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Cyclic AMP-dependent activation of ERK via GLP-1 receptor signalling requires the neuroendocrine cell-specific guanine nucleotide exchanger NCS-RapGEF2

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Abstract

Cyclic AMP activation of the Rap-Braf-MEK-ERK pathway after signalling initiated by the neuropeptide pituitary adenylate cyclase-activating peptide (PACAP), via the G_s-protein coupled receptor (G_sPCR) PAC1, occurs uniquely through the neuritogenic cAMP sensor Rap guanine nucleotide exchange factor 2 (NCS-RapGEF2) in Neuroscreen-1 (NS-1) neuroendocrine cells. We examined the expression of other Family B G_sPCRs in this cell line and assessed cAMP elevation and neuritogenesis after treatment with their cognate peptide ligands. Exposure of NS-1 cells to the VIPR1/2 agonist vasoactive intestinal polypeptide, or the GLP1R agonist exendin-4, did not induce neuritogenesis, or elevation of cAMP, presumably as a result of insufficient receptor protein expression. Vasoactive intestinal polypeptide and exendin-4 did induce neuritogenesis after transduction of human VIPR1, VIPR2 and GLP1R into NS-1 cells. Exendin-4/GLP1R-stimulated neuritogenesis was MEK-ERK-dependent (blocked by U0126), indicating its use of the cAMP→RapGEF2→ERK neuritogenic signalling pathway previously identified for PACAP/PAC1 signalling in NS-1 cells. NCS-RapGEF2 is expressed in the rodent insulinoma cell lines MIN6 and INS-1, as well as in human pancreatic islets. As in NS-1 cells, exendin-4 caused ERK phosphorylation in INS-1 cells. Reduction in RapGEF2 expression after RapGEF2-shRNA treatment reduced exendin-4-induced ERK phosphorylation. Transcriptome analysis of INS-1 cells after 1 hour of exposure to exendin-4 revealed an immediate early-gene response that was composed of both ERK-dependent and ERK-independent signalling targets. We propose that cAMP signalling initiated by glucagon-like peptide 1 (GLP-1) in pancreatic beta cells causes parallel activation of multiple cAMP effectors, including NCS-RapGEF2, Epac and protein kinase A, to separately control various facets of GLP-1 action, including insulin secretion and transcriptional modulation.

KEYWORDS
ERK, GLP-1, GLP1R, GPCR, NCS-Rapgef2, neuropeptides

1 | INTRODUCTION

G_s-protein coupled receptors (G_sPCRs) act as receptors for first messengers (neurotransmitters, incretins and other hormones and autocrine factors) by causing elevation of cAMP in ligand-responsive cells.[1,2] Although, previously, it had been assumed that cAMP signals are propagated within the cell solely via the cAMP-dependent protein kinase A (PKA), it is now known that there are also cAMP-responsive guanine nucleotide exchange factors (cAMP-GEFs) that can transduce cAMP elevation into intracellular responses following first messenger stimulation.[3-5] These findings raise important questions about which of the downstream responses to cAMP elevation are mediated by PKA and which are mediated by these newly identified GEF cAMP sensor/effectors, in a given cell type, as well as by a given G_sPCR.

For example, in the pheochromocytoma (chromaffin) cell line PC12, and a congener cell line, Neuroscreen-1 (NS-1), G_sPCR activation results in a tripartite cAMP-dependent response, leading in aggregate to cell differentiation, which includes growth arrest (mediated through the GEF Epac), neurone-specific gene expression and cell survival (mediated through PKA) and neurite extension (neuritogenesis) through a non-PKA, non-Epac signalling pathway.[6] It has subsequently been demonstrated that cAMP-dependent stimulation of neurite formation, through the Rap-Braf-MEK-ERK pathway after signalling initiated by the neuropeptide pituitary adenylate cyclase-activating peptide (PACAP) via the G_s-coupled G protein-coupled receptor PAC1, occurs uniquely through the neuritogenic cyclic AMP sensor-Rap guanine nucleotide exchange factor (NCS-RapGEF2) in neuroendocrine cells.[5-7] The present studies aimed to investigate the possibility that other family B receptors might share this signalling capability with the PAC1 receptor, and therefore that RapGEF2 might play a role in the actions of the hormones and neurotransmitters that engage these receptors, including parathyroid hormone (PTH) (receptors PTHR1 and 2), secretin (receptors SCRT1 and 2), calcitonin (CALC) (receptor CALCR), calcitonin gene-related peptide (receptor CALCRL-RAMP1), adrenomedullin (receptor CALCRL-RAMPs2/3), amylin (receptor CALCR-RAMPs1-3), glucagon (receptor GCGR), gastrin-inhibitory peptide (GIP) (receptor GIPR), vasoactive intestinal polypeptide (VIP) (receptors VIPR1 and 2), corticotrophin-releasing hormone (CRH) (receptors CRHR1 and 2), glucagon-like peptide 1 (GLP-1) (receptor GLP1R), glucagon-like peptide 2 (GLP-2) (receptor GLP2R) and growth hormone-releasing hormone (GHRH) (receptor GHRHR).[8,9]

Cyclic AMP-dependent ERK activation by GLP-1, in particular, could play an important role, alongside cAMP-dependent signalling through Epac and protein kinase A, in multi-faceted signalling required for GLP-1 modulation of glucose-stimulated insulin secretion and other functions of pancreatic beta cells.[10-23] However, the signalling components linking GLP-1 receptor engagement by GLP-1, as well as ERK activation in beta cells and insulinoma cell lines, has not been investigated. To determine whether or not the NCS-RapGEF2 pathway leading to persistent ERK activation might co-exist along with the cAMP-PKA and/or Epac pathways leading to transient ERK activation under physiologically normal conditions, we examined the role of NCS-RapGEF2 in signalling to ERK following GLP-1 receptor engagement in cellula, under conditions in which metabolic activities and functions typical of beta cells in vivo might be expected to occur.

2 | MATERIALS AND METHODS

2.1 | Drugs used

Exendin-4 and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (UO126) were purchased from Tocris (Bristol, UK). PACAP-38, glucagon,[1-29] secretin, GIP and PTH[1-34] were purchased from AnaSpec (Fremont, CA, USA). Urocortin was purchased from Sigma (St Louis, MO, USA). Drug stocks were prepared in accordance with the manufacturer's instructions, either in assay buffer or dimethysulphoxide (DMSO). Final concentrations of DMSO in cell culture experiments did not exceed 0.1%, and a 0.1% DMSO vehicle control was used whenever appropriate.

2.2 | Cell culture

Cell culture reagents were purchased from Thermo Fisher (Waltham, MA, USA), if not otherwise noted. All cell culture media contain 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.

INS-1 (rat insulinoma) cells were maintained in RPMI-1640 media supplemented with 2 mmol L^{-1} glutamine, 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 1 mmol L^{-1} sodium pyruvate; 10 mmol L^{-1} Hepes and 50 μmol L^{-1} β-mercaptoethanol. The RPMI media (catalogue #21087076; Thermo Fisher) contains 11.1 mmol L^{-1} D-glucose.

NS-1 cells were maintained as described previously[5] and grown in RPMI-1640 media supplemented with 2 mmol L^{-1} glutamine, 10% horse serum and 5% heat-inactivated FBS. Tissue culture flasks or plates were treated with collagen-1 (rat tail) for culturing NS-1 cells.

Human embryonic kidney (HEK) 293 and 293T cells were grown in Dulbecco's modified Eagle's medium with 10% FBS and 2 mmol L^{-1} glutamine.

2.3 | Establishment of stable cell lines

Neuroscreen-1 cells expressing respective human GPCR were generated by transducing NS-1 cells with a lentiviral vector (pLX304) encoding a human glucagon-like peptide-1 receptor (GLP1R; GenBank accession no. L23503), or a human vasoactive intestinal peptide receptor 1 (VIPR1; GenBank accession no. NM_004624.3), or a human VIPR2 (BC010569). The lentiviral vectors expressing different GPCRs were purchased from GeneCopoeia (Rockville, MD, USA) and all contain a blasticidin resistance gene (*bsr*) co-expressing with the individual GPCR gene. After transduction with individual lentiviral vector, the stable GPCR-expressing cell line was established

by selection with 3 μg mL^{-1} blasticidin for 2 weeks. Construction of $RapGEF2^{-/-}$ NS-1 cells has been described previously.[24]

INS-shRapgef2 cells were obtained by transducing INS-1 cells with psi-Lv-H1 lentiviral vector (GeneCopoeia) encoding shRNA construct (target sequence: GGAAGTCATTAACCAGGAA). This shRNA construct has been shown to specifically knock down Rapgef2 expression in NS-1 cells.[5] After 48 hours of transduction, INS-1 cells stably expressing shRapgef2 were selected with 1 μg mL^{-1} puromycin for 2 weeks.

HEK293_CBS and HEK293_CBS_hVIPR1, hVIPR2 and hGLP1 cells were created and maintained as described previously .[25]

2.4 | Neuritogenesis assay

Cells were seeded in six-well plates overnight. On the following day, cells were first treated with the relevant antagonist for 30 minutes before adding the respective agonist. The compound solvent was added as vehicle to control wells. After 72 hours, 20× micrographs were obtained automatically from fixed locations within each culture well, and neurite length and/or percentage of neurite-bearing cells was assessed by a rater who was blinded to the treatment plan for each plate.

2.5 | Western blotting

Cells were grown in six-well plates overnight and treated the following day with agonist or vehicle for 10 minutes if needed, then cell lysates were collected in ice-cold RIPA buffer (Pierce, Rockford, IL, USA) supplemented with HALT protease inhibitor and phosphatase inhibitor cocktails (Pierce). Human pancreatic islet (NDRI donation index number RRID SAMN17367763 and IIDP donor ID 986) and MIN6 cell lysates were prepared with Passive Lysis Buffer (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Total protein concentration was determined with a BCA kit in accordance with the manufacturer's instructions (Thermo Fisher). Western blotting was performed as described previously.[5] Briefly each lysate with 20 μg of total protein was loaded on 4%-20% Bis Tris gradient gels (Thermo Fisher) with 4× loading buffer and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis electrophoresis, then transferred onto nitrocellulose membranes. Afterwards membranes were blocked for 2 hours using 5% non-fat milk. For detection of individual protein, membranes were incubated overnight at 4°C with the respective primary antibody at a dilution of 1:1000 for each antibody. Rabbit anti-RapGEF2 primary antibody was generated in our laboratory as described previously.[24] Primary antibodies for β-actin, pERK (Thr202/Tyr204) and total ERK were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-coupled secondary antibodies were also purchased from Cell Signaling Technology. After incubation with secondary antibody membranes were washed with TBST for five time. Immunoreactive bands were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce) and visualised using a cooled charge-coupled device camera.

2.6 | cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR)

Human brain, lung and kidney total RNA was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). cDNA synthesis was performed using SuperScript III First-Strand Synthesis System (Thermo Fisher) with 1.6 μg of total RNA. To amplify each specific Rapgef2 transcript from cDNA, PCR was performed using the Takara PrimeSTAR HS DNA polymerase (Takara, Otsu, Japan) at an annealing temperature of 58°C. Sense primer, 5'-TGAGGGAGCATCAACTTAGGT-3', specific to the exon 1, or 5'-GTTCGGATTCCTTTTCTGGTC-3', specific for exon 1', was used in PCR with antisense primer 5'-CCAAGAGTTGTTCAATGTCATCA-3', which is complementary to sequences spanning the exon 2/3 boundary. The specificity of these primers has been confirmed in previous studies.[24]

2.7 | GloSensor cAMP assay

HEK293 and NS-1 cells stably expressing a cAMP biosensor in a retroviral vector pLHCX after hygromycin selection was used for the real-time measurement of the cAMP level in live cells as described previously.[25] HEK293_CBS and NS-1_CBS cells expressing, or not expressing, individual hGPCRs (VIPR1, VIPR2 or GLP1R) were seeded at a density of approximately 10,000 cells per well in 96-well plates in CO_2-independent media (Thermo Fisher) as plating media (PM) with supplements for each cell line as described above for cell cultures. The next day, cell media were aspirated and fresh substrate-containing media (PM supplemented 1.5 mg mL^{-1} D-luciferin) was added. Following 2 hours of incubation in a dark background at room temperature, luciferase activity was measured using a Glomax luminometer (Promega). VIP, exendin-4 or other peptides were then added, and real-time chemiluminescent readings were obtained continuously for 30 minutes. Luminescence intensity peaked at approximately 20 minutes following agonist addition and the luminescence at this time (in arbitrary units) was used for the dose-response analysis.

2.8 | Microarray analysis

Total RNA was extracted using miRNA extraction kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Microarray analysis was conducted in the NHGRI-NINDS-NIMH Microarray Core (Abdel Elkahloun, Director; NHGRI, Rockville, MD, USA) using its established protocols and employing RNA samples with a RIN (ie, RNA integrity number) of eight or higher and Clariom-S rat microarray chips (Affymetrix, Santa Clara, CA, USA),

with post-hybridisation, normalisation and statistical analysis as described previously .[26] Partek Genomic Suite software (Partek Inc., St Louis, MO, USA) was used to determine the differential expression of genes. The original CEL data files for all microarrays are available upon request.

3 | RESULTS

3.1 | Expression of family B GPCRs in the NS-1 neuroendocrine cell line

The expression of Family B GPCRs was inventoried in NS-1 cells, along with receptor-associated modulator proteins (RAMPs) using microarray analysis. The expression value (EV) for a given transcript, in a microarray analysis, ranks the abundance of a particular transcript relative to all other mRNA transcripts expressed by the cell (Figure 1). Higher expression values indicate a relatively greater expression of the cognate mRNA, and presumably the protein. Expression values for family B GPCRs ranged from less than six (dotted line in Figure 1), which represents a signal for transcript abundance near background for the microarray assay, to more than nine, indicating robust expression. GLP-1 receptor has an EV of 5.69, indicating a low level or lack of RNA in NS-1 cells, similar to Vipr2 with an EV of 5.61. Vipr1, as the cognate receptor for VIP together with Vipr2, has an EV of 7.31. Whether the expression level of these receptors is sufficient to bind to cognate peptide to induce signal transduction has not been established; thus, the EV ranking serves to guide, rather than establish, an expectation as to whether or not a given receptor protein is likely to be expressed, and functionally active, in a given cell type or tissue.

3.2 | Induction of neuritogenesis and elevation of cAMP by family B Gs-coupled GPCRs in NS-1 cells

Neuritogenesis induced by cAMP by the Gs-coupled GPCR PAC1 is abrogated in RapGEF2$^{-/-}$ NS-1 cells, whereas non-cAMP-dependent neuritogenesis induced by NGF is not (Figure 2). Because ERK activation, through NCS-RapGEF2, is obligatory for cAMP-dependent neuritogenesis in NS-1 cells, we were able to employ neuritogenesis as a functional assay to determine whether a given receptor was functionally linked to ERK activation upon exposure of cells to the cognate ligand. By this criterion, the family B G_sPCRs, GLP1R1, GCGR, VIPR1, VIPR2, PTH1R, PTH2R, CRHR1, CRHR2, SCRT1, SCRT2 and GIPR, are either disengaged from ERK activation and

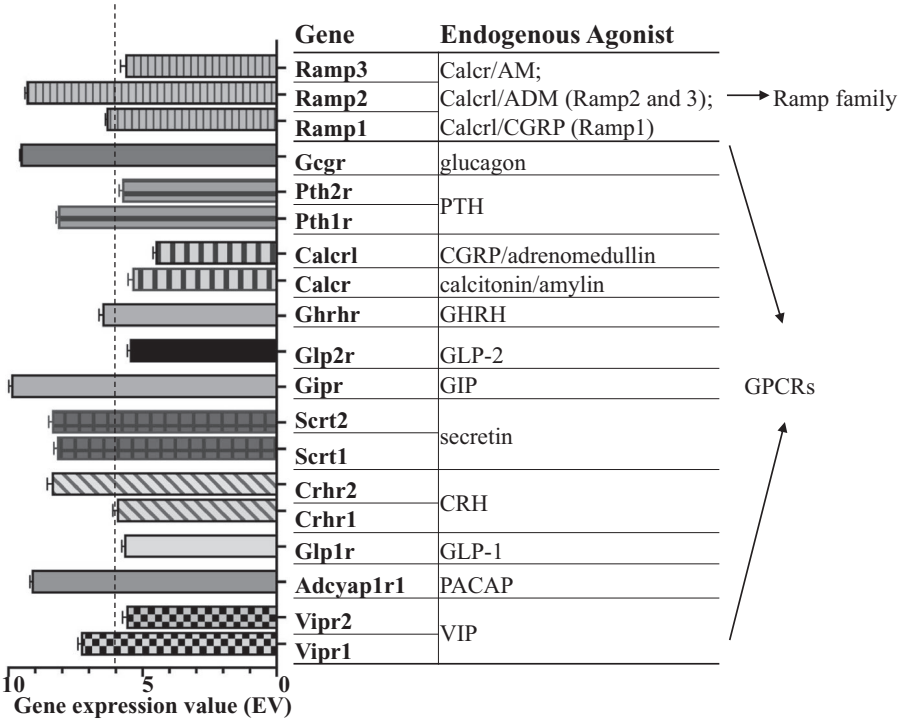


FIGURE 1 Expression of family B GPCRs in NS-1 cells. The bar graph on the left shows the expression values (EVs) for transcripts derived from NS-1 cell Clariom-S rat microarray analysis. EVs refer to rank order of expression of a given transcript relative to all other expressed transcripts in transcriptome of sample. EVs shown are the mean of three separate determinations (NS-1 cell total RNA isolates). Relative expression in NS-1 cells of transcripts for receptor activity-modifying proteins (RAMPs), necessary for membrane expression of receptor, ligand recognition and ligand-receptor coupling of some family B GPCRs (Calcrl, Calcr), is also shown. GPCRs are grouped together with similarly marked bars according to similarity of structure, as well as function in endocrine cells. AM, amylin; ADM, adrenomedullin; CGRP, calcitonin gene-related peptide; CRH, corticotrophin-releasing hormone; GHRH, growth hormone-releasing hormone; GIP, gastrin-inhibitory peptide; GLP, glucagon-like peptide; GPCR, G protein-coupled receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; PTH, parathyroid hormone; RAMP, receptor-associated modulator protein; VIP, vasoactive intestinal polypeptide

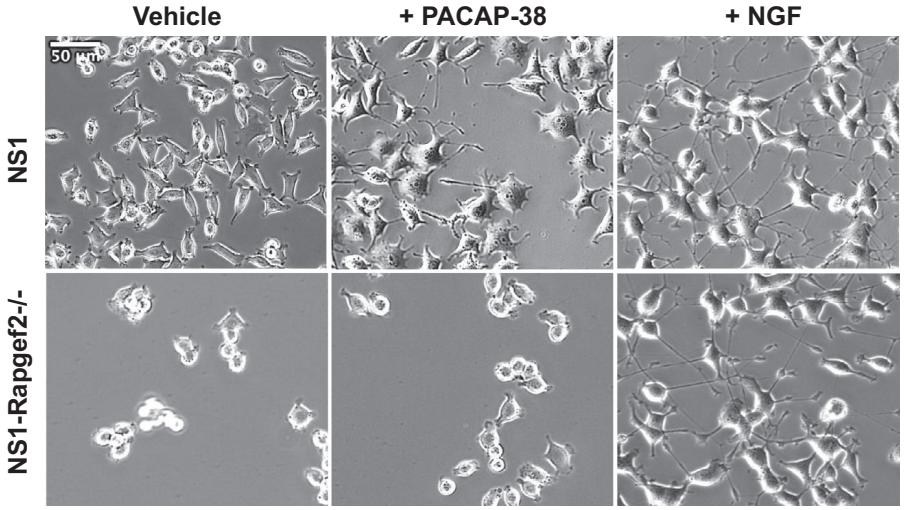


FIGURE 2 Gs-coupled GPCR-induced neuritogenesis, but not NGF-induced neuritogenesis, is abolished in RapGEF2 gene-deleted NS-1 cells (Rapgef2$^{-/-}$). Representative micrographs from three separate experiments (wells) treated with vehicle, 100 ng mL^{-1} NGF or 100 nmol L^{-1} PACAP-38

neuritogenesis in NS-1 cells, or expressed at too-low levels to support receptor function along this pathway in NS-1 cells (Figure 3A). A positive control for G_sPCR-mediated neuritogenesis is represented by the PAC1 receptor (expression value 9.14 in NS-1 cells) (Figure 3A,B), confirming previous results with both PACAP-38 and the other endogenous ligand for the PAC1 receptor, PACAP-27.[25] The same battery of ligands for these receptors was used to challenge NS-1_CBS cells, in which a biosensor for cAMP generation allows detection of Gs→adenylate cyclase engagement by ligand-occupied G_sPCR.[25] Only GCGR and GIPR supported cAMP elevation at supramaximal concentrations of ligand (Figure 2C), suggesting that other receptors were not functionally expressed in NS-1 cells, and also that GIPR and GCGR may be functionally engaged, but unable to generate cAMP for a sufficient duration or of a sufficient concentration that allows sustained activation of NCS-RapGEF2 required for neuritogenesis.

3.3 | Family B GPCR receptor engagement in cAMP elevation in HEK293 cells and neuritogenesis in NS-1 cells after transduction of hGPCRs

The results above indicate that VIPR1, VIPR2 and GLP1 receptors are insufficiently expressed in NS-1 cells to support cAMP elevation sufficient to drive neuritogenesis. Therefore, we transduced HEK293 and NS-1 cells with VIPR1R, VIPR2R or GLP1R using a lentiviral vector that increases expression levels to those comparable to endogenously expressing cells (ie, to provide stoichiometrically appropriate coupling to cellular G-proteins). A dose-dependent increase of cAMP levels in HEK293_CBS cells occurred in response to treatment with VIP after expression of hVIPR1 and hVIPR2, and by treatment with exendin-4 after expression of hGLP1R (Figure 4A). Correspondingly, increased expression of VIPR1, VIPR2 or GLP1R led to acquisition of neuritogenic activity of VIP through either of the VIPRs, as well as to the GLP1R agonist exendin-4, through GLP1R expression (Figure 4B,C). Thus, the VIPR1/2 and GLP1R are able to support cAMP-dependent neuritogenesis in NS-1 cells via the same RapGEF2- and ERK-dependent pathway as that employed by PACAP signalling via the PAC1 receptor.

3.4 | Exendin-4-dependent neuritogenesis requires ERK activation in NS-1_hGLP-1 cells

To establish that exendin-4-induced neuritogenesis in NS-1_hGLP1R cells utilises the ERK-dependent pathway previouslycharacterised for PACAP-dependent neuritogenesis, the effect of blocking ERK activation with the MEK inhibitor U0126 was examined. Exendin-4-dependent neuritogenesis in NS-1_GLPR1 cells was blocked by prior treatment with U0126, with confirmation of U0126-dependent neuritogenesis by PACAP in both parent NS-1 and NS-1_hGLP1 cells as a positive control (Figure 5A,B). Furthermore, the potency of exendin-4 to support neuritogenesis in NS-1_hGLP1 cells (Figure 5C) is similar to its potency to cause cAMP elevation in HEK293_CBS_hGLP1R cells (Figure 4A).

3.5 | RapGEF2 is present in human islets and rodent insulin-expressing cell lines, but not in non-neuroendocrine cell lines

Given the functional coupling of GLP-1 to ERK through RapGEF2 (Figure 5), it was of considerable interest to examine tissues of physiological importance to GLP-1 function through GLP1R (ie, pancreatic beta cells and insulinoma cells). A set of antibodies specific for the C-terminus and a conserved peptide contained within the N-terminus (exon 2-encoded) of RapGEF2 were used to visualise expression of the protein in human pancreatic islets and a panel of rodent cell lines. Non-endocrine cell lines such as HEK293 cells[5] do not express the protein, whereas NS1, INS-1 and Min6 cells express the full-length RapGEF2 (Figure 6A). To confirm an earlier report that RapGEF2 protein expression in adult neuroendocrine cells and tissues is

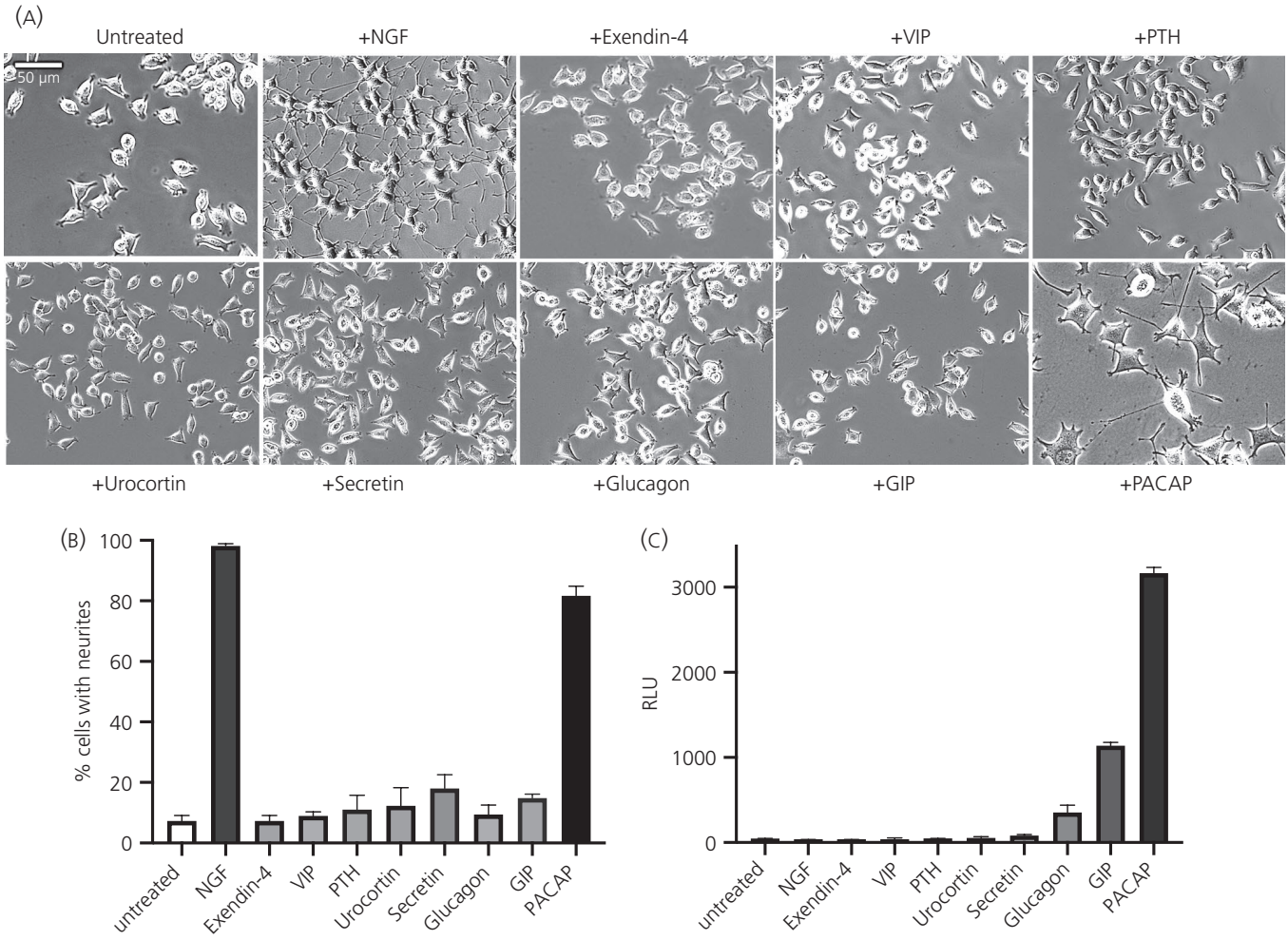
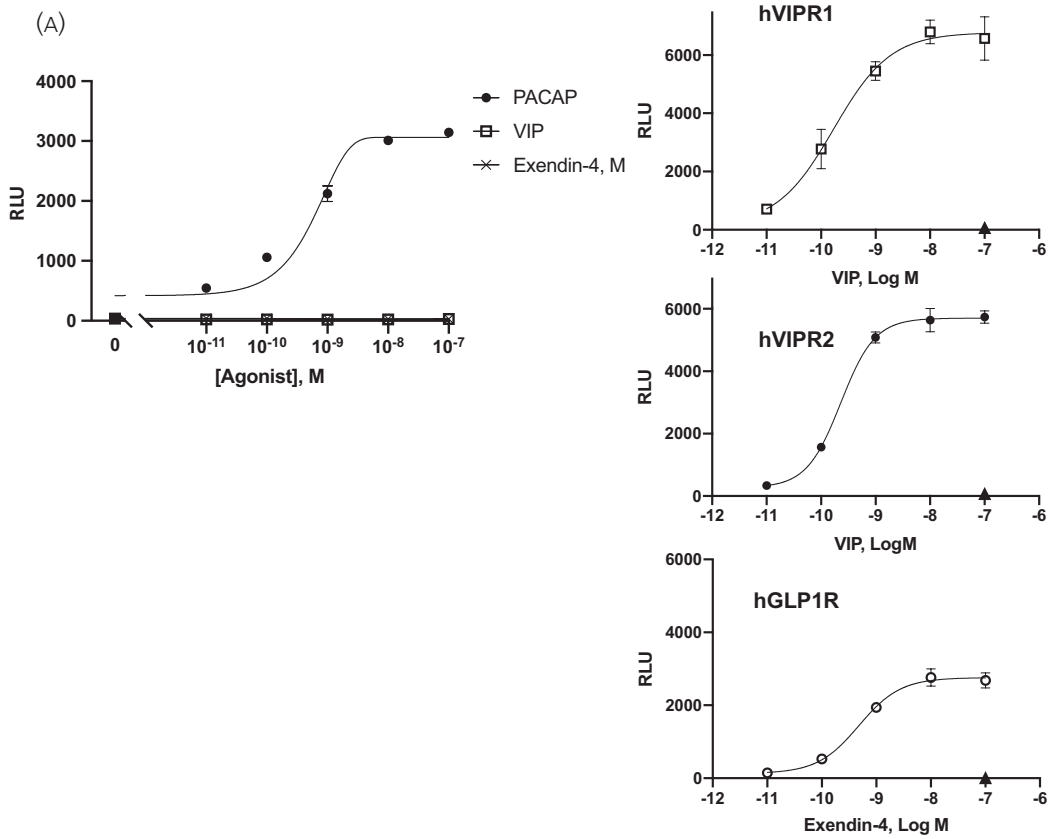


FIGURE 3 GPCR-driven neuritogenesis and cAMP elevation in NS-1 cells. A, Neuritogenesis was documented 48 hours after exposure of cells to NGF (100 ng mL^{-1}), exendin-4 (25 nmol L^{-1}), VIP (100 nmol L^{-1}), PTH (2.5 µmol L^{-1}), urocortin (1 µmol L^{-1}), secretin (10 µmol L^{-1}), glucagon (2.5 µmol L^{-1}), GIP (1 µmol L^{-1}) or PACAP-38 (100 nmol L^{-1}). Images are representative of three separate experiments. B, Quantitation of neurite formation in NS-1 cells treated with each of the peptide listed in (A). Data shown in the histograms are the mean ± SD (n = 3 per group). Percentage of cells with neurites at the length ≥ 10 µm were counted. C, Cyclic AMP generation (RLU, relative luminescence units) as measured using NS-1_CBS cells, and as described in the Materials and methods, after 20 minutes of exposure to peptides at the concentrations used for the neuritogenesis shown in (A). N = 4 from four separate wells within a single experiment; SD is indicated as error bars. Experiments were repeated twice, separately, with similar results obtained

associated with the so-called NCS-RapGEF2 transcript, differential detection of NCS- versus NN (non-neuronal)-RapGEF2 transcripts was carried out in human pancreatic islets, kidney, brain and lung tissue. NN-RapGEF2 transcripts were found in all tissues, whereas NCS-RapGEF2 transcripts were found to be copious in brain, present in pancreatic islets, present at extremely low levels in lung, and absent from kidney (Figure 6B), consistent with previous results in mouse endocrine and non-endocrine tissues.[24]

FIGURE 4 Neuritogenic activity and cAMP activation by exogenous expression of VIPR1, VIPR2 and GLP1R. A, Exogenous expression of hVIPR1, hVIPR2 and GLP1R in HEK293_CBS cells induced cAMP elevation measured in a cAMP biosensor assay in a dose-response manner. Left : VIP and exendin-4 did not induce a dose-dependent elevation of cAMP in NS-1_CBS cells, whereas PACAP did. Right : HEKCBS cells expressing hVIPR1, hVIPR2 or hGLP1R were exposed to of VIP or exendin-4 at concentrations from 0.01-100 nmol L^{-1} and cAMP elevation was measured in a cAMP biosensor assay as the relative luminescence units (RLU) signal. Note that the signal (RLU) generated by 100 nmol L^{-1} peptide in 293 cells not expressing cognate receptor is shown as a closed triangular symbol at 100 nmol L^{-1} (10^{-7} mol L^{-1}) in each graph. Dose-response curves were created using the mean of RLUs for each dose and fitted using PRISM (GraphPad Software Inc., San Diego, CA, USA) nonlinear regression. N = 6; error bars showing the SD, and with some error bars too small to be visible in the graph. In three separate experiments, the EC_{50} for VIP in HEKCBS_hVIPR1 cells ranged from 0.2 to 0.5 nmol L^{-1}, and, for VIP in HEKCBS_hVIPR2 cells, ranged from 0.02 to 0.12 nmol L^{-1}. For exendin-4, the EC_{50} of hGLPR1 expressing HEKCBS cells ranged from 0.02 to 0.47 nmol L^{-1}. B, The transduction of NS-1 cells with hVIPR1 and hVIPR2, the cognate receptors for VIP, and hGLP1R, the cognate receptor for GLP-1 and exendin-4, results in robust neurite formation after treatment with respective agonist. Micrographs taken 72 hours after exposure of cells to 100 nmol L^{-1} VIP or 25 nmol L^{-1} exendin-4. C, The percentage of cells with neurites was quantified with a bar graph to represent neuritogenesis, N = 3 separate wells; SD is shown as an error bar. $^{**}P < 0.01$ by Welch's t-test using PRISM software


(A)
RLU
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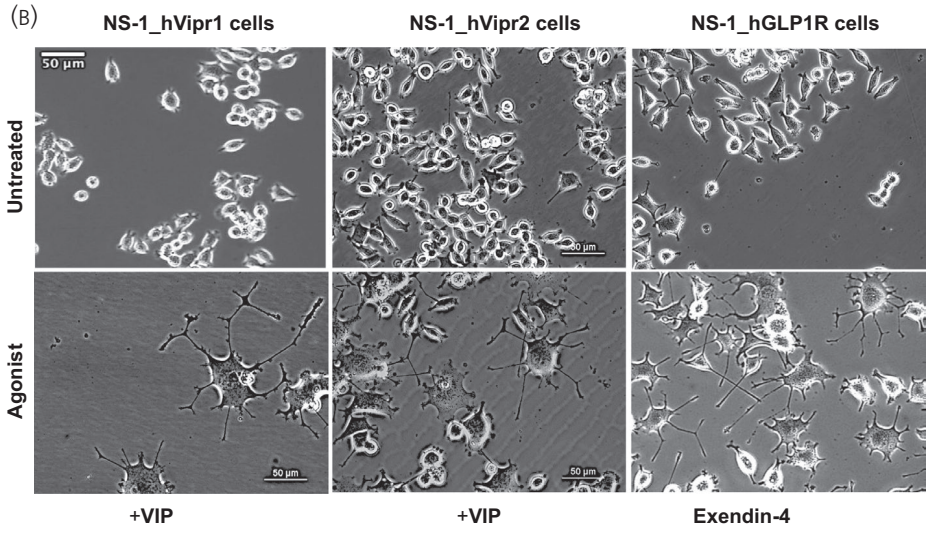
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NS-1_hVipr1 cells
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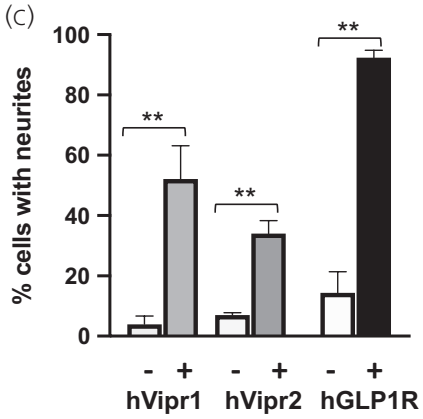
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hVipr1
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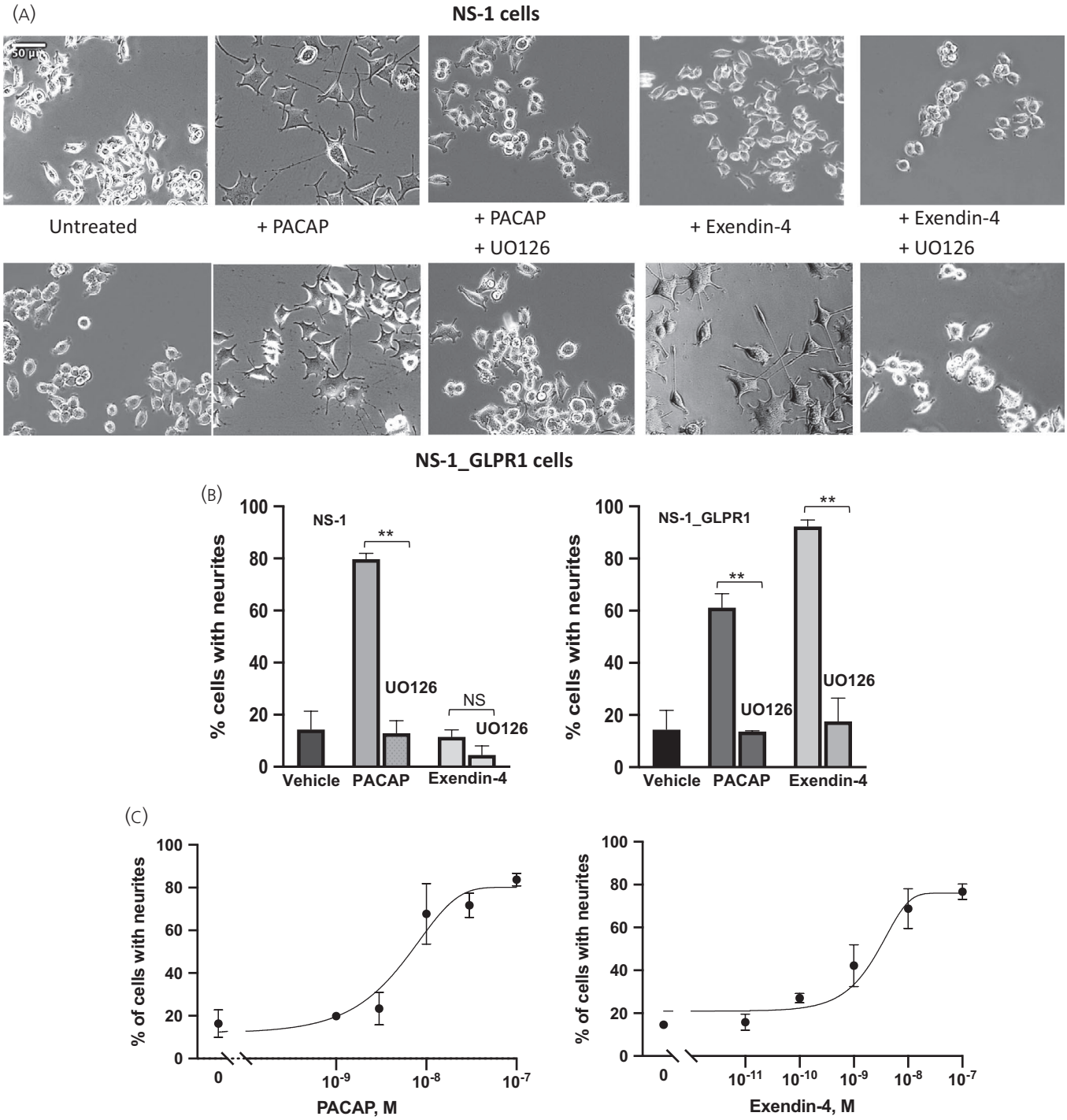

FIGURE 5 Exendin-4-dependent neuritogenesis requires ERK activation in NS-1 cells. A, NS-1 cells or NS-1_hGLP1R cells were exposed to peptide (PACAP-38, 100 nmol L^{-1}; exendin-4, 25 nmol L^{-1}) in the presence or absence of 10 µmol L^{-1} of the MEK inhibitor U0126, added 30 minutes prior to peptide exposure. B, Quantitation of neuritogenesis, as a percentage of cells with neurites, shown as a bar graph, with N = 3, representing three separate wells. NS, $P > 0.05$, $^{**}P < 0.01$ by Welch's t-test using PRISM . C, Dose-response curve for neuritogenesis induced by PACAP in NS-1 cells and of exendin-4 in NS-1_hGLP1R cells. In three separate experiments, EC_{50} for PACAP in NS-1 cells ranged from 0.5 to 20 nmol L^{-1}, and EC_{50} for exendin-4 in NS-1_hGLP1R cells ranged from 0.3 to 1.2 nmol L^{-1}

3.6 | Knockdown of RapGEF2 using shRNA results in attenuated pERK activation by exendin-4 in INS-1 cells

To determine whether or not RapGEF2 is involved in ERK signalling in INS-1 cells, we aimed to knock down the expression of RapGEF2 expression. We had previously produced a battery of shRapGEF2-encoding lentiviral vectors and investigated the level of Rapgef2 down-regulation in NS-1 cells expression using these shRapGEF2 constructs. The construct giving the most complete reduction of RapGEF2 level in previous studies[5] was selected to transduce INS-1 cells and also knocks down the RapGEF2 protein level in western

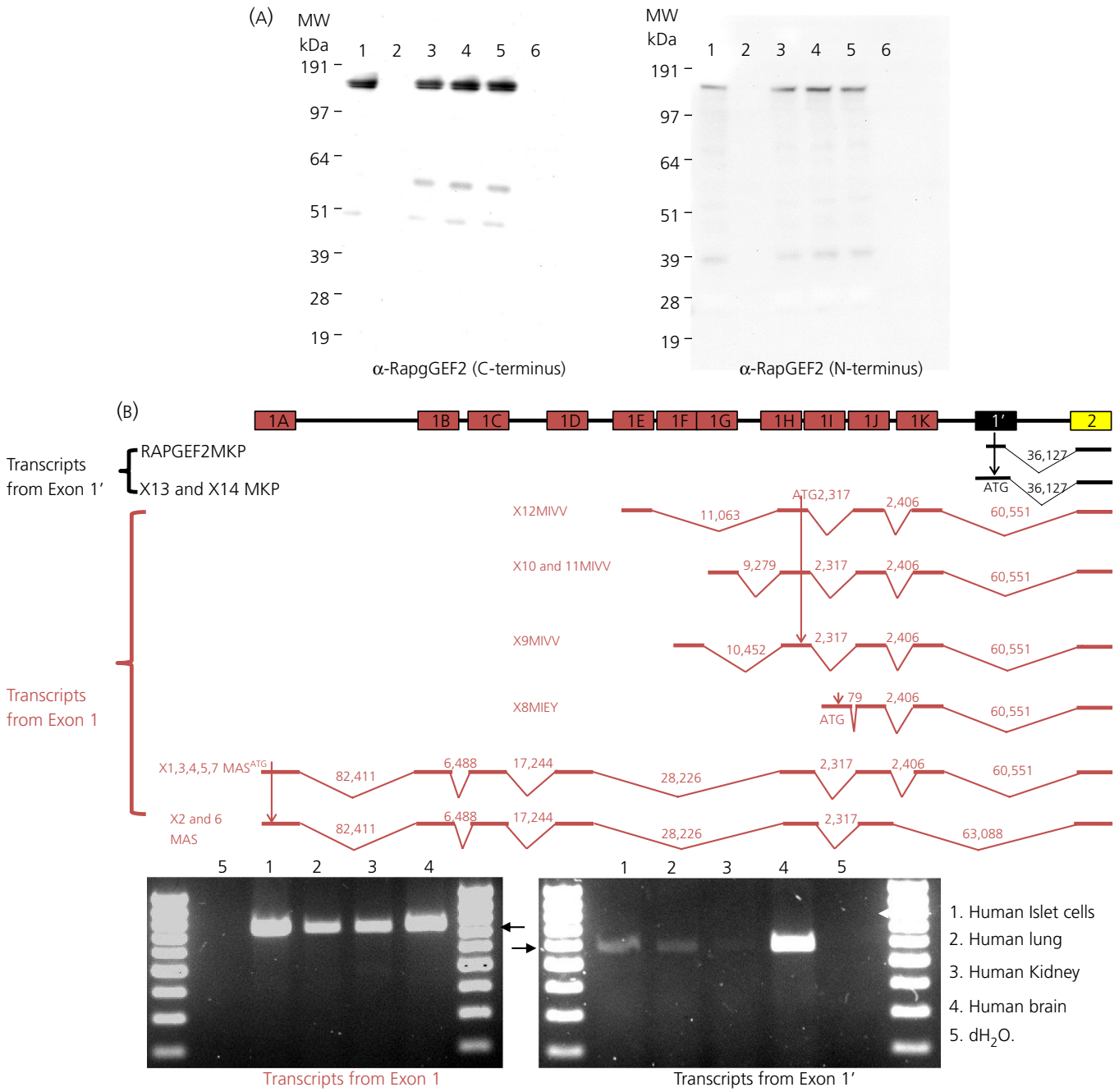

FIGURE 6 RapGEF2 expression and NCS- and NN-RapGEF2 mRNA expression in human and rodent tissues and cell lines. A, RapGEF2 is present in human islets and rodent insulin-expressing cell lines, but not in non-neuroendocrine cell lines. Lane 1, NS-1 cells; lane 2, HEK293 cells; lane 3, human pancreatic islets; lane 4, MIN6 cells; lane 5, INS-1 cells; lane 6, HEK293T cells. Protein extracts were applied in a total volume of 20 μL containing 20 μg of total protein per lane. Western blotting for a second preparation of human pancreatic islets was performed to ensure reproducibility and qualitatively identical results were obtained (not shown). B, Detection with NCS-RapGEF2- and NN-RapGEF2-specific primers of relative levels of expression of each type of transcript in human islet cell, lung, kidney and brain. Top : Schematic of human RapGEF2 gene exons 1 and 1′ and predicted transcripts (refer to https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.33).[24] Two classes of transcripts are alternatively used from the Rapgef2 gene in humans. Bottom : RT-PCR detection of the NCSRapGEF2 (exon 1′ transcript) and NN-Rapgef (exon 1 transcript)

blotting analysis (Figure 7A). INS-1-shRapGEF2 cells, in comparison with wild-type INS-1 cells, were challenged with exendin-4, and pERK levels were visualised by western blotting. As shown in Figure 7B,C, there was a robust inhibition of exendin-dependent pERK phosphorylation in these cells indicating the involvement of RapGEF2 in pERK phosphorylation signal pathway.

3.7 | ERK-dependent regulation of the INS-1 cell transcriptome by exendin-4

Exendin-4 treatment causes activation of ERK phosphorylation as shown in Figure 7B,C. To investigate the transcriptional changes resulting from this process, we treated INS-1 cells with 25 nmol L^{-1}

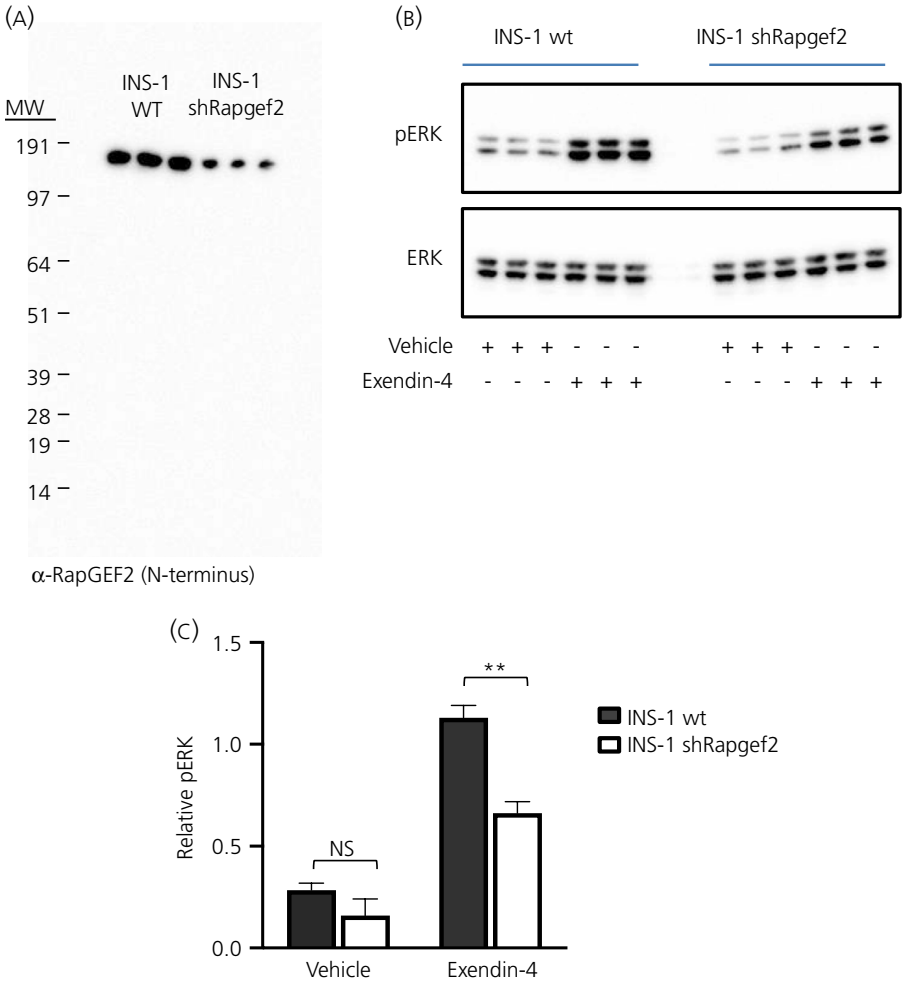

FIGURE 7 Knockdown of RapGEF2 in INS-1 cells and effect on pERK stimulation by exendin-4 in INS-1 cells. A, Western blotting with RapGEF2 antibody demonstrates knockdown of RapGEF2 protein following RapGEF2-specific shRNA expression (INS-1 shRapgef2). B, Measurement of phosphorylation of ERK in INS-1 cells with or without RapGEF2-specific shRNA expression. Cells were exposed to 25 nmol L^{-1} exendin-4 for 10 minutes. C, Quantitation of phosphorated ERK level normalised with total ERK with and without exendin-4 treatment using ImageJ. NS, $P > 0.05$, $^{**}P < 0.01$ by Welch's t-test

exendin-4 for 1 hour, and harvested RNA for microarray analysis using Clariom-S rat microarray chips from Affymetrix. Figure 8 depicts the major transcriptional changes, and their dependence on ERK signalling, induced by exendin-4 in INS-1 cells. Among forty genes up-regulated by more than two-fold ($P < 0.01$) after exendin-4 induction, four immediate-early genes, Nr4a1, Nr4a3, Fosl2 and Crem, were all robustly up-regulated (> 3.5 fold). Of these transcripts up-regulated by exendin-4, Nr4a2, Btg1, Skil, Plk2, Per1 and Dyrk3 were ERK-dependent, as evidenced by a significant down-regulation ($P < 0.05$) in induction by exendin-4 upon addition of the MEK inhibitor U0126. The notable immediate early gene (IEG) Fos was up-regulated significantly at a fold increase of 1.9 and $P < 0.002$. Similarly, the IEG Egr-1, a canonical ERK-regulated transcriptional target for GsPCR signalling, was up-regulated significantly at a fold increase of 1.7 and $P < 0.0001$, and this induction was significantly less (reduced to 1.28-fold, $P < 0.003$ when comparing treatment with exendin to treatment with exendin plus U0126). Thus, as in PC12 or NS-1 cells exposed to PACAP,[7] the agonist for a related GsPCR, PAC1, there is concomitant activation of gene expression by GLP-1 signalling through GLP1R that appears to be mediated through parallel cAMP-activated pathways, including the cAMP effectors Epac, PKA and NCS-RapGEF2 (Figure 9). ERK-dependent induction of the clock gene Per by exendin-4 may be relevant to regulation of the cell cycle and the proliferative effects of GLP-1 on beta cells; however, cultured cells were unsynchronised in the experiments conducted here and thus this aspect of ERK-dependent GLP-signalling remains to be explored. The final effect of this activation of INS-1 cells on transcription of the insulin gene at later times, or on glucose-dependent secretion of insulin, remains to be determined in further investigations.

4 | DISCUSSION

In the present study, we have demonstrated that GLP1R engagement activates ERK through a mechanism requiring cAMP-dependent activation of NCS-RapGEF2. This signaling pathway is shared by other family B G_s-coupled GPCRs including PAC1, VIPR1 and VIPR2. NCS-RapGEF2 is expressed in human pancreatic islets and rodent pancreatic islets, as well as in the rodent insulinoma cell lines MIN6 and INS-1. Activation of ERK in INS-1 cells exposed to exendin-4, a highly specific GLP1R agonist, is attenuated by shRNA directed to the NCS-RapGEF2 transcript. Finally, transcriptomic analysis of INS-1 cells exposed to exendin-4 reveals both ERK-dependent and ERK-independent gene targets in the GLP1R-activated transcriptional response. We propose that the cAMP-dependent activation of NCS-RapGEF2 is responsible for ERK phosphorylation by GLP-1, and that this signalling pathway is insulated from, and works

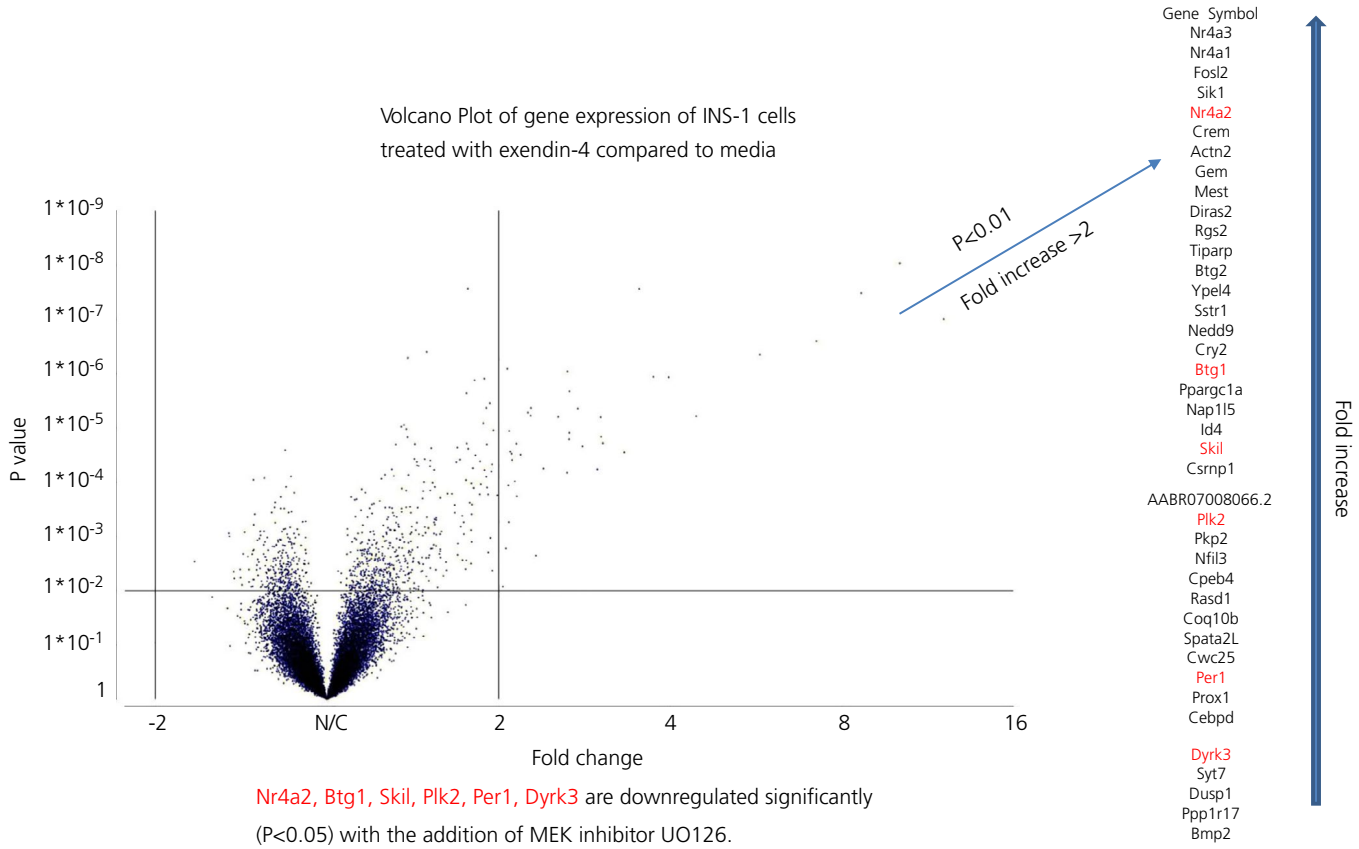

FIGURE 8 Transcriptomic analysis of INS-1 cells treated with exendin-4 with and without MEK inhibitor U0126. INS-1 cells were pretreated with 10 μmol L^{-1} U0126 or control media for 30 minutes then exposed to 25 nmol L^{-1} exendin-4 for 1 hour, followed by total RNA harvest and purification, quantification and submission to microarray analysis using Affymetrix Clariom-S rat microarray chips. The volcano plot displayed differential gene expression of INS-1 after exendin-4 treatment. Forty genes with significantly enhanced expression are listed on the right

complementarily with, cAMP signalling mediated through protein kinase A and the cAMP-dependent RapGEF, Epac, in pancreatic beta cells (Figure 9).

Beta cells of the pancreas have, as their primary professional function, the secretion of insulin in response to glucose, comprising signalling that is mediated through elevation of ATP levels and inhibition of the ATP-sensitive potassium channel K_{ATP}. Modulation of insulin release, however, is augmented by sympathetic, parasympathetic, paracrine and incretin modulation of beta cell mass, as well as insulin gene transcription, in addition to that of glucose-stimulated insulin secretion itself, allowing the precise minute-by-minute regulation of insulin action required for maintenance of appropriate blood glucose concentrations. The incretin GLP-1 allows gut-endocrine pancreas communication after a meal as a result of intestinal nutrients stimulating the release of GLP-1 from enteroendocrine L-cells located in the ileum and colon. Circulating GLP-1 in the blood then acts at its receptors on beta cells of the pancreas to raise levels of cAMP and promote insulin secretion. Cyclic AMP modulates multiple intracellular processes in the beta cell, including the control of 'stimulus-secretion-synthesis coupling', which allows the compensatory synthesis and secretory vesicle packaging of insulin to balance its export from the cell during secretion.[27] Cyclic AMP signalling requires mobilisation of third messengers, so-called cAMP sensors, or effectors, which ultimately control cellular function. For some decades, it was assumed that all of the actions of hormones activating G_sPCRs leading to cAMP elevation, including those of GLP-1 on beta cells, occurred through activation of protein kinase A, which, via phosphorylation of key serine and threonine residues on target proteins, alters their transport, enzymatic or signalling functions. However, critical cAMP-dependent functions in beta cells have also been ascribed to signalling through the cAMP-dependent guanine nucleotide exchange factor Epac and other non-PKA-dependent pathways.[10,13,15-18,20,28,29] Transcriptional effects of PKA are mediated through serine phosphorylation and activation of the transcription factor CREB, whereas cytoplasmic actions include drastic alteration in the catalytic activation of a host of intracellular proteins.

More recently, the mitogen-activated protein kinase ERK has received considerable attention as a third messenger regulated by glucose, as well as G_sPCRs, including those for GLP-1, GLP-2 and glucagon. The involvement of PKA in signalling to ERK in beta cells and beta cell lines is frequently invoked as a likely pathway for ERK activation via these receptors. Prompt ERK activation by GLP-1 or forskolin in serum-starved MIN6 cells was abolished by pre-treatment with the PKA inhibitor H89.[30] GIP-initiated ERK activation, in INS-1 cells subjected to overnight serum starvation to establish metabolic

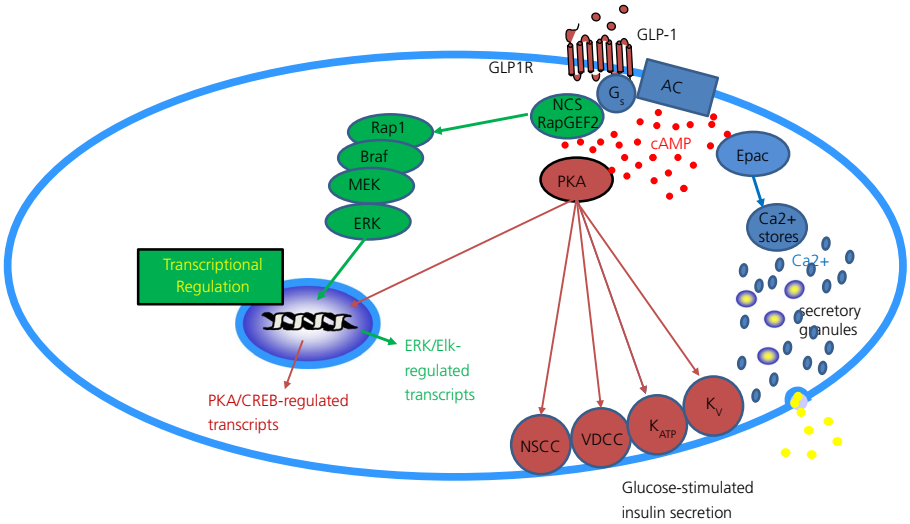

FIGURE 9 GLP1R signalling involves multiple cAMP sensor/effectors in pancreatic beta cells. G-beta-gamma activation of phosphoinositide 3-kinase may provide redundant activation of ERK and other mitogen-activated protein kinases for additional transcriptional control by GLP-1 in the beta cell. Abbreviations: AC, adenylate cyclase; Epac, exchange protein activated by cAMP; G_s, stimulatory G-protein; K_{ATP}, ATP-activated potassium channel; K_v, voltage-activated potassium channel, NSCC, non-specific calcium channels; PKA, protein kinase A; VDCC, voltage-dependent calcium channels

quiescence, was likewise reported to be mimicked by treatment with forskolin and to be blocked by H89.[31] In serum-starved MIN6 cells, prolonged ERK activation by glucagon was sensitive to blockade by H89.[14] Quoyer et al[19] reported, in MIN6 cells, ERK activation by GLP-1 that was apparently biphasic, with the early phase (5 minutes) sensitive to H89, but the later phase (20 minutes) not, suggesting another signaing pathway connecting GLP-1 receptor occupancy to activation of ERK in pancreatic beta cell lines. Because H89 is likely to block additional kinases (S6K1, MSK1 and ROCK-II) in addition to PKA,[32] a more complex mechanism for ERK activation by GLP-1 receptor occupancy has begun to emerge. Broca et al[33] examined beta-arrestin compared to PKA-mediated activation of ERK in INS-1E cells: cells were serum- and glucose-starved for 2 hours in the presence or absence of various inhibitors, prior to challenge with first messengers and measurement of phospho-ERK. Under these conditions, activation of PAC1 elicited only a rapid (5 minutes), quickly subsiding (within 20 minutes), and partially H89-sensitive activation of ERK.[33] In light of the difficulty of linking a specific cAMP effector, such as PKA, to activation of ERK by any Gs-coupled GPCR in beta cells/beta-cell like cell lines in culture, MacDonald et al[34] conceptualised ERK activation after GLP-1 receptor occupancy as occurring not via cAMP elevation, but rather via Gbeta-gamma coupling of the GLP1 receptor to phosphoinositide 3-kinase, activating ERK and leading to gene transcriptional and cell proliferation effects, whereas Gs-coupled signalling leads to activation of cAMP-GEFII (Epac) and PKA, controlling glucose-stimulated insulin secretion.

We have previously shown in NS-1 cells that GPCR→Gs→AC→cAMP→ERK signalling, stimulated over minutes to hours, is independent of PKA, leads to neuritogenesis and neuroendocrine-specific gene regulation and requires a protein factor termed the neuritogenic cAMP sensor (NCS), which is the protein product of the RapGEF2 gene.[5-7,35-37] It is important to point out that the GPCR→Gs→AC→cAMP→ERK pathway leading to neuritogenesis following persistent stimulation,[38] and requiring NCS-RapGEF2, was elucidated in cells cultured under conditions of normal metabolism (15% serum), and independently of activation of CREB by PKA.[7] By contrast, under conditions of 'metabolic quiescence' (serum starvation) Ginty et al[39] demonstrated the existence of a neurotrophin-activated ERK pathway leading to CREB phosphorylation, which does not appear to exist in cells when cultured under physiological (serum-containing) rather than pro-apoptotic (low or no serum) conditions. Several laboratories have identified a third cAMP effector, Epac, which has been implicated in signalling by G_s-coupled GPCRs in pancreatic beta cells and insulinoma cell lines.[15,40] Here, we show that the pathway to ERK activated by NCS-RapGEF2 following cyclic AMP elevation by Gs-coupled GPCR engagement, first identified for PACAP-PAC1 signalling, applies also to signalling through the GLP-1 receptor GLP1R, and is required for ERK activation by exendin-4 in insulinoma cell line(s). This finding suggests that cyclic AMP signaling in pancreatic beta cells is parcellated via the activation of at least three cyclic AMP effectors, as in PC12 and NS-1 pheochromocytoma cells. In the case of pancreatic beta cells, activation of multiple cyclic effectors including PKA and RapGEF2 allows the concerted physiological modulation of best-cell function by the critical incretin GLP-1.

A role for NCS-RapGEF2 in GLP-1 signalling can also be envisaged for the central actions of GLP-1, mainly involving appetite regulation, which occur upon activation of GLP1R in brain stem, and in various diencephalic and telencephalic nuclei.[41-43] Both ERK and immediate-early gene regulation are hallmarks of NCS-RapGEF2-dependent signalling initiated by dopaminergic signalling and are involved in appetitive behaviour regulation by psychomotor stimulants in the mouse central nervous system, making investigation of this component of GsPCR signalling potentially worthy of investigation

in the context of both peripheral/endocrine[44] and central/neuronal GLP-1 actions in vivo.

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AUTHOR CONTRIBUTIONS

Wenqin Xu: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualisation; Writing – original draft; Writing-review & editing. **Sam P Dahlke:** Data curation; Formal analysis; Methodology; Software; Validation; Visualisation; Writing – original draft; Writing – review & editing. **Andrew C Emery:** Data curation; Formal analysis; Investigation; Methodology. **Michelle Sung:** Formal analysis; Software. **Oleg G Chepurny:** Data curation; Formal analysis; Investigation. **George G Holz:** Conceptualisation; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision; Writing – review & editing. **Lee E Eiden:** Conceptualisation; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualisation; Writing – original draft; Writing – review & editing.

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