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Original article

Direct effect of hypothalamic neuropeptides on the release of catecholamines by adrenal medulla in sheep – study *ex vivo*

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Abstract

Stress causes the activation of both the hypothalamic-pituitary-adrenocortical axis and sympatho-adrenal system, thus leading to the release from the adrenal medulla of catecholamines: adrenaline and, to a lesser degree, noradrenaline. It has been established that in addition to catecholamines, the adrenomedullary cells produce a variety of neuropeptides, including corticoliberine (CRH), vasopressin (AVP), oxytocin (OXY) and proopiomelanocortine (POMC) – a precursor of the adrenocorticotropic hormone (ACTH). The aim of this study was to investigate adrenal medulla activity *in vitro* depending, on a dose of CRH, AVP and OXY on adrenaline and noradrenaline release. Pieces of sheep adrenal medulla tissue (about 50 mg) were put on 24-well plates and were incubated in 1 mL of Eagle medium without hormone (control) or supplemented only once with CRH, AVP and OXY in three doses (10^{-7}, 10^{-8} and 10^{-9} M) in a volume of 10 µL. The results showed that CRH stimulates adrenaline and noradrenaline release from the adrenal medulla tissue. The stimulating influence of AVP on adrenaline release was visible after the application of the two lower doses of this neuropeptide; however, AVP reduced noradrenaline release from the adrenal medulla tissue. A strong, inhibitory OXY effect on catecholamine release was observed, regardless of the dose of this hormone. Our results indicate the important role of OXY in the inhibition of adrenal gland activity and thus a better adaptation to stress on the adrenal gland level.

Key words: hypothalamic neurohormones, adrenal gland, adrenaline and noradrenaline release

Introduction

The fundamental process of life is maintaining homeostasis in a permanently changing environment. The hypothalamic-pituitary-adrenocortical axis (HPA) is the principal component required for adaptation. The Corticotropin-Releasing Hormone (CRH) and arginine vasopressin (AVP) are the main hypothalamic factors for releasing by stress the adrenocorticotropic hormone (ACTH) and adrenocortical glucocorticoids, most often cortisol, which are the final effectors of the HPA axis. Apart from stress

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causing activation of the HPA axis, it also initiates the sympatho-adrenal system (SAS), which results in the release of catecholamines (CA) – adrenaline (A), and to a lesser degree noradrenaline (NA) from the adrenal medulla (Knight and Baker 1983, Ungar and Philips 1983). CA are synthesized with the participation of enzymes essential for catecholamine biosynthesis: tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase, dopamine-β-hyroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT), primarily in the chromaffin cells of the adrenal medulla and in the neuronal cells. The importance of the adrenal medulla hormones and sympathetic nervous system in various pathophysiological conditions was generally determined on the basis of the measurements of plasma A and NA concentration, which reflect the activity of the adrenal medulla and the sympathetic nervous system, respectively.

It is well known that the adrenal cortex and medulla comprise a complexity of cells which have multiple contact zones and are not separated by connective tissue or interstitial membranes (Schinner and Bornstein 2005); an anatomically close contact and paracrine interactions between the cortical and chromaffin cells within the adrenal gland have been described (Connan et al. 2007). Additionally, it was established that in addition to catecholamines, in adrenomedullary cells, a variety of neuropeptides are present including CRH, AVP and proopiomelanocortine (POMC) – a precursor of ACTH (Schinner and Bornstein 2005). Many studies suggest that hypothalamic oxytocin (OXY), in contrast to HPA hormones, is involved in the stress response and reduce stress by suppressing HPA activity (Legros 2001) and takes part in neuroendocrine regulation in the stress response as a neurotransmitter or neuromodulator in the central nervous system (CNS), being capable of influencing a series of specific behaviors, for example social recognition and maternal behavior (Winslow and Insel 2006), modulation of memory consolidation and retrieval (McEwen 2004), and some affective psychopathologies, for example autism. In recent years, a considerable amount of evidence has been accumulated to show that OXY is able to decrease glucocorticoid hormone secretion from the adrenal gland and locally synthetized similarly as AVP, in the adrenal medulla (Gallo-Payet and Guillon 2008) which was supported by our own results (Wronska-Fortuna et al. 2009). The presence of OXY was demonstrated in the adrenal gland of the human, rat, cat and cow, and not only in the medulla but distributed throughout the cortex as well (Stachowiak et al. 1995). This distribution of OXY in the adrenal gland suggests its involvement in the direct regulation of the function of this endocrine gland. The early results support the hypothesis that peripheral catecholamines may at times be directly involved in the inhibition of ACTH and catecholamine secretion and also suggest that OT, which has been identified in the adrenal medulla, may have important paracrine functions in the regulation of adrenal catecholamine secretion. It has been established that the adrenocortical functions can be directly stimulated by CRH, AVP and OXY, even in the absence of ACTH (Mazzocchi et al. 1992). Considering the above information it is clear that hypothalamic neurohormones can directly modulate the activity of the adrenal hormone synthesis and/or release. The aim of this study was to investigate the adrenal medulla *ex vivo* activity depending on the dose of CRH, AVP and OXY on the release of adrenal catecholamines: adrenaline and noradrenaline.

Materials and Methods

Just after the heart and breathing stopped, the animals were decapitated, the ventral skin incised and the adrenal gland was removed, put on ice and the adhering tissues removed. A cut was then made along the adrenal gland and its layers were separated using appropriate surgical tools to cut any the whole tissue of the adrenal medulla of each gland. Adrenal glands were obtained from 5 decapitated female, 2-3 year old sheep, out of the reproductive period (April-May) at the Experimental Station of the Department of Animal Biotechnology of the Agricultural University in Cracow-Bielany. All experimental procedures were approved by the Local Bioethics Committee at the Jagiellonian University in Cracow (approval No 75/2007). The pieces of adrenal medulla tissue (about 50 mg) were placed on 24-well plates and were incubated in 1 ml of Eagle medium without hormone (control) or supplemented only once with CRH, AVP and OXY (Sigma Aldrich) in three different concentrations (10^{-7}, 10^{-8} and 10^{-9} M) in a volume of 10 µL. The pieces of adrenal medulla tissue were completely immersed in incubation medium. The incubation was carried out in a carbogen atmosphere of 95% O_2 and 5% CO_2 at 38°C in a Sanyo incubator (MCO-18AIC). The pieces of adrenal medulla tissue were moved every 30 min to other plates containing 1 ml of clean Eagle's medium. A rapid staining procedure using fluorescein diacetate has used for the detection of cell viability in cell suspension (Jones and Senft 1985).

The amount of noradrenaline and adrenaline released into the medium after 30, 60 and 90 min of incubation was measured using the radioimmunological method (RIA) in accordance with the instructions provided by the company (DRG, Germany). The sensitivity of the method for noradrenaline was 0.2

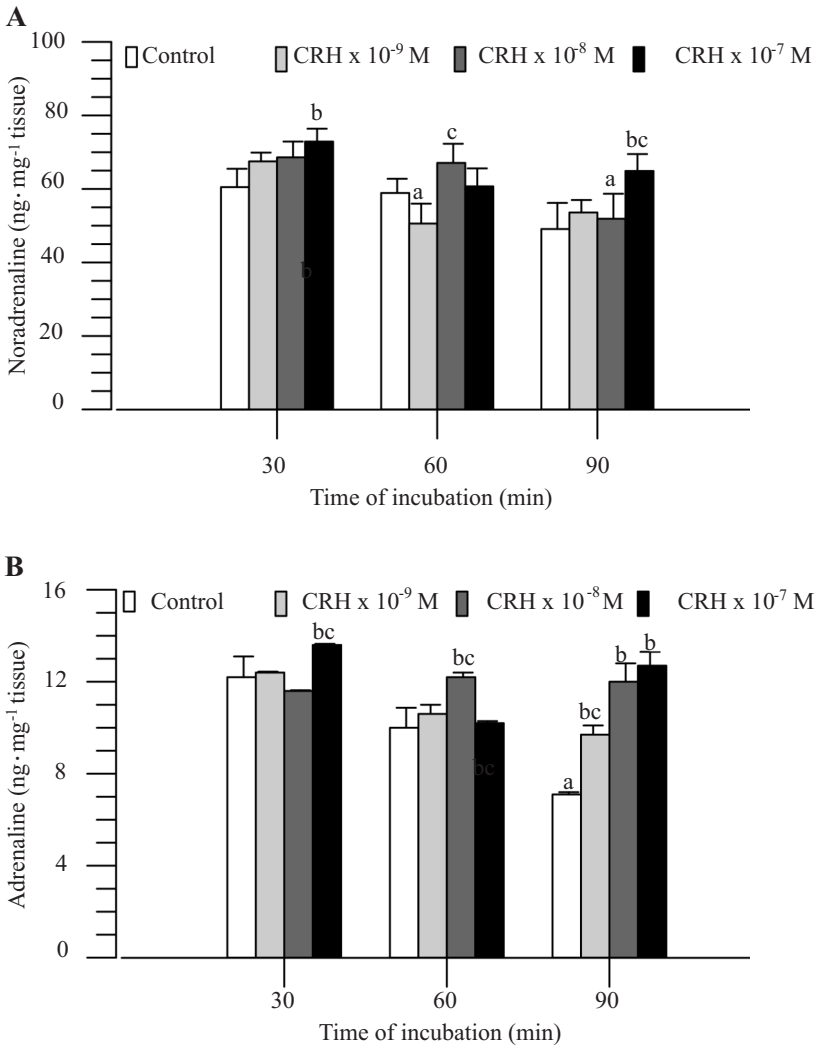

Fig. 1. Effect of **CRH** on noradrenaline (**A**) and adrenaline (**B**) *in vitro* release from sheep adrenal medulla tissue. Values are mean ± SE (n=5), letters indicate statistically significant distinction (p<0.05-0.01): a – in comparison with value estimated after first 30 min of experiment, b – in comparison with control value, c – between experimental groups.

pg/mL, intra- and interassay co-efficiton of variation were 7.0% and 6.5%, and for adrenaline 0.10 pg/mL, 8.0% and 5.5%, respectively. The concentration of the hormones in the incubation medium was calculated to 1 mg of adrenal medulla tissue.

Data were statistically analyzed by two-way ANOVA followed by Duncan's multiple range test. Log transformation was performed as needed to maintain homogeneity of variance and normality. Differences of values were considered to be significant at $p<0.05$. Calculations were performed using a Sigma Stat 2.03 program (SPSS Science Software GmbH, Germany). Results are presented as means ± SE.

Results

Incubation of adrenal tissue in clean Eagle's medium showed no significant changes in the amount of NA release into the medium within 90 minutes of the experiment (from 60.5 ± 5.0 to 49.1 ± 7.1 ng · mg^{-1} tissue; $p>0.05$; Fig. 1A, 2A, 3A).

The CRH applied in three different doses added to the medium during incubation supported, and even at the highest dose of 10^{-7}M, increased NA release from the adrenal medulla tissue, ($p\leq0.05$; Fig 1A). AVP treatment applied in three different doses decreased the noradrenaline release within in the first 30 minutes of the incubation of the adrenal medulla tissue (Fig. 2A). The poorest inhibitory effect on this CA release to the medium was noted in groups treated with AVP of 10^{-7} M. All the obtained results in the experimental groups were significantly lower as compared to the control values ($p<0.01$; Fig. 2A). In all groups, a large inhibitory (4-5-fold lower) OXY effect on NA release was observed, regardless of the applied doses of this hormone, particularly after 90 minutes of experiment ($p<0.01$; Fig. 3A).

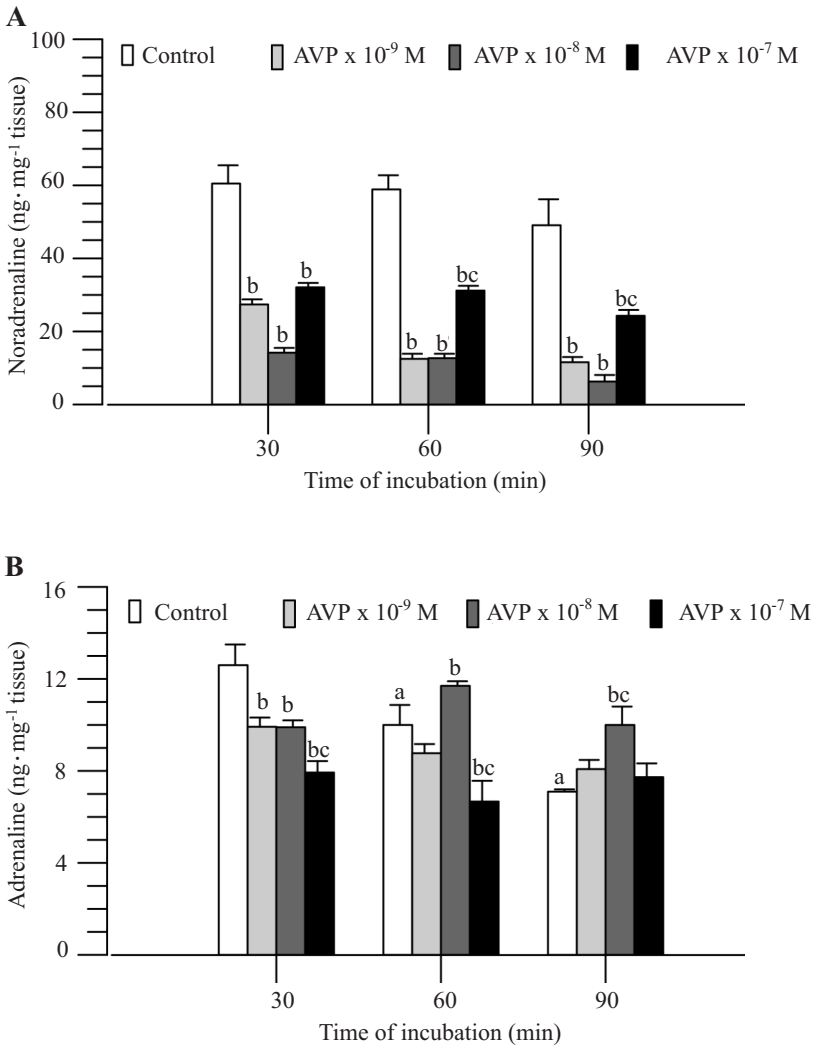

Fig. 2. Effect of **AVP** on noradrenaline (**A**) and adrenaline (**B**) *in vitro* release from sheep adrenal medulla tissue. Values are mean ± SE (n=5), letters indicate statistically significant distinction (p<0.05-0.01): a – in comparison with value estimated after first 30 min of experiment, b – in comparison with control value, c – between experimental groups.

Incubation of adrenal medulla tissues in the basic Eagle's medium clearly showed a progressive decrease of A release from 12.2 ± 0.1 ng · mg^{-1} of tissue after the first 30 minutes of the experiment to 7.1 ± 0.1 ng · mg^{-1} tissue after 90 minutes of incubation. All results obtained during the experiment were statistically significant (p<0.01; Fig. 1B, 2B, 3B).

The CRH effect on the release of adrenaline from adrenal medulla tissue was similar to that observed in the case of noradrenaline. The stimulatory effect of AVP on the adrenaline release was observed particularly after the application of AVP at a concentration of 10^{-8} M; the obtained results show an increase in A release throughout the experiment (p<0.01; Fig. 2B). After the application of OXY to the medium with adrenal medulla tissue a significant decrease in A release was observed. In all experimental groups the obtained results were similar, about 6, 5 or 4-fold lower than in control incubation after 30, 60 and 90 minutes of the experiment (p<0.01). Additionally, all the described values were similar (p>0.05).

Discussion

The results obtained in our study explicitly point to the direct action of hypothalamic neuropeptides on sheep adrenal medulla activity in both NA and A release. This directly indicates that adrenal glands are the target tissue for hypothalamic neuropeptides, which form the intensity of the stress response at the level of the adrenal glands.

The addition of CRH to the incubated adrenal medulla tissue in the medium triggers NA release, especially after the first 30 minutes of the experiment. It is well known that NA in the adrenal medulla com-

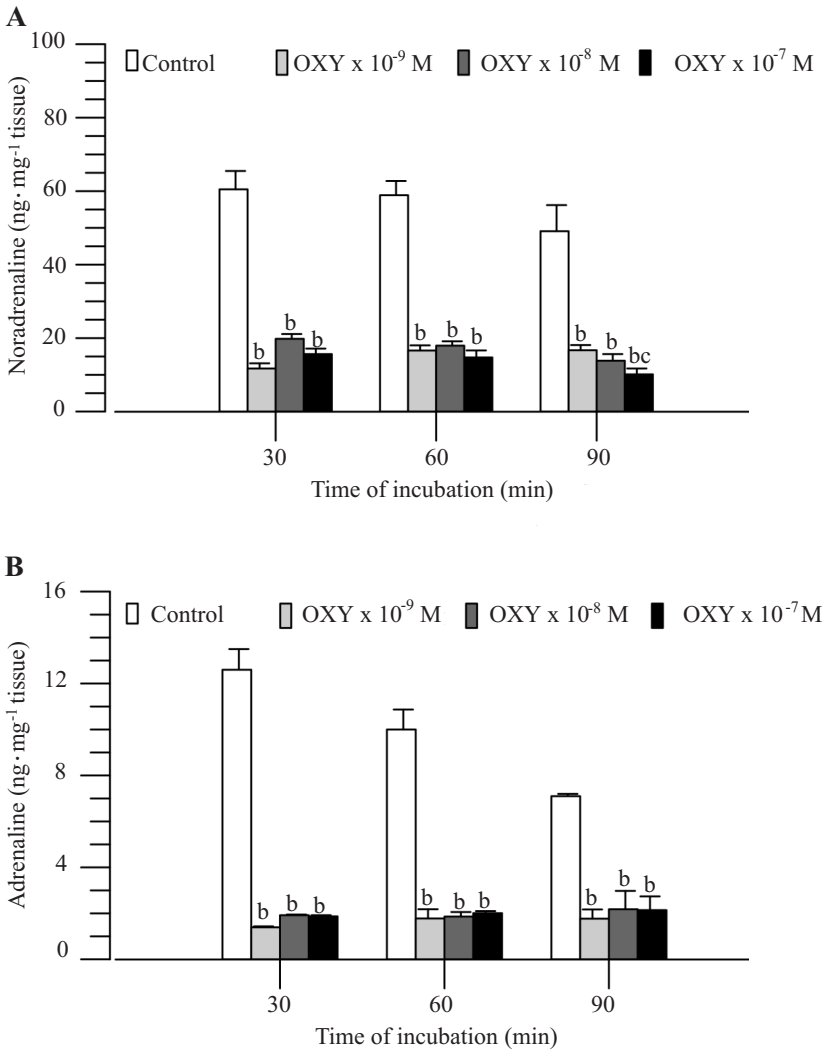

Fig. 3. Effect of **OXY** on noradrenaline (**A**) and adrenaline (**B**) *in vitro* release from sheep adrenal medulla tissue. Values are mean ± SE (n=5), letters indicate statistically significant distinction (p<0.05-0.01): a – in comparison with value estimated after first 30 min of experiment; b – in comparison with control value.

prises a substrate for A synthesis. In the successive minutes of incubation the action of CRH in higher doses more strongly affected NA release; our results indicate the unanimous effect of CRH on A secretion, and a dose-dependent effect of this neurohormone was observed after 90 minutes of the experiment. The results of numerous experiments have proven the presence of CRH in the adrenal medulla (Connan et al. 2007). The results obtained in this experiment indicate an immediate CRH influence on the amount of NA release; however, a stronger effect of this neurohormone was in relation to the A release. Each of the CRH doses used increased the A secretion, particularly after 90 minutes of adrenal medulla tissue incubation. We therefore suggest that the participation of this neurohormone in the regulation of both CA's release is connected with its effect on the phenylethanolamine N-methyltransferase (PNMT) enzyme activity, which leads to the last step of A biosynthesis – a conversion of NA to A. The results of the experiment of Kubovcakova et al. (2004) proved that from the main enzymes of CA biosynthesis – TH, DBH and PNMT – the activity of the last enzyme changes in the stress conditions in mice with CRH knockout.

The addition of CRH to the incubated adrenal medulla tissues imitates the stress effect. Tillinger et al. (2010) described the effect of the stress mechanism of the cellular neurosecretion in the adrenal medulla. These results indicate that stress increases not only CA biosynthesis, but also affects storage or uptake by the neurosecretory vesicles. During stress increase a growth of gene expression was noted but only in VMAT2 and only NA synthesizing cells. The results of the present experiment show that stimulation by CRH of the adrenal medulla tissues increases the

amount of NA, as compared with A released into the medium. This may explain the NA origin in the adrenal gland. The NA source is not only the adrenal medulla, but to a greater extent the nervous system and in part the blood of the adrenal gland which may store this CA. Since the blood flow direction in the adrenal gland is from the cortex to the medulla, its seems that the adrenal gland medulla blood should be richer in glucocorticoids, but the model of our experiment excludes such a possibility. It was noted that the presence of cortisol is necessary in CA biosynthesis (Wurtman 2002); this mainly concerns the glucocorticoids participating in the induction of expression of the promoter region PNMT.

There was also stated the presence of mRNA coding CRH in the adrenal gland (Wong 2003), also the $CRHR_1$ receptors (Yokotani et al. 2001) which indicated the possibility of direct influence of CRH one on the adrenal gland endocrine activity, including the CA's synthesis. The results of the present experiment confirm those obtained earlier and point to the increase of CA synthesis by CRH directly proportionally to the dose of neuropeptide.

It was proved also that AVP, released from the same neurosecretory nuclei as CRH, more strongly emphasizes the role of the successive structures of the HPA axis, and the *in situ* synthesis of this neurohormone (Gallo-Payet and Guillon 2008) was proved in the cells of adrenal medulla, thus indicating the possibility of the direct effect of AVP on the adrenal medulla function. Our results show a clear delay of the AVP effect on the NA release from the adrenal medulla tissue, although the highest AVP doses (10^{-7} M) to a lesser degree increases its release than the two lower doses of AVP. AVP administered at the highest dose inhibited the release of A from the adrenal medulla tissue, while the effect was not symptomatic in reference to NA release; in the successive incubations of the adrenal medulla tissue the obtained values were also higher as compared to the control, especially after the application of AVP at a concentration of 10^{-8} M.

In the present study, we found that AVP, especially in high doses attenuates A release. On the other hand, NA secretion was decreased after the application of AVP in lower doses. In our opinion, in the mechanism of control of CA release the AVP had separate ways with reference to A and NA. Another explanation is insufficient NA synthesis for maintaining A release, which may be connected with increased AVP on the activity of the earlier enzymes in CA synthesis, for example DBH, which converts dopamine (DA) to NA (Yamagushi-Shima et al. 2007) or tyrosine hydroxylase. Murat et al. (2012) demonstrated that AVP release can be stimulated by CRH, and it is possible that there is a synergism in the action between CRH and AVP also at the adrenal level.

The adrenal cortex and medulla function as two endocrine systems. "Gentle" anatomical contact and paracrine interactions between cortical and chromaffin cells inside the adrenal gland have been described (Bornstein et al. 2005). Wurtman (2002) found that glucocorticoids regulate the differentiation of chromaffin cells and CA biosynthesis. This indicates that under *in vitro* conditions cortical steroids enhance the conversion of NA to A and that steroids modulate PNMT enzyme activity.

A surprising effect of both NA and A release from the adrenal medulla after the application of OXY in three different doses was observed. The results of our experiment show explicitly an inhibitory effect of OXY on NA release and first on A secretion. The lowest dose of OXY 10^{-9} M reduced NA release 3-fold and A release 6-fold. Research results indicate that during stress an increase of OXY release from hypothalamic supraopticus nuclei (SON) and paraventricular nuclei (PVN) is observed (Engelmann et al. 2004, Bosh and Neumann 2012). Ring et al. (2005) claim that central OXY release is influenced by other neuroendocrine factors, which affect the psychological and neuroendocrine reactions of the organism. During lactation, endogenous OXY decreases glucocorticoid synthesis in rats and in humans, which in turn decreases the stress symptoms (Heinrisch et al. 2003). Many authors have demonstrated that OXY, particularly at the hypothalamus level, participates in the regulation of HPA axis activity (Engelman et al. 2004, Goldman et al. 2008). Results of their experiments indicate the possibility of local OXY synthesis, similarly to AVP, in the adrenal gland of some species. The results of our earlier studies showed changes in expression of the OXY gene and receptor in the sheep adrenal glands under stress, in the case of OXY markedly increase during adaptation process to a repeated stress factors was observed (Wronska-Fortuna et al. 2010). We showed an increase of OXY release from the sheep adrenal medulla tissue during adaptation to a repeated stress factor (Wronska-Fortuna et al. 2009).

The function of vasopressin and oxytocin in the adrenal medulla may be indicated by the inhibition of acetylcholine-stimulated CA secretion *in vitro*, although the effect requires high concentrations of either peptide; our applied model of *ex vivo* research prevented the influence of the nervous system on adrenal gland function.

OXY influences the enzymes synthetizing CA's but PNMT enzyme activity, because it this activities only leads to conversion of NA to A; our results indi-

cate that OXY decreases both NA and A release. Hormonal activation of the PNMT gene expression depends on extremely high concentrations of glucocorticoids which induce transcriptional changes *via* the glucocorticoid response elements (GREs) upstream of initiating PNMT transcription side (Wong 2003). The activation of PNMT through the sympatho-adrenal system can occur *via* the release of acetylcholine and pituitary adenylate cyclase-activating polypeptide (PACAP) from the splanchnic nerve (Wong 2006). The results clearly show that the basic CA secretion is similar in the innervated and denervated adrenal glands, indicating that there was a slight tonic sympathetic neural drive to the medulla. The employed model of our *ex vivo* experiment excluding the cortex layer of the adrenal gland and its earlier activation by the stress factors or sympathetic nerve terminal splanchnic nerve status. Thus, our results present only immanent properties of adrenal medulla tissue in the basic condition.

On the other hand Jovanovic et al. (2014) found that the repeated administration of OXY to unstressed rats produces an increase of adrenaline and noradrenaline content in the adrenal medulla but it does not apply to catecholamine release during altered homeostasis.

We suggest that the involvement of OXY on catecholamine release from the adrenal medulla is more related to the effect of OXY for its degradation, because it has been shown that the positive OXY effect on MAO and COMT activity, the major catecholamine degradation enzymes (Eisenhofer et al. 2004).

In recent years, the growing interest of researchers concerns purinergic signaling in endocrine organs; results indicate that ATP plays a major role in the synthesis, storage and release of CA's from the adrenal gland, and it was established that both AVP and OXY can increase intracellular ATP concentration (Burnstock 2014), and in consequence can increase *in vitro* catecholamine release from the adrenal medulla.

In summary, we have demonstrated in our *ex vivo* experiment that the hypothalamic neuropeptides CRH, AVP and OXY may affect catecholamine release from the sheep adrenal medulla. This indicates the existence of additional possibilities, also as a result of their local synthesis, and modulation of stress reaction intensity at the adrenal gland level.

Conclusions

1. Our results demonstrate the direct effect of hypothalamic neuropeptides on catecholamine release from the sheep adrenal medulla.

2. CRH and, to a lesser degree AVP, modulated noradrenaline and adrenaline release from the adrenal gland.

3. Our findings clearly indicate the important role of oxytocin in the inhibition of adrenal gland activity, and thus a better adaptation to stress on the adrenal gland level.

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