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RESEARCH PAPER

PACAP receptor pharmacology and agonist bias: analysis in primary neurons and glia from the trigeminal ganglia and transfected cells

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BACKGROUND AND PURPOSE

A major challenge in the development of new medicines targeting GPCRs is the ability to quantify drug action in physiologically relevant models. Primary cell models that closely resemble the clinically relevant *in vivo* site of drug action are important translational tools in drug development. However, pharmacological studies in these models are generally very limited due to the methodology used.

EXPERIMENTAL APPROACH

We used a neuropeptide system to demonstrate the applicability of using highly sensitive signalling assays in primary cells. We quantified the action of pituitary adenylate cyclase-activating peptide (PACAP)-38, PACAP-27 and vasoactive intestinal polypeptide in primary cultures of neurons and glia derived from rat trigeminal ganglia (TG), comparing our observations to transfected cells.

KEY RESULTS

PACAP-responsive receptors in rat trigeminal neurons, glia and transfected PAC_{1n} receptors were pharmacologically distinct. PACAP-38, but not PACAP-27, activated ERK in glia, while both forms stimulated cellular cAMP production. PACAP(6–38) also displayed cell-type-dependent, agonist-specific, antagonism.

CONCLUSIONS AND IMPLICATIONS

The complexity of PACAP pharmacology in the TG may help to direct, more effectively, the development of disease treatments targeting the PACAP receptor. We suggest that these methodologies are broadly applicable to other primary cell types of human or animal origin, and that our approach may allow more thorough characterization of ligand properties in physiologically relevant cell types.

Abbreviations

CREB, cAMP responsive element-binding protein; PACAP, pituitary adenylate cyclase-activating peptide; PHM, peptide histidine methionine; RAMP, receptor activity-modifying protein; TG, trigeminal ganglia; VIP, vasoactive intestinal polypeptide

Introduction

GPCRs represent a large family of membrane-spanning proteins. They have been implicated in many physiological processes and human diseases (Katritch *et al.*, 2013). Despite representing the targets of ~30% of marketed drugs, GPCRs still retain great untapped therapeutic potential (Rask-Andersen *et al.*, 2011).

GPCRs are characterized by their ability to activate signal transduction via coupling to heterotrimeric G proteins; this coupling can be promiscuous (Ritter and Hall, 2009; Couvineau and Laburthe, 2012). GPCRs can also couple to non-G-protein signalling pathways (Ritter and Hall, 2009; Couvineau and Laburthe, 2012). A recent focus in GPCR pharmacology has been the development of ligands, which preferentially activate a particular signalling pathway via that receptor. These 'biased' ligands have great therapeutic potential, allowing the preferential activation of therapeutically beneficial pathways at the expense of those that may be detrimental (Maudsley *et al.*, 2012; Kenakin and Christopoulos, 2013). Several endogenous agonists have been reported to display bias in cell culture-based models. For example, oxyntomodulin is biased towards cAMP accumulation over β-arrestin recruitment at the GLP-1 receptor (Jorgensen *et al.*, 2007).

However, proving that these observations are not simply a consequence of using overexpressed receptors in cell culture model systems is considerably more challenging. Ideally, bias should be demonstrated in physiologically relevant systems where the receptor has the capacity to couple to multiple signalling pathways, and the pathways quantified such that potential ligand bias may be demonstrated. This requires the generation of full concentration–response curves in sufficient throughput to allow investigation of multiple pathways and ligands (Kenakin and Christopoulos, 2013). Studies in primary cells are frequently limited to a single concentration or a single agonist curve for one pathway. This is further complicated by a general deficiency in quantified signalling data from relevant model systems, making it difficult to determine which pathways or signalling molecules should be investigated when assessing ligands for bias. This is particularly true for non-proliferative primary neuron models when the site of action is limited to a single ganglia or brain nuclei. The literature is lacking comprehensive examinations of pharmacology in these models. Several researchers have laboriously developed animal models genetically modified to express 'designer receptors', which signal via specific pathways (Poulin *et al.*, 2010; Wess *et al.*, 2013), or 'reporters' to detect activation of specific pathways (Dressler *et al.*, 2013). These models provide useful insight when used to investigate specific diseases or known signalling functions but they are not a substitute for pharmacological analysis.

The neuropeptide, pituitary adenylate cyclase-activating peptide (PACAP), is closely related to vasoactive intestinal polypeptide (VIP) and is a member of the secretin peptide family. PACAP and VIP are both highly conserved across mammals, displaying identical sequences between humans and rodents (Sherwood *et al.*, 2000). PACAP is expressed in two major forms: a 38-amino acid form (PACAP-38) and a 27-amino acid, C-terminally truncated variant (PACAP-27) (Miyata *et al.*, 1989; Vaudry *et al.*, 2009). PACAP-38 is apparently the major form, accounting for approximately 90% of PACAP in the nervous system (Vaudry *et al.*, 2009). However, both forms of PACAP are widely distributed throughout the body, including the central and peripheral nervous system (Vaudry *et al.*, 2009). PACAP has been implicated in a wide range of biological processes, including the regulation of circadian rhythms, reproduction and development, cognitive behaviour, pain transmission, neuroprotection and neuromodulation (Hashimoto *et al.*, 2006; Laburthe *et al.*, 2007; Rat *et al.*, 2011; Markovics *et al.*, 2012; Nakajima *et al.*, 2013).

PACAP activates three related GPCRs; the $VPAC_1$ and $VPAC_2$ receptors, which display equivalent affinity for PACAP and VIP, and the PAC_1 receptor, which displays greater affinity for PACAP than for VIP (Laburthe *et al.*, 2007; receptor nomenclature follows Alexander *et al.*, 2013). There are many PAC_1 splice variants, including N-terminal deletions and intracellular loop insertions; these modify the peptide binding and signalling properties of the receptor (Spongier *et al.*, 1993; Blechman and Levkowitz, 2013). The most commonly studied variant, PAC_{1n}, as defined by Dautzenberg *et al.*, 1999, contains the full N-terminal domain and lacks insertions into the intracellular loops (Spongier *et al.*, 1993; Pisegna and Wank, 1996; Dautzenberg *et al.*, 1999; Furness *et al.*, 2012).

There is some evidence for signal bias at PAC_{1n} receptors (Blechman and Levkowitz, 2013). Both PACAP-38 and PACAP-27 potently stimulate cAMP accumulation, whereas only PACAP-38 stimulates IP_3 turnover (Spongier *et al.*, 1993; Blechman and Levkowitz, 2013). Phosphorylation and activation of numerous other signalling proteins, including, p38, cAMP responsive element-binding protein (CREB) and ERK, have also been reported (Vaudry *et al.*, 2009). However, the physiological significance of many of these pathways is unclear and concentration–response curves are rarely constructed meaning that potential signal bias cannot be substantiated.

There is emerging interest in the potential pathological role of PACAP in migraine (Edvinsson, 2013; Kaiser and Russo, 2013). Plasma PACAP concentrations were reportedly elevated during the ictal phase of migraine (Tuka *et al.*, 2013) and infusion of PACAP-38 into migraine sufferers induces migraine-like attacks (Schytz *et al.*, 2009). VIP does not induce migraine-like attacks in people with migraine (Rahmann *et al.*, 2008), suggesting that only the PAC_1 receptor is involved in these effects (Schytz *et al.*, 2009).

We used miniaturized (high-throughput) signalling assays to quantify PACAP and related ligand potency via different signalling pathways in primary cell cultures from trigeminal ganglia (TG). We chose this system because the trigeminal nerve, which controls the sensation of pain in the head, expresses PACAP (Moller *et al.*, 1993; Kuris *et al.*, 2007) and PACAP-responsive receptors (PAC_1, $VPAC_1$ and $VPAC_2$) (Chaudhary and Baumann, 2002; Knutsson and Edvinsson, 2002; Nakajima *et al.*, 2013). However, very little is known about its signalling or pharmacology in cells derived from these ganglia. We found agonist bias in this physiologically relevant model and strengthened the evidence for the PAC_1 receptor as a target in migraine. Our methodology may facilitate further studies to quantify ligand bias in primary cell cultures.

Methods

Isolation and culture of TG neurons and glia

All animal care and experimental procedures at the University of Auckland were conducted in accordance with the New Zealand Animal Welfare Act (1999) and approved by the University of Auckland Animal Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 156 animals, supplied by the Integrated Physiology Unit (University of Auckland), were used in the experiments described here.

Isolation and culture of TG neurons and glia was performed based on previously published methods with minor modifications (Durham and Russo, 1999; Li *et al.*, 2008). Briefly, anaesthesia was induced in 3- to 5-day-old Wistar rat pups by 5% isoflurane/2 L min^{-1} O_2. Rats were killed by decapitation. The TGs were excised and collected in ice-cold dissociation buffer (25 mM HEPES buffered HBSS) and dissociated by incubation at 37°C for 30 min in dissociation buffer containing dispase II (10 mg·mL^{-1}; Invitrogen, Carlsbad, CA, USA). The dissociated cell suspension from six TG was collected by centrifugation (250 x *g*, 3 min). The pellet was then resuspended in 5 mL warm (37°C) dissociation buffer and triturated 15 times using a pipette. Large remaining fragments were removed from the suspension with a pipette and the suspension re-pelleted by centrifugation (100 x *g*, 3 min). TG cultures were then enriched for neurons or glia by differential centrifugation. The pellet was re-suspended in 0.5 mL of L15 medium and carefully layered onto 3 mL L15 medium (containing 1 mg mL^{-1} BSA), layered over 6 mL L15 medium (containing 10 mg mL^{-1} BSA). The gradient was then centrifuged (100 x *g*, 3 min) and the neuron-enriched pellet separated from the glial-enriched medium fraction. The TG glial-enriched fraction was then centrifuged (1100 x *g*, 8 min) and resuspended in high glucose DMEM media containing 8% FBS (ICPbio Ltd, Auckland, New Zealand) and penicillin/streptomycin (Invitrogen). Cells were counted using a Countess Counter™ (Invitrogen,) and plated at 20 000 cells per well in 96-well cell culture plates. TG glial cultures were maintained at 37°C/5% CO_2 in a humidified incubator for 5 days. The medium was replaced after 24 h and then every 48 h.

The TG neuron-enriched pellet was re-suspended in the culture media [L15 medium containing penicillin/streptomycin, glutamine (2 mM), glucose (50 mM), ascorbic acid (250 μM), glutathione (8 μM), mouse 2.5S nerve growth factor (NGF; Alomone Labs, Jerusalem, Israel) (10 ng·mL^{-1}) and 8% FBS] and further enriched by pre-plating for 1 h at 37°C. TG neurons were then plated (approximately 4 wells per ganglia) into 96-well (histology) or 384-well (signalling assays) poly-D-lysine/laminin-coated (BD Biosciences, Auckland, New Zealand) cell culture plates. Successful enrichment for both trigeminal ganglia neuron and glial-enriched cultures was confirmed by microscopic examination before commencing further experiments. TG neurons were plated on clear 384-well cell culture plates (Perkin-Elmer Life and Analytical Sciences, Waltham, MA, USA) to allow culture quality and neurite formation to be monitored. Cultures were maintained at 37°C in a humidified incubator for 24 h. The resulting TG neuron-enriched cultures were estimated to contain greater than 70% neurons. When used in signalling assays, the above procedure results in one rat pup yielding approximately 8 data points for neurons and 24 data points for glia. This is a significant enhancement over previous studies examining cAMP signalling, where approximately two rat pups have yielded a single data point for TG-derived neurons (Zhang *et al.*, 2007). We are unaware of any studies in cultured TG-derived glia directly measuring cAMP. However, the effect of cAMP on signalling entities has been investigated using similar animal numbers (Li *et al.*, 2008).

Cell culture and transfection

Culture of Cos7 cells (kindly donated by Associate Professor N. Birch, School of Biological Sciences, University of Auckland) was performed as previously described (Bailey and Hay, 2006). Cells were cultured in DMEM supplemented with 8% heat inactivated FBS and 5% (v/v) penicillin/streptomycin and kept in a 37°C humidified 95% air, 5% CO_2 incubator. Cells were seeded into 96-well plates at a density of 15 000 cells per well (determined using a Countess Counter™; Invitrogen) one day prior to transfection. Cells were transiently transfected using polyethylenimine as described previously (Bailey and Hay, 2006). PAC_{1n} in pcDNA3.1+ was obtained from the University of Missouri-Rolla cDNA Resource Centre (GenBank accession number AY366498). The human $VPAC_1$ receptor was as used previously (Hay *et al.*, 2006).

Signalling assays

After 24 h in culture, the medium was removed from neurons using a 27 gauge syringe and the cells were incubated in 5 μL serum and NGF free culture media containing 0.1% BSA and 1 mM 3-isobutyl-1-methylxanthine for 5 min. TG neurons were stimulated by the addition of 5 μL agonist with or without antagonists (twice the final concentration) for 30 min at room temperature. cAMP content was determined in TG neurons using the LANCE ultra-cAMP detection kit with minor modifications (Perkin-Elmer Life and Analytical Sciences). Briefly, Eu-cAMP tracer and *U*Light™-anti-cAMP reagents were added to the wells following peptide stimulation and the plates incubated for 60 min at room temperature. Fifteen microlitres of each sample was then transferred to a 384-well optiplate (Perkin-Elmer) prior to reading. All cAMP standards were incubated and treated identically to TG neuron samples.

cAMP was measured in TG glia and transfected Cos7 cells using the AlphaScreen cAMP assay kit (Perkin-Elmer) as previously described (Gingell *et al.*, 2010). These cAMP assays were also performed with 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO, USA). Plates were read using an Envision plate reader (Perkin-Elmer). All peptides were purchased from American Peptide (Sunnyvale, CA, USA) or Bachem (Bubendorf, Switzerland).

Phosphorylated ERK1/2 (pERK1/2) was measured using AlphaScreen *Surefire* assay kits (Perkin-Elmer) using the high-sensitivity protocol variation. Following peptide stimulation for 0–30 min at 37°C (ERK1/2 assays) (time courses), 7 min (Cos7 cells, neurons) or 15 min (glia) media was removed using an 8-channel aspirator (glia or Cos7 cells) or a 27 gauge syringe (neurons). Thirty microlitres (glia or Cos7) or 6 μL

(neurons) of lysis buffer was added to each well and the plates incubated with gentle shaking at room temperature for 10–15 min. Four microlitres of each sample was transferred into a 384-well optiplate for pERK1/2 measurement.

Data analysis

All data points represent the mean ± SEM combined from *n* separate experiments. Separate experiments comprise individual TG neuron, glia or transiently transfected Cos7 preparations performed in triplicate. All statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). pEC_{50} values were obtained by fitting a four-parameter logistic equation to the concentration–response curve data. To determine if the Hill slope was significantly different from one for agonist potency curves, *F*-tests were performed. For the majority of experiments, the Hill slope was not significantly different from unity. The agonist potency curves were therefore re-fitted with a Hill slope constrained to one and pEC_{50} values obtained. *F*-tests were performed within each individual data set to determine if the curve fits were significantly different. When the majority of antagonist curves were determined to have different fits, pA_2 values were calculated from pEC_{50} values obtained in the presence or absence of antagonist. Maximal cAMP responses (E_{max}) were determined and the data expressed as a percentage of the E_{max} for the associated PACAP-38 or PACAP-27 curves to enable combined data to be presented in the figures. For pERK1/2 assays, data were normalized to the media only control at the appropriate time in concentration–response curve experiments or the control value at 30 min for time courses. For statistical analysis, pEC_{50} and pA_2 values from individual experiments were combined and significant differences determined using Student's *t*-tests or one-way ANOVA with *post hoc* Dunnett's tests. Statistical significance was defined as $P < 0.05$.

Results

Preparation of TG glia and neuron cell cultures in 96- and 384-well plates

Enriched cultures of TG glia and neurons were prepared from Wistar rat pups by differential centrifugation (Figure 1). Glial cultures were grown in 96-well plates and maintained in culture for 5 days. Under these conditions, they displayed morphology consistent with satellite glia and Schwann cells, featuring elongated di- or tri-polar appearances, as shown in Figure 1. Glial fibrillary acidic protein was used to confirm the identity of these cells (Supporting Information Fig. S1).

Neurons were plated into 384-well plates. A neuron-enriched primary TG culture is shown in Figure 1, illustrating cell bodies with visible neurite projections after 24 h in culture. We estimate the cultures to be at least 70% neurons with smaller proportions of glial and other cells. The photograph was obtained from cells in a 96-well plate for imaging purposes but is typical of the morphology of the neurons in 384-well plates. Microtubule-associated protein 2 was used to confirm the identity of these cells (Supporting Information Fig. S1). In this multi-well format, we were able to proceed to pharmacology and signalling studies using time courses and concentration–response curves (Figure 1).

PACAP-responsive receptors in primary TG glia exhibit biased agonism

Glial cultures were stimulated with PACAP-38, PACAP-27 and VIP. PACAP-38 and PACAP-27 potently stimulated cAMP production in a concentration-dependent manner, which was not observed with VIP (up to 1 μM) (Figure 2A). PACAP-38 was approximately threefold more potent than PACAP-27, although both had similar efficacy [Table 1; E_{max} PACAP-38 1.81 ± 0.57 ($n = 6$) vs. PACAP-27 1.86 ± 0.52 ($n = 5$) pmol per well]. The absence of responses to VIP at the concentrations used indicates the presence of PAC_1 receptors rather than $VPAC_1$ or $VPAC_2$ receptors. To examine the pharmacology of the PACAP-responsive receptor in more detail, glial cells were treated with different concentrations of either PACAP-38 or PACAP-27 in the presence of the competitive PAC_1 receptor antagonist PACAP(6–38) (Figure 2B,C), which effectively antagonized both agonists with equal potency (Table 1).

To investigate potential agonist bias at this receptor, phosphorylation of ERK1/2 was measured. Time-course experiments showed that stimulation with 1 μM PACAP-38 increased ERK phosphorylation compared with the control group with maximal stimulation at 10–15 min (Figure 2D). In contrast, ERK phosphorylation was not significantly increased with 1 μM PACAP-27 or 1 μM VIP at any time point. To determine the potency of PACAP-38-induced ERK phosphorylation in our glial cultures, cells were stimulated for 15 min with a range of PACAP-38 concentrations. PACAP-38 induced a concentration-dependent increase in ERK phosphorylation (Figure 2E), although the potency was significantly lower than PACAP-38-induced cAMP production (~20-fold; Table 1). In parallel, concentration–response experiments with PACAP-27 and VIP failed to induce ERK phosphorylation, consistent with the time-course data (Table 1; Supporting Information Fig. S2).

PACAP-responsive receptors in primary TG neurons exhibit agonist-specific antagonism

We next examined whether functional PACAP receptors were also present in our neuron cultures, using an equivalent experimental design to the glia. PACAP-38 and PACAP-27 potently stimulated cAMP production in a concentration-dependent manner which was not observed with VIP (up to 10 μM) (Figure 3A). PACAP-38 and PACAP-27 were equipotent and had similar maximal responses [Table 1; E_{max} PACAP-38 221.3 ± 49.1 ($n = 8$) vs. PACAP-27 201.8 ± 73.8 ($n = 6$) fmol per well]. This pharmacology suggests that PAC_1, rather than $VPAC_1$ or $VPAC_2$, receptors are present in TG neurons. Subsequent antagonist studies showed that PACAP(6–38) was significantly more potent (~50-fold) as an antagonist when PACAP-27 was used as the agonist, compared to PACAP-38 (Figure 3B,C; Table 1).

To determine whether ERK was phosphorylated following activation of the PACAP receptor present in TG neurons, the time course of phosphorylation of ERK was measured. ERK phosphorylation was not elevated by 0–30 min of treatment with 1 μM PACAP-38 when compared to control-treated

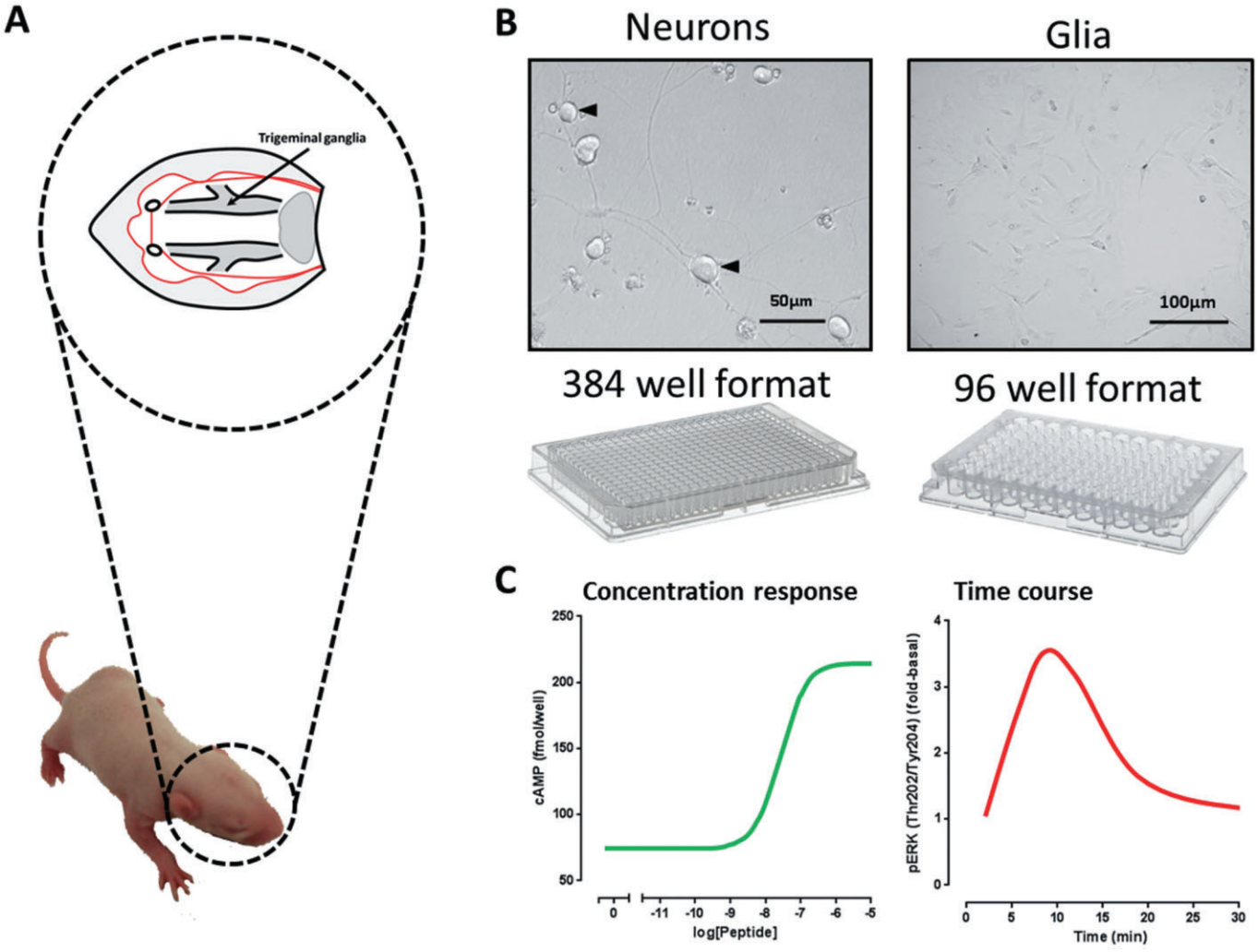

Figure 1

Experimental outline for the study of intracellular signalling in primary cultured TG-derived neurons and glia. (A) 3–5 day post-natal Wistar rat pups were killed by decapitation under anaesthesia. The skull and brain were then dissected away, exposing the TG. These were removed by cutting the connections to the brainstem and the periphery (V1 and V2/3) and teasing the structure away from the surrounding tissues. The TG were then digested in dispase II and cultures enriched for neurons or glia by differential centrifugation. (B) Enriched fractions were plated into 96- or 384-well plates and incubated for 24 h (neurons) or 5 days (glia). Two neuron cell bodies are highlighted with black arrows. (C) Concentration–response and acute time-course studies were then performed for both cell types using modified assays designed for drug discovery.

neurons (Supporting Information Fig. S3). Further, treatment with 50% FBS for 30 min did not significantly increase ERK phosphorylation in these cells, although there was an upward trend. These data suggest that there may be a limited window to detect ERK phosphorylation in these neurons and that any ERK responses may be below the limits of detection, or that ERK phosphorylation is not coupled to PAC_1 receptor activation in these cells. Although it may be possible to further explore this finding using alternative methods, the AlphaScreen method employed is a well-characterized and extremely sensitive method for the detection of ERK phosphorylation, displaying equivalent or greater sensitivity than Western blotting and other methodologies (van der Westhuizen *et al.*, 2007; Crouch and Osmond, 2008).

PAC_1 receptors in transfected Cos7 cells exhibit agonist-specific antagonism

The pharmacology of the $hPAC_{1n}$ receptor in Cos7 cells was characterized to compare with the primary cell cultures. We first established that our Cos7 cells did not endogenously express PACAP-responsive receptors when transiently transfected with the plasmid vector pcDNA3.1+ (Supporting Information Fig. S4). In Cos7 cells transiently transfected with $hPAC_{1n}$, PACAP-38 and PACAP-27 equipotently stimulated cAMP production and were more potent than VIP, peptide histidine methionine (PHM) or PACAP(6–38) (Figure 4A; Table 1). PACAP-38, PACAP-27 and VIP were full agonists at this receptor. Only weak responses to PHM were observed and curves could not be confidently fitted to the data.

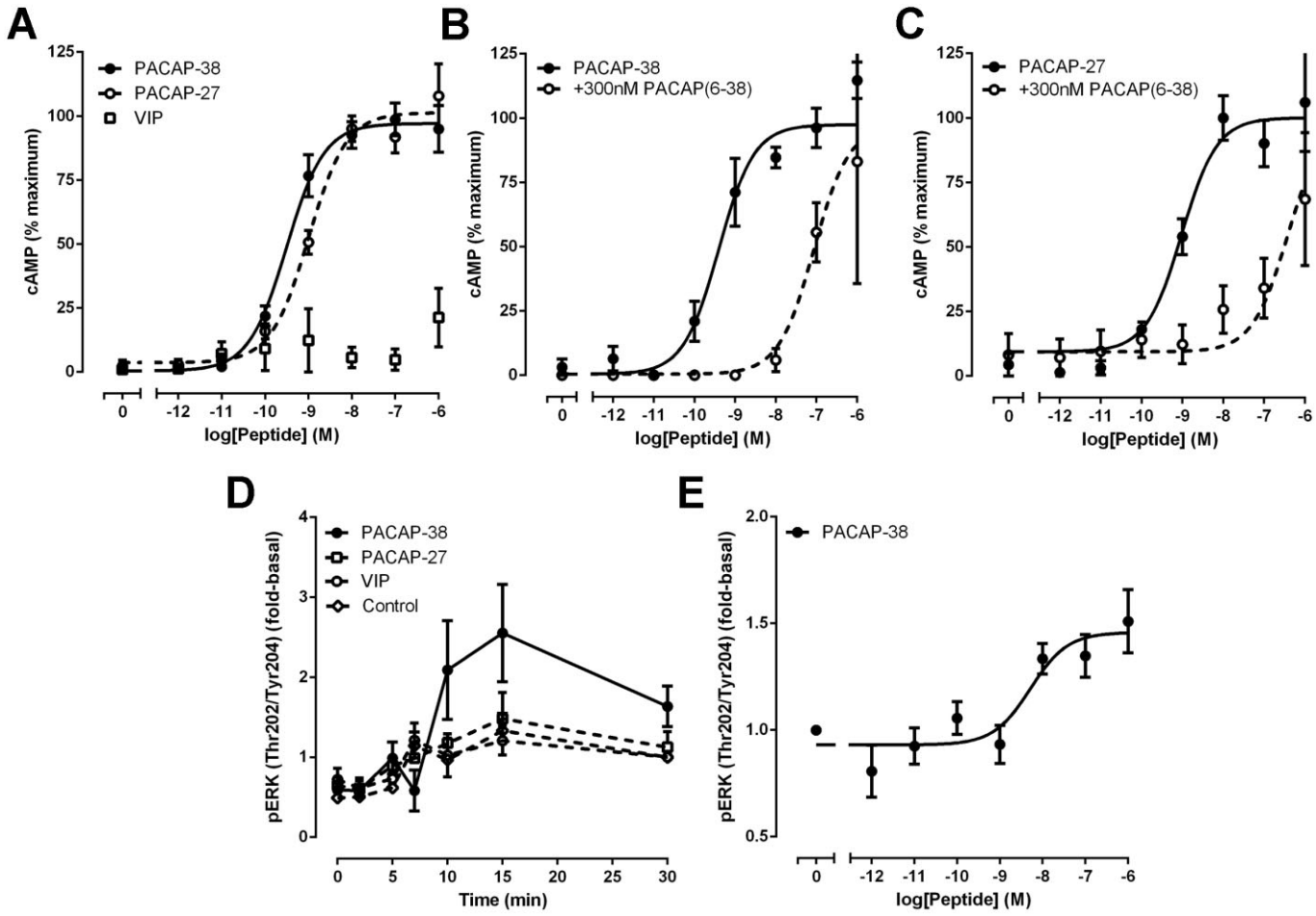

Figure 2

Intracellular signalling in TG-derived glia. (A) Stimulation of cAMP production by PACAP-38, PACAP-27 and VIP. (B, C) Stimulation of cAMP production by (B) PACAP-38 and (C) PACAP-27 in the presence and absence of PACAP(6–38). (D) Temporal stimulation of ERK phosphorylation by 1 μM PACAP-38, PACAP-27 and VIP; using two-way ANOVA followed by *post hoc* Dunnett's test, PACAP-38-induced ERK phosphorylation was significantly higher than paired control treated glia at 10 and 15 min ($P < 0.05$). (E) Concentration-response for ERK phosphorylation by PACAP-38. In all cases, each point represents the mean ± SEM of the combined data from 3 to 6 experiments, performed in triplicate.

To further elucidate the conflicting antagonist results observed in enriched cultures of glia and neurons, the ability of PACAP(6–38) to block PACAP-38 or PACAP-27 responses was also examined. Interestingly, PACAP-38 was not antagonized using 100 nM, 300 nM or 1 μM PACAP(6–38) (Figure 4). However, PACAP-27 was effectively antagonized by PACAP(6–38) at 100 or 300 nM PACAP(6–38) (Figure 4C). Interestingly, PACAP(6–38) alone induced a small increase in basal cAMP production (Figure 4A–C), consistent with its very weak partial agonist activity, as shown in Figure 4A.

ERK phosphorylation was also examined in these cells. Increased ERK phosphorylation over control was observed with 1 μM PACAP-38, PACAP-27 and VIP, with peak activation observed at 7 min (Figure 4D). Concentration–response analysis for PACAP-27 and PACAP-38 with 7 min stimulation showed that PACAP-38 and PACAP-27 were equipotent, although there was a tendency for PACAP-38 to be more potent than PACAP-27. Relatively weak responses were observed with VIP (Figure 4E; Table 1).

To compare PACAP pharmacology observed in $hPAC_{1n}$ transfected Cos7 cells with a second PACAP-activated receptor, the agonist-induced cAMP response in $hVPAC_1$ transfected Cos7 cells was also measured. At the $hVPAC_1$ receptor, PACAP-38, PACAP-27 and VIP were equipotent and significantly more potent than PHM (Figure 4F; Table 1). PACAP(6–38) at 300 nM did not antagonize PACAP-27. Interestingly, 300 nM PACAP(6–38) alone induced a small increase in basal cAMP content similar to that observed at PAC_{1n} receptors (Figure 4G).

Discussion and conclusions

We have developed and refined methods to characterize the pharmacology of PACAP-responsive receptors in neurons and glia derived from the TG, small neuronal structures that house the cell bodies of the fifth cranial nerve. The TG was selected because it is known to express PACAP (Moller *et al.*,

Table 1

Summary of agonist and antagonist potencies in rat TG-derived neurons and glia, and $hPAC_{1n}$ or $hVPAC_1$ receptor transfected Cos7 cells for cAMP production or ERK1/2 phosphorylation

Agonist	Neurons	Glia	$hPAC_{1n}$	$hVPAC_1$
		pEC_{50} (cAMP)		
PACAP-38	7.68 ± 0.14 (8)	9.44 ± 0.13 (6)	9.31 ± 0.07 (20)	9.54 ± 0.06 (7)
PACAP-27	7.35 ± 0.15 (6)	9.00 ± 0.11 (5)*	9.55 ± 0.14 (9)	9.28 ± 0.18 (4)
VIP	<5 (4)	<6 (3)	7.72 ± 0.10 (8)***,+++	9.60 ± 0.07 (4)
PHM	–	–	<6 (4)[a]	8.43 ± 0.11 (3)***,+++,###
		pA_2 (cAMP) PACAP(6–38)		
PACAP-38	6.29 ± 0.24 (3)	9.13 ± 0.23 (3)	<6.5 (3)[b]	–
PACAP-27	7.96 ± 0.22 (3)**	9.14 ± 0.23 (3)	7.88 ± 0.16 (3)	<6.5 (3)
		pEC_{50} (pERK1/2)		
PACAP-38	NR (4)	8.16 ± 0.14 (3)^^^	9.72 ± 0.43 (5)	–
PACAP-27	–	<6 (3)	9.05 ± 0.15 (5)	–
VIP	–	<6 (3)	7.81 ± 0.44 (3)	–

PACAP(6–38) was used in all antagonist experiments. Where pEC_{50} or pA_2 values could not be determined they are defined as < the highest concentration of agonist or antagonist used. Data represent mean ± SEM of (*n*) individual experiments. Comparisons were performed by one-way ANOVA followed by *post hoc* Dunnett's tests or Student's *t*-test where appropriate. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus PACAP 38; ^{+++}P < 0.001 versus PACAP 27; $^{###}P$ < 0.001 versus VIP. $^{\wedge\wedge\wedge}P$ < 0.05 for PACAP-38 (ERK vs. cAMP). –, not performed.
[a]Curves could not be confidently be fitted to the data. [b]One further experiment gave a pA2 of 7.25. NR No response at 1 μM PACAP-38 in four separate time-course experiments.

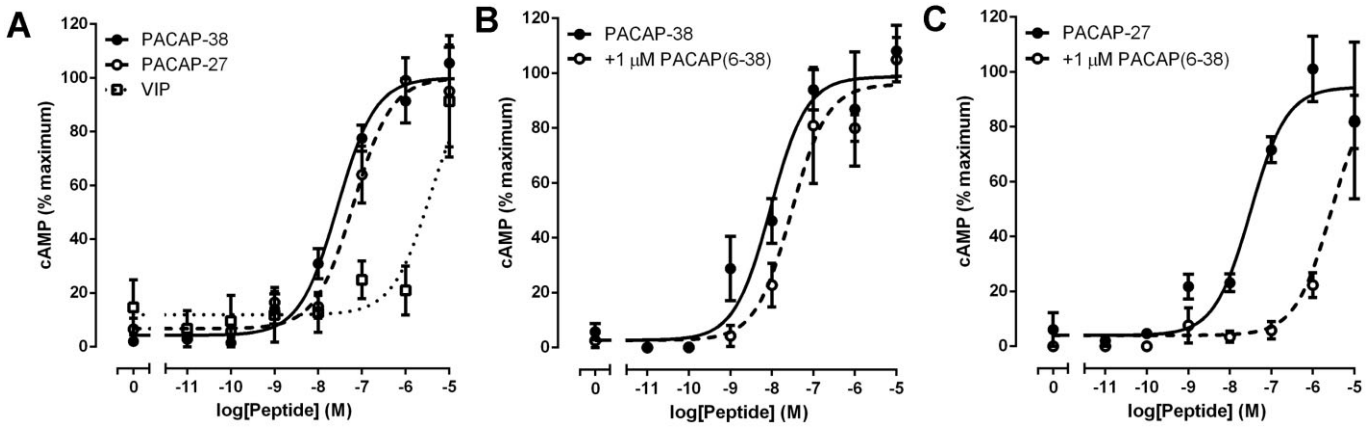

Figure 3

cAMP production in TG-derived neurons. (A) Stimulation of cAMP production by PACAP-38, PACAP-27 and VIP. (B–C) Stimulation of cAMP production by (B) PACAP-38 and (C) PACAP-27 in the presence and absence of PACAP(6–38). Each point represents the mean ± SEM of the combined data from 3 to 8 experiments, performed in triplicate.

1993) and PAC_1 receptors (Chaudhary and Baumann, 2002; Nakajima *et al.*, 2013), and there are established methods for the isolation and culture of neurons and glia from this ganglia (Durham and Russo, 1999; Bowen *et al.*, 2006; Li *et al.*, 2008). Using these cells, we conducted an examination of PACAP receptor pharmacology by constructing concentration–response curves to several agonists for two distinct signalling molecules; cAMP, a major second messenger, and ERK, an important downstream signalling protein, often reported to be downstream of cAMP following PACAP receptor activation (Vaudry *et al.*, 2009).

PACAP receptors can be identified and distinguished based on their relative affinities for endogenous ligands PACAP and VIP. The transfected $hPAC_{1n}$ receptor behaved as expected compared with other transfected cell studies; PACAP-38 and PACAP-27-induced cAMP production was greater than that produced by VIP. This pharmacology is indicative of a PAC_1 receptor (Harmar *et al.*, 2012). In

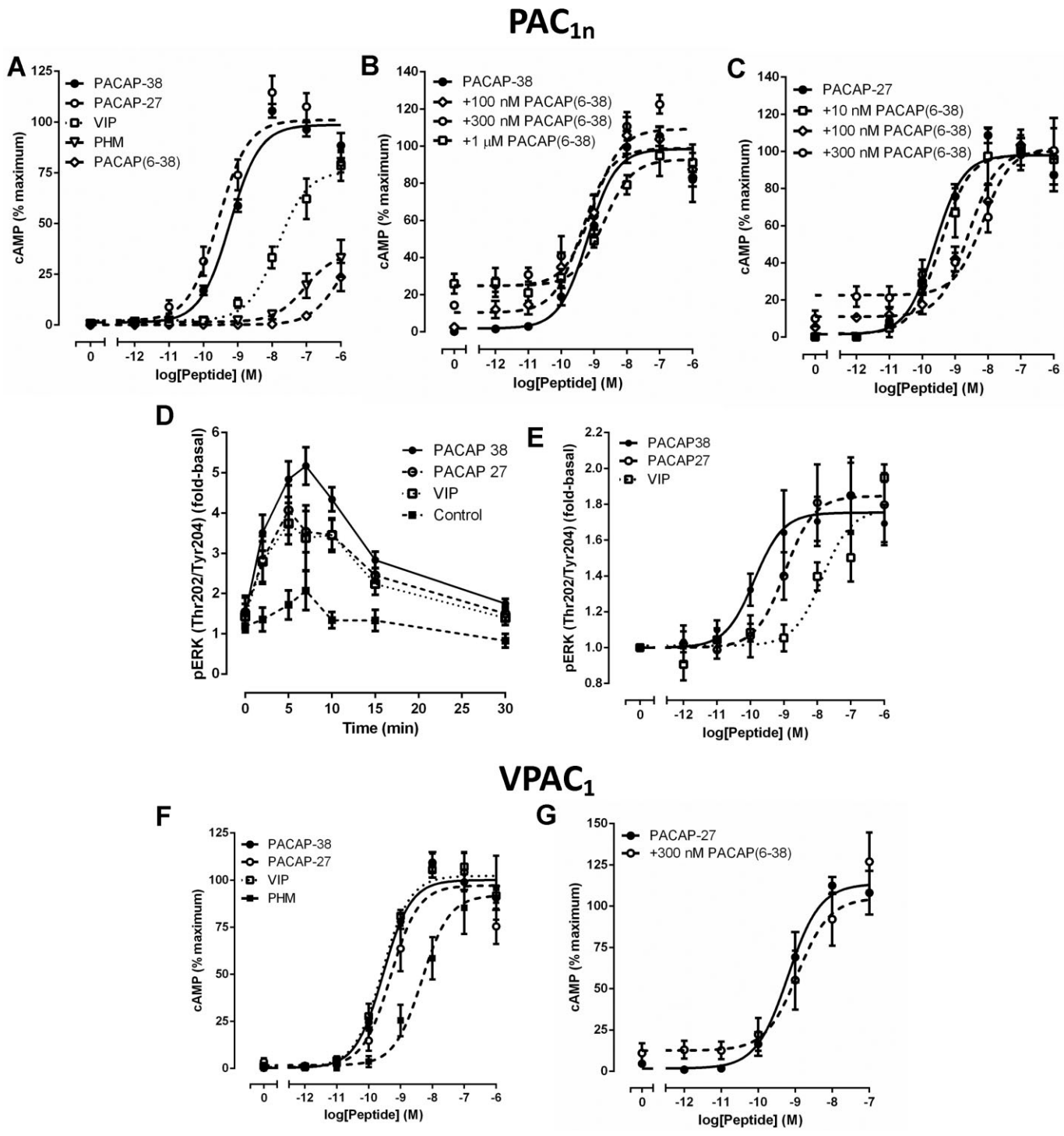

Figure 4

Intracellular signalling in Cos7 cells expressing PAC_{1n} or $VPAC_1$. (A) Stimulation of cAMP production by PACAP-38, PACAP-27, VIP, PHM and PACAP (6–38) at PAC_{1n} receptors. (B, C) Stimulation of cAMP production by (B) PACAP-38 and (C) PACAP-27 in the presence and absence of PACAP(6–38) at PAC_{1n} receptors. (D) Temporal stimulation of ERK phosphorylation by 1 μM PACAP-38, PACAP-27 and VIP at PAC_{1n} receptors; using two-way ANOVA followed by *post hoc* Dunnett's test, PACAP-38, PACAP-27 and VIP-induced ERK phosphorylation was significantly higher than paired control treated cells at 2, 5, 7, 10 and 15 min ($P < 0.05$). (E) Stimulation of ERK phosphorylation by PACAP-38, PACAP-27 or VIP at PAC_{1n} receptors. (F) Stimulation of cAMP production by PACAP-38, PACAP-27, VIP and PHM at $VPAC_1$ receptors. (G) Stimulation of cAMP production by PACAP-27 in the presence and absence of PACAP(6–38) at $VPAC_1$ receptors. Each point represents the mean ± SEM of the combined data from 3 to 20 experiments, performed in triplicate.

contrast, transfected $VPAC_1$ receptors displayed equipotent cAMP induction with PACAP-38, PACAP-27 and VIP. We observed a pattern of cAMP production, indicative of $hPAC_{1n}$ receptors for both TG-derived neurons and glia. It is interesting to note that PACAP-38 and PACAP-27 displayed nearly 100-fold greater potency at transfected $hPAC_{1n}$ receptors and TG glia than in TG neurons. This could be attributed to potential differences in PAC_1 splice variants present (Spongier *et al.*, 1993; Blechman and Levkowitz, 2013), levels of receptor expression or the microenvironment of signalling molecules and receptor regulators present in the different cell types (Chidiac and Ross, 1999; Fu *et al.*, 2004). Although signalling data in primary cultured neurons are limited, others have investigated PACAP-mediated cAMP and ERK signalling in primary cultured cerebellar granular neurons. Cerebellar granular neurons account for up to 75% of all brain neurons, making them a viable choice for pharmacological analysis when large numbers of cells are required (Llinas *et al.*, 2004). In cultured cerebellar granular neurons, PACAP-38 and PACAP-27 equipotently stimulated cAMP production (Kienlen Campard *et al.*, 1997; Villalba *et al.*, 1997), in agreement with our data in TG neurons, which are much less abundant.

To further elucidate the pharmacology of the PACAP receptor in the TG, we examined the antagonist potency of PACAP(6–38) on PACAP-38 and PACAP-27-induced cAMP accumulation. Although PACAP(6–38) displays only limited selectivity for PAC_1 over the $VPAC_2$ receptor, it is the best characterized antagonist at these receptors (Harmar *et al.*, 2012). Unexpectedly, PACAP(6–38) antagonized PACAP-27 more potently than PACAP-38 in Cos7 cells transfected with PAC_{1n}. Earlier studies have observed a similar agonist-specific antagonism by PACAP(6–38) in NB-OK-1 neuroblastoma or AR 4-J2 acinar cells, which express PAC_1 receptors (Robberecht *et al.*, 1992a; Robberecht *et al.*, 1992b). Given the important role that the C-terminal region of PACAP plays in receptor binding (Kumar *et al.*, 2011), it is possible that the loss of this region alters the behaviour of the ligand, such as altering kinetics. Interestingly, PACAP(6–27) is weaker at antagonizing PACAP activity in AR 4-J2 and rat hippocampus membranes compared with PACAP(6–38) (Robberecht *et al.*, 1992b; Hou *et al.*, 1994). We observed similar agonist-specific antagonism in TG neurons, where PACAP-38 was relatively poorly blocked compared with PACAP-27. This finding may in part explain studies where PACAP(6–38) did not antagonize PACAP-38 (Reglodi *et al.*, 2008) and further highlights the value of determining antagonist potencies from agonist concentration–response curves. These results were strikingly different from those in TG glia, where both PACAP-38 and PACAP-27 were potently antagonized by PACAP(6–38). This equipotent pattern of antagonism is similar to that observed in Y79 retinoblastoma cells (Olianas *et al.*, 1996). Thus, PACAP(6–38) antagonism observed in this study is consistent with the wide range of potency values reported in the literature. Efficient processing of PACAP-38 to PACAP-27 in TG glia cultures could explain this difference. However, glial ERK activation was observed in response to PACAP-38, not PACAP-27, suggesting that PACAP-27 was not present when PACAP-38 was used as the agonist. Further exploration of the observed PACAP(6–38) antagonist properties, using a range of antagonist concentrations in immortalized cell lines, prior to studies involving animal tissue, would be useful for discerning the apparent difference. However, the use of higher PACAP(6–38) concentrations in these cells is problematic because we observed weak partial agonist activity at 1 μM PACAP(6–38) in Cos7 cells. PACAP(6–38) is reported to have agonist activity in other studies (Baun *et al.*, 2012). We did not observe PACAP(6–38) agonism in neurons or glia and this could be a consequence of lower receptor numbers in these cell types compared with those overexpressed in Cos7 cells.

It would be interesting to consider further whether the phenomenon of agonist-specific antagonism was restricted to PACAP(6–38) or whether it might also be observed with other antagonists, such as low MW antagonists that could be developed for treating migraine or other forms of pain. These findings are particularly relevant as PACAP(6–38) displays antinociceptive behaviour (Davis-Taber *et al.*, 2008) and most research has focused on PACAP-38, the most prevalent variant. The relative importance of PACAP-27 versus PACAP-38 in physiology or pathophysiology is unknown, but our findings highlight the need to consider differences between these peptides when developing antagonists.

In stark contrast to the similarities observed across the cell types for cAMP responses, PACAP-induced ERK1/2 phosphorylation diverged radically. In Cos7 cells transfected with the $hPAC_{1n}$ receptor, 1 μM PACAP-38, PACAP-27 and VIP all displayed a rapid increase in ERK phosphorylation consistent with activation by a G-protein (DeWire *et al.*, 2007), with a peak at approximately 7 min. The concentration–response curves support this suggestion, giving a similar pattern of agonist potencies to those observed for cAMP. cAMP-mediated ERK activation is required for neurite outgrowth in PC12 cells (Gerdin and Eiden, 2007) and differentiation of SH-SY5Y neuroblastoma cells (Monaghan *et al.*, 2008). This information, combined with our data, suggests that in Cos7 cells, ERK1/2 phosphorylation may be $G_{\alpha s}$-mediated, although this is speculative.

We did not detect ERK1/2 phosphorylation in response to PACAP-38 in TG neurons over a time course of up to 30 min. ERK phosphorylation has been used as a marker of sensory neuron activation (Gao and Ji, 2009) and can be associated with nociceptive responses (Komatsu *et al.*, 2011). Further, PACAP-38 increased ERK activity with a similar potency to cAMP induction in cerebellar granular neurons (Villalba *et al.*, 1997). However, several studies in cultured TG neurons provide evidence for a lack of coupling. Stimulation of TG cultures with TNF-α or NO had no effect on ERK phosphorylation (Bellamy *et al.*, 2006; Bowen *et al.*, 2006). There are many possible explanations for this. The culture of neurons could lead to high basal ERK1/2 phosphorylation (Tajti *et al.*, 2011), *in vivo* ERK1/2 activation could occur indirectly via secondary mechanisms such as depolarization (Firner *et al.*, 2006) or our assay may not have been sufficiently sensitive, resulting in observational bias (Kenakin and Christopoulos, 2013). Interestingly, we also saw no substantial ERK1/2 phosphorylation using the *Surefire* AlphaScreen assay or fluorescent microscopy in TG neurons for calcitonin gene-related peptide, where robust cAMP, pCREB and p38 MAP kinase responses were evident using similar methodologies (CSW and DLH unpubl. obs.).

In TG-derived glia, robust agonist-induced ERK1/2 phosphorylation was observed with PACAP-38. Interestingly, the

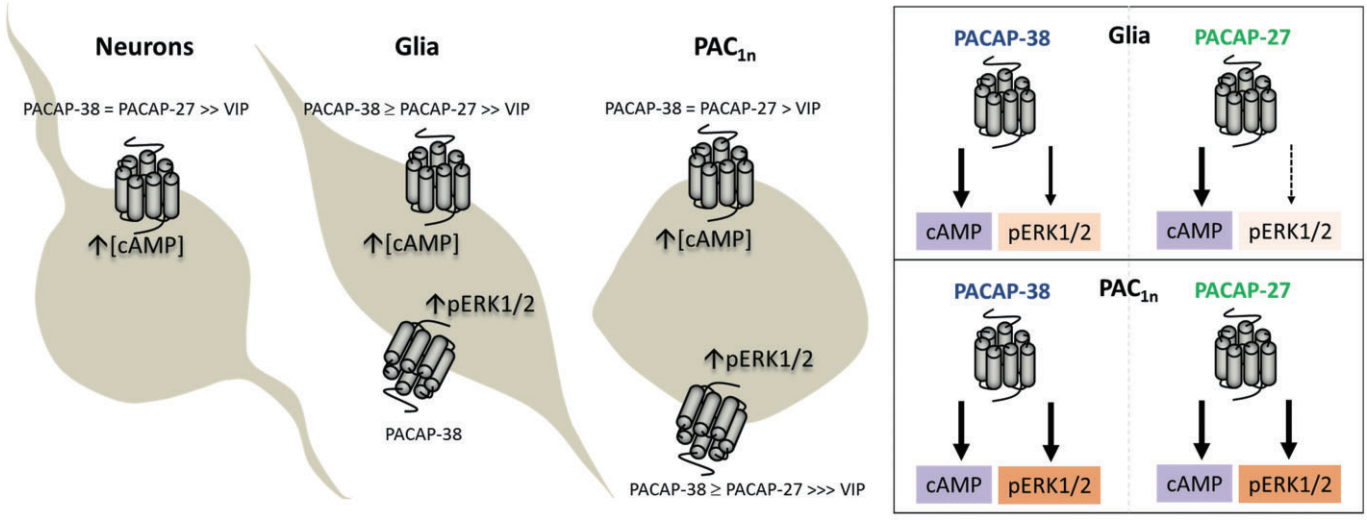

Figure 5

Schematic summary of PACAP and VIP induced signalling responses. The left panel illustrates the major results of this study. The relative potencies for each of the peptides tested are shown. Symbols indicate the following: =, equal potency; ≥, equal or less than 10-fold greater potency; >, ~10-fold greater potency; > >, ~100-fold greater potency; > > >, ~1000-fold greater potency. The right panel illustrates the observed PACAP-38 and PACAP-27 induced cAMP accumulation and ERK phosphorylation in trigeminal ganglia glia and Cos7 cells transfected with $hPAC_{1n}$. Shading intensity and arrow thickness, indicates the relative signalling induction. The dotted arrow indicates that no detectable signal was observed.

time course of activation was delayed compared with the Cos7 cells with a peak at around 15 min. It is tempting to suggest that the shift in the time course indicates that a G-protein independent pathway such as β-arrestin may be responsible for ERK1/2 phosphorylation in response to PACAP-38 in these cells (DeWire *et al.*, 2007); however, additional work would be needed to determine this. In the islet beta-cell-derived cell line, INS-1E, PACAP-38 can induce sustained ERK activation via the β-arrestin pathway suggesting that cAMP-independent ERK activation is possible for this neuropeptide (Broca *et al.*, 2009). Interestingly, only PACAP-38 increased ERK1/2 phosphorylation in glia in the time courses. This observation was supported by full concentration–response analysis. The potency for ERK1/2 phosphorylation was lower than for cAMP production for PACAP-38. This has been reported for other receptors, such as amylin receptors (Morfis *et al.*, 2008; Qi *et al.*, 2013). Most notably, PACAP-27 up to concentrations of 1 μM was unable to increase ERK1/2 phosphorylation despite being as active as PACAP-38 at increasing cAMP production in glia. This suggests that PACAP-27 is a biased agonist in this cell system (Figure 5). This type of profound bias has been referred to as 'perfect bias' but it is important to note that this is a system-dependent phenomenon because PACAP-27 did induce ERK1/2 phosphorylation in transfected Cos7 cells. Nevertheless, it is significant that we have been able to detect agonist bias for an endogenous peptide ligand in cells that endogenously express its cognate receptor. Depending on whether PACAP-27 or PACAP-38 is exposed to glia under physiological or pathophysiological conditions could determine the functional outcome.

The complex pharmacological phenotypes observed in this study highlight the need to measure signalling pathways in response to several concentrations of ligands in cells derived directly from appropriate tissues. The diversity may be due to differential expression of splice variants, which are known to alter the pharmacology of PAC_1 and other GPCRs (Furness *et al.*, 2012; Qi *et al.*, 2013). There are reportedly around 10 PAC_1 splice variants in rats. Several of these display differential intracellular coupling in cell line models. These variants include the hip and hop insertions into intracellular loop 3, which are proposed to directly modulate G-protein coupling (Spongier *et al.*, 1993; Blechman and Levkowitz, 2013). Alternatively, the presence of accessory proteins, such as receptor activity-modifying proteins (RAMPs) in the cellular background could alter receptor functionality. RAMPs can interact with the closely related $VPAC_1$ and $VPAC_2$ receptors and can alter the function and pharmacology of GPCRs (Wootten *et al.*, 2013; Christopoulos *et al.*, 2003). Interestingly, the potential impact of RAMPs on PAC_1 function has not been investigated.

Recent studies have shown that peripheral infusion of PACAP-38 can induce migraine in migraine sufferers, similar to calcitonin gene-related peptide, which is receiving considerable attention as a migraine target (Schytz *et al.*, 2010; Olesen and Ashina, 2011). The PACAP studies suggest that PACAP-38 induces migraine through a VIP-insensitive receptor, probably PAC_1. New medications for the treatment of migraine are vital to improve the quality of life for sufferers worldwide. As such, migraine has been the subject of intense investigation in both academia and the pharmaceutical industry. Typically, *ex vivo* vascular translational models have been employed for the development of migraine treatments (Eikermann-Haerter and Moskowitz, 2008). However, as the portfolio of evidence indicating that the pathological site(s) of action is neurological has evolved (Tfelt-Hansen and Olesen, 2012; Amin *et al.*, 2013), the lack of practical translational neuron models has become increasingly apparent.

Using our methods, we report novel and complex PACAP receptor pharmacology in TG glia and neurons. This strengthens the idea that the PAC_1 receptor could be a new target for migraine.

Utilizing miniaturized high-throughput techniques to investigate intracellular signalling in primary cell culture models, we provide a potentially viable platform for the development of translational models. These could be used to pharmacologically characterize the actions of drugs in small populations of disease-relevant cells. Further miniaturization and automation of these assays is likely to be possible with primary cells, increasing the scope of pharmacological analysis that can be performed. This work may also serve as a platform for linking observations of agonist bias in transfected cell models to observations in whole animals.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Histological identification of glia and neurons in enriched cultures from rat trigeminal ganglia. Primary cultures were fixed using 4% paraformaldehyde in PBS for 30 min at room temperature in cell culture plates. Cells were washed twice with PBS and stored at 4°C. For the detection of glial fibrillary acidic protein (GFAP) or microtubule-associated protein 2 (MAP2), antigen retrieval was performed by microwaving cells for 1 min in PBS. Cells were then incubated in PBS containing 0.6% hydrogen peroxide at room temperature for 20 min. Cells were washed once with PBS and blocked with 10% goat serum or rabbit serum (depending on the species of the secondary) for 1 h. Cells were incubated at 4°C overnight with specific primary antibodies raised against GFAP (1:500; Sapphire Bioscience, New South Wales, Australia) or MAP2 (1:500; Sigma-Aldrich, St. Louis, MO, USA) in PBS containing 1% goat serum. Cells were incubated with secondary antibody (1:1000; Sigma-Aldrich) at room temperature with gentle shaking for 1 h. Cells were washed in PBS and incubated with streptavidin HRP-polymer (1:1000; Sigma-Aldrich) diluted in PBS containing 1% serum and incubated at room temperature for 1 h. Cells were washed twice in PBS and visualized by incubation for 2–5 min in SIGMA*FAST*3,3′-diaminobenzidine tablets (Sigma-Aldrich) pre-dissolved in PBS. Cells were washed twice with PBS and then imaged under PBS using a Nikon Ti-E inverted microscope at l00× magnification. Images represent data from 2–3 separate preparations.
Figure S2 ERK1/2 phosphorylation in trigeminal ganglia glia. (A) Monitoring of ERK1/2 phosphorylation at 15 min with PACAP-27 or (B) VIP addition. Each point represents the mean ± SEM of the combined data from 3 experiments, performed in triplicate.
Figure S3 ERK1/2 phosphorylation in trigeminal ganglia neurons. Monitoring of ERK1/2 phosphorylation from 0–30 min following PACAP-38 or media control addition. Each point represents the mean ± SEM of the combined data from three experiments, performed in triplicate.
Figure S4 cAMP production in vector transfected Cos7 cells. Monitoring of cAMP production following the addition of PACAP-38 or VIP in Cos7 cells transfected with pcDNA3.1 (Vector). Each point represents the mean ± SEM of the combined data from 3 experiments, performed in triplicate.