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Stress hormone synthesis in mouse hypothalamus and adrenal gland triggered by restraint is dependent on PACAP signaling

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Abstract

Stress responses are elicited by a variety of stimuli and are aimed at counteracting direct or perceived threats to the well-being of an organism. In the mammalian central and peripheral nervous systems, specific cell groups constitute signaling circuits that indicate the presence of a stressor and elaborate an adequate response, ultimately restoring homeostasis. Pituitary adenylate cyclase-activating polypeptide (PACAP) is expressed in central and peripheral parts of these circuits and has recently been identified as a candidate for regulation of the stress axis. In the present experiments, we tested the involvement of PACAP in the response to a psychological stressor in vivo. We used a restraint paradigm and compared PACAP-deficient mice (PACAP^{-/-}) to wild-type controls (PACAP^{+/+}). Acute secretion of corticosterone elicited by 1h of restraint was found to be identical between genotypes, whereas sustained secretion provoked by 6h of unrelieved restraint was 48% lower in PACAP^{-/-} mice. Within the latter time frame, expression of mRNA encoding corticotropin-releasing hormone (CRH) was increased in the hypothalamus of wild-type, but not PACAP-deficient mice. Expression of hypothalamic activity-regulated transcription factors (Egr1, Fos) was rapidly and transiently induced by restraint in a PACAP-dependent fashion, a pattern that was also found in the adrenal glands. Here, abundance of transcripts encoding enzymes required for adrenomedullary catecholamine biosynthesis (TH, PNMT) was higher in PACAP^{+/+} mice after 6h of unrelieved restraint. Our results suggest that sustained corticosterone secretion, synthesis of the hypophysiotropic hormone CRH in the hypothalamus, as well as enzymes producing the hormone adrenaline in the adrenal medulla, are controlled by PACAP signaling in the mouse. These findings identify PACAP as a major potential contributor to the stimulus-secretion-synthesis coupling that supports stress responses in vivo.

Keywords

Restraint; CRH; HPA axis; pituitary adenylate cyclase-activating polypeptide; neuropeptide; stimulus-secretion-synthesis coupling

When organisms are confronted with a direct or perceived threat to their homeostasis, they react by mounting stress responses to counter the initial stimulus. In mammals, different types of stimuli (e.g. psychological vs. metabolic stressors) elicit these responses via activation of

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stressor-specific neurocircuitry (Pacak and Palkovits, 2001, Kvetnansky et al, 2009). Stress mediators such as adrenocorticotrophic hormone, adrenaline and noradrenaline are subsequently released in specific patterns, reflecting the degree of hypothalamic-pituitary-adrenocortical (HPA), adrenomedullary, and sympathetic nervous system activation (Goldstein and Kopin, 2008). Although being stressor-specific, all stress responses are centrally integrated in the paraventricular nucleus (PVN) of the hypothalamus (Herman and Cullinan, 1997, Herman et al., 2008), and the adrenal glands are their major peripheral effectors (Goldstein and Kopin, 2007, Goldstein and Kopin, 2008). The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) has recently emerged as an important candidate in regulation of central and peripheral components of the stress axis. Therefore, we tested whether the response to a psychological stressor (restraint) is altered at either of the two major stress-transducing loci, the hypothalamus and adrenal glands, in PACAP-deficient mice.

Experimental procedures

Animals and restraint paradigm

Male mice (3.4–5.4 months of age) harboring the PACAP^{-/-} allele (Hamelink et al., 2002) were used in the present study and compared to age-matched PACAP^{+/+} (wild-type). All mice represent a full backcross of the knock-out allele into the C57BL/6N strain. They were housed in a temperature- and humidity-controlled facility with 12h light/dark cycle (lights on at 6:00 AM) and had access to chow and water ad libitum. Experiments were conducted in a separate room to which animals were transferred approximately 18h before treatment. At this point, they were placed in individual home cages. All experiments were started between 9:00–10:30 AM. Mice were restrained in tapered plastic film envelopes (DecapiCones, Braintree Scientific, Braintree, USA) such that they were held immobile, with all four limbs underneath them, unable to turn around or perform significant movement. Care was taken to assure unobstructed breathing of the animals. Mice were assigned to one of four groups: untreated (non-stressed controls), 1h (restrained for 1h), 1h + 5h (restrained for 1h, then released to home cage for 5h) and 6h (restrained continuously for 6h). At the end of the treatment period, mice were decapitated while still restrained (groups 1h and 6h). Untreated (non-stressed controls) and restraint-released mice (group 1h + 5h) were picked up from their home cage by the tail, placed in DecapiCones and decapitated immediately (time from pickup to decapitation <20 seconds). Both adrenal glands per animal were quickly isolated and frozen on dry ice (solid CO₂). For sampling of hypothalamus, brains were removed from the skull and transferred to an ice-chilled rodent brain matrix (RBM-2000C, ASI Instruments, Warren, USA). Using razor blades, two coronal cuts were made, one through the optic chiasm (approx. Bregma 0.02) and the second through the mammillary bodies (approx. Bregma -3.08). From the resulting slice, a tissue block of 2 × 2 mm (width × height) was obtained by cutting just dorsal of the third ventricle and 1 mm each left and right of the midline. Tissue samples were stored at -80°C until use. All procedures were approved by the National Institute of Mental Health Animal Care and Use Committee.

Serum collection and radioimmunoassay for corticosterone

Trunk blood was collected into 1.5 ml tubes immediately after decapitation and allowed to coagulate at room temperature for approximately one hour. Samples were subsequently centrifuged (10 minutes at 10,000 × g) and serum was transferred to fresh tubes. After a second centrifugation step, samples were stored at -80°C until assayed. Corticosterone levels were determined by radioimmunoassay (Coat-A-Count, Siemens Medical Solutions Diagnostics, Los Angeles, USA) according to the manufacturer's instructions. Statistical analysis was performed as for qRT-PCR results (see below).

RNA extraction, reverse transcription, and quantitative PCR

For analysis of gene expression, adrenal (both glands per mouse) and hypothalamic tissue samples were transferred to lysis buffer (RNeasy Mini, Qiagen, Valencia, USA) and quickly homogenized using an ultrasonic processor (GE 130PB, Hielscher, Ringwood, USA). RNA extraction from homogenates was performed using a commercial kit according to the manufacturer's instructions (RNeasy Mini, Qiagen) and genomic DNA was removed by digestion with RNase-free DNase I (Roche Applied Science, Indianapolis, USA). An aliquot of each sample, corresponding to 0.5 µg RNA, was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). Quantitative PCR (samples assayed in duplicate) was carried out with 200 nmol gene-specific primers and iQ SYBR Green Supermix (Bio-Rad, Hercules, USA) on an iCycler iQ Real Time PCR System (Bio-Rad).

Calculation of transcript abundance from qRT-PCR results and statistical analysis

Absolute transcript levels were calculated as femtogram or picogram mRNA per tissue or per region, i.e. per pair of adrenal glands or per hypothalamus, based on the total RNA and cDNA aliquots used for reverse transcription and quantitative PCR, respectively, and the qPCR threshold cycles of gene-specific standard curves. Values were imported into Prism 4 (GraphPad Software Inc., La Jolla, USA) and differences between untreated and stressed mice within genotype were analyzed using one-way ANOVA with Dunnett's posttest. Differences between PACAP genotypes at each time point were analyzed by two-way ANOVA with Bonferroni posttest. Radioimmunoassay results were subjected to the same statistical tests.

Results

Restraint caused marked elevation of corticosterone secretion in both genotypes within 1h (Fig. 1A). However, only PACAP^{+/+} mice maintained high serum levels during prolonged restraint, while secretion was significantly impaired in PACAP^{-/-} (dotted line). Thus, after 6h of unrelieved restraint (group 6h), corticosterone levels were reduced by 48% compared with PACAP^{+/+} (Fig. 1A). Within the same time frame, hypothalamic levels of corticotropin-releasing hormone (CRH) mRNA increased significantly in PACAP^{+/+} mice, while no induction at all was observed in PACAP^{-/-} animals (Fig. 1B). Notably, when CRH mRNA was measured 5h after a 1h period of restraint (group 1h + 5h) no such induction was observed, suggesting that persistent stressor exposure is required to elicit compensatory CRH expression. Messenger RNAs encoding neuronal activity-regulated transcription factors were rapidly and transiently induced in the hypothalamus by restraint (Fig. 1C). Expression levels of Egr1 (early growth response 1) and Fos (FBJ osteosarcoma oncogene) peaked at 1h, at which point they were significantly higher in PACAP^{+/+} mice, returning to baseline thereafter. No difference was found between the 6h and 1h + 5h groups, indicating that the induction of activity-regulated Egr1 and Fos expression by restraint is inherently transient, even in the continued presence of the stressor.

In the adrenal glands, expression patterns of Egr1 and Fos were remarkably similar to those observed in the hypothalamus after restraint. Transcript abundance was increased in both genotypes, but the response in PACAP^{-/-} mice was significantly impaired, such that mRNA levels were <50% relative to PACAP^{+/+} animals (Fig. 2A). As in the hypothalamus, there was no significant difference between the 6h and 1h + 5h groups, although a trend towards higher expression levels after prolonged stressor exposure was evident (Fig. 2A). Transcript levels of tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT), two adrenomedullary enzymes required for catecholamine synthesis, were also PACAP-dependently induced after restraint. As in the case of hypothalamic CRH, continued presence of the stressor was required to elicit this response (compare groups 6h and 1h + 5h, Fig. 2B). Although no statistically significant upregulation was found for TH mRNA within genotypes,

abundance was higher in PACAP^{+/+} compared to PACAP^{-/-} mice after 6h of unrelieved restraint. On the other hand, PNMT mRNA was clearly induced in both genotypes, with transcript levels being significantly higher in PACAP^{+/+} adrenal glands (Fig. 2B).

Discussion

Neuropeptides are preferentially released under conditions of intense neural activation and thus represent “the language of the stressed nervous system” (Hokfelt et al., 2003). Recent evidence has implicated PACAP in the control of stress responses at multiple central and peripheral levels (Vaudry et al., 2009). PACAP-immunoreactive nerve fibers are numerous in the PVN (Hannibal, 2002), where PACAP-containing terminals innervate CRH-positive neurons (Legradi et al., 1998) and i.c.v. injection of PACAP causes upregulation of CRH mRNA (Grinevich et al., 1997). Tracer studies have demonstrated expression of PACAP in subregions of the hypothalamus and catecholaminergic neurons of the brain stem that project to the PVN and the intermediolateral column of the spinal cord (Das et al., 2007, Farnham et al., 2008). Finally, PACAP is expressed in sympathetic terminals innervating the adrenal gland (Frodin et al., 1995, Holgert et al., 1996) where it co-localizes with the vesicular acetylcholine transporter at the adrenomedullary synapse and is required for maintaining catecholamine secretion caused by sustained stress in vivo (Hamelink et al., 2002) and prolonged nerve firing in adrenal slices in vitro (Kuri et al., 2009). Thus, PACAP-containing neurons are positioned to mediate afferent signaling to the PVN, intrahypothalamic signal integration, and outflow to peripheral effectors of the stress response, thereby modulating activity of the HPA axis, adrenomedullary and sympathetic nervous systems. Functional studies in vivo further show that PACAP elicits behavioral and neuroendocrine responses characteristic of stress (Agarwal et al., 2005, Norrholm et al., 2005).

Our present experiments provide direct evidence for PACAP's involvement in mediating stress responses by using PACAP-deficient mice tested in the restraint model. First, corticosterone secretion elicited by prolonged restraint is impaired in PACAP^{-/-} mice, accompanied by a lack of induction of hypothalamic CRH mRNA. CRH is the main regulator of the HPA axis, and its biosynthesis is maintained after release during stress primarily through enhanced gene transcription (Aguilera et al., 2007). Thus, the biosynthesis of two hormones within the activated HPA axis is reduced in the absence of PACAP. Second, hypothalamic induction by restraint of the transcription factors Egr1 and Fos, both of which are markers for neuronal stimulation and have been shown to be upregulated in the parvocellular PVN during stress responses (Honkaniemi et al., 1994, Umemoto et al., 1994, Watanabe et al., 1994), is PACAP-dependent. While immediate-early gene (IEG) products such as Fos do not appear to mediate acute increases in CRH gene transcription (Imaki et al., 1996, Kovacs and Sawchenko, 1996a, Kovacs and Sawchenko, 1996b), they are indicative of excitatory input impinging on target cells within the PVN and could be involved in upregulation of CRH during prolonged stressor exposure (Yao and Denver, 2007). Therefore, future experiments will test the hypothesis that PACAP-dependent IEG products drive the long-term PACAP-dependent increase in CRH expression observed in the present study. Our data do not rule out the possibility that PACAP-dependent effects during stress originate in other brain areas, e.g. the bed nucleus of the stria terminalis feeding into the PVN (Hammack et al., 2009). However, we favor the hypothalamus itself as the site of PACAP's action, based on our IEG expression data as well as known cellular and behavioral effects of intra-PVN infusion of PACAP (Huang et al., 1996, Norrholm et al., 2005).

Restraint-induced gene expression changes in the adrenal glands provide further evidence for PACAP-dependent stimulation of the stress axis. First, induction of Egr1 and Fos is strongly blunted in PACAP^{-/-} mice. Second, abundance of TH and PNMT mRNA is higher in PACAP^{+/+} mice after prolonged restraint. The latter finding suggests that the compensatory

catecholamine synthesis that occurs during restraint and is correlated with enhanced PNMT mRNA expression (Tai et al., 2007) may also depend on PACAP. Based on the time course of transcript expression, it is tempting to speculate that PACAP-dependent upregulation of IEGs after 1h and enzyme mRNAs after 6h of restraint might be linked, given that all four candidates are known to be induced in the adrenal glands by stress, and the fact that Egr1 and AP-1 transcription factors are known regulators of TH and PNMT expression (Wong et al., 2002, Kvetnansky et al, 2009).

Conclusion

Our results provide compelling evidence that PACAP is required for sustained function of the stress axis under conditions of persistent stressor exposure. By controlling corticosterone secretion, transcriptional induction of activity-regulated genes, and key neuroendocrine factors in the hypothalamus and adrenal gland, PACAP could serve as a master integrator of stress signaling in the central and peripheral nervous system.

Acknowledgments

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List of abbreviations

ANOVA	analysis of variance
AP-1	activator protein 1
cDNA	complementary DNA
CRH	corticotropin-releasing hormone
DNA	deoxyribonucleic acid
Egr1	early growth response 1
fg	femtogram
Fos	FBJ osteosarcoma oncogene
HPA axis	hypothalamic-pituitary-adrenocortical axis
IEG	immediate-early gene
mRNA	messenger ribonucleic acid
PACAP	pituitary adenylate cyclase-activating polypeptide
PCR	polymerase chain reaction
pg	picogram
PNMT	phenylethanolamine N-methyltransferase
PVN	paraventricular nucleus of the hypothalamus
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RNA	ribonucleic acid
SEM	standard error of the mean
TH	tyrosine hydroxylase

References

- Agarwal A, Halvorson LM, Legradi G. Pituitary adenylate cyclase-activating polypeptide (PACAP) mimics neuroendocrine and behavioral manifestations of stress: Evidence for PKA-mediated expression of the corticotropin-releasing hormone (CRH) gene. *Molecular Brain Research* 2005;138:45–57. [PubMed: 15882914]
- Aguilera G, Kiss A, Liu Y, Kamitakahara A. Negative regulation of corticotropin releasing factor expression and limitation of stress response. *Stress* 2007;10:153–161. [PubMed: 17514584]
- Das M, Vihlen CS, Legradi G. Hypothalamic and brainstem sources of pituitary adenylate cyclase-activating polypeptide nerve fibers innervating the hypothalamic paraventricular nucleus in the rat. *Journal of Comparative Neurology* 2007;500:761–776. [PubMed: 17154257]
- Farnham MM, Li Q, Goodchild AK, Pilowsky PM. PACAP is expressed in sympathoexcitatory bulbospinal C1 neurons of the brain stem and increases sympathetic nerve activity in vivo. *Am J Physiol Regul Integr Comp Physiol* 2008;294:R1304–1311. [PubMed: 18272663]
- Frodin M, Hannibal J, Wulff BS, Gammeltoft S, Fahrenkrug J. Neuronal localization of pituitary adenylate cyclase-activating polypeptide 38 in the adrenal medulla and growth-inhibitory effect on chromaffin cells. *Neuroscience* 1995;65:599–608. [PubMed: 7777172]
- Goldstein DS, Kopin IJ. Evolution of concepts of stress. *Stress* 2007;10:109–120. [PubMed: 17514579]
- Goldstein DS, Kopin IJ. Adrenomedullary, adrenocortical, and sympathoneural responses to stressors: a meta-analysis. *Endocr Regul* 2008;42:111–119. [PubMed: 18999898]
- Grinevich V, Fournier A, Pelletier G. Effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on corticotropin-releasing hormone (CRH) gene expression in the rat hypothalamic paraventricular nucleus. *Brain Res* 1997;773:190–196. [PubMed: 9409720]
- Hamelink C, Tjurmina O, Damadzic R, Young WS, Weihe E, Lee HW, Eiden LE. Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. *Proc Natl Acad Sci USA* 2002;99:461–466. [PubMed: 11756684]
- Hammack SE, Cheung J, Rhodes KM, Schutz KC, Falls WA, Braas KM, May V. Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): roles for PACAP in anxiety-like behavior. *Psychoneuroendocrinology* 2009;34:833–843. [PubMed: 19181454]
- Hannibal J. Pituitary adenylate cyclase-activating peptide in the rat central nervous system: an immunohistochemical and in situ hybridization study. *J Comp Neurol* 2002;453:389–417. [PubMed: 12389210]
- Herman JP, Cullinan WE. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* 1997;20:78–84. [PubMed: 9023876]
- Herman JP, Flak J, Jankord R. Chronic stress plasticity in the hypothalamic paraventricular nucleus. *Prog Brain Res* 2008;170:353–364. [PubMed: 18655895]
- Hokfelt T, Bartfai T, Bloom F. Neuropeptides: opportunities for drug discovery. *Lancet Neurol* 2003;2:463–472. [PubMed: 12878434]
- Holger H, Holmberg K, Hannibal J, Fahrenkrug J, Brimijoin S, Hartman BK, Hokfelt T. PACAP in the adrenal gland--relationship with choline acetyltransferase, enkephalin and chromaffin cells and effects of immunological sympathectomy. *Neuroreport* 1996;8:297–301. [PubMed: 9051799]
- Honkaniemi J, Kononen J, Kainu T, Pyykonen I, Peltto-Huikko M. Induction of multiple immediate early genes in rat hypothalamic paraventricular nucleus after stress. *Brain Res Mol Brain Res* 1994;25:234–241. [PubMed: 7808222]
- Huang Q, Legradi G, Arimura A. Perfusion of the paraventricular nucleus with pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide stimulates local release of norepinephrine and its metabolite: microdialysis study in freely moving rats. *Ann N Y Acad Sci* 1996;805:737–742. [PubMed: 8993470]
- Imaki T, Shibasaki T, Chikada N, Harada S, Naruse M, Demura H. Different expression of immediate-early genes in the rat paraventricular nucleus induced by stress: relation to corticotropin-releasing factor gene transcription. *Endocr J* 1996;43:629–638. [PubMed: 9075602]
- Kovacs KJ, Sawchenko PE. Regulation of stress-induced transcriptional changes in the hypothalamic neurosecretory neurons. *J Mol Neurosci* 1996a;7:125–133. [PubMed: 8873896]

- Kovacs KJ, Sawchenko PE. Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. *J Neurosci* 1996b;16:262–273. [PubMed: 8613792]
- Kuri BA, Chan SA, Smith CB. PACAP regulates immediate catecholamine release from adrenal chromaffin cells in an activity-dependent manner through a protein kinase C-dependent pathway. *J Neurochem* 2009;110:1214–1225. [PubMed: 19508428]
- Kvetnansky R, Sabban EL, Palkovits M. Catecholaminergic systems in stress: structural and molecular genetic approaches. *Physiol Rev* 2009;89:535–606. [PubMed: 19342614]
- Legradi G, Hannibal J, Lechan RM. Pituitary adenylate cyclase-activating polypeptide-nerve terminals densely innervate corticotropin-releasing hormone-neurons in the hypothalamic paraventricular nucleus of the rat. *Neurosci Lett* 1998;246:145–148. [PubMed: 9792613]
- Norholm SD, Das M, Legradi G. Behavioral effects of local microinfusion of pituitary adenylate cyclase activating polypeptide (PACAP) into the paraventricular nucleus of the hypothalamus (PVN). *Regul Pept* 2005;128:33–41. [PubMed: 15721485]
- Pacak K, Palkovits M. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. *Endocr Rev* 2001;22:502–548. [PubMed: 11493581]
- Tai TC, Claycomb R, Siddall BJ, Bell RA, Kvetnansky R, Wong DL. Stress-induced changes in epinephrine expression in the adrenal medulla in vivo. *J Neurochem* 2007;101:1108–1118. [PubMed: 17394532]
- Umemoto S, Kawai Y, Senba E. Differential regulation of IEGs in the rat PVH in single and repeated stress models. *Neuroreport* 1994;6:201–204. [PubMed: 7703415]
- Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, Fournier A, Chow BK, Hashimoto H, Galas L, Vaudry H. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev* 2009;61:283–357. [PubMed: 19805477]
- Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* 2003;31:e154. [PubMed: 14654707]
- Watanabe Y, Stone E, McEwen BS. Induction and habituation of c-fos and zif/268 by acute and repeated stressors. *Neuroreport* 1994;5:1321–1324. [PubMed: 7919189]
- Wong DL, Anderson LJ, Tai TC. Cholinergic and peptidergic regulation of phenylethanolamine N-methyltransferase gene expression. *Ann N Y Acad Sci* 2002;971:19–26. [PubMed: 12438084]
- Yao M, Denver RJ. Regulation of vertebrate corticotropin-releasing factor genes. *Gen Comp Endocrinol* 2007;153:200–216. [PubMed: 17382944]

Fig. 1.

Impaired activation of the HPA axis in PACAP-deficient mice. **(A)** Acute corticosterone secretion is intact, but prolonged restraint reveals an insufficient response in PACAP knock-out mice (dotted line). **(B)** Hypothalamic transcript levels of CRH are increased by restraint only in wild-type mice (+/+). This response is seen after 6h of unrelieved restraint (group 6h), but not 5h after a 1h period of restraint (group 1h + 5h). **(C)** Expression of inducible transcription factors (markers of cellular activation) is also significantly reduced in PACAP-deficient animals (-/-). Serum levels of corticosterone were measured by radioimmunoassay. Transcript levels (in femtogram or picogram per tissue) were determined by qRT-PCR. Data are displayed as mean $\pm$ SEM ($n = 4-7$ male mice per time point). Pound signs indicate significant difference between untreated and post-treatment time points within each genotype (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;

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Elevation of corticosterone levels after 1h of restraint in A is significant in both genotypes ($^{##}p < 0.01$).

Fig. 2.

The transcriptional response to restraint is impaired in PACAP-deficient adrenal glands. **(A)** As in the hypothalamus, upregulation of inducible transcription factors is blunted in knock-out mice (-/-). **(B)** After 6h of unrelieved restraint (group 6h), expression levels of enzymes involved in catecholamine synthesis are significantly higher in wild-type mice (+/+). Transcript abundance (in picogram per adrenal pair) was determined by qRT-PCR. Data are displayed as mean $\pm$ SEM ($n = 4-6$ male mice per time point). Pound signs indicate significant difference between untreated and post-treatment time points within each genotype (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 in quantitative PCR

Oligonucleotide primers used in the present study. Mouse primers were retrieved from Primer Bank (<http://pga.mgh.harvard.edu/primerbank/index.html>) (Wang and Seed, 2003) or designed using Primer3 (available online, e.g. at <http://frodo.wi.mit.edu/>).

Gene	orientation	sequence (5'-3')	amplicon [bp]	Primer Bank ID
CRH	forward	CTCAGCAAGCTCACAGCAAC	141	n.a.
	reverse	GGAGCTGCGATATGGTACAGA		
Egr1	forward	AGCGAACAACCCTATGAGCAC	100	6681285a2
	reverse	TCGTTTGGCTGGGATAACTCG		
Fos	forward	CGGGTTTCAACGCCGACTA	166	6753894a1
	reverse	TTGGCACTAGAGACGGACAGA		
PNMT	forward	GACTGGAGTGTGTATAGTCAGCA	109	6679405a3
	reverse	CGATAGGCAGGACTCGCTTC		
TH	forward	CTGTCTCGGCTTTGAAAGTG	163	6678337a2
	reverse	GACGCACAGAACTGAGGAGG		