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Roles for ERK-dependent early- and late-gene expression in neuropeptide Gs-GPCR signaling for neuritogenesis

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Abstract

Gs-coupled GPCR-stimulated neuritogenesis in PC12 and NS-1 pheochromocytoma cells depends on activation of the MAP kinase ERK. Here, we examine changes in phospho-ERK stability, and the time-course of ERK-dependent gene induction, to seek transcriptional determinants for this process. Quenching of ERK activation by inhibition of MEK with U0126 at any time point for at least 24 hours following addition of PACAP resulted in arrest of neurite formation. Changes in the transcriptome profile throughout this time period revealed at least two phases of gene induction: an early phase dominated by induction of immediate-early genes, and a later phase of gene induction after 4–6 hours of exposure to PACAP and elevation of phospho-ERK levels. Genes induced by PACAP in both phases consisted in those whose induction was dependent on ERK (i.e. blocked by U0126), and some whose induction was blocked by the protein kinase A inhibitor H89. ERK-dependent 'late gene' transcripts included GPR50, previously implicated in facilitation of NGF-induced neurite formation in NS-1 cells. GPR50 induction was dependent on the cAMP-dependent guanine nucleotide exchange factor RapGEF2, previously shown to be required for PACAP-induced neuritogenesis in NS-1 cells. Expression of a GPR50-directed shRNA lowered basal levels of GPR50 mRNA and attenuated GPR50 mRNA and protein induction by PACAP, with a corresponding attentuation of PACAP-induced neuritogenesis. We conclude that Gs-GPCR-stimulated neuritogenesis is a process first triggered by immediate-early gene induction, including that of EGR1/ZIF268/NGF1A/KROX24 as previously reported. This early phase of gene induction, however, is insufficient to maintain the neuritogenic process without later-phase, ERK-dependent gene induction upon continuous exposure to the neurotrophic neuropeptide, including but probably not limited to that of GPR50. Early (EGR1) and late (GPR50) gene induction by NGF, like that for PACAP, was inhibited by U0126, but was independent of RapGEF2, confirming distinct modes of ERK activation by Gs-coupled GPCRs and neurotrophic tyrosine receptor kinases (Trks), converging on a final common ERK-dependent signaling pathway for neuritogenesis.

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Cyclic AMP; ERK; GEF; NCS-RapGEF2; Egr1-zif268; Gpr50; neuritogenesis

Introduction

It is well documented that neuritogenesis in PC12/NS-1 cells initiated either by Gs-GPCR activation by neuropeptides such as PACAP (1) or by neurotrophin receptor kinase activation by NGF (2), is driven by activation of ERK (3–7). However, not all activation of ERK by first messengers leads to neurite formation. This was first demonstrated by Marshall, who found that transient ERK activation by EGFR stimulation did not result in PC12 cell neuritogenesis, while longer-term activation by NGFR (trkA) stimulation did (7). Vaudry et al. demonstrated that neuritogenesis ensuing after exposure to the Gs-coupled receptor ligand PACAP required at least three and up to six hours of continuous extracellular exposure to the ligand (5). PACAP stimulation requires cAMP elevation, the cAMP-dependent Rap1 activator NCS-RapGEF2, and phosphorylation of ERK to effect neuritogenesis in PC12 and NS-1 cells (8, 9). Furthermore, ERK alone is sufficient for neuritogenesis if constitutively expressed in an activated, nucleus-directed form (6).

It thus appears that ERK activation must be maintained for a relatively long period of time to drive the process of neuritogenesis. However it is not known whether ERK mediates neuritogenesis initiated by neurotrophins or neuropeptides via its persistent stimulation by first messengers through a period sufficient to trigger neuritogenesis, or if first messengers initiate a switch in ERK activation to a state independent of continued first/second messenger stimulation. Results obtained with EGR1 receptors engineered for persistence in engagement with ERK stimlation suggest that for neurite formation initiated at growth hormone receptor(s), the latter is the case (10). However, the dynamics of neuritogenic signaling through Gs-coupled GPCRs, such as the PACAP-liganded PAC1 receptor, have not been examined; and the characteristics of such signaling may be relevant to the actions of neurotrophins, compared to neurotrophic neuropeptides, in the mammalian nervous system *in vivo*.

Here we have attempted to answer this question by comparing the temporal features of ERK activation by PACAP, in comparison with ERK-dependent immediate early-gene (IEG) transcription, ERK-dependent late-gene transcription, and ERK-dependence of neuritogenesis. We have identified EGR1 as the major transcript displaying ERK-dependent regulation in Neuroscreen-1 (NS-1) cells during early differentiation, and a late phase of transcription apparently independent of EGR1 required for the maintenance of neuritogenesis. Both NGF and PACAP converge on this latter pathway through persistent activation of ERK.

Materials and Methods

Cell Culture

Cell culture solutions were purchased from ThermoFisher Scientific unless otherwise noted. Neuroscreen-1 (NS-1) cells (Cellomics) were grown on flasks or plates coated with Collagen I (rat tail) as described previously (9, 11). NS-1 cells were grown in RPMI-1640 media supplemented with 10% horse serum and 5% heat-inactivated FBS (Hyclone), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Drugs and reagents

Pituitary adenylate cyclase-activating polypeptide (PACAP-38) was purchased from AnaSpec. H-89 was purchased from Calbiochem and Nerve Growth Factor (NGF) from BD Biosciences. The cAMP analog 8-CPT-cAMP purchased from Biolog Life Science Institute. 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) was obtained from Tocris. Drug stocks were prepared following manufacturers' instructions, either in assay media or dimethylsulfoxide (DMSO). Final concentrations of DMSO in cell culture experiments did not exceed 0.1%, and vehicle control with matched DMSO concentration was used as appropriate.

Establishment of stable cell lines

NS-1 RapGEF2−\− (ΔRapGEF2) cells with biallelic deletion of the cAMP binding site of rat RapGEF2 gene was constructed using CRISPR/Cas9 technique as previously described (12). Briefly a single-stranded guide RNA was designed to bind to the cAMP binding site-encoding domain of the RapGEF2 gene in a sequence-specific manner, mediating the site-specific cleavage by recruiting Cas9 to generate a double-stranded break, leading to deletion within the RapGEF2 cAMP binding site (exon 4) following DNA repair. The biallelic disruption of RapGEF2 gene was confirmed by sequencing analysis.

NS-1 cells expressing short hairpin (sh) RNAs targeting GPR50 were generated by transducing NS-1 cells with psi-Lv-H1 lentiviral vectors encoding three shRNA constructs each targeting a different GPR50 mRNA region (referred to as sh-A, sh-B and sh-C). All lentiviral particles including control particles expressing scrambled shRNA (sh-control) were purchased from GeneCopoeia (Cat#: LPP-RSH084137-LVRU6GP-050). 48 hours after transduction was initiated, NS-1 cells stably expressing each shRNA were selected with 1 μg/ml puromycin: selection was monitored by continuing until all cells in non-transfected sister wells treated with puromycin were dead. The successful and stable expression of the lentiviral vectors was confirmed by the presence of green fluorescence (GFP expression from the integrated lentiviral vector) in all selected cells.

Neuritogenesis assay

NS-1 and derivative cell lines were seeded in 6-well plates at a density of 100,000 cells per well overnight. On the following day PACAP was added at a final concentration of 30 or 100 nM. When inhibitors were employed, they were added 30 minutes before addition of PACAP, 8-CPT-cAMP, or NGF. After 48 hours or 72 hours, micrographs (20x magnification) were acquired automatically under a Nikon ECLIPSE Ti microscope from

fixed locations within each culture well, which was programed on a NIS-Elements imaging software. Neurite length or percentage of neurite-bearing cells was assessed using image J software.

Western blot

All primary and secondary antibodies were purchased from Cell Signaling Technologies. Rabbit monoclonal antibodies to EGR1, GPR50, and GAPDH were used at dilutions of 1:1000, 1:250, and 1:2000 respectively. HPR-linked anti-rabbit IgG secondary antibody was used at a dilution of 1:5000. Cell lysates were collected in cold lysis buffer (RIPA buffer supplemented with HALT protease/phosphatase inhibitor cocktail) (Pierce) and the protein concentration was determined using bicinchoninic acid (BCA) assay (ThermoFisher). 20 μg of total protein for each sample was loaded on Bullet PAGE One Precast Gel (5–15%) (Nacalai USA), electrophoresis was performed with Nacalai WEP-N Vertical Electrophoresis Cell at 250 V for ~25 minutes when the dye front reached to the bottom of the gel. Proteins were transferred to nitrocellulose membranes using the Bio-Rad TransBlot-Turbo Transfer System with standard transfer procedure for one minigel. After transfer, membrane was stained in 0.1% ponceau S solution for ~3 minutes with agitation to confirm even loading and complete transfer of proteins. To remove Ponceau staining the membrane was rinsed with distilled water then washed with TBST for 5 min before blocked in 5% nonfat milk for 1 hr at room temperature. After blocking, the membrane was incubated in specific primary antibody overnight at 4°C in 5% nonfat milk. After incubation the membrane was washed for 15 minutes in TBST for three times then incubated with HPR-linked anti-rabbit IgG secondary antibody in TBST with 5% nonfat milk for 1 hr at room temperature. The membrane was washed again for 3 × 15 minutes in TBST. For chemiluminescent imaging, immunoreactive bands were detected by SuperSignal™ West Dura Extended Duration Substrate (ThermoFisher) and imaged using Bio-Rad Chemidoc MP imaging system.

Cell-based ELISA

Phospho-ERK level was measured using a cell-based ELISA assay as described previously (13), with minor modifications. On day zero NS-1 cells were seeded in 96-well plate overnight at a density of 30,000 cells/well. On day one, cells were treated with 30 nM PACAP or vehicle for various times, medium was removed, and cells fixed with ice-cold methanol for 20 min. Cells were then permeabilized by washing three times with PBST (PBS with 0.1% Triton X-100), followed by quenching with 0.6% hydrogen peroxide (Sigma) in PBST. Following washing three times in PBST, blocking was carried out by incubation with 10% normal goat serum in PBST for one hour at room temperature. Cells were incubated in primary phospho-ERK antibody (1:500 in PBST with 5% bovine serum albumin (BSA)) overnight at 4 C with gentle agitation. On day two, plates were washed three times in PBST, twice in PBS, and cells then incubated with HRP-coupled anti-rabbit secondary antibody (1:500 in PBST with 5% BSA) for one hour at room temperature. After five washes in PBST, cells were treated with colorimetric substrate (One-Step Ultra TMB-ELISA (Thermofisher)) in the dark for 10 min. Reaction was quenched with 50 μl 4 M sulfuric acid, and absorbance at 450 nm was read.

Total RNA extraction

Total RNA was isolated using a miRNeasy kit (Qiagen) following manufacturer's instructions and stored at −80. RNA concentration was measure using NanoVue Plus spectrophotometer, only RNA samples with absorbance ratio 260/280 of ~2.0 or RNA Integrity Number (RIN) greater than 8 (determined by Bioanalyzer) were subjected to cDNA synthesis for Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) or microarray analysis. NS-1 and its derived cell lines were seeded in 6-well plates at a density of 5×10^5 per well overnight for 16 to 24 hrs. For cDNA synthesis and downstream qRT-PCR analysis to determine EGR1 expression cells were lysed for total RNA collection after one hour of PACAP or NGF treatment, for GPR50 expression cells were treated 24 hrs before total RNA purification. For microarray analysis total RNA was collected from NS-1 cells after 1, 8 or 24 hr after PACAP treatment. For all experiments MEK inhibitor U0126 or PKA inhibitor H89 was added 30 min before PACAP, NGF or 8-CPT-cAMP addition when needed.

cDNA synthesis and qRT-PCR

All reagents were purchased from ThermoFisher Scientific. cDNA synthesis was carried out using Superscript III First Strand Synthesis System with oligo(dT) as a primer following manufacturer's instructions. Real-time PCR was performed on StepOnePlus Real-Time PCR System using the TaqMan Gene Expression assay with TaqMan universal PCR master mix. Each reaction was performed in a total volume of 15 µl. Taqman probes for rat Egr1 (Assay ID: Rn00561138_m1), GPR50 (Assay ID: Rn01281711_m1) and GAPDH (Assay ID: Rn01462662_g1) were synthesized based on sequences generated by Partek Genomic Suite software, and consistent with probes used for microarray analysis. The comparative CT method ($2^{-\Delta\Delta CT}$) was used for relative quantification of the target gene. GAPDH was used as the internal control to calculate ΔCT compared to target gene. ΔΔCT was obtained by comparing ΔCT of the treated to the untreated samples of the same cell line.

Microarray analysis

Affymetrix Rat raGene 2.0 ST arrays were used to detect transcripts induced by PACAP across an eight hour timecourse and after 8-CPT-cAMP with and without inhibitors (data described in Figure 3). Affymetrix Clariom-S rat microarrays were used to interrogate transcript expression levels to detect differentially expressed early and late genes induced by PACAP in additional experiments (data described in Figures 4 and 5). The microarray assays were conducted in the NHGRI-NINDS-NIMH Microarray Core using established protocols (https://mac.nhgri.nih.gov/protocols.html) and as described previously (14). Partek Genomic Suite software (Partek Inc) was used to analyze differential transcript expression. Original CEL data files are available upon request.

Miru analysis of temporal patterns of transcript expression

The gene expression data was log2 transformed, RMA normalized (robust multi-array average) and deferentially expressed genes across all samples with and an FDR of 5% were selected. The 8195 genes that were deferentially expressed were put in MIRU analysis (Kajeka Ltd., Edinburgh, UK). MIRU network analysis tool uses unsupervised clustering

to define patterns of gene co-expression. In this approach, gene expression data are transformed into a network graph where nodes in the graph represent transcripts connected to each other due to their co-expression across multiple samples. The similarity of a node's expression signature with other nodes, or co-expression, determines its edges, in a spatial location on the 3D network graph. The networks were then clustered using a Markov clustering algorithm to subdivide the graphs into discrete sets of co-expressed genes called clusters at a correlation threshold of $r > 0.85$.

Statistical analysis

Microarray data were analyzed statistically with Partek Genomics Suite software using ANOVA with pairwise comparison within groups of interest. All other statistical analyses were performed as unpaired two-tailed Student's t-tests in GraphPad Prism.

Results

Length of the critical period for PACAP-dependent ERK activation necessary for neuritogenesis in NS-1 cells.

We examined the detailed kinetics of neurite formation and extension after exposure to PACAP in NS-1 cells (Figure 1A). After an initial lag phase of about 8 hours, there is an approximately linear increase in neurite length/cell for over 24 hours, after which neuritogenesis plateaus, consistent with previous reports for NS-1 cell differentiation (11) (Figure 1B). ERK phosphorylation over this period is prolonged: after an initial increase of several fold over the first 3–4 hours of PACAP treatment, there is a plateau of steady activation of ERK (ERK phosphorylation) for at least an additional 4–5 hours (Figure 2A). PACAP-induced neuritogenesis at 48 hours is wholly blocked by the MEK/ERK inhibitor U0126, and is unaffected by inhibition of PKA by H89 (Figure 2B), confirming ERK-dependence of PACAP-induced neuritogenesis and its independence from PKA activation as previously reported (9). In order to determine the functional necessity for this prolonged ERK activation, the MEK inhibitor U0126 was added at various times after onset of exposure to PACAP, in order to 'quench' ongoing ERK activation and measure consequent neurite formation at its maximal (48 hour) time point. As shown in Figure 2C, U0126 blocked neuritogenesis completely if added at any point during the first 12 hours of PACAP exposure, and about 50% when added at 24 hours, consistent with the approximately half-maximal level of neuritogenesis reached at 24 hours after initial exposure to PACAP. These results suggest that continuous ERK activation (phosphorylation) is required for ongoing generation of neurites, consistent with the findings of Zhang et al. using a light-activated ERK and examining NGF-induced neuritogenesis in PC12 cells, for which ERK was required, and which was mimicked by continuous induction of ERK by light-induced activation of photosensitive Raf-1 (15).

Temporal profile for PACAP- and cAMP-induced gene induction in NS-1 cells.

A detailed temporal profile of gene induction after initiation of PACAP treatment was obtained by harvesting of NS-1 cells for microarray analysis at 15, 30, 60, 90, 120, 240, 360, and 480 minutes after onset of PACAP exposure (Figure 3). MIRU analysis (not shown) identified elevent patterns of gene induction across time, with minor variations grouped

into genes up-regulated early after exposure to PACAP, and those up-regulated only after an initial period of quiescence for an hour or more. Figure 3A illustrates the two-phase pattern of gene regulation, as illustrated by behavior of the most highly-regulated (>3-fold) transcripts. Transcripts with induction of onset within minutes of PACAP application are almost exclusively genes previously designated as immediate-early genes (IEGs) based on the fact that their initial expression is very low or undetectable, their induction is prompt, and they function as transcription factors for other, downstream genes in various physiological processes, including neuronal plasticity (16–18). Transcripts with induction of onset only after one or more hours are considered 'late genes' in the context of this analysis, and are examined in greater detail in more extended experiments described below.

The signaling pharmacology of cAMP-dependent early transcription was examined by treatment of NS-1 cells with 8-CPT-cAMP for one hour. Although not all early transcripts are uniformly at peak levels of induction by PACAP at this time, all are measurably up-regulated, allowing a comparison of gene induction by PACAP compared to cAMP itself, and the effects of inhibition of ERK and PKA at doses of the MEK inhibitor U0126 and the PKA inhibitor H89, which fully inhibit these enzymes within intact cells upon extracellular application (19), and show complete (U0126) and no (H89) inhibition of PACAP-induced neuritogenesis (Figure 2B). This experiment allowed interrogation of exclusively cAMP-initiated ERK signaling in NS-1 cells, compared to that occurring by non-cAMP-dependent activation of ERK, as by the neurotrophic factor NGF (8). As shown in Figure 3B Egr1 is the only transcript identified as solely ERK-dependent induction, completely blocked by U0126, which downregulated 8-CPT-cAMP- induced a 4.3-fold in gene expression close to the basal level (1.3-fold). Orl1387 is the only gene partially regulated by PKA: H89 inhibited induction of this gene from 2.1-fold to 1.4-fold. We note here that the experimental design did not include combination of inhibitors to identify genes whose induction might be compensatorily regulated by either pathway.

Temporal profile for PACAP-induced gene induction in NS-1 cells at 1, 8 and 24 hours.

We showed that inhibition of ERK phosphorylation by U0126 blocked neuritogenesis completely when added during the first 12 hours of PACAP induction (Figure 2). Upregulation of IEGs such as ERK-dependent EGR1 is prompt and transient, with expression level that peaks within 1 hr and returns to basal level by 4 hrs (Figure 3D). Therefore IEGs may participate in the initiation of neuritogenesis, but additional transcripts regulation by either ERK directly, or ERK through IEG induction, may further participate in the neurite elongation process. To study the full scale of genes responsible for neuritogenesis, the analysis described for 8-CPT-cAMP was extended to examination of the transcriptomic profiles of NS-1 cells treated with PACAP at 1, 8 and 24 hours, and in the presence of H89 or U0126 at 24 hours to complement the pharmacological study of IEGs above, using the concentrations of each inhibitor previously employed.

Genes that were expressed 2-fold or higher than vehicle control (no treatment) after activation of PACAP were considered as upregulated (shown as "increase" in Figure 4A), whereas genes that were expressed at least 2-fold lower than vehicle control were considered as downregulated ("decrease" in Figure 4A). Over the course of 24 hr of PACAP treatment

there are more genes upregulated than downregulated at each time point (99 versus 11 at 1hr, 110 versus 46 at 8 hr, and 66 versus 20 at 24 hr) (Figure 4A). Among 99 genes upregulated at 1 hr there are 75 IEGs whose upregulation doesn't sustain at 8 hr or further (Figure 4B domain (a)), Top IEGs listed in Figure 4C (a) include the ones that were upregulated in previous microarray studies such as Nr4a3, Nr4a1, Fos and Egr1. There are more IEGs induced by PACAP than 8-CPT-cAMP, either because of non-cAMP-dependent signaling initiated by PAC1 receptor activation, or because of differences in the dynamics of cellular cAMP elevation initiated by PACAP compared to static exposure to a fixed concentration of 8-CPT-cAMP. There are 57 genes upregulated at 8 hrs only (domain (b)). Garem (or Garem1), which encodes a protein that promotes activation of the MAPK/ERK signaling pathway, was shown previously to be upregulated after 1 hr of PACAP treatment and sustained at 8 hrs. There are 10 transcripts induced at both 1 hr and 8 hr (Figure 4B and C). Among them Ofml3, which encodes a secreted scaffold protein associated with Alzheimer Disease, is upregulated continuously from 1 hr to 8 hr (Figure 3B). Btg2, which encodes an anti-proliferative protein, with a sustained overexpression until 2 hrs after PACAP as an IEG, has a basal level of expression at 8 hrs, however its induction is again significant (over 7-fold increase as shown in Figure 4C (e)) at 24 hr as a late gene. It is also upregulated to almost 2-fold with 8-CPT-cAMP (data not shown for less than 2-fold of induction), confirming its role as a cAMP-induced transcript associated with neuritogenesis. The other one of two genes upregulated at 1hr and 24 hr over 2-fold is Trib1, however, it is also upregulated at 8 hr at a level close to 2-fold (1.8) as shown in Figure 4C (e). There are 31 transcripts upregulated at both 8 and 24 hours (Figure 4B and C domain (f)). Among these, robust induction of an orphan GPCR, GPR50, is noted, which shows an over 100-fold increase of expression by 8 hours of PACAP treatment. Dynamic study of over-expression of IEGs showed that GPR50 upregulation begins as early as 4 hr after PACAP addition (Figure 3A). As it was the most highly-induced late transcript, the potential role of GPR50 in NS-1 cell neuritogenesis was further studied in subsequent experiments. Slit2 is also upregulated at both 8 and 24 hours with onset of induction at 90 minutes after onset of exposure to PACAP (Figure 3A). There are 12 genes upregulated at all time points (1, 8 and 24 hours in Figure 3C domain (g)): not all of these are also noted in the earlier 0–8 hour time course may be due in part to differences in the dynamic ranges of transcript relative quantification by the rat microarray chips (Ra-Gene versus Chlariom) used in the two experiments.

To further identify late genes involved in PACAP-induced neuritogenesis, PACAP-induced transcripts were assessed as PKA- versus ERK-dependent, depending on blockade of their induction at 24 hours of PACAP treatment, by either the MEK inhibitor U0126 or the PKA inhibitor H89, or by both (Figure 5). There are three wholly ERK-dependent transcripts. The Dclk1 transcript encodes a serine/threonine protein kinase implicated in neuronal development, and also identified as a 'latent-process' transcript in NGF-induced differentiation of PC12 cells (20). Its mRNA is upregulated ~six-fold at 8 hr and four-fold at 24 hr after PACAP treatment, and this was reduced to less than two-fold by U0126. The other two ERK-dependent transcripts are Tnfrsf22 and Dlg2. Tnfrsf22 is associated with negative regulation of the apoptotic pathway and Dlg2 encodes a postsynaptic scaffolding protein, PSD-93. There are eight late genes regulated by PKA only, including the cyclic AMP response modulator Crem and the slit guidance ligand 2 (Slit2) which functions in

axon guidance and neuronal migration (Figure 5C). Five genes regulated by both MEK and PKA include GPR50, the most highly PACAP- upregulated late gene. Induction of GPR50 mRNA is blocked more than 97% by U0126, with partial blockade (80%) by H89, supporting a potential role for Gpr50 in ERK-dependent neuritogenesis.

PACAP induction of Egr1 is transient.

Egr1 gene induction is necessary for neuritogensis, in PC12 cells (21). It is also the only 8-CPT-cAMP- upregulated early gene completely blocked by ERK inhibitor in NS-1 cells (Figure 3B). we wished to learn whether or not EGR1 protein expression was sufficiently persistent to account for ERK-dependent neuritogenesis. IEGs are not candidates for persistent effects on neuritogenesis unless the proteins persist *after* mRNA induction has peaked and subsided. We therefore examined EGR1 protein levels across the neuritogenic time span. PACAP-induced Egr1 mRNA expression peaks within 30 minutes and is sustained until returning to basal levels within four hours (Figure 3B). Egr1 protein expression lags mRNA induction, but also subsides according to a similar time course, with peak expression at 1 hr and returning to undetectable levels by six hrs (Figure 6). While EGR1-dependent gene induction may be required for maintenance of neuritogenesis, this process would occur prior to the 6–8 hour initiation of late gene induction reported here. Thus, the action of ERK to sustain neurite formation is not (only) through activation of Egr1 protein, but via direct continued nuclear effects of ERK on de novo gene transcription, including the late gene GPR50.

GPR50 mRNA expression, and co-regulation of GPR50-associated genes after PACAP

GPR50 is the most prominent late gene induced by PACAP, and we therefore examined its regulation and that of other transcripts encoding proteins reported to interact with GPR50 in mediating transcriptional activation by this protein. Two of these include RTN4 (encoding NogoA) and KAT5 (encoding TIP60) (22). We examed the dynamics of expression value (EV) of GPR50, RTN4 and KAT5 after PACAP exposure. Neither transcript is co-induced with GPR50 by PACAP and thus, although perhaps cooperative with GPR50, are not dynamically involved in neuritogenesis (Figure 7).

GPR50 regulation through the RapGEF2 pathway, and neuritogenesis

To examine the actual role of GPR50 in neuritogenesis, we tested three different shRNA constructs (sh-A, B and C) directing against GPR50 mRNA to knock down its expresson in NS-1 cells with or without induction of PACAP (Figure 8). ShRNA encoding regions were stably expressed from a lentiviral vector after transduction and integration into the genome of NS-1 cells. For knockdown of GPR50 transcript and its protein cognate in NS-1 cells, sh-RNA constructs had variable effects, with those exhibiting the largest effects also showing the greatest reduction in PACAP-induced enhancement of GPR50 levels. As shown in Figure 8A, qRT-PCR analysis of GPR50 mRNA showed markedly reduced expression when targeted with sh-A and B (50-fold of reduction compared to no treat). PACAP stimulation of NS-1 cells expressing sh-A and B still leads to 6- and 15-fold increase of GPR50 gene expresson compared to untreated NS-1 cells, which is substantially less than the over 100-fold increase in no-sh or sh-control expressing NS-1 cells. Effects on GPR50 mRNA abundance were mirrored in shRNA effects on abundance of the GPR50 protein itself.

Western blot analysis revealed that GPR50 protein is below the detectable level without PACAP stimulation, which is consistent with its low transcriptomic expression value (EV~5) in untreated NS-1 cell shown in Figure 7. Robust induction of GPR50 protein by PACAP is reduced to barely detectable levels after sh-A or B knockdown (Figure 8B). GPR50 knockdown caused a corresponding decrease in neuritogenesis after PACAP treatment, albeit impairment was not complete, i.e., sh-A or B caused a significant 50% reduction of neurite growth induced by PACAP (Figure 8C and D). Sh-C slightly reduced both PACAP-induced GPR50 mRNA and protein induction, as well as neuritogenesis, albeit not to a significant extent ($P>0.05$; Figure 8C and D).

To gain insight into the potential convergence of cAMP and neurotrophin signaling in the progression of gene regulatory events occurring during neurite formation, we compared Egr1 and GPR50 induction by either PACAP or NGF one or 24 hours after initiation of treatment, in the presence of the ERK inhibitor U0126, or in cells lacking NCS-RapGEF2 expression. As shown in Figure 9, gene upregulation of EGR1 mRNA by NGF or PACAP is similar, given the fact that both peak sharply around 1 hr, and therefore with maximal variation between experiments at this single time point. The increase of GPR50 mRNA at 24 hr induced by NGF or PACAP is also very robust and NGF-induced and PACAP GPR50 upregulation is similar (50-fold for NGF and 100-fold for PACAP). Induction of EGR1 mRNA by PACAP is eliminated by U0126 as well as RapGEF2 knockout (NS1ΔRapGEF2), whereas that of GPR50 is eliminated by U0126, and reduced about 80% with RapGEF2 knockout. NGF induction of both Egr1 and GPR50 was largely but not completely eliminated in the presence of U0126, and remained robust in RapGEF2 knockout cells compared to the corresponding effects of PACAP.

Discussion

Both PC12 and NS-1 cells have been extensively studied in attempted the transcriptomics of neuronal differentiation unto specific signaling pathways involved in it, including growth arrest, neuron-specific gene expression, and neurite formation (13, 23–30), as well as neuronal responses involved in neuroprotection (31–33), We have previously reported that exposure of PC12 cells to PACAP for six hours is required to initiate full neuritogenesis in cells measured 48 hours later, i.e. that PACAP does not act as an immediate trigger to initiate this process (34). PACAP-induced neuritogenesis is cAMP- and ERK-dependent, and requires the guanine nucleotide exchange factor RapGEF2 to propagate a signal to activation the MAP kinase ERK (8, 9, 19), which is sufficient for neurite formation (6). Likewise, neurotrophins through prolonged, but not transient ERK activation stimulate neuritogenesis (7), indicating distinct but converging signaling pathways for ligands that can mediate prolonged activation of ERK initiated either through Gs-coupled GPCRs, or receptor tyrosine kinases (10, 35).

Here, we have examined in detail the requirement for ERK activation by ‘quenching’ at various time points after initial exposure to PACAP, and compared this to the temporal program of gene activation provoked by PACAP using microarray-based transcriptome analysis. This analysis revealed that ERK activation is indeed required throughout the entire process of neuritogenesis, at least within the time frame in which this occurs in NS-1 cells.

We therefore extended our transcriptome analysis beyond that of previous studies in PC12 cells (5, 36–38), to focus more closely on the potential roles of late gene induction in the neuritogenic process. PACAP causes short-term upregulation of a cohort of genes composed almost entirely of transcription factors designated as ‘immediate-early genes’. Of these, Egr-1 has previously been reported necessary for PACAP-induced neurite formation (21); the Nr4a family is implicated in cAMP-dependent gene regulation in other endocrine cells (39, 40); Fos and Arc are markers for neuronal activation in response to a wide variety of physiological and paraphysiological stimuli (41, 42); and Klf4 and Btg2 are implicated in checkpoint regulation of cell division in differentiating neurons (43, 44).

The ERK-dependence of induction of these genes was further investigated using as stimulus the stable cAMP analog 8-CPT-cAMP, which also causes a RapGEF2-dependent induction of neuritogenesis, but is devoid of potential activation of calcium-dependent genes which may occur upon activation of Gq signaling pathway through the PAC1 PACAP receptor (45–47). Only two IEGs, EGR1 and IER3, were wholly ERK-dependent, as in PC12 cells (21). As Ier3 has been implicated in cell size increase, but not neuritogenesis, in PC12 cells (21), we focused on the time course of induction and disappearance of Egr1 protein in NS-1 cells in this study, reasoning that a connection to late-gene induction would require the presence of this protein at the time of onset of late-gene expression. It has been suggested that both Egr-1 gene induction, and activation of Egr-1 transcriptional activity by phosphorylation, are ERK-dependent (48). This could explain the need for sustained ERK activation, first for protein induction and then for protein activation, in neuritogenesis. However, Egr1 protein persists for up to four but less than six hours after initial exposure to PACAP. Thus, the continued requirement for ERK activation in neuritogenesis is not solely the induction and then continued activation of EGR1 transcriptional activity by ERK-dependent cofactor phosphorylation, as has been suggested in other systems (49). Rather, late gene induction during neuritogenesis likely requires the direct effects of ERK signaling for transcription of these genes, rather than, or in addition to, an indirect effect through sustaining Egr-1-dependent transcriptional effects.

The late phase of gene induction beginning at six to eight hours continues until at least 24 hours during the progression of neuritogenesis. 31 genes were up-regulated by 30 nM PACAP at both 8 and 24 hours, with the most robust and consistent regulation being that of the orphan GPCR GPR50 (50). GPR50 has been implicated in signaling for differentiation both as a plasma membrane receptor, of unknown liganding properties, and as a nuclear factor mediating gene transcription (22, 51–54). Furthermore, GPR50 was one of only seven late transcripts whose levels were blocked nearly or completely to baseline by U0126. In order to learn whether or not GPR50 was a candidate gene for sustaining of ERK-dependent neuritogenesis, we employed shRN-Amediated gene knockdown to block its expression, and to examine the effect of diminished GPR50 protein expression on PACAP-induced neuritogenesis. Neurite formation was impaired, but not abolished by knockdown of GPR50 gene expression, suggesting it is one, but not the only, induced protein required for completion of neuritogenesis in NS-1 cells. Furthermore, comparison of Egr1 and GPR50 induction by PACAP and NGF, and ERK-dependent and RapGEF2-dependent effects, revealed that induction of both early (EGR1) and late (GPR50) transcripts were ERK-dependent for NGF, but both ERK- and RapGEF2-dependent for PACAP.

The results obtained here support a model for the process of neuritogenesis induced by either Gs-coupled GPCR or Trk activation in which both signaling processes converge on ERK activation, albeit through separate pathways, and in which late gene activation is due not to a cascade in which ERK activation is required first for gene induction and then for protein activation, but rather one in which a continuous ERK-dependent communication with the nucleus is required to effect completion of neurite formation (Figure 10).

Do these data have implications for the role of GPCR and Trk signaling in neuronal function? Activation of the MAP kinase ERK mediates multiple signaling events and cellular functions in the mammalian nervous system, including hippocampus-dependent threat response learning (55), spatial learning (56), learned responses to psychomotor stimulants (57) and regulation of neuropeptide biosynthesis itself, in the brain (58, 59). ERK activation is required for long-term potentiation (LTP) of synaptic responses (60), a cellular mechanism underlying learning and memory. At the cellular and molecular level, ERK mediates the activation of immediate-early genes (IEGs) such as Egr1/zif268, for example in the development of cocaine preference initiated by cAMP elevation by the first messenger dopamine, acting through the Gs-coupled D1 receptor in the nucleus accumbens, the reward center of the brain (61–63). Other IEGs, such as fos, appear to be activated by Gs-coupled GPCR signaling via a separate pathway involving the transcription factor CREB, which is activated by RSK/MSK or PKA-dependent phosphorylation (64). Fos and Egr1 are also activated by other first messengers, including growth factor receptor stimulating neurotrophins such as NGF and BNDF, through non-cyclic AMP-dependent pathways (65). The implications of the present work for neuronal function is the parcellation of both early and late gene induction by Gs-coupled GPCR activation-the ERK-dependent is aimed at neuritogenesis here, while the non-ERK-dependent remains undefined. It is of interest that GPR50 induction is also partly PKA-dependent, but not sufficiently so to affect neurite formation. The implication that IEGs function not as a cascade but as a separate cohort bears consideration in assessing the role(s) of cAMP→ERK signaling in neuronal plasticity, and the relative roles of early- and late-gene induction in this process (66).

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Data Availability Statement

All original data including raw data files for microarray analysis, is available to readers upon request.

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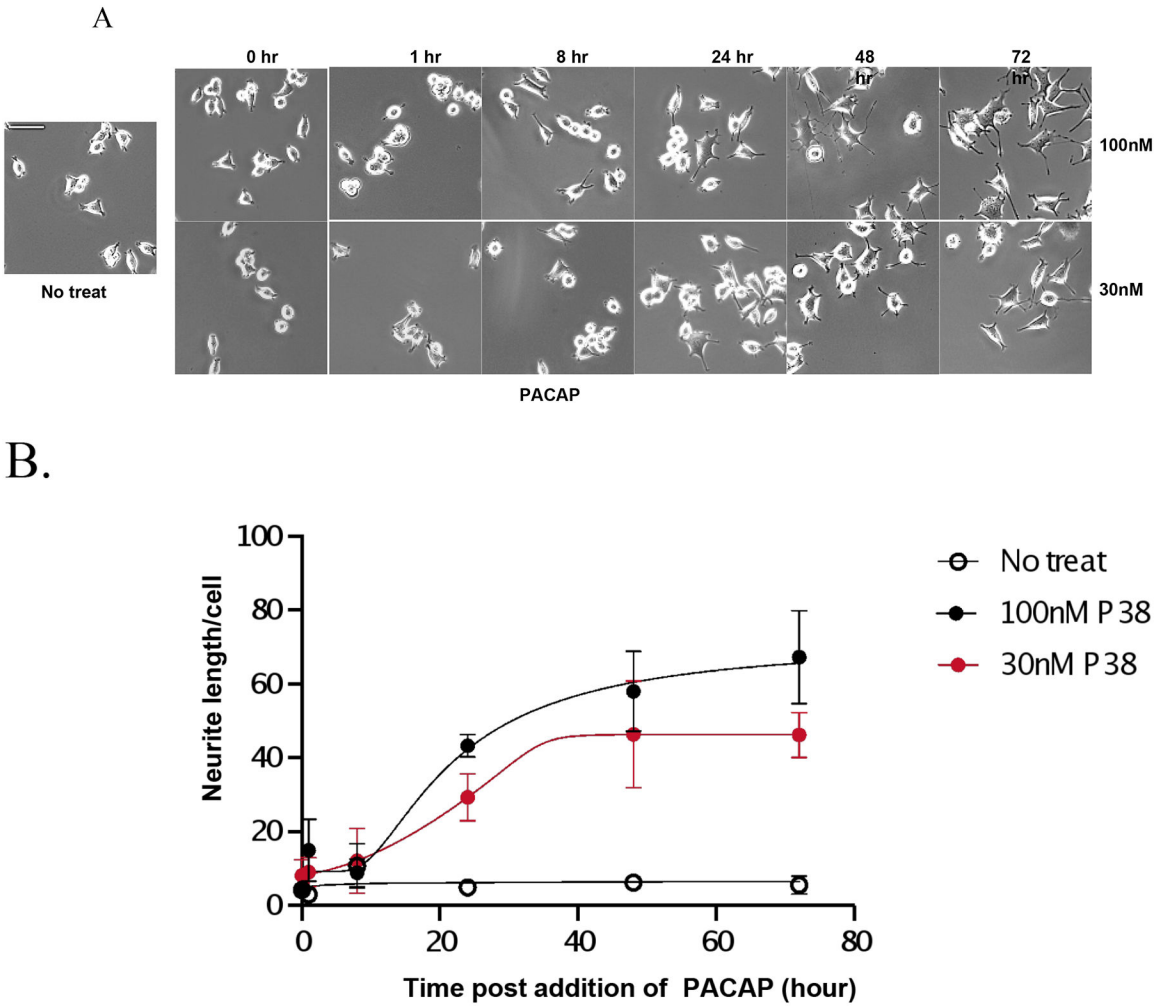
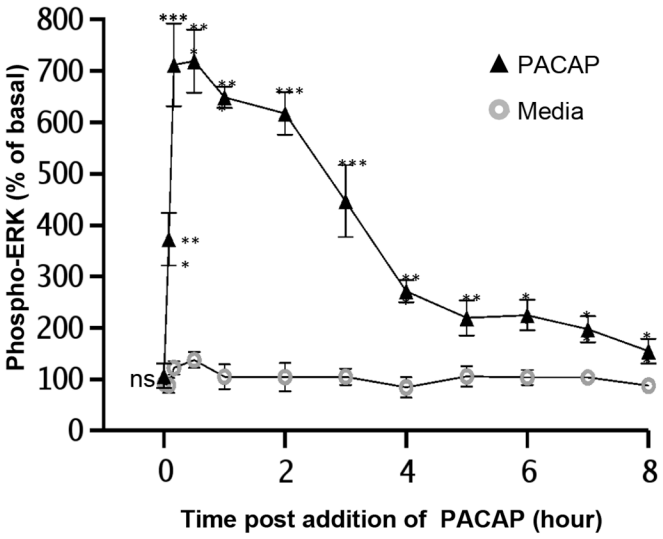


Figure 1. Time course of NS-1 neuritogenesis induced by PACAP.

NS-1 cells were treated with 30 nM or 100 nM PACAP. Images were taken at 0, 1, 8, 24, 48 and 72 hours after PACAP treatment. **A**. Representative micrographs for neurite outgrowth of NS-1 cells over the time after PACAP addition were shown. The morphology of typical NS-1 cells with no treatment is shown on the left with a scale bar representing 50 μm at top left corner of the micrograph. Top right panel, 100nM PACAP; bottom right panel, 30nM PACAP. **B**. Neuritogenesis at each time point was documented as neurite length per cell (neurites at the length ≥ 10μm were counted). N=3, S.D. is indicated as error bars.

A.



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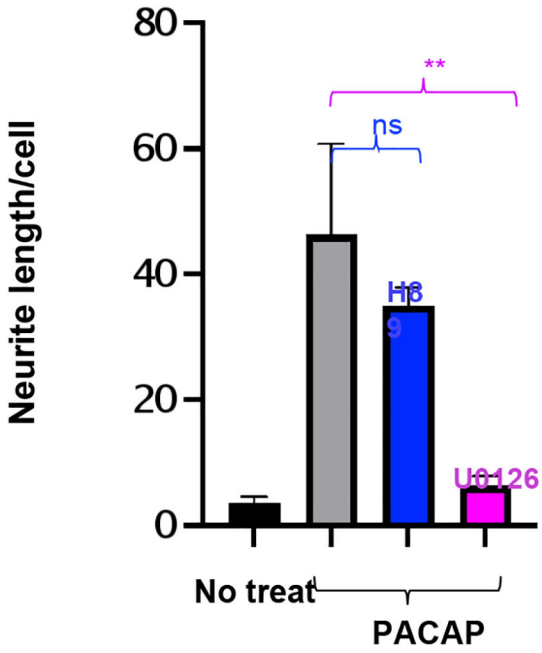


C.

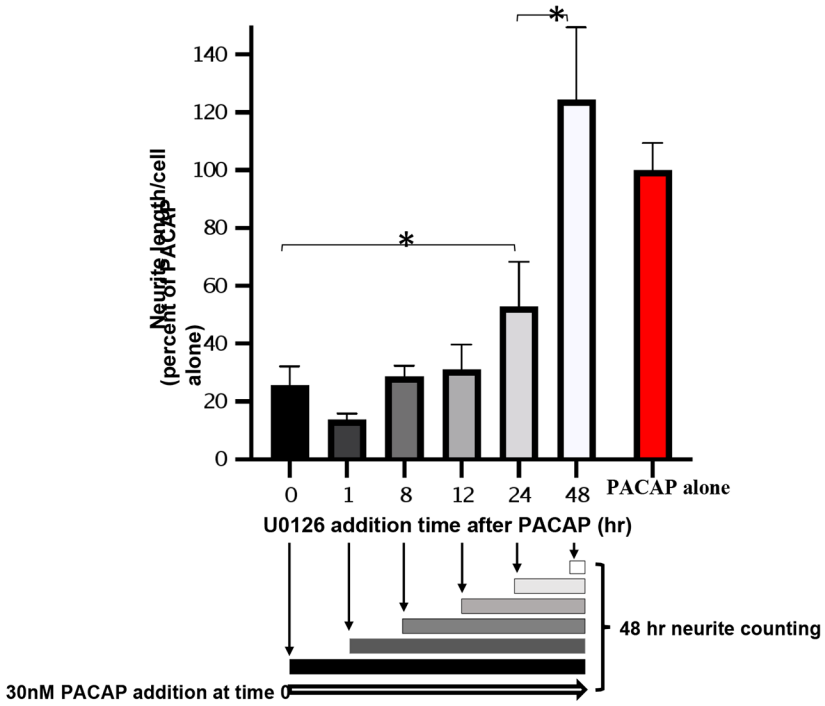
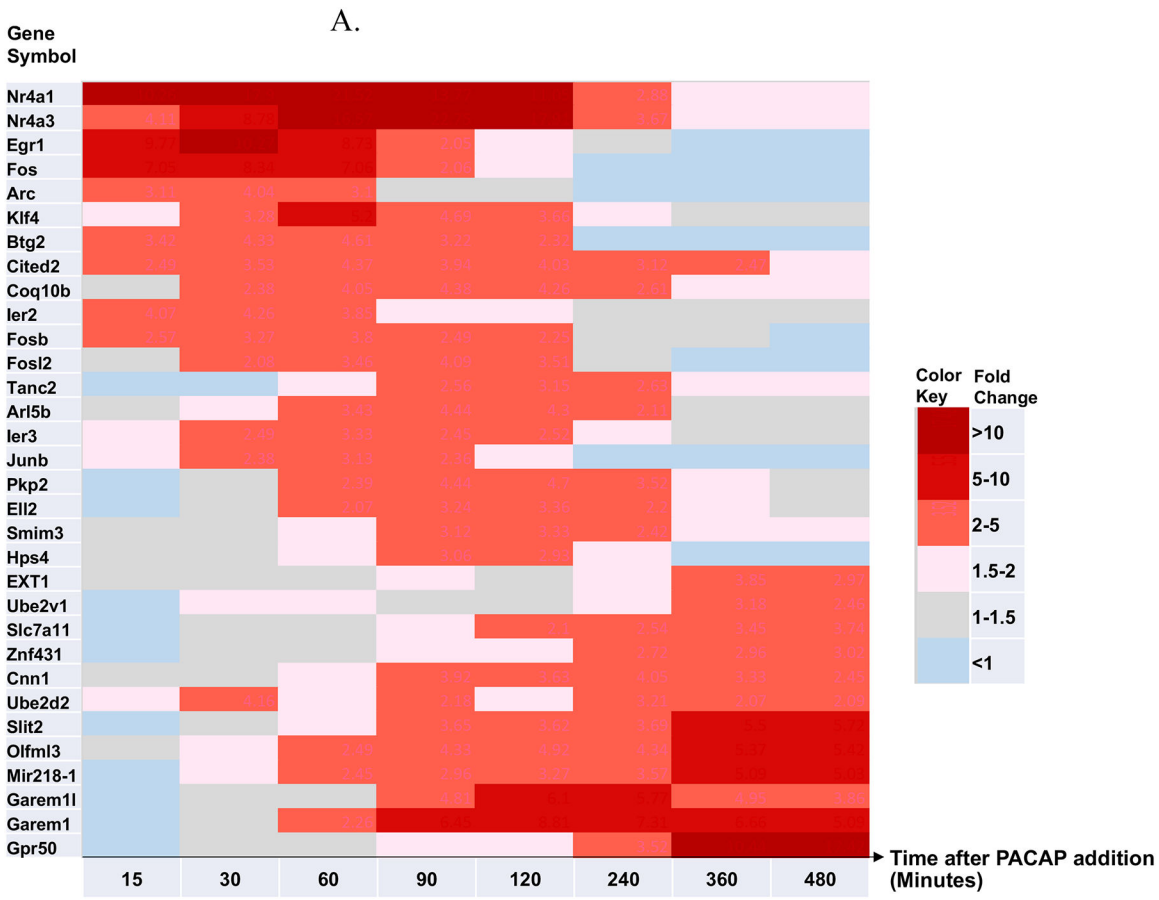


Figure 2. Phosphorylation of ERK is required to initiate neuritogenesis.

A. Phospho ERK elevation persists for hours after PACAP treatment. NS-1 cells were treated with 30 nM PACAP for 5 min, 10 min, 30 min, and hourly from 1 to 8 hours and phospho ERK level was measured using a cell-based ELISA assay. At time 0 media with or without PACAP was immediately removed after addition of PACAP (solid triangle in graph) or media (empty circle) and followed by fixation to prepare for ELISA assay. The OD_{450} reading for the wells with no addition of media or PACAP was set as basal. Phospho-ERK level was calculated as percent of basal. All values are means from triplicate wells (n=3) with error bars representing S.D. The experiment was repeated for at least three times. The statistical difference of the phospho-ERK between PACAP and media addition at corresponding times is indicated as ***p<0.001, **p<0.01 (5, 6, 7 hrs) or p=0.01 for 8 hrs. ns: not significant. **B**. PACAP-induced neuritogenesis is MEK but not PKA dependent. NS-1 cells were incubated with 10 μM MEK inhibitor U0126 or 30 μM PKA inhibitor H89 for 30 min and followed by 30 nM PACAP. Neurite length was measured at 48 hrs as described previously. n=3, S.D. is indicated as error bars. Double asterisks represent significantly different ($p < 0.01$) as compared with no inhibitor, ns: not significant. **C.** Inhibitor UO126 (10 μM) was added at time 0 (simultaneously with PACAP), 1, 8, 12, 24 and 48 hrs after addition of PACAP. Neurite length was measured for all treatments at 48 hrs after addition of PACAP. PACAP alone (red column) was used as a control. Each treatment was repeated three times (N=3), S.D. is indicated as error bars. * indicates $p < 0.05$.



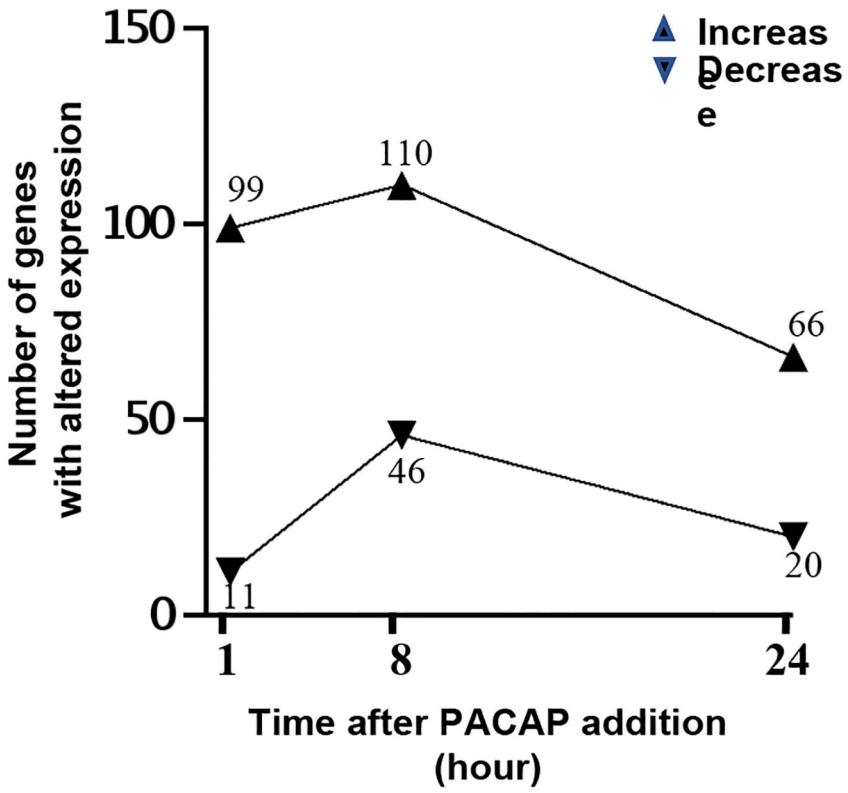
B.

Gene Symbol	Fold increase (versus no treat)		
	1 hr 8-CPT-cAMP		
	No inhibitor	UO126	H89
Nr4a1	10.5	6.9	9.0
Nr4a3	6.2	4.5	5.6
Sik1	4.7	4.2	4.4
Egr1	4.6	1.3	4.0
Fos	3.3	2.1	3.1
Mir29a	3.0	2.1	2.2
Cited2	2.8	2.4	2.5
Pde4d	2.6	2.6	2.4
Dusp1	2.5	2.8	2.5
Mir218-1	2.5	2.0	2.2
Pde4d	2.3	2.2	2.2
Klf4	2.3	1.9	2.1
Mir3565	2.2	1.7	1.8
Olr1387	2.1	1.7	1.4

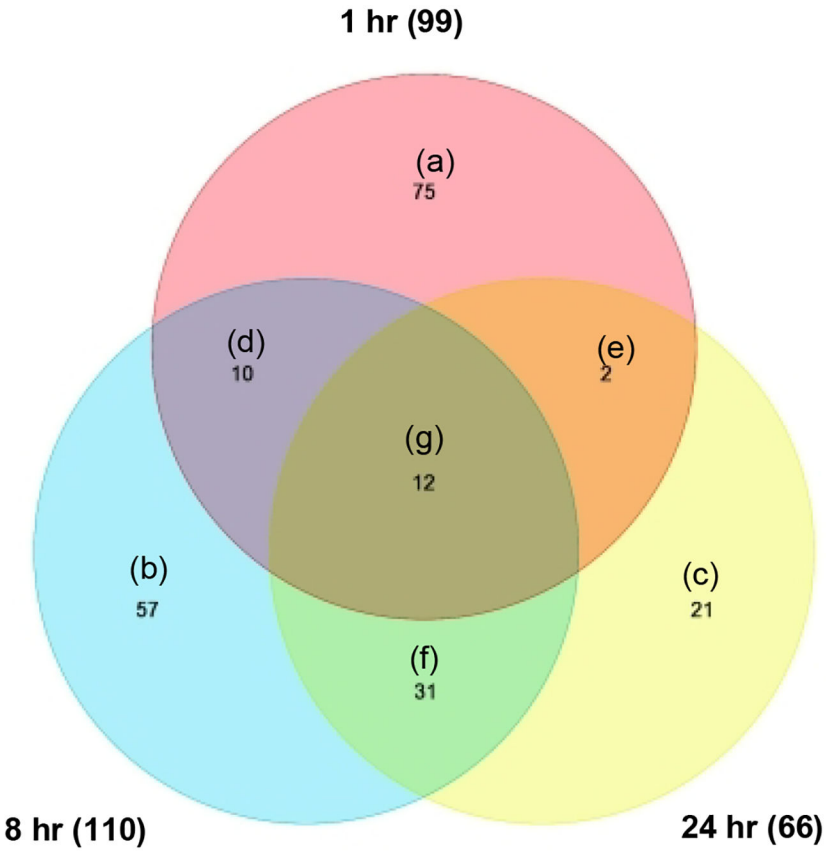
Figure 3. Temporal profile of PACAP gene induction over eight hours and signaling profile for early-regulated genes.

A. Gene expresson heatmap to identify the dynamic pattern of prominently upregulated genes across 0–8 hr time course after 100 nM PACAP treatment with Affymetrix Rat raGene 2.0 ST microarray analysis. Transcritpts regulated across the timespan were filtered for fold-regulation (>3-fold), direction of regulation (increased only) and incidence of regulation (up-regulated significantly in at least two instances across the timespan). **B**. List 14 early genes upregulated by 1 hr treatment of 8-CPT-cAMP (>2 fold, $P<0.05$) A. NS-1 cells were treated with 100 uM 8-CPT-cAMP with addition of no inhibitor (Vehicle), 30 μM U0126 or 10 μM H89 for one hr. Total RNAs were collected and subjected to Affymetrix Rat raGene 2.0 ST microarray analysis.

A.



B.



C.

(a) 1 hr of PACAP only

Gene Symbol	fold increase
Nr4a3	55.0
Nr4a1	39.9
Klf4	16.4
Fos	12.4
Fosb	9.5
Fosl2	9.2
Egr1	8.7
Arc	6.5
Csrnp1	6.0
Spata2L	5.2

(b) 8 hr of PACAP only

Gene Symbol	fold increase
Garem	4.4
Pnoc	3.4
Fmnl2	3.2
Olr1387	3.1
Sgms2	3.1
Lpo	3.1
Zcchc18	2.8
Igsf3	2.7
Serpinb1a	2.6
Prune2	2.6

(c) 24 hr of PACAP only

Gene Symbol	fold increase
Vtcn1	4.1
Adipoq	4.0
Lcn2	3.5
Plac1	3.2
Ehf	2.7
Tnfrsf22	2.5
Cym	2.5
Sidt1	2.4
Il18	2.4
Adora2b	2.3

(d) Both 1 and 8 hr

Gene Symbol	fold increase	
	1 hr	8 hr
Olfml3	8.6	7.3
Cga	6.2	2.4
Cnn1	3.9	4.4
Arl4d	3.9	2.3
Vgf	2.7	2.2
Mapkapk2	2.6	2.3
Gpat3	2.3	2.5
Gas1	2.3	2.8
Tslp	2.2	3.2
Ier3	2.0	2.0

(e) Both 1 and 24 hr

Gene Symbol	fold increase		
	1 hr	8 hr	24 hr
Trib1	3.3	1.8	2.4
Btg2	10.9	1.3	7.2

(f) Both 8 and 24 hr

Gene Symbol	fold increase	
	8 hr	24 hr
Gpr50	130.5	107.1
Npy	11.9	16.6
Sgpp2	4.5	5.7
Pde10a	7.3	4.1
Txndc5	4.8	4.1
Akr1b8	2.4	4.0
Dclk1	5.7	3.9
Vwa5a	3.1	2.9
slit2	3.1	2.7

(g) Genes upregulated through 24 hrs

Gene Symbol	fold increase		
	1 hr	8 hr	24 hr
Crem	15.1	7.4	5.2
Sik1	5.8	2.5	2.4
Plk2	5.3	4.6	5.0
ENSMUSG00000053580	4.5	2.1	2.3
Fosl1	4.2	3.2	2.6
Cebpb	4.0	2.3	2.6
Rhob	3.1	2.5	2.0
AABR07008066.2	3.0	3.1	2.6
Nabp1	2.5	2.2	2.9
Pde4d	2.5	2.6	2.2
LOC100360552	2.0	2.3	2.3
PVR	2.0	2.5	2.1

Figure 4. Summary of temporally differentially expressed genes at different times after PACAP addition.

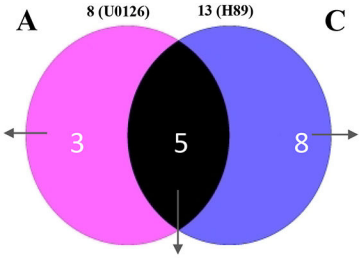
A. Total number of genes up up- and down-regulated at each time point after initiation of PACAP exposure. **B**. Venn diagram shows overlap of genes upregulated at 1 hr, 8 hr or 24 hr after PACAP addition compared to no treatment (fold increase >2, $P<0.05$). Seven domains are labeled from (a) to (g) to show number of upregulated genes overlapped between different time points. **C**. Most highly expressed genes (>2 fold, $P<0.001$) in each of the seven overlapping domains depicted in Venn diagram.

B

Genes upregulated by PACAP and blocked by U0126 only

Gene Symbol	Hours after addition of PACAP				
	1 hr	8 hr	24 hr		
			Vehicle	UO126	H89
Dclk1	1.3	5.7	3.9	1.9	3.0
Tnfrsf22	1.8	1.9	2.5	1.2	2.1
Dlg2	-1.1	2.7	2.5	1.2	2.3

A



C

Genes upregulated by PACAP and blocked by H89 only

Gene Symbol	Hours after addition of PACAP				
	1 hr	8 hr	24 hr		
			Vehicle	UO126	H89
Figf	-1.1	1.7	2.3	1.8	-2.8
Crem	15.1	7.4	5.2	2.7	1.3
Slit2	1.9	3.1	2.7	2.7	-1.1
Trpc6	1.0	1.7	2.3	3.7	-1.3
Ehf	1.1	1.6	2.7	6.2	1.0
Il18	1.1	1.4	2.4	5.6	-1.0
Plac1	1.1	1.4	3.2	10.9	1.4
Abcb1b	1.0	1.2	2.2	1.8	-1.0

D

Genes upregulated by PACAP and blocked by both U0126 and H89

Gene Symbol	Hours after addition of PACAP				
	1 hr	8 hr	24 hr		
			No inhibitor	UO126	H89
Gpr50	2.0	130.5	107.1	2.4	19.7
Npy	1.6	11.9	16.6	7.2	4.9
Sgpp2	-1.1	4.5	5.7	1.1	1.4
Txnde5	1.7	4.8	4.1	1.7	1.2
Adipoq	1.4	1.7	4.0	1.1	2.0

Figure 5. Signaling profile for late PACAP-regulated transcripts.

PACAP-induced gene upregulation at 24 hr downregulated significantly by either U0126 or H89 (gene downregulation >2 fold, $P<0.05$ compared to no inhibitor at 24 hr). NS-1 cells were treated with PACAP for 24 hours with addition of no inhibitor (Vehicle), 30 μM U0126 or 10 μM H89. Total RNA were collected and subjected to microarray analysis. **A**. Venn diagram shows overlap of genes upregulated after 24 hr of PACAP addition and downregulated by both U0126 and H89 for at least 2-fold compared to no inhibitor. **B**, **C**, and **D**: specific fold changes compared to no treatment (vehical control) for genes in Venn diagram.

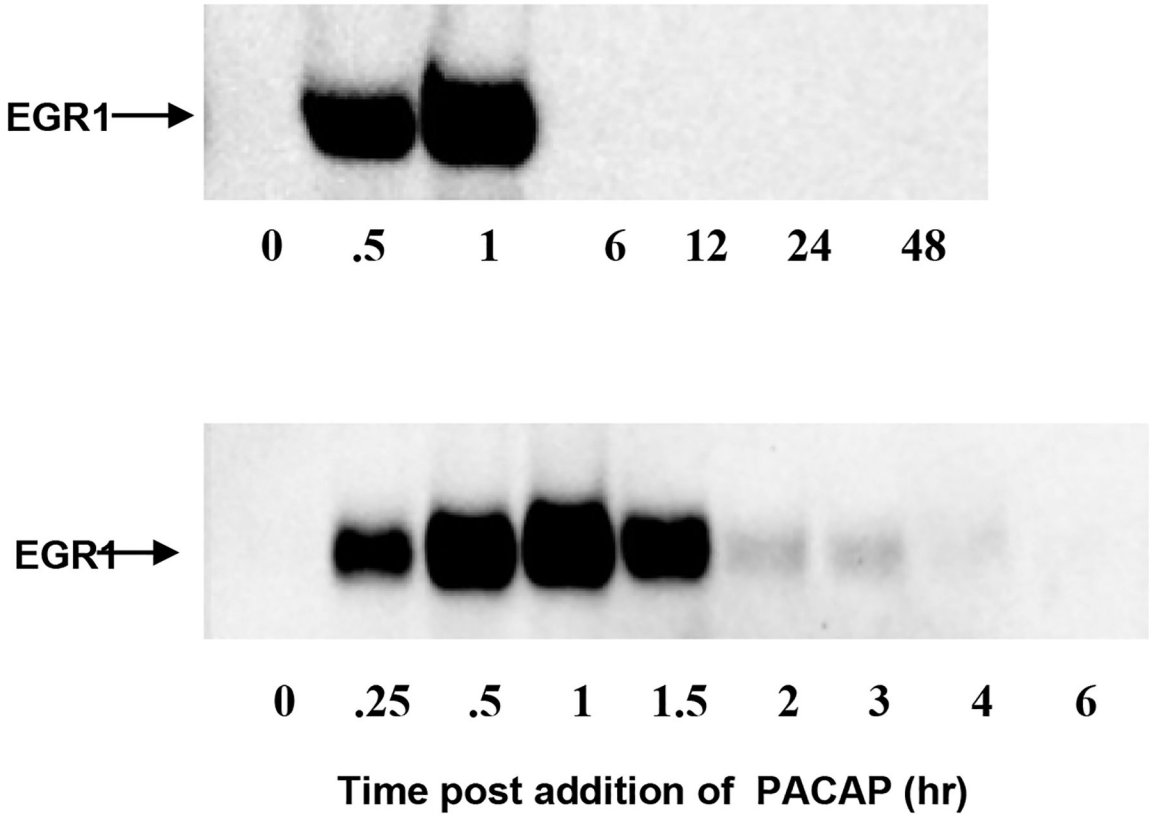


Figure 6. Time course of EGR1 protein induction following PACAP treatment in NS-1 cells.

EGR1 protein expression in NS-1 cells induced by 30 nM PACAP was evaluated by western blot at time 0, 30 min, 1, 6, 12, 24 and 48 hours after PACAP addition (top panel). More detailed temporal induction of EGR1 protein within 6 hrs PCAP treatment was shown in bottom panel. 20 ug of total protein was loaded in each well. A representative blot of three experimental repeats with similar results is shown.

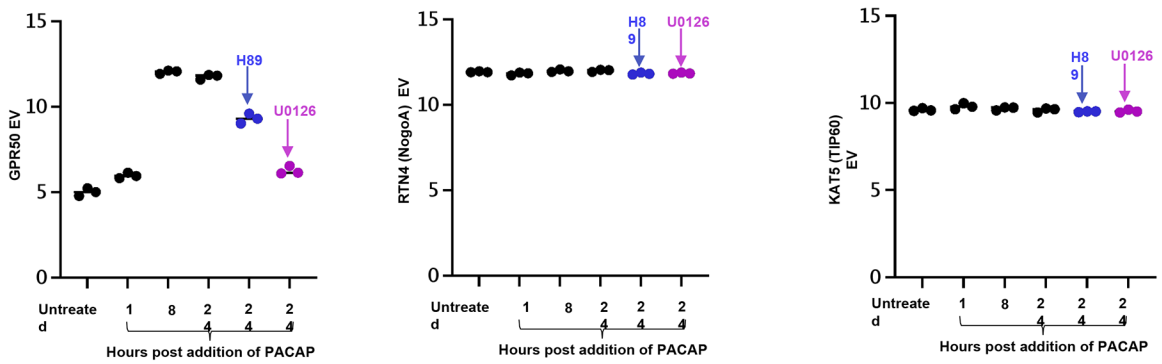
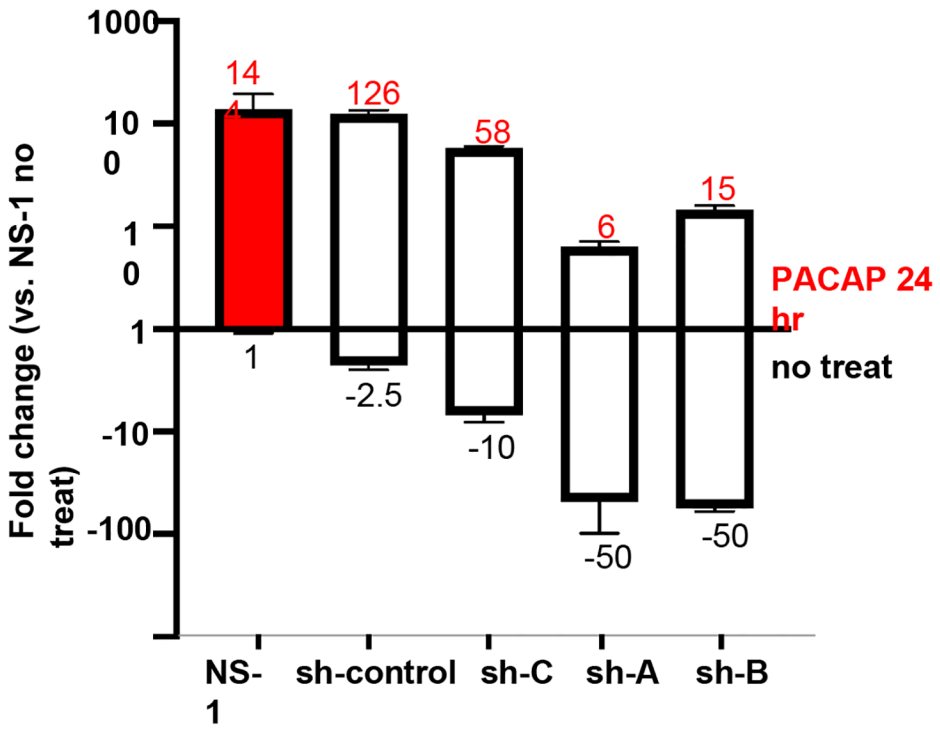


Figure 7. GPR50 does not require regulation of NOGO-A or TIP 60 gene expression to alter neurite outgrowth.

The scatter dot plots show the expression values (EVs) for GPR50, RTN4 or KATS transcripts derived from NS-1 cell Clariom-S rat microarray analysis. EVs refer to rank order of expression of a given transcript relative to all other expressed transcripts in transcriptome of sample. Individual EVs for each of the triplicate samples are shown.

A.



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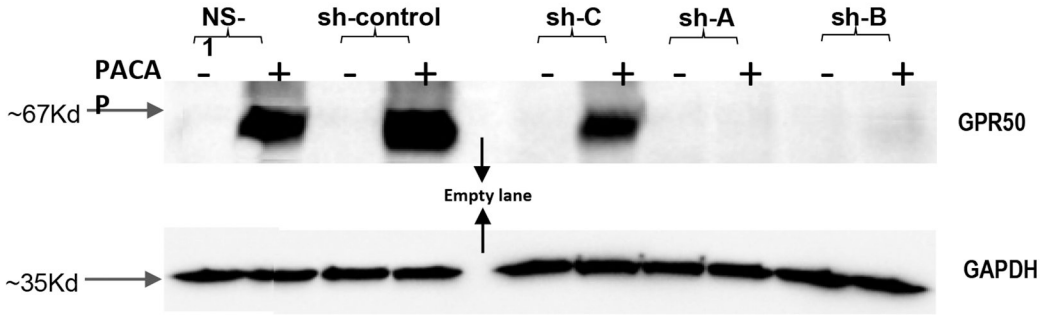

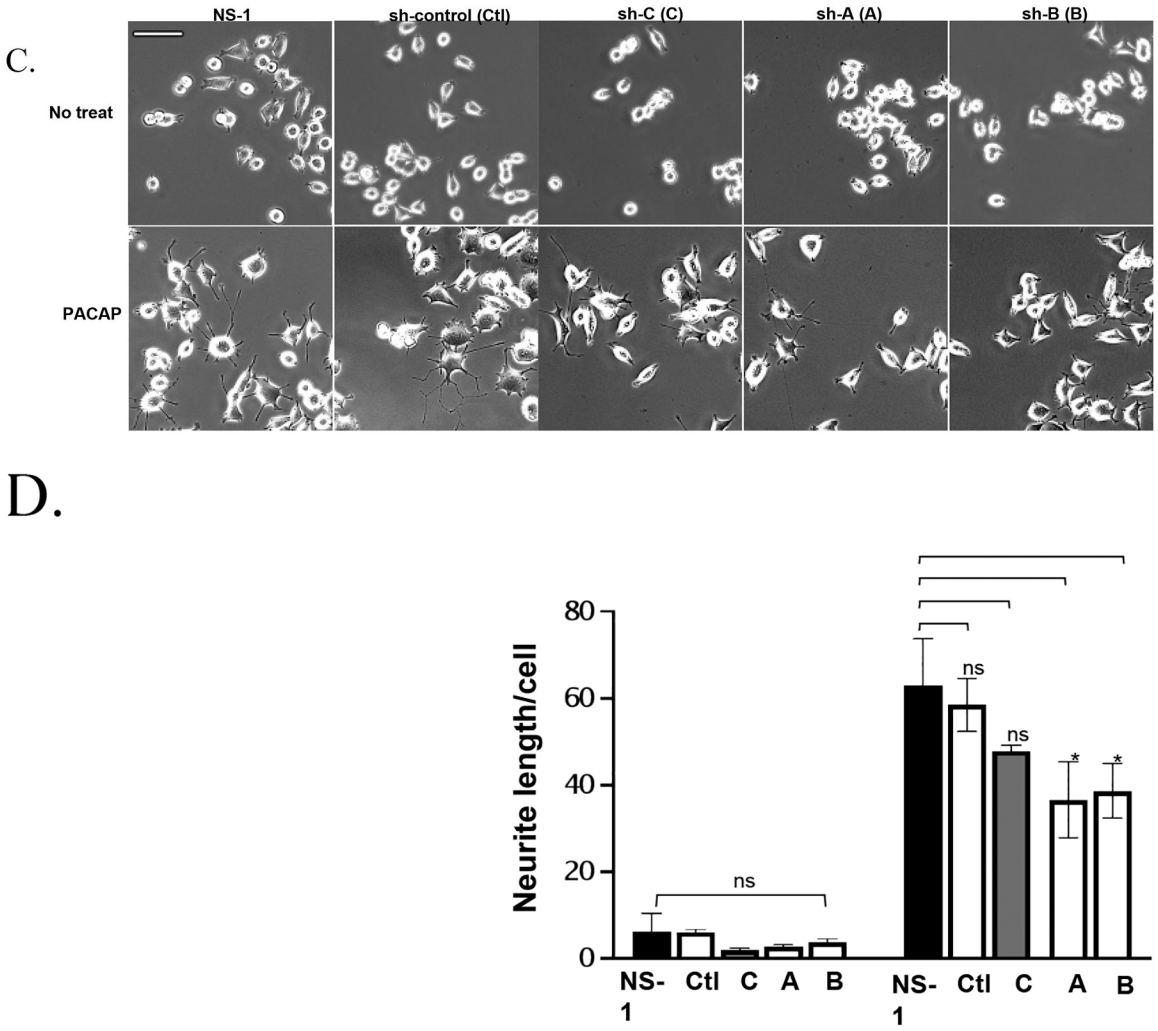

Figure 8. Knockdown of GPR50 mRNA in NS-1 cells is correlated with decreased PACAP induced GPR50 expression and neuritogenesis.

A. Short hairpin (sh) RNAs targeting GPR50 knock down GPR50 mRNA expression in NS-1 cells. Three constructs (sh-A, B and C) targeting rat GPR50 and one scrambled shRNA control (sh-control) were expressed in NS-1 cells individually using a lentiviral vector as the delivery vehicle. Cells stably expressing each shRNA were treated with PACAP for 24 hours. Relative GPR50 mRNA expression in each cell line with or without PACAP treatment was assessed using qRT-PCR. Negative numbers below the grey bars corresponding to each shRNA-transduced NS-1 cell lines (sh-control, sh-C, sh-A, and sh-B) indicate the level of GPR50 knockdown by shRNAs relative to untransduced NS-1 cells, where fold change i.e. no change is set to 1. Positive numbers above the red bars represent fold increase of GPR50 after PACAP treatment for each cell line. represents 50 μm. **B**. Reduced PACAP- induced GPR50 protein expression pattern in sh-C, sh-A, and sh-B cell lines is consistent with respective reduced transcriptional expression patten. GPR50 protein expression in each cell line with or without PACAP treatment for 24 hrs was assessed using western blot. GAPDH expression is used as loading control. The experiment is repeated for three times with similar results. **C**. Representative images of neurite formation for

each cell line with or without PACAP treatment are shown. The scale bar in the top left panel represents 50 μm. **D**. Expression GPR50 shRNA constructs A and B in NS-1 cells significantly inhibits PACAP-stimulated neurite formation. Representative images of neurite formation for each cell line with or without PACAP treatment are shown. Data are presented as mean ± SD. N=3. *$p < 0.05$. ns: not significant.

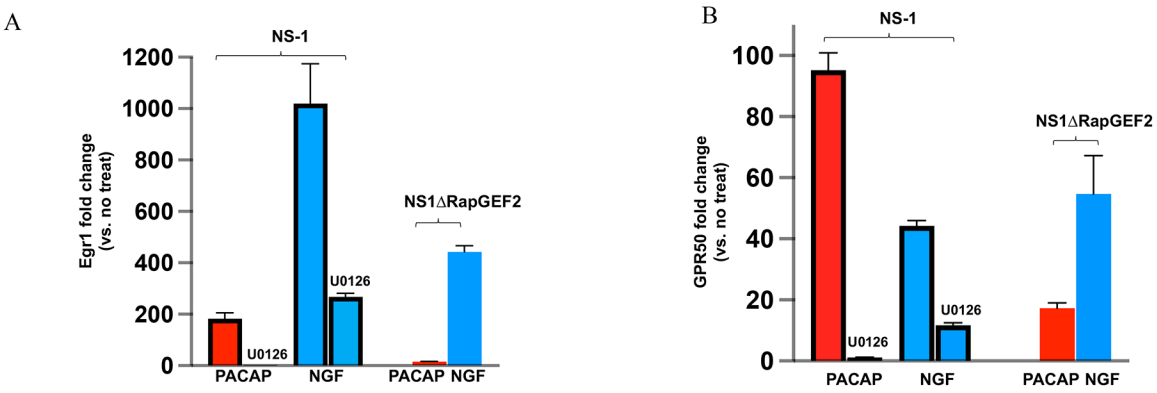

Figure 9. Rapgef 2 deletion blocks PACAP- but not NGF-induced ERK-dependent early and late gene expression.

NS1 and NS1ΔRapGEF2 cells were first treated with MEK inhibitor U0126 (10 μM) or vehicle control for 30 minutes, then cells with or without inhibitor were both treated with 30 nM PACAP or 100 ng/ml NGF. Relative Egr1 mRNA expression after one hour of PACAP or NGF treatment (Fig. A) and GPR50 expression (Fig. B) after 24 hrs of PACAP or NGF in each cell line was assessed using qRT-PCR with gene-specific primers. The normalized fold change ± SD (n=3) was calculated using the delta-delta Ct method with GAPDH as an internal control gene. PACAP- or NGF- indcued Egr1/GPR50 fold change in Y axis was obtained by comparing to vehicle control (no treat) in corresponding cell line. Red bars represent PACAP treatment; while blue bars represent NGF treatment.

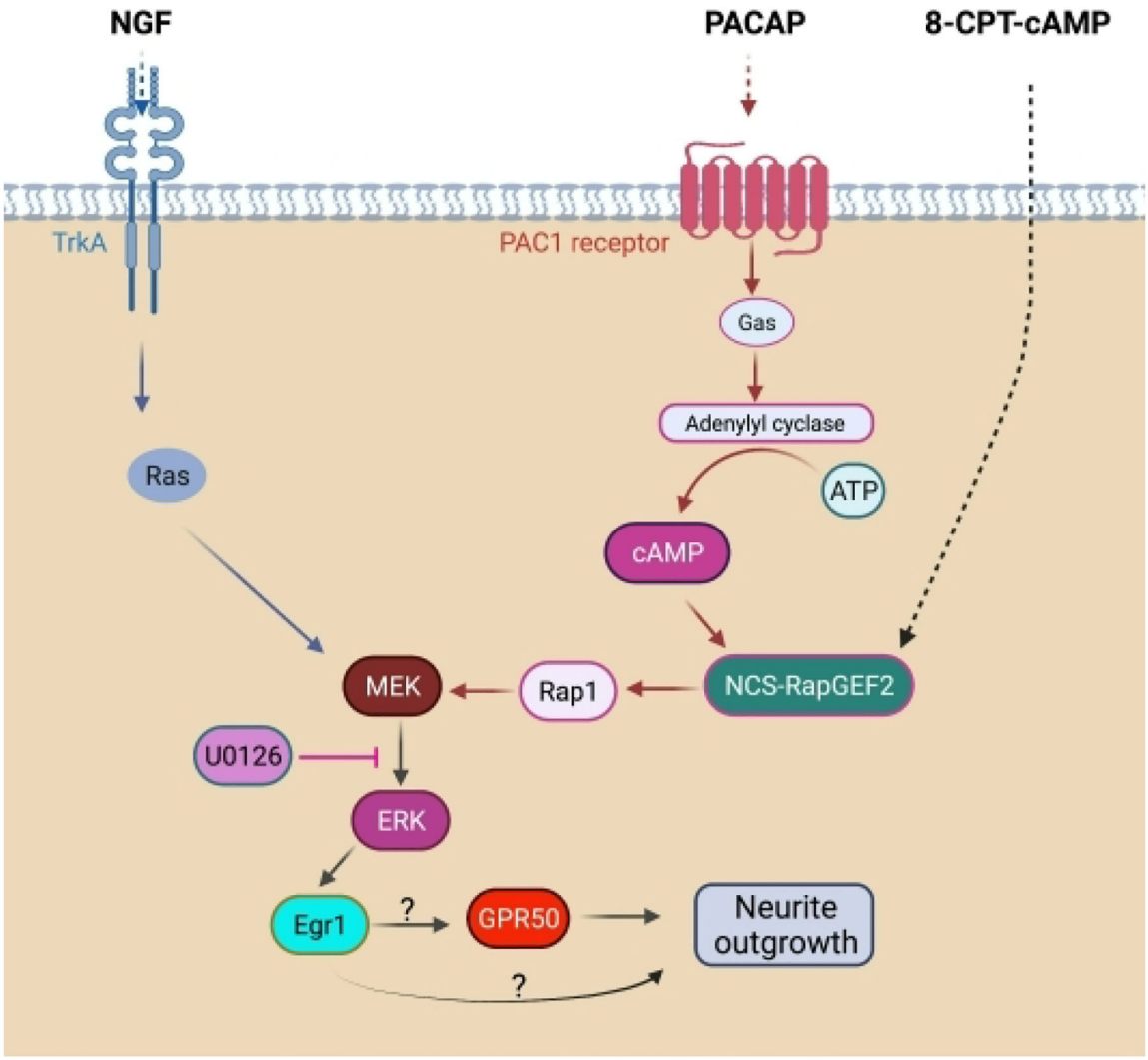

Figure 10. Neuropeptide PACAP and neurotrophin NGF activate ERK to induce neuritogenesis through different signal pathways.

Pathways depict alternative hypotheses for stepwise activation of neuritogenesis by convergent Gs-GPCR- or Trophin receptor kinase (Trk)-initiated signaling.