Neuroscience 153 (2008) 1126–1134

p11 IS UP-REGULATED IN THE FOREBRAIN OF STRESSED RATS BY GLUCOCORTICOID ACTING VIA TWO SPECIFIC GLUCOCORTICOID RESPONSE ELEMENTS IN THE p11 PROMOTER

L. ZHANG,[a1]* H. LI,[a1] T. P. SU,[b] J. L. BARKER,[c] D. MARIC,[c] C. S. FULLERTON,[a] M. J. WEBSTER,[d] C. J. HOUGH,[a] X. X. LI,[a] TRAUMATIC STRESS BRAIN STUDY GROUP AND R. URSANO[a]

[a]*Center for the Study of Traumatic Stress, Department of Psychiatry, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA*

[b]*Division of Psychiatry, School of Medicine, National Yang-Ming University and Department of Psychiatry, Taipei Veterans General Hospital, Taipei, Taiwan*

[c]*Laboratory of Neurophysiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA*

[d]*Stanley Medical Research Institute, Chevy Chase, MD 20894, USA*

Abstract—Posttraumatic stress disorder (PTSD) is one of the most common psychiatric disorders. Despite the extensive study of the neurobiological correlates of this disorder, the underlying mechanisms of PTSD are still poorly understood. Recently, a study demonstrated that dexamethasone (Dex), a synthetic glucocorticoid, can up-regulate p11, known as S100A10-protein which is down-regulated in patients with depression, (Yao et al., 1999; Huang et al., 2003) a common comorbid disorder in PTSD. These observations led to our hypothesis that traumatic stress may alter expression of p11 mediated through a glucocorticoid receptor. Here, we demonstrate that inescapable tail shock increased both prefrontal cortical p11 mRNA levels and plasma corticosterone levels in rats. We also found that Dex up-regulated p11 expression in SH-SY5Y cells through glucocorticoid response elements (GREs) within the p11 promoter. This response was attenuated by either RU486, a glucocorticoid receptor (GR) antagonist or mutating two of three glucocorticoid response elements (GRE2 and GRE3) in the p11 promoter. Finally, we showed that p11 mRNA levels were increased in postmortem prefrontal cortical tissue (area 46) of patients with PTSD. The data obtained from our work in a rat model of inescapable tail shock, a p11-transfected cell line and postmortem brain tissue from PTSD patients outline a possible mechanism by which p11 is regulated by glucocorticoids elevated by traumatic stress. Published by Elsevier Ltd on behalf of IBRO.

Key words: posttraumatic stress disorder, glucocorticoid response elements, glucocorticoid receptor, p11, prefrontal cortex.

Posttraumatic stress disorder (PTSD) is a psychiatric disorder that occurs after life threatening traumatic events, such as military combat, natural disasters, terrorist incidents, serious accidents, or violent personal assaults (Ursano, 2002; Grieger et al., 2004). PTSD is a disabling condition associated with marked deficits in social, occupational, and familial function (Howard and Hopwood, 2003). About 7.8% of the American population have experienced PTSD during their lifetime (Kessler et al., 1995). Recently, the significance of PTSD has dramatically increased because of the high prevalence of PTSD in military personnel. Studies have shown rates of PTSD in returning combat troops from the Iraq war of 15.6–17.1% (Hoge et al., 2004).

Several studies have implicated the hypothalamic–pituitary–adrenal axis (HPA axis) as the key circuit in the pathogenic processes underlying PTSD. Over the last decade, many studies have shown abnormal HPA axis activity in PTSD, but these studies do not always report changes in the same direction (Yehuda et al., 1995b; Lindauer et al., 2006). Both higher and lower concentrations of circulating glucocorticoids in PTSD patients have been reported. For example, Holocaust survivors with PTSD have low urinary cortisol excretion (Yehuda et al., 1995b). High early morning salivary cortisol levels have been reported in police officers with PTSD (Lindauer et al., 2006). Bereaved children suffering the death of a parent following the September 11, 2001, terrorist attacks had higher morning and 4:00 pm baseline cortisol concentrations than non-bereaved children (Pfeffer et al., 2006). Thus, the different stressors, the different methods used, the different patient populations recruited, and the different stages of the disorder examined in the various studies have been suggested as explanations for these diverse results.

In animal studies, traumatic stress induces a plasma glucocorticoid elevation (Vogel and Jensh, 1988), which regulates stress-related behavior (de Quervain et al., 1998; Adamec et al., 2006) and gene expression (Liberzon and Young, 1997; Roseboom et al., 2006). Acute restraint stress increases 5-HT7 receptor mRNA expression in the rat hippocampus (Yau et al., 2001). Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus (Meaney et al., 2000). Recently, a study demonstrated that dexamethasone (Dex) can up-regulate p11, an S-100 calcium-binding protein (Yao et al., 1999; Huang et al., 2003), which was down-regulated in patients with depression (Svenningsson et al., 2006), a common comorbid disorder in PTSD. These

[1] Equal contribution.

*Corresponding author. Tel: +1-301-295-0921; fax: +1-301-295-0923.
E-mail address: Lezhang@USUHS.mil.

Abbreviations: CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; Dex, dexamethasone; GR, glucocorticoid receptor, prefrontal cortex; HPA, hypothalamic–pituitary–adrenal; mut, mutant p11 promoter CAT construct; PTSD, posttraumatic stress disorder; GR, glucocorticoid receptor; PFC, prefrontal cortex.

0306-4522/08$32.00+0.00 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2008.03.022

observations led to our hypothesis that traumatic stress may alter the expression of p11 in the brain and that this alteration may be mediated by glucocorticoid receptors. To test this hypothesis, first, we conducted an experiment that mimicked traumatic stress in rats by exposing them to inescapable tail-shock, and examined p11 expression in the prefrontal cortex (PFC) and plasma levels of corticosterone. Then, we studied possible molecular mechanisms of glucocorticoid regulation of p11 induction in SH-SY5Y cells at both gene and protein levels by real-time PCR, Western blot, GRE mutation and chromatin immunoprecipitation (ChIP). Finally, we determined whether there is any change in the p11 expression levels in the postmortem PFC of patients with PTSD. The results of our studies suggest that the mechanism by which glucocorticoid regulates p11 expression occurs through GR in the promoter of p11, and substantiate an important role of glucocorticoids in traumatic stress and PTSD.

EXPERIMENTAL PROCEDURES

Real-time PCR analysis of p11 gene expression

RNA was extracted from human postmortem brain or cell lysates using TRIzol. cDNA was generated from 5 μg of total RNA for each sample using Superscript III RT (reverse transcriptase) and oligo (dT) primers (Invitrogen, Carlsbad, CA, USA) to exclude that differences in RNA-content could result also from differences in sample weights. Real-time PCR was performed on the generated cDNA product in the iQ5 system using SYBR Green (Bio-Rad, Hercules, CA, USA). The following sequences were used for human p11 mRNA analyses: forward 5′-AAATTCGCTGGGGATAAAGG-3′ and reverse 5′-AGCCCACTTTGCCATCTCTA-3′ primers. The sequences were used for rat p11 mRNA analyses: forward 5′-TGCTCATGGAAAG GGAGTTC-3′ and reverse 5′-CCCCGCCACTAGTGATAGAA-3′ primers. Beta-actin mRNA level was unchanged by Dex exposure and was used as an internal control for normalizing p11 mRNA levels in control and experimental samples. The sequences for beta-actin primers were as described by Applied Biosystems. Dilution curves confirmed the linear dependence of the threshold cycle number on the concentration of template RNAs. Relative quantitation of p11 mRNA in control and experimental samples was obtained using the standard curve method. Statistics were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

Stress procedures

All rats were handled and weighed before each study began. Animals either remained undisturbed in their home cages as controls or were exposed to inescapable tail shock. The stress protocol involved placing the rats in a Plexiglas restraining tube (23.4 cm long and 7 cm in diameter) and exposing them to 100 inescapable shocks (2.0 mA) for 5 s each, with an average intertrial interval of 60 s. The shocks were applied through electrodes taped to the tail. After stressor termination, all animals were returned to their home cages. The number and strength of the shocks were optimized to yield a model of inescapable stress as measured by changes in behavior and by elevated plasma corticosterone levels. The number of animals used and their suffering were minimized.

Tissue collection

Animals were anesthetized with a brief exposure to Haldane immediately or 48 h after inescapable tail shock. Their brains were quickly removed after decapitation. All dissections were performed on a frosted glass plate placed on top of crushed ice. Brain samples, which included prefrontal frontal cortex, hippocampus, amygdala and cerebellum were quickly frozen on dry ice and stored at −70 °C until time of use.

Plasma corticosterone assay

Rats were anesthetized with a brief exposure to Haldane and killed via rapid decapitation immediately after termination of 3-day inescapable tail shock between 8:00 and 10:00 A.M. After each animal was killed, trunk blood was collected and frozen. Rat plasma corticosterone of non-stressed control and stressed groups was measured using the DSL-10-81100 ACTIVE Rat Corticosterone Enzyme Immunoassay Kit following the manufacturer's protocol (Diagnostic Systems Laboratories, Inc., Webster, TX, USA).

Cell culture

SH-SY5Y cells, a human neuroblastoma cell line, were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM culture medium obtained from Biosource (Rockville, MD, USA) containing 10% fetal calf serum. Cells were grown in 35 mm tissue culture plates coated with type I collagen (Becton Dickinson, Bedford, MA, USA) in immunocytochemistry experiments. Six-well tissue culture plates coated with type I collagen were used for transfection studies; 75-cm^2 type I collagen-coated flasks were employed for protein expression, real-time PCR studies, and nuclear protein extraction. All experiments were done using cultures that were 90% confluent.

Treatment

Cultured SH-SY5Y cells were incubated with the specified doses of Dex for 24 h for dose-response experiments, or with 100 nM Dex for periods of 24 and 48 h. Steady-state mRNA levels and protein levels were assessed after 24 h of incubation with the specified doses of Dex for dose-response experiments or for 24 and 48 h with a dose of 100 nM. Cells incubated without Dex for the same times were used as a control. For GR translocation study, cells were treated with Dex (100 nM) for 6 h. For promoter studies, cells were incubated with Dex for 6 or 24 h prior to collection. RU486 was added to cell cultures 1 h before incubation with Dex or with medium, and was maintained for the incubation period.

Western blot

Western blot analyses were performed as described elsewhere (Zhang et al., 2003). Protein concentration in the samples was determined by Bio-Rad Protein Concentration Reagent. Equal amounts of total protein (20 μg per lane) were resolved in 10% SDS polyacrylamide gels and blotted onto PVDF membranes for immunoblotting analysis. Protein expression was detected using a 1:500 dilution of mouse anti-p11 monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA) and a 1:1000 dilution of horseradish peroxidase–conjugated goat anti-mouse IgG as a secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA). The density values are presented as means±S.D. from three experiments. The density was used to quantify immunoreactivity in terms of percentage of p11 induction relative to control (non-stressed rats or non-Dex-treated cells).

Immunostaining protocols

To evaluate GR translocation, the effect of Dex on nuclear GR was examined by immunostaining. Immunolabeling of GR was performed in Dulbecco's PBS (Quality Biologicals, Gaithersburg,

MD, USA) supplemented with 1 mg/ml BSA (PBS/BSA). Unless stated otherwise, PBS/BSA was also used as a diluent for the preparation of working stocks of all primary and secondary antibodies. All immunoreactions were performed at room temperature. Cells were fixed in 4% paraformaldehyde for 30 min and 75% alcohol for 10 min and washed twice with PBS for 15 min, then immunoreacted with a specific rabbit polyclonal anti-GR antibody and the appropriate secondary anti-rabbit IgG using the manufacturer's recommended procedure. Cells were also stained with 10 μg/ml 4′-6-diamidino-2-phenylindol (DAPI) (Sigma, St. Louis, MO, USA) for 10 min to reveal cell nuclei and then analyzed by fluorescence microscopy.

Astrocyte cultures

Astrocytes were isolated from the cerebral cortex of postnatal day 3 Sprague–Dawley rats. Briefly, the cortices were removed, washed in PBS, and placed in L-15 medium (GIBCO, Gaithersburg, MD, USA) with 50 μg/ml gentamicin (GIBCO). Tissues were transferred to 15-ml tubes and triturated by mechanical dissociation with a 10-ml pipette. Tissues were passed through 19G (3×), 22G (3×) and 25G (1×) needles. Cells in medium (DMEM, 10% FCS, and 50 μg/ml gentamicin) were thoroughly triturated. Cells from two brains were cultured in 75-cm^2 flasks pretreated with poly-D-lysine (30–70 K, Sigma, St. Louis, MO, USA) and maintained at 37 °C in a humidified, 5% CO_2 (balanced air) incubator. Cell culture medium was changed after 72 h and twice weekly thereafter until the monolayer was confluent. When the monolayer appeared confluent (7–10 days), the covers of the flasks were tightened and the flasks placed overnight on a rotary shaker at 180 rev./min at 37 °C. After 18 h the medium containing microglia and other loosely adherent cells and debris was rapidly removed. Cultures were replated with DMEM. After 3 days, astrocytes were subcultured on poly-D-lysine-coated Petri dishes (35-mm) for an additional 3 days. The sub-culture plating density was 8×10^5 cells/cm^2. The cultures consistently generated >98% astrocytes, which were identified by polyclonal GFAP or S-100β antibodies. Then, cells were harvested for real time PCR assay.

Neuronal cell cultures

Cortical neurons were prepared and pooled from E17 Sprague–Dawley embryos cortices Dissociated cells were plated on poly-L-lysine-coated plastic dishes in minimal essential medium con-

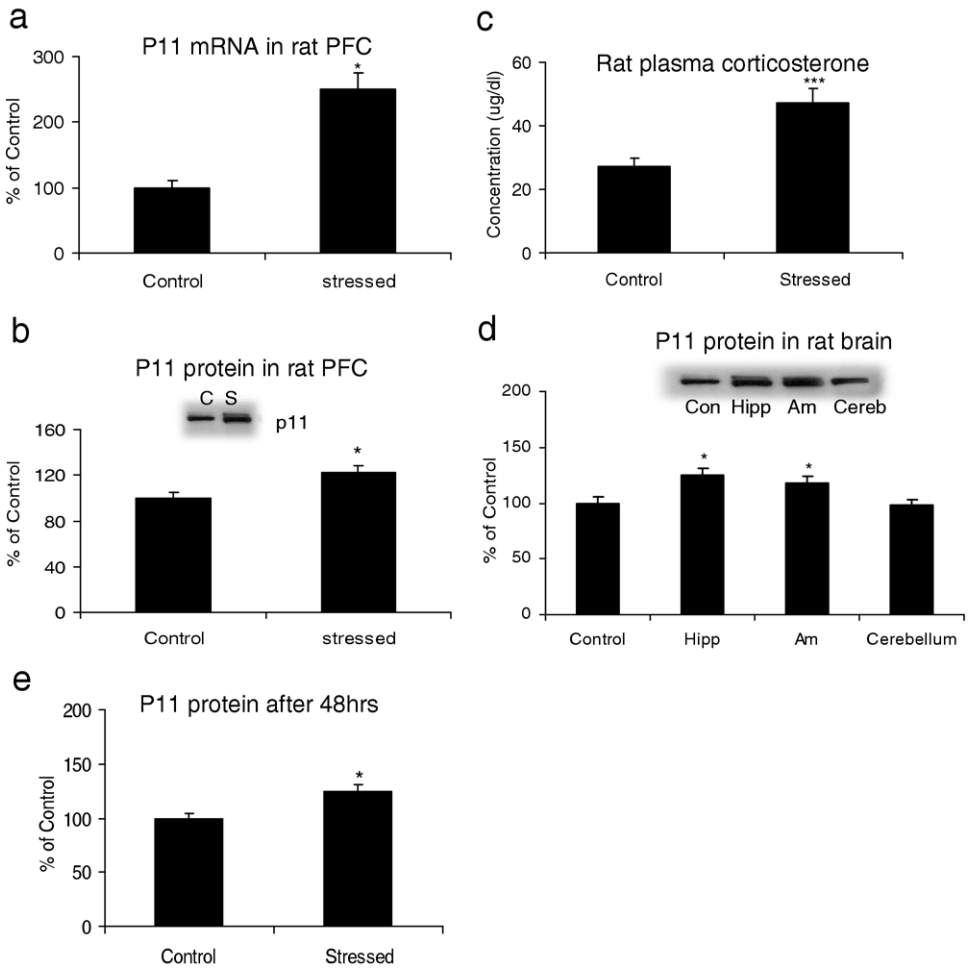

Fig. 1. Stress induces p11 up-regulation in the PFC in rats at mRNA and protein levels and elevates plasma corticosterone levels. (a) Real-time PCR analysis shows that p11 mRNA levels increase about 2.5-fold on average in the stressed group. (b) Dot slot experiments reveal that p11 protein levels in the stressed PFC increase about 30% over control, consistent with the result of real-time PCR results and indicating that stress increases p11 expression at both transcriptional and translational levels. (c) Stress increases plasma corticosterone levels in rats about 100%, as demonstrated by enzyme immunoassay. (d) p11 mRNA up-regulation also occurs in the hippocampus (Hipp) and amygdala (Am), but not in the cerebellum of stressed rats. (e) p11 Protein level in the PFC of stressed rats is significantly higher at 48 h after shock treatment compared with control. The data were analyzed by Student's *t*-test; * $P<0.05$, *** $P<0.001$.

taining 10% horse serum, 600 mg/l glucose and antibiotics. The number of cells corresponding to one cortex was plated onto one dish, resulting in a cell density of about 10,000 cells/cm^2. After 6 h, the medium was changed to Neurobasal medium containing 2% B27 supplement (Invitrogen). Cytosine arabinoside (5 mM) was added after 24 h. Cells were harvested for real time PCR assay.

Transient transfection of reporter gene plasmids

pCAT-basic vectors (Promega) containing the p11 promoter from −1499 to +89 were kind gifts from Dr. James H. Shelhamer (Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, MD, USA). Plasmids were recovered from bacterial cultures using a MaxiPrep kit (Qiagen, Valencia, CA, USA). SH-SY5Y cells were transfected with 1 μg of chloramphenicol acetyltransferase (CAT) basic vector (Clontech, Palo Alto, CA, USA) using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol. After 16 h, the medium was changed and cells were exposed to Dex, RU486, Dex+RU486 or given only medium for 6 h. Cells were washed three times with cold PBS and lysed for 30 min at room temperature. Cell lysates were collected and stored at −70 °C. CAT expressions were determined in cell lysates using the colorimetric enzyme immunoassay kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). These results were normalized to the total protein in the sample. Measurements of CAT were done in duplicate according to the manufacturer's protocol. The data are expressed as percent of control: (promoterless pCAT–basic activity or promoter–CAT activity). Transfected cells were exposed to Dex or media for 6 h. Collection of cells and CAT and total protein assays were done as described above.

Nuclear protein isolation from SH-SY5Y cells

Cells were grown in 75-cm^2 type I collagen-coated flasks to 90% confluence and were incubated with 100 nM Dex for periods of 6–24 h. The culture medium was removed and cells were washed three times with ice-cold PBS, harvested by scraping into 4 ml PBS, and centrifuged (500×g, 5 min). The pellet was dispersed in five packed cell volumes of hypo-osmotic buffer (10 mM Hepes–KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin, each 2 mg/ml). After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6% (v/v), and the nuclei were pelleted by centrifugation (5000×g, 5 min). The pelleted nuclei were dispersed in a high salt buffer (20 mM Hepes–KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin, each 2 mg/ml) to solubilize DNA-binding proteins. The suspended nuclei were gently shaken horizontally for 30 min at 4 °C and centrifuged in a microcentrifuge (12,000×g, 20 min). The supernatants containing nuclear proteins were stored at −70 °C until used for immunoprecipitation (IP). Protein concentrations were determined using a BCA assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

ChIP assays

The procedure was performed using a kit purchased from Upstate Biotechnology, according to the manufacturer's protocol. Briefly, cells were incubated with formaldehyde at a final concentration of 1% at 37 °C for 10 min. Cells were washed twice with ice-cold PBS, collected by centrifugation at 4 °C and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% SDS and protease inhibitors). Cell lysates were sonicated to give a DNA size range from 300 to 600 bp, and supernatants were diluted with dilution buffer (16.7 mM Tris–HCl, pH 8.0, 1% Triton X-100, 1.2 mm EDTA, 167 mM NaCl, 0.01% SDS, and protease inhibitors). The solutions were pre-cleared with salmon sperm DNA/protein G agarose slurry and then treated with specific antibody directed against the glucocorticoid receptor (5 μg) (ABR, Golden, CO, USA) overnight at 4 °C. Immune complexes were collected by adding a salmon sperm DNA/protein G agarose slurry. The beads were washed sequentially in the following buffer: low salt wash buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100); high salt wash buffer (20 mM Tris–HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100); LiCl wash buffer (10 mM Tris–HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet p-40, 1% deoxycholate, and 1 mM EDTA), and Tris–EDTA buffer. Immuno-complexes were extracted from the beads with 1% SDS and 0.1 M $NaHCO_3$. Cross-linking was reversed by heating the eluate at 65 °C for 4 h. The eluate was then digested with proteinase K at 45 °C for 1 h, and then further subjected to the phenol/chloroform extraction. The DNA was purified by ethanol precipitation. The human p11 promoter was amplified by PCR with the appropriate primers (forward: 5′-CAG ACA GAG CGT TCT TG-3′ and reverse: 5′-CTC CTA GGG CTA ATC TG-3′).

Human postmortem tissue and RNA extraction

Area 46 of the PFC was carefully dissected from six PTSD patients and six appropriately-matched controls. RNA was obtained from the Stanley Medical Institute. All available records, including inpatient and outpatient clinical records, were meticulously reviewed for every subject. Each reference in the chart was catalogued. Diagnoses were retrospectively established by two psychiatrists using DSM-IV criteria. The cause of death for all PTSD patients and non-PTSD controls was recorded in this study. Four patients with PTSD had depression. Two PTSD patients died from

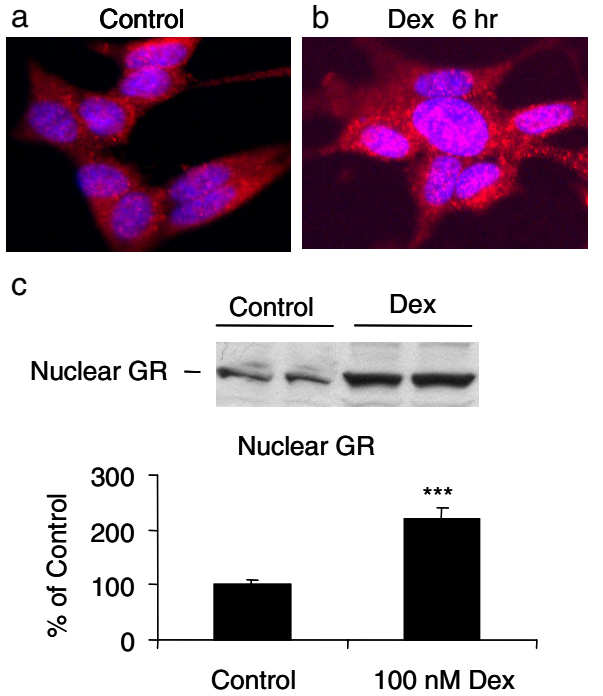

Fig. 2. Dex induces GR nuclear translocation. SH-SY5Y cells were treated with 100 nM Dex for 6 h. Images of the intracellular distributions of GR were obtained using fluorescence microscopy. (a) Under control conditions, no significant nuclear translocation occurs in the absence of Dex, suggesting that the majority of the GRs are cytoplasmic, as expected. (b) Six hours after treatment with 100 nM Dex, GR (red) nuclear (blue) translocation is significantly increased. (c) Dex at 100 nM (6 h) increases expression of GR in cell nucleus, as determined by Western blot assay. *** $P<0.001$.

suicide and four died from cardiac diseases in the PTSD group. All of the subjects in the non-PTSD control group ($n=6$) died from cardiac diseases. The medications, which were used by the patients with PTSD varied. They included carbamazepine, doxepin, risperidone, quetiapine, paroxetine, venlafaxine, olanzapine and fluoxetine. The tissue blocks dissected from the area 46 of the cortex contained primarily gray matter and a small, but presumably random, amount of white matter. The total RNA extracted from these blocks was used for the study.

Statistical analysis

GraphPad 2.0 software was used for analyzing data. Comparisons of the means between groups were performed by using the Student's *t*-test. Statistical significance was set at alpha=0.05. A positive test value generated between two means is indicative of a significant difference.

Animals

Experiments were carried out on PFC tissue obtained from stressed ($n=5$) and control ($n=5$) Sprague–Dawley 28-day male rats (Taconic Farms, Germantown, NY, USA). All animal procedures were performed in compliance with the Animal Welfare Act and Use of Laboratory Animals. The study was approved by the USUHS Animal Care and Use Committee.

RESULTS

As shown in Fig. 1, p11 mRNA expression in the PFC of the stressed rat group was increased about 2.5-fold on average (Fig. 1a), while p11 protein levels were elevated 30% relative to control group P11 levels (Fig. 1b). Levels of p11 mRNA were also significantly higher in the hippocampus and amygdala, but not in the cerebellum of the stressed rats compared with the control groups (Fig. 1d). The stress-induced p11 protein overexpression was also observed at 48 h after inescapable tail-shock treatment (Fig. 1e).

We examined the plasma level of corticosterone, a specific glucocorticoid metabolite in rats (Ottenweller et al., 1992; Cohen et al., 2003) by enzyme immunoassay. A 3-day inescapable tail shock stress doubled plasma levels of corticosterone compared with the control, as demonstrated by enzyme immunoassay (Fig. 1c).

Fig. 2a and b shows the expression of GR protein in the cell nucleus in the presence and absence of 100 nM Dex. The GR immunoreactivity in the cell nucleus increased following treatment with 100 nM Dex (Fig. 2b). Dex exposure resulted in higher nuclear GR levels, indicating that Dex induces GR nuclear translocation in SH-SY5Y cells (Fig. 2c).

Fig. 3 shows that 10–200 nM Dex treatment increased the steady-state level of p11 mRNA over 24 h in a dose-dependent manner (Fig. 3a). In addition, Dex treatment resulted in significant increases of p11 mRNA within 48 h (Fig. 3b). The effect of Dex on p11 protein was studied by Western blot in SH-SY5Y cells (Fig. 3c and d). Treatment of cells with Dex for 24 h resulted in a dose-related increase in cellular p11 protein levels (Fig. 3c). Cells treated with 100 nM Dex for 24–48 h expressed significantly ele-

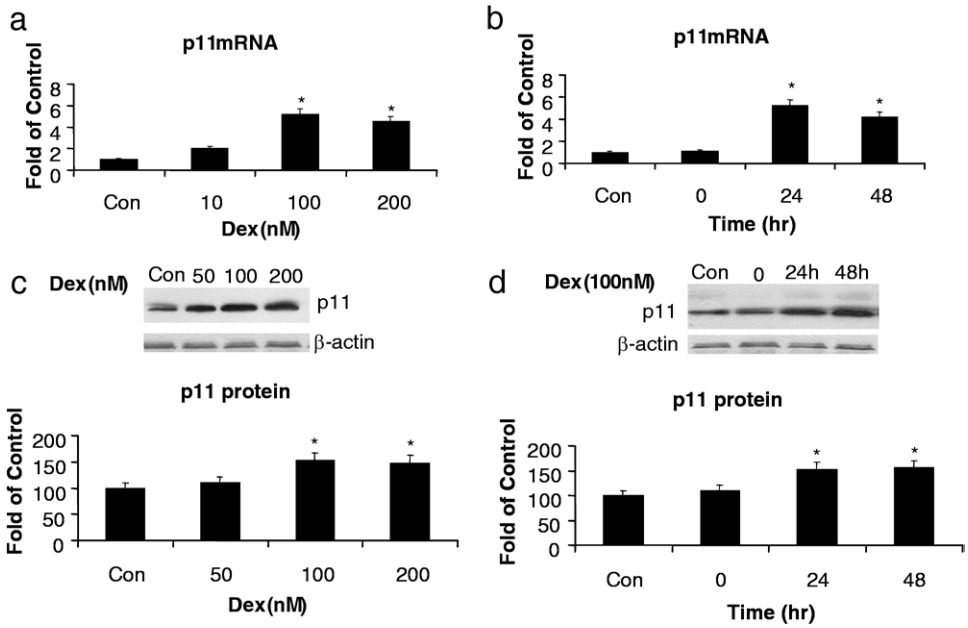

Fig. 3. Dex persistently increases p11 mRNA and p11 protein levels in a dose-dependent manner. Real-time PCR and Western blot assays were used to quantify p11 mRNA and protein levels in SH-SY5Y cells. (a) SH-SY5Y cells were grown to near confluence and treated with Dex (10–200 nM) for 0–24 h before RNA was extracted. Real-time PCR reveals a dose-dependent increase in mRNA levels with Dex treatments. (b) Cells were treated with 100 nM Dex for 0, 24 and 48 h, then p11 mRNA was quantified and examined as in panel a. Dex increases p11 transcript levels at 24 h, which are sustained for an additional 24 h. (c) Cells were grown to near confluence and then treated with Dex (0–100 nM) for 24 h, after which cell lysates were prepared from treated and untreated cells and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting. Dex increases protein levels in a dose-dependent manner. (d) Cells were treated with Dex (100 nM) for 24–48 h and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting. Dex increases p11 protein levels at 24 h, which are sustained for 48 h. Blot results shown are representative of three separate experiments. * $P<0.05$ (treatment vs. control). Con, control.

vated levels of p11 (Fig. 3d). These data suggest that Dex increased p11 expression in dose-dependent manner at the translational level.

Fig. 4a shows that basal P11 mRNA levels differ between astrocytes, being significantly higher in astrocytes. RU486 blocked the Dex-induced increase in p11 expression in SH-SY5Y cells, while RU486 alone produced no effect on p11 expression (Fig. 4b). This result implicates GR in the regulation of p11 levels by Dex.

To determine the time course of GR recruitment to the p11 promoter, a transfection study was conducted by using a reporter gene containing the p11 promoter from −1499 to +89 (Dr. J.H. Shelhamer, National Institutes of Health, Bethesda, MD, USA). SH-SY5Y cells transfected with the p11 promoter or basic vector were exposed to 100 nM Dex for 0–24 h (Fig. 5a), 0–100 nM Dex for 6 h (Fig. 5b), and RU486, RU486+Dex or Dex alone for 6 h (Fig. 5c). The increase in transcriptional activity was significantly higher in cells transfected with the p11 promoter pCAT than in control cells transfected with promoterless pCAT-basic vector (Fig. 5b). Furthermore, Dex treatment doubled CAT activation in the transfected cells. These effects were abolished at the GR level by the GR antagonist, RU486 (Fig. 5c). Our results thus indicate that Dex-induced p11 up-regulated expression involves GR binding sites on the p11 promoter. We identified three possible GRE sites (−223 to −241, −354 to −372, and −427 to −445) in the p11 promoter region (Fig. 5d). The consensus sequence of GRE is GGTACAnnnTGTTCT (Table 1).

To determine which one(s) of GRE sequences within the p11 promoter was responsive to Dex, SH-SY5Y cells were transiently transfected with three mutant p11 promoter CAT constructs (mut) (Fig. 5d). Cells transfected with mut constructs (mutGRE2 and mutGRE3) remarkably attenuated glucocorticoid-induced p11 promoter activity but mutGRE1 did not (Fig. 5d).

We quantified p11 mRNA levels in the postmortem PFC (area 46) of patients with PTSD (Fig. 6a) and in controls by real-time PCR (Fig. 6b). As shown in Fig. 6b, the p11 mRNA levels in the PFC (area 46) were significantly higher ($P<0.05$) in patients with PTSD (2.51±0.51) compared with the control group (1.14±0.29).

DISCUSSION

We examined the effect of 3 days of inescapable tail-shock p11 expression in rats and demonstrated that whether the rats were treated with a tail shock paradigm and killed immediately or 2 days later, a significant increase in p11 expression was observed in the PFC (Fig. 1). These data support the notion that traumatic stress exposure can result in p11 up-regulation at least 48 h. Additional time points will be necessary to examine these findings and their duration or return to base line. We also found that 3-day tail shock resulted in p11 up-regulation in the hippocampus and the amygdala, but not in the cerebellum, consistent with data demonstrating that these brain areas play critical roles in the response to traumatic stress in both human and rodents.

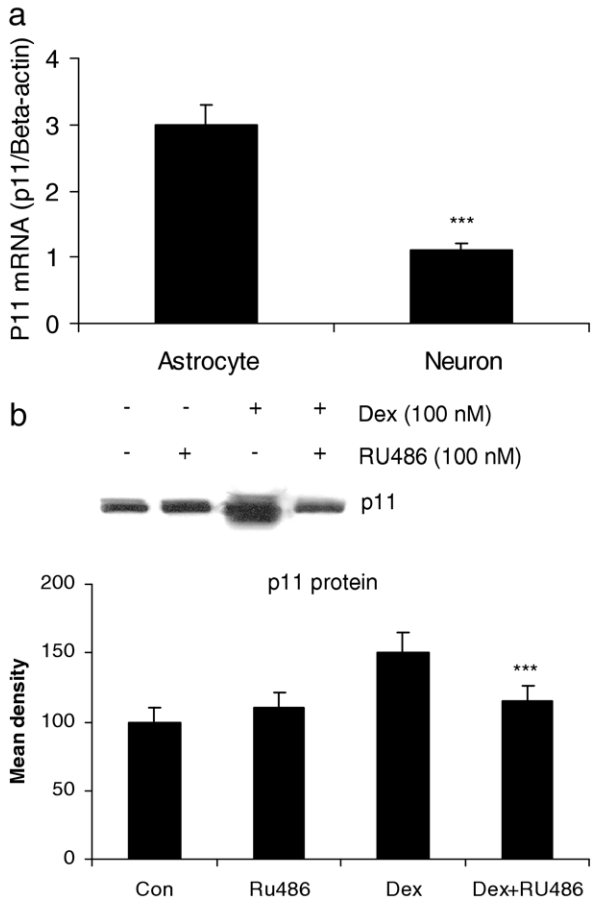

Fig. 4. Expression of p11 mRNA occurs in both astrocytes and neurons, and the GR antagonist RU486 blocks Dex-induced increases in p11 protein levels in SH-SY5Y cells. (a) Real-time PCR assays were used to quantify basal levels of p11 mRNA in cultured rat astrocytes and neurons. Basal p11 mRNA levels are significantly higher in astrocytes than in neurons. *** $P<0.001$ (astrocytes vs. neuron; $n=5$). (b) The GR antagonist RU486 blocks Dex-induced increases in p11 protein levels. Control SH-SY5Y cells received no drug treatment (Con), while others were exposed to either 100 nM RU486 or 100 nM Dex for 25 h (Dex) or RU486 together with Dex for 25 h (Dex+RU486). p11 Protein levels were determined by Western blot assay. RU486 blocks the Dex-induced increase in p11 protein levels, while having no effects on basal levels. *** $P<0.001$ (Dex vs. Dex +RU486) analyzed by the Student's *t*-test.

Several lines of evidence point to changes in HPA activity of PTSD patients and stressed animals (Yehuda et al., 1995b). For example, circulating cortisol and GR expression is changed in PTSD patients (Yehuda et al., 1995b; Lindauer et al., 2006). Under stress conditions, animals commonly exhibit an immediate burst of sympathetic nervous system activity (Resnick et al., 1995; Elzinga et al., 2003; Rasmusson et al., 2003; Lindauer et al., 2006). In addition, stress often results in a transient elevation of circulating cortisol or corticosterone as a part of the physiological or normal stress response. However, traumatic or severe stress appears to trigger higher and more sustained cortisol or corticosterone responses (Yehuda et al., 1995a) which result in pathological conditions. Our data suggest that our animal model mimicked a trau-

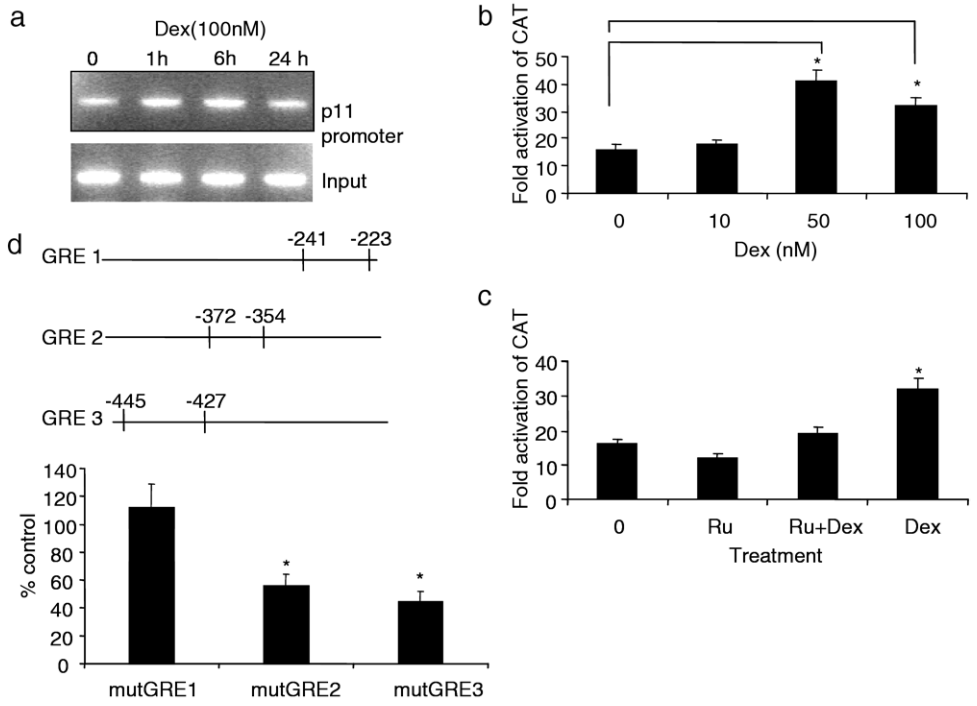

Fig. 5. Effect of Dex on GR recruitment to the p11 promoter and p11 promoter activity. (a) GRE recruitment was determined by the ChIP assay in SH-SY5Y cells, which were incubated with 100 nM Dex for 0, 1, 6 and 24 h. (b) Dex increases p11 promoter activity in transiently transfected cells with pCAT-basic vectors containing the promoter from nucleotide −1499 to +89 in a dose-dependent manner. (c) RU486 blocks Dex-induced promoter activity and marginally decreases basal promoter activity. (d) Mutational analysis of GREs in the p11 promoter reveals that GRE2 and GRE3 are the two sites in the promoter that promote glucocorticoid-activated GR up-regulation of p11, since mutation at these sites, but not at GRE1, attenuates glucocorticoid-activated GR up-regulation. Bars are means±S.D. * Significantly different from similar condition without Dex. Data are analyzed by the Student's *t*-test.

matic event resulting in an increase of plasma corticosterone levels and p11 expression. Consistent with previous reports showing that the synthetic glucocorticoid Dex up-regulates p11, our data indicate that glucocorticoids play a major role in controlling p11 expression.

We examined how glucocorticoid regulates p11 expression using the human neuroblastoma cell line SH-SY5Y and Dex. Previous studies indicate that glucocorticoid-activated GRs translocate to the nucleus, homodimerize, and bind to GREs in target gene promoters, resulting in either gene activation or repression (Chen et al., 2006a; Rogatsky and Ivashkiv, 2006). As expected, we found that exposure to Dex resulted in significant increases in both GR immunoreactivity (Fig. 2a) and GR protein levels in the nucleus, indicating that Dex induces GR nuclear translocation (Fig. 2c).

We examined Dex-induced regulation of p11 transcription in SH-SY5Y cells (Fig. 3) to determine the consequence of GR translocation. We found that Dex treatment

Table 1. Wild type and mutated sequences of the three GREs in the p11 promoter region

Oligonucleotide	Sequence[a]	Strand[b]	Nucleotide No.[c]
GRE[d]	GGTACA nnn TGTTCT		Webster, 1999
wt GRE[e]	tcTGTACA GGA TGTTCTag		Santa Cruz Biotechnology
GRE1	tcAGTAGA AAC GCACGTgc		−223–241
MutGRE1	tcAGTAGA AAC G**GT**C**C**Tgc	(+)	
GRE2	gcAAATGC AGA GGTAACcg		−354 to −372
MutGRE2	cgGTTACC AGA GGT**TCT**gc	(−)	
GRE3	gtGGCACG TGG ACACTTat		−427 to −445
MutGRE3	gtGGCACG TGG AG**TC**C**C**Tat	(+)	

[a] 5′→3′, Sense strand; n is any nucleotide. GRE sequence sites in the 15 bp palindrome are capitalized; flanking sequences are in lowercase; and mut bases are in bold type.

[b] (+) Refers to the sense strand and (−) to the complementary strand of oligonucleotide.

[c] Numbers refer to the nt position of putative GRE sites identified in the promoter by MatInspector (Genomatix Software GmbH, Germany). Flanking nucleotides are from the p11 promoter sequence (Huang et al., 2003).

[d] Consensus sequence for GRE elements (Chen et al., 2006b).

[e] Consensus wild type (wt) GRE sequence, Santa Cruz Biotechnology.

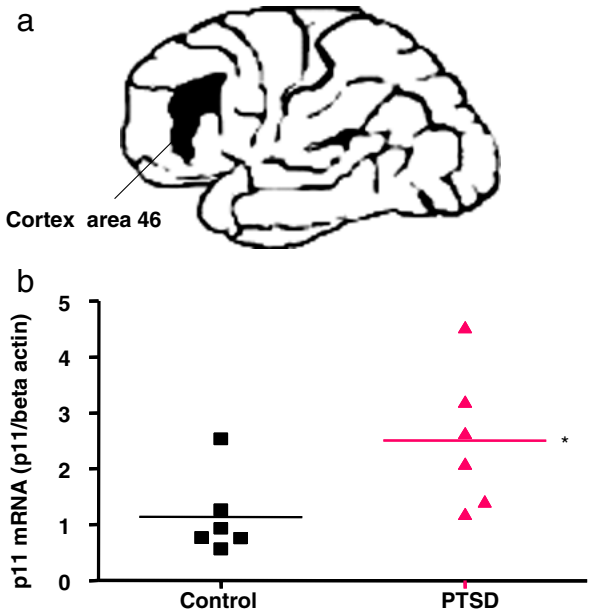

Fig. 6. p11 mRNA expression in postmortem cortex is significantly increased in patients with PTSD compared with age- and sex-matched controls. (a) A perspective of the human brain to locate area 46 in the cortex. (b) All tissue blocks have the same (output) weight. cDNA was generated from 5 mg of total RNA for each sample to exclude that differences in RNA-content could result also from differences in sample weights. p11 mRNA is significantly greater in PTSD patients at postmortem compared controls (n=6 per group, two cases died of suicide and four died of other causes in PTSD group). Data are shown as means±S.E.M., * P<0.05 (control vs. PTSD) and have been analyzed by the Student's t-test.

increased the steady-state level of p11 mRNA over 24 h in a dose-dependent manner (Fig. 3a) that could be observed as long as 48 h after Dex treatment (Fig. 3b). Dex also resulted in significant increases in the levels of p11 protein (Fig. 3c and d), indicating that Dex increased p11 expression at the translational level. The GR antagonist RU486 attenuated Dex-induced overexpression of p11 (Fig. 4b), demonstrating that Dex-induced increases in p11 expression at the transcriptional and translational levels are mediated by GR. In addition, we found that p11 was expressed in both astrocytes and neurons with basal levels of p11 mRNA being significantly higher in astrocytes compared with neurons (Fig. 4a). Although a detailed molecular mechanism needs to be explored for the differences in p11 expression between astrocytes and neurons, the data suggest that p11 expression in astrocytes is distinct from that in neurons.

To determine the role of GR in regulating p11 expression and whether GR bound directly to the p11 promoter, we conducted a ChIP study. The time course of GR ChIP demonstrated that GR was recruited to the p11 promoter over a 1–6 h period after Dex treatment and then declined at 24 h (Fig. 5a). GR appears to directly mediate the induction of transcription induced by glucocorticoid stimulation through GR response elements (GRE) in the p11 promoter.

To determine the function of GR binding to GREs of the p11 promoter, we measured p11 promoter activity. We found a remarkable increase in the transcriptional activity of cells transfected with the p11 promoter pCAT compared with control cells transfected with promoterless pCAT-basic vector (Fig. 5b). Dex treatment doubled CAT activation in the transfected cells. The Dex-induced increase in CAT activation was attenuated by the GR antagonist RU486 (Fig. 5c), confirming that Dex-induced p11 up-regulated expression involves GR binding sites on the p11 promoter.

Upon searching the p11 promoter sequence, we found three potential GREs in the p11 promoter (Fig. 5d). Previous studies have shown that in genes up-regulated by glucocorticoid, the 3′ half of the GRE palindrome is typically well conserved, while the 5′ half exhibits more divergence (Chen et al., 2006b). Glucocorticoid-activated GR binds to GREs in target genes to up- or down-regulate their expression, as reported for at least 20 mammalian genes expressed in multiple organ systems (Chameau et al., 2007). To determine which of the three GRE sequences were responsive to Dex, three mut were used (Fig. 5d). SH-SY5Y cells transfected with mutant constructs (mutGRE2 and mutGRE3) attenuated glucocorticoid-induced p11 promoter activity but mutGRE1 did not (Fig. 5d). The results indicate that the GR increases p11 promoter activity via the interaction of glucocorticoid-bound GR with GRE 2 and GRE3, but not GRE1 (Fig. 5d). A recent study reported that changes in p11 expression were associated with depression (Svenningsson et al., 2006), a frequent comorbidity symptom of PTSD (Oquendo et al., 2005; Sareen et al., 2007). Indeed, in some studies, nearly half of the veterans who had PTSD are also depressed (Kramer et al., 1994). We found that, unlike the previous report showing p11 down-regulation in the frontal cortex of patients with depression (Svenningsson et al., 2006), the expression of p11 mRNA was up-regulated in the postmortem PFC (area 46) of PTSD patients (Fig. 6). In spite of the fact that PTSD and depression share some common symptoms, these data support the perspective that they are different disorders and may have different underlying mechanisms. The difference in p11 expression in these two disorders may be related to differences in their pathogeneses. Replications with larger sample sizes are warranted to confirm and extend the pathogenetic implications of our results. In summary, the molecular mechanism underlying p11 up-regulation after traumatic stress in rats and Dex-treated cells as well as in PTSD patients may lead to a new understanding of stress-regulated p11 expression.

Acknowledgments—We thank Dr. J. H. Shelhamer of National Institutes of Health, Bethesda, MD, USA, for his kindly p11 and p11 promoter plasmids, and Eleanor Gamble and Xiaolong Jong for excellent technical support. The manuscript was improved following comments from Bruce McEwen.

Traumatic Stress Brain Study Group: Dr. David Benedek, Department of Psychiatry and Center for the Study of Traumatic Stress, Uniformed Services University of the Health Sciences, School of Medicine; Dr. Ronald Duman, Abraham Ribicoff Research Facilities Laboratory of Molecular Psychiatry, Yale University School of Medicine; Dr. Matthew Friedman, National Center for PTSD US Department of Veterans Affairs and Dartmouth Medical School; Dr. Harry Holloway, Department of Psychiatry and Center for the Study of Traumatic Stress, Uniformed Ser-

vices University, School of Medicine; Dr. Joel Kleinman, Clinical Brain Disorders Branch, Genes, Cognition and Psychosis Program, IRP, NIMH, NIH; Dr. John Krystal, Clinical Neuroscience Division, VA National Center for PTSD, VA Connecticut Health care System, West Haven, CT and Department of Psychiatry, Yale University School of Medicine; Dr. Gregory Leskin, National Center for PTSD, VA Palo Alto Health Care System; Dr. James Meyerhoff, Georgetown University School of Medicine and Division of Psychiatry and Neurosciences, Walter Reed Army Institute of Research; Dr. Elizabeth Osuch, Department of Psychiatry, University of Western Ontario, Schulich School of Medicine and Dentistry.

REFERENCES

Adamec R, Strasser K, Blundell J, Burton P, McKay DW (2006) Protein synthesis and the mechanisms of lasting change in anxiety induced by severe stress. Behav Brain Res 167:270–286.

Chameau P, Qin Y, Spijker S, Smit G, Joels M (2007) Glucocorticoids specifically enhance L-type calcium current amplitude and affect calcium channel subunit expression in the mouse hippocampus. J Neurophysiol 97:5–14.

Chen X, Oppenheim JJ, Winkler-Pickett RT, Ortaldo JR, Howard OM (2006a) Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3(+)CD4(+)CD25(+) T regulatory cells in vivo and enhances their capacity to suppress EAE. Eur J Immunol 36:2139–2149.

Chen Y, Nickola TJ, DiFronzo NL, Colberg-Poley AM, Rose MC (2006b) Dexamethasone-mediated repression of MUC5AC gene expression in human lung epithelial cells. Am J Respir Cell Mol Biol 34:338–347.

Cohen H, Zohar J, Matar M (2003) The relevance of differential response to trauma in an animal model of posttraumatic stress disorder. Biol Psychiatry 53:463–473.

de Quervain DJ, Roozendaal B, McGaugh JL (1998) Stress and glucocorticoids impair retrieval of long-term spatial memory. Nature 394:787–790.

Elzinga BM, Schmahl CG, Vermetten E, van Dyck R, Bremner JD (2003) Higher cortisol levels following exposure to traumatic reminders in abuse-related PTSD. Neuropsychopharmacology 28:1656–1665.

Grieger TA, Fullerton CS, Ursano RJ (2004) Posttraumatic stress disorder, depression, and perceived safety 13 months after September 11. Psychiatr Serv 55:1061–1063.

Hoge CW, Castro CA, Messer SC, McGurk D, Cotting DI, Koffman RL (2004) Combat duty in Iraq and Afghanistan, mental health problems, and barriers to care. N Engl J Med 351:13–22.

Howard S, Hopwood M (2003) Post-traumatic stress disorder. A brief overview. Aust Fam Physician 32:683–687.

Huang X, Pawliczak R, Yao XL, Madara P, Alsaaty S, Shelhamer JH, Cowan MJ (2003) Characterization of the human p11 promoter sequence. Gene 310:133–142.

Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CB (1995) Posttraumatic stress disorder in the National Comorbidity Survey. Arch Gen Psychiatry 52:1048–1060.

Kramer TL, Lindy JD, Green BL, Grace MC, Leonard AC (1994) The comorbidity of post-traumatic stress disorder and suicidality in Vietnam veterans. Suicide Life Threat Behav 24:58–67.

Liberzon I, Young EA (1997) Effects of stress and glucocorticoids on CNS oxytocin receptor binding. Psychoneuroendocrinology 22:411–422.

Lindauer RJ, Olff M, van Meijel EP, Carlier IV, Gersons BP (2006) Cortisol, learning, memory, and attention in relation to smaller hippocampal volume in police officers with posttraumatic stress disorder. Biol Psychiatry 59:171–177.

Meaney MJ, Diorio J, Francis D, Weaver S, Yau J, Chapman K, Seckl JR (2000) Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: the effects of thyroid hormones and serotonin. J Neurosci 20:3926–3935.

Oquendo M, Brent DA, Birmaher B, Greenhill L, Kolko D, Stanley B, Zelazny J, Burke AK, Firinciogullari S, Ellis SP, Mann JJ (2005) Posttraumatic stress disorder comorbid with major depression: factors mediating the association with suicidal behavior. Am J Psychiatry 162:560–566.

Ottenweller JE, Servatius RJ, Tapp WN, Drastal SD, Bergen MT, Natelson BH (1992) A chronic stress state in rats: effects of repeated stress on basal corticosterone and behavior. Physiol Behav 51:689–698.

Pfeffer CR, Altemus M, Heo M, Jiang H (2006) Salivary cortisol and psychopathology in children bereaved by the September 11, 2001 terror attacks. Biol Psychiatry 61:957–965.

Rasmusson AM, Vythilingam M, Morgan CA 3rd (2003) The neuroendocrinology of posttraumatic stress disorder: new directions. CNS Spectr 8:651–656, 665–667.

Resnick HS, Yehuda R, Pitman RK, Foy DW (1995) Effect of previous trauma on acute plasma cortisol level following rape. Am J Psychiatry 152:1675–1677.

Rogatsky I, Ivashkiv LB (2006) Glucocorticoid modulation of cytokine signaling. Tissue Antigens 68:1–12.

Roseboom PH, Nanda SA, Bakshi VP, Trentani A, Newman SM, Kalin NH (2006) Predator threat induces behavioral inhibition, pituitary-adrenal activation and changes in amygdala CRF-binding protein gene expression. Psychoneuroendocrinology 32:44–55.

Sareen J, Cox BJ, Stein MB, Afifi TO, Fleet C, Asmundson GJ (2007) Physical and mental comorbidity, disability, and suicidal behavior associated with posttraumatic stress disorder in a large community sample. Psychosom Med 69:242–248.

Svenningsson P, Chergui K, Rachleff I, Flajolet M, Zhang X, El Yacoubi M, Vaugeois JM, Nomikos GG, Greengard P (2006) Alterations in 5-HT1B receptor function by p11 in depression-like states. Science 311:77–80.

Ursano RJ (2002) Post-traumatic stress disorder. N Engl J Med 346:130–132.

Vogel WH, Jensh R (1988) Chronic stress and plasma catecholamine and corticosterone levels in male rats. Neurosci Lett 87:183–188.

Yao XL, Cowan MJ, Gladwin MT, Lawrence MM, Angus CW, Shelhamer JH (1999) Dexamethasone alters arachidonate release from human epithelial cells by induction of p11 protein synthesis and inhibition of phospholipase A2 activity. J Biol Chem 274:17202–17208.

Yau JL, Noble J, Seckl JR (2001) Acute restraint stress increases 5-HT7 receptor mRNA expression in the rat hippocampus. Neurosci Lett 309:141–144.

Yehuda R, Boisoneau D, Lowy MT, Giller EL Jr (1995a) Dose-response changes in plasma cortisol and lymphocyte glucocorticoid receptors following dexamethasone administration in combat veterans with and without posttraumatic stress disorder. Arch Gen Psychiatry 52:583–593.

Yehuda R, Kahana B, Binder-Brynes K, Southwick SM, Mason JW, Giller EL (1995b) Low urinary cortisol excretion in Holocaust survivors with posttraumatic stress disorder. Am J Psychiatry 152: 982–986.

Zhang L, Li PP, Feng X, Barker JL, Smith SV, Rubinow DR (2003) Sex-related differences in neuronal cell survival and signaling in rats. Neurosci Lett 337:65–68.

(Accepted 5 March 2008)
(Available online 21 March 2008)