



## Research report

## Chronic stress induces dendritic atrophy in the rat medial geniculate nucleus: Effects on auditory conditioning

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## ABSTRACT

Chronic stress induces dendritic atrophy in the inferior colliculus (IC, auditory mesencephalon) and impairs auditory avoidance conditioning. The aim of this study was to determine in Golgi preparations and in cued fear conditioning whether stress affects other auditory components, like the thalamic medial geniculate nucleus (MG) or the posterior thalamic nucleus (PO), in Sprague–Dawley rats. Chronic restraint stress produced a significant dendritic atrophy in the MG (stress:  $407 \pm 55 \mu\text{m}$ ; control:  $808 \pm 120 \mu\text{m}$ ;  $p < 0.01$ ) but did not affect auditory fear conditioning. The last result was in apparent contrast with the fact that stress impairs both the acquisition of auditory avoidance conditioned responses and the dendritic structure in two major nuclei of the auditory system. In order to analyze this disagreement, we investigated whether the stress-related freezing to tone occurring in the fear conditioning protocol corresponded to a conditioned or an unconditioned fear response, using changes in tone instead of light throughout conditioning trials. Chronic stress significantly enhanced visual fear conditioning in stressed animals compared to controls (stress:  $58.9 \pm 8.42\%$ , control:  $23.31 \pm 8.01\%$ ;  $p < 0.05$ ), but this fear enhancement was related to unconditioned fear. Conversely, chronic stress did not affect the morphology of the PO (subserving both auditory and somatosensory information) or the corresponding auditory and somatosensory unconditioned responses (acoustic startle response and escape behavior). Our results suggest that the auditory conditioned stimulus can be processed in part independently of the IC and MG in the stressed animals, and sent to the amygdala via the PO inducing unconditioned fear. Comparable alterations could be produced in major depression.

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### 1. Introduction

Chronic stress affects the hippocampus, the amygdala and the medial prefrontal cortex (mPFC), leading to impairments in learning and emotional responses [19,25]. These alterations may contribute to the cognitive deficits of major depression [39]. In addition, chronic stress produces dendritic atrophy in the rat inferior col-

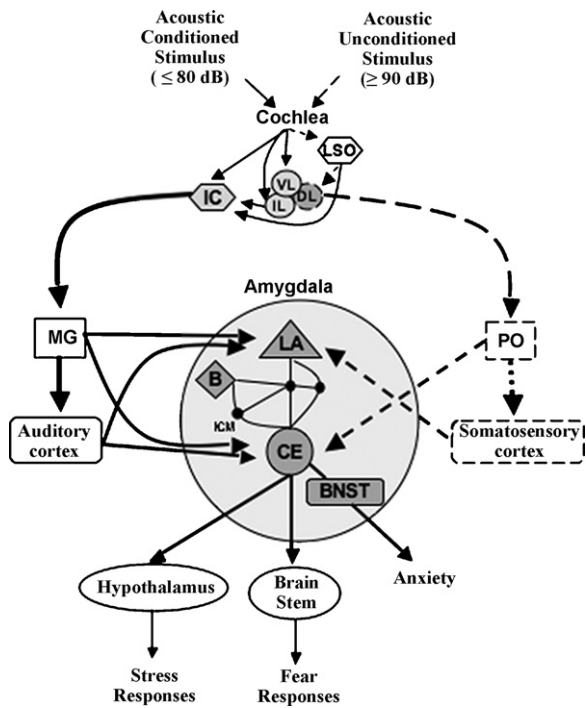
liculus (IC), a main component of the auditory nervous system, and decreases the conditioned avoidance response to auditory stimuli [8]. This indicates that the auditory pathway is sensitive to stress at least at the level of the midbrain. Auditory processing is conveyed by at least two different thalamic pathways to the amygdala, where the association between conditioned and unconditioned stimuli is supposed to take place [29,36]. The objective of this study was to determine whether the effects of stress in the auditory system are restricted to the mesencephalon, or whether additional auditory structures are affected. In addition, we analyzed which of the two thalamic pathways to the amygdala is more sensitive to stress.

Auditory stimuli lower or equal to 80 dB can acquire the ability to elicit conditioned fear responses [14,36]. Auditory stimuli are processed along a series of parallel pathways containing a large number of nuclei, which process specific aspects of the acoustic information as well as its emotional significance [24,35]. Auditory CS are conveyed centrally by the auditory nerve, which branches to

*Abbreviations:* ASR, Acoustic startle response; BLA, Basolateral complex of the amygdala; BNST, Bed nucleus of stria terminalis; CE, Central amygdaloid nucleus; CS, Conditioned stimulus; dB, decibel; IC, Inferior colliculus; ITC, Intercalated cell masses; LA, Lateral amygdala; MG, Medial geniculate nucleus; mPFC, Medial prefrontal cortex; ms, millisecond; PO, Posterior thalamic nucleus; PPI, Prepulse inhibition; s, second; US, Unconditioned stimulus.

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**Fig. 1.** Neuronal networks involved in the emotional processing of the auditory stimuli. Auditory conditioned stimuli ( $\leq 80$  dB, CS, continuous line) are received in the cochlear nucleus and sent to the ventral (VL) and intermediate (IL) nuclei of the lateral lemniscus, and then to the 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 amygdala (LA) and the central amygdaloid nucleus (CE). Information received in the LA and the basal nucleus (B) is sent to the CE through the intercalated cell masses (ICM). The CE projects to hypothalamic sites and several brain stem nuclei that participate in the stress and fear responses such as freezing. From the CE, projections are directed to the bed nucleus of stria terminalis (BNST) inducing anxiety. On the other hand, auditory unconditioned stimuli ( $\geq 90$  dB, US, dotted line) are received in the cochlear nucleus and sent to the lateral superior olive (LSO) and from there to the dorsal nucleus of the lateral lemniscus (DL). From the DL, projections are sent to the posterior thalamic nucleus (PO) and to the somatosensory cortex. The PO projects directly to the CE, inducing freezing.

distribute the acoustic information to the various cell groups in the cochlear nucleus (Fig. 1). From the cochlear nucleus the information is sent to several midbrain auditory relay nuclei, including the lateral superior olive and the nucleus of the lateral lemniscus, which converge on the IC of the midbrain (Fig. 1). The medial geniculate nucleus (MG), in the posterior thalamus, receives its main source of innervation from the IC and projects to the primary auditory cortex to process conscious and complex, higher-order information, such as the biological relevance of the acoustic stimuli [3,22,40] (Fig. 1). In addition, part of the acoustic information received in the MG is sent directly to the amygdaloid complex, providing the emotional aspects of auditory experiences [24,35,46]. Via the amygdalar pathway, neutral sensory stimuli (tones 20–80 dB, conditioned stimulus, CS) acquire the ability to elicit fear responses after being paired with an aversive unconditioned stimulus (footshock, US) [36]. The amygdaloid complex projects to several brain stem nuclei and hypothalamic sites that participate in fear responses such as freezing [29] (Fig. 1).

On the other hand, auditory stimuli higher or equal to 90 dB can acquire the ability to elicit unconditioned fear responses independently of the pathway formed by the IC–MG–auditory cortex or lateral amygdala [16,29] (Fig. 1). Acoustic information ( $\geq 90$  dB) is received in the cochlea, reaching the dorsal nucleus of the lateral lemniscus, from which it is sent to the posterior thalamic nucleus (PO), located just medial to the posterior intralaminar nucleus [16,29] (Fig. 1). The PO also receives somatosensory input, and its

efferents are sent directly to the central nucleus of the amygdaloid complex (CE) and to the primary somatosensory cortex, raising the possibility that CE receives auditory input from the thalamus, activating fear responses via this pathway [29] (Fig. 1).

First, we analyzed the effects of restraint stress on spontaneous motor activity and anxiety. Spontaneous motor activity is a measure of the whole motor activities of an animal [30]. Anxiety is an adaptive reaction induced when an animal is confronted with potential demands and dangers [15,28]. Indeed, anxiety has a key biological–adaptive role, which is highly conserved during evolution. Excessive levels of anxiety, or pathological anxiety, induce maladaptive responses [28].

Second, we used the Golgi stain to determine the effects of restraint stress in different thalamic nuclei (MG and PO), and studied both auditory conditioned and unconditioned responses in control and stressed rats, using cued fear conditioning and acoustic startle response (ASR) protocols. In addition, to analyze whether the stress-related increase in freezing to tone during auditory conditioning was elicited by a conditioned or unconditioned fear response, we used light instead of a tone in the conditioning trials (visual conditioning).

## 2. Materials and methods

### 2.1. Animals and restraint stress protocol

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-controlled room (23 °C). Rats were randomly assigned to two groups: control,  $n = 55$  and stress,  $n = 55$ , for behavioral and morphologic studies. Control animals, which were littermates of the stress-treated animals, were housed in separate cages and rooms and not subjected to any type of experimental stress. Restraint-stressed rats were placed in a plastic rat restrainer (6 cm diameter  $\times$  12 cm long; 6 cm diameter  $\times$  20 cm long as the rats grew) in their home cages for 2 h daily, beginning at 10 A.M.–noon for 15 consecutive days. Restraint occurred during the dark phase of the light cycle. All procedures related to animal experimentation were in accordance with NIH guidelines and were approved by the Institutional Animal Ethics Committee. 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 stress paradigms: percentage gain in body weight (net change in weight after experiment  $\times$  100/weight at the beginning of experiment), anxiety level as determined by performance in the elevated plus maze (see below), and relative adrenal weight (wet weight of adrenal glands in mg  $\times$  100/body weight in grams).

### 2.2. Behavioral procedures

#### 2.2.1. Spontaneous motor and locomotor activities

Twenty-four hours after completion of the stress protocol each rat was individually analyzed in the following order: spontaneous motor activity, locomotor activity and elevated plus-maze. First, each rat was placed for 30 min into a Plexiglass cage (30  $\times$  30  $\times$  30 cm<sup>3</sup>) located inside a soundproof chamber. The floor of the cage was an activity platform connected to an amplifier and an electromechanical counter (Lafayette Instrument Co, Lafayette, IN, USA) to monitor total motility of the rat, which corresponding to spontaneous motor activity. Locomotor activity was recorded with an infrared photocell activity monitor (Columbus Instruments, USA), provided with one array of 15 infrared photocells spaced 1 in. (2.54 cm) apart. Each animal was observed continuously via a video camera connected to a VHS tape-recorder. Scores of the spontaneous motor activity and locomotor activity were measured in counts (counts/30 min) generated from the electromechanical counter and the infrared photocell respectively. Video sequences were used for subsequent re-analysis.

#### 2.2.2. Elevated plus-maze

Immediately after the analysis of spontaneous motor and locomotor activities (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 (50  $\times$  10 cm<sup>2</sup> each), two closed arms (50  $\times$  10  $\times$  20 cm<sup>3</sup> each) and a central platform (10  $\times$  10 cm<sup>2</sup>), 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. At the beginning of each trial, animals were placed at the center of the maze, facing a closed arm. During a 5 min test period, we recorded the frequency of open and closed arm entries, total arm entries, and 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 [33,21]. 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. The maze was wiped clean thoroughly with 5% ethanol solution after each trial. All trials were conducted from 10 A.M. to 2 P.M.

### 2.2.3. Apparatus and stimuli

To measure fear conditioning and extinction we used one chamber of a two-way shuttle box (Model LE 916, Panlab S.L., Barcelona, Spain). Two types of CS were applied: 3000 Hz tone amplified to 80 dB, and light (10 W). The US was brief (500 ms) distributed delivery of direct current (0.5 mA) produced by a grid floor shocker (Model LE 10026; Letica, Barcelona, Spain). Both CS and US delivery was regulated by software Shutavoid (Panlab S.L., Barcelona, Spain).

In order to analyze the startle responses and prepulse inhibition (PPI), the rats were placed in a 20 × 20 × 20 cm<sup>3</sup> wire-mesh cage within a 67 × 67 × 67 cm<sup>3</sup> chamber lined with 5 cm acoustic foam. The cage was centered on a startle platform (Lafayette Instrument Co.) that uses a piezoelectric transducer to generate a continuous record of activity level. Sounds generated using an RP2.1 (Tucker-Davis Technologies) were delivered by a speaker (Optimus Bullet Horn Tweeter) mounted above the cage, about 20 cm from its center. Stimuli were adjusted for the speaker's frequency response using SigCal (Tucker-Davis Technologies). Sound intensities were measured using an ACO Pacific microphone (PS9200-7016) placed at a height approximating that of a standing rat's head.

In order to measure the escape response we used a two-way shuttle box (Lafayette Instrument Co, Lafayette, IN, USA) consisting of two stainless steel modular testing units (20 × 25 × 20 cm<sup>3</sup>). Each unit was equipped with an 18 bar insulated shock grid floor (Lafayette Instrument Co, Lafayette, IN, USA). Electric shocks were provided to the grid floor by a Master shock supply (Lafayette Instrument Co, Lafayette, IN, USA).

### 2.2.4. Auditory and visual fear conditioning procedure

We used one set of rats (control,  $n=10$ , stress,  $n=10$ ) for auditory fear conditioning and other set (control,  $n=10$ , stress,  $n=10$ ) for visual fear conditioning experiments.

One day after the analysis of spontaneous motor activity and elevated plus-maze both sets of rats were placed in the conditioning box for a 20 min acclimation period, without CS presentation (day 0). Rats were then returned to their home cages. On day 1, all rats were first exposed to 3 min acclimation period, followed by two habituation trials. For auditory fear conditioning we used 20 s tone (3 kHz, 80 dB) and for visual fear conditioning was used 20 s light (10 W) in one habituation trial. Rats did not return to their home cages. After 3 min of acclimation period, one conditioning trial was applied, consisting of two trials during which the tone or light CS (20 s) was paired with a footshock US (500 ms, 0.5 mA) that coterminated with the CS. Rats were returned to their home cages for 24 h. On day 2, rats were returned to the chamber and received one conditioning trial. Mean intertrial interval was 120 s throughout habituation and fear conditioning.

Freezing was continuously recorded during the conditioning session and later scored to determine the degree to which rats acquired the conditioned association (see Section 2.2.6). After conditioning, rats were returned to their home cages and to the colony room.

### 2.2.5. Extinction procedures

On day 3, rats were placed in the conditioning box for 3 min acclimation period, followed by extinction trials consisting of 15 CS alone. Freezing was recorded continuously during extinction trials. Consistent with the fear conditioning procedure, throughout extinction sessions the mean intertrial interval was 120 s.

### 2.2.6. 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 rest body posture [2,12]. 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 on day 3 was calculated and compared across groups.

### 2.2.7. Startle response and PPI

Two new sets of rats were used to analyze the stress effects on the ASR and PPI. One day after completing the elevated plus-maze test, rats were placed in the startle chambers and were presented 5 min background noise of 65 dB (the acclimation period). The test session consisted of two components: (1) Startle responses were elicited by 10 pulse trials consisting of 50 ms bursts of white noise at 90 dB (control,  $n=10$ , stress,  $n=10$ ) or 102 dB (control,  $n=10$ , stress,  $n=10$ ). The waveform of each response (the peak to peak voltage within 500 ms of the noise) was sampled at 10 kHz using an RP2.1 and processed using MATLAB. 2. To analyze the PPI, 10 prepulse + pulse trials were presented. Each trial, consisting 50 ms bursts of white noise at 65 dB (prepulse) preceded the onset of the 90 dB or 102 dB pulse by 100 ms. The five pulses and five prepulses were presented in random order and at intervals that again average 30 s (15–45 s).

Startle responses and PPI were measured in volts and represented as average of absolute level of startle and relative level of startle (PPI/startle response ratio), in ten pulse or prepulse + pulse trials respectively.

### 2.2.8. Escape behavior

A new set of rats (control,  $n=8$ , stress,  $n=8$ ) was used to study the escape behavior in a shock-escape paradigm. After the analysis of anxiety, each rat was individually placed in a two-way shuttle box and allowed to freely explore the apparatus for a 5 min habituation period, after which the rats were trained over 30 trials. Each trial consisted of the presentation of an aversive unconditioned stimulus, a 0.20 mA footshock, until the animal escaped to the opposite chamber, with maximum shock duration of 10 s. This aversive stimulation was the minimal shock intensity needed to elicit the unconditioned escape responses. An escape response was defined as a crossing to the opposite chamber during footshock stimulation [42,13]. If the rat failed to cross during shock delivery, it was considered as escape failure. Results are presented as the percentage of the number of escapes in 30 trials. We used a between-trial interval of 30 s.

### 2.3. Morphological data analysis

A new set of rats (control,  $n=7$ , stress,  $n=7$ ) was used to study the stress effects on the neuronal morphology of the rat MG and PO neurons. After completion of the spontaneous motor activity and elevated plus-maze test, each rat was euthanized under deep anesthesia with sodium pentobarbital. The brain was removed quickly and processed using FD Rapid GolgiStain™ kit (FD Neuro Technologies, Inc., Baltimore, MD, USA). Coronal sections were cut at 120 μm on a sliding cryostat (Microm, Walldorf, Germany). Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene, and coverslipped. Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed. Golgi studies in the rat MG show the presence of tufted, stellate and magnocellular neurons [31]. As a first stage, we restricted our morphometric analysis to the effects of stress on magnocellular neurons of the MG, because these cells constitute the major percentage of neurons present in this nucleus. The morphometric analysis was restricted to Interaural 3.8 mm and Bregma –5.2 mm [32]. On the other hand, in the PO we observed only neurons with a similar shape to interneurons [31]. We performed morphometric analysis of these neurons in control and stressed rats, in a region restricted to Interaural 5.7 mm and Bregma –3.3 mm [32]. The experimenter selected independently and at random 10 neurons per animal in the MG and the PO, 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 self-deception by the experimenter, the latter had no knowledge of whether the sample analyzed was from a control or a stressed rat, but unavoidably they knew whether the sample was from the MG or the PO nuclei. Camera lucida tracings (500X, BH-2, Olympus Co., Tokyo, Japan) were obtained from selected neurons and then scanned (eight bit grayscale TIFF images with 1200 dpi 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. In each selected neuron the dendritic length and the number of branch (bifurcation) points were determined. Ten selected neurons were averaged to get a single value of dendritic length and the number of branch points from each rat, and group means were obtained from each subject.

### 2.4. Statistical analysis

The percentage gain in body weight, relative adrenal weight, spontaneous motor activity, locomotor activity, anxiety, escape behavior and the morphological studies were analyzed by a Student's paired *t*-test. Percent freezing during habituation, fear conditioning, and extinction, and the absolute and relative level of startle were analyzed using two-way repeated-measures ANOVA [group (control, stress) × trial (habituation, conditioning, extinction) or intensity tone (90 dB and 102 dB) respectively] followed by Bonferroni post hoc comparisons test. Results were presented as the mean ± SEM. A probability level of 0.05 or less was accepted as significant.

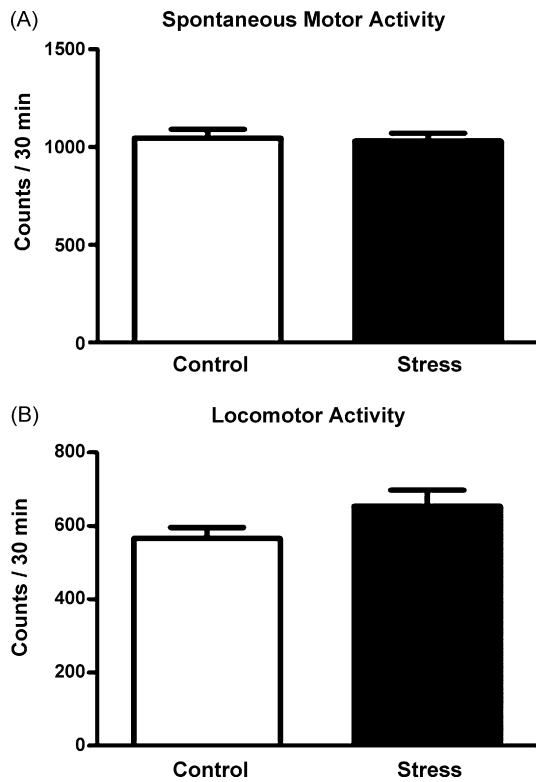
## 3. Results

### 3.1. Spontaneous motor responses and locomotor activity

Fig. 2A and B shows the effects of chronic restraint stress on spontaneous motor activity and locomotor activity respectively. Statistical analysis revealed that stress did not affect these behaviors [(spontaneous motor activity, stress: 1015 ± 34,  $n=55$ ; control: 1024 ± 35,  $n=55$ ;  $p=0.7920$ ) (locomotor activity, stress: 654 ± 44,  $n=55$ ; control: 566 ± 29,  $n=55$ ;  $p=0.1182$ )].

### 3.2. Stress markers in the experimental animals

Restraint stress induced a significant reduction in both frequency of open-arm entries (stress: 2.7 ± 0.3,  $n=55$ ; control: 4.4 ± 0.5,  $n=55$ ;  $p<0.001$ ) and time spent in open arms (stress:



**Fig. 2.** Effect of chronic restraint stress on spontaneous motor and locomotor activities in rats. Stress did not affect the spontaneous motor responses (A) and locomotor activity (B) of the experimental animals. Bars represent the total spontaneous motor activity and locomotor activity in a 30 min observation period. The values are the mean  $\pm$  SEM.

$30.8 \pm 4.2$ ,  $n = 55$ ; control:  $56 \pm 5.2$ ,  $n = 55$ ;  $p < 0.001$ ) in the elevated plus maze (Fig. 3A and B). There were no treatment differences in the number of total arm entries indicating that stress did not affect the locomotor activity (stress:  $10.2 \pm 1.0$ ,  $n = 55$ ; control:

$11.3 \pm 1.1$ ,  $n = 55$ ;  $p = 0.4754$ ) (Fig. 3C). Furthermore, the ratio of open to total arm entries was significantly lower in the stressed rats than controls (stress:  $23.9 \pm 3.0$ ,  $n = 55$ ; control:  $38.0 \pm 1.8$ ,  $n = 55$ ;  $p < 0.001$ ) (Fig. 3D). These results are indicative of an enhanced anxiety response in the stressed animals.

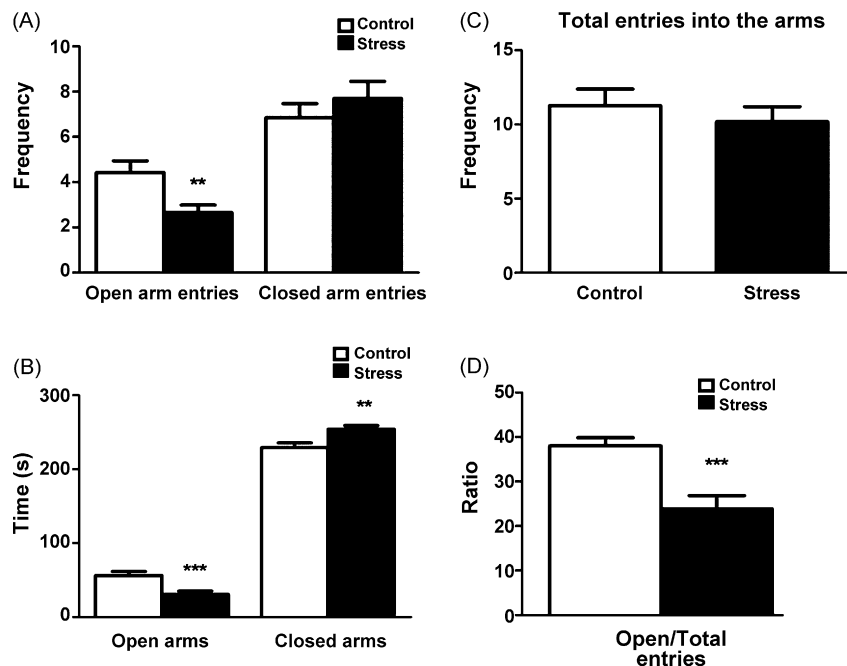
We also analyzed the effects of chronic stress on body and adrenal weights. Statistical analysis revealed a significant reduction in percentage body weight gain after 10 days of stress (stress:  $3.7 \pm 1.3\%$ ,  $n = 55$ ; control:  $11.0 \pm 1.6\%$ ,  $n = 55$ ;  $p < 0.05$ ). Finally, stress caused a significant adrenal hypertrophy (relative adrenal weight, stress:  $15.6 \pm 1.7$ ,  $n = 55$ ; control:  $10.3 \pm 1.6$ ,  $n = 55$ ;  $p < 0.05$ ). These results are consistent with earlier studies.

### 3.3. Effects of chronic restraint stress on dendritic morphology of the medial geniculate nucleus

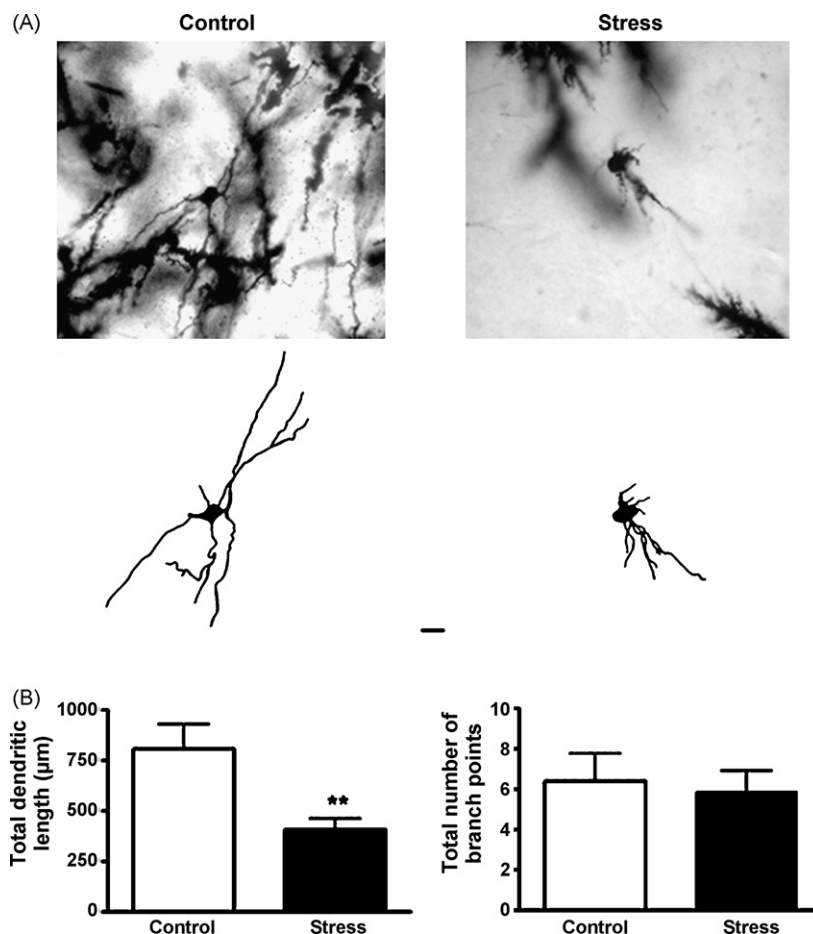
Photomicrographs of representative Golgi-impregnated magnocellular neurons of the MG from control and stress-treated animals, and their respective camera lucida drawings are shown in Fig. 4A. Total dendritic length was significantly decreased in magnocellular neurons from the MG of stressed rats ( $407 \pm 55 \mu\text{m}$ ,  $n = 7$ ), compared with the control neurons ( $808 \pm 120 \mu\text{m}$ ,  $n = 7$ , 49% difference;  $p < 0.01$ ), whereas the number of branch points did not change (Fig. 4B).

### 3.4. Auditory and visual fear conditioning

Chronic restraint stress did not significantly affect unconditioned responses to tone alone (Fig. 5A and B). During the habituation phase, there was no main effect of group on freezing ( $F_{(1,14)} = 1.68$ ,  $p < 0.05$ ) and no interaction of group and trial ( $F_{(1,14)} = 0.39$ ,  $p < 0.05$ ). Likewise, chronic stress did not significantly alter acquisition of the auditory conditioned fear response (Fig. 5A and B). Overall, freezing percentage varied significantly across trials ( $F_{(3,42)} = 32.83$ ,  $p < 0.0001$ ), with both groups acquiring the auditory conditioned fear response. No effect of group ( $F_{(1,14)} = 2.72$ ,  $p < 0.05$ ) or interaction of group and trial ( $F_{(3,42)} = 0.51$ ,  $p < 0.05$ ) was present. During the extinction phase of auditory fear condition-



**Fig. 3.** Effect of chronic restraint stress on anxiety in rats. Stress increases anxiety in the elevated plus maze. Fifteen days after restraint, stressed rats show decrease in the frequency entries (A) and in the time (B) inside onto open arms of the elevated maze. Restraint stress did not affect frequency of the total arm entries (C) and decreases the ratio of open/total arm entries (D) indicating an increase in anxiety. The values are the mean  $\pm$  SEM. Asterisk (\*) indicates significant difference relative to control rats.



**Fig. 4.** Morphometric analysis of the MG magnocellular neurons. (A) Photomicrographs and camera lucida tracings of representative Golgi-impregnated magnocellular neurons of the MG in control and stressed rats. Scale bar, 20  $\mu\text{m}$ . (B) Morphometric analysis of MG neurons from stressed and control rats. After 15 days of chronic restraint stress ( $n = 7$  animals), the total apical dendritic length of the MG magnocellular neurons was significantly reduced compared with control rats ( $n = 7$  animals) ( $p < 0.01$ ). There were no stress-induced changes observed in branch number of MG magnocellular neurons (stress,  $n = 7$  animals; control,  $n = 7$  animals).

ing, as expected, the conditioned fear response diminished with repeated presentation of tone alone in both experimental groups ( $F_{(14,196)} = 4.954$ ,  $p < 0.0001$ ) (Fig. 5A and B). However, stress did not significantly influence rate of extinction on day 3 (for main effect of group,  $F_{(1,14)} = 1.25$ ,  $p < 0.05$ ; for interaction of group and trial,  $F_{(14,196)} = 0.63$ ,  $p < 0.05$ ). Thus, control and stressed rats showed equivalent extinction learning.

Since chronic stress impairs the MG, but does not affect auditory fear conditioning, we analyzed the stress-effects on visual fear conditioning for comparison. Chronic stress did not affect the freezing percentage during the habituation phase (Fig. 5C and D). There was no main effect of group on freezing ( $F_{(1,14)} = 0.05$ ,  $p < 0.05$ ) and no interaction of group and trial ( $F_{(1,14)} = 0.02$ ,  $p < 0.05$ ). Conversely, chronic stress significantly altered the acquisition of visual conditioned fear response (Fig. 5C). Overall, percentage freezing varied significantly across trials ( $F_{(3,42)} = 20.80$ ,  $p < 0.0001$ ), with both groups acquiring a visual conditioned fear response. There was no group effect ( $F_{(1,14)} = 1.42$ ,  $p < 0.05$ ), but the interaction of group and trial was significantly altered by stress ( $F_{(3,42)} = 4.52$ ,  $p < 0.01$ ). Chronic stress significantly increased the acquisition during the second trial of the first conditioning on day 1 (stress:  $58.9 \pm 8.42$ , control:  $23.31 \pm 8.01$ ;  $p < 0.05$ ) (Fig. 5C). On day 2, stressed rats did not display a significant decrease of acquisition during the first trial of the second conditioning (stress:  $19.50 \pm 12.11\%$ , control:  $30.13 \pm 11.33\%$ ;  $p < 0.05$ ), followed by an increased acquisition during the second trial of the second conditioning (stress:  $60.25 \pm 7.90\%$ , control:  $45.44 \pm 7.08\%$ ;  $p < 0.05$ )

(Fig. 5C). During the extinction phase, both control and stressed rats showed a diminished visual conditioned fear response with repeated presentation of light alone ( $F_{(14,196)} = 1.79$ ,  $p < 0.05$ ) (Fig. 5C and D). However, stress did not affect the rate of extinction on day 3 (for main effect of group,  $F_{(1,14)} = 0.000010$ ,  $p < 0.05$ ; for interaction of group and trial,  $F_{(14,196)} = 0.24$ ,  $p < 0.05$ ). Thus, control and stressed rats showed equivalent extinction learning.

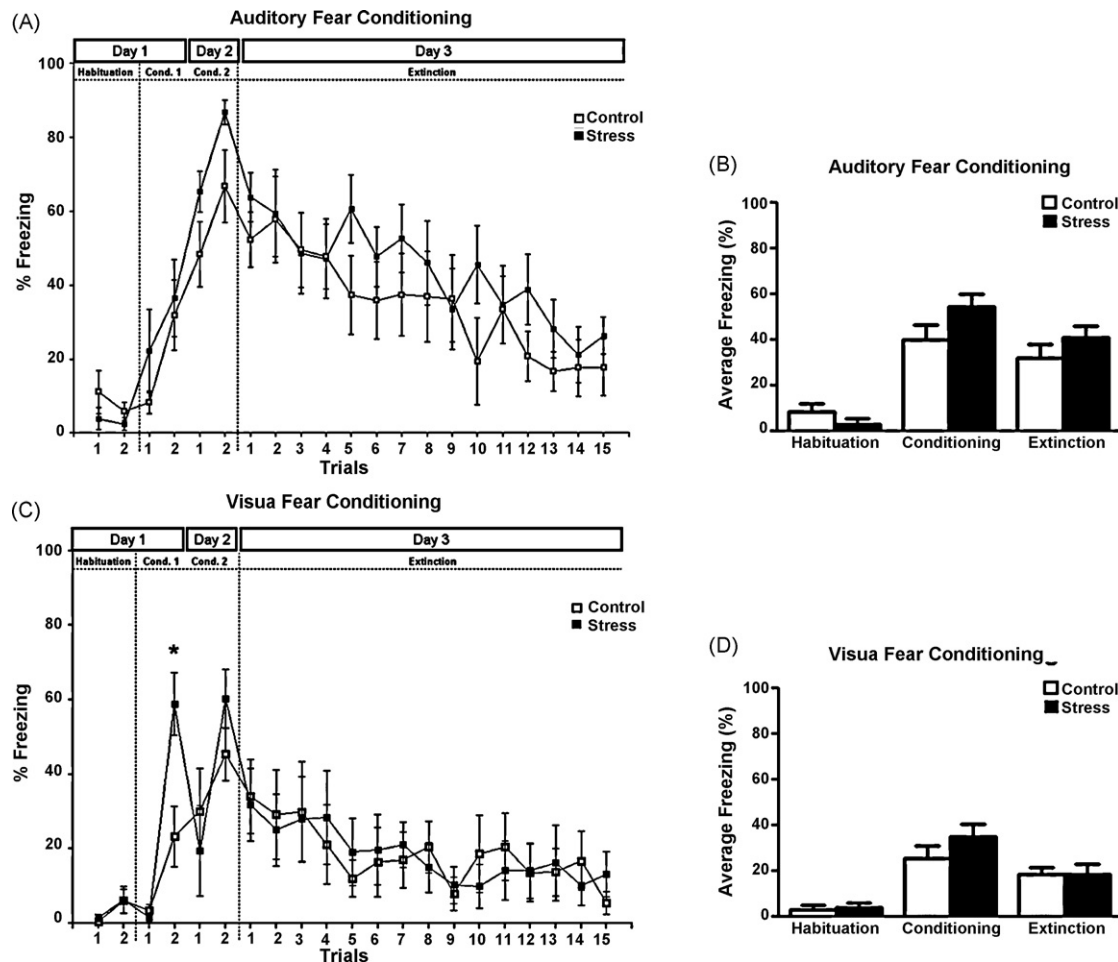
### 3.5. Effects of chronic restraint stress on dendritic morphology of the posterior thalamic nucleus

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

### 3.6. Startle response, prepulse inhibition and escape behavior

Fig. 7A shows the mean of absolute startle level elicited by 90 dB and 102 dB acoustic stimuli for control and stressed rats. A two-way ANOVA revealed that stress did not affect the startle response ( $F_{(1,14)} = 0.24$ ,  $p < 0.05$ ).

Comparable results were found in the stress-effects on relative startle level (Fig. 7B). A two-way ANOVA performed on the PPI for control and stressed rats revealed that stress did not affect the relative level of startle ( $F_{(1,14)} = 0.05$ ,  $p < 0.05$ )



**Fig. 5.** Effect of restraint stress on fear conditioning. Mean and average percent freezing to tone (A,B) and light (C,D) in control (open squares,  $n = 10$ ) versus stressed rats (filled squares) across habituation, conditioning, and trials. Vertical bars represent SEMs. Asterisk (\*) indicates significant difference relative to control rats.

Fig. 7C shows the results of the analysis of the number of escapes scored at the escape test, which demonstrated that chronic restraint stress did not produce a significant difference between groups (stress:  $66.9 \pm 9.1$ ; control:  $74.3 \pm 10.7$ ;  $p < 0.05$ ).

#### 4. Discussion

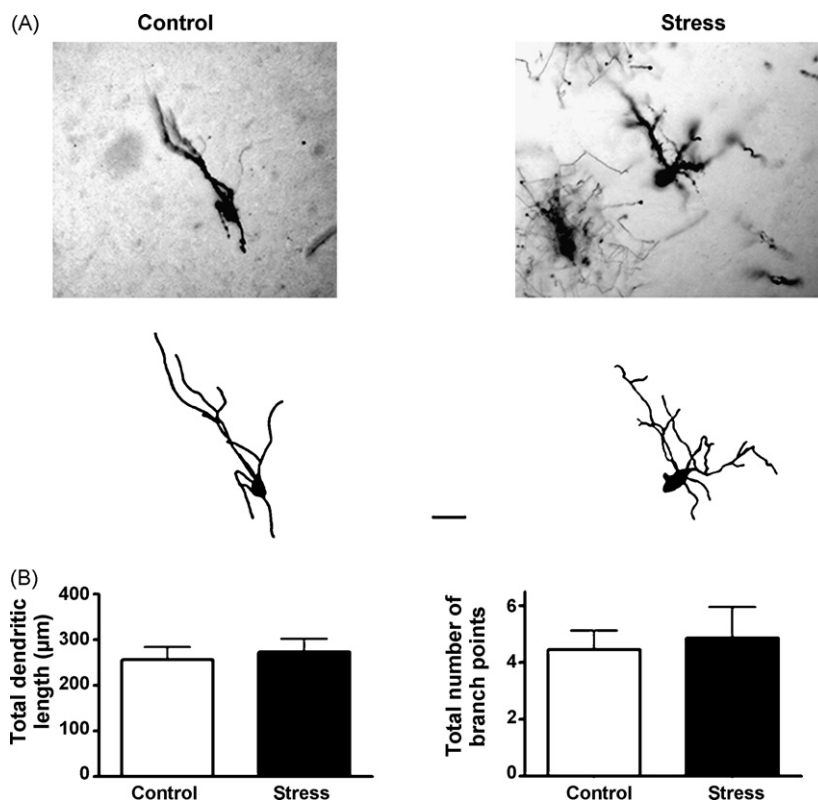
Our study investigated whether the effects of chronic stress in the auditory system are specific to the mesencephalon or whether additional auditory nuclei are affected. The first step of our investigation was to analyze whether our stress protocol was effective at triggering stress responses. Stressed rats showed an enhancement in anxiety-like behavior in the elevated plus-maze compared to the control rats (Fig. 3). The cellular substrate of this behavioral change could be the stress-related hypertrophy in the bed nucleus of the stria terminalis (BNST) in the extended amygdala, a brain area associated with the anxiety response [44]. The same treatment did not affect the spontaneous motor activity and locomotor activity (Figs. 2 and 3 respectively), indicating that the reduced exploration in the open arm of the elevated plus-maze is associated to an increase in anxiety in the stressed rats. Furthermore, chronic restraint stress produced a reduction in percentage body weight gain and significant adrenal hypertrophy. These results are similar to those in previous reports using these signs as stress markers [8,7,9].

Having established that our stress protocol was effective, we analyzed whether stress affects the morphology of the MG, related to auditory CS processing. Our results show that chronic stress

induced dendritic atrophy in the MG neurons (Fig. 4). One possible explanation for our finding is that the MG atrophy is indirectly produced by the stress-related plasticity in the amygdala. Chronic stress generates dendritic hypertrophy of the excitatory pyramidal and stellate neurons of the amygdaloid basolateral complex (BLA) [45] and of the BNST [44], while not affecting the neuronal morphology of the CE [44] (Fig. 1). We hypothesized that MG atrophy is indirectly produced by the stress-related dendritic reorganization in the BLA because the MG is massively connected only with the LA [24]. There is evidence indicating that an intact BLA is essential for developing the associative neuronal plasticity in the MG throughout aversive learning [20]. Therefore, the stress-induced plasticity in the amygdala may be propagated only to brain nuclei that are strongly connected with the LA and not with nuclei connected with the CE. Another possibility to explain our results is that the stress-related dendritic atrophy in the MG was induced by an increase of glucocorticoid receptors expressed in the MG, as proposed previously for the stress-induced hippocampal atrophy [38]. In turn, this alteration in the MG might induce the stress-related changes in the amygdala.

We also analyzed whether the stress-related MG dendritic atrophy affects *in vivo* auditory processing. Freezing during conditioning trials was not significantly enhanced by chronic stress (Fig. 5A and B). Similar results were obtained using restraint stress for both one week (3 h/day) [27] and after 21 days (6 h/day) [4,1].

It is accepted that immobilization stress is a more intense stressor than restraint [45]. In our view, both chronic immobilization stress and restraint stress are comparable because they induce



**Fig. 6.** Morphometric analysis of the PO neurons. (A) Photomicrographs and camera lucida tracings of representative Golgi-impregnated neurons of the PO in control and stressed rats. Scale bar = 20 μm. (B) Morphometric analysis of neurons shown in (A). There were no stress-induced changes in the total apical dendritic length or branch number of the PO neurons (stress,  $n = 7$  animals; control,  $n = 7$  animals).

similar morphologic and behavioral alterations, for example hippocampal atrophy and increase in anxiety-like behavior [19,45]. Then, it is possible that using a more prolonged or intense stress would significantly enhance freezing in the stressed rats when compared to control rats, through conditioning trials used in this study.

Restraint stress did not affect extinction after auditory fear conditioning (Fig. 5A and B). Nevertheless, other reports indicate that 24 h after initial extinction, restraint stress significantly impairs recall of extinction [27,1]. Two main questions were raised by our findings: (1) How may chronic stress impair essential brain areas of the auditory nervous system (MG and IC) while at the same time not affecting auditory fear conditioning?, (2) Is the stress-related enhancement of freezing to tone during fear conditioning [5] related to the acquisition of a conditioned fear or it is produced by an unconditioned fear?. To shed light on these questions we analyzed the stress effects on visual fear conditioning.

Chronic stress did not affect the unconditioned response to the light during habituation in the visual fear conditioning experiment (Fig. 5C and D). In contrast, freezing was significantly enhanced by chronic stress in the second trial of the first conditioning on day 1 (Fig. 5C). However, this alteration was not associated with the ability to elicit a conditioned fear response because during the first trial of the second conditioning (day 2), chronic stress did not affect freezing and stressed rats showed even less freezing than control rats (Fig. 5C). Should chronic stress facilitate the acquisition of conditioned fear, stressed rats would have associated the CS with the US during visual fear conditioning, and chronic stress would have produced enhanced freezing throughout the whole visual fear conditioning protocol. Therefore, the stress-related enhancement of freezing observed during visual fear conditioning was an unconditioned fear.

Control and stressed rats showed a conditioned response during the first extinction trials on day 3, and chronic stress did not affect

the extinction (Fig. 5C and D). These results support our previous finding indicating that stressed rats showed a stronger impairment in the acquisition of an auditory conditioned response through active avoidance conditioning [8]. An association between the CS and US in the LA is required to produce an emotional memory inducing an avoidance conditioned response [8]. It is, however, possible that the stress-related enhanced freezing to a tone found previously in conditioning trials [4] and recall [27,1] was an unconditioned fear. Likewise, in stressed rats the auditory CS received in the CE could be partly processed as a US. Since the main CS pathway involving the MG has been impaired by the stress, the stimulus might have used an alternative route, projecting to the CE directly via the PO or by the longer pathway PO-Somatosensory Cortex-LA-CE [34], independently of the pathways that involve the MG (IC-MG-CE and IC-MG-Auditory cortex-CE) [46] (Fig. 1). These nuclei have been involved in both somatosensory and auditory US processing [29]. Two types of US, acoustic startle stimulus and footshock, were studied to test this idea. Chronic stress did not affect these responses (Fig. 7) or the morphology of the PO (Fig. 6). A previous report showed that the dendritic structure of the CE is also spared after stress [44]. Nevertheless, two stress protocols more intensive than chronic restraint stress, chronic variable stress and psychosocial stress, showed an increase of the ASR and PPI in rats [23,47]. It is possible that stronger stressors may increase the dendritic atrophy in the IC and MG, and consequently these morphologic changes may potentiate the processing of auditory CS by the PO-CE neuronal pathway and increase the startle response (Fig. 1). In support of this idea, lesion studies in the MG and IC showed inhibited acquisition of aversive memories to tone after fear conditioning in rats [18], while lesions of the IC significantly enhance freezing to acoustic startle stimuli [17].

A potentiated PO-CE neuronal pathway could enhance the neuronal activity in the CE, because the PO sends glutamater-



**Fig. 7.** Effect of restraint stress on the startle response, prepulse inhibition, and escape behavior. The mean ( $\pm$  SEM) absolute and relative levels of startle are shown in (A) and (B) respectively. Data are represented in volts for the control and stressed rats (open and filled squares, respectively). (C) Percentage of the number of escapes of the control and stressed rats in 30 trials. The values represent the mean  $\pm$  SEM.

gic efferents to the CE [29]. Increasing the activity of the latter might result in enhancing the anxiety and fear responses in the stressed animals. The CE is likely to be involved in processing of fear responses such as freezing, while the BNST may be related to anxiety [11,10]. There is also the possibility that stress-related BLA hypertrophy increases the neuronal activity in the CE (Fig. 1). This may facilitate fear responses produced by both auditory US and CS received from the PO in the stressed [29] (Fig. 1).

The mPFC has a key role in the modulation of fear expression, mainly by descending projections to the inhibitory amygdala neurons known as intercalated cells [41,36] (Fig. 1). Daily restraint stress over a period of 7–20 days produces dendritic atrophy in the mPFC [37,5]. Longer periods of chronic stress may impair the infralimbic region of the mPFC and decrease the neuronal activity of their outputs to the amygdala, decreasing the activity of the inhibitory circuit within the amygdala and increasing the CE output (Fig. 1). Thus, fear expression induced by auditory CS and US could be enhanced by stress-related dendritic atrophy in the mPFC and BLA hypertrophy.

In conclusion, the data presented here demonstrate that the effects of chronic stress on the auditory system are more widely distributed than the auditory mesencephalon, also involving the

auditory thalamus. Stress-related enhancement of freezing in both auditory and visual fear conditioning could be in part unconditioned fear. Furthermore, the auditory US processing in the PO was not affected by chronic stress and an explanation was suggested that stress-related MG atrophy might decrease the delivery of the auditory CS to the LA. The latter stimulus may be sent in part via PO-CE pathway to the amygdala, independently of the IC and MG, establishing an association with the somatosensory US and contributing to the freezing response. Similar morphological and behavioral changes could be induced in major depression [26,43,6].

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