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Discrete signal transduction pathway utilization by a neuropeptide (PACAP) and a cytokine (TNF-alpha) first messenger in chromaffin cells, inferred from coupled transcriptome-promoter analysis of regulated gene cohorts

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Abstract

Cultured bovine adrenal chromaffin cells (BCCs) are employed to study first messenger-specific signaling by cytokines and neurotransmitters occurring in the adrenal medulla following immune-related stress responses. Here, we show that the cytokine TNF-alpha, and the neuropeptide transmitter PACAP, acting through the TNFR2 and PAC1 receptors, activate distinct signaling pathways, with correspondingly distinct transcriptomic signatures in chromaffin cells. We have carried out a comprehensive integrated transcriptome analysis of TNF-alpha and PACAP gene regulation in BCCs using two microarray platforms to maximize transcript identification. Microarray data were validated using qRT-PCR. More than 90% of the transcripts up-regulated either by TNF-alpha or PACAP were specific to a single first messenger. The final list of transcripts induced by each first messenger was subjected to multiple algorithms to identify promoter/enhancer response elements for trans-acting factors whose activation could account for gene expression by either TNF-alpha or PACAP. Distinct groups of transcription factors potentially controlling the expression of TNF-alpha or PACAP-responsive genes were found: most of the genes up-regulated by TNF-alpha contained transcription factor binding sites for members of the Rel transcription factor family, suggesting TNF-alpha-TNFR2 signaling occurs mainly through the NF-KB signaling pathway. Surprisingly, EGR1 was predicted to be the primary transcription factor controlling PACAP-modulated genes, suggesting PACAP signaling to the nucleus occurs predominantly through ERK, rather than CREB activation. Comparison of TNFR2-dependent versus TNFR1-dependent gene induction, and EGR1-mediated transcriptional activation, may provide a pharmacological avenue to the unique pathways activated by the first messengers TNF-alpha and PACAP in neuronal and endocrine cells.

Keywords

Affymetrix; Agilent; Chromaffin cell; Cyclic AMP; ERK; Gene induction; Microarray; NF- B; PACAP; PAC1; Signal transduction; TNF-alpha; Transcriptional regulation; TNFR2

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Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.peptides.2013.03.020>.

1. Introduction

Bovine adrenal medullary chromaffin cells (BCC) represent the only source of fully differentiated post-mitotic neuroendocrine cells that can currently be obtained from a mammalian species, as a relatively pure population, in amounts sufficient for correlative pharmacological, biochemical, proteomic and transcriptomic analysis [38]. These primary cells are also well-suited for the study of cell-specific pathways for receptor-mediated signaling as they are uniformly post-synaptic for a single type of input *in vivo*. Thus, all chromaffin cells presumably receive a cholinergic/PACAPergic innervation from the splanchnic nerve [15], and all express receptors for cytokines such as IL-1 beta and TNF-alpha [3]. In contrast, cultured cell preparations from other tissues including the central nervous system contain dozens to hundreds of sub-populations with differential expression of receptor types, making correspondence between first messenger stimulation, and gene regulation within receptive cells difficult if not impossible. Furthermore, mature chromaffin cells contain signaling components dedicated to the transmission of information to the nucleus in the absence of signaling for growth, differentiation, proliferation, or trans-differentiation that occur in developing or transformed neuroendocrine cells or cell lines, or perinatally derived neuronal cultures with properties vastly different from mature neurons or endocrine cells. This property of mature post-mitotic chromaffin cells is of special interest for understanding first messenger signaling required for homeostatic and transductive functions of these cells. These functions include integrative signaling during inflammation and stress, in which various hormonal outputs of the adrenal medulla, including catecholamines and neuropeptides, serve to fine-tune glucocorticoid and cytokine production elsewhere in the body [9].

PACAP and TNF-alpha are two first messengers that play important roles in integrating neural and immune information at the level of the chromaffin cell, during paraphysiological (e.g. stress) and pathological (e.g. inflammation; sepsis) events [2-4,9]. PACAP neurotransmission at the adrenomedullary synapse is mediated through the activation of PAC1, one of three receptors for the neuropeptide ([10] and references therein), while TNF-alpha signaling to chromaffin cells is mediated through the type 2 receptor (TNFR2) [26]. Chromaffin cells express only the PAC1hop receptor for PACAP [28], and only the TNFR2 for TNF-alpha [4], and therefore are a simple, homogenous cell model for gene regulation by these two first messengers. Thus, establishing principles of signaling to the nucleus, governing gene regulation by TNF-alpha/TNFR2 and PACAP/PAC1 in chromaffin cells, may ultimately also be relevant to such signaling in more complex systems, such as the central nervous system, in which these two first messengers and their cognate receptors mediate complex functions including neuroprotection, memory and learning, and allostatic adaptation to chronic stress [10,26,31,42].

PACAP and TNF-alpha exert their effects in part by signaling the activation of specific cohorts of chromaffin cell genes that are appropriate to stress transduction and immunomodulation, respectively [1,4]. Comprehensive examination of these gene cohorts can potentially reveal the transcription factors activated by each messenger, and therefore the molecular basis for their distinct nuclear signaling properties. In this study, TNF-alpha and PACAP-mediated gene regulation in BCCs has been examined using two microarray platforms developed for analysis of expression from the bovine genome to obtain for the first time a comprehensive transcriptome profile for nuclear signaling by TNF-alpha through TNFR2, and PACAP through PAC1. *Bona fide* TNF-alpha and PACAP-regulated gene populations were established by qRT-PCR-based sample validation across microarray platforms. Promoter analysis of each cohort was carried out using bioinformatics tools, which were in turn validated from empirically derived transactivator-specific target gene

lists. Results suggest that a relatively small number of distinct trans-activating proteins mediate the gene regulatory properties of each first messenger. Consequently, these are highly suggestive of the signal transduction pathways differentially activated by each first messenger that initiates signaling to the nucleus. The ability to dissect signal transduction 'signatures' for first messenger/receptor cognates in defined systems is an important first step in creating first-messenger signal transduction inventories on which a potential pharmacology for differentially enhancing cellular functions of PACAP and TNF-alpha involved in neuroprotection, memory function, and adaptation to stress might be based.

2. Materials and methods

2.1. Materials

PACAP-38 was purchased from Phoenix Pharmaceuticals (Mountain View, CA). Recombinant human TNF-alpha (cat. no. 300-01A) was purchased from Pepro Tech Inc. (Rocky Hill, NJ). Bovine microarray chips and associated reagents were obtained as described in Section 2.3.

2.2. Bovine chromaffin cell (BCC) culture and drug treatments

Primary BCC cultures were prepared as previously described [5]. Briefly, BCCs were obtained after retrograde perfusion of bovine adrenal glands with 0.1% collagenase (Worthington Biochemical, Lakewood, NJ) and 30U/ml DNase (Sigma-Aldrich, St. Louis, MO), followed by dissociation of the digested adrenal medulla in the same solution at 37°C for 30 minutes. The cells were cultured in DMEM (Invitrogen, Grand Island, NY) supplemented with 5% fetal calf serum and 100U/ml penicillin-streptomycin, 2mM glutamine, 10 µg/ml cytosine beta-D-arabinofuranoside, and 100 U/ml nystatin. Chromaffin cells were purified by differential plating in T150 flasks overnight at a density of approximately 50 million cells/50 ml/flask, and then plated in the same medium as above at a density of 1×10^6 cells/ml, 2 ml/well, in poly-D-lysine-coated 6-well plates. Twenty-four hours later, cells were treated with either 100nM PACAP or 10nM TNF-alpha (added as a 10× stock solution in medium) for 6 h.

2.3. RNA extraction, microarray hybridization and collection of data

Total RNA was isolated from cultured BCCs using RNeasy lysis buffer and spin columns (Ambion/Life Technologies/Invitrogen, CA) or RNeasy Mini Spin Columns (QIAGEN, Valencia, CA) and quantified by spectrophotometry. RNA quality and quantity was evaluated by Bioanalyzer (Agilent, Inc.) and Nano-Drop (Thermo Scientific, Inc.) respectively. Only RNA with an RNA Integrity Number (RIN) greater than 7 was used for microarray analysis. 5 µg of total RNA was used in conjunction with the Affymetrix recommended protocol for One-Cycle Target Labeling including control reagents (Affymetrix P/N 900493). The hybridization cocktail containing biotin-labeled cDNAs was added to the Affymetrix GeneChip Bovine Genome Array (P/N 900562). Hybridizations on the Affymetrix platform were performed in triplicate with labeled cDNA comparing untreated vs PACAP- or TNF-alpha-treated samples. The chips were washed and stained by the Affymetrix Fluidics Station using the standard format and protocols as described in the Affymetrix GeneChip Expression Analysis Technical Manual (P/N 702232 Rev.3). The probe arrays were stained with streptavidin phycoerythrin solution (Molecular Probes, Carlsbad, CA) and enhanced by using an antibody solution containing 0.5 mg/mL of biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA). An Affymetrix Gene Chip Scanner 3000 was used to scan the probe arrays.

For Agilent arrays, 1 µg of each sample of total RNA was reverse transcribed with T7 Oligo (dT) primer and amplified using the Ambion MessageAmp™ II Kit (P/N 1753, Ambion,

Austin, TX). The aminoallyl UTP labeled RNAs were then coupled with either Cy3 (untreated cells) or Cy5 (TNF-alpha or PACAP treated cells) mono NHS ester CyDyes (GE Healthcare, Piscataway, NJ). After purification, Cy5 labeled RNA from either PACAP or TNF-alpha treated samples were combined with Cy3 labeled RNA from the untreated samples for hybridization in triplicates with Bovine Gene Expression Microarray (design ID 015354), 4×44K oligo chips using the Agilent two color hybridization protocol (Version 5.7). The slides were then washed using the manufacturer's recommended procedures and reagents (P/N 1753). A laser confocal Agilent Technologies Scanner G2505A US14702390 (Agilent Technologies, Palo Alto, CA) was used to scan the hybridized Cy3 and Cy5 probes on the chips. The fluorescent intensities at the target locations on the array were quantified using Agilent Feature Extraction Software (Version 9.5.1.1). The expression values were normalized using the default linear-Lowess normalization.

2.4. Microarray data analysis

The Affymetrix CEL files were processed through Partek Genomic Suite following the vendor's recommendation. 'Robust Multichips Analysis' (RMA) normalization was used followed by one way ANOVA test to determine significantly up- or down-regulated genes. Transcripts that were significantly altered ($p < 0.05$, $n = 3$ for each group) by TNF-alpha or PACAP treatment 2-fold or more were selected for further analysis. Processed signals from red and green channels in Agilent studies were also analyzed using Partek Genomic Suite. After log 2 transformation and quantile normalization, a further filter was applied to remove data with very low fluorescence signals (< 200) in the Cy5 (TNF-alpha or PACAP) channels. A list of significantly regulated transcripts was obtained in Partek Genomic suite, as described above, using one way ANOVA. Latest annotations from the vendor websites (<http://www.affymetrix.com> or <http://earray.chem.agilent.com>) were used for identifying the genes that were significantly up or down regulated.

2.5. GEO deposit

The primary microarray data from this research have been deposited in MIAME format in the NCBI Gene Expression Omnibus data repository under accession number GSE24070.

2.6. Categorization of genes

For validity of comparison, and eventual merging, of data obtained using two different array platforms the fold change along with the p values for different genes were retrieved from Partek analysis for Affymetrix and Agilent arrays. JMP 8.0 was used to average fold change values for any gene that was represented by more than one probeID in either platform. Only the probes or probesets with gene symbols were used in further analysis. The genes whose expression was regulated at least 2-fold in either direction in either of the arrays at a p value of 0.05 or less were grouped into following 5 categories i.e. (1) AffyChanged_AgChanged: changed in both platforms; (2) AffyChanged_AgNC: changed in Affymetrix but not in Agilent; (3) Affy_Changed_AgNP: changed in Affymetrix but not present in Agilent, (4) AffyNC_AgChanged: changed in Agilent but not in Affymetrix and (5) AffyNP_AgChanged: changed in Agilent, not present in Affymetrix array.

2.7. Confirmation of microarray data with quantitative RT-PCR (qRT-PCR)

Five to ten representative genes were selected from each of the above categories for qRT-PCR confirmation. Approximately 2 μ g of total RNA was subjected to DNase I (RNase-free; Invitrogen) digestion and reverse transcribed using random hexamers pdN6 (Invitrogen Inc) and SuperScript II RNase H-reduced reverse transcriptase (Invitrogen Inc.) as previously described [1]. Gene-specific forward and reverse primers were then designed using Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) to generate a

product that spans across exon-exon junctions using the Splign algorithm (<http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi/>). qRT-PCR was performed using a SYBER green reaction mix to which transcribed cDNA and 10 nM of each specific primer were added prior to amplification in an iCycler real-time detection system (Bio-Rad, Hercules, CA). Fold changes in mRNA levels were determined by normalizing against a non-variable control transcript (either chromogranin A or GAPDH) using the ddCt method [23].

2.8. Creation of final annotated list of PACAP- and TNF-alpha-responsive genes

The qRT/PCR results were used to calculate an empirically-based false positive rate (transcripts indicated to have been changed by treatment based on microarray data, but not in fact changed by treatment) in each of the five categories described in Section 2.7. The final list of PACAP or TNF-alpha-responsive genes included only those categories in which there was at least a 70% concordance between the microarray and qRT-PCR data. The lists also included any specific transcripts shown to be regulated (i.e. by confirmatory qRT-PCR) regardless of categorical inclusion or exclusion, and excluded those shown not to be regulated regardless of categorical inclusion or exclusion.

2.9. Determination of enriched representation of transcription factor binding sites (TFBS) in genes encoding transcripts regulated by PACAP or TNF-alpha

Four tools for determination of TFBS over-representation in a gene list were evaluated for application to the current data sets. These were CORE_TF (http://grenada.lumc.nl/HumaneGenetica/CORE_TF/), Opossum (<http://www.cisreg.ca/cgi-bin/oPOSSUM/opossum/>), PSCAN (<http://159.149.109.9/pscan/>) and TFME (<http://bioinfo.lifl.fr/cgi-bin/TFME/tfme.py>). About 1000bp 5' to the transcription start sites in target gene sets were evaluated, as these were deemed fully representative of the regions within which response elements were captured empirically from ChIP-ChIP and ChIP-Seq studies.

The target gene sets used in the test validating ability to predict/capture TFBS over-representation based on correct prediction of the CREB-ome, NFkB-ome, EGR1-ome and REST-ome were:

1. CREB1 (<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC85336/>, kindly supplied by Rongkun Shen, OHSU),
2. NFkB (<http://bioinfo.lifl.fr/NF-kB/> and <http://www.bu.edu/nf-kb/gene-resources/target-genes/>),
3. EGR1 (<http://genomebiology.com/2009/10/4/R41>) and
4. REST (<http://genome.cshlp.org/content/16/10/1208.long/>).

About 1000 targets were selected from each set and the provided IDs were converted to Ensembl geneID and RefSeq ID using ID converter (<http://biodb.jp/#ids>). At least 500 target genes either in the form of Ref Seq or Ensembl IDs of were analyzed by each of the four transcription factor prediction tools above, to compare their relative accuracy. About 1000 base sequences upstream to the transcription start site of each gene was used for identifying over-represented TFBS in PSCAN, while Core_TF used 1000 bp upstream region as well as the first exon of each input gene, Opossum used 2000 bases at the transcription start site, and TFME used 1000 bases. 3000 random sequences are used as the background sequences for these tools. Biases based on GC content were excluded by matching random promoters with approximately equal GC content to the experimental promoters. The predictions were sorted on decreasing p value score. If the transcription factor was predicted to be the top TFBS for its target gene set, it received the highest-probability score of 1. If the corresponding transcription factor was among the five highest-probability scores for that tool, a score of 0.5 was assigned for that prediction. If the corresponding transcription factor

was not among the five highest-probability scores for that tool, a score of 0 was assigned for that prediction.

Based on this analysis, the best predictor for TFBSs was PSCAN, which was therefore used to identify the most probable transcriptional regulators for the TNF-alpha and PACAP inducible gene sets. Human RefSeq sequences for the bovine genes up-regulated by either PACAP or TNF-alpha were retrieved using ID converter (<http://biodb.jp/#ids>). In PSCAN Z scores are used to determine over-abundance as well as for p value determination.

3. Results

3.1. Distribution and fold changes for known genes

Fig. 1 summarizes the steps taken in identifying the genes regulated by either PACAP or TNF-alpha after 6h of treatment. This time point was chosen based on previous studies from this laboratory, that indicated the likelihood of capturing both immediate-early gene induction, and secondary gene regulation targeted by the latter, albeit not at the maximal amount of fold induction for either category [11].

Lists of known genes derived at the stages of data analysis depicted in Fig. 1 are found in Supplementary Tables S1-S9.

163 genes were up-regulated, and 116 genes down-regulated 2-fold or more with a p value of <0.05 by PACAP in the Affymetrix array (Table S5a) while 228 genes were up-regulated and 238 genes down-regulated by PACAP in the Agilent array (Table S6a). 94 genes were up-regulated, and 7 genes down-regulated by TNF-alpha in the Affymetrix array (Table S5b) while 128 were up-regulated and 18 down-regulated in the Agilent array (Table S6b). Note that the actual number of gene entries for up-regulated genes in Tables S5a,b and S6a,b is slightly higher than the numbers given here, because distinct entries for genes duplicated within the arrays are presented in these tables.

For both TNF-alpha and PACAP-treated cells, a somewhat higher number of significantly changed genes were obtained from the Agilent array platform than from the Affymetrix array platform. Most of the genes corresponding to transcripts up-regulated genes by either TNF-alpha or PACAP were present either in both platforms (AffyChanged_AgiChanged) or were represented in only one of the two platforms (see Tables 1 and 2). Thus, there was very little discordance per se between the two platforms, albeit representation clearly remains a problem, for the bovine transcriptome, for both. Tables S7 and S8 list the transcripts regulated by PACAP and TNF-alpha respectively (each first messenger), distributed among these five categories. PACAP and TNF-alpha appear to affect different sets of genes in bovine chromaffin cells as only about 25 transcripts were up or down regulated by both messengers.

3.2. Use of qRT-PCR to create lists of transcripts regulated by TNF-alpha and PACAP based on efficiency of discovery (empirically-derived false discovery rate) for each microarray performance category

We next sought to normalize gene numbers obtained with each platform, to an empirically-derived false discovery rate for each. To this end, genes were grouped into five different categories based on their representation, and whether or not abundance of cognate transcripts of each gene were 'changed' or 'unchanged' according to results obtained with each platform. Representative genes from each of the five categories for PACAP and TNF-alpha were tested by qRT-PCR. For PACAP up-regulated genes three categories, i.e. Affy-Changed_AgChanged, AffyChanged_AgNC and AffyNP_AgChanged, met the criteria of 70% confirmation by qRT-PCR (Table S9) and were included in the final gene list for

further analysis. For TNF-alpha the categories AffyChanged_AgChanged, AffyChanged_AgNC, AffyChanged_AgNP, and AffyNP_AgChanged were included in the final gene list based on 70% confirmation by qRT-PCR. The final lists of up and down regulated genes based on this selection are shown in Table 1 (PACAP) and Table 2 (TNF-alpha). Three hundred and seventy-three genes were modulated by PACAP (207 up-and 166 down-regulated), while one hundred and twenty-six genes were modulated by TNF-alpha (113 up- and 13 down-regulated), according to these criteria.

3.3. Determination of enriched representation of transcription factor binding sites among PACAP- and TNF-alpha-regulated genes

There are several tools available for analysis of transcription factor binding sites (TFBS) that allow prediction of enrichment for utilization of particular transcription factors in a given gene cohort. Most of these take into account the probability score of the position weight matrix (PWM), also called position-specific weight matrix (PSWM) or position-specific scoring matrix (PSSM). This score reflects the presence of specific nucleotide motifs to which specific transcription factors or transcription factor families have been reported to bind, based on empirical evidence disclosed in publications in the peer-reviewed scientific literature. A PWM assumes independence between positions in the pattern, as it calculates scores at each position independently from other positions. We were initially unable to discern good concordance among multiple tools for TFBS enrichment in our data sets, and therefore decided to qualify these tools for further use by testing them against gene lists comprised entirely of genes for which binding of a single transcription factor had been demonstrated empirically from chromatin immunoprecipitation studies. We chose four gene lists, or 'omes' comprising genes each containing at least one motif shown to bind CREB, NFKB EGR1 or REST. These four transcriptomes were chosen based on their accessibility, curation by multiple laboratories, and previous reports that known TNF-alpha and PACAP-regulated genes were likely to contain TFBS's for at least three out of four of these transcription factors in the case of PACAP (<http://www.ncbi.nlm.nih.gov/pubmed/9618900>, <http://www.ncbi.nlm.nih.gov/pubmed/18362103/>, <http://biolod.org/>), and at least one of them, in the case of TNF-alpha (http://stke.sciencemag.org/cgi/cm/stkecm;CMP_7107/, <http://atlasgeneticsoncology.org/Deep/NFKBID20033.html/>).

As described in Section 2, four different tools, PSCAN, Core-TF, Opossum and TFME were used to query lists of about 1000 target genes containing binding sites for CREB, NFKB, EGR1 or REST, and both Transfac (<http://www.gene-regulation.com/pub/databases.html/>) and Jaspar (<http://jaspar.genereg.net/>) databases were used wherever applicable. Based on the criteria described in Section 2, PSCAN performed best, and CORE_TF next best, in aggregate, for the group of 4 transcription factor 'omes' used as our validating databases (Table 3).

Using PSCAN, the TNF-alpha-regulated gene set was found to be highly enriched in binding sites for NFkB. Thus, sites binding of the REL class, i.e. NFKB1, REL, NFKB and REL were greatly over-represented in this gene set compared to either a control gene set, or the genes regulated by PACAP (Table 4). The PACAP-regulated gene set, on the other hand, was found to be enriched in binding sites for AP2, EGR1 and SP1 (Table 4). The significance of AP2 and SP1 involvement in PACAP gene regulation may be via their interaction with other transcription factors whose binding sites are over-represented among PACAP-regulated genes, such as EGR1 (Table 4).

4. Discussion

4.1. Complementary use of multiple array platforms in gene discovery and bioinformatics analysis of gene expression

We have used both Affymetrix as well as Agilent arrays to identify the transcriptome profile after 6 hr of treatment by either PACAP or TNF-alpha. As mentioned in Results, the majority of discordance between the two platforms was based on differential gene representation, rather than conflicting results. Thus, eliminating genes not represented in one or the other of the two platforms, there was 76% concordance for PACAP-regulated genes, and 86% concordance for TNF-alpha-regulated genes. Only 12% (4/32) of the overall discordance was in opposite directions of fold change, and all discordances were based on lack of significant change in one platform, with significant change in the other, i.e. truly discordant rather than contradictory. Importantly, all but three discordant genes across both data sets represented changes of less than threefold, and thus were highly likely to represent discordant precision, rather than accuracy, of altered abundance of a given transcript in either data set. Discordancies in up-regulation by PACAP of transcripts encoding *Dlck1*, *Egr1*, *PNMT* and *Gal* are noteworthy because these genes have been shown to be targets of PACAP regulation in other cell systems (see Eiden et al. [11] and references therein), with maximal up-regulation either earlier (*Egr1*, *Dlck1*) or later (*PNMT*, *Gal*) than the treatment time chosen for sampling (6 h) in the experiments reported here. This level of noncordance is somewhat lower (average 82%), than that calculated from Bosotti et al. (65%) for a similar cross-platform comparison but for a different species, almost certainly because this group's allowance of fold-change (50%) was half that used here (100%). In fact, the results obtained here are in general agreement with previously published reports that interoperability, rather than intrinsic differences among commonly used microarray platforms, account for most of the nonconcordance in measurement of changes in gene expression (mRNA abundance) in reported microarray results [8,20,25,33].

The relative incompleteness of gene annotation for the bovine compared to rodent or human transcriptome is a major driver for differences in gene expression profiles derived from the two platforms. Thus, fully half of all of the genes regulated by either PACAP or TNF-alpha are contributed from transcripts present in only one or the other of the two platforms employed here. It seems likely that different bioinformatics/annotation approaches may be leading each platform towards full representation of the bovine transcriptome by slightly different paths. If so, both are likely to end up with mutual improvement in the current situation, which is that each platform fails to identify about 25% of the genes regulated in toto, in this case by either PACAP or TNF-alpha in chromaffin cells. A practical result is that the complementary use of both platforms, at least at this time, gives a clear advantage to investigators pursuing 'gene discovery' as well as 'systems biological' approaches to transcriptome analysis, since neither platform alone performs as well as the curated sum of both, either for total genes identified uncorrected for false positive/false negative rates, or as calculated from qRT-PCR validation by category. A second consequence is that at this time, validating regulated genes by complementation with a second microarray, rather than by qRT-PCR, is not a practical option at least for this species and the arrays used here at their current state of annotation.

4.2. TNF-alpha and PACAP modulated genes are regulated by different set of transcription factors

Promoter analysis for co-expressed genes was carried out using PSCAN, and this tool was chosen over the others available based on empirical validation from known transcription factor-specific 'omes' available in the literature for CREB, NF-kB, Egr-1 and REST determined via ChIP-Seq or related experimental techniques (see [18,19,32], and URLs for

sites listed in Section 2). While it may be obvious to the reader that any choice of predictive bioinformatics tools should be based on empirical validation such as performed here, in practice this is rarely done. In part, this may be because these tools and the databases that they draw from continue to evolve rapidly. Thus, it may well be that predictions of promoter usage based on transcriptome regulation by other tools will outperform PSCAN based on validation with transcription factor sub-transcriptomes other than the CREBulome, NF-kB-ome, EGR-1-ome and REST-ome, or based on evaluation other than the simple point system employed here. Finally, empirical validation of the results obtained in this report (see Section 4.3, below) will be the ultimate vindication for the use of PSCAN rather than each of the other tools, or a consensus of all four, for analysis of PACAP and TNF-alpha signaling in endocrine cells.

The genes induced (transcripts up-regulated) by TNF-alpha in BCC were predicted by PSCAN analysis to be predominantly regulated by NFKB. This is in agreement with the well recognized role of NFKB in the signal transduction of TNF-alpha in many other systems including brain [4,7,16,24,30,36,40]. In fact, there is a paucity of evidence in the literature for TNF-alpha-initiated signaling proceeding through any other pathway besides one involving NFKB. However, there is also relatively little information about signaling in cells that contain exclusively type 2 receptors for TNF-alpha, such as chromaffin cells, so that the present study provides important new information relevant to the neuroprotective role for TNFR2, compared to that in immune activation for TNFR1, in the central and peripheral nervous systems [4,30]. The role of NFKB in transmitting signal via the type 2 receptor to the nucleus in chromaffin cells was suggested earlier based on cross-species microarray analysis [4]. The current study confirms and extends that finding. On the other hand, the dynamics of NFKB signaling in endocrine cells is likely to be considerably different from that seen in immunocytes. In the latter, in the absence of TNF-alpha stimulation, NFKB is associated with the inhibitor Ikbap in the cytoplasm, and is inactive as a transcription factor. TNF-alpha-induced activation of NFKB to allow its migration to the nucleus and activation of NFKB-responsive genes largely relies on phosphorylation-dependent ubiquitination and degradation of inhibitor of kappa B (Ikb) proteins. The inhibitor of kappa B kinase (IKK) complex, a multiprotein kinase complex is responsible for the TNF-alpha induced phosphorylation of Ikb. Hence de novo synthesis of NFKB is not a TNF by NFKB. However, in our expression analysis, NFKB2, NFKBIA, NFKBIE, NFKBIZ genes are up-regulated by TNF-alpha from 3 to 8 fold at 6 h. This suggests that the dynamics of TNF-alpha gene regulation through NFKB do not include feedback inhibition at the level of induction of Ikb gene transcription, translation, and restored 'capture' of NFKB in a cytoplasmic inhibitory complex, as in immunocytes [17,21,22,45,48]. Rather, TNF-alpha signaling through TNFR2 appears to allow a lengthier period of NFKB-mediated transcriptional stimulation, even in the face of continued IKB synthesis, than in cells of the immune system. For TNFR2 receptor stimulation, NF-kB appears to be the major final common pathway for gene regulation, so that further specificity in TNF-alpha action for neuroprotection is likely to require pharmacological specification at the receptor level (TNFR1 versus TNFR2) imparting some level of cell-specificity, or differential targeting of upstream pathways that might differentiate TNFR1-initiated compared to TNFR2-initiated activation of NF-kB. One possibility is that prolonged activation of NF-kB is neuroprotective and initiated by TNFR2 signaling, whilst NF-kB activation by TNFR1 signaling in neurons/endocrine cells, as in immune cells, is self-limited by Ikb induction and has a much shorter time scale.

PACAP has now been shown to exert a large array of pharmacological effects and biological functions in the nervous and endocrine systems [6,27]. PACAP-stimulated secretion of catecholamines and neuropeptides from chromaffin cells of the adrenal medulla is unlikely to require de novo gene transcription, but rather complex non-transcriptional effects exerted

by cytoplasmic cAMP and calcium secondary to activation of adenylate cyclase, phospholipase C, or both [28,29,39]. However, PACAP also exerts profound short- and long-term effects on transcription through both calcium and cAMP [1,14,38], and at least some of these effects are known to be mediated through Egr1 [34,35]. In vivo, up-regulation of PNMT gene expression occurring in the adrenal medulla during restraint or hypoglycemic stress is PACAP-dependent [41,44].¹ Tai et al. have demonstrated that an Egr-1 site is essential for PACAP induction of rat PNMT transcription in PC12 cells harboring a PNMT reporter gene [46]. Expression of Egr-1 is itself up-regulated by PACAP in PC12 cells with a very rapid time course, in a cAMP-dependent fashion [11,35]. PACAP up-regulated genes are also enriched in a number of additional cis-regulatory elements in addition to that responsible for binding of Egr1, consistent with the activation of multiple fourth messengers by PACAP, including CREB and Elk-1 [12,13], that act as transcription factors in endocrine as well as non-endocrine cells. In addition to Egr-1, binding sites for Sp1 and AP-2 are also over-represented among PACAP-regulated genes. Although Sp1 and AP-2 are not immediate-early genes like Egr1, nor as dynamically regulated by phosphorylation as Egr1, these transcription factors may act in tandem with the latter to provide combinatorial specificity to PACAP gene regulation in endocrine cells. Analysis of the proximity and overlap of these TFBSs in PACAP-regulated genes should provide additional insight into whether the PACAP transcriptome is comprised of genes regulated in parallel by multiple signaling pathways or in a convergent manner via a combinatorial code contained in the promoters of PACAP-responsive genes. Both mechanisms may occur we have recently found that NS-1 cells, a PC12 cell sub-clone, respond to PACAP via activation of three distinct cAMP sensors, with cAMP-dependent activation of CREB and ERK occurring in parallel to affect distinct cellular responses [12]. The lack of over-representation of CREB binding sites within genes regulated by PACAP in BCCs is somewhat surprising. Pharmacological analysis of PACAP gene regulation via cAMP elevation in PC12 cells has revealed a fairly large number of genes whose up-regulation by PACAP is blocked by H-89 [47], and a number of the genes shown to be up-regulated by PACAP in this study or previously, including EGR1, KLF5, FOSB, RAB3A, SG2, and GAL, are known to contain CREB binding sites [18]. One possibility is that while PACAP regulation of some genes occurs through CREB activation, the majority of gene regulation by PACAP occurs via calcium, and ERK, the latter signaling molecule requiring a cAMP sensor, the NCS, that does not lead to activation of CREB [12,13], as is the case for the GAL gene encoding progalanin, up-regulated in a PACAP-dependent manner in the adrenal medulla following stress [43]. A second possibility is that CREB-dependent gene regulation by PACAP occurs relatively early (0-1 h) and is not apparent at 6 h of treatment with PACAP. Importantly, more than half of the genes previously identified within the CREBulome of PC12 cells by Impey et al. were deemed to be cAMP-dependent, suggesting CREB as the major transcriptional regulator of cAMP-dependent signaling in endocrine cells [18]. Perhaps at longer times of stimulation, Egr1 may be a more important one, at least for certain first messengers such as PACAP. The implications of both of these hypotheses for neurotransmitter signaling through cAMP to the nucleus in central and peripheral neurons merits their testing, most easily and reliably performed in BCCs.

Saito et al. have recently reported a temporal correlation between PACAP and NGF stimulation of ERK, and activation of Egr1, based on simultaneous measurement of pERK, pp38, pJNK, pCREB, c-Fos, Egr1, c-Jun, JunB and FosB as a function of time after treatment with NGF and PACAP in PC12 cells [37]. Thus, two separate sets of experiments, one in neuroendocrine cells (the present report) and another in a neuroendocrine cell line

¹PNMT mRNA was 3-fold higher in PACAP-treated compared to control BCCs using either Agilent or Affymetrix bovine arrays, however the fold-increase was not significant at the p levels chosen for the data presented in Fig. 1, due to variability thought to be associated with variable levels of glucocorticoids between BCC preparations.

[37] have converged on the conclusion that PACAP signaling through ERK is selectively decoded by expression of Egr1. This in turn is quite consistent with our earlier report that neuritogenesis initiated by PACAP in PC12 cells is both ERK- and Egr1-dependent [35], and that PACAP signaling to neuroendocrine-specific genes in bovine chromaffin cells is an ERK-dependent process (A. Emery, T. Mustafa, M. Eiden and L. Eiden, *Science Signaling*, in press, 2013). It remains to be shown empirically that PACAP signaling for gene regulation in bovine chromaffin cells is abrogated by inhibition or deletion of Egr1 activity, with preservation of target gene activation by TNF-alpha.

4.3. Implications of bioinformatics analysis of receptor-specific gene regulation for translational pharmacology of peptide signaling

The work described here should have practical implications for further development of the pharmacology of both TNF-alpha and PACAP signaling for translational purposes. In the case of TNF-alpha, the gene targets of the TNFR2 signaling pathway in neuronal/endocrine cells has implications for the design of TNFR2-specific and TNFR1-specific agents for modulation of signaling in CNS diseases such as Alzheimer's disease and traumatic brain injury, in which TNF-alpha signaling through the type 1 receptor is believed to be associated with microglial activation leading to synaptic simplification and neurodegeneration, while signaling through the type 2 receptor is associated with neuroprotection [26,30]. In the case of PACAP, cAMP- and calcium-dependent signaling are believed to be differentially involved in neurite formation, apoptosis, and secretion at the cellular level, and differentiation/development, neuroprotection, and hormonal responses at the organismic level [10,27-29]. Further parsing of PACAP- and TNF-alpha-responsive genes based on correlation of pharmacological inhibition of signaling pathways with altered enrichment in promoter/enhancer motifs found in the resultant cohort of regulated genes will provide an integrative approach to the eventual development of highly selective agents targeting increasingly specific cohorts of genes controlling the 'fine function' of neurons and endocrine cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Steps in identifying the genes regulated by either PACAP or TNF-alpha.

Table 1

PACAP up-regulated genes.

AffyChanged_AgChanged				AffyChanged_AgNC				AffyChanged_AgNP				AffyNP_AgChanged				AffyNC_AgChanged			
GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC
535588	ABHD5	2.37	3.34	530414	AGPAT5	2.04	1.58	536607	ACTN2	3.03		615187	ADCYAP1	5.78		407125	EGR1	1.72	2.01
509032	ANKMY2	2.48	2.84	507966	CDC42EP2	2.43	1.72	512010	ARAP2	2.76		534032	AFAP1	2.27		281477	SCG2	1.64	9.42
780866	AQP3	4.44	9.36	282182	CITED1	2.07	1.91	538584	ARHGAP21	2.26		2334	AFF2	2.19		282521	SERPINE2	1.43	2.03
540568	ATP2A2	2.26	3.74	613449	DCLK1	7.80	1.31	100125310	B3GALNT2	3.90		616582	AJAP1	3.27		530616	TCE3	1.22	2.01
614130	BAALC	5.01	5.81	286811	EIF6	2.07	1.36	529849	C2CD4B	2.80		541209	AMOTL1	2.07					
326579	BZW2	3.86	3.84	281148	ETS2	2.11	2.19	281049	CD24	31.37		615777	ANKS1B	2.08					
280740	CA2	2.34	2.62	281758	FABP3	2.07	1.89	532067	COBLL1	2.44		507390	BCL2L11	2.26					
407218	CALCB	23.52	55.14	281160	FGFI	2.75	-1.39	540605	CREM	8.36		617701	BDNF	4.70					
338066	CD97	2.82	2.66	615823	FGFI2	2.13	1.40	515406	CSRNPI	2.47		505504	BTBD9	2.30					
538392	CDC42BPA	2.05	2.48	280799	GAL	2.91	3.35	540083	DNAIB5	2.65		515741	CAPN14	6.04					
615978	CHST2	2.28	3.96	538437	GEM	2.05	1.75	505987	GK	2.09		617510	CCK	13.34					
282196	CORO1A	3.34	3.59	513221	HMOX1	2.01	1.80	282820	HAS1	2.36		494549	DIO3	5.83					
282203	CRYBA2	18.22	15.10	510243	HMOX2	2.01	1.99	507480	HES4	2.42		530813	DOK5	2.48					
511771	CXCL14	6.29	49.12	539359	LMO7	2.46	1.86	281872	ITGA2	5.87		541166	ETV5	4.72					
541211	DXDC1	3.15	4.89	281967	PCSK1	2.05	2.40	528014	KCNE1	2.58		514601	FAM102B	2.60					
527607	EFTUD1	2.05	2.48	540135	PHLDA1	2.69	2.45	541004	NRP2	3.10		534389	FAM105A	2.53					
404130	EIF2C2	2.43	5.32	281407	PLAT	2.16	1.63	518368	PARM1	3.32		785179	FKBP1B	2.33					
540348	ELOVL1	2.18	2.30	535071	SEC23B	2.17	1.98	538477	RALA	2.00		459663	FMNL2	2.27					
516303	ERRF1	2.69	4.72	617336	SHISA2	3.39	1.58	534114	RIMS1	2.26		538532	GOLIM4	2.96					
281161	FGF2	2.48	2.53					511190	RP86KA3	3.68		506661	GPR6	2.61					
404193	FGFR2	3.89	5.83					614701	SLC22A23	2.55		716892	GRIN3A	5.14					
540819	FOSB	3.02	4.56					532774	SLC6A20	2.13		538255	HCN1	2.81					
531389	FOSL1	2.16	3.15					539465	SLC7A1	2.28		535311	HOMER1	2.97					
327681	FST	3.32	5.09					507969	SLCO4A1	2.50		510342	IRAK3	2.17					
523154	GALE	2.20	2.77					514465	SMAF2	3.61		509394	ISYNA1	2.49					
782201	GNE	2.62	3.63					504593	SPRY4	4.25		534542	KCNH7	2.18					
281203	GN2	2.06	3.46					338078	STC1	8.82		539225	MAB21L2	2.71					
281848	IGFIR	2.36	3.44					513665	STK17A	2.04		527624	MARCH4	2.02					
514529	IL27RA	2.76	2.16					36007	TFP2	2.96		533987	METTL5	2.42					
532797	IMPAD1	2.28	2.89					782528	TOR1AIP1	3.53		505007	MOB3A	2.09					
281867	INHBA	3.63	4.78					777775	TUBA4A	2.18		535171	MPP6	2.48					

AffyChanged_AgChanged				AffyChanged_AgNC				AffyChanged_AgNP				AffyNP_AgChanged				AffyNC_AgChanged			
GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC
280829	INS	2.16	3.27									526544	NCSI		2.85				
535043	ITGA6	4.67	5.77									528842	NEFH		7.80				
537201	JAK1	2.23	4.00									530407	NETO1		6.24				
535702	KLF5	5.12	6.07									281355	NPPA		14.51				
615215	MAPKAPK3	2.17	2.24									789363	NPTX2		23.02				
404180	MEST	4.90	7.82									504216	NPY		6.58				
282276	MGAT4A	3.32	3.41									521790	PI4K2B		2.50				
513155	MMID	3.93	10.20									505773	PRKX		5.72				
281226	MOCOS	3.01	5.35									527921	RASD2		5.47				
510003	NEDD4L	2.18	2.52									504255	RGS17		2.10				
353114	NNAT	3.58	4.08									535674	RIMS2		2.31				
528390	NR4A1	3.03	3.19									519541	RNFI25		3.10				
505824	NTRK2	3.27	2.95									606737	RTL1		2.06				
281368	OLR1	2.02	2.14									540852	SAMD4A		2.56				
414348	P4HA3	3.06	5.21									504658	SERINC5		4.10				
509823	PALMD	2.32	2.52									520954	SGMS2		11.50				
100124505	PDE4B	2.46	2.37									614726	SH2B3		6.15				
407183	PFKFB3	3.54	3.18									613851	SLC30A7		2.44				
525095	PIGN	2.54	2.71									506910	SLC37A3		2.49				
281408	PLAU	3.69	4.25									529134	STK32B		3.63				
504282	PLK3	4.10	6.29									541217	SYNDIG1		2.80				
617148	PPP1R14C	3.20	4.79									539419	TBX20		2.29				
282020	PTGFR	4.30	4.31									509239	TMCC3		2.10				
286810	PTPRN	2.22	2.65									514623	URB2		2.48				
616537	RAB6A	3.14	2.79									618042	VGF		5.51				
533491	RASA2	2.02	2.60									508051	WDR4		2.39				
507449	RASD1	6.39	8.30									614279	ZNF706		2.00				
510276	RASSF1	2.18	2.27																
533687	RHPN2	2.15	2.78																
533228	RILPL2	3.05	3.96																
524683	SAMHD1	3.03	3.77																
282061	SCN5A	2.39	3.02																
516019	SEMA6A	2.43	2.58																
281375	SERPINE1	2.30	2.31																

AffyChanged_AgChanged				AffyChanged_AgNC				AffyChanged_AgNP				AffyNP_AgChanged				AffyNC_AgChanged			
GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC
515854	SGK1	2.77	2.75																
282355	SLC1A5	3.04	4.63																
527491	SLC38A1	3.11	4.50																
524417	SLC41A2	2.57	3.18																
510209	SLC4A7	2.70	3.88																
787004	SMAGP	2.57	3.45																
615229	SMYD2	5.86	7.26																
539090	SPRY2	2.80	3.30																
539452	SRGAP1	2.34	2.86																
508156	SSBP4	2.10	2.04																
515911	STRA6	3.73	5.17																
539867	SYT4	2.90	2.92																
281512	TAC1	45.95	166.54																
534069	TGFB2	2.55	3.67																
407101	TJP2	3.28	3.65																
533038	TN4SF1	2.67	2.02																
515491	TNEM2	2.95	3.32																
523131	TNFAIP8L3	3.39	4.33																
574056	TNFSF8	3.78	5.19																
782450	TOR1AIP2	2.32	3.30																
525888	TOX	2.45	2.89																
521857	TRIB1	2.03	2.58																
526950	UBL3	2.11	2.64																
281564	UGDH	2.82	4.02																
515029	UPP1	3.57	5.23																
514902	VASP	2.69	3.16																
280956	VIP	115.65	973.65																
282123	VLDLR	2.37	2.52																
282124	VSNL1	4.47	5.49																
535344	ZC3H12A	2.58	2.84																
514969	ACOX2	-2.37	-2.40	281611	AKAP4	-2.10	-1.65	536925	ADCK3	-2.02	-2.66	505740	ADCY5	-2.02	-2.66				
504577	ARMCX1	-2.56	-2.99	515282	ALG13	-2.17	-1.90	280713	ADM	-2.16	-3.66	523685	AKD1	-2.16	-3.66				
768308	ARMCX6	-2.12	-2.39	514174	CPEB1	-2.17	-1.95	788039	ARL4C	-2.32	-2.63	9915	ARNT2	-2.32	-2.63				
504789	BEND6	-2.10	-2.30	511195	CYP39A1	-2.15	-2.50	504224	C11orf16	-2.94	-2.46	540113	ATP6V1E2	-2.94	-2.46				

AffyChanged_AgChanged				AffyChanged_AgNC				AffyChanged_AgNP				AffyNP_AgChanged			
GeneID		Gene symbol	Affy FC	Agilent FC		Gene symbol	Affy FC	Gene symbol		Affy FC	Gene symbol	GeneID		Gene symbol	Agilent FC
353120	BRCA1		-2.27	-2.06	503577	DBP	-2.58	-1.54	616613	C17H12orf24	-2.39	618614	BIK		-2.23
282162	CAMK2G		-2.40	-2.04	537349	DZIP3	-2.10	-2.06	517240	CC2D2A	-2.37	538758	C10H15orf41		-2.47
538772	DARS2		-2.12	-2.42	535452	FBXL4	-2.04	-1.72	538990	DBC1	-2.36	504224	C15H11orf16		-3.62
280763	DDO		-3.44	-2.95	281767	FDFI1	-2.29	-1.88	507276	DHRS12	-2.47	615337	C15H11orf63		-2.48
510124	EFHC1		-2.06	-2.51	326285	FGF10	-2.06	-2.93	100137960	DYTN	-2.12	617430	C15H11orf70		-2.08
540581	GPR61		-3.13	-5.09	512676	HACL1	-2.12	-1.69	514456	GPD1L	-2.06	616962	C16H1orf105		-2.38
281227	HMMR		-2.72	-2.75	509855	IRF9	-2.05	-1.73	407135	HTR2B	-4.01	509555	C19H17orf76		-2.49
526516	HOOK2		-2.08	-2.29	616730	KLFF1	-2.02	-1.70	781073	JPH4	-2.08	507040	C1H3orf33		-2.24
616194	HSPB11		-2.02	-2.34	535033	LIX1	-2.47	-2.04	534869	KANK1	-2.03	100140616	C20H5orf49		-2.12
533510	KLHL24		-2.30	-2.03	614509	MXI1	-2.13	-1.58	527321	KLHDC8B	-2.15	510670	C28H10orf107		-2.11
617080	LGI1		-2.37	-2.56	535203	NTHL1	-2.09	-1.80	535021	LACTB2	-2.02	783074	CAB39L		-2.49
530865	LIAS		-2.28	-2.75	516280	RIC3	-2.75	1.20	505233	LASS4	-2.08	616482	CAPS		-2.50
286883	MAP2K6		-2.53	-2.25	533764	RPE	-2.69	0.05	508378	LGP2	-2.18	515946	CBRI		-2.28
505595	MCF2L		-2.73	-3.98	282363	SLC6A2	-2.02	-2.52	520694	LINGO1	-2.50	614062	CCDC60		-2.23
613844	METTL7A		-2.04	-2.32	541012	TCP11L2	-2.11	-2.20	505750	LRIG1	-2.02	506207	CCKAR		-2.86
616069	N4BP2L1		-2.26	-2.43	504509	ZAP70	-2.12	-1.66	506766	MAP7	-2.26	510244	CDKN2D		-2.95
444987	NEIL2		-2.04	-2.66	613943	ZDHHC22	-2.46	-1.84	527679	MBNL2	-2.22	504370	CEND1		-2.23
614571	NXPH1		-2.15	-2.45					510399	MGC128008	-2.15	505233	CERS4		-2.10
407186	OMG		-4.68	-7.40					617717	NG5	-2.13	616832	DCDC1		-2.23
510583	PAH		-2.45	-2.41					537614	NR3C2	-2.74	508378	DHX58		-2.63
510532	PARP9		-2.53	-2.02					614673	NUPR1	-2.51	523187	DNAAF1		-3.44
282308	PIK3R2		-2.29	-2.48					529660	OAS2	-2.63	518852	BHHADH		-2.67
539761	PLAGL1		-2.04	-2.73					533508	ODF2L	-2.18	767880	ENO4		-2.07
509657	PTPRK		-2.52	-2.67					506308	PDXP	-2.06	524246	EPHX4		-2.67
282029	RAB3A		-2.55	-3.27					512082	PIK3IP1	-2.91	616613	FAM216A		-2.19
539884	RFX5		-2.14	-2.12					782020	PTER	-2.31	445423	FANK1		-2.55
616969	RGNEF		-2.18	-2.63					532751	PTPRD	-2.24	1112	FOXN3		-2.11
504408	RPS6KA5		-2.20	-2.37					616166	RALGPS1	-2.11	616594	GPRI37C		-3.32
527574	SALL2		-2.34	-2.72					617625	RBM43	-2.10	525734	GPRI76		-5.15
614027	SIRT3		-2.46	-2.36					538226	SESNI	-2.22	781811	HESX1		-2.80
515553	SLC25A34		-2.95	-3.74					787248	SIPA1L1	-2.52	509275	HIST1H1D		-2.13
281952	SLC6A15		-2.40	-2.24					515302	WDR76	-2.07	510569	IZUMO4		-2.48
514201	SMPD3		-2.29	-2.35								519027	KANK4		-2.18
514624	STARD10		-2.22	-2.65								526133	KCNAB1		-2.77

AffyChanged_AgChanged				AffyChanged_AgNC				AffyChanged_AgNP				AffyNP_AgChanged				AffyNC_AgChanged			
GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC
512732	STMN4	-2.93	-3.27									538479	KCNJ12		-4.77				
515940	THRSP	-2.83	-3.43									512403	LRRC36		-2.04				
538501	UNC119	-2.10	-2.63									511013	LYSMD2		-2.16				
512257	ZMYND12	-2.58	-2.18									518794	MAP6		-3.13				
												539825	MAST1		-2.26				
												616704	MCC		-2.60				
												519770	PBX4		-2.08				
												523186	PHIP		-2.09				
												523993	PMFBP1		-3.15				
												506337	PPPIR32		-2.07				
												100141235	PPPIR36		-2.17				
												617717	PRRT1		-2.33				
												511773	RBFOX3		-2.72				
												541233	RCOR2		-2.17				
												532204	RRNAD1		-2.06				
												532442	RTP4		-2.69				
												540864	SGTB		-3.13				
												510157	SLC12A5		-2.83				
												513571	SPESP1		-2.41				
												613989	SSPN		-2.04				
												506979	STOX1		-2.61				
												504393	TMEM25		-2.27				
												617572	TMIE		-2.24				
												532085	TMOD2		-2.77				
												515212	VMAC		-3.44				
												517598	XKR4		-2.12				
												538763	YDIC		-2.19				
												768236	ZC4H2		-2.13				
												504576	ZMAT1		-3.86				

The final list of genes up regulated by PACAP was calculated based on the information in table S9a. Genes from the ‘include’ groups minus the false positives and the true positives from the ‘exclude’ groups were retrieved along with their fold changes (FC) which were 2 for up regulated genes and were -2.0 for down regulated ones. *p* values from both Affymetrix and Agilent arrays were 0.05. Some of the NC designated genes did not meet the *p* value cut off criteria even though the fold change was 2.0.

TNF-alpha up regulated genes.

Table 2

AffyChanged_AgChanged				AffyChangedAgNC				AffyChangedAgNP				AffyNPAgChanged				AffyNCAgChanged			
GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC
514386	BIRC3	5.97	7.30	533050	BoLA	3.51	1.06	528597	ABTB2	2.16		532119	BDKRB1	2.37		281350	NGF	1.81	4.22
280740	CA2	2.05	2.31	338039	CASP4	3.86	3.51	786156	CCL1	3.72		509304	C1H3orf58	2.12		506182	TRAF3	1.59	2.46
281043	CCL2	12.77	15.64	281094	CSF1	3.04	1.34	281666	CCL20	14.81		526279	CASP7	2.45					
327712	CCL5	15.11	58.28	281871	ISG15	2.12	2.54	540283	CPOX	2.10		613667	CXCL3	5.04					
281048	CD14	2.04	2.33	535450	PUM1	3.35	-1.32	511671	CXCL16	3.29		281735	CXCL6	2.76					
617034	CD83	6.12	9.29					509796	DPYSL3	2.32		530813	DOK5	2.22					
281678	CEBPD	3.84	3.86					516949	GBP5	2.45		514601	FAM102B	2.04					
497199	CHLAR	2.69	3.18					514889	IFNGR2	2.20		459663	FMNL2	2.61					
507285	CHN2	2.58	2.90					615135	IPMK	2.67		513659	GBP7	6.17					
281214	CXCL2	3.42	5.50					535622	MAP3K8	2.52		541055	HTR3B	9.62					
533992	DRAM1	2.10	2.65					540817	MOBKLB	2.42		100139670	IFIH1	3.43					
541175	DUSP10	3.13	3.41					507781	NEDD4	4.41		509678	IFIH3	3.15					
530101	GFPT2	3.04	3.45					616115	NFKB1	3.82		510342	IRAK3	2.08					
513221	HMOX1	2.05	2.31					506727	NFKBIE	2.36		618448	KLK12	3.04					
281839	ICAM1	4.96	5.61					529245	ORC4	6.79		540817	MOB3B	3.22					
506759	IFI16	2.27	2.73					282328	PSMB10	2.09		617491	MPV17L	3.25					
535490	IFIH1	4.06	4.90					519269	RRAS2	2.02		510807	NFE2L3	2.34					
282258	IFNAR2	2.31	2.79					525823	SLC10A2	3.77		519541	RNF125	2.28					
280826	IL6	7.92	10.48					524959	TAP1	4.18		532442	RTP4	4.04					
280828	IL8	9.57	12.66					360007	TEPI2	2.31		532860	SLC19A2	2.09					
337917	IRF1	5.11	5.38									282375	STAT5A	2.38					
615340	IRF5	3.12	3.32									539960	STX11	2.42					
514246	JUNB	2.34	4.83									509239	TMCC3	2.06					
505563	KCNK1	2.20	2.24									508105	TNFAIP3	17.56					
280872	MX1	2.97	3.57									531850	TNFAIP8L1	2.38					
526392	NFKB2	8.45	5.29									520341	TNFRSF9	3.52					
282291	NFKBIA	6.68	6.35									506467	TREM2	2.11					
282713	NFKBIZ	3.32	3.51									508051	WDR4	2.17					
511280	NMI	2.25	2.18																
281368	OLR1	8.46	12.89																
534150	OPTN	2.11	2.47																

AffyChanged_AgChanged				AffyChangedAgNC				AffyChangedAgNP				AffyNPAgChanged				AffyNCaGChanged			
GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC
511595	PARP8	2.00	2.48																
515017	PDGFRL	2.10	2.10																
281408	PLAU	4.26	4.86																
281983	PLAUR	2.22	2.80																
338446	PPARGCIA	2.31	2.79																
282603	PRSS2	2.85	3.17																
282013	PSMB8	2.16	2.47																
510593	PSMB9	3.33	4.76																
541148	PTX3	2.25	3.67																
522670	RELB	5.00	7.64																
539884	RFX5	2.18	2.31																
540836	RGS1	14.45	25.87																
506415	RSAD2	2.32	3.04																
281474	SAX3	4.80	11.63																
508133	SDC4	2.35	2.69																
281484	SELE	4.61	7.30																
281486	SELP	3.10	3.34																
281375	SERPINE1	2.39	2.21																
521920	SULT1B1	2.00	2.19																
281534	TLR2	3.70	6.05																
515475	TMEM140	3.35	5.67																
526103	TNIP1	2.88	3.42																
281542	TP53	2.06	2.31																
359715	TRIM21	3.74	4.86																
535975	UST	2.04	2.13																
282118	VCAM1	7.36	12.89																
535344	ZC3H12A	2.12	2.17																
TNF-alpha down regulated genes																			

AffyChangedAgChanged				AffyChangedAgNC				AffyChangedAgNP				AffyNPAgChanged			
GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene symbol	Affy FC	Agilent FC
327684	NR2F1	-3.07	-3.68	286883	MAP2K6	-2.07	-1.96	506308	PDXP	-2.08		616225	ATOH8	-3.20	
519439	SOX18	-2.04	-3.63	514201	SMPD3	-2.90	-1.99					616482	CAPS	-2.07	

AffyChanged_AgChanged				AffyChangedAgNC				AffyChangedAgNP				AffyNPgChanged				AffyNCgChanged			
GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC	GeneID	Gene Symbol	Affy FC	Agilent FC
515940	THRSP		-2.37				-2.76										523187	DNAAF1	-2.22
																	538479	KCNJ12	-2.46
																	783571	LRRC3B	-2.37
																	518794	MAP6	-2.11
																	541233	RCOR2	-2.40

The final list of genes up regulated by TNF-alpha was calculated based on the information in table S9b. Genes from the 'include' groups minus the false positives and the true positives from the 'exclude' groups were retrieved along with their fold changes (FC) which were 2 for up regulated genes and were -2.0 for down regulated ones. *p* values from both Affymetrix and Agilent arrays were 0.05. Some of the NC designated genes did not meet the *p* value cut off criteria even though the fold change was 2.0.

Table 3

Assessment of transcription factor prediction tools.

Tool	CREB1	EGRI	NFKB	REST	Composite score
PSCAN	1	1	1	1	4
Core TF	1	0	1	0.5	2.5
TFME	0	0	0.5	0	0.5
Opossum	0	0	1	1	2

Four different prediction tools, i.e. CORE_TF, Genomatix, PSCAN and TFME were evaluated using upstream sequences to the Transcription start site (TSS) of experimentally derived targets for CREB1, EGRI, NFKB and REST. The predictions were sorted on increasing *p* value scores. If the correct transcription factor was predicted outright, the prediction tool was assigned a score of '1'. If the corresponding transcription factor was predicted among the five highest-probabilities, the tool got a score of '0.5'. If the corresponding transcription factor was not among the five highest-probability scores for that tool, it received a score of '0'.

Table 4

Prediction of over-abundance of transcription factor binding sites in PACAP or TNF up-regulated genes by PSCAN.

Identifier	Matrix_ID	TF_NAME	Sequence	Z-score	p-Value
PACAP					
V\$SP1_Q6	M00196	Sp1isoform2	NGGGGGCGGGGYN	3.71E+00	9.60E-05
V\$AP2GAMMA_01	M00470	AP2gamma	GCCYNNNGGS	3.60E+00	1.47E-04
V\$AP2ALPHA_01	M00469	AP2alphaA	GCCNNNRGS	3.52E+00	2.02E-04
V\$AP2_Q6	M00189	AP2gamma	MKCCSCSCNGGCG	3.43E+00	2.75E-04
V\$EGR1_01	M00243	Egr1	WTGCGTGGGCGK	3.17E+00	7.32E-04
V\$NGFIC_01	M00244	Egr4	WTGCGTGGGYGG	3.12E+00	8.40E-04
TNF V\$NFKAPPAB65_01	M00052	RelAp65	GGGATTTC	7.4992	2.47E-14
V\$CREL_01	M00053	cRel	SGGRNTTTC	7.04892	7.33E-13
V\$NFKAPPAB_01	M00054	RelAp65	GGGAMTTYCC	5.60367	9.04E-09
V\$NFKB_Q6	M00194	p50	NGGGGAMTTTCNN	4.22653	1.10E-05
V\$NFKB_C	M00208	NFkappaB2p52	NGGGACTTCCA	3.98895	3.11E-05
V\$NFKAPPAB50_01	M00051	NFkappaB1p50	GGGGATYCCC	3.85739	5.46E-05
V\$IRF7_01	M00453	IRF7A	TNSGAAWNCGAAANTNNN	3.28933	4.86E-04
V\$STAT1_03	M00496	STAT1beta	NNTTTCN	3.23163	5.95E-04
V\$PAX4_04	M00380	Pax4	RAAAAWTA(N)1SYCACNCC	3.14575	8.02E-04
V\$ISRE_01	M00258	ISGF3	CAGTTTCWCTTTYCC	3.08943	9.65E-04

950 bp of nucleotide sequences upstream of the transcription start site and 50bp downstream sequences of genes up regulated by PACAP or TNF-alpha were analyzed using PSCAN to predict over-abundance of transcription factors. Human Refseqs corresponding to the bovine gene symbols in those two categories were supplied as inputs. Only the TFBS with a Z score of >3 are shown in Table 4. Matrices with no known transcription factors were removed after checking the identification of transcription factors @ <http://www.broadinstitute.org/gsea/msigdb/search.jsp/>. The key to the nucleotide single letter codes are according to IUPAC (<http://www.bioinformatics.org/sms/iupac.html>).