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A cAMP-DEPENDENT, PKA-INDEPENDENT SIGNALING PATHWAY MEDIATING NEURITOGENESIS THROUGH Egr1 IN PC12 CELLS

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Running Title: cAMP and cell differentiation

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ABBREVIATIONS:

AC, adenylate cyclase
BSA, bovine serum albumin
DAG, diacyl glycerol
dbcAMP, N6, 2'-O-dibutyryladenosine 3', 5'-cyclic monophosphate
Egr1, early growth response 1
ERK, extracellular signal-regulated protein kinase
Ier3, immediate early response 3
MAP, mitogen-activated protein
MEK, mitogen extracellular signal-regulated kinase
NGF, nerve growth factor
PAC1, PACAP specific receptor
PACAP, pituitary adenylate cyclase-activating polypeptide
PKA, protein kinase A
PKC, protein kinase C
PLC, phospholipase C
PMA, phorbol 12-myristate acetate
Rap1, member of RAS oncogene family
SDS, sodium dodecyl sulfate
SSC, saline-sodium citrate
Vil2, villin 2
VIP, vasoactive intestinal polypeptide
VPAC1, PACAP & VIP receptor 1
VPAC2, PACAP & VIP receptor 2

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ABSTRACT

The neurotrophic peptide PACAP elevates cAMP in PC12 cells. Forskolin and dibutyryl cAMP mimic PACAP's neuritogenic and cell morphological effects, suggesting that they are driven by cAMP. Comparison of microarray expression profiles after exposure of PC12 cells to either forskolin, dibutyryl cAMP or PACAP revealed a small group of cAMP-dependent target genes. Neuritogenesis induced by all three agents is PKA-independent (not blocked by H89) and ERK-dependent (blocked by U0126), and therefore cAMP-dependent target genes potentially mediating neuritogenesis were selected for further analysis based on the pharmacological profile of their induction by PACAP i.e. mimicking that of neuritogenesis. SiRNA targeting one of these genes, Egr1, blocked PACAP-induced neuritogenesis, and siRNA targeting another, Vil2, blocked a component of the cell size increase elicited by PACAP. Neither siRNA blocked PACAP's PKA-dependent antiproliferative effects. PACAP signaling to neuritogenesis was also impaired by dominant negative Rap1 expression, but was not affected by inhibition of PKC, indicating a G-protein coupled receptor-mediated differentiation pathway distinct from the one activated by receptor tyrosine kinase ligands such as NGF, that involves both Rap1 and PKC. We have thus identified a cAMP-dependent, PKA-independent pathway proceeding through ERK that functions to up-regulate the transcription of two genes, Egr1 and Vil2, required for PACAP-dependent neuritogenesis and increased cell size, respectively. Dominant negative Rap1 expression impairs both PACAP-induced neuritogenesis and Egr1 activation by PACAP, suggesting that cAMP elevation and ERK activation by PACAP are linked through Rap1.

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Neurotrophic factors activating receptor tyrosine kinases (RTKs), such as nerve growth factor (NGF), promote neurite extension through a cAMP-independent signaling pathway involving Ras, PKC, and ERK (Ginty et al., 1991; Vaudry et al., 2002b), although other effects of NGF, such as induction of sodium channel expression, do require cAMP (Ginty et al., 1992; Yao et al., 1998). A significant literature also implicates cAMP in a broad range of neuronal differentiation responses, including neuritogenesis, survival, regeneration, repair, and expression of genes encoding neuron-specific proteins such as neurotransmitter biosynthetic enzymes, neuropeptides, receptors and ion channels (Qiu et al., 2002), albeit the effects of first messengers that regulate cAMP generation in differentiating neurons have not been studied as extensively as RTK-stimulating neurotrophins like NGF. The neuropeptide PACAP has garnered significant interest in this regard, because PACAP is neuritogenic upon exposure to PC12 cells (Deutsch and Sun, 1992), enhances neuronal survival of cerebellar granule cells (Kienlen Campard et al., 1997) and PC12 cells (Tanaka et al., 1996), and is neuroprotective after ischemic injury to the brain (Reglödi et al., 2000; Chen et al., 2006; Ohtaki et al., 2006).

PACAP signaling in PC12 cells leading to neuritogenesis is distinct from that of NGF.Both processes require ERK activation, but only NGF activation of ERK is Ras-dependent (Lazarovici et al., 1998). A number of transcripts induced by PACAP in PC12 cells are both ERK-dependent (blocked by U0126) and insensitive to PKA inhibition (by H89) (Vaudry et al., 2002a) and some of these are also induced by forskolin even in the presence of H89 (Gerdin and Eiden, 2007, and references therein). We therefore hypothesized that PACAP signaling to ERK to initiate neurite formation might proceed through a noncanonical (PKA-independent) cAMP-dependent signaling pathway. In pursuit of this hypothesis, we screened PACAP target genes in PC12 cells also induced by forskolin and dibutyryl cAMP whose regulation is both ERK–dependent and independent of activation of protein kinase A, and whose transcription might be required for various aspects of differentiation induced by PACAP.

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Rap1 is a potential regulator of ERK which can be activated by cAMP through both PKA-dependent and –independent pathways [(York et al., 1998); (Gerdin and Eiden, 2007) and references therein]. Maximal activation of total cellular Rap1 by PACAP in PC12 cells requires the participation of a number of protein kinases (Bouschet et al., 2003), and a Src-dependent activation of Rap1 initiated by cAMP and mediated by PKA has been identified in PC12 cells (Obara et al., 2004). However, the functional significance of Rap1 activation by each of these pathways, particularly for neuritogenesis, has not yet been addressed. Thus it is established in PC12 cells that PACAP elevates cAMP; that cAMP can activate Rap1; that Rap1 activation can persistently stimulate total cellular ERK; and that constitutively active ERK can drive neuritogenesis (Deutsch and Sun, 1992; Vossler et al., 1997; Yao et al., 1998; York et al., 1998; Harada et al., 2001; Stessin et al., 2006). However, a coherent signaling mechanism underlying PACAP-induced PC12 cell differentiation remains to be elucidated. Here, we address two key questions towards this end. First, which PACAP-initiated differentiating responses of PC12 cells are mimicked by cAMP, and which of these require PKA? Second, does PACAP activate a cohort of genes in PC12 cells that are also activated by elevation of cAMP alone, and does abrogation of expression of any of these transcripts affect the PACAP-induced functional differentiative responses of neuritogenesis, increased cell size, and cessation of proliferation? The experimental answers to these questions provide a mechanism for PACAP-induced neuritogenesis involving cAMP-initiated, yet PKA-independent activation of ERK, and subsequent expression of specific genes that drive distinct components of the differentiation program. These signaling mechanisms may also be relevant for cAMP-dependent signaling for differentiation by first messengers acting through other G-protein coupled receptors besides the PACl receptor activated by PACAP in PC12 cells.

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MATERIALS AND METHODS

Drugs. PACAP and VIP were purchased from Phoenix Pharmaceuticals (Mountain View, CA, USA). Chelerythrine, dbcAMP, forskolin, H89, NGF and poly-L-lysine were obtained from Sigma (Saint Louis, MO, USA). PD98059, H7 and 2',5'-dideoxyadenosine were from Calbiochem and U0126 was purchased from either Calbiochem (San Diego, CA, USA) or Promega (Charbonnières, France).

Cell culture. The PC12 cell clone PC12-G (Rausch et al., 1988) was grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 7% heat-inactivated fetal bovine serum (Sigma), 7% horse serum (BioWhittaker, Walkersville, MD, USA), 2.5% Hepes (Invitrogen), 1% glutamine (Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen) at 37°C in a 10% CO_2/90% air humidified atmosphere. Two days before treatment, cells were re-plated on poly-L-lysine coated Petri dishes (Costar, Bethesda, MD, USA). When used, inhibitors were added 30 min prior to exposure to PACAP, forskolin or dbcAMP.

Quantitative analysis of neurite outgrowth. Two days after treatment, images of PC12 cells were randomly acquired on a computer-assisted microscope (IPLab, Fairfax, VA, USA and Metamorph, Molecular Devices, Sunnyvale, CA, USA). Differentiation was investigated on more than 22,000 cells by measuring neurite length. The percentage of cells bearing neurites was quantified, the number of neurites per cell was counted and the total neurite outgrowth for each cell was measured. Neurites were defined as cell processes greater than 6 µm, to eliminate inadvertent counting of cell membrane ruffling or irregularities as neurites.

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Quantification of cell number and measurement of cell size. Two days after treatment, cells were washed with phosphate-buffered saline (PBS) and detached by incubation with Accutase (Innovative Cell Technologies, La Jolla, CA, USA) at 37°C for 15 minutes. Cell size and number were measured with a Z2 Beckman Coulter counter (Miami, FL, USA) with lower and upper limits set to 10 and 17 μm respectively. Preliminary experiments demonstrated that a 17 μm cut-off on the cell-counting instrument (above) provided the most sensitive and reliable indicator of changes in PC12 cell volume after treatment for two days with PACAP (10^{-7} M). A dose response with graded concentrations of PACAP and VIP (10^{-11} to 10^{-6} M) confirmed that a 17 μm cut-off provides results that correlated well with a direct measurement of the cell diameter (Fig. 1C).

cAMP quantification. 30 min after treatment, cAMP production was quantified with a ^{3}H-cAMP assay kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as previously described (Hamelink et al., 2002).

Western blot analysis. Proteins contained in PC12 cells were extracted in lysis buffer consisting of 1% Triton X-100, 50 mM Tris-HCl and 10 mM EDTA. The homogenate was centrifuged (14,000×g, 4°C, 15 min) and proteins contained in the supernatant were precipitated at 4°C by addition of ice-cold 10% trichloroacetic acid. The extract was centrifuged (12,000×g, 4°C, 15 min) and washed 3 times with ether/alcohol (70:30, v/v). The pellet was denatured in 50 mM Tris-HCl (pH 7.5) containing 20% glycerol, 0.7 M 2-ß-mercaptoethanol, 0.002% (w/v) bromophenol blue and 3% (w/v) SDS at 100°C for 5 min, and electrophoresed on a 10% SDS-PAGE gel. After separation, proteins were electrically transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was incubated with blocking solution (0.5% BSA and 2% milk in Tris-buffered saline containing 0.05% Tween 20) at room temperature for one hour, and developed with antibodies against phosphorylated and total cellular ERK

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(Promega) using a chemiluminescence detection kit (ECL System, Amersham). Autoradiographic films were quantified using an image analysis system (Biocom, Les Ulis, France).

Rap1 activation assay. Fusion protein GST-Ral-RBD produced in *Escherichia coli* in the presence of isopropyl ß-D-thiogalactopyranoside was a generous gift from Dr. Michel Philippe (CNRS, UMR 6187, University of Poitiers, France). The bacterial pellet was suspended in sodium-Tris-EDTA buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) in the presence of protease inhibitors (1 mg/mL lysozyme, 10 µg/mL trypsin inhibitor, 1mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin), 1 mM dithiothreitol and 1.5% N-lauryl sarcosyl. The protein was affinity purified by incubation at 4°C overnight with glutathione Sepharose 4B beads.

Rap-GTP activity was assayed as GTP-dependent binding to GST-Ral-RBD with a pull-down assay. Cells treated with PACAP were rinsed rapidly with PBS and lyzed in 10% glycerol, 1% nonidet P-40, 50 mM Tris-HCl, 200 mM NaCl, 2.5 mM $MgCl_2$, 10 mM NaF, in the presence of protease inhibitors (1 mM orthovanadate, 0.1 µM aprotinin, 250 µM PMSF, 1 µM leupeptin). For each sample, 400 µL of lysate was incubated for 1 hour at 4°C with glutathione Sepharose 4B beads coupled with GST-Ral-RBD. Beads were washed 3 times with lysis buffer before addition of electrophoresis buffer and 5% β-mercaptoethanol. The proteins were denatured at 100°C for 5 min before electrophoresis by 10% SDS-PAGE. After separation, proteins were transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated with blocking solution (0.5% BSA, 2% nonfat dry milk) at room temperature for 1 hour and developed with antibodies against Rap (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), using a chemiluminescence detection kit (ECL System, Amersham). Autoradiographic films were quantified with an image analysis system (Biocom, Les Ulis, France).

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Rap1b dominant negative vector. Rap1b DN (S17N) cloned into pcDNA 3.1(+) was a generous gift from Dr. Elisabeth Bock (Protein Laboratory, Institute of Molecular Pathology, University of Copenhagen, Denmark). The Rap1b DN insert was first subcloned into the pIRES2-eGFP vector (Clontech, Palo Alto, CA) and the Rap1b DN-IRES-eGFP was subsequently cloned into the lentivirus packageable genome pRRLsin.CMV.GFPpre.

Viral particles were generated by transient co-transfection of the packageable genome with gag/pol and vesicular stomatitis virus (VSV) envelope expression plasmids in the 293T cell line. Forty-eight hours later, the culture medium containing the viral particles was collected and filtered, and added to PC12 cells. Following overnight exposure to the viral particles, cells were washed twice with fresh medium. Forty-eight hours later, Rap1b DN-IRES-eGFP infected cells stably expressing the transduced proteins were identified under a fluorescent microscope. Cells transduced with a pRRLsin.CMV.GFPpre vector were used as a control.

RNA isolation, microarray experiments and data analysis. After six hours of treatment, total RNA was extracted with Trizol reagent (Invitrogen) and further purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA concentration was measured by absorbance at 260 nm and RNA integrity was confirmed by denaturing gel electrophoresis.

The cDNA sequences used in this study were issued from the NIA Mouse 15K cDNA clone set (see http://lgsun.grc.nia.nih.gov/cDNA/15k.html for details). PCR products generated from these clones were printed onto polylysine-coated glass slides at the National Human Genome Research Institute microarray facility. Fluorescent labeled cDNA was synthesized from 10 μg RNA from treated or untreated PC12 cells, with the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) in the presence of amine-modified random primers and aminoallyl-dUTP/dNTP. Probes were then labeled with NHS-Ester dye Cy3 or Cy5 (Amersham Pharmacia Biotech). After denaturation, purified Cy3/Cy5-labelled cDNA samples were combined and hybridized on a microarray slide in a humidified chamber (Corning, NY, USA) at

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65°C overnight in the presence of 5X saline-sodium citrate (SSC), 0.1 % sodium dodecyl sulfate (SDS), 25% formamide and polyA (25 ng/μL). Prior to scanning at 532 nm for Cy3 and 633 nm for Cy5 (Agilent Technologies, Foster City, CA, USA), slides were successively washed at room temperature in 0.5x SSC/0.1% SDS for 2 min, 0.5x SSC for 2 min (twice) and 0.06x SSC for 2 min. The two fluorescent images obtained from the scanner were analyzed using the IPLab software. The data from 37 successful experiments were entered into the FileMaker Pro 5 software (Santa Clara, CA, USA) to cluster the genes regulated in the various experimental conditions and to conduct a functional analysis. Genes were included as induced by a given treatment if a) all values in the data set had a quality index of >0.3 for the combined ratio value, and b) if the mean induction value was >1.5 fold.

Real-time PCR experiments. Total RNA was extracted with Trizol and further purified using the RNeasy Mini Kit (Qiagen). Contaminating genomic DNA was removed by treatment with DNAse I (Qiagen) and cDNA was synthesized from 5 μg of RNA using the ImProm II Reverse Transcriptase (Promega). Real-time PCR was performed on cDNA in the presence of a 1× Mastermix (Applied Biosystems, Courtaboeuf, France) containing pre-set concentrations of dNTPs, $MgCl_2$ and the SYBR Green reporter dye along with specific primers, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each primer set designed with the Primer Express software (Applied Biosystems) was used at its optimal concentration with a maximal efficacy as reported in Table 1. The cDNA-generated signals for target genes were internally corrected with that of *glyceraldehyde-3-phosphate dehydrogenase* (Gapdh) cDNA signal for variations in amounts of input mRNA. Gene expression level was then compared to a corresponding control sample group and the level of regulation was determined with the $2^{-\Delta\Delta Ct}$ method according to Applied Biosystems instructions.

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siRNA experiments. Transfection of siRNA into PC12 cells was performed with the Amaxa Nucleofector (Amaxa, Koeln, Germany) according to the instructions of the manufacturer. Briefly 2×10^6 cells were resuspended into 120 µL of Nucleofector solution containing 20 µg siRNA. Immediately after electroporation, fresh medium was added and cells were cultivated at 37°C in a 10% CO_2/90% air incubator. Several siRNA were designed and tested to inhibit *immediate early response 3* (Ier3), *villin 2* (Vil2) and *early growth response 1* (Egr1) expression (Hp flexible siRNA, QIAGEN). The sequences of the siRNA used for the experiments presented in this study were CAA CGC TAA CTC AGA ACA CTA for Ier3, AGC GAT AAT ATG GGT TTG TAA for Vil2, AAG GCG CTG GTG GAG ACA AGT for Egr1-siRNA1, ATT GTA CTA TTT GGA GTT AAA for Egr1-siRNA2 and CAA ACC AAT GGT GAT CCT CTA for Egr1-siRNA3.

The specificity of the effect of Egr1 siRNA on cell differentiation was confirmed using three different sequences (Egr1-siRNA1, 2 and 3) which all reduced Egr1 mRNA levels as well as PACAP-induced neuritogenesis. Egr1-siRNA1 was chosen for further work. The capacity of Egr1 siRNA1 to specifically reduce its cognate mRNA was confirmed by measuring its effect on induction of Egr1 mRNA, and three other mRNAs (Ier3, Odc, and Rgs2) by 10^{-7} M PACAP. Egr1 siRNA1 decreased only the expression of its cognate mRNA target. Further testing of Egr1 and Vil2 siRNAs against their cognate mRNAs likewise revealed no off target effects of these siRNAs.

Luciferase assay. PC12 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with the firefly luciferase pEgr-1-Luc plasmid (Stratagene) and the *Renilla* luciferase phRL-null vector (Promega) in the presence or absence of the Rap1b dominant negative expression vector. After transfection overnight, cells were treated 6 hours with PACAP (10^{-7} M)

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or vehicle and collected in passive lysis buffer (Promega), and relative luciferase activity was determined using the dual-luciferase reporter assay (Promega) per manufacturer's instructions.

Statistical analysis. Data are presented as the mean ± SEM from at least three independent experiments performed in triplicate, except for the histograms reporting the percentage of cells with a diameter > 17 μm in figure 11, which are the mean ± SEM from a representative experiment that has been repeated 4 times. Unless otherwise stated, statistical analyses were conducted using a Kruskal-Wallis test, followed by Dunn's post-tests or by the Mann-Whitney test using PRISM software (GraphPad Software, San Diego, CA).

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RESULTS

Concentration and duration of PACAP treatment required for PC12 cell neuritogenesis, increased cell size, and cessation of proliferation. As a prelude to identifying the signaling pathways underlying PC12 cell differentiation, the effective doses and duration of treatments required for PACAP-induced neuritogenesis, increased cell size and cessation of cell proliferation were determined. PACAP-38 (similar results were obtained with PACAP-27; data not shown) increased the percentage of cells with neurites, number of neurites per cell, and total neurite length with a maximum effect by 1 nM PACAP (Fig. 1A,B). PACAP effects on cell diameter (increased cell diameter and percentage of cells with a diameter >17 μm), and cessation of cell proliferation required somewhat higher concentrations (maximal effects were achieved by about 10 nM; Fig. 1C,D). VIP acted only at concentrations above 100 nM, consistent with the expression of PAC1, but not VPAC1 or VPAC2 receptor transcripts, in PC12-G cells (Ravni et al., 2006). Differences in PACAP potency to stimulate neuritogenesis, and to increase cell size or decrease proliferation suggest that these effects are mediated by distinct signaling pathways. The minimum time of exposure to PACAP required for a full PACAP response 48 hours later, however, was similar for neuritogenesis and cell growth arrest (Fig. 2A,B), indicating that one to six hours was an appropriate temporal window for examining cellular transcriptional changes elicited by PACAP that are likely to underlie neuritogenesis, altered cell size, or cessation of cell growth.

PACAP and cAMP share a similar inhibitor profile for separate aspects of PC12 cell differentiation. PAC1 receptors are positively coupled to adenylate cyclase (Harmar et al., 1998), and cAMP is therefore a prime candidate for the second messenger effecting changes in cell morphology and function. The cellular and biochemical profiles for PC12 cell differentiation induced by forskolin (25 μM), an adenylate cyclase stimulator, and dibutyryl cAMP (10^{-3} M),

which generates cAMP upon intracellular hydrolysis were compared to that of 100 nM PACAP. All three agents induced neuritogenesis within 48 hours of exposure that was unaffected by pre-treatment with the protein kinase A inhibitor H89, and blocked by the MEK inhibitor U0126 (Fig. 3A,B). The effect of PACAP and forskolin on cell size persisted in the presence of H89 (Fig. 3C). In contrast, the growth arrest activity of PACAP was significantly reduced, and that of forskolin totally abolished, in the presence of H89 (Fig. 3D). Finally, the adenylate cyclase inhibitor 2',5'-dideoxyadenosine inhibited PACAP-induced cAMP production (Fig. 4A) and significantly reduced the effect of PACAP on neurite outgrowth (Fig. 4B,C) and cell proliferation (Fig. 4D).

Protein kinase C is often a major contributor to neurotrophin signaling leading to neuroendocrine cell differentiation, including several of the differentiative effects of NGF on PC12 cells such as neuritogenesis (Das et al., 2004). However, the specific PKC inhibitor chelerythrine did not block PACAP-induced neuritogenesis or growth arrest (Fig. 5). Likewise, the broad spectrum (protein kinases A and C) inhibitor H7 failed to block PACAP-induced neuritogenesis (Fig. 5D). These data provide further criteria to define PACAP target genes involved in neuritogenesis and changes in cell size based on their cellular and biochemical responses to kinase inhibition, and prompted us to focus on regulation through the cAMP pathway.

MAP kinase induction by PACAP is independent of PKA. The ERK MAP kinase pathway has been shown by others to be required for PACAP-induced neurite outgrowth in PC12 cells based on inhibition with the MEK inhibitor PD98059 (Barrie et al., 1997; Lazarovici et al., 1998). Western blot experiments confirmed that PACAP induced a rapid and strong phosphorylation of ERK without affecting total ERK (Fig. 6A). Furthermore, this action of PACAP was independent of PKA in that it was unaffected by H89 (Fig. 6A). Both U0126 and PD98059 blocked PACAP-induced ERK phosphorylation (data not shown) in parallel with blockade of PACAP-induced neuritogenesis (>60 and >75% reduction in number of neurites per

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cell, and total neurite length, respectively; Fig. 6B). The effect of PACAP on the percentage of cells with a diameter above 17 μm was significantly reduced in the presence of U0126 (Fig. 6C) and there was no difference between cells treated with U0126 alone or PACAP plus U0126. The MEK inhibitor U0126 decreased cell number, as expected given the role of MAPK in cell proliferation in serum-containing medium, and thus the involvement of MAPK in PACAP signaling for growth arrest could not be reliably evaluated (Fig. 6D).

Rap1 is involved in PACAP signaling to neuritogenesis. The effects of PACAP on both neurite outgrowth and ERK activation appear to be independent of either PKA or PKC (Figs. 3-6). Based on the fact that ERK phosphorylation has been shown to involve Rap1 activation (Bouschet al., 2003), a possible ERK-dependent regulation of neurite outgrowth through Rap1 was investigated. Exposure of PC12 cells to PACAP (10^{-7} M) provoked a rapid and transient activation of Rap1, with a maximal increase observed after 30 seconds of treatment (Fig. 7A). The relatively small increase in total Rap1 activation observed may indicate that PACAP signaling reaches only a subcompartment of cellular Rap1 under our culture conditions, which do not include serum starvation prior to measurement of Rap activation.

PC12 cells transduced with a dominant negative form of Rap1 (Rap-DN) coupled with an IRES-GFP showed no morphological differences from non-transduced cells, but after 48 hours of treatment with PACAP (10^{-7} M), Rap-DN expressing cells had fewer and shorter neurites than PC12 cells not expressing Rap-DN (Fig. 7B). Blocking Rap1 signaling decreased both the number of neurites per cell and the total neurite length after PACAP treatment without affecting the percentage of cells with neurites, suggesting that neurite outgrowth, rather than neurite initiation, is the component of neuritogenesis primarily affected by Rap1-dependent signaling (Fig. 7C). The expression of GFP alone in PC12 cells had no effect on PACAP-induced neurite outgrowth (data not shown).

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PACAP, forskolin, dbcAMP and NGF regulate both common and distinct genes in PC12-G cells. The PC12 cell transcriptome was investigated after six hours of treatment with PACAP (10^{-7} M), forskolin (25 μM), or dbcAMP (10^{-3} M) in an attempt to identify cAMP-dependent target genes potentially involved in PACAP-induced neuritogenesis and increased cell size. Treatment with NGF (100 ng/mL) was used as a comparison: NGF induction of neuritogenesis is independent of cAMP (Vaudry et al., 2002b). Incubation of PC12 cells with PACAP for only six hours was sufficient to elicit the later full-length neurite outgrowth and increase in cell size observed at 48 hours (Fig. 2); therefore this time was chosen for microarray analysis. Among the 15,000 cDNAs present on the microarray, 118, 64, 48 and 133 unique transcripts were significantly induced by PACAP, forskolin, dbcAMP and NGF respectively (Fig. 8, Table 2). Twenty-seven of these transcripts were regulated in common by PACAP, forskolin and dbcAMP (Table 2A), 13 exclusively by PACAP and forskolin (Table 2A), three only by dbcAMP and PACAP (Table 2A) and eight only by forskolin and dbcAMP (Fig. 8A, Table 2A). Comparison of the PACAP and NGF transcriptomes revealed that 19 transcripts were induced in common by the two neurotrophic factors (Fig. 8B, Table 2A), and among these only three were also activated by forskolin and dbcAMP (Fig. 8C, Table 2A). Other transcripts were only induced by PACAP (Table 2B), forskolin (Table 2C), dbcAMP (Table 2C) or NGF (Table 2D).

To verify the microarray results, primers for Q-RT-PCR were designed against 17 transcripts with varying expression profiles in microarray analysis (Table 1). We chose six of the 17 genes for further analysis based on their up-regulation by all three cAMP-elevating or cAMP-mimicking agents (PACAP, dbcAMP and forskolin), and 11 additional representative transcripts from other categories in which a 1.5-fold or greater increase was seen with only one. These 17 transcripts were validated in two ways. First, transcripts induced after six hours of treatment with PACAP (10^{-7} M), forskolin (25 μM), dbcAMP (10^{-3} M) or NGF (100 ng/mL) as detected by microarray hybridization were also found to be elevated via quantification using real-time PCR (Table 3). Some transcripts, such as the *glutaredoxin* (Glrx) detected as elevated only by PACAP

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in the microarray experiments, were in fact also induced by forskolin and dbcAMP when measured using real time PCR (Table 3). Most transcripts [e.g. *GATA binding protein 2* (Gata2), *PACAP specific receptor-1* (Pac1) and *Neuropilin* (Nrp1)] that were not induced by PACAP according to microarray analysis were indeed not regulated as confirmed by real time PCR (Table 3). A Q-RT-PCR time course (Figure 9) was then carried out for all 17 transcripts to investigate the possibility of artifactual discordance in transcript regulation by PACAP, forskolin, dbcAMP or NGF based solely on the decreased sensitivity of microarray analysis compared to Q-RT-PCR (Vaudry et al., 2002a). The time course confirmed that these transcripts showed a robust up-regulation by all four pharmacological (dbcAMP, forskolin) or neurotrophic (PACAP, NGF) neuritogenic agents during the first 48 hours of treatment, at which time neuritogenesis is maximal for PACAP, dbcAMP and forskolin, and well underway for NGF. Seven of 17 transcripts (Gata2, Nrp1, Pac-1, Anx2, Homer2, Akr1b8, and Glrx) failed to fulfill this second criterion. We chose three of the remaining ten transcripts (Egr1, Vil2 and Ier3; see below) for further analysis based on the overall robustness of induction by all four agents over the first half of the 48 hour time course (Figure 9). Thus, the Q-RT-PCR time course experiment revealed that the transcript encoding *early growth response 1* (Egr1, Fig. 9), which was found to be activated only by NGF at six hours after microarray analysis, was induced earlier by cAMP and PACAP, and returned to control levels after six hours of treatment. The immediate early gene Ier3, initially thought to be differentially regulated by PACAP, forskolin and dbcAMP versus NGF was also shown to be transiently regulated by NGF, albeit its regulation by NGF was considerably less (5-fold) than that by cAMP (maximally 15-25-fold, Fig. 9). The transcript encoding villin 2 (Vil2, Fig. 9) was up-regulated modestly (2-4-fold) but consistently by all four agents with a maximum at around three hours of treatment.

Regulation of cAMP- and PACAP-dependent genes by PKA and ERK. The effects of PACAP on neuritogenesis and cell size appear to be initiated by cAMP and mediated through

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downstream signal transduction mechanisms that include ERK but not PKA, while PACAP-induced growth arrest includes a PKA-dependent, or at least an H89-inhibited, component (Figs. 3, 6). The involvement of PKA and ERK in the regulation of three prominent cAMP- and PACAP-dependent transcripts identified by microarray cluster analysis (vide supra), namely Ier3, Egr1 and Vil2, were further investigated by Q-RT-PCR in the presence and absence of H89 (10 μM) or U0126 (25 μM; Fig. 10). Ier3 induction was only slightly reduced in the presence of H89 but was strongly inhibited by U0126. Egr1 induction by PACAP was unaffected by H89 but completely blocked by U0126. Induction of Vil2 by PACAP was, like cell size, unaffected by H89 and blocked, but to a lesser degree than either neuritogenesis, or Ier3 or Egr1 induction, by U0126 (Fig. 10).

Ier3, Egr1 and Vil2 were therefore deemed to be candidates for mediating neuritogenesis or regulation of cell size during PACAP-induced differentiation of PC12 cells. Other genes potentially involved in the control of PC12 cell differentiation were identified in the present study but their function has not been tested as their regulation profile was inconsistent with involvement in those aspects of PACAP-induced cellular differentiation mediated through cAMP activation of ERK studied here. For instance *ornithine decarboxylase* (Odc1) induction by PACAP was only partially blocked by U0126 or H89 and totally blocked by co-incubation with both inhibitors (data not shown). Likewise, *growth arrest specific 1* (Gas1) induction by PACAP was only partially sensitive to H89 and not blocked by U0126 (data not shown). Two additional genes, *aldo keto reductase family 1, member B3* (Akr1b8) and *cytochrome p450 oxydoreductase* (Por) were also induced by PACAP, forskolin, dbcAMP and NGF (Table 3) in an ERK-dependent, PKA-independent manner (data not shown), but have not as yet been further studied.

Functional investigation of cAMP-dependent, PKA-independent genes regulated by PACAP as candidate mediators of PACAP signaling for neuritogenesis, cell size, and growth arrest. Based on microarray and Q-RT-PCR results, functional investigations were

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conducted on three transcripts *i.e.* Ier3, Vil2 and Egr1 that are regulated through the cAMP/ERK pathway independently of PKA (vide supra). Transfection with cognate siRNA consistently reduced Ier3, Vil2 and Egr1 induction by PACAP to the levels shown in Fig. 11A. Only Egr1 siRNA blocked PACAP-induced neurite outgrowth (Fig. 11B). This observation was confirmed by quantification of the total neurite length which was reduced by four-fold when cells were treated with PACAP in the presence of siRNA targeting Egr1 (Fig. 11E). Blocking Vil2 expression reduced the percentage of cells with a diameter above 17 μm while Ier3 and Egr1 had no effect on cell size (Fig. 11C-E). The effect of Vil2 on cell size was fractional (approximately 30%), suggesting that Vil2 may be only one of several effectors of altered cell size accompanying PACAP-induced PC12 cell differentiation. Neither Vil2, Ier3, or Egr1 silencing affected growth arrest mediated by PACAP (Fig. 11C-E), consistent with the PKA-dependent component of PACAP-induced growth arrest of PC12 cells described earlier. Among the transcripts regulated in common within the first six hours of exposure to PACAP, dibutyryl cAMP or forskolin, those wholly or partially dependent on PKA would be candidate mediators of growth arrest by PACAP, including both induced and pre-existing proteins regulated by NGF and reported to be involved in NGF-induced growth arrest in PC12 cells (Greene and Tischler, 1976).

Rap1 is involved in PACAP activation of Egr1. Silencing the expression of the cAMP- and ERK-regulated Egr1 transcript evoked the most profound and specific functional response seen in this study, inhibiting PACAP-induced neuritogenesis without affecting cell size or proliferation (vide supra). We therefore focused on the activity of this trans-activator in order to link regulation of neuritogenesis through cAMP via ERK to PACAP-dependent activation of Rap1. An Egr1-responsive reporter gene, pEgr-Luc, driving luciferase gene expression, was transfected into PC12 cells to assay functional Egr1 activation by PACAP. PACAP treatment of transfected PC12 cells induced an approximately 10-fold increase in Egr1 reporter activity compared to vehicle (Fig. 12). To test whether Rap1 is involved in this PACAP-dependent

signaling pathway to Egr1 activation, Rap1b DN was co-transfected with the Egr1 reporter prior to treatment with PACAP. Co-transfection of the Rap1b DN significantly reduced the PACAP-induced Egr1 reporter activity, indicating that either Egr1 transcription, its functional transactivation of Egr1-dependent transcription, or both, are stimulated by PACAP through Rap1 or a Rap1-like GTP-binding protein (Fig. 12).

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DISCUSSION

The PC12 cell line has been widely used to investigate the various effects of PACAP on neuronal differentiation including neuritogenesis (Deutsch and Sun, 1992; Lazarovici et al., 1998), inhibition of cell division (Deutsch and Sun, 1992), stimulation of expression of neuroendocrine-specific genes such as neuropeptide Y and tyrosine hydroxylase (Corbitt et al., 1998) and changes in electrical excitability and secretion (Taupenot et al., 1999; Osipenko et al., 2000; Grumolato et al., 2003). The aim of the present study was to elucidate the signal transduction pathways leading to specific aspects of differentiation (neuritogenesis, cell size, and cell proliferation) induced by PACAP, through stringent correlation of the regulation of signaling molecules, target genes, and functional outcomes of PACAP treatment. The major finding of this investigation, is that PACAP initiates a cAMP-dependent, PKA-independent activation of ERK that leads to *egr1* transcription and activation, and subsequent Egr1-dependent neuritogenesis. Cyclic AMP-dependent, PKA-independent activation of ERK also leads to *villin2* transcription which is in part responsible for increased cell size following PACAP treatment. Cessation of proliferation induced by PACAP is unaffected by transcription of either of these two target genes, consistent with the partial PKA-dependence of the anti-proliferative effect of PACAP on PC12 cells.

We have used cDNA microarray in conjunction with biochemical analysis to establish the existence of a cAMP-dependent, PKA-independent signaling pathway responsible for activating ERK, and a discrete set of downstream target genes in PC12 cells in response to PACAP. The functional importance of this pathway was further tested by comparing the cellular and biochemical profiles observed for inhibition of PACAP- and cAMP-initiated neuritogenesis, increase in cell size, and cessation of cell division. This concordance has led in turn to the functional identification of two transcripts, those encoding Egr1 and Vil2, whose up-regulation was demonstrated through gene silencing to be required in two distinct pathways leading to

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PACAP-mediated neurite extension and increased cell size, respectively. Vil2 codes for a protein that has been shown to be involved in microvilli formation in intestinal epithelium by regulating actin polymerization (Craig and Powell, 1980). Recently, some transcripts with a high sequence homology to Vil2 such as *pervillin* and *advillin* have been shown to promote neurite outgrowth in dorsal root ganglion and sympathetic neurons (Ravenall et al., 2002; Shibata et al., 2004), suggesting that a cohort of Vil2-related proteins may contribute to regulation of cell size during differentiation initiated by PACAP in PC12 cells. A third transcript, Ier3, that is prominently regulated by PACAP is apparently not involved in either of these processes, and may contribute to aspects of the overall differentiation program initiated by PACAP (Taupenot et al., 1999; Osipenko et al., 2000; Grumolato et al., 2003), however these linkages have not yet been uncovered. We have not identified any genes involved in the control of cell proliferation by PACAP. In this regard, future experiments will focus on transcripts such as Gas1, whose induction by PACAP requires PKA.

The pathways leading to cAMP-dependent and PKA-independent regulation of neurite outgrowth and cell size by PACAP are summarized in Figure 13. These two pathways are biochemically distinct from each other, and also from the partially PKA-dependent signal transduction pathway leading to growth arrest. The combined biochemical, microarray, and gene silencing study performed here provides compelling evidence that PACAP-induced neuritogenesis proceeds through elevation of cAMP, activation of ERK through Rap1 or a Rap1-like GTP-binding protein, and subsequent increase in Egr1 trans-activation (Figure 13).

ERK activation is critical for neuritogenesis induced by both NGF and PACAP (Barrie et al., 1997), and indeed phosphorylation of ERK is itself sufficient to induce neurite outgrowth (Robinson et al., 1998). However activation of ERK per se can occur through multiple pathways with diverse functional sequelae. In fact, it has been previously reported that cAMP, PACAP, NGF and PMA all elicit sustained activation of ERK in PC12 cells, yet neuritogenesis stimulated by NGF, but not PACAP, proceeds through activation of Ras (Young et al., 1994; Lazarovici et

al., 1998), while PMA does not stimulate neuritogenesis at all. Furthermore, stimulation of ERK by NGF or cAMP leading to transin gene activation proceeds through activation of PKA (Yao et al., 1998), but that leading to neuritogenesis stimulated by PACAP does not (this report). The best working hypothesis for these disparate effects of ERK activation by distinct signaling pathways may be that both the duration of ERK signaling, and the cellular compartment in which ERK activation occurs, provide discrete ERK signaling 'signatures' with distinct functional outcomes for the cell (MacCormick et al., 2004; Gerdin and Eiden, 2007). This hypothesis is supported by a recent report that Epac stimulates ERK through Rap1 in PC12 cells only if Epac is localized to the plasma membrane by addition of a CAAX motif, but not when Rap1 is activated through endogenous Epac stimulation by 8-chlorophenylthio-2-methyl cAMP (Wang et al., 2006). It will be important to determine if our finding of a cAMP-dependent, PKA-independent activation of ERK in the PC12 pheochromocytoma neuroendocrine cell line by PACAP corresponds to the existence of such a pathway in post-mitotic primary neuroendocrine and neuronal cells. We are currently investigating this possibility, and have observed cAMP-dependent, H89-resistant ERK activation in bovine chromaffin cells (M. J. Gerdin, unpublished observation).

Egr1 is the transcript which exhibited the highest induction after NGF, PACAP, forskolin and dbcAMP treatment, and activation of Egr1 by PACAP is dependent on the activation of Rap1. Reducing Egr1 expression with siRNA blocked the ability of PACAP to promote neuritogenesis without affecting its growth arrest effect (Fig. 13). After treatment with NGF, Egr1 acts as a transactivator to promote p35 expression which by binding to the *cyclin-dependent kinase* cdk5 induces neurite outgrowth (Harada et al., 2001). PACAP- and NGF-induced differentiation exhibit both similarities and differences in terms of mechanisms and phenotypes (Vaudry et al., 2002b). In fact, while signaling elements may be conserved in PACAP and NGF induction of neuritogenesis, our microarray analysis also shows marked differences in NGF and PACAP target gene induction. This is wholly consistent with Egr1 response element (ERE)-specific transactivation by PACAP, in contrast with the intriguing results of Levkovitz and

Baraban indicating that NGF stimulation of neurite outgrowth depends not on Egr1-dependent transactivation directly at genes containing an Egr1-response element (ERE), but rather on Egr1 activation of c-Jun via Egr1/c-Jun heterodimerization (Levkovitz and Baraban, 2002).

Our results support the view that neurotrophin-driven differentiation is a process that occurs through simultaneous activation of multiple parallel signaling events, rather than a single common 'master pathway' relying on a few common signaling intermediates. Neuritogenesis and cessation of proliferation initiated by NGF, for example, have been dissected into separate p53-dependent and –independent processes (Hughes et al., 2001). Similarly, it is now clear that PACAP signaling for neuritogenesis, cell size, growth arrest, and likely also for neuron-specific gene expression, depend on separate, parallel pathways that diverge as early as multiple downstream targets for cAMP in PC12 cells.

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This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Barrie AP, Clohessy AM, Buensuceso CS, Rogers MV and Allen JM (1997) Pituitary adenylyl cyclase-activating peptide stimulates extracellular signal-regulated kinase 1 or 2 (ERK1/2) activity in a ras-independent, mitogen-activated protein kinase/ERK kinase 1 or 2-dependent manner in PC12 cells. *J Biol Chem* **272**:19666-19671.

Bouschet T, Perez V, Fernandez C, Bockaert J, Eychene A and Journot L (2003) Stimulation of the ERK pathway by GTP-loaded Rap1 requires the concomitant activation of Ras, protein kinase C, and protein kinase A in neuronal cells. *J Biol Chem* **278**(7):4778-4785.

Chen Y, Samal B, Hamelink CR, Xiang CC, Chen Y, Chen M, Vaudry D, Brownstein MJ, Hallenbeck JM and Eiden LE (2006) Neuroprotection by endogenous and exogenous PACAP following stroke. *Regul Pept* **137**(1-2):4-19.

Corbitt J, Vivekananda J, Wang SS and Strong R (1998) Transcriptional and posttranscriptional control of tyrosine hydroxylase gene expression during persistent stimulation of pituitary adenylate cyclase-activating polypeptide receptors on PC12 cells: Regulation by protein kinase A-dependent and protein kinase A-independent pathways. *J Neurochem* **71**:478-486.

Craig SW and Powell LD (1980) Regulation of actin polymerization by villin, a 95,000 dalton cytoskeletal component of intestinal brush borders. *Cell* **22**(3):739-746.

Das KP, Freudenrich TM and Mundy WR (2004) Assessment of PC12 cell differentiation and neurite growth: a comparison of morphological and neurochemical measures. *Neurotoxicol Teratol* **26**(3):397-406.

Deutsch PJ and Sun Y (1992) The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *J Biol Chem* **267**:5108-5113.

Gerdin MJ and Eiden LE (2007) Regulation of PC12 cell differentiation by cAMP signaling to ERK independent of PKA: do all the connections add up? *Sci STKE* **2007**(382):pe15.

Ginty DD, Fanger GR, Wagner JA and Maue RA (1992) The activity of cAMP-dependent protein kinase is required at a posttranslational level for induction of voltage-dependent sodium channels by peptide growth factors in PC12 cells. *J Cell Biol* **116**(6):1465-1473.

Ginty DD, Glowacka D, DeFranco C and Wagner JA (1991) Nerve growth factor-induced neuronal differentiation after dominant repression of both type I and type II cAMP-dependent protein kinase activities. *J Biol Chem* **266**(23):15325-15333.

Greene LA and Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A* **73**(7):2424-2428.

Grumolato L, Louiset E, Alexandre D, Ait-Ali D, Turquier V, Fournier A, Fasolo A, Vaudry H and Anouar Y (2003) PACAP and NGF regulate common and distinct traits of the sympathoadrenal lineage: effects on electrical properties, gene markers and transcription factors in differentiating PC12 cells. *Eur J Neurosci* **17**(1):71-82.

Hamelink C, Lee HW, Chen Y, Grimaldi M and Eiden LE (2002) Coincident elevation of cAMP and calcium influx by PACAP-27 synergistically regulates vasoactive intestinal polypeptide gene transcription through a novel PKA-independent signaling pathway. *J Neurosci* **22**(13):5310-5320.

Harada T, Morooka T, Ogawa S and Nishida E (2001) ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat Cell Biol* **3**(5):453-459.

Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JP, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA and Waschek JA (1998) International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Revs* **50**:265-270.

Hughes AL, Messineo-Jones D, Lad SP and Neet KE (2001) Distinction between differentiation, cell cycle, and apoptosis signals in PC12 cells by the nerve growth factor mutant delta9/13, which is selective for the p75 neurotrophin receptor. *J Neurosci Res* **63**(1):10-19.

Kienlen Campard P, Crochemore C, René F, Koch B and Loeffler JP (1997) PACAP type I receptor activation promotes cerebellar neuron survival through the cAMP/PKA signaling pathway. *DNA Cell Biol* **16**:323-333.

Lazarovici P, Jiang H and Fink Jr. D (1998) The 38-amino-acid form of pituitary adenylate cyclase-activating polypeptide induces neurite outgrowth in PC12 cells that is dependent on protein kinase C and extracellular signal-regulated kinase but not on protein kinase A, nerve growth factor receptor tyrosine kinase, p21ras G protein, and pp60c-src cytoplasmic tyrosine kinase. *Mol Pharmacol* **54**:547-558.

Levkovitz Y and Baraban JM (2002) A dominant negative Egr inhibitor blocks nerve growth factor-induced neurite outgrowth by suppressing c-Jun activation: role of an Egr/c-Jun complex. *J Neurosci* **22**(10):3845-3854.

MacCormick M, Moderscheim T, van der Salm LW, Moore A, Pryor SC, McCaffrey G and Grimes ML (2004) Distinct signalling particles containing Erk/Mek and B-Raf in PC12 cells. *Biochem J* **385**:1-10.

Obara Y, Labudda K, Dillon TJ and Stork PJ (2004) PKA phosphorylation of Src mediates Rap1 activation in NGF and cAMP signaling in PC12 cells. *J Cell Sci* **117**(Pt 25):6085-6094.

Ohtaki H, Nakamachi T, Dohi K, Aizawa Y, Takaki A, Hodoyama K, Yofu S, Hashimoto H, Shintani N, Baba A, Kopf M, Iwakura Y, Matsuda K, Arimura A and Shioda S (2006) Pituitary adenylate cyclase-activating polypeptide (PACAP) decreases ischemic neuronal cell death in association with IL-6. *ProcNatl Acad Sci USA* **103**(19):7488-7493.

Osipenko ON, Barrie AP, Allen JM and Gurney AM (2000) Pituitary adenylyl cyclase-activating peptide activates multiple intracellular signaling pathways to regulate ion channels in PC12 cells. *J Biol Chem* **275**:16626-16631.

Qiu J, Cai D and Filbin MT (2002) A role for cAMP in regeneration during development and after injury. *Prog Brain Res* **137**:381-387.

Rausch DM, Iacangelo AL and Eiden LE (1988) Glucocorticoid- and nerve growth factor-induced changes in chromogranin A expression define two different neuronal phenotypes in PC12 cells. *Mol Endocrinol* **2**(10):921-927.

Ravenall SJ, Gavazzi I, Wood JN and Akopian AN (2002) A peripheral nervous system actin-binding protein regulates neurite outgrowth. *Eur J Neurosci* **15**(2):281-290.

Ravni A, Eiden LE, Vaudry H, Gonzalez BJ and Vaudry D (2006) Cycloheximide treatment to identify components of the transitional transcriptome in PACAP-induced PC12 cell differentiation. *J Neurochem* **98**(4):1229-1241.

Reglödi D, Somogyvari-Vigh A, Vigh S, Kozicz T and Arimura A (2000) Delayed systemic administration of PACAP38 is neuroprotective in transient middle cerebral artery occlusion in the rat. *Stroke* **31**:1411-1417.

Robinson MJ, Stippec SA, Goldsmith E, White MA and Cobb MH (1998) A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation. *Curr Biol* **8**(21):1141-1150.

Shibata M, Ishii J, Koizumi H, Shibata N, Dohmae N, Takio K, Adachi H, Tsujimoto M and Arai H (2004) Type F scavenger receptor SREC-I interacts with advillin, a member of the gelsolin/villin family, and induces neurite-like outgrowth. *J Biol Chem* **279**(38):40084-40090.

Stessin AM, Zippin JH, Kamenetsky M, Hess KC, Buck J and Levin LR (2006) Soluble adenylyl cyclase mediates nerve growth factor-induced activation of Rap1. *J Biol Chem* **281**(25):17253-17258.

Tanaka J, Koshimura K, Murakami Y and Kato Y (1996) Stimulatory effect of PACAP on neuronal cell survival. *Ann N Y Acad Sci* **805**:473-475.

Taupenot L, Mahata M, Mahata SK and O'Connor DT (1999) Time-dependent effects of the neuropeptide PACAP on catecholamine secretion. Stimulation and desensitization. *Hypertension* **34**:1152-1162.

Vaudry D, Chen Y, Ravni A, Hamelink C, Elkahloun AG and Eiden LE (2002a) Analysis of the PC12 cell transcriptome after differentiation with pituitary adenylate cyclase-activating polypeptide (PACAP). *J Neurochem* **83**(6):1272-1284.

Vaudry D, Stork PJ, Lazarovici P and Eiden LE (2002b) Signaling pathways for PC12 cell differentiation: making the right connections. *Science* **296**(5573):1648-1649.

Vossler MR, Yao H, York RD, Pan M-G, Rim CS and Stork PJS (1997) cAMP activates MAP kinase and elk-1 through a B-raf and rap1-dependent pathway. *Cell* **89**:73-82.

Wang Z, Dillon TJ, Pokala V, Mishra S, Labudda K, Hunter B and Stork PJ (2006) Rap1-mediated activation of extracellular signal-regulated kinases by cyclic AMP is dependent on the mode of Rap1 activation. *Mol Cell Biol* **26**(6):2130-2145.

Yao H, York RD, Misra-Press A, Carr DW and Stork PJS (1998) The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated kinases and gene expression by nerve growth factor. *J Biol Chem* **273**:8240-8247.

York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW and Stork PJS (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature* **392**:622-626.

Young SW, Dickens M and Tavare JM (1994) Differentiation of PC12 cells in response to a cAMP analogue is accompanied by sustained activation of mitogen-activated protein kinase. Comparison with the effects of insulin, growth factors and phorbol esters. *FEBS Lett* **338**(2):212-216.

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FOOTNOTE

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FIGURE LEGENDS

Figure 1: Effect of PACAP-38 and VIP on PC12 cell differentiation. (A) Microphotographs illustrating the neurite extension observed after 48 hours of treatment with PACAP (10^{-7} M) or VIP (10^{-7} M). Scale bar = 15 µm. (B) Quantification of the percentage of cells with neurites, number of neurites per cell, and total neurite outgrowth after treatment with graded concentrations of PACAP or VIP (10^{-11} to 10^{-6} M). (C) Quantification of the median diameter of PC12 cells (µm) and the percentage of cells with a diameter above 17 µm after 48 hours of treatment with graded concentrations of PACAP or VIP (10^{-11} to 10^{-6} M). (D) Quantification of the effect of graded concentrations of PACAP or VIP (10^{-11} to 10^{-6} M) on PC12 cell proliferation. * P<0.05, ** P<0.01, *** P<0.001 vs control.

Figure 2: Kinetic of the effect of PACAP on PC12 cell differentiation. (A) Cells were exposed to PACAP (10^{-7} M) for durations ranging from 1 min to 48 hours and the percentage of cells with neurites, number of neurites per cell, and total neurite outgrowth was measured 48 hours after the beginning of the treatment. (B) Cells were exposed to PACAP (10^{-7} M) for durations ranging from 1 min to 48 hours and cell quantification was performed 48 hours after the beginning of the treatment. ** P<0.01, *** P<0.001 vs control.

Figure 3: Effect of cAMP stimulators on PC12 cell differentiation. (A) Microphotographs illustrating the effect of PACAP (10^{-7} M), forskolin (25 µM) or dbcAMP (10^{-3} M) on PC12 cells after 48 hours of treatment. When indicated, a PKA inhibitor, H89 (10 µM) or a MEK inhibitor, U0126 (25 µM) was added 30 min before PACAP, forskolin or dbcAMP. Scale bar = 16 µM. (B) Quantification of the percentage of cells with neurites, number of neurites per cell and total neurite outgrowth after 48 hours of treatment of PC12 cells with PACAP (10^{-7} M) or forskolin (25 µM) in the absence or presence of H89 (10 µM). (C) Quantification of the percentage of cells with a diameter above 17 µm after 48 hours of treatment with PACAP (10^{-7} M) and forskolin (25

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μM) in the absence or presence of H89 (10 μM). (D) Quantification of the effect of a 48 hour treatment with PACAP (10^{-7} M) and forskolin (25 μM) in the absence or presence of H89 (10 μM) on cell proliferation. * P<0.05, ** P<0.01, *** P<0.001, ns vs control; # P<0.05; ns, not statistically different versus control; *NS*, not statistically different.

Figure 4: Effect of an adenylate cyclase inhibitor on PACAP-induced PC12 cell differentiation. (A) Quantification of cAMP production after treatment with PACAP (10^{-7} M) in the presence or absence of the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (ddAd, 50 μM) for 30 min. (B) Microphotographs illustrating the effect of PACAP (10^{-7} M) in the presence or absence of ddAd (50 μM) on PC12 cells after 48 hours of treatment. Scale bar = 16 μM. (C) Quantification of neurite outgrowth after treatment with PACAP (10^{-7} M) in the absence or presence of ddAd (50 μM) for 48 hour. (D) Quantification of the effect of a 48 hour treatment with PACAP (10^{-7} M) in the absence or presence of ddAd on cell proliferation. * P<0.05, ** P<0.01, *** P<0.001, ns vs control; # P<0.05 vs PACAP; ns, not statistically different vs control.

Figure 5: Effect of protein kinase C inhibitors on PACAP-induced PC12 cell differentiation. (A) Quantification of the percentage of cells with neurites, number of neurites per cell, and total neurite outgrowth after treatment with PACAP (10^{-7} M) in the presence or absence of chelerythrine (5 μM). (B) Quantification of the percentage of cells with a diameter above 17 μm after 48 hours of treatment with PACAP (10^{-7} M) in the presence or absence of chelerythrine. (C) Quantification of the effect of a 48-hour treatment with PACAP (10^{-7} M) in the absence or presence of chelerythrine on cell proliferation. (D) Quantification of total neurite outgrowth after treatment with PACAP (10^{-7} M) in the absence or presence of chelerythrine (5 μM) + H89 (10 μM) or H7 (50 μM). * P<0.05, *** P<0.001, ns vs control; # P<0.05; ns, not statistically different versus control; *NS*, not statistically different. Chel, chelerythrine.

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Figure 6: Involvement of the MAP kinase pathway in the effect of PACAP on neurite outgrowth. (A) Effects of PACAP (10^{-7} M) in the presence or absence of the PKA inhibitor H89 (10 μM) on ERK phosphorylation after 5 min of treatment. (B) Quantification of the percentage of cells with neurites, number of neurites per cell and total neurite outgrowth after treatment with PACAP (10^{-7} M) in the presence or absence of U0126 (25 μM) or PD98059 (50 μM). (C) Quantification of the percentage of cells with a diameter superior to 17 μm after a 48-hour treatment of PC12 cells with PACAP (10^{-7} M) in the presence or absence of U0126 (25 μM). (D) Quantification of a 48 hour treatment with PACAP (10^{-7} M) in the presence or absence of U0126 (25 μM) on cell proliferation. * P<0.05, ** P<0.01, *** P<0.001, ns vs control; # P<0.05, ## P<0.01, *NS* vs PACAP; ns, not statistically different versus control; *NS*, not statistically different.

Figure 7: Involvement of Rap1 in the effects of PACAP on neurite outgrowth in PC12 cells. (A) Illustration and quantification of a time-course effect of PACAP on Rap GTP loading. Cells were exposed to PACAP (10^{-7} M) for durations ranging from 30 seconds to 30 minutes. Quantifications were conducted from 4 to 5 independent experiments. (B) Typical microphotographs illustrating on green cells the effect of a GFP lentiviral expression vector (GFP) and of a Rap1 dominant negative IRES GFP lentiviral expression vector (RapDN-GFP) on PACAP-induced differentiation after 48 hours of treatment. Scale bar = 12 μm. (C) Quantification of the percentage of cells with neurites, number of neurites per cell, and total neurite outgrowth after treatment with PACAP in cells that express or not the Rap dominant negative protein. * P<0.05, *** P<0.001, ns vs control; ## P<0.01, *NS* vs PACAP; ns, not statistically different vs control; *NS*, not statistically different.

Figure 8: Venn diagrams comparing the number of genes induced by PACAP, forskolin, dbcAMP and/or NGF in PC12 cells after six hours of treatment. (A) Diagram comparing the genes induced by PACAP (10^{-7} M), forskolin (25 μM) and/or dbcAMP (10^{-3} M). The experiments

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were conducted on an array of 15,000 genes among which 27 appeared reproducibly activated by both PACAP, forskolin and dbcAMP. (B) Diagram comparing the genes induced by PACAP (10^{-7} M), and/or NGF (100 ng/mL). The experiments revealed that 20 genes were induced by both PACAP and NGF. (C) Diagram comparing the genes induced by forskolin (25 μM), dbcAMP (10^{-3} M) and/or NGF (100 ng/mL). The experiments revealed only 3 genes commonly activated by cAMP stimulators and NGF. It should be noted that these genes were also induced by PACAP (see Table 2).

Figure 9: Time-course of induction of PACAP target genes. Time-course effect of PACAP (10^{-7} M; solid black line), forskolin (25 μM; dotted black line), dbcAMP (10^{-3} M, dashed black line), NGF (100 ng/mL; solid dark grey line) and medium (solid light grey line) on the expression of various PACAP target genes A. *GATA binding protein 2* (Gata2), *neuropilin 1* (Nrp1), *adenylate cyclase activating polypeptide 1 receptor 1* (Pac-1), *annexin A2* (Anx2), *homer homolog 2 (Drosophila)* (Homer 2), *P450 (cytochrome) oxidoreductase* (Por). **B.** *aldo-keto reductase family 1, member B8* (Akr1b8), *villin 2* (Vil2), *heat shock 22kDa protein 8* (Hspb8), *early growth response 1* (Egr1), *glutaredoxin* (Glx), *protein tyrosine phosphatase 4a1* (Ptp4a1). **C.** *growth arrest specific 1* (Gas1), *antizyme inhibitor 1* (Azin1), *ornithine decarboxylase, structural 1* (Odc), *immediate early response 3* (Ier3) and *regulator of G-protein signaling 2* (Rgs2). Each time point represents the mean fold expression (± SEM) compared to the time 0 hour as measured by real time PCR. Data were corrected using *glyceraldehyde-3-phosphate dehydrogenase* (Gapdh) signal as internal control.

Figure 10: Involvement of the PKA and MAP kinase pathways in the effect of PACAP on the expression of *immediate early response 3* (Ier3), *villin 2* (Vil2) and *early growth response 1* (Egr1). Cells were preincubated for 30 min with either H89 (10 μM) or U0126 (25 μM) and then incubated for one or six hours with PACAP (10^{-7} M). The level of expression of *immediate early*

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response 3 (Ier3) and *villin 2* (Vil2) mRNA was quantified by real time PCR after six hours of treatment. The level of expression of *early growth response 1* (Egr1) mRNA was quantified by real-time PCR after one hour of treatment. Data were corrected using Gapdh signal as internal control. * $P<0.05$, ** $P<0.01$, ns vs control; # $P<0.05$, *NS* vs PACAP; ns, not statistically significant vs control; *NS*, not statistically different.

Figure 11: Involvement of *immediate early response 3* (Ier3), *villin 2* (Vil2) and *early growth response 1* (Egr1) in PC12 cell differentiation. (A) Effects of specific siRNA on Ier3, Vil2 and Egr1 mRNA expression. Cells were transfected in the absence or presence of specific siRNA targeting either Ier3, Vil2 or Egr1, and two days after transfection, cells were treated with control medium or PACAP (10^{-7} M) for six (Ier3, Vil2) or one (Egr1) hours. The level of expression of Ier3, Vil2 and Egr1 mRNA was then quantified by real-time PCR. Data were corrected using Gapdh signal as internal control. (B) Microphotographs illustrating the effect of siRNA against Ier3, Vil2 and Egr1 mRNA on cell differentiation after 48 hours of treatment with PACAP (10^{-7} M). (C-E) Quantification of the total neurite outgrowth (left), percentage of cells with a diameter greater than 17 µm (middle), and cell proliferation (right) after 48 hours of treatment with 100 nM PACAP in the absence or presence of siRNA against Ier3 (C), Vil2 (D) or Egr1 (E) mRNA. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns vs respective controls; # $P<0.05$, ### $P<0.001$, *NS* vs PACAP; ns, not statistically significant vs control; *NS*, not statistically different.

Figure 12: Involvement of Rap1 in PACAP-mediated induction of *early growth response 1* (Egr1). PC12 cells were transiently transfected with an Egr1 reporter plasmid and co-transfected with a dominant negative Rap1 or control plasmid. Total DNA per transfection was held constant by addition of the promoterless vector pGEM. Cells were then treated with PACAP (10^{-7} M) for 6 hours and Egr1 expression was measured by dual-luciferase activity. Data represent the mean±SEM of 3-7 independent experiments, analyzed by one-way ANOVA with a Tukey post-

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test with PRISM. *** p<0.001 compared to vehicle; NS vs vehicle; ##p<0.01 compared to PACAP induction of Egr1.

Figure 13: Schematic representation summarizing the intracellular events involved in the neurotrophic activities of PACAP on PC12 cells. PACAP induction of Egr1expression is required for neuritogenesis. This effect of PACAP proceeds through a cAMP/ERK-dependent, PKA-independent pathway. PACAP also increases cell size by inducing effectors such as villin 2. Finally, PACAP blocks cell proliferation in a partially PKA-dependent manner through transcription mechanisms that remain to be identified. AC, adenylate cyclase; cAMP, adenosine 3', 5'-cyclic monophosphate; DAG, diacyl glycerol; dbcAMP, N6, 2'-O-dibutyryladenosine 3', 5'-cyclic monophosphate; ddAd, 2',5'-dideoxyadenosine; Egr1, *early growth response 1*; ERK1/2, extracellular signal-regulated kinase; H89, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (PKA inhibitor); MEK, mitogen-activated protein kinase; PAC1, PACAP specific receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate acetate; Rap1, member of RAS oncogene family; U0126, MEK inhibitor. Full arrow: established regulation. Dotted arrow: assumed regulation.

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TABLES

Table 1: Sequences of primers used for real-time PCR.

	Foward Primer	Reverse Primer	Concentration
Akr1b8	**aldo-keto reductase family 1, member B8**		
	5' - CAA GCC TGG ACT GAA ACA TAA GC - 3'	5' - ATC AGT TTT TCC TGG GTG AGG TAA G - 3'	300 nM
Anxa2	**annexin A2**		
	5' - GAC ATT GCC TTC GCC TAC CA - 3'	5' - TGA CCA GAC AAG GCC GAC TT - 3'	300 nM
Azin1	**antizyme inhibitor 1**		
	5' - AAG ACG CTT TAC CCG ACT CTT TG - 3'	5' - TAT CAT CAG CTA GGT TCC CAA GGT - 3'	300 nM
Egr1	**early growth response 1**		
	5' - GGG AGC CGA GCG AAC AA - 3'	5' - GTC TCC ACC AGC GCC TTC T - 3'	300 nM
Gapdh	**glyceraldehyde-3-phosphate dehydrogenase**		
	5' - TCC CAT TCC TCC ACC TTT GA - 3'	5' - CAG GAA ATG AGC TTC ACA AAG TTG - 3'	100 nM
Gas1	**growth arrest specific 1**		
	5' - AAT ACA ATG TTT AAG GCA GTT TGG AA - 3'	5' - AGG TGT GCC CTG TGT AGA AGA AC - 3'	300 nM
Gata2	**GATA binding protein 2**		
	5' - CAC CTG TTG TGC AAA TTG TCA GA - 3'	5' - GGA TCC CTT CCT TTC TTC ATG - 3'	300 nM
Glrx	**glutaredoxin**		
	5' - CCA ATG CGA TTC AAG ATT ATT TAC A - 3'	5' – CGC CTA TGC AGT CTT TTA CCT ATG A - 3'	300 nM
Homer2	**homer homolog 2 (Drosophila)**		
	5' - CGA TGT CAC CAG GAA CAG CTA TC - 3'	5' - GGG TGA TGG TGC TGT TTA TGA TT - 3'	300 nM
Hspb8	**heat shock 22kDa protein 8**		
	5' - GCC GGA AGA ACT GAT GGT AAA G - 3'	5'-GAG ACA ATC CCA CCT TCT TGC T-3'	300 nM
Ier3	**immediate early response 3**		
	5 -'GAG GAA CCC AAC ATT GCC AA - 3'	5' - ACC TTC TTC AGC CAT CAA AAT CTG - 3'	300 nM
Nrp1	**neuropilin 1**		
	5' - CGG AGG AGT GTT CTG TCG CTA T - 3'	5' - TCC GGC CAG GAG TTT TCT G - 3'	300 nM
Odc1	**ornithine decarboxylase, structural 1**		
	5' - CCA GCA GGC TTC TCT TGG GAA - 3'	5' - CAC GAA GGT CTC AGG GTC AGT AC - 3'	300 nM
Pac-1	**adenylate cyclase activating polypeptide 1 receptor 1**		
	5' - CCC TGA CTG CTC TCC TCC TGC TGC CTA T - 3'	5' - CAG GGC AGC TCA CAA GGA CCA TCT CAC C - 3'	300 nM
Por	**P450 (cytochrome) oxidoreductase**		
	5' - ACG GGA ACT TGG AAG AGG ATT T - 3'	5' - TGG CTT CTA CCC CAA AGA ACT C - 3'	300 nM
Ptp4a1	**protein tyrosine phosphatase 4a1**		
	5' - TCG TGA AGA ACC TGG TTG CTG - 3'	5' - TTA ATG CTA GGG CAA CAA GCA C - 3'	300 nM
Rgs2	**regulator of G-protein signaling 2**		
	5' - CCG ACT TCA TCG AGA AGG AA - 3'	5' - GCA GCC ACT TGT AGC CTC TT - 3'	300 nM
Vil2	**villin 2**		
	5' - GAC GAC CGT AAC GAG GAG AA - 3'	5' - CTG GGA CAA CTC ATT GCT CA - 3'	300 nM

Akr1b8, *aldo-keto reductase family 1, member B8*; Anxa2, *annexin A2*; Azin1, *antizyme inhibitor 1*; Egr1, *early growth response 1*; Gapdh, *glyceraldehyde-3-phosphate dehydrogenase;* Gas1, *growth arrest specific 1*; Gata2, *GATA binding protein 2*; Glrx, *glutaredoxin*; Homer2, *homer homolog 2 (Drosophila)*; Hspb8, *heat shock 22kDa protein 8*; Ier3, *immediate early response 3*; Nrp1, *neuropilin 1*; Odc1, *ornithine decarboxylase, structural 1*; Pac-1, *adenylate cyclase activating polypeptide 1 receptor 1*; Por, *P450 (cytochrome) oxidoreductase*; Ptp4a1, *protein tyrosine phosphatase 4a1*; Rgs2, *regulator of G-protein signaling 2*; Vil2, *villin 2.*

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Table 2: Classification of the genes induced by a 6-h treatment with pituitary adenylate cyclase-activating polypeptide (PACAP; 10^{-7} M), forskolin (25 µM), N6, 2'-O-dibutyryladenosine 3', 5'-cyclic monophosphate (dbcAMP; 10^{-3} M) and/or nerve growth factor (NGF; 100 ng/mL). Transcripts were classified in decreasing order of magnitude of induction. Some transcripts with a ratio above 1.5 were not included in a particular category for a given treatment, if the data did not also satisfy microarray quality criteria (quality index >0.3). Bold characters indicate genes further investigated by real time PCR as reported in Table 3. (A) Genes induced by at least one neurotrophic factor, PACAP, forskolin, dbcAMP and NGF. (B) Genes induced only by PACAP. (C) Genes induced by AMPc (forskolin and dbcAMP). (D) Genes induced in presence of NGF.

A

Gene Name	Ug Nb	GB ID	PACAP		Forskolin		dbcAMP		NGF	
			Av	SEM	Av	SEM	Av	SEM	Av	SEM
PACAP Forskolin dbcAMP NGF										
Ornithine decarboxylase, structural 1 (Odc1)	Mm.34102	AU020132	9.8	1.0	4.5	0.1	3.2	0.2	3.9	0.4
Villin 2 (Vil2)	Mm.277812	AW558398	2.4	0.2	1.5	0.0	1.6	0.2	1.5	0.1
HIV-1 Rev binding protein (Hrb)	Mm.6461	C81336	2.2	0.2	1.7	0.1	1.8	0.1	1.7	0.2
PACAP Forskolin dbcAMP										
Regulator of G-protein signaling 2 (Rgs2)	Mm.28262	AU023169	10.0	3.4	3.1	1.2	1.6	0.2	1.4	0.1
Immediate early response 3 (Ier3)	Mm.25613	C87164	8.8	1.8	3.7	1.7	2.0	0.3	1.4	0.1
Antizyme inhibitor 1 (Azin1)	Mm.250214	AU016852	5.9	1.1	4.9	0.3	4.9	0.6	1.1	0.1
MAP kinase-activated protein kinase 2 (Mapkapk2)	Mm.221235	AW557658	5.9	0.6	4.2	0.2	4.4	0.5	1.4	0.1
ESTs	Mm.17000	AU016391	4.9	0.6	2.6	1.0	1.8	0.3	1.2	0.1
Protein tyrosine phosphatase 4a1 (Ptp4a1)	Mm.374437	AU022218	4.0	0.4	2.7	0.1	2.6	0.2	1.3	0.1
Transcribed locus	Mm.401636	AW548730	3.9	0.4	2.6	0.3	2.4	0.2	1.2	0.1
RIKEN cDNA 4930431J08 gene (4930431J08Rik)	Mm.86986	AW554859	3.6	0.3	2.1	0.1	1.9	0.3	1.1	0.1
Growth arrest specific 1 (Gas1)	Mm.22701	AW554898	3.5	0.4	2.5	0.4	2.3	0.4	0.9	0.1
Prickle like 1 (Drosophila) (Prickle1)	Mm.150314	AU022455	3.5	0.3	2.3	0.2	2.2	0.3	1.2	0.1
Plexin A2 (Plxna2)	Mm.392736	AW551294	3.2	0.4	1.8	0.3	1.4	0.1	1.1	0.1
RIKEN cDNA 1500041J02 gene (1500041J02Rik)	Mm.281019	AU020735	2.9	0.4	2.6	0.3	2.1	0.4	1.3	0.1
Eukaryotic translation initiation factor 1 (Eif1)	Mm.13886	AW545196	2.5	0.2	1.7	0.1	1.9	0.2	1.2	0.0
Activating transcription factor 4 (Atf4)	Mm.641	AW550463	2.2	0.2	1.9	0.1	1.9	0.2	1.2	0.1
ATPase, class VI, type 11A (Atp11a)	Mm.257837	AU024208	2.1	0.2	1.6	0.2	1.7	0.2	1.4	0.1
Transcribed locus	Mm.188460	AW543519	2.1	0.2	1.6	0.1	1.8	0.2	1.1	0.1
RIKEN cDNA 1810013L24 gene (1810013L24Rik)	Mm.390868	AU024712	2.0	0.3	1.8	0.3	1.8	0.2	1.4	0.1
Ubiquitin-conjugating enzyme E2L 3 (Ube2l3)	Mm.3074	AW551421	2.0	0.3	2.0	0.2	2.0	0.2	0.9	0.1
DNA segment, Chr 3, University of California at Los Angeles 1 (D3Ucla1)	Mm.29702	AW539563	1.9	0.2	1.6	0.1	2.0	0.3	1.1	0.1
Tubulin, beta 2b (Tubb2b)	Mm.379227	AU020799	1.9	0.2	1.7	0.1	1.6	0.1	1.1	0.1
RIKEN cDNA 2610024B07 gene (2610024B07Rik)	Mm.24685	AW536239	1.7	0.2	1.6	0.0	1.6	0.1	1.3	0.1
Nuclear distribution gene E-like homolog 1 (A. nidulans) (Ndel1)	Mm.31979	AU042890	1.7	0.2	1.6	0.0	1.6	0.1	1.4	0.1
Pyruvate dehydrogenase kinase, isoenzyme 3 (Pdk3)	Mm.12775	AW556440	1.7	0.2	2.0	0.2	2.0	0.2	0.9	0.1
Tyrosine hydroxylase (Th)	Mm.1292	C85951	1.6	0.2	1.8	0.1	1.9	0.3	1.2	0.1
PACAP Forskolin NGF										
Arsenic (+3 oxidation state) methyltransferase (As3mt)	Mm.28566	AU020528	6.0	1.2	1.9	0.4	1.4	0.2	1.9	0.1
RIKEN cDNA 5830411E10 gene (5830411E10Rik)	Mm.196290	AU019102	2.4	0.3	1.7	0.3	1.4	0.2	1.7	0.2
Annexin A2 (Anxa2)	Mm.238343	AW551165	2.4	0.3	1.6	0.1	1.4	0.2	1.5	0.1
CAMP responsive element binding protein 3-like 2 (Creb3l2)	Mm.169929	AU042737	2.1	0.4	1.8	0.3	1.4	0.3	1.7	0.2
PACAP Forskolin										
Sex comb on midleg homolog 1 (Scmh1)	Mm.388903	C86855	5.0	0.5	2.2	0.5	2.1	0.2	1.1	0.1
Plasminogen activator, tissue (Plat)	Mm.154660	AU020998	4.1	1.1	3.2	0.9	2.0	0.5	1.2	0.1
Hect domain and RLD 4 (Herc4)	Mm.234437	AW553563	4.0	0.7	1.8	0.3	1.5	0.1	1.0	0.1
LIM domain only 1 (Lmo1)	Mm.360145	AU015284	3.3	0.7	1.7	0.4	1.3	0.1	1.2	0.1
EH-domain containing 4 (Ehd4)	Mm.132226	AU044505	3.0	0.3	1.7	0.2	1.5	0.2	1.4	0.1
Heat shock protein 8 (Hspb8)	Mm.21549	AU018999	2.5	0.3	1.6	0.1	1.5	0.1	1.2	0.1
RIKEN cDNA 2410019A14 gene	Mm.24586	AW555539	2.4	0.3	1.8	0.2	1.5	0.2	1.0	0.1
Inhibitor of DNA binding 2 (Id2)	Mm.34871	AW548400	2.2	0.2	1.6	0.0	1.5	0.1	1.4	0.1
Heat shock 70kD protein 5 (glucose-regulated protein) (Hspa5)	Mm.330160	AW555441	1.9	0.4	1.6	0.1	1.6	0.2	0.9	0.1
PACAP dbcAMP										
Cytoplasmic polyadenylation element binding protein 4 (Cpeb4)	Mm.339792	AU015651	1.8	0.3	1.4	0.1	1.8	0.1	1.1	0.2
Mitogen-activated protein kinase 6 (Mapk6)	Mm.18856	AU042016	1.6	0.1	1.4	0.0	1.6	0.2	1.2	0.1
3-monooxygenase/w 5-monooxygenase activation protein, γ polypeptide (Ywhag)	Mm.233813	AU014738	1.5	0.2	1.3	0.1	1.7	0.2	1.0	0.1
Forskolin dbcAMP										
Amylo-1,6-glucosidase, 4-alpha-glucanotransferase (Agl)	Mm.237099	C77182	1.5	0.1	2.8	0.4	2.3	0.6	0.9	0.1
Cysteine and glycine-rich protein 1 (Csrp1)	Mm.196484	AA408841	2.7	0.4	2.7	0.1	2.9	0.3	1.0	0.1
Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2 (Tanc2)	Mm.22501	AU022723	1.7	0.3	2.3	0.3	2.5	0.4	1.2	0.1
RIKEN cDNA 2810407C02 gene (2810407C02Rik)	Mm.270950	AW537092	1.7	0.2	2.0	0.1	1.6	0.2	1.0	0.1
GATA binding protein 2 (Gata2)	Mm.272747	AW538547	1.0	0.1	1.9	0.1	1.9	0.2	0.9	0.1
Nucleophosmin 1 (Npm1)	Mm.6343	AW553526	1.5	0.2	1.9	0.1	1.8	0.2	0.9	0.1
Growth factor receptor bound protein 10 (Grb10)	Mm.273117	AW556824	1.2	0.1	1.7	0.1	1.9	0.2	0.9	0.1
Solute carrier family 31, member 1 (Slc31a1)	Mm.248637	AU016967	1.4	0.1	1.6	0.1	1.7	0.2	0.7	0.1
PACAP NGF										
Serine (or cysteine) peptidase inhibitor, clade B, member 1a (Serpinb1a)	Mm.20144	AW549049	5.6	1.5	1.2	0.2	1.0	0.1	2.6	0.7
Activated leukocyte cell adhesion molecule (Alcam)	Mm.288282	AW549010	3.2	0.5	1.5	0.2	1.3	0.2	1.8	0.1
Aldo-keto reductase family 1, member B3 (aldose reductase) (Akr1b3)	Mm.451	AW550812	2.4	0.2	1.4	0.1	1.2	0.1	1.7	0.1
P450 (cytochrome) oxidoreductase (Por)	Mm.3863	AU016777	2.0	0.3	1.5	0.1	1.4	0.1	1.5	0.2
Adenomatosis polyposis coli (Apc)	Mm.7883	AW550666	2.0	0.2	1.4	0.2	1.4	0.2	1.8	0.2
Methionine-tRNA synthetase 2 (mitochondrial) (Mars2)	Mm.19223	AW539214	1.9	0.1	1.4	0.1	1.5	0.2	1.5	0.1
Myeloid cell leukemia sequence 1 (Mcl1)	Mm.1639	C81342	1.8	0.2	1.3	0.1	1.5	0.2	1.6	0.2
Expressed sequence AU045404 (AU045404)	Mm.17853	AU045358	1.7	0.1	1.2	0.1	1.2	0.1	4.4	0.5
Plectin 1 (Plec1)	Mm.234912	AU042599	1.7	0.1	1.2	0.1	1.3	0.1	1.6	0.1
Aryl hydrocarbon receptor nuclear translocator-like (Arntl)	Mm.12177	AU045956	1.6	0.2	1.2	0.1	1.3	0.1	1.6	0.1
Palladin, cytoskeletal associated protein (Palld)	Mm.29933	AU045282	1.6	0.1	1.0	0.1	1.1	0.1	2.6	0.3
Tax1 (human T-cell leukemia virus type I) binding protein 3 (Tax1bp3)	Mm.371656	AW544713	1.6	0.1	1.3	0.1	1.2	0.1	1.7	0.1
Forskolin NGF										
Zinc finger, CCHC domain containing 18 (Zcchc18)	Mm.23671	615116.00	1.8	0.1	2.2	0.1	1.3	0.3	2.0	0.2

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B

Gene Name	Ug Nb	GB ID	PACAP		Forskolin		dbcAMP		NGF	
			Av	SEM	Av	SEM	Av	SEM	Av	SEM
PACAP										
Kruppel-like factor 4 (gut)	Mm.4325	AU018863	3.0	0.7	1.3	0.3	1.4	0.2	1.1	0.1
Lectin, galactose binding, soluble 3 (Lgals3)	Mm.248615	AW543680	3.0	0.4	1.3	0.0	1.3	0.2	1.3	0.1
Olfactomedin-like 3 (Olfml3)	Mm.211535	AW550633	2.8	0.6	1.6	0.3	1.1	0.2	1.7	0.3
Protein phosphatase 2, regulatory subunit B (PR 52), β isoform (Ppp2r2b)	Mm.26134	AU018728	2.7	0.6	1.2	0.2	1.1	0.3	1.5	0.1
Solute carrier family 7, member 8 (Slc7a8)	Mm.276831	AU022525	2.6	0.3	1.7	0.2	2.5	1.1	1.0	0.1
AXIN1 up-regulated 1 (Axud1)	Mm.125196	AU015509	2.4	0.5	1.2	0.2	1.2	0.1	1.3	0.1
Poliovirus receptor (Pvr)	Mm.227506	C86693	2.2	0.4	1.4	0.3	1.1	0.1	1.3	0.1
Eukaryotic translation initiation factor 2C, 2 (Eif2c2)	Mm.274482	AW554903	2.2	0.3	1.3	0.1	1.5	0.2	0.9	0.1
Mannoside acetylglucosaminyltransferase 1 (Mgat1)	Mm.196933	AW550213	2.1	0.5	1.4	0.1	1.3	0.1	1.0	0.0
Laminin, alpha 5 (Lama5)	Mm.4339	AA408762	2.1	0.2	1.6	0.4	1.4	0.2	1.5	0.3
Diacylglycerol O-acyltransferase 2 (Dgat2)	Mm.180189	AU043052	2.1	0.3	1.3	0.2	1.2	0.2	1.3	0.1
WNK lysine deficient protein kinase 1 (Wnk1)	Mm.391663	C81474	2.1	0.2	1.1	0.1	1.1	0.1	1.3	0.1
Adaptor-related protein complex 1, sigma 2 subunit (Ap1s2)	Mm.146736	AU040383	2.1	0.2	1.5	0.1	1.4	0.2	1.2	0.1
Elongation factor RNA polymerase II 2 (Ell2)	Mm.21288	AU042469	2.0	0.2	1.4	0.1	1.5	0.1	1.2	0.1
Fos-like antigen 2 (Fosl2)	Mm.24684	AU042525	2.0	0.3	1.1	0.1	1.2	0.1	1.4	0.3
ATPase, class VI, type 11A (Atp11a)	Mm.392605	AU040689	2.0	0.3	1.4	0.1	1.9	0.6	1.3	0.1
Serine/threonine kinase 40 (Stk40)	Mm.41865	AU015210	2.0	0.2	1.2	0.1	1.4	0.2	1.4	0.1
Aldo-keto reductase family 1, member B8 (Akr1b8)	Mm.5378	C77965	2.0	0.4	1.2	0.1	1.0	0.1	1.7	0.2
Testis derived transcript (Tes)	Mm.389083	C81197	1.9	0.2	1.4	0.2	1.1	0.2	1.2	0.1
UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 3 (B4galt3)	Mm.274011	AW555479	1.9	0.3	1.5	0.1	1.3	0.3	1.1	0.2
Adenomatosis polyposis coli (Apc)	Mm.7883	AU014897	1.9	0.3	1.1	0.1	1.6	0.2	1.4	0.2
Actin related protein 2/3 complex, subunit 3 (Arpc3)	Mm.275942	AW546733	1.9	0.2	1.4	0.1	1.2	0.2	1.1	0.1
Heat shock protein 1 (Hspb1)	Mm.13849	AU021579	1.9	0.2	1.2	0.1	1.2	0.1	1.0	0.1
Poliovirus receptor-related 1 (Pvrl1)	Mm.335096	AW549174	1.9	0.3	1.3	0.2	1.4	0.2	1.7	0.3
Protein C receptor, endothelial (Procr)	Mm.3243	AW545622	1.9	0.3	1.1	0.1	1.2	0.1	1.1	0.0
Phosphoserine phosphatase (Psph)	Mm.271784	AW554246	1.8	0.2	1.3	0.1	1.2	0.2	1.3	0.1
Inhibitor of DNA binding 3 (Id3)	Mm.110	AW557873	1.8	0.2	1.6	0.2	1.3	0.1	1.2	0.1
expressed sequence AW552058	Mm.198448	AW552058	1.8	0.2	1.2	0.1	1.4	0.2	1.3	0.1
ubiquitin-like 3	Mm.21846	AU042224	1.8	0.2	1.3	0.1	1.2	0.1	1.0	0.1
prostaglandin E synthase	Mm.28768	C81414	1.8	0.4	1.2	0.3	0.9	0.1	1.4	0.3
Werner helicase interacting protein 1 (Wrnip1)	Mm.286680	AU040729	1.7	0.2	1.1	0.1	1.0	0.2	1.5	0.1
Cd63 antigen (Cd63)	Mm.371552	AU020673	1.7	0.2	1.2	0.1	1.2	0.1	1.2	0.0
CCAAT/enhancer binding protein (C/EBP), gamma (Cebpg)	Mm.273090	AU022405	1.7	0.2	1.0	0.2	1.2	0.1	0.7	0.1
Tropomyosin 4 (Tpm4)	Mm.295124	AW537534	1.7	0.3	1.5	0.1	1.6	0.2	1.2	0.1
RIKEN cDNA 2310051F07 gene (2310051F07Rik)	Mm.391971	AW546247	1.7	0.2	1.2	0.0	1.4	0.2	1.1	0.1
Quininoid dihydropteridine reductase (Qdpr)	Mm.30204	AU023976	1.7	0.2	1.3	0.1	1.1	0.1	0.9	0.0
TSC22 domain family, member 1 (Tsc22d1)	Mm.153272	AU040743	1.7	0.3	0.9	0.0	0.9	0.1	0.8	0.1
Fibronectin type III domain containing 3a (Fndc3a)	Mm.205421	AA407010	1.7	0.2	1.1	0.2	1.3	0.1	1.4	0.2
Zinc finger, CSL domain containing 2 (Zcsl2)	Mm.2519	AU019309	1.7	0.2	1.1	0.0	1.1	0.1	1.1	0.0
Golgi reassembly stacking protein 2 (Gorasp2)	Mm.271950	AW551843	1.6	0.3	1.3	0.0	1.3	0.1	1.1	0.1
Glutaredoxin (Glrx)	Mm.25844	AW537328	1.6	0.2	1.5	0.3	1.4	0.2	1.3	0.2
ADP-ribosylation factor 4 (Arf4)	Mm.297768	AW554150	1.6	0.1	1.4	0.1	1.5	0.2	1.1	0.1
Ubiquitin-conjugating enzyme E2, J1 (Ube2j1)	Mm.259095	AW554710	1.6	0.2	1.4	0.1	1.3	0.2	1.3	0.1
Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform (Ppp2ca)	Mm.260288	AU018631	1.6	0.2	1.4	0.0	1.3	0.1	1.1	0.1
Rap guanine nucleotide exchange factor (GEF) 5 (Rapgef5)	Mm.227642	AU023974	1.6	0.2	1.0	0.1	1.2	0.1	0.8	0.1
Intraflagellar transport 20 homolog (Chlamydomonas) (Ift20)	Mm.358671	AU015496	1.6	0.2	1.1	0.1	1.1	0.1	1.3	0.1
Glutamyl-prolyl-tRNA synthetase (Eprs)	Mm.154511	AW557843	1.6	0.2	1.5	0.1	1.5	0.1	1.2	0.1
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked (Ddx3x)	Mm.289662	AW544374	1.6	0.1	1.4	0.1	1.4	0.1	1.3	0.1
Eukaryotic translation initiation factor 4E (Eif4e)	Mm.3941	AU016482	1.6	0.2	1.1	0.1	1.1	0.1	1.3	0.1
Cleavage stimulation factor, 3' pre-RNA, subunit 3 (Cstf3)	Mm.259876	C79534	1.6	0.2	1.4	0.1	1.3	0.1	1.2	0.1
Transmembrane protein 49 (Tmem49)	Mm.390398	C87007	1.6	0.2	1.2	0.0	1.2	0.1	1.3	0.1
Sirtuin 1 (silent mating type information regulation 2, homolog) 1 (Sirt1)	Mm.351459	AW548525	1.6	0.1	1.2	0.1	1.3	0.2	1.2	0.2
Solute carrier family 38, member 1 (Slc38a1)	Mm.103568	AW552655	1.6	0.2	1.3	0.2	1.5	0.2	1.0	0.1
Zinc finger protein 207 (Zfp207)	Mm.102253	AW553254	1.6	0.2	1.1	0.1	1.2	0.1	1.2	0.1
Pituitary tumor-transforming 1 interacting protein (Pttg1ip)	Mm.28853	AW546604	1.6	0.2	1.0	0.0	1.2	0.1	1.1	0.1
Eukaryotic translation initiation factor 1A (Eif1a)	Mm.262037	AW536208	1.6	0.3	1.0	0.1	1.2	0.1	1.2	0.1
Methylenetetrahydrofolate dehydrogenase and cyclohydrolase (Mthfd2)	Mm.443	AW558851	1.6	0.1	1.3	0.2	1.2	0.1	1.2	0.1
Ubiquitin specific peptidase 54 (Usp54)	Mm.385812	C87394	1.6	0.2	0.9	0.0	1.0	0.1	0.8	0.1
WNK lysine deficient protein kinase 1 (Wnk1)	Mm.333349	AU015196	1.5	0.1	1.1	0.0	1.1	0.2	1.2	0.1
Ninjurin 1 (Ninj1)	Mm.18503	AU024536	1.5	0.1	1.3	0.1	1.4	0.1	1.0	0.1
RIKEN cDNA 4930422I07 gene (4930422I07Rik)	Mm.259988	AU022442	1.5	0.2	1.2	0.1	1.4	0.2	1.2	0.2
Thioredoxin-like 1 (Txnl1)	Mm.19169	AU045102	1.5	0.2	1.2	0.0	1.3	0.2	1.1	0.1
Eukaryotic translation elongation factor 1 epsilon 1 (Eef1e1)	Mm.36683	AU043784	1.5	0.1	1.2	0.1	1.2	0.1	1.3	0.1

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Gene Name	Ug Nb	GB ID	PACAP		Forskolin		dbcAMP		NGF	
			Av	SEM	Av	SEM	Av	SEM	Av	SEM
Forskolin										
Ferric-chelate reductase 1 (Frrs1)	Mm.66293	C86591	1.7	0.3	2.9	1.1	2.2	0.7	1.2	0.2
Bone morphogenetic protein 6 (Bmp6)	Mm.28622	C76305	3.1	0.7	2.6	0.4	2.1	0.2	1.2	0.1
Chromogranin B (Chgb)	Mm.255241	367410.00	1.2	0.3	2.6	0.3	2.2	0.2	0.8	0.1
Peptidylglycine alpha-amidating monooxygenase (Pam)	Mm.5121	482270.00	1.2	0.4	2.2	0.3	1.9	0.3	0.7	0.1
Fibrinogen, B beta polypeptide (Fgb)	Mm.30063	552321.00	0.9	0.0	2.0	0.1	1.4	0.2	0.8	0.0
Methionine adenosyltransferase II, alpha (Mat2a)	Mm.29815	AW542928	1.6	0.3	2.0	0.2	2.1	0.3	0.9	0.1
Leucine rich repeat containing 3 (Lrrc3)	Mm.133301	316779.00	0.8	0.1	1.9	0.1	1.5	0.3	0.9	0.0
X-box binding protein 1 (Xbp1)	Mm.22718	AU040737	1.9	0.2	1.9	0.1	1.7	0.2	1.4	0.1
Gene trap ROSA b-geo 22 (Gtrgeo22)	Mm.22632	AW544177	1.6	0.2	1.8	0.0	1.9	0.1	1.1	0.1
Heat shock 70kD protein 5 (glucose-regulated protein) (Hspa5)	Mm.330160	AW537792	1.5	0.1	1.7	0.2	1.4	0.2	0.9	0.1
Frizzled homolog 7 (Drosophila) (Fzd7)	Mm.297906	AW537516	1.0	0.1	1.7	0.3	1.4	0.2	1.0	0.1
13 days embryo heart cDNA, RIKEN full-length enriched library, clone:D330042P15	Mm.417626	AU020988	1.2	0.1	1.6	0.3	1.3	0.2	1.1	0.1
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1 (B3gnt1)	Mm.258094	AU024115	1.3	0.1	1.6	0.0	1.4	0.1	1.0	0.1
RIKEN cDNA 9630050M13 gene (9630050M13Rik)	Mm.23044	AA409679	1.3	0.1	1.5	0.1	1.6	0.2	1.0	0.1
Transmembrane protein 16F (Tmem16f)	Mm.38087	AW553814	1.3	0.2	1.5	0.1	1.5	0.3	0.8	0.1
dbcAMP										
Ras homolog gene family, member Q (Rhoq)	Rn.4169	AW557645	1.4	0.2	1.4	0.1	1.9	0.2	1.1	0.1
Homer homolog 2 (Drosophila) (Homer2)	Mm.228	AA407944	1.486	0.242	1.48	0.1	1.692	0.161	1.346	0.124
RAB6, member RAS oncogene family (Rab6)	Mm.28650	AW552337	1.5	0.3	1.3	0.1	1.7	0.2	1.2	0.2
Protein tyrosine phosphatase, receptor type, F (Ptprf)	Mm.29855	AW548091	1.2	0.2	1.3	0.1	1.6	0.2	0.9	0.1
RIKEN cDNA 1700020D05 gene (1700020D05Rik)	Mm.20071	AW558842	1.5	0.6	1.4	0.3	1.6	0.2	0.9	0.1
Phosphoribosyl pyrophosphate amidotransferase (Ppat)	Mm.202337	AA408689	1.0	0.1	1.6	0.1	1.6	0.2	0.9	0.1
CAMP-regulated phosphoprotein 19 (Arpp19)	Mm.247837	AW559096	1.4	0.1	1.4	0.1	1.6	0.1	0.9	0.1
Single-stranded DNA binding protein 3 (Ssbp3)	Mm.195635	AW551939	1.3	0.1	1.3	0.1	1.6	0.1	1.1	0.1
Protein kinase, cAMP dependent regulatory, type I, alpha (Prkar1a)	Mm.30039	AW555666	1.5	0.2	1.5	0.1	1.6	0.1	1.2	0.1
T 3-monooxygenase/w 5-monooxygenase activation protein, z polypeptide (Ywhaz)	Mm.3360	AW544726	1.2	0.1	1.3	0.1	1.5	0.1	1.1	0.1

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Gene Name	Ug Nb	GB ID	PACAP		Forskolin		dbcAMP		NGF	
			Av	SEM	Av	SEM	Av	SEM	Av	SEM
NGF										
RIKEN cDNA G431001109 gene (G431001109Rik)	Mm.181490	AW545135	1.4	0.2	1.0	0.1	1.1	0.1	2.7	0.4
Tumor necrosis factor receptor superfamily, member 12a (Tnfrsf12a)	Mm.28518	C87282	1.7	0.2	1.2	0.1	1.0	0.1	2.4	0.4
Early growth response 1 (Egr1)	Mm.181959	AU017579	1.9	0.5	1.0	0.1	1.1	0.1	2.4	0.7
Kinesin family member 22 (Kif22)	Mm.286488	C81217	1.1	0.3	0.9	0.1	1.4	0.4	2.4	0.9
Keratin complex 1, acidic, gene 18 (Krt1-18)	Mm.22479	AW538107	1.4	0.1	1.1	0.1	1.3	0.1	2.4	0.2
Transient receptor potential cation channel, subfamily V, member 2 (Trpv2)	Mm.288064	AW544883	1.3	0.2	1.3	0.1	1.2	0.1	2.0	0.4
Keratin complex 2, basic, gene 8 (Krt2-8)	Mm.358618	AW542449	1.2	0.3	1.1	0.1	1.2	0.1	2.0	0.2
Syntaxin binding protein 4 (Stxbp4)	Mm.390411	AW555680	1.1	0.2	1.0	0.1	1.1	0.3	2.0	0.6
Solute carrier organic anion transporter family, member 3a1 (Slco3a1)	Mm.268798	C85055	1.0	0.2	1.1	0.1	1.4	0.1	2.0	0.2
RIKEN cDNA 9030612M13 gene (9030612M13Rik)	Mm.38813	AU040655	1.3	0.3	0.9	0.1	1.3	0.2	1.9	0.4
ATP-binding cassette, sub-family D (ALD), member 3 (Abcd3)	Mm.194462	AU040952	1.3	0.2	0.9	0.1	1.2	0.2	1.9	0.4
Adenylate cyclase activating polypeptide 1 receptor 1 (Pac-1)	Mm.44245	AW547403	1.0	0.1	0.9	0.1	1.1	0.1	1.8	0.3
Arginine-tRNA-protein transferase 1 (Ate1)	Mm.216321	585553.00	1.5	0.1	1.0	0.0	1.0	0.0	1.8	0.2
Cyclin T2 (Ccnt2)	Mm.392269	C81304	1.2	0.3	0.9	0.1	1.3	0.3	1.8	0.7
Adult male diencephalon cDNA, RIKEN full-length enriched library, clone:9330102H12	Mm.117788	C81245	1.3	0.3	1.1	0.2	1.3	0.3	1.8	0.3
Olfactory receptor 16 (Olfr16)	Mm.377103	C81149	1.2	0.3	1.0	0.1	1.3	0.3	1.8	0.3
Activating signal cointegrator 1 complex subunit 3 (Ascc3)	Mm.222497	AW555280	1.7	0.3	1.1	0.1	1.0	0.1	1.8	0.2
UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4 (B4galt4)	Mm.182377	AW555507	2.1	0.5	1.7	0.7	1.1	0.2	1.8	0.3
Methyl-CpG binding domain protein 5 (Mbd5)	Mm.235423	AW555677	1.3	0.2	1.0	0.1	1.2	0.1	1.8	0.3
EH-domain containing 2 (Ehd2)	Mm.42135	AW557507	1.4	0.3	1.0	0.1	1.3	0.3	1.8	0.3
RIKEN cDNA 1700020O03 gene (1700020O03Rik)	Mm.252967	C78682	1.1	0.2	0.9	0.0	1.3	0.2	1.8	0.3
Synaptonemal complex protein 3 (Sycp3)	Mm.297977	AW558202	1.4	0.2	1.3	0.3	1.2	0.2	1.8	0.2
UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1 (B4galt1)	Mm.15622	AU042201	1.2	0.2	1.0	0.1	1.3	0.2	1.8	0.3
Suppressor of cytokine signaling 5 (Socs5)	Mm.126885	584582.00	1.3	0.1	1.0	0.2	0.9	0.1	1.7	0.1
NACHT, LRR and PYD containing protein 9a (Nalp9a)	Mm.11889	AU022787	1.3	0.3	0.9	0.1	1.0	0.2	1.7	0.3
A disintegrin and metallopeptidase domain 10 (Adam10)	Mm.3037	AW552781	2.1	0.8	0.9	0.1	0.9	0.3	1.7	0.3
Actinin, alpha 1 (Actn1)	Mm.253564	C77473	1.1	0.1	1.0	0.1	1.1	0.1	1.7	0.1
Aldo-keto reductase family 1, member B8 (Akr1b8)	Mm.5378	C77965	2.0	0.4	1.2	0.1	1.0	0.1	1.7	0.2
Mitochondrial ribosomal protein L1 (Mrpl1)	Mm.295499	AW549179	1.4	0.2	1.2	0.1	1.3	0.2	1.7	0.4
NACHT, leucine rich repeat and PYD containing 4E (Nalp4e)	Mm.289759	AW537584	1.1	0.2	1.0	0.1	0.9	0.1	1.7	0.3
BAT2 domain containing 1 (Bat2d)	Mm.245446	AW549561	1.3	0.2	1.0	0.1	1.1	0.2	1.7	0.3
Zinc finger protein 114 (Zfp114)	Mm.246600	AU015230	1.3	0.2	1.0	0.1	1.0	0.3	1.7	0.2
Vinculin (Vcl)	Mm.279361	AW538732	0.9	0.1	0.9	0.1	1.1	0.1	1.7	0.1
Cysteine rich transmembrane BMP regulator 1 (chordin like) (Crim1)	Mm.311912	AU021760	1.1	0.2	1.0	0.1	1.2	0.1	1.7	0.3
G protein-coupled receptor kinase-interactor 2 (Git2)	Mm.195632	AA408072	1.0	0.1	1.0	0.1	1.3	0.2	1.7	0.4
Histidine decarboxylase (Hdc)	Mm.18603	AU042518	1.2	0.2	1.0	0.1	1.4	0.3	1.7	0.3
Prolactin-like protein E (Prlpe)	Mm.196424	AW538311	1.0	0.1	1.2	0.2	1.1	0.2	1.7	0.3
Progestin and adipoQ receptor family member V (Paqr5)	Mm.273267	AU040653	1.3	0.2	1.0	0.1	1.3	0.3	1.7	0.3
T-cell immunoglobulin and mucin domain containing 2 (Timd2)	Mm.234654	AU018412	1.4	0.3	1.0	0.1	1.3	0.2	1.7	0.4
RIKEN cDNA 4921517N04 gene (4921517N04Rik)	Mm.276415	AU014935	1.0	0.1	0.9	0.1	1.1	0.1	1.7	0.3
Solute carrier family 38, member 5 (Slc38a5)	Mm.6055	C81234	1.1	0.2	1.1	0.2	1.2	0.2	1.7	0.4
Phosphatidylinositol 4-kinase, catalytic, beta polypeptide (Pik4cb)	Mm.217222	AW550264	1.7	0.4	1.1	0.1	1.2	0.2	1.7	0.2
Glutathione synthetase (Gss)	Mm.252316	C81602	45.4	44.2	1.0	0.1	1.2	0.2	1.7	0.2
Elongation factor Tu GTP binding domain containing 1 (Eftud1)	Mm.238020	AU022896	1.2	0.2	1.0	0.1	1.2	0.1	1.7	0.2
TBC1 domain family, member 1 (Tbc1d1)	Mm.286353	AW555803	1.3	0.3	1.0	0.1	1.2	0.3	1.7	0.3
Restin-like 2 (Rsnl2)	Mm.196382	AU015231	1.3	0.2	0.9	0.1	1.2	0.3	1.7	0.3
DEAH (Asp-Glu-Ala-His) box polypeptide 40 (Dhx40)	Mm.260627	AW559143	1.5	0.4	1.2	0.2	1.3	0.2	1.7	0.2
Integrin alpha 3 (Itga3)	Mm.57035	AW553717	1.0	0.1	1.0	0.0	1.2	0.1	1.6	0.1
Fyn-related kinase (Frk)	Mm.332432	C85044	1.1	0.3	1.0	0.1	1.2	0.3	1.6	0.3
Pyrroline-5-carboxylate reductase 1 (Pycr1)	Mm.127731	AU015581	1.3	0.2	1.0	0.1	1.2	0.1	1.6	0.3
Phosphoribosyl pyrophosphate synthetase-associated protein 1 (Prpsap1)	Mm.25125	C85968	1.4	0.2	1.1	0.1	1.3	0.2	1.6	0.3
Periplakin (Ppl)	Mm.266875	AW553870	1.3	0.1	1.2	0.1	1.1	0.1	1.6	0.2
Beta-site APP cleaving enzyme 1 (Bace1)	Mm.220945	AU023315	1.0	0.2	1.0	0.1	1.3	0.5	1.6	0.2
RIKEN cDNA E330013P04 gene (E330013P04Rik)	Mm.245813	AU014638	1.4	0.2	1.0	0.1	1.2	0.2	1.6	0.2
Estrogen receptor 1 (alpha) (Esr1)	Mm.9213	AU018232	1.0	0.1	0.9	0.0	1.1	0.1	1.6	0.3
Deltex 3-like (Drosophila) (Dtx3l)	Mm.390852	AU042200	1.2	0.2	1.0	0.1	1.3	0.3	1.6	0.3
RIKEN cDNA 2810453I06 gene (2810453I06Rik)	Mm.383219	AU043832	1.3	0.2	0.9	0.1	1.2	0.1	1.6	0.2
Centaurin, gamma 2 (Centg2)	Mm.291135	AU017408	1.2	0.2	0.9	0.1	1.1	0.2	1.6	0.2
CD97 antigen (Cd97)	Mm.334648	420765.00	1.4	0.1	1.0	0.0	0.8	0.0	1.6	0.1
O-linked N-acetylglucosamine transferase (UDP-N-acetylglucosamine) (Ogt)	Mm.259191	C81495	1.2	0.3	1.0	0.1	1.4	0.4	1.6	0.4
RIKEN cDNA 4921524J06 gene (4921524J06Rik)	Mm.35296	C86231	1.4	0.3	1.2	0.2	1.2	0.3	1.6	0.2
Tribbles homolog 1 (Drosophila) (Trib1)	Mm.40298	AW548903	1.5	0.2	1.1	0.1	1.1	0.1	1.6	0.3
RIKEN cDNA 2810474O19 gene (2810474O19Rik)	Mm.333515	AU018783	2.0	0.4	1.2	0.1	1.2	0.3	1.6	0.2
Zinc finger protein 444 (Zfp444)	Mm.274089	AW555678	1.2	0.2	1.0	0.1	1.3	0.3	1.6	0.5
Zyxin (Zyx)	Mm.282303	AW555565	1.7	0.2	1.1	0.1	1.1	0.1	1.6	0.2
Nudix (nucleoside diphosphate linked moiety X)-type motif 19 (Nudt19)	Mm.358820	AU016790	1.4	0.2	1.0	0.1	1.0	0.2	1.6	0.2
Neuropilin 1 (Nrp1)	Mm.271745	AW549864	1.3	0.2	0.9	0.1	1.0	0.1	1.6	0.1
RIKEN cDNA 4933437F05 gene (4933437F05Rik)	Mm.79198	AA408564	1.2	0.2	1.0	0.1	1.3	0.1	1.6	0.3
Solute carrier organic anion transporter family, member 6c1 (Slco6c1)	Mm.60362	C79179	1.5	0.4	0.9	0.1	1.0	0.1	1.6	0.3
5'-nucleotidase, cytosolic II (Nt5c2)	Mm.248652	AU041566	1.4	0.3	0.9	0.1	0.9	0.2	1.6	0.2
TROVE domain family, member 2 (Trove2)	Mm.40370	AU041284	1.4	0.2	1.0	0.1	1.1	0.2	1.6	0.2
Oxoglutarate (alpha-ketoglutarate) receptor 1 (Oxgr1)	Mm.138520	AW558733	1.2	0.2	1.0	0.1	1.3	0.3	1.6	0.3
Dual specificity phosphatase 16 (Dusp16)	Mm.3994	AW551732	1.7	0.4	1.3	0.3	1.0	0.1	1.6	0.2
DNA segment, Chr 9, ERATO Doi 280, expressed (D9Ertd280e)	Mm.258310	C79755	1.2	0.3	0.9	0.1	0.9	0.1	1.6	0.2
Potassium voltage-gated channel, Shal-related family, member 2 (Kcnd2)	Mm.320691	AW554807	1.5	0.4	0.9	0.1	0.7	0.2	1.6	0.2
G protein-coupled receptor 1 (Gpr1)	Mm.103354	AU024461	1.2	0.2	1.0	0.1	1.2	0.2	1.6	0.2
Septin 7 (Sept7)	Mm.270259	C81520	1.1	0.2	1.0	0.1	1.1	0.2	1.6	0.3
Glial cell line derived neurotrophic factor family receptor alpha 1 (Gfra1)	Mm.88367	AU042498	1.0	0.1	1.0	0.1	1.3	0.2	1.6	0.2
Excision repair cross-complementing rodent repair deficiency, group 1 (Ercc1)	Mm.280913	AW544260	1.4	0.1	1.0	0.1	1.0	0.1	1.6	0.1
Exostoses (multiple) 1 (Ext1)	Mm.309395	AA407088	1.2	0.2	1.0	0.1	1.3	0.2	1.6	0.3
Transmembrane protein 64 (Tmem64)	Mm.38877	AW555938	3.5	2.7	1.0	0.1	1.0	0.1	1.6	0.1
Beta-1,4-N-acetyl-galactosaminyl transferase 2 (B4galnt2)	Mm.340702	AU040657	1.2	0.2	1.0	0.1	1.3	0.3	1.6	0.3
H19 fetal liver mRNA (H19)	Mm.14802	AW555056	1.5	0.2	1.2	0.1	1.3	0.1	1.6	0.1
ATPase type 13A5 (Atp13a5)	Mm.9823	C88250	1.4	0.2	1.0	0.1	1.2	0.3	1.6	0.2
Solute carrier family 35 (CMP-sialic acid transporter), member 1 (Slc35a1)	Mm.281885	AW539587	1.1	0.2	1.4	0.5	1.2	0.3	1.5	0.3
Synaptonemal complex protein 1 (Sycp1)	Mm.243849	AW557607	1.6	0.4	1.0	0.1	0.9	0.1	1.5	0.2
GATA binding protein 3 (Gata3)	Mm.313866	C81309	1.1	0.2	1.1	0.1	1.3	0.1	1.5	0.1
RIKEN cDNA C130037N17 gene (C130037N17Rik)	Mm.321371	AU041266	1.6	0.4	1.0	0.1	0.9	0.1	1.5	0.2
RIKEN cDNA 4631427C17 gene (4631427C17Rik)	Mm.210899	AU041120	2.2	0.7	0.9	0.1	1.0	0.1	1.5	0.2
Prefoldin 4 (Pfdn4)	Mm.28808	AU021910	1.5	0.2	1.2	0.1	1.2	0.2	1.5	0.1
GTPase activating RANGAP domain-like 1 (Garnl1)	Mm.292180	AW557194	1.5	0.3	1.1	0.1	1.2	0.1	1.5	0.2
Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2 (Slc7a2)	Mm.4676	C85183	1.1	0.3	1.0	0.1	1.0	0.2	1.5	0.3
Phosphorylase kinase alpha 2 (Phka2)	Mm.350712	AW558022	1.1	0.1	1.1	0.1	1.0	0.2	1.5	0.2
Yamaguchi sarcoma viral (v-yes) oncogene homolog 1 (Yes1)	Mm.4558	AW555652	1.8	0.4	1.1	0.1	1.7	0.7	1.5	0.2
Ubiquitin specific peptidase 47 (Usp47)	Mm.16974	AU022065	1.2	0.2	1.0	0.1	1.2	0.2	1.5	0.2
RIKEN cDNA D530005L17 gene (D530005L17Rik)	Mm.235934	AW558478	1.6	0.3	0.9	0.1	1.0	0.2	1.5	0.2
Ataxin 1 (Atxn1)	Mm.342686	C85907	1.4	0.5	0.9	0.1	0.9	0.2	1.5	0.2
RIKEN cDNA E430024C06 gene (E430024C06Rik)	Mm.392675	AU023454	1.2	0.2	1.1	0.1	1.3	0.2	1.5	0.1
RIKEN cDNA D730003I15 gene (D730003I15Rik)	Mm.16927	AU042662	1.1	0.1	0.9	0.1	1.0	0.2	1.5	0.2
Poliovirus receptor-related 3 (Pvrl3)	Mm.328072	AU016832	1.1	0.2	1.2	0.2	1.4	0.1	1.5	0.1
Wilms tumor homolog (Wt1)	Mm.389339	AW554453	1.4	0.1	1.1	0.2	1.0	0.1	1.5	0.2
Nur77 downstream gene 1 (Ndg1)	Mm.26006	AU021983	1.2	0.2	0.9	0.1	1.3	0.1	1.5	0.2
Pinin (Pnn)	Mm.22347	AW538340	1.3	0.1	1.1	0.1	1.1	0.1	1.5	0.1
Ferritin light chain 1 (Ftl1)	Mm.28251	AW555646	1.5	0.3	1.1	0.1	1.2	0.2	1.5	0.2
RIKEN cDNA A530040E14 gene (A530040E14Rik)	Mm.360513	AU045208	1.4	0.2	1.0	0.0	1.3	0.2	1.5	0.2
Transcribed locus	Mm.382067	AU016609	1.2	0.2	0.9	0.1	1.1	0.2	1.5	0.3
expressed sequence AU022928	Mm.201282	AU022928	1.0	0.2	1.1	0.2	0.8	0.1	1.5	0.3
CDNA sequence BC016495 (BC016495)	Mm.211595	AA408837	1.1	0.2	0.9	0.1	1.1	0.1	1.5	0.1
Paternally expressed 10 (Peg10)	Mm.320575	AA408654	1.1	0.2	1.4	0.4	1.2	0.1	1.5	0.1
Microfibrillar-associated protein 1 (Mfap1)	Mm.270393	C78936	1.0	0.1	1.1	0.1	1.2	0.1	1.5	0.2
Zinc finger protein 291 (Zfp291)	Mm.86588	AW554071	1.5	0.3	1.0	0.1	1.2	0.1	1.5	0.2
RIKEN cDNA 2210408F21 gene (2210408F21Rik)	Mm.59134	AU042519	1.4	0.3	0.9	0.1	1.2	0.2	1.5	0.2
Phenylalanine-tRNA synthetase-like, beta subunit (Farslb)	Mm.389145	AU042241	1.0	0.2	0.9	0.1	0.8	0.1	1.5	0.2

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Table 3: Validation of microarray results by real time PCR.

	Control	SEM	PACAP	SEM	Forskolin	SEM	dbc	SEM	NGF	SEM
Gata2	**1.19**	0.08	**1.06**	0.11	**3.21**	0.32	**1.78**	0.41	**0.69**	0.05
Nrp1	**1.23**	0.25	**1.08**	0.13	**0.41**	0.00	**0.42**	0.03	**5.26**	0.62
Pac1	**1.03**	0.11	**1.23**	0.13	**0.77**	0.05	**0.66**	0.06	**7.20**	0.81
Anx2	**1.06**	0.05	**1.35**	0.16	**1.29**	0.04	**1.70**	0.19	**1.54**	0.37
Homer2	**1.08**	0.06	**2.18**	0.13	**2.08**	0.12	**1.10**	0.37	**1.56**	0.21
Por	**0.97**	0.06	**2.46**	0.28	**2.09**	0.10	**2.58**	0.25	**3.52**	0.56
Akr1b8	**1.06**	0.05	**2.73**	0.36	**1.80**	0.19	**4.06**	1.49	**3.31**	0.96
Vil2	**1.10**	0.11	**2.86**	0.26	**2.50**	0.09	**3.40**	0.47	**2.31**	0.26
Hspb8	**1.06**	0.05	**2.95**	0.16	**2.24**	0.10	**1.25**	0.42	**1.88**	0.19
Egr1	**1.29**	0.30	**4.04**	0.36	**2.30**	0.12	**4.22**	0.66	**48.60**	13.83
Glrx	**1.10**	0.06	**6.42**	0.33	**3.57**	0.22	**4.19**	0.26	**1.14**	0.08
Ptp4a1	**1.21**	0.16	**6.64**	0.45	**4.53**	0.15	**6.61**	1.78	**2.85**	0.39
Gas1	**0.87**	0.14	**7.45**	1.63	**6.51**	0.66	**8.62**	3.09	**0.57**	0.20
Azin	**1.11**	0.08	**11.44**	2.33	**8.69**	0.44	**3.93**	1.36	**1.37**	0.14
Odc	**1.01**	0.06	**12.76**	1.75	**5.69**	0.62	**7.98**	1.89	**7.19**	1.45
Ier3	**1.11**	0.07	**33.88**	3.58	**14.34**	1.02	**15.80**	1.21	**3.17**	0.77
Rgs2	**1.35**	0.10	**76.70**	12.83	**25.66**	3.10	**29.31**	4.61	**9.67**	1.24

Table indicates mRNA induction for 17 genes, found up-regulated by microarray, after a 6-h treatment with PACAP (10^{-7} M), forskolin (25 μM), dbcAMP (10^{-3} M) and/or NGF (100 ng/mL). Genes were classified in ascending order of regulation by PACAP obtained by real-time PCR. Gata2, *GATA binding protein 2*; Nrp1, *neuropilin 1*; Pac-1, *adenylate cyclase activating polypeptide 1 receptor 1*; Anxa2, *annexin A2;* Homer2, *homer homolog 2 (Drosophila)*; Por, *P450 (cytochrome) oxidoreductase*; Akr1b8, *aldo-keto reductase family 1, member B8*; Vil2, *villin 2;* Hspb8, *heat shock 22kDa protein 8*; Egr1, *early growth response 1*; Glrx, *glutaredoxin*; Ptp4a1, *protein tyrosine phosphatase 4a1*; Gas1, *growth arrest specific 1*; Azin1, *antizyme inhibitor 1*; Odc1, *ornithine decarboxylase, structural 1*; Ier3, *immediate early response 3*; Rgs2, *regulator of G-protein signaling 2*.

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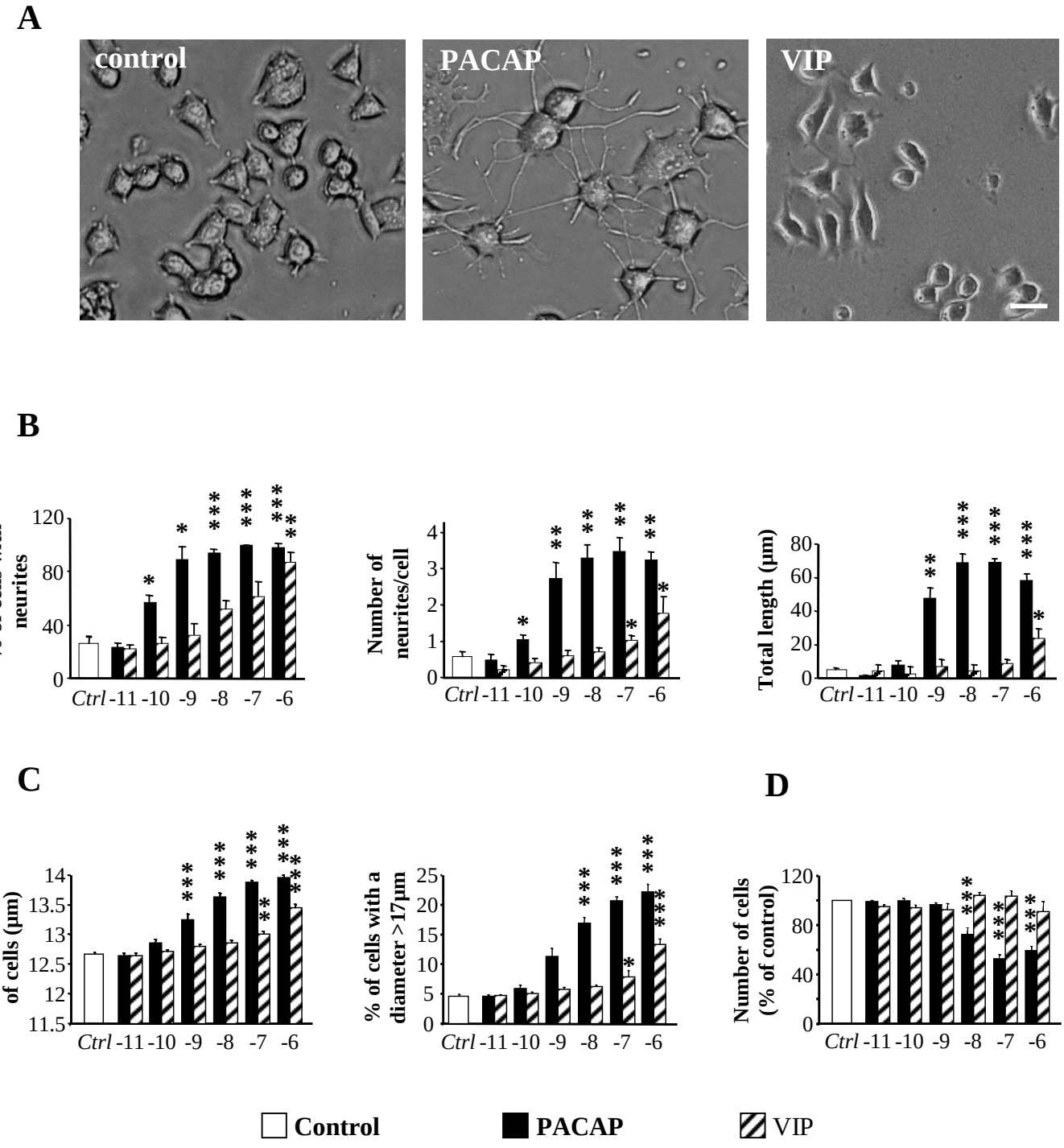

Figure 1

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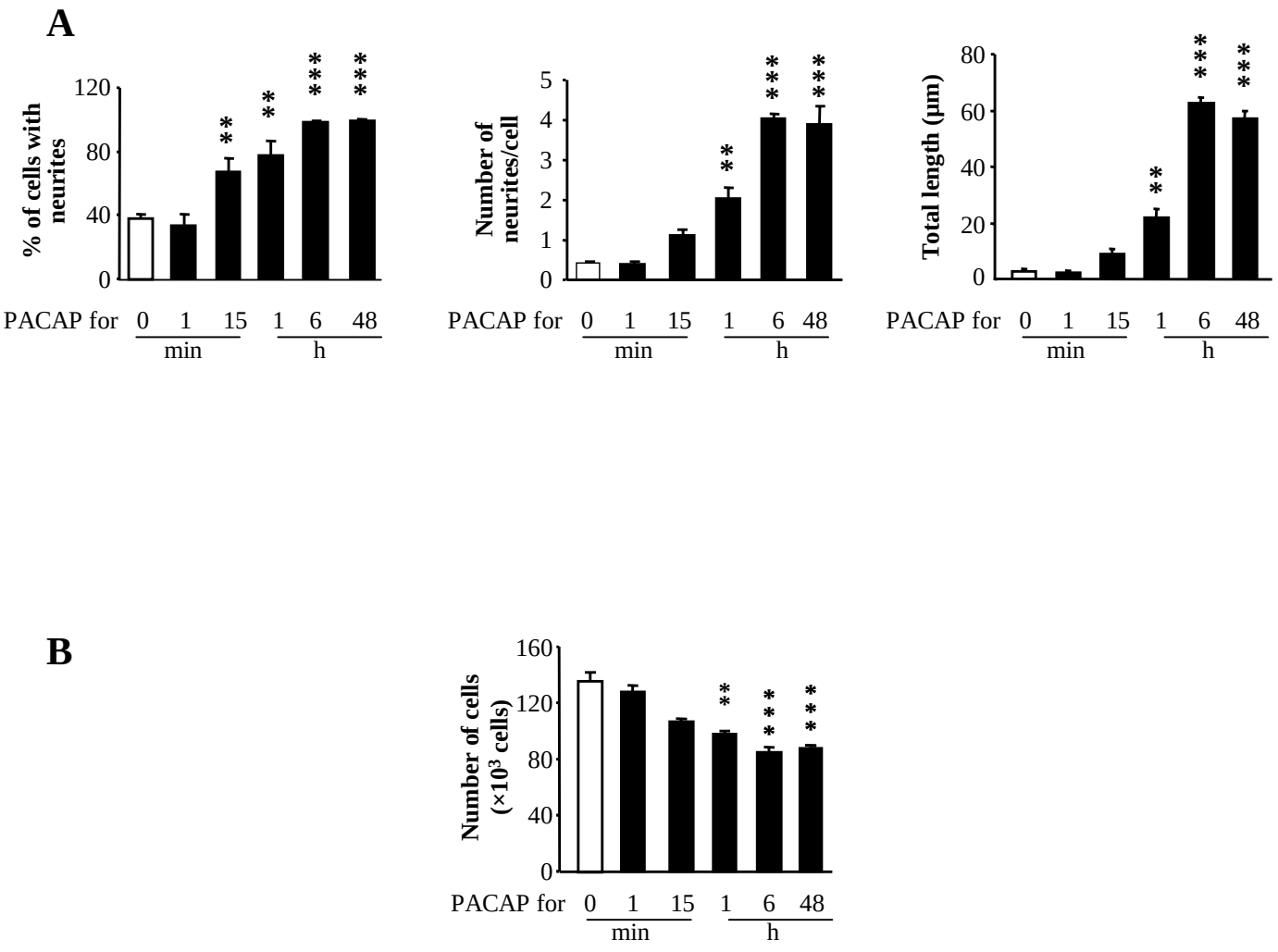

Figure 2

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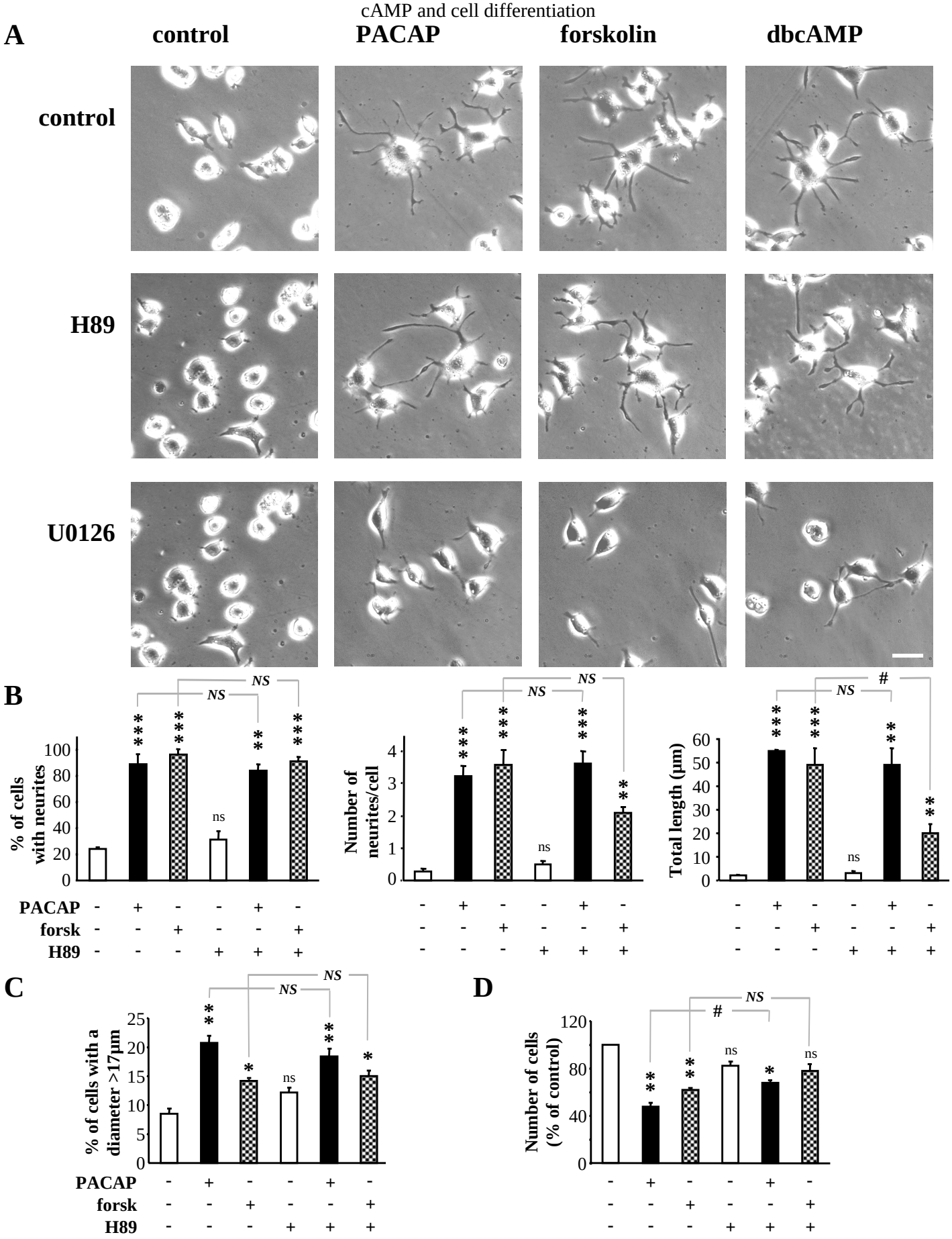

Figure 3

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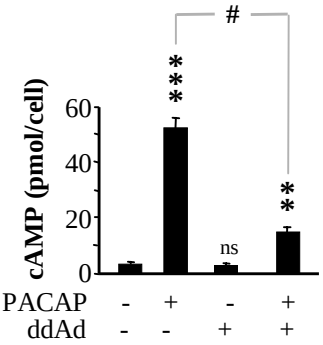
A



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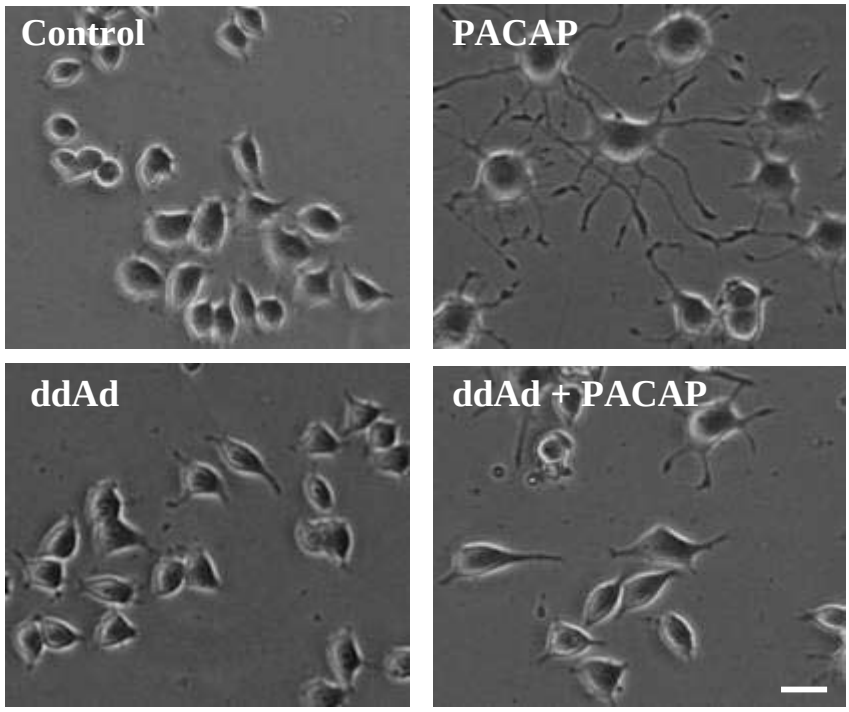

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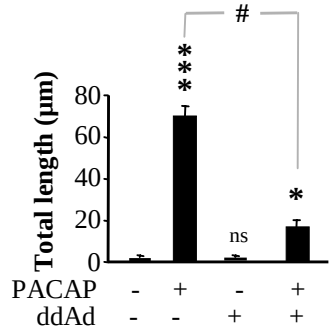

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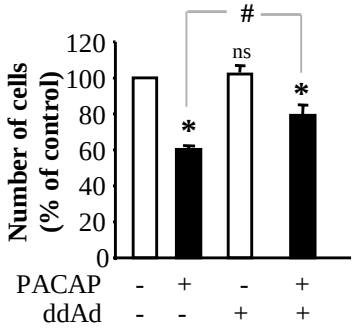

Figure 4

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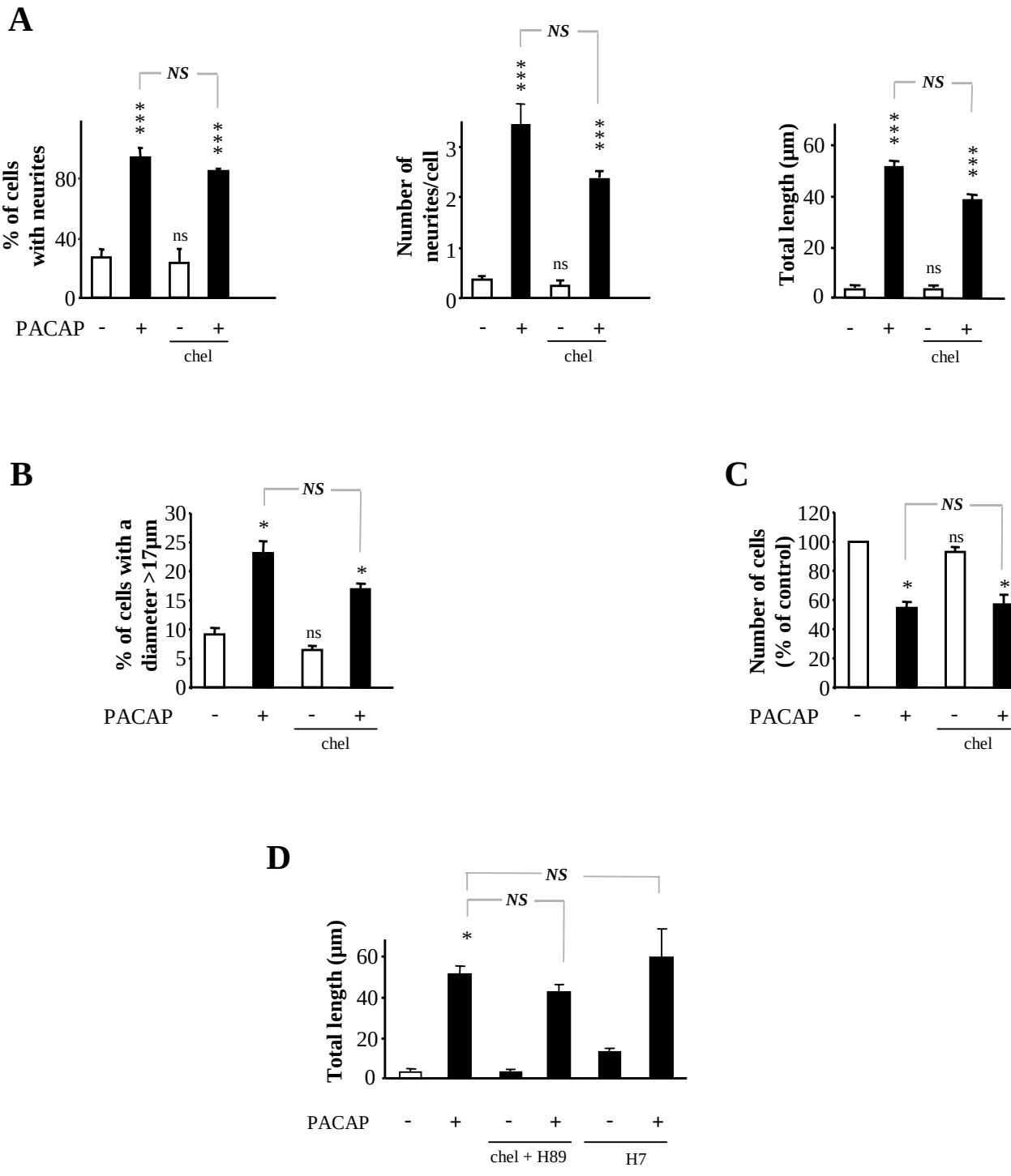

Figure 5

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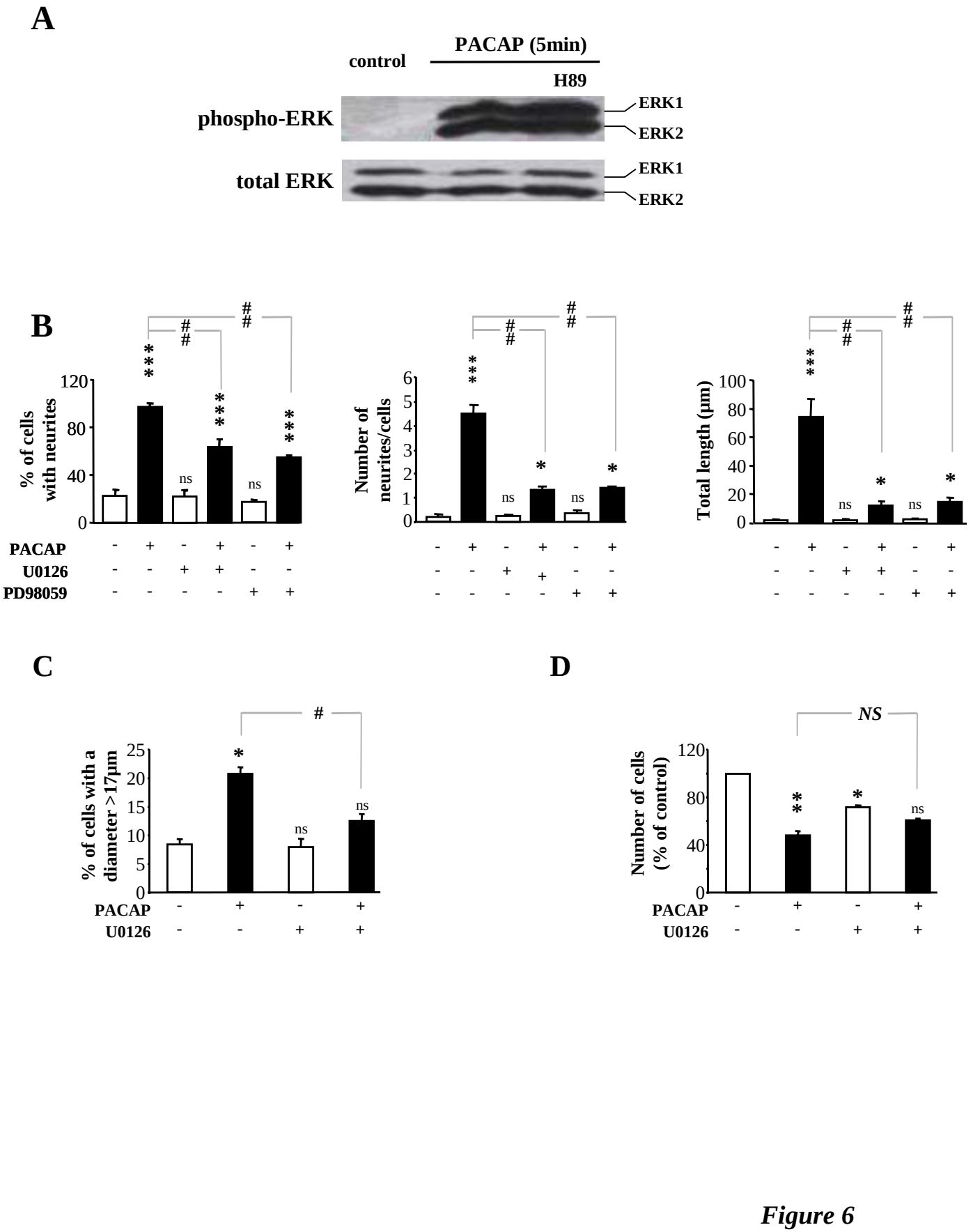

Figure 6

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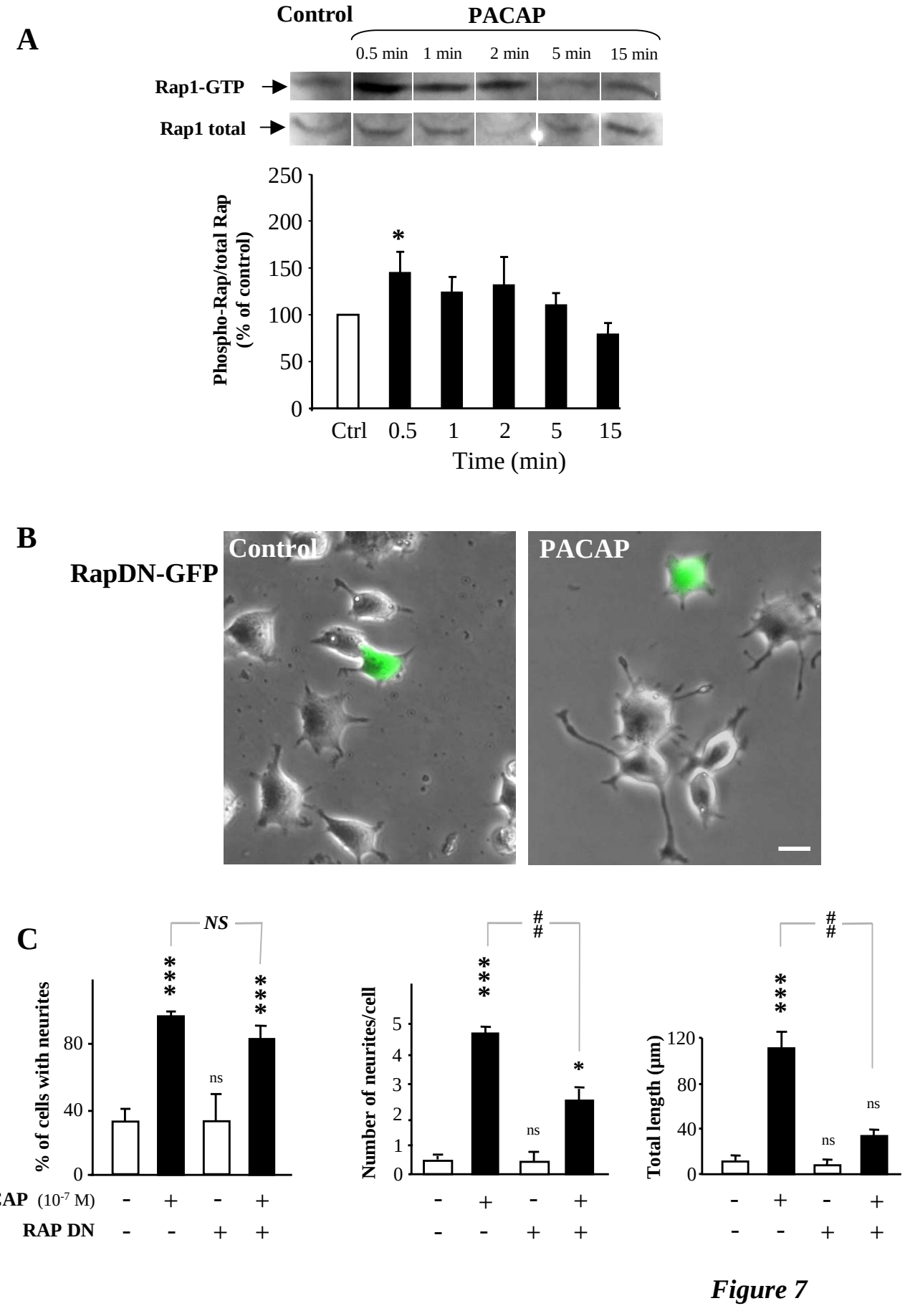

Figure 7

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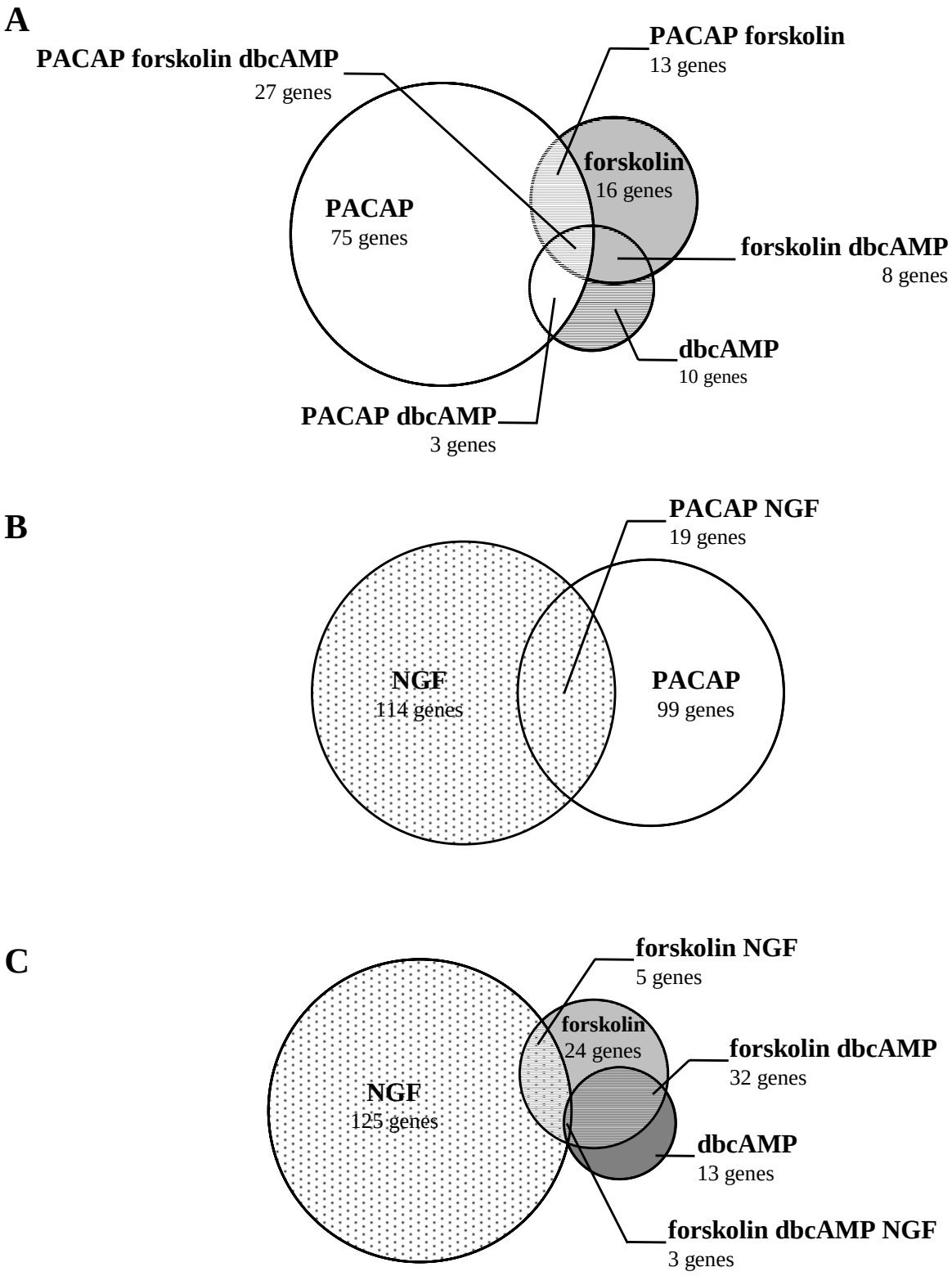

Figure 8

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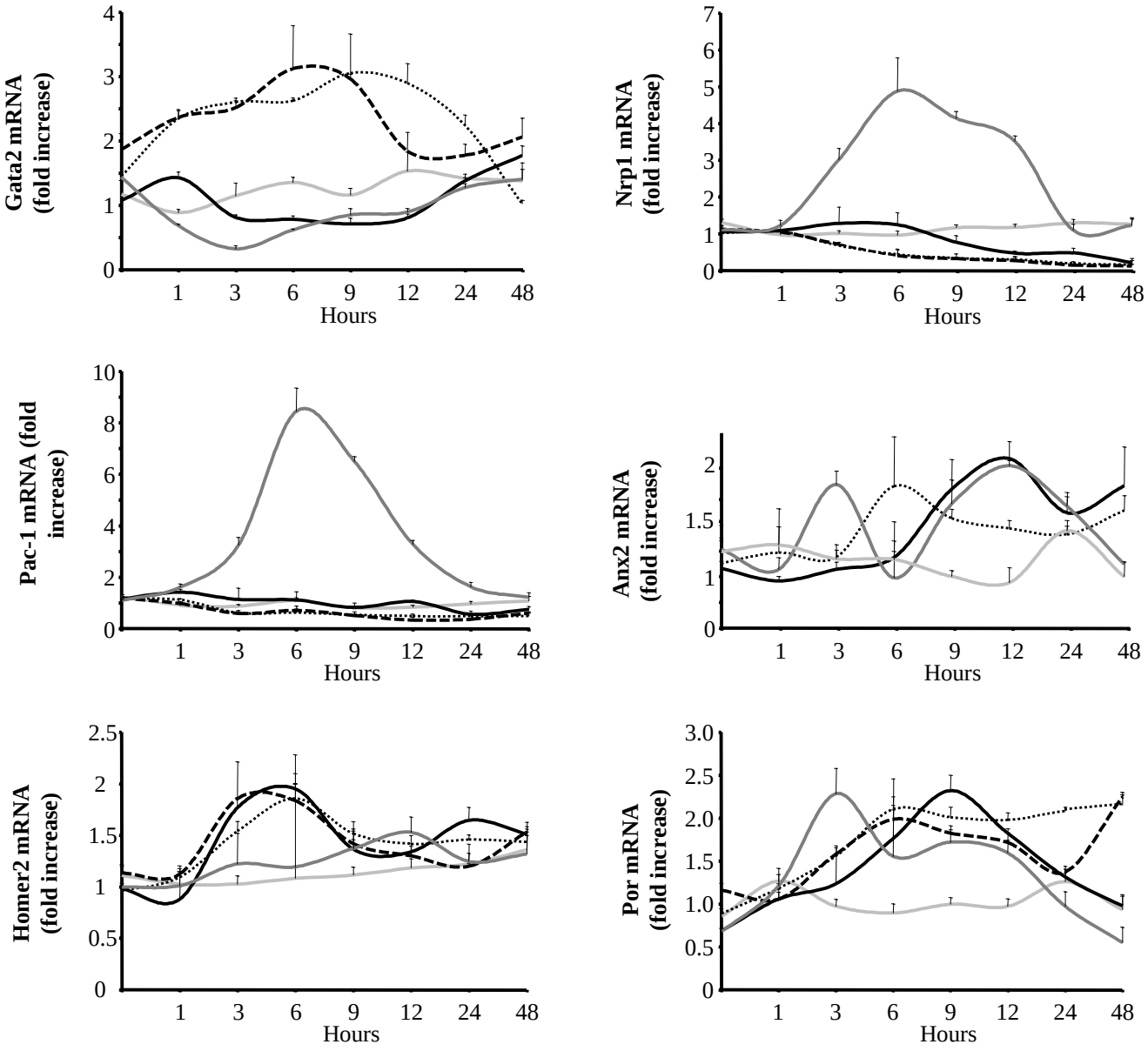

Figure 9A

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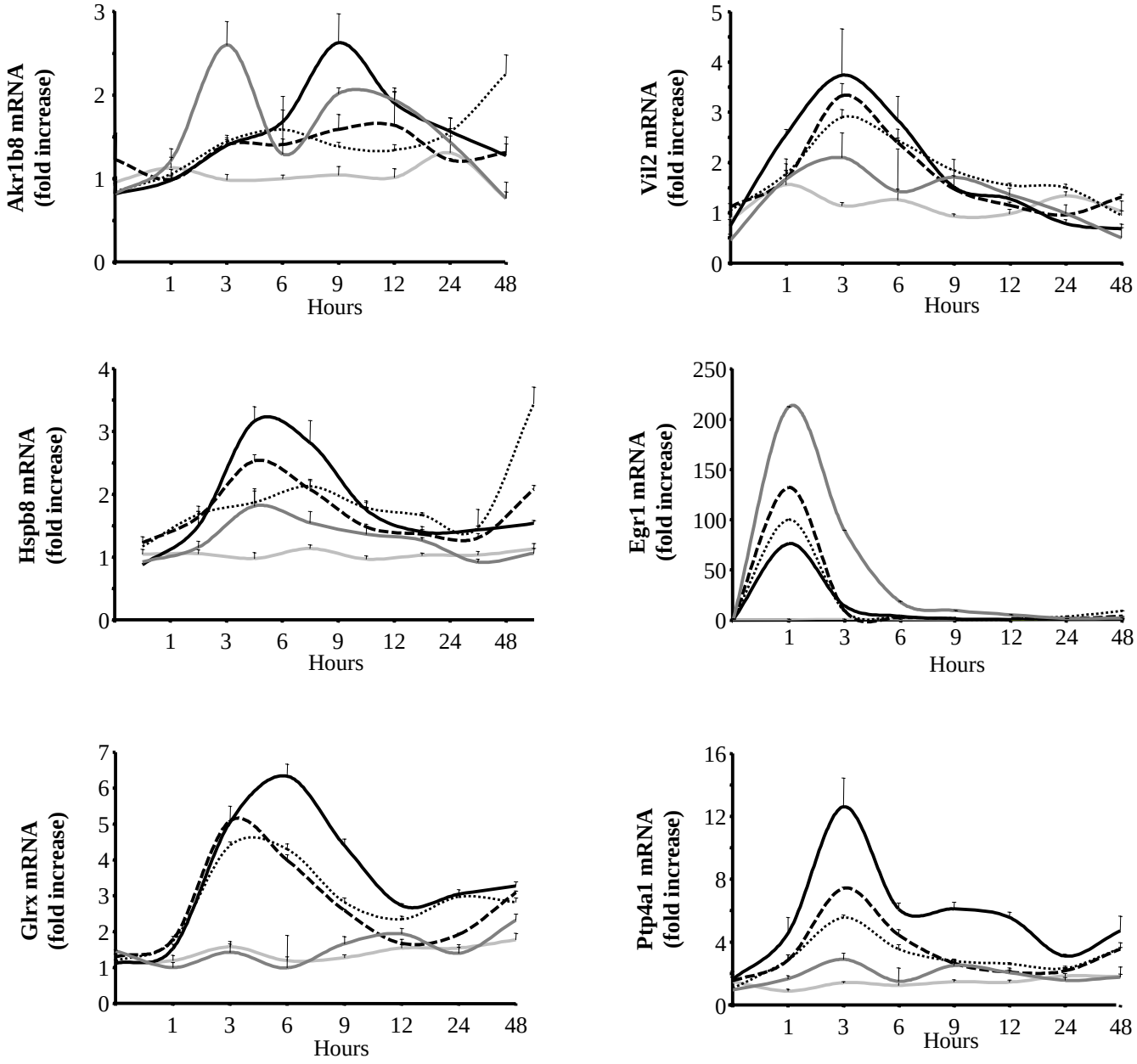

Figure 9B

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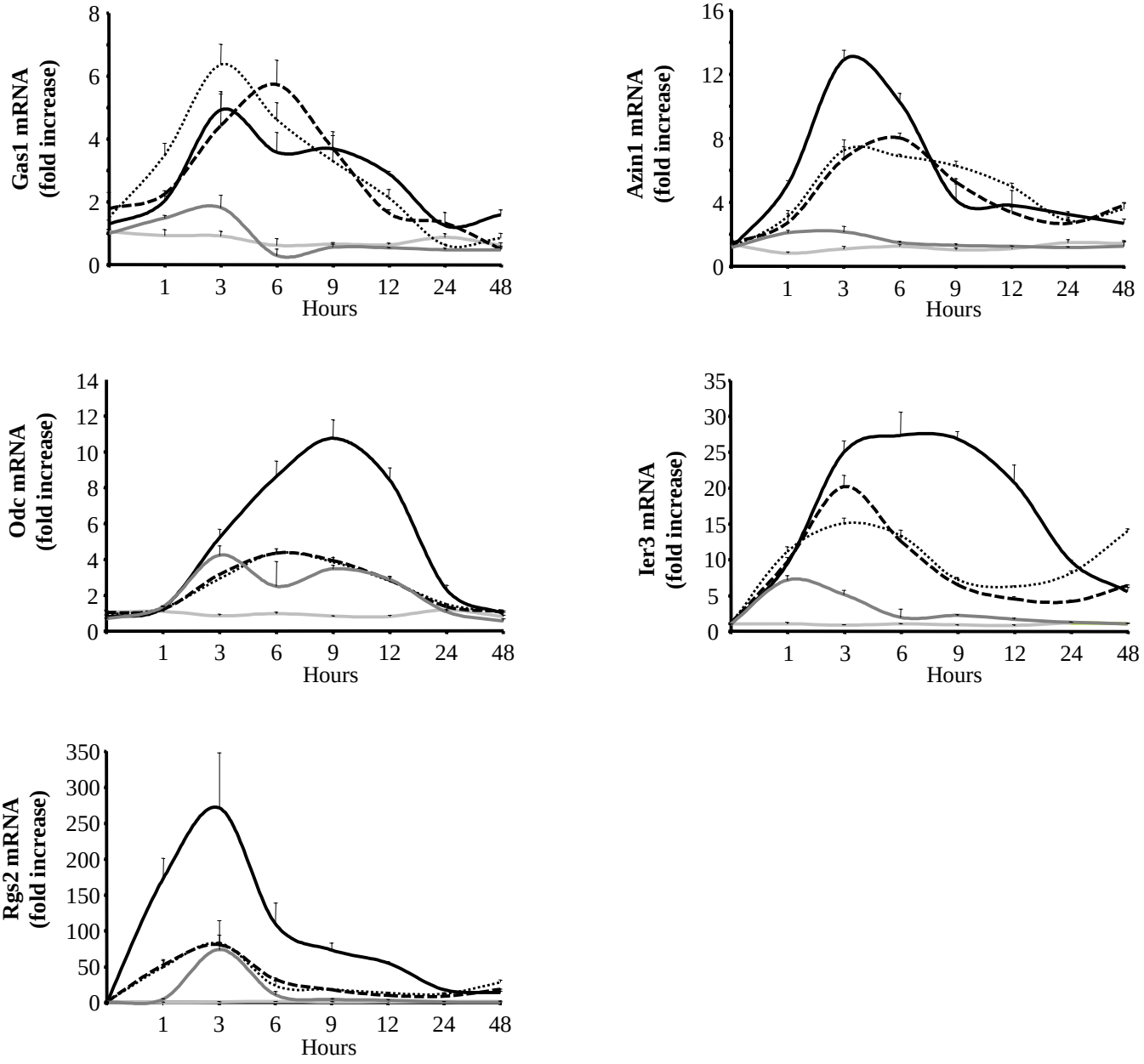

Figure 9C

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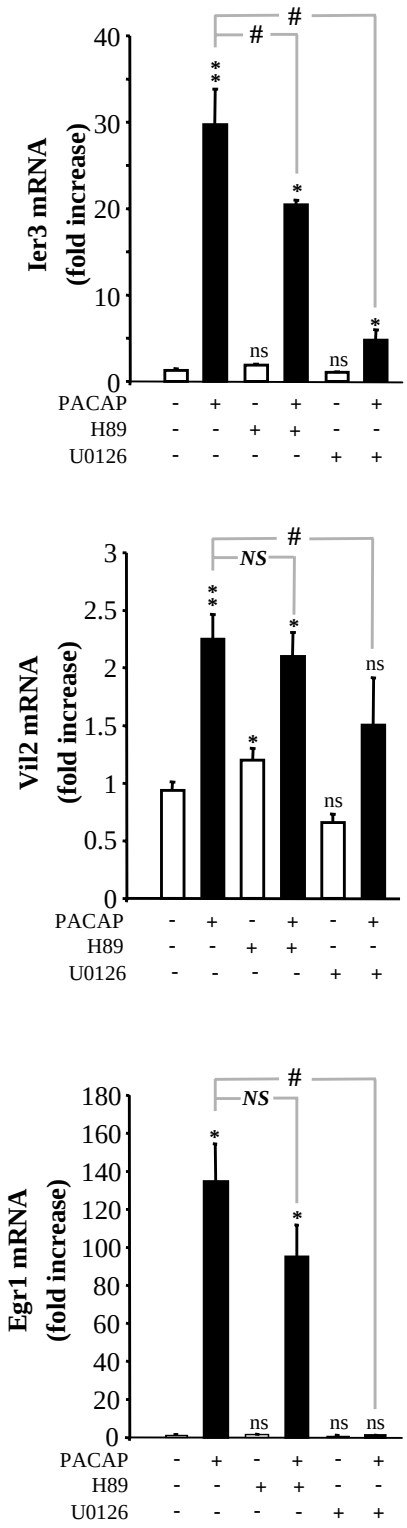

Figure 10

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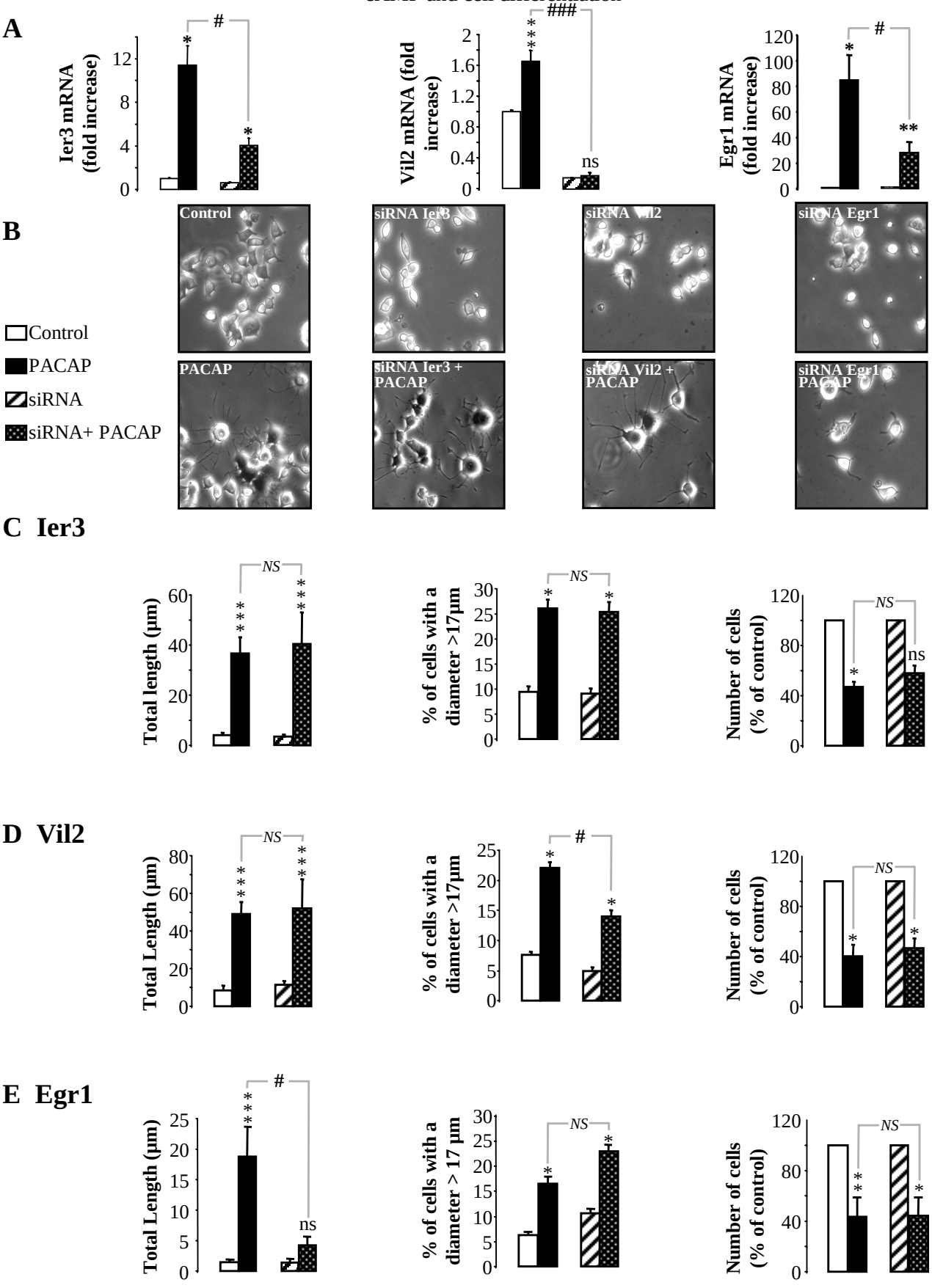

Figure 11

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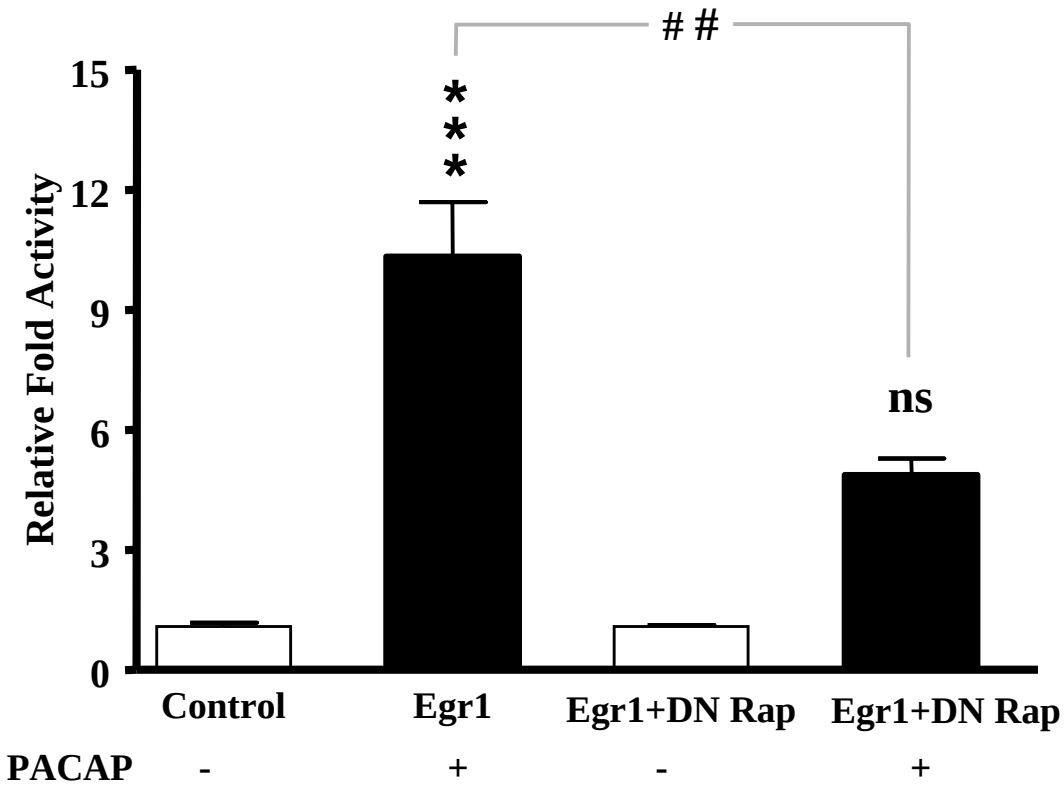

Figure 12

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cAMP and cell differentiation

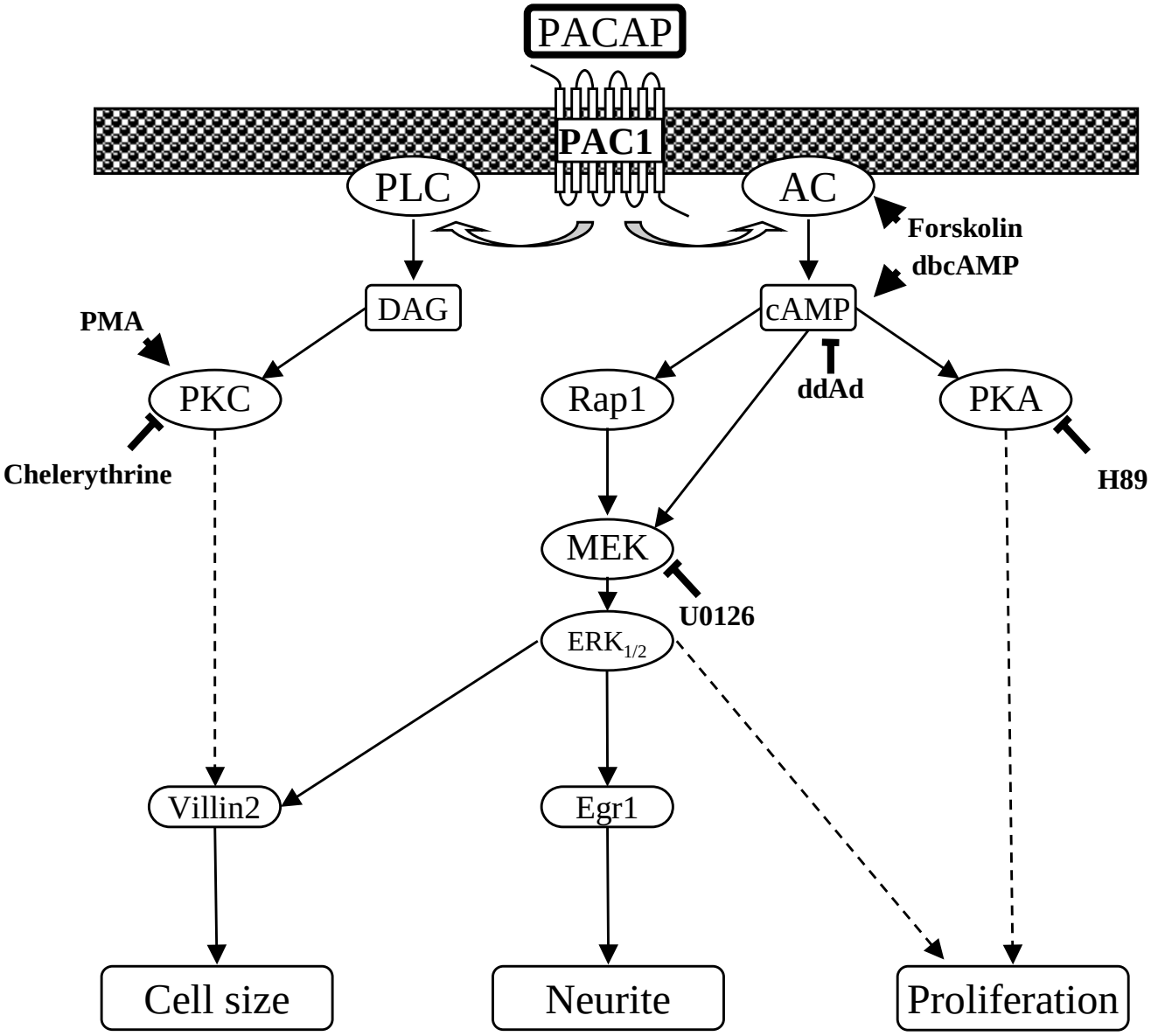

Figure 13