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Research paper

# Corticosterone treatment impairs auditory fear learning and the dendritic morphology of the rat inferior colliculus

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ABSTRACT

Stress leads to secretion of the adrenal steroid hormone corticosterone (CORT). The aim of this study was to determine the effects of chronic CORT administration on auditory and visual fear conditioning. Male Sprague–Dawley rats received CORT (400 mg/ml) in their drinking water for 10 consecutive days; this treatment induces stress levels of serum CORT. CORT impaired fear conditioning ($F_{(1,28)} = 11.52$, $p < 0.01$) and extinction ($F_{(1,28)} = 4.86$, $p < 0.05$) of auditory fear learning, but did not affect visual fear conditioning. In addition, we analyzed the CORT effects on the neuronal morphology of the inferior colliculus (flat neurons, auditory mesencephalon, a key brain area for auditory processing) and superior colliculus (wide-field neurons, related to visual processing) by Golgi stain. CORT decreased dendritic arborization of inferior colliculus neurons by approximately 50%, but did not affect superior colliculus neurons. Thus, CORT had more deleterious effects on the auditory fear processing than the visual system in the brain.



## 1. Introduction

Stress is a complex biological reaction that restores homeostasis, allowing organisms to adapt to environmental pressure (i.e., stressor) (Selye, 1936; McEwen, 2007). The stress response is mediated heavily by activation of the hypothalamic–pituitary–adrenal (HPA) axis, leading to secretion of glucocorticoids (GCs) from the adrenal gland; GCs are bound to glucocorticoid receptors (GRs) in the peripheral tissues and the brain (Herman et al., 1996, 2003; Smith and Vale, 2006; McEwen, 2007). Limbic structures like the hippocampus, amygdala and medial prefrontal cortex have high concentrations of GRs (Gray and Bingaman, 1996; Joels, 2001; Wellman, 2001). Chronic glucocorticoid (GC) treatment produces dendritic atrophy in the hippocampus (McEwen, 1992; Watanabe et al., 1992; Magariños et al., 1998) and medial prefrontal cortex (Magariños et al., 1998). Conversely, acute GC treatment induces dendritic hypertrophy in the basolateral amygdaloid nucleus and enhances anxiety and conditioned fear responses (Cordero et al., 1998; Conrad et al., 2004; Mitra and Sapolsky, 2008). The acquisition of auditory emotional memories in the amygdala is associated with neuronal plasticity in the basolateral amygdala and medial geniculate nucleus (MG, auditory thalamus) (Maren et al., 2001; Poremba and Gabriel, 2001) (Fig. 1). Both brain areas exhibit associative plasticity of spike firing during fear conditioning (Maren et al., 2001). In contrast to the MG, the lateral amygdala receives only indirect projections from the lateral geniculate nucleus (LG) of the visual thalamus (LeDoux et al., 1984; McDonald, 1998; Aboitiz et al., 2003) (Fig. 1).

Chronic stress also alters dendritic architecture and function of brain areas related to memory and emotional processing, such as the hippocampus, the amygdala and medial prefrontal cortex (Magariños and McEwen, 1995; McEwen and Chattarji, 2004). Similarly, the auditory system is sensitive to stress-induced damage. For example, in rats, chronic stress causes dendritic atrophy in the

*Abbreviations:* ACx, primary auditory cortex; BNST, bed nucleus of stria terminalis; CORT, corticosterone; CS, conditioned stimulus; dB, decibel; ELISA, enzyme-linked immunoassay; GCs, glucocorticoids; GR, glucocorticoid receptor; HPA, hypothalamus–pituitary–adrenal axis; IC, inferior colliculus; LG, lateral geniculate nucleus; MG, medial geniculate nucleus; ms, millisecond; MR, mineralocorticoid receptor; s, second; SC, superior colliculus; US, unconditioned stimulus.

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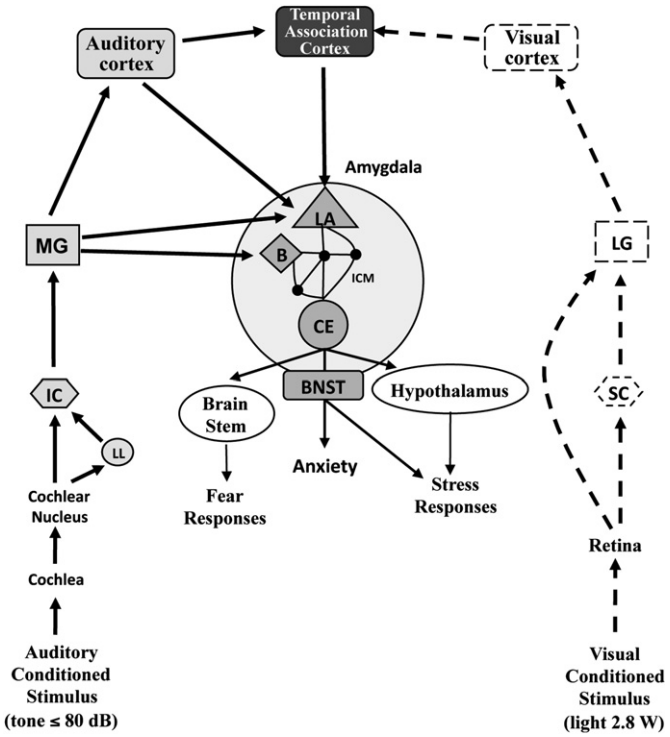


**Fig. 1.** The main ascending neuronal pathway involved in auditory and visual emotional processing in rats. The auditory and visual systems have direct and indirect connections to the amygdala. This scheme illustrates only the direct connections between the major nuclei of the auditory and visual pathway to the amygdala. Auditory conditioned stimuli (tone, CS, continuous line) are received in the cochlear nucleus and send projections to the lateral lemniscus (LL) and inferior colliculus (IC). From the IC, efferents are sent to the medial geniculate nucleus (MG) and the auditory cortex, which in turn projects glutamatergic inputs to the lateral and basal amygdaloid nuclei. Visual conditioned stimuli (light, dotted line) are received in the retina and are then sent to the lateral geniculate nucleus (LG) and the superior colliculus (SC). From the LG, projections are sent to the primary visual cortex. Information received in the lateral and basal amygdaloid nuclei is sent to the central amygdaloid nucleus through the intercalated cell masses. The central amygdaloid nucleus projects to hypothalamic sites and several brain stem nuclei that participate in the stress and fear responses (such as freezing). From the central amygdaloid nucleus, projections are directed to the bed nucleus of stria terminalis (BNST), inducing anxiety.

inferior colliculus (IC), a main component of the auditory nervous system which has a critical role in auditory fear learning (LeDoux et al., 1984). In contrast, stress does not affect superior colliculus (SC) neurons related to visual processing (Dagnino-Subiabre et al., 2005). The IC and SC are counterparts and their principal neuron types are flat neurons and wide-field neurons respectively. These neurons project to collothalamic nuclei, for example, flat neurons in the IC project to MG and wide-field neurons in the SC project to the LG (visual thalamus), thus both types of neurons are comparable (Peruzzi et al., 2000; Hilbig et al., 2000).

The stress-induced IC dendritic atrophy is correlated with auditory learning impairment. Using a two-way signaled active avoidance (2-AA) learning procedure, where rats are trained in a shuttle box to avoid a foot shock signaled by an auditory or visual stimulus, chronic stress strongly impairs the conditioned avoidance response to auditory stimuli, but does not affect visual avoidance conditioning (Dagnino-Subiabre et al., 2005). By fifteen days after stress, the IC neurons recover their structure completely, and this neural plasticity is correlated with improved auditory learning (Dagnino-Subiabre et al., 2005). A recent study using micro Positron Emission Tomography supports these findings, in that chronic mild stress induces significant decrease of glucose metabolism in the IC, but not in the SC (Hu et al., 2010).

Stress impairs other brain nuclei of the rat auditory system. Magnocellular neurons of the MG and pyramidal neurons of the primary auditory cortex (ACx) are atrophied after chronic stress (Bose et al., 2010; Dagnino-Subiabre et al., 2009). These findings raise the question of whether auditory emotional processing is affected by GCs. The objective of this study was to test whether chronic treatment with stress levels of corticosterone (CORT; the main GC of rats) affects the dendritic morphology of IC and SC neurons, and alters auditory fear learning and visual fear conditioning in rats.

## 2. Materials and methods

### 2.1. Experimental animals

Adult male Sprague–Dawley rats (180–200 g, ~50 days old at the start of the experiment) were housed in groups of three under a 12/12 light/dark cycle (lights on at 7:00 A.M.), with ad libitum access to food and water in a temperature–humidity-controlled room (21 °C, 55%). Rats were randomly assigned to two groups: vehicle-treated, $n = 60$ and CORT-treated, $n = 60$, *for* behavioral and *morphologic* studies. Vehicle animals, which were littermates of the CORT-treated animals, were housed in separate rooms and separate cages, and not subjected to any type of experimental stress. *All procedures related to animal maintenance and experiments were* approved by the Stanford University Administrative Panel on Laboratory Animal Care (APLAC), and the Institutional Animal Ethics Committee of the Faculty of Sciences-Universidad de Valparaíso (Chile). The experimental protocols were in accordance with the animal care standards in National Institutes of Health (NIH) guidelines. Efforts were made to minimize the number of animals used and their suffering. The following additional parameters were measured to monitor the overall effects of the CORT and vehicle administration: Body weight gain and anxiety level as determined by performance in the elevated plus-maze.

### 2.2. CORT treatment

Corticosterone (CORT) (Sigma–Aldrich, St. Louis, MO) was dissolved in 2.4% ethanol. Both CORT and vehicle experimental groups received the same amount of ethanol in their drinking water (2.4%). Vehicle or CORT was administered through the drinking water (400 μg CORT/ml) over a 10-day period. CORT drinking solution was made using a stock solution of CORT in 100% ethanol (16.6 mg/ml). This concentration and route of administration results in stress levels of serum CORT (Magariños et al., 1998; Conrad et al., 2004). CORT and vehicle were administered for 10 days, then removed and replaced with tap water. Behavioral and morphological endpoints were measured between 24 h and 48 h after the chronic CORT treatment. Separate sets of animals were used for behavioral, morphological and enzyme-linked immunoassay (ELISA) studies.

### 2.3. Plasma CORT measurement

We first analyzed the effects of CORT treatment on CORT plasma level. A separate set of animals was used to measure the concentration of CORT in plasma, in order to avoid the stressfulness of blood collection on morphological or behavioral experiments. One set of rats was sacrificed via decapitation at 09:00 h (vehicle, $n = 6$, CORT, $n = 6$) and other set prior to lights off at 19.00 h (vehicle, $n = 6$, CORT, $n = 6$) on day when behavior and morphological experiments were initially conducted. Blood (1 ml) was collected in heparinized microcapillary tubes and centrifuged (Model # MiniSpin Plus; Eppendorf AG, Hamburg, Germany) at 10,000 rpm for 10 min to obtain plasma and then stored at −70 °C. Total CORT was

determined by an Enzyme Immunoassay kit (Corticosterone Bio-Assay™, Catalog. # C7903-30) purchased from US Biological (Swampscott, MA). Optical density values were measured at 450 nm using a microplate reader (Model # Anthos 2010 Microplate Reader, Biochrom Ltd, UK). Samples were diluted 1:10 and then processed in duplicates and averaged final values were represented as μg/dL.

### 2.4. Behavioral testing

To rule out the possibility of CORT causing unspecific motor changes in the fear conditioning, morphologic and ELISA studies, an independent group of rats (vehicle, $n = 9$, CORT, $n = 9$) were tested for their locomotor activity and anxiety using the open field and elevated plus-maze tests respectively. These behavior tests were conducted 24 h after completion of the CORT treatment. All animals were naive to the test situations. Behavioral tests were carried out from 10.00 to 14.00 h in the test room. The activity of each rat was recorded by IP cameras (VIVOTEK, Sunnyvale CA, USA) fixed above the behavioral apparatus and connected to computer in another room outside of the vivarium. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan) and analyzed using ANY-maze video tracking system (Stoelting Co., Illinois, USA). The maze was wiped clean thoroughly with 5% ethanol solution after each trial. In all experiments, animals from vehicle and CORT were evaluated at the same time.

#### 2.4.1. Open field test

The behavior tests were conducted in a sound-proof and temperature-controlled ($21 \pm 1$ °C) room. Each rat was placed in the center of a black Plexiglass cage ($70 \times 70 \times 40$ cm) for 5 min. The noise into the open field was 40 dB (Precision sound level meter, Model # 1100, Quest Technologies, Oconomowoc, WI) and the arena was illuminated to 300 lux (measured by digital lux meter, Model # LX-1010B, Weafo Instrument Co., Shanghai, China). Time spent in the center and border zone of the arena, total distance travelled and average speed were analyzed from video recordings.

#### 2.4.2. Elevated plus-maze

Immediately after the analysis of the open field (approximately 10 s) we measured anxiety levels by using the elevated plus-maze test. Each rat was individually placed in an elevated plus-maze, consisting of two open arms ($60 \times 15$ cm each), two closed arms ($60 \times 15 \times 20$ cm each) and a central platform ($15 \times 15$ cm), arranged in a way so that the two arms of each type were opposite to each other. The maze was elevated 100 cm above the floor. The illumination was 300 lux in the open arms and 210 lux in the closed arms. At the beginning of each trial, animals were placed at the center of the maze, facing an open arm. During a 5-min test period, we recorded the frequency of open and closed arm entries, total arm entries, the amount of time spent in each section of the maze. The number of entries and time spent in the open arms, and the ratio of open to total arm entries (open/total $\times$ 100) were used as measures of the anxiety level (Dagnino-Subiabre et al., 2006a,b). Total arm entries were taken as an indicator of general locomotor activity. Entry into an arm was defined as the animal placing all four limbs onto the arm.

#### 2.4.3. Fear conditioning

*2.4.3.1. Apparatus and stimuli.* To measure fear conditioning, we used two modified observation chambers ($30 \times 24 \times 40$ cm; Med Associates, St. Albans, VT) contained in sound-proof cubicles (Med Associates). Two types of conditioned stimuli (CS) were applied: a 5 kHz tone amplified to 80 dB, with the speaker mounted in front of the pellet receptacles, or a light pulse (2.8 W), with the LED stimulus light accessory mounted above the pellet receptacles. The unconditioned stimulus (US) was a brief (500 ms) delivery of direct current (0.5 mA) produced by a grid floor shocker (Med Associates). Both CS and US delivery were regulated by computer-based operant software (MedPC-IV; Med Associates). Behavior was videotaped for analysis using a webcam (Logitech, C905, Fremont, CA) mounted to the ceiling. The fear conditioning chambers were cleaned with 5% ethanol each time a rat was removed from the chamber.

*2.4.3.2. Auditory and visual fear conditioning procedure.* We used one set of rats for auditory fear learning (vehicle, $n = 15$, CORT, $n = 15$) and another set for visual fear conditioning experiments (vehicle, $n = 15$, CORT, $n = 15$).

Fear conditioning was conducted over three days, beginning one day after the end of chronic CORT treatment. Rats were placed in the conditioning chamber for a 10-min acclimation period, without CS presentation (day 0). Rats were then returned to their home cages and colony room. On day 1, all rats were first exposed to a 3-min acclimation period, followed by five habituation trials. For auditory fear learning, we used a 20-s tone (5 kHz, 80 dB), and for visual fear conditioning, we used a 20-s light pulse (2.8 W) in one habituation trial. Rats then underwent fear conditioning, consisting of seven conditioning trials. During each, the presentation of CS coterminated with the foot shock US (500-ms, 0.5 mA). Rats were returned to their home cages for 1 h; afterwards, they were returned to the conditioning chamber and received extinction trials consisting of CS alone. To ensure comparable levels of extinction learning between vehicle and CORT groups, on day 1, extinction trials continued until the rats exhibited less than 10% (3 s) freezing on four consecutive trials. The number of trials to criterion was similar across experimental groups. After extinctions trials, rats were returned to their home cages and to the housing room.

On day 2, rats were placed in the conditioning chamber for a 3-min acclimation period, followed by extinction trials consisting of fifteen CS alone to analyze the recall of fear learned during the conditioning trials. Freezing was continuously recorded during and later scored to determine the degree to which rats acquired the conditioned association (see Measurement of freezing behavior). Mean inter-trial interval was 4 min throughout habituation, conditioning and extinction trials.

*2.4.3.3. Measurement of Freezing Behavior.* Freezing was used to measure the conditioned emotional fear response and was defined as the absence of any visible movements with the exception of respiration-related movement and non-awake or resting body posture (Monfils et al., 2009; Paré et al., 2004). For all trials, the duration of freezing during the 20-s CS was measured with a digital stopwatch by an observer blind to experimental conditions. Percent freezing (seconds spent freezing/20-s CS) during habituation, fear conditioning, and extinction were calculated.

*2.4.3.4. Sensitivity to foot shock.* One day after the completion of all extinction trials, animals were tested for sensitivity to foot shock. Rats were placed into the conditioning chamber and given unsignaled foot shocks of increasing amplitudes beginning with 0.005 mA. Foot shock was increased in 0.05 mA increments until a jumping response was induced. An observer blind with respect to experimental group assignment measured thresholds.

### 2.5. Morphological data analysis

A new set of rats (vehicle, $n = 9$, CORT, $n = 9$) was used for morphometric studies. One day after the end of vehicle and CORT administration, animals were killed under deep anesthesia with sodium pentobarbital. The brain was removed quickly and

processed using FD Rapid Golgi Stain™ kit (FD Neuro Technologies, Inc., Ellicott City, MD, USA). Both hemispheres were cut in the sagittal plane using a cryostat (Microm International) and 100-μm-thick sections were collected onto super-frost plus slides. Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene, and cover-slipped. Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed. To compare the present study with our previous results (Dagnino-Subiabre et al., 2005), we analyzed the effects of vehicle and CORT administration on the flat IC neurons and the wide-field neurons of the SC. The morphometric analysis of both types of neurons was restricted to those located between bregma −1.2 mm and 6.1 mm in the IC, and between bregma −0.1 mm and 6.8 mm in the SC. Random selection was made of 10 flat neurons and 10 wide-field neurons, in the center of the IC and SC respectively, which fulfilled the following selection criteria: (1) presence of untruncated dendrites, (2) consistent and dark impregnation along the entire dendritic field, and (3) relative isolation from neighboring impregnated neurons to avoid overlap. In order to reduce error in data acquisition and subjectivity of the experimenter, the latter was blinded to treatment (but knew whether the sample was from the IC or SC). Camera lucida tracings (BX31-U-DAL 10X, Olympus Co., Tokyo, Japan) were obtained from selected neurons and then scanned (eight-bit grayscale TIFF images with 1200 d.p.i. resolution; EPSON ES-1000C) along with a calibrated scale for subsequent computerized image analysis. Custom designed macros embedded in NIH Image 1.6 software were used for morphometric analysis of digitized images. Dendritic length and the number of branch (bifurcation) points were determined in each neuron.

### 2.6. Statistical analysis

Locomotor activity, anxiety, foot shock sensitivity, and morphological studies were analyzed by a Student's unpaired *t*-test. Body weight, CORT plasma levels and percent freezing during fear conditioning were analyzed using two-way repeated-measures ANOVA [Body weight [groups (vehicle, CORT) × Days (1, 4, 7, and 10)]; CORT plasma levels [groups (vehicle, CORT) × Hours (09.00 h, 19.00 h)]; Percent freezing [groups (vehicle, CORT) × trials (habituation, conditioning, extinction, recall)] followed by a Bonferroni post hoc comparisons test. Results are presented as the mean ± SEM. A probability level of 0.05 or less was accepted as significant.

## 3. Results

### 3.1. Effects of CORT on physiological parameters

Fig. 2A shows level of circulating CORT on morning (09:00 h) and evening (19.00 h) after 10 days of vehicle or CORT administration. A 2 × 2 mixed factor ANOVA with treatment (vehicle, $n = 6$, CORT, $n = 6$) as the between-subjects factor and time (09.00 h and 19.00 h) as the repeated measure showed no significant differences in CORT levels between experimental groups obtained at 19.00 h (CORT: 17.57 ± 2.06, $n = 6$; vehicle: 23.73 ± 3.13, $n = 6$; $p > 0.05$). However, CORT-treated animals had higher CORT levels than vehicle controls at the morning, a significant treatment by hour interaction, ($F_{(1,10)} = 7.14$, $p < 0.05$), and a significant main effect of treatment at 09:00 h (CORT = 18.33 ± 3.73, Vehicle = 5.67 ± 1.73, $p < 0.001$).

Body weight was measured daily to validate that 10 days of CORT administration reduced weight gain, as occurs with chronic stress; this was the case (Fig. 2B). A 2 × 4 mixed factor ANOVA with treatment (vehicle, $n = 9$, CORT, $n = 9$) as the between-subjects factor and day (1, 4, 7, and 10) as the repeated measure showed

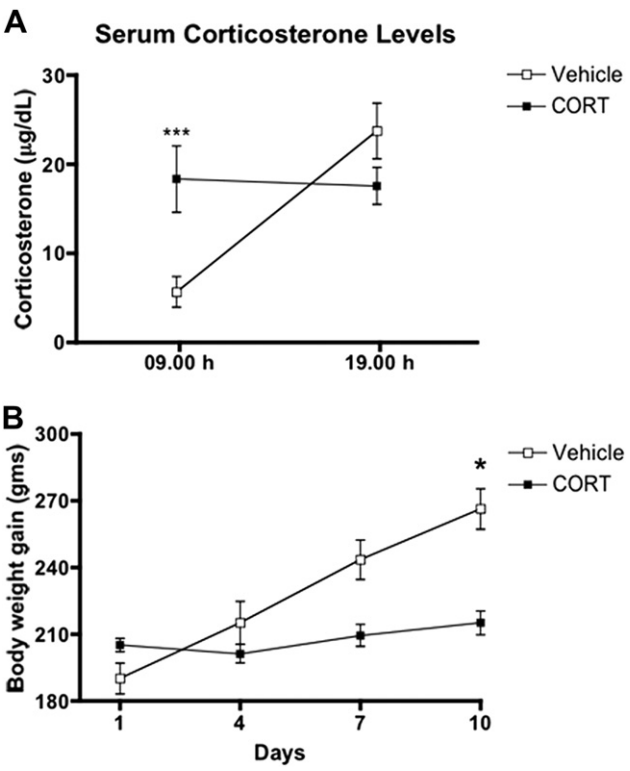


**Fig. 2.** The influence of chronic CORT treatment on CORT plasma level and body weight. (A) Rats treated with CORT in their drinking water had significantly increased serum CORT levels in the morning (09:00 h). (B) Although all rats had similar body weights at the start of the study, rats given CORT in their drinking water (CORT) failed to gain weight. In contrast, vehicle-treated rats gained weight gradually throughout the study (Vehicle). Significant treatment by day interaction, $F_{(3,48)} = 105.2$, $p < 0.0001$. Data are represented by mean ± SEM.

a significant treatment by day interaction, ($F_{(3,48)} = 105.2$, $p < 0.0001$), a significant main effect of treatment, ($F_{(1,16)} = 4.9$, $p < 0.05$), and a significant main effect of day, ($F_{(3,48)} = 189.4$, $p < 0.0001$). However, rats that received CORT showed decreased weight gain during 10 days of treatment, relative to vehicle control rats ($p < 0.05$).

### 3.2. Effects of CORT on locomotor activity and anxiety

CORT administration did not affect locomotor activity, including the total distance travelled, average speed, or time spent in central and border zone of the arena (Fig. 3). Moreover, CORT treatment did not affect measures of anxiety (i.e., the frequency of open arm entries or time spent in open arms in the elevated plus-maze, as well as the ratio of open to total arm entries) (Fig. 4). There were no treatment differences in the number of total arm entries.

### 3.3. Auditory and visual fear conditioning

CORT treatment did not significantly affect unconditioned responses to tone alone (Fig. 5A). During the habituation phase, there was no main effect of treatment on freezing ($F_{(1,16)} = 1.12$, $p > 0.05$) and no interaction of group and trial ($F_{(4,16)} = 0.36$, $p > 0.05$). In the conditioning phase, CORT treatment significantly decreased the auditory conditioned responses compared with vehicles; there was a significant difference between the CORT and vehicle groups ($F_{(1,28)} = 11.52$, $p < 0.01$), and a significant interaction of group and trial ($F_{(6,168)} = 3.64$, $p < 0.01$) (Fig. 5A). Freezing percentage varied significantly across trials ($F_{(6,168)} = 15.87$,

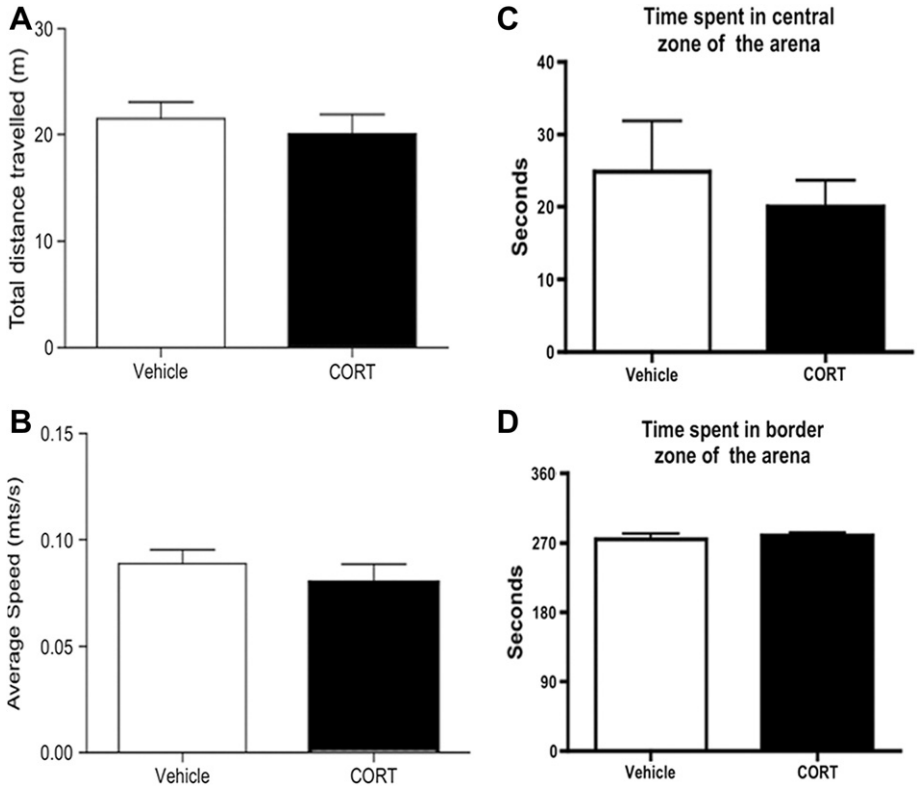


**Fig. 3.** Effect of CORT on locomotor activity in rats. CORT administration did not affect the total distance travelled (A), total average speed (B), time spent in center (C), and time spent in perimeter (D) in a 5 min observation period. Values are the mean ± SEM.

$p < 0.0001$), with both CORT and vehicle groups acquiring the auditory conditioned fear response. In the extinction phase, the interaction between the experimental groups with trials was altered by CORT administration ($F_{(14,392)} = 7.31$, $p < 0.001$) (Fig. 5A). In both groups, the conditioned fear responses were diminished with repeated presentation of tone alone ($F_{(14,392)} = 45.81$, $p < 0.0001$) (Fig. 5A), and CORT significantly reduced the extinction ($F_{(1,28)} = 4.86$, $p < 0.05$).

In the recall phase, vehicle- and CORT-treated rats showed equivalent recall of fear conditioning (group effect: $F_{(1,16)} = 0.46$, $p > 0.05$) (Fig. 5A), with such recall decreasing in both groups ($F_{(14,224)} = 6.11$, $p < 0.0001$).

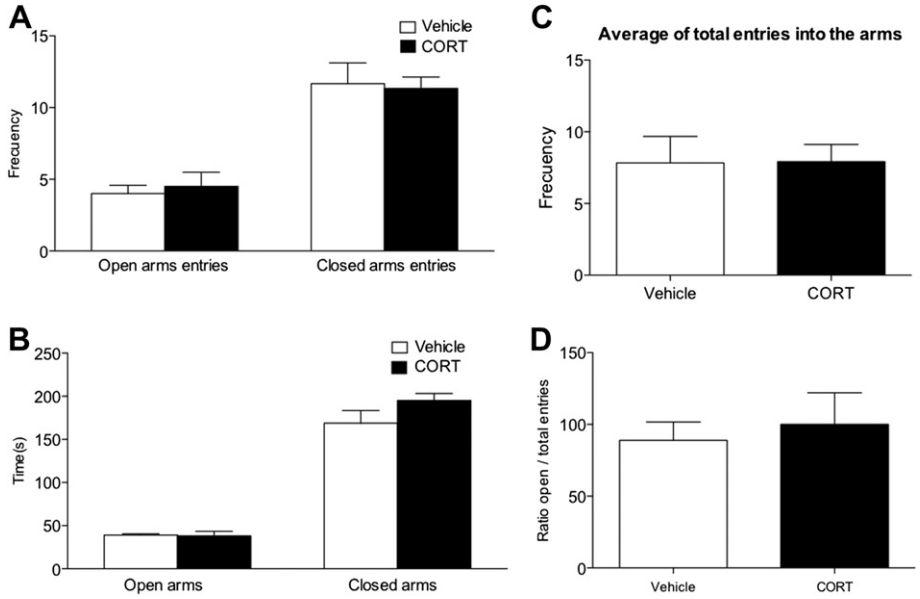


**Fig. 4.** Effect of CORT administration on anxiety. CORT did not affect the frequency of entries (A) and the time (B) spent on open arms of the elevated maze, total arm entries (C), and the ratio of open/total arm entries (D). These results indicate that CORT did not affect anxiety. Values are the mean ± SEM.

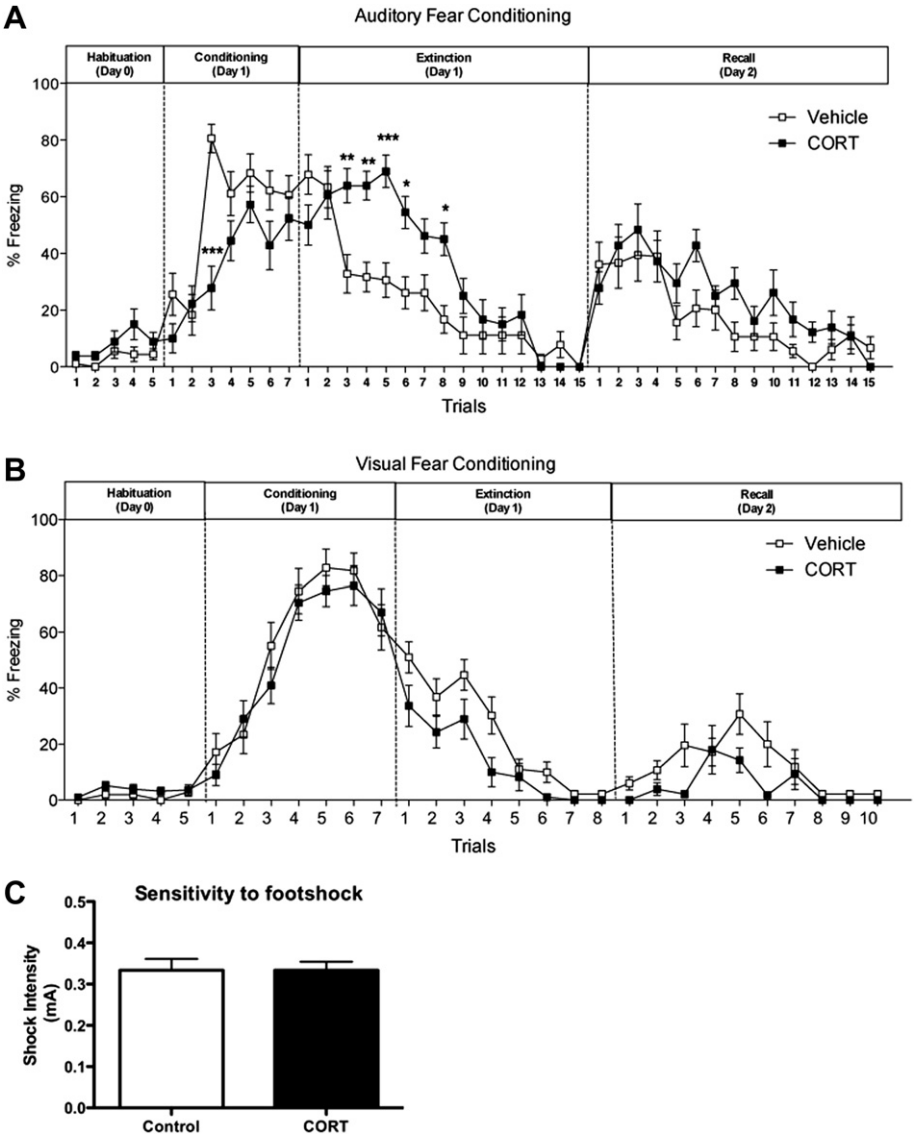


**Fig. 5.** Effect of CORT administration on fear conditioning. Mean and average percent freezing to tone (A) and light (B) in control (open squares, $n = 9$) versus CORT-treated rats (filled squares) across habituation, conditioning, extinction, recall, and trials. CORT impaired the extinction of auditory fear learning [interaction ($F_{(14,224)} = 2.51$), $p < 0.01$], but did not affect visual fear conditioning. (C) Influence of CORT on foot shock response thresholds for the experimental groups. CORT did not alter sensitivity to foot shock. Vertical bars represent SEMs.

Since CORT decreased auditory fear conditioning and extinction, we analyzed the CORT effects on visual fear conditioning and extinction for comparison. CORT did not affect the freezing percentage during the habituation phase (Fig. 5B). There was no main effect of treatment on freezing ($F_{(1,28)} = 3.88$, $p > 0.05$) and no interaction of group and trial ($F_{(4,112)} = 0.48$, $p > 0.05$). During conditioning trials, the percentage freezing varied significantly across trials ($F_{(6,168)} = 36.88$, $p < 0.0001$), with both groups acquiring a visual conditioned fear response. There were no group ($F_{(1,28)} = 0.57$, $p > 0.05$) or interaction effects ($F_{(6,168)} = 0.71$, $p > 0.05$). Both vehicle- and CORT-treated rats showed a diminished visual conditioned fear response with repeated presentation of light alone through extinction and recall phases [Extinction: ($F_{(7,196)} = 19.77$, $p < 0.001$); Recall: ($F_{(9,252)} = 5.88$, $p < 0.001$)]. For visual fear conditioning, CORT did not affect the rate of extinction and recall compared to vehicles (Fig. 5B) [Extinction: effect of treatment, ($F_{(1,28)} = 3.90$, $p > 0.05$); for interaction of group and trial, ($F_{(7,196)} = 0.21$, $p > 0.05$)] [Recall: effect of treatment, ($F_{(1,28)} = 3.82$, $p > 0.05$); for interaction of group and trial, ($F_{(9,252)} = 0.49$, $p > 0.05$)] compared to vehicles (Fig. 5B).

### 3.3.1. Foot shock sensitivity

Vehicle- and CORT-treated rats showed comparable sensitivity to lower shock intensity (CORT $= 0.33 \pm 0.02$, Vehicle $= 0.33 \pm 0.03$, $p > 0.05$) (Fig. 5C), ruling out increased sensitivity as an explanation for the CORT effect on auditory fear learning.

### 3.4. Effects of CORT treatment on dendritic morphology of the inferior and superior colliculus

Photomicrographs of representative Golgi-impregnated flat neurons of the IC from vehicle- and CORT-treated animals, and their respective camera lucida drawings are shown in Fig. 6A. CORT decreased the number of branch points in flat neurons in the IC (CORT: $2.8 \pm 0.3$, $n = 9$; Vehicle: $5.1 \pm 1.1$, $n = 9$; $p = 0.023$), but did not change total dendritic length (Fig. 6B).

Photomicrographs of representative Golgi-impregnated neurons of the SC from vehicle- and CORT-treated animals, and their respective camera lucida drawings are shown in Fig. 6A. CORT did not affect either dendritic length neurons or branch points of the SC neurons (Fig. 6B).

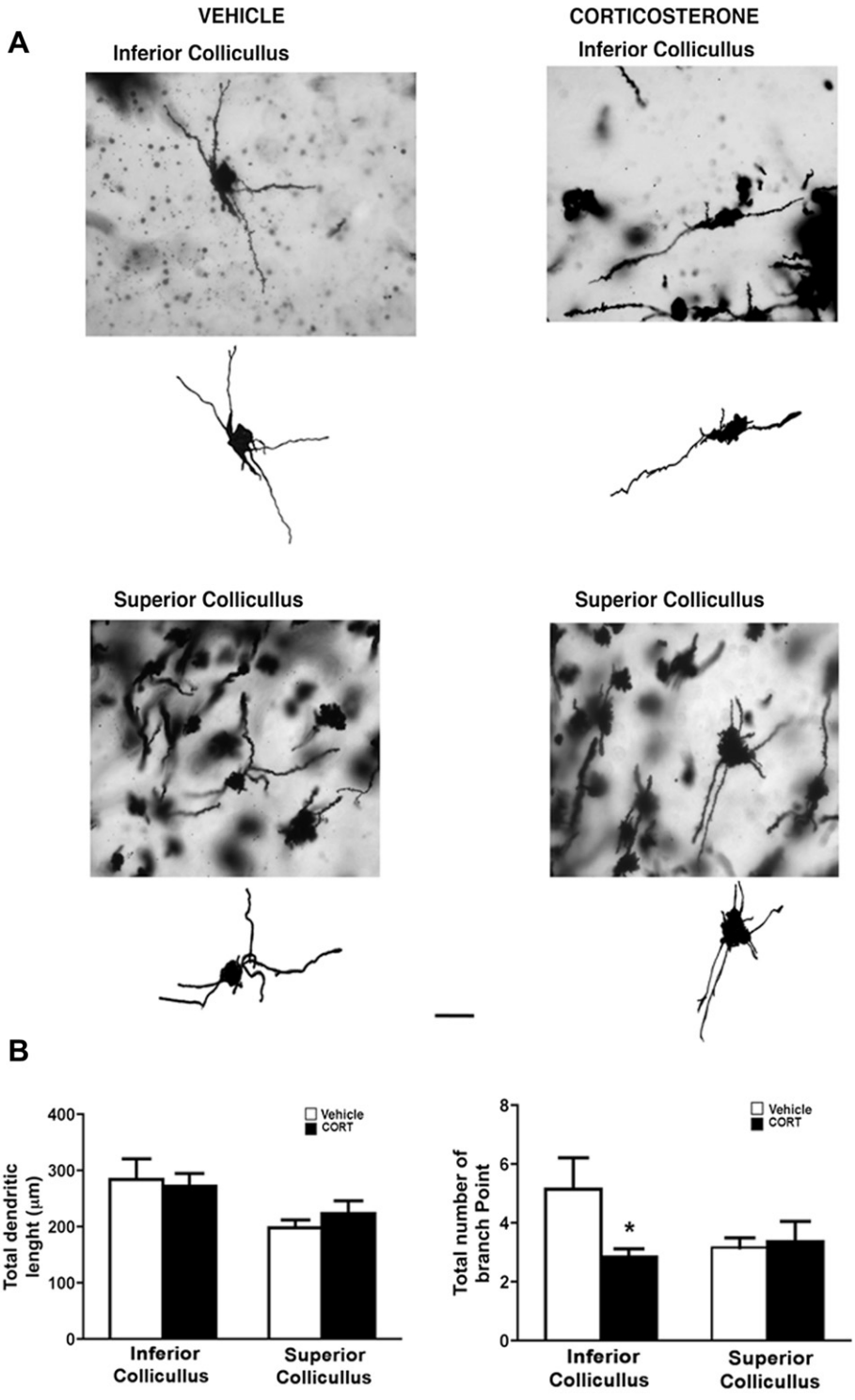


**Fig. 6.** Morphometric analyses of the IC and SC neurons. (A) Photomicrographs and camera lucida tracings of representative golgi-impregnated flat neurons of the IC and wide-field neurons of the SC, in vehicle- and CORT-treated rats. Scale bar, 20 μm. (B) Morphometric analysis of neurons from vehicle- and CORT-treated rats. After 10 days of CORT administration ($n = 9$ animals), the total branch number of the IC neurons was significantly reduced compared with vehicle-treated rats ($n = 9$ animals) ($^*p < 0.05$). There were no CORT-induced changes observed in the total dendritic length of SC neurons (CORT, $n = 9$ animals; vehicle, $n = 9$ animals). The values are the mean ± SEM. Asterisk (*) indicates significant differences relative to vehicle-treated rats.

## 4. Discussion

The present study shows that CORT administration impairs both auditory fear learning and extinction (Fig. 5), and decreases the dendritic arborization of the IC neurons (Fig. 6). On the other hand, CORT did not affect visual fear conditioning or dendritic morphology of SC neurons (Figs. 5 and 6).

### *4.1. Effects of chronic CORT administration on fear conditioning and neuronal morphology*

CORT impaired the acquisition of auditory conditioned fear responses and attenuated conditioned fear extinction (Fig. 5A). In contrast, CORT administration did not affect visual fear conditioning (Fig. 5B). These results were unlikely to be caused by changes in

sensitivity to foot shock between vehicle- and CORT-treated animals, as both groups exhibited similar foot shock threshold (Fig. 5C).

CORT-induced alterations of auditory fear conditioning and extinction could be related to decreased dendritic arborization in IC induced by CORT (Fig. 6A and B). In addition, it is possible that neuronal morphologic changes in other regions, such as in the MG or auditory cortex, may affect auditory fear learning. Dendritic atrophy in neurons of the IC, MG or auditory cortex might impair the ability to receive and deliver the auditory CS to the amygdala and decrease freezing responses through the conditioning trials (Fig. 5A). Lesion studies on the IC and MG demonstrate that an association between the auditory CS and foot shock US is necessary to acquire aversive memories (LeDoux et al., 1984). During the extinction trials, there is acquisition of a new memory concerning the failure to associate tone with foot shock, and freezing then is decreased in the extinction phase. Regardless of whether CORT treatment decreased the dendritic arborization of IC neurons, the deliver of the auditory CS to the amygdala could be decreased in the extinction trials, resulting in CORT-treated rats being slower to extinguish learned fear (Fig. 5A). These results are supported by our previous finding regarding the effects of chronic stress on auditory learning during avoidance conditioning. Fear conditioning and 2-AA are associated with auditory learning (Dagnino-Subiabre et al., 2005, 2009). Stress-induced IC dendritic atrophy correlated with auditory learning impairment in the 2-AA (Dagnino-Subiabre et al., 2005). On the other hand, CORT-induced IC dendritic atrophy also correlated with auditory learning impairment on fear conditioning (Fig. 5A).

CORT-induced impairment of fear extinction (Fig. 5A) appears to contradict previous findings related with the effects of chronic stress on fear-conditioned extinction. Stress-induced IC dendritic atrophy was not associated with extinction impairment of fear conditioned after one week (Miracle et al., 2006), 15 days (Dagnino-Subiabre et al., 2009) or 21 days (Baran et al., 2009) of restraint stress. In this respect, anxiety may affect freezing during extinction trials in fear conditioning; for example, the anxiogenic drugs Amphetamine (Vuong et al., 2010) and Yohimbine (Braun et al., 2011) blunt freezing throughout extinction (Mueller et al., 2009). Conversely, the anxiolytic effect of Fluoxetine (Ampuero et al., 2010) and Citalopram (Sun et al., 2010) correlate with increased freezing during extinction (Burghardt et al., 2007). Anxiety is regulated by the basolateral amygdala and chronic stress induces dendritic hypertrophy in this region (Vyas et al., 2002); we speculate that this morphological alteration can increase the neuronal activity in the amygdala. Stress-induced IC dendritic atrophy could decrease the deliver of auditory CS to the amygdala, thus decreasing freezing on extinction trials. This alteration could be prevented by increases of neuronal activity in the amygdala of stressed rats. In our experiments, CORT administration did not affect anxiety (Fig. 4); consequently CORT-treated rats were slower to extinguish learned fear (Fig. 5). Effects of IC dendritic atrophy on auditory fear learning can be measured in behavioral paradigms that induce low levels of freezing in the animals. In support of this idea, control rats subjected to 2-AA have less freezing, as compared to fear conditioning (Bose et al., 2010; Choi et al., 2010), and thus stressed rats show decreased auditory fear learning when subjected to 2-AA (Dagnino-Subiabre et al., 2005). In contrast, stressed rats subjected to fear conditioning did not differ in freezing responses on conditioning trials (Dagnino-Subiabre et al., 2009). In conclusion, effects of IC dendritic atrophy on auditory fear learning could be measured in less aversive behavioral paradigms due to the low levels of freezing that these induces on rats.

Neither CORT administration nor restraint stress affected SC morphology (Dagnino-Subiabre et al., 2005, 2009); as would thus be expected because CORT treatment did not affect visual fear learning (Fig. 5B). It is difficult to determine the impact of CORT on visual extinction and recall trials because the animals showed less freezing on the last conditioning trial with respect to the preceding trial (Fig. 5B). Apparently the rats in the last visual conditioning trial began to adapt to the unconditioned stimulus, as has been reported previously (Bouton et al., 2008).

The dendritic lengths of IC flat neurons measured in this study were smaller than other Golgi analyses of dendritic morphology in IC neurons (Malmierca et al., 1993, 1995, 2011). These studies used female *Rattus novegicus* rats (Malmierca et al., 2011), in contrast to our *Sprague–Dawley* males. Ovarian hormones and strain type might affects neuronal morphology; as precedent, 17 β-estradiol increases the apical dendritic length of hippocampal CA1 pyramidal neurons in female rats (McLaughlin et al., 2010), and the total dendritic length of CA3 neurons from *Wistar* rats is greater than in *Sprague–Dawley* rats (Magariños and McEwen, 1995). Therefore, it is possible that the IC flat neurons from female *R. novegicus* are bigger than comparable neurons in male *Sprague–Dawley* rats.

### 4.2. Possible cellular mechanisms underlying dendritic changes in the inferior colliculus

The CORT effects in the IC may be due to direct actions via the GR in such neurons. GR are expressed in the IC (Mazurek et al., 2010) and the ultradian release of CORT from the adrenal glands regulates the activation of GR in neurons (Stavreva et al., 2009). CORT binds to cytosolic GR, inducing GR dimerization and translocation to the nucleus, thereby increasing the gene expression of pro-plasticity genes, such as neuronal cell adhesion molecules, NCAM and L1 (De Kloet et al., 1998; Sandi, 2004; Meltser and Canlon, 2011). Moreover, the CORT–GR complex increases the nuclear translocation of NF-kB, increasing expression of neurotrophins such as BDNF and NT-3 in IC neurons (Reichardt, 2006). These molecules are implicated in neurite extension, cell survival and synaptic plasticity (Kiss et al., 2001). In contrast, both chronic CORT administration (Fig. 2A) and chronic mild stress affects the ultradian CORT release and increases the plasma CORT levels (Grippo et al., 2005; Ushijima et al., 2006). These alterations are correlated with a significant decrease of glucose metabolism in the IC after chronic mild stress, but not in the SC, suggesting that the IC could be more sensitive to stress and higher level of CORT compared to the SC (Hu et al., 2010). Chronic stress down regulates GR expression in the IC at the time that plasma CORT levels have returned to baseline (Mazurek et al., 2010). Therefore, chronic CORT administration could down regulate the GR expression in the IC and decrease the levels of pro-plasticity proteins; this may lead to decreased dendritic arborization of the IC neurons. We propose that chronic CORT treatment may have either none or an opposite effect in the SC.

Dexametasone and RU 486 are agonist and antagonist of GR respectively (Jadavji et al., 2011). In this context, we speculate that the circadian level of GR agonists, might increase the neuronal activity in the IC, conversely, GR antagonist, may inhibit the GR and NF-kB nuclear translocation and decrease the expression of neurotrophic factors in the IC neurons, which in turn may affect the neuronal morphology of the IC (as shown in Fig. 6). Regardless of whether chronic CORT treatment down regulates GR expression in the IC, we speculate that the mineralocorticoid receptor (MR) antagonist Spironolactone (Kumar et al., 2007) could have a possible neuroprotective effects on IC neurons. Spironolactone might decrease the availability of MR in the cytoplasm of IC neurons, increasing the probability that CORT binds to GR. As a result, the GR–CORT complex and NF-kB nuclear translocation up regulate the expression of protective neurotrophic factors and pro-plasticity proteins in IC neurons.

### 4.3. Auditory and visual pathways, and their connectivity with the amygdala: the possible role in the CORT effects on fear conditioning

Other possible explanations for our finding is that CORT-induced IC plasticity is indirectly produced by morphologic changes propagated from upper or lower levels of the amygdala–auditory pathway (for example, from the basolateral amygdala or cochlear nucleus, respectively; Fig. 1). There exists evidence indicating that an intact basolateral amygdaloid nucleus is essential for developing the associative neuronal plasticity in the MG throughout aversive learning (Maren et al., 2001). In addition, CORT produces dendritic hypertrophy of the spiny pyramidal-like neurons of basolateral amygdaloid nucleus (Mitra and Sapolsky, 2008). It is possible that the chronic CORT administration in the present study produces plasticity in the basolateral amygdaloid nucleus; while not sufficient to enhance anxiety, this is sufficient to produce morphologic changes in the MG. This process may be propagated to even lower levels in the auditory pathway and influence plasticity at the mesencephalic level in the IC. In contrast to the MG, the neighboring LG of the visual thalamus does not directly project to the lateral amygdala (McDonald, 1998). During classical visual fear conditioning, the expression of conditioned fear is produced directly from both the SC by the lateral posterior nucleus–lateral amygdala pathway and the retina by the LG-primary visual cortex–temporal association cortex–lateral amygdala pathway (Doron and Ledoux, 1999; Shi and Davis, 2001). It is probably the case that such projections are not as robust as the auditory projections from the MG to the lateral amygdala (LeDoux et al., 1990). In this context, the CORT-induced structural changes in lateral amygdala may not be propagated to the LG of the visual thalamus. On the other hand, there are direct projections from the basal amygdala to IC (Marsh et al., 2002). Therefore, CORT administration can increase the NMDA-receptor-mediated synaptic currents in the basolateral amygdala, which in turn, could directly reduce NMDA-receptor-mediated synaptic currents in the IC. NMDA-induced increases in intracellular calcium concentrations regulate BDNF expression by the aryl hydrocarbon receptor and BDNF is known to be a key regulator of dendritic morphology (Lin et al., 2009; Lakshminarasimhan and Chattarji, 2012); thus, CORT administration can increase both BDNF expression and growth of dendrites in the basolateral amygdala, while down regulating BDNF and inducing dendritic atrophy in the IC (Lakshminarasimhan and Chattarji, 2012).

The IC is strongly innervated by afferents from the cochlear nucleus (Fig. 1); thus, neuronal plasticity in the cochlea might be propagated to upper levels of the auditory pathway. GRs are expressed in the hair cells, spiral ganglion neurons and the spiral ligament of the cochlea (Meltser et al., 2009; Tahera et al., 2006; ten Cate et al., 1992, 1993; Zuo et al., 1995). Also GR have a protective role after acute stress and acoustic trauma (Meltser and Canlon, 2011; Kraus and Canlon, 2012). Thus, we propose that both chronic CORT administration and chronic restraint stress have negative effects on the cochlea; this may be due to down regulation of GR mRNA expression and to decreased expression of neurotrophic factors in the cochlea. Moreover, it is possible that a mixture of direct and indirect effects of CORT on the IC could be necessary to produce plasticity in the IC neurons.

### 4.4. Conclusions

The data presented here demonstrate that CORT treatment impaired auditory fear learning and the extinction of auditory conditioned fear, without affecting the visual fear conditioning. Additionally, CORT treatment decreased dendritic arborization of the IC neurons, a major auditory nucleus, but did not affect the SC. Overall, these results show a sensory modality-specific CORT effect on auditory fear processing in the rat brain. Potentially, similar behavioral and morphological changes could be induced by psychosocial stress on sound processing in humans (Simoens et al., 2007).

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