
D
E
F
G
H
ACTH pg/ml
corticosterone (µg/dl)
IMOR30
IMO R30
WS R30
WSR30
D1
D2
D3
D4
D5
D7
D10
D13

Figure 6

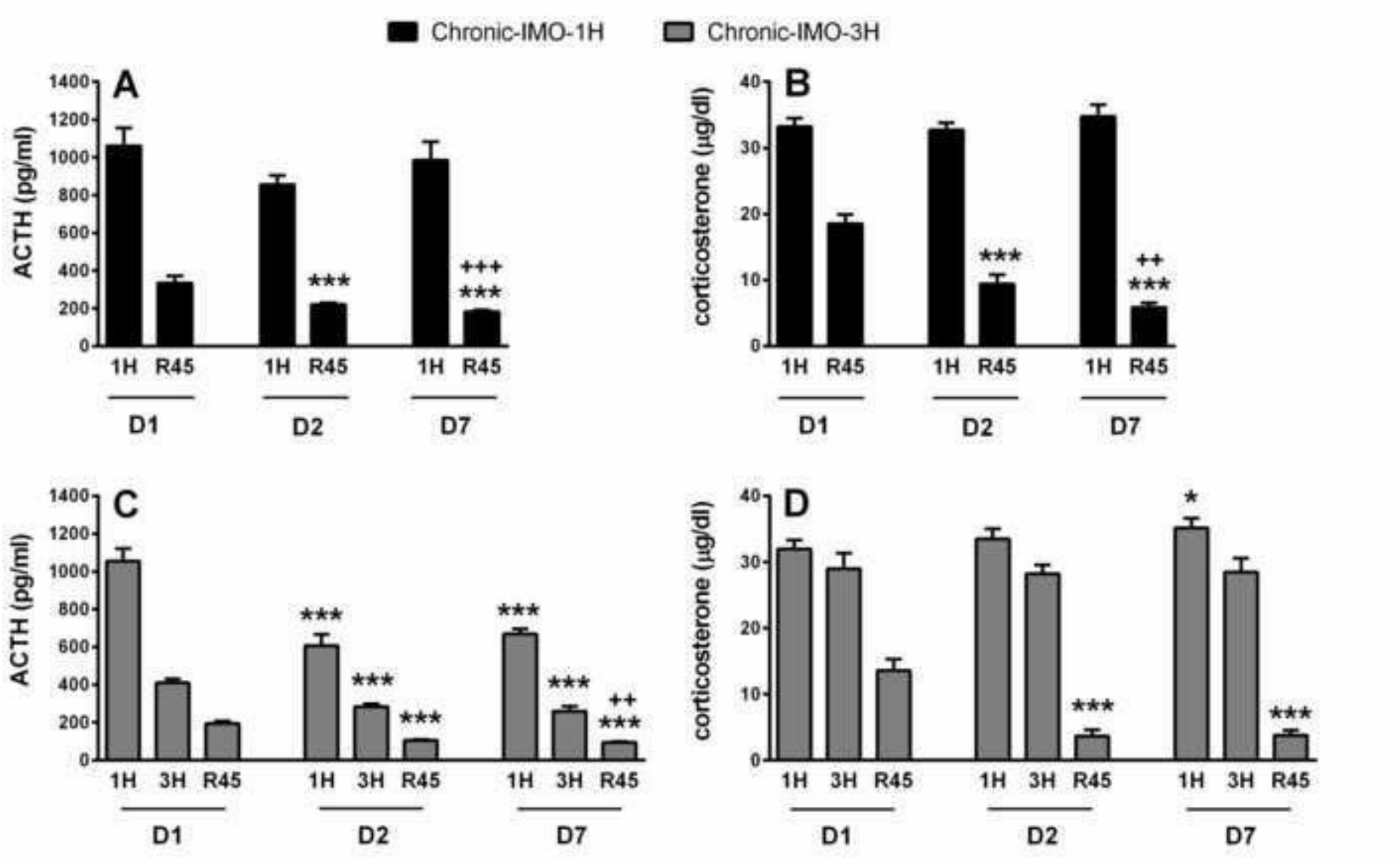
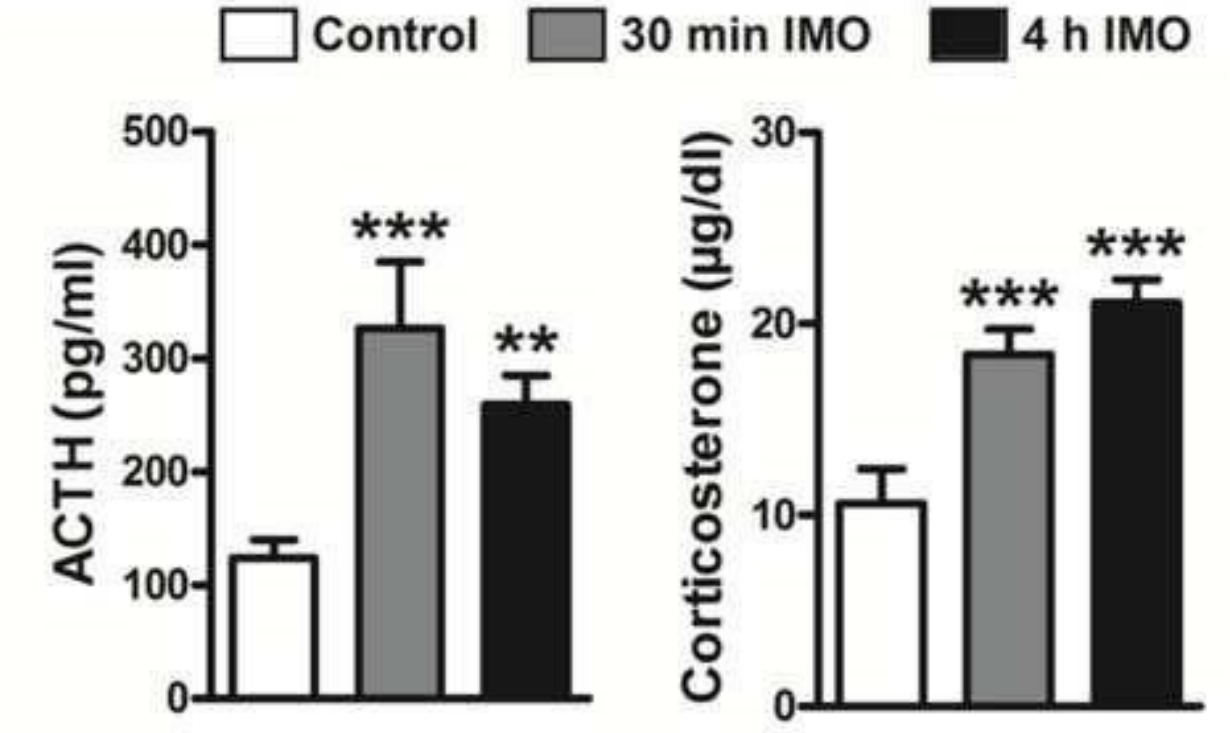

Chronic-IMO-1H
Chronic-IMO-3H
A
ACTH (pg/ml)
1400
1200
1000
800
600
400
200
0
1H R45 1H R45 1H R45
D1 D2 D7
***
+++
***
B
corticosterone (µg/dl)
40
30
20
10
0
1H R45 1H R45 1H R45
D1 D2 D7
***
++
***
C
ACTH (pg/ml)
1400
1200
1000
800
600
400
200
0
1H 3H R45 1H 3H R45 1H 3H R45
D1 D2 D7
***
***
***
***
***
++
***
D
corticosterone (µg/dl)
40
30
20
10
0
1H 3H R45 1H 3H R45 1H 3H R45
D1 D2 D7
*
***
***

Figure 7


Control
30 min IMO
4 h IMO
ACTH (pg/ml)
500
400
300
200
100
0
***
**
Corticosterone (µg/dl)
30
20
10
0
***
***