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Role of Activatory and Inhibitory Neuropeptides in the Regulation of the Hypothalamo-Pituitary-Adrenal System

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PUBLICATIONS

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II. **Jászberényi, M.**; Bujdosó, E.; Telegdy, G. Effects of Brain Natriuretic Peptide on Pituitary-Adrenal-Activation in Rats. Life Sci 66: 1655-1661; 2000. (impact factor: 1.808)

III. **Jászberényi, M.**; Bujdosó, E.; Pataki, I.; Telegdy, G. Effects of Orexins on the Hypothalamo-Pituitary-Adrenal System. J Neuroendocrinol 12: 1174-1178; 2000. (impact factor: 2.598)

IV. **Jászberényi, M.**; Bujdosó, E.; Telegdy, G. The Role of Neuropeptide Y in Orexin-Induced Hypothalamic-Pituitary-Adrenal Activation. J Neuroendocrinol 13: 438-441; 2001. (impact factor: 2.598)

2. Original publications cited in the text

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II. Pataki, I.; **Jászberényi, M.** and Telegdy, G. Hyperthermic Effect of Centrally Administered Natriuretic Peptides in the Rat. Peptides 20: 193-197; 1999. (impact factor: 1.867)

III. Bujdosó, E.; **Jászberényi, M.;** Tömböly, C.; Tóth, G.; Telegdy, G. Effects of Endomorphin-1 on Open-Field Behavior and on the Hypothalamic-Pituitary-Adrenal System. Endocrine 14: 221-224; 2001. (impact factor: 1.609)

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II. **Jászberényi, M.;** Bujdosó, E. and Telegdy, G. The Effects of Brain Natriuretic Peptide on the Hypothalamo-Pituitary-Adrenal Activation in Rats. Regul Pept 80 (3) 1999.

III. **Jászberényi, M.;** Bujdosó E.; Pataki I.; Telegdy G. The Role of Neuropeptide Y in the Action of Orexin-A on the Hypothalamo-Pituitary-Adrenal System. Eur J Neurosci Vol. 12 Suppl. 11, 417. 2000.

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4. Presentations

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VII. **Jászberényi M.,** Bujdosó E., Pataki I., Telegdy G. The role of neuropeptide Y in the action of orexins on the hypothalamo-pituitary-adrenal system. Trabajos del Instituto Cajal (Abstract -Joint Meeting of the VIth International Conference on Hormones, Brain and Behavior and The Society for Behavioral Neuroendocrinology), Madrid, 2000.

VIII. **Jászberényi, M.,** Bujdosó, E., Kiss, E., Pataki, I., Telegdy, G. The role of neuropeptide Y in the mediation of orexin-induced hypothermia. A Magyar Idegtudományi Társaság VIII. Konferenciája, Szeged, 2001.

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XI. **Jászberényi, M.,** Bujdosó, E., Telegdy, G. Nitric oxide displays a stressor-dependent effect on the activation of the hypothalamo-pituitary-adrenal axis. IBRO International Workshop Debrecen, 2002.

ABBREVIATIONS

ACTH	adrenocorticotropin hormone
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
CCK	cholecystokinin
CNP	C-type natriuretic peptide
CNS	central nervous system
CRH	corticotropin-releasing hormone
GPCRs	G protein-coupled receptors
HPA	hypothalamo-pituitary-adrenal
icv.	intracerebroventricular
ip.	intraperitoneal
LPS	lipopolysaccharide
MCH	melanocyte concentrating hormone
NPY	neuropeptide Y
PVN	paraventricular nucleus
PRL	prolactin
rpm.	revolutions per minute
TRH	thyrotropin-releasing hormone

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1 INTRODUCTION

1.1 The complexity of stress response

1.1.1 The stress theory in the light of recent discoveries

Adaptability is one of the basic principles that govern the evolution of the organism. The homeostasis of living creatures is unceasingly challenged by their always changing environment. This interaction inevitably puts a considerable burden ("stress") on the organism and it is of key importance to react successfully to the outer nocuous stimuli.

It was some common features of these reactions that Selye János recognized and formulated as a species and stimulus independent phenomenon: "Stress is the non-specific response of the body to any demand upon it"[179]. He coined the term "general adaptation syndrome" and meticulously elaborated on the theory setting up three characteristic stages[178]. The "alarm reaction" is the first stage and was thought to consist of two phases: "shock" and "counter-shock". The latter can be characterized principally by the responses of the adrenal cortex ("corticoids") and the adrenal medulla ("adrenalines"). The second stage is of the "resistance" with increased ability to withstand the given "stressor" but with decreased resistance to other agents. In case the harmful stimulus persists the balanced state would end with the depletion of "adaptation energy" and the third stage of "exhaustion" would ensue. The stress response in all stages was viewed as a stereotyped reaction of four major components: enlargement of the adrenal glands, involution of the thymus and the lymph nodes, peptic ulceration of the stomach and decrease in the circulating number of the eosinophils.

The ingeniously simple but comprehensive aspect of the theory made it immensely popular for the public but at the same time provoked much debate in the scientific community and proved to be a fertile ground for much further research. Even at the beginning of the "career" of the stress theory such a "bigwig" as Walter Cannon heavily criticized the doctrine of "non-specificity"[58]. Cannon disputed that a stereotyped response can be adaptive and like

many others he felt the whole theory an elegant and witty oversimplification. Mason[127] also pointed out that depending on the type of the stressor the activity of the pituitary-adrenocortical system can increase, decrease or remain unaffected. Since the doctrine of "non-specificity" was of key importance to preserve the attractive comprehensiveness of the theory, Selye started to refine his theory incorporating more and more "variable" into the "equation". During this "adaptation of the adaptation syndrome" he made an attempt, with coming up with the so called "internal conditioning factors" (genetic, nutritional etc.), to explain how different patients react with different "stress disease" for the same challenge, and even in his last papers he insisted on the "core" hypothesis of his theory[180]: "Stress is the sum of the nonspecific biologic phenomena (including damage and defense), and consequently, a stressor agent is by definition nonspecific since it produces stress".

In the past few decades huge body of evidence has been accumulated for that the doctrine of non-specificity is untenable in many regards. Selye emphasized that both stress and stress responses are non-specific i.e. after removing the specific components, a nonspecific syndrome would remain. In terms of the inciting agents the stressors should be viewed only as "eustressors", which play a stimulative role or "distressors", which inevitably bring on "diseases of adaptation". Further, according to Selye's doctrine, the stress responses, despite their individual features, always share the aforementioned four basic non-specific symptoms. However, it does not seem to be the case regarding either the input (stress) or the output (stress response) of this homeostatic function. First, numerous publications have disclosed that different stressors activate highly individual neural pathways and strikingly different pattern of neural loci[60, 73, 74, 120, 128, 157, 160, 175, 176]. Painful stimuli and stress-related visceral impulses utilize the sensory neural routes, and trigger the release of CRH through the catecholaminergic pathways of the brainstem and the centres of the pons and the midbrain[120, 157, 175]. The impulses from the circumventricular organs activated by the changes in the osmotic milieu reaches the PVN through the forebrain afferents[176]. Environmental or "psychological" stressors such as the restraint stress activates the limbic system - mainly the ventral amygdalofugal pathway and the central amygdaloid nucleus[69, 207] - and appear to reach the perinuclear GABAergic interneurones[74, 160]. Second, diverse sympathoneural and adrenomedullary responses have been demonstrated during exposure to different stressors.

Breier *et al.* demonstrated that hypoglycaemia elicits a predominant adrenomedullary and HPA activation with little sympathoneural response[18], while in the case of hypothermia sympathoneural activation prevails[100, 215]. Other hormonal responses (oxytocin, vasopressin, angiotensin II) also vary according to the type of stress[6, 55]. CRH is the predominant mediator of the responses to acute stimuli, while vasopressin plays a role in the mediation of responses to chronic or repeated ones[1, 12] and appears to be an especially important mediator of the immune reaction evoked stress response[46]. As CRH release mainly depends on the phosphorylation of the cAMP-response element binding protein, while the release of vasopressin seems to be linked to the expression of the Nerve Growth Factor-induced transcriptional regulators[106], the second messenger responses and immediate-early gene activation patterns brought on by psychological, physical or immune stress paradigms also show remarkable variations. Therefore, the theory of final common pathway[67] needs revising. Besides, the findings of the past few decades revealed that not only the regulation of ACTH release but also the control of CRH secretion displays a bewilderingly complicated picture. Numerous neurotransmitters - such as catecholamines[23], serotonin[79] and nitric oxide[34, 131, 158] - and neuropeptides - such as NPY[187, 202], neurotensin[162] and opioids[21, 22, 56] - control the central processes of different stress responses. Nitric oxide seems to inhibit the HPA activation elicited by immune signals[203], while may mediate the stress response to the physico-emotional stimuli[99]. The opioids also play very important roles in the regulation of stress response[146], which appears to be species-dependent. In humans, they seem to attenuate HPA activation[33], whereas in rodents their effect is stimulatory[21, 22, 117, 185]. Further, both enkephalin and neurotensin mRNA expression in the PVN shows strong stressor-dependency[27]. These data reinforce the hypothesis that various stress paradigms in different species may elicit a quite specific pattern of neuronal activation beside some common and aspecific components of the stress reaction.

1.1.2 The importance of neuropeptides in the control of the HPA axis

In the last few decades, numerous studies have shed light on the importance of different neuropeptide families in the regulation of diverse homeostatic functions. In

endocrine control[8, 114], thermoregulation[90, 148, 149], food-intake[94, 165], behaviour[14, 16, 198] etc. the role of neuropeptides can not be overestimated. It is the complex interplay among the neuropeptide-positive neurones that appears to provide a considerable flexibility to the control of - among others - the HPA system. The function of CRH[206], vasopressin[107, 130], PRL[130], oxytocin[87], neurotensin[162] and the opioids[21, 22, 56, 117, 146, 185] has been thoroughly investigated in this regard. The interaction among the various neuropeptide circuits, the significance of multi-level feed-back mechanisms also has been demonstrated in the control of the stress response[8, 109, 133, 161], and the links between the HPA system and energy homeostasis appears to be of special importance[25, 211].

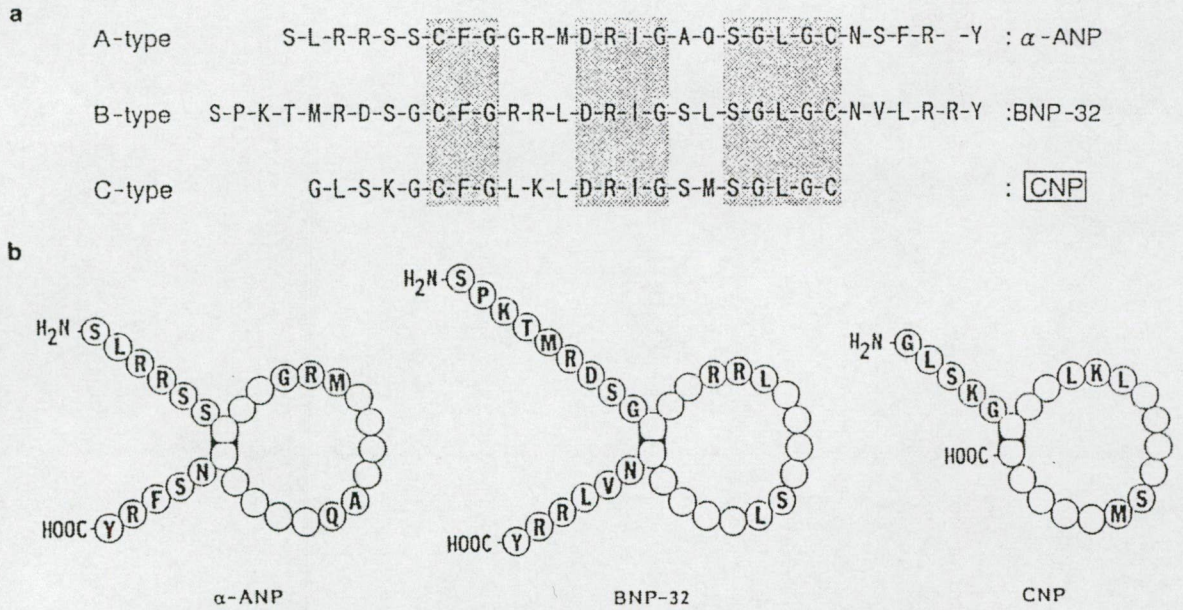
1.2 The natriuretic peptide family

1.2.1 Structure, signal transduction and distribution

The family of natriuretic peptides is made up of three structurally related members: ANP - isolated from rat cardiomyocytes[36] -, BNP and CNP; the latter ones were discovered in porcine brain[188, 190]. Despite their name, neither the distribution of these peptides is confined to a specific organ nor their function can be limited to their first described volume regulatory action[36].

The analysis of the chemical structure of the natriuretic peptides reveals that they share some important features. All of them contain a 17 amino acid sequence flanked by two cysteine residues[36, 188, 190], which region is closed by a disulphide bridge forming the highly conserved ring structure thought to be essential for their biological activity[188]. In the case of ANP and BNP the N-terminus and the C-terminus protrude from the ring, while the molecule of the CNP contains only one N-terminal extension and, lacking the C-terminal one, ends at the second cysteine residue. The N-terminal and C-terminal branches are considerably variable among them, and the species-specific structural variations of a given natriuretic peptide are also confined to these regions[36, 188, 190]. The N-terminal and C-terminal extensions in porcine ANP consist of 6 and 5 amino acids, respectively[36], while porcine BNP contains 3 and 6 residues in the corresponding branches[188]; the N-terminal extension of porcine CNP

comprises 5 amino acids. The natriuretic peptides derive from precursor molecules named pre-pro-ANP, -BNP and -CNP which consist of 152, 131 and 126 molecules, respectively. The processing of the pre-pro molecules yields pro-ANP, pro-BNP and pro-CNP of 127, 106 and 103 residues, respectively[24, 102, 103, 197]. The alternative processing of pro-BNP produces BNP-32, an N-terminal six amino acid extended form of the peptide[189]. The pro-hormones appear to represent the storage form of the natriuretic peptides[199].



Ill. 1 The primary and secondary structure of the natriuretic peptides

a.) Amino acid sequences of ANP, BNP-32 and CNP. Intramolecular disulfide linkage is formed between two cysteine residues in each peptide. Identical residues among the three peptides are shaded. One letter amino acid code: A: alanine, C: cysteine, D: aspartic acid, F: phenylalanine, G: glycine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: arginine, S: serine, T: threonine, V: valine, Y: tyrosine

b.) Schematic representation of ANP, BNP-32 and CNP structures. Identical residues among the three peptides are indicated by open circles. (Sudoh 1990)

The action of the natriuretic peptides is mediated by the membrane-bound guanyl cyclase system, which activates cGMP dependent phosphodiestherases, protein-kinases and ion-channels[7, 184]. The natriuretic peptides interact with three receptors; all of which contain a well-characterized extracellular ligand-binding domain[191]. It is the A-type and B-type receptors that natriuretic peptides generate cGMP through[184]. The rank order of potency for cGMP production via the A-type receptor ANP > BNP >> CNP, while the affinity sequence for the B-type receptor appears to be CNP >> BNP > ANP[191]. Therefore the A-type receptor

was designated as selective for ANP and the B-type as selective for CNP. The precise nature of the transducing mechanism of the C-type natriuretic peptide receptor is not firmly established[72, 111]. All known members of the natriuretic peptide family bind to it, and several publications demonstrated that the C-type receptor may also take part in the transmission of natriuretic peptide signaling via the inhibition of adenylyl cyclase through a pertussis toxin-sensitive G protein[5]. Nevertheless, it is well-accepted that the C-type receptor and endopeptidase 24.11 may serve as a clearance receptor complex for all the natriuretic peptides; this function being verified by binding assays and endopeptidase 24.11 inhibitors[30].

Although ANP was first isolated from cardiocytes, later findings showed it a quite ubiquitous peptide. In the tissues the pro-ANP dominates while the bioactive 28 residue form is to be found in the circulation[136]. The highest concentration of pro-ANP was detected in the cardiac tissue and the aorta[209]. Remarkably high concentration of the prohormone can be found in the intestine, followed by lung, liver and kidney. It was also detected in the adrenal medullary chromaffin cells, and in the anterior pituitary[132]. In the CNS moderate concentrations of ANP immunoreactivity are present in the hypothalamus (preoptic and periventricular nuclei) the septum and in the circumventricular organs, with low concentrations in the thalamus, mesencephalon, cerebral cortex, hippocampus and cerebellum[98, 220, 222].

Marumo[126] demonstrated that the distribution pattern of BNP in the peripheral tissues is quite similar to that of ANP. However, the determination of the BNP and ANP contents of the heart, kidney, liver and plasma showed that BNP concentration was approximately one order of magnitude lower than that of ANP. In the CNS the opposite is true. Even Sudoh *et al.*[188] and later Ueda *et al.*[204] pointed out that the brain concentration of BNP is 13 times higher than that of ANP in the pig. Further studies reported[173] that BNP immunoreactivity involves many central structures of autonomic and endocrine control (lateral hypothalamus, supraoptic and paraventricular nuclei, midbrain, brainstem), which display little ANP positive staining.

CNP has a quite unique distributional pattern among the natriuretic peptides. On the one hand several studies showed that CNP is the major natriuretic peptide in the CNS[96, 134] in both rodents and humans, and especially high expression of CNP mRNA could be demonstrated by *in situ* hybridization analysis in the hypothalamus[70]. On the other hand CNP

represents only a minor proportion of natriuretic peptide immunoreactivity in the peripheral tissues[134, 205] suggesting that this natriuretic peptide functions in the central nervous system. The distribution of the natriuretic peptide receptors reinforces this hypothesis. High density of ANP and BNP binding sites were localized in the kidney and the heart[145]. In contrast, the A-type receptor could be localised only in discrete brain regions such as the hypothalamic nuclei, the circumventricular organs and the brainstem[20, 71, 85], which distribution considerably overlaps that of the angiotensin II receptor. This suggests that it be the control of cardiovascular homeostasis that A-type natriuretic peptide receptor mediation plays an important role in. In the CNS the B-type receptor appears to prevail[71, 104]; in the peripheral tissues the expression of this receptor is moderate compared with that of the A-type[118].

1.2.2 Peripheral and central actions of the natriuretic peptide family

ANP and BNP, sharing the A-type receptor, appear to be equally important in the regulation of the cardiovascular system and volume regulation. They show prominent natriuretic-diuretic, hypotensive and smooth-muscle relaxant activities[188]. On the contrary, CNP possesses much less prominent peripheral actions[190]; these findings might be explained by the different receptor affinity of the natriuretic peptides[191], and the tissue specific expression of the receptors[71, 104, 118].

The role of the natriuretic peptides in the central regulation of water and salt intake, blood pressure, behaviour, thermoregulation and hormone secretion has been demonstrated by countless publications. Both ANP and BNP exhibit prominent antidipsogenic action and blunt salt appetite after icv. administration[51, 85, 86, 143]. However, CNP appears to stimulate water intake under similar conditions[171]. ANP and BNP also exert a central inhibitory effect on vasopressin secretion[167, 216] and - although in relatively high concentration - inhibit the angiotensin and endothelin-induced pressor response[26, 122, 182, 183]. CNP proved to be much more potent in this regard. It suppressed vasopressin secretion in a two-orders of magnitude lower concentration than ANP and BNP[217]. Centrally administered CNP is also a more effective inhibitor of pressure response[28].

The picture outlined by behavioural studies appears to be even more complicated. On the one hand both ANP and BNP seem to be unequivocally anxiolytic and facilitate memory[198]. On the other hand the effect of CNP on anxiety-related behaviour displays marked dose-dependence[16, 186] and different neurotransmitters may mediate its action than those of ANP and BNP[14, 16].

Recent publications have demonstrated that natriuretic peptides may act as endogenous pyrogens[148], and they appear to play an essential role in the control of neuroendocrine processes. Their actions on vasopressin secretion is directly linked to their volume-regulatory actions[167, 216, 217]. Besides, they seem to regulate the release of numerous pituitary hormones. ANP activates growth hormone release[142], while it inhibits luteinizing hormone[169], PRL[170] and oxytocin[154] secretion. On the contrary, CNP stimulates both growth hormone release[181] and PRL secretion[172] and seems to be an autocrine regulator of gonadotropes[129]. Samson *et al.*[172] demonstrated that the neuroendocrine effects of ANP or CNP on PRL secretion are expressed on different cellular elements of the HPA axis, which data are in harmony with the concept, that the A-type and B-type receptor mediated processes may diverge significantly.

1.2.3 The role of the natriuretic peptides in the regulation of the HPA system

It has been shown that ANP can regulate, the HPA axis both at a pituitary[40, 50, 105] and a hypothalamic level[15, 82, 195]. ANP proved to inhibit the basal and CRH, vasopressin or ether-stress stimulated ACTH release *in vivo*[50, 105]. *In vitro* experiments have shown that the inhibitory action of ANP on ACTH release is dependent on cGMP regulated K^+ channels[7, 40] and disclosed that ANP also inhibits the basal and stimulated CRH secretion[15, 82, 195].

Although the concentration of BNP in the brain is much higher[204] than that of ANP, and CNP appears the major natriuretic peptide in the CNS[96], only several publications dealt with the function of these natriuretic peptides in the regulation of the HPA system[15, 54]. Moreover, the highly region-specific expression of the natriuretic peptides[96, 98, 173, 220, 222] and their receptors[20, 71, 72, 85, 104, 191] raised the possibility that they might be differently involved in the regulation of the afferent and efferent pathways of various stress responses. *In vitro* experiments investigating the action of the natriuretic peptides on the CRH content of brain

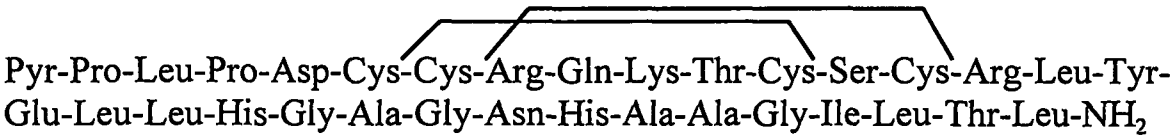
regions (hypothalamus, amygdala etc.) that are involved in the regulation of anxiety-related behaviour also suggested a diverse impact of ANP, BNP or CNP on the mediation of the inputs of the HPA system[15, 54]. These data urged the systematic investigation of the actions of BNP and CNP on different stress paradigms so that they could be compared with that of ANP.

1.3 The orexins

1.3.1 Chemical nature, their receptors and distribution

In 1998 a systematic biochemical search for endogenous peptide ligands for multiple orphan GPCRs led to the identification of a completely new family of neuropeptides[165]. As the mRNA for the precursor of these peptides specifically expressed in the lateral hypothalamus, a region classically implicated in the central regulation of feeding[147], they were termed orexin-A and orexin-B after the greek word ορεξισ, which means appetite. Both are processed from a 130-amino acid precursor prepro-orexin, and express no meaningful similarity to any known peptides[165]. Moreover, they differ markedly in structure, as orexin-A is a 33-amino acid residue peptide containing two intrachain disulphide bonds, an N-terminal pyroglutamyl residue and a C-terminal amidation, while orexin-B is a 28-amino acid residue linear peptide with a free N-terminus and displays only 46 % homology to orexin-A. These two peptides can activate two distinct GPCRs, $orexin_1$ and $orexin_2$. Both orexin-A and orexin-B have high affinities for $orexin_2$, suggesting that it is a nonselective orexin receptor. In contrast, $orexin_1$ is selective for orexin-A, binding orexin-B with an affinity 3 orders of magnitude lower[165]. These two receptors are much more similar to each other than they are to other GPCRs. The amino acid identity between the sequences of $orexin_1$ and $orexin_2$ is 64 %, which is much higher than their similarity to other neuropeptide receptors such as NPY_2 (26 % identity), TRH (25 %) and CCK-A (23 %). Both receptor genes have proved to be highly conserved among the species and these findings have strengthened the hypothesis that the orexins and their receptors represent another class of small regulatory peptides.

Orexin A (human, bovine, rat, mouse)



Orexin B (rat, mouse)

H-Arg-Pro-Gly-Pro-Pro-Gly-Leu-Gln-Gly-Arg-Leu-Gln-Arg-Leu-Leu-Gln-
Ala-Asn-Gly-Asn-His-Ala-Ala-Gly-Ile-Leu-Thr-Met-NH_2

Ill. 2 The primary structure of the orexins

These two neuropeptides and their receptors display a specific tissue distribution, which strikingly resembles that of the MCH, another important regulator of feeding[94]. Northern-blot analysis of adult rat tissues showed that prepro-orexin mRNA activity is abundantly expressed in the hypothalamus[41, 165]. Studies[41, 144], using a polyclonal anti-orexin serum, have demonstrated prominent granular immunoreactivity within widely spaced large polymorphic neurones exclusively in the dorsal lateral hypothalamic area.

Although most of the orexin positive perikarya are confined to the lateral hypothalamus, dense projection of orexin-positive nerve terminals is to be found in both hypothalamic and extrahypothalamic structures[144, 151]. Orexin positive fibers were observed throughout the hypothalamus (tuberal region, preoptic area, supraoptic nucleus, perifornical nucleus, ventromedial hypothalamic nucleus, arcuate nucleus, the PVN, etc.), and the fibers projecting out of the hypothalamus form four different pathways[151]: the dorsal and ventral ascending and the dorsal and ventral descending tracts. The densest extrahypothalamic projections reach the paraventricular and central medial nucleus of the thalamus, the zona incerta, the mesencephalic central grey, the nucleus of the solitary tract, the area postrema, the locus coeruleus and the raphe nuclei.

Again, in sharp contrast with the well-defined localization of the orexin-positive perikarya, the orexin receptors are widely and strongly expressed in the brain[76, 165] and the distribution of the receptor protein[76] was in agreement with the mRNA pattern revealed by *in*

situ hybridization[119]. Immunosignals were observed in the cerebral cortex, amygdala, basal ganglia, hippocampal formation, and various other subcortical nuclei in the hypothalamus, thalamus, midbrain and reticular formation. In particular, robust immunosignals were present in many hypothalamic and thalamic nuclei and in the locus coeruleus. The expression of the orexin$_1$ and orexin$_2$ mRNAs in some brain regions exhibited distinct distribution patterns[119, 125]. Within the hypothalamus, expression for the orexin$_1$ mRNA was largely restricted to the ventromedial and dorsomedial hypothalamic nuclei, while high levels of orexin$_2$ mRNA were contained in the PVN, ventromedial nuclei, and arcuate nucleus as well as in mammilary nuclei. In the amygdala, orexin$_1$ mRNA was expressed throughout the amygdaloid complex with robust labelling in the medial nucleus, while orexin$_2$ mRNA was only present in the posterior cortical nucleus of amygdala.

1.3.2 Biological activity

As the orexins are synthetized in the feeding center[41, 147, 165], and the vast majority of orexin neurones express leptin receptors[65] the first functional assays tried to assess the role of the orexins in the regulation of feeding. Numerous studies[45, 165, 166] have been published and demonstrated the food intake enhancing effect of the orexins. Therefore these neuropeptides seem to be among the central regulators of feeding.

The orexins also have been identified as mediators of various homeostatic and endocrine functions. Hagan *et al.*[64] demonstrated that orexin-A increases arousal and locomotion through the activation of locus coeruleus cell firing. It has been concluded that orexin-A might orchestrate the sleep-wake cycle. Later experiments[31, 115] with knockout mice and dogs have reinforced these ideas and lead to the elaboration of a stunningly new hypothesis: the abnormalities of the orexin system may play a crucial role in the pathophysiology of narcolepsy. Chemelli *et al.*[31] has reported that orexin knockout mice exhibit a phenotype strikingly similar to human narcolepsy patients, and modafinil, an anti-narcoleptic drug has proved to activate orexin-containing neurones. At the same time canine narcolepsy was identified as a disease caused by mutation in the orexin$_2$ gene[115]. These results opened novel potential therapeutic approaches for narcoleptic patients.

Due to the efforts of countless teams that "jumped on the bandwagon" to clarify the function of this newly discovered system, further homeostatic actions of the orexins were discovered. The orexins have turned out to possess prominent neuroendocrine effects. Both orexin-A and -B stimulate luteinizing hormone secretion[101, 156] and a significant participation of orexin-A in the preovulatory PRL surge was disclosed[101]. Further studies revealed that orexin-A acts on the hypothalamus to inhibit TRH release[135]. Besides, the orexins seem to regulate drinking behaviour[108] and they were "implicated" even in the modulation of nociceptive transmission[13].

1.3.3 The integrated regulation of feeding and stress response

The hypothalamus plays a central, integrative role in the control of neuroendocrine, autonomic and energy homeostasis. Fasting evokes a complex response of the HPA system, altering both the basal function and the responsivity to stressors. The rhythm in HPA activity follows the rhythm in food consumption[3, 214]. The HPA response to insulin-induced acute hypoglycaemia, a well-known stress paradigm, reflects the strong functional relationship between the hypothalamic feeding centres and the HPA system[63, 91]. Neuropeptides such as CRH, MCH and NPY have been found to be important regulators of both the HPA system and feeding behaviour[66, 92, 94, 206, 210].

The integrated action of the PVN and the arcuate nucleus appears to play a crucial role in the regulation of both stress response[6, 206] and feeding behaviour[94] through the secretion of neuropeptides such as CRH and NPY[66, 68, 94, 206, 210].

Recent studies have demonstrated that insulin-induced acute hypoglycaemia, a well-established stress paradigm[63, 91], can activate the orexin-containing cells in the lateral hypothalamus[61, 139]. Although orexin-containing neurones have not yet been detected in the PVN and the arcuate nucleus, abundant projections from the lateral hypothalamus to the PVN have been identified[38, 80, 144]. Histological studies have verified the functional activation of these neuronal circuits. Icv. infusion of the orexins greatly increased the expression of *c-fos*, a marker of neuronal activation, in the PVN and the arcuate nucleus[38, 45]. Moreover, van den Pol

et al.[208] has reported that the orexins increase the synaptic activity of hypothalamic neuroendocrine cells.

The anatomical and functional connections between the orexin neurones of the lateral hypothalamus and the PVN-arcuate nucleus complex support the hypothesis that the orexin neurones can mediate a complex endocrine and homeostatic response to fasting and insulin-induced hypoglycaemia. The prominent impact of the orexin system on arousal[64, 83, 194] also suggests that these neurones might take part in the regulation of the "alarm" reaction.

The characteristic distribution of the orexin neurones[83, 165] also raises the possibility that the orexins do not activate or inhibit homeostatic and endocrine processes directly, but rather through the release of other neuropeptides. Indeed, the effects of orexins on feeding[165] and hormone secretion[64, 84, 135] markedly resemble those of NPY[37, 110, 187, 201, 202, 212], and recent studies[43, 89, 218], involving the use of NPY antagonists, have revealed that the hyperphagia elicited by the orexins is at least partially mediated by NPY. Previously published data[80, 151] provided histological evidence for such an interaction between the orexin and the NPY system demonstrating that orexin axons synapse on the NPY cells in the arcuate nucleus. Because the NPY-positive neurone population of the arcuate nucleus also plays a considerable role in the regulation of not only feeding but also the HPA system[112, 116, 123, 187, 202], it raised the possibility that NPY signaling is involved in the action of the orexins on the HPA system, too.

1.4 The aim of our experiments

The purpose of our investigations was to reveal the role of two neuropeptide families - the natriuretic peptides and the orexins - in the regulation of the HPA system and also to clarify the mediation of their actions. In the light of the above-mentioned remarkable progress in stress research during the development our experiments the idea of "stressor specificity" always was taken into consideration.

1 4.1 The natriuretic peptides

To clarify the questions outlined above:

1. We investigated the *in vivo* effects of icv. administered BNP and CNP on the basal corticosterone secretion.

2. Their effects on the HPA responses to ether stress, electric shock and restraint also were tested to examine whether these peptides show any challenge specific action.

3. A comparison was made between the dose response curves of BNP and CNP to estimate the relative importance of the A-type and B-type receptor mediated processes in the regulation of stress response.

1.4.2 The orexins

Both *in vivo* and *in vitro* experiments were designed to test the effects of orexin-A and orexin-B on the HPA system and to investigate the sites mediating their actions.

1. The orexins were administered icv. in an attempt to characterize their central effect.

2. Rats were pretreated with the CRH antagonist α-helical CRH_{9-41} to elucidate whether these peptides activate the pituitary ACTH secretion or stimulate the hypothalamic CRH release.

3. By means of icv. pretreatment with an NPY antagonist[11] or NPY antiserum we set out to elucidate the possible role of NPY in the action of orexin-A and -B.

4. Ip. administration of the peptides and an *in vitro* system of perifused adrenal slices were used to investigate the peripheral action of the orexins.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 The tested peptides and the substances used in the *in vivo* experiments

- Rat CNP_{22}, porcine BNP_{32}, rat orexin-A and rat orexin-B. All the peptides were purchased from Bachem (Switzerland).
- ACTH ($\beta^{1\text{-}24}$ corticotropin, Ciba-Geigy, Vienna).
- CRH antagonist α-helical $CRH_{9\text{-}41}$.
- NPY antagonist - a full sequence peptide (D-Trp^{32})-NPY, which proved to be highly specific inhibitor of hypothalamic actions of NPY both *in vitro* and *in vivo*[11].
- NPY antiserum. It shows 100 % specificity to human/rat NPY (produced by the Yanaihara Institute Inc.).
- Normal rabbit serum generously donated by Miklós Vecsernyés (Department of Endocrinology, University of Szeged, Hungary).
- Saline (sodium chlorine inj. of 0.9 %, Biogal, Hungary).

2.1.2 Substances used in the *in vitro* experiments

- The components used for the Krebs' buffer preparation: NaCl, KCl, $MgSO_4$, $NaHCO_3$, glucose, KH_2PO_4 and $CaCl_2$ of analytical grade (Reanal, Hungary).
- Mixture of 5 % CO_2 and 95 % O_2 for continuous gassing.

2.1.3 The materials used for corticosterone assay

Ethyl alcohol, methylene chloride and sulfuric acid of analytical grade (Reanal, Budapest).

2.2 Animals

Rats were kept and handled during the experiments in accordance with the instructions of the University of Szeged Ethical Committee for the Protection of Animals in Research. Male Wistar rats weighing 150-200 g were used. The rats were kept in their home cages at a constant room temperature (23 ± 1 °C) on a standard illumination schedule, with 12-h light and 12-h dark periods (lights on from 6.00 a.m.). Commercial food and tap water were available *ad libitum*. The rats were allowed a minimum of 1 week to acclimatize before surgery, and to minimize the effects of nonspecific stress the rats were handled daily.

2.3 Surgery

To allow icv. peptide administration, the rats were implanted with a 10 mm long stainless steel cannula (prepared from hypodermic Luer needle of 20 G x 1.5 inch) aimed at the right lateral cerebral ventricle under Nembutal (35 mg/kg, ip.) anaesthesia. The stereotaxic coordinates were 0.2 mm posterior; 1.7 mm lateral to the Bregma; 3.7 mm deep from the dural surface, according to the atlas of Pellegrino *et al.*[150]. Cannulae were secured to the skull with dental cement and acrylate. The rats were used after a recovery period of at least 5 days. One day before the experiments the animals were transferred from the animal house to the laboratory so that they could habituate to their new environment. All tests were carried out between 8.00 and 10.00 a.m.

After completion the experiments methylene blue was injected into the ventricle of the decapitated heads and the brains were dissected to verify the correct positioning and the permeability of the cannulae. Only data from rats with accurate placement were considered in the statistical evaluation.

2.4 Corticosterone assay

The plasma corticosterone concentration was determined by the fluorescence assay described by Zenker and Bernstein[223] as modified by Purves and Sirett[155].

2.4.1 Sample collection

During the *in vivo* experiments trunk blood was collected from the animals into heparinized tubes and centrifuged for 10 min at 3000 rpm. Two hundred µl of plasma were transferred to centrifuge tubes. In the case of the perifusion system 200 hundred µl aliquots of the medium were transferred to centrifuge tubes.

2.4.2 Extraction

A reagent blank of 200 µl of distilled water and 2 corticosterone standards of the same volume containing 25 µg or 50 µg, respectively were prepared. Five ml of methylene chloride was delivered with an automatic pipette to each tubes and rocked for 30 min to allow for complete extraction of corticosterone by the solvent. The extract is centrifuged for 10 min at 3000 rpm. to eliminate any aqueous phase. Approximately 3.2 ml of the lower hydrophobic phase was aspired with a glass syringe then transferred into another centrifuge tube.

2.4.3 Fluorescent reaction

Four ml fluorescent reagent [stable mixture of 2.4 volumes of sulfuric acid and 1.0 volume of 50 % (v/v) aqueous ethyl-alcohol] was added to the extract. The tubes were shaken vigorously for 15 min, centrifuged at 3000 rpm. for 10 min and was allowed to stand at room temperature for 2 hours, which permitted the maximum development of fluorescence from corticosterone.

2.4.4 Measurement

Emission intensity was measured from the lower sulfuric acid layer with Hitachi 204-A fluorescent spectrophotometer at 456 nm extinction and 515 emission wavelength. The concentration of corticosterone of the samples was calculated from the values of the standards, and in the *in vivo* experiments was expressed as µg/100 ml. In the *in vitro* experiments, the amount of corticosterone secreted was expressed in terms of 100 mg adrenal tissue, for a period of 1 hour $\left(\frac{\mu g}{100\ mg \cdot hour}\right)$.

2.5 Experimental protocols

2.5.1 Effect of BNP and CNP on the activation of the HPA axis

2.5.1.1 Peptide treatment

BNP or CNP, dissolved in saline were injected icv. to conscious rats with Hamilton microsyringe over 30 s in a volume of 2 µl, immobilization of the rats being avoided during handling. The control animals received the same volume of saline. In order to obtain comparable data, we used equimolar doses of the natriuretic peptides (from 20 ng to 4.0 µg of CNP and from 32.5 ng to 6.5 µg of BNP).

2.5.1.2 Stress procedures

Thirty minutes after the peptide administration, the animals were subjected to one of the following stimuli: ether stress, electric foot-shock and restraint.

- For the ether stress, the animals were placed for 1.5 min in a jar containing an ether-dampened paper at the bottom. Unconsciousness always occurred within this period of breathing the ether-saturated atmosphere.
- In the case of the electric shock, the rats were placed in a shock-box (a wooden box with a stainless steel grid floor) and exposed to unescapable shocks. Electric foot-shocks (1 mA A.C., 50 Hz) were delivered to the paws by a shocker (Master Shocker, Lafayette Instrument Co., USA) using direct output. The current was turned on for 5 sec, then off for 10 sec for a period of 1 min
- For the restraint procedure, the animals were placed for 30 min in a 20 cm long, 6 cm wide non-transparent plastic tube, which was closed at either end with a 2 cm wide metal plate allowing air supply from both ends.

The rats were decapitated 30 min after the stress procedures and approximately 3 ml trunk blood was transferred for the corticosterone assay.

2.5.2 Effect of the orexins on the HPA system

2.5.2.1. In vivo experiments

To clarify the central and peripheral effects of the orexins, we used both icv. and ip. peptide administration. In the case of icv. administration, equimolar doses of orexin-A, orexin-B or α-helical CRF_{9-41} were injected to the animals.

For peripheral administration, equimolar doses of orexins or ACTH were dissolved in 0.5 ml saline and injected ip. Control animals received 0.5 ml saline ip.

In order to obtain trunk blood for corticosterone assay, in every experiment the rats were sacrificed by decapitation and approximately 3 ml blood was collected.

2.5.2.1.1 Effect of icv. administered orexin-A and -B on the basal corticosterone level

Rats received different icv. doses of either orexin-A (from 2.8 pmol to 280 pmol), orexin-B (from 28 pmol to 560 pmol). The doses of the orexins were selected with a view to finding the minimal statistically effective concentration and identifying the concentration, which evokes the maximal response. Control rats received saline alone. Each rat was returned to its home cage and maintained in a non-stressful environment till they were decapitated 30 min after the icv. injection.

2.5.2.1.2 Effect of α-helical CRF_{9-41} on the HPA response elicited by the orexins

For this experimental setting, we selected the most effective doses of the orexins (140 pmol and 280 pmol for orexin-A and orexin-B, respectively), and animals were divided into four treatment groups. Thirty min before orexin administration, group II and IV received an equimolar dose of α-helical CRF_{9-41} icv., and group I and III received saline alone. Afterwards group III and IV were treated with orexin, while vehicle was injected to groups I and II. The rats were killed 30 min after the second treatment.

2.5.2.1.3 Effect of (D-Trp^{32})-NPY on the orexin evoked HPA system activation

Four treatment groups were set up. Thirty minutes before orexin administration, groups II and IV received different concentrations of (D-Trp^{32})-NPY (from 280 pmol to 560 pmol) icv.,

whereas group I and III received saline alone. The doses of the antagonist were selected with a view to finding the minimal and maximal statistically effective concentrations. Groups III and IV subsequently received the most effective concentrations of orexin-A or orexin-B (140 or 280 pmol, respectively) icv., while groups I and II received saline alone. Each rat was returned to its home cage and maintained in a non-stressful environment until it was decapitated 30 min after the second icv. injection.

2.5.2.1.4 Effect of NPY antiserum on the orexin-induced HPA response

To further scrutinize the mediation of the action of the orexins different dilutions of NPY antibody (groups II and IV) or normal rabbit serum (groups I and III) were injected icv. 24 h before orexin administration. The time intervals for antiserum administration were chosen on the basis of previous data obtained with other antisera[174]. The rats were further treated with either orexin-A (140 pmol) or orexin-B (280 pmol) (groups III and IV), while vehicle was injected in groups I and II. The rats were killed and trunk blood was collected 30 min after the orexin treatment.

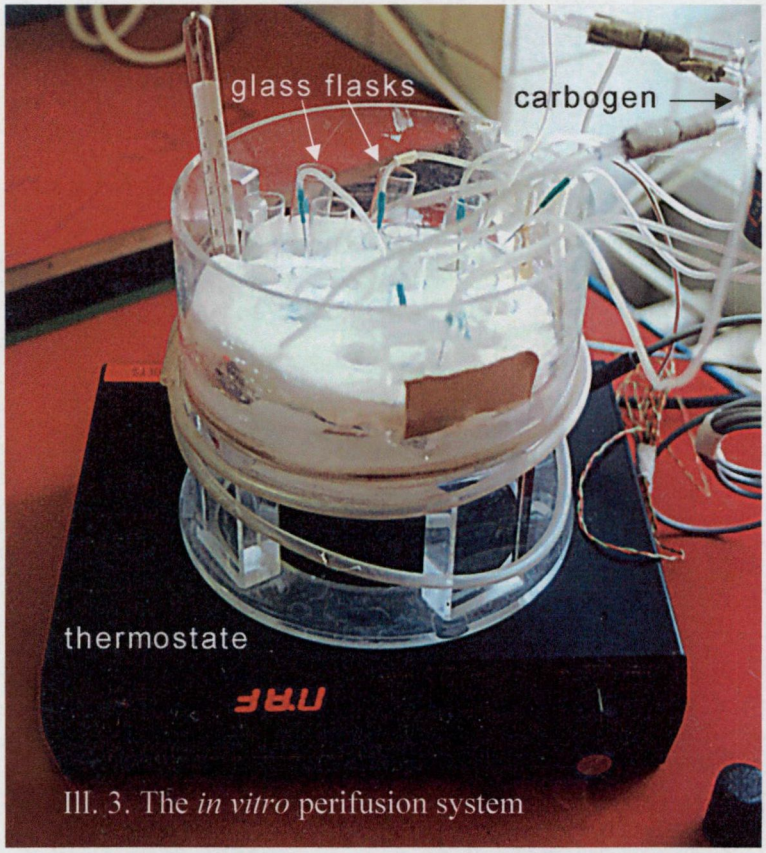
2.5.2.1.5 Effect of peripherally administered orexins on the adrenal corticosterone release

In this study, rats received an equimolar dose of orexin-A, orexin-B or ACTH (280 pmol) ip. Saline was injected ip. into the controls. Trunk blood was collected 30 min later for corticosterone assay.

2.5.2.2. Action of the orexins on the corticosterone secretion of adrenal slices in a perifusion system

We used the *in vitro* system described by Saffran and Schally[164] as a starting-point in developing our experimental design. Rats weighing 200-250 g were decapitated and the adrenals were removed and cleaned from the adhering fat and capsule with fine surgical forceps and a blade. The adrenals were weighed on a micro torsion balance (fresh wet weight about 12-16 mg) and each pair was immediately transferred to a separate Petri dish containing ice-cold, Krebs' solution (113 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.5 mM glucose, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, pH=7.4) as incubation medium. The

adrenals were rapidly, and as evenly as possible, cut into approximately 500 µm slices with a surgical blade. The slices from each pair were placed in a separate glass flask (Ill. 3). The flasks, containing 5 ml incubation medium, were submerged in a water-bath at 38 °C and constantly and gently gassed through a single-use needle (30 G; 0.3 x 13) with a mixture of 5 % CO_2 and 95 % O_2; the pH was maintained at 7.4. The adrenals were preincubated for 1 h, at the end of which the medium was sucked out and discarded. In 5 ml of fresh medium, different doses of orexin-A, orexin-B or ACTH (or the medium alone as a control) were added to the adrenal. The samples were incubated for 30 min, after which 200 µl aliquots of the medium were transferred into centrifuge tubes for corticosterone assay.



Ill. 3. The *in vitro* perifusion system

2.6 Statistical analysis

All data are given as means ± S.E.M. Statistical analysis of the results was performed by one way analysis of variance (ANOVA). In order to reveal the stressor-dependent action of the natriuretic peptides and to compare their effectiveness two-way ANOVA was carried out to unveil stressor x treatment interactions. If the ANOVA revealed the unequality of means the groups were compared with Tukey's *post hoc* test for multiple comparisons with unequal cell size (Spjotvoll-Stoline). A probability level of 0.05 was accepted as indicating a statistically significant difference.

3 RESULTS

3.1. Effects of the natriuretic peptides on the basal corticosterone release and the HPA activation evoked by ether-stress, electric-shock and restraint

3.1.1 Effect of BNP on the corticosterone response induced by different stressors

BNP in the doses applied (0.0325, 0.325, 3.25 or 6.5 μg) did not influence the basal corticosterone secretion (Figs. 1-3). Ether stress (Fig. 1), electric shock (Fig. 2) and restraint (Fig. 3) all increased the plasma corticosterone concentration approximately 4-fold.

BNP caused a dose-dependent decrease in the ether stress-induced corticosterone response (Fig. 1). The highest dose of BNP (6.5 μg) inhibited the corticosterone response by 42.8 % and this effect proved highly significant ($F(9, 84) = 110.13$, $p < 0.005$). Even in a dose of 0.325 μg, BNP decreased the plasma corticosterone level to a statistically significant extent as compared to the stressed animals ($p < 0.05$).

The 6.5 μg dose of BNP reduced the corticosterone response to electric shock (Fig. 2) by 35.7 % ($F(7, 63) = 70.2$, $p < 0.005$) and the dose of 3.25 μg likewise elicited significant inhibition ($p < 0.01$).

The effect of BNP on the corticosterone response to restraint (Fig. 3) was less marked. Only the highest dose of BNP (6.5 μg) was able to attenuate (by 28.8 %) the restraint-induced increase in plasma corticosterone level to a statistically significant extent ($F(5, 41) = 60.4$, $p < 0.005$).

3.1.2 Effect of CNP on the corticosterone response elicited by different stressors

CNP in a dose of 2 μg or 4 μg had no action on the basal level of plasma corticosterone (Figs. 4-6). Ether stress (Fig. 4), electric shock (Fig. 5), and restraint (Fig. 6) caused the characteristic increase in plasma corticosterone.

CNP exerted a dose-dependent inhibition of ether stress-induced secretion of corticosterone (Fig. 4). The effect was significant ($F(5, 24) = 36.6$; $p < 0.01$) even at a dose of 0.02 μg: the corticosterone response was inhibited by 58 % by 0.2 μg and abolished by 2 μg

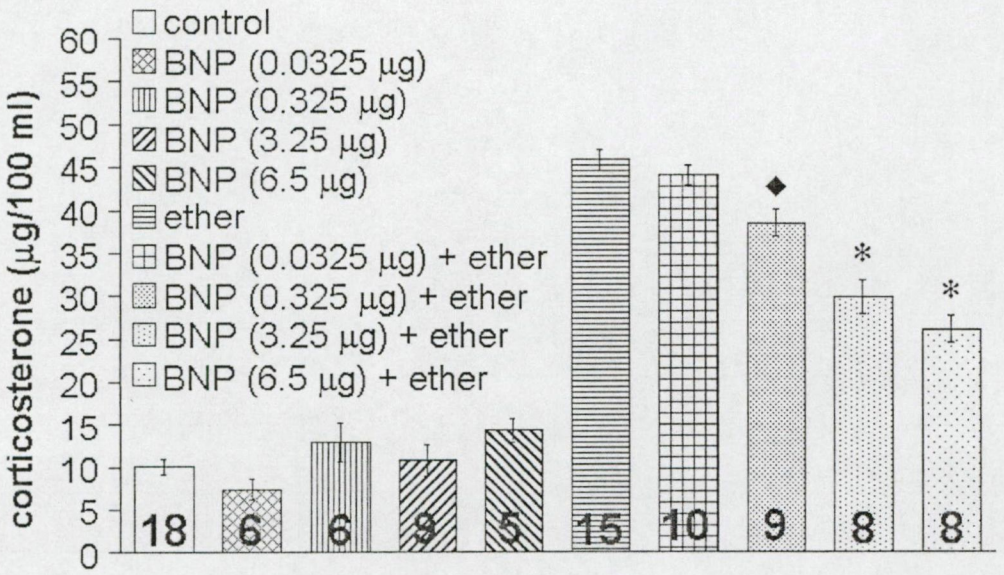


Fig. 1. The effect of brain natriuretic peptide on the plasma corticosterone levels evoked by ether stress. Symbols: ◆: $p < 0.05$ vs. ether; *: $p < 0.005$ vs. ether. Figures within bars are the numbers of animals used.

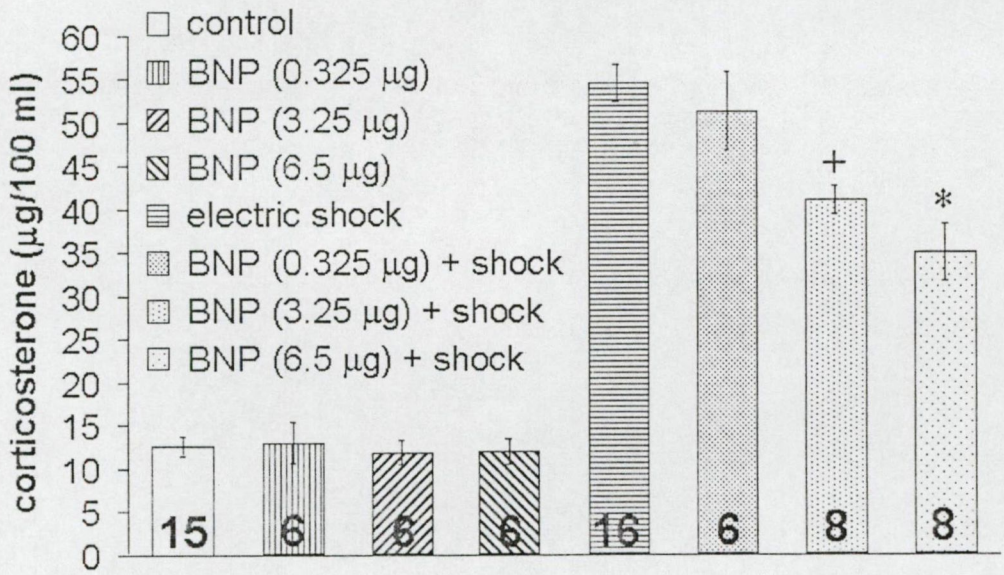


Fig. 2. The effect of brain natriuretic peptide on the electric shock-induced increase in plasma corticosterone level. Symbols: +: $p < 0.01$ vs. electric shock ; *: $p < 0.005$ vs. electric shock. Figures within bars are the numbers of animals used.

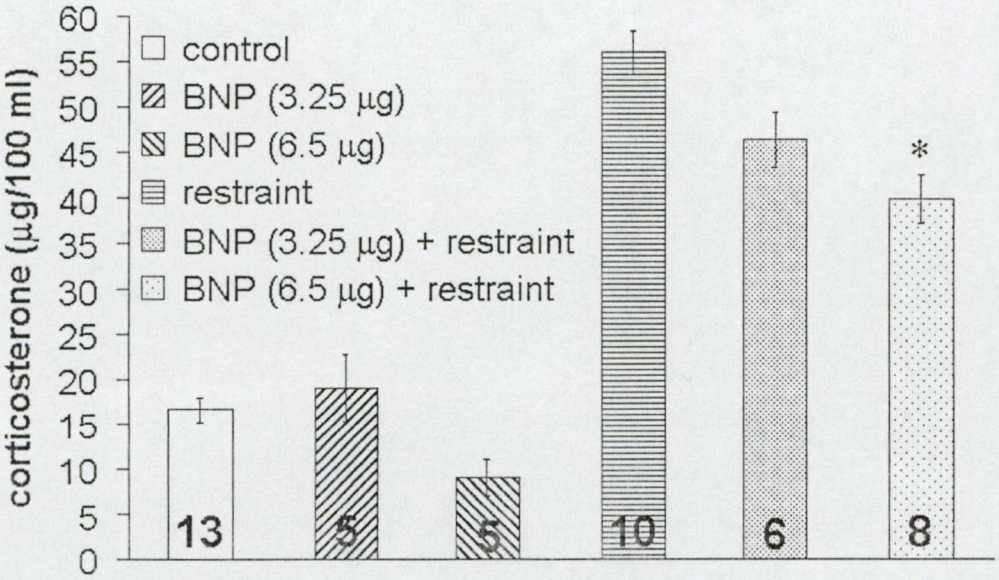


Fig. 3. The effect of brain natriuretic peptide on the plasma corticosterone level induced by restraint. Symbols: *: $p < 0.005$ vs. restraint. Figures within bars are the numbers of animals used.

CNP (both $p < 0.001$) so that there was no difference from controls ($p = 0.1$ and 0.9, respectively).

The effects of CNP on the corticosterone response to electric shock were less marked (Fig. 5). The 2 μg dose, which abolished the response to ether, decreased the corticosterone response by only 24 % ($F (5, 32) = 79.75$; $p < 0.005$ vs. electric shock); the 4 μg dose decreased the response by 34 % ($p < 0.001$ vs. electric shock) but to a level still significantly above that in controls ($p < 0.001$ vs. control).

The effects of CNP on the corticosterone response to restraint stress were also less marked (Fig. 6). Only the highest dose of CNP (4 μg) was able to diminish the restraint-induced increase in plasma corticosterone in a statistically significant manner ($F (5, 40) = 39.89$; $p < 0.01$); the effect of 2 μg CNP was not significant.

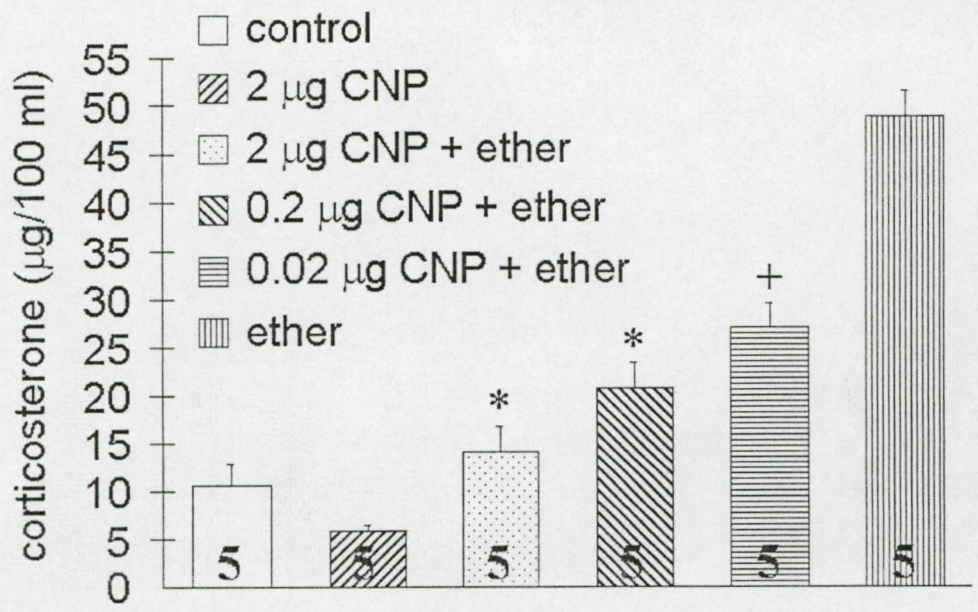


Fig. 4. The effect of C-type natriuretic peptide on ether stress induced plasma corticosterone levels. Symbols: +: $p<0.01$ vs.ether; *: $p<0.001$ vs. ether. Figures within bars are the numbers of animals used.

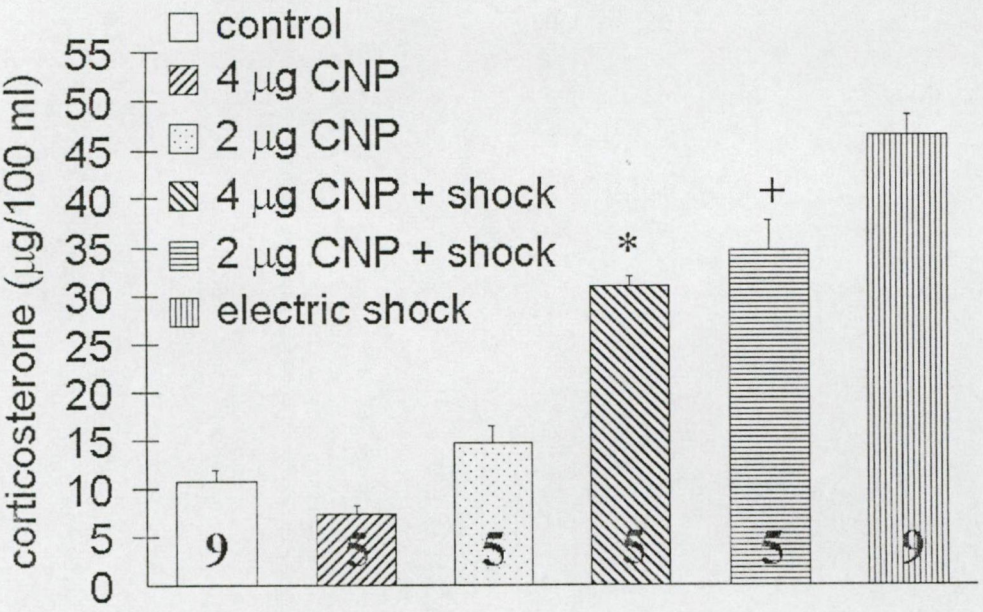


Fig. 5. The effect of C-type natriuretic peptide on plasma corticosterone evoked by electric shock. Symbols: +: $p<0.005$ vs. electric shock; *: $p<0.001$ vs. electric shock. Figures within bars are the numbers of animals used.

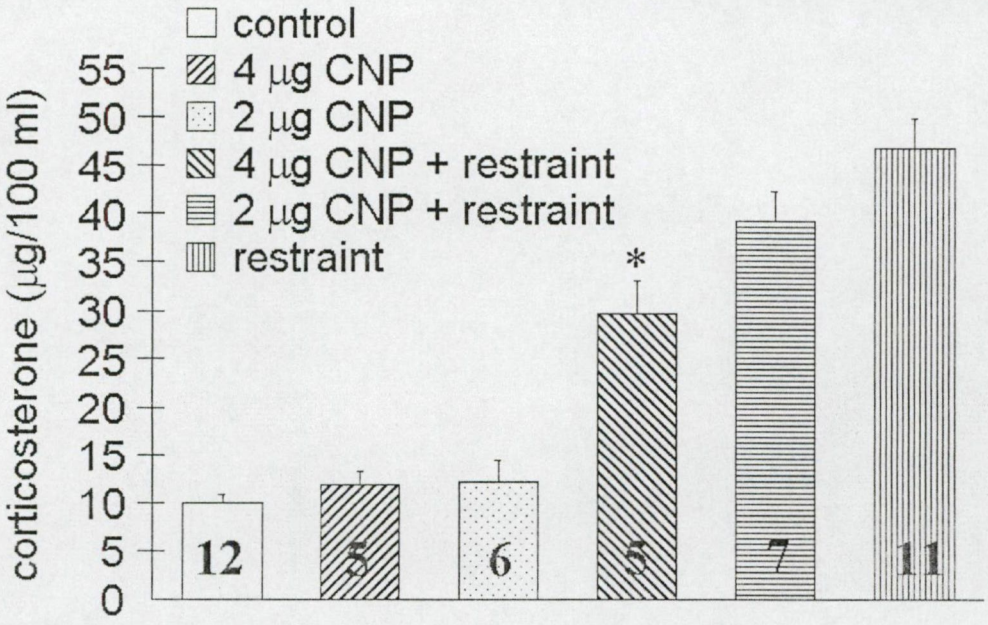


Fig. 6. The effects of C-type natriuretic peptide on plasma corticosterone evoked by restraint. Symbol: *: p = 0.01 vs. restraint. Figures within bars are the numbers of animals used.

3.1.3 Comparison of the action of BNP and CNP

Although both peptides proved to inhibit the HPA response to different stressors, only in the case of CNP did the statistical analysis reveal stressor-specific action. Comparing the effects of 2 μg CNP on the HPA responses evoked by different stressors, two-way ANOVA unveiled significant stressor x treatment interaction ($F(2, 36) = 9.06$; $p < 0.001$). One-way ANOVA ($F(2, 14) = 16.67$; $p < 0.001$) and the *post hoc* comparison disclosed that CNP blocked much more effectively the corticosterone response to ether-stress than to restraint or electric shock ($p < 0.01$ ether + 2 μg CNP *vs.* restraint + 2 μg CNP; $p < 0.05$ ether + 2 μg CNP *vs.* electric shock + 2 μg CNP).

The statistical analysis of the effects of the equimolarly applied BNP or CNP on the ether stress evoked corticosterone response also unveiled significant stressor x treatment interaction ($F(2, 64) = 49.8$; $p < 0.001$). One-way ANOVA ($F(3, 23) = 23.49$; $p < 0.001$) and Tukey's test showed the action of BNP on the ether stress evoked HPA activation significantly less pronounced than that of CNP ($p < 0.001$ ether + 2 μg CNP vs. ether + 3.25 μg BNP).

3.2. Effect of the orexins on the HPA axis

3.2.1. Effects of orexin-A and orexin-B administered icv. on basal corticosterone release

Orexin-A increased corticosterone secretion in a dose-dependent manner (Fig. 7). The most effective dose (140 pmol) elevated the plasma concentration of corticosterone by 208 % as compared to the control ($F(8, 88) = 4.62$; $p < 0.001$). A higher dose of orexin-A (280 pmol) proved less effective, evoking only a 149 % increase ($p < 0.05$) versus (vs.) the control. Orexin-B likewise caused a dose-dependent increase (Fig. 7). However, it peaked at a dose of 280 pmol and resulted in only a 189% increase above control levels ($p < 0.005$).

3.2.2. Effects of α-helical CRH_{9-41} on HPA activation evoked by orexins

The CRH antagonist α-helical CRH_{9-41} completely abolished the corticosterone response evoked by 140 pmol orexin-A ($F(5, 65) = 12.38$; $p < 0.005$ α-helical CRH_{9-41} + 140 pmol

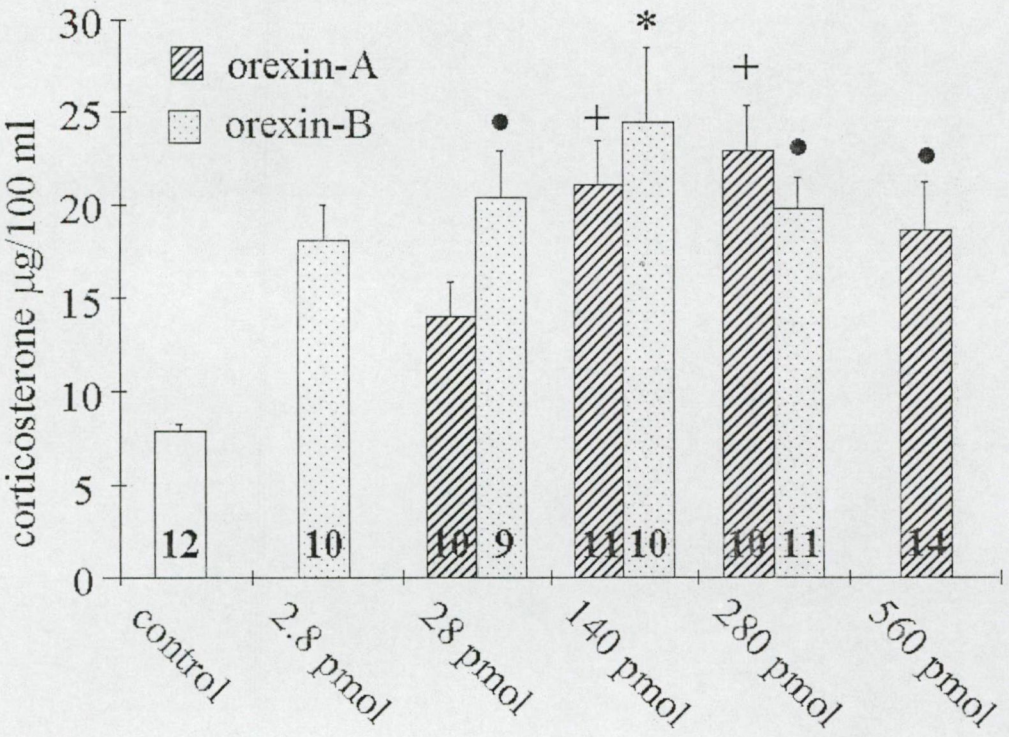


Fig. 7. Effects of icv. administered orexin-A or orexin-B on plasma corticosterone concentration. Symbols: *: $p<0.001$ vs. control, +: $p<0.005$ vs. control, ●: $p<0.05$ vs. control. Figures within bars are numbers of rats used.

orexin-A vs. 140 pmol orexin-A) (Fig. 8). The response induced by orexin-B was similarly inhibited by α-helical CRH_{9-41} ($p < 0.001$ α-helical CRH_{9-41} + 280 pmol orexin-B vs. 280 pmol orexin-B) (Fig. 8).

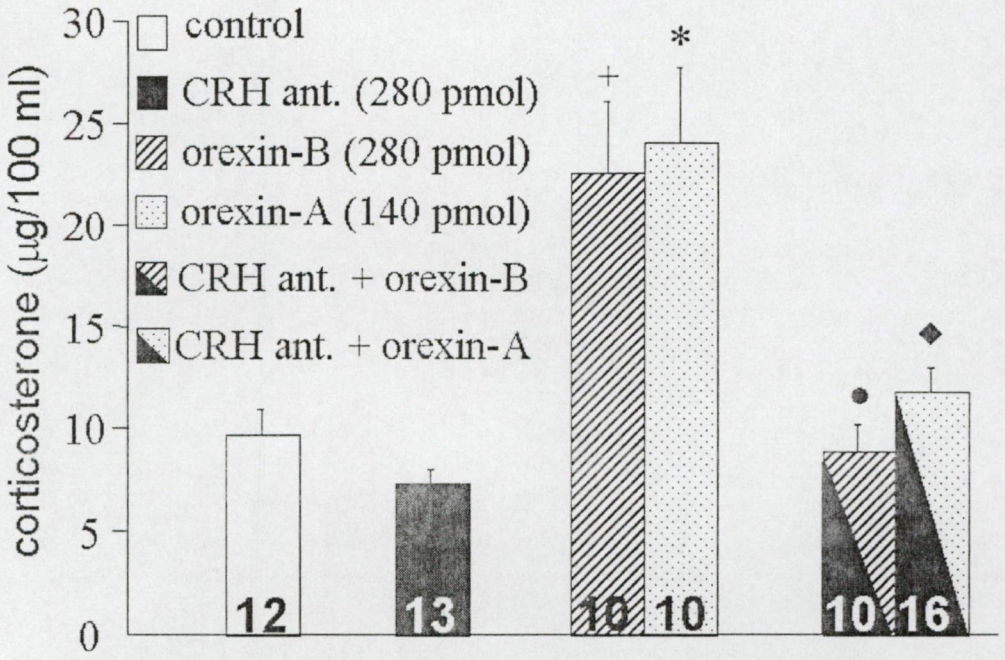


Fig. 8. Effects of icv. administered α-helical CRH_{9-41} on the plasma corticosterone release evoked by orexin-A or orexin-B. Symbols: *: $p<0.001$ vs. control, +: $p<0.005$ vs. control, ●: $p<0.001$ vs. orexin-B, ◆: $p<0.005$ vs. orexin-A. Figures within bars are numbers of rats used.

3.2.3. Effects of NPY antagonist on HPA activation elicited by orexins

Both orexin-A and orexin-B elicited a pronounced increase in the plasma concentration of corticosterone relative to the control (121 % and 98 %, respectively), the effect proving statistically significant ($F(11, 181) = 7.01$, $p < 0.001$ and $p < 0.001$, respectively vs. the control) (Fig. 9). (D-Trp^{32})-NPY pretreatment diminished the orexin-induced corticosterone response in a dose-dependent manner. The doses of 280 pmol and 420 pmol revealed a tendency to attenuation, but only the highest dose (560 pmol) brought about a statistically significant inhibition ($p < 0.05$ vs. 140 pmol orexin-A and 280 pmol orexin-B), diminishing the orexin-A or orexin-B-induced HPA activation by 42 % and 46 %, respectively (Fig. 9).

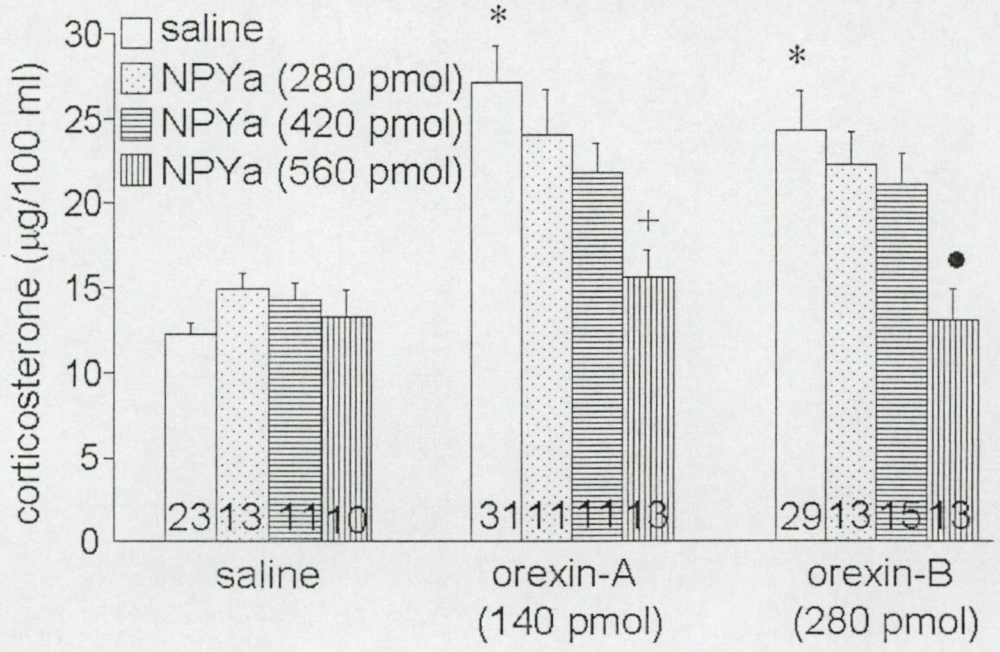


Fig. 9. Effects of NPY antagonist on corticosterone release evoked by orexins. Abbreviation: NPYa: NPY antagonist. Symbols: *: $p<0.001$ vs. control; +: $p<0.05$ vs. orexin-A; ●: $p<0.05$ vs. orexin-B. Numbers within bars are numbers of animals used.

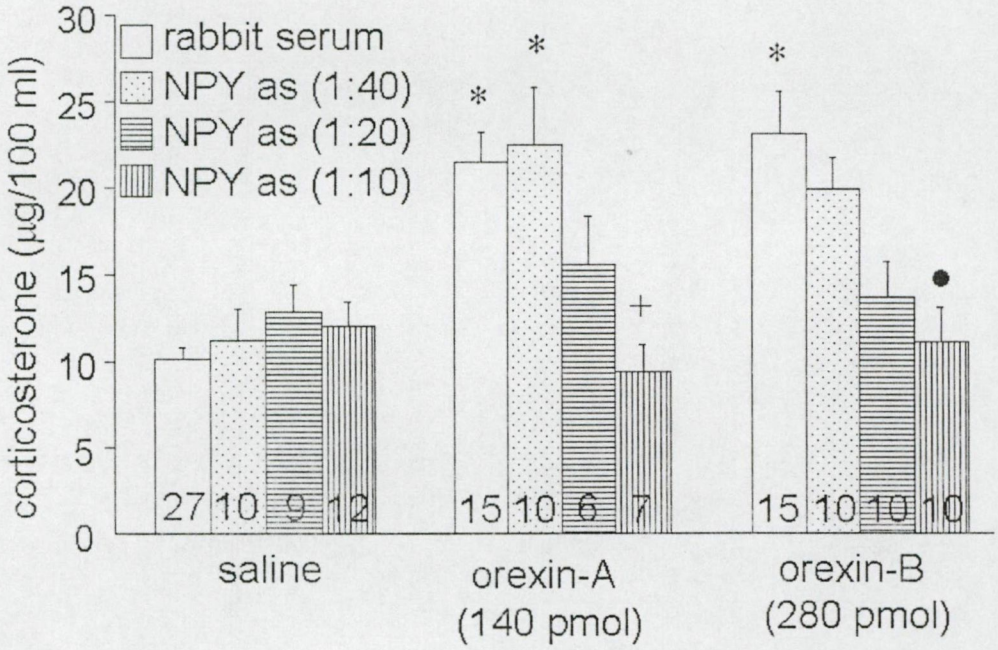


Fig. 10. Effects of NPY antiserum on corticosterone release evoked by orexins. Abbreviations: NPYas: NPY antiserum; 1:40, 1:20, 1:10: dilutions of antiserum; rabbit serum: normal rabbit serum. Symbols: *: $p<0.01$ vs. control; +: $p<0.05$ vs. orexin-A; ●: $p<0.01$ vs. orexin-B. Numbers within bars are numbers of animals used.

3.2.4 Effects of NPY antiserum on orexin-induced HPA activation

The NPY antiserum dose-dependently inhibited the corticosterone response elicited by the orexins. Even a dilution of 1:20 resulted in a marked inhibition of the orexin-A and orexin-B-evoked HPA activation (27 % and 40 % decrease, respectively), but only a dilution of 1:10 caused a statistically significant inhibition of the HPA response evoked by orexin-A (56 %, $F(11, 129) = 7.41$ $p < 0.05$ vs. 140 pmol orexin-A) or orexin-B (51 %, $p < 0.01$ vs. 280 pmol orexin-B) (Fig. 10).

3.2.5 Comparison of effects of ip. administered orexins and ACTH on basal corticosterone release

The basal plasma concentration of corticosterone was not modified by exposure to either orexin-A or orexin-B. By contrast, an equimolar dose of ACTH elicited 127 % increase; $F(3, 16) = 7.61$, $p < 0.01$ vs. control) (Table 1).

	control	orexin-A (280 pmol)	orexin-B (280 pmol)	ACTH (280 pmol)
mean ± S.E.M.	14.99 ± 1.61 (5)	14.13 ± 2.11 (5)	18.86 ± 3.46 (5)	34.05 ± 5.1 (5) *

Table 1. Effects of ip. administration of orexins and ACTH on corticosterone secretion. Corticosterone concentration is given as µg/100 ml. Numbers in brackets are numbers of animals used. Symbol: * : $p < 0.01$ vs. control.

3.2.6 Comparison of effects of orexins and ACTH on the corticosterone release of perifused adrenal slices

The basal secretion of corticosterone from isolated adrenals *in vitro* was not significantly influenced by exposure to orexin-A or by exposure to orexin-B. Exposure to ACTH (280 pmol) produced the expected significant increase in secretion (Table 2).

	control	orexin-A		orexin-B		ACTH
		280 pmol	2.8 nmol	280 pmol	2.8 nmol	280 pmol
mean ± S.E.M.	3.22 ± 0.76 (5)	2.71 ± 0.43 (5)	3.29 ± 0.9 (5)	2.52 ± 0.51 (5)	5.28 ± 2.32 (5)	13.00 ± 4.3* (5)

Table 2. Effects of orexins and ACTH on corticosterone release in an *in vitro* system. Values represent the corticosterone released by 100 mg adrenal tissue per hour (µg / 100 mg x hour). Numbers in brackets are numbers of animals used. Symbol: * : $p < 0.05$ vs. control.

4 DISCUSSION

Present data have clearly shown that neither BNP nor CNP affects the basal release of corticosterone. This finding is in harmony with previous publications[50, 121], which demonstrated that through icv. administration neither BNP nor anti-ANP serum influence the basal ACTH secretion. Our data support the view that hypothalamic or pituitary cells bearing natriuretic peptide receptors do not exert a permanent facilitating action on the HPA axis.

Both peptides dose-dependently inhibited the hormonal response to three stressful stimuli, which confirms the view that natriuretic peptides have a prominent impact on the central regulation of the HPA axis. Several papers showed the A-type receptor specific natriuretic peptides to have an unambigous inhibitory action on the pituitary ACTH release evoked by CRH or vasopressin[62, 105], which support the concept that they may have a prominent impact on the HPA system as circulating hormones[50]. On the other hand, the publications regarding their effect on the hypothalamic part of the HPA system revealed conflicting data[15, 50, 54, 82, 121, 195]. Makino *et al.*[121] demonstrated the inhibitory action of centrally administered BNP on endothelin evoked ACTH release. In contrast, Fink *et al.*[50] found a marked facilitatory effect of intra-atrial but not icv. administration of anti-ANP serum on the ACTH response to stress and concluded that ANP acts at the level of pituitary as a peripheral regulator. However, the *in vitro* studies showed the inhibitory action of ANP and BNP both at a pituitary[7, 40, 62] and a hypothalamic[15, 54, 82, 195] level. Electrophysiological experiments[2, 219] revealed the inhibitory action of ANP and BNP even on the firing rate of hypothalamic neurons, and their action proved to be mediated by cGMP-dependent protein-kinases. The different concentrations reached by *in vitro* or *in vivo* administration might serve as a reasonable explanation for the discrepancy between the data of the *in vitro* and *in vivo* experiments: higher effective doses may result in the activation of not only the A-type but, aspecifically, the B-type receptors.

The complex action of the CNP circuitry on the HPA axis is also revealed by several publications. The intricate organization of the B-type receptor positive network is reflected by the marked time- and stimulus-dependent effect of icv. administered CNP in the sheep. While

in normal sheep it evokes hypotension and after an abrupt initial increase in the corticosterone response a long-lasting supression ensues[28], CNP appears to augment the HPA response to hemorrhage[29]. Not only its hypothalamic actions represent equivocal data but we also have conflicting findings regarding the action of CNP at a pituitary level. Mulligan *et al.*[141] reported a lack of CNP effect on the basal or stimulated ACTH release from equine pituitary cells *in vitro*. On the other hand, Guild[62] demonstrated CNP to exert a robust inhibitory influence on CRH-stimulated ACTH secretion from mouse pituitary preparations. Such a discrepancy suggests a species dependent role of CNP in the regulation of the ACTH release; the opioids show similar species specific action on the HPA system[21, 22, 33, 117, 185].

Although both BNP and CNP attenuated the stimulated corticosterone release in our experiments, their actions showed some remarkable differences. BNP indistinctively attenuated the response to ether, electric shock and restraint, while CNP displayed marked stressor specificity i.e. one order of magnitude lower concentration (0.2 μg) elicited the same inhibition of ether stress induced HPA response as in the case of electric shock or restraint, and 2 μg CNP completely abolished the corticosterone release evoked by ether. The stressor specific action of CNP can be attributed to the different expression of the B-type receptor in the afferent pathways of various stress paradigms and cast doubt on its direct inhibitory action on the paraventricular CRH release. On the other hand, the indistinctive inhibitory effect of the A-type receptor specific BNP presumably reflects that it acts only on the hypothalamic[54, 82, 121] or pituitary[7, 40, 50, 105] cells of the final common pathway in the HPA system. Histological studies confirm this concept[20, 71, 104]. While high B-type receptor expression can be observed in all the structures (preoptic-hypothalamic structures, limbic cortex, olfactory bulb, amygdala, brainstem)[71, 104] that take part in the organization of the input of the applied stressors (ether, restraint, electric shock) and the CNP neurones represent the most abundant type of the natruretic peptide positive cells in the brain[96, 134], BNP and A-type receptor expression[20, 71, 85, 98, 173, 220, 222] is practically restricted to those hypothalamic regions (subfornical organ, supraoptic and paraventricular nuclei) that represent the output of the HPA axis. Moreover, the remarkably high contribution of the dominant ACTH co-secretagogues[107, 130] vasopressin to the ACTH secretion in the case of ether-stress[55] suggests that the CNP circuitry exert considerable

inhibition on the vasopressin-mediated ACTH release. In contrast, the actions of BNP may sign a uniform inhibition of CRH and/or ACTH secretion.

This hypothesis is in harmony with the theory of "stressor specificity" and the "state-of-the-art" classification of the stress pathways. Electric footshock and restraint belong to the processed, neurogenic or psychological stressors which represent a multi-modal, brain-constructed form that rely on somatosensory or nociceptive inputs plus involve a distinct, affective emotional component[73, 177]. Unlike the systemic stressors (e.g. hemorrhage, hypoglycaemia, hyperosmotic challenge, immune stimuli or endotoxic shock) that target homeostatic parameters, the processed stressors are prominently regulated by limbic/cortical structures (such as the hippocampus and prefrontal cortex). Systemic stressors triggered by interoceptive stimuli predominantly activate the brainstem/circumventricular organ systems that project directly to the paraventricular nucleus without being channeled through cortical structures[73]. Lesion[42, 75, 107] and immediate early gene mapping[113] studies unequivocally provided huge bulk of evidence for such separate "handling" of the different inputs to the HPA axis.

Despite the differences in central processing, both types of stressors utilize the sensory neural routes (e.g. catecholaminergic pathways of the brainstem and the centers of the pons and the midbrain)[74, 175]. Denervation experiments clearly showed that either cord section or denervation of the hind limb prevents painful stimuli from evoking ACTH secretion[120, 157]. In contrast, ether inhalation seems to represent a different stress paradigm. It may act directly on the medial basal hypothalamus, because it can induce the activation of the pituitary-adrenal axis in spite of complete hypothalamic deafferentation[48, 49, 60, 128]. Recent studies based upon immediate early gene mapping have revealed that it may also invoke chemoreceptive/respiratory pathways in the brainstem eliciting an activation pattern which stunningly resemble that of systemic stressors[47]. In contrast with novelty-stress ether evokes more circumscribed, although more robust, CNS activation without eliciting *c-fos* induction in limbic sites such as the medial amygdala and the lateral septum.

On the basis of some recent behavioural studies[88, 138, 213] an even more complicated picture needs outlining. These studies showed that unlike ANP and BNP[14, 16, 138] CNP at a dose-range from 0.5 μg to 5 μg possess a distinct anxiogenic property and propose that this

action is mediated by CRH[88]. Like the opposite effects of ANP and CNP on water uptake, salt appetite and PRL secretion[170, 171, 172] might be explained by the different distribution of the specific natriuretic peptide receptors in the CNS[71, 104, 118]. To be more elusive, CNP in smaller concentrations (100-200 ng)[16] appears to have anxiolytic properties pointing toward a bidirectional non-linear dose-response curve[88].

In the aforementioned behavioural experiments[88, 138, 213] CNP did not have an impact on ACTH or corticosterone release 30 min after the peptide administration and, no significant response could be established even 30 min later either, which findings are in agreement with our results regarding the action of CNP on the basal corticosterone secretion. Further, some apparent discrepancies between the results of the behavioural and the endocrinological studies pertaining to the effects of CNP on CRH secretion do not necessarily represent contradiction. Different parts of the CRH circuitry of the brain appear to be involved in the control of behavioural or neuroendocrine processes: the CRH positive cells of the parvocellular PVN governs the stress response and show *c-fos* activation regardless of the type of stress[177]. On the other hand, the amygdala appears to be the source of CRH that controls anxiety-related behaviour [59], and its different parts play stressor-dependent modulatory roles in the processing and the mediation of the impulses to the PVN[35, 39, 177]. This hypothesis is supported by the findings of Pich *et al.*[152]: the blockade of pituitary-adrenal axis activation by CRH immunoneutralization did not affect the behavioural response to social defeat stress in rats. Besides, the neuropeptide profile of the HPA system and the stress-related behavioural processes displays conspicuous differences, too. For instance, while vasopressin plays a vital role in the control of the HPA axis[55, 107], it does not seem to have anxiogenic properties[152]. Therefore, the CNP neurones might possess a dose dependent effect on the parts of the CRH system that controls behaviour. In contrast, their impact on the HPA system is made up of their different influence on the B-type receptors of the afferent stress pathways and they do not necessarily influence directly the paraventricular CRH release. On the other hand, ANP and BNP might exert an unambiguous inhibitory action on both parts of the CRH circuitry through the A-type receptor system.

Fasting and its experimental analogue insulin-induced hypoglycaemia represent another well-known stress paradigm. In the presented experiments the orexins, the putative

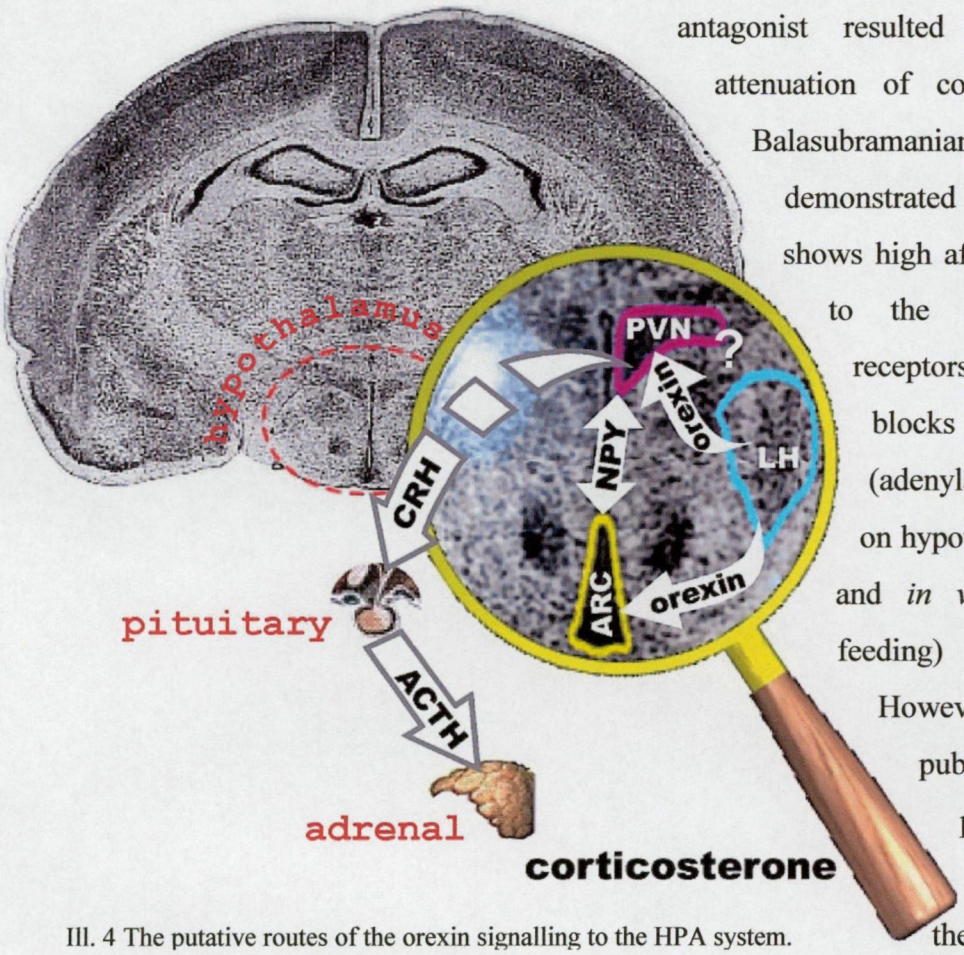
coordinators of feeding related homeostatic and neuroendocrine responses[165] , elicited a dose-dependent increase in corticosterone release. It is noteworthy that orexin-B was less effective in equimolar concentrations than orexin-A, which finding might be explained by the differences in the chemical structure of the orexins. Both the two intrachain disulphide bonds and the posttranslational modification of the N terminus appear to make orexin-A less susceptible to inactivating peptidases[165]. Indeed, orexin-B was demonstrated to be rapidly degraded in blood and its bioavailability in the brain is approximately 20 % of orexin-A after 2 min peripheral infusion[97]. Besides, as both orexin-A and orexin-B have high affinities for $orexin_2$, but $orexin_1$ appears selective for orexin-A[165], $orexin_1$ might predominate in the activation of the HPA axis. These data are in harmony with previous studies, that have demonstrated that the effect of orexin-A on nutritional homeostasis[45, 165] is more potent and longer lasting than that of orexin-B. Discrepancies were also disclosed by endocrinological and behavioural studies: the effect of orexin-B on TSH secretion and grooming and searching behaviour[83, 93] appears to be considerably different from orexin-A, which data may reflect diverse actions through the $orexin_1$ and $orexin_2$ receptors.

Both peptides furnish dose-response curves, with a downturn phase. This resembles the action of orexin-A on feeding[45], and the bell-shaped dose-response curve of NPY on food-intake, memory and body temperature[17, 32, 52]. Neuropeptides characteristically elicit such a dose-response curve, which feature may reflect functional antagonism in post-receptorial signal transduction[153] such as receptor phosphorylation by a G protein-coupled receptor kinase [53]. The inhibitory action of less-specific receptors[81] at higher concentrations or the postsynaptic down-regulation of the receptor synthesis[9] also might be taken into consideration.

The actions of the most effective dose of both orexin-A and orexin-B could be completely abolished by the preliminary administration of α-helical CRH_{9-41}. As the amino acid sequences of the orexins exhibit little similarity to the sequences of any other peptides[165, 196, 206] present results with the CRH antagonist suggest that the orexins should not act on the pituitary CRH receptors, but rather bring about hypothalamic CRH secretion. Nambu *et al.*[144] reported that the anterior and intermediate lobes of the pituitary do not contain orexin-positive fibers, which data provide histological support to our findings. Besides, the complete

inhibition of the stimulatory action of the orexins by the application of α-helical CRH_{9-41} implies that other ACTH secretagogues such as vasopressin might not play a major role in the orexin-induced HPA activation.

Pretreatment with NPY receptor antagonist $(D\text{-}Trp^{32})\text{-}NPY^{11}$ dose-dependently inhibited the orexin-induced HPA activation, although only the highest concentration of the antagonist resulted in a pronounced attenuation of corticosterone release. Balasubramaniam *et al.*[11] demonstrated that $(D\text{-}Trp^{32})$-NPY shows high affinity and specificity to the hypothalamic NPY receptors and competitively blocks both the *in vitro* (adenylate cyclase inhibition on hypothalamic membranes) and *in vivo* (stimulation of feeding) actions of NPY. However, a recent publication raised the possibility that NPY can act through the orexin receptors[95], therefore to strengthen our results NPY antiserum was also used to neutralize the peptide. The antiserum, which has a 100 % specificity to human/rat NPY, brought about a highly significant inhibition of the action of the orexins and a 1:10 dilution of the antiserum appeared to neutralize the releasable pool of NPY entirely. Our results reinforce the findings of histological studies, which demonstrated orexin-positive nerve terminals on the NPY neurones of the arcuate nucleus[80, 151], and verified abundant NPY-positive projections from the arcuate nucleus to the PVN[19, 80, 112, 116]. Moreover, immunocytochemical studies documented the presence of NPY perikarya in the PVN too, and a close apposition of orexin-positive fibres to the paraventricular NPY



Ill. 4 The putative routes of the orexin signalling to the HPA system.

neurone population[80]. The histological data and our present findings suggest that, the orexin neurones of the lateral hypothalamus[139] presumably give rise to NPY secretion in the arcuate nucleus and/or the PVN: the released NPY stimulates the paraventricular CRH neurones and consequently activates the HPA axis[187]. The results of these experiments also suggest the exclusive role of NPY neurones in the transmission of orexin signalling to the HPA system, because both the NPY antagonist and the NPY antiserum, in the highest concentrations, almost completely blocked the corticosterone release evoked by the orexins.

The data obtained with the ip. administration of the orexins demonstrated that neither orexin-A nor orexin-B had a direct impact on the basal corticosterone release of the adrenal gland, whereas ACTH elicited a significant response. An *in vitro* adrenal perifusion system also was used to ensure that these peptides did in fact reach the adrenal glands. As Kastin *et al.*[97] have recently published that orexin-A crosses the blood-brain barrier, only such an *in vitro* system appeared to provide the completely separated examination of the adrenal function; in this experimental setting, centrally-mediated actions of the orexins could be excluded. However, even though different doses of the peptides were applied, we could not demonstrate any effects of the orexins on corticosterone release. These findings are in harmony with the results of histological studies, which reported that these novel neuropeptides are practically confined to the central nervous system[165].

On the other hand, Malendowicz *et al.*[124] using pharmacological concentrations of the orexins [5-10 nmol/kg, which is approximately 5-10 times higher than the applied concentration of ACTH in their studies and the dose (280 pmol/animal) of the orexins tested in our experiments] showed that the orexins do activate the corticosterone secretion of the adrenals. Nevertheless, the findings of Kane *et al*[95], who disclosed a cross-reactivity among orexin-A, and other neuropeptides such as NPY and secretin in a receptor binding study raises the possibility that the orexins in a pharmacological dose might modulate the NPY or VIP/secretin receptors of the local splanchnic regulator system of the adrenals[77, 78, 124] and bring on the release of steroid hormones.

Griffond[61] proposed that the orexin neurones may belong to the division of glucose-sensitive neurones[147], and could play important roles in the hypoglycaemia-induced endocrine and behavioural responses. Beside their role in the HPA activation, their stimulatory action on

anxiety-related behavioural paradigms such as grooming, burrowing, searching and locomotion provides further evidence for the hypothesis that orexin neurones regulates the CRH positive network in the CNS[83, 192]. The activation of this circuitry induces the behavioural profile of the "alarm reaction"[44, 137, 140], or when CRH reaches the anterior pituitary through the median eminence, it activates ACTH secretion[206]. The orexin-induced CRH and glucocorticoid secretion also appears to modulate nutritional homeostasis[94]: CRH decreases food intake[68, 140], raising the possibility that it functions as a negative feed-back regulator[94], while adrenal glucocorticoids have a significant impact on the output of orexigenic and anorexigenic signals[94] and as catabolic hormones cause the mobilization of energy stores.

Some recent publications[4, 163] support our findings and strengthen the hypothesis that the orexin neurones activate the HPA axis and may establish a connection between two systems of vital importance in the control of homeostasis: nutrition and stress response. The NPY mediation of the HPA activation elicited by the orexins has also been reinforced lately [163] by *in vivo* and *in vitro* experiments. However, the significance of the intercalated NPY neurones of the PVN and the arcuate nucleus in the transmission of other orexin-regulated functions such as control of body-temperature[10, 17, 90, 193, 221] and thyrotropin secretion[37, 135, 201] still needs to be clarified.

5 SUMMARY

In the presented experiments, the role of BNP and CNP in the control of the HPA response to different stress paradigms has been established. The action and the mediation of the orexin signalling - the putative link between fasting and HPA activation - in the control of stress reaction has also been outlined.

The main findings of the presented studies:

1. BNP after icv. administration inhibits the HPA response in a dose-dependent manner, but does not affect the basal corticosterone release.

2. Centrally applied CNP in equimolar doses shows stressor-specific and dose-dependent inhibition of the HPA response, without affecting the basal corticosterone release. It attenuates the HPA response evoked by electric shock and restraint but completely abolishes that evoked by ether stress.

This data suggest that BNP attenuates the HPA response acting on the components of the final common pathway (CRH neurons or ACTH secreting cells), but CNP has an additional and remarkable impact on CNS regions that relay the inputs of the stress response, too.

3. Orexin-A and orexin-B prominently activate the HPA system if administered icv. Both peptides elicit dose-response curve and orexin-A proved more effective at equimolar doses.

4. Neither orexin-A nor orexin-B influences the corticosterone response after peripheral administration and they do not evoke corticosterone response from perifused adrenal slices either.

5. The previous icv. application of the CRH antagonist α-helical CRH_{9-41} completely abolishes the HPA response induced by the orexins, which with the findings of the peripheral experiments argues for the central site of action of the orexins.

6. Both the NPY antagonist (D-Trp^{32})-NPY administered icv. 30 min before the orexin treatment and the icv. application of NPY antiserum 24 h before the experiments significantly inhibit the corticosterone release elicited by the orexins. These data provide evidence for the considerable role of NPY mediation in the orexin-induced HPA activation.

We hope that our data have provided further evidence for the importance of neuropeptides in the complex and flexible regulation of the HPA axis, and have broadened the concept of "stressor-specificity". Present findings also confirmed that the peptide-peptide interplay in the hypothalamus may form the neurochemical background of the adaptability of the HPA system and the versatility of the stress response. It goes without saying that our results also furnish a fertile ground for later investigation of the intricate mechanisms that govern the release and mediate the action of the natriuretic peptides and the orexins. Further experiments are needed to clarify the underlying biochemical processes: the analysis of the involvement of other mediators and the application of the recently discovered orexin-antagonist SB 334867[159] and the non-peptide natriuretic peptide antagonist isatin[57]. Histological studies (e.g. detection of the immediate-early gene expression of the involved structures with *in situ* hybridization) could verify the putative connections between the PVN and the CNS regions that secrete these activatory and inhibitory neuropeptides.

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APPENDIX