PACAP Protects Against Inflammatory-Mediated Toxicity in Dopaminergic SH-SY5Y Cells: Implication for Parkinson s Disease

Dwayne Brown [1]

Andrea Tamas [2]

Dora Reglodi [2]

Yousef Tizabi [1,*]

Phone +202-806-9719 Fax +202-806-4453 Email ytizabi@howard.edu

[1] Department of Pharmacology, Howard University College of Medicine, 520 W Street NW, Washington, DC, 20059 USA

[2] Department of Anatomy, PTE-MTA Lendulet" PACAP Research Team, University of Pecs, Pecs, Hungary

Abstract

There has been a growing recognition of the role of neuroinflammation caused by microglia-exaggerated release of inflammatory mediators in the pathogenesis of Parkinson s disease (PD). Pituitary adenylate cyclase activating polypeptide (PACAP) is an endogenous 38 amino acid containing neuropeptide that has been shown to possess neurotrophic as well as neuroprotective properties. In this study, we sought to determine whether PACAP could protect SH-SY5Y dopaminergic cells against toxicity induced by inflammatory mediators. For this purpose, THP-1 cells which possess microglia-like property were stimulated by a combination of lipopolysaccharide (LPS) and interferon gamma (IFN-γ), and the media containing inflammatory mediators were isolated and applied to SH-SY5Y cells. Such treatment resulted in approximately 54 % cell death as well as a reduction in brain-derived neurotrophic factor (BDNF) and phosphorylated cyclic AMP response element-binding protein (p-CREB). Pretreatment of the SH-SY5Y cells with PACAP (1-38) dose-dependently attenuated toxicity induced by the inflammatory mediators. PACAP effects, in turn, were dose-dependently blocked by the PACAP receptor

antagonist (PACAP 6-38). These results suggest protective effects of PACAP against inflammatory-induced toxicity in a cellular model of PD that is likely mediated by enhancement of cell survival markers through activation of PACAP receptors. Hence, PACAP or its agonists could be of therapeutic benefit in inflammatory-mediated PD.

Keywords

PACAP
Inflammation
SH-SY5Y cell line
Neuroprotection
Neurotrophic factors
Apoptosis
Parkinson s disease

Introduction

Parkinson s disease (PD) is a progressive neurodegenerative disease with physical manifestations of motor dysfunctions, resulting from damage of dopaminergic neurons in the substantia nigra (Samii et al. **2004**). Currently, the most effective treatment is the administration of levodopa (L-Dopa), which replenishes dopamine levels in the brain. However, chronic therapy causes motor and psychiatric side effects (Dong-Kug and Kyoungho, **2007**). Therefore, it is essential to understand the etiological and pathological factors implicated in PD in order to find an effective treatment. Epidemiological and pathological evidence indicate that chronic inflammation contributes to deterioration of dopaminergic neurons in the substantia nigra (McGeer et al. **2001**; Chen et al. **2003**). Moreover, elevated levels of inflammatory cytokines in PD suggest their involvement in the degenerative process. This contention is further supported by studies showing a positive correlation between gene polymorphisms that enhance certain cytokines and an increased risk for PD (McGeer et al. **2001**; Schulte et al. **2002**).

Microglia or cells that can behave like microglia (e.g., the human monocytic cell: THP-1) are significant generators of inflammatory mediators, as they possess appropriate receptors that permit them to respond to cytokine stimulation (Raghavan and Bjorkman **1996**; Theus et al. **2004**). Microglial activation is a key host defense mechanism. However, over activation of microglia can cause neurotoxicity (Klegeris et al. **2003**). Lipopolysaccharide (LPS), gram-negative bacteria derived endotoxin is commonly used to activate the inflammatory response of microglia cells (Nakamura et

commonly used to activate the inflammatory response of microglia cells (Nakamura et al. 1999; Liu and Hong, 2003). LPS activation of microglia causes release of pro-inflammatory factors such as tumor necrosis factor (TNF-alpha), interferon gamma (INF-γ), and nitric oxide (NO) along with a host of neurotoxic substances that result in dopaminergic neuronal death (Chao et al. 1995). LPS also decreases the number of tyrosine hydroxylase-positive cells in primary mesencephalic cultures as well as increasing cytokine output (Gayle et al. 2002).

Several studies propose that targeting the inflammatory pathway could have therapeutic advantage in the treatment of PD (Wersinger and Sidhu, 2002; Barcia et al. 2003; Carta and Pisanu, 2013; Chinta et al. 2013). Pituitary adenylate cyclase activating polypeptide (PACAP), originally isolated from the sheep hypothalamic extract, is an endogenous neuropeptide with neuroprotective effects (Somogyvari-Vigh and Reglodi 2004). Indeed, it has been shown that PACAP has protective effects in a rat or mouse model of Parkinson s disease (6-OHDA or MPTP treatment, respectively) (Reglodi et al. 2004; 2006; 2011; Wang et al. 2008). Moreover, it was recently reported that PACAP deficiency in mice may enhance microglial inflammatory response and sensitize nigrostriatal dopaminergic neurons to paraquat-induced damage (Watson et al. 2013). PACAP exerts its effects via the specific PAC1 receptor, and the VPAC1/2 receptors, which also bind vasoactive intestinal peptide (Vaudry et al. 2009). It has been suggested that at least some of the protective effects of PACAP are due to enhancement of the phosphorylation of CREB (cyclic AMP response element-binding protein) as well as upregulation of brain-derived neurotrophic factor (BDNF) (Frechilla et al. 2001; Yaka et al. 2003; Racz et al. 2006; Rat et al. 2011). Moreover, PACAP s ability to reduce caspase-3 levels (main mediator of apoptosis) is considered a major contributor to its protective properties (Vaudry et al. 2000, 2002; Botia et al. 2011; Brown et al. 2013).

PACAP is released within the lymphoid organs following antigenic stimulation and modulates the function of inflammatory cells through specific receptors (Ganea and Delgado, 2002). Since the balance between pro- and anti-inflammatory factors plays an essential role in the successful control of inflammation, it has been suggested that PACAP plays a significant role in the inflammatory process by inhibiting the in vitro and in vivo production of pro-inflammatory cytokines and by stimulating the production of anti-inflammatory cytokines (e.g., IL-10) (Delgado and Ganea 2001; Ganea and Delgado, 2002, Martinez et al. 2002). Based on these findings, it has been suggested that PACAP may offer a novel therapeutic approach in the treatment of neurodegenerative diseases including Parkinson s disease (Delgado and Ganea 2001).

In this study, we first sought to determine whether PACAP might have protective effects against inflammatory-mediated toxicity and whether these effects were mediated by PACAP receptors. For this purpose, we utilized human neuroblastoma-derived cell line (SH-SY5Y) that express high levels of dopaminergic activity and are used extensively as a cellular model to study mechanism(s) of toxicity and protection in nigral dopaminergic neurons (Storch et al. **2002**; Maruyama et al. **2004**; Naoi et al. **2004**; Copeland et al. **2007**; Das and Tizabi, **2009**; Ramlochansingh et al., **2011**, Brown et al. **2013**). Moreover, since neurotrophic factors have been implicated in neurotoxicity and PACAP-mediated protection, the levels of brain-derived neurotrophic factor (BDNF) and phosphorylated cyclic AMP response element-binding protein (p-CREB) were also evaluated.

Methods

Drugs

Lipopolysaccharide, PACAP (1-38) and its antagonist (PACAP 6-38) as well as other analytical reagents were purchased from Sigma Chemical Company (Sigma Aldrich, St. Louis, MO). IFN-γ was purchased from Peprotech, Rocky Hill, NJ. Antibodies for Western blot analyses were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

Cell Culture

SH-SY5Y cells purchased from American Type Culture Collection (ATCC) in Manassas, VA and were cultivated in a 1:1 mixture of Dulbeccos Modified Eagle Medium and Ham s F12 supplemented with 10 % fetal bovine serum, penicillin/streptomycin (1000 IU/ml), and gentamicin (50 ug/ml) at 37^{O}C in an incubator. The cells (passage 9) were harvested approximately 5 days later when confluent and plated in a 96-well plate ($1.6x10^4$ cells/well). Cells were allowed to adhere to plate for 24 h. The cells we used here were undifferentiated and similar to the ones used in the previously published report (Brown et al., **2013**). It is of relevance to note that the response of the differentiated cells might be different from the undifferentiated cells; and in some cases, the differentiated SH-SY5Y cells may develop resistance to toxicants (Schneider et al. **2011**; Jantas et al. **2013**). THP-1 cells were also purchased from ATCC and cultivated in ATCC formulated RPMI-1640 medium supplemented with 2-mercaptoethanol (0.05mM) and 10 % fetal bovine serum at 37^{O}C in an incubator. The cells (passage 4) were harvested approximately 3 days later when confluent and plated in 12-well plate. PACAP and PACAP antagonist treatments as well as the controls were performed in medium containing FBS since the

viability of the SH-SY5Y cells is compromised in the absence of FBS.

Conditioned media from LPS- and/or LPS+IFN-gamma stimulated THP-1 cells were used to provide inflammatory mediators that were applied to SH-SY5Y cells. Control cells were exposed to medium derived from THP-1 cells in the absence of inflammatory stimulation

LPS, IFN-γ, and LPS+IFN-γ stimulation of THP-1 cell Treatment

THP-1 cells were grown in 12-well plates and were stimulated with different concentrations of LPS for 24 h. The cells were then spun down, and the cell free supernatant was added to SH-SY5Y cells in 96 wells for 72h. THP-1 cells grown in 12-well plates were stimulated with different concentrations of IFN-γ for 24 h. The cells were then spun down, and the cell free supernatant was added to SH-SY5Y cells in 96 wells for 72h. THP-1 cells in 12-well plates were stimulated with different concentrations of LPS+ IFN-γ for 24 h, following which the supernatant obtained was applied to SH-SY5Y cells as described for LPS or IFN-γ alone.

PACAP and PACAP Antagonist Treatment

To determine the protective effects of PACAP, 2 h prior to the addition of the inflammatory mediators, various concentrations of PACAP were added. Again, after 72 h, cell viability was determined as described below. To determine the effects of PACAP antagonist on protective effects of PACAP, 2 h prior to PACAP, cells were treated with PACAP6-38, followed by the addition of the conditioned media obtained from LPS+IFN-γ stimulated THP-1 cells. Cell viability was determined after 72 h (see below).

MTT Assay for Cell Viability

Determination of cell viability was done by 3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The yellow MTT tetrazolium salt (0.5 mg/ml) was dissolved in phosphate-buffered saline (PBS) with 10 mM HEPES, and 30 l of this reagent was added to each well for 3 h and was incubated at 37°C. The live cells cause a reduction of the yellow salt to insoluble purple formazan crystals. The wells were then aspirated, and 50 ul of dimethyl sulfoxide (DMSO) was added to the wells to solubilize the crystals, and the plates were placed in a shaker for an hour. The plates were then read spectrophotometrically at 570 nm in a plate reader. The data were then analyzed and represented as percent cell viability.

Cell Flow Cytometry

Cell flow cytometry was used to detect apoptosis vs necrosis by measuring and sorting cells by fluorescent labeling of markers on cell surface. The cells were grown and treated following the same procedure listed under drug treatment above. The cells (approximately $2x10^6$ cells/ml) were harvested and washed twice with cold PBS and then gently suspended in a solution that consisted of 100 l Annexin V-Fluos labeling solution, 5 l of fluorescein isothiocyanate labeled by Annexin V-FITC, and 5 l of propidium iodide (PI). Afterward, the cells were incubated in the dark at room temperature for 15 minutes before adding 500 l Annexin V-Fluos labeling solution to each well. Finally, 20 ul of this mixture (containing approximately 40,000 cells) was subjected to flow cytometry using a cellometer machine (Nexcelom, Lawrence, MA) followed by analysis of apoptotic and necrotic fraction using FCS express software.

BDNF, P-CREB, and Caspase-3 measurement

Western blot was used to determine the levels of BDNF and p-CREB in SH-SY5Y cells post treatment with inflammatory mediators. Briefly, cells were homogenized in lysis buffer (10 mM Tris-buffer, 5 mM EDTA, 150 mM NaCl, 0.5 % Triton X-100 (v/v) with protease inhibitors (Sigma-Aldrich, St. Louis, MO). The protein concentration in each sample was determined using a BCA protein Assay Kit (Pierce Biotechnology Inc., IL), and equal protein amount (as confirmed by β-actin) was loaded in each immunoblot. The proteins were separated using 12 % SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membranes were blocked with a blocking reagent (5 % nonfat milk in TBS buffer) for ½ h and incubated at 4°C overnight with the primary antibody against BDNF, p-CREB, and cleaved caspase-3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA 1:1000). The membranes were washed with TBST (TBS buffer with 1 % Tween-20) and blocked with the blocking reagent. Membranes were then incubated for 1 h at room temperature in Goat Anti-Rabbit-HRP conjugated secondary antibody (1:3000 in TBS, Bio-Rad Laboratories, CA). The membranes were then washed in the TBST washing solution and then visualized using enhanced chemiluminescent kits (Bio-Rad Laboratories, CA). The intensity of the protein bands on the gel was quantified using ChemiDoc XRS system (Bio-Rad Laboratories, CA).

Statistical Analysis

Statistical difference between treatment groups was determined by one-way ANOVA followed by post-hoc Tukey comparison test to determine which groups differed. Significant difference was considered a priori at $p < 0.05$. Data were analyzed using

Graphpad Prism 3 (San Diego, CA), and are expressed as mean ± SEM.

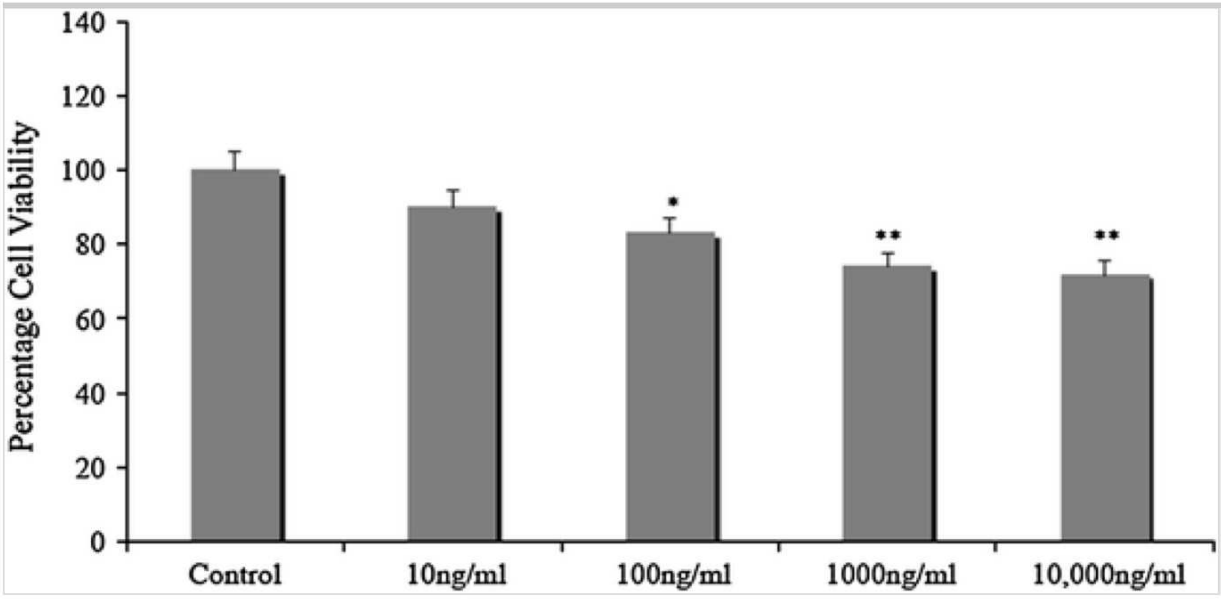
Results

Lipopolysaccharide (LPS)-mediated effect

The effects of conditioned media obtained from LPS-stimulated THP-1 cells on SH-SY5Y toxicity are shown in Figure 1. Conditioned media (CM) obtained from lower concentration of LPS (10 ng/ml) did not have any significant effect on cell viability. However, CM from LPS at 100 ng/ml resulted in approximately 18 % toxicity ($p<0.05$) and from LPS at 1000 ng/ml, resulted in approximately 30 % toxicity ($p<0.01$). CM from higher concentrations of LPS (e.g., 10,000 ng/ml) did not add significantly to the toxicity. Hence, CM from the lower concentration of 1000 ng/ml LPS was used in subsequent studies. In preliminary studies where LPS was directly applied to the SH-SY5Y cells, much higher concentration of LPS (approximately 300 fold higher) was needed to bring similar toxicity. Hence, to determine the effects of inflammatory mediators, THP-I cells were exposed to much lower concentrations of LPS, and the cell free media obtained from these cells was applied to SH-SY5Y cells.

Fig. 1

Concentration-response effect of LPS (L)-stimulated THP-1 cell media on SH-SY5Y cells. MTT assay was conducted 72 h after addition of the conditioned media. Values are mean ± SEM, n = 5. * $P<0.05$ ** $P<0.01$, compared to control



Interferon Gamma (IFN-γ)-mediated effect and LPS+ IFN-γ mediated effect

CM obtained from lower concentration of IFN-γ (1 ng/ml)-stimulated THP-1 cells did not have any significant effect on cell viability of SH-SY5Y cells (Figure 2). However, CM from IFN-γ at 5 ng/ml resulted in approximately 13 % toxicity ($p<0.05$) and from IFN-γ at 30 ng/ml, resulted in approximately 33 % toxicity ($p<0.01$). Hence, CM from this IFN-γ concentration was used in subsequent studies. Conditioned media (CM) obtained form LPS 1000 ng/ml + IFN-γ 30 ng/ml resulted in the highest toxicity approximately 54 % ($p<0.001$) (Figure 3). Hence, this concentration was used in subsequent studies. It is of relevance to note that the conditioned media obtained from the THP-1 cells might also contain some residual concentrations of LPS and IFN-γ. Thus, the protection observed by PACAP pretreatment includes protection against direct stimulation by residual LPS and IFN-γ on SH-SY5Y cells, although the effects of residual LPS and IFN-γ might be minimal due to their relatively low concentrations.

Fig. 2

Concentration-response effect of IFN-γ (I)-stimulated THP-1 cell media on SH-SY5Y cells. MTT assay was conducted 72 h after addition of conditioned media. Values are mean ± SEM, n = 5. * $P<0.05$, **$P<0.01$, compared to control

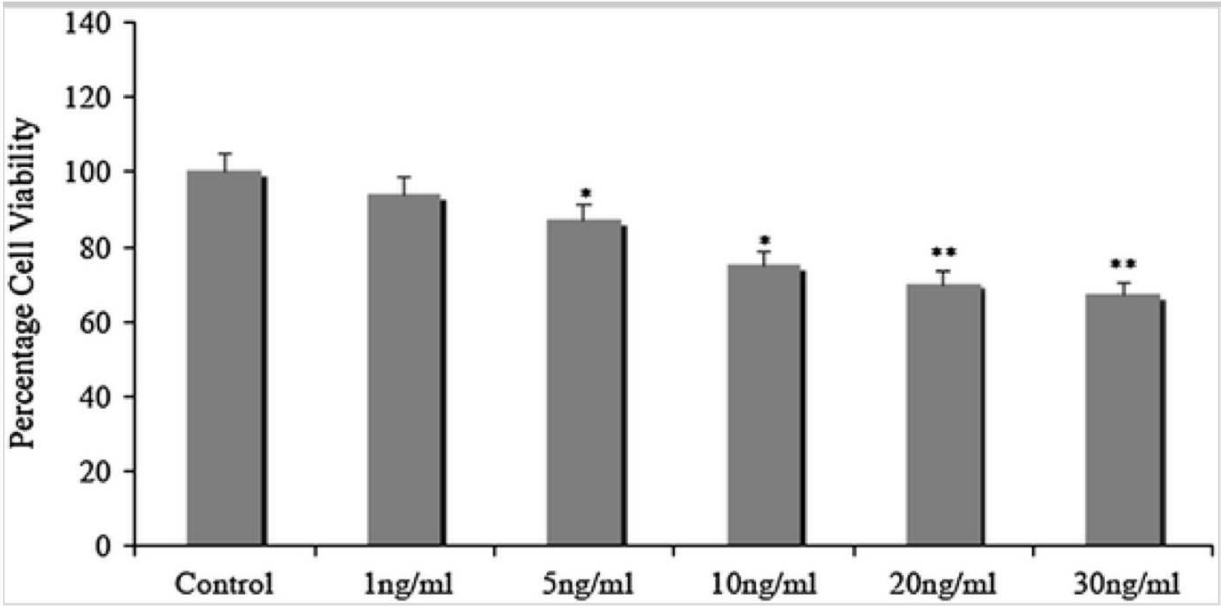
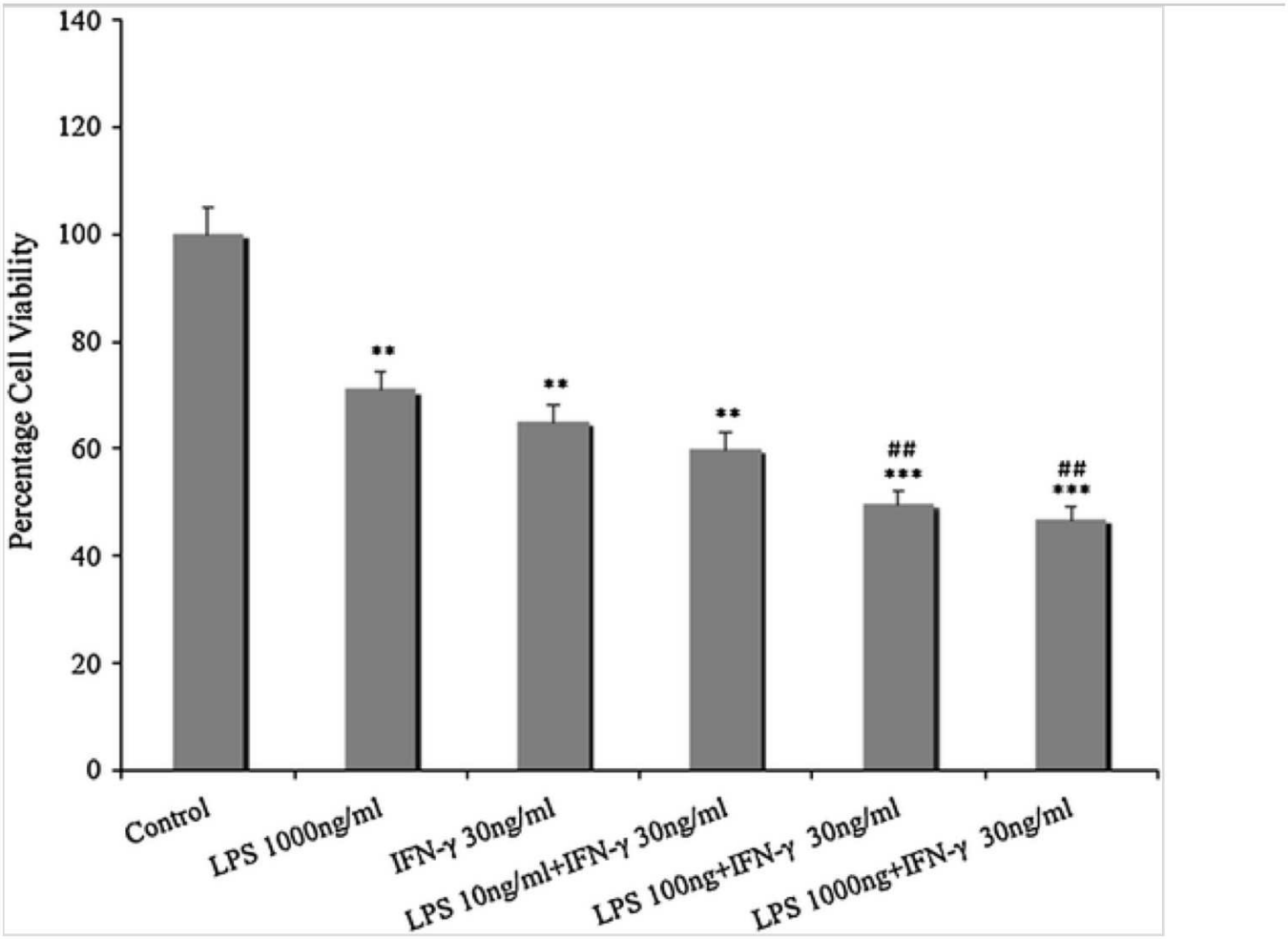


Fig. 3

Concentration-response effect of LPS + IFN-γ (L+I)-stimulated THP-1 cell media on SH-SY5Y cells. MTT assay was conducted 72 h after addition of conditioned media. Values are mean ± SEM, n = 5. **$P<0.01$, ***$P<0.001$ compared to control. ## $p<0.01$ compared to LPS or IFN-γ alone

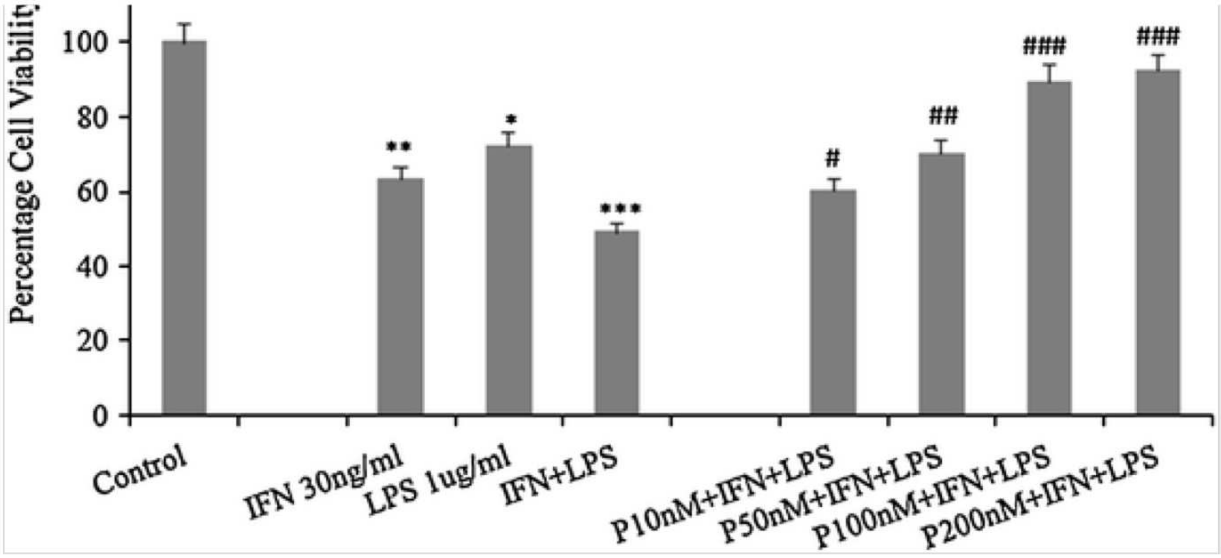


Effect of PACAP pretreatment

PACAP pretreatment provided a concentration-dependent protection against toxicity induced by the CM obtained from the combination of LPS (1000 ng/ml) + IFN-γ (30 ng/ml) in SH-SY5Y cells (Figure 4). Thus, 10 nM PACAP resulted in approximately 34 % protection ($p<0.05$), whereas 200 nM PACAP resulted in full protection ($p<0.001$). Thus, this concentration of PACAP was used in subsequent studies. PACAP by itself had no effect on cell viability (data not shown).

Fig. 4

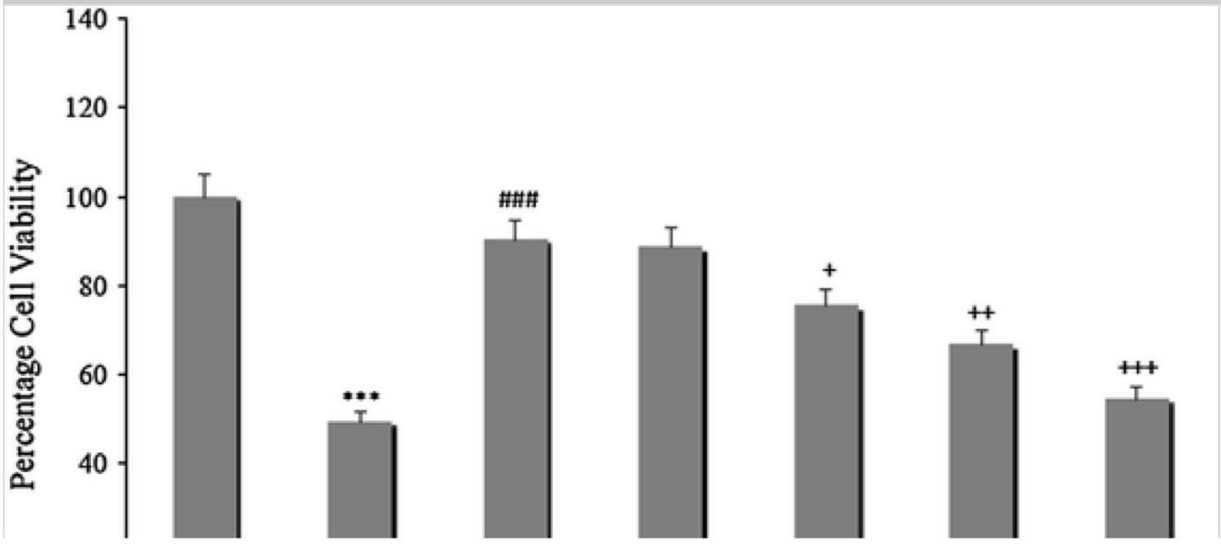
Concentration-response effect of PACAP pretreatment of SH-SY5Y cells against media obtained from THP-1 cells stimulated with LPS+IFN-γ (L+I). MTT assay was conducted 72 h after addition of conditioned media. Values are mean ± SEM, n = 5. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to control. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared to L+I

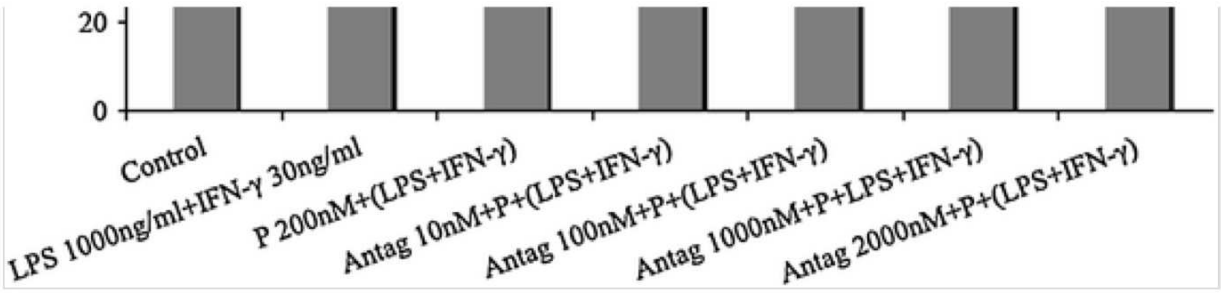


In order to check whether this effect is mainly mediated by the PAC1 receptors, PACAP antagonist (PACAP 6-38) was applied at various concentrations to antagonize the PACAP protection (200 nM) against toxicity induced by the CM obtained from a combination of LPS (1000 ng/ml) + IFN-γ (30 ng/ml) in SH-SY5Y cells (Figure 5). At 20 nM PACAP, antagonist did not affect PACAP protective capacity, but at 2 M it completely blocked the effect of PACAP ($p<0.001$). PACAP antagonist by itself had no effect on cell viability (data not shown).

Fig. 5

Concentration-response effect of PACAP antagonist (Antag) on protective effects of PACAP (P) in SH-SY5Y cells against media obtained from LPS+IFN-γ (L+I) stimulated THP-1 cells. PACAP antagonist was applied 2 h prior to PACAP, which was applied 2 h prior to L+I. Cell viability was determined 72 h after treatment by MTT assay. Values are mean ± SEM, n = 5. ***$P<0.001$ compared to control,, ###$P<0.001$ compared to L+I, $^{+}P<0.05$, $^{++}p<0.01$, $^{+++}P<0.001$ compared to P+L+I



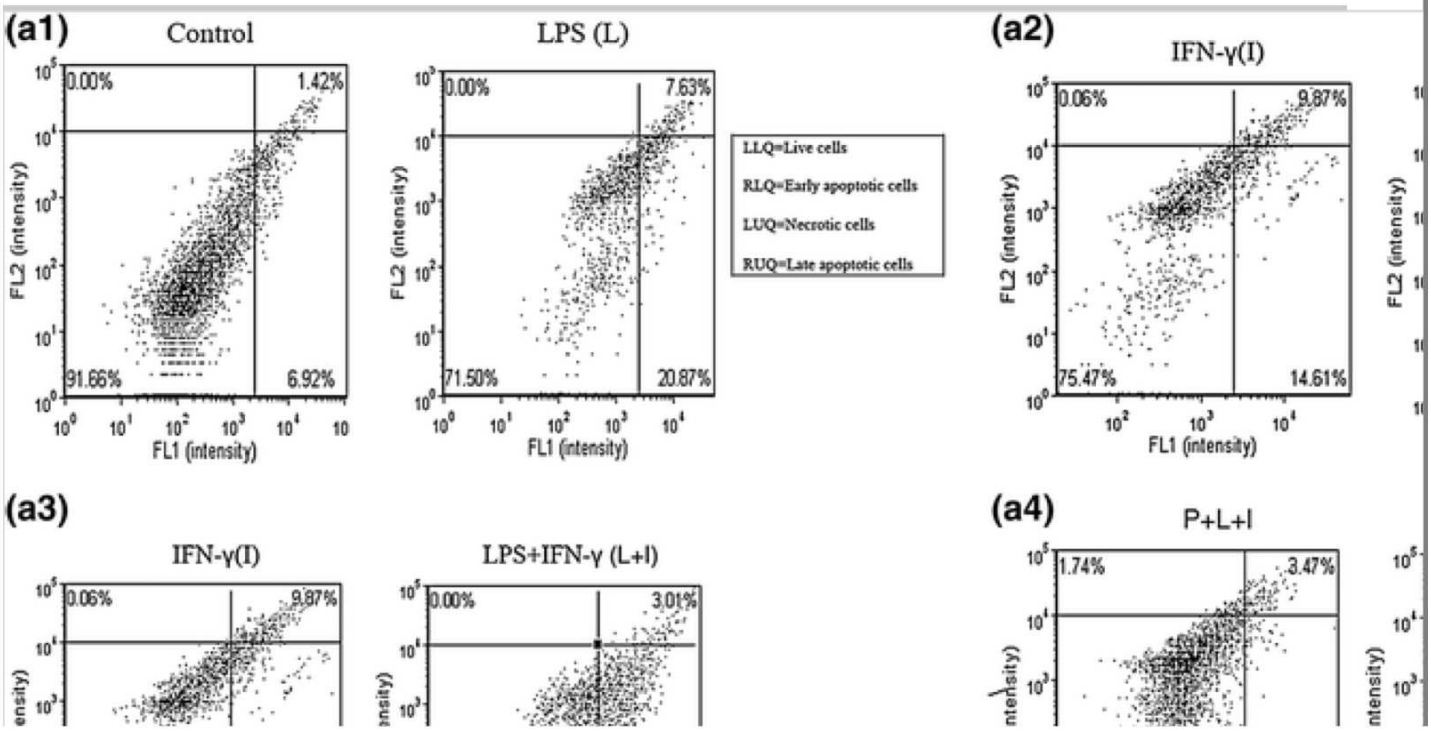


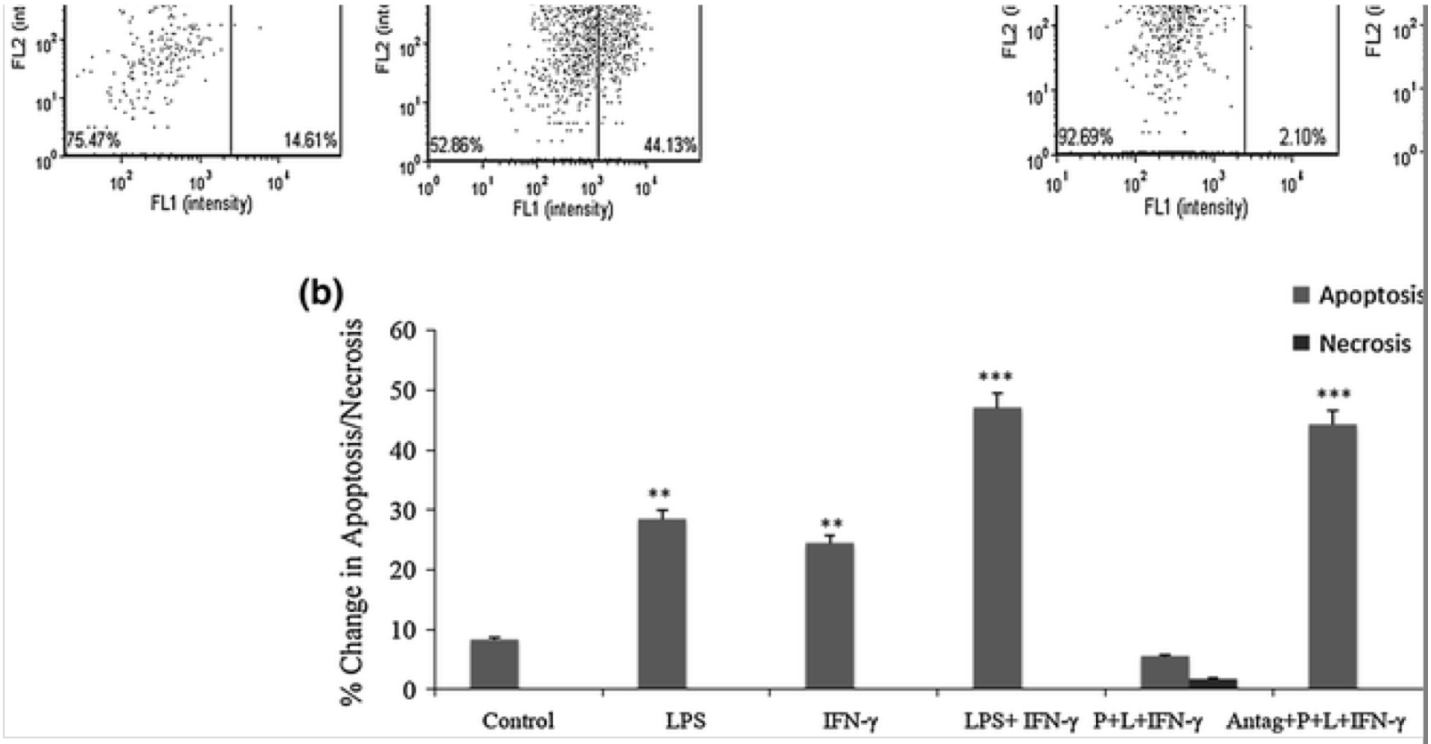
Effects of PACAP on apoptosis

Cell flow cytometry indicates that the toxicity of SH-SY5Y cells caused by the addition of conditioned media obtained from LPS+IFN-γ-stimulated THP-1 cells was due to apoptotic mechanism. These effects were blocked by PACAP (200nM), and PACAP antagonist (2 M), in turn, blocked the effects of PACAP (Figure 6).

Fig. 6

Effects of PACAP (P, 200 nM), and PACAP antagonist (Antag, 2 M) on LPS+IFN-γ (L+I)-induced apoptosis/necrosis as determined by cell flow cytometry (6a, cell flow diagram, 6b, % change in apoptosis/necrosis). Living cells ($FITC^-/PI^-$) are represented in lower left quadrant; early apoptotic cells ($FITC^+/PI^-$) are represented in lower right quadrant; late apoptotic cells ($FITC^+/PI^+$) are represented in upper right quadrant; and necrotic cells ($FITC^-/PI^+$) are represented in upper left quadrant. Antagonist was applied 2 h before PACAP, which was applied 2 h prior to L+I. Cell flow analysis was performed 72 h after treatment. Values are mean ± SEM, n = 5. **P<0.01, ***P<0.001 compared to control



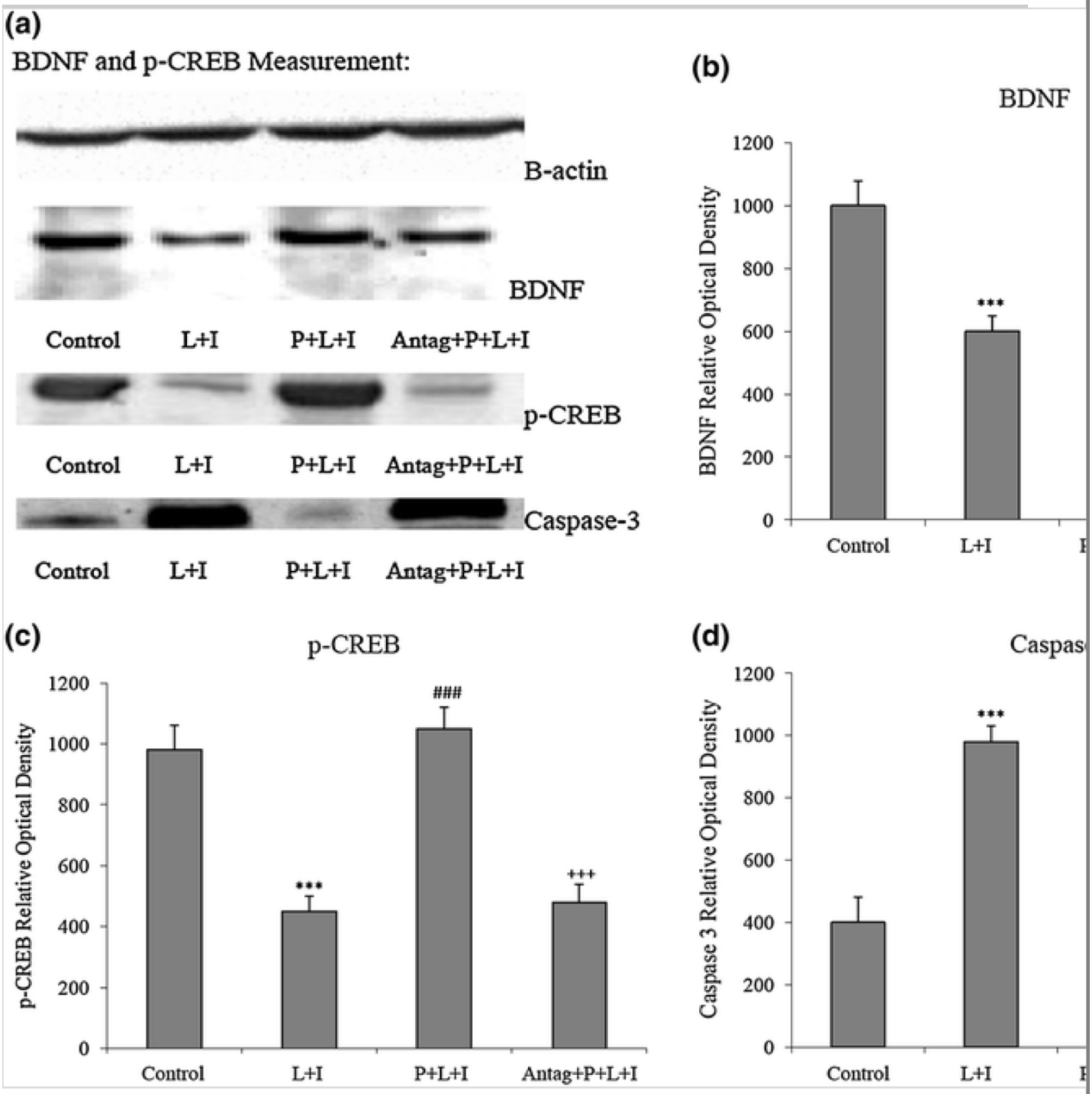


Effect of PACAP on BDNF, p-CREB, and caspase-3 expression

Conditioned media obtained from LPS (1000 ng/ml) + IFN-γ (30 ng/ml)-stimulated THP-1 cells resulted in approximately 40 % reduction in BDNF ($p<0.001$) (Figure 7a,b) and approximately 55 % reduction in p-CREB ($p<0.001$) levels (Figure 7a,c). PACAP pretreatment (200nM) completely blocked the effect of LPS+IFN-γ treatment. PACAP antagonist (2 M), in turn, blocked the effects of PACAP. PACAP or its antagonist did not have any effect of their own on levels of BDNF or p-CREB (data not shown), consistent with their lack of effect on cell viability. Conditioned media obtained from LPS (1000 ng/ml) + IFN-γ (30 ng/ml)-stimulated THP-1 cells resulted in approximately 60 % increase in caspase-3 levels ($p<0.001$). PACAP pretreatment (200nM) completely blocked the effect of LPS+IFN-γ treatment (Figure 7d). PACAP antagonist (2 M), in turn, blocked the effects of PACAP. PACAP or its antagonist did not have any effect of their own on levels of caspase-3 (data not shown).

Fig. 7

Effects of PACAP (P, 200 nM), and PACAP antagonist (Antag, 2 M) on LPS+IFN-γ (L+I)-induced changes in BDNF, p-CREB, and caspase-3 as determined by western blot (7a, gel representative). Antag was applied 2 h before P, which was applied 2 h prior to L+I. Figs 7b, c, and d represent BDNF, p-CREB, and caspase3 relative optical densities, respectively. Values are mean ± SEM, n = 5. ***$P<0.001$ compared to control, $^{###}P<0.001$ compared to L+I, $^{+++}P<0.001$ compared to P+L+I



Discussion

The findings from this study suggest that PACAP or its receptor agonist could be of therapeutic potential in retarding inflammation-induced neuronal degeneration, particularly in relationship to Parkinson s disease. This suggestion is based on concentration-dependent protective effects of PACAP against inflammatory-mediated toxicity in SH-SY5Y dopaminergic cells. Thus, application of inflammatory factors isolated from THP-1 cells following exposure to LPS+IFN-γ resulted in significant toxicity in SH-SY5Y cells that was inhibited by PACAP pretreatment. The effects of PACAP were, in turn, inhibited by its receptor antagonist. Moreover, the cell flow

PACAP were, in turn, inhibited by its receptor antagonist. Moreover, the cell flow results indicate apoptosis as a primary mechanism of toxicity, which is likely caspase-dependent as reflected in elevated caspase-3 levels. These results are consistent with previous findings of PACAP protection against hypoxia-induced inflammatory response (~~SUK~~Suk et al, **2004**) as well as in primary rat mesencephalic neuron-glia cultures where protective effects of PACAP against LPS-induced neurotoxicity were observed (Yang et al. **2006**).

The mediators of toxicity in our paradigm are likely consisting of cytokines such as interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNF-α), and nitric oxide (NO). This is based on the fact that human monocytic leukemia cell line, THP-1, has many similarities with human monocyte-derived macrophages which are used extensively to study inflammation-induced PD. THP-1 cell is a single, round suspension cell with distinct monocytic markers (Tsuchiya et al, **1980**). When differentiated, these cells become adherent macrophages with microglia-like properties and display increased phagocytic activity (Theus et al, **2004**). Additionally, this cell line possesses Fc (Fragment crystallizable region) and C3b (erythrocyte complement receptor), which plays a major role in inflammation and also produces lysozymes. Important to this study, THP-1 cells can be differentiated into macrophage-like cells using LPS. When stimulated with LPS, THP-1 cells are able to secrete and respond to cytokines, implying the presence of appropriate cytokine receptors on this cell line (Klegeris et al, **2003**). Along with the release of cytokines, THP-1 release a host of other neurotoxic materials including nitric oxide, which leads to the formation of reactive nitrogen species and ultimately death of cells (Ohira et al. **2012**). Interestingly, exposure of these cells to a combination of LPS and IFN-γ resulted in much higher toxicity than induced by each alone, consistent with previous findings (Klegeris et al, **2003**), and hence permitting further investigation of PACAP protective effects. In this regard, it would also be of significant interest to determine whether pretreatment of the THP-1 cells with PACAP will inhibit the release of inflammatory mediators.

This study also revealed that PACAP receptor antagonist blocked the effect of PACAP, which suggests that PACAP s effect is receptor mediated. It is of importance to note that although PACAP6-38 is a PAC1/VPAC2 receptor antagonist, it is often regarded as a PAC1 receptor antagonist in neuronal cells. Moreover, it is well known that most of the neuroprotection elicited by PACAP is mediated via the PAC1 receptor (Somogyvari-Vigh and Reglodi, **2004**; Vaudry et al. **2009**). On the other hand, VIP, the peptide showing the closest structural resemblance to PACAP, binds to PAC1 receptors with very little affinity (Vertongen et al, **1996**). This is most likely the reason behind the lower neuroprotective efficacy of VIP. Thus, in most studies, where the

effect of both peptides in neuroprotective paradigms involving apoptosis were compared, VIP was either effective only at 10-100 times higher concentrations than PACAP or was not effective at all (Somogyvari-Vigh and Reglodi, **2004**; Szabadfi et al. 2013).

The result also confirms that the toxicity of SH-SY5Y cells induced by the conditioned media obtained from THP-1 cells stimulated with LPS + IFN-γ is via apoptosis, a finding in agreement with previously suggested mechanism of inflammatory-mediated toxicity (Beatie, **2004**; Haanen and Vermes, **1995**; Kuwano and Hara, **2000**). Our findings of an increase in caspase-3 also suggest that the apoptotic process is likely caspase-dependent, although this latter contention needs to be verified by further analysis of other caspases as well as the caspase-3 activity. These findings are in accordance with several previous observations that show that PACAP is a strong antiapoptotic peptide, increasing antiapoptotic molecules, and inhibiting proapoptotic signaling (Racz et al. **2006**, Reglodi et al. **2011**; Somogyvari-Vigh and Reglodi, **2004**). Among others, PACAP inhibits caspase-3 activation in cerebellar granule cells exposed to ceramide or alcohol toxicity, retinal cells under glutamate-induced excitotoxicity, and also in PC12 cells exposed to amyloid-toxicity (Vaudry et al. **2009**; Somogyvari-Vigh and Reglodi, **2004**). It is also of relevance to note that most neuroblastoma-derived cell lines express PACAP and its receptors (Vertongen et al, **1996**), which might provide endogenous protection against some toxicants. However, the concentration of exogenous PACAP applied to these cells ensures adequate protection against insults induced by CM containing significant inflammatory mediators.

Our findings of PACAP reversal of the decreases in BDNF and p-CREB induced by the conditioned media indicate a central role of neurotrophic factors in neurotoxicity induced by inflammatory mediators, and protection by PACAP. Thus, it may be suggested that phosphorylation of CREB by PACAP results in BDNF induction, which can lead to reduced caspase-3 levels and/or activity, and therefore inhibition of neuronal apoptosis and toxicity. Since other neuroprotective agents (e.g., curcumin) may act via a similar mechanism (Qualls et al. **2013**), it would be of interest to determine whether combination of such drugs may lead to an additive or synergistic neuroprotection.

In summary, PACAP or PAC1 receptor agonists, by enhancing the neurotrophic pathway and inhibiting apoptosis, may be of therapeutic benefit in PD.

Acknowledgment

Supported by NIH/NIGMS 2 SO6 GM08016-39 and Howard University College of Medicine Bridge Fund~~(YT)~~ (YT) and KTIA NAP 13-1-2013-0001, ~~KTIA_NAP_13-1-2013-0001,~~ MTA Momentum Program, TAMOP4.2.2.A-11/1/KONV-2012-0024), Arimura Foundation, and OTKA K104984 (AT, DR)

References

Barcia C, Fernandez Barreiro A, Poza M, Herrero MT (2003) Parkinson s disease and inflammatory changes. Neurotox Res 5:411 418

Beatie MS (2004) Inflammation and apoptosis: Linked therapeutic targets in spinal cord injury. Trends Mol Med 10:580 583

Blesa J, Phani S, Jackson-Lewis V, Przedborski S (2012) Classic and new animal models of Parkinson s disease. J Biomed Biotechnol 2012:845618

Botia B, Jolivel V, Burel D, Le Joncour V, Roy V, Naassila M, Bénard M, Fournier A, Vaudry H, Vaudry D (2011) Neuroprotective effects of PACAP against ethanol-induced toxicity in the developing rat cerebellum. Neurotox Res 19:423 434

Brown D, Tamas A, Reglodi D, Tizabi Y (2013) PACAP protects against salsolinol-induced toxicity in dopaminergic SH-SY5Y cells: implication for Parkinson s disease. J Mol Neurosci 50:600 607

Copeland RL, Das JR, Kanaan YM (2007) Antiapoptotic effects of nicotine in its protection against salsolinol-induced cytotoxicity. Neurotox Res 12:61 69

Chao CC, Hu S, Ehrlich L, Peterson PK (1995) Interleukin-1 and tumor necrosis factor-alpha synergistically mediate neurotoxicity: involvement of nitric oxide and of N-methyl-D-aspartate receptors. Brain Behav Immun 9:355 365

Chen H, Zhang SM, Hernan MA, Schwarzschild MA, Colditz GA, Speizer FE, Ascherio A (2003) Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. Arch Neurol 60:1059 1064

Chinta SJ, Lieu CA, Demaria M, Laberge RM, Campisi J, Andersen JK (2013) Environmental stress, ageing and glial cell senescence: a novel mechanistic link to Parkinson s disease? J Intern Med 273:429 436

Das JR, Tizabi Y (2009) Additive protective effects of donepezil and nicotine against salsolinol-induced cytotoxicity in SH-SY5Y cells. Neurotox Res 16:194 204

Delgado M, Ganea D (2001) Inhibition of endotoxin-induced macrophage chemokine production by vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide in vitro and in vivo. J Immunol 167:966 975

Dong-Kug C, Kyoungho S (2007) Glial reaction in Parkinson s diseases: inflammatory activation signaling of slia as a potential therapeutic target. Curr Signal Transduction Therapy 2:77 90

Frechilla D, Garcia-Osta A, Palacios S, Cenarruzabebeitia E, Del Rio J (2001) BDNF mediates the neuroprotective effect of PACAP-38 on rat cortical neurons. NeuroReport 12:919 923

Ganea D, Delgado M (2002) Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) as modulators of both innate and adaptive immunity. Crit Rev Oral Biol Med 13:229 237

Gayle DA, Ling Z, Tong C, Landers T, Lipton JW, Carvey PM (2002) Lipopolysaccharide (LPS)-induced dopamine cell loss in culture: roles of tumor necrosis factor-alpha, interleukin-1beta, and nitric oxide. Brain Res Dev Res 133:27 35

Haanen C, Vermes I (1995) Apoptosis and Inflammation. Mediators Inflamm 4:5 15

Jantas D, Roman A, Kuśmierczyk J, Lorenc-Koci E, Konieczny J, Lenda T, Lasoń W (2013) The extent of neurodegeneration and neuroprotection in two chemical in vitro models related to Parkinson s disease is critically dependent on cell culture conditions. Neurotox Res 24:41 54

Klegeris A, Bissonnette CJ, McGeer PL (2003) Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor. Br J Pharmacol 139:775 786

Kuwano K, Hara N (2000) Signal transduction pathways of apoptosis and inflammation induced by the tumor necrosis factor receptor family. Am J Respir Cell Mol Biol 22:147 149

Liu B, Hong JS (2003) Role of Microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J Pharamacol Exp Ther 304:1 7

Martinez C, Abad C, Delgado M, Arranz A, Juarranz MG, Rodriguez-Henche N, Brabet P, Leceta J, Gomariz RP (2002) Anti-inflammatory role in septic shock of pituitary adenylate cyclase-activating polypeptide receptor. Proc Natl Acad Sci USA 99:1053 1058

Maruyama W, Yi H, Takahashi T, Shimazu S, Ohde H, Yoneda F, Iwasa K, Naoi M (2004) Neuroprotective function of R-(-)-1-(benzofuran-2-yl)-2-propylaminopentane, [R-(-)-BPAP], against apoptosis induced by N-methyl(R)salsolinol, an endogenous dopaminergic neurotoxin, in human dopaminergic neuroblastoma SH-SY5Y cells. Life Sci 75:107 117

McGeer PL, Yasojima K, McGeer EG (2001) Inflammation in Parkinson s disease. Adv Neurol 86:83 89

Naoi M, Maruyama W, Nagy GM (2004) Dopamine-derived salsolinol derivatives as endogenous monoamine oxidase inhibitors: occurrence, metabolism and function in human brains. Neurotoxicology 25:193 204

Nakamura Y, Si QS, Kataoka K (1999) Lipopolysaccharide-induced microglial activation in culture: temporal profiles of morphological change and release of cytokines and nitric oxide. Neurosci Res 35:95 100

Ohira H, Fujioka Y, Katagiri C, Yano M, Mamoto R, Aoyama M, Usami M, Ikeda M (2012) Butyrate enhancement of inteleukin-1β production via activation of oxidative stress pathways in lipopolysaccharide-stimulated THP-1 cells. J Clin Biochem Nutr 50:59 66

Qualls Z, Brown, D, Ramlochansingh C, Hurley LL, Tizabi Y (2013) Protective effects of curcumin against rotenone and salsolinol-induced toxicity: implications for Parkinson s disease. Neurotox Res (E-pub ahead of print)

Racz B, Tamas A, Kiss P, Toth G, Gasz B, Borsiczky B, Ferencz A, Gallyas F Jr, Roth E, Reglodi D (2006) Involvement of ERK and CREB signaling pathways in the protective effect of PACAP in monosodium glutamate-induced retinal lesion

the protective effect of PACAP in monosodium glutamate induced retinal lesion. Ann N Y Acad Sci 1070:507 511

Raghavan M, Bjorkman P (1996) Fc receptors and their interactions with immunoglobulins. Annu Rev Cell Dev Biol 12:181 222

Ramlochansingh C, Taylor RE, Tizabi Y (2011) Toxic effects of low alcohol and nicotine combinations in SH-SY5Y cells are apoptotically mediated. Neurotox Res 20:263 269

Rat D, Schmitt U, Tippmann F, Dewachter I, Theunis C, Wieczerzak E, Postina R, van Leuven F, Fahrenholz F, Kojro E (2011) Neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) slows down Alzheimer s disease-like pathology in amyloid precursor protein-transgenic mice. FASEB J 25:3208 3218

Reglodi D, Kiss P, Lubics A, Tamas A (2011) Review on the protective effect of PACAP in models of neurodegenerative diseases in vitro and in vivo. Curr Pharm Des 17:962 972

Reglodi D, Lubics A, Kiss P, Lengvari I, Gaszner B, Toth G, Hegyi O, Tamas A (2006) Effect of PACAP in 6-OHDA-induced injury of the substantia nigra in intact young and ovariectomized female rats. Neuropeptides 40:265 274

Reglodi D, Tamas A, Lubics A, Szalontay L, Lengvari I (2004) Morphological and functional effects of PACAP in 6-hydroxydopamine-induced lesion of the substantia nigra in rats. Regul Pept 123:85 94

Samii A, Nutt JG, Ransom BR (2004) Parkinson s disease. Lancet 363:1783 1793

Schneider L, Giordano S, Zelickson BR, Johnson MS, Benavides GA, Ouyang X, Fineberg N, Darley-Usmar VM, Zhang J (2011) Differentiation of SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress. Free Radic Biol Med 51:2007 2017

Schulte T, Schols L, Muller T, Woitalla D, Berger K, Kruger R (2002) Polymorphisms in the interleukin-1 alpha and beta genes and the risk for Parkinson s disease. Neurosci Lett 326:70 72

Somogyvari-Vigh A, Reglodi D (2004) Pituitary adenylate cyclase activating polypeptide: a potential neuroprotective peptide. Curr Pharm Des 10:2861 2889

polypeptide: a potential neuroprotective peptide. Curr Pharm Des 10:2861–2889

Storch A, Ott S, Hwang YI, Ortmann R, Hein A, Frenzel S, Matsubara K, Ohta S, Wolf HU, Schwarz J (2002) Selective dopaminergic neurotoxicity of isoquinoline derivatives related to Parkinson s disease: studies using heterologous expression systems of the dopamine transporter. Biochem Pharmacol 63:909–920

Szabadfi K, Danyadi B, Kiss P, Tamas A, Fabian E, Gabriel R, Reglodi D (2012) Protective effects of vasoactive intestinal peptide (VIP) in ischemic retinal degeneration. J Mol Neurosci 48:501–507

Suk K, Park JH, Lee WH (2004) Neuropeptide PACAP inhibits hypoxic activation of brain microglia: a protective mechanism against microglial neurotoxicity in ischemia. Brain Res 1026:151–156

Theus SA, Cave MD, Eisenach KD (2004) Activated THP-1 cells: an attractive model for the assessment of intracellular growth rates of Mycobacterium tuberculosis isolates. Infect Immun 72:1169–1173

Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K (1980) Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 26:171–176

Vaudry D, Gonzalez BJ, Basille M, Pamantung TF, Fontaine M, Fournier A, Vaudry H (2000) The neuroprotective effect of pituitary adenylate cyclase-activating polypeptide on cerebellar granule cells in mediated through inhibition of the CED3-related cysteine protease caspase-3/CPP32. Proc Natl Acad Sci USA 97:13390–13395

Vaudry D, Pamantung TF, Basille M, Rousselle C, Fournier A, Vaudry H, Beauvillain JC, Gonzalez BJ (2002) PACAP protects cerebellar granule neurons against oxidative stress-induced apoptosis. Eur J Neurosci 15:1451–1460

Vaudry D, Falluel-Morel A, Bougault S, Basille M, Burel D, Wurtz O, Fournier A, Chow BK, Hashimoto H, Galas L, Vaudry H (2009) Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacol Rev 61:283–357

Vertongen P, Devalck C, Sariban E, De Laet MH, Martelli H, Paraf F, Hélardot P,

Robberecht P (1996) Pituitary adenylate cyclase activating peptide and its receptors are expressed in human neuroblastomas. J Cell Physiol 167:36 46

Wang G, Pan J, Tan Y, Sun XK, Zhang YF, Zhou HY, Ren RJ, Wang XJ, Chen SD (2008) Neuroprotective effects of PACAP27 in mice model of Parkinson s disease involved in the modulation of K (ATP) subunits and D2 receptors in the striatum. Neuropeptides 42:267 276

Watson MB, Nobuta H, Abad C, Lee SK, Bala N, Zhu C, Richter F, Chesselet MF, Waschek JA (2013) PACAP deficiency sensitizes nigrostriatal dopaminergic neurons to paraquat-induced damage and modulates central and peripheral inflammatory activation in mice. Neuroscience 240:277 286

Wersinger C, Sidhu A (2002) Inflammation and Parkinson s disease. Curr Drug Targets Inflamm Allergy 1:221 242

Yaka R, He D, Phamluong K, Ron D (2003) Pituitary adenylate cyclase-activating polypeptide (PACAP(1-38)) enhances N-methyl-D-aspartate receptor function and brain-derived neurotrophic factor expression via RACK1. J Biol Chem 278:9630 9638

Yang S, Yang J, Yang Z, Chen P, Fraser A, Zhang W, Pang H, Gao X, Wilson B, Hong JS, Block ML (2006) Pituitary adenylate cyclase-activating polypeptide (PACAP) 38 and PACAP4-6 are neuroprotective through inhibition of NADPH oxidase: potent regulators of microglia-mediated oxidative stress. J Pharmacol Exp Ther 319:595 603