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Effects of C-type natriuretic peptide on pituitary–adrenal activation in rats

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THE effects of C-type natriuretic peptide (CNP) on the hypothalamo–pituitary–adrenal system response to different stressors was studied. Various doses of CNP (0.2, 2, 4 μg) were injected into the lateral cerebral ventricle of freely moving rats 30 min before stress and activation of the adrenal was measured by plasma corticosterone. CNP did not affect basal corticosterone secretion in the doses applied, but inhibited in a dose-dependent manner the increase in plasma corticosterone induced by ether stress, electric shock and restraint. CNP exerted a more profound inhibitory effect on the response to ether stress than on that to electric shock or restraint. These results suggest that CNP acts centrally and to a different extent on the responses to different stresses. *NeuroReport* 9: 2601–2603 © 1998 Rapid Science Ltd.

Key words: C-type natriuretic peptide; Electric shock; Ether stress; Pituitary–adrenal system; Restraint

Introduction

Atrial natriuretic peptide (ANP), which is present in the brain,[1,2] is able to inhibit pituitary [p-]adrenal activation.[3–10] C-type natriuretic peptide (CNP) can also be detected in the brain,[11,12] and seems to be the most abundant type of natriuretic peptide in the central nervous system.[13] Since both ANP and CNP are members of a family of peptides which share homologous sequences in chemical structure,[11,14] involvement of CNP in the control of the pituitary[p-]adrenal axis seemed likely. The present experiments therefore explored the effect of CNP introduced into the lateral cerebral ventricle on basal corticosterone secretion, and on the changes induced by three types of stress: ether stress, electric foot-shock and restraint.

Materials and Methods

The animals were kept and handled during the experiments in accordance with the instructions of the Albert Szent-Györgyi Medical University Ethical Committee for the Protection of Animals.

Adult male Wistar rats weighing 150–200 g were housed 5 per cage on a 12:12 h light:dark cycle with access to a standard diet. The rats were handled daily for 1 week before the experiment to reduce the effects of non-specific stress. In order to allow intracerebroventricular (i.c.v.) peptide administration, the rats were implanted with a stainless steel cannula introduced into the right cerebral ventricle 1 week before the experiment, at coordinates 0.2 mm posterior and 1.7 mm lateral to the bregma, and 3.7 mm deep from the dural surface, according to the atlas of Pellegrino *et al.*[15] Surgery was performed under pentobarbital (Nembutal 35 mg/kg, ip.) anesthesia. Cannulas were secured with dental acrylic cement. The rats were allowed a minimum of 5 days to recover from the surgery before peptide treatment. Rat CNP-22 was purchased from Bachem (Cal, USA). All experiments started at 08.00 h.

Different doses (from 20 ng to 4.0 μg) of CNP dissolved in saline, or saline alone (control animals), was injected i.c.v. in a volume of 2 μl, immobilization of the animals being avoided during handling. Thirty min after the i.c.v. injection, the animals were exposed to different stressors. For ether stress, rats were removed from their cages and placed individually for 1.5 min in a large closed jar containing paper towels saturated with diethyl-ether. Electric foot-shock was given to rats placed in a shock-box (a wooden box with a stainless steel grid floor). Animals were exposed to an unescapable foot-shock of 1 mA for 5 s every 15 s for a total of 1 min. Restraint involved placing the animals in a plastic tube, which was closed at either end with a metal plate, for 30 min.

The animals were sacrificed by decapitation in a separate room 30 min after the stress procedures. Approximately 3 ml trunk blood was collected into beakers containing 0.2 ml heparin. The plasma

corticosterone level was measured by the fluorescence assay described by Zenker and Berstein[16] and modified by Purves and Sirett.[17] At the same time, to verify the permeability of the cannulas, methylene blue was injected into each decapitated head and the brains were dissected. Only animals with correctly located cannulas were used for evaluation.

Values are presented as means ± s.e.m. Data were evaluated statistically using the ANOVA procedure followed by Tukey's *post hoc* test. A probability level of $p < 0.05$ was considered to be statistically significant.

Results

CNP in a dose of 2 μg or 4 μg had no significant effect on the basal level of plasma corticosterone (Figs 1–3). Ether stress (Fig. 1), electric shock (Fig. 2) or restraint (Fig. 3) all caused an approximately 4-fold increase in plasma corticosterone. CNP caused a dose-dependent inhibition of ether-stress-induced secretion of corticosterone (Fig. 1). The effect was significant ($p < 0.001$) even at a dose of 0.02 μg: the corticosterone response was inhibited by 58% by 0.2 μg and abolished by 2 μg CNP (both $p < 0.0001$) so that there was no difference from controls ($p = 0.096$ and 0.93, respectively). The effects of CNP on the corticosterone response to electric shock were less marked (Fig. 2). The 2 μg dose, which abolished the response to ether, decreased the corticosterone response by only 24% ($p < 0.005$); the 4 μg dose decreased the response by 34% ($p < 0.001$) but to a level still significantly above that in controls ($p < 0.0001$). The effects of CNP on the corticosterone response to restraint stress were also less marked (Fig. 3). Only the highest dose of CNP (4 μg) was able to diminish the restraint-induced increase in plasma corticosterone in a statistically significant manner ($p < 0.01$); the effect of 2 μg CNP was not significant.

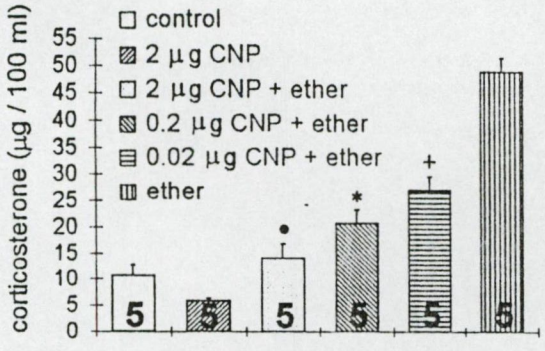

FIG. 1. The effect of C-type natriuretic peptide on ether stress-induced plasma corticosterone levels. $+p = 0.0002$ *vs* ether; $*p = 0.0001$ *vs* ether; $p = 0.0001$ *vs* ether. Figures within the bars represent the number of animals used.

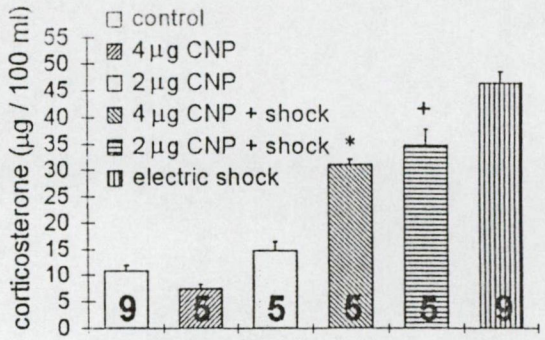

FIG. 2. The effect of C-type natriuretic peptide on plasma corticosterone levels evoked by electric shock. $+p = 0.0046$ *vs* electric shock; $*p = 0.0003$ *vs* electric shock. Figures within the bars represent the number of animals used.

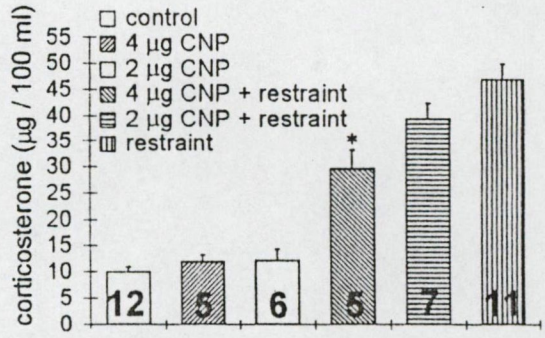

FIG. 3. The effect of C-type natriuretic peptide on plasma corticosterone levels evoked by restraint. $*p = 0.0099$ *vs* restraint. Figures within the bars represent the number of animals used.

Discussion

The chemical similarity between ANP and CNP[11,14] suggests that they might also share biological activity. ANP inhibits ACTH secretion at the pituitary level,[4–9] and may also inhibit the release of corticotrophin releasing hormone (CRH).[3,10]

We have demonstrated that i.c.v. administered CNP can decrease the secretion of corticosterone induced by three different stresses, but it is not able to affect the basal secretion. This probably occurs by a central neuropeptide action of CNP on the hypothalamo–pituitary CRH–ACTH axis. This hypothesis is supported by the findings of Charles *et al.*,[18] who showed i.c.v. administered CNP to suppress the adrenocortical response to hypotension in the sheep. Mulligan *et al.*[19] reported a lack of CNP effect on the basal or stimulated ACTH release from equine pituitary cells *in vitro*, which suggests that CNP does not act at the pituitary to inhibit ACTH secretion. In contrast, Fink *et al.*[8] have demonstrated a marked facilitatory effect of intra-atrial but not i.c.v. administration of anti-ANP serum on the ACTH response to stress and conclude that ANP acts at the level of pituitary. All these findings suggest

that ANP acts as a hormone on the pituitary, but CNP acts centrally. Furthermore, the concentration of CNP in the cerebrospinal fluid is one order of magnitude greater than that of ANP.[13]

In our experiments, CNP influenced the hormone responses brought about by ether, electric shock and restraint to different extents. It is clear from a number of experiments that different stressful stimuli activate the pituitary–adrenal system by different pathways. Ether 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.[20–22] On the other hand, electric shock and other painful stimuli, such as s.c. injection of capsaicin or traumatic stress, trigger ACTH release through sensory neural pathways: either cord section or denervation of the hind leg prevented ACTH release.[23–25]

Other hormonal responses (oxytocin, vasopressin) also vary according to the type of stress.[26,27] It appears that ether and other stressful stimuli activate the hypothalamo–pituitary stress axis by different routes, and that the hormonal responses that they produce are not the same. CNP could exert its inhibitory effects at a number of different central sites and so affect the secretion of various hormones differently.

Conclusion

These data demonstrate that CNP administered i.c.v. to rats is able to block ether-stress-induced activation of the pituitary–adrenal axis by a central action *in vivo*. However, it can only attenuate the response evoked by electric shock, and is even less effective in diminishing the restraint-induced corticosterone response. This discrepancy may be explained by differences in the anatomical or biochemical organization of the pathways involved in the various stress responses.

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EFFECTS OF BRAIN NATRIURETIC PEPTIDE ON PITUITARY-ADRENAL ACTIVATION IN RATS

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Summary

The aim of this study was to characterize the effects of brain natriuretic peptide (BNP) on the hypothalamo-pituitary-adrenal (HPA) responses to different stress paradigms (ether stress, electric shock and restraint). Rats were subjected to the stressful stimuli after intracerebroventricular administration of BNP (32.5 ng - 6.5 μg) and plasma corticosterone was used as an indicator of the HPA activation. BNP did not modify the basal secretion, but inhibited the stress-induced rise in plasma corticosterone in a dose-dependent manner. BNP proved most effective in decreasing the corticosterone response to ether stress and attenuated the electric shock and restraint-induced HPA activation to a lesser extent. These results confirm the view that BNP takes part in the regulation of the HPA system.

Key Words: brain natriuretic peptide, pituitary-adrenal system, ether stress, restraint stress, electric shock

Brain natriuretic peptide (BNP) is the second member of the natriuretic peptide family first isolated from porcine brain (1) and its concentration was shown 13 times higher than that of atrial natriuretic peptide (ANP) in the central nervous system (CNS) (2). BNP with 26 or 32 amino acid residues exhibits a marked sequence homology to (ANP) (1). Natriuretic, hypotensive and smooth muscle relaxant activities of BNP seem to be very similar to those of ANP (1,3). Natriuretic peptides are distributed in discrete brain regions of different species (1,2,4-6) and take part in the regulation of hormone secretion (7-13), water drinking (14) and behaviour (15). Saper *et al.* (6) demonstrated that BNP is present in the CNS of the rat in a distribution, which is different from that of ANP. Moreover Brown *et al.* (16) localized high affinity binding sites for ANP and BNP in various brain regions of the rat such as area postrema and hypothalamic nuclei. Since other studies presented contradictory data (17,18), the reported effects of centrally administered BNP (11-15) in the rat may reflect either physiological or pharmacological action. Studies indicate that ANP can regulate, mainly as an inhibitor, the hypothalamo-pituitary-adrenal (HPA) axis at a pituitary (7,8) and hypothalamic levels (9,10). Former results propose that the HPA response to various stressful stimuli may involve different neural pathways. Ether presumably directly stimulates the medial basal hypothalamus, because it activates the pituitary-adrenal axis in spite of complete hypothalamic deafferentation (19,20).

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Electric shock and other painful stimuli, trigger ACTH secretion through sensory neural pathways, because either cord section or denervation of the hind leg prevents the release of ACTH (21). Restraint represents the environmental or "psychological" stress paradigm, which evokes the stress reaction through the ventral amygdalofugal pathway and the central amygdaloid nucleus (22,23). Previous studies showed that not only ACTH but oxytocin or vasopressin responses induced by restraint or ether also vary according to the applied stress paradigm (24,25). Furthermore, our previous findings (26) showed that C-type natriuretic peptide (CNP) abolished the corticosterone response evoked by ether stress, but caused only less marked inhibition of the HPA response brought about by restraint and electric shock. As previous reports examined the action of BNP only on the basal (12) and endothelin-induced HPA activation (11), in the present study we have investigated the *in vivo* effects of intracerebroventricularly (i.c.v.) administered BNP on HPA responses to ether stress, electric shock and restraint to clarify whether this peptide shows any challenge specific action similar to that of CNP (26).

Methods

Animals and Surgery

The animals were kept and handled during the experiments in accordance with the instructions of the Albert Szent-Györgyi Medical University Ethical Committee for the Protection of Animals in Research. Male Wistar rats weighing 150-200 g upon arrival were used. The animals were kept in their home-cages at a constant room temperature 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 animals were allowed a minimum of 1 week to acclimatize before surgery. In order to minimize the effects of nonspecific stress the rats were handled daily. The rats were implanted with a stainless steel 20G1.5 Luer cannula (10 mm long) aimed at the right lateral cerebral ventricle under Nembutal (35 mg/kg, intraperitoneal) anesthesia. 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.* (27). Cannulas were secured to the skull with dental cement and acrylate. After decapitation in order to verify the correct positioning and the permeability of the cannulas, methylene blue was injected into each decapitated head and the brains were dissected. Results from animals with incorrectly positioned cannulas were excluded from the statistical evaluation.

Peptide administration

The rats were used after a recovery period of at least 5 days. All experiments were carried out between 8.00 and 10.00 a.m. In order to obtain more comparable data, the applied doses of BNP (from 32.5 ng to 6.5 μg) were equimolar to the CNP concentrations used in our previous experiments (26). BNP was dissolved in saline, and was injected i.c.v. in a volume of 2 μl to conscious, freely-moving rats. The control animals received the same volume of saline.

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 ether stress, the animal was 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.

Corticosterone assay

The rats were decapitated 30 min after the stress procedures and approximately 3 ml trunk blood was collected in heparinized tubes. The plasma corticosterone level was determined by the fluorescence assay described by Zenker and Berstein (28), as modified by Purves and Sirett (29).

Reagents and Drugs

During the experiments, the following substances were used: diethyl ether, methylene chloride and sulfuric acid of analytical grade (Reanal, Budapest), and porcine BNP ($pBNP_{32}$) (Bachem, Germany).

Statistical analysis

All data are given as means ± S.E.M. Statistical analysis of the results was performed by one-way ANOVA followed by Tukey's *post hoc* comparison test. A probability level of 0.05 was accepted as indicating a statistically significant difference.

Results

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.

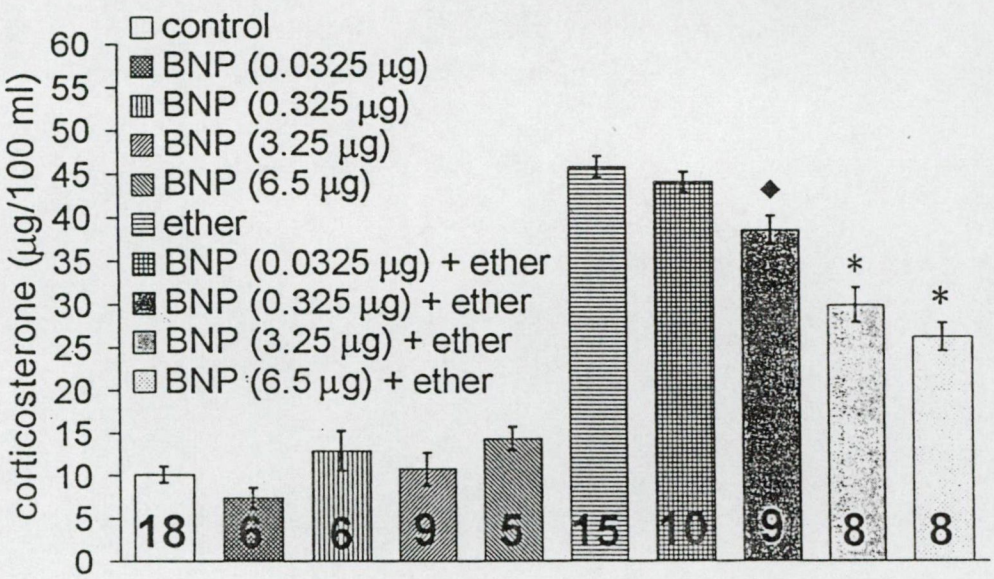

Fig. 1.

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.

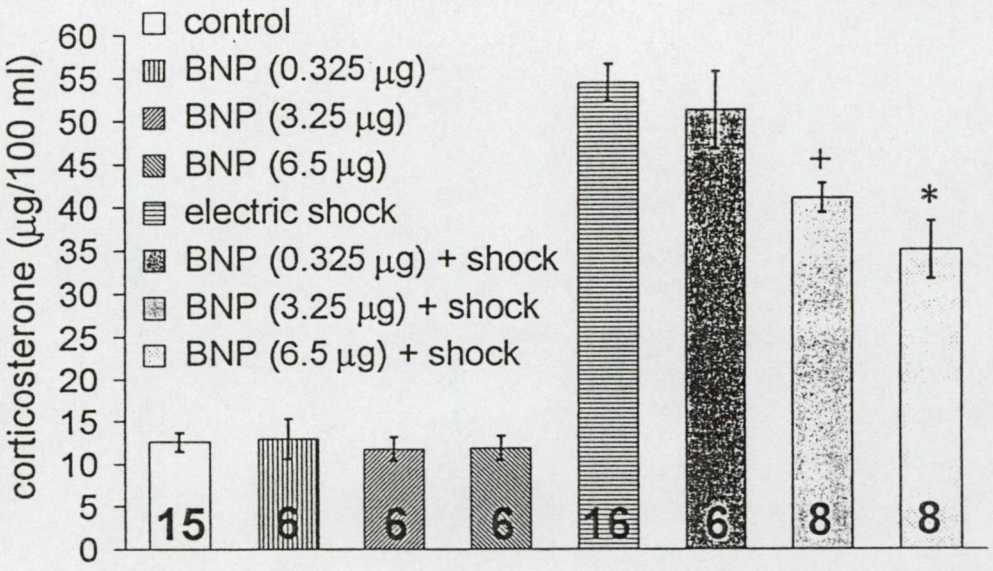

Fig. 2.
The effect of brain natriuretic peptide on the electric shock-induced increase in plasma corticosterone level. Symbols: +: $p < 0.01$ vs. electric shock ; *: $p < 0.005$ vs. electric shock. Figures within bars are the numbers of animals used.

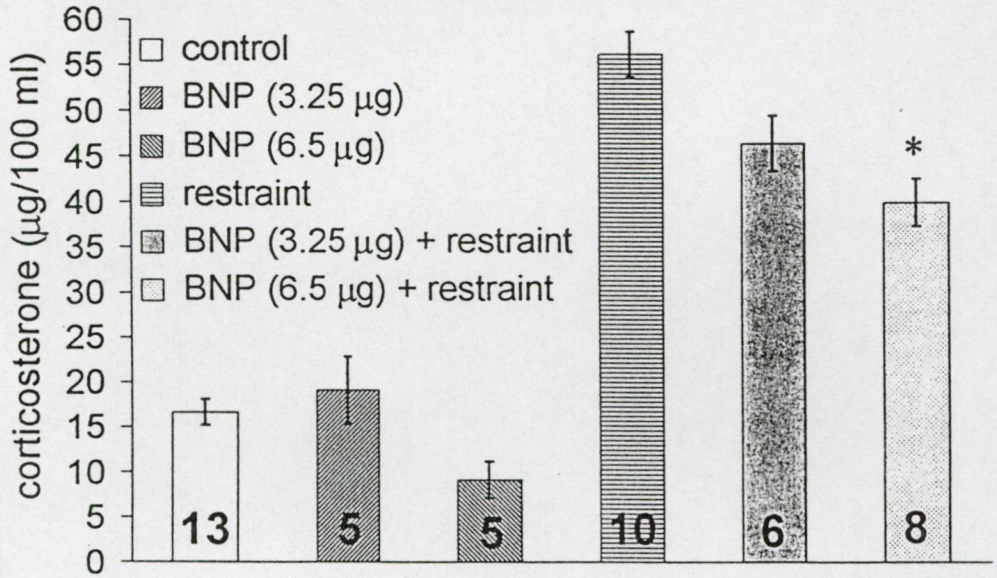

Fig. 3.
The effect of brain natriuretic peptide on the plasma corticosterone level induced by restraint. Symbols: *: $p < 0.005$ vs. restraint. Figures within bars are the numbers of animals used.

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 ($p < 0.01$). However, the dose of 0.325 μg, which proved effective in the case of ether stress, failed to produce a significant inhibition ($p = 0.99$).

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 ($F(5, 41) = 60.4$, $p < 0.005$).

Discussion

Previous findings showed that ANP and BNP share biological activity in extracerebral tissues (1,3) and the central effects of BNP on vasopressin secretion (13), water-drinking (14) and learning behaviour (15) are quite similar to those of ANP. Other results demonstrated that ANP decreased hypothalamic CRF release (9,10). However several investigators demonstrated that ANP inhibited the HPA system preferentially at a pituitary level (7,8).

The present data have clearly shown that centrally administered $pBNP_{32}$ does not affect the basal release of corticosterone, but dose-dependently inhibits the hormonal response to three stressful stimuli *in vivo*. The action site of i.c.v. administered BNP on the stress response is not established. However we have strong evidence that high density binding sites for BNP exists in the subfornical organ, supraoptic and paraventricular nuclei (16), which structures regulate pituitary ACTH secretion. This inhibitory effect of BNP on the HPA axis is very probably mediated by cyclic guanosine monophosphate (cGMP) (30), and previous studies (31,32) demonstrated that cGMP-dependent protein-kinases might play an important role in the action of BNP on the HPA system..

Nevertheless our previous results (26) revealed that the effect of CNP is more marked and more stressor-specific than that of BNP. These findings can be explained by the different distributions of natriuretic peptides (5,6,16-18) and their receptors (33,34) in the CNS, and by the fact that ANP and BNP prefer the A-type (NPR-A), while CNP the B-type (NPR-B) natriuretic peptide receptor (35). High NPR-B expression can be observed in all the structures (preoptic-hypothalamic structures, amygdala, brainstem) (34) that take part in the organization of the anatomical pathways of the applied stressors (ether, restraint, electric shock). However BNP binding (16) is practically restricted to the subfornical organ, supraoptic and paraventricular nuclei, which can explain the fact that the action of BNP is less dependent on the type of the applied stress paradigm than that of CNP. Despite the different distribution of endogenous sources of ANP and BNP (6) these structurally related peptides possibly have an impact on the common pathway of the stress response either at a hypothalamic or at a pituitary level (7-11), while CNP as the most abundant natriuretic peptide in the CNS (5) may inhibit the ascending stress-pathways to a different extent.

The results provide evidence for an inhibitory modulation of BNP on the activated HPA system. We conclude that BNP does not influence the basal corticosterone secretion, but in a dose-dependent manner diminishes the corticosterone response to ether stress, electric shock and restraint. Thus, the present publication, together with our previous results (26), strongly support the hypothesis that natriuretic peptides may play an important role in the regulation of stress response.

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Effects of Orexins on the Hypothalamic-Pituitary-Adrenal System

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Key words: stress response, hypothalamic-pituitary-adrenal system, orexin-A, orexin-B.

Abstract

The effects of the recently identified neuropeptides orexin-A and orexin-B on the hypothalamic-pituitary-adrenal (HPA) system were investigated. An *in vivo* system was used to assess the central effects of both orexin-A and orexin-B. Different doses of the orexins (2.8–560 pmol) were administered intracerebroventricularly (i.c.v.) to adult male rats, and plasma corticosterone was used as an index of the degree of the activation of the HPA system. Both peptides exhibited a clear dose–response action, although orexin-B proved to be less effective than orexin-A. Pretreatment with the corticotropin-releasing hormone (CRH) antagonist α-helical CRH_{9-41} completely prevented the action of the orexins. Orexin-A, orexin-B or adrenocorticotropic hormone (ACTH) was further administered intraperitoneally (i.p.). While ACTH evoked a significant adrenal response, the orexins did not influence the basal secretion. Adrenal slices, oxygenized and perifused with Krebs' solution, were also treated with orexin-A, orexin-B or ACTH. Both orexins failed to modify the release of corticosterone, but ACTH induced a marked adrenal response. This study suggests that these appetite-regulating peptides might activate the HPA system at a central level but neither orexin-A nor orexin-B appears to modulate directly the adrenal corticosterone release.

The hypothalamus plays a central, integrative role in the control of neuroendocrine, autonomic and energy homeostasis. Fasting evokes a complex response of the hypothalamic-pituitary-adrenal (HPA) system, altering both the basal function and the responsivity to stressors. The rhythm in HPA activity follows the rhythm in food consumption (1, 2). Insulin-induced acute hypoglycemia, which is a potent activator of the HPA axis, reflects the strong functional relationship between the hypothalamic feeding centres and the HPA system (3, 4). Neuropeptides such as corticotropin-releasing hormone (CRH), melanin concentrating hormone (MCH) and neuropeptide Y (NPY) have been found to be important regulators of both the HPA system and feeding behaviour (5–9).

Two novel neuropeptides, orexin-A and orexin-B, have been identified as mediators of homeostatic and endocrine hypothalamic functions (10–13). Both are processed from a 130-amino acid precursor, prepro-orexin and they differ markedly in structure and function (10). These two neuropeptides and their receptors display a specific tissue distribution, which resembles that of MCH (9). Prepro-orexin mRNA activity is abundantly expressed in the lateral hypothalamus (10, 14), and Nambu *et al.* (15), using an antiorexin serum, demonstrated the same distribution pattern of the orexin-immunoreactive neurones. Although most of these neurones are located in the hypothalamus, a dense projection of orexin-positive nerve terminals is found in both hypothalamic and extrahypothalamic structures (15, 16). Because orexins are synthesized in the lateral hypothalamus (10, 14) [an area classically regarded as the feeding centre (17)] and enhance food intake (10, 18), and because the vast majority of orexin neurones express leptin receptors (19), these neuropeptides seem to be among the central regulators of feeding.

The integrated action of the paraventricular nucleus (PVN) and the arcuate nucleus appears to play a crucial role in the regulation of both stress response (5, 20) and feeding behaviour (9) through the secretion of neuropeptides such as CRH and NPY (5–7, 9, 21). Recent studies have demonstrated that insulin-induced acute hypoglycemia, a well-established stress paradigm (3, 4), can activate the orexin-containing cells in the lateral hypothalamus (22, 23). Although orexin-containing neurones have not yet been detected in the PVN and the arcuate nucleus, neuronal projections from the lateral hypothalamus to the PVN have been identified (11, 15,

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24). Histological studies have verified the functional activation of these neuronal circuits: i.c.v. infusion of orexins greatly increased the expression of c-*fos*, a marker of neuronal activation, in the PVN and the arcuate nucleus (18, 24). Moreover, van den Pol *et al.* (25) reported that 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 orexin neurones can mediate a complex endocrine and homeostatic response to fasting and insulin-induced hypoglycemia. The present experiments were designed to test the effects of both orexin-A and orexin-B on the HPA system and to investigate the sites mediating their action. The orexins were administered i.c.v. in an attempt to characterize their central effect. Rats were pretreated with CRH antagonist α-helical CRH_{9-41} to elucidate whether these peptides activate directly the pituitary adrenocorticotropic hormone (ACTH) secretion or through the stimulation of hypothalamic CRH release. Intraperitoneal administration of the peptides and an *in vitro* system of perifused adrenal slices were used to investigate the peripheral action of the orexins.

Materials and methods

Animals

The animals 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 upon arrival were used. The rats were kept in their home-cages at a constant room temperature on a standard illumination schedule with 12-h light and 12-h dark periods (lights on from 06.00 h). 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.

Experimental protocols

In vivo experiments

To clarify the central and peripheral effects of the orexins, we used both i.c.v. and i.p. peptide administration. In the case of i.c.v. administration (experiment 1, experiment 2), the rats were implanted with a stainless steel 20-gauge 1.5 Luer cannula (10 mm long) aimed at the right lateral cerebral ventricle under Nembutal (35 mg/kg i.p.) anaesthesia. The stereotaxic coordinates were 0.2 mm posterior; 1.7 mm lateral to bregma; 3.7 mm deep from the dural surface, according to the atlas of Pellegrino *et al.* (26). Cannulae were secured to the skull with dental cement and acrylate. The rats were used after a recovery period of at least 5 days. All experiments were carried out between 08.00 h and 10.00 h. Peptides, dissolved in saline, or saline alone (control animals) were injected i.c.v. to conscious rats with Hamilton microsyringe over 30 s in a volume of 2 µl, immobilization of the rats being avoided during handling. In order to obtain comparable data, we used equimolar doses of orexin-A, orexin-B or α-helical CRF_{9-41}. For peripheral administration (experiment 3), equimolar doses of orexins or ACTH were dissolved in 0.5 ml saline and injected i.p. Control animals received 0.5 ml saline i.p. 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 in heparinized tubes. In the case of i.c.v. administration, methylene blue was injected into each decapitated head 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 for the statistical evaluation.

Experiment 1: Rats received different i.c.v. doses of either orexin-A (from 2.8–280 pmol) or orexin-B (from 28–560 pmol). The doses of the orexins were selected with a view to finding the minimal statistically effective concentration and identifying the concentration evoking the maximal response. Control rats received saline alone. Each rat was returned to its home cage and maintained in a nonstressful environment till they were decapitated 30 min after the i.c.v. injection.

Experiment 2: 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} i.c.v., 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.

Experiment 3: In this study, rats received an equimolar dose of orexin-A, orexin-B or ACTH (280 pmol) i.p. Saline was injected i.p. into the controls. Trunk blood was collected 30 min later for corticosterone assay.

In vitro experiments

We used the *in vitro* system described by Saffran and Schally (27) 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 approximately 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. 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-gauge; 0.3 × 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 adrenals. The samples were incubated for 30 min, after which 200 µl aliquots of the medium were transferred to centrifuge tubes for corticosterone assay.

Corticosterone assay

The plasma corticosterone concentration was determined by the fluorescence assay described by Zenker and Bernstein (28), as modified by Purves and Sirett (29). In the *in vivo* experiments, corticosterone concentration 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 h (µg/100 mg/h).

Reagents and drugs

For the experiments, the following substances were used: ethyl alcohol, methylene chloride and sulphuric acid of analytical grade (Reanal, Budapest) for the corticosterone assay; and NaCl, KCl, $MgSO_4$, $NaHCO_3$, KH_2PO_4, $CaCl_2$ and glucose (Reanal, Budapest) for the Krebs' buffer preparation. The peptides applied were rat orexin-A, rat orexin-B (both from Bachem, Switzerland), ACTH (β^{1-24} corticotropin, Ciba-Geigy, Vienna) and α-helical CRH_{9-41} (Bachem).

Statistical analysis

All data are given as means ± SEM Statistical analysis of the results was performed by one-way ANOVA followed by Tukey's *post hoc* comparison test. A probability level of 0.05 was accepted as statistically significant.

Results

In vivo *experiments*

Study 1: Effects of orexin-A and orexin-B administered i.c.v. on basal corticosterone release

Orexin-A increased corticosterone secretion in a dose-dependent manner (Fig. 1). The most effective dose (140 pmol) elevated the plasma concentration of corticosterone by 208% 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 ($P < 0.05$) *vs* the control.

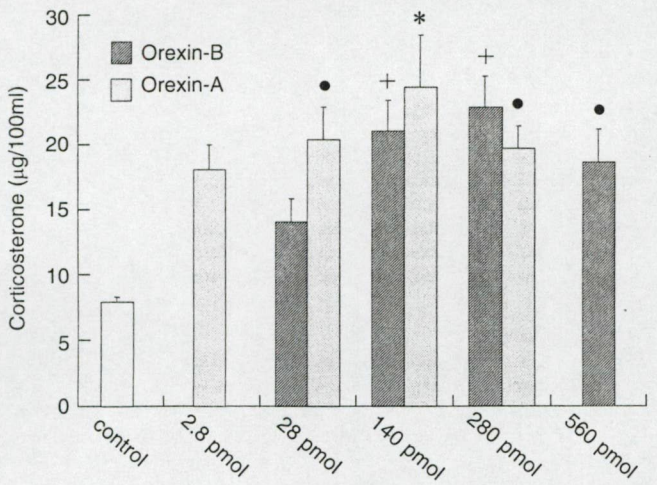

FIG. 1. Effects of i.c.v. administered orexin-A or orexin-B on plasma corticosterone concentration. *P < 0.001 *vs* control, + P < 0.005 *vs* control, ●P < 0.05 *vs* control. Figures within bars indicate the number of rats used.

Orexin-B likewise caused a dose-dependent increase (Fig. 1). However, it peaked at a dose of 280 pmol and resulted in only a 189% increase above control levels (P < 0.005).

Study 2: Effects of a-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 *vs* 140 pmol orexin-A) (Fig. 2). The response induced by orexin-B was similarly inhibited by α-helical CRH_{9-41} (P < 0.001 *vs* 280 pmol orexin-B) (Fig. 2).

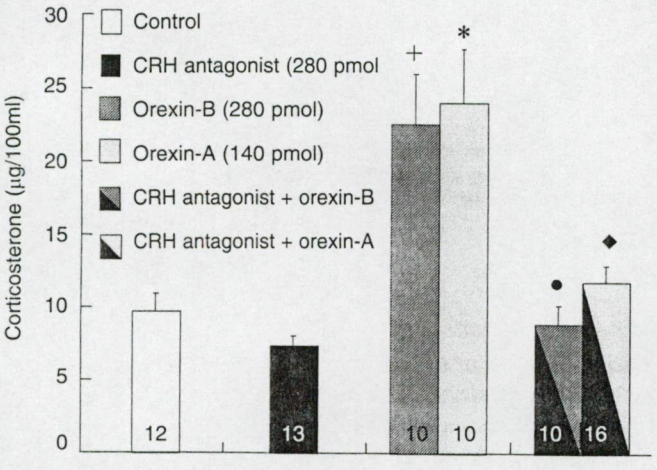

FIG. 2. Effects of i.c.v. administered α-helical CRH_{9-41} on the plasma corticosterone release evoked by orexin-A or orexin-B. *P < 0.001 *vs* control, + P < 0.005 *vs* control, ●P < 0.001 *vs* orexin-B, ◆P < 0.005 *vs* orexin-A. Figures within bars indicate the number of rats used.

Study 3: Comparison of effects of i.p. administered orexins and ACTH on basal corticosterone release

The basal plasma concentration of corticosterone (15 ± 1.61 μg/100 ml) was not modified by exposure to either orexin-A (280 pmol: 14.1 ± 2.11 μg/100 ml) or orexin-B (280 pmol: 18.9 ± 3.46 μg/100 ml). By contrast, an equimolar dose of ACTH elicited 127% increase (34.05 ± 5.1 μg/100 ml; $F_{3,16} = 7.61$, P < 0.01 *vs* control; n = 5 in all cases).

In vitro *experiments*

The basal secretion of corticosterone from isolated adrenals *in vitro* (3.2 ± 0.8 μg/100 mg/h) was not significantly influenced by exposure to orexin-A (280 pmol: 2.7 ± 04 μg/100 mg/h; 2.8 nmol: 3.3 ± 09 μg/100 mg/h) or by exposure to orexin-B (280 pmol: 2.5 ± 05 μg/100 mg/h; 2.8 nmol: 5.3 ± 2.3 μg/100 mg/h). Exposure to ACTH (280 pmol) produced the expected significant increase in secretion (13 ± 4.3 μg/100 mg/h; $F_{5,24} = 3.78$, P < 0.05 *vs* control; n = 5 in all cases).

Discussion

Previous studies have demonstrated that the orexins posses prominent neuroendocrine effects. Pu *et al.* (12) observed that both orexin-A and -B stimulated luteinizing hormone secretion. Hagan *et al.* (13) showed that i.c.v. applied orexin-A inhibited prolactin and growth hormone release and evoked a dose-dependent corticosterone secretion. The present experiments were designed to extend our understanding of the role of both orexin-A and -B in the regulation of the HPA system. We attempted to identify the site of action of the orexins and compared the effects of these related peptides, which are quite distinct in their chemical nature and differ in their physiological actions (10, 18, 30, 31). Orexin-A, i.c.v., elicited a dose-dependent increase in corticosterone release, in agreement with the findings of Hagan *et al.* (13). Orexin-B administered i.c.v. also activated the HPA system in a dose-dependent manner. However, it is noteworthy that orexin-B was less effective than orexin-A. Orexin-A is a 33-amino acid residue peptide with two intrachain disulphide bonds; and both termini of the peptide are blocked post-translationally. In contrast, orexin-B, a 28-residue linear peptide with a free *N*-terminus, appears more susceptible to inactivating peptidases (31). These two peptides can activate two distinct G-protein-coupled cell surface receptors, $orexin_1$ 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 three orders of magnitude lower (10). Previous studies have demonstrated that the effect of orexin-A on nutritional homeostasis (10, 18) is more potent and longer-lasting than those of orexin-B. Furthermore, the effect of orexin-B on behavioural phenomena appears to be considerably different from orexin-A (30).

Both peptides furnish dose–response curves, with a down-turn phase. This resembles the action of orexin-A on feeding (18), and the bell-shaped dose–response curve of the food intake-stimulating effect of NPY (32), and presumably reflects

functional antagonism in postreceptorial signal transduction (33).

To investigate whether orexins evoke the activation of the HPA system at a hypothalamic or at a pituitary level, rats were pretreated i.c.v. with the CRH antagonist α-helical CRH_{9-41}. 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}. These results, and the fact that the amino acid sequences of the orexins exhibit little similarity to the sequences of any other peptides (5, 10), suggest that orexins might not act on the pituitary CRH receptors, but rather bring about hypothalamic CRH secretion. These data are in accordance with the anatomical findings. Nambu *et al.* (15) reported that the anterior and intermediate lobes of the pituitary do not contain orexin-positive fibres. Besides, the complete inhibition of the stimulatory action of orexins by the application of CRH antagonist α-helical CRH_{9-41} implies that vasopressin mediation might not play a major role in the orexin induced HPA activation.

Further studies were undertaken to investigate whether orexins influence the function of the peripheral part of the HPA system. The present data clearly demonstrate that neither orexin-A nor orexin-B has a direct impact on the basal corticosterone release of the adrenal gland. Orexins administered i.p. failed to influence the basal glucocorticoid release, whereas ACTH elicited a significant response. We also used an *in vitro* adrenal perifusion system to ensure that these peptides did in fact reach the adrenal glands. Kastin *et al.* (31) recently demonstrated that orexin-A but not orexin-B crosses the blood–brain barrier, and this *in vitro* system provided a completely separate examination of adrenal function. Therefore, in this experimental setting, centrally mediated actions of 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 secretion. These findings are in accordance with the results of recent histological studies, which reported that these novel neuropeptides are practically confined to the central nervous system (10).

Our results, taken together with previous findings (9, 13, 30), support the concept that the orexins participate in the regulation of the PVN and the arcuate nucleus, activating the cells of these nuclei through synaptic contacts, or act as neurohormones, being discharged into the ventricular circulation (34). Griffond (23) proposed that the orexin neurones may belong to the glucose-sensitive neurones of the lateral hypothalamus (17), and might play important roles in the hypoglycaemia-induced endocrine and behavioural responses. The stimulatory action of orexin-A on grooming behaviour provides further evidence for the hypothesis that orexin neurones control the physiological functions of the PVN (30). The well-established mediator of these processes is CRH; when released from the PVN, this increases grooming (35), or when it reaches the anterior pituitary through the median eminence, it activates ACTH secretion (5). Furthermore, orexin-induced CRH and glucocorticoid secretion appear to regulate appetite (9). CRH decreases food intake (21, 36), raising the possibility that it functions as a negative feed-back regulator (9), while adrenal glucocorticoids seem to modulate the output of orexigenic and anorexigenic signals (9) and, as catabolic hormones, cause the mobilization of energy stores.

It has not yét been clarified whether the orexin containing cells act directly on the CRH-positive cells of the PVN or through the activation of other hypothalamic neurones. Several lines of evidence suggest that the orexin, NPY and CRH neurones interact in the hypothalamus, integrating the functions of the HPA axis and the hypothalamic feeding system (6, 7, 11). Further studies are required to elucidate the function and interaction of these neuropeptides in the regulation of the HPA system.

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The Role of Neuropeptide Y in Orexin-Induced Hypothalamic-Pituitary-Adrenal Activation

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Key words: hypothalamic-pituitary-adrenal system, neuropeptide Y, orexin-A, orexin-B.

Abstract

The role of neuropeptide Y (NPY) in the mediation of orexin-induced hypothalamic-pituitary-adrenal (HPA) activation was investigated in the rat. The HPA system was stimulated by intracerebroventricular (i.c.v.) administration of orexin-A or orexin-B (140 or 280 pmol, respectively), and the plasma concentration of corticosterone was used as an index of the degree of activation. i.c.v. pretreatment with NPY antagonist or NPY antiserum (30 min or 24 h before orexin administration, respectively) inhibited the orexin-induced corticosterone release. The inhibitory actions of the antagonist and the antiserum were revealed by the dose–response curve; the highest concentrations practically abolished the HPA activation evoked by the orexins. These data suggest that the HPA system-stimulating effect of the orexins may be mediated by NPY.

Recent studies have demonstrated that the members of a novel family of neuropeptides, the orexins (1), can activate the hypothalamic-pituitary-adrenal (HPA) system (2–4) through the release of corticotropin releasing hormone (CRH). The orexin immunoreactive neurones are located in the lateral hypothalamus (1, 5), a structure described as the feeding centre (6), and orexins stimulate food intake (1, 7). However, despite the specific and rather limited distribution of orexin neurones in the central nervous system (CNS) (1, 5), orexin-A and orexin-B have impacts on numerous behavioural, endocrine and autonomic processes (1, 2, 8, 9). The dense projection of orexin neurones to other hypothalamic and extrahypothalamic structures (10, 11), and the widespread distribution of orexin receptors in the CNS (1, 12) appear to form the anatomical basis of such diverse actions. Nevertheless, the characteristic distribution of orexin neurones (1, 5) raises the possibility that the orexins do not activate or inhibit CNS processes directly, but rather through the release of other neuropeptides. The effects of orexins on feeding (1) and hormone secretion (2–4, 9) markedly resemble those of NPY (13–18) and recent studies (19, 20), involving the use of NPY antagonist, have revealed that the hyperphagia elicited by orexins is at least partially mediated by NPY. Orexin axons synapse on the NPY cells in the arcuate nucleus, a nucleus regarded as one of the most important regulators of feeding (10, 21). Because the NPY-positive neurone population of the arcuate nucleus also plays a considerable role in the regulation of the HPA system through the activation of the CRH neurones in the paraventricular nucleus (PVN) (14, 17, 22–25), it seemed worthwhile to investigate whether the NPY neurones are involved in the action of the orexins on the HPA system. By means of i.c.v. pretreatment with an 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* (26)] or NPY antiserum, we aimed to elucidate the possible role of NPY in orexin-A and orexin-B-induced HPA activation.

Materials and methods

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 under controlled lighting conditions (12 : 12 h light : dark cycle; lights on at 06.00 h). 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.

To allow i.c.v. peptide administration, the rats were implanted with a 10-mm long stainless steel cannula (prepared from hypodermic Luer needle of 20-G × 1.5 inch) aimed at the right lateral cerebral ventricle under Nembutal (35 mg/kg. i.p.) anaesthesia. The stereotaxic coordinates were 0.2 mm posterior; 1.7 mm lateral to the Bregma; 3.7 mm deep from the dural surface.

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according to the atlas of Pellegrino *et al.* (27). Cannulae were secured to the skull with dental cement and acrylate. The rats were used after a recovery period of at least 5 days. All experiments were carried out between 08.00 h and 10.00 h. Orexin-A or orexin-B dissolved in saline, or saline alone (control animals), was injected i.c.v. to conscious rats with a Hamilton microsyringe over 30 s in a volume of 2 µl, immobilization of the rats being avoided during handling. The doses of orexins applied were the concentrations that had proved the most effective in our previous experiments (4). To scrutinize the mediation of the action of the orexins, different dilutions of (D-Trp^{32})-NPY (26), as an NPY antagonist or NPY antiserum were applied i.c.v. (30 min or 24 h before orexin treatment, respectively). The time intervals for antagonist and antiserum administration were chosen on the basis of previous data obtained with other antagonists and antisera (28). The doses of the antagonist and the antiserum were selected with a view to finding the minimal and maximal statistically effective concentrations. To obtain trunk blood for corticosterone assay, in both experiments, rats were killed by decapitation and approximately 3 ml blood was collected in heparinized tubes. Methylene blue was next injected into each decapitated head 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.

Corticosterone assay

Plasma concentrations of corticosterone were determined by the fluorescence assay described by Zenker and Bernstein (29) as modified by Purves and Sirett (30).

Statistical analysis

All data are given as means ± SEM. Statistical analysis of the results was performed by one-way ANOVA, followed by Tukey's post-hoc comparison test for unequal cell size. $P<0.05$ was considered statistically significant.

Reagents and drugs

For the corticosterone assay, the following substances were used: ethyl alcohol, methylene chloride and sulphuric acid of analytical grade (Reanal, Budapest). The peptides applied were rat orexin-A, rat orexin-B, (D-Trp^{32})-NPY (Bachem, Switzerland) and NPY antiserum (Yanaihara Institute Inc., Japan).

Results

Study 1: Effects of NPY antagonist on HPA activation evoked by orexins

Rats were divided into four treatment groups. Thirty minutes before orexin administration, groups II and IV received different concentrations of (D-Trp^{32})-NPY (from 280 pmol to 560 pmol) i.c.v., whereas group I and III received saline alone. Groups III and IV subsequently received equimolar doses of orexin-A or orexin-B (140 or 280 pmol, respectively) i.c.v., while groups I and II received saline alone. Each rat was returned to its home cage and maintained in a nonstressful environment until it was decapitated 30 min after the second i.c.v. injection. 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, versus the control)]. (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 ($P<0.05$ versus 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. 1).

Study 2: Effects of NPY antiserum on orexin-induced HPA activation

Different dilutions of NPY antibody (groups II and IV) or normal rabbit serum (groups I and III) were injected i.c.v. 24 h before orexin administration. 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 30 min later, as above. 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%, $F(11,129)=7.41$ $P<0.05$ versus 140 pmol orexin-A] or orexin-B (51%, $P<0.01$ versus 280 pmol orexin-B) (Fig. 2).

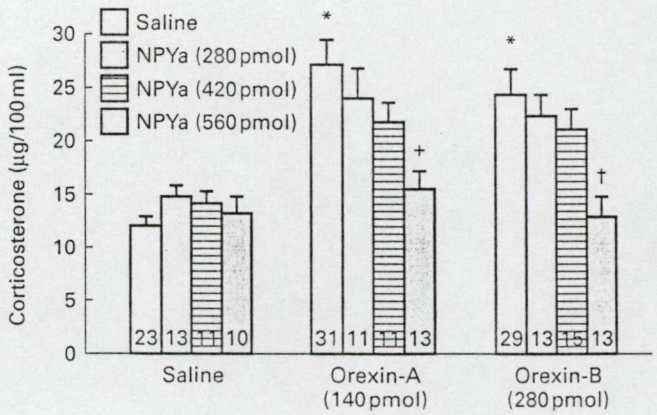

FIG. 1. Effects of neuropeptide Y (NPY) antagonist on corticosterone release evoked by orexins. NPYa: NPY antagonist. *$P<0.001$ versus control; +$P<0.05$ versus orexin-A; †$P<0.05$ versus orexin-B. Numbers within bars indicate the number of animals used.

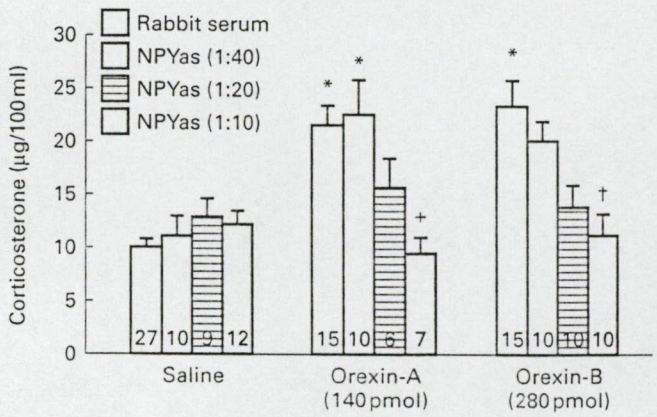

FIG. 2. Effects of neuropeptide Y (NPY) antiserum on corticosterone release evoked by orexins. NPYas, NPY antiserum; 1 : 40, 1 : 20 and 1 : 10: dilutions of antiserum; rabbit serum: normal rabbit serum, respectively. *$P<0.01$ versus control; +$P<0.05$ versus orexin-A; †$P<0.01$ versus orexin-B. Numbers within bars indicate the number of animals used.

Discussion

The present experiments were designed to elucidate the mediation of the action of the orexins on the HPA system, and to clarify the function of NPY in this process. Pretreatment with NPY receptor antagonist (D-Trp^{32})-NPY (26) 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.* demontrated that (D-Trp^{32})-NPY shows high affinity and specificity to the hypothalamic NPY receptors and competitively blocks the *in vitro* (adenylate cyclase inhibition on hypothalamic membranes) and *in vivo* (stimulation of feeding) actions of NPY (26). However, a recent study raised the possibility that NPY can act through the orexin receptors (31), therefore to strenghten our results NPY antiserum was 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 further support histological data that the orexin neurones, projecting from the lateral hypothalamus, relay the impulses from the feeding centre of the lateral hypothalamus to the HPA system: orexin-positive nerve terminals can be demonstrated on the NPY neurones of the arcuate nucleus (10, 21), and abundant NPY-positive projections from the arcuate nucleus to the PVN have been verified (21, 24, 25, 32). Moreover, immunocytochemical studies have documented the presence of NPY perikarya in the PVN too, and a close apposition of orexin-positive fibres to the paraventricular NPY neurone population (21). The histological data and our present findings suggest that (i) the hypoglycaemia-activated orexin neurones (33) presumably give rise to NPY secretion in the arcuate nucleus and/or the PVN and (ii) the released NPY stimulates the paraventricular CRH neurones and consequently activates the HPA axis (17). CRH may act as a feedback regulator of appetite (34), while the glucocorticoids play a permissive role in the central regulation of feeding (35) and elicit the characteristic catabolic processes of starvation.

The results obtained in the present study clearly reflect that NPY mediation plays an exclusive role 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.

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