DIFFERENTIAL EFFECTS OF ENVIRONMENTAL ENRICHMENT AND ISOLATION HOUSING ON THE HORMONAL AND NEUROCHEMICAL RESPONSES TO STRESS IN THE PREFRONTAL CORTEX OF THE ADULT RAT: RELATIONSHIP TO WORKING AND EMOTIONAL MEMORIES

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ABREVIATIONS:

EC: environmental enrichment conditions.

GRs: Glucocorticoid receptors.

IC: impoverished conditions.

n.s.: non significant.

PFC: Prefrontal cortex.

ABSTRACT

The present study was designed to investigate the modulation of the stress responses by the environmental conditions and its putative neurobiological mechanisms. For that an integrative study on the effects of environmental enrichment and isolation housing on 1/ the corticosterone, dopamine and acetylcholine responses to acute restraint stress in the prefrontal cortex (PFC) of the awake rat; 2/ the mRNA levels of glucocorticoid receptors (GRs) in the PFC; and 3/ the behavioral responses to stress, related to the PFC (habituation to a novel environment, spatial-working memory and inhibitory avoidance response) was performed. Male Wistar rats were maintained from 3 to 6 months of age in two different conditions: enriched (EC) or impoverished (IC). Animals were stereotaxically implanted with bilateral guide cannulae in the PFC to perform microdialysis experiments to evaluate the concentrations of corticosterone, dopamine and acetylcholine. EC animals showed lower increases of corticosterone and dopamine but not of acetylcholine than IC animals in the PFC in response to acute restraint stress (20min). In the PFC, GR mRNA levels showed a trend towards an enhancement in EC animals. EC reduced the days to learn the spatial working memory task (radial-water maze). Spatial working memory, however, was not different between groups in either basal or stress conditions. Inhibitory avoidance response was reduced in EC rats. The changes produced by EC in the neurochemical, neuroendocrine and behavioral parameters evaluated suggest that EC rats could show a better coping during an acute stress challenge.

Keywords: corticosterone, dopamine, acetylcholine, prefrontal cortex, spatial working memory, inhibitory avoidance.

1. INTRODUCTION

Stress activates a set of endocrine, neurochemical, and behavioral responses aimed to restore the actual or potentially threatened homeostasis (Bruce S 2000; Sapolsky et al. 2000). These stress responses can be modulated by environmental conditions (Szyf et al. 2008). Animals reared in environmental enrichment conditions (EC) show a lower reactivity to different stressors compared to standard or isolation conditions (Fox et al. 2006; Segovia et al. 2009). In fact, EC animals show lower anxiety levels (Fernández-Teruel et al. 1997; Sztainberg et al. 2010), a faster habituation to a novel environment (Zimmermann et al. 2001; Schrijver et al. 2002; Segovia et al. 2008a) and a better recovery from psychosocial stress (Schloesser et al. 2010; Lehmann and Herkenham 2011). These behavioral effects of EC are thought to be mediated, at least in part, by a lower release of plasma corticosterone under stress conditions (Mlynarik et al. 2004; Moncek et al. 2004). In the rat, acute stress leads to the release of corticosterone from the adrenal cortex through the activation of the hypothalamo-pituitary-adrenal (HPA) axis (Antoni 1986). Corticosterone can bind to glucocorticoid receptors (GRs), which are expressed in several areas of the brain (Meaney and Aitken 1985; Reul and Kloet 1985; McEwen et al. 1986). Among those brain areas is the prefrontal cortex (PFC), where corticosterone modulates the activity of the HPA axis (Jankord and Herman 2008) and also influences working and emotional memories (Lupien et al. 2007; Roozendaal et al. 2009b; Barsegyan et al. 2010). Since several studies have shown that brain levels of corticosterone may not mirror plasma levels (Lengvári and Liposits 1977; Croft et al. 2008; Droste et al. 2009; Garrido et al. 2012a), it is not known whether EC also reduces the increases of corticosterone in PFC during stress. Enriched animals also show enhanced levels of GRs in the hippocampus (Olsson et al. 1994) but their levels have not been evaluated in the PFC. Measuring corticosterone levels and

GRs in PFC could help to assign a role to corticosterone on the behavioral effects of EC.

The PFC is thought to integrate the information about the stressor, thus coordinating neurochemical, hormonal and behavioral responses aimed to cope with a stressful situation (Sullivan 2004; Herman et al. 2005; Robbins 2005). Different acute stressors increase the release of dopamine and acetylcholine in the PFC (Thierry et al. 1976; Abercrombie et al. 1989; Mark et al. 1996; Del Arco et al. 2007). These neurotransmitters play a role in modulating working memory and attention (Sarter and Bruno 1997; Williams and Castner 2006), which have been suggested to be relevant to cope with stress (Sarter and Bruno 1997; Hains and Arnsten 2008). Animals reared in EC show a reduced response to acute handling (a mild stress challenge) of the dopaminergic but not the cholinergic systems in the PFC (Del Arco et al. 2007; Segovia et al. 2008; Segovia et al. 2008). It is not known whether these differences are observed under more intense stress protocols, such as restraint, which produces a more reliable increase of brain free corticosterone compared to handling (Croft et al. 2008; Garrido et al. 2012a). Whether the effects of EC are dependent on the intensity of the stressor would be relevant to ascertain the role of dopamine and acetylcholine in the optimisation of cortical circuits that are necessary for copying behaviors (Robbins 2005; Segovia et al. 2009; Mora et al. 2012).

The effects of EC on the stress-induced increases of corticosterone, dopamine and acetylcholine in the PFC could lead to differences in behavioral parameters modulated by them in this brain area, such as working memory and consolidation of aversive memories (Goldman-Rakic 1995; Williams and Castner 2006; Roozendaal et al. 2009a; Barsegyan et al. 2010). More specifically, elevated dopamine levels in response to stress

in the PFC are thought to be involved in the impairment in working memory produced by acute stress (Goldman-Rakic 1995; Williams and Castner 2006). In a previous work of our laboratory no effects of EC were found in the performance of a working memory task in a T-maze both in basal and under acute stress conditions (Segovia et al. 2008a). However, an enhanced complexity of the task (i.e.: increasing memory load through the use of a radial maze instead of a T-maze) could help to reveal an effect of EC on working memory performance during stress. Moreover, to our knowledge no studies have been aimed to investigate the effects of EC on the consolidation of aversive memories (inhibitory avoidance) in the adult rat.

The aim of this study was to perform an integrative study on the effects of EC on a range of neurochemical, neuroendocrine and behavioral parameters related to the responses to an acute stress. Hence, the effect of EC on 1/ the hormonal (corticosterone) and neurochemical (dopamine and also acetylcholine) responses to acute restraint stress in the PFC of the awake rat; 2/ the GR mRNA levels in the PFC; 3/ behavioral responses to stress and related to the PFC (habituation to a novel environment, spatial-working memory and inhibitory avoidance response) was studied. The results of these experiments will help to clarify the modulation of the stress responses by the environmental conditions and its putative neurobiological mechanisms.

2. METHODS

2.1. Animals and housing conditions

Young (3 months) male Wistar rats were housed during 12 weeks in two different conditions: in large methacrylate cages of 120x100x60 cm (10–12 animals per cage) containing 2 running wheels, a rearrangeable set of plastic tunnels, an elevated platform,

and different objects changed every 5–6 days (EC group); or in standard Plexiglas cages of 55x35x20cm (1 animal per cage; IC group). Animals were provided with food and water *ad libitum*, and maintained in a temperature-controlled room under a 12:12h light-dark cycle (lights on at 20:00). All experiments were conducted during the dark period. Three different sets of animals were used for microdialysis experiments, mRNA quantification and behavioral experiments. The animals remained in EC or IC conditions when the experiments were performed. All experiments were carried out in our laboratory at the Universidad Complutense of Madrid and followed the Spanish regulations for the protection of laboratory animals (RD1201/2005).

2.2. Microdialysis experiments

Under Equithesin (2.5mg/kg i.p.) anaesthesia rats were stereotaxically implanted with bilateral guide-cannulae to accommodate microdialysis probes in the medial PFC (Del Arco and Mora 2002), according to the following co-ordinates from bregma: -3.2mm rostral; +0.8mm medial; -2mm from the top of the skull, with the incisive bar set at -3.3mm (Paxinos and Watson 1998). Six to seven days after surgery dual-probe microdialysis experiments were carried out in freely moving animals. Microdialysis probes, constructed in our own workshop, were of concentric design with an active dialysis membrane (5000Da, Hospal, Barcelona, Spain) of 4mm in length. The probes were perfused with artificial CSF consisted of (in mM): NaCl 137; CaCl2 2.4; KCl 3; MgSO4 1; NaH2PO4 0.5; Na2HPO4 2; glucose 3; containing the inhibitor of the dopamine transporter nomifensine (5μM) and the acetyl cholinesterase inhibitor neostigmine (0.5μM), at a flow rate of 2μl/min. After basal concentrations of neurotransmitters were established (3h perfusion period), 20min samples were collected and immediately stored at -80ºC until analyzed. The first three samples were used as a

control (basal levels) and then it followed the stress period (20min of restraint stress, see 2.3.). The experiments were performed from 9:00 to 17:00.

2.3. Acute restraint stress

Free movement of the rats was restrained for 20 minutes by wrapping them tightly using a cloth tied with adhesive tape. This procedure was used to study the effects of an acute moderate stress during microdialysis experiments and on radial arm water maze performance.

2.4. HPLC analysis

Dopamine analysis

Dopamine was analysed by reverse-phase HPLC and electrochemical detection (HP1049A, Agilent, Palo Alto, USA). Samples were injected in a Rheodyne injector (20µl loop) running in a C18 column of 4µm particles, and 3.9mm×150mm (Nova-Pak, Waters, Milford, MA). The mobile phase consisted of 0.1M acetate-citrate buffer (pH=4.35 adjusted with HCl and NaOH 1N), 1mM EDTA, 4.7mM sodium octyl sulphonate, and 15% methanol. The mobile phase was re-circulated at a flow rate of 1ml/min. These conditions allowed dopamine to be detected at 5.5min. Dopamine was measured by a coulometric detector (Coulochem II model 5200, ESA). Conditioning cell (ESA 5021) was set at 0mV and analytical cells (ESA 5011) at +275mV (cell 1) and −250mV (cell 2). Chromatograms were processed using the Millenium software (Waters). The limit of detection for dopamine (20µl samples) was 0.15 nM.

Acetylcholine analysis

Acetylcholine content of samples was analyzed by cation-exchange HPLC and electrochemical detection (Hernandez et al. 2003). Samples were injected in an auto sampler (Hewlett Packard, series 1100,Spain) running in a microbore column of 10mm particles and 530x1mm (Unijet microbore Ach/Ch analytical column. BAS, West Lafayette, IN). The mobile phase consisted of 50mM phosphate buffer, 0.5mM EDTA, and ProClin 150 microbiocide Reagent 5ml/l (BAS), pH=8.5 adjusted with NaOH 1N). The mobile phase was not re-circulated and the flow rate maintained at 0.15ml/min. These conditions allowed acetylcholine to be detected at 6.7min. Acetylcholine was hydrolyzed by acetylcholinesterase to choline in a post-coulmn enzyme reactor (Unijet microbore Ach/Ch IMER, BAS); Choline was oxidized by choline oxidase to produce hydrogen peroxide that was detected by an electrochemical detector (Hewlett Packard 1049A, Spain) equipped with a platinum electrode at +500mV. The limit of detection for acetylcholine (8 µl samples) was 5nM.

2.5. Real-time PCR

A separate set of animals was used for mRNA expression determination of GRs in brain tissue. Rats reared in EC and IC conditions (12 weeks) were killed by decapitation between 09:00 and 11:00, and brains were frozen immediately by -20ºC isopentane (Sigma-Aldritch, Spain) and dry ice and stored at -80ºC. Tissue from the medial PFC was collected and stored again at -80ºC. Total RNAs were purified from PFC tissue by the single step procedure of Chomczynski and Sacchi (Chomczynski and Sacchi 1987) using Tri-Reagent (Sigma, Spain). The concentration and purity of RNA extracted were determined by an automated electrophoresis system (Experion^{TM}, Bio-Rad, USA). 1µg of total RNA extracted from PFC tissue was reverse transcribed into first strand cDNA using GoScript^{TM} Reverse Transcription system (Promega, Spain). Real-time PCR was

performed in ABI Prism equipment using the SYBR Green PCR master mix (Applied Biosystems, UK) and 300nM concentrations of specific primers. The primers used for the determination of the concentration of GR mRNA were: 3´ CACCCATGATCCTGTCAGTG and 5´ AAAGCCTCCCTCTGCTAACC. Amplification of the 18S rRNA was used for normalization of cDNA loading in the PCR. Primers for 18S were CCAGTAAGTGCG GGTCATAAG C and CCTCACTAAACCATCCAATCGG. The amount of targets, normalized to the endogenous reference (18S) and relative to the calibrator, was defined by the Ct (threshold cycle) methods (Livak and Schmittgen 2001). In the samples of the medial PFC, random primer cDNA (dilution 1:10) gave cycle threshold values of around 23 for GRs transcripts. In the case of 18S rRNA, a dilution of 1:1000 gave cycle threshold values around 17. In all runs melting curves were performed to make sure that only the corresponding DNA fragment was amplified.

2.6. Behavioral tests

Every animal underwent three different behavioral tasks, in the following order: 1/ Exposure to open field; 2/ Radial arm water-maze task; 3/ Inhibitory avoidance task. The animals remained in EC or IC conditions during the time in which experiments were performed.

2.6.1. Open field

Spontaneous locomotion was evaluated in non-habituated animals in open field arenas (MED Associates Inc., VT, USA). The open field apparatus consisted of a Plexiglas box (80x80x45cm) equipped with two horizontal rows of eight infrared light sensitive photocell beams located at 5 and 15 cm, respectively, from the basement, allowing the

detection of horizontal and vertical (rearing) motor activity. Interruptions of the photocell beams were registered automatically by a computer software connected to the open field apparatus (MED Associates Inc., VT, USA). Each rat was placed in the center of the open field and allowed to explore the open field during 60min. All animals were evaluated between 15:00 and 18:00h and the arena was wiped with 70% ethanol immediately before every new measurement to avoid odor cues.

2.6.2. Radial arm water-maze task

To test spatial-working memory a dark gray Plexiglas maze made up of 8 equally arms (40x20x40cm) radiating from a central area (60cm diameter) and filled with clear water (22±1ºC) was used. At the end of 7 out of 8 arms there was a platform (Plexiglas, 15x18cm) submerged 2cm above the water. The other arm was considered as the starting arm. The apparatus was set on a 75cm high table in a slightly lit room (two bulbs of 40W in two opposite corners) and was surrounded by extra maze cues (different color figures, posters, doors and the experimenter). The experiments were performed between 13:00 and 18:00, six days a week.

The experimental protocol used was modified and adapted to aquatic conditions from the delayed spatial win-shift paradigm of Seamans and Phillips (1994). Briefly, rats had to learn to enter in arms not visited previously, where they had to find a submerged platform to escape from the water. Before starting the training of the task, animals were habituated to handling (3 consecutive days, 2-3 minutes per rat). On the day 0, rats were allowed to explore the maze for 2min. None of the arms contained platforms during this session of free exploration. Learning steps of the task were as follows: (1) On the first day, all arms apart from the starting arm contained a platform. Animals were introduced in the starting arm and they had to swim to an arm that contained a submerged platform.

If the animal did not reach a platform in less than 2min it was conducted to the nearest arm which contained a platform. When an animal reached a platform, it was placed in a plastic holding cage (27x27x23cm), the platform was removed from the maze and after an inter-trial interval of 30s the animal was again introduced in the starting arm. This sequence was followed until the rat reached the seven platforms. Two types of errors were recorded: working memory errors (the first entrance in an arm without platform) and perseverative errors (the second and following entrances in an arm without platform). Animals performed the task in this way for 9 days and then they were submitted to the next step of learning; (2) From this step of learning the task consisted of a *training phase* and a *test phase*. Before the training phase, a set of three arms was randomly blocked by guillotine-doors. When animals visited the four free-access platforms, the guillotine-doors were removed, and the test phase began. No within phase-delay was applied in this step of learning. Errors were scored as entries in arms without platform during the test phase. Two types of errors were scored during the test phase: *across-phase errors* (the first entry into an arm that was visited during the training phase) and *within-phase errors* (a re-entry into an arm that had been entered earlier during the test phase). The learning criterion during this step of learning was a mean of 1 or less across-phase errors during three consecutive days. When each animal reached that learning criterion, it was submitted to the next step; (3) In the last step of learning the within-phase delay was increased to 20min. Animals had to make again a mean of 1 or less *across-phase errors* memory errors within three consecutive days to reach the learning criterion. The following day after reaching the learning criterion the spatial-working memory was evaluated under acute restraint stress (see 2.3.) during the entire within-phase delay (20min). Spatial-working memory was also evaluated

applying within-phase delays of 60min (3 consecutive days) and 300min (3 consecutive days).

2.6.3. Inhibitory avoidance task

One month after the radial arm water-maze task was terminated (over 20 weeks of housing), animals were tested for fear-related memory in an inhibitory avoidance apparatus consisting of a shuttle-box divided into two compartments, separated by a guillotine-door. The starting compartment (light compartment, 45x45x19cm) was made of white opaque plastic, it had an open roof and was well lit by one overhead 60W bulb; the shock compartment (dark compartment, 25x24x19cm) was made of black plastic, it had a closed (removable) roof, no illumination and an electrified grid floor. The inhibitory avoidance test was carried out as follows. On the *training day*, animals were placed in the light compartment and allowed to explore the whole apparatus (guillotine-door open) over a period of 300s. Five hours later (between 15:00 and 18:00), animals were re-exposed to the apparatus and latency to enter the dark compartment was recorded (*training latency*). When the animals placed their four paws on the dark compartment the guillotine-door was lowered and a single foot-shock (0.6mA, 2s) was delivered. After 10s animals were removed from the dark compartment and a blood sample was taken in a different room. Blood samples (150μL) were taken by tail-nick immediately after and 30min after the foot-shock delivered in the inhibitory avoidance apparatus. On the *testing day* (48h after the foot-shock), rats were re-exposed to the light compartment and retention of the inhibitory avoidance response was recorded as the latency (*retention latency*), up to a maximum of 300s to enter the dark compartment. None of the animals reached that maximum latency. Shock was not delivered at the retention test trial.

2.7. Corticosterone assays

Blood samples were collected in heparinized vials in less than 2 minutes. Vials containing blood samples were centrifuged for 10 minutes at 15,000 rpm to obtain plasma samples. Total corticosterone levels in plasma from inhibitory avoidance experiments and dialysate free corticosterone levels from microdialysis experiments (15µl samples) were measured using a radioinmunoassay kit (MP Biomedicals, Inc.). In the case of dialysate corticosterone levels, samples were not prior diluted, a different standard curve was used, and the volume of corticosterone-I^{125} was reduced 4 times to increase the sensitivity of the kit. Dialysate levels were not corrected for probe recovery. Although the basal levels of free corticosterone were low, they were above the detection limit of the assay. The inter- and intra-assay coefficient of variance were 6.5% and 4.4% respectively.

2.8. Histology

At the end of microdialysis experiments animals were anesthetized with an overdose of equithesin and perfused intracardially with 0.9% saline followed by 10% formalin. The brain was removed and the placement of the microdialysis probes was verified in sections cut with a cryostat microtome and viewing lens. Figure 1 shows an schematic representation of the location of the microdialysis probes in the medial PFC.

2.9. Statistical analysis

To analyse motor activity, radial-arm water-maze performance, inhibitory avoidance response, plasma corticosterone and dialysate concentrations of acetylcholine, dopamine and corticosterone, a two-way analysis of variance (ANOVA) with repeated measures design was used to perform planned comparisons (*a priori* analysis), considering Time

and Group (EC or IC) as within- and between-subject factors, respectively. For the analysis of dialysate concentrations, absolute dialysate values were normalized by subtracting basal concentrations (average of three sample values) to each post-basal sample. Student t-test for independent samples was performed to analyse basal levels of dopamine, acetylcholine and corticosterone in dialysates, mRNA receptor quantification and days of learning in the radial-arm water-maze. Statistical analyses were performed with STATISTICA software. Statistical signification was considered in all cases $p<0.05$.

3. RESULTS

3.1. Effects of environmental conditions on the basal and stress-induced extracellular concentration of corticosterone in the PFC

Basal extracellular concentrations of corticosterone in the PFC were 0.53±0.11ng/ml for IC (n=16) and 0.37±0.04ng/ml for EC group (n=16). There was a trend for EC group to show lower basal corticosterone levels but it did not reach statistical signification ($t_{1,30}$=1.87; p=0.071).

Acute stress produced an average increase of extracellular corticosterone in the PFC of 0.45±0.11ng/ml in IC rats and 0.20±0.08ng/ml in EC rats (Figure 2). The two-way ANOVA showed a significant effect of Time ($F_{3,90}$=7.50; $p<0.001$) and Group ($F_{1,30}$=4.84; p=0.036). The interaction Group x Time did not reach statistically signification ($F_{3,90}$=2.20; n.s.). Planned comparisons showed that acute stress increased extracellular concentrations of free corticosterone in the PFC (minutes 80-120) in IC ($F_{1,30}$=25.27; $p<0.001$) and EC rats ($F_{1,30}$=4.78; p=0.036) (Figure 2). EC rats showed

lower levels of corticosterone in response to stress than IC rats 20 minutes after the stress exposure ($F_{1,30}$=5.68; p=0.024).

3.2. Effects of environmental conditions on the basal and stress-induced extracellular concentrations of dopamine and acetylcholine in the PFC

Basal extracellular concentrations of dopamine in the PFC were 0.33±0.06nM for IC (n=9) and 0.45±0.05nM for EC group (n=13). Basal extracellular concentrations of acetylcholine were 27.97±3.90nM for IC (n=8) and 22.15±3.52nM (n=8) for EC group. Environmental conditions did not modify these parameters (Dopamine :$t_{1,20}$=-1.49; n.s.; Acetylcholine: $t_{1,14}$=1.11; n.s.).

Acute stress produced an average increase of extracellular dopamine in the PFC of 0.28±0.10nM in IC rats and 0.06±0.02nM in EC rats (Figure 3A). The two-way ANOVA showed a significant effect of Time ($F_{3,63}$=11.70; p<0.001) and Group ($F_{1,21}$=7.76; p=0.011) on the levels of dopamine in the PFC. There was a trend for the interaction Group x Time to reach statistical signification ($F_{3,63}$=2.48; p=0.069). Planned comparisons showed that acute stress increased extracellular concentrations of dopamine (minutes 80-100) in the PFC of IC ($F_{1,21}$=22.79; p=0.001) but not EC rats ($F_{1,21}$=0.61; n.s.). The increases of dopamine were lower in EC than IC rats immediately after ($F_{1,21}$=14.00; p=0.001) and 20 minutes after the stress exposure ($F_{1,21}$=6.83; p=0.016).

Acute stress produced an average increase of acetylcholine in the PFC of 15.43±3.17nM in IC rats and 9.07±2.90nM in EC rats. The two-way ANOVA showed that Time ($F_{3,42}$=3.88; p=0.015) but not Group ($F_{1,14}$=1.88; n.s.) modified significantly the extracellular concentration of acetylcholine in the PFC. The interaction Group x Time

was not statistically significant ($F_{3,42}$=0.21; n.s.). Planned comparisons showed that acute stress increased the extracellular concentration of acetylcholine in the PFC in both IC ($F_{1,14}$=25.74; p<0.001) and EC rats ($F_{1,14}$=8.90; p=0.010) (Figure 3B).

3.3. Effects of environmental conditions on GR mRNA levels in the PFC

There was a non significant trend for EC rats to show enhanced levels of GRs mRNA levels in the PFC ($t_{1,20}$=-1.79; p=0.088)(Figure 4).

3.4. Effects of environmental conditions on spontaneous motor activity

The two-way ANOVA showed that horizontal activity was significantly modified by Time ($F_{11,220}$=45.16; p<0.001) and Group ($F_{1,20}$=6.62; p=0.018). The interaction Group x Time was also statistically significant ($F_{11,220}$=2.14; p=0.019), produced by the faster habituation of EC rats to the open field (see Figure 5A for a point to point analysis). Vertical activity was modified by Time ($F_{11,220}$=29.34; p<0.001) but not by Group ($F_{1,20}$=0.54; n.s.) (Figure 5B). The interaction Group x Time did not reach statistical signification ($F_{11,220}$=1.43; n.s.). As shown in Figure 5B, EC animals showed higher levels of vertical activity during the first 5 minutes of exposure to the open field ($F_{1,20}$=5.55; p=0.028).

3.5. Effects of environmental conditions on spatial working-memory

The two-way ANOVA showed that the number of working memory errors during the first step of learning was modified by Time ($F_{7,54}$=8.10; p<0.001) and Group ($F_{1,22}$=6.60; p=0.017) and there was a trend for the interaction Time x Group ($F_{7,154}$=1.87; p=0.078). Planned comparisons showed that EC rats showed lower working memory errors on days 2 ($F_{1,22}$=8.25; p=0.009) and 8 ($F_{1,22}$=6.90; p=0.015)

(Figure 6A). The number of perseverative errors during the first step of learning was modified by Time ($F_{7,154}$=5.29; p<0.001) and Group ($F_{1,22}$=5.37; p=0.030). The interaction Group x Time was not statistically significant ($F_{7,154}$=0.93; n.s.). Planned comparisons showed that, on day 5, EC rats made less perseverative errors than IC rats ($F_{1,22}$=7.50; p=0.011) (Figure 6B).

EC rats needed less days to reach the learning criterion of the last step of training ($t_{1,18}$=7.02; p<0.001) (Figure 7A). In fact, 3 out of 12 IC rats (25% of total) were excluded from the study, while only 1 out of 12 EC rats (8.3% of total) was excluded from it. Once the animals reached the learning criterion, the number of across-phase errors was modified by Delay ($F_{2,36}$=23.75; p<0.001) but not by Group ($F_{1,14}$=1.14; n.s.) (Figure 7B). The interaction Group x Delay was not statistically significant ($F_{2,36}$=1.21; n.s.). Planned comparisons showed that the 300min delay increased the number of across-phase errors in both IC ($F_{1,18}$=26.56; p<0.001) and EC rats ($F_{1,18}$=12.01; p=0.003) compared to 20min delay. Within-phase errors were not modified by Group ($F_{1,18}$=2.79; n.s.), Delay ($F_{2,36}$=1.56) or Group x Delay interaction ($F_{1,36}$=0.23; n.s.)(not shown).

Acute stress significantly increased the number of across-phase errors ($F_{1,18}$=7.41; p=0.014) but they were not modified by Group ($F_{1,18}$=0.19; n.s.) (Figure 7C). The interaction Group x Stress was not statistically significant ($F_{1,18}$=1.65; n.s.). Acute restraint stress increased the number of across-phase errors in IC ($F_{1,18}$=7.30; p=0.014) but not in EC rats ($F_{1,18}$=1.14; n.s.). Within-phase errors remained at very low levels and they were not modified by stress ($F_{1,18}$=0.77; n.s.), Group ($F_{1,18}$=0.77; n.s.) or Group x Stress interaction ($F_{1,18}$=0.25; n.s.)(not shown).

3.6. Effects of environmental conditions on the inhibitory avoidance task and plasma corticosterone in response to foot-shock

Latency to enter the dark compartment of the inhibitory avoidance apparatus was modified by Shock ($F_{1,19}$=9.95; p=0.005) and Group ($F_{1,19}$=5.54; p=0.029). The interaction Time x Group was statistically significant ($F_{1,19}$=5.95; p=0.025) (Figure 8A). Planed comparisons showed that on the testing day, IC rats but not EC rats showed a higher latency to enter the dark compartment compared to the training day (IC group: $F_{1,19}$=16.43; p=0.001; EC group: $F_{1,19}$=0.24; n.s.). EC rats showed a lower retention of the inhibitory response than IC rats ($F_{1,19}$=5.79; p=0.026).

Plasma corticosterone levels were modified by Shock ($F_{1,19}$=35.80; p<0.001) but not by Group ($F_{1,19}$=0.83; n.s.). The Group x Shock interaction did not reach statistical signification ($F_{1,19}$=1.60; n.s.). Planed comparisons showed that the foot-shock delivered in the dark compartment of the inhibitory avoidance apparatus increased plasma corticosterone levels both in IC ($F_{1,19}$=27.58: p<0.001) and EC rats ($F_{1,19}$=10.62; p=0.004)(Figure 8B).

DISCUSSION

The aim of the present study was to investigate the effects of EC on several neurochemical, neuroendocrine and behavioral measures related to the responses to an acute stress. This study shows for the first time that EC reduces the stress-induced levels of corticosterone in response to an acute stress in the PFC. Moreover this study confirms, through the use of a different protocol of acute stress (Segovia et al. 2008a, b) that the increase of dopamine but not acetylcholine in the PFC in response to acute stress is lowered by EC in young adult rats. In line with this lower reactivity to stress,

EC rats show an enhanced learning capacity on a spatial-working-memory task under stressful (aquatic) conditions and a faster habituation to a novel environment. Furthermore, EC rats showed a reduced latency to enter the dark compartment in an inhibitory avoidance task. EC, however, did not change working memory either under basal or stress conditions and it did not modify the stress-induced increase of corticosterone after a foot-shock. On the whole, these results suggest that EC conditions lead to lower neurochemical and hormonal responses to stress, which could reflect a better coping behavior under stress conditions.

Acute stress and corticosterone in the PFC

Acute stress (20 min, restraint) increased the extracellular levels of free corticosterone in the PFC, as it has been previously shown using the microdialysis technique (Kitchener et al. 2004; Thoeringer et al. 2007; Droste et al. 2009; Garrido et al. 2012a). The stress-induced increase of corticosterone in the PFC was significantly lower in EC rats, which suggests a lower reactivity to stress of the HPA axis in these animals. This result agrees with previous studies examining the effect of EC on the stress-induced increases of plasma corticosterone (Mlynarik et al. 2004; Moncek et al. 2004; Peña et al. 2009; Sztainberg et al. 2010). Our results extend these findings to the free levels of corticosterone in the brain, where it acts to modulate the HPA axis activity and behavioral adaptation to stress (Bruce S 2000; Herman et al. 2005; Sandi and Pinelo-Nava 2007). Moreover, measuring free levels of corticosterone in the brain is a relevant issue since several studies have shown that brain levels may not mirror plasma levels of corticosterone, either in basal or under stress conditions (Lengvári and Liposits 1977; Croft et al. 2008; Droste et al. 2009; Garrido et al. 2012a). However, in the case of EC versus IC rats it seems that plasma and free corticosterone levels in the brain do run in

parallel because the results of other studies on plasma corticosterone and those showed in the present study on free corticosterone in the brain suggest a lower stress-induced increase in EC animals.

The mechanism by which EC rats show a lower increase of corticosterone in response to an acute stress remains unexplained. It has been suggested that since the PFC and also the hippocampus exert a negative control on the release of corticosterone under stress conditions (Jacobson and Sapolsky 1991; Diorio et al. 1993; Sullivan 2004; Herman et al. 2005; Radley et al. 2006), EC animals would show an enhanced expression of GRs in those brain areas, thus leading to a more effective corticosterone signal, which would drive a faster recovery of basal levels of corticosterone under stress conditions (Larsson et al. 2002). In line with this hypothesis, EC rats showed a trend towards enhanced GR mRNA levels in the PFC. Also similar results have been reported for the hippocampus (Olsson et al. 1994).

Acute stress, dopamine and acetylcholine in the PFC

The extracellular concentrations of dopamine and acetylcholine were increased by acute moderate stress (restraint) in the PFC, as it has been previously shown using different stressors (Thierry et al. 1976; Abercrombie et al. 1989; Feenstra et al. 1995; Mark et al. 1996; Day et al. 2001; Del Arco et al. 2007; Mora et al. 2007; Segovia et al. 2008a, b). The stress-induced increase of dopamine but not acetylcholine was lower in EC rats, which suggests a lower reactivity of the mesocortical dopaminergic system to stress in these animals. These differences seem not to depend on the intensity of the stress protocol since similar results were also obtained using handling, a milder stress challenge (Segovia et al. 2008a,b). Since different studies have shown that corticosterone can modulate dopamine levels in the PFC (Imperato et al. 1989;

Mizoguchi et al. 2004; Ago et al. 2009), it is possible that the reduced increases of dopamine are secondary to the lower increases of corticosterone observed in the PFC of the EC rats. This would be in agreement with the reduction of stress-evoked dopamine release after blockade of GRs locally within PFC (Butts et al. 2011). As shown in the Results section, restraint produces a reliable increase of free corticosterone in PFC (Garrido et al. 2012a). In contrast, we have observed that handling does not increase free corticosterone in PFC using microdialysis (unpublished results), which is in agreement with previous studies (Croft et al. 2008). These findings do not support the possibility of a role of corticosterone in the effects of EC on dopamine responses, since EC reduces these responses to both restraint and handling.

The activity of the dopaminergic mesocortical system has been proposed to be modulated by the amygdala (Davis et al. 1994; Goldstein et al. 1996). It is therefore possible that changes in the amygdala produced by EC could lead to a lower reactivity of this system in response to the presence of a stressor. This lower reactivity of the dopaminergic system in EC rats could be related to a better coping strategy displayed by these animals in response to a stress situation (Carlson et al. 1993; Horger and Roth 1996; Berridge et al. 1999; Bland et al. 2003).

By contrast to dopamine, stress-induced levels of acetylcholine in the PFC were not modified by EC in young adult rats, as we showed in a previous study (Segovia et al. 2008b). However, rats of 15 and 24 months of age maintained in EC conditions showed reduced increases of acetylcholine in the PFC in response to acute stress (Segovia et al. 2008b). Therefore, it is possible that the cholinergic system is less sensitive to EC conditions than the dopaminergic system, needing a longer period to be modified by the environmental conditions.

Spontaneous motor activity

EC rats showed lower total levels of horizontal activity, which are the result of a faster habituation during the 60min of exposure to the open field apparatus (Figure 5A). This result has been consistently shown in several studies (Zimmermann et al. 2001; Schrijver et al. 2002; Elliott and Grunberg 2005; Segovia et al. 2008a) and suggests an enhanced ability of EC rats to habituate to a novel environment. This lower motor activity shown by EC rats in a novel environment has been suggested to be related to an increased exploratory efficacy of those animals, due to their higher possibilities to explore a changing environment in their usual conditions of life (Zimmermann et al. 2001; Schrijver et al. 2002), a possibility reinforced by the enhanced vertical activity of EC rats during the first 5min of exposure to the open field (Figure 5B). This last result has also been observed during an object recognition test (Zimmermann et al. 2001; Lee et al. 2003). Additionally, EC rats could experience a lower reactivity to stress that would facilitate habituation to the novel environment. In line with this suggestion, it has been proposed that glucocorticoids can modulate motor activity in a novel environment (Oitzl et al. 1994; Sandi et al. 1996). Therefore, a lower increase of corticosterone in EC rats in response to their exposure to the open field could lead to the observed differences.

Spatial working memory

The protocol used in this study to evaluate spatial working memory is an aquatic version of the spatial delayed win-shift protocol used by Seamans and Phillips (1994). Although different radial arm water-maze paradigms have been used in several studies (Diamond et al. 1999; Bimonte et al. 2003; Shukitt-Hale et al. 2004), this is the first time that the delayed win-shift paradigm has been performed under aquatic conditions.

The performance of this task is dependent on PFC integrity (Floresco et al. 1997; Seamans et al. 1998) and, interestingly, the learning protocol used in this study also allows to measure spatial memory in a less complex way because in the first step of learning there are no delays or arms blocked, which resembles the common protocols used to evaluate spatial memory in the radial arm maze (Paul A 2004).

Animals of both experimental groups were able to reach the learning criterion, however, EC reduced the time needed to reach it (Figure 6B), which is in accord with a previous study (Richard C 1999). During the first step of training EC rats made less working memory and perseverative errors than IC rats (Figures 6A and 6B). Interestingly, some studies have found a lower number of errors in EC animals only on the first days of training of this task (Leggio et al. 2005; Galani et al. 2007; Hoffmann et al. 2009). This lower number of errors could reflect an enhanced cognitive flexibility of EC rats, since during the first days rats have to avoid re-entering in a previously visited arm against their natural tendency to do it. Interestingly, the PFC plays a key role in cognitive flexibility (Birrell and Brown 2000; Ragozzino 2002; Robbins and Roberts 2007), which could be the main role of the PFC in the performance of the radial-maze win-shift paradigm (Gisquet-Verrier and Delatour 2006; Rich and Shapiro 2007). Therefore, the faster learning of the task by EC rats could be due to changes in the PFC that would enhance their ability to adapt to the changes faced across the different steps of learning of the task.

There were no differences between EC and IC rats in the performance of the task with the different delays used (20, 60 and 300min) (Figure 6C). This result suggests that EC conditions do not modify working memory, and it agrees with a previous study of our laboratory using an aquatic version of the T-maze, which implied lower delays (10-

100s) and egocentric rather than visual cues to perform the task (Segovia et al. 2008). Furthermore, the highest delay used in this study (300min) increased the number of across-phase errors made by both experimental groups, which confirms that this aquatic version of the radial-arm maze is delay-dependent.

Acute restraint stress during the delay period increased the number of across-phase errors in both experimental groups. This increase, however, reached statistical significance only in IC rats. Different studies have shown that different acute stressors lead to a deficit in working memory (Diamond et al. 1996; Murphy et al. 1996; Arnsten and Goldman-Rakic 1998; Del Arco et al. 2007; Park et al. 2008; Segovia et al. 2008a). This effect of acute stress on working memory has been suggested to be related to either an over activation of D1 receptors in the PFC (Williams and Castner 2006) or to an over activation of GRs (Park et al. 2006; Barsegyan et al. 2010). However, in spite of the lower increases of corticosterone and dopamine in the PFC of EC rats in response to acute stress obtained in the microdialysis experiments (Figures 2 and 3A), there were no differences in the effect of restraint stress on spatial working memory. This result agrees with a previous work of our laboratory (Segovia et al. 2008a), in which a different task (T-maze) and a different stressor (novel environment) were used. As a whole, these results suggest that EC does not modify working memory performance either in basal or under acute stress conditions.

Inhibitory avoidance response

EC rats showed a lower latency to enter the dark compartment than IC rats 48h after the foot-shock. In fact, latency of EC rats on the test phase was not statistically different from the latency on the training phase. A very recent study in mice has shown that EC (3 weeks) increases the latency 24h after the training phase (Leger et al. 2012).

However, the disagreement in the results could be explained by the differences in the EC protocol and in the species used. Although we cannot completely discard a memory deficit in the EC animals, the lower latency of EC animals to enter the dark compartment on the test phase could reflect a lower reactivity to the foot-shock on the training phase. Different studies have shown that corticosterone mediates the consolidation of aversive memory (Roozendaal et al. 2009a). In our study, however, the levels of corticosterone 30min after the foot-shock were not different between EC and IC rats (Figure 8B). Alternatively, a study showed that the consolidation of the inhibitory avoidance response needs the activation of the dopaminergic ventral tegmental neurons (Rossato et al. 2009). It is possible that a lower activation of the ventral tegmental area in EC rats, as it has been shown in the case of restraint stress, could lead to the reduced memory consolidation observed in EC animals.

It is also of interest to note that while restraint stress led to differences in the increases of prefrontal corticosterone levels between EC and IC rats, the foot-shock did not reveal this difference between both groups. The possibility exists for these differences being due to the nature of the stressor (more psychogenic in the case of restraint, more physical in the case of foot-shock). In fact, it has been shown that PFC control over the HPA axis activity is exerted under psychogenic stressors rather than under physical stressors (Diorio et al. 1993; Jones et al. 2011). Nonetheless, the possibility of differences beyond the unique time point of stress evaluated (30min after stress) cannot be excluded.

Final considerations

The results of this study show that lower corticosterone and dopamine increases in the PFC fit well with a better coping with stressful events of EC animals. The enhanced

learning capacity under stressful (aquatic) conditions and the faster habituation to a novel environment are in line with this suggestion.

Acute stress increases the neuronal activity (measured as cFos expression) of the PFC (Cullinan et al. 1995; Weinberg et al. 2010). Interestingly, animals able to control a stress situation show reduced increases of dopamine and serotonin in the PFC (Carlson et al. 1993; Berridge et al. 1999; Bland et al. 2003) and these effects are thought to depend on the ventromedial PFC activation (Amat et al. 2005; Maier et al. 2006). Moreover, animals allowed to display coping behaviors (i.e., chewing while exposed to novelty or restraint) show lower stress-induced increases of corticosterone (Hennessy and Foy 1987) and an enhanced expression of cFos in the PFC (Coco and Weiss 2005; Stalnaker et al. 2009). Last, the activation of the medial PFC by a local picrotoxin microinjection reduces the increase of corticosterone in response to acute restraint stress (Weinberg et al. 2010; Garrido et al. 2012b). Therefore, as previously proposed (Segovia et al. 2009), EC may induce changes in the PFC leading to the increase of the activity of this brain area in response to acute stressors that could explain, at least in part, the results obtained in this study. In line with this hypothesis it has been recently reported that EE improves the resilience from a psychosocial stress and this effect is produced by an enhanced activity of the prelimbic cortex in EE mice (Lehmann and Herkenham 2011).

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Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS.

Figure 1. Schematic representation showing the location of microdialysis probes in the PFC. Locations of guide cannulas (grey area) and membrane of the microdialysis probes (black area) are shown. Modified from Paxinos and Watson (1998).

Figure 2. Temporal profile of the effect of restraint stress (20min, shaded area) on the free corticosterone dialysate concentrations in the PFC of IC and EC rats. Data (mean ±SEM) are shown as percentage values related to basal corticosterone concentrations. The number of animals is shown in parenthesis. ***$p<0.001$ compared to basal levels; #$p<0.05$ compared to IC group (planned comparisons in a two-way ANOVA).

Figure 3. Temporal profile of the effect of restraint stress (20min, shaded area) on the dopamine (A) and acetylcholine (B) dialysate concentrations in the PFC of IC and EC rats. Data (mean ± SEM) are shown as percentage values related to basal dopamine concentrations. The number of animals is shown in parenthesis. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to basal levels; #$p<0.05$; ##$p<0.01$ compared to IC group (planned comparisons in a two-way ANOVA).

Figure 4. mRNA GRs levels in the PFC of IC and EC animals. Data (mean ± SEM) are shown as absolute values. Number of animals is shown in parenthesis.

Figure 5. Temporal profile of horizontal activity (A) and rearing (B) of IC and EC rats during 60min in an open field. Data (mean ± SEM) are shown as absolute values The number of animals is shown in parenthesis. *p<0.05, ***p<0.001 compared to IC group (planned comparisons in a two-way ANOVA).

Figure 6. Spatial (A) and perseverative (B) errors of IC and EC rats on the performance of a non-delayed version of a radial arm water-maze. Data (mean ± SEM) are shown as absolute values. The number of animals is shown in parenthesis. *p<0.05 compared to IC group (planned comparisons in a two-way ANOVA).

Figure 7. A) Days to reach the learning criterion of IC and EC rats on a spatial delayed win-shift version of the radial arm maze under aquatic conditions. ***p<0.001 (Student´s t-test); Effect of delay (B) and acute restraint stress (C) on across-phase errors. Data (mean ±SEM) are shown as absolute values. The number of animals is shown in parenthesis. *p<0.05 compared to 20min delay (B) or pre-stress (C) (planned comparisons in a two-way ANOVA).

Figure 8. A) Latency of IC and EC rats to enter the dark compartment of a inhibitory avoidance apparatus; B) Plasma corticosterone levels produced immediately and 30min after the foot-shock delivered to IC and EC rats in the dark compartment of the inhibitory avoidance apparatus. Data (mean ±SEM) are shown as absolute values. The number of animals is shown in parenthesis. **p<0.01, ***p<0.001 compared to training phase (A) or basal levels (B); #p<0.05 compared to IC rats (planned comparisons in a two-way ANOVA).

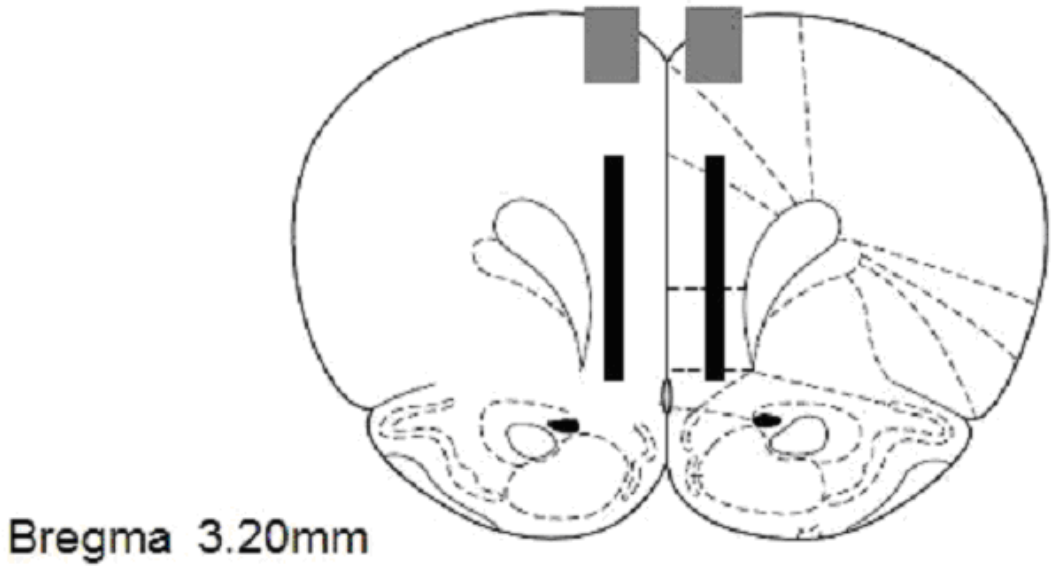

Fig.1

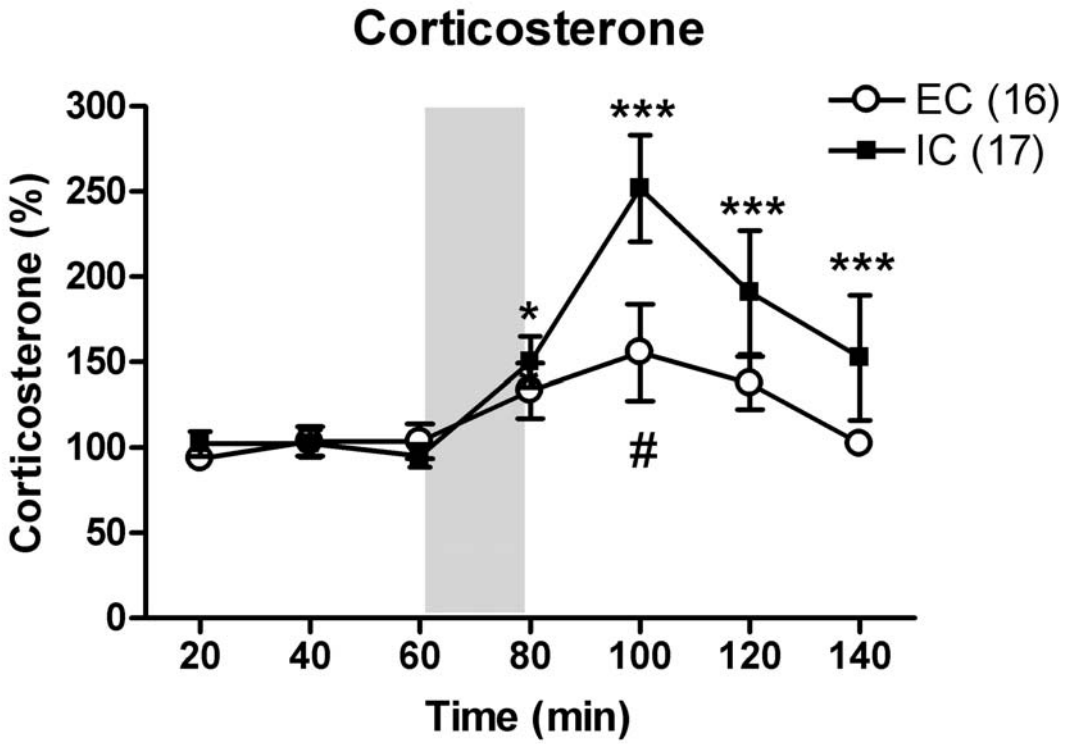
Corticosterone
EC (16)
IC (17)
Corticosterone (%)
300
250
200
150
100
50
0
20
40
60
80
100
120
140
Time (min)
*
***
***
***
#

Fig 2

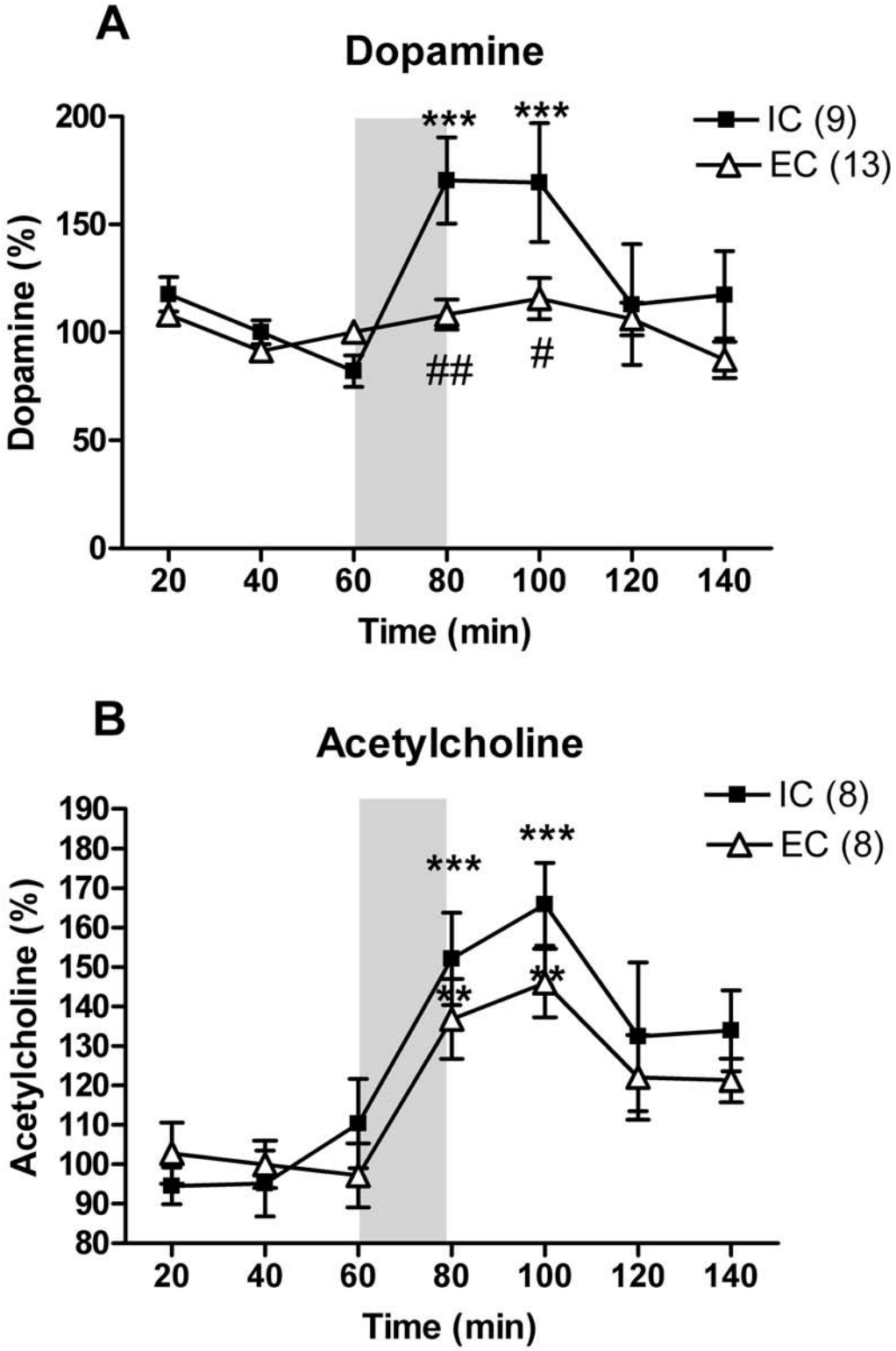
A
Dopamine
IC (9)
EC (13)
Dopamine (%)
200
150
100
50
0
***
***
##
#
20
40
60
80
100
120
140
Time (min)
B
Acetylcholine
IC (8)
EC (8)
Acetylcholine (%)
190
180
170
160
150
140
130
120
110
100
90
80
***
***
**
**
20
40
60
80
100
120
140
Time (min)

Fig 3

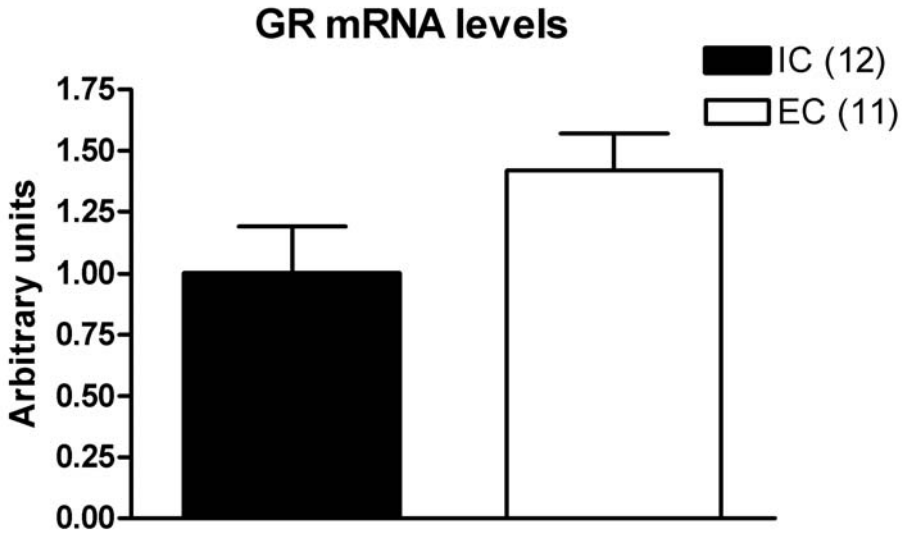
GR mRNA levels
IC (12)
EC (11)
Arbitrary units
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00

Fig 4

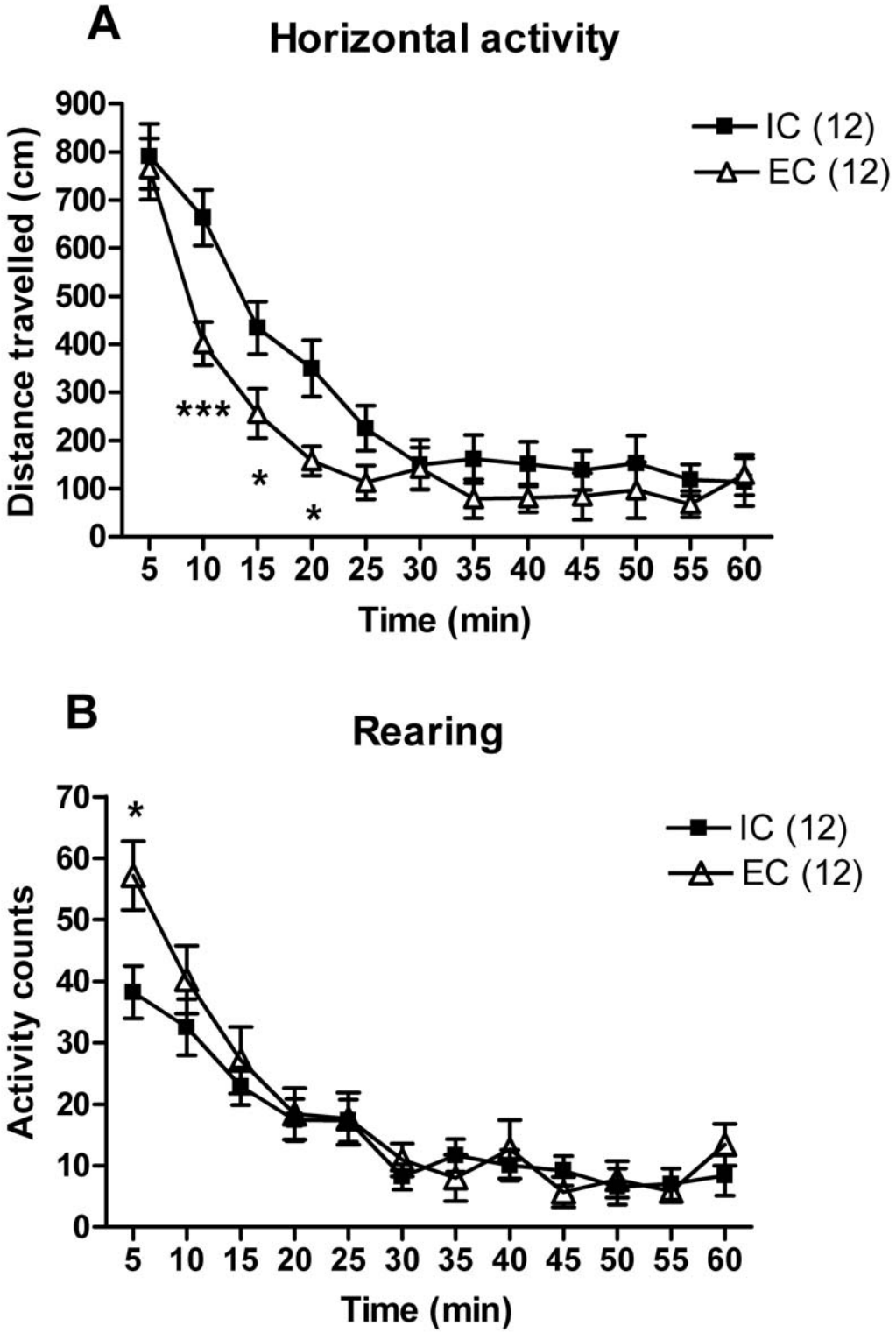
A
Horizontal activity
Distance travelled (cm)
900
800
700
600
500
400
300
200
100
0
IC (12)
EC (12)
***
*
*
5 10 15 20 25 30 35 40 45 50 55 60
Time (min)
B
Rearing
Activity counts
70
60
50
40
30
20
10
0
*
IC (12)
EC (12)
5 10 15 20 25 30 35 40 45 50 55 60
Time (min)

Fig. 5

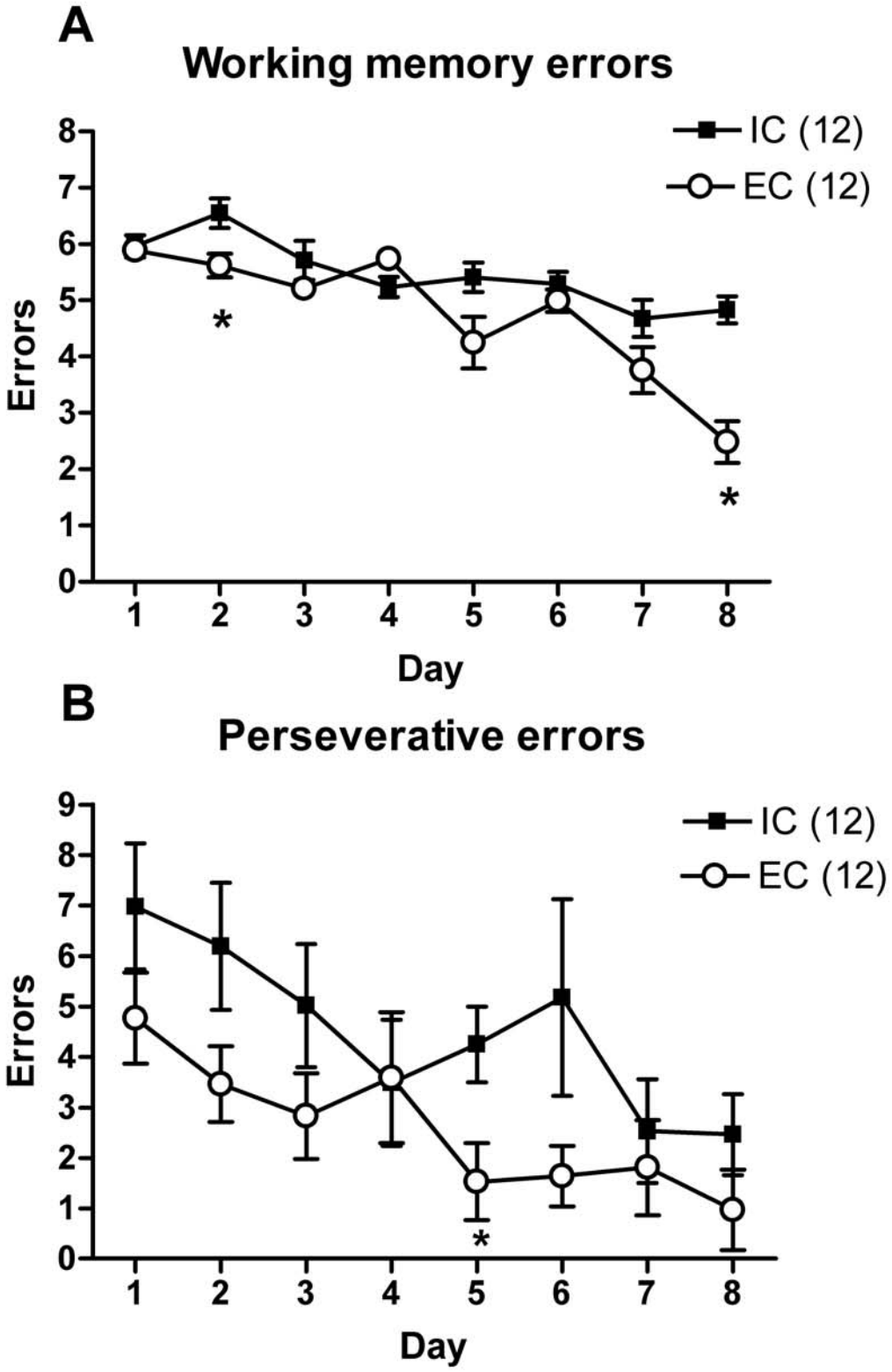
A
Working memory errors
IC (12)
EC (12)
Errors
Day
B
Perseverative errors
IC (12)
EC (12)
Errors
Day

Fig. 6

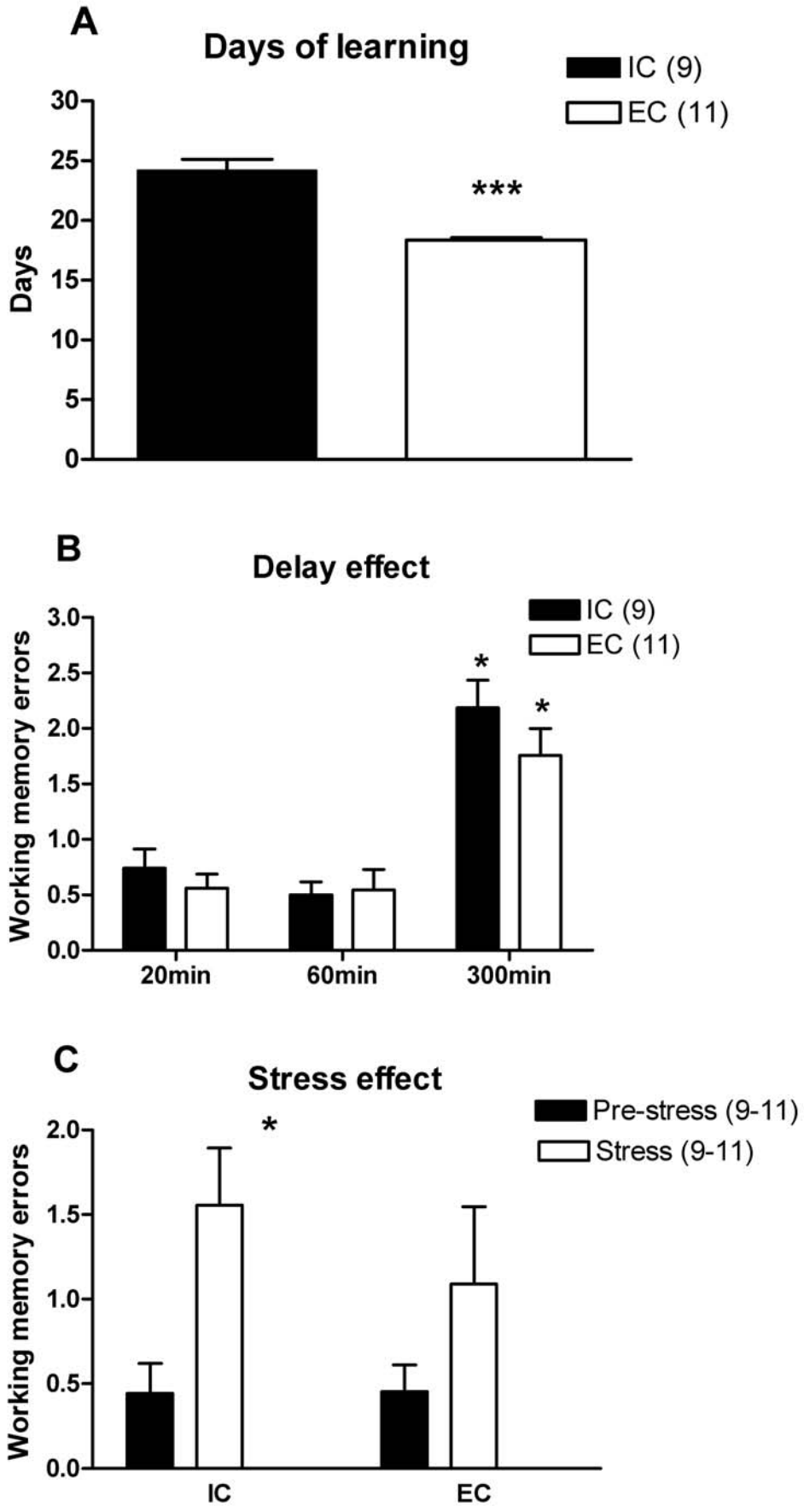
A
Days of learning
IC (9)
EC (11)
Days
30
25
20
15
10
5
0
***
B
Delay effect
IC (9)
EC (11)
Working memory errors
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
20min
60min
300min
C
Stress effect
Pre-stress (9-11)
Stress (9-11)
Working memory errors
2.0
1.5
1.0
0.5
0.0
*
IC
EC

Fig. 7

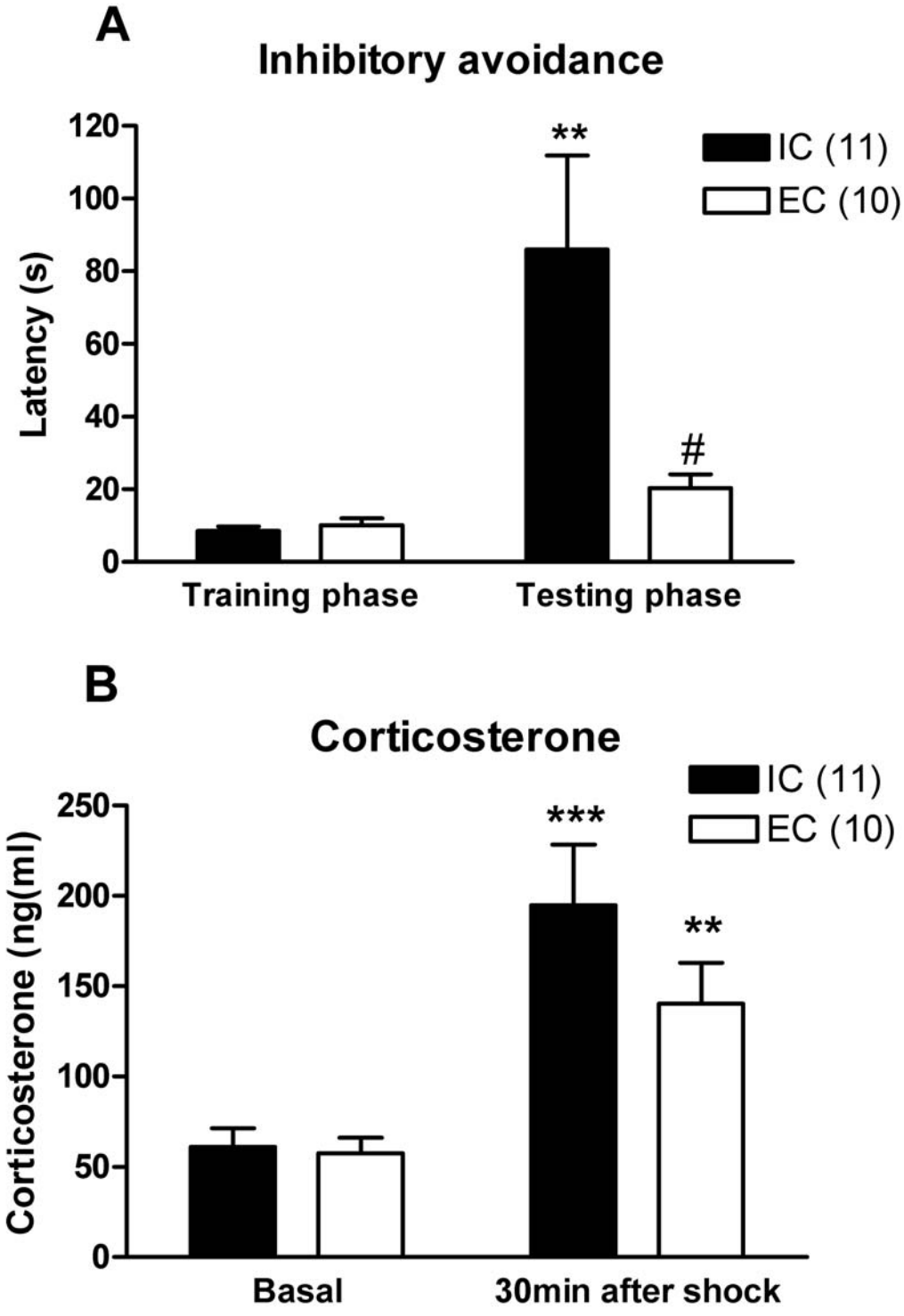
A
Inhibitory avoidance
Latency (s)
120
100
80
60
40
20
0
**
#
IC (11)
EC (10)
Training phase
Testing phase
B
Corticosterone
Corticosterone (ng(ml)
250
200
150
100
50
0
***
**
IC (11)
EC (10)
Basal
30min after shock

Fig. 8