CHRONIC STRESS IMPAIRS ACOUSTIC CONDITIONING MORE THAN VISUAL CONDITIONING IN RATS: MORPHOLOGICAL AND BEHAVIOURAL EVIDENCE

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Abstract—Chronic stress affects brain areas involved in learning and emotional responses. These alterations have been related with the development of cognitive deficits in major depression. The aim of this study was to determine the effect of chronic immobilization stress on the auditory and visual mesencephalic regions in the rat brain. We analyzed in Golgi preparations whether stress impairs the neuronal morphology of the inferior (auditory processing) and superior colliculi (visual processing). Afterward, we examined the effect of stress on acoustic and visual conditioning using an avoidance conditioning test. We found that stress induced dendritic atrophy in inferior colliculus neurons and did not affect neuronal morphology in the superior colliculus. Furthermore, stressed rats showed a stronger impairment in acoustic conditioning than in visual conditioning. Fifteen days post-stress the inferior colliculus neurons completely restored their dendritic structure, showing a high level of neural plasticity that is correlated with an improvement in acoustic learning. These results suggest that chronic stress has more deleterious effects in the subcortical auditory system than in the visual system and may affect the aversive system and fear-like behaviors. Our study opens a new approach to understand the pathophysiology of stress and stress-related disorders such as major depression.

Key words: stress, atrophy, inferior colliculus, plasticity, depression.

Stress has been shown to have profound effects in neuronal morphology and function, in several forebrain systems including limbic structures and the prefrontal cortex. Studies in animal models of chronic stress and stress hormones have demonstrated stress-induced dendritic remodeling of CA3 pyramidal neurons, decrease in adult neurogenesis in the dentate gyrus and reduction of total hippocampal volume (Magariños and McEwen, 1995; McEwen, 1999; Czéh et al., 2001). All these alterations are reversible post-stress. It has been proposed that these morphological changes might interfere with the negative regulation of the stress response that is induced by the hippocampus via the hypothalamic–pituitary–adrenal (HPA) axis (Jacobson and Sapolsky, 1991; McEwen, 1999). More recently, it has been shown that in rats, the amygdala and prefrontal cortex are also morphologically affected by stress (Wellman, 2001; Vyas et al., 2002; Radley et al., 2004). These structures also regulate the HPA axis, and particularly the amygdala has a critical role in fear and anxiety (LeDoux, 1995). The stress-induced morphologic alterations in hippocampus, amygdala and prefrontal cortex are related to learning, memory and emotional response impairments (McEwen and Chattarji, 2004). In humans, neuroimaging studies have shown hippocampal volume atrophy, reduction of gray and white matter volumes in the prefrontal cortex and decreased volume in the amygdala of depressed patients (Sheline et al., 1996; McEwen and Chattarji, 2004). Postmortem studies in the brains of patients with major depression showed reductions in neuronal size and/or decreased density of glial cells in the orbitofrontal, dorsolateral, and subgenual prefrontal cortex (Rajkowska et al., 1999; Manji et al., 2003). These alterations may contribute to the cognitive deficits of major depression (Sapolsky, 2001).

In this article, we investigated whether other, nontelencephalic brain components could also be targets of stress-induced damage. Specifically, we chose mesencephalic regions related with the auditory system (inferior colliculus, IC) and visual system (superior colliculus, SC). The IC is a main structure of the central auditory nervous system (Pollak et al., 2003), in which several parallel subcortical pathways converge. The IC is related to aversive behavior, which is responsible for the organization of fear and anxiety-like behaviors (Brandão et al., 1994). The emotional interpretation of auditory stimuli is mediated by limbic areas, like the amygdala, that receive indirect projections from both the IC and SC, via posterior thalamic nuclei (LeDoux et al., 1983; Aboitiz et al., 2003). Auditory and visual information are also conveyed to the cerebral cortex via the medial geniculate and the lateral geniculate bodies of the thalamus.

We found that in rats, chronic immobilization stress induced a significant dendritic atrophy in IC neurons, but did not produce neuronal alterations in the SC. Consider-

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Abbreviations: ANOVA, analysis of variance; BLA, basolateral amygdala; HPA, hypothalamic–pituitary–adrenal; IC, inferior colliculus; MGN, medial geniculate nucleus; SC, superior colliculus.

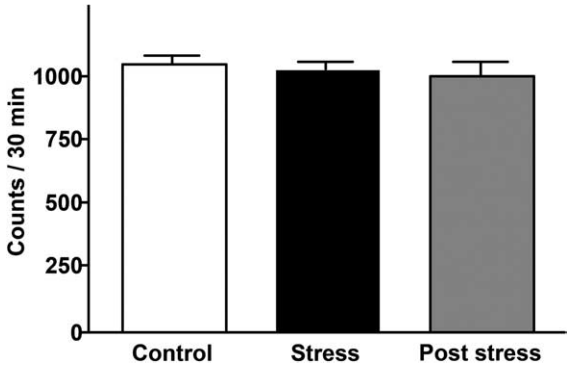

Fig. 1. Effect of chronic immobilization stress on spontaneous motor responses in rats. Stress does not affect the motor activity of the experimental animals. Bars represent the total spontaneous motor activity in a 30-min observation period. The values are the mean±S.E.M.

ing this, we hypothesized that stress might have different effects on acoustic and visual learning. In fact, we observed that stressed rats showed a stronger impairment in acoustic conditioning than in visual conditioning. Fifteen days post-stress the IC neurons completely restored their structure, which is correlated with an improvement in acoustic learning in the avoidance conditioning test. Our results suggest that the effects of stress in the brain may be more extended than previously thought and affect non-limbic areas receiving sensory information, each of these related to specific behavioral responses.

EXPERIMENTAL PROCEDURES

Animals and immobilization stress protocol

Male rats (Sprague–Dawley; 285–310 g; 3 months old) were housed in groups of three under a 12-h light/dark cycle (lights on at 7:00 A.M.) with *ad libitum* access to food and water in a temperature-controlled room. Rats were randomly assigned to three groups: control, $n=28$, stress, $n=31$ and post-stress (15 days after the term of stress), $n=28$, for conditioning and morphologic experiments. Control animals, which were littermates of the stress-treated and post-stress animals, were housed in separate cages and rooms and not subjected to any type of stress. 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. We used the same immobilization stress protocol previously described by Vyas et al. (2002), which demonstrated that complete immobilization of rats (2 h/day, 10 A.M.–noon) for 10 days in immobilization cages, induces hippocampal atrophy, similar to what is found after 21 days (6 h/day) of repeated restraint stress (Magariños and McEwen, 1995). The rodent immobilization cages were made in our laboratory, and their dimensions were length: 18 cm, wide: 6 cm, and high: 6 cm. The cages allow the complete immobilization of the animals, but they can breathe without problems in the cages and may urinate and defecate without being in constant contact with their waste. 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×100/weight at the beginning of experiment), anxiety level as determined by performance in the elevated plus maze, relative adrenal weight (wet weight of adrenal glands in mg×100/body weight in grams), and presence of ulcers in gastric mucosa (Brzozowski et al., 2000).

Spontaneous motor activity

Twenty-four hours after completion of the stress protocol each rat was individually analyzed in the following order: spontaneous motor activity, elevated plus-maze and active avoidance conditioning. First, each rat was placed into a Plexiglas cage (30×30×30 cm) and the spontaneous motor activity was monitored during a period of 30 min and evaluated as described previously (Díaz-Véliz et al., 2004). The floor of the cage was an activity platform (Lafayette Instrument Co., Lafayette, IN, USA) connected to an electromechanical counter. In order to avoid the influence of disturbing noises the platform was placed into a soundproof chamber. Each animal was observed continuously via a Sony video camera connected to a VHS tape recorder. Scores were generated from live observations, while video sequences were used for later reanalysis when necessary.

Elevated plus-maze

After the analysis of spontaneous motor activity 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 (50×10 cm each), two closed arms (50×10×20 cm each) and a central platform (10×10 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. 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: a) the number of open arm entries, b) the number of closed arm entries, c) the time spent in open arms, and d) the time spent in closed arms. 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 between 10 A.M. and 2 P.M.

Active avoidance conditioning

Immediately after completion of the elevated plus-maze test, each rat was subjected to the active avoidance conditioning test (Díaz-Véliz et al., 2004) to analyze whether stress has different effects on acoustic and visual learning. Each rat [acoustic experimental group: control, $n=10$, stress, $n=11$ and post-stress, $n=10$; visual experimental group: control, $n=10$, stress, $n=12$ and post-stress, $n=10$] was individually placed in a two-way shuttle box (Lafayette Instrument) composed of two stainless steel modular testing units. Each unit was equipped with an 18-bar insulated shock grid floor, two 28-V DC lights and a tone generator (Mallory Sonalert 2800 Hz; Lafayette Instrument). Electric shocks were provided to the grid floor by a Master shock supply (Lafayette Instrument). The rats were trained over 50 trials after a 5-min period of habituation. Each trial consisted of the presentation of an auditory (2800 Hz tone) or a visual (28 V light) stimulus that after 5 s overlapped with a 0.20-mA footshock until the animal escaped to the opposite chamber, with maximum shock duration of 10 s. A conditioned avoidance response was defined as a crossing to the opposite chamber within the first 5 s after the tone or light.

Morphological data analysis

A new set of rats (control, $n=8$; stress, $n=8$, post-stress, $n=8$) was used in this experiment. Immediately after completion of the active avoidance conditioning test, animals were killed under deep anesthesia with sodium pentobarbital after completion of the stress protocol. The brain was removed quickly and processed using FD Rapid GolgiStain^{TM} kit (FD Neuro Technologies, Inc., Ellicott City, MD, USA). Both hemispheres were cut in the sagittal plane using a cryostat (Microm, Walldorf, Germany) and 60-μm-thick sections were obtained. Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene, and cover-

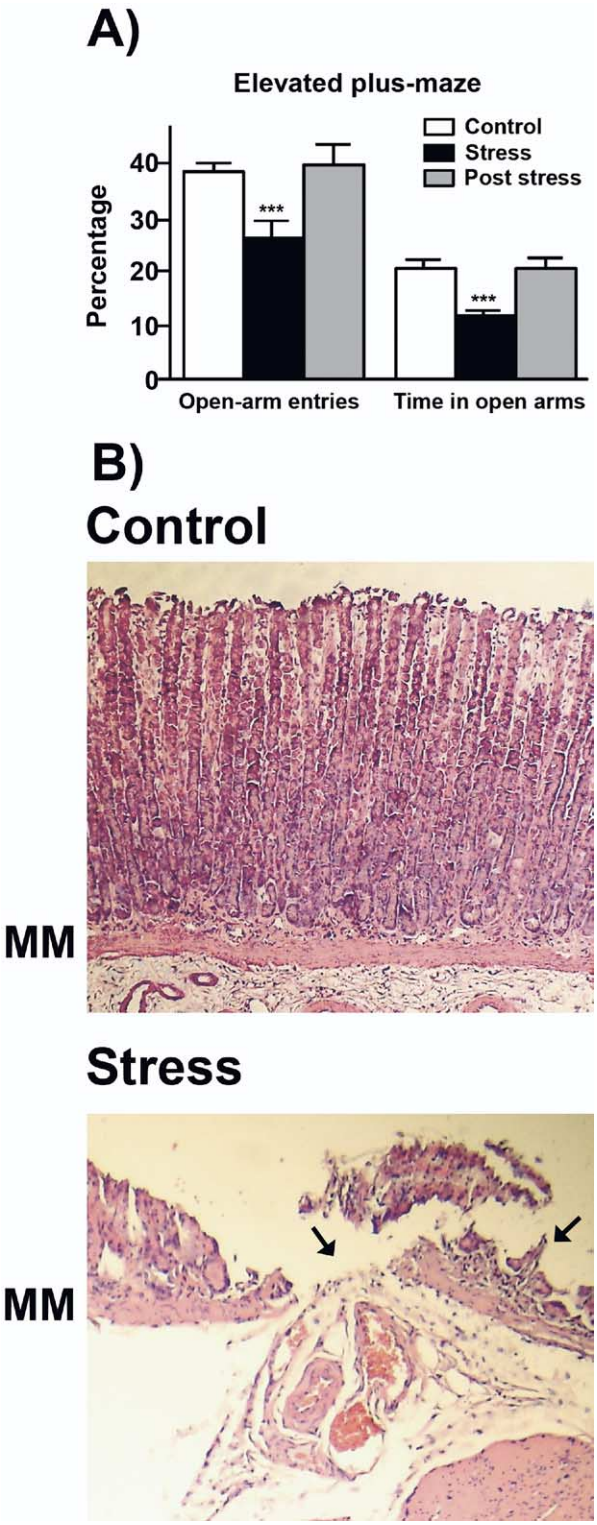

Fig. 2. Behavioral and morphological indicators of stress in the experimental animals. (A) Stress increases anxiety in the elevated plus maze. Ten days after chronic immobilization, stressed rats show decreases in the time inside and in the entries onto open arms of the elevated maze, indicating an increase in anxiety. Data represent the means±S.E.M. of 28 rats for control group, 31 for stress group and 28 for post-stress group. Comparisons were made by one-way ANOVA followed by Newman Keuls post hoc test (*** $P<0.001$ compared with control group). (B) Microscopic appearance of gastric mucosa in stressed animals. Note that following of the stress protocol (stress), the gastric mucosa exhibits a discontinuation of the surface epithelium and the muscularis mucosae (MM) (arrows) that is related to mucosal ulceration. Hematoxylin and eosin, magnification 260×.

slipped. Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed. Previous reports suggest that the central nucleus of the IC has two types of neurons based on the shape and orientation of the dendritic tree (Peruzzi et al., 2000). In rats, the principal neuron of the IC is flat (called the disc-shaped neuron in other species) with dendrites that parallel the fibro-dendritic laminae and project to the thalamus. A second, less common type of neuron with a different dendritic morphology is named in rats as less-flat neuron (Peruzzi et al., 2000). Likewise, typical neurons of the superficial layers of the SC have been classified on the basis of their dendritic morphology as wide-field type (whose target is in the lateral posterior nucleus of the thalamus) and of the narrow-field type (that project to the lateral geniculate nucleus) (Hilbig et al., 2000). As a first stage, we performed our morphometric study analyzing the effect of stress on the flat neurons of the IC and the wide-field neurons of the SC because both cell types project to collothalamic nuclei and are thus comparable. The morphometric analysis of the flat and wide-field 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. Three different experimenters selected independently and at random 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 self-deception in the experimenters, the latter had no knowledge of whether the sample analyzed was from a control or a stressed rat, but they knew whether the sample was from the superior or the IC. Camera lucida tracings (500×, BH-2, 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. In each selected neuron the dendritic length and the number of branch (bifurcation) points were determined.

Statistical analysis

Behavioral data were analyzed using the statistical tests one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test. The morphological studies of the IC and SC neurons were analyzed using a Student's *t*-test. Results were expressed as the mean±S.E.M. values. A probability level of 0.05 or less was accepted as significant.

RESULTS

Spontaneous motor responses

Fig. 1 shows the effects of chronic immobilization stress on spontaneous motor activity. The one-way ANOVA revealed that stress did not affect the motor activity of the rats (control: 1062±39, $n=28$; stress: 1042±33, $n=31$; post-stress: 1033±44, $n=28$).

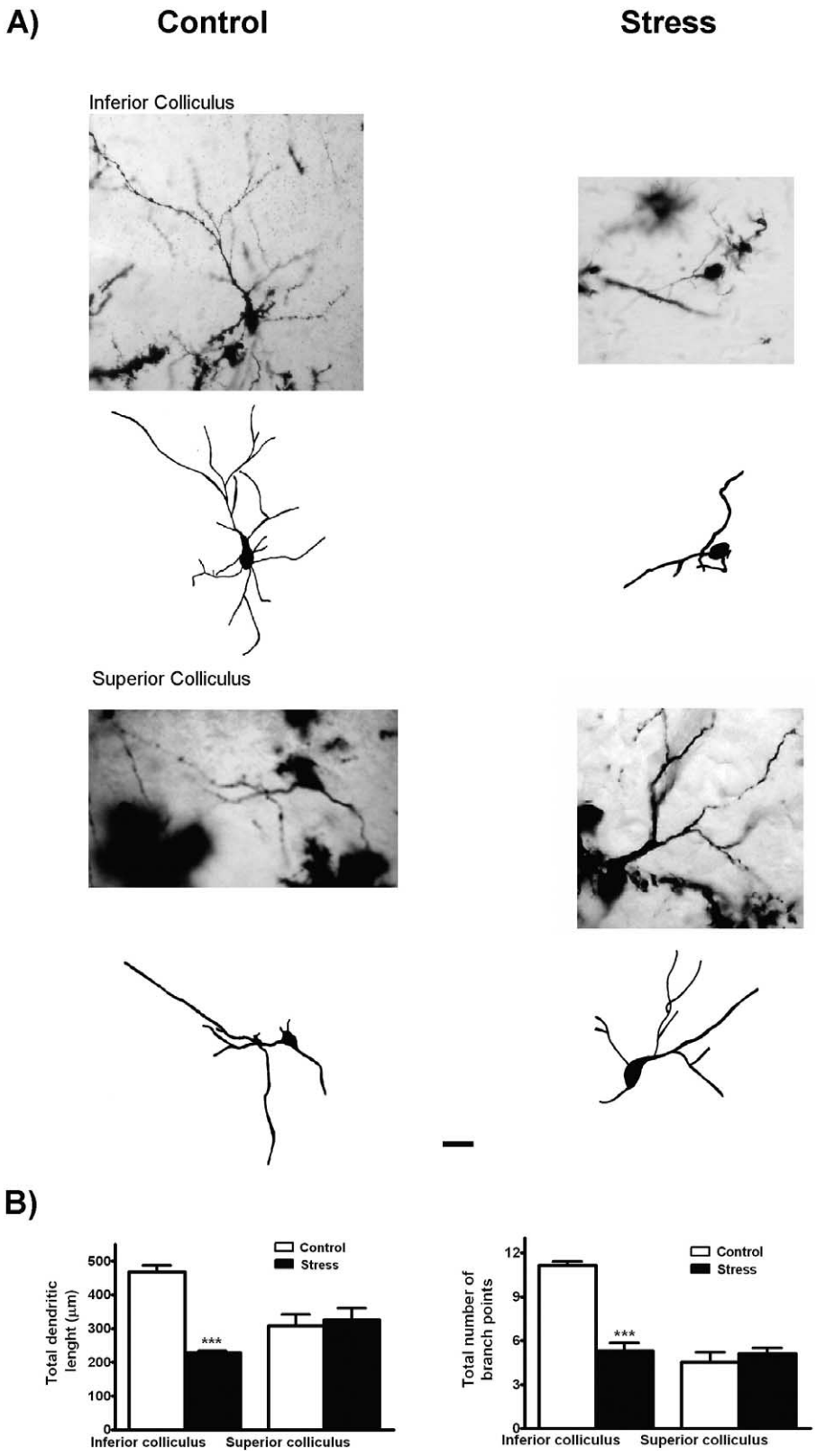

Fig. 3. Different effects of chronic stress on the neuronal morphology of the IC and SC. (A) Photomicrographs and camera lucida tracings of representative Golgi-impregnated flat IC and wide-field SC neurons from control and stressed rats. Scale bar=20 μm. (B) Morphometric analysis of flat IC and wide-field SC neurons. After 10 days of chronic immobilization stress (n=80 cells; n=8 animals), the total dendritic length and the total number of dendritic branches of flat IC neurons was significantly reduced compared with control rats (n=80 cells; n=8 animals). There were no stress-induced changes observed (stress, n=80 cells; n=8 animals; control, n=80 cells; n=8 animals) in total dendritic length or branch number of wide-field type neurons of the SC.

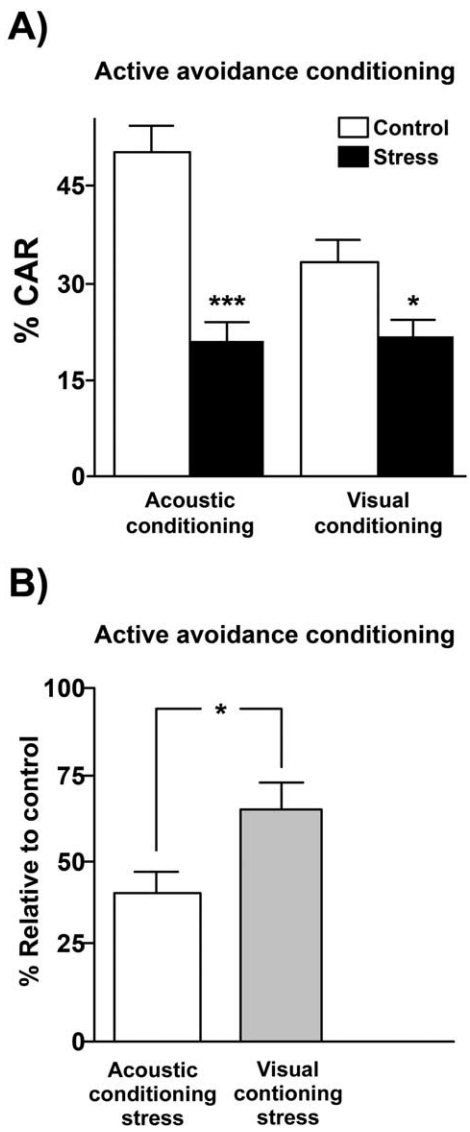

Fig. 4. Stress-effects on visual and acoustic conditioning. The values are the means±S.E.M. of 20–23 animals on each group. Bars represent the percentages of conditioned avoidance (% CAR) responses for 50 trials. For statistical comparisons were used one-way ANOVA followed by post hoc Newman-Keuls test. (A) Stress decreases the % CAR response when using auditory (*** $P<0.001$) or visual (* $P<0.05$) stimuli. (B) Stress produces a more dramatic decrease in acoustic conditioning than in visual conditioning (* $P<0.05$). Percentages relative to control of the acoustic and visual conditioning were obtained from its respective % conditioned avoidance response control values show in A.

Stress markers in the experimental animals

A significant reduction in both percentage of open-arm entries, $F(2.68)=10.35$; $P<0.0001$ (stress: 26±2.6%, $n=31$; control: 39±1.5%, $n=28$; post-stress: 40±3.0%, $n=28$; $P<0.001$) and percentage time spent in open arms, $F(2.68)=12.95$; $P<0.0001$ (stress: 12±1.2%, $n=31$; control: 21±1.4%, $n=28$; post-stress: 21±2.3%, $n=28$; $P<0.001$) was found in the elevated plus maze that is indicative of an enhanced anxiety response in the stressed animals (Fig. 2A). In addition, acute gastric lesions were observed in the stressed animals (Fig. 2B). Stress induced desquamation of the surface epithelium, appearance of necrotic rest, and unspecific acute inflammation in the gastric mucosa, features that are usually related with chronic ulcerated lesions. We also analyzed the effects of chronic stress in body and adrenal weights. After 10 days of stress, statistical analysis revealed a significant reduction in percentage body weight gain (stress: 4.2±2.5%, $n=31$; control: 10.1±1.8%, $n=28$; post-stress: 21.7±4.5%, $n=28$; $P<0.01$) and a significant adrenal hypertrophy (relative adrenal weight, stress: 14.3±0.5, $n=31$; control: 9.8±0.5, $n=28$; post-stress: 9.4±0.4, $n=28$; $P<0.01$). These results indicate a correct stress protocol.

Effects of chronic immobilization stress on dendritic morphology of the inferior and SC neurons

Photomicrographs of representative Golgi-impregnated IC flat and SC wide-field neurons from control and stress-treated animals, and their respective camera lucida drawings are shown in Fig. 3A. In the flat neurons of the IC, we observed a stress-induced reduction in the dendritic length (51%; $P<0.001$; 229±6 μm against 468±19 μm in controls), and in the total number of branch points (52%; $P<0.001$; 5.3±0.6 against 11.1±0.3 in controls) (Fig. 3B). On the other hand, we did not detect stress-induced differences in neuronal morphology of the wide-field type neurons in the SC (Fig. 3A, B). There were no observable apoptotic profiles after stress treatment (data not shown). The one-way ANOVA revealed no significant differences between the three experimenters that performed the analysis [IC: dendritic length of the controls ($P=0.9822$), branch points of the controls ($P=0.9683$), dendritic length of the stressed animal ($P=0.6068$), branch points of the stressed animal ($P=0.9558$); SC: dendritic length of the controls ($P=0.5648$), branch points of the controls ($P=0.6487$), dendritic length of the stressed animal ($P=0.4223$), branch points of the stressed animal ($P=0.5247$)]. The values of dendritic length and branch points are the average of the three experimenters.

Effects of chronic immobilization stress on acoustic and visual conditioning

Stress significantly decreases the acquisition of conditioned avoidance response compared with control animals in both acoustic ($P<0.001$) and visual conditioning ($P<0.05$) (Fig. 4A). However, the effect of stress was stronger on acoustic conditioning than on visual conditioning ($P<0.05$; % relative to control; Fig. 4B). No significant differences were observed in the footshock thresholds among the experimental groups (0.25±0.05 mA).

Post-stress analyses

Photomicrographs of representative Golgi-impregnated IC flat neurons from control, stress-treated and post-stress animals, and their respective camera lucida drawings are shown in Fig. 5A. After stress, we observed a significant increase in both the dendritic length (55%; $P<0.001507$±32 μm against 229±6 μm in stressed neurons), and the total number of

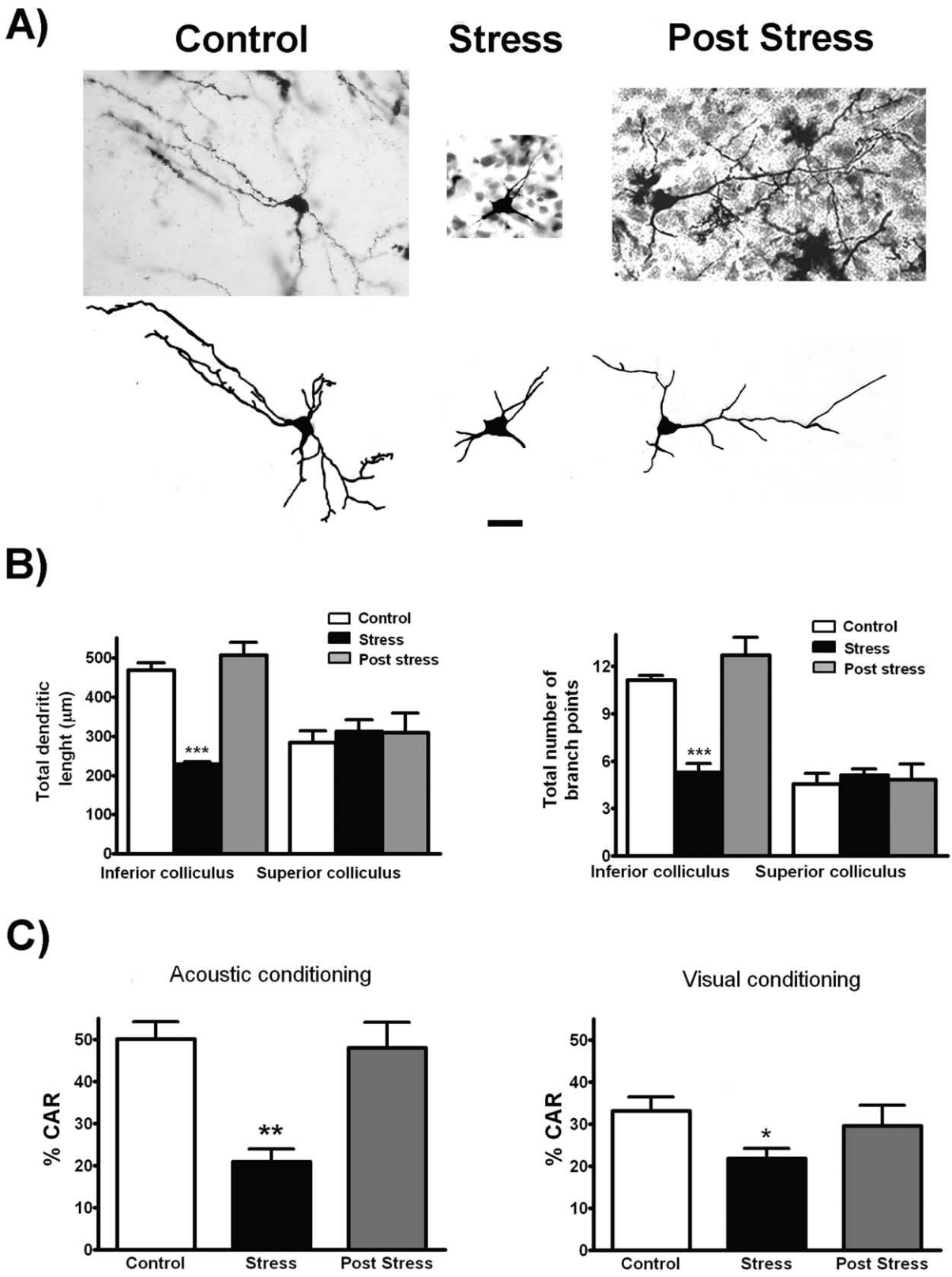

Fig. 5. Adaptive plasticity in the IC after chronic stress. (A) Photomicrographs of representative Golgi-impregnated IC flat neuron from control, stress and post-stress-treated animals (*top*). Camera lucida tracings of representative Golgi-impregnated IC flat neurons (*bottom*). Scale bar=20 μm. (B) Morphometric analysis of the IC flat neurons. Plots of median values for total dendritic length and number of branch points of the control, stress and post-stress flat neurons of the IC. Fifteen days post-stress the dendritic structure of the IC flat neurons was completely restored. (C) Post-stress effects on conditioned avoidance response of the acoustic and visual conditioning experimental group. Control and stress groups are the same that in Figs. 3B and 4A. Each bar represents the mean±S.E.M. of the percentages of conditioned avoidance response (% CAR) for 50 trials.

branch points (58%; $P<0.00112.7\pm1.1$ against 5.3 ± 0.6 in stressed neurons) (Fig. 5B). We did not detect morphologic change between control and post-stress wide-field type neurons of the SC These results show that 15 days post-stress the dendritic structure of the IC flat neurons was completely restored. The post-stress adaptive plasticity of the IC is correlated with an improvement in the acoustic and visual learning during avoidance conditioning test [acoustic conditioning (stress: $20.9\pm3.1\%$, $n=11$; control: $50.2\pm4.0\%$, $n=10$, and post-stress $48.0\pm6.0\%$, $n=10$, $P<0.01$); visual conditioning (stress: $21.8\pm2.4\%$, $n=12$; control: $33.1\pm3.4\%$, $n=10$, and post-stress $29.7\pm4.8\%$, $n=10$; $P<0.05$)] (Fig. 5C).

DISCUSSION

In this study, we analyzed the effect of stress on auditory and visual structures and behaviors. In stressed rats, we observed IC (auditory) neuronal atrophy but no effect on SC (visual) neuronal structure (Fig. 3), while the same treatment produced a stronger impairment on acoustic than on visual conditioning and did not affect spontaneous motor activity in any experimental group (Figs. 1 and 4). This observation is in our view remarkable and implies that the auditory pathway is more susceptible to stress than the visual pathway. Other brain components like the hippocampus and the amygdala are known to participate in conditioning (Conejo et al., 2005) and are targets of the stress response (McEwen and Chattarji, 2004). It is thus highly possible that damage to these structures is also involved in the stress-induced behavioral impairments reported here (especially in visual conditioning). However, the stronger effect on acoustic conditioning than on visual conditioning may be explained by additional damage to the IC during stress. IC atrophy may reduce the ability of those neurons to receive and integrate auditory afferent signals and reduce the capacity of the IC to send auditory information to the auditory cortex and limbic areas via the thalamus (Pollak et al., 2003; Aboitiz et al., 2003). In this context, neuronal electrophysiological characteristics, such as buildup-pauser or rebound discharge patterns, are associated to simple or complex branching patterns of the IC neurons (Peruzzi et al., 2000). Therefore, it is possible that IC atrophy impairs the discharge patterns of the latter neurons, while the adaptive post-stress plasticity in the IC could be related with an increased ability to perceive afferent signals in the IC (Fig. 5). Note that stress-induced atrophy is quantitatively different in the IC and in the hippocampus [51% and 29% (Magariños and McEwen, 1995), respectively]. It is possible that this discrepancy results from different susceptibilities to stress, due to functional and morphologic differences between the IC flat neurons and the hippocampal CA3 neurons.

One possibility to explain our findings is that IC atrophy is indirectly induced by the stress-related morphologic alteration in the hippocampus and the amygdala. Evidence indicates that an intact basolateral amygdala (BLA) is essential for the development of associative neuronal plasticity in the medial geniculate nucleus (MGN) during aversive learning (Maren et al., 2001), and might also influence plasticity at mesencephalic levels like the IC. Chronic stress-induced hypertrophy of BLA pyramidal-like neurons (Vyas et al., 2002) may produce an increase in the local excitatory activity in the BLA. Increase of excitatory activity associated with change in intracellular calcium concentration may be related with both neural plasticity (Johnston, 2004) and neurotoxicity (Sapolsky, 2000), which may induce morphologic changes in the MGN efferents to the BLA, which may retrogradely affect the morphology of MGN neurons. This process may be propagated to even lower levels in the auditory pathway such as the IC. For some yet unknown reason the retrograde effect of amygdalar hypertrophy may be less effective in the visual mesencephalon than in the auditory mesencephalon. Although there are visual projections from the SC to the BLA via the lateral posterior nucleus of the thalamus (Doron and LeDoux, 1999), it is possible that these are not as robust or as plastic as those involved in the auditory projection from the IC to the amygdala via the medial geniculate body and surrounding regions (LeDoux et al., 1990).

Auditory stimuli can trigger aversive behaviors, perhaps more likely than visual stimuli (Azrin, 1958; Reed et al., 1996; Macedo et al., 2005). The IC and BLA are related to the aversive system, which is responsible for the organization of fear and anxiety-like behaviors (Brandão et al., 1994). It is known that the auditory receiving medial geniculate body of the thalamus projects intensely to the BLA (LeDoux et al., 1990), and that fear conditioning plasticity in the auditory thalamus requires amygdalar indemnity (Maren et al., 2001). Therefore, the auditory system seems to be particularly linked to the amygdalar system during fear conditioning, and stress-related alterations in the amygdala may produce downstream effects in the thalamus (Maren et al., 2001) and, according to our results, even in the IC. In turn, IC atrophy induced by stress may affect the auditory perception of aversive signals and may impair the regulation of emotional and cognitive behaviors, affecting environmental adaptation.

CONCLUSION

In conclusion, this study confirms that chronic stress also affects brain areas receiving sensory information in rats. The stress-related learning impairment observed in this report might also occur in humans and have a role in the development of depressive disorders. Our study opens a new approach to understand the pathophysiology of the stress and stress-related psychiatric disorders such as posttraumatic stress disorder and major depression.

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