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Chronic electroconvulsive shock treatment elicits up-regulation of CRF and AVP mRNA in select populations of neuroendocrine neurons

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The effects of repeated electroconvulsive seizures (ECS) on expression of mRNAs coding for corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) in neuroendocrine neurons of the hypothalamo-pituitary-adrenocortical (HPA) axis and hypothalamo-neurohypophysial system (HNS) were assessed via semi-quantitative in situ hybridization histochemical analysis. Measures of mRNA content were accompanied by measurement of peptide- and hormone-expression in the relevant neuroendocrine systems. Following 7 daily ECS treatments, CRF mRNA was significantly increased in the medial parvocellular paraventricular nucleus (PVN) of treated rats relative to controls. CRF peptide content of whole PVN homogenates was decreased to 50% of control levels. Changes in CRF message and peptide levels were accompanied by increases in pituitary ACTH content and by elevated plasma corticosterone, suggesting ECS elicits long-term up-regulation of the HPA axis. AVP mRNA in the medial parvocellular PVN, which is known to up-regulate in response to HPA challenge by adrenalectomy, was not increased by ECS. Chronic ECS causes a clear up-regulation of HNS neurons of the supraoptic nucleus, characterized by increased AVP mRNA content, decreased AVP peptide content, and depletion of neurohypophysial AVP. However, no changes were observed in magnocellular vasopressinergic neurons of the PVN, indicating that magnocellular SON and PVN neurons respond differentially to stimulation by ECS. The data indicate that ECS is a potent stimulus for activation of select components of both the HPA axis and the HNS. As such, ECS provides a useful tool for examining mechanisms underlying neuroendocrine processes.

INTRODUCTION

Among the salient physiological consequences of electroconvulsive seizure treatment (ECS) is an activation of the hypothalamo-pituitary-adrenocortical (HPA) system, the principal neuroendocrine system responsible for control of circulating glucocorticoids. Numerous literature reports have suggested that in man, ECS produces an up-regulation of the HPA axis, characterized by hypersecretion of pro-opiomelanocortin (POMC)-derived peptides from the anterior pituitary gland[1,5,8,32] and glucocorticoids from the adrenals[1,32]. Recently, our laboratory has shown that ECS elicits both short- and long-term up-regulation of the HPA axis in the rat[32]. Up-regulation of the HPA axis following ECS mimics that seen following various stress regimens, indicating that the endocrine effects of ECS may be executed by systems governing stress regulation.

It is generally agreed that a rather circumscribed population of hypothalamic corticotropin releasing factor (CRF) neurons are primarily responsible for central integration of ACTH release (cf. ref. 2). These neurons, localized to the medial parvocellular

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subdivision of the hypothalamic paraventricular nucleus (PVN), send projections to the median eminence[3,26], whereby CRF is released into the hypophysial portal circulation upon receipt of an appropriate stimulus (e.g. stressor) and causes the release of ACTH by anterior pituitary corticotrophs (cf. ref. 2, for review). Following removal of glucocorticoid negative feedback (adrenalectomy), which elicits a profound increase in pituitary ACTH production and secretion, CRF messenger RNA (mRNA) and peptide content are markedly increased in medial parvocellular PVN neurons[13,20,22,29,34], confirming a primary role for CRF in hypothalamic drive of the HPA axis. Adrenalectomy-induced increases in CRF production and peptide expression in medial parvocellular PVN neurons are accompanied by a massive induction of arginine vasopressin (AVP) mRNA[7,31] and peptide expression[13,20] in CRF perikarya of the PVN, as well as a marked increase in colocalization of AVP and CRF in terminal boutons in the external lamina of the median eminence[30]. The importance of coexpression and apparent cosecretion of AVP and CRF by parvocellular neurosecretory neurons is highlighted by a well-documented synergistic interaction between CRF and AVP in promoting ACTH release by corticotrophs[10].

The literature summarized above supports a putative role for CRF and AVP originating in the medial parvocellular PVN in neuronal regulation of HPA responses to physiological or psychological stressors. The precise role played by these ACTH secretagogues is presently unclear, with some important issues in need of clarification. For instance, it remains to be determined whether stress results in long-term up-regulation of CRF production, as occurs following drive of the HPA axis by adrenalectomy. It is likely that repeated stress will elicit an increase in CRF message, in that CRF is released in response to a variety of stressors. However, elevations of circulating glucocorticoids resulting from stressful episodes are capable of exhibiting negative feedback effects at multiple levels of the HPA axis[11], including the PVN itself[14,21]. Clearly, chronic effects at the CRF neuron will represent an integration of stress activation vs feedback inhibition of this neuronal population. Along similar lines, it is not yet known whether induction of AVP mRNA in CRF neurons represents a 'physiological' amplification system for ACTH release by CRF under conditions of stress, or is primarily a concomitant of severe glucocorticoid depletion. In these studies, we have attempted to address these issues, utilizing chronic ECS as a model of repeated HPA activation. ECS was chosen as a stressor due to its ability to rapidly and reliably elicit widespread activation of the HPA axis in rats, with minimal evidence for across-trial habituation[32]. The overall synthetic tone of PVN neuropeptidergic neurons was inferred from measures of mRNA coding for AVP and CRF, using semi-quantitative in situ hybridization histochemistry. In situ hybridization histochemistry allows precise anatomical localization and differentiation of mRNA levels in tightly defined brain regions; for our purposes, it permits a ready differentiation between the effects of ECS in parvocellular vs adjacent magnocellular neurosecretory systems of the PVN in the same histological specimen. Measures of CRF and AVP mRNA were accompanied by measurement of CRF content in PVN homogenates, POMC peptides in the pituitary and plasma and determination of plasma corticosterone, to verify the efficacy of ECS in inducing up-regulation of endocrine components of the HPA axis.

In addition to co-expression and putative cosecretion of AVP and CRF in select hypothalamic parvocellular neurons, AVP is synthesized in neighboring magnocellular neurosecretory neurons in the PVN, which comprise one component of the classical hypothalamo-neurohypophysial system (HNS). The HNS secretes AVP directly into the systemic circulation, whereby its physiological effects on vascular smooth muscle and renal water resorption are conveyed. However, the effects of ECS on this vital neuroendocrine system remain to be characterized. Therefore, in the present study, in situ hybridization analysis of AVP mRNA expression were conducted on magnocellular neurons of the PVN and SON. Measurements of AVP mRNA were accompanied by determination of brain and pituitary AVP content.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats, weighing 250–300 g at the time of experimentation, were housed 6 per

cage and had ad libitum access to food and water. Animals were housed on a 12:12 h light:dark cycle, with lights on at 06.00 h.

Treatment

Between 09.00 and 10.30 h ECS-administered (ECS) rats were removed from their home cages and subjected to transauricular ECS (80 μA, 0.2 s, 60 Hz). Control (CON) rats were removed from their home cages and replaced, receiving no ECS. ECS rats received treatment on each of 7 consecutive days. On the eighth day, *24 h following the last treatment,* ECS and CON rats were killed by rapid decapitation and brains and pituitaries rapidly removed. For some subjects (6 ECS and 5 CON), brains were immediately frozen in liquid isopentane at −50 °C for in situ hybridization analysis. Additional brains were dissected on ice to yield small tissue blocks containing, respectively, the PVN and SON for AVP and CRF radioimmunoassay. Pituitary glands from all animals were dissected into anterior and neurointermediate lobes and frozen on dry ice. Blood was collected into tubes containing EDTA, spun for 5 min at 1500 × *g*, acidified and frozen on dry ice until analysis.

In situ hybridization

Brains were sectioned at 10 μm in a Bright cryostat (−20 °C), thaw-mounted onto polylysine-coated slides and stored at −80 °C until processed for in situ hybridization histochemistry. Tissue was sampled for in situ procedures at 50 μm intervals; an additional series was stained for Nissl substance with thionine to establish a cytoarchitectonic context for analysis. The in situ protocol consisted of removing sections directly from the −80 °C freezer and immersing them immediately in cold buffered 4% formaldehyde. Tissue was fixed for 1 h and washed 3× in phosphate-buffered saline (20 mM). Sections were deproteinated with 0.2 N HCl for 20 min at room temperature and washed in 2× SSC for 20 min prior to hybridization.

^{35}S-labeled RNA probes complementary to proAVP mRNA (T.G. Sherman; subcloned in pGEM4) and proCRF mRNA (courtesy R. Thompson, J. Douglass and E. Herbert; subcloned in pSP64) were produced using the SP6 transcription system (Promega). The proAVP probe was a 197 bp cRNA coding for the C-terminal region of the proAVP molecule, which bears no homology with proOxytocin. The proCRF probe was a 353 bp cRNA derived from a rat CRF cDNA clone, including the peptide coding region (exon 2) of the rat CRF gene. ^{35}S-labeled and unlabeled S-UTP was added to the transcription reaction in amounts calculated to yield specific activities estimated at 0.94×10^4 Ci/mmol probe and 2.54×10^4 Ci/mmol probe for AVP and CRF cRNA probes, respectively.

Probe was diluted in a standard hybridization buffer (75% formamide, 10% dextran sulfate, 3× SSC, 50 mM sodium phosphate buffer, pH 7.4, 1× Denhardt's, 0.1 mg/ml yeast tRNA and 0.1 mg/ml sheared salmon sperm DNA) sufficient to yield 2,000,000 dpm/30 μl buffer. 30 μl aliquots were then applied to each slide, the slides coverslipped, and coverslips sealed with rubber cement. Slides were placed in plastic boxes containing moistened foam and the boxes sealed. Sections were incubated at 55 °C for 48 h, at which time coverslips were removed, the slides rinsed in 2× SSC and immersed in fresh 2× SSC for 20 min. Tissue was then reacted with RNAse A (200 μg/ml) at 37 °C for 30 min to degrade any remaining single-stranded RNA. Sections were then washed successively in 2×, 1× and 0.5× SSC for 10 min each, followed by a 1 h wash in 0.5× SSC at the hybridization temperature. Sections were then exposed to Kodak XAR X-ray film overnight, and subsequently emulsion-dipped, along with slide-mounted brain-paste standards containing known amounts of ^{35}S-labeled L-methionine, in Kodak NTB2 nuclear emulsion. Emulsion-dipped sections and standards were exposed for 10 days (AVP) or 3 weeks (CRF), with final batch development based on grain densities of dipped slides developed at regular intervals.

Image analysis

Semi-quantitative analysis of in situ hybridization autoradiographs was conducted utilizing Loats image analysis software. Analysis was performed on 6 ECS and 5 CON animals, using 8.2 ± 0.4 PVN sections/animal for CRF analysis of the PVN, and 12.7 ± 1.5 PVN sections/animal for AVP analysis of the PVN, and 22.7 ± 2.1 sections/animal for AVP analysis of the SON. Emulsion-dipped sections and ^{35}S-labeled standards of known radioactivity were digitized and

subjected to areal analysis of optical density over the areas of interest in the images, those being the medial parvocellular and immediately adjacent posterior magnocellular divisions of the PVN and the SON. The image analytic scheme is presented in Fig. 1, which illustrates the criteria used to select appropriate regions of interest. Briefly, quantified areas

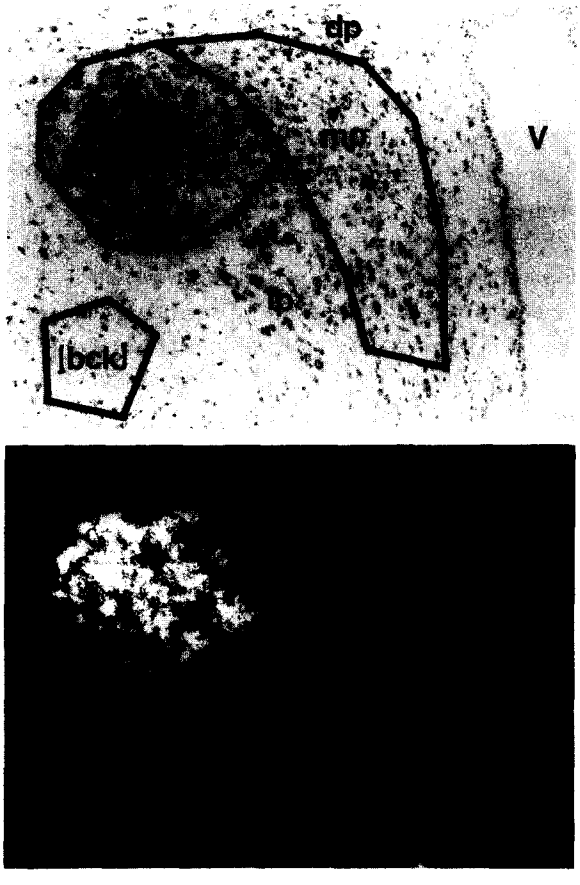

Fig. 1. Example illustrating the analytical scheme employed for semi-quantitative in situ hybridization analysis of AVP and CRF mRNAs in the paraventricular nucleus. Shown are neighboring sections from the same animal, one stained for Nissl substance with thionine (upper panel) and the other hybridized with an antisense RNA probe for AVP mRNA (lower panel). Sections are separated by 30 μm. At this level of the PVN, posterior magnocellular (PM) and medial parvocellular (mp) divisions were defined (as shown) from Nissl-stained sections and the corresponding regions outlined on digitized images of autoradiographs by a manually operated cursor, using a polygonal sampling mode (illustrated in upper panel). Care was taken to avoid including the dorsal parvocellular (dp) PVN, lateral parvocellular (lp) PVN or periventricular zone (pz) in the sampling procedure. Background regions were digitzed over cellular regions distinct from the PVN and free from any evidence of positive hybridization signal (bck).

were defined on the basis of PVN and SON cytoarchitecture (based on parallel Nissl-stained series) and by patterns of grain localization within the section of interest. Determination of disintegration values for areas selected was derived from a standard curve of best fit relating optical density values of the digitized images of ^{35}S-labeled standards with corresponding disintegration values. Radioactivity of standards was determined by counting adjacent standard sections in a Beckman beta counter, and subsequent dpm values corrected for time of exposure and area of standard. The optical density of all samples fell within the range of the standard values.

For statistical analysis, Nissl-stained sections were used to group SON and PVN sections from individual animals into different anatomical levels, using the Paxinos and Watson coordinate system[18]. For the SON, coronal sections were grouped into 1 of 4 levels, corresponding to ranges spanning 1000–1200, 1200–1400, 1400–1600, and 1600–1800 μm posterior to bregma. For the magnocellular PVN, sections were grouped into 4 levels corresponding to ranges spanning 1600–1700, 1700–1800, 1800–1900, and 1900–2000 μm posterior to bregma. As both parvocellular CRF and AVP mRNA-containing neurons were widely scattered at the 1900–2000 μm level, quantitative data are not reported for this region in analysis of the parvocellular PVN.

Radioimmunoassay

Radioimmunoassay for AVP in PVN, SON and neurointermediate lobe (NIL) was performed according to previously published methods[25]. PVN and SON punches were diluted 1/1000 in assay buffer for analysis, and NIL samples 1/10,000. The minimal detectable amount of AVP was 1 pg/assay tube. Cross-reactivity with oxytocin was 0.02%.

For CRF radioimmunoassay, a single 50 μl and duplicate 25 μl aliquots of PVN and SON homogenates were tested. The antibody to CRF was generously provided by Dr. John Olschowka, University of Rochester. It was raised in rabbits against a conjugate of human/rat CRF and bovine thyroglobulin (antiserum 455 (ref. 16)) and used at a final dilution of 1/1,500,000. The assay buffer was 0.1 M phosphate-buffered saline containing 0.5% bovine serum albumin (pH 7.6). Synthetic CRF (Peninsula)

was used as the standard. ^{125}I-CRF (Amersham, 5000 cpm) was added to the assay mixture after 24 h of incubation at 4 °C. Following an additional 72 h of incubation, antibody-bound CRF was separated from unbound CRF by the addition of 1 ml of 95% cold ethanol and subsequent centrifugation (3000 rpm, 4 °C, 45 min). The supernatant was discarded and the pellets containing bound tracer were counted in a gamma counter. In the absence of CRF, the assay conditions resulted in binding of 45% of the tracer. The assay has a minimum detection limit of 5 pg per assay tube, the IC_{50} was 32 pg per assay tube, and the intra-assay coefficient of variation was 4.5%. All samples for CRF content were evaluated in the same assay. Lack of cross-reactivity of this antibody with a variety of peptides has been demonstrated previously[16].

Radioimmunoassay of ACTH was performed on anterior pituitary samples. Plasma samples were extracted prior to assay with Sep-Pak C_{18} cartridges as previously described[6] and subsequently assayed for β-endorphin immunoreactivity. Both radioimmunoassays and antibodies have been described previously[33]. Some aspects of the β-endorphin assay deserve comment. The β-endorphin antibody (Brenda) is a mid-portion antibody raised against β-endorphin(1–31). It was used at a final dilution of 1/40,000. [^{125}I]β-endorphin (human) was used as the radiolabeled tracer. β-endorphin(1–31) (camel) was used for construction of a standard curve. Under these conditions, this antibody shows 100% cross-reactivity with β-lipotropin, 80% cross-reactivity with *N*-acetylated β-endorphin(1–31) and 50% cross-reactivity with *N*-acetylated β-endorphin(1–27), the primary species of β-endorphin secreted by rat intermediate lobe. Previous studies from our laboratory indicate that plasma β-endorphin values obtained using this antibody reliably parallel ACTH levels[33].

Plasma corticosterone was measured using a standard competitive protein binding assay[17], using [^{3}H]corticosterone as the radiolabeled tracer.

TABLE I

HPA axis hormones and peptides: effects of ECS

	Control	*ECS*
Pituitary ACTH (pmol/gland)	122 ± 7 (7)	210 ± 16 (7)*,**
Plasma β-endorphin (fmol/ml)	71 ± 12 (12)	55 ± 11 (11)
Plasma corticosterone (μg/dl)	3.3 ± 1.1 (18)	7.5 ± 1.1 (17)**

* Values represent mean ± standard error of the mean; numbers in parentheses represent number of animals/group.

** Significantly different from control, $P < 0.05$, two-tailed *t*-test.

RESULTS

Hypothalamo-pituitary-adrenocortical axis

Table I summarizes the effects of ECS on hormonal components of the HPA axis 24 h after the final ECS treatment. Analysis of POMC-derived

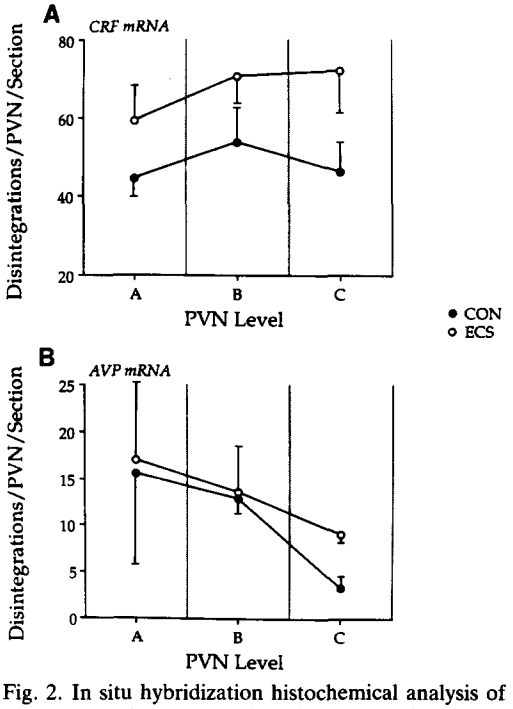

Fig. 2. In situ hybridization histochemical analysis of corticotropin releasing factor (CRF) mRNA (A) and arginine vasopressin (AVP) (B) in the medial parvocellular subdivision of the hypothalamic PVN. The medial parvocellular subdivision was divided into 3 levels for analysis, with level A including sections from 1600–1700 μm, level B sections from 1700–1800 μm, and level C sections 1800–1900 μm posterior to bregma, using the coordinate system of Paxinos and Watson. CRF mRNA showed a clear-up regulation in response to ECS, showing increased expression across the entire medial parvocellular PVN (overall group effect: $F_{1,26} = 6.81$, $P < 0.02$). In contrast, AVP mRNA did not change following ECS.

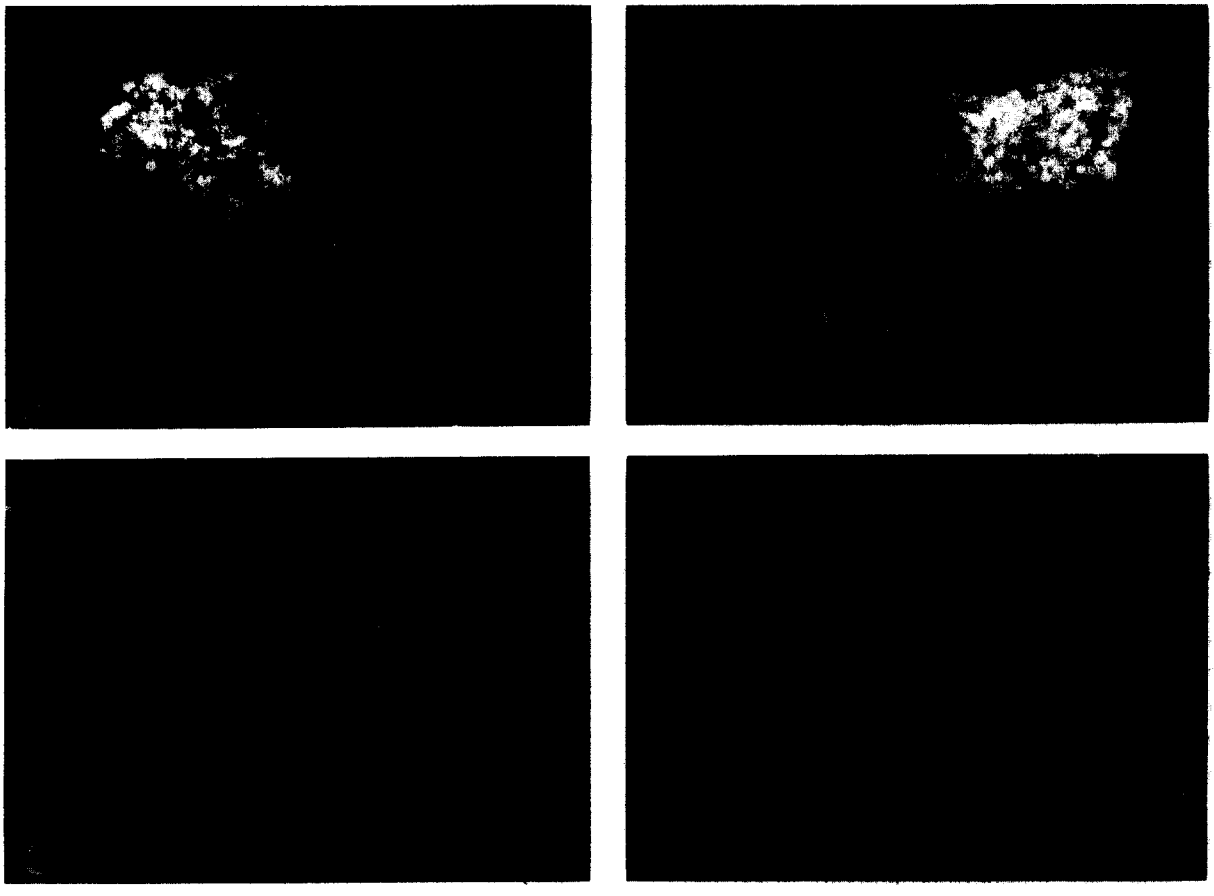

Fig. 3. Autoradiographs of representative emulsion-dipped sections through the PVN of ECS (A,C) and control (B,D) rats, hybridized with ^{35}S-labeled cRNA probes complementary to AVP (A,B) and CRF (C,D) mRNA. All sections are from approximately the same rostrocaudal level (PVN level B). In A and B, it is evident that no substantial differences in hybridization density can be detected between the ECS (A) and control (B) groups in either the posterior magnocellular PVN (pm) or the medial parvocellular PVN (mp). Cells scattered in the medial parvocellular PVN probably represent scattered magnocellular neurons normally present in this region. In C and D, however, an increase in hybridization signal for CRF mRNA is evident in ECS (C) sections relative to control (D) tissue. Positive hybridization signal for CRF mRNA is primarily associated with the medial parvocellular subgroup of the PVN. Magnification bar = 100 μm.

peptides in pituitary and plasma revealed a significant increase in pituitary ACTH content in the ECS group (t_{12} = 5.64, $P < 0.001$), indicative of an increased synthesis/storage of POMC-derived peptides in the anterior pituitary. Circulating β-endorphin did not differ significantly between the two groups, indicating that peptide levels had returned to resting values. Yet, plasma corticosterone levels were significantly increased in animals receiving ECS relative to handled controls (t_{33} = 2.67, $P < 0.02$), suggesting a long-term potentiation of adrenocortical glucocorticoid secretion.

Results of quantitative in situ analysis for AVP and CRF mRNAs are shown in Fig. 2. Neurosecretory neurons of the HPA axis undergo an up-regulation in response to ECS, as evident from a significant increase in CRF mRNA across the 3 levels of the medial parvocellular PVN sampled ($F_{1,26}$ = 6.81, $P < 0.02$) (Fig. 2). AVP mRNA content across the same 3 levels of the medial parvocellular PVN was, however, unaffected by ECS (Fig. 2). Neither CRF nor AVP mRNA varied as a consequence of region of the PVN sampled.

Autoradiographs of labeled CRF and AVP mRNA in the medial parvocellular PVN in the ECS and CON groups are illustrated in Fig. 3. Qualitative differences can be observed between ECS and CON rats in overall density of CRF mRNA labeled over

TABLE II

Hypothalamic and pituitary CRF content

	Control	*ECS*
PVN (fmol/nucleus)	49.8 ± 0.2 (6)	26.1 ± 6.7 (6)*,**
SON (fmol/nucleus)	23.1 ± 3.4 (6)	30.1 ± 6.5 (6)
NIL (fmol/pituitary lobe)	59.2 ± 7.6 (6)	67.4 ± 3.8 (6)

* Values represent mean ± standard error of the mean; numbers in parentheses represent number of animals/group.

** Significantly different from control, $P < 0.05$, two-tailed t-test.

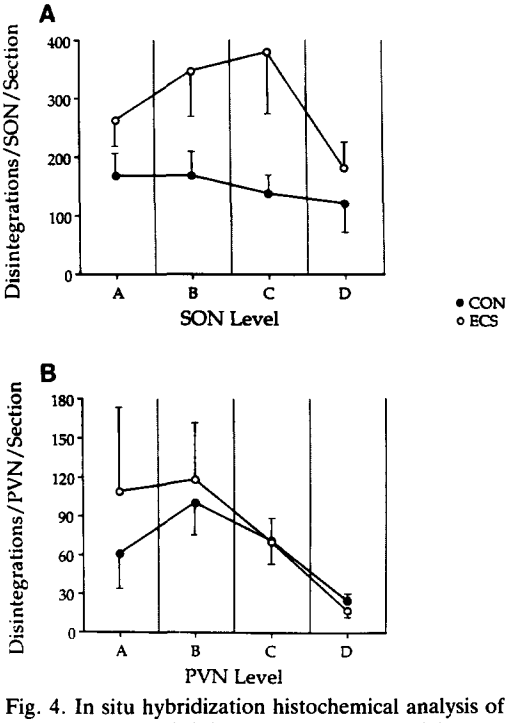

Fig. 4. In situ hybridization histochemical analysis of arginine vasopressin (AVP) (B) in magnocellular nuclei comprising the hypothalamo-neurohypophysial system, including the supraoptic nucleus (SON) (A) and the posterior magnocellular subdivision of the paraventricular nucleus (PVN) (B). The SON was divided into 4 levels for analysis: level A included sections between 1000 and 1200 μm, level B, sections between 1200 and 1400 μm, level C, sections between 1400 and 1600 μm, and level D, sections between 1600 and 1800 μm posterior to bregma, using the Paxinos and Watson coordinate system. Over all SON levels, AVP mRNA was significantly increased in the ECS group relative to controls (significant group effect: $F_{1,33} = 6.88$, $P < 0.02$). Differences were particularly pronounced over levels B and C, which contain the densest populations of AVP neurons. The posterior magnocellular PVN was divided also into four levels, with level A including sections from 1600 to 1700 μm, level B sections from 1700 to 1800 μm, and level C sections 1800 to 1900 μm, and level D sections from 1900 to 2000 μm posterior to bregma. In contrast to the SON, no change in AVP mRNA was observed in this neuronal population following chronic ECS treatment.

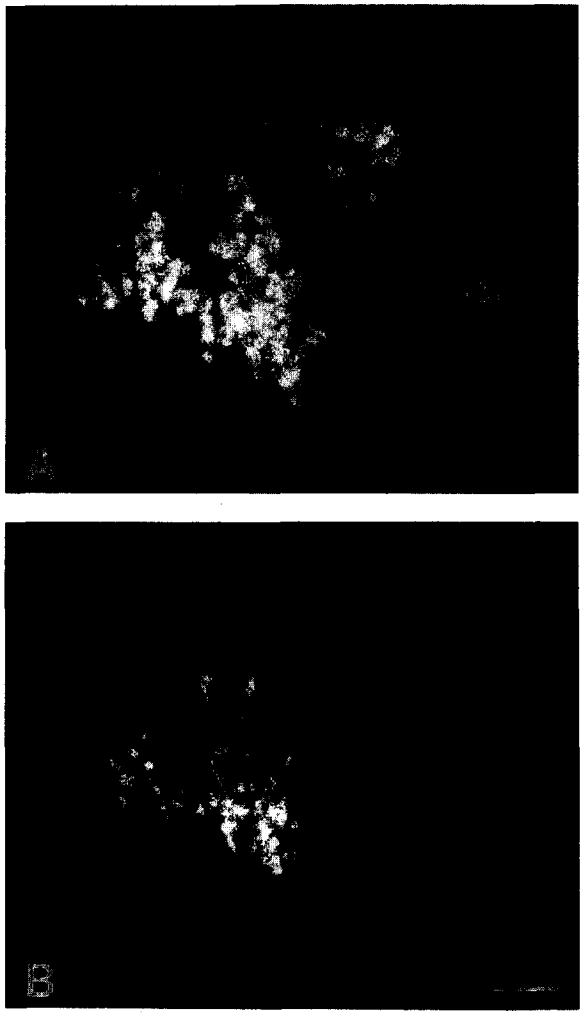

Fig. 5. Autoradiographs of representative emulsion-dipped sections through the SON of ECS (A) and control (B) rats, hybridized with ^{35}S-labeled cRNA probes complimentary to AVP mRNA. Both sections are taken from approximately the same rostrocaudal level (SON level C). A considerably more dense hybridization signal is observed over the SON of ECS tissue when compared with sections from control rats. Magnification bar = 100 μm.

the PVN (Fig. 3C,D). However, no changes in AVP mRNA were observed in the medial parvocellular zone of the PVN (Fig. 3A,B). There is no evidence to suggest an induction of AVP mRNA in medial parvocellular PVN neurons; most positively labeled cells seen scattered throughout this area are isolated magnocellular neurons (based on neuronal size and density of hybridization, utilizing phase-contrast microscopy).

Radioimmunoassay for CRF peptide in homoge-

nates of the PVN region (Table II) indicate that chronic ECS elicits a decrease in CRF in the PVN ($t_{10} = 3.39$, $P < 0.01$). No changes were observed in AVP content of the PVN; however, it should be noted that homogenates of the PVN sample both the magnocellular and parvocellular PVN, and given the overwhelming preponderance of AVP in magnocellular neurons it is unlikely that any change in parvocellular content would be detectable.

Hypothalamo-neurohypophysial system

Examination of magnocellular nuclei comprising the hypothalamo-neurohypophysial system revealed an interesting and somewhat divergent pattern of changes following chronic ECS (Fig. 4). AVP mRNA content in the 4 sampled regions of the posterior magnocellular subdivision of the PVN was not changed by ECS. However, AVP mRNA content was significantly increased across the SON regions sampled ($F_{1,33} = 6.88$, $P < 0.02$) (Fig. 4). Enhancement of SON AVP mRNA levels following ECS was most pronounced in levels B and C, corresponding to regions of the SON where AVP is particularly abundant.

Autoradiographs of sections hybridized with labeled antisense AVP cRNA probes for the ECS and CON groups are presented in Figs. 3 and 5. As seen in Fig. 3A,B, AVP mRNA does not appear increased in the posterior magnocellular division of the PVN in the ECS group relative to controls. In contrast with the magnocellular PVN, marked increases in detected AVP mRNA can be observed in the SON of ECS animals (Fig. 5).

CRF mRNA was present only in scattered neurons of the SON and magnocellular divisions of the PVN, and was not quantifiable using areal density measures. No qualitative differences were evident between the ECS and CON groups in magnocellular CRF mRNA content.

Radioimmunoassay for AVP in homogenates of the PVN and SON suggest that AVP peptide is regulated differentially in the two nuclei following ECS (Table III). AVP peptide was significantly decreased in the SON of ECS rats relative to control animals ($t_{28} = 2.76$, $P < 0.01$), while no changes in peptide content were observed in PVN. Content of AVP in the NIL was significantly diminished in the ECS group ($t_{53} = 4.75$, $P < 0.001$), indicating an apparent long-term depletion of neurohypophysial stores in response to ECS. In contrast to changes in neurohypophysial AVP, CRF immunoreactivity did not change in the SON or NIL following ECS.

TABLE III

Hypothalamic and pituitary vasopressin content

	Control	*ECS*
PVN (pmol/nucleus)	6.2 ± 0.9 (18)	5.2 ± 0.8 (13)*
SON (pmol/nucleus)	20.6 ± 1.9 (17)	14.0 ± 1.0 (13)**
NIL (pmol/pituitary lobe)	878 ± 34 (29)	636 ± 38 (26)**

* Values represent mean ± standard error of the mean; numbers in parentheses represent number of animals/group.

** Significantly different from control, $P < 0.05$, two-tailed *t*-test.

DISCUSSION

Results of this study indicate that chronic ECS effects an up-regulation in neurons regulating activation of the HPA axis. CRF mRNA content is increased by approximately 40% in the medial parvocellular PVN of the ECS group. In combination with decreases in PVN CRF content, the data suggest that PVN CRF neurons are in a state of accelerated production and release of CRF as a result of chronic ECS. The significant changes in CRF peptide content and CRF mRNA levels are accompanied by significant elevations of pituitary ACTH stores and circulating corticosterone. The long-term augmentation of CRF mRNA production, in the face of elevated glucocorticoid levels, suggests that the CRF neuron plays an important role in maintaining activation of the HPA axis under conditions of chronic stress. Elevated glucocorticoid levels appear unable to return CRF mRNA expression to normal, indicating that CRF gene expression may be controlled by sources other than, or in addition to, circulating glucocorticoids following chronic challenge with ECS.

The observed increase in CRF mRNA expression probably represents a summation of conflicting physiological information stemming from competition between the repeated drive of CRF neurons by ECS and inhibition of CRF gene expression by glucocorticoids. An analogous situation occurs with

the POMC gene upon chronic stress; increased drive of the pituitary leads to initiation of POMC mRNA expression, while increased glucocorticoid secretion inhibits transcription, leading to a relatively small (30%) overall increase in anterior pituitary POMC mRNA[24]. Interestingly, chronic stress results in a two-fold increase in anterior pituitary POMC peptide stores[24] and increased releaseability of POMC peptides[33], indicating that the limited increase in mRNA in the POMC system may be of considerable physiological significance. These data, coupled with the ability of the HPA axis to physiologically 'amplify' signals at successive points between brain and adrenal, underline the potential for relatively small changes in brain CRF mRNA to profoundly influence the overall tone of the HPA axis.

CRF mRNA in the medial parvocellular PVN of ECS rats was consistently greater than control at all anterior–posterior levels of the nucleus examined. These data indicate that CRF induction by ECS does not topographically select any individual area of PVN, and thus that in response to the chronic ECS stimulus, medial parvocellular CRF neurons may act in concert to stimulate the HPA axis.

In contrast with the observed elevation of CRF mRNA content, no change was seen in AVP mRNA content in the medial parvocellular PVN, where AVP is co-localized with CRF following removal of circulating steroids[13,20,31]. The lack of an induction of AVP in medial parvocellular PVN neurons implies that chronic ECS does not recruit production of AVP in this neuronal population. The failure of ECS to induce AVP mRNA in CRF-containing nuclei suggests either (1) that the AVP induction in these neurons is critically dependent on circulating glucocorticoid levels rather than neuronal stimulation, and hence will not occur under conditions of normal or elevated glucocorticoids, (2) that the drive of the HPA axis by ECS was not sufficient to elicit long-term up-regulation of parvocellular AVP neurons, or (3) that parvocellular PVN AVP induction may be physiologically associated with *acute* stress responses, and rapidly returns to baseline following removal of the stressor (perhaps as a result of glucocorticoid feedback).

It should be noted that although the data do not demonstrate significant changes in parvocellular AVP mRNA under conditions of chronic stress, the possibility remains that the in situ hybridization procedure employed is not sufficiently sensitive to detect changes in AVP-producing cells in this region. For instance, small changes may be undetectable either due to a level of expression which is beneath the detection limits imposed by the selected in situ hybridization conditions, or to increased variability imposed by scattered magnocellular neurons localized within the medial parvocellular PVN. The physiological relevance of any such undetectably small change in AVP mRNA should not be summarily dismissed, given the known synergism between CRF and AVP in promotion of ACTH release[10].

Chronic ECS also provides a significant stimulus to the hypothalamo-neurohypophysial system (HNS), as is evident from a significant increase in SON AVP mRNA. The increase in SON AVP message is particularly pronounced over levels B and C, regions known to be particularly rich in AVP-containing magnocellular neurons[19]. Activation of the HNS is further corroborated by the observed depletion of NIL AVP peptide, suggesting that this stimulus serves to promote extensive release of secretable pools of AVP at the neurohypophysial vasculature. The decrease in SON peptide content in ECS animals, in conjunction with neurohypophysial depletion and increased SON AVP mRNA levels, indicates that AVP is being rapidly synthesized and perhaps rapidly released by SON neurons. Activation of the SON component of the HNS by ECS suggests a hypersecretion of AVP into the circulation, perhaps contributing to increases in arterial blood pressure seen following ECS[4]. One of the most striking points to be made about up-regulation of SON AVP neurons is the chronic nature of this effect: perikaryal and terminal depletion and increased AVP mRNA synthesis are occurring 24 h following the last ECS. Continued synthesis and release of AVP following cessation of ECS may reflect a characteristic response of magnocellular neurosecretory neurons to chronic stimulation; previous studies indicate that while plasma osmolality returns to normal fairly quickly following cessation of chronic osmotic stimulation (salt loading), hypothalamic and pituitary AVP content and hypothalamic AVP mRNA take considerably longer to return to baseline levels[35]. These data seem to

indicate that the HNS maintains high rates of peptide synthesis for a substantial time period following stimulation, perhaps to replenish depleted pituitary stores.

Perhaps more perplexing than the chronic increase in SON AVP mRNA content is the apparent lack of any observable effect of ECS on AVP mRNA or AVP peptide levels in magnocellular PVN neurons. This neuronal population, like that of the SON, is believed to project predominantly to the neurohypophysis, where it is involved primarily with fluid and electrolyte balance. In addition, neuronal afferents to SON and magnocellular PVN are remarkably similar in scope and magnitude[27]. Although it has been documented that the SON contains a considerably greater number of AVP neurons and correspondingly greater amounts of AVP[9,19], it is reasonable to expect that functionally related AVP neurons of the PVN should experience an up-regulation of similar magnitude. The apparent discrepancy in AVP mRNA expression between magnocellular PVN and SON neurons in response to ECS represent a rather novel indication of differentiation between neurons commonly held to be a functionally homogeneous pool. While this discrepancy is difficult to explain given the input–output systems and common function of the two nuclei, two salient differences between the SON and PVN may lie at the core of this phenomenon. First, unlike magnocellular PVN neurons, SON AVP neurons appear to retain a significant population of glucocorticoid neurons[12]. In that glucocorticoids are increased by chronic ECS, as shown above, genomic effects of internalized glucocorticoids may be sufficient to induce long-term changes in synthesis and perhaps release of AVP by SON neurons. Secondly, SON neurons are localized close to the surface of the brain. If the depolarizing stimulus is propagated along the surface of the brain with a greater intensity than that in deeper structures, the cell-body depolarization occurring in SON neurons may be significantly greater than that of PVN neurons, yielding an effectively more intense stimulus for release.

CRF peptide content in the SON or NIL is not affected by ECS. Combined with a failure to observe substantial increases in CRF mRNA in SON, these data indicate that CRF and AVP are differentially regulated in the hypothalamo-neurohypophysial system. These observations agree with anatomical data showing colocalization of CRF in oxytocin but not AVP-containing magnocellular neurons[27]. Interestingly, it is well-known that oxytocin neurons are osmotically sensitive and that both oxytocin and CRF mRNAs increase dramatically in the SON upon chronic salt loading[15]. The lack of change in SON CRF mRNA, accompanied by the relatively limited increase in AVP mRNA in this region (in comparison to 8–10 fold changes seen following osmotic stimulation)[23], suggest that stimulation provided by ECS is modest with respect to the dynamic range of response available to neurons associated with the HNS.

Increases in pituitary ACTH and plasma corticosterone following ECS strongly mimic changes in HPA axis function seen with chronic stress[24], and accent the value of ECS as a model of chronic stress. ECS has significant advantages over other stress paradigms, in that it is a relatively short-lived stressor which produces long-lasting endocrine changes comparable in magnitude with more prolonged stress regimens. In addition, the combined observations of involvement of the HPA axis in human depressive illness[28] and the well-known efficacy of ECS as remedial treatment for major depression suggest that interactions between depression and the HPA axis may hinge on processes susceptible to influence by ECS, and mandate further characterization of effects of ECS on neuroendocrine systems.

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