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# PACAP and VIP regulate hypoxia-inducible factors in neuroblastoma cells exposed to hypoxia

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two related peptides acting as neurotransmitters/neuromodulators in central and peripheral nervous system. They are also involved in cancer showing a controversial role. Particulary, they are implicated in neuroblastoma differentiation (NB). This pediatric tumor can evolve to a malignant metastatic disease or spontaneously regress towards a benign form, known as ganglioneuroblastoma/ganglioneuroma. A negative hallmark of neoplasia progression is represented by hypoxic microenvironment. Low oxygen tension induces activation of hypoxia-inducible factors (HIFs) promoting cells proliferation and metastasis formation. Moreover, HIFs trigger vascular endothelial growth factor (VEGF) release favouring high-risk NB phenotype development.

In the present work, we have investigated for the first time, if PACAP and VIP interfere with NB differentiation through modulation of hypoxic/angiogenic process. To this end, we analyzed their effect in malignant undifferentiated and all-trans retinoic acid (RA) differentiated SH-SY5Y cells, representing the benign form of this tumor.

Our results have suggested tha both peptides, but predominantly VIP, induce NB differentiation into benign form by regulating HIFs, VEGF and VEGFRs expression and distribution.

All these data give new insight regarding PACAP/VIP regulatory role in NB progression.

## 1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two structurally related neuropeptides exerting their functions through activation of three different G-protein coupled receptors known as PAC1, VPAC1 and VPAC2. PACAP binds to all three receptors with high affinity, whereas VIP binds with high affinity to VPAC1 and VPAC2, but displays a 1000-fold lower affinity towards the PAC1 receptor (Arimura and Shioda, 1995).

PACAP and VIP act either as neurotransmitters or neuromodulators in the peripheral and central nervous system (CNS) by mediating various biological prˆocesses. Here, they are involved in neuronal death, inflammatory response, cell division and modulation of immune system (Uchida et al., 1996; Waschek, 2013; D'Agata et al., 1996; D'Agata and Cavallaro, 1998; Cavallaro et al., 1995; Castorina et al., 2008, 2010; Giunta et al., 2012; Canonico et al., 1996).

Their protective effect has been shown in neurodegenerative and ocular diseases (Reglodi et al., 2004, 2011; Maugeri et al., 2017a and b; Lamine et al., 2015; D'Amico et al., 2015a). Moreover, several studies have also described their involvement in different cancers. Based on tumor phenotype, they exert contrasting biological functions by stimulating cell proliferation or inhibiting mass growth (Giunta et al., 2010; Castorina et al., 2008; Maugeri et al., 2016a; Moody et al., 2016).

Particularly, previous papers have demonstrated that PACAP and VIP promote neuroblastoma (NB) differentiation (Wollman et al., 2002; Monaghan et al., 2008; Heraud et al., 2008). The latter is the most common tumor of early childhood affecting 10.2 per million children under 15 years of age (Maris, 2010). It is characterized by

*Abbreviations:* CNS, central nervous system; DFX, desferrioxamine mesylate salt; HIFs, hypoxia inducible factors; HRE, hypoxia-response element; NB, neuroblastoma; PACAP, pituitary adenylate cyclase-activating polypeptide; PHD, prolyl-hydroxylase domain; RA, all-trans retinoic acid; VIP, vasoactive intestinal peptide; VEGF, vascular endothelial growth factor; Vhl, vehicle

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unpredictable evolution since it can evolve to an highly aggressive metastatic disease or spontaneously regress to a less malignant form known as ganglioneuroblastoma/ganglioneuroma (Brodeur and Nakagawara, 1992). Negative neoplasia progression has been related to presence in tumor mass of hypoxic regions. In these areas, low oxygen tension promotes cells dedifferentiation towards an immature stem phenotype triggering fatal outcome (Jogi et al., 2002; Lu and Kang, 2010).

The hypoxia-inducible factors (HIFs) represent the key players of this process by mediating the adaptive response of NB cells to hypoxic microenvironment. These factors are heterodimeric complexes including an oxygen-labile α- and a more stable β-subunit (ARNT) (Semenza, 1999a,b). Humans express three HIF-α genes: HIF-1α, HIF-2α and HIF-3α.

During hypoxia, the decreased activity of the prolyl-hydroxylase domain (PHD) enzymes allows the stabilization of HIF-1α/2α which translocate into the nucleus to dimerize with HIF-1β.

The role displayed by HIFs in malignancy has been widely described (Lu and Kang, 2010). Previous studies have demonstrated that HIF-1α enhances progression of NB by promoting cells invasiveness during hypoxia (Chen et al., 2015) whereas Herrmann et al. (2015) have suggested the involvement either of HIF-1α or HIF-2α in metastasis induction. On contrary, HIF-3α subunit acts as a negative regulator of the hypoxic response by reducing, in opposition, HIF-1α/2α overexpression (Maynard et al., 2007). The latter induce transcription of a wide variety of genes including vascular endothelial growth factor (VEGF), whose upregulation is linked to unfavorable histology and aggressiveness of NB (Langer et al., 2000; Fukuzawa et al., 2002). This angiogenic factor represents a fundamental mediator of tissue vascularization by contributing to cells' survival, metastasis development and disease's poor outcome (Meitar et al., 1996).

VEGF binds to VEGFR-1 and VEGFR-2 receptors, both involved in phosphatidylinositol 3-kinase (PI3K)/Akt oncogenic pathway activation (Opel et al., 2007).

Previous studies have suggested that PACAP and VIP promote NB differentiation towards the benign form by stimulating cells' neuritogenesis (Monaghan et al., 2008; Heraud et al., 2008). Furthermore, VIP and a stable PACAP analog reduce MYCN amplification, a biomarker linked to advanced stage of malignancy (Chevrier et al., 2008).

Considering the importance of hypoxic process in promoting tumor aggressivity, here, we have investigated for the first time the effect of these neuropeptides in the modulation of HIFs in a model in vitro of malignant undifferentiated and all-trans retinoic acid (RA) differentiated SH-SY5Y cells representing the benign form of this tumor. Since hypoxic event is strictly linked to angiogenesis, we have also investigated whether PACAP and VIP interfere to NB progression by modulating VEGF and related receptors expression.

The results have demonstrated that both peptides, but predominantly VIP, affect hypoxic/angiogenic process in NB. Our data add new insights explaining the role of PACAP and VIP in this cancer.

## 2. Materials and methods

### 2.1. Cell culture and differentiation

The human NB cell line SH-SY5Y, obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA), was grown in a mixture of 1:1 Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 K Nutrient Medium supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100-μg/ml streptomycin, and incubated at 37 °C in 5% $CO_2$, as previously described (D'Amico et al., 2014). To realize an in vitro model of less aggressive neuroblastoma tumor we differentiated SH-SY5Y cells by using 10 μM *all-trans* retinoic acid in complete growth medium (RA) (Sigma Cat n. 302-79-4) for 7 days by changing the medium every two days, as described by Maugeri et al. (2016b).

### 2.2. Treatments

Pituitary adenylate cyclase activating polypeptide-38 (PACAP38, 100 nM; cat no. A1439, Sigma-Aldrich) and vasoactive intestinal peptide (VIP, 100 nM; cat no. V3628, Sigma-Aldrich), were added to SH-SY5Y cells for 24 h in normoxic or hypoxic condition.

Cells grown under hypoxia were exposed for 24 h to 100 μM desferrioxamine mesylate salt (DFX) (Sigma-Aldrich), a hypoxia-mimetic agent, which induces hypoxia by inhibiting the HIF PHDs (Epstein et al., 2001; Hirsilä et al., 2005). As compared to cells incubation method in hypoxic chamber, this offers the advantage to the experimentator to open the culture plate/dish/flask many times without affecting the hypoxic condition.

### 2.3. Western blot analysis

Western blot analysis was performed according to the procedures previously described by D'Amico et al. (2015b). Proteins were extracted with buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics, Monza, Italy) using a Teflon-glass homogenizer and then sonicated twice for 20 s using an ultrasonic probe, followed by centrifugation at 10,000 ×*g* for 10 min at 4 °C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen,Carlsbad, CA, USA). About 30 μg of protein homogenate were diluted in 2× Laemmli buffer (Invitrogen), heated at 70 °C for 10 min and then separated on a Biorad Criterion XT 4–15% Bis-tris gel by electrophoresis and then transferred to a nitrocellulose membrane. Blots were blocked using the Odyssey Blocking Buffer (Li-Cor Biosciences, Nebraska, USA). The transfer was monitored by a prestained protein molecular weight marker (BioRad Laboratories, Segrate (MI), Italy). Immunoblot analysis was performed by using appropriate antibodies: rabbit anti-PACAP (H-76, cat no. sc-25439, Santa Cruz Biotechnology, Texas, U.S.A. 1:200), mouse anti-VIP (H-6, cat no. sc-25347, Santa Cruz Biotechnology; 1:100), rabbit anti-PAC1 receptor (H-55, cat no.sc-30018, Santa Cruz Biotechnology; 1:300), rabbit anti-VPAC1 (H-130, cat no. sc-30019, Santa Cruz Biotechnology; 1:200), rabbit anti-VPAC2 (H-50, cat no. sc-30020, Santa Cruz Biotechnology; 1:200), mouse anti- HIF-1alpha (cat.n. NB100-105, Novus Biologicals, Littleton, USA 1:500), rabbit anti- HIF-2alpha (cat.n. NB100-122, Novus Biologicals, 1:500), rabbit anti-HIF-3alpha (H-170) (cat n.sc-28707, Santa Cruz Biotechnology; 1:200), goat anti-VEGF (cat n.sc-1836, Santa Cruz Biotechnology; 1:200), rabbit anti-VEGF-R1 (cat n.sc-316, Santa Cruz Biotechnology; 1:200), mouse anti- VEGF-R2 (cat n.sc-6251, Santa Cruz Biotechnology; 1:200), and rabbit anti-β-tubulin (cat n.sc-9104, Santa Cruz Biotechnology; 1:500). The secondary antibody goat anti-rabbit IRDye 800CW (cat #926-32211; Li-Cor Biosciences), goat anti-mouse IRDye 680CW (cat #926-68020D, Li-Cor Biosciences) and donkey anti-goat IRDye 800CW (cat #926-32214; Li-Cor Biosciences) were used at 1:15,000, 1:20,000 and 1:15,000, respectively. Blots were scanned with an Odissey Infrared Imaging System (Odyssey). Densitometric analyses of Western blot signals were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Values were normalized to β-tubulin, which served as loading control, as previously described by Maugeri et al. (2017c). For each gene has been detected β-tubulin expression in a parallel immunoblot.

### 2.4. Immunolocalization

To determine cellular distribution of HIF-1α and VEGF proteins, immunofluorescence analysis was performed on control and RA treated cells as previously described by Maugeri et al. (2016c). They were cultured on glass cover slips, fixed in 4% paraformaldehyde in PBS (15′ at room temperature), permeabilized with 0.2% Triton ×100, blocked with 0.1% BSA in PBS, and then probed with anti- HIF-1α (1:50) and