Chronic stress decreases the expression of sympathetic markers in the pineal gland and increases plasma melatonin concentration in rats

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Abstract

Chronic stress affects brain areas involved in learning and emotional responses. Although most studies have concentrated on the effect of stress on limbic-related brain structures, in this study we investigated whether chronic stress might induce impairments in diencephalic structures associated with limbic components of the stress response. Specifically, we analyzed the effect of chronic immobilization stress on the expression of sympathetic markers in the rat epithalamic pineal gland by immunohistochemistry and western blot, whereas the plasma melatonin concentration was determined by radioimmunoassay. We found that chronic stress decreased the expression of three sympathetic markers in the pineal gland, tyrosine hydroxylase, the p75 neurotrophin receptor and α-tubulin, while the same treatment did not affect the expression of the non-specific sympathetic markers Erk1 and Erk2, and glyceraldehyde-3-phosphate dehydrogenase. Furthermore, these results were correlated with a significant increase in plasma melatonin concentration in stressed rats when compared with control animals. Our findings indicate that stress may impair pineal sympathetic inputs, leading to an abnormal melatonin release that may contribute to environmental maladaptation. In addition, we propose that the pineal gland is a target of glucocorticoid damage during stress.

Keywords: depression, epithalamus, melatonin, pineal gland, stress.

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Several studies have shown that the hippocampus is involved in the regulation of the stress response via the hypothalamic-pituitary-adrenal (HPA) axis and is susceptible to stress-related damage evidenced, for example, in dendritic remodeling of CA3 pyramidal neurons and a decrease in adult neurogenesis in the dentate gyrus of rats (Magariños and McEwen 1995; McEwen and Chattarji 2004). Hippocampal volume reductions, which have been observed in patients with major depression, may be related to emotional and memory impairments (Sheline *et al.* 1996; Duman *et al.* 1999). More recently, it has been reported that, in addition to the hippocampus, the amygdala and prefrontal cortex in rats are also morphologically affected by stress (Vyas *et al.* 2002; Radley *et al.* 2004). These alterations may contribute to the

cognitive deficits of major depression (Sapolsky 2001; McEwen and Chattarji 2004). In this study, we investigated whether other, non-telencephalic brain components could

also be targets of stress-induced impairment. We chose a diencephalic component, the epithalamic pineal gland (PG), because this gland expresses a high density of the glucocorticoid receptor (Warembourg 1975; Meyer *et al.* 1998) and may be a target of stress-induced damage by glucocorticoids, the adrenal steroids secreted during stress (Sapolsky 2000).

The PG is a phototransducer neuroendocrine organ that plays a central role in the rhythmic secretion of melatonin, the night signal in all vertebrates (Simonneaux and Ribelayga 2003). Interactions between melatonin and the HPA system of the stress response have been demonstrated (Kellner *et al.* 1999). Melatonin receptors are present in brain areas that participate or are affected in the stress response, such as the adrenal glands and the hippocampus, whose activity is modulated by melatonin (Musshoff *et al.* 2002; Torres-Farfan *et al.* 2003). The PG receives photic information from the retina via the suprachiasmatic nucleus and the sympathetic neurons from the superior cervical ganglion (SCG), which, in this organ, is translated into hormonal information (Simonneaux and Ribelayga 2003). Rhythmic melatonin secretion from the PG has been related to important biological processes such as the modulation of neurotransmitter release, especially serotonin and dopamine (Simonneaux and Ribelayga 2003). In this line, melatonin has been associated with the regulation of cognitive and emotional processes such as memory and anxiety (Laudon *et al.* 1989; Boatright *et al.* 1994; Hemby *et al.* 2003).

In the rat PG, melatonin content and arylalkylamine *N*-acetyltransferase (AA-NAT) activity increase in response to acute physical immobilization and forced swimming (Lynch *et al.* 1973; Vollrath and Welker 1988; Wu *et al.* 1988; Funk and Amir 1999). In addition, electron microscopy studies have determined that immobilization stress induces degeneration of the pinealocytes (Martinez *et al.* 1992; Milin *et al.* 1996; Milin 1998). Likewise, psychosocial stress induces a drastic increase in 6-sulfatoxymelatonin (the principal melatonin metabolite) excretion in subordinate tree shrews (Fuchs and Schumacher 1990), while in humans, serum melatonin concentration is reduced in depressive patients (Brown *et al.* 1985; Frazer *et al.* 1986; Almay *et al.* 1987). At the present time, little is known about how stress affects pineal sympathetic innervation and pineal gland, and its roles in the stress response remains to be clarified.

In this context, we investigated the effects of chronic immobilization stress in rats on the expression of pineal sympathetic markers and plasma melatonin concentration, both of which were substantially modified.

Materials and methods

Animals and immobilization stress protocol
Male rats (Sprague-Dawley; 285–310 g; 3 months old) were housed in groups of three under a 12/12 light/dark cycle (lights on at 07.00 hours), with *ad libitum* access to food and water, in a temperature-controlled room. Rats were randomly assigned to two groups: control, $n = 49$; stress, $n = 49$. Control animals, which were littermates of the stress-treated 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, 10.00–12.00 hours) for 10 days in immobilization cages, induces hippocampal atrophy similar to that found after 21 days (6 h/day) of repeated restraint stress (Magariños and McEwen 1995). The dimensions of the rodent immobilization cages, constructed in our laboratory, were: length 18 cm, width 6 cm and height 6 cm. While the cages allowed complete immobilization of the animals, they can breathe without problems in the cages, and could 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 milligrams × 100/body weight in grams), and presence of ulcers in the 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: first spontaneous motor activity and immediately after the elevated plus-maze. Each rat was placed in a plexiglass cage (30 × 30 × 30 cm), then 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 in a soundproof chamber. Each animal was observed continuously via a Sony video camera connected to a VHS tape recorder (Sony Corporation, Mexico DF, Mexico). Scores were generated from live observations, while video sequences were used for later re-analysis when necessary.

Elevated plus-maze
Following the analysis of spontaneous motor activity, anxiety levels were measured 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 such a way that the two arms of each type were opposite 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: (i) the number of open arm entries; (ii) the number of closed arm entries; (iii) the time spent in open arms; (iv) 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 thoroughly wiped clean with 5% ethanol solution after each trial. All trials were conducted between 10.00 and 14.00 hours.

Immunohistochemistry of tyrosine hydroxylase

Three independent experiments were performed to analyze the expression of tyrosine hydroxylase (TH), a pineal sympathetic marker (Kohn *et al.* 1999), in control and stressed rats. In each experiment, rats were randomly assigned to two groups: control, $n = 6$ and stress, $n = 6$. When the behavioral study was complete, the rats were killed by an overdose of sodium pentobarbital and perfused intracardially with 0.9% saline followed by 4% buffered formaldehyde solution. The rats were then decapitated and the PGs were immediately removed, post-fixed in a somogyi fixative for 2 h and cryopreserved in 30% sucrose. The PGs were cut serially in 15 μm frozen sections on a cryostat (Microm, Walldorf, Germany), and each PG was completely represented on three slides (anterior, medial and posterior parts). Sections were washed for 10 min in 1× phosphate-buffered saline (PBS), pH 7.4, and non-specifically blocked with 1% milk plus 0.3% Triton X-100 in PBS, pH 7.4. Subsequently, the slides were incubated overnight at 4°C with a commercially available monoclonal antibody directed against TH (1 : 1000; Sigma-Aldrich Corp., St. Louis, MO, USA). The slides were washed three times in PBS for 10 min, then incubated for 2 h in a biotynilated pan-specific antibody (1 : 300; BA-1300, Vectastain, Vector Laboratories, Burlingame, CA, USA). After three 10 min washes in PBS, the slides were incubated for 1 h with a streptavidin Texas red stain (1 : 1000; SA-5001, Vector Laboratories) and again washed three times in PBS. Finally, the slides were coverslipped using DABCO Sigma mounting medium (Sigma). Observations were made with fluorescence microscopy, and the images were captured using a Nikon microscope (Model OPTIPHOT-2, Melville, NY, USA) equipped with a Coolpix photographic camera (Nikon, Ville, NY, USA). Fluorescence of each slide was measured in pixels using NIH IMAGE ANALYSIS software. Fluorescence is proportional to TH immunolabeling (Kohn *et al.* 1999). Finally, for each PG, the average pixels of the slides from the anterior, medial and posterior parts were calculated. This value corresponded to the total pixels of the complete PG. The results of each independent experiment were expressed as percentage increase in TH immunoreactivity relative to the control. Comparisons were made only between slides that were processed together.

Western blot analysis

A different set of rat groups (control, $n = 6$ and stress, $n = 6$) was used for western blot analysis of the sympathetic markers TH, neurotrophin receptor [p75(NTR)] and α-tubulin, and non-sympathetic markers (Erk1 and Erk2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kohn *et al.* 1999). When the behavioral study was complete, rats were killed with an overdose of sodium pentobarbital. PGs from control and stress groups were placed in ice-cold homogenization buffer consisting of 320 mM sucrose, 5 mM HEPES, pH 7.4, containing a protease inhibitor mixture [phenylmethyl sulfonyl fluoride (1 mM), aprotinin (10 μg/mL), leupeptin (5 μg/mL) and pepstatin A (1 μg/mL)], and homogenized for biochemistry analysis. The tissue was rocked for 10 min at 4°C and centrifuged at 8000 *g* at 4°C for 10 min. The supernatant fluid was collected and the precipitate was normalized for protein concentration using a bicinchoninic acid (BCA) Protein Assay kit (Pierce Chemical Co., Rockford, IL, USA).

For electrophoretic analysis, equal amounts (50 μg) of PG protein from control and stress groups were boiled in loading buffer [0.31 M Tris-HCl, 11.5% sodium dodecyl sulfate (SDS), 50% glycerol, 25% β-mercaptoethanol and bromophenol blue], in a final volume of 30 μL, for 5 min. Samples were then separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 5–20% gels for TH, p75(NTR), α-tubulin, Erk1, Erk2 and GAPDH, under fully reducing conditions, and transferred to nitrocellulose membranes for 1.5 h at 0.35 A . The membranes were then washed three times for 10 min with PBS, and blocked in 5% non-fat milk in PBS for 1.5 h at 20–25°C. For immunodetection, nitrocellulose membranes were incubated overnight at 4°C with primary antibodies. Antibodies against the following antigens were used: anti-TH (1 : 1000; Sigma-Aldrich); anti-p75(NTR) (1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-α-tubulin (1 : 1000; Sigma-Aldrich); anti-Erk1 and anti-Erk2 (1 : 500; Santa Cruz Biotechnology); and anti-GAPDH (1 : 1000; Santa Cruz Biotechnology). The membranes were washed four times for 10 min in PBS-TWEEN and then incubated for 1 h at 20–25°C with the following secondary antibodies: 1 : 5000 goat anti-rabbit (Calbiochem-Novabiochem Corporation, San Diego, CA, USA) for anti-p75(NTR; 1 : 3000 anti-mouse (Calbiochem-Novabiochem Corporation) for anti-TH; 1 : 5000 anti-mouse (Calbiochem-Novabiochem Corporation) for anti-α-tubulin; 1 : 5000 goat anti-rabbit IgG horseradish peroxidase (HRP) (Santa Cruz Biotechnology) for anti-Erk1 and anti-Erk2; 1 : 10 000 goat anti-rabbit IgG HRP (Santa Cruz Biotechnology) for anti-GAPDH. Immunoreactivity was visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Buchler, Braunschweig, Germany).

Blood sample collections

A further group of rats was used to analyze the effect of stress on plasma melatonin concentration. Animals were randomly grouped as follows: control ($n = 20$) and stress ($n = 20$). At the end of the behavioral study, the experimental rats were housed in groups of five under a 12/12 light/dark cycle (lights on at 07.00 hours) with *ad libitum* access to food and water. Blood samples were collected into heparinized glass tubes from the femoral artery of each animal, under deep anesthesia with sodium pentobarbital, at 15.00 hours, 23.00 hours, 01.00 hours, 04.00 hours and 07.00 hours (at each time point, control, $n = 5$ and stress, $n = 5$). Blood samples were centrifuged at 500 *g* for 10 min, then plasma was separated and stored in aliquots at − 20°C until melatonin determinations.

Melatonin radioimmunoassay (RIA)

The melatonin concentration in the plasma was measured by radioimmunoassay (Fraser *et al.* 1983) using a highly specific melatonin antiserum (G/S/704 8483, Stockgrand Ltd, Guildford, UK) and [*O*-methyl-^{3}H]melatonin, 85 Ci/mmol (AB, SE-751 84, Amersham Biosciences Uppsala, Sweden) as tracer, following the manufacturer's recommendations. The range of the standard curve was 0.78–50 pg/tube (3.4–215 fmol per tube) and the intra- and inter-assay coefficients were less than 15%. Plasma samples were extracted with diethyl ether prior to assaying. Values were not corrected for recovery, as it was over 90%.

Statistical analysis

The behavioral studies were analyzed by a non-parametric Chi-square test. The immunohistochemistry and radioimmunoassay studies were analyzed using a Mann–Whitney *U*-test. The percentage gain in body weight and relative adrenal weight were analyzed

by a Student's *t*-test. Results were expressed as the mean ± SEM values. A probability level of 0.05 or less was accepted as significant.

Results

Spontaneous motor responses

Figure 1 shows the effects of chronic immobilization stress on spontaneous motor activity. Stress did not affect the motor activity of the rats (control: 1075 ± 76, $n = 49$; stress: 1073 ± 65, $n = 49$).

Stress markers in the experimental animals

A significant reduction in both percentage of open-arm entries, $p < 0.01$ (stress: 25.8 ± 3.5%, $n = 49$; control: 40.8 ± 1.9%, $n = 49$) and percentage of time spent in open arms, $p < 0.05$ (stress: 11.7 ± 1.8%, $n = 49$; control: 21.8 ± 1.7%, $n = 49$), was recorded in the elevated plus-maze, which is indicative of an enhanced anxiety response in the stressed animals because stress did not affect the motor activity (Figs 1 and 2a). In addition, acute gastric lesions were observed in all the inspected stressed animals ($n = 2$ for each of the immunohistochemistry, western blot and RIA studies) (Fig. 2b). The stress-induced desquamation of the surface epithelium, appearance of necrotic rests and unspecific acute inflammation in the gastric mucosa are features that are usually related to chronic ulcerated lesions. We also analyzed the effects of chronic stress on body and adrenal weights. After 10 days of stress, statistical analysis revealed a significant reduction in percentage body weight gain (stress: − 3.8 ± 2.7%, $n = 49$; control: 7.8 ± 3.2%, $n = 49$; $p < 0.001$) and a significant adrenal hypertrophy (relative adrenal weight, stress: 18.1 ± 1.1, $n = 49$; control: 16.1 ± 1.2, $n = 49$; $p < 0.05$). These results indicate a correct stress protocol.
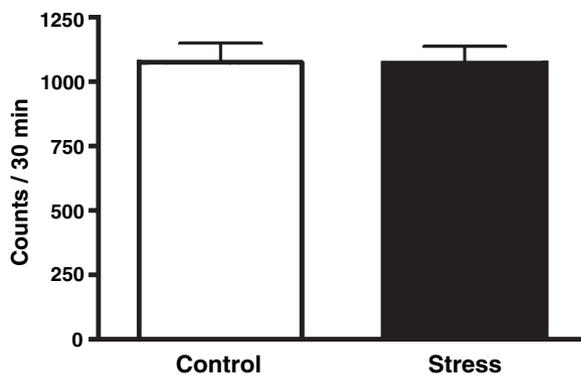

Fig. 1 Effect of chronic immobilization stress on spontaneous motor responses in rats. Stress does not affect the motor activity of the experimental animals. Bars represent the total spontaneous motor activity in a 30 min observation period. The values are the mean ± SEM.
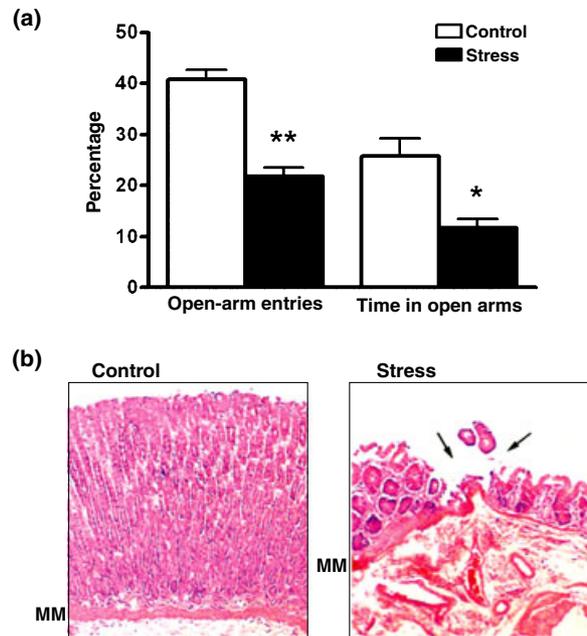

Fig. 2 Physical and behavioral parameters of stress in the experimental animals. (a) Stress increases anxiety in the elevated plus-maze. At 10 days after chronic immobilization stress, rats show decreases in the time and entries into the open arms of the elevated maze, indicating an increase of anxiety in these animals. Data represent the means ± SEM of 49 rats for the control group and 49 for the stress group. Comparisons were made using non-parametric Chisquare test; *$p < 0.05$, compared with control group. (b) Microscopic appearance of gastric mucosa in control and stressed animals. Note that following the stress protocol (stress), the gastric mucosa exhibits a discontinuation of the surface epithelium (arrows) that is related to mucosal ulceration. Hematoxylin and eosin staining, magnification 260×.

Chronic immobilization stress decreases the expression of sympathetic markers in the PG

TH-positive label was significantly decreased (approximately 40%) in the PG of stressed rats with respect to unstressed control rats (Figs 3a and b). To test whether the sympathetic innervation of the PG was specifically reduced, the protein levels of TH and other sympathetic markers [p75(NTR) and α-tubulin] and of non-specific sympathetic axon markers (Erk1 and Erk2) were immunodetected (Kohn *et al.* 1999). Western blot analysis of equal amounts of protein from PGs obtained from control and stressed animals revealed that TH, p75(NTR) and α-tubulin levels were decreased in the stressed rats relative to controls (Fig. 4), in agreement with the immunohistochemical results. The levels of the non-specific sympathetic markers and GAPDH did not change in control and stressed animals (Fig. 4).

Effect of chronic stress on plasma melatonin concentration

Chronic stress increased total plasma melatonin concentration between 15.00 hours and 07.00 hours with respect to
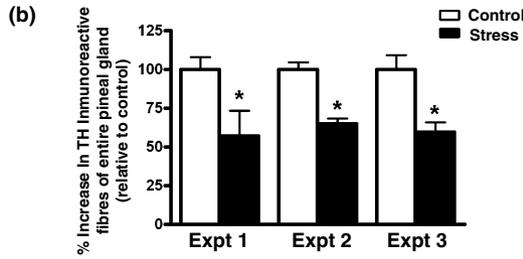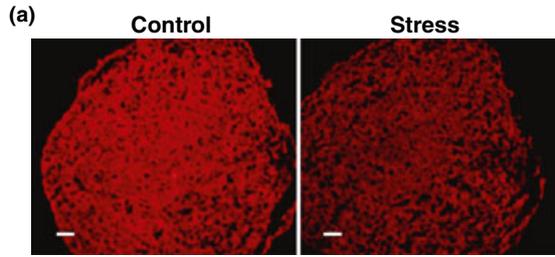
(a)

(b)

Fig. 3 Effects of chronic stress on sympathetic innervation of the PG. (a) Immunohistochemical analysis of TH, a specific marker for sympathetic axons, in sections of the PG from control and stressed rats. Photomicrographs showing a significant loss of TH-positive label in the PG of rats that were subjected to stress, compared with control rats. Bar = 10 μm. (b) Semi-quantitative analysis of the relative amount of PG area covered by TH-immunoreactive fibers in control and stressed animals, obtained using sections similar to those shown in (a). The amount of TH-positive innervation in the stressed PG was significantly lower than that seen in control PGs (*$p < 0.05$).
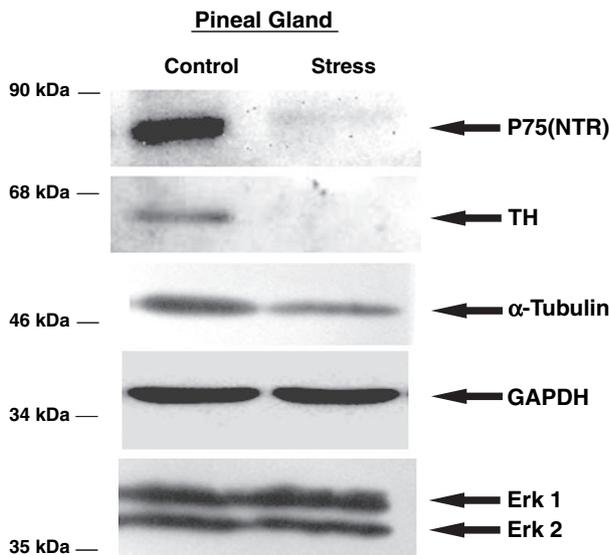
Pineal Gland

Fig. 4 Expression of sympathetic axonal markers is decreased in PGs of stressed rats. Western blot analysis of pineal sympathetic markers [p75(NTR), tyrosine hydroxylase (TH) and α-tubulin], non-sympathetic markers [ERK1 (Erk1) and ERK2 (Erk2)] and GAPDH in equal amounts of protein from the PGs of control and stressed animals. Blots for pineal sympathetic markers show that these proteins are decreased in the PGs of rats subjected to stress. The expression of non-sympathetic markers did not change in control and stressed rats.

control animals (stress: 302 ± 43 pg/mL, $n = 25$; control: 231 ± 32 pg/mL, $n = 25$) (Figs 5a and c). Nocturnal plasma melatonin concentration at 23.00 hours was significantly higher ($p < 0.05$) in stressed rats than in control rats (stress: 365 ± 73 pg/mL, $n = 5$; control: 149 ± 24 pg/mL, $n = 5$; $p < 0.05$) (Fig. 5b).
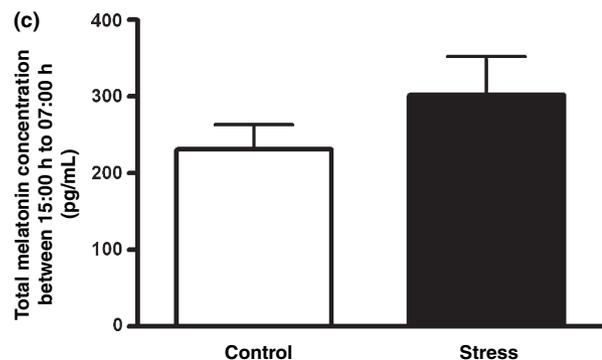
(a)

(b)

(c)
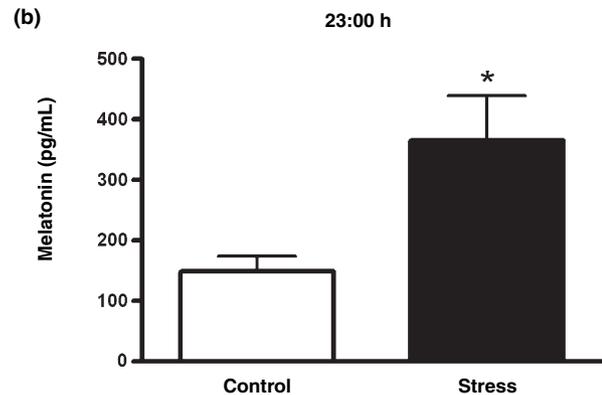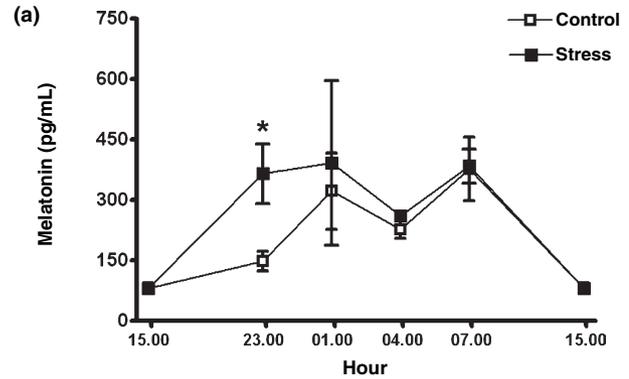
Fig. 5 Effects of chronic stress on plasma melatonin concentration. (a) Circadian profile of plasma melatonin concentration in control (open squares) and stressed (filled squares) rats. (b) Plasma melatonin concentration in control and stressed rats at 23.00 hours. Plasma melatonin concentration in stressed rats was significantly lower than control rats ($p < 0.05$). (c) Total plasma melatonin concentration between 15.00 hours and 07.00 hours with respect to control animals. Values are mean ± SEM ($n = 5$ in each group).

Discussion

In the present study, we analyzed the effect of chronic stress on pineal sympathetic markers and on plasma melatonin concentration in rats. Our results indicate that chronic stress decreased the expression of TH, p75(NTR) and α-tubulin (sympathetic markers), while the same treatment did not affect Erk1, Erk2 (non-specific sympathetic markers) and GAPDH expression (Figs 3 and 4). These results are compatible with sympathetic denervation of the PG. In addition, stress significantly increased plasma melatonin concentration during the dark phase (23.00 hours, $p < 0.05$) (Fig. 5). This may result from stimulation of pinealocyte β-receptors by the high levels of circulating norepinephrine (NE), which is secreted from the adrenal medullae during stress (Vogel and Jensh 1988).

Previous reports have shown that chronic immobilization stress increases dendritic arborization in stellate neurons of the basolateral amygdala, which could be the cellular substrate of the enhancement in anxiety-like behavior in the elevated plus-maze (Fig. 2a) (Vyas *et al*. 2002; McEwen and Chattarji 2004). The same stress treatment did not affect spontaneous motor activity (Fig. 1), indicating that poor performance in the elevated plus-maze is related to an increase in anxiety in stressed rats. In this line, some reports suggest that different types of maze and the inter-test interval may have an impact on behavioral performance (Bellani *et al*. 2006). Other reports suggest that in mice, the testing interval in various types of maze has no impact on performance (Paylor *et al*. 2006). We believe that testing motor activity in rats does not influence performance in the elevated plus-maze because the motor activity test is a non-aversive task in relation to other tests such as the water maze (Morris 1984). Conversely, if the elevated plus-maze test is performed before the motor activity test, it is possible that the plus-maze test affects the performance of the motor activity test, because the plus-maze has been reported to be anxiogenic (Rodgers *et al*. 1996; Ouagazzal *et al*. 1999). Moreover, chronic immobilization stress produced reduction in percentage body weight gain, significant adrenal hypertrophy and acute gastric lesions (Fig. 2b). These results are similar to those from previous reports using the same signs as stress markers (Magariños and McEwen 1995; Vyas *et al*. 2002).

Having established the efficacy of our stress regime, the novel and interesting observations of the present study came from our analyses of the effect of chronic immobilization stress on the expression of sympathetic markers in the rat PG and the plasma melatonin concentration. In this line, destruction of sympathetic terminals in the PG with 6-hydroxydopamine, or bilateral superior cervical ganglionectomy, does not block but rather, potentiates the increases in melatonin content and AA-NAT activity in the PG following physical immobilization in rats (Lynch *et al*. 1973, 1977).

Conversely, stress-induced increase in pineal AA-NAT activity was blocked by bilateral adrenalectomy (Lynch *et al*. 1977). These findings suggest that synthesis of melatonin in the PG may be regulated both by the pineal sympathetic innervation and via the circulating NE secreted from the adrenal medulla. Stress increased the levels of circulating NE in rats (Vogel *et al*. 1988), and the stress-related high plasma melatonin concentration is probably produced through stimulation of pinealocyte β-receptors by the high levels of circulating NE. We believe that our immunohistochemical and western blot studies suggest that stress could induce sympathetic denervation of the PG. It is difficult to produce direct evidence that stress produces sympathetic denervation in the PG. Loss of nerve terminals could be assessed by measuring labeled NE uptake as a function of uptake blockers or alpha 2-adrenergic treatments. However, it is difficult to determine which cell types are in fact contributing to the differences in NE uptake because, in addition to the pineal sympathetic neurons, the phagocytes (Balter and Schwartz 1977; Cosentino *et al*. 1999), peptidergic neuron-like cells (Al-Damluji and Kopin 1996) and pineal neurons (Simonneaux and Ribelayga 2003) of the PG take up NE. Secondly, stress may affect the expression of pineal adrenergic receptors or the interaction between NE and the pineal adrenergic receptors. Finally, it is difficult to discriminate in these experiments whether or not stress is affecting NE metabolism in the pineal sympathetic axon terminals, for example, affecting monoamine oxidase expression which may induce an increase in NE release from the axon terminals. Another proposed method for providing functional evidence that stress produces pineal sympathetic denervation is to inject NE into the rat, which could stimulate the pineal gland if it is truly denervated, as indicated by melatonin production or arylalkylamine *N*-acetyltransferase activity. For this experiment, a new stressor would be applied when the NE was injected, possibly affecting the expression of several neuropeptides that regulate melatonin secretion in the brain or induce an increase of NE in the adrenal glands. Thus, this procedure might produce changes in melatonin secretion independently of sympathetic innervation.

In general, the regulation of the synthesis and secretion of melatonin in the PG is complex and not completely understood. In addition to the dense sympathetic innervation of the PG, other fibers originating from various central structures (especially the habenular nuclei, paraventricular nucleus, thalamic intergeniculate leaflet, dorsal raphe and lateral hypothalamus) innervate the rodent PG (Simonneaux and Ribelayga 2003). Therefore, various neural, endocrine and paracrine inputs participate in the regulation of melatonin synthesis and release. As the melatonin levels measured in the stress group at the other time points in the dark phase did not differ from the control, we propose that some of these inputs could inhibit the synthesis or release of melatonin at

these time points, leading to a cancellation of the activating effects of circulating NE.

As mentioned above, immobilization stress induces functional impairment in the rat pinealocytes (Martinez *et al.* 1992; Milin *et al.* 1996; Milin 1998). In our view, the PG may be a target site for glucocorticoid damage during stress because, like other regions such as the hippocampus that are sensitive to stress, this gland expresses a high density of glucocorticoid receptor, which is also higher than other regions that are not affected by glucocorticoids, such as the intermediate and posterior lobes of the pituitary (Warembourg 1975; Meyer *et al.* 1998). Prolonged glucocorticoid secretion during chronic stress may have deleterious effects in the PG. Proposed mechanisms include inhibition of glucose transport, and a faster decline of ATP concentrations and metabolism in pinealocytes, similar to that proposed in hippocampal stress damage (Sapolsky 2000). Sympathetic denervation may be another of the deleterious effects of stress on the PG, that is normally highly irrigated and innervated by sympathetic neurons of the SCG. Chronic stress increases the synthesis and release of NE from the adrenal medulla and activates the sympathetic-adrenergic-noradrenergic system of the stress response (Vogel *et al.* 1988; Tafet and Bernardini 2003). The net effect of both changes may be an increase in NE concentration between sympathetic axon terminals and the NE receptor-expressing pinealocyte plasmatic membranes. We propose that the increase in NE surpasses the NE uptake capacity of the sympathetic axon terminal and the biodegradation capacity of NE through *o*-metilation catalyzed by catechol-*o*-methyltransferase in the PG, increasing the NE catechol group oxidation and free radical production, and favoring the retraction or neurodegeneration of sympathetic neurons. A similar process is found with L-dopa and dopamine (Dagnino-Subiabre *et al.* 2000a,b).

Impairments in the pineal sympathetic innervation and the rhythmic secretion of melatonin may, in turn, affect the information transmitted to brain areas that regulate the limbic-HPA and sympathetic-adrenergic-noradrenergic systems, because the hippocampus, an important regulator of both, has high levels of melatonin receptors (Musshoff *et al.* 2002). *In vivo* experiments of SCG ganglionectomy and pineal sympathetic denervation with 6-hydroxy-dopamine in mammals showed several effects, such as alterations in the normal photoperiodic control of reproduction and in the immune system (Cardinali *et al.* 1981a,b; Aldegunde *et al.* 1985; Magnusson and Kanje 1998). Different models of chronic stress induced similar effects associated with *in vivo* alterations of melatonin secretion (Lynch *et al.* 1977; Vollrath and Welker 1988; Persengiev *et al.* 1991). If the PG is denervated by chronic stress, the light and darkness information received by the retina may not reach other cells of the body because a key neuronal circuit that translates the neurochemical message

of the photoperiod into a hormonal message is interrupted. Likewise, pineal denervation may have enormous biological implications, because rhythmic melatonin secretion participates in the modulation of several biological processes, such as the regulation of cellular metabolism by the circadian cycle (Simonneaux and Ribelayga 2003) and the release of neurotransmitters, e.g. serotonin and dopamine (Simonneaux and Ribelayga 2003). These changes may be a related to alterations in brain areas that are enriched in melatonin receptors and that are regulated by melatonin, like the hippocampus and amygdala (Miguez *et al.* 1991a,b; Musshoff *et al.* 2002).

There is evidence, especially at the clinical level, that stress induces depressive symptoms and is an important cause of the development of several depressive disorders, including major depression (Gold *et al.* 1988; Post 1992; Tafet and Bernardini 2003). Sleep disturbances such as insomnia (Jindal and Thase 2004), and reduced nocturnal peak of pineal melatonin secretion, are almost always present in depressed patients (Pacchierotti *et al.* 2001). It is possible that in major depression, decreased melatonin levels are a consequence of the low plasma NE concentration observed (Koenigsberg *et al.* 2005).

In conclusion, chronic stress specifically decreased sympathetic marker expression in the PG and increased circulating melatonin levels in rats. Both alterations may affect the capacity to process the emotional interpretation of external stimuli by the hippocampus and amygdala. Furthermore, this study proposes that the PG may be a target of glucocorticoid damage during stress, and that the stress-related alterations in rhythmic melatonin secretion may play a key role in the development of stress-related disorders. If this is true, medical treatment to achieve normal rhythmic melatonin secretion could become an effective approach to prevent the development and/or progression of some of the depressive symptoms in chronically stressed subjects.

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