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PACAP: A master regulator of neuroendocrine stress circuits and the cellular stress response

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Abstract

The neuropeptide PACAP is an informational molecule released from stress-transducing neurons. It exerts post-synaptic effects required to complete hypothalamo-pituitary-adrenocortical (HPA) and hypothalamo-splanchnico-adrenomedullary (HSA) circuits activated by psychogenic and metabolic stressors. PACAP-responsive (in cell culture models) and PACAP-dependent (in vivo) transcriptomic responses in the adrenal gland, hypothalamus, and pituitary upon activation of these circuits have been identified. Gene products produced in response circuits during stress include additional neuropeptides and neurotransmitter biosynthetic enzymes and neuroprotective factors. Major portions of HPA and HSA stress responses are abolished in PACAP-deficient mice. This deficit occurs at the level of both the adrenal medulla (HSA axis) and the hypothalamus (HPA axis). PACAP-dependent transcriptional stress responses are conveyed through non-canonical cyclic AMP- and calcium-initiated signaling pathways within the HSA circuit. PACAP transcriptional regulation of the HPA axis, in the hypothalamus, is likely to be mediated via canonical cyclic AMP (cAMP) signaling through protein kinase A.

Keywords

PACAP; stress; HPA axis

The stress response may be defined operationally as the response of the neuroendocrine network to systemic or environmental perturbations outside of the normal physiological range. Stress responses in mammals are mediated through specific neural and neuroendocrine circuits. The propagation of signaling through these circuits is itself a cellular stressor. In other words, a 'stressed neuron' is one propagating an organismic stress response through a neuronal stress circuit. Thus, the cellular stress response is a point of entry to identifying targets for pharmacological modulation of organismic stress perception and processing.

Neuropeptides play a special role in the stress response. As informational molecules stored in large dense-core vesicles, neuropeptides are released preferentially at higher firing rates, as occur during neurotransmission activated by stress in both the central and peripheral nervous systems. Fast and slow transmission are the two major modes of intercellular communication that underlie nervous system function. Fast transmission by glutamate, GABA, and acetylcholine involves direct gating of plasma membrane ionic channels for

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point-to-point communication, and slow transmission, by dopamine, neuropeptides, adenosine, glutamate and acetylcholine¹ at metabotropic receptors, involves the mobilization of second messengers like calcium and cAMP, producing the long-term changes underlying cellular plasticity that encode experience.

Bloom et al. made the acute observation that neuropeptides represent the language of stress because the enhanced neuronal firing required to message systemic or environmental stressors to the brain, and execute homeostatic signaling to the periphery, is characterized not only by increased release of classical neurotransmitters from small synaptic vesicles, but the *selective* release of informational molecules, among them neuropeptides, from large dense-core vesicles (LDCVs). Acting mainly through metabotropic, rather than ionotropic post-synaptic receptors, neuropeptides convey information for post-synaptic adaptation through long-term secretory as well as gene-encoded changes, rather than short-term electrical changes, in the post-synaptic cell [1,2].

Can microarray analysis of neuropeptide signaling help us understand, in an unbiased fashion, the ‘emergency response’ role of neuropeptides such as PACAP during stress? This short review will examine this question and demonstrate how microarray analysis in combination with informed measurement of key proteins involved in transcription and hormone biosynthesis in the neuroendocrine stress axes have helped to uncover the role of PACAP as a key regulators of signaling in major stress transducing regions of the brain and in the periphery (hypothalamus and adrenal gland). The role of PACAP in mediating stress responses may also be connected to its emerging function in neuroprotection during episodes of hypoxia, ischemia, and traumatic injury, and this connection is also briefly explored here.

PACAP as an “emergency response” peptide

Pituitary adenylate cyclase-activating polypeptide (PACAP) was discovered in 1989 by Miyata, Arimura, Coy and co-workers in a screen for hypothalamic hypophysiotropic hormones elevating cAMP in perfused pituitary [3]. It is an ancient and well-conserved peptide that exists in a 38-amino acid and a 27-amino acid form processed from a prohormone precursor [4]. Processing of PACAP to its 27- and 38 forms (the latter predominates), occurs through the processing enzymes PC1 and 2 and the amidating enzyme PAM [5,6] except in gonads where PC4 is used [7]. An analog of PACAP in *Drosophila* has been shown by Zhang, Feany and others to function both in the brain and at the neuromuscular junction through a dual signaling pathway involving both ion channel opening and cAMP [8,9], and this dual signaling paradigm characterizes PACAP signaling in mammalian nervous system as well [4]. PACAP is a neurotrophic factor, promoting the survival of cortical neuronal progenitor cells [10], cortical neurons [11], dorsal root ganglion cells [12], cerebellar granule cells [13], and peripheral sympathetic neurons [14]. PACAP elicits both PC12 cell [15] and pluripotent stem cell differentiation [16]. It is also neuroprotective in ischemia *in vivo* and in response to oxidative stress in granule cell culture [17], and increases cell survival in response to multiple stressors including environmental toxins, hypoxia, and excitotoxins in various cell culture systems [13,18,19]. PACAP is also cardioprotective in response to cardiotoxin and ischemic insult [20]. Arimura remarked at a meeting of the Editorial Board of *J. Mol. Neurosci.* New Orleans several years ago that the 10-fold higher levels of PACAP in brains of the diving turtle [21], a species chronically

¹Glutamate and acetylcholine are released from SSVs and act as both fast and slow transmitters depending on receptor interaction; DA is released from both SSVs and LDCVs and acts as a slow transmitter in both cases. Neuropeptides are uniquely released from LDCVs, in part because their prohormones and processing enzymes do not traffic to SSVs.

exposed to brain ischemia, was his inspiration for the investigation of PACAP action as a neuroprotective agent in stroke (vide infra).

PACAP: a neurotransmitter at the adrenomedullary synapse

PACAP was shown to potently activate catecholamine secretion and biosynthesis in the adrenal medulla ([22], and references therein) and later to be present in neurons innervating the adrenal medulla [23,24]. Hamelink et al. [25] demonstrated how PACAP acts as an emergency response peptide in paraphysiological situations. PACAP, present at the adrenomedullary synapse, is absolutely required for survival from prolonged hypoglycemia—mice without PACAP do not survive insulin administration, while wild-type mice become comatose but recover. This is because activation of tyrosine hydroxylase to provide enhanced catecholamine synthesis and release to allow gluconeogenesis to reestablish euglycemia (Figure 1) does not occur in PACAP-deficient mice. Induction of TH and PNMT mRNA after insulin is also PACAP-dependent (Stroth et al., in preparation), and PACAP-dependent induction of mRNAs encoding TH and PNMT also occurs in adrenal gland during restraint stress (Figure 2; from [26]). It is noteworthy that effects of stress on TH and PNMT are time-dependent—occurring only after six hours although catecholamine release begins much earlier, and is prolonged, during immobilization [27].

In order to determine if PACAP induces additional cellular plasticity associated with stress transduction at the adrenomedullary synapse, we performed microarray experiments in cultured chromaffin cells exposed to PACAP for six hours, corresponding to the period of prolonged stress in vivo causing up-regulation of TH and PNMT mRNA. We wished in particular to test the hypothesis that prolonged neuronal stress transduction, itself potentially stressful to neurons due to prolonged calcium elevation and metabolic demand during prolonged episodes of secretion, is accompanied by induction of calcium-neuroprotective proteins in the post-synaptic cell. A number of growth factors, cytokines and neuropeptides are induced by prolonged exposure of chromaffin cells to PACAP [28]. Among these is stanniocalcin-1 (Stc1). Stc1 is a 56 kD protein which interacts with a mitochondrial receptor to enhance mitochondrial calcium uptake and protect cardiomyocytes and neurons against toxicity associated with excessive calcium uptake [29-34].

Signaling pathways for PACAP-regulated stress-responsive genes

PC12 cells have been a convenient model for gathering sufficient numbers of a pure population of cells to perform combined pharmacological, biochemical, and microarray analysis of signaling pathways underlying PACAP action, and tie together PACAP-initiated cellular signaling pathways, inducible genes, target genes, and cellular outcomes (see Figure 3). In particular, this is a model system in which the *transitional transcription* (gene expression that occurs *during* the transition from state A to state B), often difficult to define in vivo, can be systematically analyzed. Thus, in previous studies, we determined that PACAP expression leading to a full neuritogenic response of PC12 cells can be obtained by PACAP exposure for 6 hours [35], and chose this time point for initial work, while also performing time courses to establish the temporal order of gene transcriptional changes occurring during the process of differentiation [36]. The pharmacology of neuritogenesis was defined using inhibitors of PKA, ERK, and other putative nodes in PACAP signaling pathways (Figure 3). This in turn to allow categorization of induced genes based on their pharmacological profile, as potential actors in PACAP-initiated processes including proliferation, neurite extension, cell size, growth arrest, survival following serum withdrawal, as well as further alterations in the downstream transcriptome of the cell itself, including those encoding late or end-stage differentiation-associated proteins, both pan-neuronal and specific to particular sublineages such as noradrenergic, cholinergic, etc. This

strategy allowed the characterization of Egr1 as a PACAP-dependent gene required for neuritogenesis [37].

The signaling pathway through which Egr1 gene induction occurs in response to PAC1 receptor activation is a unique cAMP-initiated, but protein kinase A-independent one, first characterized in bovine chromaffin cells [38]. Egr1 transcription is also up-regulated in the adrenal gland after restraint stress [26]. We have identified additional genes, including Stc1 and galanin (GAL), that are elevated in bovine chromaffin cells and in stress and also regulated by the non-canonical cAMP pathway as determined by resistance to H89 inhibition and blocked by U0126 (Stroth et al., in preparation). In contrast, available evidence suggests that PACAP regulation of CRH biosynthesis at the level of the hypothalamus involves canonical (PKA-dependent) cAMP signaling [39].

PACAP and neuroprotection: linkage to stress-related signaling?

PACAP-deficient mice show aberrant locomotor activity and lack of adaptation to novelty in exploratory behavior, and PAC1 receptor-deficient mice show impaired social investigation reminiscent of autistic behaviors in humans [40,41]. We have also shown that PACAP is neuroprotective in ischemic insult ([42,43]; Figure 4) in mice, as previously demonstrated by Reglodi et al. in the rat [44]. Furthermore, lack of PACAP expression in cerebral cortex increases the size of the ischemic lesion after middle cerebral artery occlusion (MCAO), a mouse model for stroke, and enhances the neurological damage caused by MCAO, while treatment with PACAP (even when administered an hour after the ischemic event) significantly decreases lesion size and improves neurological outcome [43]. Ohta et al. have also determined that lack of endogenous PACAP exacerbates tissue damage after stroke, and further, that the protective effects of PACAP seem to require patent regulation of IL-6 gene expression, suggesting that induction of IL-6 by PACAP may contribute to its neuroprotective actions [45]. Because PACAP does not induce IL-6 in cortical neuronal cultures (Holighaus, unpublished observations), it is most likely that PACAP's effects on IL-6 induction are mediated through astrocytes or perhaps even microglial cells in the brain in situ. However, PACAP does induce neuroprotective genes, including BDNF [11], and the neuro- and cardioprotective protein Stc1 (Holighaus unpublished observations), directly in cultured cortical neurons, as first observed in PC12 cells expressing physiological levels of the PAC1hop receptor [28]. Indeed PACAP regulation of Stc1 occurs in cerebrocortical neuronal cultures, and appears to use the non-canonical cAMP pathway identified in bovine chromaffin cells for regulation of Stc1, GAL and other genes (Holighaus, unpublished observations).

PACAP and the HPA and HSA stress axes

The importance of PACAP at the splanchnicoadrenomedullary has been documented [25], as summarized in Figure 1. At the same time, PACAP deficiency does not affect elevation of CORT levels 2 hours after induction of hypoglycemia by administration of insulin (i.e. conditions under which PACAP is absolutely required for full epinephrine secretion, maintenance of blood glucose, and survival) [25]. It was thus initially surprising to us to find that following restraint stress in mice, induction of immediate early genes in hypothalamus; elevation of ACTH secretion; induction of steroidogenic enzyme mRNA in adrenal cortex; and elevation of plasma CORT were all (Figure 5) significantly attenuated in PACAP-deficient mice ([26]; Stroth et al., in preparation).

Induction of CRH mRNA in hypothalamus by restraint stress, as shown in Figure 5, is completely dependent on the presence of PACAP. It is not yet known whether PACAP release from neurons directly innervating CRH neurons of the paraventricular nucleus controls CRH gene transcription in stress, although several anatomical lines of evidence

suggest this is the case [46-48]. In addition, reports of PACAP expression in the parvocellular neurons themselves, suggest that PACAP in addition to regulating CRH biosynthesis, could be co-released with CRH to regulate ACTH secretion directly [49,50]. It will be of particular interest to elucidate the roles of PACAP in promoting HPA axis activation in acute and chronic (prolonged) stress. Our initial observations suggest that initial (acute) HPA responses are relatively independent of PACAP, while prolonged stress responses are sustained by PACAP-dependent mechanisms (see Figure 5C), possibly reflecting adequacy of pre-stored CRH for acute responses, and a need for stimulus-secretion-synthesis coupling to sustain HPA axis activation in response to stress.

Conclusions and future prospects

Several conclusions about PACAP involvement in stress transduction and neuroprotection during stress can be inferred from the data obtained so far, both from our own laboratory and that of others. However, several questions remain to be addressed, as enumerated below, before the role of PACAP in these functions emerges with sufficient clarity to exploit therapeutically in traumatic brain injury, post-traumatic stress disorder and other anxiety disorders.

First, PACAP regulates stress hormone biosynthesis in adrenal gland via up-regulation of at least two enzymes involved in epinephrine biosynthesis, TH and PNMT. Second, PACAP acts at the level of the hypothalamus to control induction, though not basal levels, of CRH mRNA. Third, PACAP control of stress hormone biosynthesis appears to be specialized for *prolonged* versus *acute* stress axis activation. Fourth, at least in its effects on the chromaffin cell of the adrenal medulla, and possibility also in cerebrocortical neurons, PACAP utilizes a novel ERK-mediated cAMP-dependent/PKA-dependent signaling pathway to activate a 'stress response transcriptome' that includes other neuropeptides, neuroprotective factors, and prohormone convertases. Finally, it is noteworthy that PACAP modulation of stress response in the brain may extend even beyond the HPA and HSA axes. May and colleagues have characterized PACAP induction of a cohort of neuropeptides in cultured post-ganglionic sympathetic neurons in culture [14,51]. These results imply an action of PACAP to mediate stress responses specific to the sympathetic nervous system (SNS) although these have not yet been investigated *in vivo* despite the critical role of SNS activation in acute and chronic homeostatic and allostatic responses to a wide range of stressful stimuli [27,52-54]. Hammack and colleagues have observed that PACAP expression in the bed nucleus of the stria terminalis is greatly enhanced by prolonged stress, and have postulated a potential anxiogenic role for PACAP in the limbic system [55,56]. The effects of PACAP at these different levels of stress response are summarized in Figure 6.

As a master regulator of stress responses throughout the neuroaxis, PACAP may represent only a 'jack of all trades' in mediating neuroprotective, neurotransmission, and gating functions in hypothalamic, hippocampal, amygdalar, and peripheral nervous transduction of homeostatic and allostatic stress responses. GAL, CRH, adenosine, and other neuropeptides may play more specialized roles at particular neuroanatomical locations and under specific stress conditions. PACAP does, however, deserve further attention as a major multi-level stress regulator whose modulation could affect deleterious sustained stress leaving intact the acute (fight or flight) stress response helpful for survival.

In summary, PACAP is paradigmatic as a neuropeptide regulator in stress, and some more general conclusions can also be drawn about microarray analysis of neuropeptide-regulated genes from the accumulating information about PACAP signaling. Microarray analysis reveals—in an unbiased way—gene targets related to mechanisms, biomarkers, and potential drug targets for stress-related disease. These may overlap, and may also function to reinforce

each other in translational and reverse-translational approaches to understanding and treatment of stress disorders (Figure 7). Signaling pathways activated in stress may be dissected pharmacologically to allow manipulation of individual components, e.g. neuroprotection, stress hormone biosynthesis, neurotrophin production. Finally, deciding when neuropeptide *agonists* or *antagonists* should be sought as stress management therapeutics remains a key question, and one that can be addressed in part by microarray analysis of neuropeptide signaling to the nucleus in a variety of physiological contexts.

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Figure 1. PACAP a neurotransmitter at the mouse adrenomedullary synapse in vivo

A. PACAP-38 and a marker for cholinergic neurons, VACHT, are co-localized in neuronal cell bodies the intermediolateral column (IMLC) of the spinal cord, from whence splanchnic sympathetic innervation of the adrenal gland originates, and the splanchnicoadrenomedullary nerve terminals innervating chromaffin cells of the adrenal medulla.

B. PACAP-deficient mice treated with insulin (2 units/kg) die within hours (black) unless rescued with i.p. PACAP (blue) or glucose (red). Wild-type mice invariably survive insulin challenge.

C. Following insulin administration, wild-type mice show decrease blood levels of glucose (filled squares) compared to saline injection (open squares) while PACAP-deficient mice show a more profound hypoglycemia (filled circles) than wild-type after insulin compared to saline (open circles).

D. Lack of compensatory glucose elevation in PACAP-deficient mice can be explained by a less pronounced elevation in plasma epinephrine after insulin administration in PACAP-deficient compared to wild-type mice (symbols mean the same as in C.). E. Decreased epinephrine levels in blood may be explained in part by lack of epinephrine release which is stimulated by PACAP, but also a failure of repletion of released epinephrine due to a failure of PACAP knock-out mice to up-regulate tyrosine hydroxylase (not shown) resulting in adrenomedullary depletion of epinephrine in the stress PACAP-deficient, but not in the stressed wild-type, adrenal gland. Adapted from Hamelink et al., Proc. Natl. Acad. Sci. USA 99: 461-466, 2002.

Figure 2. PACAP-dependent induction of mRNAs encoding TH and PNMT in adrenal gland during restraint stress

Restraint stress, as well as insulin shock (Stroth et al., in preparation), results in up-regulation of both tyrosine hydroxylase (TH) and phenylethanolamine methyltransferase (PNMT) mRNA in adrenal gland after 6 hours, and this up-regulation is absent in PACAP-deficient mice. Note that 1 hour of restraint stress is not sufficient for PACAP-dependent induction of TH and PNMT mRNA, either when measured immediately following the stressor (1h) or after 5 hours of non-restraint following on the 1 hours restraint stress (1h + 5h). As indicated in the key, data obtained from wild-type C57Bl/6 mice are shown in *grey*, while those obtained from PACAP-deficient mice of the same strain are shown in *black*. Adapted from Stroth and Eiden, Neuroscience 165: 1025-1030, 2010.

Figure 3. Analysis of scheme used to identify signaling pathways to activation of key genes controlling various facets of cellular function in PC12 cells

PACAP signaling through the PAC1 receptor activates Gq- and Gs-dependent signaling that results in gene activation. Protein products of induced genes are required for various cellular functions including increased cell size, growth arrest, neuritogenesis, activation of specialized genes for chemical coding of neurotransmission (TH, NPY, sodium and calcium channels, etc), and protection from cell death. By correlating the effects of specific inhibitors on the total PC12 cel transcriptome using microarray, with their effects on cellular phenotype (neuritogenesis, growth arrest, etc), linkages between signaling pathways, gene induction, and cellular function can be established, and then tested with siRNA treatment directed at candidate genes underlying function. Examples of this approach are discussed in text, and see Ravni et al., *Mol. Pharmacol.* 73: 1688-1708, 2008.

Figure 4. PACAP an endogenous neuroprotectant in stroke in the mouse

A. The effects of middle cerebral artery occlusion (MCAO) on ischemic lesion size and neurological deficits were compared in wild-type and PACAP-deficient mice in which PACAP or saline was administered one hour after MCAO, and lesion size and neurological function assessed 24 hours later.

B. Effects of PACAP administered either intravenously (i.v., red bars) or intracerebroventricularly (i.c.v., red hatched bars) one hour after MCAO on neurological outcome (change in number of walking faults-higher number equals greater recovery/less damage) assessed at 24 hours post-MCAO.

C. Effects of PACAP administered either intravenously or intracerebroventricularly (symbols as in B.) one hour after MCAO on neurological outcome (larger infarct volume size reflects greater extent of brain damage) assessed at 24 hours post-MCAO

Adapted from Chen et al., Regul Pept. 137: 4-19, 2006 and see this reference for additional details.

Figure 5. Induction of immediate early genes in adrenal gland, CRH mRNA in hypothalamus, and corticosterone in blood after restraint stress is PACAP-dependent

A. PACAP-dependent regulation of immediate early genes in hypothalamus following restraint stress.

B. Regulation of CRH mRNA in hypothalamus during restraint is abolished in PACAP-deficient mice.

C. Sustained corticosterone secretion induced by restraint stress requires PACAP.

Adapted from Stroth and Eiden, Neuroscience 165: 1025-1030, 2010.

Figure 6. PACAP is a master regulator of stress circuits in brain and periphery

Depiction of the levels of the central and peripheral nervous system at which PACAP acts to modulate the efferent limb of systemic and processive stress responses at the levels of the HPA and HAS axes, and afferent (or processing nodes) within the limbic system that integrate processive stress responses, such as angiogenesis. The involvement of PACAP in the output of the sympathetic nervous system (independently of the HSA axis) is not yet clear (see text).

Figure 7. Gene expression analysis reveals mechanism, biomarkers, and therapeutic targets in stress