Chronic stress induces upregulation of brain-derived neurotrophic factor (BDNF) mRNA and integrin α5 expression in the rat pineal gland

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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. Moreover, stress induces deleterious actions on the epithalamic pineal organ, a gland involved in a wide range of physiological functions. The aim of this study was to investigate whether the stress effects on the pineal gland are related with changes in the expression of neurotrophic factors and cell adhesion molecules. Using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot, we analyzed the effect of chronic immobilization stress on the BDNF mRNA and integrin α5 expression in the rat pineal gland. We found that BDNF is produced in situ in the pineal gland. Chronic immobilization stress induced upregulation of BDNF mRNA and integrin α5 expression in the rat pineal gland but did not produce changes in β-actin mRNA or in GAPDH expression. Stressed animals also evidenced an increase in anxiety-like behavior and acute gastric lesions. These results suggest that BDNF and integrin α5 may have a counteracting effect to the deleterious actions of immobilization stress on functionally stimulated pinealocytes. Furthermore, this study proposes that the pineal gland may be a target of glucocorticoid damage during stress.

Keywords:
Stress
BDNF
Integrin α5
Pineal gland
Depression

1. Introduction

Chronic stress induces increased levels of adrenal glucocorticoids and morphologic alterations in limbic areas (McEwen and Chattarji, 2004). The hippocampus, a main structure for spatial learning and memory, is susceptible to stress, and glucocorticoid damage produces dendritic remodeling of CA3 pyramidal neurons and a decrease in adult neurogenesis in the dentate gyrus (Sapolsky et al., 1991; Magariños and McEwen, 1995; McEwen, 1999). More recently, it has been shown that in addition to the hippocampus, the amygdala and prefrontal cortex are morphologically affected by stress and corticosterone in rats (Wellman, 2001; Vyas et al., 2002; Radley et al., 2004). These alterations are related to learning, memory,

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and emotional response impairments (McEwen and Chattarji, 2004). In humans, postmortem and brain imaging studies have revealed atrophy or loss of neurons in the hippocampus and prefrontal cortex of both depressed and anxious patients (Sheline et al., 1996; Shah et al., 1998; McEwen and Chattarji, 2004). Furthermore, hippocampal volume reductions have been related to emotional and memory impairment in major depression (Duman et al., 1999).

Stress has also been found to affect the epithalamic pineal gland, which is known to secrete melatonin, a hormone involved in a wide range of physiological functions (Simonneaux and Ribelayga, 2003). Electron microscopy studies have determined that immobilization stress induces pinealocyte degeneration (Milin et al., 1996; Milin, 1998). These alterations are related with significant increases of rat pineal melatonin levels (Vollrath and Welker, 1998). In tree shrews, psychosocial stress induces a drastic increase of 6-sulfatoxymelatonin (a main melatonin metabolite) excretion in subordinate animals (Fuchs and Schumacher, 1990), while in humans, sleep disturbances, such as insomnia (Jindal and Thase, 2004), and reduced nocturnal peak of pineal melatonin secretion are almost always present in depressed patients (Brown et al., 1985; Frazer et al., 1986; Pacchierotti et al., 2001). These studies suggest that the pineal gland may be target of stress damage by the glucocorticoid stress hormones because this gland expresses high density of the glucocorticoid receptor (Warembourg, 1975; Sarrieau et al., 1988; Meyer et al., 1998a,b).

Melatonin receptors are present in regions 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). Rhythmic melatonin secretion from the pineal 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).

Stress-related morphologic alterations may be induced by impairments in the neurotrophic factor expression and/or in the neurotrophin signaling transduction pathway. Stress and corticosterone treatment have been found to decrease brain-derived neurotrophic factor (BDNF) mRNA levels in the rat hippocampus and prefrontal cortex (Smith et al., 1995; Nibuya et al., 1999), and BDNF concentration is decreased in the plasma of depressed patients (Karege et al., 2002). BDNF has been shown to protect the central nervous system under a variety of insults and has been considered as a protecting factor in experimental models of depression (Altar, 1999).

Moreover, integrins have been also suggested to play a role in brain damage reparation, especially in response to cellular injury such as focal ischemia (Ellison et al., 1999). Integrins are a family of transmembrane glycoprotein receptors that couple intracellular cytoskeletal elements with extracellular matrix molecules. Integrins exist as $\alpha\beta$ heterodimers that associate at their extracellular domains. Both α and β subunits contribute to the ligand binding domain. The integrin $\alpha 5$ is a key molecular component of the matrix remodeling process after cellular insult (Ellison et al., 1999).

In this context, we decided to study the stress effect on BDNF and integrin $\alpha 5$ expression in the rat pineal gland because both have a key role in the protection to cellular injury in the central nervous system. Thus, the main objective of the present study is to determine whether previously described degenerating pinealocytes and pineal function impairment induced by immobilization stress are associated with changes of BDNF and integrin $\alpha 5$ expression in the rat pineal gland.

2. Results

2.1. *Spontaneous motor responses*

Fig. 1 shows the effects of chronic immobilization stress on spontaneous motor activity. Stress did not affect the motor activity of the rats (control: 1137 ± 67, $n = 12$; stress: 1111 ± 52, $n = 12$) (Fig. 1).

2.2. *Stress markers in the experimental animals*

The behavioral response to chronic stress was investigated comparing the performance of stressed and control rats in the elevated plus-maze test. We observed consistent changes in anxiety levels after exposure to stress. A significant reduction in percentage of open-arm entries (stress: 25.8 ± 3.5%, $n = 12$; control: 40.7 ± 1.7%, $n = 12$; $P < 0.05$) and percentage of time spent in open arms (stress: 15 ± 0.6%, $n = 12$; control: 22 ± 1.4%, $n = 12$; $P < 0.001$) was found in stressed animals. These results are indicative of an enhanced anxiety response in rats exposed to stress compared with control animals (Fig. 2A) and cannot be related to changes in locomotor activity because there was no significant difference in the total number of arm entries. In addition, acute gastric lesions were observed in the stressed animals (Fig. 2B). Stress induced desquamation of the surface epithelium in the stomach of stressed rats that is related with chronic ulcerated lesions. We also analyzed the effects of chronic stress in body and adrenal weights. Statistical analysis revealed a significant reduction in percentage body weight gain ($P < 0.001$; Student's t test)

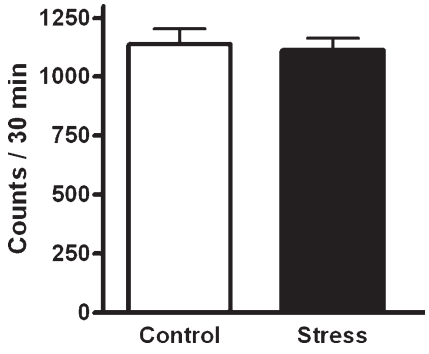

Fig. 1 – Effect of chronic immobilization stress on spontaneous motor responses in rats. Stress does not affect the motor activity of the experimental animals. Bars represent the total spontaneous motor activity in a 30-min observation period. The values are the mean ± SEM.

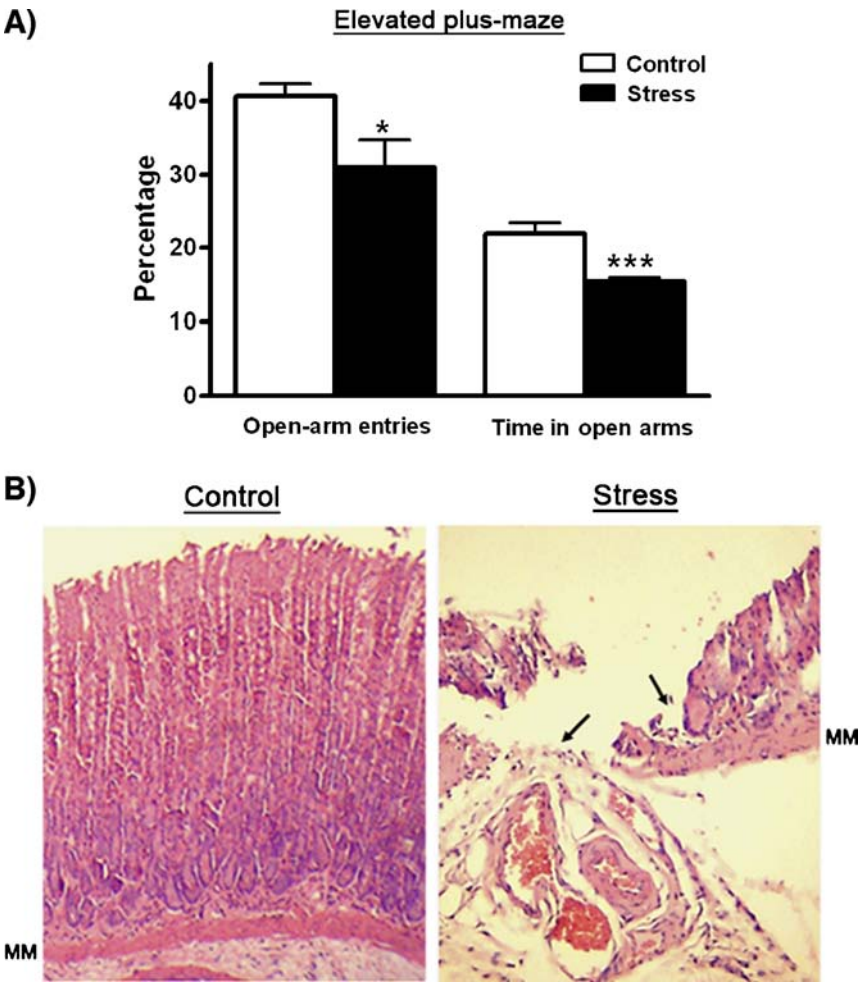

Fig. 2 – Behavioral and morphological indicators of stress in the experimental animals. (A) Stress increases anxiety in the elevated plus maze. After 10 days of chronic immobilization, rats show decreases in the time inside and in the number of entries onto open arms of the elevated maze, indicating an increase of anxiety. Results were expressed as percentage of entries and time spent in open arms over the total arm entries and time (closed plus open arms). Data represent the means ± SEM of 12 rats for control group and 12 for stress group. Comparisons were made using Student's *t* test ($^{*}P < 0.05$ and $^{***}P < 0.001$ compared with control group). (B) Microscopic appearance of gastric mucosa in stressed animals. Note that following 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×.

after 10 days of stress (stress: –3.5 ± 2.9%, $n = 12$; control: 7.9 ± 3.2%, $n = 12$). Finally, stress caused a significant adrenal hypertrophy (relative adrenal weight, stress: 19 ± 1.3, $n = 12$; control: 16.6 ± 1.3, $n = 12$; $P < 0.05$; Student's t test) in the stressed rats. These results indicate that the stress protocol used here was effective in inducing a physiological response.

2.3. *Effects of chronic immobilization stress on BDNF mRNA and integrin α5 expression in the rat pineal gland*

BDNF is synthesized in situ in the rat pineal gland and chronic immobilization stress induced BDNF mRNA overexpression (Fig. 3). Expression of the housekeeping gene β-actin did not change in the pineal gland of the stressed rats (Fig. 3).

Western blot analysis of equal amounts of protein from control and stressed pineal glands revealed that the integrin α5 level was increased in the stressed rats relative to controls (Fig. 4). In addition, GAPDH did not change its expression in control and stressed animals (Fig. 4).

3. Discussion

In this study, we analyzed the effect of chronic immobilization stress on BDNF mRNA and integrin α5 expression in the rat pineal gland. Our results indicate that BDNF mRNA and integrin α5 expression are upregulated in the pineal gland of the stressed rats, while the same treatment did not affect β-actin mRNA and GAPDH expression. The results suggest that basal gene expression in the pineal gland was not affected, while BDNF and integrin α5 overexpression correspond to a specific molecular response to chronic stress. Also, the results suggest that BDNF may have a protecting role in the pineal neurons during stress and a cellular repairing role after the

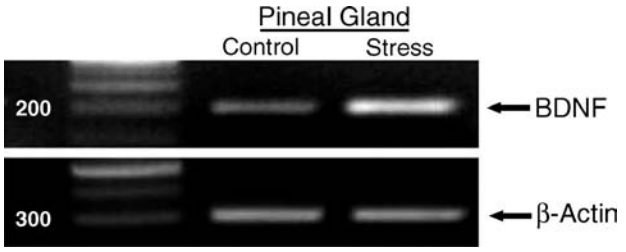

Fig. 3 – Effects of chronic immobilization stress on BDNF expression in the rat pineal gland. The figure shows agarose gels in which the amplification products of BDNF and β-actin of rat pineal glands from animals subjected to stress and control were resolved. Chronic stress induced BDNF overexpression in the pineal gland of stressed rats and did not change the expression of β-actin in controls and stressed animals. Molecular size markers are shown to the left (100 bp DNA Ladder, Gibco BRL).

stress protocol. On the other hand, integrin α5 may have similar roles as BDNF but in all cells types of the pineal gland.

Previous reports showed 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) (McEwen and Chattarji, 2004; Vyas et al., 2002). The same stress treatment did not affect the 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 addition, 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 in previous reports using these signs as stress markers (Takagi and Okabe, 1968; 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 BDNF and integrin α5 expression in the pineal gland. Previous reports show that BDNF is present in the pineal gland during the period of sympathetic target innervation and in the adult rat (Kohn et al., 1999). Fig. 3 shows that BDNF is produced in situ in the pineal gland. Macrophages and neurons of the pineal gland may produce BDNF mRNA (Batchelor et al., 1999; Dougherty et al., 2000; Moller et al., 2002). Specifically, macrophages are able to increase their BDNF synthesis after cellular injury fulfilling a protecting role in the neurons affected (Imamura et al., 2003; Shibata et al., 2003). Nevertheless, our results contrast with previous reports indicating a decrease in BDNF expression in the hippocampus (Hansson et al., 2003). Addressing this point, we consider that during stress, the upregulation of BDNF mRNA expression in the pineal gland could be induced by corticosterone-induced specific activation of some BDNF gene promoter in the pineal macrophages or in neurons, perhaps through activation of glucocorticoid responsive elements (Tabuchi et al., 2000, 2002). This process can be independent of the c-fos/c-jun complex activation, which is activated in the hippocampus by corticosterone, inducing downregulation of glucocorticoid receptors and of BDNF expression in response to acute, as well as repeated immobilization stress (Timmusk et al., 1993; Hansson et al., 2003).

Integrins are additional proteins relevant to cell survival and restoration of the normal brain architecture during brain damage (Milin, 1998; Ellison et al., 1999). Specifically, integrin α5 is expressed in macrophages and neurons and is a key molecular component of the matrix remodeling process after cellular injury (Ichikawa et al., 1996; Pinkstaff et al., 1999; Jin et al., 2004). It is possible that the basal integrin α5 expression in the pineal gland is produced by the macrophages and neurons of the pineal gland (Fig. 4). Pinealocytes make up 90% of all pineal cells (Moller et al., 2002).

As mentioned, immobilization stress induces functional impairment in the rat pineal gland (Martinez et al., 1992; Milin et al., 1996; Milin, 1998). Glucocorticoid receptors are highly expressed in the pineal gland respect to other brain areas and modulate melatonin production (Warembourg, 1975; Sarrieau et al., 1988; Meyer et al., 1998b; Benyassi et al., 2001). In this line, the pineal gland may be a target site of glucocorticoid damage during stress through inhibition of glucose transport and an acceleration of the decline of ATP concentrations and metabolism in pinealocytes, perhaps in a way similar to that proposed in hippocampal stress damage (Sapolsky, 2000). Chronic stress increases plasma corticosterone levels only on the first day of stress protocol (Cook and Wellman, 2004), which is when most pineal damage may occur. At this time, the pineal BDNF and integrin α5 proteins could be used to protect the pineal cells from the deleterious actions of immobilization stress. Pineal neurons can be protected by BDNF, and integrin α5 might have a protective role in all pineal cell types, by enhancing the transmembrane link between the extracellular matrix and the cytoskeleton, which is crucial for cell viability, thus increasing cellular survival during stress (Bellail et al., 2004). It is possible that BDNF and integrin α5 proteins are decreased on the first days of the stress protocol. After this time, the BDNF synthesis could be activated, and the BDNF mRNA upregulated even after the stress protocol. Both, BDNF and integrin α5, may play a role in pineal damage reparation induced by stress.

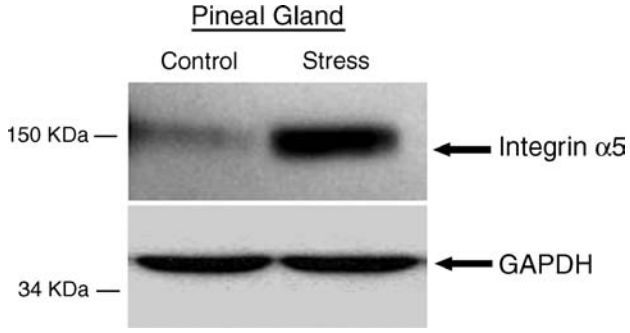

Fig. 4 – Effects of chronic immobilization stress on integrin α5 expression. Western blot analysis of integrin α5 expression in equal amounts of protein from pineal glands of control and stressed rats. Blots for integrin α5 show that this protein is increased in the pineal glands of rat subjected to stress. GAPDH does not change its expression in control and stressed rats.

In conclusion, this is the first study showing evidence that chronic immobilization stress stimulates the expression of cell survival molecules in the rat pineal gland. The upregulation of BDNF and integrin α5 expression in the pineal gland may occur as a counteracting effect to the deleterious actions of immobilization stress on pineal cells. Future studies are needed to establish whether the molecular mechanisms related to stress damage in the hippocampus and pineal gland are similar. Finally, we propose that the pineal gland may be target of glucocorticoid damage during stress in subjects chronically stressed and in patients with major depression.

4. Experimental procedures

4.1. *Experimental animals*

Male rats (Sprague-Dawley) of 285–310 g (3–3.5 months old) (Biological Sciences Faculty, Catholic University, Santiago, Chile) were used for all experiments. Rats were housed in groups of three under a 12/12 light/dark cycle (lights on at 7:00 AM) with ad libitum access to food and water in a temperature-controlled room. Control animals, which were littermates of the stress-treated animals, were housed in separate cages and rooms. All procedures related to maintenance and experimentation of animals were in accordance with the guidelines of the National Commission of Scientific Research and Technology of Chile (CONICYT) and were approved by the animal care committee of the Pontificia Universidad Católica de Chile.

4.2. *Immobilization stress protocol*

We used the same immobilization stress protocol previously described by Vyas et al. (2002), which demonstrated that complete immobilization (2 h/day, 10 AM–noon) for 10 consecutive days in rodent immobilization cages, without access to food and water, induces a significant dendritic atrophy of the apical CA3 neurons in the rat hippocampus, similar to that found after 21 days (6 hr/day) of repeated restraint stress (Magariños and McEwen, 1995). Rats were randomly assigned to two groups: control and stress. Control animals were not subjected to any type of stress. The rodent immobilization cages were made in our laboratory. The dimensions of the immobilization cages were length: 18 cm, wide: 6 cm, and height: 6 cm. The cages allow the complete immobilization of the animals. The rats 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), relative adrenal weight (wet weight of adrenal glands in mg × 100/body weight in grams), anxiety, and presence of ulcers in gastric mucosa (Brzozowski et al., 2000).

4.3. *Spontaneous motor activity*

Between 22 h and 26 h after completion of the stress protocol, each rat was individually analyzed in the following order: spontaneous motor activity and elevated plus-maze. Spontaneous motor activity was tested during 30 min only once. Each rat was individually placed into a plexiglass 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., 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. The monitoring of spontaneous motor activity was performed between 10 AM and 14 PM, under a light condition.

4.4. *Elevated plus-maze*

Immediately after completion of the analysis of spontaneous motor activity, we measured anxiety levels by using the elevated plus-maze test. The rats were transported directly from the activity monitoring apparatus to the elevated plus maze. 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 AM and 2 PM. Results were expressed as percentage of entries and time spent in open arms over the total arm entries and time (closed plus open arms) in order to rule out differences due to changes in locomotor activity.

4.5. *Reverse transcription-polymerase chain reaction (RT-PCR) analysis*

Rats subjected to chronic immobilization stress protocol ($n = 6$) and control animals ($n = 6$) were used to study the BDNF expression in the pineal gland using the RT-PCR technique. When the behavioral study was complete, each rat was sacrificed with an overdose of sodium pentobarbital and perfused intracardially with 0.9% saline solution. Afterwards, the pineal glands were removed immediately, and total RNA was isolated by using RNeasy Midi kit (QIAGEN Inc., Valencia, CA, USA). cDNA was synthesized from 1 μg of total RNA using ThermoScript reverse transcriptase. The PCR was performed in 50 μl of reaction sample containing cDNA (corresponding to 75 ng of total RNA), 2.5 μM of each oligonucleotide primer, 0.4 mM of dNTP, 2 mM $MgCl_2$ and 2 U of Taq polymerase (Gibco BRL, Grand Island, NY). The PCR reaction was performed in three steps: (i) 95 °C for 5 min; (ii) 30 cycles at 95 °C for 50 s, 57 °C for 50 s, 72 °C for 50 s; (iii) one cycles at 72 °C for 10 min. The following set of primers was used for the amplification of

BDNF cDNA: primers 5′-GGTCACAGCGGCAGATAAAAAGAC-3′ (upstream) and 5′-TTCGGCATTGCGAGTTCCAG-3′ (downstream) designed from the cDNA sequence of rat BDNF (GenBank accession no. BC087634). Beta-actin expression was used as housekeeping gene. Primers for β-actin were 5′-TTGGGTCAACTTCTCAGCACG-3′ (upstream) and 5′-AGGACAGGGGCTCCATTTAGAC-3′ (downstream) (GenBank accession no. V01217). The PCR-amplified region of the BDNF gene spanned the interval between pair bases 519–542 and 687–706, which resulted in a fragment of 188 bp; and for the β-actin gene it was between bases 408–431 and 744–720, resulting in a fragment of 293 bp. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and photographed.

4.6. *Western blot analysis*

A new set of rats, including control ($n = 6$) and stress ($n = 6$) groups, were used for Western blot analysis of both integrin α5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). When the behavioral study was complete, rats were sacrificed with an overdose of sodium pentobarbital. Rat pineal glands of control and stressed animals were placed in ice-cold homogenization buffer [20 mM Tris, 150 mM NaCl, 0.5% NP-40, 2 mM EGTA, 2 mM EDTA, pH 7.4, containing phosphatase inhibitor mixture (sodium pyrophosphate (30 mM), sodium Fluoride (100 mM), sodium orthovanadate (2 mM)), containing protease inhibitor mixture (phenylmethylsulfonyl fluoride (10 μg/ml), aprotinin (10 μg/ml), leupeptin (5 μg/ml), and pepstatin (1 μg/ml)] and homogenized for biochemistry analysis. The tissue was rocked for 5 min at 4 °C and centrifuged at 8000 × *g* at 4 °C per 10 min. The supernatant was collected, and lysates were normalized for protein concentration using a BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL).

For electrophoretic analysis, equal amounts (1.5 μg) of protein from control and stressed rat pineal glands were boiled in loading buffer (62.5 M Tris–HCl, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.006% bromophenol blue), in a final volume of 30 μl, for 5 min. Samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 6% gel under fully reducing conditions and transferred on to nitrocellulose membranes for 2 h at 0.3 amps. After that, the membranes were washed three times for 10 min with PBS and were blocked in nonfat milk-Tween (0.61% Tris–HCl, 0.59% NaCl, 1% Tween, 5% nonfat milk, pH 7.7) for 1.5 h at room temperature. For immunodetection, nitrocellulose membranes were incubated overnight at 4 °C with primary antibodies. Antibodies against the following antigens were used: anti-integrin α5 (1:500; molecular weight: 150 kDa; Santa Cruz biotechnology, Santa Cruz, CA), anti-GAPDH (1:1000; molecular weight: 37 kDa; Santa Cruz biotechnology, Santa Cruz, CA). The membranes were washed three times for 10 min in nonfat milk-Tween and then incubated with secondary antibody [1:5000 rabbit anti-goat IgG HRP (Santa Cruz biotechnology, Santa Cruz, CA) for integrin α5; 1:10,000 goat anti-rabbit IgG HRP (Santa Cruz biotechnology, Santa Cruz, CA) for GAPDH] for 1 h at room temperature. Immunoreactivity was visualized using the ECL detection system (Amersham Buchler).

4.7. *Statistical analysis*

Data of behavioral studies were analyzed by using an independent groups Student's *t* test. Significance was accepted at the $P < 0.05$ significance level. All data were expressed as mean ± SEM values.

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