Differential Effects of Neuropeptide Y on the Growth and Vascularization of Neural Crest–Derived Tumors

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Abstract
Neuropeptide Y (NPY) is a sympathetic neurotransmitter recently found to be potently angiogenic and growth promoting for endothelial, vascular smooth muscle and neuronal cells. NPY and its cognate receptors, Y1, Y2 and Y5, are expressed in neural crest–derived tumors; however, their role in regulation of growth is unknown. The effect of NPY on the growth and vascularization of neuroendocrine tumors was tested using three types of cells: neuroblastoma, pheochromocytoma, and Ewing’s sarcoma family of tumors (ESFT). The tumors varied in expression of NPY receptors, which was linked to differential functions of the peptide. NPY stimulated proliferation of neuroblastoma cells via Y2/Y5Rs and inhibited ESFT cell growth by Y1/Y5-mediated apoptosis. In both tumor types, NPY receptor antagonists altered basal growth levels, indicating a regulatory role of autocrine NPY. In addition, the peptide released from the tumor cells stimulated endothelial cell proliferation, which suggests its paracrine angiogenic effects. In nude mice xenografts, exogenous NPY stimulated tumor vascularization of ischemic tissues. Taken together, this is the first report of NPY being a growth-regulatory factor for neuroendocrine tumors, acting both by autocrine activation of tumor cell proliferation or apoptosis and by angiogenesis. NPY and its receptors may become targets for novel approaches in the treatment of these diseases, directed against both tumor cell proliferation and angiogenesis. (Cancer Res 2005; 65(5): 1719-28)

Introduction
Neural crest–derived tumors synthesize and release neural-specific peptides, such as 36 amino acid neuropeptide Y (NPY). This sympathetic neurotransmitter and its receptors are highly expressed in tumors derived from the autonomic nervous system, including those originating from sympathetic neurons (neuroblastoma and pheochromocytoma) as well as Ewing’s sarcoma family of tumors (ESFT) with a putative origin from parasympathetic neurons (20, 21). The angiogenic activities of NPY seem involved in proliferation of vascular smooth muscle cells and neuronal cells (19). However, there is growing evidence that the peptide is also a growth factor for various types of cells, such as vascular smooth muscle cells and neuronal cells (20, 21). Recently, we have discovered that NPY is also a potent angiogenic factor, which stimulates endothelial cell proliferation and migration, capillary tube formation, and revascularization of ischemic tissues (22–24). NPY acts via multiple Gi/o-protein-coupled receptors, designated as Y1 to Y5 (25). The serine protease, dipetidyl peptidase IV (DPPIV), converts the full-length NPY1–36 to a shorter form, NPY3–36, which is no longer able to bind to the Y1R, but retains affinity for all other receptors. Hence, DPPIV functions in the NPY system as a "receptor switch" shifting the functions of the peptide from Y1R to non-Y1R mediated (26). The NPY receptors vary in their cellular distribution and mediate different functions of the peptide. For example, Y1 is the main receptor involved in proliferation of vascular smooth muscle cells and neuronal precursors (20, 21). The angiogenic activities of NPY seem responsive to NPY1–36, whereas NPY3–36 fails to activate the receptor (27).

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to be mediated mainly by Y2R because they are severely impaired in Y2 knockout mice (27, 28).

Neuroendocrine tumor cells, particularly those derived from the autonomic nervous system, often express both NPY and its receptors, which suggests that the peptide may modify their proliferation in an autocrine manner. Moreover, if secreted by these tumors, NPY may also stimulate endothelial cell proliferation and thus promote angiogenesis in a paracrine fashion. Hence, the purpose of this study was to determine if NPY is released from neural crest–derived tumors and if it regulates their growth either directly by inducing tumor cell proliferation or indirectly by stimulating angiogenesis.

Materials and Methods

**Materials.** NPY and its analogues were purchased from Bachem Peninsula Laboratories (San Carlos, CA), Y1R antagonist, H409/22 acetate, and Y5R antagonist, CGP71683A (Novartis International AG, Basel, Switzerland) were gifts from AstraZeneca (Möln达尔, Sweden), and Y2R antagonist, BII0246TF, from Boehringer Ingelheim Pharma (Ingelheim, Germany). Selective receptor agonists, Y2 (IAhV2-29) and Y5 (cM10), were gifts from Dr. Annette Beck-Sickinger (University of Leipzig, Leipzig, Germany) and DPPIV inhibitor, P32/98, from Probiodrug (Halle, Germany).

**Cell Culture.** Human neuroblastoma, ESFT (SK-N-MC), and rat pheochromocytoma PC12 cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured according to the supplier's recommendations. Additional ESFT cell lines were previously reported (TC32, TC71, SKES, and A4573; ref. 29). Human dermal microvascular endothelial cells (HMVEC) at passage 4 were purchased from Cambrex (East Rutherford, NJ) and cultured according to the supplier's recommendation.

**Neuropeptide Y ELISA.** A total of 10⁶ cells were seeded onto a 100-mm tissue culture dish. After 6 days, culture media were collected, cells washed with ELISA assay buffer containing 0.1% Tween 20, and then trypsinized, counted, and lysed. NPY concentration in the cells, washing buffer, and culture medium was determined using Neuropeptide Y Enzyme Immunoassay Kit (Bachem Peninsula Laboratories, San Carlos, CA).

**Reverse Transcription–PCR.** RNA was isolated using RNaseasy Mini Kit (Qiagen Inc., Valencia, CA) and digested with DNase I using DNA-free (Ambion, Austin, TX). cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR reactions were done as previously described (24) with modified NPY primers: 5′-TACCCCTCTCAAGCCGGCAAA-3′, 5′-CATTTTCTGTGCCTTCTCAT-3′. In each sample, 18s rRNA served as a control. Real time reverse transcription–PCR (RT-PCR) was done using iCycler iQ Detection System (Bio-Rad Laboratories, Hercules, CA). The PCR reactions were carried out using TaqMan Universal PCR Master Mix and predesigned primers and fluorescein-labeled probes (Applied Biosystems, Foster City, CA). The results were calculated by the comparative Ct method using ß-actin as an endogenous reference gene, according to the Applied Biosystems ABI PRISM 7700 User Bulletin #2.

**Immunocytochemistry.** Cells were grown on coverslips, fixed with 4% paraformaldehyde, stained with rabbit polyclonal anti-NPY and mouse monoclonal anti-tyrosine hydroxylase antibody (ImmunoStar, Hudson, WI) according to the manufacturer's recommendations, and counterstained with methyl green.

**Immunohistochemistry.** Tissue samples were embedded in OCT compound (Sakura Finetek USA, Torrance, CA), snap frozen in liquid nitrogen, and cut into 5-μm sections. NPY expression was visualized using biotinylated goat anti-rabbit (1:200) (DakoCytomation, Glostrup, Denmark) and rabbit anti-Y2R (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were then incubated with streptavidin–alkaline phosphatase and detected with nitroblue tetrazolium dye. For double labeling, samples were incubated with rabbit polyclonal anti-NPY (1:100) (ImmunoStar, Hudson, WI) and mouse monoclonal anti-Y2R (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, followed by biotinylated goat anti-rabbit (1:200) and horse anti-mouse IgG (1:200) (Jackson ImmunoResearch, West Grove, PA). Sections were counterstained with hematoxylin.

**Western Blot.** Detection of NPY receptors was done using polyclonal rabbit antibody against Y1 and Y5Rs (gift from Dr. Janice Urban, Department of Physiology and Biophysics, The Chicago Medical School, Chicago, IL) and goat polyclonal antibody against Y2R (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Cell Cycle Analysis.** Cells were plated onto 100-mm plates (500,000 cells per plate), cultured for 2 days, and growth arrested in serum-free media for 24 hours. Then, the cells were stimulated with 10⁻⁷ mol/L NPY for desired periods, trypsinized, fixed in 75% ethanol, and stained with propidium iodide according to the standard procedures. Flow cytometric analysis was done on FACSort (Becton Dickinson, Franklin Lakes, NJ) and data analyzed using CellQuest and ModFit LT software packages (Verity Software House Inc., Topsham, ME).

**Mitogen-Activated Protein Kinase Activation.** Mitogen-activated protein kinase (MAPK) was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

**Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling.** Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) reaction was done using in situ Cell Detection Kit (Roche Diagnostic, Indianapolis, IN). The cells were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) and counted using Metamorph software (Universal Imaging Corporation, Downingtown, PA).

**Histochemistry.** Tissue samples were stained as above, signal converted to visible light using AP converter, and tissues counterstained with hematoxylin.

**Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling.** Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) reaction was done using In situ Cell Detection Kit (Roche Diagnostic, Indianapolis, IN). The cells were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) and counted using Metamorph software (Universal Imaging Corporation, Downingtown, PA).

**Nude Mice Xenograft Model.** Nude mice between the ages of 7 to 10 weeks old (National Cancer Institute) were given s.c. injections into their right flank of 5 × 10⁶ SK-N-BE(2) or SK-N-MC cells suspended in 0.1 mL of media. Slow-release pellets containing either placebo or NPY (10⁻⁷ mol/L), the receptor antagonists (10⁻⁶ mol/L), and DPPIV inhibitor (10⁻⁵ mol/L) for 24 hours. Caspase 3/7 activity was measured using Apone- homogenous caspase 3/7 reagent (Promega).

**Statistical Analysis.** Data are presented as means ± SE for the absolute values or percent of control and analyzed by one-way repeated-measures ANOVA followed by Dunnett’s t test using SigmaStat 2.03 software (SPSS Science, Chicago, IL). P < 0.05 was considered statistically significant for the indicated n per group.
Results

Neural Crest–Derived Tumors Express and Release Neuropeptide Y

Expression of the NPY system and functions of the peptide in neural crest–derived tumors were compared in three model cell lines: pheochromocytoma (PC12), neuroblastoma [SK-N-BE(2)], and ESFT (SK-N-MC). The sympathetic origin of neuroblastoma and pheochromocytoma cells was confirmed by positive staining for tyrosine hydroxylase (Fig. 1A). No tyrosine hydroxylase was detected in ESFT SK-N-MC cells derived from parasympathetic nerves. NPY mRNA was detected by RT-PCR in all three cell lines (Fig. 1A). In contrast, no NPY expression was found in breast cancer cells MCF7 and MDA-MB-231 and glioblastomas (U-87MG and C6; data not shown). The intracellular level of the peptide, measured by ELISA and confirmed by NPY immunostaining, was higher in SK-N-BE(2) than in SK-N-MC cells, which was associated with increased release of NPY observed in neuroblastoma cells (76 ng per 10^6 cells versus 3.7 ng per 10^6 cells in ESFT cells). In PC12, all synthesized NPY was bound to the cell membrane and no peptide was detected in the cells or culture medium. To determine if the observed differences in NPY synthesis and release are specific for these tumor types, real-time RT-PCR and NPY ELISA were done on the panels of neuroblastoma and ESFT cell lines. NPY expression levels were generally higher in neuroblastoma than in ESFT cells and, in four of five neuroblastoma cell lines, accompanied by significant release of the peptide (Fig. 1B).

Neuroblastoma and ESFT Conditioned Medium Stimulates Proliferation of Endothelial Cells

We tested the effect of neuroblastoma and ESFT-conditioned medium on proliferation of HMVECs to determine if NPY released from neuroendocrine tumors stimulates their vascularization. Conditioned medium, obtained from SK-N-BE(2) and SK-N-MC cells cultured for 24 hours in low-serum medium, contained NPY at a concentration of 7.8 × 10^{-9} and 1.6 × 10^{-9} mol/L, respectively, and stimulated HMVEC proliferation in a dose-dependent manner (Fig. 1C). The effect of SK-N-BE(2)-conditioned medium was completely blocked by a mixture of NPY receptor antagonists (Y1, Y2, and Y5 at concentrations of 10^{-7} mol/L, each), whereas proliferative activity of SK-N-MC-conditioned medium was only partially reduced. The antagonists also blocked the mitogenic effect of synthetic NPY in HMVEC, but did not affect proliferation stimulated by basic fibroblast growth factor (10^{-7} mol/L; Fig. 1C).

Neuroblastoma and ESFT Cells Vary in Neuropeptide Y Receptor Expression

Before evaluating the direct effects of NPY on tumor cell proliferation, we determined NPY receptor expression in the three tumor cell lines by RT-PCR and Western blot. SK-N-BE(2) cells expressed Y2 and Y5Rs, SK-N-MC expressed Y1 and Y5Rs, whereas PC12 expressed all three receptors (Fig. 2). Expression of the Y1R in SK-N-MC and PC12 cells was accompanied by the presence of DPPIV mRNA (Fig. 2). To determine if the observed differential receptor pattern is cell type specific, real time RT-PCR on the panel of neuroblastoma and ESFT cell lines was done (Table 1). All five investigated neuroblastoma cell lines predominantly expressed Y2R, SK-N-BE(2) and IMR-32 cells additionally expressed low levels of Y5 and Y1R. All ESFT cell lines expressed high levels of both Y1 and Y5Rs, whereas Y2 mRNA was almost undetectable. Expression of DPPIV protease was diverse among the neuroblastoma cells; however, all ESFT cells expressed high levels of the DPPIV message.

Neuropeptide Y Regulates Growth of Neural Crest–Derived Tumors in a Receptor-Dependent Manner

Neuroblastoma, ESFT, and pheochromocytoma cells were treated with NPY at concentrations ranging from 10^{-12} to 10^{-7} mol/L for 48 hours to test if differential receptor expression determines the effects of NPY on tumor cell proliferation. NPY stimulated growth of SK-N-BE(2) neuroblastoma cells up to 60% above the control, with two peaks at 10^{-11} and 10^{-7} mol/L (Fig. 2). In contrast, in SK-N-MC cells, the peptide decreased the number of viable cells by 30% below the control, whereas no effect was observed in PC12 cells. The above results were confirmed by cell count (data not shown).

Specific receptor agonists and antagonists were used to determine which NPY receptors are responsible for the stimulatory and inhibitory effects of NPY in the above cells (Fig. 2). In SK-N-BE(2) cells, the mitogenic effect of 10^{-7} mol/L NPY, which corresponds to the peak of its activity, was mimicked by both Y2 and Y5 specific receptor agonists ([A]b^2) NPY and cM10, respectively) at the same concentrations. The combination of both agonists did not augment the mitogenic effect. Y2 and Y5 receptor antagonists (BIIE0246TF and CGP71683A, respectively), at a concentration of 10^{-6} mol/L, added separately and in combination, not only blocked the mitogenic effect of NPY but also decreased the number of viable cells to 30% of the basal levels, even in the absence of exogenous peptide. Y1R antagonist did not reduce NPY-induced proliferation of SK-N-BE(2) cells.

In SK-N-MC cells, effects of receptor agonists and antagonists were opposite to those observed in neuroblastoma cells (Fig. 2). Leu\textsuperscript{31}Pro\textsuperscript{34}PYY, a selective Y1 and Y5R agonist, mimicked the inhibitory effect of NPY in these cells, whereas the Y5 agonist alone had no effect. In addition, the Y2 agonist did not alter growth of SK-N-MC cells. As in SK-N-BE(2), treatment with the respective antagonist (Y1, H409/22 acetate, and Y5, CGP71683A) not only blocked the agonist effects but also significantly increased the number of viable cells (up to 150% above the control), even without exogenous NPY. The Y2R antagonist did not alter inhibitory effect of NPY in SK-N-MC cells.

In PC12 cells, NPY (Fig. 2), as well as selective receptor agonists (data not shown), did not affect cell growth. However, the Y1R antagonist and, to lesser extent, Y2 and Y5 antagonists significantly increased the number of viable cells (163%, 130%, and 135% of control, respectively).

Mechanisms of Neuropeptide Y Growth-Regulatory Actions in Neural Crest–Derived Tumors

Neuropeptide Y Stimulates Proliferation of SK-N-BE(2) Cells via p44/42 Mitogen-Activated Protein Kinase Activation. Cell cycle analysis of serum-starved SK-N-BE(2) cells was done to determine if the growth-stimulatory effect of NPY observed in neuroblastoma cells is directly associated with increased proliferation. NPY (10^{-7} mol/L) significantly increased the percent of cells in S phase (from 18.6 ± 2.1% in control to 28.9 ± 0.9% in NPY-treated cells) with a peak observed 24 hours after stimulation (Fig. 3A). The proliferative effect of NPY was associated with activation of p44/42 MAPK. The first early peak of p44/42 MAPK activation occurred at 5 minutes and was followed by the second, late peak of sustained activity starting 1 hour after stimulation. NPY-induced p44/42 phosphorylation was blocked by both Y2 and Y5 but not Y1R antagonist.

Neuropeptide Y Induces Apoptosis of SK-N-MC Cells. Cell cycle analysis of SK-N-MC cells was done to determine the mechanisms of growth-inhibitory effect of NPY in ESFT cells. In 0.25% serum media, no significant change in percent of cells in

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S phase was observed after NPY treatment (10^{-7} \text{ mol/L}) over the course of 24 hours (Fig. 3B). There was also no significant p44/42 MAPK activation within 90 minutes from NPY treatment (data not shown). However, cell cycle analysis done 24 hours after stimulation revealed 2-fold increase in sub-G1 phase in the NPY-treated culture as compared with the control. In addition, SK-N-MC cell death induced by prolonged culture in serum-free conditions (72 hours) was further augmented by the selective Y1 and Y5R agonist, Leu^{31}Pro^{34}PYY, whereas Y5 and Y1R antagonists dramatically increased cell survival (Fig. 3B), which suggested that NPY may be apoptotic for these cells.

To test this hypothesis, SK-N-MC cells were treated with 10^{-7} \text{ mol/L} NPY or selective receptor agonists in 10% serum media for 24 hours and apoptosis was measured by TUNEL assay. NPY caused a 5-fold increase in the percent of TUNEL-positive cells (Fig. 3B). The effect of NPY was mimicked by the selective Y1 and Y5 agonist, Leu^{31}Pro^{34}PYY, whereas the Y5 agonist, cM10, increased apoptosis levels up to 2-fold. NPY had no effect on the apoptotic rate of SK-N-BE(2) cells (data not shown).

The apoptotic effect of NPY in SK-N-MC cells was confirmed by caspase 3/7 activity assay. After 24 hours, 10^{-7} \text{ mol/L} NPY increased activity of the caspases up to 40% above the control (Fig. 3B).

Figure 1. Expression and release of NPY in neural crest-derived tumors. NPY mRNA was detected by RT-PCR in three tumor cell lines: neuroblastoma (SK-N-BE(2)), ESFT (SK-N-MC), and pheochromocytoma (PC12). The levels of NPY (intracellular, membrane bound, and released to the culture medium (ELISA)) are presented as nanograms of the peptide per 10^6 cells. NPY expression was confirmed by immunostaining (right), whereas sympathetic origin of SK-N-BE(2) and PC12 cells by positive immunostaining for tyrosine hydroxylase. Magnification x250. B. expression of NPY in neuroblastoma (NB) and ESFT cell lines was detected by real-time RT-PCR and presented as expression relative to β-actin mRNA levels. NPY release after 24 hours culture (ELISA) is presented as fold increase in the peptide levels, as compared with the control culture media (per 10^6 cells). C. HMVECs were treated with SK-N-BE(2)- and SK-N-MC–conditioned media or growth factors: NPY or basic fibroblast growth factor (bFGF), with or without a mixture of NPY receptor antagonists (Y1, Y2, and Y5; 10^{-7} \text{ mol/L each}). Proliferation levels were measured by MTS assay and results presented as percent of the low-serum control. Columns, means from three separate experiments, six wells per treatment each; bars, SE. *, statistically significant difference as compared with NPY receptor antagonists–treated cells (P < 0.05).
Because both Y1 and Y5Rs seemed necessary for the apoptotic actions of NPY in SK-N-MC cells, we tested the possibility that DPPIV may prevent this effect by cleaving the full-length NPY(1-36) to NPY(3-36), which is inactive at Y1R. The DPPIV inhibitor, P32/98, increased NPY-induced activation of caspases 3/7 to 60% above the control and this was significantly decreased by both Y1 and Y5 antagonists (Fig. 3B).

Neuropeptide Y Exerts Opposite Effects on the Growth of Neuroblastoma and ESFT Tumors In vivo. The overall effect of NPY on the growth of neural crest–derived tumors in vivo was determined using nude mice xenograft model. SK-N-BE(2) and SK-N-MC cells were injected s.c. into nude mice in the presence of NPY (1 g/14 days) or placebo slow-release pellets. Unexpectedly, in the initial experiment, NPY implanted simultaneously with SK-N-BE(2) cells induced death of five of six mice within 2 weeks, whereas no deaths were observed in the untreated tumor-bearing group (data not shown). No metastases were detected by the standard histopathologic analysis in the lungs and livers of the dead mice. However, there were also no signs of infections; thus, the exact cause of death remains unclear. Hence, to determine the effect of NPY on SK-N-BE(2) tumor growth, the above protocol was modified by inserting the NPY pellets after the tumors reached a volume of ~0.3 mL. NPY treatment dramatically increased tumor growth (up to 16-fold increase in tumor volume as compared with 4-fold observed in the placebo group; Fig. 4A), whereas no significant difference in apoptosis was observed (Fig. 4B). The apoptotic cells were concentrated in the areas distant from blood vessels. Neuroblastoma tumors were highly vascularized, which was further increased by NPY treatment (1.9-fold increase in area of CD31-positive vessels as compared with the placebo group; Fig. 4C). Increased vascularization of NPY-treated SK-N-BE(2) tumors was associated with a change of the vessel character from large vessels...
observed in the control group to the large number of small vessels seen in the NPY group, which was visualized by Masson’s trichrome and CD31 staining (Fig. 4C). However, despite their small size, vessels present in the NPY-treated tumors were surrounded by smooth muscle α-actin–positive cells, indicating the presence of pericytes. In the control group, smooth muscle α-actin content was much lower even in the bigger vessels.

In mice bearing SK-N-MC tumors, NPY given simultaneously with the cell injections dramatically reduced tumor growth rate (Fig. 4A), which led to the lower volume and weight of tumors after 30 days of treatment (4- and 2-fold decrease, respectively). The growth inhibition was accompanied by a 20-fold increase in apoptotic cells, measured by TUNEL staining (Fig. 4B). However, similar to SK-N-BE(2) xenografts, NPY caused an increase in SK-N-MC tumor vascularization despite overall growth reduction (Fig. 4C).

**Discussion**

Neural crest–derived tumors, neuroblastoma, and ESFT are clinically challenging diseases, which, despite aggressive chemotherapy, too often lead to the death of the patients. Here, we report that NPY, previously known only as neuronal differentiation marker, plays an important role in the regulation of tumor growth. The peptide stimulates proliferation of neuroblastoma cells via Y2/ Y5Rs and p44/42 MAPK pathway and induces apoptosis in ESFT cells via Y1/Y5Rs, which is associated with caspase activation. In both cell types, selective receptor antagonists not only blocked the effects of NPY but also strongly modified basal growth levels, indicating the critical role of NPY as their autocrine growth regulator. Moreover, NPY released from the tumor cells increased proliferation of HMVECs, suggesting that the peptide also stimulates tumor vascularization in a paracrine manner.

Among the pleiotropic functions of NPY, its proliferative actions are only now being appreciated. The peptide stimulates proliferation of neuronal precursors via Y1R (20), vascular smooth muscle cells via both Y1 and Y5 (21), whereas its mitogenic effect in endothelial cells and angiogenic actions are Y2 and Y5R mediated (22, 24, 27, 28). In many cell types, both Y1- and Y2R-dependent proliferative effects of NPY are associated with activation of p44/42 MAPK (20, 31). Here, we showed that the same signaling pathway is involved in Y2/Y5-mediated mitogenic actions of the peptide in SK-N-BE(2) cells. The bimodal fashion of mitogenic response to NPY, with high and low affinity peak, which is observed in neuroblastoma cells, has been also shown for endothelial and vascular smooth muscle cells and seems to be characteristic for various actions of the peptide (21, 23). The predominant expression of the Y2R observed in all investigated neuroblastoma cell lines suggests that Y2-mediated mitogenic effect of NPY is characteristic for this type of tumor.

Neuroblastomas, derived from sympathetic neurons, synthesize and release NPY, which distinguishes them from other neural crest–derived malignancies, such as tumors of the central nervous system (primitive neuroectodermal tumors and astrocytomas; ref. 4). High NPY expression is often associated with elevated circulating levels of the peptide in patients with neuroblastoma (2, 4, 17, 32), which have been found mainly in children >1 year of age with high-grade neuroblastomas (stage III and IV), in association with poor clinical outcome and MYCN amplification (7, 33, 34). These high-grade tumors are also very highly vascularized because, in neuroblastoma, the degree of vascularization directly correlates with advanced stage and metastases (10–12). The findings of the present study suggest that NPY released from neuroblastomas enhances their growth by stimulation of both tumor cell proliferation and angiogenesis. Interestingly, brain-derived neurotrophic factor, which is an important growth-stimulator for aggressive neuroblastomas, enhancing tumor cell survival, invasiveness, and angiogenesis in an autocrine fashion (35–37), also stimulates NPY expression (38). Thus, we postulate that in aggressive neuroblastomas, NPY mediates the growth-stimulatory actions of brain-derived neurotrophic factor. Interestingly, both NPY-induced angiogenesis and its mitogenic effect in neuroblastoma cells are mediated by the same Y2R, which suggests that blocking the Y2 pathway could be a potential, bidirectional treatment for these diseases (Fig. 5). Therapeutic strategies targeting both angiogenesis and tumor cell growth by using low doses of cytostatics combined with angiostatic agents have been already proven to be effective in neuroblastomas (39). Targeting NPY-mediated angiogenesis in childhood malignancies is further supported by the fact that the peptide does not seem to be involved in developmental angiogenesis because both Y2 and NPY knockout mice have no defects in organogenesis, despite severely impaired NPY-induced angiogenesis in ischemia and retinopathy (22, 27, 28).

ESFT cells, which are believed to be of parasympathetic origin (1, 13), release significantly less NPY than the sympathetic neuroblastomas. Despite this, NPY is a crucial angiogenic factor for ESFT tumors because the proliferative effect of SK-N-MC–conditioned media on HMVEC was significantly, although not completely, reduced by NPY receptor antagonists. This partial blockage may be due to the presence of other growth factors, such as insulin-like growth factor I, which is secreted from ESFT, but not neuroblastoma cells (40). Although the NPY secretion per 10^6 of SK-N-MC cells was much lower than in SK-N-BE(2), due to higher proliferation rate and cell number, the SK-N-MC–conditioned

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**Table 1. NPY receptor expression in neuroblastoma and ESFT cell lines**

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<thead>
<tr>
<th>Neuroblastoma cell lines</th>
<th>Y1R</th>
<th>Y2R</th>
<th>Y5R</th>
<th>DPP1IV</th>
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<th>Y2R</th>
<th>Y5R</th>
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**NOTE:** +, expression level above 10^6 of the β-actin mRNA levels; +/−, expression level below 10^6 of the β-actin mRNA levels; −, no expression detectable.
media contained nanomolar concentration of the peptide, well within the range of endothelial cell responsiveness to NPY (10^{-12} to 10^{-8} mol/L; refs. 23, 24). In vivo, no difference in area of CD31-positive vessels were found between SK-N-BE(2) and SK-N-MC tumors, despite hemorrhagic appearance of neuroblastoma xenografts. Thus, hemorrhage observed in neuroblastomas may be due to increased permeability of the vessels caused by imbalance between factors promoting vascular instability, such as vascular endothelial growth factor, and maturation (e.g., angiopoietin 1). Weaker smooth muscle α-actin staining of vessels in the placebo-treated neuroblastomas suggesting more immature character, as compared with those in the NPY-treated tumors, supports this hypothesis. The angiogenic activity of NPY is known to be in part mediated by vascular endothelial growth factor (22). However, the

Figure 3. Mechanisms of NPY growth-regulatory actions. A. NPY stimulates proliferation of SK-N-BE(2) via p44/42 MAPK activation. Left, cell cycle analysis. SK-N-BE(2) cells were serum starved for 24 hours, treated with NPY at 10^{-7} mol/L for 24 hours, and evaluated by flow cytometry. NPY significantly increased percent of cells in S phase. Representative of three separate experiments. Right, activated p44/42 MAPK detected with phospho-p44/42 antibody normalized to β-actin levels (Western blot). Serum starved SK-N-BE(2) cells were treated with NPY at 10^{-7} mol/L for desired periods. Selective receptor antagonists at 10^{-6} mol/L were added 15 minutes before 5 minutes stimulation with NPY at 10^{-7} mol/L. The results are presented as fold increase in phosphorylated protein levels as compared with the nonstimulated control. Columns, mean from three separate experiments; bars, SE. * statistically significant change as compared with the control (P < 0.05). B. NPY-induced apoptosis in SK-N-MC cells. Top left, cell cycle analysis. SK-N-MC cells were serum starved for 24 hours, treated with NPY at 10^{-7} mol/L, and evaluated by flow cytometry. No significant change in S fraction was detected. Representative of three separate experiments. Top right, cell survival (MTS assay). Serum deprivation for 72 hours induced cell death, which was augmented by Y1 and Y5R agonist (10^{-7} mol/L) and decreased by Y1 and Y5R antagonists (10^{-6} mol/L each). Bottom left, apoptosis (TUNEL assay). After 24 hours treatment in 10% serum-containing media, NPY or selective Y1/Y5 or Y5 agonists (10^{-7} mol/L each) stimulated the number of the apoptotic TUNEL-positive nuclei (fold increase in percent of control). Columns, mean three experiments, six microscopic fields each; bars, SE. Bottom right, caspase 3/7 activation confirmed the apoptotic activity of NPY; this effect was augmented by DPPIV inhibitor P32/98 (10^{-5} mol/L) and blocked by both Y1 and Y5R antagonists (10^{-6} mol/L). Columns, mean percent of control from three separate experiments, three wells per treatment each; bars, SE.
peptide is also strongly mitogenic for vascular smooth muscle cells (21, 31). Thus, the final angiogenic effect of NPY and character of new vessels may be tumor specific, with neuroblastomas favoring increased permeability, possibly due to vascular endothelial growth factor activity. Interestingly, additional treatment of neuroblastomas with NPY apparently changed the balance between tumor-derived factors and augmented the proliferative effect of the peptide on vascular smooth muscle cells and possibly pericytes, giving the vessels the appearance of being more mature.

In contrast to Y2-expressing neuroblastomas, ESFT cells express high levels of Y1 and Y5Rs, which may be a functional method to distinguish these two types of tumors. Although Y1Rs have been extensively studied in ESFT cell lines (3, 41), sparse and conflicting results have been reported on the role of NPY in their growth (42, 43). Our study strongly supports the inhibitory role of autocrine NPY in ESFT cells. We have found that endogenous NPY stimulates apoptosis in SK-N-MC cells, whereas Y1/Y5R antagonists increase cell survival. Similar growth-inhibitory activities have been found for other NPY peptide family members, such as peptide YY, which inhibits proliferation of breast, prostate, and pancreatic cancer cells (44–46).

Interestingly, despite having this deadly autocrine loop, SK-N-MC cells retain a high proliferation rate. ESFT cells may be protected from NPY-induced cell death by several mechanisms, including a low release of the peptide and secretion of other growth-promoting factors, such as insulin-like growth factor I (40). However, our data implicate DPPIV as a key player in this

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**Figure 4.** Effect of NPY on neuroblastoma and ESFT tumor growth in vivo. A. Tumor growth rate. SK-N-BE(2) cells (5 × 10⁶) were injected s.c. into nude mice. After the tumors reached a size of ~0.3 mL, slow-release pellets with either placebo or NPY (1 μg/14 days) were inserted <1 cm from the tumors. NPY significantly increased tumor growth rate as compared with the placebo-treated group. In SK-N-MC tumors, NPY slow-release pellet given at the time of s.c. cell injections (5 × 10⁶ cells) reduced tumor growth rate within 30 days. In both experiments, the tumors were measured periodically and the volume was calculated based on the formula: 0.44 × length × width². The results are expressed as mean ± SE of fold increase of the initial tumor volume (on the day of pellet insertion for SK-N-BE(2) tumors or when a measurable tumor was first detected for SK-N-MC). B. Apoptosis (TUNEL staining). In SK-N-BE(2) xenografts, no significant difference in the apoptotic rate between placebo and NPY group was observed, whereas in SK-N-MC xenografts, NPY caused 20-fold increase in apoptosis measured by the density of TUNEL-positive cells. The photograph shows an area of high apoptosis in NPY-treated tumors, Magnification ×600. C. Tumor vascularization. In both SK-N-BE(2) and SK-N-MC tumors, NPY treatment increased tumor vascularization, which was visualized by Masson’s trichrome staining (top) and immunostaining with CD31 antibody (gray, middle) and measured as area of CD31-positive vessels. * statistically significant difference between NPY and placebo group, n = 6 (P < 0.05). Arrows, blood vessels. Magnification ×250. Bottom, costaining for CD31 (blue) and smooth muscle α-actin (SMA, red). Despite small size, CD31-positive vessels in the NPY-treated xenografts are surrounded by area of strong smooth muscle α-actin staining, whereas in control group the vessels consist mainly from endothelial cells. Magnification ×600.
process. This membrane protease accompanies Y1Rs in all investigated ESFT cell lines. The enzyme cleaves the full-length NPY1-36 to its shorter form, NPY3-36, which is inactive at Y1Rs, but retains a high affinity to all other receptors (26). Our data suggest that both Y1 and Y5Rs need to be activated to inhibit growth of ESFT cells. Thus, DPPIV, by converting NPY to a non-Y1 agonist, may prevent its apoptotic actions (Fig. 5). Indeed, DPPIV inhibitor augmented NPY-induced caspase 3/7 activation. In addition, by cleaving NPY, the protease shifts its activity from Y1-mediated to those dependent of other receptors, such as Y2-mediated angiogenesis.

In PC12 pheochromocytoma cells, which expressed all three NPY receptors, the peptide activity seems to be in between that found in SB-N-BE(2) and SK-N-MC cells. Whereas NPY had no direct effect on proliferation, selective receptor antagonists increased the number of viable cells, suggesting that NPY may, similar to SK-N-MC cells, be growth inhibitory. The lack of exogenous NPY effect can be explained by saturation of the Y1R with the endogenous peptide suggested by high levels of these receptors and membrane-bound NPY. The clinical reports regarding the role of NPY in pheochromocytomas are contro-versial. High levels of NPY mRNA have been found in benign tumors, whereas its plasma levels are elevated in patients with malignant pheochromocytomas (16, 17). The latter raises the possibility that even if NPY does not promote cell proliferation directly, it may facilitate tumor growth and metastases indirectly by stimulating angiogenesis. This hypothesis is supported by the fact that nerve growth factor, known to stimulate NPY expression in PC12 cells, increases vascularization of PC12 xenografts via a vascular endothelial growth factor pathway, which is also a mediator of NPY-induced angiogenesis (22, 47).

In summary, this is the first report of the differential and multifaceted effects of NPY on growth of neural crest–derived tumors. We found that NPY both regulates proliferation of neuroendocrine tumor cells in an autocrine manner and induces tumor vascularization via its Y2 receptors. The overall growth-regulatory effect of the peptide is critically dependent on tumor type and its receptor expression pattern. Thus, NPY and its receptors may become targets for novel, bidirectional therapies in the treatment of neuroendocrine tumors, directed against both tumor cell proliferation and angiogenesis.

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