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Microarray analyses of pituitary adenylate cyclase activating polypeptide (PACAP) - regulated gene targets in sympathetic neurons

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Abstract

The high and preferential expression of the PAC₁(*short*)HOP1 receptor in postganglionic sympathetic neurons facilitates microarray studies for mechanisms underlying PACAP-mediate neurotrophic signaling in a physiological context. Replicate primary sympathetic neuronal cultures were treated with 100 nM PACAP27 either acutely (9 h) or chronically (96 h) before RNA extraction and preparation for Affymetrix microarray analysis. Compared to untreated control cultures, acute PACAP treatment modulated significantly the expression of 147 transcripts of diverse functional groups, including peptides, growth factors/cytokines, transcriptional factors, receptors/signaling effectors and cell cycle regulators, that collectively appeared to facilitate neuronal plasticity, differentiation and/or regeneration processes. Some regulated transcripts, for example, were related to BDNF/TrkB, IL-6/Jak2/Socs2 and TGF/follistatin signaling; many transcripts affected bioactive peptide and polyamine biosynthesis. Although chronic PACAP treatments altered the expression of 109 sympathetic transcripts, only 43 transcripts were shared between the acute and chronic treatment data sets. The PACAP-mediated changes in transcript expression were corroborated independently by quantitative PCR measurement. The PACAP-regulated transcripts in sympathetic neurons did not bear strong resemblance to those in PACAP-treated pheochromocytoma cells. However, many PACAP-targeted sympathetic transcripts, especially those related to peptide plasticity and nerve regeneration processes, coincided significantly with genes altered after peripheral nerve injury. The ability for sympathetic PAC₁(*short*)HOP1 receptors to engage multiple downstream signaling cascades appeared to be reflected in the number and diversity of genes targeted in a multifaceted strategy for comprehensive neurotrophic responses.

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Keywords

PACAP; PAC₁ receptor; sympathetic; superior cervical ganglion; neurotrophic

1. Introduction

Many neural crest derivatives that form the peripheral nervous system express and/or respond to peptides of the vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase activating polypeptide (PACAP) family. VIP and PACAP are prototypic members of a superfamily composed of six genes encoding nine bioactive peptides, which include growth hormone releasing hormone, peptide histidine-methionine, secretin, glucagon, glucagon-like peptide-1 and -2, and glucose-dependent insulintrophic peptide [56]. The two peptides share high amino acid homology from exon/gene duplication events along the same evolutionary scheme and consequently, display similarities not only in structure but also in biological activities. There are two $\forall$ -amidated forms of PACAP from alternative posttranslational processing of the precursor molecule; PACAP38 has 38 amino acid residues [proPACAP(131-168)], while PACAP27 corresponds to the amino terminus of PACAP38 [proPACAP(131-157)]. PACAP27 exhibits 68% amino acid identity with VIP [1,40,56]. The cloning of cDNAs encoding three distinct G-protein-coupled receptors for PACAP and VIP has provided a molecular basis for understanding the complexity of PACAP and VIP signaling. Only PACAP peptides exhibit high affinity for the PAC₁ receptor, whereas VIP and PACAP appear equipotent at VPAC1 and VPAC2 receptors [1,35,36,56,64,69]. Unlike VPAC receptors which appear to be coupled preferentially to adenylyl cyclase, PAC₁ receptor isoforms demonstrate differences in PACAP peptide potency and downstream signaling from alternative splicing events [46,57]. Alternative exon splicing in the amino-terminal extracellular domain (*short* vs *very short* variants) can dictate PACAP27 and PACAP38 selectivity; other receptor variants arising from the alternative splicing of two 84 bp HIP and HOP cassettes in the region encoding the third cytoplasmic loop exhibit differential patterns of adenylyl cyclase and phospholipase C (PLC) activation by PACAP peptides. Further, the ability for the PAC₁HOP1 receptor isoform to engage these and other downstream signaling cascades, including MEK/ERK and PI3K/Akt, may represent a coordinate multifaceted cellular strategy for neuroprotection and regeneration [53].

Given the potency, efficacy and distribution of PACAP in the autonomic nervous system, PACAP most likely represents the noncholinergic regulator of sympathetic function. Dense PACAP immunoreactivity in the superior cervical ganglion (SCG) has been identified in preganglionic neuronal projection fibers from the intermediolateral cell column of thoracic spinal cord [4]; transection of the central sympathetic trunk severely diminishes PACAP staining in SCG [34]. While only a minor population of sympathetic neurons expresses PACAP endogenously under normal physiological conditions, SCG PACAP expression can be induced dramatically upon chronic depolarization *in vitro* or axotomy *in vivo* as components of neurophenotypic plasticity responses [10,41]. From immunocytochemical, *in situ* hybridization and molecular analyses, nearly all sympathetic neurons express the PACAP-selective PAC₁(*short*)HOP1 receptor isoform coupled to multiple intracellular signaling cascades [9]. SCG VPAC receptors, by contrast, appear to be restricted to nonneuronal background cells. PACAP stimulation of sympathetic PAC₁(*short*)HOP1 receptors increases potently and efficaciously neuronal cyclic AMP and IP₃ production, and mitogen-activated protein kinase (MAPK) phosphorylation [3,9]. In good accord with other studies, PACAP signaling has been shown to promote sympathetic neuroblast survival, proliferation, differentiation and neurite outgrowth [15,37,44], facilitate neuronal survival from apoptotic signals [13,51], augment catecholamine/neuropeptide Y production and secretion [39], and increase neuronal excitability/depolarization in a PLC/IP₃-dependent manner by regulating a nonselective cationic channel that may be related to Trp [5]. The many neurotransmitter and

neurotrophic properties of PACAP have been appreciated in many neuronal systems, and yet how PACAP/PAC₁ receptor-mediated signaling is able to transduce and coordinate these pleiotropic responses is still not well understood. Although the study of specific PACAP/PAC₁ receptor-mediated responses can be obscured in many cellular systems from PAC₁/VPAC receptor subtype coexpression, the preferential expression of PAC₁(*short*)/HOP1 receptors in sympathetic postganglionic neurons offers a direct means to delineate more precisely the consequences of multifactorial intracellular signal integration, and establish the identities of the relevant downstream gene targets that participate in the pleiotropic responses in a physiological context. Accordingly, primary SCG sympathetic neuronal cultures were exposed to PACAP peptides under short and chronic treatment paradigms for microarray analyses to assess for changes in gene expression profiles. Among transcripts distinguished by functional attributes, PACAP stimulated significant numbers of peptides/posttranslational processing enzymes, growth factors/cytokines, transcriptional factors, cell cycle regulators and receptor signaling components. The alterations in gene expression patterns shared similarities with those after peripheral nerve injury, and offered important insights to PACAP-mediated mechanisms for neuronal survival, plasticity and regeneration.

2. Method

2.1. Neuronal cell cultures and treatments

Primary SCG sympathetic neuronal cultures were prepared as described previously [9,10,39]. Neonatal Sprague-Dawley rat SCGs (postnatal day 1 - 3) were enzymatically dispersed to produce a pooled population of cells and plated at a density of 1.5×10^4 neurons/cm² onto collagen-coated 35 mm (9.6 cm²) or 16 mm (2 cm²) multiwell plates for microarray or PCR analyses, respectively. After 48 h, the cultures were treated with 10 :M cytosine β -D-arabinosylfuranoside to eliminate nonneuronal cells, and maintained in defined complete serum-free medium containing 50 ng/ml nerve growth factor for 5 days before treatments with PACAP peptides. Since previous studies demonstrated that PACAP27 and PACAP38 are equipotent at sympathetic PAC₁ receptors, all treatments were performed with PACAP27. Sympathetic neuronal cultures were treated with 100 nM PACAP acutely for 9 h or chronically for 96 h in a reverse treatment paradigm. The 96 h PACAP cultures were treated first and the PACAP-containing medium was completely replaced after 48 h. The acute PACAP cultures were treated with peptide 9 h before study termination; all wells including the control untreated cultures were harvested at the conclusion of the experiment. NGF was included in the defined serum-free medium to avoid potential confounding apoptotic events. Under normal basal culture conditions, previous studies demonstrated that there are no changes in gene expression during the four day study window [10,23]. For all studies, triplicate culture dishes were prepared for untreated control and peptide-treated groups.

2.2. Microarray assay and analyses

For the microarray studies, total RNA from control and PACAP-treated sympathetic neuronal cultures from individual culture dishes was prepared using the RNA STAT-60 total RNA/mRNA isolated reagent (Tel-Test B, Inc., Friendswood TX, USA) [9,10]; each individual sample presented a yield of approximately 30 :g total RNA based on yield and quality assessments using an Agilent 2100 bioanalyzer. To validate the treatment, 2 :g of total RNA from each sample was reverse transcribed and the cDNA templates submitted for semiquantitative PCR of transcripts known to be regulated by PACAP signaling. Upon validation of the sympathetic PACAP-mediated responses, the remaining total RNA from each sample was submitted to the Vermont Cancer Center Microarray Facility for target preparation using standard Affymetrix protocols. Briefly, 10 :g total RNA from each sample was reverse transcribed using an oligo-dT primer coupled to a T7 RNA polymerase binding sequence. Following double-stranded cDNA preparation, biotinylated-cRNA was synthesized using T7

polymerase, and hybridized to Affymetrix rat genome U34A oligonucleotide arrays for 16 h. The arrays were first incubated with a streptavidin-conjugated to phycoerythrin, followed by sequential incubation with a biotin-coupled polyclonal anti-streptavidin antibody and streptavidin-phycoerythrin as an amplification step. After washing and laser scanning, the collected data were submitted to the University of Vermont Bioinformatics Core Facility for analyses.

2.3. Microarray statistics

The Affymetrix rat genome U34 oligonucleotide array represents sequences to 8799 known transcripts and expressed sequence tags (ESTs). The oligonucleotides on the array are arranged in multiple probe pairs corresponding preferentially to the 3'-UTR regions of the target mRNA. Each probe pair consists of a 25 oligonucleotide perfect match (PM) sequence to the target region and a duplicate 25 oligonucleotide sequence with a single base mismatch (MM). After hybridization, PM/MM signal intensity was assigned to each probe from the scanned images using Affymetrix GeneChip Operating Software (GCOS). Background corrected probe statistics and normalized probe-level intensities were performed with BioConductor tools using the Qspline methods of Workman [67], and Robust Multichip Average (RMA) statistics were calculated for each probe set and sample using the method of Speed [8,29]. RMA expression statistics from the 9 samples were modeled with one three-level factor: Control (CTL), 9H treatment (9 H), or 96H treatment (96 H). For each probe set in each of the three binary comparisons (CTL v 9 H, CTL v 96 H, and 9 H v 96 H), we calculated the magnitude of the response, expressed as the change in the RMA statistic, ΔRMA , or as "fold change", $FC = \text{sgn}(\Delta RMA)2^{|\Delta RMA|}$. We also calculated the p -value associated with each probe set and binary comparison as well as the false discovery rate using the method of Storey [59,60]. Visual representations of the comparisons were prepared as two-dimensional volcano plots with M ($\log_2$ fold change) as the abscissa and $-\log_{10} p$ as the ordinate. The implementation of Boolean filters, considering fold changes and p values, to the data spreadsheets facilitated the identification of regulated gene targets.

2.4. Reverse transcription-polymerase chain reaction (PCR)

Total RNA from cultured sympathetic neurons was prepared as described above. Complementary DNA was synthesized using the SuperScript II Preamplification System (GIBCO-Invitrogen, Grand Island, NY, USA) and oligo-dT or random primers [9,10]. Amplification of single-stranded cDNA was performed in a 13 :l reaction volume consisting of 15 mM Tris-HCl, pH 8.0, containing 50 mM KCl, 2.5 mM $MgCl_2$, 200 :M deoxynucleotide triphosphates, 0.5 :M primers, 0.5 :l of cDNA template and 0.3 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Norwalk, CT) with the following cycling parameters: (1) initial denaturation and enzyme activation 95° C, 10 min; (2) denaturation and enzyme activation 94° C, 45 s; annealing, 58° C (primer specific, Table 1), 30 s; extension 72° C, 45 s (22 - 27 cycles); and (3) final extension, 72° C; 5 min. VIP and PACAP primers used in these studies were characterized previously [23]; these and other oligonucleotide primers are listed in Table 1. The amplified cDNA product was resolved on 1.6% agarose gels, stained with ethidium bromide and viewed under UV illumination. Verification of the PCR products was performed using direct sequencing of the amplified products. Routine controls included cDNA synthesis in the absence of either RNA or reverse transcriptase; amplification with omission of template, primers or DNA polymerase failed to yield products. The same cDNA templates from each experiment were amplified for β -actin as internal controls.

2.5. Real-time quantitative PCR and data analyses

Some of the PACAP-regulated transcripts were also analyzed by quantitative PCR for comparisons with the microarray data. To prepare real-time quantitative PCR standards, the

amplified cDNA products were ligated directly into pCR2.1 TOPO using the TOPO TA cloning kit [23] (Invitrogen, Carlsbad, CA). The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). To estimate the relative expression of the target transcripts, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically.

Real-time quantitative PCR was performed essentially as described [21-23] but using SYBR Green I detection. cDNA templates from individual culture wells, diluted 5-fold to minimize the inhibitory effects of the reverse transcription reaction components, were assayed using SYBR Green I JumpStart™ Taq Ready Mix (Sigma, St. Louis, MO) containing 3.5 mM MgCl₂, 200 :M dATP, dGTP and dCTP, 400 :M dTTP, 0.64 U Taq DNA polymerase and 300 nM of each primer in a final 25 :l reaction volume. The real-time quantitative PCR was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using the following standard conditions: (1) serial heating at 50° C for 2 min and 94° C for 2 min; and (2) amplification was performed over 40 cycles at 94° C for 15 s and 62° C for 40 s. The amplified product from these amplification parameters was subjected to SYBR Green I melting analysis by ramping the temperature of the reaction samples from 62 to 95° C. A single DNA melting profile was observed under these dissociation assay conditions demonstrating amplification of a single unique product, free of primer dimers or other anomalous products [21-23].

For data analyses, a standard curve was constructed by amplification of serially diluted plasmids containing the target sequence [21-23]. Data were analyzed at the termination of each assay using the Sequence Detector 1.7a software (Applied Biosystems, Foster City, CA). In standard assays, default baseline settings were selected. The increase in SYBR Green I fluorescence intensity (ΔR_n) was plotted as a function of cycle number and the threshold cycle (C_T) was determined by the software as the amplification cycle at which the ΔR_n first intersects the established baseline. Transcript levels in each sample were calculated from the C_T by interpolation from the standard curve to yield the relative changes in expression. Data were typically normalized to 18S RNA levels. Neurons from three individual cultures were prepared for each treatment paradigm; template from each culture well was prepared separately and represented one point for real-time quantitative PCR analyses.

3. Results

3.1. Acute sympathetic transcript responses to PAC₁ receptor activation

The preferential high expression of PAC₁(*short*)HOP1 receptor isoform in nearly all postganglionic sympathetic neurons of the SCG facilitated analyses of the downstream regulated gene targets that may participate in PACAP-mediated neurotransmission and neurotrophic functions. Using our sympathetic neuronal culture model, the neurons were treated with 100 nM PACAP27 either acutely (9 h) to estimate the immediate peptide-mediated responses, or chronically (96 h) to examine the long term dynamics that might sustain the changes in functional attributes. To capture as many relevant genes of interest as possible and facilitate data comparisons with previous work, intermediate analytical stringencies were adopted to broaden the scope of the responses. When the data sets were filtered for fold changes ≥ 1.5 (either up or down), 165 target genes were identified for the CTL v 9 H comparison, and 119 genes for the CTL v 96 H comparison. Even when the second criterion of p values < 0.05 was also imposed on transcript expression level, the criteria were satisfied by 147 genes for the CTL v 9 H data set, and 109 genes for the CTL v 96 H comparative group, suggesting that there were very few false positives from the experimental designs. In visual 2-dimensional representations of the data in volcano plots, where log₂fold change (M) for each gene was expressed as a function of $-\log_{10}p$, all of the upregulated genes were noted in the upper right

quadrant, and all of the downregulated transcripts in the upper left quadrant of each graph (Figure 1). Bounded by the stringency criteria in the CTL v 9 H plot, the stimulated transcripts (135 genes), by inspection, far outnumbered those that were diminished (12 genes) by acute PACAP treatment (Figure 1A). Notably, some of the largest functional ontology groups augmented by PACAP/PAC₁ receptor signaling related to peptides/growth factors/cytokines and the posttranslational processing enzymes that may participate in their biosynthesis (total 23 genes or 17% of the upregulated transcripts; Table 2). The transcripts for subtilisin/kex (also prohormone convertase PC1) and neuron specific endothelin-converting enzyme-like (Ecell1; also damage induced neuronal endopeptidase (DINE)) represented some of the key precursor endoproteolytic processing enzymes; the regulated increase in peptidylglycine $\forall$ -amidating monooxygenase (PAM) mRNA expression was critical for the generation of bioactive $\forall$ -amidated peptides. A large number of sympathetic peptides including VIP, PACAP, galanin and tachykinins was induced by PACAP; PACAP appeared autoregulatory as suggested by previous work and stimulated the expression of some peptides not typically associated with sympathetic function, such as neuromedin U and TRH. In parallel, transcript levels for several growth factors (i.e., brain derived neurotrophic factor (BDNF) and fibroblastic growth factor (FGF)) and cytokines (i.e., interleukin-6 (IL-6), interleukin-1 β (IL-1 β), and follistatin) were also augmented. The diversity in receptors and signaling effector transcripts (29 genes; 21% of upregulated transcripts) was expansive to include receptor tyrosine kinase receptors to complement the increase in growth factor expression, and downstream signaling components related to adenylyl cyclase, MAPK, cytokine and small Ras/Rho GTPase pathways. The number of PACAP-upregulated transcriptional factor and immediate early genes was also considerable (22 genes; 16% of upregulated transcripts) and included the novel transcripts immediate early response 3 (Ier3; also PACAP-regulated gene (PRG1)) and pleiomorphic adenoma gene-like 1 (Plagl1; also lost-on-transformation (LOT1)/zinc finger gene involved in apoptosis and cell cycle control 1 (Zac1)), which have been associated with PACAP/PAC₁ receptor function in cellular growth responses. The preponderance of cell cycle regulator transcripts acutely increased by PACAP, such as cyclin-dependent kinase inhibitor 1a (Cdkn1a), B-cell translocation gene-1 (BTG), meteorin (Metrnl) and delta-like 1 homolog (Dlk1), were antiproliferative in nature and appeared consistent with the differentiation functions of Zac1. Other transcriptional factor gene targets included those related to cyclic AMP and MAPK signaling (CREB/CREM), cell survival and regeneration (Atf3), and development/differentiation processes (Klf4/Klf9). The PAC₁(short)HOP1 receptor isoform can be coupled to multiple second messenger pathways in many neuronal systems and the resulting collaborations among downstream signaling elements may be reflected in the marked increases in transcriptional factor expression for PACAP-mediated pleiotrophic responses.

3.2. Altered sympathetic transcript expression following chronic PACAP stimulation

The 109 PACAP-regulated genes identified after chronic treatment were lower in number compared to those after acute peptide exposure (compare upper right quadrant of Figure 1A and 1B). From the stringency criteria, 66 transcripts in the data set were uniquely regulated upon chronic peptide treatment; 43 transcripts were upregulated and 23 transcripts were downregulated after 96 h of PACAP exposure (Table 3). The remaining 43 gene transcripts appeared common to both the acute and chronic PACAP treatment data sets (Table 2). Among these, the expression of only 3 transcripts, VIP, endothelin converting enzyme-like 1 (Ecell1/DINE) and amphiregulin (Areg), continued to increase in the chronic treatment paradigm. While the induction of VIP after acute 9 h PACAP exposure was 28-fold, for example, VIP expression augmented nearly 44-fold by 96 h of treatment. Thirty-four transcripts, common to both the acute and chronic treatment data sets, maintained the same elevated expression levels through long term PACAP treatment. These included transcripts encoding select peptides, carriers, and intracellular signaling molecules. By contrast, a large proportion of the 135 transcripts acutely upregulated by PACAP either returned to control levels or diminished to a

lower expression plateau upon chronic peptide exposure, which may have reflected receptor desensitization and/or internalization processes. This was especially evident in the receptor, growth factor/cytokine, transcriptional factor and cell cycle ontology groups to suggest the transient nature of some sympathetic PACAP responses. Significantly, among the 19 transcriptional factor transcripts increased after acute PACAP treatment, only 6 transcripts remained elevated in the 96 h treatment paradigm, which included *Plagl1* (*LOT1/Zac1*), *Cebpb*, *Fosl* (*Fra-1*), *Cited 1* (*Cbp/p300 interacting transactivator 1*) and *Atf3*. Although nearly all acutely regulated cell cycle transcripts returned to basal levels, others, such as growth arrest and damage-inducible 45 alpha (*Gadd45*, alpha), were uniquely upregulated for cell differentiation after chronic PACAP exposure. The induction of new transcripts related to cytoskeleton formation and synaptogenesis after long term peptide treatment may reflect PACAP-mediated increases in fiber outgrowth and vesicle formation for secretory mechanisms.

3.3. Quantitative PCR assessment of PACAP gene targets

Quantitative PCR measures were performed as an independent means of corroborating the changes and dynamics of PACAP-regulated gene targets identified in the microarray studies. From the prominent functional groups, several sympathetic peptides, growth factors/cytokines and receptors were selected based on variations in expression patterns for preliminary semiquantitative analyses (Figure 2). Consistent with the microarray data sets, VIP, and galanin transcript expression were sustained upon chronic PACAP treatment paradigms. The expression of PACAP-stimulated PACAP and IL-6 transcripts was increased during the acute treatment phase, but diminished to a lower plateau upon continued peptide exposure (Figure 2A and 2B); the increase in BDNF and *trkB* transcript expression, by contrast, was transient and returned to near control levels with long term peptide treatments. Interestingly, the mRNA levels for the low affinity neurotrophin p75 receptor were diminished only upon chronic PACAP exposure. From these results, quantitative PCR analyses of a subset substantiated the temporal changes in transcript expression observed in the semiquantitative PCR and microarray studies. Except for BDNF and p75 transcript expression, the magnitude of the PACAP-mediated responses, as measured by quantitative PCR, was greater than microarray estimations (Figure 2B). Evident from quantitative mRNA measures, for example, VIP transcripts levels increased nearly 50-fold after 9 h acute peptide exposure and more than 120-fold after 96 h long term PACAP treatment; these levels were 2-fold greater than changes by microarray assessments. Yet, despite the large changes in sympathetic VIP transcript expression, the population of VIP-expressing neurons induced by PACAP was constant. While VIP-immunoreactive neurons could not be appreciated in control untreated cultures, 12% of the neuronal population was VIP-positive after PACAP treatment; sympathetic neuronal VIP staining was also prominent in fibers and varicosities which appeared consistent for peptide release processes (data not shown). As the number of VIP-immunoreactive neurons did not change during the chronic treatment paradigm, the dramatic temporal increases in transcript levels represented strikingly heightened VIP mRNA expression in individual neurons.

4. Discussion

Unlike many central neuronal systems which express predominantly the PAC₁null (neither HIP nor HOP) receptor isoform, nearly all postganglionic sympathetic neurons of the SCG have been shown to express the PAC₁(*short*)HOP1 receptor variant. As PAC₁(*short*)HOP1 receptor activation and integration of multiple intracellular signaling cascades have been suggested to be important in transducing the many trophic properties of PACAP, microarray studies of sympathetic PAC₁(*short*)HOP1 receptor signaling can be especially revealing of the downstream mechanisms and gene targets underlying these events. Both acute and chronic treatment paradigms were performed in these studies to better assess the early and late PACAP-

mediated gene responses, respectively. Although semiquantitative PCR analyses of cDNA templates from treated cultures for increased VIP and PACAP mRNA expression [47] confirmed PACAP-mediated responses prior to sample submission for microarray analyses, the induction of several other transcripts on the genechips further corroborated the specificity of the peptide response. Consistent with previous work, for example, PACAP stimulated transcripts related to sympathetic NPY and catecholamine production [39]. Chronic 96 h PACAP27 treatments increased neuropeptide Y transcript expression 1.5-fold ($p < 0.0005$) which agreed well with previous Northern blot measurement; in parallel, tyrosine hydroxylase mRNA levels were also augmented 1.4-fold ($p < 0.0005$), but the changes just narrowly missed criteria for inclusion in the data set. Significantly, mRNA for GTP cyclohydrolase, a rate-limiting enzyme for the synthesis of tetrahydrobiopterin, an essential cofactor for tyrosine hydroxylase and catecholamine biosynthesis, was increased 2-fold throughout the acute and chronic PACAP treatment paradigms. These results suggested that PACAP may facilitate or maintain the differentiated sympathetic catecholaminergic/NPY phenotype at multiple regulatory sites. The expression of other transcripts for catecholamine biosynthetic enzymes, including dopa decarboxylase, dopamine β -hydroxylase and phenylethanolamine-N-methyl transferase, was unchanged by PACAP.

Among the over 200 genes under either acute and/or chronic PACAP regulation, only 3 transcripts, VIP, Ecel1/DINE and Areg, demonstrated continued increased expression upon long term PACAP treatment. Interestingly, all 3 transcripts have been associated with injury-induced plasticity and function. Among trophic peptides, VIP has been well shown to promote neuronal survival under a variety of adverse conditions and to be induced in many injury paradigms in the peripheral nervous system [11,24,28,42,65,66,68]. The membrane-bound zinc metallopeptidase Ecel1/DINE is preferentially expressed in the nervous system and highly induced in several axotomy systems, presumably for neuroprotection [31,33]. More recently, Areg has been proposed to behave as an autoregulatory survival factor in sensory neurons [45]. Hence, the observed increases in sympathetic Ecel1/DINE and Areg expression in these studies appeared consistent with PACAP trophic programs.

While any collection of transcripts within the current data sets can be constructed for functional attributions, only certain transcript groups will be emphasized for conciseness. The PACAP-regulated transcripts encoding peptides, growth factors/cytokines and processing enzymes are prodigious in number and functional diversity. PACAP was self-regulatory, consistent with previous work [27,47]. In addition to augmenting VIP transcripts, PACAP induced efficaciously a number of other sympathetic peptides, including galanin, substance P and somatostatin. The regulated and transient developmental expression of somatostatin and enkephalins in sympathetic ganglion has been described [25,32,58]. Although the expression of other PACAP-induced peptides, especially TRH and neuromedin U, has not been well described in sympathetic neurons, these peptide transcripts may have been identified as consequences of temporal postnatal development. Alternatively, as with enkephalin mRNA, the detection of regulated transcripts may be independent of peptide production [25]. The various sympathetic peptides demonstrated different transcript expression patterns in response to PACAP. As described above, VIP transcript expression was among few that continued to increase upon chronic PACAP treatment. The heightened expression of most peptide mRNAs was sustained with chronic peptide exposure (i.e., galanin and somatostatin); the expression of others, including PACAP and substance P, diminished with treatment time. While the mechanisms underlying the variable peptide expression patterns were unclear, similar temporal dynamics have been observed in axotomy studies [7,28,52]. The increase in peptide production was complemented by increased expression of a number of posttranslational processing enzymes. Whether Ecel1/DINE expression related to peptide processing or degradation was unclear [33], but the subtilisin/kex-like proprotein convertase PC1, a endoproteolytic enzyme, has been well studied to cleave at dibasic amino acids and release the processed peptide from

its precursor molecule [55]. Many expressed peptides, including VIP, PACAP, galanin, neuromedin U, and TRH, are $\forall$ -amidated for full biological activity, and the increase in PAM expression, an essential $\forall$ -amidating enzyme, appeared sustained to maintain bioactive peptide production [50].

The number of sympathetic peptide transcripts induced was matched by an equally diverse group of growth factors, cytokines and chemokines. The increased expression of IL-6, follistatin, TGF $\forall$, BDNF, FGF14 and amphiregulin among others, represented at least 4 different receptor/intracellular signaling pathways implicated in neuronal survival, differentiation and plasticity. Of particular note, the transcripts for the neurotrophin BDNF and its cognate TrkB tyrosine kinase receptor were upregulated coordinately 3.0- and 1.7-fold, respectively, following acute PACAP treatment. While PACAP regulation of BDNF expression has been implicated in central neuronal systems [49], many previous studies have shown that sympathetic neurons do not respond to TrkB signaling [2,6]. The current observations appeared novel and suggested that sympathetic BDNF and TrkB expression may be induced coordinately, under specific developmental or physiological states, to promote survival, synaptic plasticity or related neurotrophic functions. Unlike the transient increase in TrkB mRNA which returned to basal levels after chronic peptide treatment, the increase in BDNF transcripts declined 50% to a different expression plateau during the same period. Significantly, other components of Trk signaling were also regulated, but in a reciprocal manner by PACAP. After long term PACAP treatment, expression of the neurotrophin low affinity p75 receptor transcript was diminished 1.5-fold from untreated control levels. In attenuating the proapoptotic or axonal repulsion signals attributed to p75 signaling, the response appeared to reinforce the trophic effects of BDNF and TrkB. Contrary to the TrkB response, PACAP also diminished TrkA transcript expression upon chronic peptide treatment. Although the decrement in TrkA mRNA was small (1.2-fold decrease) and did not meet one of the criteria for inclusion in the data set, the decrease was significant ($p < 0.0167$). Other neurotrophin signaling components, including NGF, NT-3 and TrkC, were not regulated by PACAP by microarray analyses.

PACAP also appeared to have complex regulatory effects on other receptor signaling cascades. From acute or chronic peptide treatments, PACAP increased transcript expression of IL-1 β , IL-6, IL-1 receptor-like 1 (IL1rl1), and the downstream cytokine signaling effector janus kinase 2 (Jak2). While IL-6 belongs to the ciliary neurotrophic factor (CNTF)/leukemia inhibitor factor (LIF)/cardiotrophin-1 (CT-1) family of neuropoietic cytokines, to implicate roles in neurophenotypic differentiation and plasticity [17,19], the sympathetic effects of IL-6, in the absence of a soluble IL-6 receptor, appeared modest in previous studies to challenge current understandings its physiological contributions [38]. Within the same pathway, PACAP also augmented transcripts for suppressor of cytokine signaling 2 (Socs2); but whether the increase represented transient compensatory mechanisms to dampen cytokine signaling or an independent means to facilitate neuronal fiber outgrowth remains to be further elucidated [62]. PACAP-mediated increases in TGF $\forall$, follistatin and Smad1 transcripts implicated modulation of bone morphogenetic protein (BMP) signaling elements. Surprisingly, PACAP not only increased the expression of adenylyl cyclase 7 (AC7), but also protein kinase C*, which binds and phosphorylates AC7 for cyclase activation [43]. While this may present one basis for sustained PAC₁ receptor intracellular signaling, PACAP also induced protein kinase inhibitor-3 (Pki3) and several cyclic AMP- and MAPK-selective phosphodiesterase/phosphatase transcripts suggesting mechanisms to balance the responses.

The transcriptional factor/immediate early gene transcripts affected by acute PACAP stimulation appeared equally extensive in scope, and two of the gene targets, Plag1 (LOT1/Zac1) and Ier3 (PRG1), were especially notable from their response magnitude and associations with PACAP/PAC₁ receptor function. Other PACAP-regulated transcriptional

factors also included CREB/CREM, Fos, Fra-1/2, Cebpb and Cited1/2, which may have been anticipated from cyclic AMP and related downstream signaling events. Together, the plethora of transcriptional factors induced may be consistent with the diversity in altered cellular programs necessary for PACAP-mediated neurotrophic responses. While the expression of most PACAP-induced transcriptional factor/immediate early gene transcripts was transient reflecting the immediacy of the response, PACAP sustained the induction of Atf3 transcripts, a transcriptional factor augmented after axotomy and other neuronal injuries [61]. Atf3, in conjunction with c-jun expression, has been associated with neuronal survival and regeneration responses, and the ability for PACAP to induce Atf3 may have echoed those neurotrophic functions [48]. If the analytical criteria were relaxed, the transcriptional factors induced by PACAP would have included multiple Jun members, including c-jun (>1.2-fold induction at $p < 0.0300$) to suggest additional mechanisms for PACAP-mediated fiber outgrowth.

Other transcripts in the microarray data sets can be organized within distinct pathways for specific functions, and one group of note is composed of transcripts for arginase 1 (Arg1), ornithine decarboxylase (ODC) and antizyme inhibitor 1 (Azin1) which participate in polyamine biosynthesis. Arginase 1 catalyzes the conversion of arginine to ornithine, and the rate limiting ODC enzyme facilitates ornithine catalysis to putrescine as substrate for downstream synthesis of the polyamines spermidine and spermine. Azin1 is highly similar to ODC and in binding to the antizyme, facilitates ODC activity and prevents ODC degradation. The application of exogenous polyamines to injured SCG or facial motoneurons *in vivo* facilitated neurite outgrowth and regeneration which could be recapitulated upon upregulation of Arg1 expression in neurons cultured on nonpermissive substrates [7,12,16,20]. In conjunction with their fiber outgrowth and regeneration properties, activation of the polyamine pathway has been suggested to be antiapoptotic and promote central neuron survival after insults [18]. Hence, the ability for PACAP/PAC₁ receptor signaling to upregulate polyamine biosynthesis can be significant within the totality of the neurotrophic program.

As adrenal medullary chromaffin cells are also derived from the neural crest, there were expectations that the PACAP-responsive genes from sympathetic and pheochromocytoma cells would be similar. Indeed, upon inspection of results from previous studies [26,30,54,63], some targets such as Erg1, Fos, Klf4, Ier3 and Odc1 appear common between PC12 and sympathetic neurons, and may have unique functional dimensions in many neuronal systems requiring more detailed studies. Yet, the similarities were limited suggesting that PACAP-regulated genes may be cell type-specific. This was especially evident in our more recent PACAP array studies with retinal neuroblasts which had little resemblance to our sympathetic data sets (data not shown). Alternatively, the culture conditions between PC12 and primary neurons may be sufficiently different to impact target responses. Surprisingly however, the PACAP-regulated transcripts in the currents studies, bore marked similarities to the changes in gene expression following axotomy [7]. Many of the peptide, growth factor/cytokine, transcriptional factor, and cell survival genes between the two studies were identical. Allowing some relaxation in the fold-change criterion, more than 40% of the altered transcripts identified following SCG axotomy were also regulated in the same direction in the PACAP-treated sympathetic cultures. Although not as abundant, many of the PACAP-regulated transcripts in sympathetic neurons were also common to genes regulated in dorsal root ganglia after sciatic nerve transection [14]. Given that the *in vivo* axotomy microarray studies almost certainly reflected altered gene expression not only in neurons but also in nonneuronal cells, the similarities in gene data sets between injured neurons and PACAP-treated sympathetic neuronal cultures appeared even more striking.

There may be several underlying reasons for the parallels. For one, PACAP/PAC₁ receptor signaling pathways may intersect with those following neuronal injury. As PAC₁ receptor activation can stimulate elements related to cytokine signaling, for example, a significant subset

of the injury-induced transcripts would be shared by PACAP signaling. However, another possibility is that some alterations in neuronal gene expression after injury reflect secondary responses to PACAP function. PACAP expression in sympathetic neurons can be tonically inhibited by target-derived factors [47], and the release of that inhibition after axotomy may stimulate PACAP production and signaling as an integral component to initiate a comprehensive injury response. Given the exceptional abilities for PACAP to drive diverse transcript expression with high efficacy, the mechanism has important attractions. Nevertheless, the compendia of sympathetic transcripts regulated by PACAP recapitulate those for neuronal differentiation, repair and regeneration. The PAC₁(*short*)HOP1 receptor is able to engage multiple intracellular signaling cascades and the current data set suggest that the number of downstream effectors may be broader than previously suspected. Some important consequences of that signaling may be reflected in the number and diversity of targeted genes which position PACAP uniquely in a nexus of cellular strategies to promote neuronal survival and regeneration. In understanding and harnessing PACAP/PAC₁ receptor function, there may be important therapeutic avenues to stimulate endogenous cellular repair.

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Figure 1.

Volcano plot demonstrate differential regulation of PACAP-targeted genes. Sympathetic transcripts identified on the Affymetrix U34 microarray to be regulated by 9 h acute (A) or 96 h chronic (B) peptide treatments were represented as correlation between robust multichip average expression index (M , $\log_2$ fold change) and p value ($-\log_{10}p$) for each of the 8799 genes. Criteria boundaries are shown for each plot. Transcripts upregulated following PACAP treatment are designated by open circles in the upper right quadrants; transcript expression diminished by PACAP are shown in upper left quadrants. Compared to panel A, the decrease in number of gene targets in the upper right quadrant of panel B reflects the transient nature of

many induced transcripts after chronic PACAP treatment. The transcript in the extreme upper right of each plot is VIP.

Figure 2.

PCR studies corroborate PACAP-regulated transcript response magnitude and temporal dynamics shown by microarray analyses. Sympathetic cultures were treated with 100 nM PACAP27 and prepared for semiquantitative (A) and quantitative (B) PCR analyses as described in Methods. The peptide treatment times corresponded to those submitted for the microarray experiments to facilitate comparisons; the transcripts analyzed were representative of peptide, growth factor and receptor transcripts that demonstrated temporal differences in expression. The expression patterns of select transcripts after PACAP treatment followed those described in the gene chip studies; from quantitative PCR, the magnitude of the responses was

frequently underestimated in the array data. Data from the quantitative PCR studies represent fold-changes compared to untreated control cultures ($n = 3$) $\forall$ SEM.

Table 1**PCR oligonucleotide primers**

	Sequence	T _a (°C)	Product size (bp)
PACAP	5'-ATGCCTCTCTGGTTGTGATTC-3' 5'-CGCTATTTCGGCGTCCTTTGTT-3'	57.0	606/612
VIP	5'-AACAACCAAGTGATTACATTA-3' 5'-GTGAGTGATTACGGAAAGAGT-3'	49.4	675
Galanin	5'-GCCAACAAGGAGAAGAGAGG-3' 5'-ATCAAAGCAGAGAACAAGTG-3'	55.3	407
BDNF	5'-GTGACAGTATTAGCGAGTGGG-3' 5'-GGGTAGTTTCGGCATTGC-3'	59.4	213
TrkB	5'-TGACGCAGTCGCAGATGCTG-3' 5'-TTTCCTGTACATGATGCTCTCTGG-3'	62.6	245
p75	5'-TGCTGCTGCTGCTGATTCTA-3' 5'-ACAAGGTCTTGCTCTGGAGGA-3'	65.0	628
IL-6	5'-TTCCCTACTTCACAAGTC-3' 5'-CTAGGTTTGCCGAGTAGA-3'	54.4	564

Table 2
PACAP-regulated sympathetic transcripts after acute and chronic treatments

Transcripts	9h Fold Δ	p value	96h Fold Δ	p value
Peptides/hormones/transmitters				
Vip	28.1	<0.0001	143.8	<0.0001*
Neuromedin U	15.7	<0.0001	11.6	<0.0001
PACAP	7.5	<0.0001	3.3	<0.0001*
Tachykinin1	6.4	<0.0001	2.7	<0.0001*
Galanin	3.5	<0.0001	4.6	<0.0001
Somatostatin	3.1	<0.0001	2.8	<0.0001
Proenkephalin-rs	2.8	<0.0001	2.5	<0.0001
Prepronociceptin	2.4	<0.0001	2.1	<0.0001
TRH	2.3	<0.0001	1.2	<0.0001*
CGRP alpha	2.3	<0.0001	nc	
glycoprotein hormones, alpha subunit	1.6	<0.0005	nc	
Peptide processing enzymes				
proprotein convertase subtilisin/kexin type 1; PC1	2.8	<0.0001	1.6	<0.0016*
Peptidylglycine amidating monooxygenase	1.7	<0.0038	1.9	<0.0016
endothelin converting enzyme	1.6	<0.0002	3.5	<0.0001*
Growth factors/cytokines/chemokines				
interleukin 6	5.1	<0.0001	2.7	<0.0001*
folliculin	3.7	<0.0001	1.9	<0.0002*
brain derived growth factor	3.0	<0.0016	1.6	<0.0005*
chemokine (CXC) ligand 1	2.0	<0.0012	1.6	<0.0079
fibroblastic growth factor 14	2.0	<0.0126	nc	
amphiregulin	1.7	<0.0006	3.7	<0.0001*
interleukin 1beta	1.7	<0.0016	nc	
neuregulin 1	1.6	<0.0131	nc	
transforming growth factor alpha	1.5	<0.0001	nc	
Receptors/effector/Associated Proteins				
nuclear receptor subfamily 4, groupA, member 3	5.7	<0.0001	nc	
nuclear receptor subfamily 4, groupA, member 2	2.5	<0.0001	nc	
somatostatin receptor 1	2.2	<0.0160	nc	

Transcripts	9h Fold Δ	p value	96h Fold Δ	p value
RAR-related orphan receptor beta (predicted)	2.1	<0.0002	nc	
fibroblastic growth factor receptor 1	1.9	<0.0002	nc	
endothelin receptor type B	1.8	<0.0002	nc	
nuclear receptor subfamily 4, group A, member 1	1.8	<0.0005	nc	
plasminogen activator, urokinase receptor	1.8	<0.0001	1.2	<0.0017*
neurotrophic tyrosine kinase receptor, type 2 TrkB	1.7	<0.0001	nc	
dopamine receptor D1A	1.6	<0.0001	nc	
glutamate receptor interacting protein 1	1.6	<0.0065	nc	
poliovirus receptor	1.6	<0.0114	nc	
neurexin 3	1.5	<0.0007	1.3	<0.0060
<i>opioid receptor-like 1</i>	-1.5	<0.0022	nc	
<i>pyrimidinergic receptor P2Y, G-protein coupled, 6</i>	-1.7	<0.0008	nc	
<i>chemokine (C-X-C motif) receptor 4</i>	-2.9	<0.0001	-1.9	<0.0003*
Transcriptional Factors				
pleiomorphic adenoma gene-like 1, Zalc1	7.1	<0.0001	1.3	<0.0322*
cAMP responsive element modulator	6.6	<0.0001	2.2	<0.0001*
early growth response 1	2.7	<0.0001	nc	
Fos-like antigen 2, Fra-2	2.7	<0.0005	nc	
FBJ murine osteosarcoma viral oncogene homolog	2.3	<0.0001	nc	
JunB	2.3	<0.0012	nc	
CCAAT/enhancer binding protein (C/EBP) beta	2.2	<0.0007	1.7	<0.0059
Chp/p300-interacting transactivator 2	2.1	<0.0025	nc	
myelocytomatosis viral oncogene homolog	2.1	<0.0011	nc	
Fos-like antigen 1, Fra-1	2.0	<0.0001	1.3	<0.0005*
hypoxia inducible factor 1, alpha subunit	1.9	<0.0309	nc	
Kruppel-like factor 4	1.8	<0.0071	nc	
cAMP responsive element binding protein 1	1.7	<0.0377	nc	
basic helix-loop-helix domain containing, class B2	1.6	<0.0070	nc	
Chp/p300-interacting transactivator 1	1.6	<0.0001	1.3	<0.0010
Kruppel-like factor 9	1.6	<0.0009	nc	
activating transcription factor 3	1.5	<0.0009	1.6	<0.0003
Mad homolog 1	1.5	<0.0439	nc	

Transcripts	9h Fold Δ	p value	96h Fold Δ	p value
nuclear factor 1/A	1.5	<0.0085	nc	
<i>ISL1 transcription factor, LIM/homeodomain 1</i>	-1.6	<0.0010	nc	
Immediate early genes				
Homer1	4.5	<0.0001	nc	
immediate early response 3, PACAP responsive gene (PRG1)	3.1	<0.0001	1.6	<0.0018
Intracellular Signal Transduction Molecules				
interferon-related developmental regulator	2.7	<0.0002	1.4	<0.0368*
dual specificity phosphatase 1, Mkp-1	2.5	<0.0001	1.7	<0.0010*
protein kinase inhibitor beta, cAMP dependent, catalytic	2.2	<0.0014	2.1	<0.0013
v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.9	<0.0088	1.6	<0.0303
Ras homolog gene family, member B	1.8	<0.0014	nc	
Ras-related associated with diabetes	1.7	<0.0057	nc	
suppressor of cytokine signalin 2	1.7	<0.0349	nc	
adenylyl cyclase 7	1.6	<0.0001	1.7	<0.0001
phosphodiesterase 4D, cAMP specific	1.6	<0.0110	nc	
dual specificity phosphatase 5, MAP-kinase phosphatase, CPG 21	1.5	<0.0003	nc	
protein tyrosine phosphatase, receptor type N	1.5	<0.0018	1.9	<0.0002
RAB3C	1.5	<0.0225	1.8	<0.0035
<i>dapper homolog 2, antagonist of beta catenin (predicted)</i>	-1.5	<0.0024	nc	
Channels/carriers				
solute carrier family 18 (vesicular monoamine), member 2	2.1	<0.0439	1.6	<0.0130
solute carrier family 1 (glial high affinity glutamate transporter), member	2.1	<0.0280	nc	
solute carrier family 2 (facilitated glucose transporter), member 3	2.0	<0.0146	2.5	<0.0130
calcium channel, voltage-dep. L type alpha1D	1.9	<0.0001	1.1	<0.0447*
Aquaporin 3	1.7	<0.0002	1.2	<0.0235
solute carrier family 16 (monocarboxylic acid transporters), member 1	1.7	<0.0004	1.5	<0.0061
solute carrier family 7 (cationic amino acid transporter), member1	1.5	<0.0096	1.5	<0.0121
<i>potassium intermediate/small conductance calcium activated channel</i>	-1.5	<0.0100	-1.3	<0.0385
<i>sodium channel, voltage-gated type I, alpha</i>	-1.5	<0.0025	nc	
Cell Cycle/Proliferation/Differentiation				
myeloid differentiation primary response gene 116	1.8	<0.0002	nc	
BCL2-like 11	1.6	<0.0224	nc	

Transcripts	9h Fold Δ	p value	96h Fold Δ	p value
B-cell translocation gene 1, anti-proliferative	1.5	<0.0077	nc	
cyclin-dependent kinase inhibitor 1A	1.5	<0.0040	nc	
delta-like 1 homolog	1.5	<0.0138	1.4	<0.0305
meteorin, glial cell differentiation regulator-like	1.5	<0.0047	nc	
proliferating cell nuclear antigen	1.5	<0.0073	nc	
Cytoskeleton/Synapse/function organization				
neuronal pentraxin 1	3.2	<0.0001	1.1	<0.0500*
synaptotagmin IV	2.4	<0.0013	1.7	<0.0124*
plakophilin 4	1.5	<0.0003	1.4	<0.0006
Spire homolog 1	1.5	<0.0001	1.2	<0.0052
brain-enriched guanylate kinase-associated	1.7	<0.0123	nc	
<i>contactin 3</i>	-1.8	<0.0003	-1.7	<0.0006
<i>nexilin</i>	-1.6	<0.0066	nc	
<i>supervillin_predicted</i>	-1.6	<0.0278	nc	
<i>tubulin, alpha 1</i>	-1.5	<0.0405	nc	
Matrix				
matrix metalloproteinase 10	4.2	<0.0001	nc	
tissue inhibitor or metalloproteinase 1	1.8	<0.0007	1.5	<0.0037
matrix metalloproteinase 13	1.6	<0.0001	nc	
secreted acidic cysteine rich glycoprotein, osteonectin	1.5	<0.0400	nc	
Metabolism/homeostasis				
cytochrome P450, family 1, subfamily B, polypeptide 1	3.4	<0.0001	1.9	<0.0026*
glutamate-cysteine ligase, catalytic subunit	2.8	<0.0010	nc	
protein phosphatase 2C, magnesium dependent, catalytic subunit	2.7	<0.0003	nc	
heme oxygenase (decycling) 1	2.5	<0.0001	1.9	<0.0007
neuraminidase 2	2.4	<0.0001	nc	
protease, serine, 35	2.4	<0.0025	nc	
glutaminase 2 (liver, mitochondrial)	2.3	<0.0001	1.2	<0.0065*
NAD(P)H dehydrogenase, quinone 1	2.2	<0.0001	1.4	<0.0019*
GTP cyclohydrolase 1	2.1	<0.0005	1.9	<0.0009
peroxisome biogenesis factor 5 (predicted)	2.1	<0.0003	1.3	<0.0375*
antizyme inhibitor 1	1.9	<0.0330	nc	

Transcripts	9h Fold Δ	p value	96h Fold Δ	p value
ribosome associated membrane protein 4	1.9	<0.0370	nc	
acyl-CoA synthetase long-chain family member 5	1.8	<0.0200	nc	
ADP-ribosylation factor-like 4A	1.8	<0.0056	nc	
protein tyrosine phosphatase 4a1	1.8	<0.0011	1.3	<0.0489
ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	1.8	<0.0001	1.2	<0.0041*
adenosine monophosphate deaminase 3	1.7	<0.0259	nc	
glutamate-ammonia ligase (glutamine synthase)	1.7	<0.0293	nc	
beta galactosidase alpha 2,6 sialyltransferase 1	1.7	<0.0076	1.5	<0.0319
ferredoxin 1	1.6	<0.0035	nc	
heat shock 27 kDa protein 1	1.6	<0.0040	nc	
Insulin induced gene 1	1.6	<0.0110	nc	
ornithine decarboxylase 1	1.6	<0.0005	1.2	<0.0435
pyruvate dehydrogenase phosphatase isoenzyme 2	1.6	<0.0001	nc	
proline rich 2	1.6	<0.0103	1.4	<0.0340
ubiquitin specific protease 12 (predicted)	1.6	<0.0296	nc	
arginase 1	1.5	<0.0021	nc	
ADP-ribosylation factor-like 5A	1.5	<0.0303	nc	
eukaryotic translation initiation factor 2 alpha kinase 3	1.5	<0.0087	nc	
glutathione S-transferase A3	1.5	<0.0146	nc	
hexokinase 2	1.5	<0.0010	nc	
isopentenyl-diphosphate delta isomerase	1.5	<0.0030	nc	
plasminogen activator, tissue	1.5	<0.0218	nc	
phosphatidic acid phosphatase type 2B	1.5	<0.0023	nc	
prostaglandin-endoperoxide synthase 2, COX-2	1.5	<0.0357	1.7	<0.0150
ribonuclease, RNaseA family 4	1.5	<0.0216	nc	
superoxide dismutase 3, extracellular	1.5	<0.0160	nc	
<i>dihydropyrimidine dehydrogenase</i>	-1.5	<0.0195	nc	
Miscellaneous				
cysteine-rich secretory protein 1	10.2	<0.0001	3.0	<0.0008*
decay accelerating factor 1	2.4	<0.0004	1.8	<0.0032*
ERBB receptor feedback inhibitor 1	2.4	<0.0003	1.5	<0.0200*
VFG nerve growth factor inducible	2.2	<0.0001	1.8	<0.0005

Transcripts		9h Fold Δ	p value	96h Fold Δ	p value
emerin	Emd	2.1	<0.0001	1.2	<0.0111*
villin 2	vil2	1.5	<0.0056	nc	

Fold change (fold Δ) in the acute (9h) and chronic (96h) transcript sets, and the corresponding *p* values represent data compared to untreated control cultures (n = 3); Transcripts in italics are downregulated after PACAP treatment. nc, not changed from control cultures; asterisk (*) designate statistical difference between 9h and 96h PACAP-treated cultures.

Table 3
Sympathetic transcripts uniquely regulated by PACAP after chronic treatment

Transcript		96h Fold Δ	p value
Peptides/hormones/transmitters			
neuropeptide Y	Npy	1.5	<0.0005
prolactin-like protein B	Prlpb	1.5	<0.0031
Peptide Processing enzymes			
cathepsin k	Ctsk	-1.5	<0.0016
proprotein convertase subtilisin/kexin type 5	Pcsk5	-1.9	<0.0011
Growth factors/cytokines/chemokines			
pleiotrophin	Ptn	-1.5	<0.0043
chemokine (C-X3-C) ligand 1	Cx3cl1	-2.0	<0.0020
Insulin -like growth factor 1	Igf1	-2.1	<0.0012
Receptors/Effectors/Assoc. Proteins			
purinergic receptor P2X, ligand-gated ion channel 2	P2rx2	2.1	<0.0011
5-hydroxytryptamine (serotonin) receptor 2B	Htr2b	1.9	<0.0003
interleukin 1 receptor-like 1	Il1rl1	1.6	<0.0265
TYRO3 protein tyrosine kinase 3	Tyro3	-1.5	<0.0345
nerve growth factor receptor (TNFR superfamily member 16),	Ngfr	-1.5	<0.0099
cannabinoid receptor 1	Cnr1	-1.6	<0.0145
secretin receptor	Sctr	-1.7	<0.0049
olfactory receptor 59	Olr59	-1.7	<0.0001
Transcriptional factors			
zinc finger protein 180	zfp180	1.5	<0.0009
POU domain, class3, transcription factor 1	Pou3f1	-1.7	<0.0017
Intracellular signal transduction molecules			
protein tyrosine phosphatase, non-receptor type 5	Ptpn5	1.9	<0.0001
protein tyrosine phosphatase, receptor type O	Ptpro	1.9	<0.0011
protein kinase C, delta	Prkcd	1.8	<0.0002
ras homolog gene family, member V	RhoV	1.7	<0.0004
Janus kinase 2	Jak2	1.5	<0.0374
RAS protein-specific guanine nucleotide-releasing factor 1	Rasgrf1	1.5	<0.0031
guanine nucleotide binding protein (G protein) gamma 8	Gng8	-1.7	<0.0007
Channels/carriers/pumps			
solute carrier family 2 (facilitated glucose transporter member 1	Slc2a1	1.8	<0.0198
amiloride-sensitive cation channel1 neuronal (degenerin)	Accn1	1.7	<0.0002
ATPase, H+ transporting, lysosomal V0 subunit A1	Atp6v0a1	1.6	<0.0032
solute carrier family 24 (sodium/potassium/calcium exchange,	Slc24a2	1.6	<0.0250
cellular retinoic acid binding protein 2	Crabp2	1.5	<0.0176
solute carrier family 7 (cationic amino acid transporter, y+ system)	Slc7a3	1.5	<0.0014
Cell cycle/proliferation/differentiation			
cell death-inducing DNA fragmentation factor, alpha subunit	Cidea-pred.	1.9	<0.0018
growth arrest and DNA-damage-inducible 45 alpha	Gadd45a	1.6	<0.0123
cyclin B1	Ccnb1	-1.5	<0.0012

Transcript		96h Fold Δ	p value
<i>cell division cycle 2 homolog A (S. pombe)</i>	<i>Cdc2a</i>	-1.5	<0.0179
Cytoskeleton/synapse/junction organization			
synaptosome-associated protein 25	Snap25	2.2	<0.0229
secretogranin 2	Seg2	1.7	<0.0005
synaptotagmin V	Syt5	1.7	<0.0005
lin-7 homolog a ©. elegans)	Lin7a	1.6	<0.0030
secretogranin III	Seg3	1.6	<0.0083
thymosin beta-like protein	Tmsb1l	1.5	<0.0123
<i>PDZ and LIM domain 3</i>	<i>Pdlim3</i>	-1.5	<0.0013
<i>neurexophilin 1</i>	<i>Nxph1</i>	-1.6	<0.0166
<i>myosin, heavy polypeptide 1, skeletal muscle adult</i>	<i>Myh1</i>	-1.6	<0.0004
Matrix/adhesion			
embigin	Emb	1.7	<0.0021
<i>elastin</i>	<i>Eln</i>	-1.7	<0.0026
<i>fibulin</i>	<i>Fbln5</i>	-1.7	<0.0055
<i>spondin 1</i>	<i>Spon1</i>	-1.7	<0.0119
Metabolism/homeostasis			
glutathione S-transferase, mu 1	Gstm1	1.8	<0.0002
glutathione peroxidase 3	Gpx3	1.8	<0.0048
argininosuccinate lyase	Asl	1.7	<0.0001
heterogeneous nuclear ribonucleoprotein A/B	Hnrpab	1.7	<0.0001
UDP-glucose ceramide glucosyltransferase	Ugcg	1.6	<0.0427
cystathionase (cystathionine gamma-lyase)	Cth	1.6	<0.0014
beta galactoside alpha 2,6 sialyltransferase 1	St6gal1	1.5	<0.0319
acyl-coenzyme A oxidase 1, palmitoyl	Acox1	1.5	<0.0089
UDP glycosyltransferase 1 family, polypeptide A6	Ugt1a6	1.5	<0.0059
branched chain aminotransferase1, cytosolic	Bcat1	1.5	<0.0082
eukaryotic translation initiation factor 4E binding protein 1	Eif4ebp1	1.5	<0.0100
<i>topoisomerase (DNA) 2 alpha</i>	<i>Top2a</i>	-1.6	<0.0002
Misc.			
Purkinje cell protein 4, PEP19	Pcp4	2.0	<0.0034
Cd59	Cd59	1.7	<0.0133
regulated endocrine-specific protein 18	Resp18	1.6	<0.0004
Cd200 antigen	Cd200	1.6	<0.0339
Cd24	Cd24	1.5	<0.0490
<i>alanine and arginine rich domain containing protein</i>	<i>Aard</i>	-1.5	<0.0279
<i>lamin B1</i>	<i>Lmnbl</i>	-1.5	<0.0284

Fold change (fold Δ) for the chronic (96h) transcript set and the corresponding p values represent data compared to untreated control cultures (n = 3); Transcripts in italics are downregulated after PACAP treatment. nc, not changed from control cultures.