Neuromedin U enhances proliferation of ACTH-stimulated adrenocortical cells in the rat

MARCIN TREJTER[1], DIEGO GUIDOLIN[2], MAGDALENA NOWAK[1], VERONICA MACCHI[2], MARIOLA MAJCHRZAK[1], RAFFAELE DE CARO[2] and LUDWIK K. MALENDOWICZ[1]

[1]Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan PL-60781, Poland; [2]Department of Human Anatomy and Physiology, Section of Anatomy, University of Padua, Padua I-35121, Italy

Received September 5, 2008; Accepted November 6, 2008

DOI: 10.3892/ijmm_00000106

Abstract. Neuromedin U (NMU) is a brain-gut peptide involved in the regulation of the hypothalamic-pituitary-adrenal axis and adrenocortical cell proliferation. In this study, we investigated the effects of NMU8 (three subcutaneous injections of 6.0 nmol/100 g, 24, 16 and 8 h before autopsy) on the adrenal glands of rats treated for 2 or 4 days with a low (2 μg/100 g body weight/24 h) or a high (8 μg) dose of adrenocorticotropic hormone (ACTH). As revealed by RT-PCR, ACTH treatment did not prevent expression of NMUR1 in rat adrenal cortex. At day 4 of ACTH administration, the weight of adrenals was lower than at day 2. NMU8 administration prevented ACTH-induced increases of adrenal weight at day 2 of the experiment. ACTH plasma concentrations were increased in all ACTH-administered rats. NMU8 administration increased ACTH plasma concentration at day 2 of the lower ACTH dose-treated group while it reduced the ACTH plasma level at day 4 in the higher ACTH dose-administered rats. In all groups of ACTH-treated rats, NMU8 changed neither aldosterone nor corticosterone plasma concentrations. In the zona glomerulosa (ZG), NMU8 increased metaphase index at days 2 and 4 in the lower ACTH dose-treated group and had no statistically significant effect in rats treated with the higher ACTH dose. In the zona fasciculata (ZF), NMU8 administration increased metaphase index at day 2 in the lower ACTH dose-treated group but reduced metaphase index at day 4 in the higher dose ACTH-administered rats. NMU8 reduced the number of cells per unit area both in ZG and ZF at day 2 in the higher ACTH dose-treated rats. In the remaining groups NMU8 did not produce statistically significant changes in the number of cells per unit area. Thus, our findings demonstrate that exogenous NMU may stimulate proliferation primarily of the cortical ZG cells in rats administered with ACTH, although at high doses of exogenous corticotropin an opposite effect occurred.

Correspondence to: Professor L.K. Malendowicz, Department of Histology and Embryology, Poznan University of Medical Sciences, 6 Swiecicki St., Poznan PL-60781, Poland
E-mail: lkm@amp.edu.pl

Key words: neuromedin U, adrenocorticotropic hormone, corticosterone, aldosterone, adrenal growth, proliferative activity

Introduction

Neuromedin U (NMU), neuromedin S (NMS) and NMU receptors (the peripheral NMUR1 and the central NMUR2) are involved in the regulation of a variety of physiological systems (reviewed in ref. 1) and play a pivotal role in the regulation of the hypothalamic-pituitary-adrenal axis (HPA) (1-15). Recent data indicate that NMU is also involved in regulation of adrenocortical growth.

The main mechanisms responsible for adrenocortical growth *in vivo* are hyperplasia and hypertrophy, however a variety of endocrine and neural inputs may be involved in adrenal enlargement. Dallman (16) distinguishes four types of adrenal cortex growth and each of them is differentially regulated. Enucleation-induced adrenocortical regeneration resembles growth of the gland at early embryogenesis and depends on several neural and endocrine signals (17-21), while unilateral adrenalectomy-induced compensatory growth is primarily neurally mediated (16,22-25). Adrenocortical growth related to maturation is probably regulated by numerous growth factors while adrenocorticotropic hormone (ACTH)-induced growth primarily depends on adrenocortical cell proliferation (16,26). Recently, we demonstrated the stimulating effects of exogenous NMU on enucleation-induced adrenocortical regeneration (27), and on proliferation of cultured rat adrenocortical cells (28). On the contrary, this neuropeptide was found not to interfere with adrenocortical growth related to maturation (28) and was observed to inhibit unilateral adrenalectomy-induced compensatory adrenal growth (29). Therefore, the present study was undertaken to investigate the effects of NMU on ACTH-induced adrenal growth in rats.

Materials and methods

Animals and reagents. Female Wistar rats (final body weight 120-130 g) were kept under a 14:10 h light-dark cycle (illumination onset at 6:00 a.m.) at 23˚C, and maintained on a standard diet and tap water *ad libitum*. The study protocol was

approved by the Local Ethics Committee for Animal Studies. NMU8 was purchased from Bachem Feinchemikalien AG (Bubbendorf, Switzerland), ACTH (Synacthen depot) from Ciba (Basle, Switzerland), and vincristine from Gedeon-Richter (Budapest, Hungary). All other chemicals and reagents were provided by Sigma-Aldrich or POCh (Gliwice, Poland).

Experimental design. Groups of rats (n=8) were administered with subcutaneous ACTH injections of 2 or 8 μg/100 g body weight/24 h, for 2 or 4 days. Four appropriate groups were administered 3 subcutaneous injections (24, 16 and 8 h before sacrifice) of 6.0 nmol/100 g body weight of NMU8, dissolved in 0.2 ml 0.9% saline. The other four groups (control rats) received subcutaneous injection of the vehicle. Three hours before sacrifice all rats were administered with an intra-peritoneal (i.p.) injection of 0.1 mg/100 g body weight of vincristine. The animals were decapitated at 11:00 a.m., and their trunk blood was collected in the presence of EDTA (1 mg/ml). Plasma was separated and stored at -36˚C for hormone assay. Adrenals were removed, weighed, fixed in Bouin's solution and embedded in paraffin for metaphase-index assay and morphometric estimations.

Metaphase index and morphometry. Sections (6 μm) were stained with hematoxylin and eosin and metaphase index (number of vincristine-arrested metaphase cells per 1,000 cells) was calculated at a magnification of x400, by counting 5,000 cells in the zona glomerulosa (ZG) or zona fasciculata (ZF) of the adrenal gland (30-32). The number of nuclei of adrenocortical cells was counted at a magnification of x400 in 50 fields (area of one field: 0.003 mm^2) of each adrenal, and the number of nuclei per mm^2 was calculated.

NMUR1 gene expression (RT-PCR). NMUR1 gene expression in the adrenal cortex was examined as detailed previously (27-29). The following primers were used: sense (481-500), 5'-GCCATCTGGGTCTTCGCTAT-3' and antisense (797-816), 5'-CACCTGTCTGCGTTCCCTAT-3' (336 bp; accession number, AF242873). The primers were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

Hormone assay. Plasma ACTH concentrations were measured by RIA, using a commercial kit purchased from Diagnostic Product Co., Los Angeles, CA, USA, as previously detailed (27,28,33,34). ACTH RIA: sensitivity 8 pg/ml, intra- and inter-assay CVs 7 and 9%, respectively. Cross-reactivity: ACTH (1-24), 100%; α-MSH, 0.3%; the remaining pituitary hormones, <0.001%.

Aldosterone and corticosterone were extracted from plasma and their concentrations were measured by RIA, using [1,2,6,7-^{3}H]-aldosterone and [1,2,6,7-^{3}H]-corticosterone (Amersham, UK; S.A.; 1.96 Tbq/mmol) and antisera developed in rabbit (Sigma, St. Louis, MO, USA) (19,27,28,33,34). Aldosterone RIA sensitivity was 5 pg/ml and the cross reactivity was: aldosterone, 100%; 17-iso-aldosterone and other steroids, <0.1%. Intra- and inter-assay variations amounted to 5 and 7%, respectively. Corticosterone RIA sensitivity was 50 pg/ml and cross-reactivity was: corticosterone and cortisol, 100%; 11-deoxy-corticosterone and progesterone, 2%, other steroids, <0.001%. Intra- and inter-assay variations amounted to 7 and 9%, respectively.

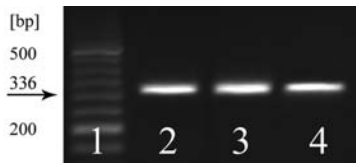

Figure 1. Expression of the neuromedin U receptor 1 (NMUR1) gene in the adrenal cortex of ACTH-treated rats. PCR products were separated by electrophoresis in 1.2% agarose gel and stained with ethidium bromide. Amplification by means of RT-PCR with specific primer RNA revealed the presence of reaction products with the expected size of 336 bp. Lanes: 1, marker; 2. adrenal cortex of intact rat; 3, adrenal cortex of rat treated for 2 days with ACTH (8 μg/100 g/day); 4, adrenal cortex of rat treated for 4 days with ACTH (8 μg/100 g/day).

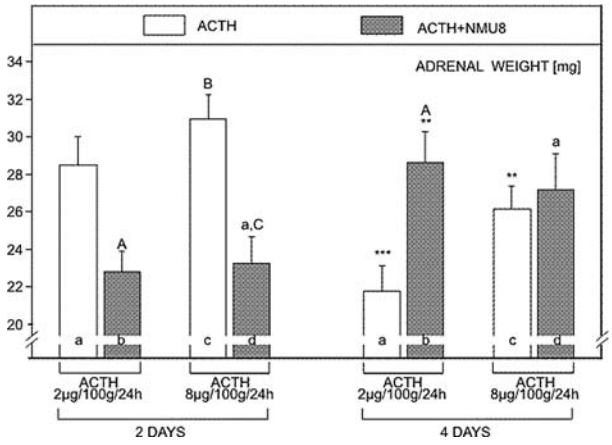

Figure 2. Effects of neuromedin U8 (NMU8) (6 nmol/100 g body weight/per injection) applied 24, 16 and 8 h prior to autopsy, on adrenal weight of mature female rats treated with ACTH (2 or 8 μg/100 g/day) for 2 or 4 days. Three hours before autopsy the rats were i.p. injected with vincristine at the dose of 0.1 mg/100 g. Bars represent means and ±SE. Each group n=8. Letters in bars (a,b,c,d) indicate appropriate group for statistical comparisons. Statistical comparison of differences by Duncan's multiple range test: as compared to the indicated group at the same day the differences are significant at: lower cases $p<0.05$; capitals $p<0.01$. Comparison with the analogous group at day 2 of the experiment using Student's t test: the differences are significant at: $^{*}p<0.05$; $^{**}p<0.02$; $^{***}p<0.01$; $^{****}p<0.001$.

Statistics. Data are expressed as means ± SEM and the statistical significance of the differences among the experimental groups was estimated using ANOVA, followed by Duncan's multiple range tests and the unpaired Student's t-test.

Results

RT-PCR study revealed the presence of NMUR1 mRNA in adrenal cortex of rats treated with ACTH for 2 or 4 days, as well as in the glands of intact animals (Fig. 1). Amplification by means of RT-PCR using specific primer RNA revealed the presence of reaction products with an expected size of 336 bp.

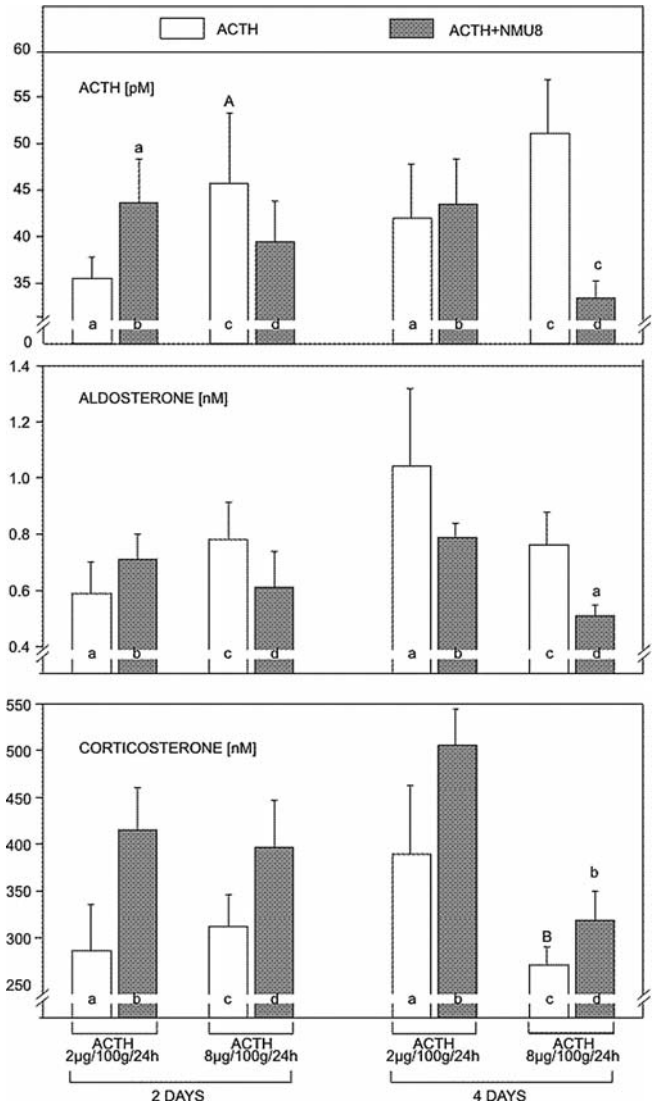

Figure 3. Effects of neuromedin U8 (NMU8) (6 nmol/100 g body weight/per injection) applied 24, 16 and 8 h prior to autopsy, on plasma ACTH (pM); aldosterone (nM) and corticosterone (nM) levels, in mature female rats treated with ACTH (2 or 8 μg/100 g/day) for 2 or 4 days. Three hours before autopsy the rats were i.p. injected with vincristine at the dose of 0.1 mg/100 g. Detailed explanations are provided in Fig. 2. The basal plasma ACTH concentration in the rat was 15-20 pM.

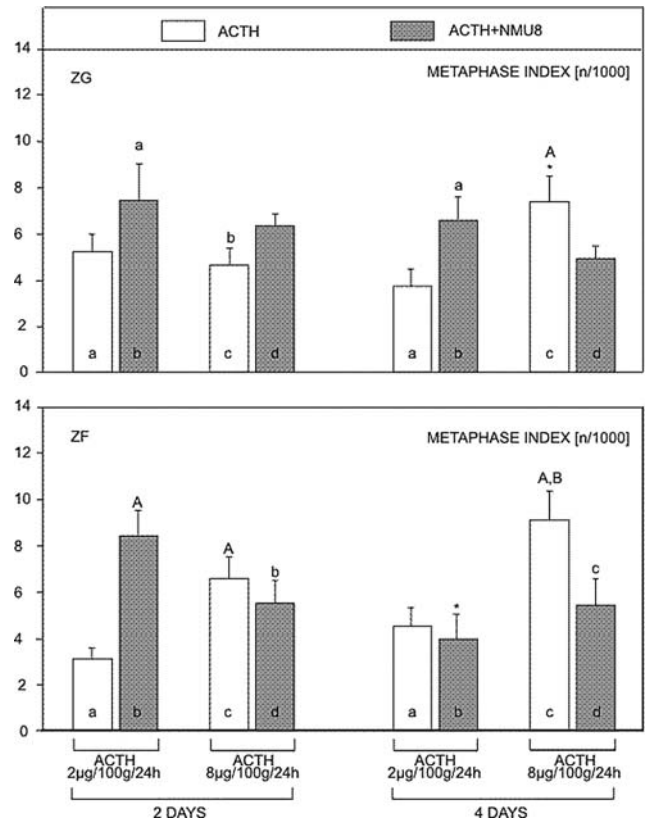

Figure 4. Effects of neuromedin U8 (NMU8) (6 nmol/100 g body weight/per injection) applied 24, 16 and 8 h prior to autopsy, on adrenal metaphase index (n/1000) in zona glomerulosa (ZG) and zona fasciculata (ZF) of mature female rats treated with ACTH (2 or 8 μg/100 g/day) for 2 or 4 days. Three hours before autopsy the rats were i.p. injected with vincristine at the dose of 0.1 mg/100 g. Detailed explanations are provided in Fig. 2.

As shown in Fig. 2, the adrenal weight at day 4 of the experiment for both lower and higher dose ACTH-treated rats was lower than at day 2. NMU8 administration prevented ACTH-induced increases of adrenal weight at day 2 in both the lower and the higher ACTH dose-treated rats. On the contrary, NMU8 further increased adrenal weight at day 4 in the lower ACTH dose-treated animals and did not affect adrenal weight at day 4 in rats administered with the higher dose of ACTH.

ACTH plasma concentrations were increased in all ACTH-administered rats; basal ACTH plasma levels being 15-20 pM in rats receiving no ACTH treatment. NMU8 administration increased ACTH plasma concentration at day 2 in the lower ACTH dose-treated group but reduced ACTH plasma level at day 4 in the higher ACTH dose-administered rats. In all groups of ACTH-treated rats NMU8 did not change aldosterone and corticosterone plasma concentrations (Fig. 3).

In the ZG, NMU8 increased metaphase index at days 2 and 4 in the lower ACTH dose-treated group, and had no statistically significant effect in rats treated with the higher ACTH dose. In the ZF, NMU8 administration increased metaphase index at day 2 in the lower ACTH dose-treated group but reduced metaphase index at day 4 in the higher ACTH dose-administered rats (Fig. 4). NMU8 reduced the number of cells per unit area both in ZG and ZF at day 2 in the higher ACTH dose-treated rats. In the remaining groups, NMU8 did not produce statistically significant changes in the number of cells per unit area (Fig. 5).

Discussion

Within the HPA axis, individual components of NMU, NMS and NMU receptors system show rather tissue-specific expression. In the hypothalamus, NMS and NMUR2 genes are highly expressed; in adenohypophysis, NMU, NMUR1 and NMUR2 (at a relatively low level) are present while in the adrenal cortex and medulla only NMUR1 gene expression is found (10,35-48). Within the HPA axis, NMU stimulates CRH synthesis and secretion, enhances pituitary ACTH and adrenal corticosterone and aldosterone secretion. Furthermore,

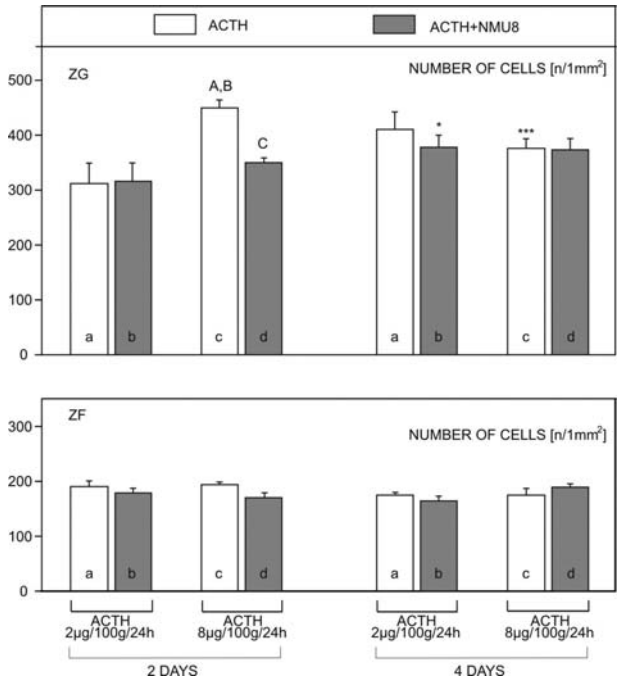

Figure 5. Effects of neuromedin U8 (NMU8) (6 nmol/100 g body weight/per injection) applied 24, 16 and 8 h prior to autopsy, on number of nuclei of adrenocortical cells per unit area (n/1 mm^2) in zona glomerulosa (ZG) and zona fasciculata (ZF) of mature female rats treated with ACTH (2 or 8 μg/100 g/day) for 2 or 4 days. Three hours before autopsy the rats were i.p. injected with vincristine at the dose of 0.1 mg/100 g. Detailed explanations are provided in Fig. 2.

NMU directly affects adrenocortical cell steroidogenesis, an effect probably dependent on the presence of the CRH-ACTH system of the adrenal medulla (1-15,28).

This study aimed to investigate whether NMU affects ACTH-induced adrenocortical growth rats. Earlier we found NMUR1 mRNA in the rat adrenal cortex, while NMU, NMS and NMUR2 mRNAs were not found (48). In this study we found that adrenal cortex of ACTH-treated rats was also provided with NMUR1 mRNA.

It is well documented that *in vivo* ACTH administration or its hypersecretion causes primarily adrenocortical cellular hypertrophy, which is subsequently followed by hyperplasia (16,31,49-57). On the contrary, the direct *in vitro* effect of ACTH involves inhibition of adrenocortical cell proliferation and stimulation of the cell specialized functions, that is corticosterone secretion (28,34,58-60).

Our observations revealed that after 2 days of ACTH administration the adrenal gland weight was heavier than after 4 days of treatment with ACTH. As is known, the adrenal cortex is an endocrine tissue with a rich vascular supply and ACTH results in a potent stimulation of adrenal blood flow (61-64). ACTH-induced increased adrenal blood flow is transient and, therefore, the changes in the weight of adrenals may be connected with altered response of the cortical vasculature to chronically elevated blood corticotropin levels. In ACTH-treated rats, NMU8 either reduced (at day 2 in both the lower and the higher ACTH dose-treatment groups) or increased (at day 4 in the lower ACTH dose-administered rats) the adrenal weight. The above discussed NMU-evoked changes in the adrenal weights may be connected with the polypeptide effects on adrenal vasculature or its stimulatory action on a sympathetic nervous system (65-67).

Effects of NMU8 on ACTH plasma concentrations varied depending on ACTH doses and days of treatment. NMU8 increased plasma corticotropin concentrations at day 2 in the lower ACTH dose-treated rats while it reduced the ACTH level at day 4 in the higher ACTH dose-administered animals. Moreover, plasma aldosterone and corticosterone levels of ACTH-treated rats were not significantly altered by NMU8 injections. Despite the lack of major alterations in hormone levels, NMU8 exerted a stimulating effect on proliferative activity of rat adrenocortical cells. Such a proliferative response was observed in the ZG at days 2 and 4 in the lower ACTH dose-treated animals and in the ZF at day 2 in the lower ACTH dose-administered rats. The opposite effect was found in the ZF at day 4 in the higher ACTH dose-treated rats. Thus, the stimulating effects of NMU8 on ZG cell proliferation in ACTH-treated rats was evident at days 2 and 4 of the experiment and in animals administered with the lower ACTH dose (2 μg/100 g body weight/24 h) while such a response was not observed in rats administered with the higher ACTH doses. Changes in the rate of ZG cell proliferative activity in ACTH-NMU8-treated rats were not paralleled by changes in plasma ACTH concentrations. These findings suggest that the stimulating effect of NMU8 on ZG cell proliferation in the rat is independent of ACTH action on the adrenal cortex. However, large corticotropin doses have appeared to prevent the stimulating effects of NMU8 on mitotic activity in the cortical ZG. It remains to be established whether these differences depend on altered sensitivity of cells in rats administered with different doses of ACTH. Also we cannot exclude the possibility that these changes may be dependent on activation of local compensatory mechanisms in rats treated with high ACTH doses. The stimulating effect of NMU8 on ZG cells in ACTH-treated rats was similar to that evoked by ACTH alone. ZG of adrenal cortex is a proliferative region provided with stem cells, and newly formed cells from that region differentiate and migrate centripetally through the ZF into the zona reticularis (ZR), where they undergo apoptosis (31,51-53,56,68-74).

As shown by the number of nuclei profile of adrenocortical cells per unit area, enhanced proliferative activity of the ZG cells of ACTH- and NMU-treated rats was not accompanied by cellular hyperplasia. Thus, our present findings demonstrate that exogenous NMU may stimulate primarily proliferation of the cortical ZG cells in ACTH-administered rats, although at high doses of exogenous corticotropin an opposite effect occurred.

In conclusion, our recent studies reveal that NMU exerts a potent and complex modulatory effect on the proliferative activity of rat adrenal cortex. In primary adrenocortical cell cultures NMU directly stimulates proliferation of cells, while *in vivo* immature rat adrenocortical cells do not respond to NMU administration (28). NMU also exerts a potent proliferogenic effect on enucleation-induced adrenal growth (27) and, as documented by present results, on ACTH-induced adrenocortical growth. On the contrary, NMU exerts a potent inhibitory effect on unilateral adrenalectomy-induced compensatory adrenal growth (29). Accumulatively, our data suggest that NMU effects on proliferation of adrenocortical

cells *in vivo* are mediated neither by adrenal medulla nor by alteration in blood ACTH concentrations, which are not modified in compensatory adrenal growth and adrenocortical regeneration. Conversely, it could be hypothesized that *in vivo* NMU effects on proliferative activity of rat adrenocortical cells may be mediated primarily via the nervous system.

Acknowledgements

This study was conducted in partial fullfilment of the PhD thesis requirement for Dr Marcin Trejter of the Poznan University of Medical Sciences, Poland.

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