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Stimulation of neuroendocrine differentiation in prostate cancer cells by GHRH and its blockade by GHRH antagonists

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Abstract

Prostate cancer is the second leading cause of cancer-related deaths among men in developed countries. Neuroendocrine prostate cancer, in particular, is associated with an aggressive phenotype and a poor prognosis. Neuroendocrine cells produce and secrete peptide hormones and growth factors in a paracrine/autocrine manner which promote the progression of the disease. Recent studies have demonstrated that extracellular vesicles or exosomes are released by prostate cancer cells, supporting the spread of prostate cancer. Hence, the aim of this study was to investigate the effect of growth hormone-releasing hormone (GHRH) on neuroendocrine differentiation (NED) in the androgen-dependent prostate cancer cell line LNCaP and the molecular mechanisms underlying these effects. GHRH induced an increase in the percentage of neurite-bearing cells and in the protein levels of Neuron-Specific Enolase. Both effects were blocked by the GHRH receptor antagonist MIA-690. In addition, pretreatment of these cells with the calcium chelator BAPTA, the EGFR inhibitor AG-1478 or the HER2 inhibitor AG-825 reduced the effect of GHRH, suggesting that the GHRH-induced stimulation of NED involves calcium channel activation and EGFR/HER2 transactivation. Finally, PC3-derived exosomes led to an increase in NED, cell proliferation and cell adhesion. Altogether, these findings suggest that GHRH antagonists should be considered for in the management of neuroendocrine prostate cancer.

Keywords: GHRH; LNCaP cells; PC3 cells; Neuroendocrine differentiation; Exosomes; Prostate cancer.

Abbreviations

PCa, Prostate cancer

CRPCa, castration resistant prostate cancer

NEPCa, neuroendocrine prostate cancer

NE, Neuroendocrine

NED, neuroendocrine differentiation

GHRH, growth hormone-releasing hormone

GHRH-R, growth hormone-releasing hormone receptors

VIP, vasoactive intestinal peptide

EVs, extracellular vesicles

FBS, fetal bovine serum

BrdU, bromodeoxyuridine

NSE, neuron-specific enolase

PBS, phosphate buffered saline

EDTA, ethylenediaminetetraacetic acid

SE, standard error

PKA, protein kinase A

ERK, extracellular signal-regulated kinases

MAPK, mitogen-activated protein kinase

MEK, mitogen-activated protein kinase kinase

PI3K, phosphoinositide 3-kinase

EGFR, epidermal growth factor receptor

HER2, human epidermal growth factor receptor 2

Introduction

Prostate cancer (PCa) is the second leading cause of cancer-related deaths among men in developed countries [1]. Androgen deprivation therapy is widely used for the management and treatment of aggressive and metastatic prostate tumours. However, these tumours acquire resistance and develop into castration-resistant prostate cancer (CRPCa), which is difficult to treat. One consequence of prolonged systemic androgen blockade is the increasing occurrence of neuroendocrine prostate cancer (NEPCa), associated with an aggressive phenotype and a poor prognosis [2, 3].

Neuroendocrine (NE) cells constitute only 1% of total epithelial cells in the prostate gland. In prostate tumours, neuroendocrine-differentiated (NED) areas have been associated with tumour progression and the development of castration-resistant cancer [3–5]. These NE cells produce and secrete peptide hormones and growth factors in a paracrine/autocrine manner in order to sustain the lack of expression of androgen receptors.

Growth hormone-releasing hormone (GHRH) is a peptide secreted by the hypothalamus that regulates GH synthesis and secretion; the ectopic production of GHRH by tumour cells, however, extends its role to carcinogenesis. Dr. Schally's group has synthesized several classes of potent GHRH receptor (GHRH-R) antagonists capable of inhibiting growth, tumorigenicity, and metastases of a wide range of human cancers such as prostate, breast, and ovarian cancer [6–9]. Vasoactive intestinal peptide (VIP), structurally similar to GHRH, is a neuropeptide present in the human prostate gland secreted by autonomous nerves and produced by prostate cells. Our group has previously demonstrated that VIP induces neuroendocrine differentiation in prostate cancer cells [10–13].

There is a complex system of communication between tumour cells and the surrounding

microenvironment. Such interactions are promoted by soluble and membrane-associated factors secreted by the tumour cells [14]. These include extracellular vesicles (EVs) or exoxomes, with sizes ranging from 30 to 150 nm. In this regard, neoplastic and metastatic PCa cells have been reported to release prostasomes, supporting the spread of PCa [15].

The purpose of the present study was to analyse the effects of GHRH on neuroendocrine differentiation in the androgen- responsive prostate cancer cells LNCaP as well as the intracellular mediators involved. In addition, we assessed whether PC3 cell-derived exosomes are involved in processes such as cell proliferation, cell adhesion and neuroendocrine differentiation in LNCaP cells.

Materials and methods

Peptides

The GHRH-R antagonist MIA-690 was synthesized in Dr. Schally's laboratory. GHRH(1–29)NH2 was purchased from PolyPeptide (Strasbourg, France). EGFR tyrosine kinase inhibitor (AG-1478) was acquired from Calbiochem (Darmstadt, Germany). HER-2 tyrosine kinase inhibitor (AG-825) was from Tocris Bioscience (Bristol, United Kingdom). 2'-amino-3′- methoxyflavone (PD98059) and N-(2-(p-bromocinnamylamino) ethyl)-5-isoquinolinesulfonamide (H89) was from Alexis (San Diego, CA). Wortmannin (W), 1 ,2-bis(o-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA/ AM), and 8-Chloroadenosine 3′,5′-cyclic-monophosphate (8-Cl-cAMP) were acquired from Sigma Aldrich (Madrid, Spain).

Cell culture

Two human prostate cancer cell lines were used in this study: an androgen-responsive prostate cancer cell line, LNCaP, and a castration-resistant prostate cancer cell line, PC3. These cell lines, which represent both stages of prostate cancer, were obtained from the American Type

Culture Collection. LNCaP and PC3 cells were grown and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B (Life Technologies, Carlsbad, CA, USA). The culture was performed in a humidified 5% CO_2 environment at 37 °C. After reaching 70–80% confluency, the cells were washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin/0.2% ethylenediaminetetraacetic acid (EDTA), and seeded at 30,000–40,000 cells/cm^2. The culture medium was changed every 3 days.

Detection of NE morphology in LNCaP cells

LNCaP cells were allowed to attach to 24-well plates at a density of 4×10^5 cells/well with complete medium for 24 h. Then, the medium was changed to 10% serum-supplemented medium (control cells), with or without 0.1 μM GHRH and/or 0.1 μM of the GHRH-R antagonist MIA-690. After 2 h of treatment, cells were photographed using a Nikon Optiphot-2 (inverted and phase contrast microscopy). NE-differentiated cells were considered as those bearing neurites of at least twice the length of the cell body.

Preparation of cell lysates

LNCaP and PC3 cells (1.5–3× 10^6 cells) were washed with ice- cold PBS and then harvested, scraped with ice-cold PBS, and pelleted by centrifugation at 500×g for 5 min at 4 °C. For preparation of cell lysates, cells were kept on ice for 30 min in a solution containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl, 1 mM EDTA, 2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml pepstatin. Afterwards, cells were pelleted by centrifugation at 4,000×g for 5 min at 4 °C.

Western blot assays

Proteins (30 μg) from cell lysates were denatured by heating. They were then resolved by 10% SDS-PAGE, and blotted onto a nitrocellulose membrane (BioTrace/NT) overnight in 50 mM

Tris–HCl, 380 mM glycine, 0.1% SDS, and 20% methanol. Rabbit anti-NSE (ICN Biomedical, Aurora, OH) was added, followed by incubation for 1 h at room temperature. The signals were detected after incubation with the corresponding secondary antiserum, using an enhanced chemiluminescence reagent (Pierce). An antibody against β-actin was used as the loading control.

Isolation of PC3 cell-derived exosome

Human PC3 cells were grown in culture medium with exosome-free serum for 72 h. Thus, any serum added to the cell culture medium should be depleted of exosomes by ultracentrifugation at 120,000 x g 16 h at 4 °C prior to use. The extracellular medium was centrifuged at 300 x g for 10 min at 4 °C to pellet the cells. The supernatant was centrifuged at 16,500 x g for 20 min at 4 °C to further remove cells and cell debris. The supernatant was then filtered through a 0.22 μm filter to remove particles larger than 200 nm, and ultracentrifuged at 120,000 x g for 70 min at 4 °C to pellet the exosomes. The pellets enriched with exosomes were resuspended in a small volume of medium with exosome-free serum.

Proliferation of LNCaP cells

LNCaP cells (2×10^5) were grown in 6-well plates. After 24 h, the culture medium was removed and replaced with RPMI-1640 medium containing 1% antibiotic/antimycotic for 16 h. The cells were then subjected for 24 h to different treatments. In the last 30 min of incubation, cells were pulsed with 10 μ M bromodeoxyuridine (BrdU). Afterwards, cells were washed with PBS, fixed with ice- cold absolute ethanol, and stored at −20 °C for 30 min. The fixative was removed by centrifugation and the cell pellets were washed with PBS. DNA was partially denatured by incubation with 1 M HCl for 30 min at room temperature. The cells were then washed three times with PBS containing 0.05% Tween-20 (pH 7.4) and 0.1%

BSA and incubated with 20 μl of anti-BrdU monoclonal antibody conjugated with FITC (BD Bioscience) in the dark for an additional 30 min. In preparation for flow cytometry analysis, the cells were stained with propidium iodide (PI) staining solution: PI (50 μg/ml) and RNase (10 μg/ml) in PBS. The number of BrdU-positive cells was counted with the use of the Cyflogic program (Version 1.2.1.).

LNCaP cell adhesion

Concentrated type-I collagen solution was diluted with 10 mM acetic acid and coated onto 96-well plates for 1 h at 37 °C. Plates were washed twice with PBS (pH 7.4). Cells were harvested with 0.25% trypsin/0.2% EDTA and collected by centrifugation, resuspended in RPMI medium with 0.1% (*w*/*v*) BSA (pH 7.4) and treated with 0.1 μM GHRH for 30 min. The cells were then plated at 2.5×10^4 cells per 100 μl. The assay was terminated at 80 min by aspiration of the wells. Cell adhesion was quantified by adding MTT (1 mg/ml) followed by a 1 h-incubation at 37 °C. Isopropanol (50 μl) was added to each well to dissolve the formazan precipitates and the absorbance was measured at 540 nm, with a reference wavelength at 630 nm.

Data analysis

Quantification of band densities was performed using the Quantitive One Program (Bio-Rad, Alcobendas, Spain). Data were subjected to one-way ANOVA and statistical significances were determined by Bonferroni's multiple comparison test. Each experiment was repeated at least three times. Data are shown as the means of individual experiments and represented as the mean ± SE. $P < 0.05$ was considered statistically significant.

Results

Effects of GHRH and a GHRH receptor antagonist, MIA-690, on NE differentiation in LNCaP cells

LNCaP cells were grown in serum-supplemented medium (control cells) in the presence or absence of GHRH (0.1 μM) and/or MIA-690 (0.1 μM) for different time-periods (2, 4 and 6 h). As shown in Fig. 1a, cell exposure to 0.1 μM GHRH significantly increased the percentage of neurite-bearing cells in a time-dependent manner, as compared with control cells. By contrast, the GHRH-R antagonist MIA-690 did not induce NE differentiation in LNCaP cells but was able to block completely the induction of NE differentiation by GHRH in LNCaP cells at all the time-periods studied.

The protein levels of Neuron-Specific Enolase (NSE) were measured by Western blotting in order to correlate the expression of this neural biomarker with the changes in NE differentiation induced by GHRH and MIA-690. The results shown in Fig. 1b indicate that GHRH significantly increased NSE levels by up to 90.8%. Treatment with MIA-690 did not alter NSE levels at any of the time-periods studied. On the other hand, the combination of GHRH and MIA-690 caused a reduction in NSE levels as compared with those obtained after incubation with GHRH alone. There were no significant changes, however, when compared with the control group.

Evaluation of intracellular mediators involved in GHRH-mediated NE differentiation in LNCaP cells

The signalling pathways involved in the stimulatory effect of GHRH on NE differentiation (Fig. 2a) and NSE expression (Fig. 2b) were evaluated in LNCaP cells. Thus, we used the following agents: a calcium chelator (BAPTA/AM), the cAMP analogue (8-Cl-cAMP), and the protein kinase inhibitors H89 for protein kinase A (PKA), PD98049 for

extracellular signal-regulated kinases (MEK) 1/2 and wortmannin for phosphatidylinositol 3-kinase (PI3K). Cells were pre-treated with each of these agents for 30 min and then incubated with 0.1 μM GHRH for 2 h, since NE differentiation was observed at that time-period. Only BAPTA/AM was able to reduce the percentage of neurite-bearing cells (Fig. 2) and to block the stimulatory effect of GHRH on NSE expression (Fig. 2b). The other agents had no effects on the GHRH-mediated increase of neurite-bearing cells nor on the stimulated NSE levels.

Involvement of HER2 and EGFR transactivation in NE differentiation induced by GHRH in LNCaP cells

To explore whether the GHRH effect on NED in LNCaP cells is exerted through HER2 or EGFR transactivation, the following specific tyrosine kinase inhibitors were used: AG-1478 for EGFR and AG-825 for HER2. LNCaP cells were pre-treated with each inhibitor at 10 μM for 30 min and then incubated with GHRH 0.1 μM for 2 h. Neurite formation and NSE levels were then assessed. The results show that transactivation of both EGFR and HER2 was involved in GHRH-mediated NED (Fig. 3).

Effect of PC3 cell-derived exosomes on NE differentiation in LNCaP cells

We evaluated the phenotypic changes induced by PC3-derived exosomes in LNCaP cells with or without GHRH pre-treatment. Human PC3 cells were grown in a medium with exosome-free serum for 72 h in the presence (+) or absence (−) of GHRH. Afterwards, the exosomes were isolated from the culture medium. LNCaP cells were treated with both types of exosomes derived from PC3 cells: (+) GHRH-PC3-exosomes or (−) GHRH-PC3-exosomes. After 24 h, there was a 32–37% increase in the number of LNCaP cells bearing neurite outgrowths (Fig. 4a). In addition, both exosomes derived from PC3 cells led to an increase (26–38%) in the NSE expression levels as compared with the control group. There were no significant changes,

however, between both treatments (Fig. 4b).

Effects of PC3 cell-derived exosomes on cell proliferation and cell adhesion in LNCaP cells

The effects of PC3 cell-derived exosomes on cell proliferation and cell adhesion of LNCaP cells were assessed by BrdU incorporation and adhesion to type-I-collagen assays, respectively. Treatment with both types of exosomes derived from PC3 cells significantly increased LNCaP cell proliferation of by 23–26% as compared with control cells (Fig. 5a). The exosomes derived from PC3 cells that had been pre-treated with GHRH caused a significant increase in LNCaP cell proliferation at 2 h, which remained for a further 22 h. By contrast, PC3 cell-derived exosomes without GHRH-pretreatment caused a rise in cell proliferation at 4 h, which remained for 4 h. Furthermore, both types of PC3 cell-derived exosomes resulted in a significant increase (25–34%) of LNCaP cell adhesion at 80 min (Fig. 5b).

Discussion

Neuroendocrine differentiation is a process that contributes to castration-resistant prostate cancer (CRPCa) [3]. Our study shows, for the first time, that GHRH can act as an autocrine/paracrine factor that induces an effect on the acquisition of a NE morphology in an androgen-dependent human prostate cancer cell line, LNCaP. GHRH has been previously implicated in several relevant processes associated with the progression of prostate cancer [16]. VIP, structurally related to GHRH, induces neuroendocrine differentiation in these cells in a similar way via PKA, extracellular signal-regulated kinases (ERK) 1/2, PI3K and/or calcium channels [10–13]. In our study, MIA-690 blocked GHRH-induced NED at different time-periods, contributing to a reduction of the NED phenotype, confirming a specific role for the neuropeptide GHRH.

We investigated the molecular mechanisms underlying the inducing effect of GHRH on the

NE phenotype. GHRH is known to activate different pathways in tumour cells such as PKA, MAPK, PI3K and/or calcium channels [17–20]. Our group previously described the involvement of EGFR/HER2-transactivation induced by GHRH in various tumour processes such as cell proliferation, migration or adhesion [16]. Here, we demonstrate that GHRH may induce NED in LNCaP cells via the activation of calcium channels and EGFR/HER2 transactivation. In line with this, there are studies showing that NED of prostate cancer cells is linked to the overexpression of voltage-gated T-type Ca^{2+} channels [21]. In addition, EGFR appears to be involved in the pathways leading to NED in prostate cancer cells [22].

NE cells secrete peptide hormones and growth factors to sustain tumour growth in a paracrine manner, promoting prostate cancer progression [4]. It has been shown that NED can be mediated by the release of exosomes from CRPCa cells exposed to IL-6 [23]. Our study demonstrates that CRCPa cell-derived exosomes can induce NED in androgen-responsive prostate cancer cells. Furthermore, both types of exosomes derived from PC3 cells increased LNCaP cell proliferation and adhesion. The exosomes released by the GHRH-pretreated PC3 cells promoted proliferation of LNCaP cells at a shorter time-period than those released by untreated PC3 cells. In addition, this effect on cell proliferation was maintained for a longer time. However, neither NED nor cell adhesion were modified by the pre-treatment with GHRH. This suggests that the exosomes released after exposure to the neuropeptide are not involved in NED nor in cell adhesion in LNCaP cells.

In summary, the present study supports a role for GHRH as a pro-tumoral factor in prostate cells. This neuropeptide induces NED by interaction with GHRH receptors, an effect involving intracellular calcium and EGFR/HER2 transactivation. More studies are needed to clarify whether the composition of the CRPCa cell-derived exosomes determines the effects of GHRH on NED, cell proliferation and cell adhesion in LNCaP cells. Our findings should be helpful for the

improvement of neuroendocrine prostate cancer therapy.

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Compliance with ethical standards

Conflict of interest

The author Laura Muñoz-Moreno declares that she has no conflict of interest. The author María J. Carmena declares that she has no conflict of interest. The author Andrew V. Schally declares that he has no conflict of interest. The author Juan C. Prieto declares that he has no conflict of interest. The author Ana M. Bajo declares that she has no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure legends

Fig. 1 GHRH induces neuroendocrine (NE) phenotype in LNCaP cells at short period of treatment. **a** Left: Control, serum-deprived cells and serum-deprived cells grown in the presence of GHRH (0.1 μM), GHRH-R antagonist MIA-690 (0.1 μM) or their combination for 2 h, 4 h and 6 h are presented. A representative experiment of five others is shown. Right: Percentage of NE cells in LNCaP cultures. **b** Immunoblot analyses of neuronal-specific enolase (NSE) protein expression in LNCaP cell lysates of control and serum-deprived LNCaP cells after treatment with GHRH (0.1 μM), GHRH-R antagonist MIA-690 (0.1 μM) or their combination are presented. The intensities of the bands were quantified by densitometric scanning, and β-actin was used as a loading control. The bands correspond to a representative experiment of three others. Quantitative results are the mean ± SEM of four separate experiments. **, $P < 0.01$; ***, $P < 0.001$ comparing values with the corresponding control condition; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ comparing values with those in GHRH-treated cells alone.

Fig. 2 Evaluation of intracellular mediators involved on GHRH-mediated NE differentiation in LNCaP cells. **a** Top: Serum-deprived LNCaP cells grown in the presence of 0.1 μM GHRH, the inhibitors: BAPTA/AM, H89, PD98058 (PD), 8-pCPT, wortmannin (W) or in combination for 2 h. A representative experiment of five others is shown. Botton: Percentage of NE cells in LNCaP cultures. **b** Immunoblot analysis of neuronal-specific enolase (NSE) protein expression in LNCaP cell lysates of control and serum-deprived LNCaP cells after treatment with GHRH (0.1 μM), the inhibitors or in combination are presented. The intensities of the bands were quantified by densitometric scanning, and β-actin was used as a loading control. The bands correspond to a representative experiment of three. Quantitative results are the mean

± SEM of four separate experiments. *, $P < 0.05$; ***, $P < 0.001$ comparing values with the corresponding control condition; ##, $P < 0.01$; ###, $P < 0.001$ comparing values with those in GHRH-treated cells alone.

Fig. 3 Involvement of transactivations of HER2 and EGFR on NE differentiation induced by GHRH. **a** Left: Serum-deprived cells grown in the presence of 0.1 μM GHRH, the specific tyrosine kinase inhibitors of EGFR (10 μM AG-1478) and HER2 (10 μM AG-825) or in combination for 2 h are presented. A representative experiment of five others is shown. Right: Percentage of NE cells in LNCaP cultures. **b** Immunoblot analysis of neuronal-specific enolase (NSE) protein expression in cell lysates of control and serum-deprived LNCaP cells after treatment with GHRH (0.1 μM), the specific inhibitors of the tyrosine kinase activity (10 μM) or in combination. The intensities of the bands were quantified by densitometric scanning, and β-actin was used as a loading control. The bands correspond to a representative experiment of three others. Quantitative results are the mean ± SEM of four separate experiments. ***, $P < 0.001$ comparing values with the corresponding control condition; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ comparing values with those in GHRH-treated cells alone.

Fig. 4 Involvement of PC3 cell-derived exosomes on NE differentiation. Human PC3 cells were grown in medium with exosome-free serum for 72 h in the presence (+) or absence (−) the GHRH. **a** Top: Serum- deprived LNCaP cells grown in the presence of 2 μg/ml or 5 μg/ml of each type of PC3 cells-derived exosomes: PC3-exosomes (−) GHRH and PC3-exosomes (+) GHRH. A representative experiment of five is shown. Bottom: Percentage of NE cells in LNCaP cultures. **b** Immunoblot analysis of neuronal-specific enolase (NSE) protein expression in LNCaP cell lysates after treatment with both types of PC3 cell-derived exosomes (2 or 5 μg/ml) are shown. The intensities of the bands were quantified by densitometric scanning, and β-actin

was used as a loading control. The bands correspond to a representative experiment of three. Quantitative results are the mean ± SEM of four separate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ comparing values with the corresponding control condition.

Fig. 5 Effect of PC3 cell-derived exosomes (2 μg/ml and 5 μg/ml) on cell proliferation (**a**) and cell adhesion (**b**) as assessed by BrdU incorporation and collagen adhesion, respectively, in LNCaP cells. PC3-exosomes (−) GHRH and PC3-exosomes (+) GHRH. Data are mean ± SEM of five independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ comparing values with the corresponding control condition.

Figure 1

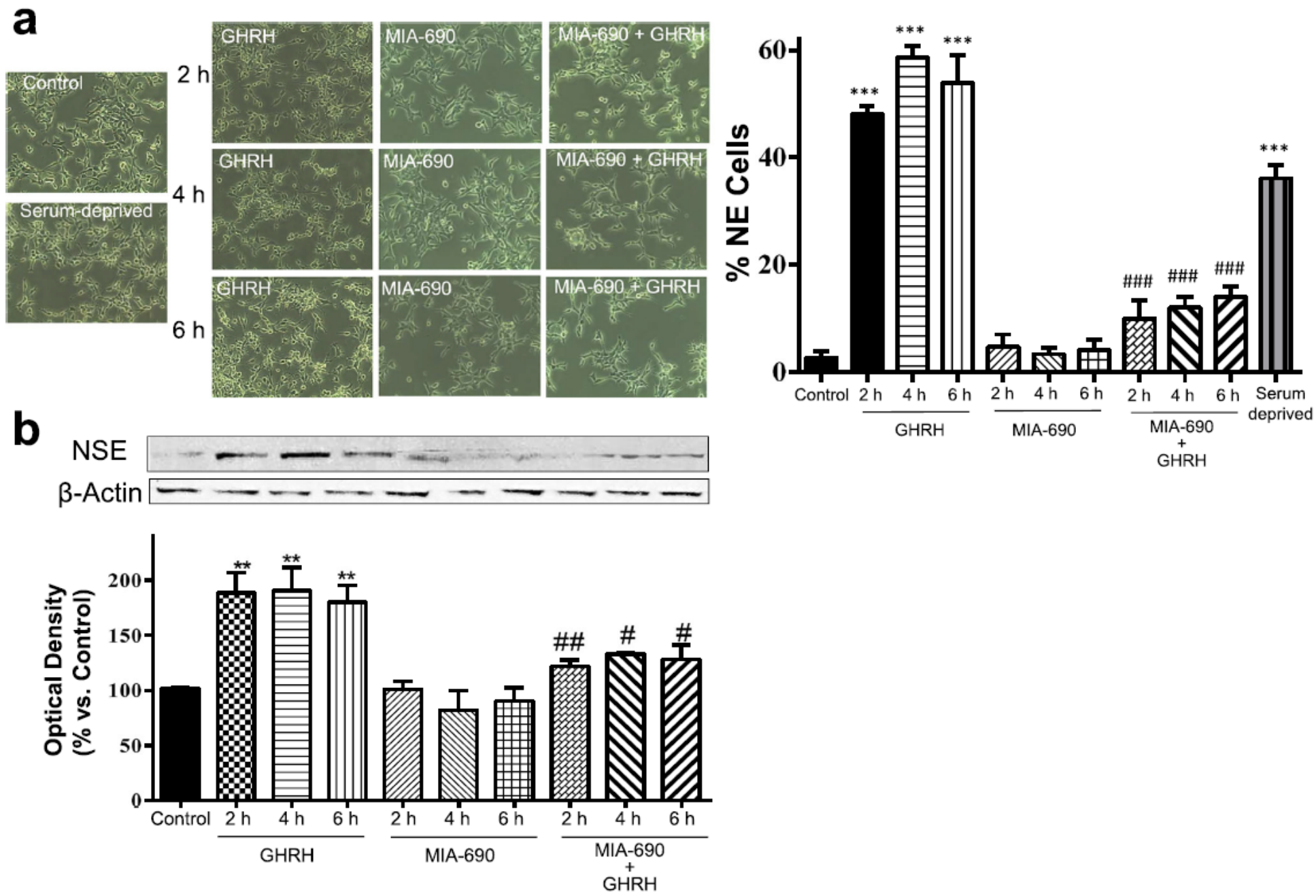
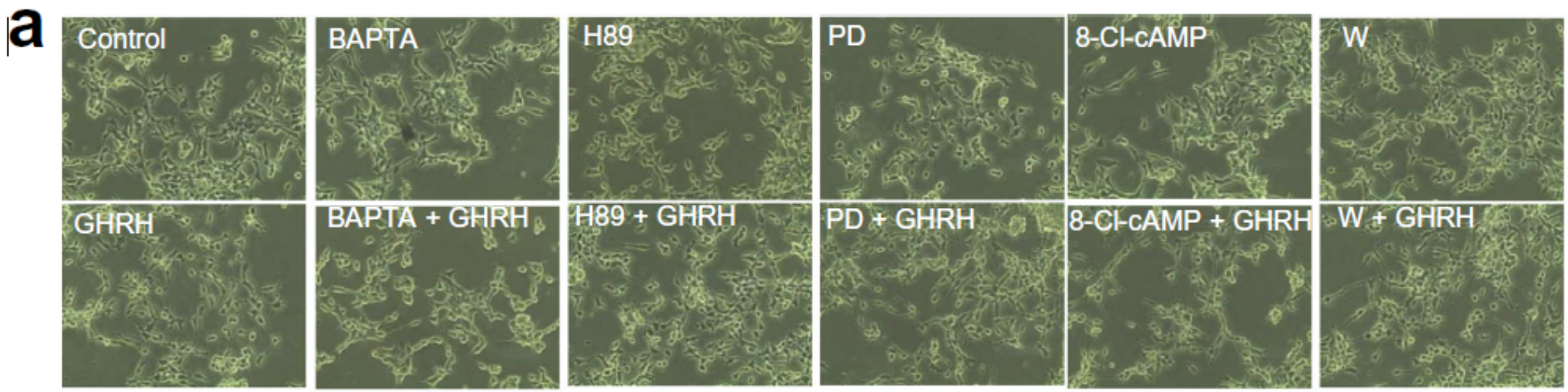
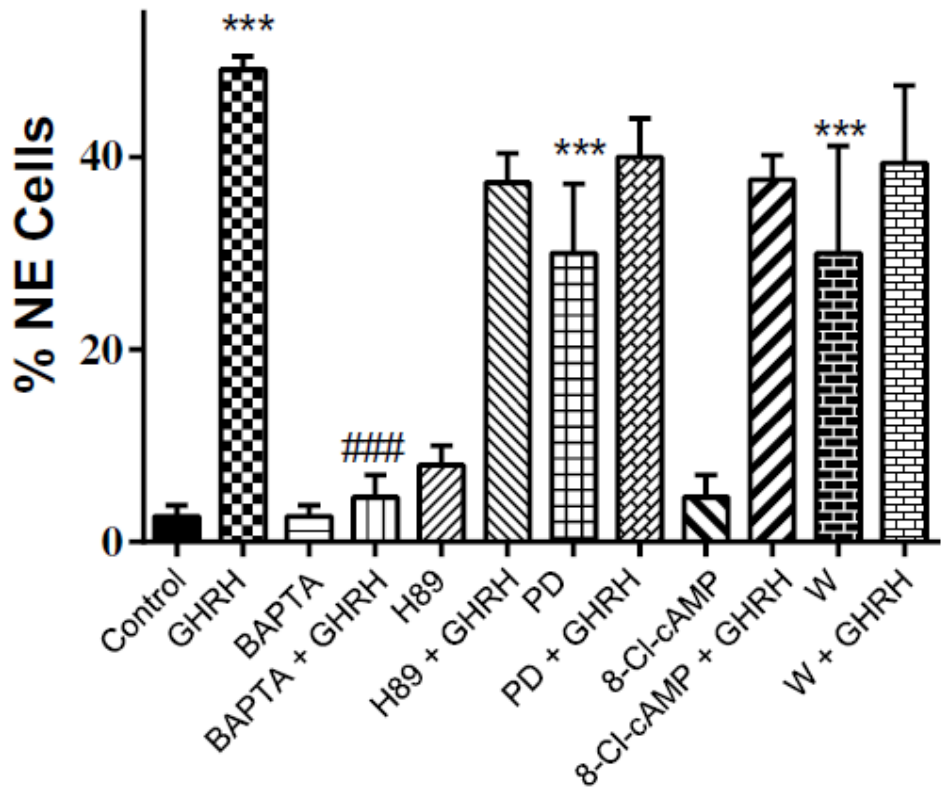

a
Control
Serum-deprived
2 h
4 h
6 h
GHRH
MIA-690
MIA-690 + GHRH
% NE Cells
60
40
20
0
***
###
Control
2 h
4 h
6 h
GHRH
MIA-690
MIA-690 + GHRH
Serum deprived
b
NSE
β-Actin
Optical Density (% vs. Control)
200
150
100
50
0
**
##
#
Control
GHRH
MIA-690
MIA-690 + GHRH

Figure 2


a
Control
BAPTA
H89
PD
8-Cl-cAMP
W
GHRH
BAPTA + GHRH
H89 + GHRH
PD + GHRH
8-Cl-cAMP + GHRH
W + GHRH


% NE Cells
0
20
40
***
###
***
***
Control
GHRH
BAPTA
BAPTA + GHRH
H89
H89 + GHRH
PD
PD + GHRH
8-Cl-cAMP
8-Cl-cAMP + GHRH
W
W + GHRH

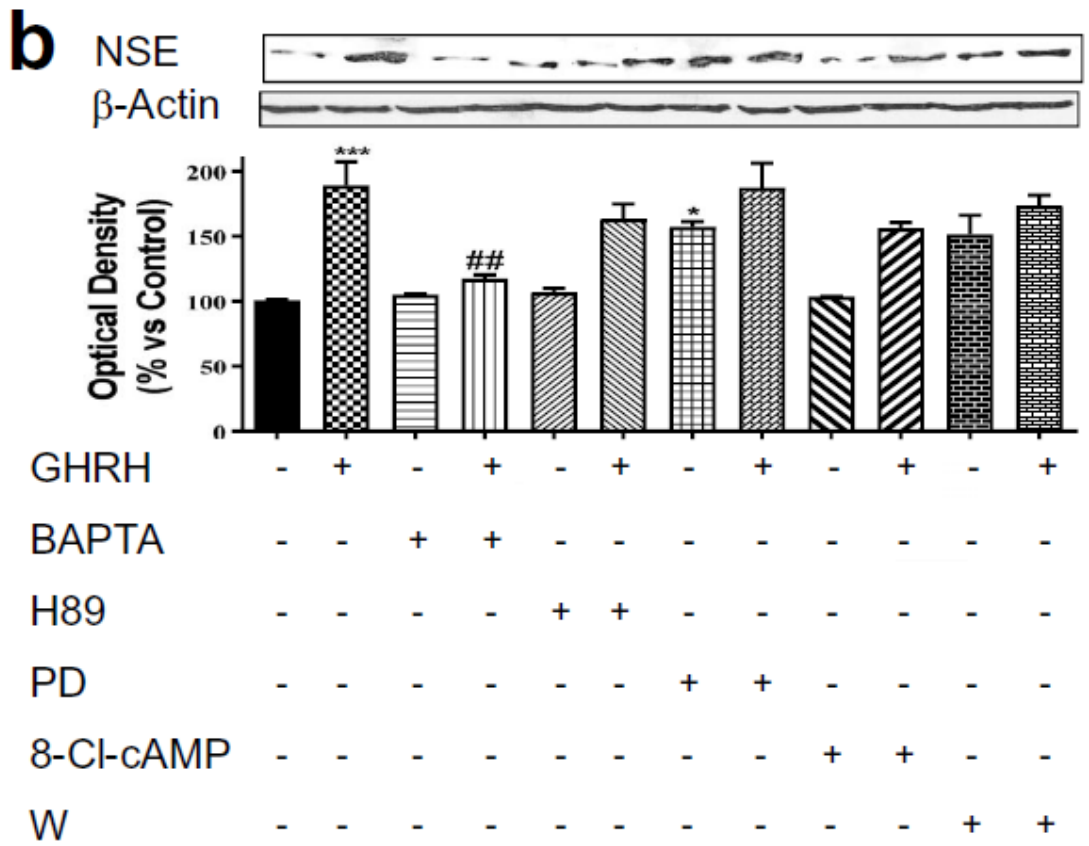
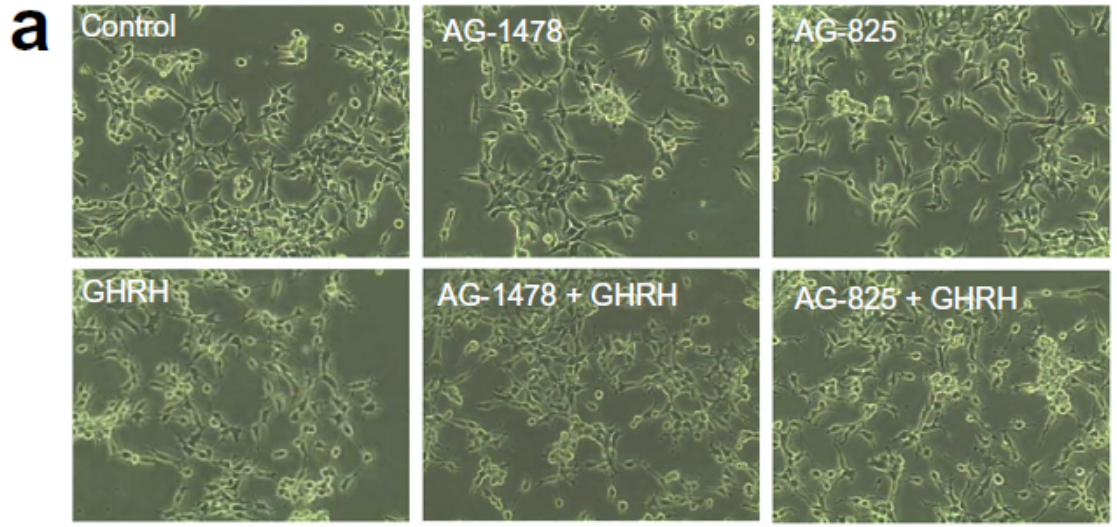
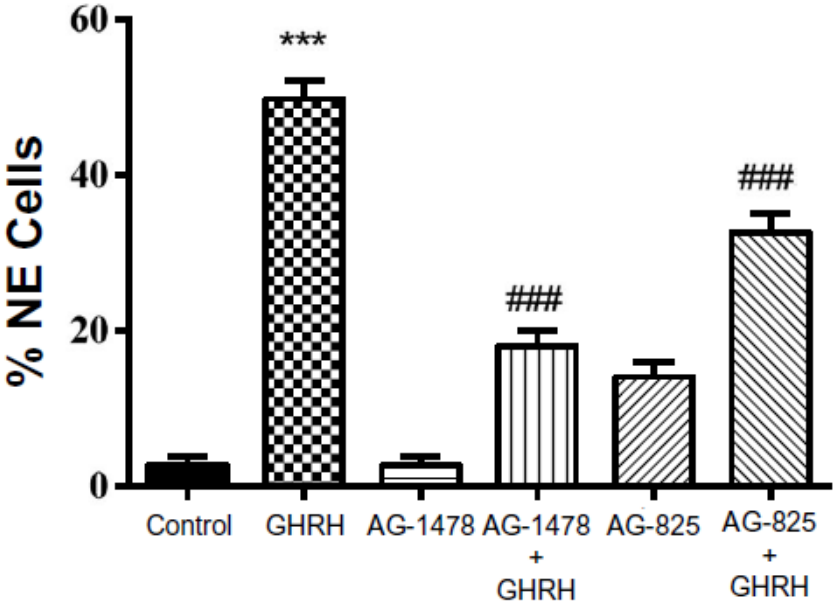

b
NSE
β-Actin
Optical Density (% vs Control)
0
50
100
150
200
***
##
*
GHRH
BAPTA
H89
PD
8-Cl-cAMP
W

Figure 3


a
Control
AG-1478
AG-825
GHRH
AG-1478 + GHRH
AG-825 + GHRH


% NE Cells
60
40
20
0
***
###
###
Control
GHRH
AG-1478
AG-1478 + GHRH
AG-825
AG-825 + GHRH

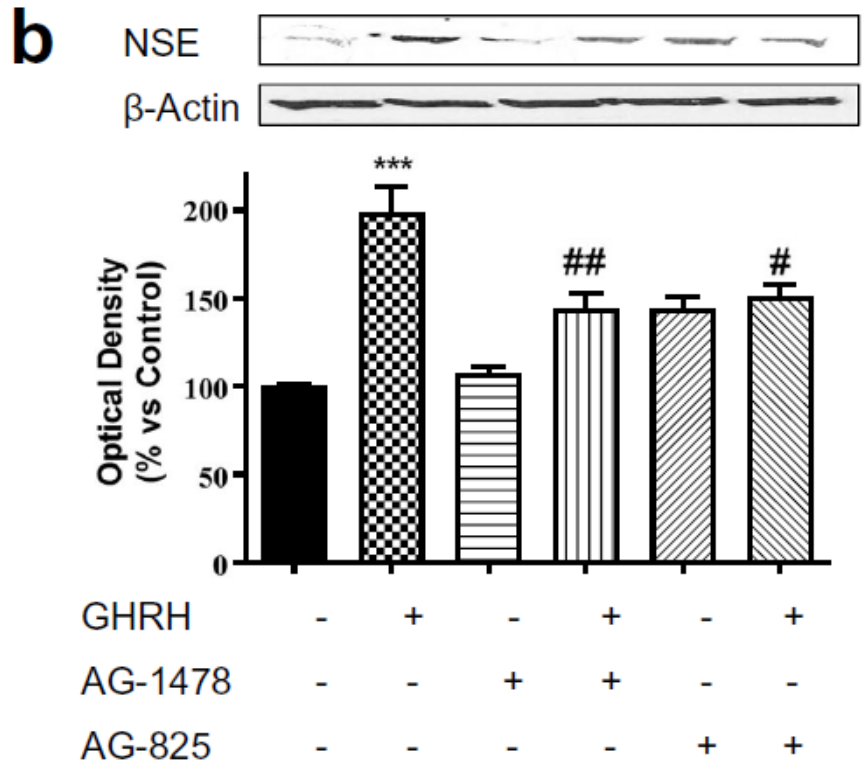

b
NSE
β-Actin
Optical Density (% vs Control)
200
150
100
50
0
***
##
#
GHRH - + - + - +
AG-1478 - - + + - -
AG-825 - - - - + +

Figure 4

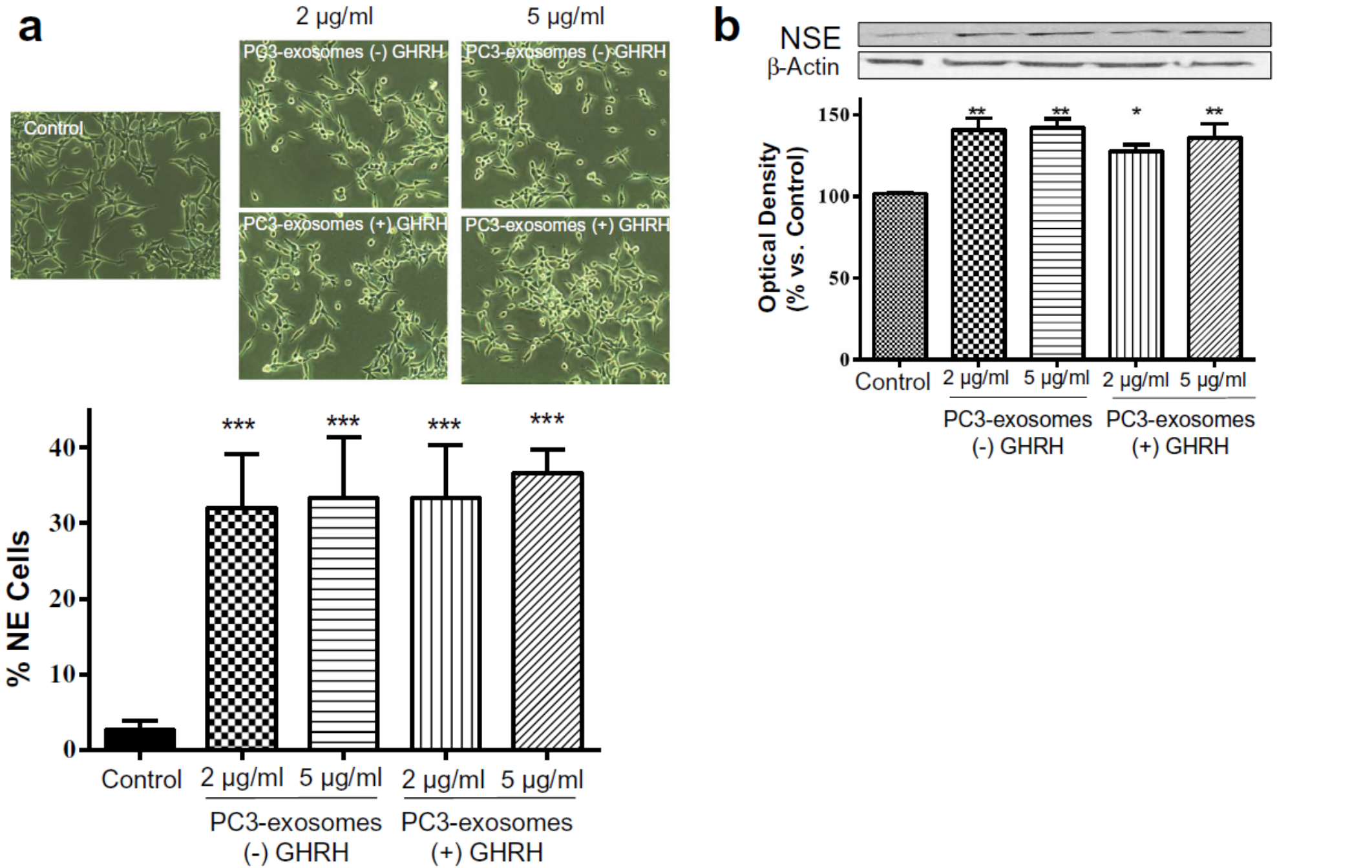
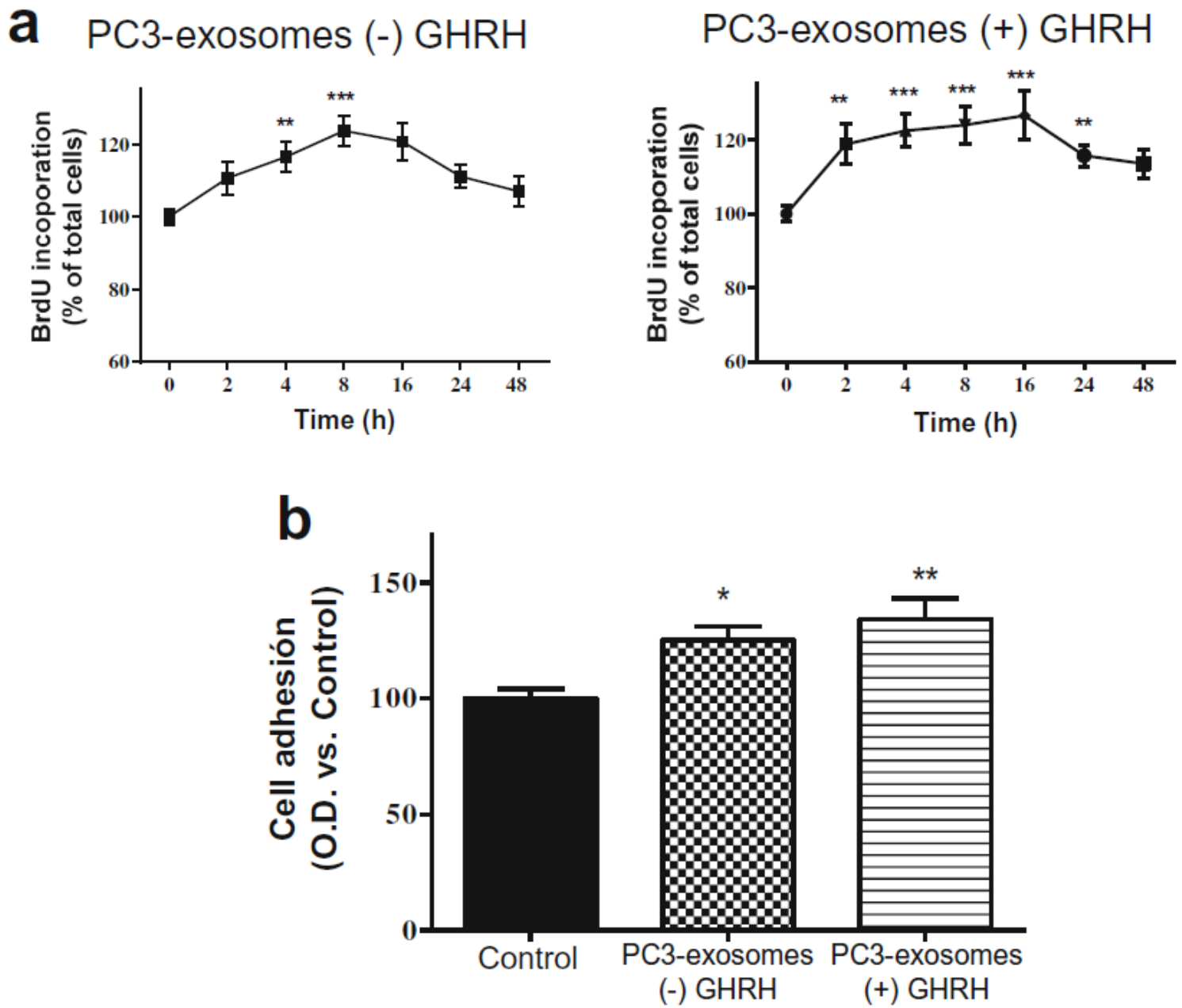

a
2 µg/ml
5 µg/ml
Control
PC3-exosomes (-) GHRH
PC3-exosomes (-) GHRH
PC3-exosomes (+) GHRH
PC3-exosomes (+) GHRH
% NE Cells
0
10
20
30
40
***
***
***
***
Control
2 µg/ml
5 µg/ml
2 µg/ml
5 µg/ml
PC3-exosomes (-) GHRH
PC3-exosomes (+) GHRH
b
NSE
β-Actin
Optical Density (% vs. Control)
0
50
100
150
**
**
*
**
Control
2 µg/ml
5 µg/ml
2 µg/ml
5 µg/ml
PC3-exosomes (-) GHRH
PC3-exosomes (+) GHRH

Figure 5


a
PC3-exosomes (-) GHRH
PC3-exosomes (+) GHRH
BrdU incoporation (% of total cells)
Time (h)
b
Cell adhesión (O.D. vs. Control)
Control
PC3-exosomes (-) GHRH
PC3-exosomes (+) GHRH