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International Journal of Cancer

Neuropeptide Y stimulates proliferation and migration in the 4T1 breast cancer cell line

Philip J. Medeiros[1], Baraa K. Al-Khazraji[1], Nicole M. Novielli[1], Lynne M. Postovit[2], Ann F. Chambers[1,3,4,5] and Dwayne N. Jackson[1,6]

[1] Department of Medical Biophysics, The University of Western Ontario, London, Ontario Canada
[2] Department of Anatomy and Cell Biology, The University of Western Ontario, London, Ontario, Canada
[3] London Regional Cancer Program, London, Ontario, Canada
[4] Department of Pathology, The University of Western Ontario, London, Ontario, Canada
[5] Department of Oncology, The University of Western Ontario, London, Ontario, Canada
[6] Department of Biomedical Engineering, The University of Western Ontario, London, Ontario Canada

Stress has long been thought of to be associated with increased risk of cancer. Chronic stress is associated with elevated levels of sympathetic neurotransmitter (norepinephrine and neuropeptide Y: NPY) release and immunosuppression. The expression of NPY receptors has been reported in human breast carcinomas. Recently, activation of the NPY Y5 receptor was shown to stimulate cell growth and increase migration in human breast cancer cells; however the effects of NPY have yet to be investigated in a murine model of breast cancer. Thus, the specific aims of the current study were to: (*i*) characterize NPY receptor expression in 4T1 breast cancer cells and orthotopic tumors grown in BALB/c mice and (*ii*) investigate the impact of NPY receptor activation on 4T1 cell proliferation and migration *in vitro*. Positive expression of NPY receptors (Y1R, Y2R and Y5R) was observed in cells and tumor tissue. As well, NPY treatment of 4T1 cells promoted a concentration-dependent increase in proliferation, through increased phosphorylation of ERK 1/2. Using NPY receptor antagonists (Y1R:BIBP3226, Y2R:BIIE0246 and Y5R:L-152,804), we found the proliferative response to be Y5R mediated. Additionally, NPY increased chemotaxis through Y2R and Y5R activation. These data are in congruence with those from human cell lines and highlight the 4T1 cell line as a translatable model of breast cancer in which the effects of NPY can be studied in an immunocompetent system.

Recent studies have demonstrated that stress is correlated with increased breast cancer risk. Chronic stress leads to elevated sympathetic neurotransmitter release (norepinephrine and neuropeptide Y: NPY) which is associated with immunosuppression, and has been positively correlated with increased incidences of infection and cancer.[1–5] Sympathetic nerves arising from the lateral and anterior cutaneous branches of the second to sixth intercostal nerves abundantly innervate the human breast, thereby providing a constant supply of sympathetic ligands to the breast microenvironment. Interestingly, women with family histories of breast cancer are reported to have greater sympathetic neurotransmitter release under normal conditions.[6] As such, the pathological contributions of these neurotransmitters to breast cancer development and progression have been a growing area of investigation.

In mammalian systems, NPY is the most abundantly expressed peptide of the pancreatic polypeptide family, exhibiting pleiotropic actions throughout the body. The 36-amino acid peptide is most widely expressed in the central and peripheral nervous systems, playing major roles in cognitive function, feeding behavior and cardiovascular regulation. NPY activates a group of six NPY G protein-coupled receptors (Y1R–Y6R) of which the Y1R, Y2R and Y5R are most abundantly expressed in humans, and have been most extensively studied.[7] In addition to regulating several physiological functions, NPY has been shown to promote proliferation, vascularization and stimulate migration in several cell types and tissues.[8–11]

NPY Y1R, Y2R and Y5R expression has been reported in several breast cancer cell lines.[12,13] In human primary breast carcinomas, 85% exhibited Y1R expression, while only 24%

Key words: Neuropeptide Y, 4T1 mammary carcinoma, breast cancer, Y1 receptor, Y2 receptor, Y5 receptor, chemotaxis
Additional Supporting Information may be found in the online version of this article.
Grant sponsors: Natural Sciences and Engineering Research Council, the Canadian Institutes of Health Research, Canadian Breast Cancer Research Alliance, Academic Development Fund from the University of Western Ontario, Canadian Breast Cancer Foundation, Translational Breast Cancer Research Unit of the London Regional Cancer Program, Canada Research Chairs Program
DOI: 10.1002/ijc.26350
History: Received 18 Nov 2010; Accepted 26 Jul 2011; Online 5 Aug 2011
Correspondence to: Dwayne N. Jackson, Ph.D., Department of Medical Biophysics, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, Ontario, Canada N6A 5C1, Tel.: +519-661-2111, E-mail: dwayne.jackson@schulich.uwo.ca

expressed the Y2R subtype.[12] Recently, Y5R expression was found in the MCF-7, T47D, MDA MB-231, MDA MB-468, HS578T and BT-549 cell lines.[13] Activation of Y5R in BT-549 cells was shown to stimulate cell growth through increased mitogen-activated protein kinase (MAPK) activity and concomitant with increased extracellular signal-regulated kinase (ERK 1/2) phosphorylation.[13] In contrast, Y1R activation in MCF-7 cells has been reported to inhibit estrogen-induced proliferation.[14] The Y2R has also been shown to increase cellular proliferation, however current studies have been limited to neuroendocrine tumors, which highly express the receptor.[15,16]

The contribution of NPY to the development of metastasis has been a question of increasing importance. Neuropeptides have been reported to exhibit chemotactic potency and stimulate migratory activity of immune,[17] endothelial[18] and breast cancer cells.[13,19] Substance P and bombesin have been observed to increase migratory activity in MDA MB-468 cells[19] and more recently NPY stimulation was reported to increase the migration of MDA MB-231 cells through Y5R activation.[13]

The 4T1 murine mammary cancer cell line is a well-established model of breast cancer. It has been used extensively to study anti-metastatic and anti-angiogenic therapies,[20,21] however the effects of NPY on this breast cancer model have yet to be examined. Therefore, in the current study, using qPCR, Western blot and immunohistochemical analyses we examined the expression of Y1R, Y2R and Y5R in 4T1 cells *in vitro* and orthotopic tumors. Positive expression for each of the three receptors led our group to test whether NPY would stimulate proliferation and/or migration in this model. Using NPY receptor antagonists, we provide evidence of Y5R mediated proliferation and Y2R and Y5R mediated chemoattraction to NPY treatment. These data complement and parallel recent findings in human cell lines, and highlight the 4T1 cell line as a breast cancer model in which the effects of NPY can be studied in an immuno-intact *in vivo* model.

Material and Methods

Reagents and drugs

Fetal bovine serum (FBS), culture media, Hank's Balanced Salt Solution (HBSS) and trypsin were purchased from Invitrogen (Invitrogen Canada Inc., Burlington, ON, Canada). RNA isolation was performed using PureZol© (Aurum Total RNA Mini Kit, Bio-Rad Laboratories, Hercules, CA) and cDNA was synthesized using a RT^2 First-Strand cDNA synthesis kit (QIAGEN Inc, Mississauga ON, Canada). Primer pairs corresponding to target genes for mouse *NPY Y1R* (Cat no. PPM042483E), *NPY Y2R* (Cat no. PPM04278A), *NPY Y5R* (Cat no. PPM04629A) and *GAPDH* (Cat no. PPM02946E) were purchased from QIAGEN (Mississauga, ON, Canada).

Cell lysis buffer (M-PER, Mammalian Protein Extraction Reagent), protease and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail) were purchased from Pierce (Rockford, IL). For immunohistochemistry, immunocytochemistry and Western blot experiments, we used primary antibodies specific to mouse NPY Y1R, Y2R, Y5R (Cat no. NPY1R, Cat. no. ab73897, NPY2R, Cat. no. ab31894, NPY5R, Cat. no. ab43824, Abcam Inc., Cambridge, MA), phospho-ERK 1/2 and total ERK 1/2 (Cell Signaling Technology, Inc.). For immunohistochemistry secondary incubation, we used biotinylated goat anti-rabbit IgG (H+L) affinity purified secondary (Cat. no. BA-1000, Vector Laboratories Inc., Burlingame, CA). For Western blots, we used secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit IgG, Cat no. A0545, Sigma Aldrich, St. Louis, MO, USA). NPY (non-specific YR agonist), peptide YY (3–36) (PYY_{3-36}; Y2R/Y5R agonist), [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (Y5R agonist), BIBP3226 (Y1R antagonist), BIIE0246 (Y2R antagonist) and L-152,804 (Y5R antagonist), were from Tocris (MO). Cell proliferation was measured using a tetrazolium-based assay from Promega (CellTiter 96® AQ_{ueous} One Solution, Madison, WI).

Cell culture

4T1 cells, a gift from Dr. Fred Miller (Wayne State University, MI), were cultivated in high glucose Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% sterile FBS. Cells were incubated at 37°C and 5% carbon dioxide. At approximately 80% confluency, cells were washed with HBSS and passaged using 0.25% trypsin-EDTA treatment for dissociation.

Quantitative RT-PCR

Total RNA was isolated from $\sim 5 \times 10^6$ 4T1 cells using PureZol© following manufacturer's protocols. Five micrograms of RNA was reverse-transcribed in a 25μl reaction. Primer pairs were used to amplify the target genes for mouse *NPY Y1R, NPY Y2R, NPY Y5R* and *GAPDH*.

Relative quantification of target mRNA was performed using SYBR® green quantitative RT-PCR detection (CFX 96 real-time PCR detection system, Bio-Rad, Hercules, CA). Twenty-five microliter reactions were conducted with equal amounts of cDNA templates from each sample (triplicate) according to the manufacturer's protocol. The protocol was: 95°C for 10 min, 40 cycles at 95°C for 15 sec, 60°C for 60 sec. At the end of the protocol, a stepwise increase in temperature from 65°C to 95°C was performed and dissociation curves were constructed to ensure purity of PCR products. Data were analyzed with CFX manager software (Bio-Rad, Hercules, CA). Target gene expression was quantified by measuring threshold cycle. Experiments were repeated three times.

Orthotopic tumor model

Seven female BALB/c mice, purchased from Charles River Laboratories (Saint-Constant, QC, Canada) at 7–9 weeks of age, were used in the current study. Animals were housed in the animal care facility at The University of Western Ontario.

They were maintained on Harlan 2018, Teklad Global 18% Protein Rodent Diet and water *ad libitum* and kept on a 12 hr light/dark cycle. All experimental procedures were carried out with the approval of the Council on Animal Care at The University of Western Ontario.

After three passages, 4T1 cells were prepared for injection. Cells were washed and resuspended in sterile HBSS. The solution was filtered with a 40-μm cell strainer and cell viability was assessed by trypan blue staining and counting with a hemocytometer. The cell suspension was diluted to 10^3 cells/μl for injection.

Animals were anesthetized with isofluorine/oxygen gas mask (2–3% isofluorine, 1 l/min) and hair was removed from the lower abdominal region using commercial hair remover (Nair®). A sagittal cut (∼1 cm) was made at the lower abdomen and skin was separated from the underlying muscle, to expose the underlying mammary fat pad. One hundred microliters (10^5 cells) of cell suspension was carefully injected into the right inguinal mammary fat pad. Wound clips were then applied to close the skin incision. Animals were returned to their cage for recovery and wound clips were removed 7 days later. Animals were then housed in cages without disturbance for 29 days. Twenty-nine days post-injection, all mice were sacrificed after induction of anesthesia using ketamine (100 mg/kg) and xylazine (25 mg/kg) followed by cervical dislocation. Mammary tumors were removed from each animal, sectioned in half, one half flash frozen in liquid nitrogen and the other fixed in 4% paraformaldehyde.

Western blot analysis

Cultured cells from a 25 cm^2 flask were washed twice in ice cold HBSS and then lysed in 500 μl lysis buffer containing protease (104 mM AEBSF, 80 μM aprotinin, 2.1 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A and 1.4 mM E-64) and phosphatase (Halt Phosphatase Inhibitor Cocktail) inhibitors. Cells were scraped and then lysed by sonication. One hundred milligrams of tumor tissue was immersed in ice-cold lysis buffer (50 mM Tris, 150 mM sodium chloride, 5 mM ethylene glycol tetraacetic acid, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1% Triton-X 100, pH = 7.5) and mechanically homogenized. Cell and tissue lysates were centrifuged for 15 min at 14,000 rpm at 4°C. Supernatant was collected and stored at −80°C until protein concentration was determined.

A Bradford assay[22] was performed to determine total protein concentration of samples. Fifty micrograms of protein from each sample and a positive control (mouse brain lysate) were loaded on a 4–12% gradient gel and separated by SDS-PAGE. After electrophoresis, proteins were transferred at a constant voltage to polyvinylidene fluoride membranes. Membranes were blocked for 1 hr in 5% milk in Tris-Buffered Saline + Tween-20 (0.5%) (TTBS) at 4°C. Membranes were then incubated in primary antibody specific to mouse NPY Y1R, Y2R, and Y5R in 5% milk TTBS at a concentration of 1:1,000 at 4°C overnight. Membranes were washed in TTBS then incubated in secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit IgG, 1:2,000) in 2% milk in TTBS for 1 hr. Membranes were washed three times and bands were detected using an Immun-Star WesternC© chemiluminescent kit (Bio-Rad, Hercules, CA) and imaged with the ChemiDoc XRS System (Bio-Rad, Hercules, CA). Membranes were washed, stained for total protein using Amido black, and imaged using the ChemiDoc XRS. Densitometric band analysis was performed with Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA).

Immunocytochemistry and immunohistochemistry

Cells (10^5) were grown on sterile glass coverslips for 48 hr, then fixed in 4% paraformaldehyde for 5 min at room temperature. Cells were washed in phosphate-buffered saline (PBS; 2.68 mM KCl, 1.46 mM KH_2PO_4, 137 mM NaCl and 6.48 mM Na_2HPO_4), and permeabilized in 0.25% (v/v) Triton-X 100 (in PBS) for 20 min at room temperature followed by PBS washes. Following 10 min blocking in 1.5% normal goat serum (in PBS), excess blocking reagent was removed and replaced with primary antibody specific to NPY Y1R, Y2R or Y5R ([Y1R]: 2μg/ml, [Y2R]: 2μg/ml, [Y5R]: 5μg/ml) for 1 hr at room temperature. Cells were washed, incubated in secondary antibody (biotinylated goat anti-rabbit secondary, 4 μg/ml in 0.1% blocking serum in PBS) for 30 min at room temperature, washed and incubated in 1:1 avidin:biotin complex (ABC) (Vectastain Elite ABC® Kit (Standard), Cat. no. PK-6100, Vector Laboratories Inc., Burlingame, CA) solution for 30 min. After washing, cells incubated in 3,3′-diaminobenzidine (DAB Peroxidase Substrate Kit, 3,3′-diaminobenzidine, Cat. no. SK-4100, Vector Laboratories Inc., Burlingame, CA) for 8 minutes, were washed in ddH_2O, counterstained in Harris' hematoxylin, dehydrated, cleared and mounted.

Tumors were immersed in 4% paraformaldehyde solution in PBS, followed by paraffin processing and tissue embedding. Tumor tissues were sectioned (5 μm thickness) and sequentially placed in triplicate on glass slides in staggered formation (with a non-specific binding control section on each slide). Following deparaffinization, tissues were placed in antigen retrieval for 30 min at 75°C (10 mM sodium citrate buffer, 0.05% Tween-20, pH = 6.0), washed in PBS, and blocked and permeabilized for 1 hr in blocking solution (1.5% normal goat serum, 0.2% Triton-X 100 (in PBS)) at room temperature. Endogenous biotin-binding proteins were blocked with Avidin D for 15 min, washed, then incubated in Biotin solution for 15 min at room temperature (Avidin/Biotin Blocking Kit, Cat. no. SP-2001, Vector Laboratories Inc., Burlingame, CA). Following overnight incubation in primary antibody specific to Y1R, Y2R and Y5R (same concentrations as above) sections were washed in PBS, and incubated in biotinylated goat anti-

rabbit secondary antibody (4 μg/ml in 0.1% blocking serum in PBS) for 2 hr at room temperature. Slides were washed in PBS and incubated for 40 min in 1:1 avidin:biotin-complex followed by a PBS wash. Slides were then treated for 40 min in 4:1 avidin:biotin followed by a PBS wash, and subsequently 40 min in 1:4 avidin:biotin. Slides were treated with DAB for 8 min and washed off with ddH_2O. Slides were counterstained in Harris's hematoxylin, cleared, dehydrated and mounted.

Cell proliferation experiments

Twenty-five hundred cells were seeded per well of 96-well plates and incubated for 24 hr in DMEM supplemented with 10% FBS. After 24 hr, media was replaced with experimental medium (DMEM + 2% FBS) containing 10^{-11} to 10^{-6}M NPY (Tocris, MO). The proliferative effect of NPY was tested using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) based assay (CellTiter 96® AQ_{ueous} One Solution, Madison, WI). Proliferation was measured after 24 hr, 48 hr and 72 hr of NPY treatment. These experiments were conducted in triplicate (per concentration) and repeated at least four times. These experiments were repeated with [cPP^{1-7}, NPY^{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]-hPP.

In order to elucidate the contribution of each respective receptor to NPY-stimulated proliferation, receptor antagonist experiments were conducted. Twenty-four hours after cells were seeded, experimental media containing NPY (10^{-8}M, peak effective dose) and one of three antagonists (BIBP3226, BIIE0246, L-152,804) were added. For each drug, increasing concentrations (10^{-8}–10^{-4}M) were added to wells and cell proliferation was measured at 24 hr. These experiments were repeated in the absence of NPY to ensure antagonist treatment had no toxic or anti-proliferative effects. Drugs were reconstituted in HBSS and all treatments were compared to control cells that were incubated in experimental media containing an equal volume of HBSS as peptide, agonist or antagonist treated cells.

NPY-stimulated ERK 1/2 phosphorylation

NPY receptor activation has been shown to stimulate MAPK activity, leading to increased ERK 1/2 phosphorylation (pERK 1/2) and cell proliferation. To confirm that our NPY treatment stimulated MAPK activity in the 4T1 cell line, a time-point experiment was conducted to examine the effect of NPY treatment (10^{-8}M) on pERK 1/2. 4T1 cells (5×10^4) were seeded in 60 mm diameter tissue culture dishes in culture media and left to incubate for 24 hr. Cells were then growth-arrested for 24 hr with experimental DMEM plus 0.1% BSA. Experiments were conducted where individual dishes were treated with experimental media containing NPY (10^{-8}M), and [cPP^{1-7}, NPY^{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (10^{-9}M) for 2, 5, 15, 30 and 60 min ($n = 4$/time point). Additionally, blockade experiments were conducted where cells were treated with experimental media containing NPY or [cPP^{1-7}, NPY^{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]-hPP and one of three antagonists (BIBP3226, BIIE0246, L-152,804). After treatments, cells were washed three times in ice cold HBSS, 100 μl of lysis buffer was added to the dish and cells were scraped. Lysates were centrifuged for 15 min at 14,000 rpm at 4°C. Supernatant was collected and stored at 4°C until protein concentration was determined. Samples were then analyzed by Western blot for levels of pERK 1/2 and total ERK 1/2 expression using antibodies specific to each respective protein (rabbit anti-mouse phospho-p44/42 MAPK (pERK 1/2) 1:1,000 and rabbit anti-mouse p44/42 MAPK (ERK 1/2) 1:1,000). Experiments were repeated four times and densitometric analysis was conducted for each blot. ERK 1/2 phosphorylation was quantified as the level of pERK 1/2 normalized to total ERK 1/2.

Live/Dead® viability assay

To assess the effects of experimental drug treatments on cell viability, we used a two-color fluorescence cell viability assay (Live/Dead® Viability Kit, Molecular Probes, Eugene, OR). We repeated experimental conditions from proliferation experiments (96-well format), cells were then treated with calcein acetoxymethyl ester (1 μM) and ethidium homodimer (2 μM) according to manufacturer's protocol. Fluorescence was measured on a microplate spectrofluorimeter (calcein acetoxymethyl ester excitation filter: 485 nm; emission: 530 nm, ethidium homodimer excitation filter: 530nm; emission: 645nm). Data were expressed as a percent change of control.

Chemotaxis assays

Cell chemotaxis was assessed using a modified Boyden chamber apparatus with a 12-well plate and cell culture inserts with polyethylene terephthalate (PET) membranes and 8 μm pores (BD Biosciences). 4T1 cells (2×10^4) were plated in the upper chamber in serum-free media. To examine the chemotactic potential of NPY, PYY_{3-36} and [cPP^{1-7}, NPY^{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]-hPP, cells were exposed to a range of peptidergic-treated media (10^{-11}–10^{-6}M) placed in the bottom chamber. To examine receptor-specific effects of NPY on chemotaxis, cells were incubated separately in Y1R, Y2R or Y5R antagonists for 30 min. After incubation, cells were then added to the upper chamber in wells containing a range of Y-receptor antagonist treated serum-free media (10^{-8}–10^{-4}M), while NPY treated media (10^{-8}M, previously determined peak effective dose) was added to the bottom chamber. All experiments were repeated four times. After 24 hr of incubation, non-migrated cells were scraped from the top of the membrane with a cotton swab, migrated cells (on the bottom of the membrane) were then fixed in methanol and stained with 4′,6-diamidino-2-phenylindole (DAPI). The membranes were carefully removed, mounted on slides and imaged using fluorescence microscopy (5× magnification Zeiss Axiovert 200, Zeiss Axiocam HRc camera).

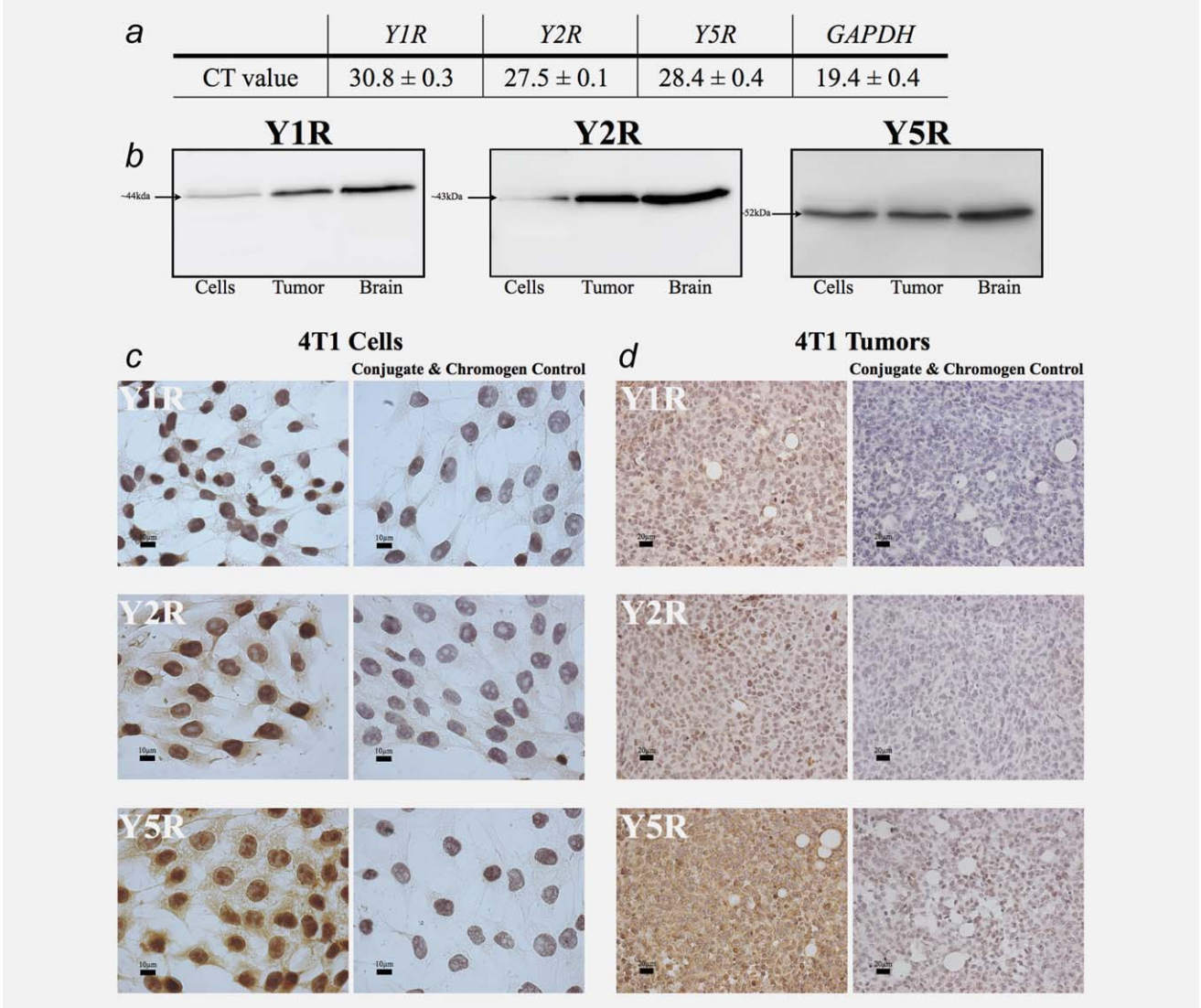

Figure 1. Y1, Y2 and Y5 receptor expression. Quantitative RT-PCR indicated NPY receptor mRNA expression in the 4T1 cell line [(*a*) CT values]. Western blot analysis of cell and tumor lysates provided evidence of Y1R, Y2R and Y5R expression in this model (brain as a postive control) (*b*). Immunohistochemical analysis (DAB and hematoxylin) of cells (*c*) and tumor sections (*d*) confirmed NPY receptor expression. 4T1 cells exhibited positive cytoplasmic and membranous staining of receptors; the Y5R subtype exhibiting the greatest expression. In tumors, staining was observed on tumor cell membranes and was also detected on blood vessel endothelium [(*c*) scale bar = 10 μm; (*d*) scale bar = 20 μm].

Migrated cells were quantified using Matlab® based software (five fields of view per condition), and was conducted by a blinded experimenter.

Statistical analyses

Statistical analysis was performed using GraphPad software (Version 4.0a, GraphPad Software Inc.), data are presented as mean ± SEM. Non-linear regression analysis was performed on proliferation and chemotaxis experiments to test whether the effects of NPY were concentration-dependent and to determine the peak effective NPY dose to be used in antagonist experiments. Statistical differences between treatments were evaluated by one-way ANOVA, followed by Tukey's HSD test. The level of statistical probability was set at $p < 0.05$.

Results

NPY receptor expression in the 4T1 cell line

Quantitative real-time PCR revealed expression of mRNA for *Y1R, Y2R* and *Y5R* in the 4T1 cell line (Fig. 1*a*). Additionally, receptor protein expression was confirmed by Western blot analysis (Fig. 1*b*). Immunocytochemical staining revealed positive Y1R, Y2R and Y5R expression in 4T1 cells (Fig. 1*c*). Membranous and cytoplasmic staining was observed, with the Y5R exhibiting the most prevalent

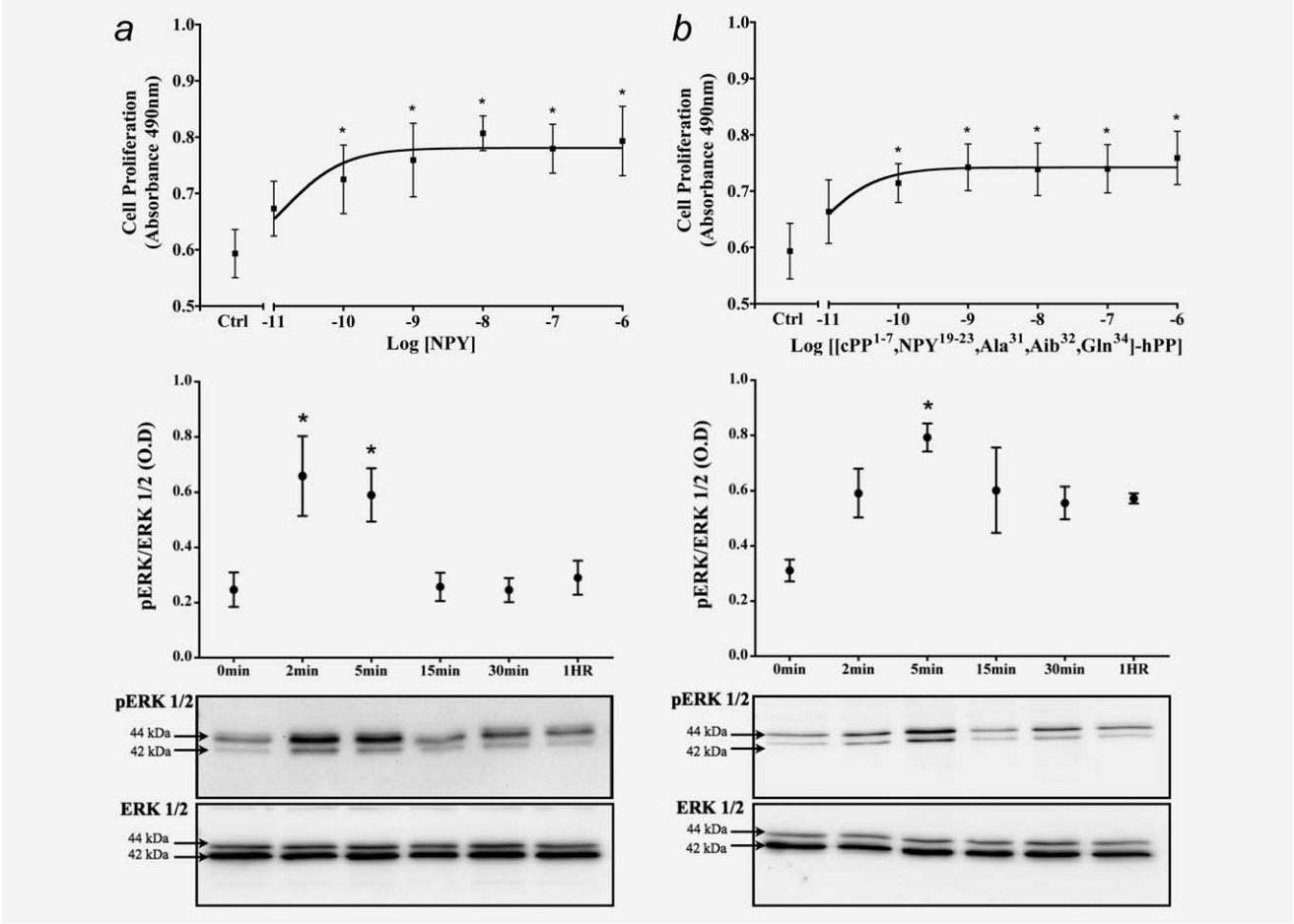

Figure 2. Effect of peptidergic treatment on proliferation and ERK1/2 phosphorylation of 4T1 cells. Proliferation (MTS assay) and ERK 1/2 phosphorylation (Western blot) were measured in cells treated with media containing NPY (*a*) and [cpp$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (*b*). A concentration-dependent increase in proliferation was observed after 24 hr of peptide treatment ($r^2 = 0.94$ and 0.97, respectively). Peptide treatment significantly increased proliferation compared to cells in experimental medium (Ctrl; DMEM + 2% FBS) ($n = 4$, $p < 0.05$). Western blot analysis was used to examine ERK 1/2 phosphorylation in cells treated with NPY (*a*) and [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (*b*) for 2 min, 5 min, 15 min, 30 min and 1 hr. Peptide treatment stimulated an increase in pERK 1/2 at 2 min (NPY) and 5 min (NPY and [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP) after which pERK 1/2 levels returned to non-treated levels (0 min). Densitometric data are presented as pERK 1/2 normalized to total ERK 1/2. Data are presented as mean ± SEM ($n = 4$ *Represents differences from Ctrl. $p < 0.05$, one-way ANOVA).

expression. Y1R, Y2R and Y5R expression was also observed in histological sections (Fig. 1*d*), receptor expression was observed on tumor cell membranes and was detected on blood vessel endothelium.

Cell proliferation experiments

NPY treatment stimulated a concentration-dependent ($r^2 = 0.94$) increase in proliferation measured at 24 hr (Fig. 2*a*), 48 hr and 72 hr (Supporting Information). When compared to control cells, a proliferative effect of NPY was observed at 10^{-10}M and was maximal at 10^{-8}M ($p < 0.05$). Similarly, treatment with the Y5R specific agonist, [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP also stimulated a concentration-dependent increase in proliferation ($r^2 = 0.97$) after 24 hr, at a concentration of 10^{-10}M and peak concentration of 10^{-9}M ($p < 0.05$, Fig. 2*b*).

Effect of NPY on phosphorylation of ERK 1/2

After 24 hr of serum-free incubation, low basal levels of pERK 1/2 were observed (0 min: Fig. 2). NPY treatment significantly increased pERK 1/2 levels after 2 min and 5 min incubations (Fig. 2*a*, $p < 0.05$), and by 15 min pERK 1/2 levels returned to basal levels. Y5R agonist, [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP treatment caused an increase in pERK 1/2 levels after 5 min ($p < 0.05$, Fig. 2*b*). Increased pERK 1/2 activity could not be blocked by BIBP3226 and BIIE0246 at concentrations from 10^{-8} to 10^{-4}M (Figs. 3*a* and 3*b*: 10^{-4}M and 10^{-5}M, respectively). NPY and Y5

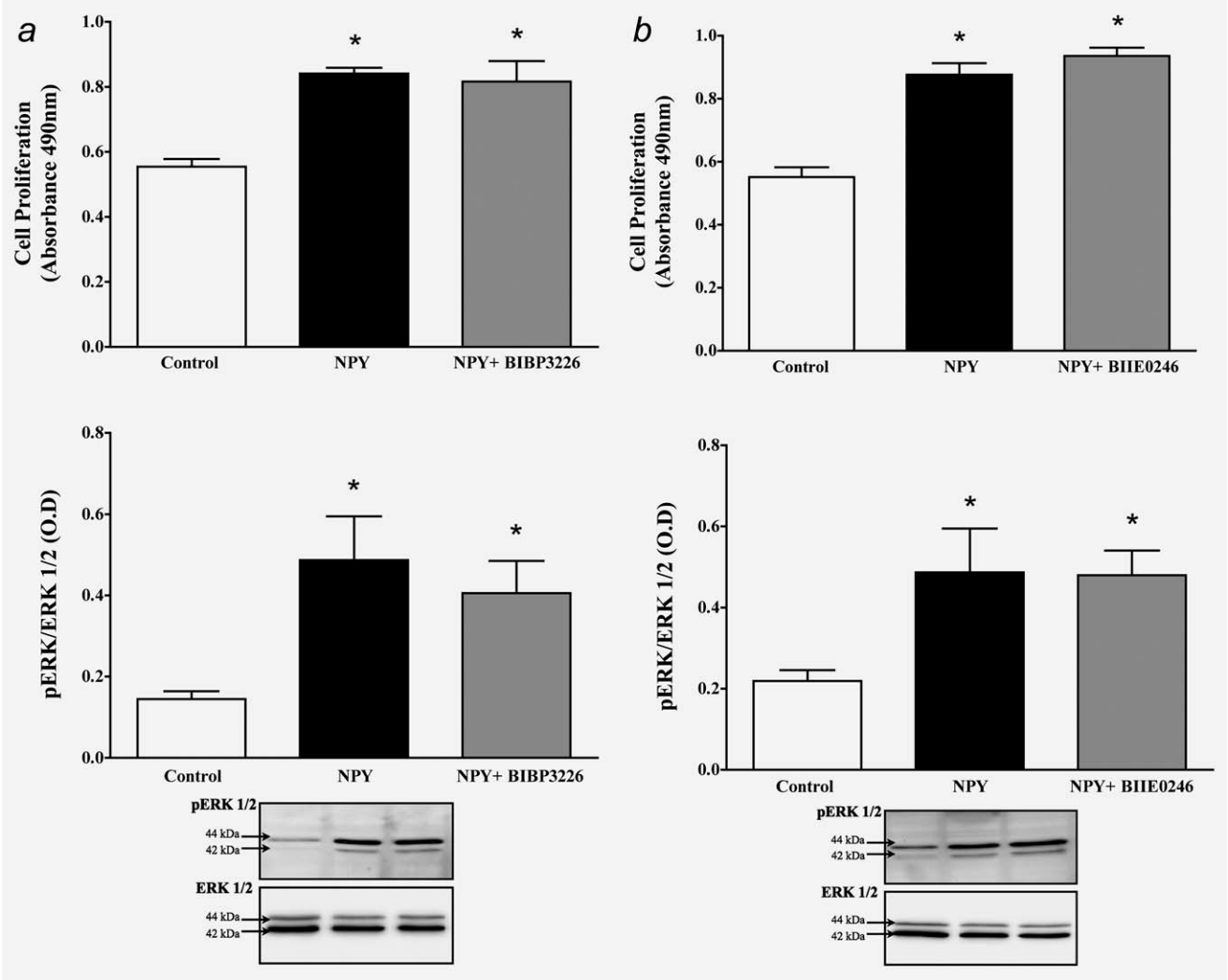

Figure 3. Effect of NPY receptor activation and blockade (Y1R, Y2R and Y5R) on cell proliferation and ERK 1/2 phosphorylation. Cells were treated with NPY (10^{-8}M) and one of three antagonists [(*a*) Y1R:BIBP3226; (*b*) Y2R:BIIE0246; (*c*) Y5R:L-152,804; 10^{-8}–10^{-4}M]. NPY-stimulated proliferation was not blocked by Y1R antagonism or Y2 antagonism (Y1R:10^{-4}M Y2R:10^{-5}M). Y5R antagonism (10^{-4}M) abolished the proliferative effects of NPY (*$p < 0.05$). Y5R agonist treatment [(*d*), [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP 10^{-9}M] caused an increase in proliferation which was blocked by L-152,804. ERK 1/2 phosphorylation increased when cells were treated with NPY and [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP; this effect was blocked by L-152,804 treatment. Panels depict peak antagonist concentrations used, all experiments were repeated four times ($n = 4$), data are presented as mean ± SEM (*Represent differences from Control $p < 0.05$, one-way repeated measures ANOVA).

agonist stimulated increased pERK 1/2 activity and was blocked by L-152,804 (10^{-4}M) ($p < 0.05$, Figs. 3*c* and 3*d*).

Effect NPY re1ceptor blockade (Y1, Y2 and Y5) on cell proliferation

The independent contributions of Y1R, Y2R and Y5R activation on proliferation were tested by receptor-specific antagonist supplementation. Cells were incubated with media containing NPY (10^{-8}M, peak proliferative concentration) and BIBP3226, BIIE0246 or L-152,804 (10^{-8}–10^{-4}M). At all time points, NPY treatment led to increased proliferation compared to vehicle containing media (Control) ($p < 0.05$, Fig. 3). Of the three antagonists tested, only the Y5R antagonist (L-152,804) inhibited the proliferative effect of NPY ($p < 0.05$, Fig. 3*c*). Compared to control, there was no change in NPY stimulated proliferation in cells exposed to Y1R antagonism (BIBP3226) (Fig. 3*a*: 10^{-4}M) or Y2R antagonism (BIIE0246) (Fig. 3*b*: 10^{-5}M). Y5R agonist treatment (10^{-9}M) also caused an increase in proliferation which was blocked by L-152,804 (Fig. 3*d*: 10^{-4}M). Control experiments conducted with each antagonist in the absence of NPY, we observed BIIE0246 to be toxic at 10^{-4}M, while no toxic or proliferative effects were observed from L-152,804 or BIBP3226 treatment at concentrations (10^{-8}–10^{-4}M) used in blockade experiments.

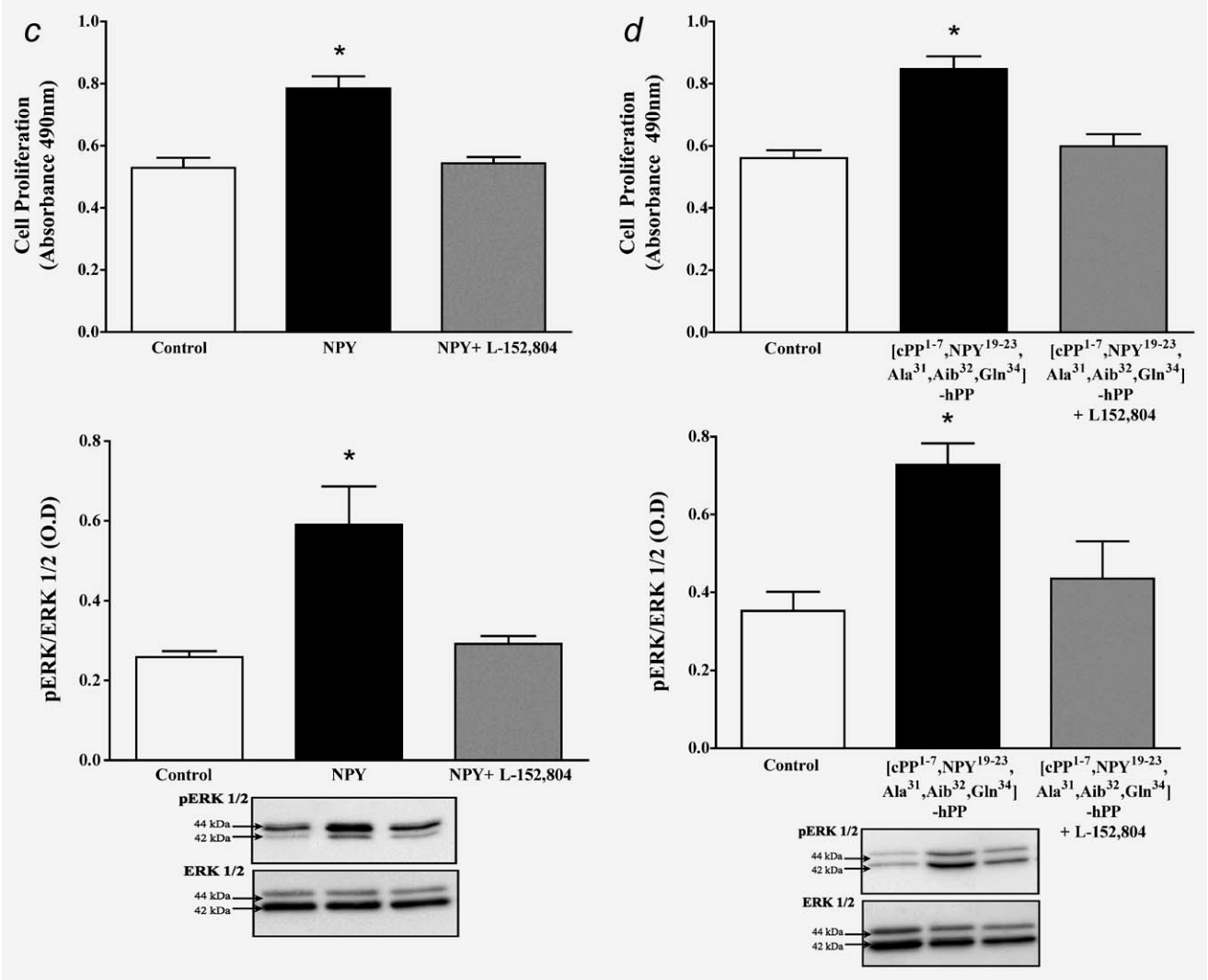

Figure 3. (Continued)

Cell viability

NPY treatment (10^{-8}M) led to an increase in the percent of live cells compared to control and had no effect on cell death ($p < 0.05$, Fig. 4). Treatment with [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (10^{-9}M) led to a similar increase in the percent of live cells. Compared to control cells, independent treatment with the receptor antagonists BIBP3226 (10^{-4}M), BIIE0246 (10^{-5}M) or L-152,804 (10^{-4}M) had no effect on cell viability or cell death.

NPY-stimulated chemotaxis

NPY induced a concentration-dependent increase in chemotaxis with a peak concentration of 10^{-8}M (Fig. 5*a*). At NPY concentrations of 10^{-9}–10^{-6}M, chemotaxis was significantly augmented compared to untreated cells (Ctrl). Similarly, PYY_{3-36} (Y2R/Y5R agonist) and Y5R agonist treatment stimulated a concentration-dependent increase in chemotaxis (Figs. 5*b* and 5*c*). Treatment of cells with Y1R antagonist (BIBP3226) had no effect on NPY-stimulated chemotaxis (Fig. 6*a*), whereas Y2R blockade (BIIE0246) attenuated NPY-mediated increases in cell chemotaxis at 10^{-6}M ($p < 0.05$, Fig. 6*b*). In addition, NPY-mediated increases in cell chemotaxis were attenuated when cells were treated with Y5R antagonist (L-152,804) at all concentrations tested (10^{-8}–10^{-4}M) ($p < 0.05$, Fig. 6*c*).

Discussion

A neuroendocrine influence on breast cancer progression has been a postulate of recent interest; in the current study, we have presented data supporting this postulate in the 4T1 breast cancer model. We report for the first time the expression of the Y1R, Y2R and Y5R in the 4T1 cell line, in culture and in primary tumors. Additionally, we observed NPY-stimulated proliferation through Y5R activation, and signaling *via* ERK 1/2 phosphorylation. Finally, we have shown that NPY is a potent chemoattractant acting through Y2R and Y5R activation.

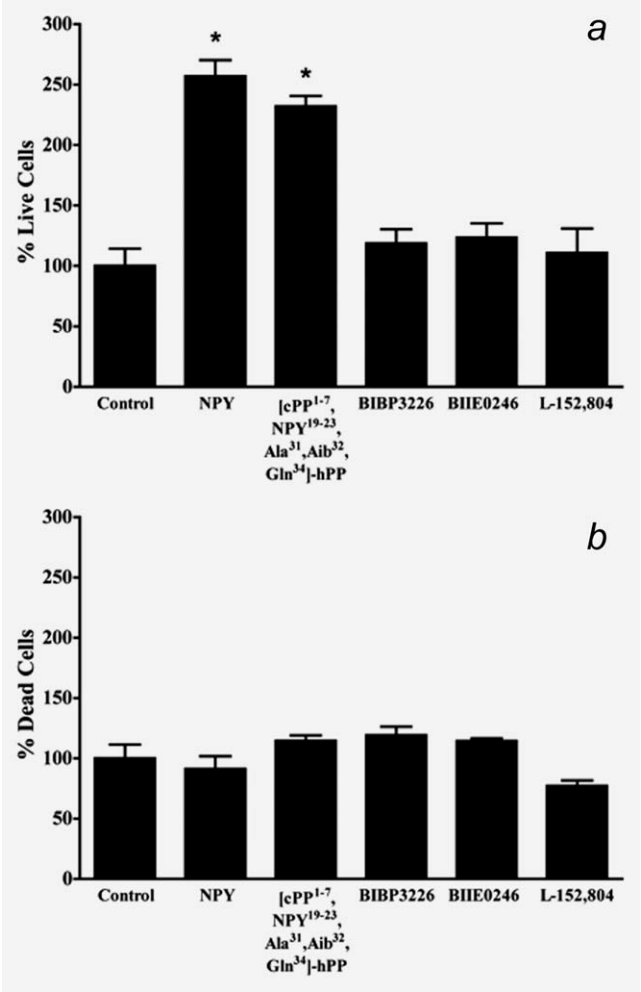

Figure 4. Effect of peptide and antagonist treatments on cell viability. The effect of experimental drug treatments (from proliferation experiments) on cell viability was assessed with a Live/Dead® viability assay. Cells were treated for 24 hr with NPY (10^{-8}M), [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (Y5R agonist; 10^{-9}M), BIBP3226 (10^{-4}M), BIIE0246 (10^{-5}M) or L-152,804 (10^{-4}M). Calcein (Live, emission: 530 nm) and ethidium (Dead, emission: 645 nm) fluorescence was measured and compared to control cells (DMEM + 2% FBS). NPY and Y5R agonist treatment led to an increase in the percent of live cells and there was no effect of these drug treatments on cell death. Data are expressed as a percent change of control (mean ± SEM; $n = 6$; *Represents differences from Control $p < 0.05$, one-way ANOVA).

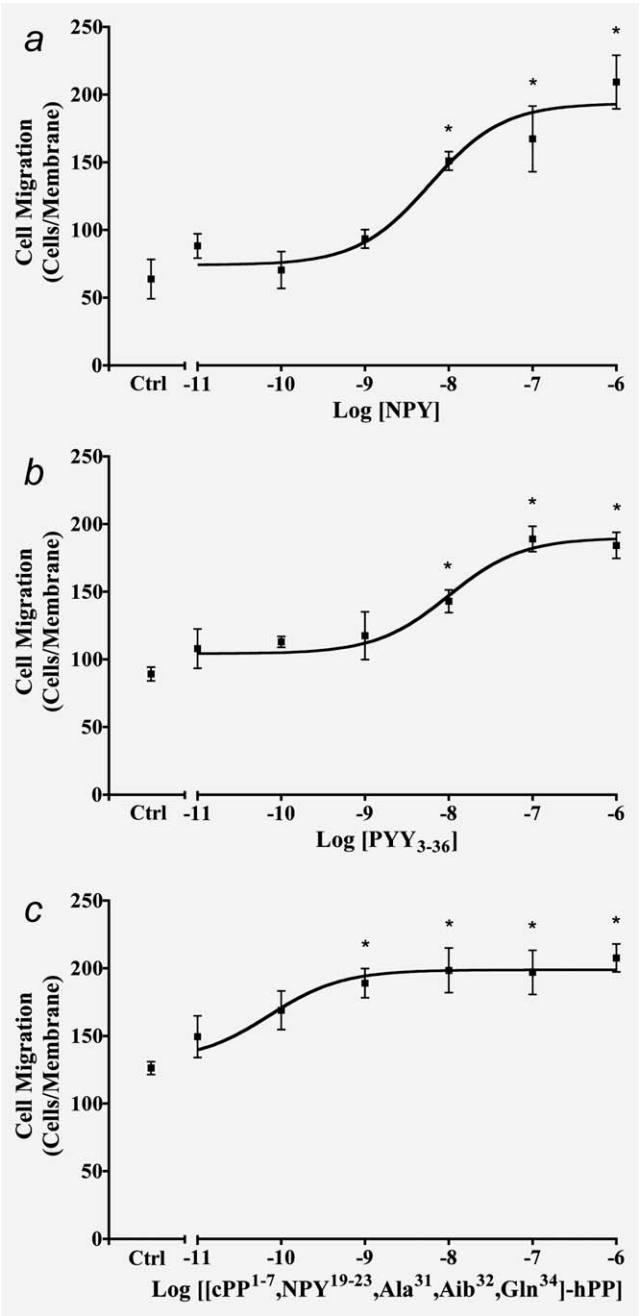

Figure 5. Peptidergic stimulated chemotaxis of 4T1 cells. Cell chemotaxis was assessed using a modified Boyden chamber. Cells were seeded (2×10^4 cells) in serum-free media in the top chamber and were exposed to (*a*) NPY, (*b*) PYY$_{3-36}$ (Y2R/Y5R agonist), (*c*) [cPP$^{1-7}$, NPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (Y5R agonist) (10^{-11}–10^{-6}M) in DMEM in the lower chamber. After 24 hr migrated cells were fixed, stained with DAPI and imaged. Cells were counted by Matlab® based software. Peptide treatment elicited a concentration-dependent increase in chemotaxis compared to control cells. Data are presented as mean ± SEM, ($n = 4$, *Represent differences from Control (Ctrl); $p < 0.05$, one-way ANOVA).

The expression of NPY receptors (Y1R, Y2R and Y5R) in the 4T1 cell line observed in the current study is congruent with others reporting the presence of NPY receptors in several different carcinomas including prostate,[23] ovarian[24] and breast.[12,13] Reubi *et al.*[12] reported expression of Y1R in 85% of the breast carcinomas sampled, while 24% were positive for Y2R. In all cases, Y2R positive tumors also expressed Y1R. Furthermore, in the same study, normal breast tissue expressed Y2R only. Y5R expression in breast cancer tumors

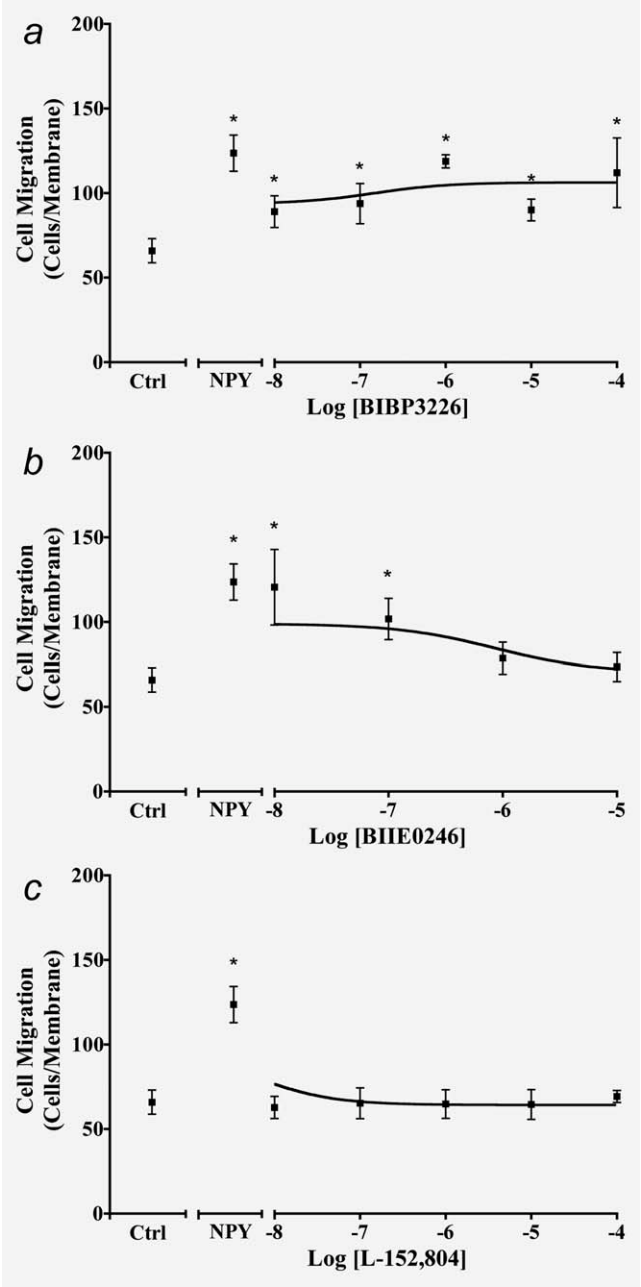

Figure 6. Effect of NPY receptor activation and blockade (Y1R, Y2R and Y5R) on chemotaxis. NPY receptor antagonists (*a*) Y1R:BIBP3226; (*b*) Y2R:BIIE0246 and (*c*)Y5R:L-152,804) were added to the upper chamber (10^{-8}–10^{-4}M) and NPY treated media (10^{-8}M) was added to the bottom chamber. Treatment of cells with BIBP3226 had no effect on NPY-stimulated chemotaxis, while BIIE0246 and L-152,804 inhibited the chemotactic effects of NPY. The data are presented as the mean ± SEM, ($n = 4$, *Represent differences from Control (Ctrl)× $p < 0.05$, one-way ANOVA).

was not investigated in the aforementioned study; however, a recent publication from Sheriff *et al.*[13] reported Y5R transcript and protein expression in several human breast cancer cell lines. In the 4T1 cell line, we observed Y1R, Y2R, and Y5R expression and our *Y1R* and *Y5R* mRNA levels paralleled those recently reported by Sheriff *et al.*[13] Based on our immunohistochemical observations Y5R expression predominates over Y1R and Y2R expression in 4T1 cells and tumors.

NPY has been shown to stimulate proliferation in prostate cancer,[25] neuroblastomas[26] and breast carcinomas.[13,14] In the current study, we observed a concentration-dependent increase in proliferation with NPY treatment, with proliferation occurring in physiological concentrations of NPY (10^{-10}–10^{-8}M).[19] Additionally, Y5R agonist treatment induced a concentration-dependent increase in proliferation. Y1R (BIBP3226) or Y2R (BIIE0246) antagonism did not attenuate NPY-stimulated proliferation; however, Y5R blockade (using L-152,804) produced concentration-dependent attenuation of NPY's effects. In support of these data, Y5R blockade inhibited the proliferative effects of the specific Y5R agonist treatment. This set of experiments illustrates the predominant role that the Y5R plays in NPY mediated proliferation in this cell line. These findings follow those reported in neuroblastomas and human breast cancer cells.[13,27] Y5R activation has been shown to stimulate MAPK activity, leading to increased ERK 1/2 phosphorylation and cell proliferation.[13,28,29] Sheriff *et al.*,[13] reported that Y5R activation led to an increase in pERK 1/2 in BT-549 cells, an effect that was inhibited by Y5R blockade. We observed a similar increase of pERK 1/2 levels after 2 min and 5 min of NPY treatment and Y5R agonist treatment, a response that could only be blocked by Y5R antagonism, further supporting that Y5R activation promotes NPY-stimulated proliferation.

Neuropeptides have been implicated to be involved in the development of metastasis.[19] The migratory propensity of MDA MB-231 cells has been shown to increase when treated with NPY, a response that was mediated by Y5R activation.[13] In our current study, we observed that NPY acts as a concentration-dependent chemoattractant and it appears that NPY-mediated chemotaxis occurs through Y2R and Y5R activation. Accordingly, chemotaxis increased when cells were exposed to PYY_{3-36} (Y2R/Y5R agonist) and [cPP^{1-7}, NPY^{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (Y5R agonist). Similar NPY-stimulated migration has been reported in endothelial cells as a result of Y2R and Y5R activation.[18]

The immune system influences microenvironmental interactions between tumor cells and the host-tissue; such interactions mediate primary tumor development and play an important role in the metastatic cascade. Thus, in many human cell xenograft models in immunocompromised mice, metastatic development can often be variable and sometimes unpredictable.[30,31] The 4T1 murine mammary carcinoma represents a syngenic model that effectively metastasizes and exhibits similar metastatic characteristics to those clinically observed.[20,32,33] Positive Y2R expression in this model contrasts the recent report that many human breast cancer cell lines lack this receptor.[13] Based on the work of others,[12] we acknowledge that the Y2R effects observed in the current study may be relevant to a small subset of human breast

carcinomas. However, our current findings highlight the complexity and heterogeneity of the NPY system in breast cancer. As it stands, this model enables the investigation of all three receptors (Y1R, Y2R, and Y5R) and their influence on breast cancer tumor progression.

Overall, we report the presence of Y1R, Y2R and Y5R receptors in the 4T1 murine breast cancer cell line for the first time. More importantly, we have shown that NPY (at physiological levels) is a potent proliferative and chemotactic agent in the 4T1 cell line, acting through the Y5R and Y2R/Y5R respectively. Our findings support the accumulating evidence demonstrating the pathological contribution of NPY and its respective receptors in the development and progression of breast cancer. Given our current findings and previously reported interactions between stress, the sympathetic nervous system, the immune system and tumor cells, we propose the 4T1 cell line as a syngenic model for future *in vivo* investigations addressing the integrated functional effects of NPY on breast cancer growth/progression and metastasis.

Acknowledgements

Authors are grateful to Michael Wakem for technical assistance and G.M.Z. for motivational support. P.J.M. is the recipient of a Doctoral Fellowship from the Canadian Breast Cancer Foundation and Studentship from the Translational Breast Cancer Research Unit of the London Regional Cancer Program. A.F.C. is Canada Research Chair in Oncology, supported by the Canada Research Chairs Program.

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