# Neuropeptide Y as a presynaptic modulator of norepinephrine release from the sympathetic nerve fibers in the pig pineal gland

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## Abstract

Norepinephrine (NE) released from the sympathetic nerve endings is the main neurotransmitter controlling melatonin synthesis in the mammalian pineal gland. Although neuropeptide Y (NPY) co-exists with NE in the pineal sympathetic nerve fibers it also occurs in a population of non-adrenergic nerve fibers located in this gland. The role of NPY in pineal physiology is still enigmatic. The present study characterizes the effect of NPY on the depolarization-evoked $^3$H-NE release from the pig pineal explants.

The explants of the pig pineal gland were loaded with $^3$H-NE in the presence of pargyline and superfused with Tyrode medium. They were exposed twice to the modified Tyrode medium containing 60 mM of $K^+$ to evoke the $^3$H-NE release via depolarization. NPY, specific agonists of $Y_1$- and $Y_2$-receptors and pharmacologically active ligands of $\alpha_2$-adrenoceptors were added to the medium before and during the second depolarization. The radioactivity was measured in medium fractions collected every 2 minutes during the superfusion.

NPY (0.1 – 10 μM) significantly decreased the depolarization-induced $^3$H-NE release. Similar effect was observed after the treatment with $Y_2$-agonist: $NPY_{13-36}$, but not with $Y_1$-agonist: $[Leu^{31},Pro^{34}]$-NPY. The tritium overflow was lower in the explants exposed to the 5 μM NPY and 1 μM rauwolscine than to rauwolscine only. The effects of 5 μM NPY and 0.05 μM UK 14,304 on the depolarization-evoked $^3$H-NE release were additive.

The results show that NPY is involved in the regulation of NE release from the sympathetic terminals in the pig pineal gland, inhibiting this process via $Y_2$-receptors.

**Key words**: pig, pineal gland, sympathetic nervous system, neuropeptide Y, norepinephrine

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## Introduction

The mammalian pineal gland plays, via the rhythmic secretion of melatonin, an important role in synchronization of several body functions with the environmental light-dark cycle (Pevet 2000, Simonneaux and Ribelayga 2003). Moreover, melatonin, due to its physicochemical properties and various modes of action on target cells, is also involved in numerous processes, which are not considered as closely related to the circadian rhythmicity, such as the protection against free radicals, ossification or tumor formation (Garcia et al. 2014, Jardim-Perassi et al. 2014, Wang et al. 2014).

The sympathetic input is crucial for the precise regulation of rhythmic melatonin secretion in the mammalian pineal gland, as it constitutes the last step of multisynaptic pathway connecting this organ with the suprachiasmatic nucleus and the retina (Drijfhout et al. 1996a, Larsen et al. 1998). In addition to the sympathetic innervation, the pineal gland receives also nerve fibers from other peripheral ganglia and from the brain (Mzller et al. 1996, 1999, Matsushima et al. 1999).

Among neurotransmitters occurring in the pineal nerve endings, norepinephrine (NE) and neuropeptide Y (NPY) are present in the highest amounts (Williams et al. 1989, Møller 1990, Møller et al. 1990, 1996, 1998, 2014, Zhang et al. 1991, Cozzi et al. 1992, Olcese 1994, Reuss and Olcese 1995, Drijfhout et al. 1996a, Mikkelsen and Møller 1999, Simonneaux and Ribelayga 2003). Both substances co-exist in the sympathetic nerve fibers, but NPY also occurs in a small population of non-sympathetic fibers, probably of the central origin (Hakanson et al. 1986, Zhang et al. 1991, Møller et al., 1996, Møller 1999). Unquestionably, NE is the essential factor stimulating the secretory activity of pinealocytes in all mammals, however, the mechanisms of adrenergic regulation of melatonin synthesis exhibit strong species heterogeneity (Coon et al. 1995, Schomerus 2000, Stehle et al. 2001, Simonneaux and Ribelayga 2003, Lewczuk et al. 2005, 2014, Chattoraj et al. 2009). In contrast to NE, the role of NPY in the pineal gland physiology is poorly recognized, since the obtained experimental data are not univocal (Vacas et al. 1987, Olcese 1991, Harada et al. 1992, Simonneaux et al. 1994a,b, Ribelayga et al. 1998).

The pineal gland of the domestic pig, which is the subject of the present study, shows several species-specific morphological features (Wyrzykowski et al. 1987, Lewczuk et al. 1994). Pinealocytes in the pig distinguish from these cells in other mammalian species by the presence of numerous cytoplasmic dense bodies called MBB (Wyrzykowski et al. 1987).

The morphology and relative volume of MBB change in different physiological stages (Wyrzykowski et al. 1987, Przybylska et al. 1990) and under experimental conditions (Lewczuk and Przybylska-Gornowicz 1997a, 2000b). Also, the specificity of pig pineal physiology is well documented (Lewczuk and Przybylska-Gornowicz 1997b, 2000a). The species-characteristic features include the specific pattern of plasma melatonin rhythm (Lewczuk and Przybylska-Gornowicz 1997b, 2000a) and the unique mechanism of adrenergic stimulation of melatonin synthesis (Lewczuk 2002b).

The pig pineal is richly innervated by the nerve fibers showing immunoreactivity to NPY (Kaleczyc et al. 1994, Przybylska-Gornowicz et al. 1995, Bulc et al. 2013). These fibers are located in the capsule and connective septa of the organ as well as in the parenchyma, where they form a network of variable density. The majority of NPY-positive fibers demonstrate immunoreactivity to catecholamine-synthesizing enzymes: tyrosine hydroxylase (TH) and dopamine-β-hydroxylase, therefore they could be classified as belonging to the sympathetic innervation. However, some NPY-positive and TH-negative fibers are present in the rostral part of the gland and in the stalk, and they are probably of the central origin. In contrast to the rodents, the well-developed sympathetic innervation of the pineal gland was observed in 70- and 90-day-old pig fetuses (Bulc et al. 2013). The density of these fibers increases during the first 20-days of postnatal development (Przybylska-Gornowicz et al. 1995). The study of C-terminal flanking peptide of neuropeptide Y (CPON), which is one of the three peptides formed by the proteolytic cleavage of the preproNPY molecule, showed the presence of dense network of CPON-immunoreactive nerve fibers in the pig pineal gland. The distribution pattern of these fibers is similar to the NPY-positive fibers localization (Przybylska-Gornowicz et al. 1997). It also should be noted, that besides the numerous sympathetic nerve fibers, the pig pineal gland also contains the moderate number of fibers showing immunereactivity to somatostatin (Przybylska-Gornowicz et al. 2000a), substance P (Przybylska-Gornowicz et al. 2000b) as well as vasoactive intestinal peptide and peptide histidine isoleucine (Nowicki et al. 2007). In addition, sparse vasopressin-positive nerve fibers were also demonstrated in pineal gland of this species (Przybylska-Gornowicz et al. 2002).

The above mentioned results demonstrate that NPY is the second, after NE, the most abundant neurotransmitter in the pig pineal gland. The functional significance of this peptide was not yet investigated in the pig pineal. The experiments performed on the rat pineal suggest the role of NPY in the modulation of

noradrenergic transmission (Vacas 1987, Simonneaux et al. 1994a,b).

The aim of the present study was to determine the effect of NPY on NE release from the sympathetic nerve fibers in the pig pineal gland.

## Materials and Methods

### Chemicals

The drugs were purchased as follows: UK 14,304 and rauwolscine hydrochloride from Tocris Cookson Ltd (UK); aprotinin, pargyline hydrochloride, desipramine hydrochloride, and ascorbic acid from SIGMA (USA); NPY, $NPY_{13-36}$ peptide and NPY[$Leu^{31}$, $Pro^{34}$] peptide from Peninsula Laboratories (UK). Levo-[7-$^3$H]-norepinephrine (spec. act. 15Ci/mmol) was obtained from NEN Life Sciences Products (USA). Tissue Solubiliser and DMSO – from MP Biomedicals (USA). All other used chemicals were of analytical grade. Tyrode medium consist of NaCl 137mM, KCl 2.68mM, $CaCl_2$ 1.78mM, $MgCl_2$ 1.04mM, $NaH_2PO4$ 0.42mM, $NaHCO_3$ 11.9mM, $Na_2EDTA$ 0.069mM, ascorbic acid 0.061mM and glucose 5.55mM. Peptide solutions were prepared in Tyrode medium with aprotinin. Stock solution (2mM) of UK 14,304 was prepared in DMSO.

### Animals and tissues

Investigations were performed during spring in Poland. Female crossbred pigs (3.5 months of age, 35 kg of body weight, n=19) were purchased from a commercial piggery, 7-14 days before the experiments started. Animals were kept in a room, in which natural lighting from windows was supplemented between 06:00 and 20:00 with fluorescent illumination, providing a 500 lx light intensity at the level of animal heads. The gilts had free access to standard food and water. The pigs were euthanized at 17:00. The pineal glands were removed 3-5 minutes after the heart stopped beating, cleaned of adherent tissue, then immediately used in the experiments. The protocol for animal experiments was approved by the Local Ethical Commission in Olsztyn, Poland.

### Experimental protocols

The pineal glands were divided into three pieces. The explants were incubated for 60 minutes in Tyrode medium containing $^3$H-norepinephrine (0.5 μM) and pargyline (100 μM) in a humidified atmosphere of 80% $O_2$ and 5% $CO_2$. Then, the pieces were flushed with Tyrode medium, placed into a nylon net and transferred to separate perfusion chambers (chamber volume – 0.5 ml, total volume of perfusion set – ca 1.4 ml). Tyrode medium containing desipramine (10 μM) was perfused at a flow rate of 0.4 ml/min. The medium was gased with a mixture of 95% $O_2$ and 5% $CO_2$. The medium and chambers were maintained at 37.5°C. Medium fractions were collected every 2 minutes. After perfusion the pieces were digested with Tissue Solubiliser. The content of $^3$H in the medium and the tissue pieces was measured using a liquid scintillation method.

The explants preloaded with $^3$H-norepinephrine were exposed twice (after 110 minutes of perfusion – S1 and 180 minutes of perfusion – S2) to the modified Tyrode medium containing 60 mM of $K^+$ for 14 minutes. Twenty minutes before and during the second stimulation the following drugs were added to the medium: experiment I: NPY at concentrations 0.01-10 μM; experiment II and III: $Y_1$-receptor agonist – $NPY_{13-36}$, or $Y_2$-receptor agonist – NPY [$Leu^{31}$, $Pro^{34}$] at concentrations 0.01-1 μM; experiment IV: $\alpha_2$-antagonist – rauwolscine (1 μM), NPY (5 μM) or rauwolscine (1 μM) and NPY (5 μM); experiment V: $\alpha_2$-agonist – UK 14,304 (0.05 μM), NPY (5 μM) or UK 14,304 (0.05 μM) and NPY (5 μM); and experiment VI: UK 14,304 (1 μM), NPY (5 μM) or UK 14,304 (1 μM) and NPY (5 μM). The control explants were treated with medium containing vehiculum and aprotinin (100 μg/ml).

### Data analysis

The fractional tritium release rate was calculated as the amount of radioactivity released into the fraction over the total radioactivity present in the tissue at the start of this fraction collection. The high potassium-evoked tritium overflow (S2 and S1) was calculated by subtraction of the basal release (estimated as the mean fractional release during 10 minutes before stimulation) from each fractional release value during 30 minutes after the start of perfusion with the high $K^+$ Tyrode medium and then by summing the obtained differences. The ratios of S2/S1 were calculated for the control and experimental explants. Additionally, the levels of mean basal tritium release during 10 minutes before the first and the second depolarization were compared for the determination of drug action on spontaneous release of tritium.

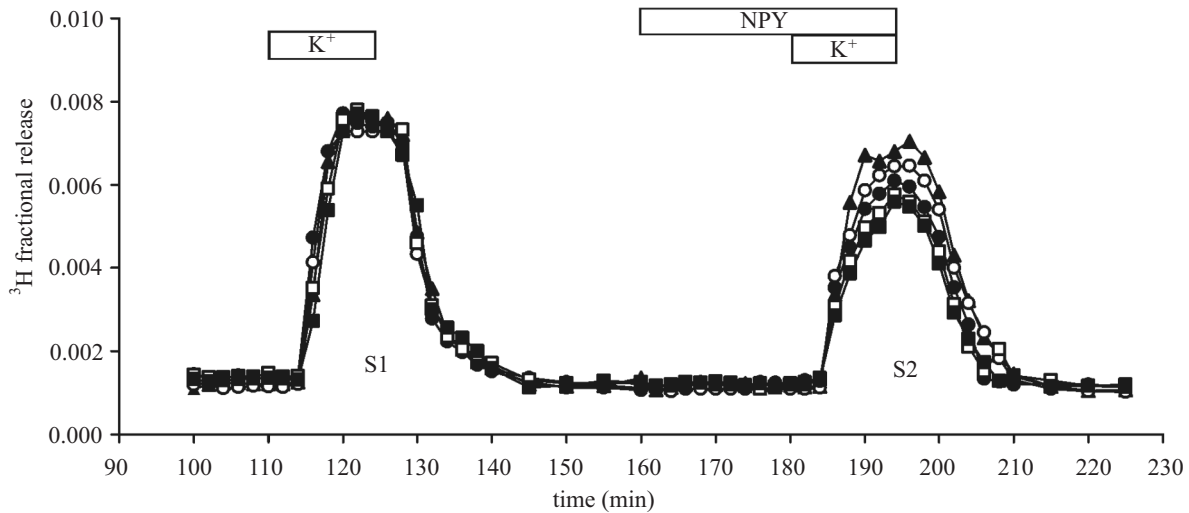The data was analyzed using one-way ANOVA followed by Duncan test.

Fig. 1. The effect of different concentrations of NPY (open circles 0.01 μM, closed circles 0.1 μM, open squares 1 μM, closed squares 10 μM, triangles – control) on the tritium fractional release from the explants of the pig pineal gland preloaded with ³H-norepinephrine (Experiment I). The tissue pieces were exposed twice, after 110 (S1) and 180 (S2) minutes of the perfusion, to the modified Tyrode medium containing 60 mM of K⁺. The duration of each depolarization period was 14 minutes. NPY was added to the medium 20 minutes before and during the second depolarization (S2). The presented data are means from four independent experiments.

## Results

Neuropeptide Y at concentrations of 0.1-10 μM significantly decreased the depolarization-induced overflow of tritium (Fig. 1, 2A). The maximum inhibition reached about 20% and was noted after the treatment with peptide at a concentration of 10 μM. NPY at a concentration of 0.01 μM did not change the depolarization-evoked overflow of tritium.

The depolarization-induced overflow of tritium was significantly lower in the medium containing NPY$_{13-36}$ at concentrations of 0.1 – 10 μM than in the control medium (Fig. 2C). [Leu$^{31}$,Pro$^{34}$]-NPY peptide did not change the depolarization-induced tritium overflow (Fig. 2B). None of these peptides changes significantly the basal release of tritium.

The treatment with rauwolscine (1 μM) increased the depolarization-evoked overflow of tritium by more than two-fold (Fig. 3). The depolarization-induced tritium overflow was lower from the pieces of the pineal gland treated simultaneously with 5 μM NPY and 1 μM rauwolscine than from the explants exposed solely to rauwolscine.

The depolarization-induced tritium outflow was reduced by 18.9% after the treatment with UK 14,304 at a concentration of 0.05 μM and by 20,9% after the treatment with NPY at a concentration of 5 μM (Fig. 4A). The exposition of explants simultaneously to 0.05 μM UK 14,304 and 5 μM NPY resulted in the reduction of depolarization-induced tritium outflow by 40.2%.

UK 14,304 at a concentration of 1 μM decreased the depolarization-induced tritium outflow by 47.65% as compared to the control group (Fig. 4B). There were no significant differences in the level of depolarization-induced overflow of tritium between the explants exposed solely to UK 14,304 at a concentration of 1 μM and those exposed simultaneously to UK 14,304 at a concentration of 1 μM and NPY at a concentration of 5 μM.

## Discussion

The wide occurrence of NPY in the nerve fibers of the mammalian pineal gland is well documented, however, the role of this peptide in the pineal physiology still remains unexplained. The present study focuses on the influence of NPY on the pineal noradrenergic transmission at the presynaptic level. The method used in our investigation was previously validated in the experiments with the rat and pig pineal explants (Chuluyan et al. 1991, Lewczuk 2002a). It was demonstrated that the depolarization-evoked tritium overflow from the pig pineal explants was abolished by the treatment with the neuronal uptake inhibitor – desipramine performed before and during loading with tritium labeled NE, the removal of Ca²⁺ from the medium used for perfusion and significantly decreased in the presence of Cd²⁺ (Lewczuk 2002a). ³H-norepinephrine represents more than 90% of tritium released from various tissues (including the
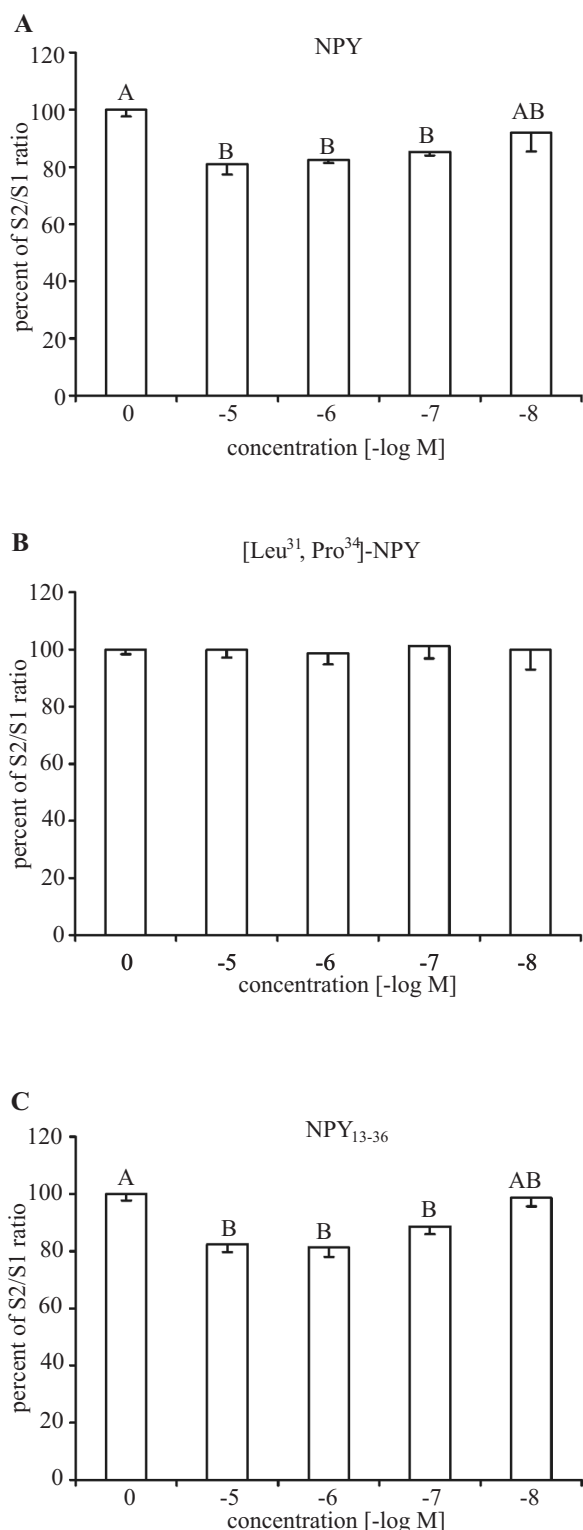
**A** NPY



**B** [Leu³¹, Pro³⁴]-NPY



Fig. 3. The effect of NPY and rauwolscine (Experiment IV) on the depolarization-evoked overflow of tritium from the explants of the pig pineal gland preloaded with ³H-norepinephrine, expressed as percent of the control S2/S1 value (means ± SEM, n=4). The tissue pieces were treated with rauwolscine (1 μM), NPY (5 μM) or rauwolscine (1 μM) + NPY (5 μM), which were added to the medium 20 minutes before and during the second depolarization (S2). Values signed with different letters are significantly different at p≤0.05. RAU – rauwolscine.
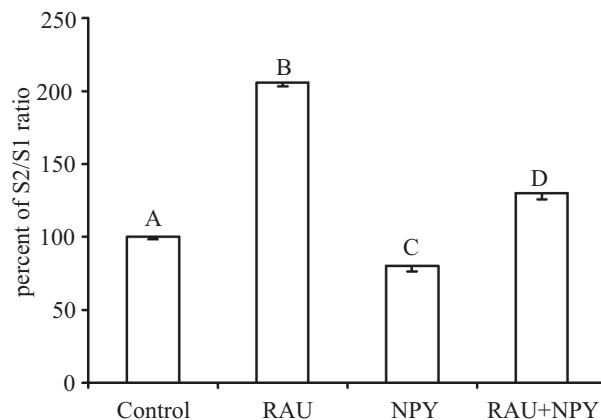
**C** NPY₁₃₋₃₆



Fig. 2. The effect of different concentrations of NPY (**A** – Experiment I), [Leu³¹, Pro³⁴]-NPY peptide (**B** – Experiment II) and NPY₁₃₋₃₆ peptide (**C** – Experiment III) on the depolarization-evoked overflow of tritium from the explants of the pig pineal gland preloaded with ³H-norepinephrine, expressed as percent of the control S2/S1 value (means ± SEM, n=4). The peptides were added to the medium 20 minutes before and during the second depolarization (S2). Values signed with different letters are significantly different at p≤0.05.

pig pineal gland) preloaded with this ³H-labeled catecholamine in the presence of pargyline (Maura et al. 1985, Chuluyan et al. 1991, our unpublished data). In view of the above presented data, the depolarization-induced release of tritium is a sensitive and valid index of NE release from the sympathetic nerve fibers in the pig pineal gland.

Our results demonstrate that NPY decreases by about 20% the depolarization-induced release of NE from the sympathetic nerve fibers in the pineal gland of the domestic pig. The use of selective agonists of NPY-receptors (Wahlestedt et al. 1986, Fuhlendorff et al. 1990) enabled us to establish that the action of NPY on the NE release occurs via $Y_2$-receptors, as the inhibitory effect was observed after the treatment of explants with $Y_2$-receptor agonist – NPY₁₃₋₃₆ peptide, but not with $Y_1$-receptor agonist – [Leu³¹, Pro³⁴]-NPY peptide.

In the previous investigations, it has been shown that the release of NE from the sympathetic nerve fibers in the rat and pig pineal glands is regulated by presynaptic $\alpha_2$-adrenoceptors (Simonneaux et al. 1994a, Lewczuk 2002a). To determine the possible interactions between this autoregulation and the NPY action, the pig pineal explants were treated with rauwolscine ($\alpha_2$-antagonist) or UK 14,304 ($\alpha_2$-agonist) together with NPY. The obtained results show that NPY decreases the depolarization-induced release of NE from the explants simultaneously exposed to rauwolscine. Moreover, the effects of UK 14,034 at a concentration of 0.05 μM and NPY at a concentration of 5 μM on NE release were additive. In contrast,
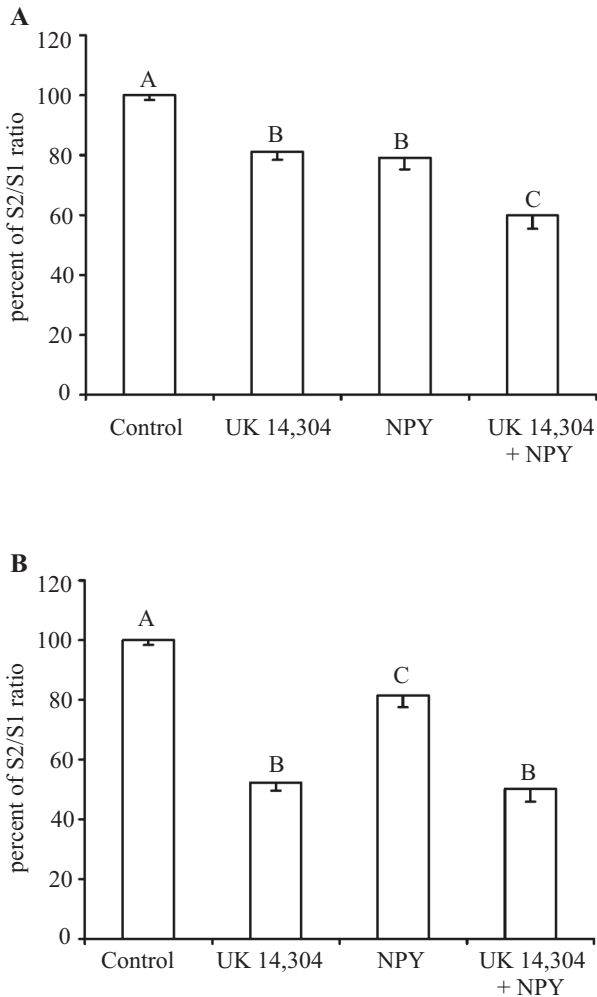
**A**



**B**



Fig. 4. The effect of NPY and two different concentrations of UK14,304: 0.05 μM (**A** – Experiment V) and 1 μM (**B** – Experiment VI) on depolarization-evoked overflow of tritium from the explants of the pig pineal gland preloaded with ³H-norepinephrine, expressed as percent of the control S2/S1 value (means ± SEM, n=4). The tissue pieces were treated with UK 14,304 (0.05 / 1 μM), NPY (5 μM) or 14,304 (0.05 / 1 μM) + NPY (5 μM), which were added to the medium 20 before and during the second depolarization (S2). Values signed with different letters are significantly different at p≤0.05.

there was no difference in the release of tritium induced by depolarization in explants treated solely with UK 14,304 at concentration 1 μM and those exposed to both NPY at concentration 5 μM and UK 14,304 at concentration 1 μM. The lack of more robust response is probably related to powerful inhibitory effect of UK 14,304 at this concentration, which is much stronger than the impact of NPY. The obtained data demonstrate that NPY and NE act on the sympathetic nerve fibers independently via Y$_2$-receptors and α$_2$-adrenoceptors.

Up till now, role of NPY in the presynaptic regulation of NE release in the pineal gland has been studied only in rats. The obtained results have consistently shown that NPY decreases depolarization-induced release of this catecholamine in the rat pineal (Vacas et al. 1987, Simonneaux et al. 1994a,b). Simonneaux et al. (1994a,b) demonstrated that NPY inhibited by about 45% the potassium-evoked NE release from rat pineal, and that this process occurred, similar as in the pig pineal via Y$_2$-receptor. Vacas et al. (1987) reported the inhibition of the NE release by about 15%. In view of the literature data and our results the presynaptic modulation of neurotransmission is definitely the best known action of NPY in the mammalian pineal gland. It is worth to note that the regulation of melatonin secretion is completely different in rat and pig pinealocytes (Stehle et al. 2001, Lewczuk 2002b, Lewczuk et al. 2005).

The data concerning the post-synaptic action of NPY in the mammalian pineal gland are not univocal. The *in vitro* studies of rat pinealocytes have shown that NPY inhibits by 20 – 30% the increase in cAMP level induced by the adrenergic stimulation (Olcese 1991, Harada et al. 1992, Simonneaux et al. 1994a,b). This effect is mediating by postsynaptic Y$_1$-receptors (Olcese 1991, Simonneaux et al. 1994b). The studies on other putative intracellular mediators have shown that NPY does not change the basal and NE-stimulated conversion of inozytol (Olcese 1994). On the other hand, this peptide causes the increase in the intracellular concentration of Ca$^{2+}$ in rat pinealocytes (Simonneaux et al. 1994a,b). So far, the results concerning the effect of NPY on melatonin synthesis in the rat pineal gland are inconclusive. Some studies demonstrated that in rats pinealocytes NPY stimulated the basal melatonin release or potentiated the NE-induces melatonin synthesis (Vacas et al. 1987, Simonneaux et al. 1994b), while other studies reported an inhibition of NE-induces melatonin secretion (Olcese et al. 1991, Rekasi et al. 1998). NPY increases HIOMT activity in rat pinealocytes during short and long-term exposition (Ribelayga et al. 1998). As concerning other species, effect of NPY on pinealocyte activity has been studied in the sheep. NPY does not reduce the isoproterenol-induced cAMP increase as well as basal and NE-stimulated secretion of melatonin in the organotypic culture of ovine pineal explants (Morgan et al. 1988, Williams et al. 1989).

The current state of knowledge points out, that the local, intra-pineal presynaptic modulation of NE release should be considered as an important regulatory mechanism integrating different neuronal and paracrine signals. Beside α$_2$-autoreceptors, the release of NE from the nerve endings in the rat pineal gland is modulated by NPY, substance P, acetylcholine, γ-aminobutyric acid and melatonin (Rosenstein et al.

1990, Chuluyan et al. 1991, Simonneaux et al. 1994b, Drijfhout et al. 1996b, Mukda et al. 2009). The above presented list of substances is probably much longer, because at least 30 different metabotropic and ionotropic receptors have been found to control the amount of transmitter being released from the sympathetic nerve terminals, located in various body structures (Boehm and Kubista 2002). Moreover, the effect of melatonin on the NE release seems to be dependent of the stage of diurnal rhythm (Chuluyan et al. 1991). The pineal hormone impaired the depolarization-evoked release of NE in the pineals taken from the rats euthanized during scotophase at 24:00 and 4:00, but remained inactive in the glands excised during photophase at 14:00 and 20:00 (Chuluyan et al. 1991).

In conclusion, the results obtained show that NPY takes part in the regulation of NE release from the sympathetic nerve fibers in the pineal gland of the domestic pig, inhibiting this process via $Y_2$-receptors.

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