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Summary

Androgenic-anabolic steroid (AAS) misuse has been associated with depression. It has been proposed that stress has a role in depression and that serotonin is involved in both endocrine responses to stress and depressive physiopathology. Although reports demonstrate that AAS chronic administration modifies components of stress-responsive hypothalamic-pituitary- adrenal axis (HPAA), no study has evaluated AAS effect on the response to stressful stimuli. We studied the effects of the subchronic administration (once a day for 14 days in rats) of a supratherapeutical dose of nandrolone decanoate (ND) on HPAA and cortical serotoninergic system response to acute restraint stress (RS). Acute RS produced the following effects: increase in CORT (in blood) and ACTH (both in blood and in pituitary corticotropes), GR depletion in hippocampus and hypothalamus cytosol and GR translocation in hippocampus nuclear fraction, cortical serotonin re-uptake stimulation and hippocampus cytosolic ERK2 activation. ND by itself, i.e. in non-stressed rats, did not modify these parameters, except for a decrease of plasma CORT and ACTH levels and an increase in hippocampus cytosolic phospho-ERK1/2. On the contrary, in stressed rats ND affected stress-induced plasma ACTH increase and prevented all other above reported stress effects, except the increase in pituitary ACTH positive cell density. Our results show that the prolonged administration of a supratherapeutical dose of ND in rats, albeit did not affect in a notable way HPAA and serotonin transporter activity in the absence of stress, may deregulate the stress-induced hormonal cascade which plays a crucial role in depressive psychopathology.

Keywords: nandrolone decanoate, acute stress, HPAA, serotonin re-uptake, ERK1/2, JNK1.

1. Introduction

Anabolic-androgenic steroids (AAS) are among the substances more frequently misused by athletes in order to increase muscle mass, enhance lean body mass and improve physical performance. AAS abuse is not restricted to professional athletes, but it also involves amateurs and adolescents who use high doses of these drugs to improve physical appearance and/or for their euphoriant effect (Kindlundh et al., 2001). Therefore, AAS misuse involves a large and heterogeneous group of people. Chronic AAS abuse may be associated with numerous physical and psychological adverse effects(Kicman, 2008). With regard to psychological and psychiatric symptoms, AAS abuse has been associated with mania, major depression and aggression (Bahrke et al., 1992; Pope and Katz, 1994). It has been suggested that corticotrophin releasing factor in the amygdala may play an important role in mediating psychiatric symptoms similar to those associated with AAS abuse and withdrawal (Koob and LeMoal, 1997; Kreek and Koob, 1998). It has been observed that androgens interfere with stress-induced corticosterone high levels probably by acting at multiple sites (Goel et al., 2011); the inhibitory effects of androgens on hypothalamic-pituitary-adrenal (HPA) stress axis may be mediated by reductions in hypothalamic corticotropinreleasing hormone (CRH) levels, adrenocorticotropin hormone (ACTH) content in the pituitary or adrenal weights (Kitay, 1963; Bingaman et al., 1994; Goel and Bale, 2010). Moreover, androgens increase glucocorticoid receptor expression in the hippocampus, which may enhance the negative feedback of the HPA axis in males (Ahima and Harlan, 1992). Many of these effects seem to be obtained by means of an interaction of gonadal hormone with the stress-5-HT circuitry (Goel et al., 2011). Some years ago Schlussman et al. (2000) found that the chronic administration of a high dose of AAS alters proopiomelanocortin and corticotrophin releasing factor mRNA levels in the brain and circulating levels of corticosterone (CORT) and ACTH in rats. Furthermore, it has long been proposed that stress is involved in psychiatric disorders such as depression and that the serotonin system plays an important role in both the regulation of autonomic and endocrine responses to stressful stimuli (Lowry, 2002) and depressive physiopathology (Nemeroff and Owen, 1994). Schlussman's

findings (2000) suggest that chronic administration of AAS may disrupt components of the stressresponsive hypothalamic—pituitary—adrenal axis (HPAA) but, to our knowledge, no data are available on this issue to date. The aim ofthe presentstudy wasto investigate the effects of a prolonged treatment with a supratherapeutical dose of nandrolone decanoate (ND) on HPAA and cortical serotoninergic system response to acute restraint stress (RS) in rats. In particular, we evaluated circulating CORT and ACTH levels, anterior pituitary ACTH cells density, glucocorticoid receptor (GR) number, affinity and subcellular distribution in hypothalamus and hippocampus, hippocampal extracellular signalrelated kinase (Erk1/2), as well as c-Jun N-terminal kinase (JNK1) activation and cortical synaptosomal serotonin transporter (SERT) activity.

2. Materials and methods

2.1. Animals

A total of 70 male Sprague-Dawley rats (Harlan Laboratories, San Pietro al Natisone, Udine, Italy) 2 months old with a body weight of 300 g were housed in groups of two, in polycarbonate cages with ad libitum access to food and tap water, at a 12:12 light-dark cycle (lights on at 0700 h) and controlled room temperature (20-24° C) and humidity (50-60%). They were allowed 1 week acclimatization before the experiment and handled daily during the week prior to the experiment. To avoid circadian variability, all testing was performed between 1000 h and 2400 h, when plasma hormones are at a relatively low levels. Ethical permission for the studies was granted by the Turin University Bioethical Committee; all experimental procedures were performed in accordance with the EC Directive 86/609/EEC and with the Italian law regulating experiments on animals. Furthermore, all efforts were made to minimize animal suffering and to reduce the number of animals used. A first group of 32 rats was treated once a day for 14 days with an IM injection of 0.5 mL of peanut oil containing 15 mg/ kg of ND (Deca-Durabolin1). On the basis of Pope data (Pope and Katz, 1988) Lindblom et al. (2003) consider this dose well corresponding to the abuse of AAS in humans. Sixteen of these rats (N) remained in the cages until sacrifice, while the remaining 16 (N

+ S) were exposed, 24 h after the last injection and immediately before sacrifice, to restraint for 1 h, a physical stress with putative psychological aspects. A second group of 32 rats received once a day the vehicle of ND (0.5 mL oil IM) for 14 days. These animals were subdivided in a subgroup of 16 vehicle-treated rats (V) that stayed in the cages until sacrifice and served as control, and in a second 16 rat subgroup (V + S) that underwent the same acute stress protocol as above.The remaining group of six rats was used as basic control and received daily injection (IM) for 14 days of normal saline to check possible peanut oil effects. For RS,rats were placed into polypropylene restraint tubes for 1 h (Sweerts et al., 1999). On the 15^{th} day of the experiment (i.e.immediately after acute stress protocolforstressed animals), all rats were sacrificed by decapitation; trunk blood was collected and centrifuged to obtain plasma samples for measurement of CORTand ACTH levels; samples were stored at -80°C until use. The brain was rapidly removed and dissected on an ice-cold plate; hypothalamus (for GR binding assay), hippocampus (for GR binding assay, GR protein cellular distribution and assessment of kinasephosphorylation in cytosolic and nuclear extract preparations), cortex (for evaluation of serotonin re-uptake) and pituitary (forthe evaluation of ACTH cells) were collected, immediately frozen in liquid N_2 and stored at -80°C until use.

2.2. Plasma CORT levels

2.2.1. HPLC analysis and chromatographic conditions

CORT was detected and quantified in plasma samples using an HPLC system (High Pressure Liquid Chromatography, VWRHitachi system, LaChrom Elite) as described by Gatti et al. Briefly, separation of CORT and dexamethasone (DXT), used as internal standard (IS), was achieved with a C18 reversed-phase column (LiChroCART1 250-4 HPLC Cartridge, LiChrospher1 100, RP-18, 5 mm) preceded by a guard column. Chromatography separation was carried out by isocratic elution, with methanol-water (70/30, v/v) mobile phase (flow rate 0.8 mL/min) at 30 °C; process of each chromatographic analysis ended in 8 min. The sample injection volume was 50 mL. The EzChrom Elite software integration was employed for CORT quantitative analysis measuring the absorbance

at 240 nm wavelength. The lowest limit of quantification was 0.01 mg/mL (2005), with minor modifications.

2.2.2. Preparation of standards and plasma extraction

Stock solutions of CORT and DXTwere prepared by dissolving an accurately weighed amount of drug in ethanol to obtain a final concentration of 1 mg/mL. Working solution of IS was made with DXT (50 mg/mL) in ethanol. The calibration standards (0.01-5 mg/mL) were prepared with appropriate dilutions of CORT stock solution in blank plasma; a blank sample was included. Solid phase extraction (SPE) columns (100 mg/1.5 mL) were used to perform CORT extraction from plasma. Each plasma sample or standard plasma sample (0.5 mL), spiked with 25 mL of 50 mg/mL IS solution, was applied to a SPE column previously equilibrated with methanol and water. After sample injection in SPE, the washing steps were: 1 mL of water followed by 2 mL of acetone water solution (1/4, v/v) and 1 mL of hexane. One milliliter of diethyl ether was then added and the eluate was completely desiccated. Each dried sample was subsequently resuspended with 200 mL of mobile phase and then injected into HPLC for the analysis. Our analytes retention times were 6.2± 0.1 min for CORT, and 5.2± 0.1 min for DXT. A linear regression was used for all curves in order to obtain the best fit for all calibration points. Regression coefficient (r^2) of all calibration curves was higher than 0.99 for CORT in order to allow a correct drug quantification in plasma samples.

2.3. Plasma ACTH levels

Plasma ACTH was measured using an enzyme-linked immunosorbent assay (ELISA)-ACTH (GenWay Biotech, Inc., San Diego, CA), following the manufacturer's instructions. The intra-assay coefficient of variation was 6.1%; the inter-assay coefficient of variation was 8.7%. The detection limits were approximately 1-500 pg/mL.

2.4. Immunohistochemical analysis

Sections of rat pituitary tissue, serial to those used for conventional hematoxylin and eosin stain, were tested by means of immunohistochemistry without any antigen retrieval procedure using a polyclonal antibody to human/rat ACTH (DakoCytomation, Glostrup, Denmark) diluted 1:2. A biotinfree, dextran chain detection system (EnVysion, Dako) was used according to a standard procedure, and diaminobenzidine was employed as a chromogen. A neutral observer evaluated the density of ACTH positive cells (D) by counting the number of cells detectable in each section (n) by 2-D disector method (Geuna, 2005) and applying the Abercrombie formula described by Hedreen (1998):

$$D = \frac{n}{A \cdot t} \cdot \frac{t}{t + h}$$

where A is the total area of bioptic tissue in the histological section, t is the mean thickness of the section and h is the mean diameter of the ACTH-positive cells.

2.5. GR binding assay

GR binding levels in cytosolic hypothalamus and hippocampus preparations were measured using the procedure previously described by Miller et al. (1990), with partial modifications. Briefly, tissues from three animals of each group were homogenized with a motor-driven pestle in a buffer solution (10 mM Tris, 1 mM EDTA, 20 mM MoNa2O4, 10% glycerol, 5 mM dithiothreitol, pH 7.4) at 4 8C. The homogenate was then ultracentrifuged (105,000 g) for 60 min at 4 8C. The supernatant (cytosol) was incubated for 18-24 h in the presence of [1,2,4,6,7-^{3} H]-DXT (0.6-10 nM, S.A. 23 Ci/mmol, GE Healthcare Europe, Milano, Italia) at 4 °C. Binding in the presence of an excess of unlabeled DXT (2.5 mM) was used for determining non-specific binding. After incubation, the unbound hormone was removed by treatment with dextran-coated charcoal. Proteins were determined with the Bradford method (Bradford, 1976), with bovine serum albumin as a protein reference. The results obtained by Scatchard analysis were expressed as femtomoles of hormone specifically bound per milligram of cytosol protein.

2.6. Western blot analysis

2.6.1. Cytosolic and nuclear extract preparations

Cytosolic and nuclear extracts were prepared as described by Spencer et al. (2000). Hippocampus tissues from four animals ofthe same group were homogenized with a hand-held dounce glass-on-glass tissue grinder in a 50 mM Tris buffer (pH 7.2) containing 6 mM MgCl2, 1 mM EDTA, 10% sucrose, 1 mM phenylmethyl-sulfonyl fluoride, 5 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotinin. The homogenates were centrifuged for 5 min at 2000x *g* at 4 °C. The resulting supernatant and pellet were further processed to generate cytosol and nuclear extract, respectively. For the cytosol preparation, the supernatant was ultracentrifuged (105,000 x *g*) for 1 h at 4 °C and the final supernatant was used as the cytosolic tissue fraction. For the nuclear extract preparation, the pellet was washed twice by resuspension in 0.5 mL of homogenization buffer. The washed pellet was then resuspended in 0.25 mL homogenization buffer containing 0.5 M NaCl. This suspension was then incubated for 1 h in an ice bath with frequent vortexing. Subsequently, the tissue suspension was centrifuged (8000x *g)* for 10 min at 4 8C. The final supernatant was used as the tissue nuclear extract. Protein concentrations for each cytosolic and nuclear samples were determined according to the Bradford method (Bradford, 1976).

2.6.2. GR protein

GR protein cellular distribution in cytosolic and nuclear extract preparations were determined by Western blotting (WB) as previously described (Racca et al., 2005). Briefly, equivalent amounts of protein of each sample (30 mg) were resolved by SDS-PAGE on 8% polyacrylamide gels and transferred to nitrocellulose membrane (HybondTM ECLTM, GE Healthcare Europe, Milano Italia) for immunoblotting. Blots were probed with primary rabbit polyclonal anti-GR (1:650, Santa Cruz sc-1002), and anti-actin (1:2000, Sigma-Aldrich). Blots were then probed with anti-rabbit secondary polyclonal antibody conjugated with horseradish peroxidase. Protein levels were detected using enhanced chemiluminescence ECL WB detection reagent (GE Healthcare Europe, Milano Italia) and bands were visualized through Kodak Image Station 440 CF (Eastman Kodak Company,

Rochester, NY, USA). Image analyses were performed by the Kodak 1D 3.5 software (Eastman Kodak Company). Quantification of GR protein was performed by calculating the ratio between GR and actin protein expression; the latter was selected as reference housekeeping protein.

2.6.3. ERK1/2 and JNK1

For the assessment of kinase phosphorylation, equivalent amounts of protein of each sample (30 mg) were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membrane (HybondTM ECLTM, GE Healthcare Europe, Milano Italia) for immunoblotting. Blots were blocked for 1 h in a PBS-buffered saline solution with 0.1% Tween-20 and 5% low fat milk. Blots were incubated with anti-phospho-ERK1/2 (1:1000, Thr202/Tyr204, Cell Signaling 9106) and anti-phospho-JNK (1:200, Santa Cruz sc-6254) primary antibodies overnight at 4 8C and then washed 10 min x 3 in PBS. Blots were incubated with horseradish-peroxidase-labeled anti-rabbit secondary antibody for 1 h at room temperature, washed 10 min x 3 in PBS, treated with ECL reagent and analyzed by the Kodak 1D 3.5 software (Eastman Kodak Company). Then, blots were stripped with 0.4 M NaOH for 5 min, washed, re-blocked and incubated for 1 h at room temperature with anti-ERK1/2 (1:1000, Cell Signaling 9107) and anti-JNK1 (1:220, Santa Cruz sc-571) primary antibodies which recognized total antigen protein. Subsequently, blots were incubated with horseradish-peroxidase-labeled anti-rabbit secondary antibody, the total protein of ERK1/2 and JNK1 were visualized by treatment with ECL reagent. The above procedures were repeated and the antigen of actin was visualized by binding to the proper primary and secondary antibodies. Quantification of total Erk1/2 and JNK1 was performed by calculating the ratio between each kinase and actin protein (housekeeping protein) expression. Phospho-ERK1/2 and phospho-JNK relative protein level was calculated from the ratio of phosphorylated kinase/total protein kinase.

2.7. Measurement of serotonin (5-HT) re-uptake

2.7.1. Experiments ‘‘ex vivo’’: effects of stress and ND on SERT activity

The measurement of 5-HT uptake was performed as previously described by Bonanno and Raiteri (1987) with some adjustments. Cortex samples, stored as described, were homogenized with a glass/Teflon Potter in a sodium-phosphate buffer (Na2HPO4 80 mM, NaH2PO4 20 mM, pH 7.2) with 0.32 M sucrose, and centrifuged at 1000 xg for 5 min at 4 8C. Supernatant fraction wasthen ultracentrifuged at 12,000 xg for 20 min at 4 8C, and the obtained pellet (containing synaptosomes) was resuspended with a Krebs buffer (125 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1.2 mM CaCl2, 22 mM NaHCO3, 1 mM NaH2PO4) containing 10 mM glucose. The protein assay was performed according to the Lowry method (Lowry et al., 1951). Samples obtained were incubated (100 mL, with a concentration of 0.6—0.8 mg of proteins/tube) with 50 mL [3 H]-5-HT (0.0625—0.5 mM, S.A. 17.8 Ci/mmol, GE Healthcare Europe, Milano Italia) and 50 mL Krebs buffer, with tubes in 37 8C water (total reuptake) or in ice (non-specific re-uptake). After 5 min, the reaction was stopped with 15 mL of ice-cold Krebs buffer and subsequent filtration using Whatman GF/B filters. Filters were put into vials with 4 mL Biofluor (Packard, Milano, Italy) and cpm were read with a Packcard liquid scintillation counter. Vmax and Km were obtained by Lineweaver—Burk linearization.

2.7.2. Experiments ‘‘in vitro’’: effects of CORT on SERT activity

To evaluate if CORT was able to directly affect 5-HT-transporter, we measured 5-HT uptake in cortical synaptosomes obtained from rats of the group V in the presence or absence of corticosterone at two concentrations, the first one corresponding to stress-induced CORT plasma levels in our study (0.3 mg/mL) and the second one higher (1 mg/mL). The measurement of 5-HT uptake was performed according to the protocol described in the previous paragraph.

2.7.3. Experiments ‘‘in vitro’’: effects of ND on 5-HTtransporter activity

To evaluate if ND was able to affect directly 5-HT-transporter activity, we measured 5-HT uptake in cortical synaptosomes obtained from rats of the group V + S in the presence or absence of: 1230 mg/mL ND (concentration derived by the ND dose/rat assuming a total drug bioavailability and

distribution into central nervous system); 100 nM paroxetine (PX, Millan et al., 2010), a well known selective serotonin reuptake inhibitor, or the association ND + PX. All procedures were conducted as described above.

2.8. Statistical analysis

Treatment and stress effects on the parameters considered in the study were analyzed using two-way ANOVA models. Multiple comparisons were further performed with Tukey‘s post hoc test. The effects of CORTon SERTactivity were analyzed by one-way ANOVA. Differences were considered statistically significant when p-value < 0.05. All data were showed in figures and expressed as mean and relative 95% confidence interval (95%CI).

3. Results

Results obtained in rats receiving saline are not shown because they were similar to those obtained in vehicletreated rats (V).

3.1. Plasma CORT levels

As reported in Fig. 1, ND decreased CORT levels in nonstressed as well as in stressed rats while RS increased plasma CORT in treated and untreated rats (NS and VS) in comparison with non-stressed animals (N and V). This evaluation of data is supported by statistical analysis that led to significant effects for treatment ($F = 11.52$, $p < 0.001$) and stress ($F = 11.21$, $p < 0.001$), but not for the interaction.

3.2. Plasma ACTH levels

As regards ACTH plasma levels (Fig. 2) the statistical analysis revealed a significant effect of ND treatment ($F = 1270.07$, $p < 0.001$), stress ($F = 92.12$, $p < 0.001$) and interaction ($F = 63.27$, $p < 0.001$). The post hoc comparisons showed that RS produced marked increases in plasma ACTH

levels in the groups V + S and N + S in comparison to V and N, respectively ($p < 0.001$ in both comparisons). In ND treated rats, the induction of ACTH release in response to RS was preserved, however the plasma ACTH concentrations were very significantly decreased as compared to V + S rats ($p < 0.001$ N + S vs. V + S). In non-stressed rats ND decreased ACTH levels but the variation was not statistically significant, probably due to the high hormone levels variability.

3.3. ACTH expression in pituitary

As shown in Fig. 3, RS significantly increased density of ACTH positive cells in rat anterior pituitary ($F = 24.78$, $p < 0.001$). ND administration did not modify cell density in non-stressed rats and no interaction between ND and stress was observed.

3.4. GR binding assay in hypothalamus and hippocampus

3.4.1. Hypothalamus

The two-way ANOVA for GR levels (Fig. 4A) evaluated by binding assay on cytosolic preparations from hypothalamus showed a significant effect for RS ($F = 19.01$, $p < 0.001$), ND treatment ($F = 16.00$, $p < 0.001$) and interaction ($F = 19.80$, $p < 0.001$). Post hoc comparisons showed that RS decreased GR levelsin ratsin basal conditions($p < 0.001$ V + S vs. V), ND treatment did not modify this parameter and prevented RSinduced GR levels reduction ($p > 0.98$ N + S vs. N). No significant modification was observed in Kd values (Fig. 4B).

3.4.2. Hippocampus

In this tissue, the two-way ANOVA for GR levels (Fig. 5A) showed a significant effect for RS ($F = 12.50$, $p < 0.001$) and ND ($F = 6.40$, $p = 0.03$) with a marginal statistical significant interaction ($F = 3.57$, $p = 0.07$). Post hoc comparisons results overlapped with those obtained in hypothalamus for stress as well as for ND treatment in the absence or presence of stress. No significant modification was observed in Kd values (Fig. 5B).

3.5. GR protein cellular distribution in hippocampus

As regards GR content in cytosol hippocampal fraction (Fig. 6B) no statistical significant differences among groups were observed. In nuclear hippocampal fractions (Fig. 6C) statistical analysis revealed a significant effect for RS ($F = 6.15$, $p = 0.04$) and ND treatment ($F = 6.15$, $p = 0.04$) with a borderline statistical significant interaction ($F = 4.79$, $p = 0.057$). Post hoc comparisons showed that RS increased GR levels in V group ($p < 0.001$ V + S vs. V), ND administration was devoid of any effect on GR cellular distribution in the absence of RS whereas it decreased RS-induced GR nuclear translocation $p = 0.99$ N + S vs. N).

3.6. Measurement of 5-HT re-uptake

3.6.1. Ex vivo experiments

The evaluation of [^{3}H]-5-HT uptake on synaptosomal preparations from cortical areas revealed that RS induced a strong activation of Vmax (Fig. 7A) [$F = 15.11$, $p < 0.001$]. In addition, Vmax values showed a significant effect for ND ($F = 21.54$, $p < 0.001$) and for interaction ($F = 14.24$, $p < 0.001$) due to the capacity of ND to prevent the RS-induced Vmax increase (post hoc test: $p < 0.001$ V + S vs. V and $p = 0.73$ N + S vs. N). As regards Km (Fig. 7B), we observed a significant reduction of SERT affinity after stress (increase of Km value, [$F = 7.92$, $p = 0.02$]) a marginal statistical significant decrease in ND group ($F = 3.58$, $p = 0.08$) and a significant interaction ($F = 18.40$, $p < 0.001$). Post hoc comparisons showed that ND did not affect SERT affinity in the absence of stress but prevented RS-induced Km increase ($p = 0.004$ N + S vs. V + S).

3.6.2. In vitro experiments: effects of CORT on SERT activity

Experiments in vitro showed that both concentrations of CORT had no effect on SERT activity (Vmax, $F_{2,9} = 0.41$, $p = 0.67$) and affinity (Km, $F_{2,9} = 0.07$, $p = 0.93$) in cortical synaptosomes obtained from rats of the group V (Fig. 8A and B).

3.6.3. In vitro experiments: effects of ND on 5-HT transporter activity

The in vitro evaluation of [^{3}H]-5-HT uptake on synaptosomes of rat cortex from group V + S (Fig. 9A and B) in the presence or absence of ND, PX or ND+ PX revealed main effects of ND ($F = 76.09$, $p < 0.001$), PX ($F = 186.63$, $p < 0.001$) and their interaction ($F = 15.56$, $p = 0.002$) on Vmax and Km (ND: $F = 20.536$, $p < 0.001$; PX: $F= 24.063$, $p < 0.0001$; interaction: $F = 17.288$, $p < 0.001$). Post hoc comparisons showed that ND caused a significant decrease of Vmax and Km ($p < 0.001$ ND treated vs. untreated, for both parameters). PX caused, as expected, a strong inactivation of SERTmediated re-uptake and a reduction of increased Km ($p < 0.001$ PX treated vs. untreated, for both parameters). As regards the association ND + PX, the effects on Vmax were much more evident and statistically significant.

3.7. ERK expression in hippocampus

Fig. 10A reports the results obtained for total ERK and phospho-ERK expression in hippocampus. No significant difference among the groups was observed in the levels of total ERK1 and ERK2 in both cytosol and nuclear hippocampus fractions (densitometric analysis not shown). In cytosolic hippocampus fraction, the two-way ANOVA on phospho-ERK1/ERK1 (Fig. 10B) and phospho-ERK2/ERK2 ratio (Fig. 10C) showed a significant effect for stress ($F = 1040.78$, $p < 0.001$ and $F = 747.81$, $p < 0.001$ respectively), treatment ($F = 1016.87$, $p < 0.001$ and $F = 3760.77$, $p < 0.001$ respectively) and for interaction ($F = 859.16$, $p < 0.001$ and $F = 2399.29$, $p = < 0.001$ N vs. V), RS did not modify this ratio but inhibited ND effect ($p < 0.001$ N + S vs. N). With regard to cytosolic phospho-ERK2/ERK2 ratio (Fig. 10C), post hoc comparisons showed that RS increased this value ($p < 0.001$ V + S vs. V), ND was much more effective than RS in this respect ($p < 0.001$ N vs. V) and RS partially reversed ND-induced increase ($p < 0.001$ N + S vs. N). No significant stress, treatment and interaction effects were found for phospho-ERK1/ERK1 and phospho-ERK2/ERK2 ratio in nuclear hippocampus fraction (Fig. 10D and E).

3.8. JNK1 expression in hippocampus

WB analysis in cytosol and nuclear hippocampus fractions showed no significant differences among the groups in both JNK1 total levels (analysis not shown) and phospho-JNK1/ JNK1 ratio (Fig. 11).

4. Discussion

We have investigated the effect of the anabolic androgenic steroid ND on HPAA and cortical serotoninergic system activity in response to an acute RS. A significant increase in CORT circulating levels was observed following RS, which shows that our stress model was effective on HPAA. CORT plasma levels were also affected by the subchronic administration of ND with a slight but significant decrease in the circulating CORT 24 h following the last ND injection. This result is in agreement with data reported by Alsio et al. (2009) who observed a significant reduction in rat CORT plasma levels following 15 mg/kg ND administration for 14 days, although the decrease observed in theirstudy was more higher than that in our. Data from the literature show that ND differently affects CORT plasma level depending on duration of treatment, dose administered and time of sacrifice after the last injection. For example, Schlussman et al. (2000) observed a decrease in circulating CORT levels 24 h after the last injection of 45 mg/kg for 14 days, while the administration of 15 mg/kg ND for 3 days was followed by a significant increase in CORTcirculating levels 1 h afterthe last injection, but the hormone returned to the control levels 24 h later. In addition, Matrisciano et al. (2010) recently reported an increase in plasma CORT levels 24 h after the last s.c. ND injection administered once a day for 28 days at the dose of 5 mg/kg. It is to note that Matrisciano et al. (2010) used a different routes of administration with a consequent different ND pharmacokinetics. In our study acute stress and ND seem to affect CORT plasma concentrations by acting at different levels, since no significant interaction between stress and treatment resulted for this parameter by the two way ANOVA. It is well established that stress modulates circulating CORT by acting on HPAA: upon receipt of a stressful stimulus, brain initiates an ACTH surge to promote adrenocortical activation. The ACTH surge is initiated by

hypophysiotrophic neurons in the paraventricular hypothalamic nucleus (PVN) that produce ACTH releasing factors. It is known that corticotropin releasing factor is the primary but not the only regulator of ACTH release by pituitary and that PVN neurons are under control not only of sensory afferents which are relayed via brainstem loci, but also of limbic brain regions, such as the hippocampus, amygdala and prefrontal cortex. In view of this multifactorial control mechanism, we can speculate that in unstressed conditions ND affects CORT adrenal release by acting on HPAA, targeting central signals different from those involved in response to stress, or directly at adrenal level. In this regard, Alsio et al. (2009) identified a decreased expression of two key enzymes involved in CORT synthesis (5a-reductase I and 11b-OHase) in adrenals of rats submitted to a prolonged ND treatment. This observation suggests that chronic treatment with ND could impair CORT adrenal release by acting on the gene transcription of enzymes involved in CORT synthesis. As regards ACTH release, we observed that ND was able to decrease basal plasma ACTH levels (although in a not statistically significant way) and to blunt the strong RS-induced ACTH increase. These data suggest that a subchronic administration of ND is able to interfere with ACTH release in response to stress. It has been demonstrated that the hypothalamic—pituitary-gonadal axis exerts considerable effects on HPAA activity under both basal conditions and stress. In particular, it has been observed that plasma ACTH response to RS was increased in male rats by gonadectomy and decreased by testosterone replacement (Viau et al., 1999). Therefore, we can suppose that the high levels of circulating androgen following the daily administration of ND may exert an inhibitory control on both basal and stimulated ACTH release. Very interestingly, acute stress increased pituitary ACTH positive cell density in the same way in vehicle- and in NDtreated animals. This different response of plasma and pituitary ACTH levels to a modification of hormone blood milieu was also reported by Viau et al. (1999), who observed in male rats that adrenalectomy markedly increased both plasma and pituitary ACTH levels and that gonadectomy reduced this adrenalectomy effect only at plasma level. It is well-known that GRs are involved in the negative feedback that glucocorticoids exert at both hypothalamus and hippocampus levels (Bradbury et al., 1994; de Kloet

et al., 1998). Therefore, to further investigate ND effects on HPAA, we measured GR binding levelsin cytosolic hypothalamus and hippocampus preparations in stressed and nonstressed rats. Acute RS in vehicle-treated rats was followed by a decrease in cytosolic GR levels in both hypothalamus and hippocampus tissues. The GR decrease in hippocampus resulted from GR activation as it occurs after acute glucocorticoid treatment. In fact, when the stress decreased cytosolic GR binding capacity there was a concurrent increase in GR level in the nuclear fraction. ND treatment impaired the effects of stress on both cytosol GR binding capacity and intracellular distribution. Similarly, ND treatment completely abolished the cortical SERT activation induced by acute RS. The 5-HT neuronal system plays an important role in regulating the HPAA response to stress (Lorens and Van de Kar, 1987; Calogero et al., 1990) and modulates the release of stress hormones, including corticosterone (Wetzler et al., 1996; Klaassen et al., 2002). On the other hand, changes in extracellular levels of 5-HT in different brain areas after exposure to several stressors have been shown (Carrasco and Van de Kar, 2003). In particular, RS has been associated with increased synthesis or metabolism of 5-HT in some limbic regions (Dunn, 1999). The critical role played by CORT in stress-induced 5-HT neural system activation has been substantiated by experimental procedures, such as adrenalectomy and exogenous CORT administration, which demonstrated that the adrenal steroid hormone exerts a stimulating influence on tryptophan hydroxylase activity and 5-HT turnover in brain (Singh et al., 1990). With regard to ND influence on 5-HT neuronal system, subchronic treatment with supraphysiological doses of ND has been associated with increased levels of 5-HT in the cerebral cortex, as well as in hypothalamus and hippocampus (Kurling et al., 2005). In our experiments, we found that ND treatment was devoid of any effect on 5-HT re-uptake in nonstressed animals while it completely impaired the increase of SERT activity induced by acute RS. In our study CORT at concentrations equivalent (or higher) to those detected in stress conditions appears devoid of direct effects on SERT activity. The cortical SERT activation observed after RS probably resulted from the stress-induced increase of 5-HT concentration into the synaptic cleft. The lack of SERTactivation after stress in ND-treated rats

could be linked to the lower increase of CORT in stressed rats pre-treated with ND and consequently to the reduction of the stimulating influence of CORT on 5-HT neuronal system. This interpretation is supported by the absence of intracellular GR redistribution in the same group of animals suggesting that stress-induced plasma CORT increase in these rats is not so high to activate GR machinery. However, our in vitro data showing that ND is able to affect directly RS-activated 5-HT re-uptake suggest that ND may act on SERT activity by both direct and indirect mechanisms. It has been reported that the activities of some mitogenactivated protein kinases (MAPKs) are altered in response to various acute stimuli, such as electroconvulsive shock and other stressors, but the elicited changes are both kinase and brain-region specific (Alessandrini et al., 1999; Oh et al., 1999; Gioia et al., 2001). Therefore, we studied the phosphorylation of ERK (p-ERK1 and p-ERK2), as well as JNK1 (p-JNK1) in hippocampus after RS, ND treatment or their association. Our findings showed that acute RS increased cytosolic p-ERK2 levels. Furthermore, we observed that ND treatment increased not only cytosolic p-ERK2 level (to a higher degree than stress), but also pERK1. When ND treatment was combined with stress, ND effects were partially reverted. Rossbach et al. (2007) showed an increase of ERK phosphorylation in rat hippocampal synaptoneurosomes 24 h after a single high dose (15 mg/kg) of ND. The kinase activation was not significant if ND treatment was prolonged for 14 days, indicating adaptation to high steroid levels. In our study, ERK activation was evident in rat hippocampus cytosol fraction following a subchronic treatment, so we can assume that adaptation does not involve the whole hippocampus, but only its synaptoneurosome fraction. Although active ERK is mostly associated with transcriptional regulation in the cell nucleus, we observed an increase of p-ERK content only in the cytosolic hippocampus fraction. However, it has been reported that also cytoplasmic proteins are substrates of ERK and the functional targets of ERK signaling depend on the precise location of ERK within differentsubcellular compartmentsin brain regions(Bhat et al., 1998). In particular, electroconvulsive shock treatmentresult in an increase of ERK content in the cytoplasm of hippocampal neurons and in both the nucleus and cytoplasm of cortical neurons. This suggests that

unique functional pathways of ERK-mediated signal transduction are present within different brain regions. As reported by Jovanovic et al. (1996), ERK cytoplasmic substrates include proteins whose phosphorylation affects synapse formation. Studies have demonstrated that MAPKs in neurons are recruited when the brain undergoes synaptic plasticity and remodelling and supports their role in rapid synaptic functional events, such as neurotransmission (Grewal et al., 1999; Vanhoose et al., 2002). Therefore, itseemsreasonable to hypothesize that RS and ND could interfere with intracellular processes in which ERK have a role in coupling extracellular stimuli to biochemical events occurring in the cytoplasm. ND-induced hippocampal ERK activation was lower when steroid treatment was combined with acute RS. It is evident that stress and ND interfere each other also on ERK activation, however the mechanisms involved are not clear on the basis of our experiments. Our results demonstrate that ND treatment does not affect JNK phosphorylation level. The lack of RS influence on JNK activation was already observed in several stressrelevant brain areas (Meller et al., 2003). In conclusion, we have shown that a subchronic administration of ND, at doses considered equivalent to those abused by humans, modifies the effects of acute stress on HPAA and abolishes RS-induced SERT activities in cortical synaptosomal preparations. ND effects on stress-induced HPAA activation are probably the consequence of an ND action at central level as suggested by the significant decrease of plasma ACTH response to RS in ND-treated rats. However we can hypothesize that ND may affect HPAA response to acute RS acting also at adrenal level, as previously reported (Alsio et al., 2009). Studies in rodents have demonstrated lower HPAA stress responses in males than in females. These different HPAA stress responsivities between sexes has been ascribed to circulating androgens with the involvement of both androgen receptor (enhancement of inhibitory signals, Goel et al., 2011) and estrogen receptor-b (reduction of excitatory inputs, Lund et al., 2006; Handa et al., 2009). We can hypothesize that in our study ND, as a testosterone derivative, exerts its action like testosterone, with similar inhibitory effects on the HPAA. On the other hand it is not possible to exclude the involvement of additional mechanisms. Furthermore, our results show that acute stress, as well as ND, are able to increase ERK activity in

the cytosol of hippocampus, suggesting their involvement in hippocampal intracellular processes. Our data, taken together, demonstrate that ND abuse actively interferes with the HPAA stress response. ND high dose subchronic administration appears to develop both direct and indirect effects on HPAA and desensitize the feedback action of the hormonal cascade induced by stress according to Goel et al. (2011). Although several evidences associate depression to stress-induced HPAA hyperactivity (Swaab et al., 2005) and in this respect androgens could be protective, we suggest that the above mentioned desensitization in HPAA by high doses of ND for a long time may disrupt the adaptive responses to stress and may explain the AAS abuse-induced depressive psychopathology.

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Figure 1.

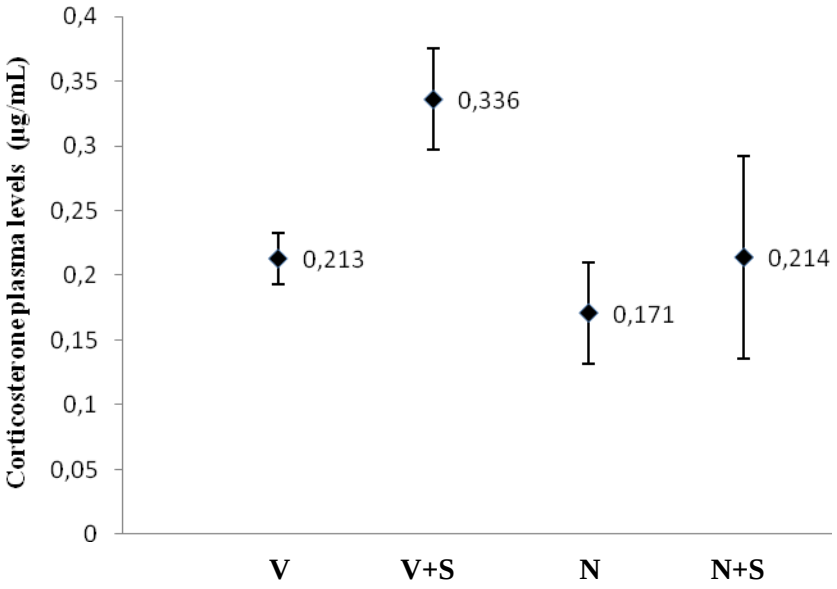

Figure 1. Circulating levels of corticosterone in plasma samples. V=vehicle, V+S=vehicle+stress, N=15mg/kg/day nandrolone decanoate (ND), N+S=15mg/kg/day ND+stress. Results are means and relative 95% confidence intervals; n=10 values/group.

Figure 2.

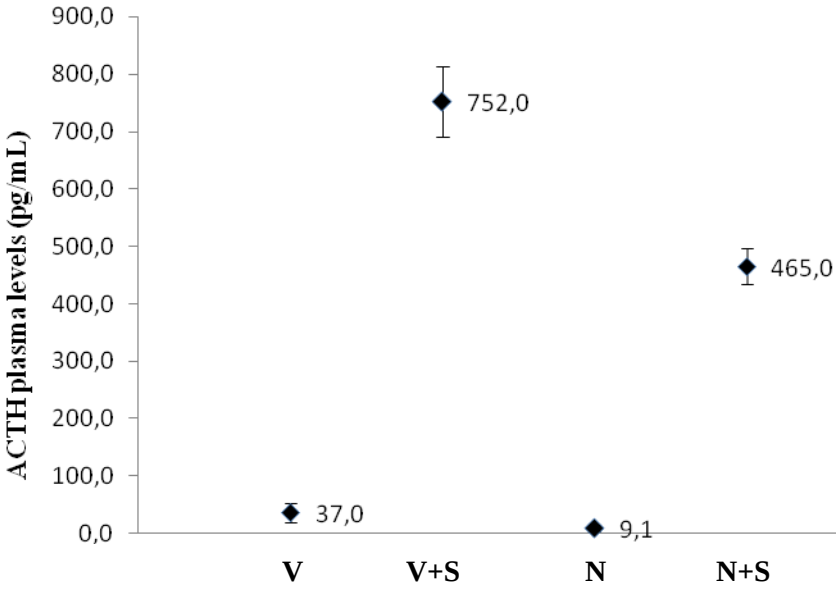

Figure 2. Circulating levels of adrenocorticotropin (ACTH) in plasma samples V=vehicle, V+S= vehicle+stress, N=15mg/kg/day nandrolone decanoate (ND), N+S=15mg/kg/day ND+stress. Results are means and relative 95% confidence intervals; n=6 values/group.

Figure 3.

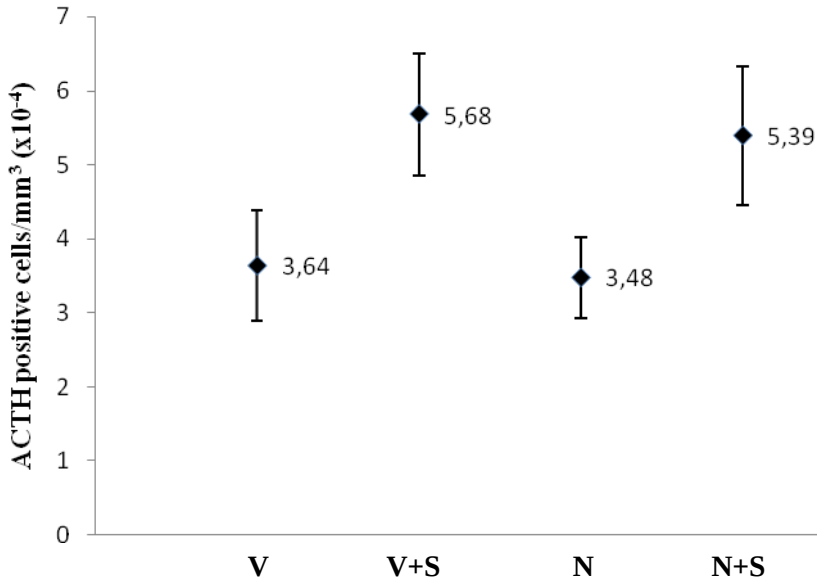

Figure 3. ACTH positive cells in rat anterior pituitary. V=vehicle, V+S=vehicle+stress, N =15mg/kg/day nandrolone decanoate (ND), N+S=15mg/kg/day ND+stress. Results are means and relative 95% confidence intervals; n= 4 values/group.

Figure 4.

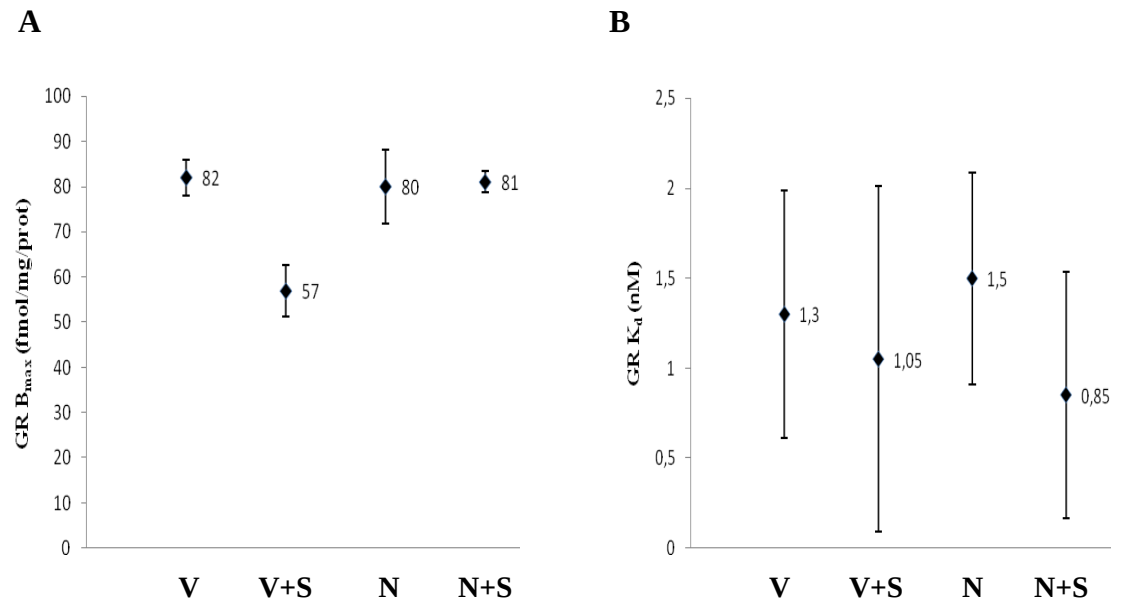

Figure 4. GR evaluation in cytosolic preparations from hypothalamus. V=vehicle, V+S=vehicle+stress, N=15mg/kg/day nandrolone decanoate (ND), N+S=15mg/kg/day ND+stress. Results are means and relative 95% confidence intervals; n=4 values/group. In (A): GR levels (B_{max}). In (B): GR affinity (K_d).

Figure 5.

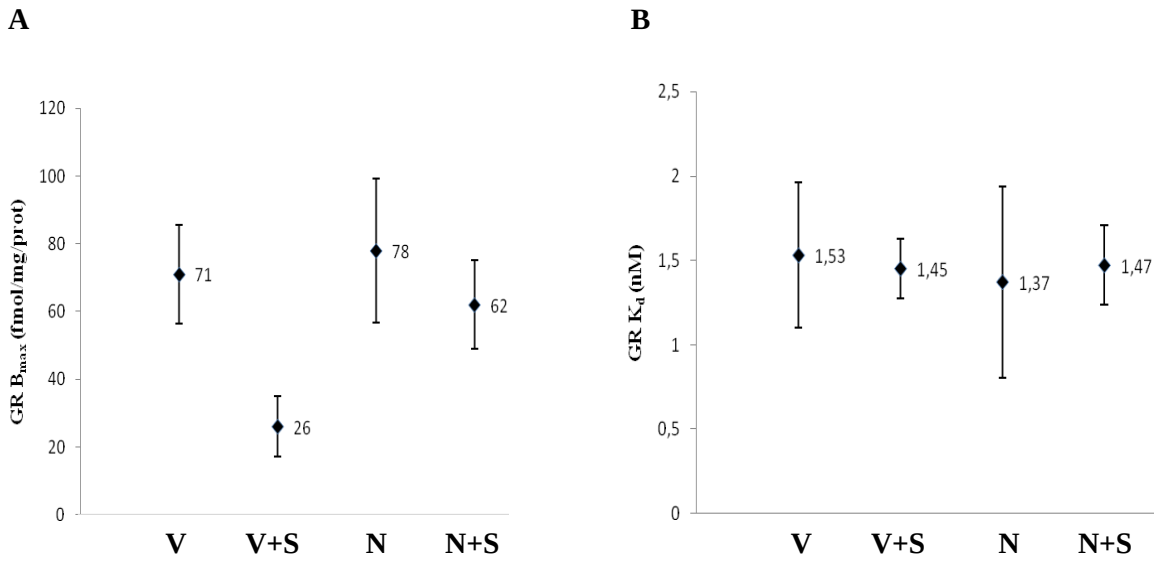

Figure 5. GR evaluation in cytosolic preparations from hippocampus. V=vehicle, V+S=vehicle+stress, N=15mg/kg/day nandrolone decanoate (ND), N+S=15mg/kg/day ND+stress. Results are means and relative 95% confidence intervals; n=4 values/group. In (A): GR levels (B_{max}). In (B): GR affinity (K_d).

Figure 6.

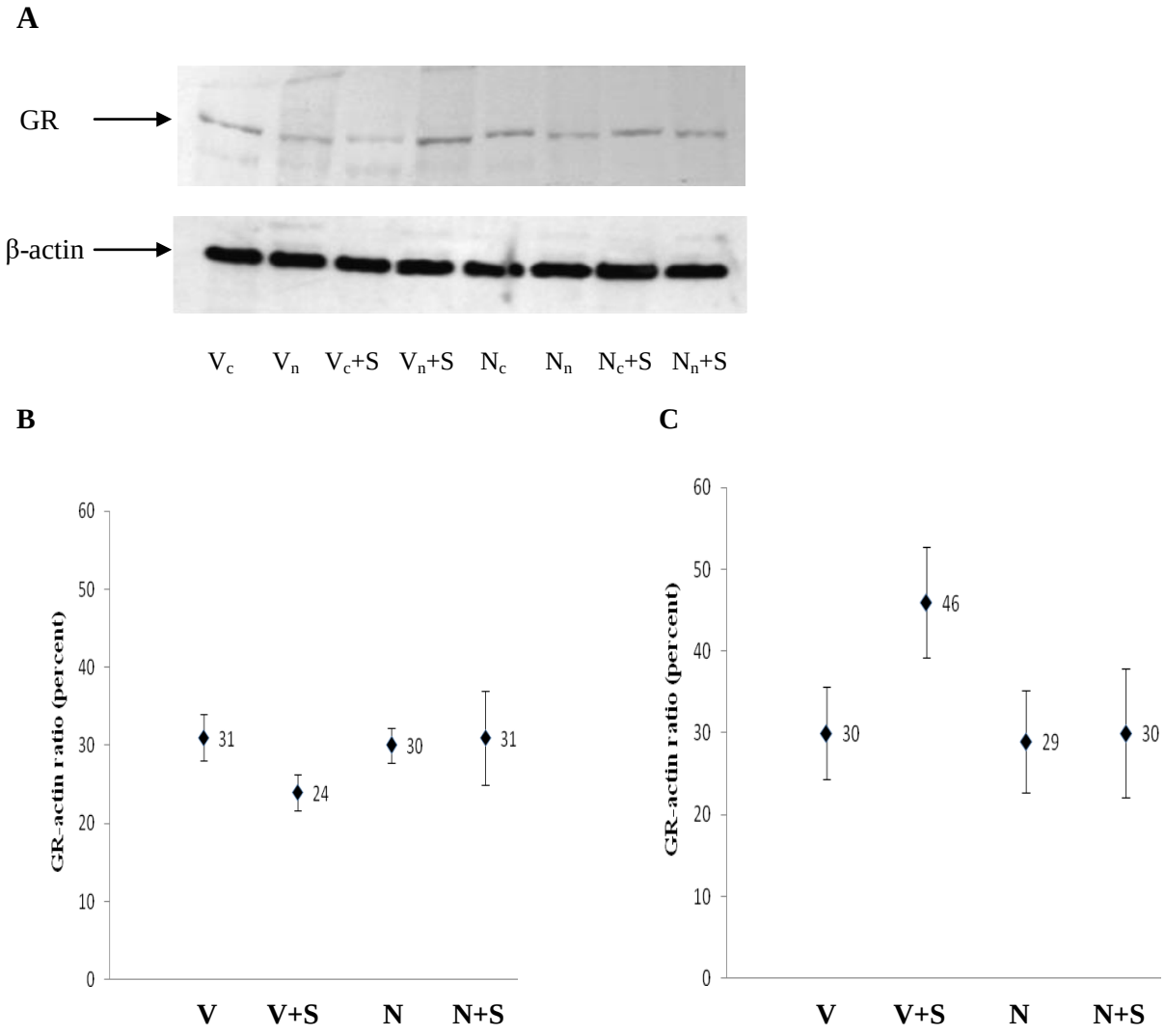

Figure 6. Representative western blot for glucocorticoid receptor (GR) levels in both the cytosolic (c) and nuclear (n)hippocampus fraction (A). V = vehicle, V + S = vehicle + stress, N = 15 mg/kg/day nandrolone decanoate (ND), N + S = 15 mg/kg/day ND + stress. In (B and C) densitometric analyses in cytosol and nucleus respectively. Results are expressed as means and relative 95% confidence intervals of GR/β-actin ratio;*n* = 3 values/group.

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Figure 7.

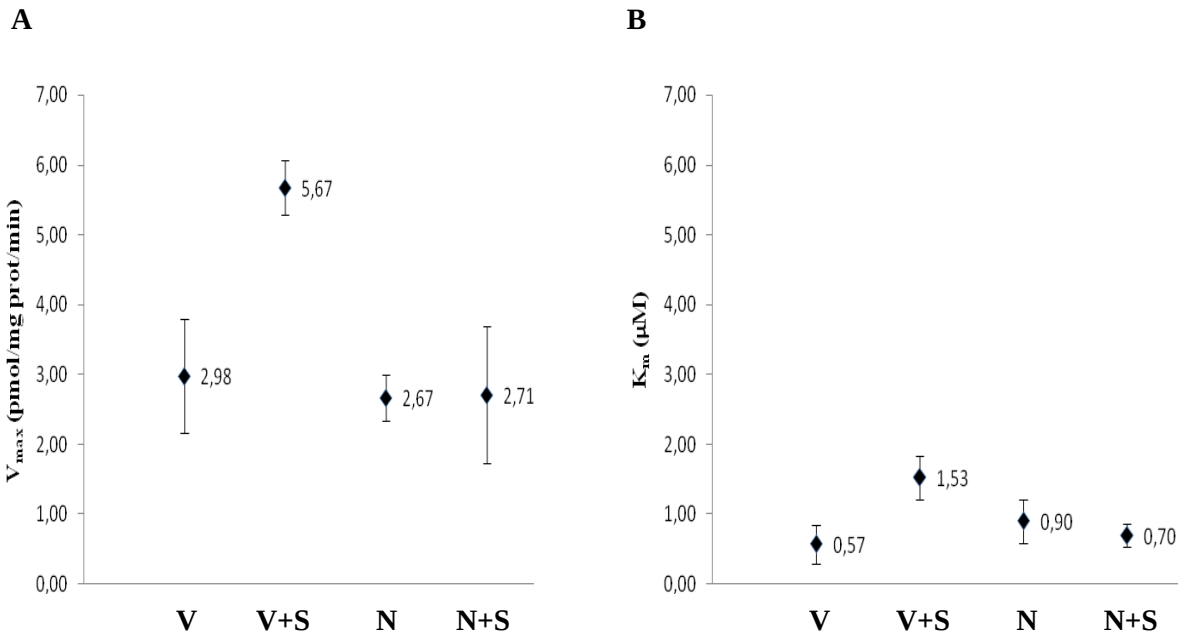


Figure 7. [^{3}H]-serotonin re-uptake on synaptosomal preparations from cortical areas. V=vehicle, V+S=vehicle+stress, N=15mg/kg/day nandrolone decanoate (ND), N+S=15mg/kg/day ND+stress. Results are means and relative 95% confidence intervals; n=4 values/group. In (A): V_{max}. In (B): K_m.

Figure 8.

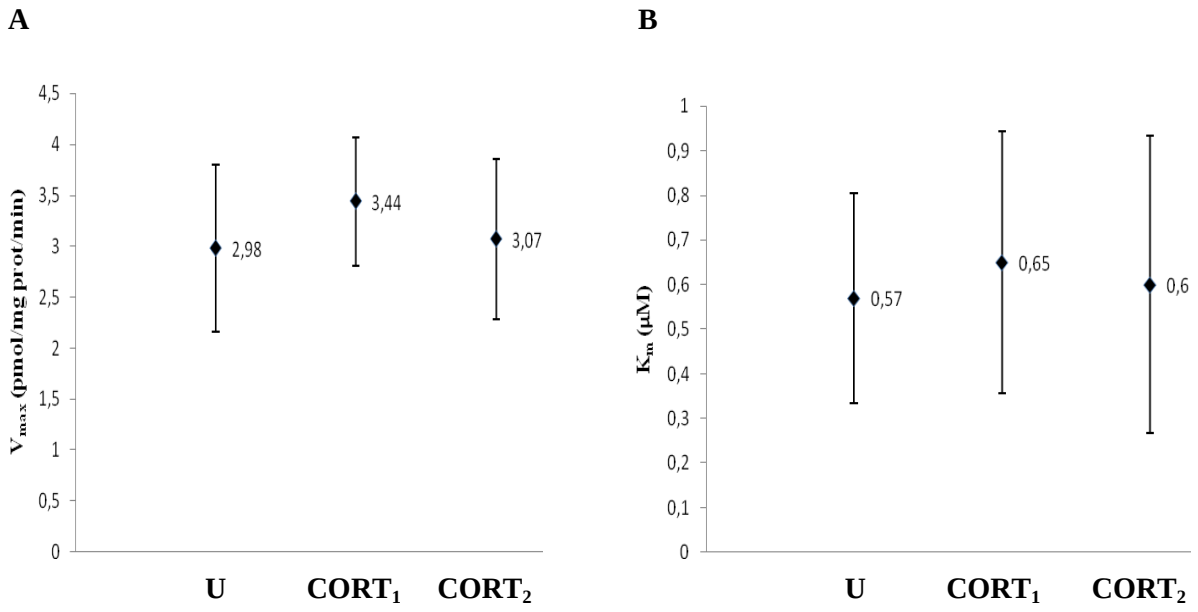


Figure 8. In vitro effect of corticosterone on [^{3}H]-serotonin re-uptake on synaptosomal preparations from cortical areas. U=untreated, $CORT_1$=0.3µg/mL corticosterone, $CORT_2$=1µg/mL corticosterone. Results are means and relative 95% confidence intervals; n=4 values/group. In (A): V_{max}. In (B): K_m.

Figure 9.

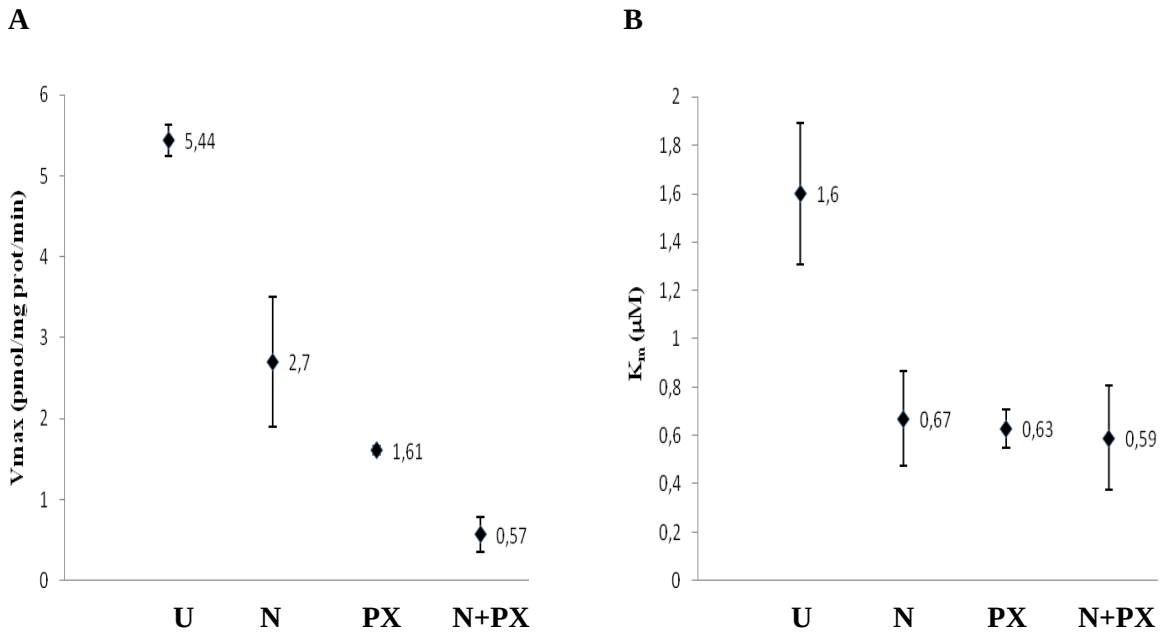
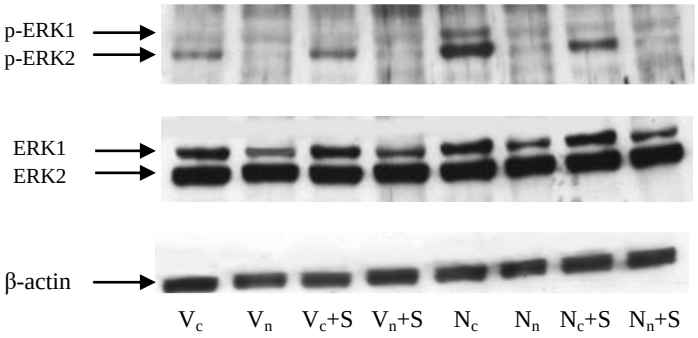


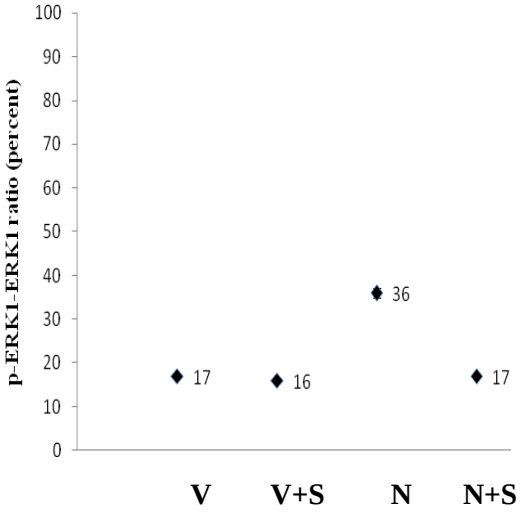
Figure 9. In vitro effect of nandrolone on [^{3}H]-serotonin re-uptake on synaptosomal preparations from cortical areas. U=untreated, N=1230µg/mL nandrolone, PX=100nM paroxetine, N+PX= 1230µg/mL nandrolone+100nM paroxetine. Results are means and relative 95% confidence intervals; n= 4 values/group. In (A): V_{max}. In (B): K_m.

Figure 10

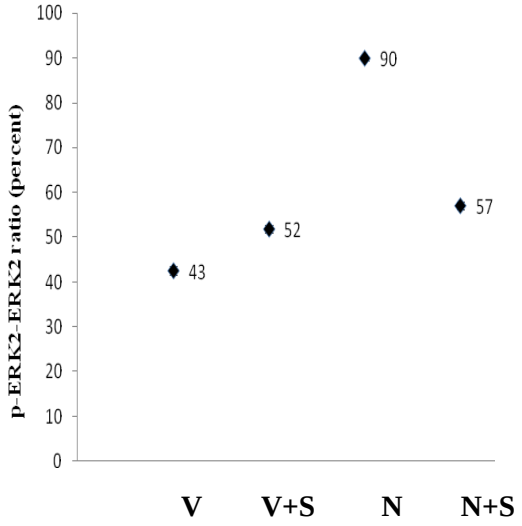
A



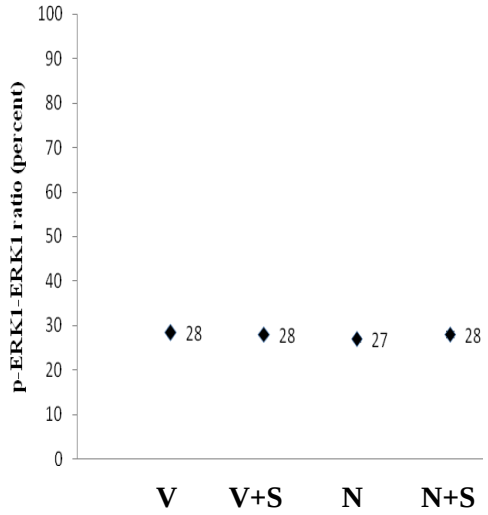
B



C



D



E

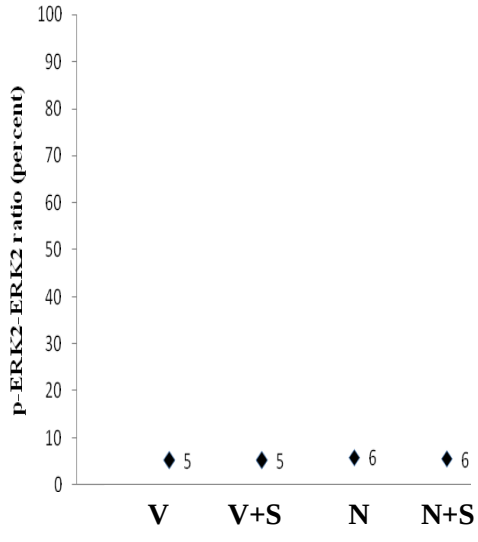


Figure 10. Representative western blot for ERK1/2 and phospho-ERK1/2 expression in both the cytosolic (c) and nuclear (n) hippocampus fraction (A). V = vehicle V + S = vehicle + stress, N = 15 mg/kg/day nandrolone decanoate (ND), N + S = 15 mg/kg/day ND + stress. In (B) densitometric analysis of phospho-ERK1/ERK1 ratio in cytosol. In (C) densitometric analysis of phospho-ERK2/ERK2 ratio in cytosol. In (D) densitometric analysis of phospho-ERK1/ERK1 ratio in nucleus. In (E) densitometric analysis of phospho-ERK2/ERK2 ratio in nucleus. Results are expressed as means and relative 95% confidence intervals; *n* = 3 values/group.

Figure 11

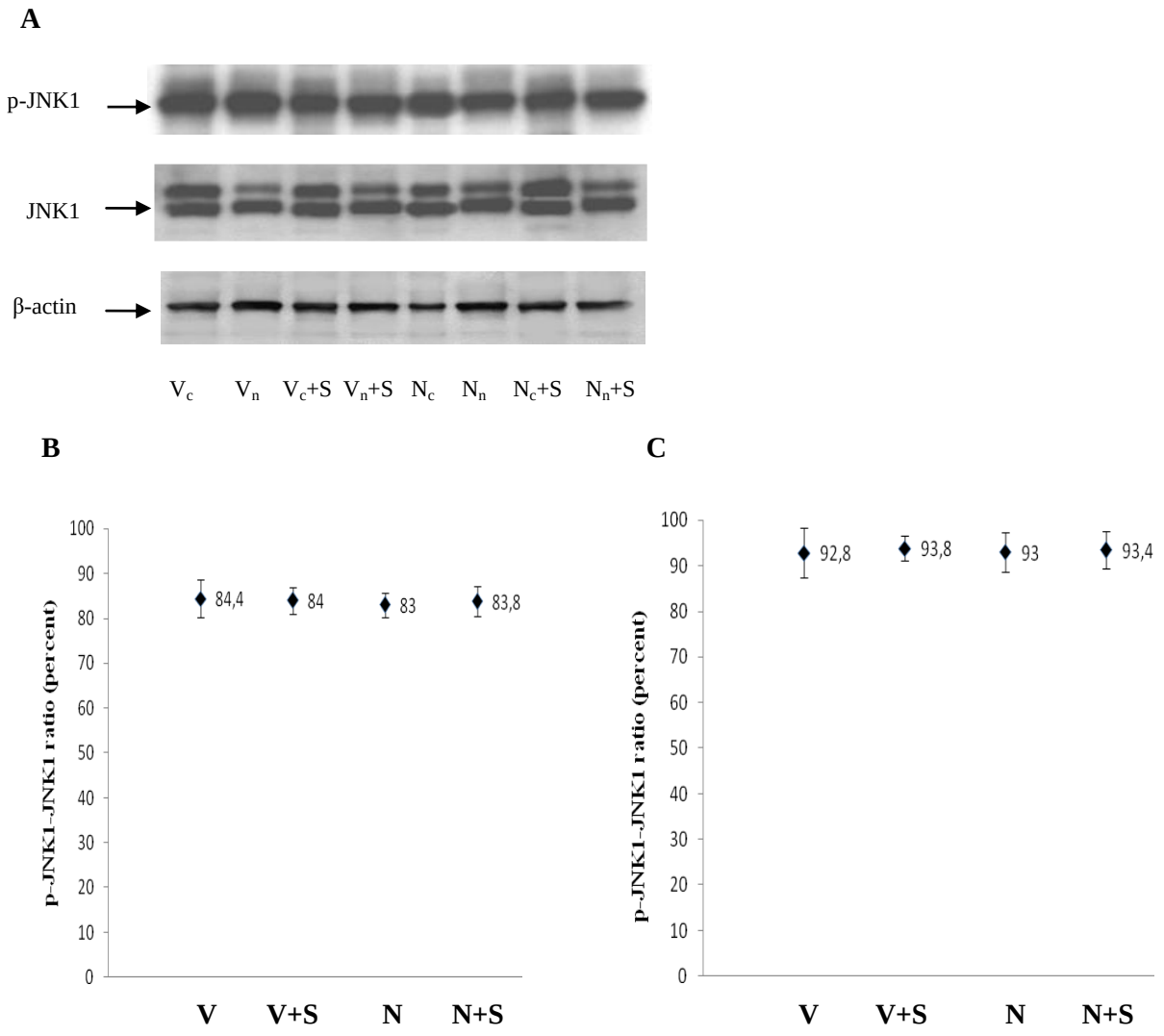


Figure 11. Representative western blot for JNK1 and phospho-JNK1 expression in both the cytosolic (c) and nuclear (n) hippocampus fraction (A). V = vehicle, V + S = vehicle + stress, N = 15 mg/kg/day nandrolone decanoate (ND), N + S = 15 mg/kg/day ND + stress. In (B and C) densitometric analyses of phospho-JNK1/JNK1 ratio in cytosol and nucleus respectively. Results are means and relative 95% confidence intervals; *n* = 3 values/group.