

# Neuropeptide B (NPB) and neuropeptide W (NPW) system in cultured rat calvarial osteoblast-like (ROB) cells: NPW and NPB inhibit proliferative activity of ROB cells

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**Abstract.** Neuropeptides B (NPB) and W (NPW) have been identified as endogenous ligands of two G-protein-coupled receptors, neuropeptides B/W receptor 1 (NPBWR1, formerly known as GPR7) and neuropeptides B/W receptor 2 (NPBWR2, formerly known as GPR8). In rodents where NPBWR2 is absent, its counterpart is named the similar to neuropeptides B/W receptor 2 (similar to NPBWR2, formerly GPR8-like). Both NPB and NPW play a role in the control of feeding, neuroendocrine axis functions, memory and learning processes as well as in pain regulation. The present study aimed to investigate the expression of NPB, NPW, NPBWR1 and the similar to NPBWR2 genes in cultured rat calvarial osteoblast-like (ROB) cells and the effects of both peptides on proliferative activity and osteocalcin secretion by ROB cells. Classic RT-PCR technique revealed the presence of ppNPB mRNA, ppNPW mRNA, and NPBWR1 mRNA, but not similar to NPBWR2 mRNA in ROB cells. QPCR revealed gradual (days 7, 14 and 21 of culture) increase of the ppNPB gene expression, while expression of ppNPW gene was the highest at day 14 and was comparable to that seen in freshly isolated cells. In ROB cells, expression of NPBWR1 gene was notable at day 7 of culture, lower at day 21, and negligible at day 14. Neither NPB nor NPW changed osteocalcin secretion by cultured osteoblast-like cells while both neuropeptides inhibited their proliferative activity. Results of the present study suggest that the systems of NPW, NPB and NPBWR1 directly regulate proliferative activity of cultured rat calvaria osteoblast-like cells. The physiological significance of this osteoblastic system remains unclear, and requires further investigation.

## Introduction

Neuropeptide B/W receptor 1 (NPBWR1) and neuropeptide B/W receptor 2 (NPBWR2) are two structurally related orphan

receptors linked to protein G. In rodents NPBWR2 is absent, and its counterpart is named similar to neuropeptide B/W receptor 2 (NPBWR2 like). Endogenous ligands of these receptors have been identified as neuropeptide B (NPB) and W (NPW). NPB is 29 amino acid residues long and uniquely modified with bromine. NPW has been identified in two molecular forms of 23 and 30 amino acids (NPW23 and NPW30, respectively) (1-6).

Both at the mRNA and protein levels, the systems of NPB, NPW and their receptors were found to be widely distributed. Expression of its components was observed by others in the brain and alimentary tract (2,7-14). Both NPB and NPW affect food intake and energy expenditure (15-18). Those peptides and their receptors are also present in the neuroendocrine system and regulate its function. In this regard, involvement of both NPB and NPW in regulating hypothalamo-pituitary-adrenocortical axis is well recognised (17,19-27).

Numerous neuropeptides involved in energy homeostasis are also recognized as regulators of bone metabolism in animals and humans. Recent data suggest that bone and energy metabolisms exert reciprocal regulations and such an inter-relationship was observed between osteocalcin and insulin (28). NPB, NPW and their receptors are expressed in pancreatic islets of the rat and NPW affects insulin secretion both *in vivo* and *in vitro* (20,25,29). To our knowledge there are no data on the presence of NPB and NPW and their receptors in the major cell types of the bone. Therefore the present work aimed to examine the expression of that system in cultured rat calvaria osteoblast-like cells (ROB) and its possible role in regulating ROB cell functions.

## Materials and methods

**Chemicals.** NPB29 and NPW23 were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). If not otherwise stated, the remaining reagents were from Sigma-Aldrich (St. Louis, MO, USA) or POCh (Gliwice, Poland).

**Animals.** Two day-old Wistar rats, born in our animal facilities, were used. The local ethics committee for animal studies approved the study protocol.

**ROB cell culture.** The technique was that described earlier (30-34) with minor modifications. Briefly, calvarias of 8 2-day-old rats were placed in DMEM (Gibco, UK), and the

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Table I. QPCR analysis of NPB, NPW, NPBWR1, similar to NPBWR2 and hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNAs in cultured ROB cells.

cDNA	Genbank accession number	Primer or probe	Primer or probe sequence (5'-3') (Universal probe library number)	Position	PCR product size (bp)
NPB	NM 153293	S	CGGGATCCCACCACTACTC	80-98	245
		A	GAAGACGTCCGCCTTACACT	305-324	
NPW	NM 153294	S	ACCTGAGCAGTCGTAAGCTTT	489-510	150
		A	CATCGGTTCTTGAGACGGTC	619-638	
NPBWR1	NM 001014784	S	CATCTGCGCCCTCTATATCA	660-679	220
		A	GAAGTAAGAGATGCCGATGACC	858-879	
similar to NPBWR2	XM 230970	S	CAGCTCTCTTAGCCCAAGTGTAAAG	24-48	180
		A	TGGTTCAGGGCAAACACATTAGTTA	179-203	
HPRT	NM 012583	S	CAGTCAACGGGGGACATAAAAG	391-412	146
		A	ATTTTGGGGCTGTACTGCTTGA	515-536	
		probe	TGGTGGAG (22)	417-426	

Oligonucleotide sequences for sense (S) and antisense (A) primers are shown.

connective tissue was removed. Calvarias were then cut into small fragments, which were dissociated to cell suspensions by enzymatic digestion with 0.1% collagenase-I (Sigma-Aldrich) for 30 min at 37°C. ROB cells were harvested by centrifugation and resuspended in DMEM supplemented with NaHCO<sub>3</sub>, 6% fetal calf serum (FCS) and antibiotic-antimycotic solution. Cells were then plated in culture dishes (Nalge Nunc International, Denmark, 1x10<sup>4</sup> cells/dish), and cultured for 21 days at 37°C in an humidified atmosphere of 95% air - 5% CO<sub>2</sub>, medium was changed every other day (35). The experiments were performed on freshly isolated cells (QPCR only) and cells were harvested at days 7, 14 and 21 of culture. Before sampling, cells were maintained in medium without FCS for 48 h. In such medium, osteoblasts were exposed for 48 h to various concentrations of NPB and NPW.

**Characterization of ROB cell culture by double immunofluorescence microscopy.** Isolated osteoblasts were placed into LabTek™ Slide Chamber™ (Nalge Nunc International), and incubated as described above. Immunofluorescence was performed on ROB cells cultured for 14 days. Cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min, at room temperature. Then slides were washed 3 times for 5 min in PBS and subsequently preincubated in PBS with 5% bovine albumin for 30 min. In a humidified chamber they were incubated for 18 h in 4°C with primary antibodies against osteonectin and collagen 1α (Acris Antibodies GmbH, Germany) in concentrations of 1:400. After incubation, slides were washed in PBS 3 times for 5 min. As secondary antibodies goat anti-rabbit MFP488 (absorption wavelength, 503 nm; emission wavelength, 524 nm) and goat anti-mouse MFP590 (absorption wavelength, 597 nm; emission wavelength, 624 nm) (MoBiTech, Göttingen, Germany), respectively, in concentration of 1:1,000 (60 min, room temperature), were used. Slides were then washed in PBS 3 times for 5 min, coverslips were mounted with a drop of mounting medium for fluorescence with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Reactions were documented using Axiolmager Z1 microscope with ApoTome system (Carl Zeiss AG, Germany).

**Osteocalcin determination.** Osteocalcin concentrations in the culture medium were estimated using a rat osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA, USA). The sensitivity of the assay was 0.5 ng/ml, and inter- and intra-assay CVs, 7 and 4%, respectively.

**Cell proliferation.** Proliferation rate of ROB cells was measured by two methods. In the first method, the EZ4U Nonradioactive Cell Proliferation and Cytotoxic Assay kit (Biomedica, Vienna, Austria) was employed (36). Cultured cells were incubated for 90 min with EZ4U, and formazan derivatives production, which is linearly related to the cell number, was measured at 490 nm wavelength in a microplate autoreader EL-13 (Bio-Tek Instruments, Winooski, VT, USA). It is worthy to emphasize that EZ4U assay system is highly compatible with the standard <sup>3</sup>H-thymidine incorporation assay. In the second method, cell proliferation was studied using a colorimetric bromodeoxyuridine (BrdU) kit (5-Bromo-2'-deoxy-uridine Labeling and Detection Kit III, Roche Diagnostics). Cells were incubated with BrdU for 6 h and subsequently fixed with methanol (30 min, -20°C). After fixative removal, cells were incubated with nuclease (30 min, 37°C). After washing, cells were incubated with Anti BrdU-POD (30 min, 37°C). Cells were washed 3 times, incubated with substrate for 10 min and then extinction was measured at 490 nm.

**Conventional RT-PCR and QPCR.** Methods applied were described earlier (21,37-41). Applied primers for studies of NPB, NPW, NPBWR1 and similar to NPBWR2 gene expression (Table I) were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. The hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene was used as a reference to normalize data.

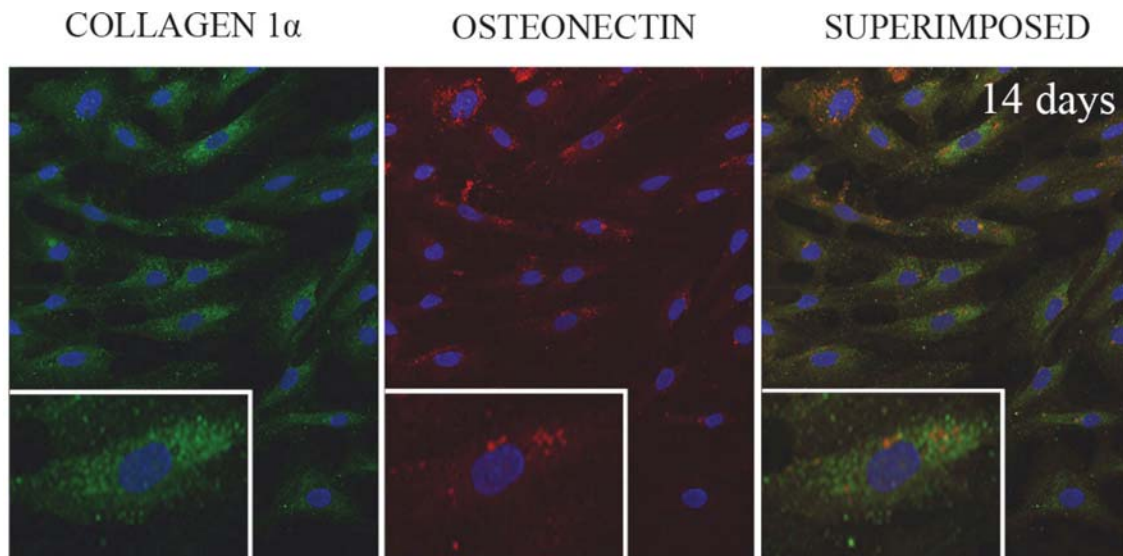


Figure 1. Double immunofluorescence microscopy illustrating the colocalization of collagen 1 $\alpha$  and osteonectin in cultured calvaria rat osteoblast-like cells during differentiation (day 14) stage. Cultures were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature and double labeled with primary antibodies against osteonectin and collagen 1 $\alpha$ , and followed by goat anti-rabbit MFP488 and goat anti-mouse MFP590 secondary antibodies. Coverslips were mounted in medium for fluorescence with DAPI. Reactions were documented using Axiomager Z1 microscope with ApoTome system. Collagen 1 $\alpha$ , green; osteonectin, red; nuclei, blue. Colocalization was determined by merging green and red pictures to give yellow/orange stained cells. Magnification  $\sim$ x400, inserts  $\sim$ x1000.

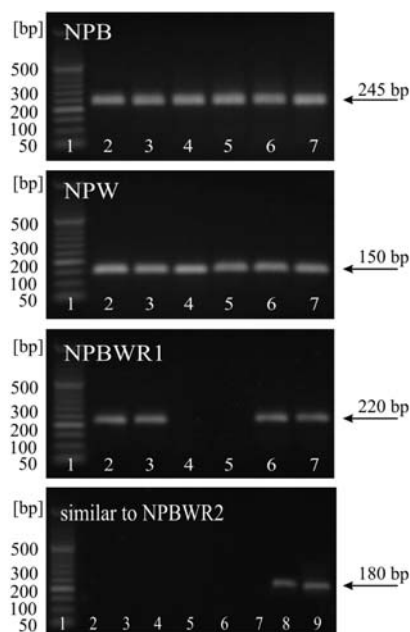


Figure 2. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat specific primer from RNA of ROB cells cultured for 7, 14 and 21 days. Lane 1, size marker (O'Range Ruler 50 bp DNA Ladder; MBI Fermentas, Lithuania); lanes 2 and 3, day 7; lanes 4 and 5, day 14; lanes 6 and 7, day 21. Lines 8 and 9, positive controls (adrenal cortex) for similar to NPBWR2 receptor. Reaction products with expected size are seen: NPB, 245 bp; NPW, 150 bp; NPBWR1, 220 bp; similar to NPBWR2, 180 bp.

**Statistics.** Data are expressed as the means  $\pm$ SEM and their statistical comparison was done by the unpaired Student's t-test.

## Results

As shown by double immunofluorescence microscopy, all cultured ROB cells contain collagen 1 $\alpha$  and osteonectin, typical

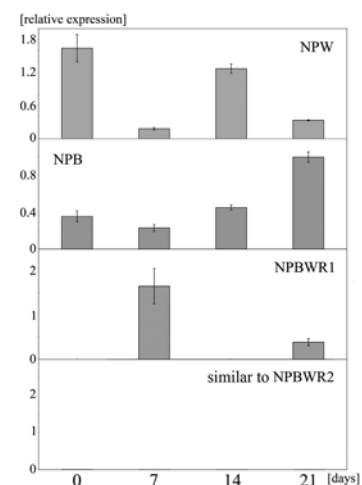


Figure 3. QPCR analyses of NPB, NPW, NPBWR1 and similar to NPBWR2 gene expression in the ROB cells at days 7, 14 and 21 of culture and in freshly isolated cells (day 0). All samples were amplified in triplicates, and HPRT gene expression was used as reference to normalize data. In each group n=3. Bars present means and SE is indicated.

markers of osteoblasts (Fig. 1). Such a pattern of immunofluorescence was observed at days 7, 14 and 21 of culture. Both peptides were mainly present in secretory granules, a number of which differed markedly among individual cells. As evidenced by merging green and red pictures (to give yellow/orange colour), both collagen 1 $\alpha$  and osteonectin express a very high degree of colocalization.

Classic RT-PCR technique revealed the presence of ppNPB and ppNPW mRNA at days 7, 14 and 21 of ROB cell culture (Fig. 2). NPBWR1 mRNA, on the other hand, was present at days 7 and 21 of culture, while similar to NPBWR2 mRNA was not observed. The similar to NPBWR2 mRNA was expressed in the rat adrenal cortex, an organ used as a positive

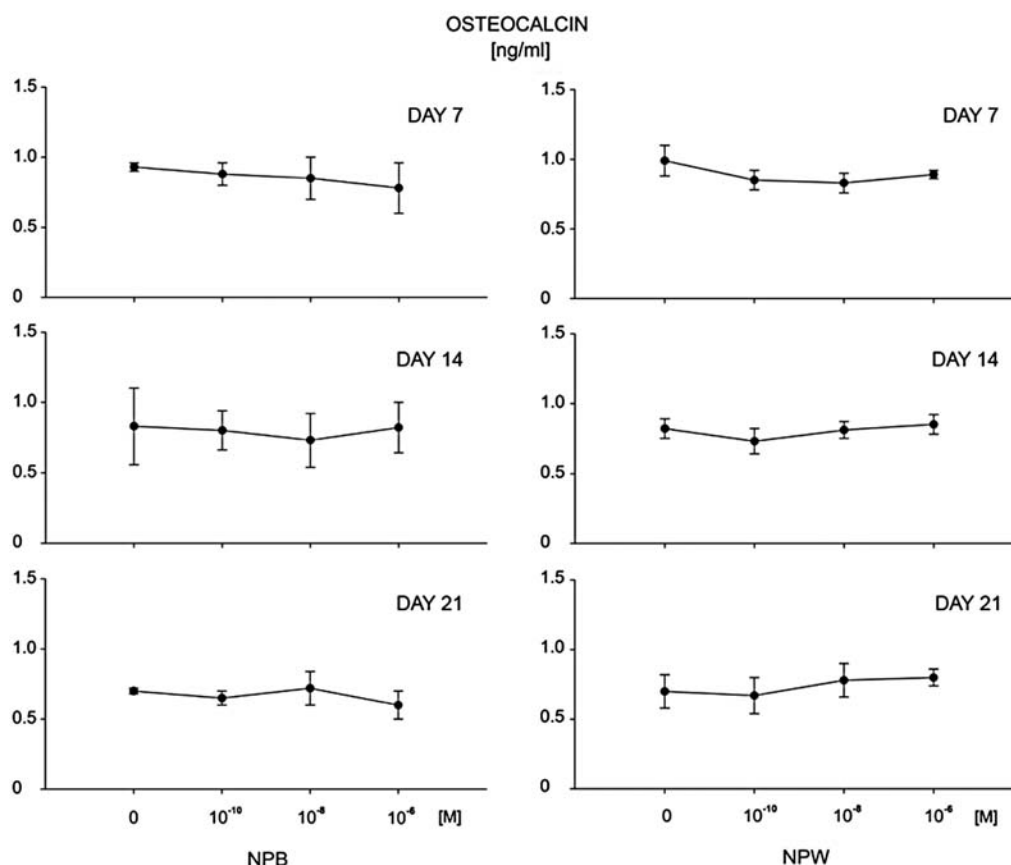


Figure 4. Effects of NPB and NPW on osteocalcin secretion by cultured ROB cells. Cells were exposed to tested compounds for 48 h in FCS stripped of medium. Neuropeptides were added to culture 48 h before medium collecting at days 7, 14 and 21. Data are expressed as means  $\pm$  SEM,  $n=6$ . Statistical comparisons (in relation to control) were done by the unpaired Student's *t*-test, all differences statistically nonsignificant.

control. With specific primers, RNA amplification by means of RT-PCR revealed the presence of reaction products with expected sizes: NPB, 245 bp; NPW, 150 bp; NPBWR1, 220 bp, and similar to NPBWR2, 180 bp.

In freshly isolated rat calvaria osteoblasts, QPCR revealed relatively high expression of NPB and NPW genes while expression of NPBWR1 and similar to NPBWR2 genes was not identified (Fig. 3). Expression of NPB gene increased with the time of culture and at day 21 reached  $\sim 2.5\times$  higher level than seen in freshly isolated cells. When compared with freshly isolated osteoblasts, NPW gene expression at days 7 and 21 of culture was notably lower while at day 14 was similar to that seen in freshly isolated cells. Relatively high expression of NPBWR1 gene appeared at day 7 of culture, lower at day 21, and expression was negligible at day 14. Expression of similar to NPBWR2 gene was not found throughout the entire period of isolation and culture of ROB cells.

To study whether NPB or NPW affect osteocalcin production, cultured ROB cells were exposed for 48 h to various concentrations of neuropeptides ( $10^{-10}$ - $10^{-6}$  M). Neither NPB nor NPW changed osteocalcin concentrations in media analyzed at days 7, 14 and 21 of culture (Fig. 4). On the other hand, NPW at the highest concentration applied ( $10^{-6}$  M) exerted a notable inhibitory effect on proliferative activity of ROB cells at days 7, 14 and 21 of culture, while NPB exerted a similar effect only at day 14 (Fig. 5). It should be emphasised that both, MTT and BrdU incorporation methods gave very similar results.

## Discussion

To our knowledge, the present study is the first to demonstrate the presence of ppNPB and ppNPW mRNA in freshly isolated and cultured rat calvaria osteoblast-like cells. In freshly isolated cells however, we did not find the presence of either NPBWR1 or the similar to NPBWR2 mRNA, and such a pattern of expression of both neuropeptides and their receptors in studied cells is puzzling. Furthermore, expression of either the NPB or NPW genes was found throughout the entire time of ROB cell culture, while NPBWR1 gene was expressed only in distinct periods of observations (days 7, proliferative stage and 21, mineralization stage) and that of the similar to NPBWR2 gene was absent.

As evidenced by double immunofluorescence microscopy, our studied cultures were composed exclusively of cells expressing collagen 1 $\alpha$  and osteonectin, standard markers of osteoblasts (30,43-46). In such cultures the reciprocal expression of NPW and NPBWR1 genes in freshly isolated and cultured ROB cells suggests an inverted interrelationship between the two genes. The studied osteoblast-like cells, high expression of the NPW gene at days 0 and 14 of the experiment was accompanied by very low or negligible expression of NPBWR1. This finding suggests a potent down-regulation of NPBWR1 by NPW. No such interrelationship was observed between the NPB and NPBWR1 genes expression. On the contrary, expression of the NPB gene gradually increased in the course of culture and was not related to expression of the remaining studied genes.



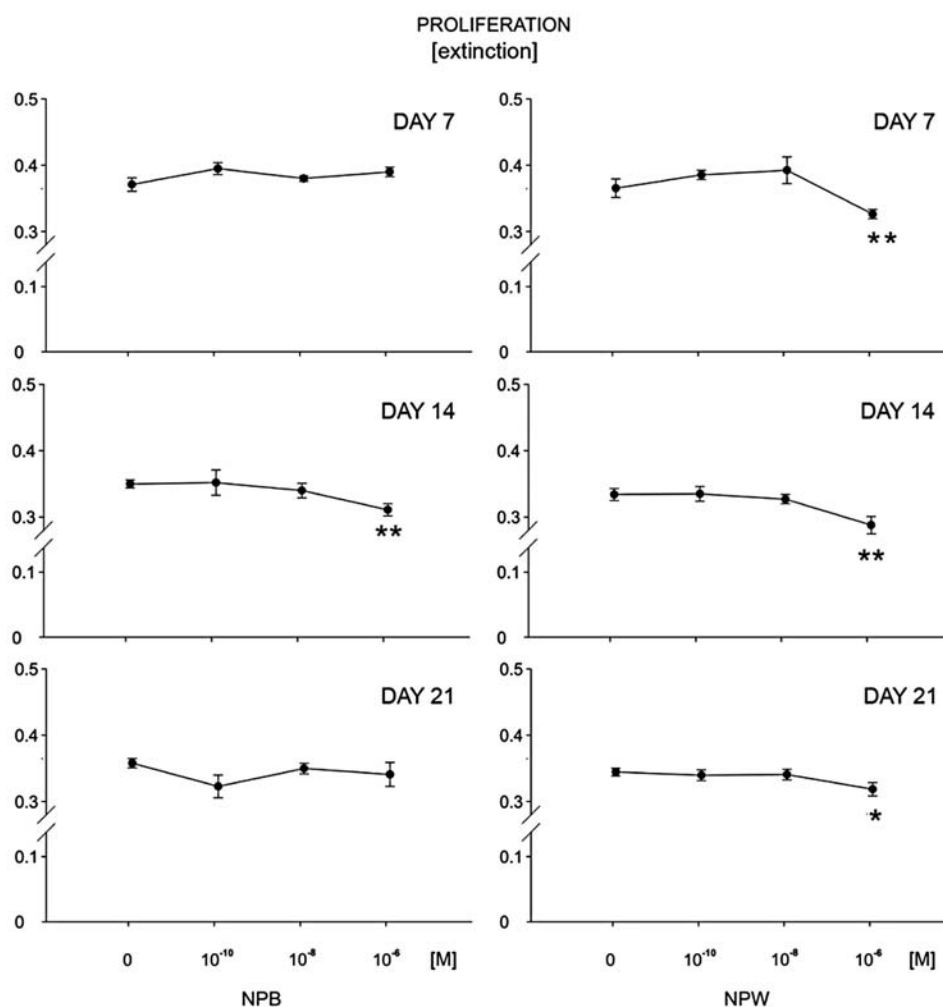


Figure 5. Effects of NPB and NPW on proliferative activity (extinction) of cultured ROB cells. BrdU incorporation method. Cells were exposed to tested compounds for 48 h in FCS stripped of medium. Neuropeptides were added to culture 48 h before medium collecting at days 7, 14 and 21. Data are expressed as means  $\pm$  SEM, n=6. Statistical comparisons (in relation to control) were done by the unpaired Student's t-test, \*p<0.05, \*\*p<0.02.

High structural homology of both neuropeptides (NPB and NPW) and their receptors (NPBWR1 and the similar to NPBWR2) suggests that each ligand and receptor are partially overlapping and also have specific roles in this signaling system. It is well documented that NPB activates and binds to both human NPBWR1, and with lower affinity to NPBWR2 (5,6). Likewise, NPW23 and NPW30 interact with those receptors, although with lower affinity. To test the possible roles of both neuropeptides in regulating ROB cell function, we exposed cultured cells for 48 h to various concentrations of NPB and NPW. In the FCS free culture medium both tested neuropeptides did not change osteocalcin secretion. Osteocalcin is one of the abundant noncollagenous matrix molecules in adult bone, and it is a specific marker for studying osteoblast differentiation (46,47). Thus, obtained data suggest that neither NPB nor NPW affects the specialized function (osteocalcin secretion) of cultured ROB cells. On the contrary, exposure to rather high NPW concentration ( $10^{-6}$  M) resulted in potent inhibition of ROB cell proliferative activity at days 7, 14 and 21, while a similar effect with NPB was found only at day 14. Thus, the obtained results suggest an inhibitory effect of NPW and NPB on the non-differentiated function of ROB cells, with NPW being more active. In this context it should be emphasised

that notable differences in NPW and NPB actions on functioning of various cells were observed in adrenocortical and pancreatic islet cells (20,23-25). The present data expand these earlier observations.

Concomitant expression of neuropeptides and their NPBWR1 receptor suggests that both NPB and NPW paracrinally modulate biological functions of ROB cells. In this context it is noteworthy that NPW30 is present in blood and in the systemic vein of the rat its concentration is  $\sim 0.42 \pm 0.7$  fmol/ml (7). This finding suggests that NPW also affects rat osteoblast functions via endocrine mechanism. However, we can not rule out the other possible mode of NPW and NPB action on osteoblasts *in vivo*.

Recent reports indicate that peptides involved in regulation of energy homeostasis affect both bone formation and remodelling. These compounds may act via endocrine, paracrine or autocrine routes, as well as through the central nervous system. This last mode of action is mediated via the sympathetic system and was demonstrated among others for leptin and neuromedin U (48-55). As mentioned previously, NPB and NPW also belong to neuropeptides involved in regulation of energy homeostasis. Icv NPB or NPW administration affects food intake and energy expenditure (4,6,14,16,18,19). In this

regard, targeted disruption of the GPR7 gene leads to metabolic defects and adult-onset obesity and similar effects have been observed in NPB-null mice (15,56). Moreover, icv administration of NPW stimulates plasma catecholamine concentrations in conscious rats and similar effects were observed at the adrenal medulla level (24,26,27). Thus, it cannot be excluded that *in vivo* both NPB and NPW may also affect bone cell activity acting via the central nervous system.

In conclusion, our results suggest that the system of NPW, NPB and NPBWR1 directly regulates proliferative activity of cultured rat calvaria osteoblast-like cells. The physiological significance of this osteoblastic system remains unclear, and requires further investigation.

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