Published in final edited form as:

Mol Cell Neurosci. 2010 December ; 45(4): 345–354. doi:10.1016/j.mcn.2010.07.007.

PACAP protects against TNF α -induced cell death in olfactory epithelium and olfactory placodal cell lines

Shami Kanekar¹, Mahendra Gandham¹, and Mary T Lucero^{1,2,3,§}

¹Department of Physiology, University of Utah, Salt Lake City, UT 84108

²Utah Brain Institute, Salt Lake City, UT 84108

³Utah Center on Aging, University of Utah, Salt Lake City, UT 84112

Abstract

In mouse olfactory epithelium (OE), pituitary adenylate cyclase activating peptide (PACAP) protects against axotomy-induced apoptosis. We used mouse OE to determine whether PACAP protects neurons *during* exposure to the inflammatory cytokine TNF α . Live slices of neonatal mouse OE were treated with 40 ng/ml TNF α $\pm$ 40 nM PACAP for 6 hours and dying cells were live-labeled with 0.5% propidium iodide. TNF α significantly increased the percentage of dying cells while co-incubation with PACAP prevented cell death. PACAP also prevented TNF α -mediated cell death in the olfactory placodal (OP) cell lines, OP6 and OP27. Although OP cell lines express all three PACAP receptors (PAC1, VPAC1, VPAC2), PACAP's protection of these cells from TNF α was mimicked by the specific PAC1 receptor agonist maxadilan and abolished by the PAC1 antagonist PACAP6–38. Treatment of OP cell lines with blockers or activators of the PLC and AC/MAPKK pathways revealed that PACAP-mediated protection from TNF α involved both pathways. PACAP may therefore function through PAC1 receptors to protect neurons from cell death during inflammatory cytokine release *in vivo* as would occur upon viral infection or allergic rhinitis-associated injury.

Introduction

During CNS injury, microglia become activated and release cytokines such as TNF α and IL1 β (Clausen *et al.*, 2008). Cytokines then cause a series of inflammatory responses in the tissue that are often detrimental, and can result in exacerbation of the original injury. Several studies have documented the ability of pituitary adenylate cyclase activating polypeptide (PACAP) to reduce expression and release of TNF α and other cytokines following lipopolysaccharide stimulation of microglial cell lines (Kim *et al.*, 2000) or purified macrophage cultures (Delgado. *et al.*, 2003b). PACAP belongs to the vasoactive intestinal peptide (VIP)-secretin-glucagon superfamily of peptides (Kieffer & Habener, 1999) and acts through G-protein coupled PAC1, VPAC1 and VPAC2 receptors to increase cAMP and reduce TNF α expression in microglia (Kim *et al.*, 2000). We used the murine olfactory epithelium (OE) as a model to analyze the effect of PACAP administration during cytokine release. Unlike in the CNS, the neurons of the OE are directly exposed to the external environment and are

© 2010 Elsevier Inc. All rights reserved.

[§]Corresponding author, Mary T Lucero, 420 Chipeta Way Ste 1700, Department of Physiology, University of Utah, Salt Lake City, UT 84108, Phone 801-585-5601, Fax 801-581-3476, mary.lucero@utah.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

continually damaged by airborne toxins and xenobiotics. In response to the continual damage, the OE is able to regenerate and does so throughout adulthood (Graziadei & Graziadei, 1979). Both TNF α and TNF α receptors (TNFR1 and TNFR2) are expressed in the OE (Farbman *et al.*, 1999) and TNF α can induce apoptosis in explants of the embryonic OE (Suzuki & Farbman, 2000). TNFR2 activates pro-survival signaling pathways, while TNFR1 contains a cytoplasmic death domain which allows recruitment of several death domain and other proteins which can then activate caspases and induce apoptosis (McCoy & Tansey, 2008).

PACAP is expressed in the OE (Hegg *et al.*, 2003a; Hansel *et al.*, 2001) and protects against axotomy-induced apoptosis of neonatal rat olfactory sensory neurons (OSNs) in primary cultures (Hansel *et al.*, 2001). In addition, PACAP protects against axotomy-induced apoptosis of adult mouse OSNs via a phospholipase C (PLC) and calcium-dependent reduction in A-type potassium channels (Han & Lucero, 2005). While axotomy is a well-characterized model for study of olfactory neurodegeneration, it differs considerably from apoptosis induced by direct damage to the OE. Studies using cytokine-induced apoptosis more closely mimic an inflammatory response to direct damage to the OE and are likely to yield more physiologically-relevant information.

In this study, we therefore used a live slice model of the OE (Hegg *et al.*, 2003a) to demonstrate that PACAP can protect against cytokine-induced cell death. As this model does not distinguish between effects of PACAP on neurons or immune cells, the majority of our study involved using two olfactory placodal (OP) cell lines to examine the effects of PACAP on neuronal cells in the absence of immune cells. OP6 and OP27 are olfactory neuronal precursor cell lines clonally derived from the olfactory placode of embryonic mice (Illing *et al.*, 2002). These cell lines are well-characterized and shown to be a good model to study both immature and mature olfactory neuron function. OP27 cells contain the expression profile of immediate neuronal precursors (INPs) while OP6 cells express markers of immature receptor neurons (IRNs) (Illing *et al.*, 2002). When placed in differentiating conditions, both OP cell lines develop a bipolar neuronal morphology, express odorant receptors, upregulate G_{olf} and OMP, and down regulate NST in a manner similar to olfactory neurons *in vivo* (Illing *et al.*, 2002; Regad *et al.*, 2007; Pathak *et al.*, 2009).

We examined the role of PACAP receptors and identified PAC1R subtypes expressed by the OP cell lines. The PACAP receptors PAC1, VPAC1 and VPAC2 have different agonist and antagonist profiles. Maxadilan is a potent vasodilatory peptide isolated from sandfly saliva (Lerner *et al.*, 1991) which functions by activating the PAC1 receptor (PAC1R) (Lerner *et al.*, 2007). Unlike PACAP itself, maxadilan binds specifically to PAC1R, and does not activate either of the VPAC receptors (Moro & Lerner, 1997). PACAP6–38 on the other hand is a truncated version of PACAP which functions as a PACAP receptor antagonist at both PAC1 and VPAC2 receptors, but does not block VPAC1 (Moro *et al.*, 1999). Maxadilan and PACAP6–38 were therefore used to probe receptor specificity in this study. In addition, the PAC1 receptor occurs as several subtypes resulting from alternative splicing of mRNA encoding the first extracellular domain (EC1) (Pantaloni *et al.*, 1996), and the third intracellular cytoplasmic loop (IC3) (Spengler *et al.*, 1993). The most common EC1 variants are called normal (N, with no deletion) and short (S, with a 21 AA deletion), and these primarily regulate agonist binding (Pantaloni *et al.*, 1996). IC3 splice variants occur as the regular form (R) without insertions, or with the inserted cassettes of Hip, Hop1, Hop2, or HipHop1/2 (Spengler *et al.*, 1993). The IC3 splice variants govern coupling to either or both of the adenylyl cyclase (AC) or phospholipase C (PLC) transduction pathways (Spengler *et al.*, 1993; Ushiyama *et al.*, 2007). We determined the PAC1R splice variants expressed by the OP cells to aid in identifying the second messenger systems activated by PACAP in this model.

In the present work, we use live-labeling with propidium iodide (PI) to show that TNF α induces cell death in neuronal precursors, and that TNF α -mediated cell death is reduced by PACAP via PAC1 receptor binding and activation of either the PLC or the AC/MAPKK second messenger systems. We also show that TNF α induced caspase activity is blocked by PACAP treatment in OP cells. These studies suggest that PACAP can be neuroprotective even if it does not precede TNF α release.

Results

PACAP protects against TNF α -induced cell death in live slices of the OE

We treated live slices of OE from P1 mice with 40 ng/ml TNF α , TNF α +40 nM PACAP or vehicle for 6 hrs, and then live-labeled for 20 mins with 0.05% propidium iodide (PI), a dye that only enters dead or dying cells. Slices treated with TNF α (Fig. 1B, 1E) exhibited a significantly higher percentage of PI labeling ($38 \pm 6\%$, Fig. 1G) than vehicle controls ($10 \pm 3\%$, $p=0.04$, Fig. 1A, 1D). Co-incubating slices in both TNF α and PACAP (Fig. 1C, 1F) significantly reduced the percentage of PI labeling compared to TNF α alone ($p=0.003$) to a value that was not different from control ($7 \pm 1\%$, $p=0.5$, Fig. 1G). While TNF α treatment affected cells in all layers of the OE (Fig. 1E), the majority of PI labeling was in the basal to lower middle layers of the OE (Fig. 1D–F), which contain mainly basal progenitor cells, immediate neural precursors and immature neurons. These immature OE cells may thus be more vulnerable to the cumulative damaging effects of slicing and cytokine treatment than the mature neurons and sustentacular cells in the apical OE. PACAP reduced TNF α -induced PI labeling throughout the OE, demonstrating that PACAP can protect olfactory epithelial cells against cell death induced by TNF α , a cytokine endogenous to the OE (Farbman *et al.*, 1999).

OE slice experiments however were relatively qualitative and did not differentiate between necrotic cell death and apoptosis nor distinguish between the different cell types of the olfactory mucosa. To focus our studies on neuronal cells of the OE, we assayed olfactory placodal cell lines of neuronal lineage.

OP6 and OP27 cells express TNF α , PACAP and their receptors

OP6 and OP27 cells are clonal olfactory neuronal precursor cell lines derived from E10 mouse olfactory placode (Illing *et al.*, 2002). Prior to using the OP cell lines in the apoptosis assay, we first determined whether they express receptors for the cytokine TNF α as well as PACAP and its receptors. RT-PCR showed that both OP6 and OP27 cells express both TNFR1 and TNFR2 (Fig. 2). RT-PCR also showed that OP6 and OP27 cells express PACAP and its receptors PAC1, VPAC1 and VPAC2 (Fig. 2). These data therefore indicate that the OP cell lines could function as an appropriate model system to examine a potential role for PACAP in protection against TNF α -induced neuronal cell death.

PACAP protects olfactory placodal cell lines against TNF α -induced cell death

To test the effects of PACAP on survival of cells of neuronal lineage, we cultured OP6 and OP27 cells for 5 hrs in the presence or absence of 40 ng/ml TNF α $\pm$ 40 nM PACAP. The percentage of PI-labeled to total cells was calculated for cultures treated with TNF α , TNF α +PACAP or vehicle. Under control conditions, PI labeled cells comprised $4 \pm 0.7\%$ of total in OP6 cells (Fig. 3A, 3G) and $2.3 \pm 0.4\%$ of total in OP27 cells (Fig. 3B, 3H). Treatment with TNF α significantly increased the level of cell death to $5.7 \pm 0.8\%$ in OP6 cells (Fig. 3C, 3G, $n=15$, $p=0.003$) and $3.5 \pm 0.6\%$ in OP27 cells (Fig. 3D, 3H, $n=15$, $p=0.004$). TNF α therefore increased cell death 42% above control in OP6 cells and 52% above control in OP27 cells.

Co-incubating cells with TNF α and PACAP reduced the percentage of PI-labeled cells down to or below that seen in control: to $2.9 \pm 0.5\%$ in OP6 cells (Fig. 3E, 3G) and $2.3 \pm 0.4\%$ in

OP27 cells (Fig. 3F, 3H). TNF α +PACAP treatment significantly lowered cell death from that seen with TNF α alone ($n=15$, $p<0.001$ (OP6); $p=0.002$ (OP27)). PACAP therefore protects against TNF α -induced cell death in the olfactory neuronal precursor cell lines, OP6 and OP27.

To examine whether PACAP treatment alone reduced cell death in the absence of TNF α , a subset of experiments included treatment of OP cells with PACAP at 40 nM (Fig. 3I). Treatment with PACAP alone did not significantly alter the level of dying cells compared to matched controls in either OP6 ($3.1 \pm 0.7\%$ vs $3.2 \pm 0.8\%$, $n = 6$, $p=0.41$) or OP27 cells ($2 \pm 0.7\%$ vs $1.9 \pm 0.7\%$, $n = 6$, $p=0.88$).

Caspase assay

To test whether TNF α induced necrotic or apoptotic cell death, we measured caspase activity in OP6 cells treated for 5 hours with TNF α , TNF α +PACAP or vehicle alone. OP6 cells treated with 40 ng/ml TNF α showed a significant increase in activity of the initiator caspase, caspase 8, (Kaushal & Schlichter, 2008) compared to control conditions: $115 \pm 3\%$ of that seen in control ($n = 3$ independent experiments, $p=0.03$). Inclusion of 40 nM PACAP with TNF α prevented the increase in caspase 8 activity ($93 \pm 11\%$ of control; $n = 3$, $p=0.58$). Induction of activated caspase 8 by TNF α and prevention of this induction by co-treatment with PACAP implies that both TNF α and PACAP mediate their effects at least partially, if not fully, on apoptotic cell death.

PACAP functions through PAC1R activation

To define the mechanism by which PACAP protects against TNF α -mediated cell death in olfactory placodal cells, we first looked at whether PACAP's neuroprotective function is receptor-mediated. We co-incubated OP cells with TNF α +PACAP and the PAC1/VPAC2 specific antagonist PACAP6–38 at either 40 nM or 100 nM (Fig. 4A). In OP6 cells, TNF α +PACAP reduced the PI-labeled cells from $5.5 \pm 0.8\%$ in TNF α alone to $2.9 \pm 0.7\%$. Adding 40 nM PACAP6–38 to TNF α +PACAP did not significantly alter the level of PI-labeled OP6 cells from TNF α +PACAP ($2.6 \pm 0.5\%$, $n = 6$, $p=0.10$, Fig. 4A), while at 100 nM, the antagonist significantly reduced the protective effect of PACAP ($4.2 \pm 0.6\%$, $n = 7$, $p=0.004$). In OP27 cells, TNF α +PACAP reduced the PI-labeled cells from $3.8 \pm 0.9\%$ in TNF α alone to $2.4 \pm 0.6\%$. PACAP6–38 was highly effective in blocking PACAP's protection of OP27 cells, increasing the level of PI-labeled cells back to that seen with TNF α : $3.4 \pm 0.6\%$ at 40 nM and $4 \pm 1\%$ at 100 nM ($n = 6$, $p=0.04$; $n = 7$, $p=0.03$, Fig. 4B). Controls consisting of treating cells with PACAP6–38 alone at 40 nM or 100 nM or with TNF α +PACAP6–38 (100 nM), did not significantly alter the percentage of dying cells from that seen in matched controls or TNF α treatment respectively (Supplemental Data, Table 1).

We then proceeded to define whether both PAC1 and VPAC2 are involved in PACAP-mediated protection from TNF α -induced cell death in the OP cells. We used the PAC1 specific agonist maxadilan to see if TNF α -induced cell death could be suppressed solely by activating PAC1. Co-incubating OP6 cells with TNF α and maxadilan (100 pM) significantly reduced PI-labeled cells from $6.8 \pm 1\%$ with TNF α alone to $4.3 \pm 0.9\%$ ($n = 7$, $p=0.002$, Fig. 4C). This is not significantly different from the level of dying cells seen with TNF α +PACAP ($4.1 \pm 0.9\%$, $p=0.22$, Fig. 4C). In OP27 cells, maxadilan also functioned similarly to PACAP, significantly reducing PI labeling from $3.8 \pm 0.7\%$ in TNF α alone to $2.8 \pm 0.7\%$ ($n = 7$, $p=0.03$) vs. $2.7 \pm 0.7\%$ seen with TNF α +PACAP ($p=0.71$, Fig. 4D). Co-incubating cells with TNF α + maxadilan and the antagonist PACAP6–38 significantly increased the level of dying cells to $7.3 \pm 2\%$ in OP6 cells ($n = 3$, $p=0.02$) and $4 \pm 0.7\%$ in OP27 cells ($n = 3$, $p=0.02$). Maxadilan alone did not alter the level of dying cells from that seen in control (Supplemental Data Table 1). These data indicate that in OP cells, PACAP is functioning at least in part through PAC1 receptors. Since the PAC1 receptor occurs as several different splice variants which couple differentially with

second messenger systems, we next looked at expression of PAC1R splice variants in the two cell lines.

OP6 and OP27 cells express different splice variants of PAC1R

We used RT-PCR to examine OP6 and OP27 cells for the presence of the dominant variants of PAC1R: Extracellular domain 1 (EC1) variants normal (N) or short (S) with third intracellular domain (IC3) variants regular (R), Hip, Hop1, Hop2 or HipHop (Ushiyama *et al.*, 2007) (Fig. 5A, 5B). We found that both the N/R and N/Hop1 variants were expressed in both OP6 and OP27 cells (Fig. 5B). Of the other EC1 normal variants, OP6 cells also expressed N/Hop2. Of the EC1 short variants, OP6 and OP27 expressed the S/Hop2 variant, while OP6 cells also expressed S/R and S/Hop1 variants. Neither cell line expressed the IC3 insertion Hip in any combination: thus the N/Hip, N/HipHop, S/Hip or S/HipHop variants were absent in both OP6 and OP27 cells (Fig. 5B). OP27 cells therefore expressed only three PAC1R splice variants: N/R, N/Hop1 and S/Hop2. OP6 cells express these subtypes as well as N/Hop2, S/R and S/Hop1. These expressed splice variants have been shown to activate both the phospholipase C (PLC) and adenylyl cyclase (AC) pathways while the absent Hip variant only activates the AC pathway (Spengler *et al.*, 1993).

PACAP protects through the PLC signaling pathway

We next set out to identify the signaling mechanism by which PACAP protects against TNF α -induced cell death. Previous studies showed that PACAP reduces A-type K current and caspase activity through activation of PLC, and not AC, in mature OSNs in culture (Han & Lucero, 2005). Here we used phorbol 12-myristate 13-acetate (PMA) to directly activate protein kinase C (PKC), an effector protein downstream of PLC. Co-incubation of TNF α with PMA at 10 nM or 50 nM for 5 hrs significantly reduced the level of dying cells seen in both cell lines (Fig 6A, B). The level of PI-labeled OP6 cells dropped from $6.7 \pm 1\%$ in TNF α treatments to $2 \pm 0.8\%$ with 10 nM PMA ($n = 3$, $p=0.03$) and $1.9 \pm 1\%$ with 50 nM PMA ($n = 3$, $p=0.004$, Fig 6A). In OP27 cells, co-incubation of TNF α with 10 nM PMA reduced the level of PI-labeled cells from $2.9 \pm 0.5\%$ with TNF α treatment to $1.3 \pm 0.3\%$ ($n = 3$, $p=0.06$) while TNF α + PMA at 50 nM reduced the level to $0.7 \pm 0.1\%$ ($n = 3$, $p=0.05$, Fig 6B). PMA alone at 10 nM or 50 nM did not significantly alter the level of dying cells from that seen in control (Supplemental Data Table 2, $p>0.05$). Activating the PLC pathway using PMA thus significantly reduces TNF α -induced cell death, thereby mimicking PACAP's effect against TNF α -induced cell death in both OP cell lines.

Next, to examine whether blocking PLC activity could prevent PACAP's function, we co-incubated cells with TNF α +PACAP and the PLC pathway blocker U-73122. Co-incubating cells with 100 μ M U-73122 reduced the effect of PACAP on TNF α -induced cell death in both cell lines (Fig 6A, B). U-73122 significantly increased the level of cell death from $2.8 \pm 0.6\%$ in TNF α +PACAP-treated OP6 cells to $5.3 \pm 1.5\%$ with U-73122 ($n = 5$, $p=0.04$), which was not significantly different from TNF α treatment alone ($6.7 \pm 1.2\%$, $p=0.18$). In OP27 cells, cell death increased significantly from $1.7 \pm 0.3\%$ in TNF α +PACAP to $2.1 \pm 0.4\%$ when combined with U-73122 ($n=5$, $p=0.03$). However the level of cell death was still significantly lower than in TNF α alone ($2.8 \pm 0.5\%$, $p=0.02$), implying that U-73122 did not completely block PACAP's protection of OP27 cells. U-73122 alone or TNF α +U-73122 did not significantly alter the percentage of dying cells from that seen in paired control or TNF α treatments respectively (Supplemental Data Table 2). These studies suggest that activating the PLC pathway is sufficient for protection from TNF α and that inhibiting the PLC pathway reveals an incomplete block of PACAP protection suggesting involvement of additional pathways.

PACAP protects through the adenylate cyclase (AC) signaling pathway

In several models for CNS damage and disease, PACAP has been shown to be neuroprotective primarily by activation of protein kinase A or MAPKK through the AC pathway (Villalba *et al.*, 1997). Since the PLC blocker did not eliminate PACAP's inhibitory effect, we next looked at the potential role of AC in OP cells. Incubating cultures with TNF α and the AC activator forskolin (FSK, 20 μ M) reduced the level of PI-labeled cells from $6.1 \pm 1\%$ in TNF α treated OP6 cells to $2.5 \pm 0.5\%$ with FSK ($n = 6$, $p=0.02$, Fig 7A), which is not significantly different from values for TNF α +PACAP treatment: $2.9 \pm 0.6\%$ ($p=0.2$). In OP27 cells, adding FSK to the TNF α treatment changed the level of PI-labeled cells from $2.9 \pm 0.7\%$ with TNF α alone to $1.6 \pm 0.5\%$ with TNF α +FSK ($n = 6$, $p=0.007$, Fig 7B), which was not significantly different from that seen with TNF α +PACAP ($1.8 \pm 0.5\%$, $p=0.6$). FSK alone did not significantly alter the level of dying cells from that seen in control (Supplemental Data Table 3). These data indicate that AC pathway activation can mimic the protective effect of PACAP on TNF α treatment of OP cells.

We then used the AC blocker SQ22536 (300 μ M) to attempt to eliminate PACAP's function in OP cells by specifically blocking AC. In OP6 cells, SQ22536 abolished PACAP's protective effect, increasing the level of cell death from: $2.9 \pm 0.6\%$ with TNF α +PACAP to $5.4 \pm 1\%$ when SQ22536 was added (Fig 7A, $n = 6$, $p=0.03$). SQ22536 also significantly blocked PACAP function in OP27 cells, increasing cell death from $1.8 \pm 0.5\%$ with TNF α +PACAP to $2.3 \pm 0.6\%$ with the AC blocker (Fig. 7B, $n = 6$, $p=0.04$). SQ22536 alone or TNF α +SQ22536 did not significantly alter the level of dying cells from that seen in control or TNF α respectively (Supplemental Data Table 3). PACAP therefore can also function in this model of cell death through activation of the AC pathway.

To further investigate the second messenger pathways used by PACAP in its protective role against TNF α -induced cell death, we looked at the effect of incubating cells with the MEK1/2 inhibitor, U-0126 in conjunction with TNF α +PACAP. Adding 10 μ M U-0126 significantly reduced PACAP's protective function by increasing the level of PI-labeled OP6 cells from $5.4 \pm 0.8\%$ in TNF α +PACAP to $6.1 \pm 1\%$ with the MEK1/2 inhibitor (Fig 7C, $n = 5$, $p=0.05$). In OP27 cells, adding U-0126 increased the level of PI-labeled cells from $3.3 \pm 0.8\%$ in TNF α +PACAP to $6.2 \pm 1\%$ with the MEK1/2 inhibitor (Fig 7D, $n = 5$, $p=0.04$). Incubation of OP cells with U-0126 alone or with TNF α +U-0126 did not significantly alter the level of cell death from that seen in control or TNF α treatments respectively (Supplemental Data Table 3). PACAP can therefore use the MAPKK pathway to protect against TNF α -induced cell death in OP cell lines.

Discussion

We showed that PACAP can protect olfactory sensory neurons against cell death induced by TNF α , a cytokine endogenous to the olfactory epithelium. PACAP protected cells in live OE slices as well as olfactory placodal cells of neuronal lineage against TNF α -induced cell death. Assays to measure caspase activity indicated that caspase 8, an apoptosis initiator caspase is significantly increased by 5 hours of TNF α treatment and this increase is blocked by PACAP, suggesting that TNF α -induced cell death involves apoptosis. However since the majority of experiments were performed using PI which does not distinguish between apoptosis and necrosis, we conservatively describe our results in terms of cell death rather than apoptosis. Our studies show that PACAP mediated this protection via activation of PAC1R and signaling through adenylate cyclase and/or phospholipase C. We also showed that the olfactory placodal cell lines OP6 and OP27 express all three PACAP receptors and several PAC1R isoforms. To our knowledge, these data are the first to show PACAP-mediated protection against TNF α -induced cell death in olfactory neuroepithelium or olfactory neuronal cell lines.

PACAP and Receptor expression in OP6 and OP27 cells

PACAP belongs to the secretin-glucagon superfamily of peptides (Kieffer & Habener, 1999) and occurs as either a 38 or a 27 amino acid peptide. The 38 amino acid peptide (PACAP38) is the predominant form of PACAP and is the focus of the present work. PACAP27 is formed through post-translational modification and represents only 10% of the PACAP peptide measured in various brain regions (Mikkelsen *et al.*, 1995). Both isoforms of PACAP and the closely-related peptide VIP bind to three GPCRs (PAC1R, VPAC1 and VPAC2) with PACAP38 and PACAP27 having a 500 to 1000 fold higher affinity than VIP at PAC1R and similar affinities as VIP at the VPAC receptors (Spengler *et al.*, 1993). The presence of mRNA for PACAP and all three of its receptors in the OP cell lines suggests that PACAP signaling is important in olfactory neuronal precursors.

We found that PACAP significantly inhibited TNF α -induced cell death through a PAC1R-specific mechanism in both cell lines. Although VPAC1 and VPAC2 also bind PACAP, the effects of TNF α were significantly reduced by maxadilan, a PAC1R selective agonist. These studies suggest that PAC1R activation alone is sufficient to mimic the neuroprotective effects of PACAP.

Interestingly, we found differences in the efficacy of PACAP6–38, a PAC1R antagonist between the two OP cell lines: PACAP6–38 was effective at significantly blocking PACAP activity at a lower dose (40 nM) in OP27 cells compared to OP6 cells. In addition, although PACAP equally eliminated the TNF α -induced component of cell death in OP6 and OP27 cells, TNF α +PACAP treatments in the 2 cell lines differed relative to their respective controls. PACAP alone did not significantly alter cell death over that seen in control. However, in the presence of TNF α , PACAP reduced the level of cell death significantly below control levels in OP6 cells ($p=0.003$) but not in OP27 cells ($p=0.79$). OP cells express both types of TNF α receptor, TNF1 and TNF2. TNFR1 activates the apoptotic pathway, while TNFR2 activation can be protective against apoptosis. It is possible that when cells are treated with TNF α +PACAP, TNF α , acting through the TNF2 receptors, synergizes with PAC1 receptor activation thereby increasing the protection against apoptosis, while in the absence of PACAP, TNF α binding to TNF1 receptors overcomes any beneficial effect of TNF α binding to TNF2 receptors, and the end result is increased cell death. The different levels of maturation between OP6 and OP27 cells may also contribute to this differential effect in baseline neuroprotection.

Supporting the idea of multiple roles for PACAP in OP cells, we found 6 PAC1R splice variants in OP6 cells and 3 PAC1R splice variants in OP27 cells. OP6 cells expressed N/R, N/Hop1, S/Hop2, N/Hop2, S/R and S/Hop1 while OP27 cells expressed N/R, N/Hop1, and S/Hop2. The R and Hop1/2 splice variants strongly activate both PLC and AC pathways, while Hip which is absent from OP cells, exclusively activates the AC pathway (Spengler *et al.*, 1993). Differential expression of PAC1R variants alone can regulate neuronal function through differential coupling to second messenger systems (Nicot & Diccio-Bloom, 2001), implying that the splice variants present in OP cells allow for PACAP's activation of the AC and PLC pathways.

PACAP mediates neuroprotection by AC and PLC pathways

PACAP has varied functions in a wide range of tissues (Vaudry *et al.*, 2009). PACAP has been shown to be protective to cells in tissues including the heart (Gasz *et al.*, 2006), kidney (Li *et al.*, 2008), immune system (Delgado *et al.*, 2003a), and the nervous system (Shioda *et al.*, 2006). In the nervous system, PACAP's protective role includes increasing proliferation and survival of both neurons and glia (Waschek, 2002), reducing the level of programmed cell death during development (Falluel-Morel *et al.*, 2007) and protecting neurons against cytotoxicity induced by glutamate (Morio *et al.*, 1996), ethanol (Vaudry *et al.*, 2002b), ischemia

(Unal-Cevik *et al.*, 2004), oxidative stress (Vaudry *et al.*, 2002a) and bacterial or viral infections (Kim *et al.*, 2000). PACAP has been shown to be neuroprotective in several systems, including *in vitro* models for apoptosis using cerebellar granule cells (Falluel-Morel *et al.*, 2007), rat cortical neurons (Morio *et al.*, 1996) or PC12 cells (Onoue *et al.*, 2002), as well as in *in vivo* models for Parkinson's disease (Reglodi *et al.*, 2004). PACAP primarily functions in these systems through activation of protein kinase A or MAPKK through the AC pathway (Dejda *et al.*, 2005; Villalba *et al.*, 1997). The present study also showed involvement of the AC/MAPKK pathway in the PACAP-mediated protection of OP cells. Forskolin significantly increased cell survival in the presence of TNF α while the AC inhibitor SQ22536 and the MEK1/2 inhibitor U-0126 reduced the protective effects of PACAP.

In contrast to the predominance of the AC pathway for neuroprotection in other systems, the PLC pathway was also implicated in the neuroprotective effects of PACAP on OP cells. The protein kinase C agonist PMA mimicked the effects of PACAP while the PLC antagonist U-73122 inhibited the effects of PACAP in both cell lines. Previous work showed that PACAP exclusively utilizes the PLC pathway in reducing axotomy-induced apoptosis of mature OSNs (Han & Lucero, 2005; Han & Lucero, 2006). It will be interesting to directly determine whether the differences in second messenger pathway efficacy are due to differences between axotomy and TNF α or to the difference in maturation between the OP cell lines and mature neurons.

Conclusions

Collectively, our studies show that PACAP is effective at reducing TNF α -induced PI labeling (a marker for cell death) in slices of neonatal olfactory epithelium as well as in two cell lines of neuronal progenitors derived from olfactory placode. A recent transgenic model for chronic rhinosinusitis shows that chronic OE expression of TNF α leads to a gradual loss of OSNs over 42 days (Lane *et al.*, 2010). This surprisingly slow time course for TNF α -induced neuronal death may reflect the ability of endogenous PACAP to protect against TNF α -mediated cell death in the OE *in vivo*. In our OE slice studies, TNF α accelerated the rate of death to hours rather than days, likely due to higher concentrations and the summed insults inherent with slice preparation. Remarkably, PACAP blocked the TNF α -induced PI labeling in OE slices suggesting protection from cell death. The success of PAC1R activation in reducing cytokine insult both *in situ* and in cell lines suggests that PACAP might be a promising therapeutic for protecting neurons and/or neuronal progenitors from inflammatory damage by TNF α .

Experimental Methods

Chemicals

PACAP38 and PACAP6–38 were obtained from Bachem Chemicals (Torrance, CA). TNF α , phorbol 12-myristate 13-acetate (PMA), U-73122, U-0126, forskolin, SQ22536 and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. Propidium iodide (PI) was obtained from Invitrogen (Carlsbad, CA). Maxadilan was a generous gift from Dr. Ethan Lerner (Massachusetts General Hospital, Boston, MA).

OE live slice experiments

All animal procedures were conducted under the guidelines of the NIH Guide for Care and Use of Laboratory Animals and were approved by the University of Utah Institutional Animal Care and Use Committee. Coronal olfactory epithelial slices (250 μ m thick) were prepared from postnatal day 1 (P1) C57/B16 mice as previously described (Hegg *et al.*, 2003b). Slices were cut and collected in ice cold Ringer's solution (in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES and 10 Glucose), and then transferred immediately into assay medium (DMEM/F12 + 0.5% BSA) with TNF α (40 ng/ml), TNF α +PACAP (40 nM) or assay medium

alone (vehicle control). The total slices obtained from each mouse were divided equally between the 3 treatments. Slices were exposed to treatments for 6 hrs at 37°C in a tissue culture incubator (Suzuki & Farbman, 2000) and then live-labeled for 20 mins with 0.5% PI to identify dying cells. Slices were then fixed with 20% Formal-Fixx (Thermo Scientific, Waltham, MA) for 20 mins and stained with 10 μ M SYTOX-Green (Molecular Probes, Carlsbad, CA) to label cellular nuclei. (A total of 9–11 slices were cut from the OE of one mouse, these were split between the 3 treatments, and therefore 2–3 slices were imaged per treatment per mouse.) Slices were then imaged at identical settings across all treatments using a Zeiss confocal LSM510 argon-krypton laser scanner attached to a Zeiss Axioskop 2FS microscope. Regions of interest (ROIs) were drawn around the dorsal recess and septum, and fluorescence intensity was measured using LSM510 software. The percentage of dying cells (PI-labeling) versus total cells (SYTOX-Green-labeling) was determined by dividing the total number of pixels labeled with PI by those labeled with SYTOX-Green $\times$ 100 (threshold pixel intensity = 100; bin width = 6). The ratio of PI/SYTOX-Green pixels in the 3 treatments is expressed as mean $\pm$ SEM, $n = 3$ mice; independent Student's T test, $p \leq 0.05$. This assay provides an overall qualitative assessment of the effects of TNF α and PACAP on cell death in live OE slices and does not yield information on actual cell counts.

Tissue culture

The olfactory placodal (OP) cells lines OP6 and OP27 were obtained from the laboratory of Dr. A. J. Roskams (Univ. British Columbia, Vancouver, BC). Cells were grown in a tissue culture incubator at 33°C in DMEM/F12 media supplemented with 10% FBS and 1% anti-mycotic/antibiotic (Invitrogen, Carlsbad, CA). All treatments were added to assay media which consisted of DMEM/F12 with 0.5% BSA. While these cells can be induced to differentiate, all of our studies were done on undifferentiated OP6 and OP27 cells as determined by the absence of cells with mature neuronal morphology: an apical ciliated dendrite and basal axon.

Cell death assays

Initial assays were done to determine the optimal conditions under which cell death could be induced by TNF α . OP6 and OP27 cells were treated to several concentrations of TNF α for 24 hrs: 1, 2, 5, 10, 20 and 40 ng/ml (data not shown). The 40 ng/ml treated cultures had the highest level of cell death. To determine the optimal time of TNF α exposure, cells were treated to 10 ng/ml or 40 ng/ml TNF α for periods of 1, 2, 3, 4, 5, 12 and 24 hrs (data not shown). The optimal treatment was found to be 40 ng/ml of TNF α for 5 hrs, similar to that used in previous studies inducing apoptosis in organotypic cultures of the embryonic mouse OE (Suzuki & Farbman, 2000). All assays were done in parallel using both OP6 and OP27 cells, with 3–4 coverslips assayed per treatment per experimental trial. Experiments were repeated a minimum of three times.

Cells were plated in culture media at 1.5×10^5 cells per well on 12 mm glass coverslips placed in 24 well tissue culture plates. The next day, cells were treated with 40 ng/ml TNF α , TNF α + PACAP (40 nM), or assay media (vehicle control). In addition to these treatments, we tested blockers and activators of various pathways that might be involved in PACAP's actions on TNF α -mediated cell death. Control treatments included treating with the test agents alone, or with vehicle (DMSO) for test agents, and were without significant effects (Supplemental data, Tables 1–3). After a 5 hr exposure period at 33°C in a tissue culture incubator, media was replaced with 0.5% PI in assay media for 20 mins to label nuclei of dying cells. After rinsing with PBS, cells were fixed with 20% Formal-Fixx for 15 mins. Cells were then rinsed with PBS and stained with DAPI to label all cellular nuclei. Coverslips were then mounted onto slides using Vectastain mounting media (Vector Labs, Burlingame, CA).

Cell count analysis

Slides were blinded and images taken using a Zeiss Axioplan2 microscope, attached AxioCam camera and AxioScan software. Both DAPI and PI labeling were imaged from 5 representative areas on each of the 3–4 coverslips used per treatment. For each image, total cells (DAPI-labeled cells) and dead/dying cells (PI labeled cells) were counted using ImageJ software (NIH). The total number of cells in each image ranged from approximately 600–1200 cells. The percentage of dying cells (PI cells/DAPI cells $\times$ 100) was calculated for each treatment. Data are presented as mean $\pm$ SEM, with n = number of times an experiment (trial) was performed per cell line.

Statistical significance was set at $p \leq 0.05$. To control for conditions that could vary between trials, treatment comparisons were made within the same trial using the paired sample T-test. Unlike the independent sample T-test, which assumes a correlation of zero between two groups, the paired T-test assumes that a correlation in the treatments within each trial is non-zero. To verify this assumption, a Pearson's correlation coefficient was computed within trial treatment pairs. As expected, there was a high correlation between treatments done within the same trial: in 15 trials, the correlation coefficient for control vs TNF α treatments was $r=0.83$, $p<0.001$, in OP6 cells and $r=0.84$, $p<0.001$, in OP27 cells; between TNF α and TNF α +PACAP treatments was $r=0.86$, $p<0.001$, in OP6 cells and $r=0.89$, $p<0.001$, in OP27 cells. The large r values verified that the paired T-Test was appropriate. Consistent with paired significance testing, data are presented with the within trial control, TNF α and TNF α +PACAP treatment values for each subset of experiments.

Caspase assays

To identify the activated caspases, we used a fluorometric caspase assay kit as per manufacturer's instructions (Biovision, Mountain View, CA). Cells were plated at 0.6×10^5 – 0.6×10^6 cells/ well in 96 well plates and treated with TNF α , TNF α +PACAP or vehicle [DMEM/F12 + 0.5% BSA] for 5hrs as described above. Plates were read using a SpectraMax Gemini EM microplate spectrophotometer (Molecular Devices). Caspase assays were done in duplicate, and repeated 3 times using OP6 cells. Statistical analysis was done using paired Student's T-test at $p \leq 0.05$.

RT-PCR

Total RNA was extracted from OP6 and OP27 cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Genomic DNA was eliminated by digesting with RNase-free DNase I. The total RNA concentration was determined by measuring the optical density at 260 nm. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo dT primers. A total amount of 50 ng cDNA was used for all PCR reactions. The final reaction mixture of 25 μ L contained 0.6 μ M each of forward and reverse primers, 1.5 mM MgCl₂, 1 mM dNTPs and 5 units of platinum taq DNA Polymerase (Invitrogen, Carlsbad, CA). All the PCRs were done for 40 cycles at an annealing temperature of 63°C and extension at 72°C.

Primers used were PACAP forward primer- 5'-gtatgggataataatgcatagcagtg-3'; PACAP reverse primer: 5'-ctggtcgtgaagcctcgtct-3'; VIP forward primer: 5'-agtgtgctgttctctcagtcg-3'; VIP reverse primer: 5'-gccattttctgctaagggattct-3'; PAC1R forward primer: 5'-gcttcctgaatggggagtgta-3'; PAC1R reverse primer: 5'-ggtgcttgaagtcctatgtg-3'; VPAC1 forward primer: 5'-gatgtgggacaacctcacctg-3'; VPAC1 reverse primer: 5'-tagccgtgaatgggggaaac-3'; VPAC2 forward primer: 5'-gacctgctactgctggttg-3'; VPAC2 reverse primer: 5'-cagctctgcacattttgtctct-3'; TNFR1 forward primer: 5'-acaccctacaaccggaacc-3'; TNFR1 reverse primer: 5'-tgagccttctgtcatagtattct-3'; TNFR2 forward primer: 5'-cgggagaagaggatagctt-3'; TNFR2 reverse primer: 5'-tcggacagtcactcaccaagt-3'; ACTIN

forward primer: 5'-ggctgtattccctccatcg-3'; ACTIN reverse primer: 5'-ccagttggaacaatgccatgt-3'.

To amplify the various combinations of PAC1R splice variants in the first extracellular (EC1) domain (normal and short) with the different splice variants in the third intracellular (IC3) domain (regular, HIP, HOP1, HOP2 and HIP/HOP), primer combinations (8 total) were designed based on published work (Ushiyama *et al.*, 2007). The schematics in Fig. 5A show the design of the combination primers. The PCR products of these combination primer sets had expected sizes of 741–891 bp.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

List of abbreviations

AA	amino acid
AC	adenylate cyclase
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
dNTP	deoxynucleotide triphosphate
dT	deoxythymidine
EC1	first extracellular domain of PAC1R sequence
FBS	fetal bovine serum
FSK	forskolin
GPCR	G-protein-coupled receptor
IC3	third intracellular domain of PAC1R sequence
INP	immediate neuronal precursor
IRN	immature receptor neuron
K	potassium
MAPKK	mitochondrial activated protein kinase kinase
N	normal variant of EC1 domain of PAC1R
<i>n</i>	number of animals used, or cell-based experiments done
NST	neuron specific tubulin
OE	olfactory epithelium
OMP	olfactory marker protein
OP	olfactory placodal
OSN	olfactory sensory neuron
P1	post-natal day 1
PACAP	pituitary adenylylate cyclase-activating peptide
PAC1R	PACAP-specific receptor

PCR	polymerase chain reaction
PI	propidium iodide
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PKC	protein kinase C
R	regular variant of IC3 domain of PAC1R
ROI	regions of interest
RT-PCR	reverse transcriptase polymerase chain reaction
S	short variant EC1 domain of PAC1R
TNFα	tumor necrosis factor α
TNFR1, TNFR2	TNF α receptors
VIP	vasointestinal peptide
VPAC1, VPAC2	receptors for VIP
VS	very short variant of EC1 domain of PAC1R

Acknowledgments

We thank Dr. A.J. Roskams for providing OP6 and OP27 cell lines; Dr. E. Lerner for providing maxadilan; Dr. W. Michel for use of his microscope and helpful suggestions on the manuscript; Dr. A. Greig for technical support. Funded by NIH NIDCD DC002994, CSS/ARRA supplements to SK, AG, and NIA 1K07AG028403-01A1 from the Utah Center on Aging.

References

1. Clausen BH, Lambertsen KL, Babcock AA, Holm TH, Dagnaes-Hansen F, Finsen B. Interleukin-1beta and tumor necrosis factor-alpha are expressed by different subsets of microglia and macrophages after ischemic stroke in mice. *J. Neuroinflammation* 2008;5:46. [PubMed: 18947400]
2. Dejda A, Sokolowska P, Nowak JZ. Neuroprotective potential of three neuropeptides PACAP, VIP and PHI. *Pharmacol. Rep* 2005;57:307–320. [PubMed: 15985713]
3. Delgado M, Abad C, Martinez C, Juarranz MG, Leceta J, Ganea D, Gomariz RP. PACAP in immunity and inflammation. *Ann. N.Y. Acad. Sci* 2003a;992:141–157. [PubMed: 12794054]
4. Delgado M, Leceta J, Ganea D. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the production of inflammatory mediators by activated microglia. *J. Leukoc. Biol* 2003b;73:155–164. [PubMed: 12525573]
5. Falluel-Morel A, Chafai M, Vaudry D, Basille M, Cazillis M, Aubert N, Louiset E, de Jouffrey S, Le Bigot JF, Fournier A, Gressens P, Rostene W, Vaudry H, Gonzalez BJ. The neuropeptide pituitary adenylate cyclase-activating polypeptide exerts anti-apoptotic and differentiating effects during neurogenesis: focus on cerebellar granule neurones and embryonic stem cells. *Journal of Neuroendocrinology* 2007;19:321–327. [PubMed: 17425606]
6. Farbman AI, Buchholz JA, Suzuki Y, Coines A, Speert D. A molecular basis of cell death in olfactory epithelium. *J. Comp Neurol* 1999;414:306–314. [PubMed: 10516598]
7. Gasz B, Racz B, Roth E, Borsiczky B, Ferencz A, Tamas A, Cserepes B, Lubics A, Gallyas F Jr, Toth G, Lengvari I, Reglodi D. Pituitary adenylate cyclase activating polypeptide protects cardiomyocytes against oxidative stress-induced apoptosis. *Peptides* 2006;27:87–94. [PubMed: 16095757]
8. Graziadei PP, Graziadei GA. Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. *Journal of Neurocytology* 1979;8:1–18. [PubMed: 438867]

9. Han P, Lucero MT. Pituitary adenylate cyclase activating polypeptide reduces A-type K⁺ currents and caspase activity in cultured adult mouse olfactory neurons. *Neuroscience* 2005;134:745–756. [PubMed: 16019148]
10. Han P, Lucero MT. Pituitary adenylate cyclase activating polypeptide reduces expression of Kv1.4 and Kv4.2 subunits underlying A-type K(+) current in adult mouse olfactory neuroepithelia. *Neuroscience* 2006;138:411–419. [PubMed: 16426762]
11. Hansel DE, Eipper BA, Ronnett GV. Regulation of olfactory neurogenesis by amidated neuropeptides. *Journal of Neuroscience Research* 2001;66:1–7. [PubMed: 11598996]
12. Hegg CC, Au E, Roskams AJ, Lucero MT. PACAP is present in the olfactory system and evokes calcium transients in olfactory receptor neurons. *Journal of Neurophysiology* 2003a;90:2711–2719. [PubMed: 12761277]
13. Hegg CC, Greenwood D, Huang W, Han P, Lucero MT. Activation of purinergic receptor subtypes modulates odor sensitivity. *Journal of Neuroscience* 2003b;23:8291–8301. [PubMed: 12967991]
14. Illing N, Boolay S, Siwoski JS, Casper D, Lucero MT, Roskams AJ. Conditionally immortalized clonal cell lines from the mouse olfactory placode differentiate into olfactory receptor neurons. *Mol.Cell Neurosci* 2002;20:225–243. [PubMed: 12093156]
15. Kaushal V, Schlichter LC. Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra. *Journal of Neuroscience* 2008;28:2221–2230. [PubMed: 18305255]
16. Kieffer TJ, Habener JF. The glucagon-like peptides. *Endocr.Rev* 1999;20:876–913. [PubMed: 10605628]
17. Kim WK, Kan Y, Ganea D, Hart RP, Gozes I, Jonakait GM. Vasoactive intestinal peptide and pituitary adenylyl cyclase-activating polypeptide inhibit tumor necrosis factor- α production in injured spinal cord and in activated microglia via a cAMP-dependent pathway. *Journal of Neuroscience* 2000;20:3622–3630. [PubMed: 10804204]
18. Lane AP, Turner J, May L, Reed R. A genetic model of chronic rhinosinusitis-associated olfactory inflammation reveals reversible functional impairment and dramatic neuroepithelial reorganization. *Journal of Neuroscience* 2010;30:2324–2329. [PubMed: 20147558]
19. Lerner EA, Iuga AO, Reddy VB. Maxadilan, a PAC1 receptor agonist from sand flies. *Peptides* 2007;28:1651–1654. [PubMed: 17681401]
20. Lerner EA, Ribeiro JM, Nelson RJ, Lerner MR. Isolation of maxadilan, a potent vasodilatory peptide from the salivary glands of the sand fly *Lutzomyia longipalpis*. *Journal of Biological Chemistry* 1991;266:11234–11236. [PubMed: 2040631]
21. Li M, Maderdrut JL, Lertora JJ, Arimura A, Batuman V. Renoprotection by pituitary adenylate cyclase-activating polypeptide in multiple myeloma and other kidney diseases. *Regulatory Peptides* 2008;145:24–32. [PubMed: 17935800]
22. McCoy MK, Tansey MG. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. *J.Neuroinflammation* 2008;5:45. [PubMed: 18925972]
23. Mikkelsen JD, Hannibal J, Fahrenkrug J, Larsen PJ, Olcese J, McArdle C. Pituitary adenylate cyclase activating peptide-38 (PACAP-38), PACAP-27, and PACAP related peptide (PRP) in the rat median eminence and pituitary. *Journal of Neuroendocrinology* 1995;7:47–55. [PubMed: 7735297]
24. Morio H, Tatsuno I, Tanaka T, Uchida D, Hirai A, Tamura Y, Saito Y. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neurotrophic factor for cultured rat cortical neurons. *Ann.N.Y.Acad.Sci* 1996;805:476–481. [PubMed: 8993427]
25. Moro O, Lerner EA. Maxadilan, the vasodilator from sand flies, is a specific pituitary adenylate cyclase activating peptide type I receptor agonist. *Journal of Biological Chemistry* 1997;272:966–970. [PubMed: 8995389]
26. Moro O, Wakita K, Ohnuma M, Denda S, Lerner EA, Tajima M. Functional characterization of structural alterations in the sequence of the vasodilatory peptide maxadilan yields a pituitary adenylate cyclase-activating peptide type 1 receptor-specific antagonist. *Journal of Biological Chemistry* 1999;274:23103–23110. [PubMed: 10438479]
27. Nicot A, Diccico-Bloom E. Regulation of neuroblast mitosis is determined by PACAP receptor isoform expression. *Proc.Natl.Acad.Sci.U.S.A* 2001;98:4758–4763. [PubMed: 11296303]
28. Onoue S, Endo K, Ohshima K, Yajima T, Kashimoto K. The neuropeptide PACAP attenuates beta-amyloid (1–42)-induced toxicity in PC12 cells. *Peptides* 2002;23:1471–1478. [PubMed: 12182949]

29. Pantaloni C, Brabet P, Bilanges B, Dumuis A, Houssami S, Spengler D, Bockaert J, Journot L. Alternative splicing in the N-terminal extracellular domain of the pituitary adenylyl cyclase-activating polypeptide (PACAP) receptor modulates receptor selectivity and relative potencies of PACAP-27 and PACAP-38 in phospholipase C activation. *Journal of Biological Chemistry* 1996;271:22146–22151. [PubMed: 8703026]
30. Pathak N, Johnson P, Getman M, Lane R. Odorant receptor (OR) gene choice is biased and non-clonal in two olfactory placodal cell lines, and OR RNA is nuclear prior to differentiation of these cell lines. *Journal of Neurochemistry* 2009;108:486–497. [PubMed: 19012738]
31. Regad T, Roth M, Breidenkamp N, Illing N, Papalopulu N. The neural progenitor-specifying activity of FoxG1 is antagonistically regulated by CK1 and FGF. *Nature Cell Biology* 2007;9:531–540.
32. Reglodi D, Lubics A, Tamas A, Szalontay L, Lengvari I. Pituitary adenylyl cyclase activating polypeptide protects dopaminergic neurons and improves behavioral deficits in a rat model of Parkinson's disease. *Behavioural Brain Research* 2004;151:303–312. [PubMed: 15084446]
33. Shioda S, Ohtaki H, Nakamachi T, Dohi K, Watanabe J, Nakajo S, Arata S, Kitamura S, Okuda H, Takenoya F, Kitamura Y. Pleiotropic functions of PACAP in the CNS: neuroprotection and neurodevelopment. *Ann.N.Y.Acad.Sci* 2006;1070:550–560. [PubMed: 16888224]
34. Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH, Journot L. Differential signal transduction by five splice variants of the PACAP receptor. *Nature* 1993;365:170–175. [PubMed: 8396727]
35. Suzuki Y, Farbman AI. Tumor necrosis factor- α -induced apoptosis in olfactory epithelium in vitro: possible roles of caspase 1 (ICE), caspase 2 (ICH-1), and caspase 3 (CPP32). *Experimental Neurology* 2000;165:35–45. [PubMed: 10964483]
36. Unal-Cevik I, Kilinc M, Can A, Gursoy-Ozdemir Y, Dalkara T. Apoptotic and necrotic death mechanisms are concomitantly activated in the same cell after cerebral ischemia. *Stroke* 2004;35:2189–2194. [PubMed: 15256676]
37. Ushiyama M, Ikeda R, Sugawara H, Yoshida M, Mori K, Kangawa K, Inoue K, Yamada K, Miyata A. Differential intracellular signaling through PAC1 isoforms as a result of alternative splicing in the first extracellular domain and the third intracellular loop. *Molecular Pharmacology* 2007;72:103–111. [PubMed: 17442841]
38. Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, Fournier A, Chow BK, Hashimoto H, Galas L, Vaudry H. Pituitary adenylyl cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol.Rev* 2009;61:283–357. [PubMed: 19805477]
39. Vaudry D, Pamantung TF, Basille M, Rousselle C, Fournier A, Vaudry H, Beauvillain JC, Gonzalez BJ. PACAP protects cerebellar granule neurons against oxidative stress- induced apoptosis. *European Journal of Neuroscience* 2002a;15:1451–1460. [PubMed: 12028355]
40. Vaudry D, Rousselle C, Basille M, Falluel-Morel A, Pamantung TF, Fontaine M, Fournier A, Vaudry H, Gonzalez BJ. Pituitary adenylyl cyclase-activating polypeptide protects rat cerebellar granule neurons against ethanol-induced apoptotic cell death. *Proc.Natl.Acad.Sci.U.S.A* 2002b;99:6398–6403. [PubMed: 11972030]
41. Villalba M, Bockaert J, Journot L. Pituitary adenylyl cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway. *Journal of Neuroscience* 1997;17:83–90. [PubMed: 8987738]
42. Waschek JA. Multiple actions of pituitary adenylyl cyclase activating peptide in nervous system development and regeneration. *Developmental Neuroscience* 2002;24:14–23. [PubMed: 12145407]

Figure 1. PACAP protects against TNFα-induced cell death in the olfactory epithelium

Live coronal slices of OE from P1 mice were treated for 6 hrs with (A, D) vehicle control, (B, E) 40 ng/ml TNFα, or (C, F) 40 ng/ml TNFα and 40 nM PACAP. (A–C) Confocal projections (10 Z-stack images) of the dorsomedial septal area of the OE are shown, with dying cells labeled with PI (red) and all cells labeled with SYTOX-Green (scale bar = 200 μm). (D–F) Higher magnification images of the OE (confocal projections of 3 Z-stack images) demonstrate that a majority of the dying cells (red) are seen in the basal part of the OE, particularly in the ISN and BC layers. (SC: sustentacular cells, OSN: olfactory sensory neurons, ISN: immature sensory neurons, BC: basal cells; dotted line delineates the basement membrane. Scale bar = 30 μm) (G) Shown are the percentages of PI-pixel intensities vs. SYTOX-Green pixel intensities averaged across all slices and animals for each treatment (control [C]: 10 ± 3%; TNFα [T]: 33 ± 2%; TNFα + PACAP [T+P]: 7 ± 1 %; *n* = 3 mice. * = significantly different from control, Student's T-test, *p*=0.04; # = significantly different from TNFα, Student's T-test, *p*=0.003.

Figure 2. OP6 and OP27 cells express PACAP, and receptors for both TNF α , and PACAP
 In both OP6 and OP27 cells, RT-PCR showed expression of PACAP and all three PACAP receptors: PAC1, VPAC1 and VPAC2. Expression of TNF receptors, TNFR1 and TNFR2 was also seen in both cell lines (6 = OP6, 27 = OP27). Negative controls consisted of omitting the reverse transcriptase enzyme (-RT). Actin was used as a loading control.

Figure 3. PACAP protects against TNF α -induced cell death in OP cells

OP6 cells and OP27 cells were treated for 5 hrs in vehicle control (A, B) 40 ng/ml TNF α (C, D) or 40 ng/ml TNF α + 40 nM PACAP (E, F). The percentage of dying cells (PI-labeled, red) to total cells (DAPI-labeled, blue) was determined for each treatment: control [C], TNF α [T] and TNF α +PACAP [T+P] in OP6 cells (G) and OP27 cells (H). Data is presented as mean $\pm$ SEM, $n = 15$ independent experiments. * = significantly different from control; # = significantly different from TNF α ; paired Student's T-test, $p \leq 0.05$. (I) Treatment of OP cells with 40 nM PACAP [P] alone did not change the percentages of cell death from control values [C] ($n = 6$, paired Student's T-test, $p \leq 0.05$).

Figure 4. The PAC1 receptor mediates the effects of PACAP

Percentages of PI-labeled cells versus total cells are shown for OP6 cells (A) and OP27 cells (B) incubated for 5 hrs with vehicle control [C], 40 ng/ml TNF α [T], 40 ng/ml TNF α + 40 nM PACAP [T+P] in the presence and absence of the PAC1R antagonist PACAP6–38 [T+P+P6–38] at 40 nM ($n = 6$) or 100 nM ($n = 7$) and then live-labeled with PI and DAPI. Data is presented as mean $\pm$ SEM. * = significantly different from control; # = significantly different from TNF α ; + = significantly different from TNF α +PACAP; paired Student's T-test, $p \leq 0.05$. (C, D) Percentages of PI-labeled versus total cells are shown for OP cells incubated for 5 hrs with vehicle control [C], 40 ng/ml TNF α [T], 40 ng/ml TNF α + 40 nM PACAP [T+P], and 40 ng/ml TNF α + the PAC1R agonist maxadilan [T+P+Max] (100 pM, $n = 7$) in the presence and absence of the PAC1R antagonist PACAP6–38 at 100 nM [T+P+Max+P6–38] ($n = 3$) and then live-labeled with PI and DAPI. Data is presented as mean $\pm$ SEM, * = significantly different from control; # = significantly different from TNF α ; + = significantly different from TNF α + maxadilan; paired Student's T-test, $p \leq 0.05$.

Figure 5. OP6 and OP27 express a different panel of PAC1R variants

(A) A schematic diagram of PAC1R variants and primer design. Primers were created to identify variants in the extracellular 1 (EC1) and the third intracellular loop (IC3) domains of PAC1R. EC1 domain primers identified the normal (N) and the short (S, with a 21AA deletion) versions of EC1 domain variants. IC3 domain primers identified the regular (R) version, and the IC3 insertions of Hip, Hip/Hop, Hop1 or Hop2. Primers for each EC1 domain were used in combination with that for each IC3 domain to identify expression of the 10 dominant isoforms of PAC1R. (B) Both OP6 and OP27 cells expressed the N/R, N/Hop1 and S/Hop2 variants. In addition, OP6 cells expressed the N/Hop2, S/R and S/Hop1 variants. The IC3 insertions of Hip and Hip/Hop were not expressed in either cell line. Negative controls (-RT) consisted of omitting the reverse transcriptase enzyme. Actin was used as a loading control.

Figure 6. PACAP functions through the PLC pathway

Percentages of PI-labeled cells versus total cells are shown for OP6 cells (A) and OP27 cells (B) incubated for 5 hrs with vehicle control [C], 40 ng/ml TNF α [T], 40 ng/ml TNF α + 40 nM PACAP [T+P] in the presence and absence of the PLC pathway antagonist U-73312 [T+P+U] at 100 μ M ($n = 5$). In addition, 40 ng/ml TNF α was co-incubated with the PKC activator, PMA at 10 nM or 50 nM [T+PMA] ($n = 3$). Data is presented as mean $\pm$ SEM, * = significantly different from control; # = significantly different from TNF α ; + = significantly different from TNF α +PACAP; paired Student's T-test, $p \leq 0.05$.

Figure 7. PACAP functions through the AC pathway

(A, B) Percentages of PI-labeled cells versus total cells are shown for OP6 cells (A) and OP27 cells (B) incubated for 5 hrs with vehicle control [C], 40 ng/ml TNF α [T], 40 ng/ml TNF α + 40 nM PACAP [T+P] in the presence and absence of the AC pathway antagonist SQ77536 [T+P+SQ] at 300 μ M. In addition, cells were treated with 40 ng/ml TNF α + the AC activator forskolin at 20 μ M [T+FSK]. (C, D) Percentages of PI-labeled cells versus total cells are shown for OP6 cells (C) and OP27 cells (D) incubated for 5 hrs with vehicle control [C], 40 ng/ml TNF α [T], 40 ng/ml TNF α + 40 nM PACAP [T+P] in the presence and absence of the MEK1/2 antagonist U0126 at 10 μ M [T+P+U0126]. Data is presented as mean $\pm$ SEM, $n = 6$; * = significantly different from control; # = significantly different from TNF α ; + = significantly different from TNF α +PACAP; paired Student's T-test, $p \leq 0.05$.