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Expression of mRNA and proteins for testicular steroidogenic enzymes and brain and pituitary mRNA for glutamate receptors in rats exposed to immobilization stress ☆

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Abstract

The objectives of this study were to determine whether stress attenuates the pituitary LH response to excitatory amino acids by altering expression of glutamate receptor 1 (GluR1) and *N*-methyl-D-aspartic acid (NMDA) receptor mRNA levels in the hypothalamus or pituitary, and assess whether stress influences testicular levels of mRNA or protein for steroidogenic enzymes. Three hours (h) of immobilization stress was associated with a greater than 7-fold increase in serum corticosterone, and a marked reduction in serum testosterone (T) concentrations. Stress did not significantly alter hypothalamic or pituitary GluR1 and NMDA receptor mRNA levels. Although transcript levels for $P450_{SCC}$ and $P450_{17\alpha}$ mRNA in the testis were unchanged in stressed rats, western blotting of testicular fractions revealed reduced amounts of $P450_{SCC}$ and 3β-HSD, but not $P450_{17\alpha}$. The data suggest that immobilization stress reduces T production by suppressing the translation of transcripts for $P450_{SCC}$ and 3β-HSD, but the attenuated LH response of stressed animals to NMDA is not mediated by altered hypothalamic or pituitary expression of GluR1 and NMDA receptor levels.

1. Introduction

Exposure of adult male rats to acute immobilization stress is characterized by a reduced LH response to administration of the excitatory amino acid agonist, *N*-methyl-D-aspartic acid (NMDA), and lower basal and gonadotropin-stimulated testosterone (T) production [1–4]. The decline in T production during immobilization stress is associated with suppressed activity of key enzymes in the testicular steroidogenic pathway, i.e., 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase/17,20 lyase ($P450_{17\alpha}$) [5–7]. These data provide some interesting information concerning the mechanisms by which stress alters the activity of the hypothalamic-pituitary-testicular (HPT) axis in the male, but these modulatory mechanisms have not been fully defined.

The present study was undertaken in our continuing effort to better understand this process. The study had two major objectives. The first was to determine whether the attenuated LH response to NMDA in stressed rats resulted wholly or in part from alterations in hypothalamic or pituitary expression of glutamate receptor 1 (GluR1) and/or NMDA receptors. The second was to assess whether the reduced testicular activities of 3β-HSD or $P450_{17\alpha}$ were the result of changes in expression (mRNA content) or translation (protein content) of these two enzymes.

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2. Materials and methods

2.1. Animals and experimental protocol

Adult male rats (250 g) purchased from Harlan Sprague–Dawley (Indianapolis, Indiana) were housed two animals per cage and provided with water and standard rat pellets (Ralston Purina, St Louis, Missouri) ad libitum. Animals were maintained in a 14 h light and 10 h dark schedule at 23–26°C. Animals were handled daily during a 1-week period of acclimation to the animal room conditions before experimentation.

Rats were assigned randomly to the stress or control group. Stressed rats were immobilized for 3 h as previously described [6]. Control rats remained unhandled throughout the stress period. Immediately following the stress period, animals (stressed and control) were subjected to rapid decapitation in an area immediately adjacent to the animal room as we have described previously [6]. Trunk blood was collected for measurement of serum concentrations of T and corticosterone. The hypothalamus, anterior pituitary and testes were rapidly excised and frozen in liquid nitrogen for determination of hypothalamic and pituitary GluR1 and NMDA receptor mRNA levels, and testicular levels of side-chain cleavage enzyme ($P450_{SCC}$), 3β-HSD and $P450_{17\alpha}$ mRNA and protein. The experiment was terminated at 3 h to measure excitatory amino acid receptor expression because this was precisely the time when sensitivity to NMDA (relative to LH) was reduced in our previous report [1].

2.2. Northern blot analysis

Total RNA from brain, anterior pituitary or testis from control or stressed animals was isolated with RNAzol (Biotecx Laboratories, Inc., Houston, TX). The RNA was reconstituted in 0.1% Sarcosyl with β-mercaptoethanol and quantified spectrophotometrically at 260 nm.

Total RNA (20 μg) was denatured by glyoxylation and electrophoresed on a 1.5% agarose gel with TEA buffer (10 mM Tris, 0.03 mM EDTA, 5 mM sodium acetate), then blotted to a nylon membrane by capillary action in 10X SSC (1X SSC = 150 mM NaCl, 15 mM sodium citrate) for 24 h [9]. After the transfer, the membrane was rinsed in 50 mM NaOH for 15 s, followed by 1X SSC, 0.2 M Tris–HCl, pH 7.5 for 30 s. The membrane was allowed to dry overnight and the gel was stained with ethidium bromide (5 μg/μl) to ensure complete transfer of RNA.

The cDNA probe for $P450_{17\alpha}$ was obtained from Dr H. R. Fevold (University of Montana, Missoula, MT) and the cDNA for NMDA receptors was kindly provided by Dr S. Nakanishi (Kyoto University, Kyoto, Japan). The GluR1 cDNA probe was obtained from Jim Bolter (Salk Institute, San Diego, CA) and the cDNA probe for β-actin was provided by Dr N. Shimizu (University of Chicago, Chicago, IL). The cDNA probe for cyclophilin was obtained by using RT-PCR amplification of a 196 bp fragment of brain cyclophilin mRNA (nt 59–254, gb M19533). The amplified product was gel purified before ^{32}P labeling by the Oligolabeling Kit (Pharmacia Biotech, Inc., Piscataway, NJ).

Hybridization was carried out in 50% (v/v) formamide, (10% (w/v) dextran sulfate, 50 mM Tris, pH 7.5, 1% (w/v) SDS, 1 M NaCl, and 100 μg/ml sheared denatured salmon sperm DNA for 16–24 h at 42°C. The membranes were washed once with 0.2X SSC, 0.1% SDS at room temperature, twice with 0.2X SSC, 0.1% SDS at 60°C, and once with 2X SSPE (0.36 M NaCl, 20 mM NaH_2PO_4, 20 mM EDTA) at room temperature. Each membrane was autoradiographed and scanned by a digitizing computer program (Alpha Innotech Corp., San Leandro, CA). β-actin or cyclophilin was used to standardize the RNA extraction and transfer.

2.3. Preparation of testicular mitochondrial and microsomal fractions

Mitochondrial and microsomal fractions were prepared from control and stressed animals as previously described [7]. Briefly, testes were decapsulated and homogenized in a buffer (0.01 M potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose, 2.0 mM EDTA, and 20% glycerol). The homogenate was centrifuged at 1200 *g* for 20 min at 4°C and the supernatant was removed. The supernatant was spun at 12,000 *g* for 20 min at 4°C and the resultant mitochondrial pellet was washed, resuspended in homogenizing buffers and stored at −70°C until assayed for $P450_{SCC}$. The postmitochondrial supernatant was further centrifuged at 105,000 *g* for 60 min at 4°C and the resultant microsomal pellet was washed, resuspended in homogenizing buffers and stored at −70°C until assayed for 3β-HSD and $P450_{17\alpha}$. Protein content in the microsomal and mitochondrial fractions from control and stressed rats were determined using Bradford assays [10].

2.4. Western blot analysis

Testicular mitochondrial (60 μg for $P450_{SCC}$) and microsomal (250 μg for 3β-HSD or $P450_{17\alpha}$) fractions from control and stressed rats were solubilized and denatured in a buffer (0.5 M Tris–HCl pH 6.8, 4% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue), and electrophoresed by SDS-

PAGE [11]. Electrophoresed proteins were transferred to Polyscreen PVDF membrane.

The membrane was blocked with 5% nonfat dry milk for 1 h. After washing in PBS containing 1% Tween 20 twice (5 min each time), the membrane was incubated with purified human placental anti 3β-HSD (1:2000 dilution) [12], anti $P450_{SCC}$ (1:2000 dilution) [13] or anti $P450_{17\alpha}$ (1:5000 dilution) for 1 h at room temperature. The membrane was washed 5 times (15 min for the first wash and 5 min/wash thereafter) to remove unbound antibodies. Anti-rabbit IgG conjugated with horseradish peroxidase (1:16,000 dilution; Sigma Immunochemicals, St Louis, MO) was used as secondary antibody. Finally, the membrane was washed 5 times (as indicated above) and then incubated with chemi-luminescent detection reagent for 30 s. It was wrapped in a saran wrap and exposed to reflection autoradiography film for 30 s, before processing with Kodak M35A X-Omat. Tubulin was used as control and the results were normalized for tubulin. The intensity of the signal emitted recognizing 3β-HSD protein were quantitatively analyzed using Bio Image 1-D analyzers. A similar procedure was utilized for $P450_{SCC}$ and $P450_{17\alpha}$. The primary antibodies for 3β-HSD, $P450_{SCC}$ and $P450_{17\alpha}$ were provided by Dr V. Luu-The (CHUL Research Center, Quebec City, Quebec, Canada), Dr M. J. Soares (University of Kansas, Kansas City, KS) and Dr D. B. Hales (The University of Illinois at Chicago, Chicago, IL), respectively. Tissues utilized for protein analyses ($P450_{SCC}$, 3β-HSD and $P450_{17\alpha}$) were obtained from the same set of animals.

2.5. Hormone measurement

Plasma T concentrations were measured as previously described [14], using a commercial radioimmunoassay (RIA) kit purchased from Diagnostic Products Corporation (Los Angeles, CA). The sensitivity of the assay was 0.2 ng per assay tube, and intra- and inter-assay coefficients of variation were 4.6 and 5.7%, respectively.

Plasma corticosterone levels were measured using a commercial kit obtained from ICN Biomedicals, Inc. (Cason, CA) as previously reported [14]. The sensitivity of the assay was 25 ng per assay tube, and intra-assay coefficient of variation was 6.9%.

2.6. Statistical analysis

t-test was used to assess differences between control and stressed animals for plasma T and corticosterone levels, testicular mRNA levels for $P450_{SCC}$ and $P450_{17\alpha}$, and amounts of $P450_{SCC}$, 3β-HSD and $P450_{17\alpha}$ proteins. Expression of hypothalamic or pituitary GluR1 and NMDA receptor mRNA levels were analyzed separately using *t*-test. Differences were regarded as significant at the 95% confidence level ($P < 0.05$).

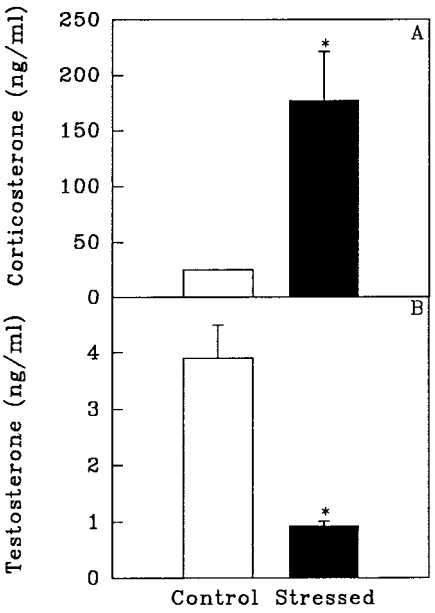

Fig. 1. Effect of immobilization stress (3 h) on mean ± SE plasma corticosterone (A) and testosterone (B) concentrations in control and stressed animals ($N = 6$ rats/group). $^*P < 0.01$ or better, significantly different from the control group.

3. Results

3.1. Effect of immobilization stress on serum T and corticosterone concentrations

Immobilization stress elevated serum corticosterone concentrations more than 7-fold ($P < 0.01$, Fig. 1A), but suppressed serum T levels by more than 80% ($P < 0.007$, Fig. 1B).

3.2. Effect of immobilization stress on hypothalamic and pituitary mRNA levels for GluR1 and NMDA receptors

Acute immobilization stress had no effect on hypothalamic mRNA levels for GluR1 and NMDA receptors as shown in Fig. 2A and B (mean densitometric value ± SE). There were no differences in β-actin expression between control and stressed rats. Pituitary mRNA levels for GluR1 and NMDA receptors in stressed animals also did not differ significantly from the control levels, although there was a tendency for pituitary mRNA receptor levels to be reduced in stressed animals (Fig. 2C and D, respectively).

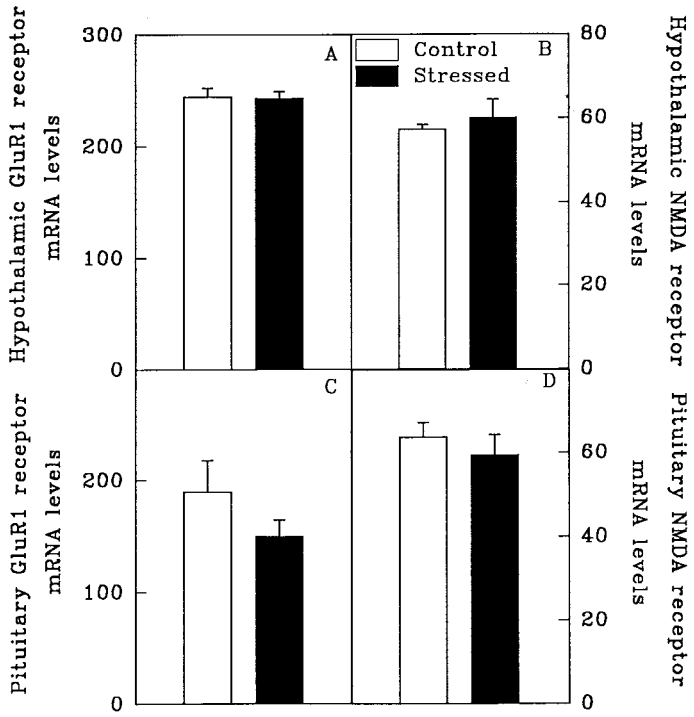

Fig. 2. Northern blot analysis of hypothalamic (A) GluR1 and (B) NMDA, and pituitary (C) GluR1 and (D) NMDA receptors mRNA levels after 3 h of stress exposure in control and stressed animals. Mean ± SE densitometric data was normalized for β-actin. Total RNA was isolated from four individual rats per experimental group.

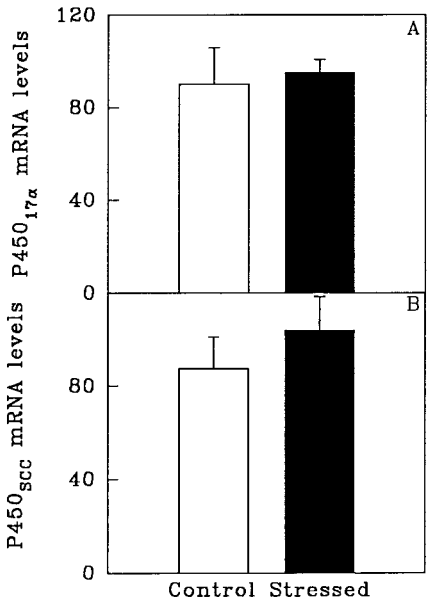

Fig. 3. Northern blot analysis of testicular (A) $P450_{17\alpha}$ and (B) $P450_{SCC}$ mRNA levels in control and stressed animals. Mean ± SE densitometric data was normalized for β-actin. Testes were removed immediately following the stress period or from control rat killed at corresponding times, and subjected to blot hybridization as described in the materials and methods. Total RNA was isolated from four individual rats per experimental group.

3.3. Effect of immobilization stress on testicular $P450_{17\alpha}$ and $P450_{SCC}$ mRNA

Although stress elevated plasma corticosterone and reduced T levels, the overall steady state mRNA levels for $P450_{17\alpha}$ and $P450_{SCC}$ were not altered by stress whether the data were expressed as % β-actin (Fig. 3A and B) or % cyclophilin (data not shown). The levels of expression of β-actin and cyclophilin did not differ between stressed and control animals, suggesting that the lack of differences in mRNA for these steroidogenic enzymes between control and stressed tissues was unrelated to changes in expression of the housekeeping genes.

3.4. Effect of immobilization stress on testicular contents of $P450_{SCC}$, 3β-HSD and $P450_{17\alpha}$

Although transcript levels for $P450_{SCC}$ and $P450_{17\alpha}$ were unaffected by stress, western blotting of testicular fractions (Fig. 4A) revealed reduced amounts of $P450_{SCC}$ (Fig. 4B; $P < 0.05$) and 3β-HSD (Fig. 4C; $P < 0.01$), but not $P450_{17\alpha}$ (data not shown) proteins in stressed rats. Tubulin levels were not different between the control and stressed animals.

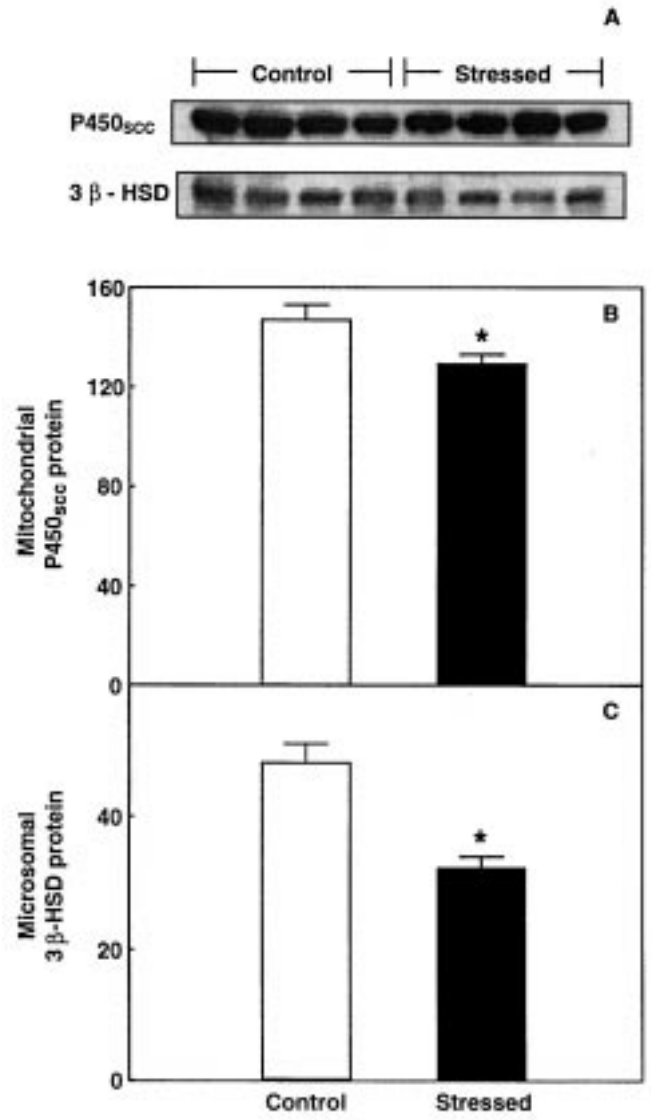

Fig. 4. Effect of immobilization stress (3 h) on western blot analysis of testicular (A) $P450_{SCC}$ and 3β-HSD proteins. Panels B ($P450_{SCC}$) and C (3β-HSD) represent mean ± SE densitometric data for control and stressed animals. Mitochondrial and microsomal fractions were isolated from testes of control and stressed rats, and were subjected to SDS-PAGE and subsequently analyzed by western blotting as described in the materials and methods. Blot was probed with anti-rabbit IgG antiserum. From left to right in panel A, rows 1 and 2, lanes 1–4 represent control and lanes 5–8 represent stressed animals. Each lane contained 60 μg of mitochondrial $P450_{SCC}$, or 250 μg of microsomal 3β-HSD proteins. The blot was quantified by laser densitometry and normalized for tubulin. *Significantly different from the corresponding control value.

4. Discussion

Testosterone produced by testicular Leydig cells plays a vital role in male reproduction, including the initiation, maturation and maintenance of spermatogenesis, accessory gland function and libido. The working hypothesis for these studies is that stress may affect sexual function by acting directly on the testis and/or indirectly through the HPT axis to suppress T production. The results from the current study showed that one means by which immobilization stress suppresses T production is by modulating post-transcriptional events associated with the production of the key steroidogenic enzymes. Testicular protein content of both $P450_{SCC}$ and 3β-HSD but not $P450_{17\alpha}$ enzymes was reduced by immobilization stress. However, stress was not associated with a decrease in expression of the gene coding for $P450_{SCC}$ and $P450_{17\alpha}$ enzymes. It appears that one way in which stress suppresses T production is by reducing the activity [5,6] and protein contents (present study) of key steroidogenic enzymes. However, recent evidence suggests that cholesterol translocation across the mitochondrial membrane by steroidogenic acute regulatory protein may be the rate-limiting step for T biosynthesis [8]. Whether or not this regulatory protein is altered by stress remains a topic for future investigation.

The regulation of enzymatic activity in a tissue is very complex and involves transcription, translation and activation. Steady state mRNA levels usually are good indicators of transcription although differences in mRNA stability and degradation may reduce their value. Western blots for protein content with the above limitations are good indicators of translation, however, they cannot indicate enzyme activity which may be influenced by activators and/or repressors. The results of our previous studies [5,6] showed that the $P450_{17\alpha}$ activity was decreased, but in the present study $P450_{17\alpha}$ mRNA levels and protein levels were unchanged after stress. This is in contrast to $P450_{SCC}$ in which the mRNA levels were unchanged after stress but the protein content was decreased (present study) and the activity was unchanged [3]. Finally with respect to 3β-HSD, our previous study [7] and this study show that both the protein level and the enzyme activity were decreased. Payne and Sha [15] have reported that in the mouse Leydig cell, steady state levels of $P450_{SCC}$, 3β-HSD and $P450_{17\alpha}$ are differentially regulated. There is high constitutive expression of $P450_{SCC}$ and 3β-HSD mRNA, while expression of $P450_{17\alpha}$ mRNA is totally dependent on cAMP stimulation. Furthermore, newly synthesized protein is required for cAMP induction of 3β-HSD and $P450_{17\alpha}$ mRNA levels, but not for $P450_{SCC}$ mRNA [15]. The results of our previous studies and this study, extend the concept of differential control in translation and activation of key steroidogenic enzymes in the testes.

Hales and Payne [16] also demonstrated that corticosterone or the synthetic glucocorticoid, dexamethasone, repressed both basal and cAMP-induced synthesis of $P450_{SCC}$ protein as well as steady state levels of $P450_{SCC}$ mRNA, but the glucocorticoid-mediated decrease of $P450_{SCC}$ synthesis was prevented

by the antiglucocorticoid RU-486. In addition, Orr and Mann [14] demonstrated that in vivo pretreatment of adult male rats with RU486 partially reversed the stress effect of immobilization on serum concentrations of T, and in vitro coincubation of testicular interstitial cells from control animals with RU486 reversed the effect of corticosterone or dexamethasone on testicular steroidogenesis in a dose-dependent manner. These reports and elevated corticosterone levels (present study) suggest that glucocorticoids might be involved in mediating some of the stress-induced changes in testicular steroidogenesis.

Other evidence implicates corticosterone in the negative regulation of T production in rats. Serum T levels were elevated after adrenalectomy (ADX) when compared to control or ADX rats that received corticosterone [17]. The capacity of Leydig cells from ADX rats to produce T was increased compared to controls, indicating that corticosterone at physiological concentrations exerts a negative control on T production by Leydig cells. In humans, elevated cortisol decreases serum T without changing LH levels [18]. In addition, glucocorticoid receptors have been reported to be present on Leydig cells [19] and repression of $P450_{SCC}$ is mediated by these receptors [16]. Although evidence showed that 11β-hydroxysteroid dehydrogenase (11β-HSD) oxidation predominates over reduction in Leydig cells, protecting these cells from glucocorticoid-mediated inhibition of T biosynthesis [20,21]; it appeared the stress-induced/increased degradation of T concentrations associated with a reduction in $P450_{17\alpha}$ activity took place when the intracellular corticosterone exceeded the capacity of 11β-HSD to inactivate it [21,22].

Glucocorticoids can act through nuclear receptors to regulate the genes for steroidogenic enzymes, but this may take longer than the 3 h treatment period used in the present study. Glucocorticoids may also operate through other mechanisms (non-genomic effects) by direct effects on the plasma membrane to regulate steroidogenic enzymes [23,24]. The non-genomic effects of steroids are characterized by fast onset, direct actions on enzyme functions, and rapid recovery upon glucocorticoid removal, effects that do not require RNA and protein synthesis [23,25]. A potential mechanism of these non-genomic effects includes the fact that steroids may affect the coupling between the membrane receptors and regulatory protein, or between a regulatory protein and adenylate cyclase. The current study suggests that stress-induced changes in glucocorticoid levels may alter key steroidogenic enzymes via non-genomic mechanisms.

As yet, the mechanism by which stress reduces the amount of testicular enzyme protein is not completely understood. One possibility is reduced translation of the mRNA transcripts. The fact that there were no differences in testicular mRNA levels for $P450_{SCC}$ and $P450_{17\alpha}$ enzymes between control and stressed rats suggests that the effect of acute stress was on regulation of post-transcriptional events and consequently reduced protein expression. Another possibility is that there is increased degradation of steroidogenic enzymes in Leydig cells during stress. For example, testicular P450 enzymes are susceptible to degradation by oxygen-derived free radicals [26,27]. Finally, there may be a number of inhibitors induced by stress that govern enzymatic activity.

The current study also shows that changes in the serum LH response to excitatory amino acid analogue, NMDA, in stressed rats is not the result of alterations in GluR1 and NMDA receptor expression in the hypothalamus and pituitary. mRNA levels for both GluR1 and NMDA receptor did not differ between control and stressed animals. Thus, the mechanism responsible for the attenuated LH response of stressed rats to NMDA will require further definition.

In summary, one mechanism by which immobilization stress suppresses testicular T production is by regulating post-transcriptional events leading to a reduction in the amounts of $P450_{SCC}$ and 3β-HSD and decreasing the activity of $P450_{17\alpha}$. The level of transcription of $P450_{SCC}$ and $P450_{17\alpha}$ does not appear to be altered by immobilization stress. The decreased activity of $P450_{17\alpha}$ in the presence of unchanged protein levels may be due to degradation products still recognized by the antibody or the presence of as yet unidentified inhibitors.

Acknowledgements

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