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Citation Information

Fan, Yan; Chen, Ping Ping; Li, Ying; Cui, Kui; Noel, Daniel M.; Cummins, Elizabeth D.; Peterson, Daniel J.; Brown, Russell W.; and Zhu, Meng-Yang. 2014. Corticosterone Administration up-Regulated Expression of Norepinephrine Transporter and Dopamine B-Hydroxylase in Rat Locus Coeruleus and Its Terminal Regions. *Journal of Neurochemistry*. Vol.128(3). 445-458. https://doi.org/10.1111/jnc.12459 ISSN: 1471-4159

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Corticosterone Administration up-Regulated Expression of Norepinephrine Transporter and Dopamine Β-Hydroxylase in Rat Locus Coeruleus and Its Terminal Regions

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J Neurochem. Author manuscript; available in PMC 2015 February 01.

Published in final edited form as:
J Neurochem. 2014 February ; 128(3): 445–458. doi:10.1111/jnc.12459.

Corticosterone administration upregulated expression of norepinephrine transporter and dopamine β-hydroxylase in rat locus coeruleus and its terminal regions

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Abstract

Stress has been reported to activate the locus coeruleus (LC)–noradrenergic system. In the present study, corticosterone (CORT) was orally administrated to rats for 21 days to mimic stress status. *In situ* hybridization measurements showed that CORT ingestion significantly increased mRNA levels of norepinephrine transporter (NET) and dopamine β-hydroxylase (DBH) in the LC region. Immunofluorescence staining and western blotting revealed that CORT treatment also increased protein levels of NET and DBH in the LC, as well as NET protein levels in the hippocampus, the frontal cortex and the amygdala. However, CORT-induced increase of DBH protein levels only appeared in the hippocampus and the amygdala. Elevated NET and DBH expression in most of these areas (except for NET protein levels in the LC) was abolished by simultaneous treatment with combination of corticosteroid receptor antagonist mifepristone and spironolactone (s.c. for 21 days). Also, treatment with mifepristone alone prevented CORT-induced increases of NET expression and DBH protein levels in the LC. In addition, behavioral tasks showed that CORT ingestion facilitated escape in avoidance trials using an elevated T-maze, but interestingly, there was no significant effect on the escape trial. Corticosteroid receptor antagonists failed to counteract this response in CORT-treated rats. In the open-field task, CORT treatment resulted in less activity in a defined central zone compared to controls and corticosteroid receptor antagonist treatment alleviated this increase. In conclusion, the present study demonstrates that chronic exposure to CORT results in a phenotype that mimics stress-induced alteration of noradrenergic phenotypes, but the effects on behavior are task-dependent. As the sucrose consumption test strongly suggests CORT ingestion-induced depression-like behavior, further elucidation of underlying mechanisms may improve our understanding of the correlation between stress and the development of depression.

Keywords

Norepinephrine transporter; Dopamine β-hydroxylase; Corticosterone; Rat brain; Locus coeruleus; animal behavior

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1. Introduction

It is well known that both chronic stress and dysfunctional noradrenergic systems are causatively involved in the pathophysiology of major depressive disorder (MDD). Animal studies have revealed that prolonged exposure to various stressors resulted in the motivational, neuroendocrine, anhedonic, and behavioral characteristics observed in human depression (Brady 1994, Sapolsky 1996). On the other hand, deficiencies of noradrenergic transmission have been thought to be one factor for the etiology of depression (Schatzberg *et al.* 1995, Charney 1998). Furthermore, many lines of evidences have revealed that the interaction between chronic stress and the noradrenergic system may contribute to the development of depression. For example, animal studies have shown that the brain noradrenergic system is rapidly activated by different stressors (Korf *et al.* 1973, Anisman *et al.* 1979, Abercrombie *et al.* 1987, Ritter *et al.* 1998), which results in an increase of norepinephrine (NE) release from noradrenergic terminals (Pacak *et al.* 1995, Smagin *et al.* 1997, Rosario *et al.* 1999), and this can lead to an overall reduction of brain NE levels (Weiss *et al.* 1980, Carboni *et al.* 2010). Nevertheless, the underlying mechanisms are far from being fully understood. Exploring the molecular links of the interaction between chronic stress and alteration of the noradrenergic system is worthwhile for elucidating the biological basis of depression and identifying new treatments.

Stress triggers multiple biological reactions in different organisms and systems including the release of several stress-related hormones. Among them, as the final effector of the hypothalamus-pituitary-adrenal (HPA) axis, glucocorticoids have been implicated in most of the reported stress-induced physiological changes in brains (McEwen, 1999) through their ubiquitously distributed intracellular receptors (Bamberger *et al.* 1996). It has been suggested that prolonged stress-induced hypersecretion of glucocorticoids may form part of the intrinsic mechanism underlying the development of depression (Carrasco and Van de Kar, 2003). Thus, understanding glucocorticoid-induced modulation on neural systems may further clarify the relationship between stress and depression.

Generally, the central noradrenergic system is one of the targets modulated by glucocorticoids (Dallman *et al.* 2006). The noradrenergic system acts as an arousal and alerting system to enhance organismic function and behaviors. Therefore, interaction between glucocorticoid and noradrenergic systems may play an important integrative function in coping and adaptation to stress. Both the NE transporter (NET) and dopamine β-hydroxylase (DBH, EC 1.14.17.1) are the important endophenotype of the noradrenergic system. NET has the primary function for reuptake of NE from presynaptic terminals of noradrenergic nerves, by which NE transmission is inactivated at the synapse (Barker *et al.* 1995). DBH is an enzyme that catalyzes the oxidation of dopamine to NE (Friedman *et al.* 1965). Both NET and DBH play an essential role for maintaining the transformational homeostasis and normal functions of the noradrenergic system. Therefore, the modulation of glucocorticoids on the noradrenergic system may be mediated by affecting the expression of these two phenotypes.

In a previous study, we found that chronic social defeat (CSD) significantly increased mRNA and protein levels of the NET in the locus coeruleus (LC), hippocampus, frontal cortex and amygdala. The latter three regions are the projection areas of the LC neurons. CSD-induced increases in NET expression were abolished by adrenalectomy or treatment with corticosteroid receptor antagonists, suggesting the involvement of corticosterone (CORT) and corticosteroid receptors in this upregulation (Chen *et al.* 2012). Further, CSD also increased the expression of DBH in the same brain regions (Fan *et al.* 2013). In order to further verify that CORT secreted during stressful events accounts for this effect, adult Fischer 344 rats were treated with a dose relevant to stress-induced plasma CORT

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concentration, and the expression of NET and DBH in the LC as well as the main NE terminal regions were examined. Meanwhile, two behavioral tasks, elevated T-maze and open-field, were performed to examine whether there is a parallel behavioral change after CORT treatment. The present findings demonstrate that chronic treatment with CORT upregulated expression of NET and DBH in the LC and its terminal regions, which was mediated through corticosteroid receptors. The expressional alteration of NET and DBH induced by CORT was accompanied by increased stressful behavior. These results are similar to those observed in the CSD model, and indicated that there is an integrative interaction between chronic stress, through CORT, and the noradrenergic system.

Material and methods

Animals

Male Fischer 344 rats, weighing 200-250g at the beginning of the experiment, were purchased from Harlan Laboratories Inc. (Indianapolis, IN, USA). All animal procedures were approved by the Animal Care and Use Committee of East Tennessee State University, and complied with the NIH Guide for the Care and Use of Laboratory Animals. Rats were maintained on a 12-h light/dark cycle (lights on at 07:00 am) with *ad-libitum* access to food and tap water except as specifically described below. After an acclimation period of 5 days, rats were randomly assigned to experimental groups.

Oral administration with CORT

Animal chow was available *ad libitum*, but drinking water was replaced with a solution containing 100 μg/ml CORT (Sigma, St. Louis, MO) at 9:00 am of each day for 21 days, which was freshly prepared daily. Selection of such concentration of CORT was based on past works, which resulted in an increased plasma CORT level approaching stress induced levels in animals (Karatsoreos *et al.* 2010, Donner *et al.* 2012). Because of its hydrophobic characteristics, CORT was first dissolved in 100% ethanol, and then diluted in regular tap water to a final ethanol concentration of 2.4% ethanol. Control rats were given a 2.4% ethanol solution alone. This vehicle containing 2.4% ethanol itself neither activated the hypothalamic-pituitary-adrenal axis, nor affected other measurements including organ weight (adrenal, spleen and thymus), as well as neuronal measurements (Magarinos *et al.* 1998, Nacher *et al.* 2004a, Nacher *et al.* 2004b, Gourley *et al.* 2009). Some groups of rats were injected daily with mifepristone (10 mg/kg, daily, s.c.) or spironolactone (15 mg/kg, daily, s.c.); either alone or in combination around the similar time (9:00 am) as the replacement of CORT drinking solution daily. Also, a separate set of rats (N=6) was injected daily with either mifepristone or spironolactone without CORT ingestion. All these compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). The doses of these antagonists were chosen on the basis of previous reports (Ratka *et al.* 1989, Ni *et al.* 1995, Haller *et al.* 1998, Macunluoglu *et al.* 2008) and preliminary pilot data. Rats in the untreated control and CORT alone groups were injected with vehicle. After 3-weeks of exposure to CORT animals were sacrificed on the 22nd day by rapid decapitation and trunk blood was collected for measurement of plasma CORT (around 9:30 am). After rats were sacrificed, brains were removed and rapidly frozen in 2-methyl-butane on dry-ice, then stored at −80° C until dissection. Trunk blood from rats was quickly collected and prepared for plasma CORT measurement with the protocol described previously (Chen et al., 2012).

Sucrose consumption test and plasma CORT determination

The methods for these two tests are the same as described before (Chen et al. 2012). Briefly, the sucrose consumption test was during a one hour window between 06:30 pm and 07:30 pm using a two bottle test (either 1% sucrose solution or tap water). This test was carried out weekly on the same day of the week (Thursday) throughout the three week CORT ingestion.

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Sucrose consumption is often used as a measurement of anhedonia in animal models of stress (Willner *et al.* 1987, Papp *et al.* 1991). Plasma CORT was later measured by a radioimmunoassay using a commercial kit according to the manufacturer's instructions [ImmuChem radioimmunoassay kit, MP Biomedicals, LLC in Orangeburg, NY (formerly ICN Pharmaceuticals, Costa Mesa, CA)].

In situ hybridization

The *in situ* hybridization method is the same as described previously (Zhu *et al.* 2002). Briefly, slides, where brain sections (16 μm) around the brain stem LC region were mounted on, were fixed with 4% (w/v) paraformaldehyde followed by acetylation with acetic anhydride. Lipids were extracted by washing with increasing concentrations of alcohol (50, 70, 95 and 100% [vols]). The [^{35}S]-labeled cRNA probes (Perkinelmer, MA) were transcribed *in vitro* from cDNAs for rat NET or DBH in pGEM-3Zf vectors with T7 RNA polymerase. After incubation with hybridization solution containing the radiolabeled probes at 55°C for 3-5 hours, sections were then washed extensively and apposed to Biomax autoradiographic films (Kodak; Rochester, NY). For higher-resolution analyses, sections were also dipped in Kodak NTB2 emulsion (Fisher, Pittsburgh, PA) and quantitatively analyzed with the Bioquant Nova program (R.M. Biometrics, Inc.; Nashville, TN). The specificity of cRNA probes was tested using three criteria. First, sense probes synthesized from each cDNA were used to perform *in situ* hybridization in parallel with antisense probes. There were no specific signals on these slides. Second, antisense probes were used on control slides from the cerebellum and cortex and no hybridization signals were detected. Third, antisense probes were hybridized to slides that were treated with RNase A (20 μg/mL) and no hybridization signal was detected (data not shown).

Detection of protein levels

Western blotting was performed to measure protein levels of NET and DBH in the samples in a similar manner as previously reported (Chen et al., 2012). Briefly, samples were microdissected or micropunched from rat hippocampus, frontal cortex and amygdala, and processed by homogenization and centrifugation. Protein concentrations were measured. Equal amounts of samples (30 μg of protein per lane) were loaded on 10% SDS-polyacrylamide gels for electrophoresis. Protein bands in gels were transferred to polyvinylidene diflouride membranes by electro-blotting. The membranes were in turn incubated with a polyclonal primary antibody against NET from rabbit (1:330 dilution; Alpha Diagnostic Intl. Inc, San Antonio, TX) or DBH from rabbit (1:500 dilution; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) overnight at 4°C, as well as secondary antibodies in the next day after washing (horseradish peroxidase-conjugated anti-rabbit IgG, 1:3000; Amersham Biosciences, Little Chalfont, UK). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham; Piscataway, NJ). Bands were detected by G:Box Imaging (Fyederick, MD, USA), or exposed on films and scanned by Quantity One imaging devices (Bio-Rad, Hercules, CA). Band densities were then quantified by imaging software (Molecular Dynamics IQ solutions, Molecular Dynamics, Inc., Sunnyvale, CA). Before the experiment, the antibody specificity was tested by using blocking peptides and results showed there was no band at the known molecular weight for the target after addition of the blocking peptide. Also, a linear standard curve was created from optical densities (ODs) of bands with a dilution series of total proteins prepared from brain tissues. OD values of NET or DBH were compared with those of the standard curve to ensure that detection was in the linear range of measurement. The quantitated value normalized with β-actin immunoreactivities, which were determined on the same blot, to assess equal protein loading. Normalized values were then averaged for all replicated gels and used to calculate the relative changes of the same gel.

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Immunofluorescence staining with NET and DBH

Rats were transcardially perfused under anesthesia with pentobarbital (40 mg/kg, i.p.) using 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Brains were stored in 10% sucrose followed by 30% sucrose and then sectioned at 30 μm using a sliding microtome. Immunofluorescence staining was performed using commercial polyclonal antibody from rabbit against NET (Alpha Diagnostic Intl. Inc, San Antonio, TX) or DBH (Protos Biotech Corp., New York, NY, USA). After washing in phosphate-buffered saline (PBS) and preincubated in 5% bovine serum albumin in PBS supplemented with 0.2% Triton-X 100 for 1 h at room temperature, brain sections were incubated in primary antibodies (for NET: 1:330 dilution; for DBH: 1:500 dilution, in PBS containing 0.2% Triton-X 100) overnight at 4°C, followed by incubation with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG, from Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature, which was subsequently followed by four rinses with 0.1M PBS. Slides were then coverslipped using Citifluor mounting medium. In order to reduce inter-animal staining variability, each immunofluorescence stain was performed on all tissue sections at the same time by an investigator blinded to conditions. Immunofluorescence labeling of NET and DBH was identified under a Leica TCS SP2 confocal microscope system (Leica Microsystems Inc., Bannockburn, IL, USA). Specimens were scanned sequentially to avoid crosstalk between fluorochromes, and a maximum LC image was obtained from each series. These digital images were taken with identical exposure times to allow the comparison of fluorescence intensity between images. Under matched imaging conditions, the negative control showed no visible staining (see below). Images were then semiquantitatively analyzed by using ImageJ software (Rasband, US National Institutes of Health, Bethesda, http://rsbweb.nih.gov/ij, 2010). Reference background levels were obtained from non-immunoreactive portions of brain sections adjacent to the LC region by determining the optical density on a 0–255 grayscale (0 being white and 255 black). The area fraction of immunofluorescence was quantified at three levels of each section and two sections from each rat. To validate the specificity of these polyclonal antibodies, some slides were processed to determine the specificity of this immunostaining by 1) using blocking peptides in which the antibody with the blocking peptide was incubated, and 2) negative control testing in which the primary antibody was not applied. In both tests, no positive immunostaining was found in these slides (data not shown).

Behavior tests to measure explorative and anxiety-related behavior

Two behavioral tests were performed during the last two days of CORT administration paradigm (21st and 22nd day): the elevated T-maze task and open-field. The order in which the tasks were performed was balanced across subjects. The elevated T-maze is a modified version of the more commonly used elevated plus maze, except the maze itself has only three arms, with the stem of the T-maze enclosed by Plexiglas lateral walls (40 cm high) that was perpendicular to two open arms. A total of four trials were given: a single baseline trial, followed by two avoidance trials, subsequently followed by an escape trial following the protocol of Trivedi and Coover (Trivedi *et al.* 2004). On the baseline and avoidance trials, the animal is placed at the end of the stem, and time is recorded as to the latency for the animal to visit the open arms. On the escape trial, the animal is placed at the end of one of the two open arms (balanced across subjects) and time is recorded for the animal to visit the walled arm.

For the open-field task, animals were placed into a square locomotor arena measuring 40 cm on a side, and all trials lasted for 10 min each. Activity was measured through AnyMaze software (Stoelting, Wood Dale, IL). This software allows for the analysis of several dependent measures. For this experiment, locomotor activity, clockwise and

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counterclockwise turning, as well time spent in immobile were measured. In addition, measurement includes visits to and time spent in a defined central zone in the arena.

Statistics

All experimental data are presented in the text and graphs as the mean ± SEM. The unpaired Student's *t*-test was used to analyze data of plasma CORT measurement in the experiment of CORT administration (Fig. 1A). The repeated measures analysis of variance (ANOVA) was used for the data in Fig. 1B and behavioral tests. For all other analysis, an one-way ANOVA was used, followed by *post hoc* Newman-Keuls tests for pairwise comparisons ($p<0.05$).

Results

Plasma CORT measurement and sucrose consumption test

To define the efficacy of CORT administration in inducing hormonal alteration, plasma CORT levels were measured in collected samples. As shown in Fig. 1A, CORT ingestion significantly increased plasma CORT levels ($p<0.01$). Also, the sucrose consumption test was performed to evaluate depressive effects of CORT ingestion in rats. As shown in Fig. 1B, the intake of the sucrose solution was not significantly changed in untreated animals over the whole duration of the experiment ($p>0.05$). However, it was significantly reduced in the first and second week of CORT treatment ($F_{3,31}$=7.54, $p<0.01$). Although the sucrose consumption in the third week of CORT ingestion also showed a reduced level, it did not reach statistical significant level when compared to the baseline (0). Nevertheless, it was still significantly lower when compared to that at the 3rd week time point of the non-treated animals ($P<0.05$).

CORT ingestion increases mRNA and protein levels of NET and DBH in the rat LC

To investigate the influence of CORT ingestion on the expression of NET and DBH in the LC of rats, as well as whether this influence was mediated by corticosteroid receptors, *ex vivo in situ* hybridization was performed to measure mRNA levels of NET and DBH. First, to examine whether these antagonists alone affected the expression of the noradrenergic phenotypes, a separate set of rats were injected with vehicle (the control), mifepristone or spironolactone in the absence of CORT ingestion. As showed in Figs. 2A and 3A, compared to the mRNA level of NET and DBH in the LC of the control rats, injection of mifepristone or spironolactone alone did not produce any significant alteration of NET ($F_{2,17}$ = 0.48, $p>0.05$) or DBH ($F_{2,17}$ = 0.53, $p>0.05$) mRNA levels. It indicates that without stress hormones' binding, glucocorticoid and mineralocorticoid receptors are not activated and their antagonistic functions are not supposed to be working. In contrast, CORT ingestion significantly affected mRNA levels of these noradrenergic phenotypes ($F_{4,27}$ = 3.06, $p<0.05$ for NET; $F_{4,34}$ = 3.45, $p<0.05$ for DBH). *Post hoc* tests revealed that CORT treatment increased mRNA levels of NET or DBH in the LC region, respectively, by 281.5% or 163.6% ($P<0.05$), compared to controls. Furthermore, mifepristone and spironolactone, alone or in combination, were treated to these CORT-ingested rats. The analysis showed that treatment with glucocorticoid receptor antagonist mifepristone, or combination of the two antagonists blocked CORT-induced elevation of NET mRNA levels ($p<0.05$, respectively, Fig. 2B). In contrast, CORT-induced increase of DBH mRNA levels in the LC was completely abolished only by the treatment with combination of mifepristone and spironolactone (Fig. 3B, $p<0.05$).

Alteration of NET/DBH protein levels in the LC caused by CORT ingestion was also examined by inmmunofluorescence staining. The separate experiment to examine effects of mifepristone or spironolactone on protein levels of NET and DBH in the absence of CORT demonstrated these antagonists alone did not result in any significant alteration on

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immunoreactive activities of NET and DBH in the LC (Figs. 4A and 5A; $F_{2,16} = 0.33$, p>0.05 for NET; $F_{2,17} = 0.41$, p>0.05 for DBH). However, CORT ingestion markedly influenced immunoreactive levels of these two phenotypes ($F_{4,44} = 8.37$, p<0.01 for NET; $F_{4,34} = 4.07$, p<0.01, for DBH). Further examination revealed that CORT ingestion significantly increased protein levels of NET and DBH about 74.4% and 71.7%, (both p<0.01) respectively, compared to those of the control (Figs. 4 and 5). These increased protein levels were also attenuated by administration of mifepristone alone (P<0.05 for both NET and DBH), or combination of mifepristone and spironolactone (p<0.05 only for DBH).

CORT ingestion increases protein levels of NET and DBH in the hippocampus, frontal cortex and amygdala

Western blotting was performed on samples from the hippocampus, frontal cortex and amygdala to explore whether CORT ingestion alters NET/DBH protein levels in these terminal areas of the LC. Treatment of rats with mifepristone or spironolactone in the absence of CORT did not cause significant changes in protein levels of NET or DBH in these areas (Table 1). As shown in Fig. 6, CORT ingestion significantly affected NET protein levels in these terminal regions ($F_{4,39}$=3.26, p<0.05 for the hippocampus; $F_{4,39}$=4.06, p<0.01 for the frontal cortex; $F_{4,34}$=8.97, p<0.001 for the amygdala). However, while CORT ingestion similarly changed DBH protein level in the hippocampus ($F_{4,39}$=3.01, p<0.05) and amygdala ($F_{4,39}$=3.16, p<0.05), there was no significant alteration for DBH protein levels in the samples from the frontal cortex ($F_{4,39}$=1.01, p>0.05). The posteriori Newman-Keuls test revealed that the CORT-induced increase of protein levels of NET (in all three regions) or DBH (in the hippocampus and amygdala) was abolished by treatment with combination of mifepristone and spironolactone.

CORT ingestion resulted in behavioral changes

Two behavioral tasks were used to analyze CORT treatments: the elevated T-maze and open-field. In order to gain insight whether corticosteroid receptor antagonists alone resulted in any behavioral alteration, a separate experiment was carried out, in which rats were injected with mifepristone (10 mg/kg, daily, s.c.) or spironolactone (15 mg/kg, daily, s.c.) in the absence of CORT ingestion for 21 days and the control rats were injected with the vehicle. The elevated T-maze task showed that either mifepristone or spironolactone significantly increased Avoidance T1 ($F_{2,15} = 8.52$, p<0.004) and Avoidance T2 ($F_{2,15} = 5.63$, p<0.01) (60.00 is the maximal value for such test), while leaving the escape trial unchanged (Table 2). A longer latency to explore the open arms in these groups treated with antagonists alone represent an increased fear, indicating that these antagonists alone may have anxiogenic effects. Nevertheless, the open-field tests showed that there was not any significant alteration after treatment with either mifepristone or spironolactone in the absence of CORT ingestion (Table 2).

As the biochemical measurements above showed that elevated NET and DBH expression in most of the brain areas was abolished by simultaneous treatment with combination of corticosteroid receptor antagonist mifepristone and spironolactone, we only examined effects of the cocktails (combination of mifepristone and spironolactone, which were daily injected for 21 days) in the behavior tests for the CORT-involved experiment (Table 3). The order of these tasks was counterbalanced across subjects, and each task was completed within a single day. A 2 × 5 repeated measures ANOVA of the avoidance trials on the elevated T-maze revealed a significant main effect of group ($F_{2,23} = 4.25$, p<0.01) and trial ($F_{2,23} = 6.96$, p<0.01). There was no significant effect on analysis of the escape trial. Controls had a shorter latency to explore the open arms compared to all other groups, presumably due to a lack of learned fear of the open arms, whereas fear of the open arms appears to be enhanced in CORT treated subjects, as would be expected, indicating an

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anxiogenic effect caused by CORT. Interestingly, the result of the group of cocktails plus CORT was not significantly different from that of the CORT group, indicating that a combination of these antagonists failed to block the avoidance response in CORT treated subjects. For the open-field task, significant group differences were revealed on horizontal activity ($F_{2,23} = 5.80$, $p<0.01$) and the number of visits made to a central zone in the arena ($F_{2,23} = 4.56$, $p<0.05$). In terms of horizontal activity, animals administered with the antagonists demonstrated a significant increase in horizontal activity compared to the CORT-treated group and controls. Analysis of visits to the central zone revealed that the CORT-treated group made less visits to the central zone than the control. This result appears to indicate that combination of antagonists was effective in blocking CORT-related behaviors in the open-field. Note that this effect cannot be accounted for by horizontal activity, because the CORT group was equivalent to the controls on the horizontal activity measure. Thus, it appears that CORT ingestion induced anxiety as indicated by both of these tasks. However, the effects of the antagonists were task dependent, in that they were only effective in blocking effects of CORT on visits to the center zone in the open- field.

Discussion

Our previous study demonstrated that chronic social defeat in rats up-regulated the expression of the noradrenergic phenotype in the LC and its terminal regions (Chen et al. 2012). In the present study, we attempted to mimic the stress state through chronic administration of CORT to rats. Results showed that CORT ingestion significantly increased both mRNA and protein levels of NET/DBH in the rat LC. The same treatment also increased NET protein levels in LC projection regions such as the hippocampus, frontal cortex, and amygdala, as well as DBH protein levels in the hippocampus and amygdala. Elevated NET and DBH expression in most of these areas (except for NET protein levels in the LC) was abolished by treatment with combination of corticosteroid receptor antagonist mifepristone and spironolactone. Also, treatment with mifepristone alone prevented CORT-induced increases of NET expression and DBH protein levels in the LC. Furthermore, this CORT-induced expressional upregulation of the noradrenergic phenotypes was accompanied by specific anxiety-like behavioral alterations, as demonstrated by inhibitory avoidance of the open arm and reduced center zone entries. Taken together, these studies confirmed that there is a functional linkage between chronic stress and the noradrenergic system through action of CORT on the expression of noradrenergic phenotypes.

Dysregulation in the function of the HPA axis activity and central noradrenergic system is a common feature of many stress-related mental disorders including major depression and anxiety disorders (Bunney *et al.* 1965, Thomson *et al.* 2008). Animal studies have demonstrated that stress-induced activation of the LC-NE system (Abercrombie & Jacobs 1987) alters the release and metabolism of NE in the noradrenergic neuronal cell bodies and their terminal regions (Pacak et al. 1995, Smagin et al. 1997). However, whether an abnormal HPA axis, represented by hypercortisolemia, and a disturbed noradrenergic system has a causative relationship is not completely clear. Our previous studies demonstrated that chronic stress increased expression of NET (Chen et al. 2012) and DBH (Fan et al. 2013) in the LC and its main projection regions, indicating that chronic stress activated the noradrenergic system by action on noradrenergic phenotypes. To further clarify this relationship, the present study was carried out through oral administration of CORT to mimic the chronic stress. Results revealed that CORT ingestion upregulated NET and DBH expression in most central noradrenergic neurons (except for DBH protein levels in the frontal cortex, discussed below). The results not only confirmed the similarity between the CORT ingestion and the CSD models, but also lead us to postulate that elevated NET (increased reuptake of intracellular NE) and DBH (increase NE synthesis to compensate the stress-induced release of NE) induced by CORT ingestion may be a necessary response for

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integrating homeostasis, an important adaptive modification of the central NE system reacted to chronic stress (Goddard *et al.* 2010). It may be worth mentioning that tyrosine hydroxylase as a rate-limiting enzyme plays an important role for the biosynthesis of NE. However, DBH also is a key factor to determine the rate of NE synthesis (Kobayashi *et al.* 1994, Kim *et al.* 2002), as disruption of the DBH gene has been reported to block the synthesis of NE (Sabban 2007, Kvetnansky *et al.* 2008). The present study demonstrated that CORT ingestion markedly increased mRNA and protein levels of DBH in the LC, and DBH protein levels in the hippocampus, and amygdala. These results indicate that in response to CORT exposure, upregulated expression of DBH in the LC and some terminal regions may increase synthesis of NE. However, as NE concentration in the brain has not been measured in the present study, this notion has to be verified by further experiments.

Stressful life events are potent factors that trigger, induce or exacerbate episodes of depression. During chronic stress, several hormones and systems are involved. Stress-induced release of glucocorticoids, as the final effector of the HPA axis, and subsequent activation of corticosteroid receptors in the brain play a crucial role in mounting the adaptive response to stress. Development of a model that produces a persistent, varied behavioral alteration would be a major advance for understanding the neurobiology of depression. To extend our previous work using CSD animal models, non-invasive CORT ingestion was performed to verify the previous observation. The results showed that oral administration of 100 μg/ml CORT for 3 weeks resulted in a blood concentration of CORT about 56 μg/dl, a level corresponding to the stress status found in animal studies (Sapolsky *et al.* 1995). Also, sucrose consumption, an analog of "anhedonia-like" symptoms in stress paradigm, exhibited a very similar pattern to that in stress model rats (Chen et al. 2012), indicating that chronic non-invasive administration of CORT can mimic the CSD model to induce "anhedonia-like" depressive-like behavior. It is worth pointing out that while reduced sucrose consumption in the third week of CORT treatment was absolutely lower than that in the controls ($p<0.05$), it did not reach the statistical significance when compared to the baseline before the treatment. Nevertheless, sucrose consumption in the chronic social defeat rat model showed a consistently decreased state throughout CSD (Chen et al. 2012). This discrepancy between the CORT ingestion and CSD regime may be accounted by the different experimental paradigm used in these two studies. In the previous study, a paradigm of 4 week stress with variable defeat session schedules was used for the purpose to avoid the habituation of rats to a regular schedule. In contrast, in the present study, CORT was administrated daily. A habituation may occur during this period. The curve of the sucrose consumption can verify this phenomenon: the lowest sucrose consumption rate appeared in the first week of CORT ingestion, which seemed to be attenuated with time.

In the present study, CORT ingestion significantly increased expression of NET in the LC region and its major terminal regions, as well as the expression of DBH in the most of these regions. However, no significant changes were found in DBH protein levels in the frontal cortex after CORT ingestion. In contrast, CSD regimen upregulated DBH protein levels in all major terminal regions (Fan et al. 2013). Currently we do not have a satisfactory explanation for this discrepancy of DBH protein levels between the present study and previous CSD experiment. While the technical factors related to measurement may be considered, the similar phenomenon has been reported previously. For example, chronic stress significantly reduced expression of brain-derived neurotrophic factors in the frontal cortex (Roceri *et al.* 2004, Mao *et al.* 2010, Li *et al.* 2012), whereas chronic administration of CORT had no significant effect on mRNA and protein levels of the same factor in the same brain region (Jacobsen *et al.* 2006). One of the possibilities for such different effect on DBH expression between chronic stress and CORT treatment may be explained by the fact that chronic stress is involved by more hormones and signal transduction pathways (Kwon *et al.* 2007). Multiple hormones and their activated signal transduction pathways may produce

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relatively stronger effects on the DBH gene, which has several *cis*- and *trans*-regulatory elements in the prompter regions (Shaskus *et al.* 1992, Afar *et al.* 1996), than that caused by CORT treatment alone. In addition, given that DBH expression levels are lower in cortical regions (Schroeter *et al.* 2000), the effect from CORT ingestion on DBH in the frontal cortex may be smaller than other regions, although DBH expression there still shows a potential enhancement.

Regarding behavior, the corticosteroid receptor antagonists increased overall horizontal activity. Although there are no studies to show chronic effects of the combination of these antagonists on behavioral activity, one study has shown that an acute dose of mifepristone had no effect on behavioral activity (De Vries *et al.* 1996). These antagonists have been shown to alleviate motor impairments caused by stress and CORT treatment, whereas either antagonist alone produced no significant differences compared to controls (Jadavji *et al.* 2011). Studies using the immunohistochemical technique have shown the presence of glucocorticoid receptors in the motor cortex, basal ganglia, and cerebellum (Ahima & Harlan, 1990; Ahima *et al.* 1991), thus rendering these motor brain regions sensitive to the actions of glucocorticoid receptors. By contrast, mineralocorticoid receptors in the motor system appear to be restricted to cortical areas, but are present in the motor cortex (Roland *et al.* 1995). Therefore, blockade of both receptors appears to produce a synergistic effect on specific behavioral activity in the status of stress or CORT treatment. Regardless, the task dependent effects of the CORT antagonist cocktail emphasizes that these tasks measure different types of behaviors related to stressful events, but also highlight the complexity of behavioral testing. A recent review (Ramos 2008) nicely summarizes and emphasizes the importance of different behavioral tasks related to emotionality, anxiety, and stress to provide a comprehensive profile of the effects of stress on behavior.

CORT appears to have increased anxiety as measured on the elevated T-maze, which was not alleviated by the corticosteroid receptor antagonist cocktail. This may be due to the fact that the cocktail was given only once daily, but oral administration of CORT was continuing throughout the day over the entire three-week period. Thus, the antagonist cocktail did not sufficiently block the effects of CORT on anxiety as tested on the elevated T-maze. It was noteworthy that elevated T-maze tasks showed that either mifepristone or spironolactone in the absence of CORT ingestion resulted in a significant increase in Avoidance tests (Table 2), indicating that these antagonists alone may have potential anxiogenic effects. Although these results may explain the reason why the cocktail failed to reverse CORT-induced anxiety-like behavior (Table 3), they are in conflict to the available reports in the literature. It was reported that treatment with spironolactone or other MR antagonists, either by infusion into the brain or systematic administration, exerted anxiolytic effect, but not for GR antagonists (Korte *et al.* 1995, Smythe *et al.* 1997, Bitran *et al.* 1998, Hlavacova *et al.* 2010). Other studies also showed that treatment with mifepristone had no any anxiogenic effects (Calfa *et al.* 2006, Auger *et al.* 2008). Currently, we do not have a satisfactory explanation for such conflict. Different species of rats (Fischer 344 in the present study verse Wistar or others in those reports) may account for these differences, as it is well established that individual rats exhibit marked differences in behavioral responses to a novel environment (Kabbaj *et al.* 2000). Also, another attributable factor for such conflict may be the difference in the treatment period: 21 days in the present study verse 3 to 11 days in those reports. Since biochemical measurements in the current study showed that these antagonist administered alone did not cause a significant alteration for expression of NET and DBH in the absence of CORT, these potential anxiogenic effects may not be related to these phenotypes in the brain. As the study to use antagonists alone to test their behavioral effects is limited, further studies are warranted to pursue this issue.

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The reason of possible non-involvement of noradrenergic phenotypes in these antagonists-induced anxiogenic effects is not known, as even the degree that noradrenergic systems modulate behavioral state remained ambiguous. The LC-NE system is a critical component of the neural architecture. As such, it appears reasonable to propose that dysregulation of this system might contribute to dysregulation of a variety of cognitive and affective processes, resulting in specific behavioral alteration such as anxiety. However, the data in the literature show some difference. For example, rats with 6-OHDA-induced LC ablation did not show any signs of impairment in learning and performance of feat-motivated tasks (Mason *et al.* 1979). Also, noradrenaline depleted rats were more reluctant to leave a familiar place and took longer to consume the food pellets in an unfamiliar place, suggesting an increase in fear following the lesion. Moreover, there was no difference in the behavioral parameters (elevated plus-maze, light-dark box and open-field test) between DBH (−/−) and DBH (+/−) mice (Cryan *et al.* 2001, Marino *et al.* 2005). Therefore, while we think the alteration of CORT-induced noradrenergic phenotype alterations possibly account for the specific behavioral changes in the present study, there is another notion that the LC-NE system may be viewed as a general and global modulator of neuronal circuits that guide behavioral action (Itoi 2008, Itoi *et al.* 2010).

In summary, the present study demonstrates that non-invasive CORT administration significantly upregulated NET and DBH expression in the LC and its main terminal regions and it was mediated by corticosteroid receptors, a similar phenomenon to that of CSD regime as reported previously (Chen et al. 2012). In addition, the CORT ingestion not only induced anhedonia-like behavior, but also anxiety behavioral alteration. Taken together with previous observations, the results of the present study indicate that CORT plays a primary role in the chronic stress-induced activates the LC-NE system, which may be related to the pathophysiology of stress-precipitated psychiatric disorders.

Acknowledgments

This work is supported by NIH grant MH080323 and NARSAD Young Investigator Award. The authors thank Mrs. Hobart Zhu and Chris Hill for their helpful comments on the manuscript. The authors have no conflicts of interest.

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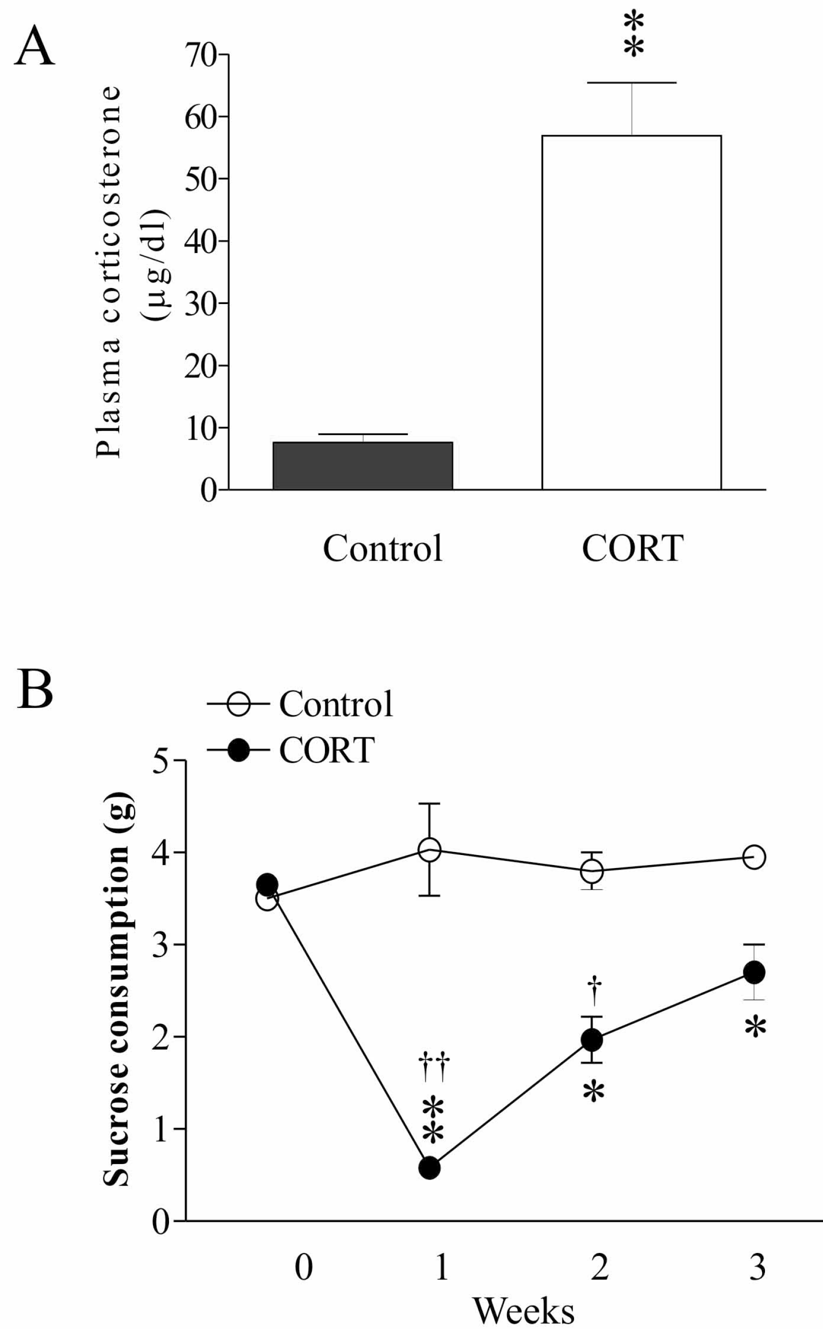

Figure 1.

Effects of CORT ingestion (n=8/group) on plasma CORT concentrations (A) and sucrose consumption (B). * *p<0.05*, ** *p<0.01*, compared to the control (A) or corresponding time point in the control group (B); † p<0.05, †† *p<0.01*, compared to the baseline of sucrose consumption (0 week).

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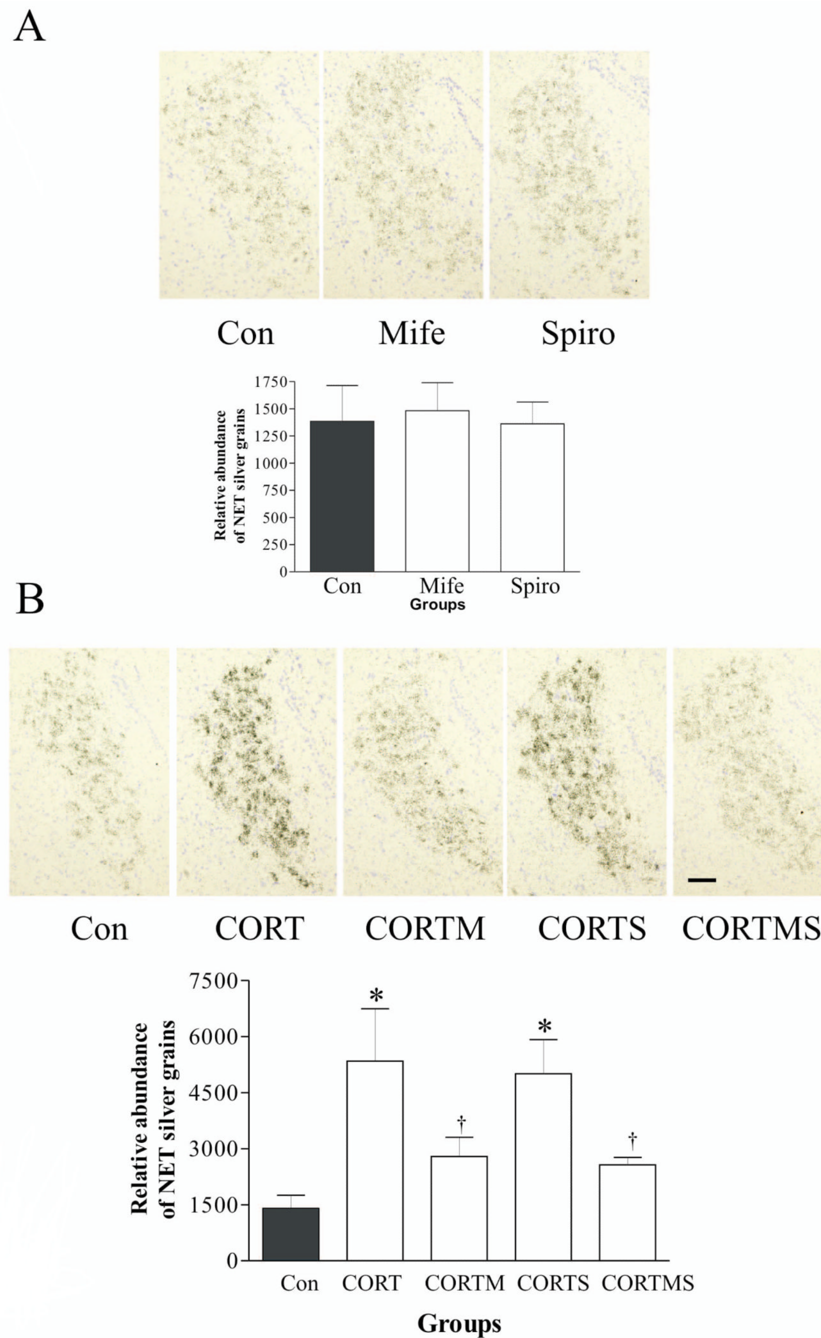

Figure 2.

Effects of corticosteroid receptor antagonists alone (A, n=6/group), CORT ingestion, and CORT ingestion plus treatment with corticosteroid receptor antagonists (B, n=8/group) on NET mRNA in the LC. *Upper panel* in A and B: NET mRNA in LC tissues of rats detected by *in situ* hybridization. Coronal brain sections were taken at 9.7 mm posterior from bregma (corresponding to Plate 58 in the brain atlas) (Paxinos *et al.* 2005). *Lower panel* in A and B: Quantitative analyses of mRNA in slides. * $p<0.05$, compared to the control; † $p<0.05$, compared to the CORT ingestion group. Con: control; CSDM: CORT plus treatment with mifepristone; CSDS: CORT plus treatment with spironolactone; CSDMS: CORT plus

treatment with both mifepristone and spironolactone; Mife: mifepristone; Spiro: spironolactone. Scale bar: 50 μm for all images.

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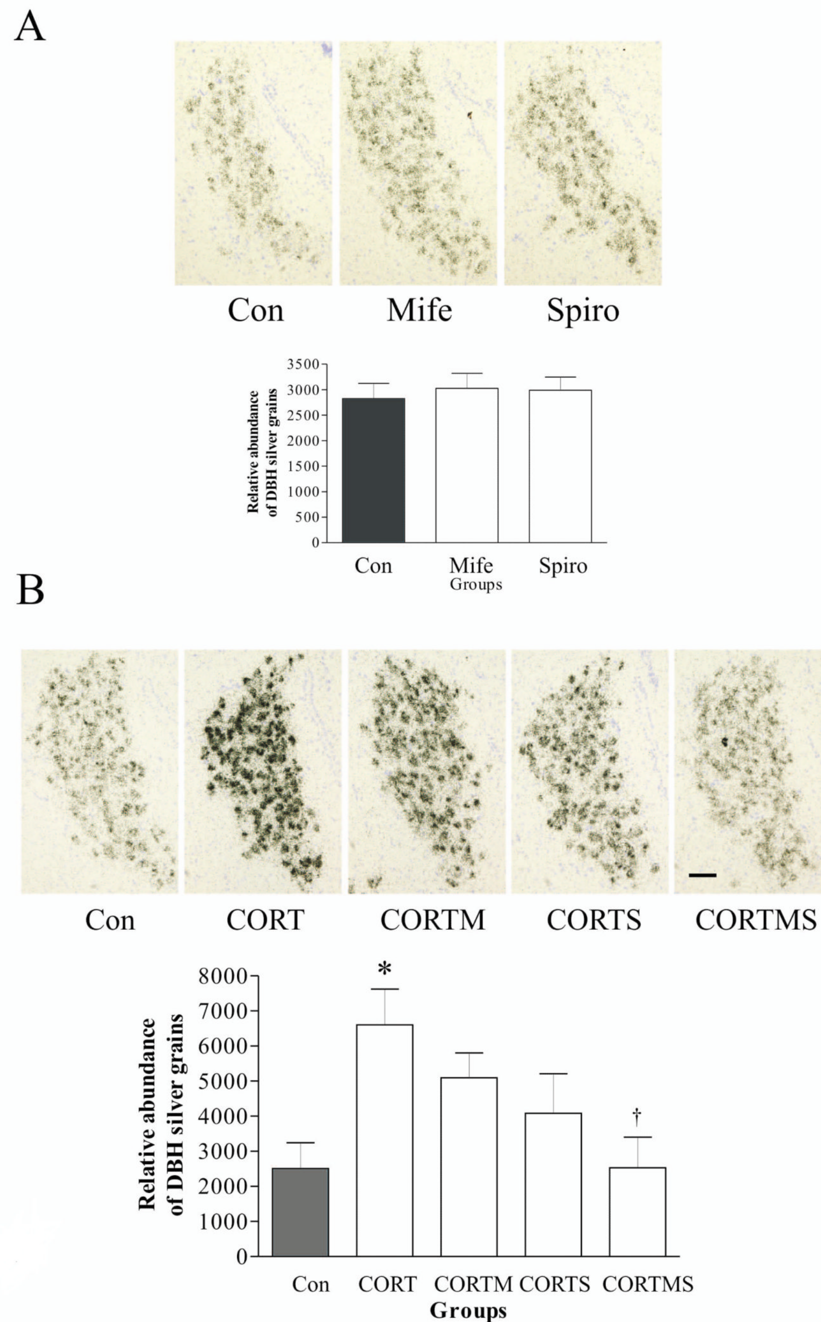

Figure 3.

Effects of corticosteroid receptor antagonists alone (A, n=6/group), CORT ingestion, and CORT ingestion plus treatment with corticosteroid receptor antagonists (B, n=8/group) on DBH mRNA levels in the LC. *Upper panel* in A and B: DBH mRNA in the LC detected by *in situ* hybridization. *Lower panel* in A and B: quantitative analysis of DBH mRNA obtained in slides, * $P<0.05$, compared to the control, † $P<0.05$, compared to the CORT ingestion group. See Fig. 2 for abbreviations. Scale bar: 50 μm for all images.

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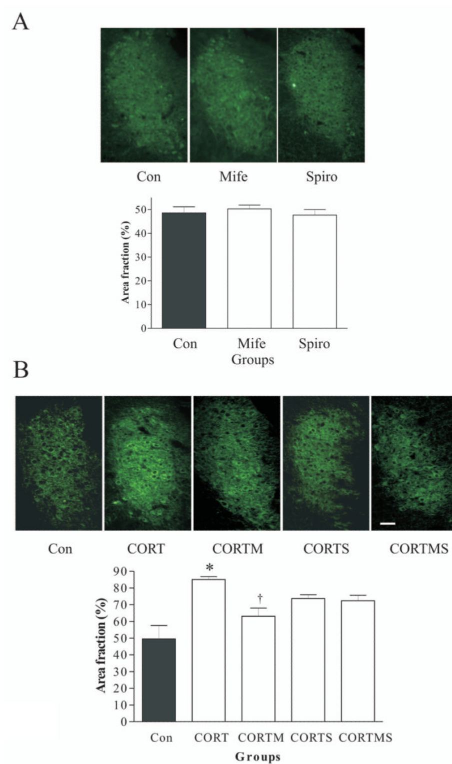

Figure 4.

Effects of corticosteroid receptor antagonists alone (A, n=6/group), CORT ingestion, and CORT ingestion plus treatment with corticosteroid receptor antagonists (B, n=6/group) on NET-immunoreactivity in the LC. The top panels in A and B are representative micrographs of NET immunofluorescence in the LC region from experimental rats. The bottom panels in A and B show measurements of the area fraction of NET immunofluorescence in the LC region. * $P<0.05$, compared to the control; † $P<0.05$, compared to the CORT ingestion group. See Fig. 2 for abbreviations. Scale bar: 50 μm for all images.

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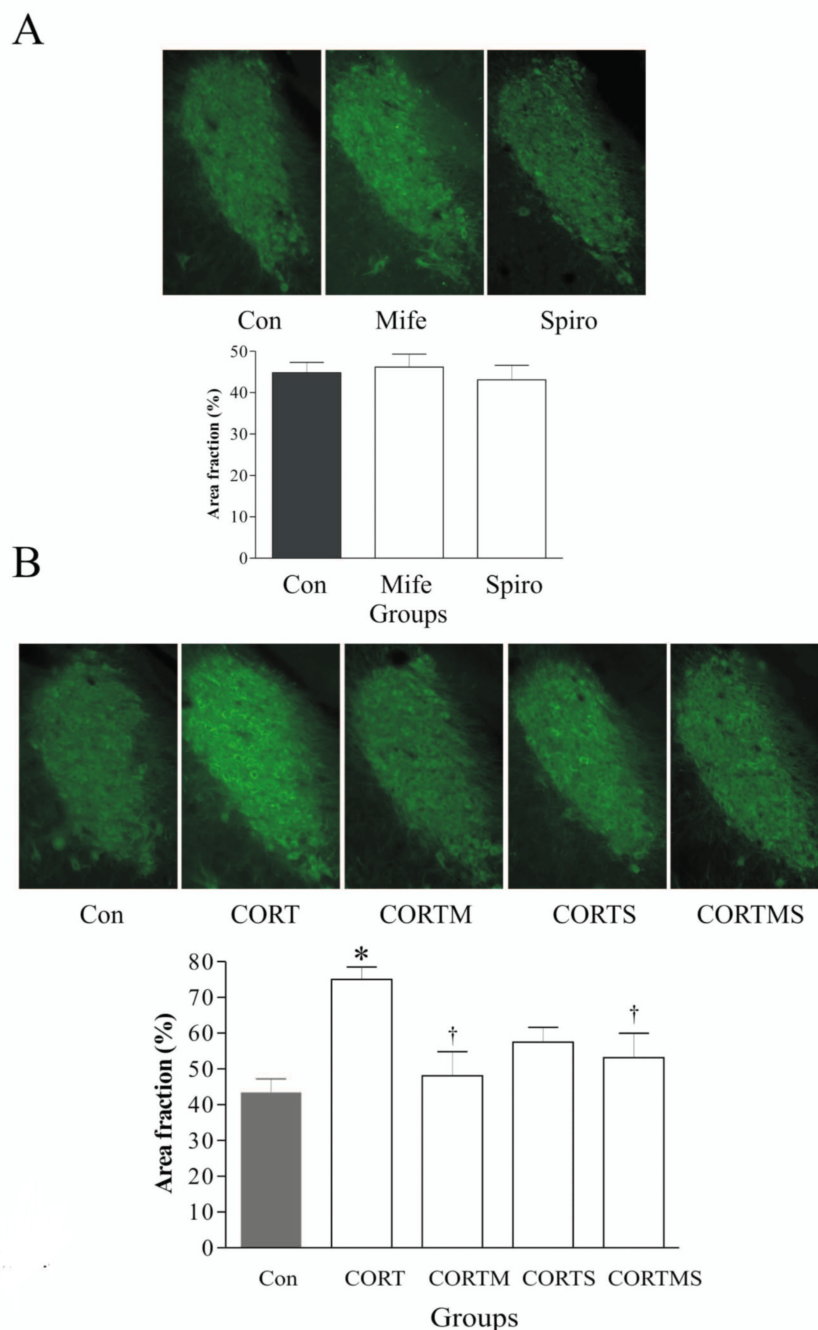

Figure 5.

Effects of corticosteroid receptor antagonists alone (A, n=6/group), CORT ingestion, and CORT ingestion plus treatment with corticosteroid receptor antagonists (B, n=6/group) on DBH-immunoreactivity in the LC. The top panels are representative micrographs of DBH immunofluorescence in the LC region from experimental rats. The bottom panels show measurements of the area fraction of DBH immunofluorescence in the LC region. * $P<0.05$, compared to the control; † $P<0.05$, compared to the CORT ingestion group. See Fig. 2 for abbreviations. Scale bar: 50 μm for all images.

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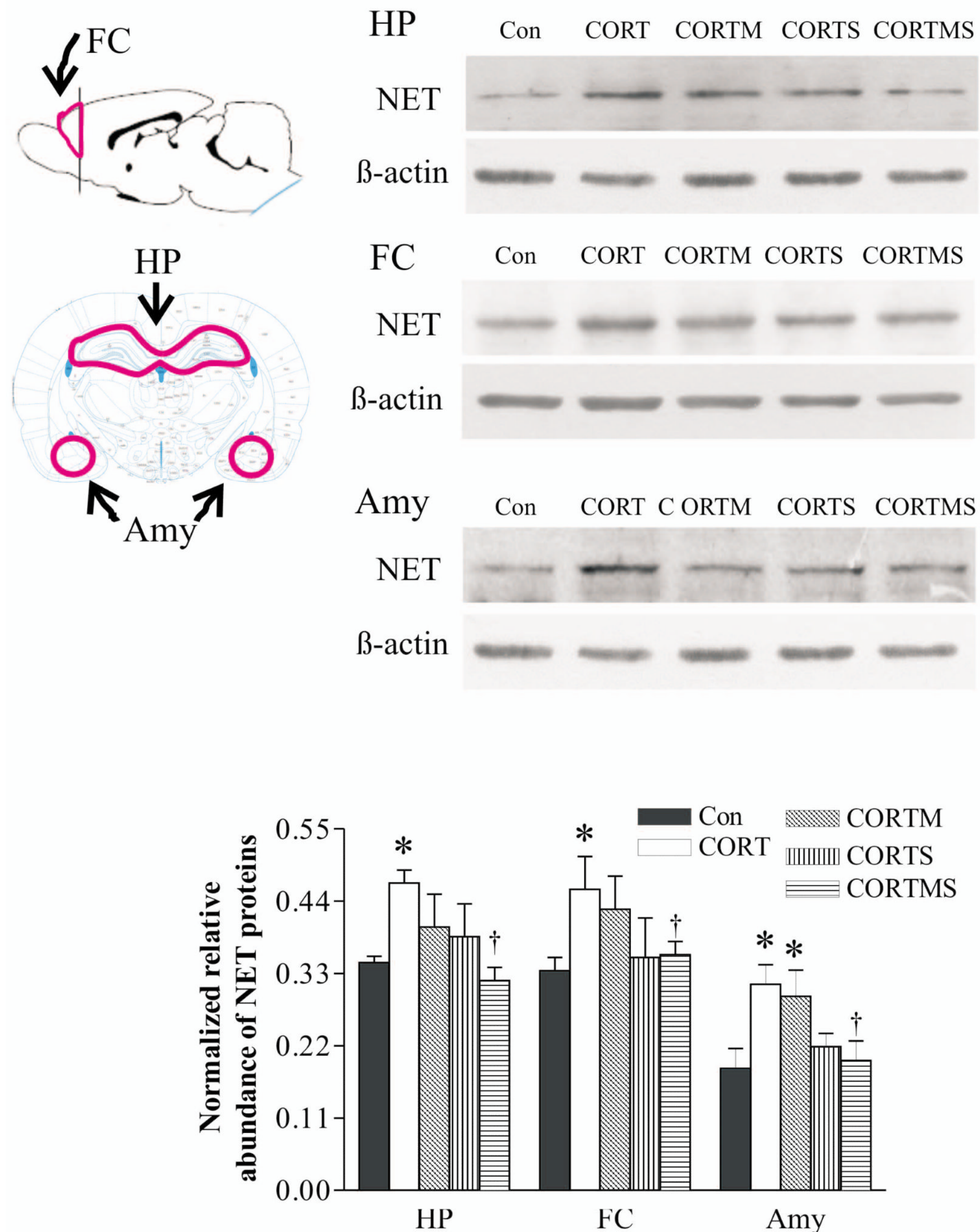

Figure 6.

Effect of CORT ingestion, and CORT ingestion plus treatment with corticosteroid receptor antagonists on NET protein levels in the LC terminal regions. The upper left panel is the neuroanatomical figure to depict the microdissection/micropunch regions used for western blotting. The upper right figures show autoradiographs obtained by western blotting of NET in the hippocampus (HP), frontal cortex (FC) and amygdala (Amy) (n=6-8/group). The lower graph shows quantitative analysis of band densities. Values of NET bands were normalized to those of β-actin probed on the same blot. *$P<0.05$, compared to the control group; † $p<0.05$, compared to the CORT group. See Fig. 2 for abbreviations.

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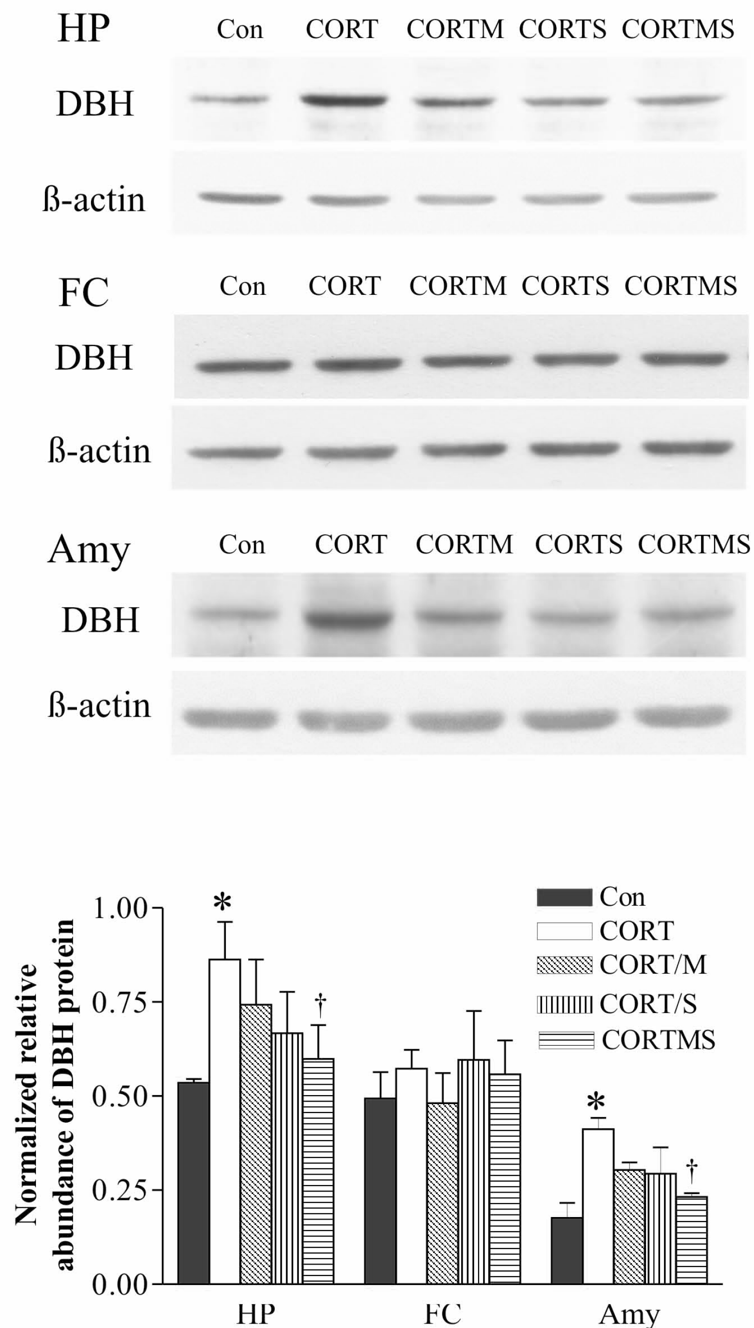

Figure 7.

Effect of CORT ingestion, and CORT ingestion plus treatment with corticosteroid receptor antagonists on DBH protein levels in the LC terminal regions. The upper figures show autoradiographs obtained by western blotting of NET in the hippocampus (HP), and amygdala (Amy) (n=6-8/group). The lower graph shows quantitative analysis of band densities. Values of NET bands were normalized to those of β-actin probed on the same blot. *$P<0.05$, compared to the control group; † $p<0.05$, compared to the CORT group. See Fig. 2 for abbreviations.

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Table 1

Effects of corticosteroid receptor antagonists on protein levels of NET and DBH in the hippocampus (Hipp), frontal cortex (FC) and amygdala (Amy) in the absence of CORT

Group	NET			DBH		
	Hipp	FC	Amy	Hipp	FC	Amy
Control (n=6)	0.318± 0.01	0.347±0.02	0.179±0.02	0.555±0.01	0.486±0.06	0.183±0.03
Mifepristone (n=6)	0.326± 0.07	0.352±0.01	0.187±0.06	0.503± 0.03	0.472±0.04	0.192±0.04
Spironolactone (n=6)	0.306± 0.09	0.349±0.02	0.173±0.03	0.548± 0.07	0.497±0.03	0.176±0.05

Value donated are means±SE and are expressed as the relative abundance of proteins which were normalized by the value of ß-actin. There is no statistical significant difference among the groups.

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Table 2

Behavior tests for mifepristone and spironolactone in the absence of CORT as measured by elevated t-maze (top) and open-field (bottom)

Group	Mean Avoidance T1(s)	Mean Avoidance T2(s)	Mean Escape Trial (s)	N
Control	35.67 ± 8.76	44.253 ± 9.86	37.69 ± 11.90	6
Mifepristone	60.00[*†]	60.00[*]	32.20 ±11.20	5
Spironolactone	60.00[*]	60.00[*]	44.00 ± 3.64	5

Group	Horizontal Activity Counts	Center Zone Entries	N
Control	552.33 ± 41.63	28.17 ± 2.78	6
Mifepristone	479.00 ± 39.90	33.40 ± 2.33	5
Spironolactone	466.20 ± 47.10	37.50 ± 10.17	5

N: the number of rats in each group.

[*] p<0.05, , compared to the control.

[†] There are no standard errors. 60 was the maximal value for each trial.

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Table 3

Behavior tests for CORT and CORT plus mifepristone and spironolactone (Antagonists) as measured by elevated T-maze (top) and open-field (bottom).

Group	Mean Avoidance T1(s)	Mean Avoidance T2 (s)	Mean Escape Trial (s)	N
Control	26.78 ± 9.20	38.33 ± 7.90	40.00 ± 6.50	8
CORT	40.11 ± 8.00*	58.30 ± 1.70*	41.20 ± 6.10	9
CORT Antagonists	41.38 ± 8.60*	58.50 ± 1.50*	50.71 ± 5.96	9

Group	Horizontal Activity Counts	Center Zone Entries	N
Control	627.00 ± 46.70	33.67 ± 3.40	8
CORT	617.00 ± 97.50	12.50 ± 3.40*	9
CORT Antagonists	959.30 ± 76.70*†	32.30 ± 3.39†	9

N: the number of rats in each group.

*p<0.05, compared to the control.

†p<0.05, compared to the CORT group.