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Cycloheximide treatment to identify components of the transitional transcriptome in PACAP-induced PC12 cell differentiation

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) promotes neurite outgrowth, reduces proliferation and inhibits apoptosis of PC12 cells. We have partially characterized the transcriptome changes induced by PACAP after 6 h of treatment, when commitment to differentiation has occurred. Here, we have investigated the effects of a 6-h treatment with PACAP (10^{-7} M) in the presence of cycloheximide (5 μM) to identify, via superinduction, components of the transitional transcriptome initially induced by PACAP and potentially participating in the regulation of late-response genes required for differentiation. Approximately 100 new transcripts were identified in this screen, i.e. as many individual genes as make up the 6-h PACAP differentiation transcriptome itself. Six known transcripts in this cohort were then measured at several time points between 0 and 6 h by real-time PCR to determine whether these transcripts are induced early following PACAP treatment in the absence of cycloheximide, and therefore may be of functional importance in differentiation. Five out of the six transcripts were indeed induced by PACAP alone soon (between 30 min and 3 h) after cell treatment. *β*-Cell translocation gene 2, antiproliferative (Btg2), serum/glucocorticoid-regulated kinase (Sgk), nuclear factor for the κ chain of B-cells (NFκB), seven in absentia homologue 2 (Siah2) and FBJ osteosarcoma related oncogene (Fos) showed a 2.5–200-fold induction by PACAP between 15 min and 3 h, and mRNA levels returned either to baseline or near baseline after 6 h. This work provides new information concerning genes whose transient regulation early after PACAP exposure may contribute to the expression of the differentiated transcriptome in PC12 cells, and should help to elucidate the molecular mechanisms involved in the control of nerve cell survival and differentiation.

Keywords

cycloheximide; early response genes; microarray; PACAP; transitional transcriptome

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The PC12 cell line was selected from a rat adrenal pheochromocytoma (PC) based on its ability to differentiate (cease proliferation and extend branching varicose processes) in the presence of nerve growth factor (NGF; Greene and Tischler 1976). Treatment of PC12 cells with pituitary adenylate cyclase-activating polypeptide (PACAP) also induces growth arrest, promotes neuritogenesis (Deutsch and Sun 1992), changes cell size (Ravni *et al*., in preparation), inhibits apoptosis (Vaudry *et al*. 2000) and increases the expression of a battery of genes specific to neuroendocrine cell secretory function. We characterized the transcriptome of PC12 cells committed to PACAP-induced differentiation by collecting RNA samples after a 6-h exposure to PACAP, sufficient for the unfolding of the differentiation program over a subsequent 48–72-h period (Vaudry *et al*. 2002a). RNA from PACAP-treated and -untreated cells was hybridized using two-colour technology on 15 000-element cDNA microarray slides (Vaudry *et al*. 2002a). These experiments revealed 73 genes robustly regulated by PACAP, with 71% of the transcripts increased and 29% decreased in abundance.

We then measured the dependence on new protein synthesis of both up- and down-regulated messenger RNA by re-examining PACAP induction in the presence of cycloheximide (Wettstein *et al*. 1964), to determine whether *de novo* protein synthesis was required for the transcription of genes regulated by PACAP. Most of the transcripts induced within a 6-h period did not themselves require the induction of new protein synthesis. However, some did, including transcripts such as *annexin A2* and *actin-related protein 2/3 complex*, which are likely to be important for the eventual production of the differentiated PC12 cell phenotype (Vaudry *et al*. 2002a). These results suggested that identification of transitional transcripts – those encoding gene products required for the unfolding of the differentiation program but not part of the final differentiated cell transcriptome – might be required for a full understanding of the molecular events of PC12 cell differentiation by PACAP.

In addition to inhibiting protein synthesis required to effect neurite elongation and other cellular changes attending differentiation, cycloheximide and similar agents normally stabilize short-lived mRNA, such as those encoding immediate early genes like *activity-regulated cytoskeleton-associated protein* (*Arc*) and *FBJ osteosarcoma-related oncogene* (*Fos*), so that these normally transiently expressed transcripts can be detected for longer periods following their initial induction (Vaudry *et al*. 1998; Ichikawa *et al*. 2003). These transcripts also often exhibit mRNA superinduction in the presence of a translational inhibitor (Vaudry *et al*. 1998). These messengers are usually associated with rapid mRNA turnover and often contain AU-rich elements (Caput *et al*. 1986), which are thought to confer instability, in their 3′ untranslated regions (3′UTR; Roshak *et al.* 1996).

Superinduction is commonly attributed to decreased mRNA turnover, although increased nuclear signalling may also explain this effect (Edwards and Mahadevan 1992). Based on this concept of superinduction, microarray analyses were conducted to identify genes that are regulated after a 6-h treatment with PACAP in the presence of cycloheximide, but not initially shown to be up-regulated at the 6-h time point by PACAP alone. These transcripts represent potential immediate early genes, the protein products of which may act in a transient manner within the first 6 h of treatment to further transactivate genes effector for

neurite extension, morphological alterations, electrical excitability, cessation of proliferation, protection from apoptosis or newly synthesized prohormones, the secretory vesicle payloads of differentiated neuroendocrine cells.

Following the detection of transcripts elevated after 6 h of treatment with PACAP in the presence of cycloheximide, a potential involvement of members of this cohort in the PACAP transitional transcriptome was tested by examining the regulation of these transcripts by PACAP alone between 0 and 6 h of treatment. Our results indicate that the cycloheximide microarray screen, paired with a detailed time-resolved analysis of individuals transcripts after exposure to neurotrophic factors is a comprehensive approach to identify transcripts of the transitional transcriptome of differentiating neuroendocrine cells.

Materials and methods

Cell culture and treatments

PC12-G cells were plated at a density of 70 000 cells/mL (140 cells/mm^2) on poly-L-lysine-coated plates and cultured at 37°C in 10% CO_2 and 90% air atmosphere. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 7% heat-inactivated fetal bovine serum, 7% horse serum, 2.5% HEPES, 1 × glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin antibiotic. When necessary, cells were pre-incubated in either the absence or the presence of cycloheximide (5 μM) 30 min before treatment with either control medium or PACAP (10^{-7} M).

RNA extraction, probe preparation and microarray hybridization

After 6 h of treatment, RNA samples were harvested from the cells with Trizol reagent and further purified using the RNeasy Maxi Kit (Qiagen, Valencia, CA, USA). Fluorescently labelled cDNAs were synthesized from RNA by oligo(dT)-primed reverse transcription in the presence of either Cy3- or Cy5-2′deoxy-uridine 5′triphosphate (dUTP). After denaturation, purified Cy3/Cy5-labelled probes were combined and hybridized on a microarray slide containing over 15 000 mouse cDNA sequences (Tanaka *et al*. 2000) in the presence of 2 × Denhart's solution, 3.3 × saline sodium citrate (SSC) and 0.5% sodium dodecyl sulfate (SDS) in a humidified chamber at 65 °C overnight. Prior to scanning (Agilent Technologies, Foster City, CA, USA), slides were successively washed at room temperature (22°C) in 0.5 × SSC/0.1% SDS for 2 min, 0.5 × SSC/0.01% SDS for 2 min and 0.06 × SSC for 2 min. Image analysis was performed with the IPL_{AB} software (Scanalytics, Fairfax, VA, USA). The two fluorescent images (red and green channels) obtained from the scanner constituted the raw data from which differential gene expression ratio and quality control values were calculated. All data were entered into a relational database, using the FileMakerPro 5 software (FileMaker, Santa Clara, CA, USA). Each spot on the microarray was assessed for quality control in the IPLAB software. Any genes regulated in self–self hybridizations (either control vs. control or PACAP vs. PACAP hybridizations) were excluded from further consideration.

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Reverse transcription and real-time PCR

Transcripts used for real-time PCR were extracted using the same protocol as for the microarray, except that during RNA purification DNA was removed by incubation of the samples with DNAse (Qiagen). cDNAs were synthesized from 4 μg of total RNA with ImProm II Promega kit (Promega, Charbonnières-les-Bains, France). Real-time PCR was performed on obtained cDNA in the presence of 1 × SYBR Green Mastermix (Applied Biosystems, Courtaboeuf, France) containing preset concentrations of dNTPs, $MgCl_2$ and buffers, along with adequate concentrations of reverse and forward primers (Table 1).

Conventional RT-PCR

PCR analysis of the PACAP receptors expressed in PC12-G cells was conducted using the same cDNA as for real-time PCR. RNAs isolated from hypothalamus and lung tissues were also added as positive controls for PACAP specific receptor (PAC1), PACAP and VIP receptor 1 (VPAC1) and PACAP and VIP receptor 2 (VPAC2) expression. Amplification of the PAC1, VPAC1 and VPAC2 receptors was conducted as previously described (Basille *et al*. 2000). The pairs of primers design to amplify the PAC1, VPAC1 and VPAC2 mRNAs are indicated in Table 1. The specificity and size of the amplicons was verified after migration on a 3% agarose gel in TBE and visualized under ultraviolet illumination after ethidium bromide staining.

Statistical analysis

For microarray experiments, data are presented as mean ± SEM from three independent experiments for control vs. PACAP plus cycloheximide, and from 12 independent experiments for control vs. PACAP. For real-time PCR, data are presented as mean ± SEM from three independent experiments performed in triplicate. Statistical analyses of the data were performed using one-way analysis of variance followed by a Tukey's multicomparison post-test.

Results

Effects of PACAP plus cycloheximide on gene expression in PC12 cells

The extensive neurite sprouting seen 48 h after continuous exposure to PACAP can also be observed 48 h later, even if PC12 cells only receive an initial 6-h exposure to PACAP (Vaudry *et al*. 2002a). This suggests that some genes must be regulated very rapidly in these cells. In order to identify some early mRNA that would be induced transiently, PC12 cells were incubated with PACAP in the presence of cycloheximide. In control conditions, PC12-G cells express the hop and hip-hop variants of the PAC1 receptor but none of the VPAC receptors (Figs 1a-d). Treatment with cycloheximide does not affect the expression pattern of the PACAP receptors (Figs 1a-d) and does not change the expression level of the PAC1 receptor (Fig. 1e), which indicates that the results observed are not caused by a modification of the PACAP receptors. After 6 h of treatment, 166 transcripts were induced by at least two-fold. Among these genes, 106 had not been previously found to be regulated by PACAP alone at 6 h. Eight exhibited an average level of activation by PACAP plus cycloheximide that exceeded 10-fold (Table 2). According to the previous microarray experiments, some of

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the transcripts activated by PACAP plus cycloheximide such as *serum/glucocorticoid-regulated kinase* (*Sgk*) were not increased at all by PACAP alone at 6 h, whereas others, including either Cbp/p300-interacting transactivator with Glu/Asp-rich carboxterminal domain 1 (*Cited1*) or *Fos*, were increased by between 1.7- and 1.9-fold, which remains under the threshold limit of a two-fold induction that was applied to the previous analysis (Table 2). The highest induction by PACAP plus cycloheximide was observed for *β-cell translocation gene 2, antiproliferative* (*Btg2*; a 21-fold increase) followed by those observed for *early growth response 1* (*Egr1*), *Cited1* and *Fos* (Table 2). In contrast to the components of the transitional transcriptome discussed here, up-regulation of some classical PACAP-induced transcripts such as *tyrosine hydroxylase* mRNA, seen induced by PACAP alone in PC12-G cells, are not enhanced in the presence of cycloheximide suggesting that these mRNAs are probably regulated in an indirect manner.

Functional classification of the genes activated by PACAP plus cycloheximide

To facilitate further analysis, the genes identified in the present study were classified according to the GeneOntology (GO) references (http://www.geneontology.org/) using the Unigene accession number provided for each clone (Fig. 2, Table 2). Among the 106 mRNA regulated by PACAP plus cycloheximide but not by PACAP alone after 6 h of treatment, 63 genes had an inferred function and were assigned to 17 categories as indexed in the biological process level three of the GO database (Fig. 2). The three most abundantly populated categories were, in descending order: cellular physiological processes, metabolism and morphogenesis, and cell differentiation and development. As PACAP can either promote cell differentiation or inhibit apoptosis, it is interesting to note in Table 2 the presence of several genes controlling growth arrest, i.e. *growth arrest and DNA-damage-inducible 45 gamma* (*Gadd45g*), morphogenesis/cell differentiation, i.e. *signal transducer and activator of transcription 3* (*Stat3*), embryonic development, i.e. *ring finger protein 2* (*Rnf2*) or cell death, i.e. *Sgk*.

Three genes have no known role in the GO and eight have an identified function but are not present in the biological process level two categories (Table 2). Finally, 32 genes regulated by PACAP plus cycloheximide (30%) were not referenced in the GO database. A majority of these genes code for RIKEN cDNA (50%), and the others for genes that have not yet been entered in the database. Some of the transcripts without GO annotation, such as *growth arrest specific 5* (*Gas5*), are nevertheless likely candidates for controlling aspects of the neurotrophic effects of PACAP.

Verification of gene induction by real-time PCR

Six genes with varying expression profiles were selected and their expression level was quantified by real-time PCR to validate the microarray results (Table 1). Primer efficacy was addressed by measuring the slope of a standard curve taken from a serial dilution of control cDNA using the *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) mRNA as an internal standard. The real-time PCR results confirmed the microarray data as all the genes tested by quantitative PCR were indeed significantly induced after 6 h of treatment with PACAP plus cycloheximide (Fig. 3). Real-time PCR experiments also revealed that *nuclear factor for κ chain of B-cells* (*NFκB*) and *Fos* were induced to 2.3- and 3.3-fold levels, respectively, by

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PACAP alone (Fig. 3), but this was not significant according to our statistical analysis. Incubation of the cells with cycloheximide alone increased the expression level of the genes tested from 1.9- (*Sgk*) to 209-fold (*Fos*) (Fig. 3) and, for five genes out of six, the addition of PACAP resulted in a synergistic and highly significant increase in transcript levels. Maximum expression was observed for *Fos*, which was induced to a level of 630-fold higher than the control level after 6 h of exposure to PACAP plus cycloheximide. The only gene not activated by PACAP in the presence of cycloheximide was the *activity-dependent neuroprotective protein* (*Adnp*). *Adnp* is known to be regulated by PACAP, but this gene seems to be induced through the VPAC2 receptor which is not expressed in PC12 cells (Zusev and Gozes 2004).

The possible effect of PACAP alone on the expression of the six genes tested by real-time PCR was investigated by exposure to PACAP (10^{-7} M) for durations ranging from 2 min to 6 h, with additional time points of 12, 24 and 48 h measured after the first 6-h period (Fig. 4). This time-course experiment revealed that all the genes activated by PACAP in the presence of cycloheximide, with the exception of *Adnp* were also induced by PACAP alone. The activation of some transcripts such as *Fos* and *Btg2* occurred within 10 mins, whereas it took 2 h for *NFκB* to be induced. Maximum expression observed after 1–3 h of treatment with PACAP was only 2.5 over control for *Sgk*, and exceeded 200-fold over control for *Fos* (Fig. 4). After 6 h of treatment, all transcripts in this group returned to basal levels of expression.

Discussion

Recent studies performed on PC12 cells after 0.5, 6 and 48 h revealed that several populations of early as well as late-response genes are induced by PACAP after various times of treatment, highlighting the difficulty of selecting a single and optimal time point for microarray experiments (Vaudry *et al*. 2002a; Grumolato *et al*. 2003; Ishido and Masuo 2004). In a previously published study (Vaudry *et al*. 2002a) we chose a time point of 6 h to examine the effect of PACAP on PC12 cell transcriptome because the removal of PACAP before 6 h resulted in a reduced neuritogenic response at the subsequent assessment at 48 h. The withdrawal of PACAP at 6 h produced the same response at 48 h as that seen with PACAP present during the entire 48-h period. This strongly suggests that PACAP triggers the differentiation program by, but not before, 6 h of treatment, which 48 h later results in neurite extension, cessation of cell proliferation and the production of a battery of transcripts required for full expression of the neuroendocrine phenotype. The design of our previous study overlooked, however, those transcripts transiently contributing to the regulatory cascade leading to full differentiation. So-called immediate early genes, for example, are likely to be found within the cohort of genes making up the transitional transcriptome. Immediate early genes are defined as those which encode proteins that act mainly as transacting factors for transcription, in order to regulate either the expression of the genes encoding the final protein effectors of the cellular processes under investigation (Morgan and Curran 1995).

The present study offers a useful approach to identify genes regulated in a transient manner that may not be detected at a given single time point, which most microarray studies are restricted to for practical considerations. The real-time PCR experiments indicated that

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PACAP can activate some genes such as *Btg2* within 10 min of treatment. PCR validation conducted on six different genes also confirmed the microarray results and revealed that five transcripts out of six were actually also regulated by PACAP alone. This observation indicates that most of the genes presented in Table 2 are likely to be induced by PACAP alone as part of the transitional transcriptome(s) leading to PC12 cell differentiation. As the regulation of all genes of interest can be easily validated by real-time PCR, as illustrated for the six transcripts examined here, the important question will now be the identification of the function of each of these genes during PC12 cell differentiation, and specifically either their role in the induction of downstream targets at the transcriptional level or the enhancement of effector function through transient effects on phosphorylation, proteolysis or other modes of protein activation.

Effects of PACAP on transcription factors

The functional classification performed with the GO database highlighted 15 transcription factors known to be regulated rapidly and transiently. Some of these messengers encoding CREB binding protein (Crebbp) and Stat3 have already been shown to regulate the expression of other late response genes such as either tyrosine hydroxylase or neuropeptide Y in PC12 cells (Ghee *et al*. 1998; Muraoka *et al*. 2003). Conversely, some genes, which have previously been shown to be strongly induced by PACAP after 6 h of treatment, such as the *immediate early response 3* (*Ier3*), contain in their promoter region functional binding sites for NFκB (Schafer *et al*. 1998; Vaudry *et al*. 2002a). As this transcription factor is activated within 2 h of treatment with PACAP it may participate in the induction of *Ier3*. NFκB is usually associated with either Tumor Necrosis Factor-α (TNF-α) or Fas ligand-induced apoptosis, but it has also been demonstrated that inhibition of this transcription factor by sodium salicylate leads to PC12 cell death (Kiss *et al*. 2004). In addition, NFκB inhibitors exacerbate oxidative stress-induced cell death and play a role in B-cell lymphoma protein 2 (Bcl-2) protection (Jang and Surh 2004). These observations suggest that the genes regulated through NFκB may potentially control the anti-apoptotic effects of PACAP.

Some of the early response genes regulated by PACAP are likely to convey its neurotrophic effects in PC12 cells (Tanaka *et al*. 1997). For instance, *Fos*, previously identified as transiently up-regulated by PACAP in cultured cerebellar granule neurones (Vaudry *et al*. 2000), was shown to be strongly induced by cycloheximide plus PACAP but also by PACAP alone in PC12 cells. The involvement of *Fos* in the neurotrophic effects of PACAP is supported by the fact that blocking *Fos* expression in PC12 cells inhibits the differentiation induced by NGF (Gil *et al*. 2004). *Jumonji* (*Jarid 2*), another gene regulated by PACAP in the presence of cycloheximide, encodes a protein required for proper neural tube formation (Takeuchi *et al*. 1995) and the reduction of cardiac cell proliferation via the repression of *cyclin D1* expression (Toyoda *et al*. 2003). Based on this information, *Jumonji* is a likely candidate for involvement in the effects of PACAP on either cell proliferation or neuritogenesis, and work is in progress to test this hypothesis directly through the abrogation of *Jumonji* induction during PACAP-induced neuronal differentiation of PC12 cells.

Treatment with PACAP plus cycloheximide also increased the transcript for *activating transcription factor 3* (*Atf3*), a protein that can stimulate cell proliferation (Tamura *et al*.

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2005), enhance cell survival (Francis *et al*. 2004) or promote apoptosis (Hai and Hartman 2001) after exposure to stress agents. The variability in the effects of this protein, which is usually increased when cells are exposed to stress signals such as ischemia, alcohol, UV radiation or axotomy, could be a result of the fact that the ATF3 homodimer is a transcriptional repressor, whereas heterodimeric association with Jun proteins produces a transcriptional activator (Hai and Hartman 2001). In particular, overexpression of ATF3 in *c-Jun*-activated PC12 cells promotes *heat shock protein 27* (*Hsp27*) expression (Benn *et al*. 2002), which in turn rescues cell survival and induces neurite outgrowth (Nakagomi *et al*. 2003). Co-expression of *Atf3* with *c-Jun* significantly enhances *c-Jun*-mediated neurite sprouting, suggesting that these two transcription factors initiate an axonal regeneration program in response to axotomy (Pearson *et al*. 2003). Altogether these results suggest that *Atf3* could mediate some of the neurotrophic effects of PACAP in PC12 cells, and the possible regulation of *Atf3* by PACAP is also supported by the fact that this protein belongs to the ATF/CREB family of transcription factors known to be induced via the cAMP pathway (Gao *et al*. 2004).

Genes potentially involved in cell neuroprotection and differentiation

PACAP has been shown to reduce PC12 cell proliferation, promote neurite outgrowth and inhibit apoptosis (Vaudry *et al*. 2002b). It was thus interesting to classify the genes according to their putative function in order to identify those potentially involved in the neurotrophic effects of PACAP. Apart from the genes involved in the control of mRNA transcription, a significant proportion of other messengers could also control either apoptosis or cell proliferation. In particular, it has been shown that *Btg2*, which is induced by more than 60-fold by PACAP, inhibits cell proliferation when overexpressed in either NIH3T3 or PC12 cells (Duriez *et al*. 2004). *In vivo* overexpression of *Btg2* inhibits cyclin D1 expression and increases the synthesis of the transcription factor *Math1*, which is required for neurogenesis (Canzoniere *et al*. 2004). Finally, it has been reported that in PC12 cells *Btg2* potentiates NGF-induced differentiation and protects these cells from apoptosis elicited by NGF deprivation (Corrente *et al*. 2002). As in PC12 cells both PACAP and NGF initiate differentiation and inhibit apoptosis (Vaudry *et al.* 2002b), *Btg2* is likely to have the same role when activated by PACAP as by NGF.

In the presence of cycloheximide, PACAP also activated *Sgk*, to a level of more than 10-fold, which has been reported to inhibit apoptosis of human (Mikosz *et al*. 2001) and rat mammary tumour cells (Webster *et al*. 1993). Besides its neuroprotective effect *Sgk* also promotes dendrite outgrowth in neurones (David *et al*. 2005), thereby supporting the possible involvement of this protein kinase in the neurotrophic effects of PACAP on PC12 cells.

Perhaps the most intriguing of the transiently regulated transcripts discovered here is *Siah2*. The structurally related *Siah1* has recently been strongly implicated in NO-mediated apoptotic signalling through *Gapdh* in PC12 cells upon neurotrophin withdrawal and macrophage cell death following exposure to lipopolysaccharides (LPS) (Hara *et al*. 2005), and may be involved in *Gapdh*-dependent cerebellar granule cell death during development (Ishitani *et al*. 1996; Hara and Snyder 2006). Whether *Siah2* modulates this pathway or

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participates in a parallel cell-survival pathway initiated by exposure to PACAP, remains to be investigated.

In conclusion, the present report has identified by microarray about one hundred genes activated by PACAP in the presence of cycloheximide that had not previously been shown to be regulated by PACAP alone. The real-time PCR experiments have revealed that a significant proportion of these genes is transiently induced by PACAP alone. To the best of our knowledge, this is the first study using this approach to characterize early response genes regulated by neuropeptides. Some of the proteins encoded by mRNA identified in the present study are likely to participate in the effects of PACAP on PC12 cell differentiation. Further analysis of the transduction pathways regulating these genes, investigation of their functional significance in PC12 cells, and the determination of their role in other cell types will provide a better understanding of the mechanisms involved in the neurotrophic effects of PACAP during development and in pathological conditions.

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Abbreviations used

Adnp	activity-dependent neuroprotective protein
Arc	activity-regulated cytoskeleton-associated protein
Atf3	activating transcription factor 3
Btg2	β-cell translocation gene 2, antiproliferative
Cited1	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxterminal domain 1
Crebbp	CREB binding protein
Egr1	early growth response 1
Fos	FBJ osteosarcoma related oncogene
Gadd45g	growth arrest and DNA-damage-inducible 45 gamma
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Gas5	growth arrest specific 5
GO	GeneOntology
Hsp27	heat shock protein 27
Ier3	immediate early response 3
NFκB	nuclear factor for the κ chain of B-cells
NGF	nerve growth factor

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PACAP	pituitary adenylate cyclase-activating polypeptide
Rnf2	ring finger protein 2
SDS	sodium dodecyl sulfate
Sgk	serum/glucocorticoid-regulated kinase
Siah2	seven in absentia homologue 2
SSC	saline sodium citrate
Stat3	signal transducer and activator of transcription 3
3′UTR	3′ untranslated regions

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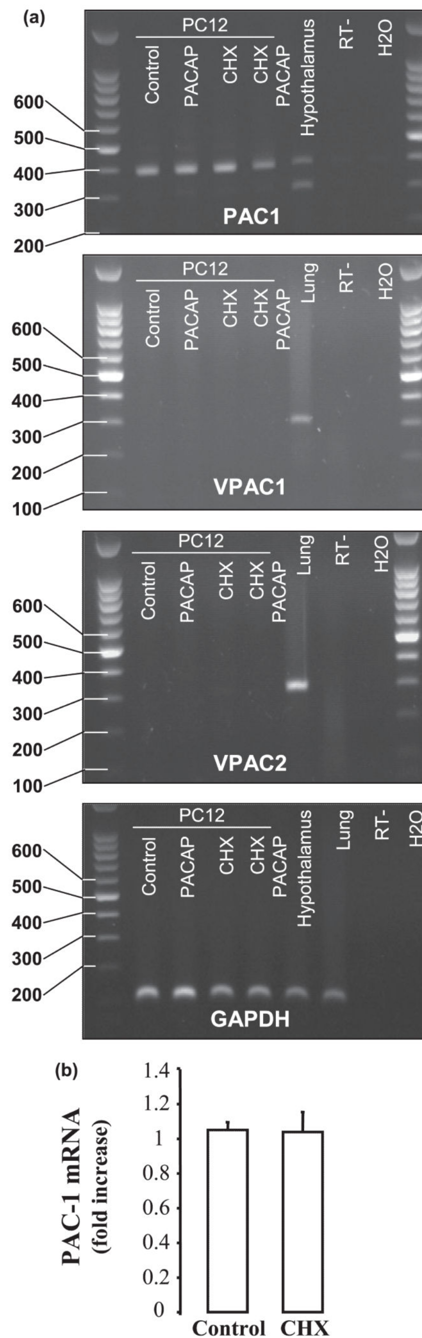

Fig. 1.

Expression of pituitary adenylate cyclase-activating polypeptide (PACAP) receptors mRNA in PC12 cells. (a) Expression of PAC1, VPAC1, VPAC2 and GAPDH receptor isoforms in either the absence or the presence of PACAP and/or cycloheximide. cDNA from hypothalamus and lung tissues were used as positive controls for PAC1 and VPAC receptors, respectively. (b) Quantification by real-time PCR of the effect of cycloheximide on PAC1 receptor expression after 30 min of treatment with cycloheximide.

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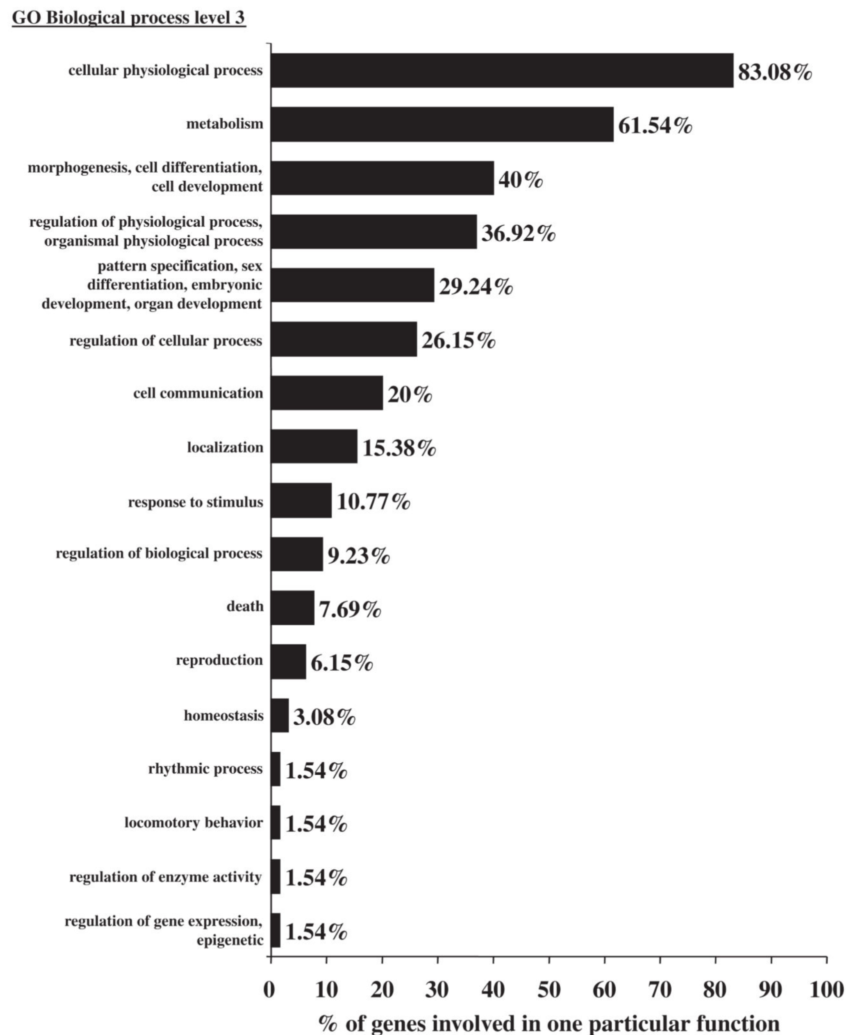
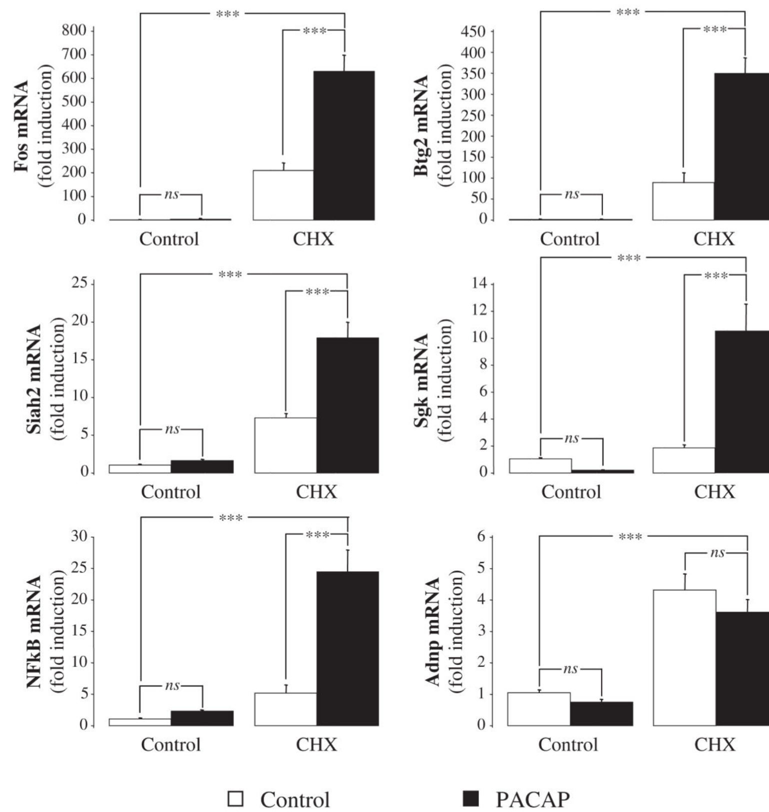


Fig. 2.

Functional classification according to the GeneOntology (GO) reference of the mRNA activated by pituitary adenylate cyclase-activating polypeptide (PACAP) plus cycloheximide (biological process level three). Cluster analysis was conducted with the FatiGO application (http://www.fatigo.org). As illustrated with *FBJ osteosarcoma-related oncogene (Fos)*, which belongs to six categories indexed in GO biological process level three (pattern specification, sex differentiation, embryonic development and organ development; metabolism; morphogenesis, cell differentiation and cell development; regulation of physiological process, organismal physiological process; regulation of cellular process and finally cellular physiological process), one particular gene can be assigned to several GO annotations.



	Control	*SEM*	PACAP	*SEM*	CHX	*SEM*	CHX PACAP	*SEM*
Fos	1.00	*0.10*	3.32	*1.45*	209.41	*30.48*	629.60	*67.01*
Btg2	1.02	*0.07*	1.41	*0.12*	89.47	*23.14*	350.06	*35.64*
Siah2	1.05	*0.05*	1.66	*0.11*	7.30	*0.52*	17.90	*2.00*
SGK	1.04	*0.07*	0.21	*0.03*	1.86	*0.19*	10.54	*1.98*
NFkB	1.07	*0.09*	2.30	*0.11*	5.17	*1.25*	24.45	*3.41*
Adnp	1.05	*0.08*	0.075	*0.08*	4.31	*0.50*	3.62	*0.38*

Fig. 3.

Independent verification of representative genes regulated by pituitary adenylate cyclase-activating polypeptide (PACAP) in PC12 cells. Quantification of FBJ osteosarcoma-related oncogene (Fos), β-cell translocation gene 2, antiproliferative (Btg2), seven in absentia 2 (Siah2), serum/glucocorticoid-regulated kinase (Sgk), ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homologue, yeast) (NFκB) and *activity-dependent neuroprotective protein (Adnp)* expression levels by real-time PCR after 6 h of treatment with either PACAP (10^{-7} M) alone or in the presence of cycloheximide (5 μM). *Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* mRNA was used as an internal standard. ****p* < 0.001; *ns*, not statistically different.

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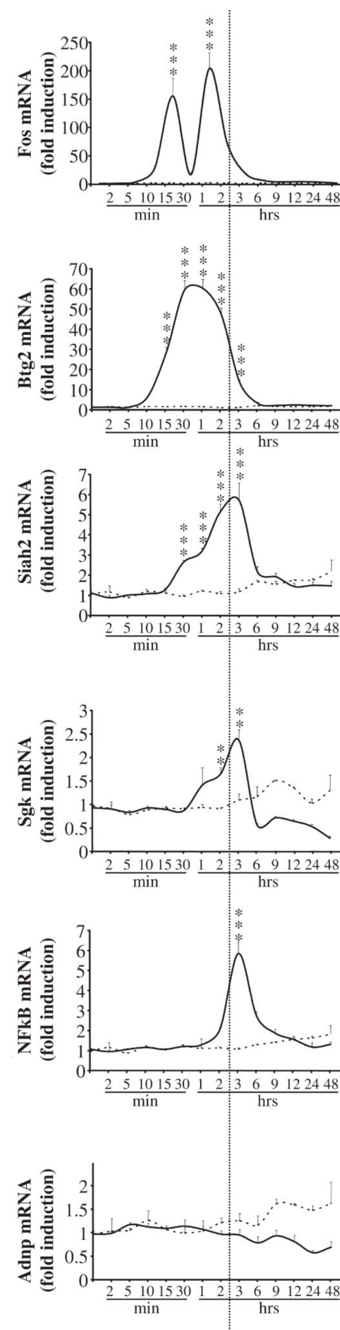

Fig. 4.

Time-course of pituitary adenylate cyclase-activating polypeptide (PACAP) effect on gene expression. PC12 cells were exposed to PACAP (10^{-7} M) for durations ranging from 2 min to 48 h and the gene-expression profile was measured by real-time PCR for FBJ osteosarcoma-related oncogene (Fos), β-cell translocation gene 2, antiproliferative (Btg2), seven in absentia 2 (Siah2), serum/glucocorticoid-regulated kinase (Sgk), nuclear factor for kappa chain of B-cells (NFκB) and *activity-dependent neuroprotective protein (Adnp). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* mRNA was used as an internal

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standard. Solid lines correspond to the mRNA expression in PACAP-treated cells and dashed lines correspond to the mRNA expression in cells treated with control medium. ** $p < 0.01$; *** $p < 0.001$; unmarked data points, not statistically different from control.

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Table 1

Information about the primers used for conventional and real-time PCR

	Foward primer	Reverse primer	Amplicon size (bp)	T_m (°C)	Final concentration (nM)
Gapdh	**Glyceraldehyde-3-phosphate dehydrogenase**				
	CAGCCTCGTCTCATAGACAAGATG	CAATGTCCACTTTGTCACAAGAGAA	106	81	300
Fos	**FBJ osteosarcoma related oncogene**				
	GCCAAGTGCCGGAATCG	AGTTGATCTGTCTCCGCTTGGA	62	83	300
Btg2	**β-cell translocation gene 2, antiproliferative**				
	CGAGCAGAGACTCAAGGTTTTCA	ATAGCCGGAGCCCTTGGA	103	81	300
Siah2	**Seven in absentia 2**				
	AAGGTCGCCTCGGCAGTT	GGACGGTATTCACAGATGTCTTCA	110	81	300
Sgk	**Serum/glucocorticoid regulated kinase**				
	GGGACAACGTCCACCTTCTGT	CAGGCCATAGAGCATCTCATACAA	129	84	300
NFκB	**Nuclear factor of kappa light chain gene enhancer in B-cells, p105**				
	AGGATTTCGATTCCGCTACGT	CCAACTGAACGATAACCTTTGCA	140	81	300
Adnp	**Activity-dependent neuroprotective protein**				
	TTGGGTTGGAATACTGTAAAGAACATATA	CCGATAGTCCTGATTTTTTGTAAGAGA	134	74	500
PAC1	**PAC1 receptor**				
	CCCTGACTGCTCTCCTCCTGCTGCCTAT	CAGGGCAGCTCACAAGGACCATCTCACC	213	82	300
PAC1	**PAC1 receptor** *				
	CTTGTACAGAAGCTGCAGTC	GGTGCTTGAAGTCCATAGTG	short 281	80	1000
			hop 365	81	
			hip-hop 449	81	
VPAC1	**VPAC1 receptor** *				
	GCCCCCATCCTCCTCTCCATC	TCCGCCTGCACCTCACCATTG	298	81	1000
VPAC2	**VPAC2 receptor** *				
	GTCACCTTTGCCCTCTCCATCA	GCCTCTCCACCTTCTTTTCAGT	296	80	1000

*Primers used for conventional PCR.

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Table 2

Functional classification of the genes induced by a 6-h treatment with pituitary adenylate cyclase-activating polypeptide (PACAP) plus cycloheximide, but not by PACAP alone

Gene name	Ug cluster	GB ID	Control vs. CHX + PACAP	Control vs. PACAP
Development/physiological process/cellular process/regulation of biological process				
β-cell translocation gene 2, antiproliferative (Btg2)	Mm.239605	C87946	21.54	1.72
Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1 (Cited1)	Mm.2390	AU019144	15.21	1.86
FBJ osteosarcoma related oncogene (Fos)	Mm.246513	AU015150	13.50	1.90
Growth arrest and DNA-damage-inducible 45 gamma (Gadd45g)	Mm.281298	C86281	7.86	1.14
Jumonji, AT rich interactive domain 2 (Jarid2)	Mm.25059	C80019	5.12	1.23
Nuclear factor for kappa chain of B-cells	Mm.256765	AU024604	4.50	1.15
Signal transducer and activator of transcription 3 (Stat3)	Mm.249934	AU043374	3.78	2.03
DNA methyltransferase 3B (Dnmt3b)	Mm.89772	AW537957	2.58	1.37
Development/physiological process/behaviour/cellular process				
Platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit (Pafah1b1)	Mm.56337	AU021752	3.11	1.57
Development/physiological process/cellular process				
Seven in absentia 2 (Siah2)	Mm.2847	AU017586	6.35	1.21
Tescalcin (Tesc)	Mm.273285	AU023528	3.51	1.16
Guanine nucleotide binding protein, alpha 13 (Gna13)	Mm.193925	AW556943	2.74	1.17
Nucleoporin 50 (Nup50)	Mm.28379	AW556935	2.74	1.33
Wingless-related MMTV integration site 4 (Wnt4)	Mm.20355	AW549398	2.59	0.98
Tousled-like kinase 2 (Arabidopsis) (Tlk2)	Mm.126976	AW553714	2.50	1.08
Development/physiological process				
SID1 transmembrane family, member 2 (Sidt2)	Mm.200859	AU046146	6.94	1.24
Tuftelin interacting protein 11 (Tfip11)	Mm.172947	AW547124	4.52	1.35
Development/cellular process				
Plakophilin 2 (Pkp2)	Mm.2252	AW538171	12.11	1.12
SKI-like (Skil)	Mm.15406	AU014590	7.11	1.37
Myeloid differentiation primary response gene 116 (Myd116)	Mm.4048	AW536864	4.77	1.43
Interferon-related developmental regulator 1 (Ifrd1)	Mm.168	C87178	4.67	0.96
Development				
Ring finger protein 2 (Rnf2)	Mm.31512	AW539513	2.27	1.15
Physiological process/cellular process/regulation of biological process				
Early growth response 1 (Egr1)	Mm.181959	AU017579	16.58	1.92
RIKEN cDNA 4930563E22 gene (Med31)	Mm.159496	AU045064	5.08	0.94
Activating transcription factor 3 (Atf3)	Mm.2706	C86078	3.76	1.31

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Gene name	Ug cluster	GB ID	Control vs. CHX + PACAP	Control vs. PACAP
YY1 transcription factor (Yy1)	Mm.3868	AU017017	3.15	1.30
CREB binding protein (Crebbp)	Mm.132238	AW552828	2.80	1.16
Acyl-CoA synthetase long-chain family member 4 (Acsl4)	Mm.143689	AU019232	2.69	1.67
GTPase activating RANGAP domain-like 1 (Garnl1)	Mm.292180	AW548663	2.64	0.95
Activity-dependent neuroprotective protein (Adnp)	Mm.201322	AW554081	2.51	1.23
General transcription factor IIF, polypeptide 1 (Gtf2f1)	Mm.24632	AW556946	2.48	1.20
Physiological process/cellular process				
ADP-ribosylation factor-like 4 (Arl4)	Mm.12723	AU021341	11.71	1.21
Serum/glucocorticoid regulated kinase (Sgk)	Mm.28405	AU042681	10.52	1.06
RIKEN cDNA 2810012H18 gene (2810012H18Rik)	Mm.281741	AU044772	4.49	1.26
RIKEN cDNA 9130230 N09 gene (B3gnt1)	Mm.258094	AU024115	4.33	1.30
Paraspeckle protein 1 (Pspc1)	Mm.20129	AU045828	4.25	1.03
Protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein 2 (Ppfibp2)	Mm.2817	AU041064	4.08	1.43
Prostaglandin I2 (prostacyclin) synthase (Ptgis)	Mm.2339	AW559113	3.92	1.18
SAR1a gene homologue 1 (S. cerevisiae) (Sara1)	Mm.6698	AW544555	3.68	1.37
Protein tyrosine phosphatase 4a2 (Ptp4a2)	Mm.193688	C88125	3.60	1.33
Centromere autoantigen A (Cenpa)	Mm.290563	AU021358	3.52	1.29
RIKEN cDNA A130048E20 gene (Rev3l)	Mm.288788	AW547620	3.36	0.95
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R) (Elavl1)	Mm.119162	C80193	3.35	1.42
G protein-coupled receptor associated sorting protein 1 (Gprasp1)	Mm.271980	AW556585	3.16	1.09
CDC like kinase 4 (Clk4)	Mm.239354	AW554767	3.07	1.28
Splicing factor, arginine/serine-rich 6 (Sfrs6)	Mm.24042	AW559147	3.05	0.89
Sperm-associated antigen 9 (Spag9)	Mm.260737	AW553401	3.02	1.37
RIKEN cDNA C920008N22 gene (Hbxap)	Mm.211743	AW556115	3.02	1.09
RNA binding motif protein 18 (Rbm18)	Mm.205937	C87520	2.88	1.37
Ubiquilin 1 (Ubqln1)	Mm.182053	C81478	2.75	1.08
RIKEN cDNA 2900046G09 gene (2900046G09Rik)	Mm.196512	AW550265	2.72	1.17
Chromodomain helicase DNA binding protein 1 (Chd1)	Mm.8137	AU045087	2.66	1.09
RIKEN cDNA B930096F20 gene (Stx5a) syntaxin 5a	Mm.153061	AW555342	2.56	1.08
Farnesyltransferase, CAAX box, alpha (Fnta)	Mm.3496	AW556923	2.46	1.25
DNA segment, Chr2, ERATO Doi 435, expressed (D2Ertd435e)	Mm.283361	C86136	2.24	1.26
FUS interacting protein (serine-arginine rich) 1 (Fusip1)	Mm.10229	AW537256	2.21	1.14
RNA binding protein gene with multiple splicing (Rbpms)	Mm.323997	AU019051	2.20	1.27
Tubulin, gamma 1 (Tubg1)	Mm.142348	AW539270	2.13	1.29
Amyloid beta precursor protein (cytoplasmic tail) binding protein 2 (Appbp2)	Mm.271997	AW544549	2.12	1.23
Physiological process				
Calponin 1 (Cnn1)	Mm.4356	C86052	4.16	1.28
CCR4 carbon catabolite repression 4-like (S. cerevisiae) (Ccrn4l)	Mm.86541	AU043840	2.88	1.11

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Gene name	Ug cluster	GB ID	Control vs. CHX + PACAP	Control vs. PACAP
Cellular process				
G-protein signalling modulator 2 (AGS3-like, C. elegans) (Gpsm2)	Mm.226941	AW537963	11.96	1.32
Phosphodiesterase 8A (Pde8a)	Mm.322891	C87459	4.61	1.15
Ras association (RalGDS/AF-6) domain family 1 (Rassf1)	Mm.12091	AU044980	2.56	1.41
Unknown				
DiGeorge syndrome critical region gene 6 (Dgcr6)	Mm.27155	AU045383	10.12	1.48
Calmodulin binding transcription activator 1 (Camta1)	Mm.318846	AU024273	6.98	1.02
RIKEN cDNA F830004D09 gene (Eml4)	Mm.295565	AW556280	2.51	1.27
No function in level 2				
PHD finger protein 17 (Phf17)	Mm.286285	AW551496	8.61	1.64
Oocyte specific homeobox 2 (Obox1)	Mm.358932	AU046150	3.85	1.34
Ciliary neurotrophic factor receptor (Cntfr)	Mm.272210	AU018997	2.93	1.26
Ly1 antibody reactive clone (Lyar)	Mm.28560	AU044713	2.91	1.32
Lymphocyte antigen 6 complex, locus G6C (Ly6g6c)	Mm.215096	AU016360	2.74	1.07
Glypican 1 (Gpc1)	Mm.297976	AW555635	2.47	1.34
Zinc finger, A20 domain containing 2 (Za20d2)	Mm.292405	AU043297	2.40	1.27
Mitochondrial tumour suppressor 1 (Mtus1)	Mm.149438	AW552927	2.20	0.92
Without GO annotation				
RIKEN cDNA 1500041J02 gene (1500041J02Rik)	Mm.281019	AW538623	6.59	1.79
MYST histone acetyltransferase monocytic leukaemia 4 (Myst4)	Mm.248967	C85086	5.55	0.97
RIKEN cDNA 9130229H14 gene (9130229H14Rik)	Mm.266884	C80126	5.31	1.10
RIKEN cDNA 1110003E01 gene (1110003E01Rik)	Mm.10709	AU023219	5.30	1.48
Downstream of Stk11 (Dos)	Mm.44231	C85710	4.66	1.13
Myocyte enhancer factor 2D (Mef2d)	Mm.28184	C86932	4.55	1.14
RIKEN cDNA 1110007L15 gene (1110007L15Rik)	Mm.319134	AW555473	4.42	1.31
H3 histone, family 3B LOC433382	Mm.18516	AW539780	4.13	0.76
Fyn proto-oncogene (Fyn)	Mm.4848	AW552119	3.93	0.97
RIKEN cDNA 1500011J06 gene (1500011J06Rik)	Mm.276341	AW557796	3.80	1.77
Trk-fused gene (Tfg)	Mm.235108	AU021269	3.78	1.22
Ring finger protein 139 (Rnf139)	Mm.4537	AW538451	3.74	1.42
Growth arrest specific 5 (Gas5)	Mm.270065	AW546280	3.51	1.28
RIKEN cDNA 2510001I10 gene (2510001I10Rik)	Mm.29432	C76468	3.47	1.19
RIKEN cDNA 5830415L20 gene (5830415L20Rik)	Mm.240265	AU023795	3.43	1.15
RIKEN cDNA 2410002M20 gene (2410002M20Rik)	Mm.157534	AW544376	3.23	1.27
Wdr45-like (Wdr45l)	Mm.103986	AW552315	3.18	0.98
RIKEN cDNA 2810017D21 gene (Pum1) pumilio 1 (Drosophila)	Mm.34701	AU044062	3.14	0.93
RIKEN cDNA 5830435K17 gene (5830435K17Rik)	Mm.155687	AU019572	2.91	1.25
Protein phosphatase 1, regulatory (inhibitor) subunit 12A (Ppp1r12a)	Mm.207499	AU015467	2.77	1.27

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Gene name	Ug cluster	GB ID	Control vs. CHX + PACAP	Control vs. PACAP
Enhancer of polycomb homologue 2 (Drosophila) (Epc2)	Mm.29167	AU040241	2.69	0.93
RIKEN cDNA 1110067M05 gene (1110067M05Rik)	Mm.341886	AU015454	2.55	1.06
RIKEN cDNA 4121402D02 gene (Casc3)	Mm.40120	AW556296	2.54	1.13
RIKEN cDNA A730098D12 gene (A730098D12Rik)	Mm.196325	C86491	2.53	1.10
RIKEN cDNA 1700037H04 gene (1700037H04Rik)	Mm.27711	AU041152	2.49	0.99
WD repeat domain 26 (Wdr26)	Mm.289082	AW540967	2.47	0.94
Eukaryotic translation termination factor 1 (Etf1)	Mm.329353	AU016000	2.42	1.75
DNA segment, Chr 1, Brigham & Women's Genetics 1363 expressed (D1Bwg1363e)	Mm.260577	AW553790	2.41	1.08
Plakophilin 4 (Pkp4)	Mm.260938	AW549051	2.39	0.84
Muscleblind-like 2 (Mbnl2)	Mm.238266	AU041504	2.28	1.00
RIKEN cDNA 9630050M13 gene (9630050M13Rik)	Mm.23044	AA409679	2.23	1.27
RIKENcDNA 3321401G04 gene (3321401G04Rik)	Mm.24652	AW558113	2.16	1.65

Classification was performed according to the GeneOntology biological process level 2 reference.