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Tonic, but not phasic corticosterone constrains stress activated extracellular-regulated-kinase 1/2 immunoreactivity within the hypothalamic paraventricular nucleus

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Abstract

The negative feedback actions of corticosterone (CORT) depend on both phasic and tonic CORT secretion patterns to regulate hypothalamic pituitary adrenal (HPA) axis activity. How these two different CORT secretion patterns influence specific intracellular signal transduction pathway activity within the cellular elements of the HPA axis has not been determined. For example, it is unknown whether CORT has suppressive actions over signal transduction events within medial parvocellular paraventricular nucleus (PVN) corticotropin-releasing hormone (CRH) neurons and if these suppressive actions are responsible for alterations in PVN transcriptional processes and neurohormone secretion associated with stress. The extracellular-regulated kinase (ERK) is a stress activated intracellular signaling molecule that is potentially subject to glucocorticoid negative feedback regulation. We tested the ability of CORT to modulate levels of the active (phosphorylated) form of ERK (pERK1/2) in the PVN of rats. Acute psychological stress (restraint) produced a rapid increase in the number of PVN pERK1/2 immunopositive cells within CRH neurons. Absence of tonic CORT via adrenalectomy (ADX) produced no change in basal pERK1/2 cell counts, but augmented the increased pERK1/2 cell counts elicited by acute restraint. Treatment of ADX rats with CORT in the drinking water normalized this enhanced pERK1/2 response to stress. In contrast, treatment of ADX rats with a phasic increase in CORT 1 hour prior to restraint had no effect on pERK1/2 cell counts, despite substantially suppressing stress-induced PVN *crh* gene expression and ACTH secretion. This tonic CORT inhibition of stress-induced activation of ERK1/2 may involve both alteration of the activity of stress-dependent neural inputs to PVN CRH neurons and alteration within those neurons of stress-dependent intracellular signaling mechanisms associated with ERK activation.

Keywords

pERK1/2; CRH; ACTH; PVN

Introduction

Glucocorticoids potently suppress stress-induced hypothalamic-pituitary-adrenal (HPA) axis activity (negative feedback) as measured by ACTH secretion (1). Both phasic and tonic glucocorticoid secretion patterns contribute to the regulation of basal and stimulated HPA-axis activity (2, 3). Tonic glucocorticoid actions (sometimes referred to as permissive

actions) generally refer to effects from the long-term presence (hours to days) of basal circulating glucocorticoids. Tonic glucocorticoid actions produce a somewhat constant modulation over physiological function, such as HPA axis hormone secretion (3). In contrast, phasic glucocorticoid actions (sometimes referred to as suppressive and stimulating actions) can be attributed to the effects that stem from a rapid and transient increase in glucocorticoids elicited by an acute exposure to stress. Phasic glucocorticoid actions produce additional dynamic modulation of physiological function including HPA axis activity (3). Tonic glucocorticoid negative feedback effects have been studied by long-term removal of endogenous glucocorticoids (adrenalectomy) combined with various replacement regimens of exogenous glucocorticoids (4–6). Phasic glucocorticoid negative feedback effects have been studied by pretreating subjects with an exogenous dose of glucocorticoids prior to HPA axis stimulation (7–9). The underlying cellular and molecular mechanisms by which tonic and phasic glucocorticoids act at specific anatomical sites to regulate HPA axis activity remains largely undetermined. Glucocorticoid negative feedback within the hypothalamic element of the HPA axis suppresses both the coupling of cellular excitation with neurohormone secretion and gene induction (10, 11). Nevertheless, it is largely unknown whether tonic or phasic glucocorticoid activity inhibits specific stress-induced intracellular signal transduction events within CRH neurons.

Extracellular signaling-regulated kinase (ERK) is one member of a family of mitogen-activated protein kinases (MAPK), and it is a functionally versatile intracellular signaling molecule (12). For example, ERK activity is responsive to an array of intercellular stimuli (e.g. classic neurotransmitters, growth factors, cytokines and hormones), and as a result the active (phosphorylated) forms of the two isoforms of ERK (phospho-p44/phospho-p42 or pERK1/2) participate in regulation of a variety of cellular processes (e.g. cell proliferation, cell differentiation, cell death, as well as neuronal plasticity and neuroexcitability). Interestingly, pERK1/2 may be an important intracellular mediator of stress-induced HPA axis activity. For example, mice challenged with 30 minutes of restraint displayed an increase in PVN pERK1/2 cell counts (13), and others have found that intravenous infusion of rats with a physiological stressor (2-deoxy-D-glucose) produced an increase in pERK1/2 immunoreactivity that was present in PVN CRH neurons (14, 15). In some instances pERK1/2 can selectively activate specific transcription factors, such as Elk-1 (16). Also pERK1/2 can indirectly activate the transcription factor cAMP response element binding protein (CREB) through phosphorylation of other signaling intermediates such as ribosomal-s6-kinase (17). CREB is a primary transcription factor responsible for induction of PVN *crh* gene expression (18), therefore pERK1/2 may contribute to stress-induced PVN *crh* gene expression. pERK1/2 also plays a role in maintaining enhancement of neuronal excitability (19), consequently pERK1/2 may be a mediator of stress-induced alterations in CRH neuronal excitability and subsequent neurohormone secretion. These examples of pERK1/2 cellular function overlap with cellular processes believed to be altered by glucocorticoid negative feedback, and therefore make pERK1/2 a candidate target for glucocorticoid regulation within CRH neurons.

In this study we characterized both basal and stimulated PVN pERK1/2 immunoreactivity in response to tonic and phasic glucocorticoid manipulations. We first, determined if pERK1/2 levels can be rapidly increased in rat PVN CRH neurons by an acute exposure to a psychological stressor (restraint). We then examined whether tonic changes in corticosterone (CORT) exposure may influence PVN pERK1/2 levels by removing endogenous CORT via adrenalectomy (ADX) and providing some of those rats with exogenous CORT replacement in their drinking water. This particular CORT water replacement regimen normalizes the augmented HPA axis response that ADX rats display when challenged with acute stress (5, 20). We also examined whether restraint-stimulated PVN pERK1/2 levels were subject to modulation by a 1 hour phasic CORT pretreatment. We have previously shown that this

particular phasic CORT pretreatment is sufficient to inhibit stress-induced ACTH secretion and PVN *crh* hnRNA expression (8). Phasic glucocorticoid negative feedback is evident when exogenous glucocorticoids are administered either within a few minutes (fast negative feedback) or in a moderately short interval of time (~1 hour or more; short-term delayed negative feedback) prior to the onset of stress (8, 9). Short-term delayed glucocorticoid negative feedback effects differ from fast negative feedback effects in that their inhibitory actions depend on alterations in *de novo* protein synthesis (21, 22). Short-term delayed glucocorticoid negative feedback may be especially important for the regulation of the HPA axis in the context of recurring acute episodes of stress or chronic stress, and it is this mode of phasic glucocorticoid negative feedback that we examined in this study.

The phosphorylated form of ERK1/2 may be used as an experience dependent marker of recent neuronal PVN activation (14) in a similar fashion that immediate early genes are conventionally used (23). Additionally, pERK1/2 may serve as a stress-induced intermediate signaling molecule for both *crh* gene expression and hormone release in PVN CRH neurons. Consequently, measuring changes in pERK1/2 cell counts may provide specific information about the actions of CORT (tonic and phasic) within the PVN by localizing those effects to events that modulate ERK activation.

Materials and Methods

Subjects

Young adult (250–320g) male Sprague-Dawley rats (Harlan Labs, Indianapolis, IA) were housed two per-cage (polycarbonate tubs, 47 cm × 23 cm × 20 cm) and were maintained on a 12 hour light/dark cycle with rat chow and tap water available ad libitum unless otherwise indicated. All test day manipulations were performed during the first half of the rat's light period when basal HPA axis activity is at its diurnal trough. Rats were given two weeks to acclimate to the facility before exposure to experimental procedures. All experiments were conducted in accordance with ethical procedures and were approved by the University of Colorado Institutional Animal Care and Use Committee.

Surgery

In experiments 2–4, rats received either bilateral adrenalectomy (ADX) or were sham-ADX. During surgery all rats were anesthetized with halothane. Bilateral incisions were made through the skin and peritoneal wall in close proximity to each kidney. Sham-ADX rats underwent the same surgical procedure as ADX rats except adrenal glands were not removed. All ADX and sham-ADX rats had a post-surgical recovery period of 4 days. ADX rats were given 0.9% saline water ad libitum unless indicated otherwise.

Restraint Stress

Stress challenge consisted of placing rats in clear plexiglas tubes (23.5 cm in length and 7 cm in diameter; with multiple air holes). The size of the tube restricted lateral, forward and backward movement but did not interfere with breathing. Restraint is widely accepted as a psychological stressor within the stress neurobiology field. This stressor categorization has not only a conceptual basis, but is also supported by neurocircuit activity studies (24).

Experimental procedures

Experiment 1a and b: time-course for stress-induced PVN pERK1/2 cell counts, and colocalization of stress-induced pERK1/2 in CRH neurons—1a:

The time-course experiment examined 5 treatment groups of adrenal intact rats (N = 34). Four groups of rats (n = 6) were placed in restraint for either 5, 10, 20 or 30 minutes.

Immediately after restraint these rats were killed by guillotine decapitation. The 5th group of rats (n = 10) were used as a no stress control comparison group, and were left undisturbed in their home cages within a closed room separate from the restrained rats. **1b:** The colocalization experiment examined 2 treatment groups of adrenal intact rats (N = 12). Stress challenged rats (n = 6) were restrained for 15 minutes and then decapitated. Non-stressed rats (n = 6) were left undisturbed in their home cages in a room separate from restrained rats and killed at the same time of day as stress challenged rats.

Experiment 2: effects of long term absence of endogenous CORT (ADX) on basal and stress-induced ACTH secretion and PVN pERK1/2 cell counts—This experiment examined 4 separate treatment groups (2 × 2 between subjects factorial design; n = 4; N = 16 rats) with treatment factors of tonic CORT condition (sham-ADX or ADX) and stress challenge (no stress or 15 minute restraint). On the test day rats were either restrained for 15 minutes or left undisturbed in their home cage prior to decapitation.

Experiment 3: effects of tonic CORT treatment of ADX rats on basal and stress-induced ACTH secretion and PVN pERK1/2 cell counts—This experiment examined 6 separate treatment groups (3 × 2 between subjects factorial design; n = 6; N = 36 rats) with treatment factors of tonic-CORT condition (sham-ADX, ADX, or ADX + CORT) and stress challenge (no stress or 15 minute restraint). After surgery, ADX + CORT rats were given ad libitum a drinking solution of 0.9% saline that contained CORT and 0.1% ethanol. The CORT drinking solution was made by dissolving CORT (Steraloids Inc. Newport, RI, USA) in absolute ethanol (25 mg/ml) and then diluting that solution 1:1000 into 0.9 % saline tap-water, to give a final CORT concentration of 25 µg/ml and ethanol concentration of 0.1%. ADX rats were given ad libitum a drinking solution containing 0.9% saline and 0.1% ethanol. Sham-ADX rats were given ad libitum a drinking solution of tap-water containing 0.1% ethanol. On the test day rats were either restrained for 15 minutes or left undisturbed in their home cage prior to decapitation.

Experiment 4: effects of a phasic 1 hour CORT pretreatment of ADX rats on basal and stress-induced ACTH secretion, PVN pERK1/2 cell counts and PVN crh gene expression—This experiment examined 4 treatment groups (2 × 2 between groups factorial design; n = 6; N = 24 rats) with treatment factors of stress challenge (no stress or 15 minute restraint) and 1 hour CORT pretreatment (CORT or vehicle). All rats were ADX and were given 0.9% saline ad libitum. On the test day, rats were pretreated 1 hour before stress challenge with CORT (2.5 mg/kg, i.p.) or vehicle (10% ethanol, 30% propylene glycol and 60% sterile saline, i.p.). One hour after drug pretreatment stressed rats were restrained for 15 minute and then were decapitated. The remaining rats were returned to their home cage after injection and left undisturbed until decapitation at a post injection time matched to that of the stress challenged rats.

Brain tissue processing

Due to the high level of PVN pERK1/2 immunoreactivity present in brains of rats that had been deeply anesthetized and perfused with buffer followed by 4% paraformaldehyde at the time of death (our unpublished observations), we instead used a guillotine to decapitate unanesthetized rats and adopted a whole brain postfixation method suitable for immunohistochemistry (14, 15). In brief, brains were placed in 0.1 M sodium acetate buffered 4%-paraformaldehyde (PFA; pH 6.5) and stored over night (12–15 hours; 4 ° C). Brains were transferred to a 4% PFA solution buffered with sodium borate (pH 9.5; 4 ° C). After 4–5 days, brains were transferred to cryoprotectant (0.1 M sodium acetate/20% glycerol, 4 ° C). After two days in cryoprotectant, brains were flash frozen in –30 ° C isopentane chilled with dry ice and stored at –80 ° C. Coronal brain sections (30 µm thick)

were cut on Leica Microsystems cryostat (model 1850, Bannockburn, IL, USA) through the extent of the PVN (approximately 1.80 mm caudal from bregma), and stored at 4 °C in cryoprotectant.

Immunohistochemistry

pERK1/2 immunoperoxidase histochemistry—Tissue sections were immunostained for pERK1/2 using a standard immunoperoxidase procedure (25). In experiments 2 and 3, pERK1/2 was detected with a monoclonal primary antibody (rabbit anti-phospho p44/p42 MAPK, 1:500 dilution; catalog #4376, Cell Signaling Technology, Danvers, MA). In experiments 1 and 4, pERK1/2 was detected with a polyclonal primary antibody (rabbit anti-phospho p44/p42 MAPK, 1:4000 dilution; #9101 Cell Signaling Technology). The same secondary antibody was used for all immunoperoxidase assays (goat anti-Rabbit IgG, 1:500; #BA1000, Vector Labs, Southfield, MI). After incubation with ABC complex reagents (1:500; Vector Labs) a dark purple precipitate was generated using 0.2 mg/ml diaminobenzidine (Sigma; #D5905, St. Louis, MO) and 6 mg/ml nickel ammonium sulfate (Fisher Scientific; #N48-500, Pittsburgh, PA) as substrates for the peroxidase mediated reaction. Sections were then rinsed and mounted in 0.1M Tris buffer (pH 7.4) onto 10% gelatin coated slides, dried, delipidized, and coverslipped. We chose to use two separate anti-pERK1/2 antibodies in order to validate that the specific pattern of pERK1/2 immunoreactivity that we observed in the PVN would generalize across two separate antibody preparations. Both anti-pERK1/2 antibodies yielded similar results in terms of the relative number of pERK1/2 immunoreactive cells under basal and stress conditions, however the monoclonal rabbit antibody (#4376, Cell Signaling Technology) tended to produce higher background immunostaining.

pERK1/2 and CRH co-localization immunofluorescence histochemistry—PVN pERK1/2 was detected with rabbit primary antibody (anti-phospho p44/p42 MAPK, 1:4000 dilution; #9101 Cell Signaling Technology) and secondary antibody (Alexa-594 anti-rabbit IgG, 1:500 dilution; #A-11021 Invitrogen, Carlsbad, California). PVN CRH peptide was detected with guinea pig primary antibody (anti-CRH monoclonal antibody, 1:20000 dilution; #T-5007 Peninsula Laboratories, member of the Bachem group, Torrance, California) and secondary antibody (Alexa-488 anti-guinea pig IgG, 1:500 dilution; #A-11073 Invitrogen, Carlsbad, California). Floating tissue sections were incubated overnight (4 °C) in a 0.01 M phosphate buffered saline (PBS; pH 7.4) solution containing both primary antibodies, 0.3% triton-X and 1.5% normal goat serum (Vector Labs; #PCN-5000). Sections were then rinsed and incubated for 2 hours in a 0.01 M PBS solution containing both secondary antibodies, 0.3% triton-X and 1.5% normal goat serum. Sections were then rinsed and mounted onto gelatin coated slides, with addition of a few drops of the nuclear 4', 6-diamidino-2-phenylindole fluorescent stain. As an immunostaining control procedure, some sections were treated the same as described above except that either one or both primary antibodies were omitted from the overnight incubation solution. For those control sections, no distinct cellular immunostaining was evident when applying the appropriate fluorescent viewing conditions for the absent antibody.

Quantification

pERK1/2 immunoperoxidase cell counts—Bright field photomicrographs (200X; Zeiss microscope, Axioimager M1) of peroxidase immunostained PVN sections were captured using Axio-Vision imaging software (version 4.6, Zeiss). At least 4 separate PVN hemispheric images per brain were captured and scored by individuals that were blind to treatment group assignments throughout this process. The target region of interest (ROI) was the medial parvocellular PVN region as determined by matching digitized rat hypothalamic structures to rat brain atlas images (26). Immunopositive cells within the PVN were

manually counted using NIH-ImageJ (freeware, <http://rsbweb.nih.gov/ij/>) touch-count feature which allowed the investigator to mark each cell within the ROI that was judged to be immunopositive. A threshold grey level for immunopositive cells was chosen that was approximately 50% of the maximum grey level cell signal over background.

pERK1/2 immunofluorescence cell counts—2-color channel fluorescent (mercury bulb illumination) photomicrographs (200X; Zeiss microscope: Axioimager M1) of PVN sections were captured using Axio-Vision imaging software as noted above. At least 4 separate PVN hemispheric images per brain were captured for co-localization assignments. For assessment of co-localization of pERK1/2 immunoreactivity with CRH immunoreactive neurons, a software application (Adobe Photoshop-CS3, San Jose, CA) was used to view and tag immunopositive cells within a red channel image layer that displayed fluorescently labeled pERK1/2 cells and a green channel image layer that displayed fluorescently labeled CRH cells. Each image layer was separately marked with red-dots over pERK1/2 immunopositive cells or green-dots over CRH immunopositive cells. The two marked layers were then merged, and the number of cells containing both red and green marks was determined. The region of interest for analysis was restricted to an outline of the CRH immunopositive cell region within the PVN. Cell counts were performed by an individual that was blind to the treatment group assignment for each image.

ACTH radioimmunoassay and CORT ELISA

Trunk blood was collected into EDTA-containing vacutainer tubes (Becton-Dickinson; Franklin Lakes, New Jersey, USA), placed on wet ice and then centrifuged for 15 minute (4 °C). Plasma was then aliquoted into microfuge tubes and snap-frozen on dry ice. The blood processing procedure was completed within 45 minute after decapitation. ACTH (pg/ml) was determined in duplicate (100 µl plasma) by competitive radioimmunoassay protocol as described previously (20). Radio-labeled ¹²⁵I ACTH-Tracer was obtained from DiaSorin (Cat # 20515, Stillwater MN) and primary ACTH anti-serum Rb 7 (diluted to a final concentration of 1:30,000) was provided courtesy of Dr. William Engeland, University of Minnesota. The detection limit for this assay was 15 pg/ml; the intra-assay coefficient of variability was 6% and inter-assay coefficient of variability was 6%.

Plasma CORT measurement was conducted using 20µl of plasma with an enzyme immunoassay kit (Assay Design, Ann Arbor, MI, USA) according to manufacturer's instructions. Sensitivity for the CORT assay was 27 pg/mL. The intra-assay and inter-assay coefficients of variability for the corticosterone assay were approximately 4% and 6%, respectively.

CRH hnRNA in situ hybridization and quantification (experiment 4)

Coronal PVN brain sections (post fixed and processed as described above) were floated in 0.5 M PBS and mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Tissue was then further fixed in 4% PFA for 30 minute at room temperature and then processed as previously published (21). Generation of ³⁵S-UTP labeled cRNA probes for *crh* hnRNA used a cDNA template corresponding to a 510 nucleotide intronic sequence (genomic location 104765147 to 104765657, Ensembl data base; <http://uswest.ensembl.org/index.html> plasmid containing cDNA provided courtesy of Dr. R. T. Thompson, University of Michigan. *crh* hnRNA probes were subsequently linearized from pGEM-3 vectors (Promega, Madison, WI) with the restriction endonuclease HindIII and transcribed using T7 RNA polymerase. The identity of *crh* cloned DNA was verified by DNA sequencing (University of Colorado Molecular, Cellular and Developmental Biology sequencing facility). After the hybridization assay procedure, slides were exposed to x-ray film (Kodak Biomax MR film) for 20 days.

Semiquantitative analyses were performed on digitized images from x-ray films using the linear range of the gray values obtained from an acquisition system (Northern Lights lightbox, model B 95, Ontario, Canada; CCD camera, model XC-77, Sony, Tokyo, Japan; image capture with National Institutes of Health scion Image v1.59 software) as previously described (27). Signal pixels of the region of interest were defined as those with a gray value of 3.5 standard deviations above the mean gray value of background (lateral hypothalamus or corpus callosum). The PVN region was determined by matching digitized rat hypothalamic structures to rat brain atlas diagrams (26). The product of the number of pixels and the average pixel values above the set background were then computed, providing an integrated densitometric measure of arbitrary units (integrated gray level). Quantification and analysis of images were performed by individuals that were blind to treatment condition assignments.

Statistical Analysis

Statistical analyses were conducted using the SPSS statistical program 10.5 (Chicago, IL, USA) for Macintosh operating system. One way analysis of variance (ANOVA) (experiment 1a), two way ANOVA (experiments 2–4), or Student's t-test (experiment 1b) were used to determine main effects and interactions for all treatment factors. In cases where there was an overall significant F-test, pairwise comparisons of interest were assessed by Fisher's least significant difference test (FLSD); $\alpha = 0.05$. Data presented in graphs represent group means $\pm$ standard error of the mean.

Results

Experiment 1a. Restraint rapidly increased ACTH and CORT secretion, as well as PVN pERK1/2 cell counts

Restraint significantly increased ACTH ($F_{4,31} = 9.7$, $P < 0.05$) and CORT ($F_{4,31} = 46.3$, $P < 0.05$) secretion, and restraint increased the number of cells within the PVN that were immunoreactive for pERK1/2 ($F_{4,22} = 4.6$, $P < 0.05$) (Fig. 1). Posthoc tests indicate that hormone secretion and pERK1/2 immunoreactivity were increased within 5 minute after restraint onset. Both CORT levels and pERK cell counts remained elevated throughout the duration of 30 minute restraint. Mean ACTH levels had started to decline at the 30 minute time-point, however the 30 min levels were not significantly different from the peak levels present at the 10 minute time-point.

Experiment 1b. Approximately 50% of restraint-stimulated pERK1/2 immunoreactive cells were colocalized with PVN CRH immunoreactive cells

To assess the extent to which restraint-stimulated increases in pERK1/2 cell counts occurred within PVN CRH neurons, we used a double-label CRH and pERK1/2 immunofluorescence histochemistry procedure. Restraint (15 min) increased both single labeled PVN pERK1/2 immunoreactive cell counts ($t_{(11)} = 3.3$, $P < 0.05$) and doubled labeled PVN pERK1/2 and CRH immunoreactive cell counts ($t_{(11)} = 3.4$, $P < 0.05$) (Fig. 2). Approximately 50% of stress-stimulated pERK1/2 immunoreactivity was colocalized within PVN CRH neurons. There was no effect of restraint on the total number of CRH neuron counts (data not shown).

Experiment 2. Long-term absence of endogenous CORT increased basal and stress-stimulated ACTH secretion, and stress-stimulated pERK1/2 cell counts within the PVN

As expected, ACTH secretion was increased by 15 min of restraint challenge (stress challenge: $F_{1,12} = 15.8$, $P < 0.05$) (Fig 3a). In addition, basal and stress-stimulated ACTH secretion were substantially increased in ADX rats compared to sham-ADX rats (tonic CORT condition: $F_{1,12} = 34.01$ $P < 0.05$) (Fig. 3a). PVN pERK1/2 immunoreactivity was

increased after 15 min of restraint stress compared to non-restrained rats regardless of surgical condition (stress challenge: $F_{1,10} = 25.8$, $P < 0.05$) (Fig 3b). There was also an overall effect of ADX on PVN pERK1/2 cell counts (tonic-CORT condition: $F_{1,10} = 13.3$, $P < 0.05$) (Fig 3b). Although statistical analysis supported only a trend for a significant stress challenge by tonic CORT condition interaction ($p = .09$), posthoc tests indicated that there was a greater effect of stress on PVN pERK1/2 cell counts in restrained ADX rats compared to sham-ADX rats. Non-stressed PVN pERK1/2 immunoreactive cell numbers were comparably low in both sham-ADX and ADX rats (Fig 3b).

Experiment 3. CORT replacement (via drinking water) of ADX rats normalized basal and stress-stimulated ACTH secretion and stress-stimulated PVN pERK1/2 cell counts

As was the case in experiment 2, 15 min of restraint increased ACTH secretion regardless of tonic CORT condition (stress challenge: $F_{1,30} = 45.12$, $P < 0.05$). ADX led to significantly greater basal and stress-stimulated ACTH secretion (tonic CORT condition: $F_{2,30} = 28.01$, $P < 0.05$) (Fig 4a). Treatment of ADX rats with a tonic CORT drinking water regimen was sufficient to normalize both basal and stress-stimulated ACTH secretion (stress challenge by tonic CORT condition interaction: $F_{2,30} = 9.52$, $P < 0.05$, followed by FLSD *post hoc* tests; Fig 4a). Also, consistent with experiment 2, PVN pERK1/2 immunoreactive cell counts were increased after 15 min of restraint stress (stress challenge: $F_{1,28} = 86.1$, $P < 0.05$), and this increase was greater in ADX rats (tonic CORT condition: $F_{2,28} = 5.2$, $P < 0.05$) (Fig 4b and 4c). Interestingly, treatment of ADX rats with CORT drinking water normalized the magnitude of stress-stimulated PVN pERK1/2 cell counts (stress challenge by tonic CORT condition interaction: $F_{2,28} = 11.3$, $P < 0.05$, followed by FLSD *post hoc* tests; Fig 4b and 4c). Our CORT water replacement regimen resulted in low, but detectable basal levels of circulating plasma CORT in non-stressed ADX rats ($M = 40.4$ ng/ml, $SEM = 1.4$) and ADX Stressed rats ($M = 20.4$ ng/ml, $SEM = 0.5$). These CORT levels were comparable to basal plasma CORT levels of non-stressed sham-ADX rats ($M = 50.0$ ng/ml, $SEM = 0.4$), whereas stressed sham-ADX rats had an expected large increase in CORT levels ($M = 320.0$ ng/ml, $SEM = 4.1$). ADX rats that were not given CORT in their drinking water had plasma CORT levels below the detection limits of the assay.

Experiment 4: A phasic increase in CORT 1 hour prior to restraint challenge suppressed stress-stimulated ACTH secretion and stress-induced PVN *crh* gene expression, but not stress-stimulated PVN pERK1/2 cell counts

Restraint (15 min) significantly increased ACTH secretion ($F_{1,20} = 7.0$, $P < 0.05$), PVN pERK1/2 immunoreactive cell number ($F_{1,20} = 23.4$, $P < 0.05$) and PVN *crh* hnRNA levels ($F_{1,20} = 17.4$, $P < 0.05$) (Fig. 5a–d). CORT pretreatment (1 hour) significantly inhibited basal and stress-stimulated ACTH secretion (phasic CORT treatment: $F_{1,20} = 40.1$, $P < 0.05$, and stress by phasic CORT treatment interaction: $F_{1,20} = 11.4$, $P < 0.05$) and it inhibited stress-induced *crh* hnRNA expression (phasic CORT treatment: $F_{1,20} = 25.4$, $P < 0.05$, and stress by phasic CORT treatment interaction: $F_{1,20} = 17.5$, $P < 0.05$) (Fig. 5a–c). In contrast, CORT pretreatment had no effect on stress-stimulated PVN pERK1/2 immunoreactive cell numbers (Fig. 5d).

Discussion

From these experiments we determined that CORT constrains in a tonic, but not phasic manner the extent to which the intracellular signal transduction protein ERK1/2 is activated by an acute psychological stress challenge in the rat PVN. Thus, the long-term absence of endogenous CORT (via ADX) augmented restraint-stimulated PVN pERK1/2 immunoreactivity, and treatment of ADX rats with CORT in the drinking water was sufficient to normalize pERK1/2 immunoreactive levels. As previously reported (21), a

short-term phasic CORT treatment 1 hour prior to restraint substantially suppressed basal and restraint-stimulated ACTH plasma levels, and suppressed stress-induced PVN *crh* hnRNA levels. However, we found that this short-term phasic CORT pretreatment did not suppress the increase in PVN pERK1/2 immunoreactivity produced by restraint. This study also served to further characterize the dynamic response of a MAPK pathway in the PVN of rats to stress. We found that exposure of rats to an acute psychological stressor (restraint) resulted in a rapid (within 5 minutes) increase in the number of PVN pERK1/2 immunopositive cells, and that this increase was maintained over a 30 minute period of restraint. Approximately half of restraint-induced pERK1/2 immunopositive cells within the medial parvocellular portion of the PVN were colocalized within CRH neurons.

This specific MAPK pathway may play an important role in the coupling of CRH neuron excitation with both gene induction as well as neurohormone secretion. A number of genes are rapidly induced in the PVN with acute stress (20, 28, 29). Khan et al. (14) provide evidence for stress-stimulated activation of ERK1/2 in CRH neurons to depend on noradrenergic alpha-1 receptors located in the PVN. Increased norepinephrine in the PVN is associated with *crh* and *c-fos* gene induction (14, 30, 31). Moreover, ERK1/2 activity is upstream of activation of several transcription factors (e.g. CREB, AP-1, ELK and NGFI-B) that are instrumental in the activity-dependent expression of most, if not all, of the genes that have been found to be rapidly induced in the PVN by acute stress (32–34). One research group has recently demonstrated that inhibition of ERK1/2 phosphorylation reduces the firing rate of CRH neurons in response to norepinephrine, suggesting that pERK1/2 also plays a functional role in the modulation of neurohormone release from these neurons (35). Similarly, pERK1/2 may also function as a dynamic intermediate signaling molecule for gene expression and hormone release within corticotrophs. For instance, CRH receptor stimulation of corticotrophs recruits the MAPK pathway for the modulation of *pomc* gene transcription, and pERK1/2 is thought to participate in the mediation of stimulated ACTH secretion (36).

Given the accumulating evidence for an important role of this MAPK pathway in HPA axis function, the focus of this study was on characterization of the regulatory influence of glucocorticoids on basal and stimulated pERK1/2 levels within the hypothalamic element of the HPA axis. By comparing the regulatory effects of glucocorticoids on PVN pERK1/2 levels with other activity-dependent HPA axis related measures, we can also make some inferences about which aspects of this neuroendocrine system are affected by changes in tonic and phasic glucocorticoids.

We first consider the implications of our data for understanding tonic glucocorticoid regulation of basal HPA axis activity. Although ADX produces a large increase in basal ACTH levels, the absence of tonic CORT activity did not change basal PVN pERK1/2 cell counts. We have previously found that the basal expression of several experience dependent genes (*c-fos*, *zif268* and *ngfi-b* genes) within the PVN also remain unaltered by the loss of tonic CORT activity (6, 20). In contrast, the basal expression of the neurohormone encoding genes, *crh* and *avp* upregulate in the PVN with ADX (20, 37). There is evidence for both the *crh* and *avp* genes to be directly regulated in a negative fashion by glucocorticoids, and consequently their basal expression may be tonically constrained by endogenous glucocorticoids (38, 39). There is not evidence for a direct glucocorticoid repressive effect on the expression of *c-fos*, *zif268* and *ngfi-b* genes within the PVN. Thus, the absence of an increase in the basal expression of these genes after ADX may reflect an absence of net excitatory drive to CRH neurons. The parallel absence of increased basal pERK1/2 levels is consistent with this interpretation. Consequently, the large increase in basal ACTH in ADX rats may be due to removal of tonic CORT inhibition that occurs directly at the level of neurohormone production and basal secretion independent of changes in basal CRH neuron

firing rate. Alternatively, it may reflect a direct inhibitory effect at the level of the anterior pituitary, as is supported by some recent studies (40, 41).

Whereas the tonic presence of CORT may not alter basal CRH neuron activity, tonic CORT clearly constrains the stress reactivity of CRH neurons. In this study we establish that the absence of tonic CORT activity in ADX rats challenged with restraint showed an exaggerated increase in PVN pERK1/2 cell counts and ACTH secretion. We have previously demonstrated that ADX rats exposed to restraint have an exaggerated increase in the expression of five genes within the PVN that are rapidly induced by acute stress (*c-fos*, *crh*, *avp*, *zif268*, and *ngfi-b*) (6, 20). Stress-stimulated Fos protein levels in the PVN are also increased in ADX rats (42). Together, these studies indicate that the loss of systemic tonic CORT regulation leads to an amplified stress-stimulated response of intracellular PVN activity (i.e. increased signal transduction, gene transcription and protein translation). Whether the augmented stress-induced HPA axis activity that results from ADX is a consequence of increased extrinsic drive to PVN neurons or an increase in responsivity of those neurons to stimulation remains to be determined. The absence of tonic CORT activity does not alter stress-induced *c-fos* mRNA or protein expression in a number of brain regions outside of the PVN including the hippocampus (42–44). Interestingly, the loss of tonic CORT increases the extent to which noradrenergic inputs in the PVN suppress GABAergic transmission (45). This decreased GABAergic activity could result in an unrestricted stress-induced excitatory drive to PVN CRH neurons of ADX rats.

HPA axis hormone secretion is substantially regulated by tonic CORT activity (2, 3). For example, both basal and stress-stimulated ACTH plasma levels are increased by the removal of endogenous glucocorticoid activity via adrenalectomy (4, 5, 20). Interestingly, treatment of ADX rats with a constant low dose of CORT normalizes basal but not stress-stimulated ACTH secretion patterns (4). In contrast, the augmented stress levels of ACTH that result from the long-term absence of CORT activity can be normalized by treatment of rats with CORT containing drinking water (5, 6). This replacement regimen closely mimics endogenous circadian CORT secretion patterns (5). Consistent with these previous studies, we found that providing ADX rats free access to CORT containing drinking water was sufficient to not only normalize the exaggerated ACTH plasma secretion patterns, but also the increased stress-stimulated PVN pERK1/2 cell counts. This shared profile for tonic CORT regulation of stress-stimulated ACTH secretion and PVN pERK activation may reflect a common cellular mechanism exerted by CORT at multiple levels of the HPA axis. Alternatively, tonic CORT may regulate stress reactivity at sites central to or within the PVN, resulting in an effect that is then expressed throughout the downstream HPA axis anatomical elements.

In addition to the tonic regulatory effects of CORT on the HPA axis, there is a temporally dynamic inhibition of the axis in response to a phasic increase in CORT. These inhibitory effects are evident within minutes (fast feedback) or after a delay, such as those present after the 1 hour pretreatment interval used in this study (Keller-Wood and Dallman, 1984). In previous studies we found that 1 hour systemic CORT pretreatment inhibited not only stress-stimulated ACTH secretion, but also stress-induced *crh* gene expression in the PVN and stress-induced *pomc* gene expression in the anterior pituitary (8, 46). We also found (21) that the inhibition of stress-stimulated ACTH secretion by 1 hour CORT pretreatment depended on de novo protein synthesis, which is consistent with the notion that the underlying suppressive mechanism of delayed glucocorticoid negative feedback is distinct from that present during glucocorticoid fast feedback (2). In this study we found that the suppressive effects of 1 hour CORT pretreatment on stress-induced PVN *crh* gene transcription and ACTH secretion was not associated with a suppressive effect on PVN pERK1/2 activity. One hour CORT pretreatment also does not inhibit stress-induced PVN *c-*

fos gene expression (8, 21). The disassociation between the ability of phasic CORT to inhibit stimulated *crh* hnRNA expression 1 hour after treatment, but not stimulated PVN pERK1/2 activity or *cfos* mRNA expression, suggests that this CORT treatment does not alter at least some of the stress-induced neural input to PVN neurons or the corresponding stress-induced intracellular signaling activity of those neurons.

It is likely that the suppressive actions of 1 hour CORT pretreatment on stimulated ACTH secretion is a result of CORT negative feedback actions that directly alter PVN and corticotroph hormone secretion. Studies (*in vivo* and *in vitro*) support a direct inhibitory effect of glucocorticoids on corticotroph intracellular processes necessary for corticotroph ACTH hormone release (22, 40, 47). Two recent studies also provide evidence for glucocorticoids to act directly at the level of the PVN to inhibit stress-stimulated neurohormone secretion. Evanson et al. (2010) found that microinfusion of dexamethasone in the PVN produces a rapid inhibition of stress-stimulated ACTH secretion. We have recently found that microinfusion of CORT into the PVN 1 hour before stress challenge also inhibits ACTH secretion (10). Although, the mechanisms underlying this direct phasic glucocorticoid inhibitory action at CRH neurons remains to be established, they appear to not depend on altered MAPK pathway activity.

There are a couple of caveats to note when considering the absence of an effect of phasic CORT manipulation on stress-stimulated pERK1/2 activity that we observed in this study. First, we examined pERK1/2 activity at one particular time point (1 hour after CORT treatment), but cellular and molecular effects of CORT can vastly change with timing (21, 48), therefore, stimulated PVN pERK1/2 activity may be more responsive to phasic CORT activity within other negative-feedback time-intervals (e.g. more rapid or delayed time frames). Second, the ultradian pattern of endogenous CORT secretion may have differential functional effects on CRH neuron function that are not evident with our acute and long-term pharmacological CORT manipulations (49, 50). Nonetheless, our findings further illustrate the differential tonic and phasic effects of CORT on the cellular and systems-level function of the HPA axis. The ERK1/2 MAPK pathway appears to be a key intracellular signal transduction pathway for the coupling of CRH neuron excitation with gene induction and neurohormone secretion. Modulation of PVN ERK1/2 activation may be a molecular process by which glucocorticoids tonically regulate stress reactivity of the HPA axis.

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Figure 1.

(A–C) Restraint significantly increased ACTH and CORT secretion, and increased PVN pERK1/2 cell counts in adrenal intact rats. Rats not challenged with restraint displayed low basal ACTH, CORT and PVN pERK1/2 cell counts. Five minutes of restraint rapidly increased ACTH, CORT and pERK1/2 cell counts. The increased ACTH and CORT hormone levels and pERK1/2 cell counts remained elevated over the course of a 30 minute restraint challenge. *, represents a difference compared to unstressed rats (time 0); $p < 0.05$, FLSD.

Figure 2. Colocalization of basal and stress-stimulated pERK1/2 cell counts in PVN CRH neurons. (A) Both the number of single labeled (pERK1/2 alone) and double labeled (pERK1/2 and CRH) PVN pERK1/2 immunopositive cells were increased in response to 15 minute of restraint. Approximately 50% of stress-stimulated immunofluorescence pERK1/2 was colocalized within PVN CRH neurons. *, represents a significant stress effect compared to non-stressed group ($p < 0.05$, FLSD). (B) Displays a representative merged immunofluorescence image of a single PVN hemisphere section from either a non-stressed (left) rat or stressed (right) rat. Single labeled pERK1/2 immunoreactive cells are labeled with red immunofluorescence (gray arrows), single labeled CRH immunoreactive cells are labeled with green immunofluorescence (blue arrow heads), and pERK1/2+CRH doubled immunoreactive cells are evident by yellow/orange immunofluorescence (pink arrow heads). Scale bar = 100 μ m.

A. **B.** **Figure 3.**

ADX resulted in a marked increase in both basal and stress-stimulated ACTH secretion, as well as stress-stimulated PVN pERK1/2 cell counts. (A) ACTH secretion was increased by 15 minutes restraint. Both basal and stress-stimulated ACTH secretion were substantially increased in ADX rats. (B) PVN pERK1/2 cell counts were also increased by restraint. ADX rats displayed a greater increase in PVN pERK1/2 cell counts compared to sham-ADX rats. *, represents a significant stress effect compared to the no stress group within the same surgical condition ($p < 0.05$, FLSD); #, represents a significant tonic CORT condition (ADX) effect compared to the sham group within same stress condition ($p < 0.05$, FLSD).

Figure 4.

Tonic CORT replacement in drinking water of ADX rats normalized basal and stress-induced ACTH secretion, and normalized stress-induced PVN pERK1/2 cell counts. (A–B) Restraint (15 minutes) increased ACTH secretion and PVN pERK1/2 cell counts, and ADX augmented these stress-stimulated effects. Tonic CORT water replacement of ADX rats normalized basal and stress-stimulated ACTH secretion to levels comparable to those displayed by sham-ADX rats. Tonic CORT water replacement also normalized the increase in stress-stimulated PVN pERK1/2 cell counts. *, represents a significant stress effect compared to no stress group within the same tonic CORT condition ($p < 0.05$, FLSD); #, represents a significant tonic CORT condition effect compared to sham rats within the same stress condition ($p < 0.05$, FLSD); +, represents a significant CORT replacement (ADX +CORT water) effect compared to non-replaced ADX rats within the same stress condition ($p < 0.05$, FLSD). (C) Shows representative photomicrographs from each treatment group for pERK1/2 immunoperoxidase histochemistry in one hemisphere of the PVN (3rd ventricle on the right side of no stress condition images and on the left side of stress condition images). Scale bar = 100 μ m.

Figure 5.

Phasic increase in CORT 1 hour prior to restraint challenge suppressed stress-stimulated ACTH secretion and PVN *crh* gene expression, but not stress-stimulated PVN pERK1/2 cell counts in ADX rats. (A) Basal and restraint-stimulated ACTH secretion was suppressed 1 hour after phasic CORT treatment. (B–C) Stress-induced PVN *crh* gene expression was suppressed by this CORT pretreatment. (C) Shows representative autoradiograms for *in situ* hybridization of PVN *crh* hnRNA expression. Images are centered over the PVN containing ventral-medial portion of coronal brain sections. (D) Phasic CORT pretreatment had no effect on stress-stimulated PVN pERK1/2 cell counts. *, represents a significant stress effect compared to non-stressed rats within the same phasic CORT condition ($p < 0.05$, FLSD); +, represents a significant phasic CORT effect compared to vehicle treated rats within the same stress condition ($p < 0.05$, FLSD).