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Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Controls Stimulus-Transcription Coupling in the Hypothalamic- Pituitary-Adrenal Axis to Mediate Sustained Hormone Secretion During Stress

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Abstract

External and internal stimuli that threaten homeostasis trigger coordinated stress responses through activation of specialised neuroendocrine circuits. In mammals, the hypothalamic-pituitary-adrenal (HPA) axis mediates responses to stressors such as restraint, ultimately enhancing adrenocortical hormone secretion. Pituitary adenylate cyclase-activating polypeptide (PACAP) has been implicated in central control of the HPA axis, and we have recently shown PACAP-dependent expression of corticotropin-releasing hormone (CRH) and secretion of corticosterone in response to restraint. We now provide a more detailed analysis of PACAP-dependent HPA axis stimulation in the mouse, indicating that the hypothalamic paraventricular nucleus (PVN) is the primary site of action. We demonstrate by quantitative PCR and *in situ* hybridisation that upregulation of mRNAs encoding CRH and inducible transcription factors from the Nr4a family (Nr77, Nr1) in the PVN is PACAP-dependent. Furthermore, CRH hnRNA is rapidly upregulated in cultured hypothalamic neurones after treatment with PACAP. Induction of Nr4a factors (Nr77, Nr1) in response to restraint is attenuated in the pituitary gland of PACAP-deficient mice. In the adrenal glands, restraint elicits a marked PACAP-dependent increase in adrenocortical mRNA levels of all three Nr4a transcription factors, SF-1 (steroidogenic factor 1; Nr5a1), steroidogenic acute regulatory protein (StAR) and steroid 21-hydroxylase. Taken together, our results show that PACAP controls HPA responses to restraint primarily at the level of the hypothalamus by upregulating CRH, possibly involving transcription factors such as Nr77 and Nr1. Subsequent adrenocortical steroidogenesis also appears to involve PACAP-dependent stimulus-transcription coupling, suggesting a mechanism by which PACAP exerts control over HPA axis function during stress.

Keywords

PACAP; stress; HPA axis; CRH; Nr4a

Introduction

Stress can be defined as a condition where changes in the internal or external environment of an organism elicit patterned responses that aim to compensate for the effects of the initial stimulus (stressor) and restore homeostasis. These compensatory responses are generated by neuroendocrine circuits that monitor internal and external variables and counter discrepancies through activation of various effectors (1). In mammals, stress responses are mediated by the hypothalamic-pituitary-adrenal (HPA) axis, adrenomedullary hormonal system, and sympathetic nervous system, the chief effector molecules of which are glucocorticoids, epinephrine, and norepinephrine, respectively (2). After exposure to stressors such as hypoglycemia, hemorrhage or physical restraint, release of these effector molecules into the circulation occurs rapidly but transiently, with different stressors eliciting different response patterns (2). Because virtually all bodily functions are affected by the aforementioned molecules, basal activity and responsiveness of the respective neuroendocrine circuits must be tightly controlled to ensure proper conditions at rest and following stimulation (3). Within the HPA axis, synthesis and secretion of hypothalamic corticotropin-releasing hormone (CRH), pituitary adrenocorticotrophic hormone (ACTH) and adrenocortical steroid hormones, such as cortisol (in humans) and corticosterone (in rodents), is therefore subject to complex regulation.

We have recently shown that the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) is necessary for sustained function of the HPA axis during stress responses evoked by restraint. Thus, CRH mRNA induction is abolished and corticosterone secretion is diminished in PACAP-deficient mice (4). Since CRH gene transcription is required for restoration of releasable peptide pools (5), and upregulation of the inducible transcription factors Egr1 and Fos in hypothalamus and adrenal glands is blunted in PACAP-deficient mice (4), it appears that responses to acute stressor exposure require PACAP-dependent stimulus-transcription coupling within stress-reactive neuroendocrine circuitry. Evidence has accumulated that the Nr4a family of orphan nuclear receptors (Nur77 [Nr4a1], Nurr1 [Nr4a2] and Nor1 [Nr4a3]) participates in stimulus-transcription coupling throughout the HPA axis, both as targets and mediators of hormonal effects. Thus, Nur77 and Nurr1 enhance transcriptional activity of the CRH and proopiomelanocortin (POMC) promoters, while also being upregulated by CRH treatment in pituitary cells, thereby mediating expression of hypothalamic and pituitary stress hormones (6–9). Within the rodent hypothalamus *in vivo*, stress induces Nur77 expression in the paraventricular nucleus (PVN), where CRH-positive neuroendocrine neurones are located (10–15). Because CRH-positive neurones in the PVN are crucial activators of HPA responses during stress (16), Nr4a transcription factors are likely to be involved in the central control of these responses. This is further supported by the observation that CRH induces Nur77 expression in the PVN *in vivo*, potentially as a means of autoregulation (17).

In the periphery, all three Nr4a factors have been shown to participate in transcriptional regulation of steroid biosynthetic enzymes, such as steroid 21-hydroxylase, also known as P450c21 (18, 19). Together with SF-1 (steroidogenic factor 1; Nr5a1), another member of the nuclear receptor superfamily, they furthermore regulate expression of StAR (steroidogenic acute regulatory protein), a factor that mediates the rate-limiting step in steroid production (20, 21). Following stressor exposure or ACTH injection, rapid and pronounced expression of Nur77 and Nurr1 occurs in the rodent adrenal cortex (22, 23), suggesting that Nr4a-mediated transcriptional control of steroidogenic factors is indeed recruited *in vivo* to provide for increased corticosterone biosynthesis during stress.

We wished to address the role of PACAP in activation of the HPA axis, CRH transcription, as well as ACTH and corticosterone secretion, and to determine whether it might involve

upregulation of Nr4a family transcription factors. Accordingly, we measured the PACAP-dependence of Nr4a gene transcription in wild-type and PACAP-deficient mice, which have previously been shown to exhibit an impaired cortisone response to restraint stress (4, 24). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and *in situ* hybridisation (ISH) were used to measure expression of Nr4a factors and their potential target genes in hypothalamus, pituitary and adrenal glands. Our results support the notion that PACAP controls HPA axis activation centrally via direct effects on the PVN. In addition, our data provide *in vivo* evidence for the involvement of Nr4a transcription factors in expression of key neuroendocrine genes within the HPA axis.

Materials and Methods

Animals

Male mice between 3.4 and 6.7 months of age were used in the present study. Experimental groups each represented the entire range, such that potential age-related effects were controlled for. After a complete backcross of the PACAP knock-out allele (25) into the C57BL/6N strain, PACAP^{-/-} and age-matched PACAP^{+/+} mice were obtained via homozygous breeding pairs. All mice were bred and housed in a temperature- and humidity-controlled facility with 12 h light/dark cycle (lights on at 06:00 AM) and had access to chow and water *ad libitum*. Approximately 18 h before experiments, the mice were transferred to a separate room and placed in individual home cages. Experiments were started between 09:00 AM and 12:15 PM. All experimental procedures were approved by the National Institute of Mental Health Animal Care and Use Committee, and every effort was made to minimise the number of mice used in this study.

Restraint stress

Restraint was carried out in tapered plastic film envelopes (DecapiCones, Braintree Scientific, Braintree, USA) as previously described (4). Mice were continuously restrained for various durations (groups 1 h, 3 h and 6 h) in their home cage, or restrained for 1 h, subsequently released into their home cage, and euthanised 5 h thereafter (group 1 h + 5 h). Untreated mice (non-stressed controls) were picked up from their home cage by the tail, placed in DecapiCones and decapitated immediately (time from pickup to decapitation <20 seconds). Mice from the continuous restraint groups were decapitated while still restrained. Restraint-released mice (group 1 h + 5 h) were picked up and decapitated in the same manner as untreated animals.

Sampling of mouse tissues

For RNA extraction, pituitary and adrenal glands (both adrenals per mouse pooled) were quickly excised and frozen on dry ice (solid CO₂). Samples of hypothalamus for RNA extraction were obtained as previously described (4). Briefly, the brain was removed from the skull, transferred to an ice-chilled rodent brain matrix (RBM-2000C, ASI Instruments, Warren, USA), and a coronal section (approximately encompassing Bregma 0.02 to -3.08) was generated. From this section, a tissue block was obtained by cutting dorsal of the third ventricle and 1 mm each left and right of the midline. For *in situ* hybridisation, individual adrenal glands were embedded in O.C.T. compound (Tissue-Tek, Sakura Finetek, Torrance, USA) and dropped into liquid nitrogen. Whole brain samples for *in situ* hybridisation were dropped into isopentane (2-methylbutane), which had been placed in a container on dry ice and cooled to -50°C, for rapid freezing without cracking of the tissue. All tissue samples were stored at -80°C until use.

Preparation of tissue sections and *in situ* hybridisation (ISH)

Brains and adrenal glands (left gland per mouse) were embedded in O.C.T. compound (Tissue-Tek, Sakura Finetek) and cut at 14 μ m thickness using a cryostat. Frozen tissue sections were thaw-mounted onto microscope slides (Fisherbrand Superfrost Plus, Fisher Scientific, Pittsburgh, USA), allowed to air-dry at room temperature, and either processed directly or stored at -80°C . Sections were fixed in 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 60 min, washed in PBS (3×10 min), permeabilised in 0.4% Triton X-100 in PBS for 10 min, washed again in PBS for 5 min and rinsed in H_2O . Subsequently, acetylation was carried out in TEA buffer (1% triethanolamine in PBS, pH 8.0) containing 0.25% acetic anhydride, followed by washing in PBS for 10 min and rinsing in H_2O . Slides were then dehydrated by rinsing in 50% and 70% isopropanol and allowed to air-dry. For production of complementary RNA (cRNA) probes, fragments of CRH (nucleotides 346–753), Nur77 (nucleotides 936–1385), Nurr1 (nucleotides 1748–2179) and Nor1 (nucleotides 1064–1518) were generated by PCR (see Table 1B for primers and target RefSeqs) and subcloned into the pGEM-T vector (Promega, Madison, USA), DNA preparations of which were subjected to PCR using SP6 and T7 primers. The resulting amplicons thus contained the respective bacterial promoter sequences and were used for *in vitro* transcription with SP6 and T7 RNA polymerases (Roche Diagnostics, Mannheim, Germany) in the presence of ^{35}S -labelled UTP (Perkin Elmer, Boston, USA). After treatment with RNase-free DNase I (Roche) and column purification (Micro Bio-Spin P-30 Tris Chromatography Columns, Bio-Rad, Hercules, USA), sense and antisense cRNA probes were adjusted to 50,000 dpm/ μ l in hybridisation buffer (10% dextran sulfate, 600 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA disodium salt, 0.05% tRNA [Invitrogen, Carlsbad, USA], $1 \times$ Denhardt's solution [USB Corporation, Cleveland, USA], 100 $\mu\text{g}/\text{ml}$ sonicated salmon sperm DNA [SABiosciences, now Qiagen], 50% formamide). The resulting hybridisation solution was applied to the tissue sections, which were covered with glass cover slips and incubated overnight at 60°C in a humidified chamber. Posthybridisation consisted of washes in $2 \times$ and $1 \times$ SSC (20 min each), incubation in RNase buffer (containing 40 $\mu\text{g}/\text{ml}$ RNase A and 2000 U RNase T1; Roche), further washes in SSC ($1 \times$, $0.5 \times$, $0.2 \times$), one wash in H_2O , dehydration and air-drying. For regional detection of the ^{35}S -labelled cRNA probes, autoradiography film (Kodak Biomax MR; Carestream Health, Woodbridge, USA) was exposed to the tissue sections and automatically developed (Kodak X-OMAT 2000A, Carestream Health) about 24 h later. Resulting autoradiograms were scanned at high resolution (2400 or 4800 dpi) on a conventional flatbed scanner (Scanjet 4890, Hewlett-Packard; Palo Alto, USA) and visually inspected. For analysis at the cellular level, tissue sections were coated with autoradiography emulsion Type NTB (Carestream Molecular Imaging, New Haven, USA), exposed for up to 92 h at 4°C , and developed and fixed using Developer D-19 and Fixer (Kodak; obtained through Ted Pella Inc., Redding, USA) according to manufacturer's instructions. Cresyl violet was used for counterstaining. Photographic images were taken through a microscope (Eclipse 50i, Nikon Instruments, Melville, USA) connected to a CCD camera (Retiga 1300i Fast 1394, QImaging, Surrey, Canada) and captured using iVision software (version 4.0.14, BioVision Technologies, Exton, USA) running on an Apple Macintosh computer. For darkfield visualisation of silver grains representing hybridisation signals, a Darklite illuminator was used (Micro Video Instruments, Avon, USA).

Primary culture of rat hypothalamic neurones

Fetal Sprague Dawley rats, embryonic day 18, were used to obtain primary cultures of hypothalamic neurones. Fetuses were rapidly removed from pregnant rats after CO_2 sedation and decapitation. Fetuses were decapitated, and hypothalamic tissues dissected and collected in ice-cold buffer (pH 7.4, containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 25 mM HEPES buffer, and 100 $\mu\text{g}/\text{ml}$ gentamicin). Tissues were digested for 1.5 h with collagenase

type 2 (1 mg/ml; Worthington, Lakewood, NJ) dissolved in the above buffer, supplemented with 1 mg/ml glucose, 4 mg/ml BSA, and 0.2 mg/ml DNase. The cell suspension was filtered through a 40 μ m cell strainer (BD Biosciences, Bedford, USA) and centrifuged at $200 \times g$ for 10 minutes. Cells were washed twice in buffer and once in plating medium (D-MEM/F12, 100 μ g/ml gentamicin, and 10% heat-inactivated fetal bovine serum). Cells were plated at a density of 1×10^6 cells per well in 6-well plates coated with poly-L-lysine. To support neurone growth, medium was changed to Neurobasal Medium (Invitrogen) with B27 supplement (Invitrogen), 0.5 mM L-glutamate, and 100 μ g/ml gentamicin on the second day. From days 5 to 9 (i.e. for 5 days) cells were cultured in the presence of 5 μ M cytosine arabinoside (Sigma), a selective inhibitor of DNA synthesis, which was added to the Neurobasal Medium in order to prevent glial proliferation. On day 10, cells were changed into serum-free/B27 supplement-free Neurobasal Medium containing 0.1% BSA for 2 h before treatment with PACAP-38 (Phoenix Pharmaceuticals, Burlingame, USA) or forskolin (Calbiochem/EMD Chemicals, Gibbstown, USA).

Serum collection and hormone measurements

Immediately after decapitation, trunk blood was collected into 1.5 ml polypropylene tubes and allowed to coagulate at room temperature for approximately 1 h. Samples were subsequently centrifuged (10 min at $10,000 \times g$) and the serum was transferred to fresh tubes. After removing residual debris via a second centrifugation step, samples were immediately frozen and stored at -80°C until assayed. ACTH (ELISA, MD Bioproducts, St Paul, USA) and corticosterone (EIA, Assay Designs, Ann Arbor, USA) were measured using commercially available kits according to the manufacturer's instructions. Samples were diluted 1:2 in calibrator A (for ACTH ELISA) or 1:10 in assay buffer (for corticosterone EIA) and results were calculated accordingly. Statistical analysis was performed as for qRT-PCR results (see below).

RNA extraction and qRT-PCR

Samples of hypothalamus, pituitary or adrenal glands (both adrenals per mouse) were transferred to lysis buffer (RNeasy Mini, Qiagen, Valencia, USA) and immediately homogenised using an ultrasonic processor (GE 130PB, Hielscher, Ringwood, USA). A commercial kit was used for RNA extraction (RNeasy Mini), and genomic DNA was removed by digestion with RNase-free DNase I (Roche). Reverse transcription (SuperScript II Reverse Transcriptase, Invitrogen) was carried out using 0.5 μ g RNA per sample. For quantitative PCR (samples assayed in duplicate), 2 μ l of cDNA were mixed with iQ SYBR Green Supermix (Bio-Rad) and run on an iCycler iQ Real Time PCR System (Bio-Rad) in the presence of 200 nM gene-specific primers (see Table 1A). Samples from hypothalamic primary cultures were processed similarly, with the exception that a different DNase I (Qiagen) and reverse transcriptase (SuperScript III; Invitrogen) were used, and quantitative PCR was carried out on a different thermal cycler (7900 HT Fast Real-time PCR system; Applied Biosystems, Foster City, USA).

Calculation of transcript abundance from qRT-PCR

Transcript expression levels in mouse tissues were calculated from qRT-PCR results, taking into account the total RNA and cDNA aliquots used for reverse transcription and quantitative PCR, respectively. Absolute values were derived from gene-specific standard curves (serial dilutions of purified cDNA fragments). For rat hypothalamic neurones, relative expression changes were calculated using the ddCt method (26) with GAPDH as reference gene.

Statistical analysis

Statistical analysis was carried out in Prism 4 (GraphPad Software Inc., La Jolla, USA). For mouse tissues, one-way ANOVA with Dunnett's posttest was used to compare untreated controls with mice after various durations of restraint. Differences between PACAP genotypes at each time point were analysed by two-way ANOVA with Bonferroni posttest. Where experiments involved only one treatment period, effects and differences were assessed by one-way ANOVA with Newman-Keuls posttest (comparison of all four groups). Results from measurements of serum ACTH and corticosterone were analysed using the same tests. For hypothalamic neurones, differences between groups were calculated by one-way ANOVA, followed by Fisher's LSD method for pairwise multiple comparisons. The cutoff for significance was set at $p < 0.05$ in all cases.

Results

Hormonal responses of the HPA axis to restraint are blunted in PACAP-deficient mice

Restraint elicited marked activation of the HPA axis (Figure 1). Within 1 h, circulating levels of ACTH and corticosterone were increased in both genotypes. After 6 h, ACTH was no longer significantly elevated, whereas corticosterone levels were still higher than in untreated mice (Figure 1A). Peak responses of ACTH and corticosterone, as well as the sustained phase of corticosterone secretion, were significantly blunted in PACAP-deficient mice. Thus, after 1 h of restraint, concentrations of ACTH (PACAP^{+/+} 152 vs. PACAP^{-/-} 38 pg/ml) and corticosterone (PACAP^{+/+} 282 vs. PACAP^{-/-} 163 ng/ml) were 75% and 42% lower, respectively, compared to wild-types. After 6 h of restraint, serum corticosterone concentrations in PACAP-deficient mice were >60% lower compared to wild-types (PACAP^{+/+} 188 vs. PACAP^{-/-} 74 ng/ml). In another experiment, we measured ACTH and corticosterone 30 minutes after onset of restraint, to determine whether secretory responses are intact after briefer stressor exposure (Figure 1B). Again, serum concentrations of both hormones were lower in PACAP-deficient mice, although the differences were clearly less pronounced compared to prolonged restraint (ACTH 42% lower, corticosterone 33% lower).

Restraint induces PACAP-dependent upregulation of CRH and Nr4a transcription factor mRNA in the hypothalamic paraventricular nucleus

To quantify changes in gene expression occurring in the hypothalamus in response to restraint, RNA was extracted and transcript levels were measured using qRT-PCR (Figure 2). Confirming our recently published results (4), CRH mRNA was significantly upregulated in the hypothalamus exclusively in wild-types (PACAP^{+/+}), while PACAP-deficient mice (PACAP^{-/-}) showed no induction at all. In wild-type mice, mRNA levels were elevated after 1 h, 3 h and 6 h of restraint. In addition, mRNAs encoding inducible transcription factors from the Nr4a family of nuclear receptors were differentially upregulated. In the hypothalamus, Nur77 and Nor1 were induced after 1 h of restraint, and this response was significantly attenuated in PACAP-deficient mice. Nurr1, the third family member, was unaffected by treatment and equally abundant in both genotypes (Figure 2).

To determine the localisation of PACAP-dependent expression after stress, *in situ* hybridisation with cRNA probes for CRH, Nur77 and Nor1 was carried out on brain sections encompassing the hypothalamus including PVN. Sense probes were used as negative controls and yielded no specific signal (data not shown). Regional analysis of autoradiography films confirmed that stress-induced increases in CRH mRNA occurred within the expected area, namely the medial parvocellular part of the PVN. These increases after 1 h of restraint did not occur in PACAP-deficient mice, whereas expression in untreated mice was not significantly different between genotypes (Figure 3A), confirming

the quantitative results obtained by qRT-PCR (Figure 2). Serial sections labelled with probes for Nur77 and Nor1 were analysed at the cellular level using emulsion autoradiography and showed hybridisation signals in the same area, overlapping with CRH-positive cell groups in the PVN. These signals increased after restraint and were clearly more abundant in wild-type mice after 1 h (Figure 3B). In addition to the PVN, hybridisation signals for Nor1 were also visible in the suprachiasmatic nucleus of the hypothalamus (data not shown) and in cells lining the third ventricle (Figure 3B), with the latter occurring only in stressed wild-type mice. Nur77 and Nor1 mRNA expression in the hypothalamus appeared somewhat diffuse, whereas CRH hybridisation was restricted to the PVN, with a high relative intensity of expression per cell.

PACAP induces expression of CRH hnRNA in hypothalamic neurones

Primary cultures of rat hypothalamic neurones were used to test whether PACAP could directly stimulate CRH gene transcription in these cells. Concentration-response experiments showed that 45 minutes of exposure to 1 nM PACAP-38 significantly induced CRH hnRNA compared to untreated controls, with induction being maximal at 3 nM (Figure 4A). PACAP-induced CRH expression peaked after 60 minutes and remained elevated until 120 minutes after beginning of treatment, subsequently returning to baseline (Figure 4B). Forskolin (1 μ M) caused an increase in CRH transcription that was very similar to the increase caused by 4 nM PACAP-38 (Figure 4C).

Inducible expression of Nr4a transcription factor mRNA in the pituitary gland is attenuated in PACAP-deficient mice

Expression of the Nr4a transcription factors was measured by qRT-PCR in pituitary gland extracts from wild-type and PACAP-deficient mice (Figure 5). Nur77 and Nurr1 were robustly induced by 1 h of restraint only in wild-types, returning to baseline thereafter. In contrast, Nor1 was not statistically significantly upregulated in either genotype, but showed higher expression levels in pituitaries from PACAP-deficient mice after 6 h of restraint. POMC mRNA was not significantly affected by restraint in either genotype (data not shown).

PACAP is required for restraint-induced stimulus-transcription coupling and steroidogenic gene expression in the adrenal glands

In the adrenal glands, all three Nr4a transcription factors were PACAP-dependently induced, with maximal mRNA expression levels at 1 h being about 50% lower in PACAP-deficient mice, as measured by qRT-PCR (Figure 6). While expression of Nurr1 and Nor1 returned to baseline after the initial peak, Nur77 was still elevated after 3 h of restraint in wild-types and significantly higher than control after 6 h in PACAP-deficient mice. PACAP-dependent induction of all three factors occurred in the adrenal cortex, with some degree of zonal differences (Figure 7). Thus, Nur77 was strongly induced throughout the cortex, whereas Nurr1 and Nor1 were most prominently expressed in the outer layers. Hybridisation signals for Nur77 and Nurr1 also appeared to increase after stress in the adrenal medulla, although far less pronounced (Figure 7). Another transcription factor from the nuclear receptor superfamily, SF-1 (Nr5a1), also exhibited a PACAP-dependent expression pattern in response to restraint (Figure 6). In wild-type mice, expression in the adrenal glands was robustly elevated as early as 1 h after stressor exposure, reaching a maximum at 3 h. In contrast, PACAP-deficient mice displayed only a modest increase at 1 h that was maintained throughout the time course of the experiment, to the effect that peak levels were more than 50% lower compared to wild-types.

To determine whether PACAP-dependent corticosterone secretion is driven through activation of the biosynthetic pathway, we measured transcript levels of factors involved in

adrenocortical steroidogenesis (Figure 8). Steroidogenic acute regulatory protein (StAR) is the rate-limiting factor for steroidogenesis, controlling the transport of cholesterol to the mitochondrial site of steroid hormone biosynthesis. StAR was significantly induced in response to restraint in a PACAP-dependent fashion. Expression was elevated in wild-types at all time points tested, with a maximum at 3 h. After 1 h and 3 h of restraint, StAR levels were significantly higher in adrenal glands from wild-type compared to PACAP-deficient mice. Expression of mRNA encoding steroid 21-hydroxylase (P450c21) was rapidly induced 1.7-fold in wild-type mice after 1 h of restraint, subsequently returning to baseline. No induction occurred in PACAP-deficient mice (Figure 8).

Discussion

Hypophysiotropic neurones within the hypothalamic PVN are key regulators of the HPA axis, expressing and releasing CRH in response to a variety of stressors (16). PACAP-containing nerve terminals innervate these neurones (27), and intracerebroventricular (i.c.v.) administration of PACAP results in enhanced CRH mRNA synthesis (28) as well as corticosterone secretion (29). Thus, PACAP has emerged as a candidate for central control of the HPA axis. Strongly supporting this notion, we recently demonstrated that sustained corticosterone secretion is diminished, and CRH mRNA upregulation is abolished, in PACAP-deficient mice exposed to restraint (4). Our present results suggest that PACAP-dependent stimulus-transcription coupling is required for fully functional stress responses, and link Nr4a gene regulation throughout the HPA axis to a signaling cascade initiated by up-regulation of CRH mRNA in the hypothalamus. The site of action of PACAP in controlling CRH synthesis is likely to be the PVN itself, as PACAP also induces CRH gene transcription in hypothalamic neurones in culture (Figure 4).

The Nr4a family of orphan nuclear receptors is rapidly upregulated in a variety of cells and tissues in response to diverse stimuli, mediating a wide range of physiological functions via ligand-independent effects on gene transcription (30). Monomeric and homo- or heterodimeric DNA binding of Nur77, Nurr1 and Nor1 to their shared response elements (NBRE and NurRE) activates target gene promoters, with specific combinations of Nr4a factors allowing for gene- and sequence-specific response patterns (31). Of great interest with respect to HPA axis function is the finding that all three Nr4a factors also antagonise glucocorticoid receptor-mediated negative feedback, both through direct protein-protein interactions with the glucocorticoid receptor (GR) and competition for binding to DNA response elements (9, 32). PACAP-dependent upregulation of Nur77 and Nor1 in the PVN observed in our present study (Figures 2 and 3) could thus potentially affect CRH expression in two ways, i.e. directly, via activation of the CRH promoter (7), and/or indirectly, by inhibiting GR-mediated negative feedback (9, 32). The latter scenario has previously been proposed for the AP-1 transcription factors Fos and Jun (15), and restraint-induced expression of Fos in CRH-positive cells of the PVN is strongly attenuated in PACAP-deficient mice (24). Supporting the notion that PACAP directly controls Nr4a expression in the hypothalamus, we have previously found Nr4a factors to be rapidly induced by PACAP in a neuroendocrine cell line expressing the PACAP-specific PAC1 receptor (33), and preliminary results suggest this is also the case in rat hypothalamic neurones (data not shown).

In mouse pituitary cells and the pituitary-derived cell line AtT-20, mRNAs encoding the three Nr4a factors have been shown to be induced by treatment with CRH (6, 7, 31). Thus, PACAP-dependent upregulation of Nr4a factors *in vivo*, as reported here (Figure 5), may serve as a surrogate marker for PACAP-dependent CRH release and activation of the pituitary gland. Furthermore, overexpression of Nur77 in AtT-20 cells antagonises GR-mediated inhibition of ACTH secretion (34), indicating that PACAP-dependent upregulation

of Nr4a factors could maintain elevated levels of circulating ACTH by blocking glucocorticoid negative feedback. Although Nor1 appeared to be expressed at higher levels in PACAP-deficient pituitaries after 6 h of restraint, results were quite variable and thus difficult to interpret.

In the adrenal glands, the effector organs of the HPA axis, we found all three Nr4a family members to be PACAP-dependently upregulated (Figures 6 and 7). Rapid and pronounced increases in adrenocortical Nur77 and Nurr1 expression during stress are consistent with the literature (22, 23) whereas, to our knowledge, we are the first to describe stressor-induced increases in Nor1 abundance. Expression patterns were somewhat different among the factors, with Nur77 showing strong upregulation throughout the cortex, while Nurr1 and Nor1 were most prominently induced in the outer layers (Figure 7). This pattern is also in agreement with previous reports and identical with that elicited by ACTH injection (22), suggesting that PACAP-dependent Nr4a induction in adrenocortical cells is a consequence of the PACAP-dependent ACTH secretion reported here.

All of the factors required for adrenocortical steroid biosynthesis, including those that produce corticosterone, are targets for ACTH-mediated signalling, and most of them are subject to transcriptional control by SF-1 (35). In addition to the Nr4a factors, as described above, SF-1 mRNA was significantly and PACAP-dependently induced in the present experiments, with expression reaching a peak after 3 h of restraint (Figure 6). Steroid 21-hydroxylase (P450c21), upregulated in wild-types after 1 h of restraint, was unresponsive in PACAP-deficient mice, i.e. also clearly PACAP-dependent (Figure 8).

Of greatest interest regarding adrenocortical function and corticosterone production, we found mRNA encoding steroidogenic acute regulatory protein (StAR), the limiting factor for all steroidogenic processes, to be PACAP-dependently induced in the adrenal glands in response to stress (Figure 8). StAR mRNA and protein production are tightly linked and tightly coupled to steroid biosynthesis in steroidogenic cells (36), and transcriptional activators include SF-1 as well as the Nr4a family (20, 21, 37). Since Nur77, Nurr1 and Nor1 were all PACAP-dependently induced in the adrenocortical compartment, in addition to SF-1, our data suggest that their upregulation and subsequent increases in StAR provide the link between the PACAP-dependent ACTH release and PACAP-dependent sustained corticosterone secretion demonstrated in the present paper. Historically, it was thought that the adrenal cortex stores little of its secretory products (38), whereas recent data raise the possibility that some form of storage occurs (39). Our results clearly indicate that ongoing adrenocortical steroidogenesis is necessary for maintenance of corticosterone output during persistent stressor exposure, and that PACAP is required for this response. We and others have noted variations in the PACAP-dependence of the initial corticosterone elevation among cohorts of mice following restraint, ranging from no PACAP-dependence at 1 h to >40% reduction at 1 h in PACAP-deficient mice [(4), (24), and Figure 1 in this report]. What is invariant is that the PACAP-dependence of the initial (30–60 min) corticosterone elevation is less than the PACAP-dependence at later times (>60 min). This is consistent with a brief initial elevation triggered by release of previously stored CRH in hypothalamic nerve terminals, which is independent of PACAP, followed by increasing PACAP-dependence of corticosterone elevation, reflecting the role of hypothalamic PACAP in stimulus-secretion-transcription coupling in CRH neurones after more prolonged stressor exposure.

It was recently shown that corticosterone secretion induced by systemic injections of CRH or ACTH is not different between PACAP-deficient and wild-type mice (24), suggesting that pituitary and adrenal glands are largely intact in the absence of PACAP. Taken together with what we proposed based on our previous findings (4) and present data, this strengthens

the concept of PACAP acting centrally to regulate the stress response induced by restraint. In other words, CRH neurones of the PVN appear to be the principal site at which PACAP-dependent control of HPA output occurs by means of ongoing stimulus-secretion-transcription coupling.

In conclusion, we show that PACAP is required to maintain output of the HPA axis during prolonged periods of stimulation. Our data indicate that this is mediated via direct PACAP-dependent activation of CRH-positive hypophysiotropic neurones in the PVN, followed by ACTH release, ACTH-stimulated regulation of adrenocortical gene expression, and finally corticosterone production and secretion. In addition, our results suggest that these PACAP-dependent effects involve induction of Nr4a orphan nuclear receptors in the PVN, pituitary and adrenal cortex, supporting the view that these factors play an integrative role throughout the HPA axis.

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Figure 1.

Secretion of ACTH and corticosterone elicited by restraint is blunted in PACAP-deficient mice. (A) Hormone levels in serum were measured after 1 h and 6 h of restraint in PACAP-deficient and wild-type mice ($N = 5-7$ per time point). Values are means $\pm$ standard error of the mean (SEM). Pound signs indicate significant difference from the respective genotype's control (untreated). One-way analysis of variance (ANOVA) with Dunnett's multiple comparison test ($^{##}p < 0.01$). Asterisks indicate significant difference between genotypes. Two-way ANOVA with Bonferroni posttest ($^{**}p < 0.01$). (B) The early phase of secretion was assessed by measuring serum hormone levels after 30 minutes of restraint ($N = 8-10$ per time point). Values are means $\pm$ SEM. Pound signs indicate significant difference from the respective genotype's untreated control ($^{##}p < 0.01$, $^{###}p < 0.001$). Asterisks indicate significant difference between genotypes ($^{*}p < 0.05$, $^{**}p < 0.01$). One-way ANOVA with Newman-Keuls multiple comparison test.

Figure 2.

Stimulus-transcription coupling induced in the hypothalamus by restraint depends on PACAP. Transcript levels were measured by qRT-PCR. Data are displayed as means $\pm$ SEM ($N = 5-6$ per time point). Pound signs indicate significant difference from the respective genotype's control (untreated). One-way ANOVA with Dunnett's multiple comparison test ($^{\#}p < 0.05$, $^{##}p < 0.01$). Asterisks indicate significant difference between genotypes. Two-way ANOVA with Bonferroni posttest ($^{**}p < 0.01$, $^{***}p < 0.001$).

Figure 3.

Restraint-induced expression of Nr4a transcription factors overlaps with CRH in the hypothalamic paraventricular nucleus (PVN). Frozen coronal sections of brains from wild-type (+/+) and PACAP-deficient mice (-/-) were hybridised with ³⁵S-UTP-labelled cRNA probes for CRH, Nur77 and Nor1, and hybridisation was visualised by autoradiography. (A) Expression of CRH mRNA in the hypothalamus, as assessed on autoradiography films, is limited to the PVN. No obvious differences exist between untreated wild-type and PACAP-deficient mice, whereas restraint-induced upregulation occurs only in wild-types. Dotted box indicates the region excised for RNA extraction and qRT-PCR (see “Materials and Methods”, and compare Figure 2 for quantitative results). (B) Sections were coated with autoradiography emulsion subsequent to in situ hybridisation. After exposure (68 h for CRH, 92 h for Nur77, 90 h for Nor1) and development, silver grains representing specific hybridisation signals were visualised using darkfield microscopy. The PVN from one hemisphere is depicted for each mouse. Note expression of Nor1 in cells lining the third ventricle, which was only observed in stressed (restraint 1 h) wild-type mice. Scale bar in B = 100 μm.

Figure 4.

PACAP induces CRH gene transcription in hypothalamic neurones. (A) Concentration-response curve of CRH hnRNA expression after 45 minutes of treatment with PACAP-38. (B) Time course of CRH transcription after exposure to 3 nM PACAP-38. (C) Comparison of effects elicited by PACAP-38 (4 nM) or forskolin (1 μ M). Abundance of CRH hnRNA was measured in primary cultures of rat hypothalamic neurones by intronic qRT-PCR. Data are expressed as fold changes (means $\pm$ SEM) compared to untreated controls and represent pooled values from 2–5 experiments, each condition being assayed in duplicate per experiment. Pound signs indicate values that are significantly different from control. One-way ANOVA with Fisher's LSD test ($\#p < 0.05$, $##p < 0.01$, $###p < 0.001$).

Figure 5.

Stimulus-transcription coupling induced in the pituitary gland by restraint depends on PACAP. Transcript levels of Nr4a transcription factors were measured by qRT-PCR. Data are displayed as means $\pm$ SEM (N = 5–6 per time point). Pound signs indicate significant difference from the respective genotype's control (untreated). One-way ANOVA with Dunnett's multiple comparison test ([#]p<0.05, ^{##}p<0.01). Asterisks indicate significant difference between genotypes. Two-way ANOVA with Bonferroni posttest (*p<0.05, ***p<0.001).

Figure 6.

Upregulation of inducible transcription factors is blunted in PACAP-deficient adrenal glands. Transcript levels were measured by qRT-PCR. Data are displayed as means $\pm$ SEM (N = 5 per time point). Pound signs indicate significant difference from the respective genotype's control (untreated). One-way ANOVA with Dunnett's multiple comparison test ([#]p<0.05, ^{##}p<0.01). Asterisks indicate significant difference between genotypes (Two-way ANOVA with Bonferroni posttest (***)p<0.001).

Figure 7. PACAP-dependent induction of Nr4a transcription factors occurs in the adrenal cortex. Frozen sections of adrenal glands from wild-type (+/+) and PACAP-deficient mice (-/-) were hybridised with ³⁵S-UTP-labelled cRNA probes for Nur77, Nurr1 and Nor1. Sections were coated with autoradiographic emulsion, and after exposure (44 h for Nur77, 48 h for Nurr1, 68 h for Nor1) and development, silver grains representing specific hybridisation signals were visualised using darkfield microscopy. Scale bar = 500 μ m.

Figure 8.

Induction of steroidogenic acute regulatory protein (StAR) and steroid 21-hydroxylase (P450c21) in the adrenal glands during stress is PACAP-dependent. Transcript levels were measured by qRT-PCR. Data are displayed as means $\pm$ SEM (N = 5–6 per time point). Pound signs indicate significant difference from the respective genotype's control (untreated). One-way ANOVA with Dunnett's multiple comparison test ([#]p<0.05, ^{##}p<0.01). Asterisks indicate significant difference between genotypes. Two-way ANOVA with Bonferroni posttest (^{*}p<0.05, ^{**}p<0.01, ^{***}p<0.001).

Table 1

Primers used for qRT-PCR and generation of cRNA probe templates. (A) Primers were retrieved from Primer bank (<http://pga.mgh.harvard.edu/primerbank/index.html>) (40) or designed using Primer3 (available online, e.g. at <http://frodo.wi.mit.edu/>). All primers are based on mouse target sequences, except for rat primers rCRH. Rat primers for CRH amplify hnRNA by targeting the intron of the CRH gene. (B) Primers were designed using Primer3 and the indicated official reference sequences (RefSeq). * PCR amplification of subcloned fragments using SP6 and T7 primers yields different amplicon sizes for each fragment. Thus, no amplicon size is indicated for these two primers. n.a., not applicable.

Table 1A: Primers for qRT-PCR			
primer	sequence (5'-3')	amplicon [bp]	Primer Bank ID
CRH-F	CTCAGCAAGCTCACAGCAAC	141	n.a.
CRH-R	GGAGCTGCGATATGGTACAGA		
rCRH-F	TCAATCCAATCTGCCACTCA	150	n.a.
rCRH-R	TAAGCTATTCGCCCGCTCTA		
GAPDH-F	CCATCACTGCCACCCAGAAGA	164	n.a.
GAPDH-R	GACACATTGGGGGTAGGAACA		
Nur77-F	TTGAGTTCGGCAAGCCTACC	100	6754216a1
Nur77-R	GTGTACCCGTCCATGAAGGTG		
Nurr1-F	AACACTGAAATTACTGCCACCA	138	7305325a2
Nurr1-R	TTCTACCTTAATGGAGGACTGCT		
Nor1-F	AGGATTCACTGATCTCCCAA	140	7657397a1
Nor1-R	GATGCAGGACAAGTCCATTGC		
SF-1-F	GCCCTGTTGGATTACACCTTG	135	20522231a2
SF-1-R	GTTGCCCAAATGCTTGTGGTA		
StAR-F	ATGTTCTCGCTACGTTCAAG	122	31543776a1
StAR-R	CCCAGTGCTCTCCAGTTGAG		
P450c21-F	TACAGACCCTTCACGACTGTG	102	6753570a2
P450c21-R	GGTTGGGGAGGAACCTGAG		
Table 1B: Primers for generation of cRNA probe templates			
primer	sequence (5'-3')	amplicon [bp]	target RefSeq
CRH-F2	ATCCGCATGGGTGAAGAATA	408	NM_205769.1
CRH-R2	AAGCGCAACATTTTCATTTC		
Nur77-F2	CAATGCTTCGTGTCAGCACT	450	NM_010444.1
Nur77-R2	TCTGCCCACTTTCGGATAAC		
Nurr1-F2	AGTCTGATCAGTGCCCTCGT	432	NM_013613.1
Nurr1-R2	AATGCAGGAGAAGGCAGAAA		
Nor1-F2	GTGTCGGGATGGTTAAGGAA	455	NM_015743.2
Nor1-R2	CTGAAGTCGATGCAGGACAA		
SP6	CGATTTAGGTGACACTATAG	*	n.a.
T7	TAATACGACTCACTATAGGG	*	n.a.