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The Central Effects of Orexin-A in the Hypothalamic-Pituitary-Adrenal Axis *In Vivo* and *In Vitro* in Male Rats

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Abstract

Orexin-A is synthesized in the posterolateral hypothalamus and immunoreactive fibres project to many central nervous system structures, including the paraventricular nucleus, which is rich in corticotropin releasing factor (CRF) neurones and neuropeptide Y (NPY) innervation. We investigated the central effects of orexin-A on the hypothalamic-pituitary-adrenal (HPA) axis by measuring plasma concentrations of corticosterone and adrenocorticotropic hormone (ACTH) *in vivo*. We explored the potential neuropeptide pathways involved by investigating the effects of orexin-A on CRF, NPY, arginine vasopressin (AVP) and noradrenaline release from hypothalamic explants *in vitro.* Intracerebroventricular (i.c.v.) injection of orexin-A (3 nmol) in male rats stimulated increases in plasma concentrations of corticosterone between 10 and 40 min after injection, and of plasma ACTH at 20 and 90 min after injection. Orexin-A significantly stimulated CRF and NPY release from hypothalamic explants *in vitro*. Orexin-A did not stimulate CRF release in the presence of the selective NPY Y1 receptor antagonist, BIBP3226. BIBP3226 alone did not alter CRF release from hypothalamic explants. Orexin-A had no effect *in vitro* on the release of other neuropeptides, AVP and noradrenaline, involved in the central regulation of the HPA axis. These results suggest that orexin-A is involved in activation of the HPA axis, and that these effects could be mediated via the release of NPY.

Orexin-A was first identified in 1998 as a novel peptide ligand for a previously identified, G protein coupled 'orphan' receptor (1). Orexin-A is a 33 amino acid peptide, with two intrachain disulphide bonds, an *N*-terminal pyroglutamyl residue and an amidated *C*-terminus. Orexin-A is synthesized almost exclusively in the posterolateral hypothalamus and perifornical area, from a 130 amino acid precursor prepro-orexin (1). Hypocretin 1 (Hcrt-1) was independently identified in 1998 using subtraction cDNA cloning (2), and has an identical amino acid sequence to orexin-A but with an additional five amino acids at the *N*-terminus. Compared with hypocretins 1 and 2 and orexin-B, orexin-A has the greatest potency at orexin receptors OX_1R and OX_2R (1, 3, 4).

Orexin-A immunoreactive fibres project widely to intra- and extra-hypothalamic sites (5, 6), including the paraventricular nucleus (PVN) which is rich in corticotropin releasing factor (CRF) neurones and is important in the control of the hypothalamic-pituitary-adrenal (HPA) axis (5–7). There is also dense orexin-A immunoreactive innervation of the midbrain dorsal raphe, the locus coeruleus (6, 8, 9) and the arcuate nucleus (10), areas which influence the HPA axis through noradrenergic, adrenergic and neuropeptide Y (NPY) neuronal projections to the PVN. Furthermore, orexin-A administered intracerebroventricularly (i.c.v) increases c-*fos* expression in hypothalamic nuclei known to be involved in the regulation of the HPA axis (5), and has been reported to stimulate plasma corticosterone and adrenocorticotropic hormone (ACTH) release (9, 11) in rats. However, the mechanism mediating the effect of orexin-A on the HPA axis remains to be established.

Orexin-A acts at both OX_1R and OX_2R, which are expressed in all areas involved in HPA axis regulation. OX_2R is predominantly expressed in the PVN (12) and OX_1R mRNA is expressed at high levels in the monoaminergic locus coeruleus, the dorsal/median raphe nuclei and the arcuate nucleus. Both receptors are also densely expressed in the hippocampus and associated areas (12), which also send projections synapsing on the paraventricular CRF neurones.

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Evidence suggests that noradrenaline and NPY are involved in the central regulation of the HPA axis (13, 14). Both stimulate CRF release into the hypophysial-portal circulation in conscious sheep and both increase CRF mRNA expression when administered i.c.v. (15–17). Noradrenergic fibres from the A_2 region in the brainstem project primarily to areas of the parvocellular PVN rich in CRF neurones, whereas A_1 neurones terminate preferentially on vasopressinergic cell bodies in the PVN (18, 19). NPY neurones are widely distributed in the brain, but the PVN and arcuate nucleus contain the highest density of NPY stained nerve endings and cell bodies, respectively (20). Furthermore, NPY innervation is densest in areas of the parvocellular PVN containing principally CRF and TRH neurones, and direct synaptic contacts between NPY axon terminals and CRF cell bodies have been demonstrated (21).

We investigated the effects of orexin-A on the HPA axis *in vivo* in male rats. We then explored the effects of orexin-A on potential neuropeptides, neurotransmitters and releasing factors involved, by examining the effects of orexin-A on NPY, noradrenaline, CRF and arginine vasopressin (AVP) release *in vitro*.

Materials and methods

Peptides

Orexin-A was purchased from Peninsula (St Helens, Merseyside, UK).

Animals

Male Wistar rats (CBS, Imperial College, London, UK), weighing 250–300 g, were individually caged under controlled temperature (21–23°C) and light (12-h light/dark cycle, lights on at 07.00 h), with access to food (RM1 diet, SDS Ltd, Witham, UK) and water *ad libitum*. The animal procedures undertaken were all approved by the British Home Office Animals Scientific Procedures Act 1986 (Project Licence no. PIL 90/1077).

Intracerebroventricular (i.c.v.) cannulation and injection

Rats were anaesthetized and cannulated as previously described (22). Permanent 22-gauge stainless steel cannulae (Plastics One Inc., Roanoke, VA, USA) were inserted into the third ventricle according to the coordinates (0.8 mm back from bregma, midsagittal line) obtained from the Paxinos and Watson Rat Brain Atlas (23). Following a 7-day recovery period, rats were handled daily for 1 week to minimize stress. Correct positioning of the cannula was confirmed by a positive dipsogenic response to angiotensin II (150 ng). All compounds were dissolved in 0.9% saline and injected in a 10-µl volume and substances were administered via a 26-gauge stainless steel injector.

In vivo *study: time course effect of i.c.v. administration of orexin-A on plasma ACTH and corticosterone*

Orexin-A (3 nmol) or saline was administered i.c.v. in the early light phase (09.00–11.00 h) (n = 9–10 per group). Following injection, rats were returned to their home cages from which all chow had been removed, a standard protocol established previously in our department (24). At 10, 20, 40 or 90 min after injection, rats were killed by decapitation because this method minimizes stress and its metabolic consequences. The dose of orexin-A injected was determined from previously published studies, which demonstrated significant alteration in food intake and the hypothalamic-gonadal axis (3, 25), and from a dose–response study by Hagan *et al*. (9). Trunk blood was collected in plastic lithium heparin tubes containing 0.6 mg aprotinin. Plasma was separated by centrifugation, immediately frozen on dry ice and stored at −70°C until plasma ACTH and corticosterone were measured by immunoradiometric assay (IRMA) and radioimmunoassay (RIA), respectively. Due to cost limitations of the study, ACTH measurements were confined to the 20- and 90-min time-points.

In vitro *studies: release of CRF, NPY, AVP and noradrenaline from hypothalamic explants.*

The static incubation system used was a modification of a previously described method (26). Male Wistar rats were killed by decapitation and the whole brain immediately removed. The brain was mounted with ventral surface uppermost and placed in a vibrating microtome (Biorad Microfield Scientific Ltd, Southmead, Dartmouth, UK). A slice was taken from the base of the brain to a depth of 1.7 mm from the surface of the median eminence, to include the paraventricular and the arcuate nucleus, and the lateral cortical areas trimmed away. A single hypothalamus slice/explant was immediately transferred into an individual tube containing 1 ml of artificial cerebrospinal fluid (aCSF) [20 mM $NaHCO_3$, 126 mM NaCl, 0.09 mM Na_2HPO_4, 6 mM KCl, 1.4 mM Ca_2Cl, 0.09 mM $MgSO_4$, 8 mM glucose, 180 µg/ml ascorbic acid and 100 µg/ml aprotinin (Trasylol R, Bayer, Haywards Heath, UK)] gassed with 95% O_2 and 5% CO_2. The tubes, each containing a single hypothalamic explant, were placed in a water bath maintained at 37°C. After an initial 2-h preincubation period, the hypothalamic explants were incubated for 45 min in 600 µl aCSF (basal period) before being challenged for 45 min with 100 nM orexin-A in 600 µl aCSF with or without 1000 nM BIBP3226 (test period) or 1000 nM BIBP3226 in 600 µl aCSF alone. Finally, the viability of the tissue was verified by a 45-min exposure to 56 mM KCl solution with isotonicity maintained by substituting K^+ for Na^+. At the end of each period, aCSF was collected and stored at −20°C until CRF, NPY and AVP were measured by RIA. Noradrenaline was measured by high-pressure liquid chromatography (HPLC) with electrochemical detection based on previously described methods (27). Hypothalamic explant experiments were each carried out a minimum of three times, using 30 hypothalami on each occasion (n = 90 per experiment). Hypothalami releasing less than 150% of basal peptide following exposure to 56 mM KCl solution were assumed not to be viable, and were not included in the final data.

Hormone measurement

Antibody to CRF was raised in a rabbit immunized against CRF 41 (Peninsula Laboratories) conjugated to bovine serum albumin by glutaraldehyde and used at final dilution of 1 : 380000. ^{125}I-[Tyr] CRF was labelled by the iodogen method and purified by HPLC. The CRF assay was performed in a total volume of 350 µl, with phosphate EDTA buffer containing 0.1% BSA and 0.2% tween (1 : 10) (pH 7.4). Separation was with sheep anti-rabbit antibody and centrifugation at 2500 r.p.m. Bound and free fractions were separated and counted for 180 s. The assay sensitivity was 2fmol/assay tube, with 95% confidence limit. No cross-reactivity was observed (0.01%) with known hypothalamic peptides including, NPY, CART, galanin, agouti-related protein, orexins, substance P, CRF and growth hormone-releasing hormone. Intra- and interassay variation was less than 10%.

The NPY assay was performed as previously described (28). AVP was assayed using reagents and methods from Biogenesis (Poole, UK). There was no cross-reactivity with orexin-A in any of the above radioimmunoassays. Intra- and interassay coefficients of variation were approximately 8% and 12%, respectively, at the mid-points of the standard curves for the above assays.

Corticosterone was measured using a radioimmunoassay kit from ICN Biomedicals, Inc. (Irvine, CA, USA) for which intra- and interassay coefficients of variation were less than 10% and 7%, respectively. Using this corticosterone RIA kit, the baseline range for corticosterone in rats is 50–400 ng/ml and the lower limit of detection is 5 ng/ml, as specified in the kit.

ACTH was assayed using IRMA coated tube system purchased from Euro-Diagnostica B.V. (Arnhem, The Netherlands). The intra- and interassay coefficients of variation were both less than 4%, and the sensitivity of the assay was 1.0 pg/ml, as specified in the kit. There was no cross-reactivity with orexin-A in either the corticosterone RIA or the ACTH IRMA.

Statistical analysis

All data are presented as mean ± SEM. Statistical difference between control and experimental groups was determined by ANOVA, followed by post-hoc LSD (Systat 8.0, Evanston, IL, USA). Data from static incubation with hypothalamic explants were expressed as pmol/l and analysed by a paired *t*-test between the basal and test period. $P < 0.05$ was considered statistically significant.

Results

In vivo *study: time course effect of i.c.v. administration of orexin-A on plasma ACTH and corticosterone*

Following i.c.v. administration of orexin-A (3 nmol), the plasma concentration of corticosterone was significantly elevated at 10, 20 and 40 min (20 min saline 148 ± 14 ng/ml versus orexin-A 294 ± 24 ng/ml, P = 0.00005, Fig. 1), and that of ACTH was significantly raised at 20 and 90 min (20 min saline 11 ± 2 pg/ml versus orexin-A 32 ± 5 pg/ml, P < 0.005; 90 min saline 6 ± 1 pg/ml versus orexin-A 17 ± 5 pg/ml, P < 0.05, Fig. 2), relative to saline controls. There was a rise in basal corticosterone at 90 min, which was not significantly different to basal corticosterone measured at other time-points. The absence of chow, following i.c.v. injection, for a longer time period may have caused a mild degree of stress in this group.

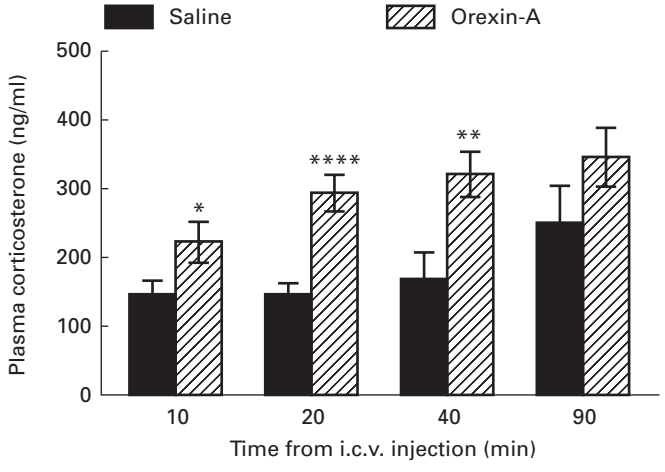

FIG. 1. The time course effect of intracerebroventricular (i.c.v.) orexin-A on plasma corticosterone. Orexin-A was injected into the third ventricle in male rats in the early light phase (n = 9–10 per group). Plasma corticosterone was measured 10, 20, 40 and 90 min following i.c.v. injection. *P < 0.05, **P < 0.005, ****P < 0.00005 versus saline control.

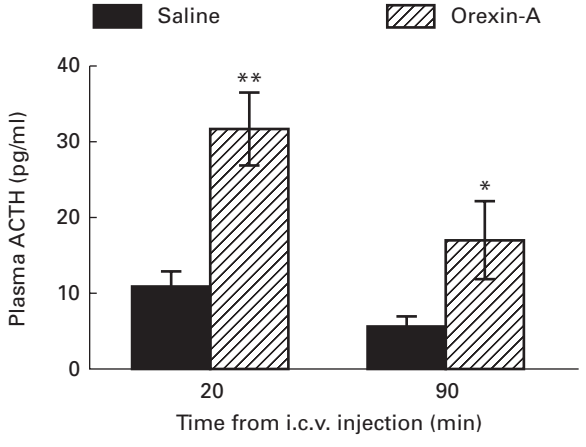

FIG. 2. The time course effect of intracerebroventricular (i.c.v.) orexin-A on plasma adrenocorticotropic hormone (ACTH). Orexin-A was injected into the third ventricle in male rats in the early light phase (n = 9–10 per group). Plasma ACTH was measured 20 and 90 min following i.c.v. injection. *P < 0.05, **P < 0.005 versus saline control.

In vitro *studies: release of CRF, NPY, AVP and noradrenaline from hypothalamic explants*

Effect of orexin-A on CRF, NPY, AVP and noradrenaline release

Orexin-A significantly stimulated CRF [basal 1.4 ± 0.2 pmol/explant versus orexin-A (100 nM) 3 ± 0.3 pmol/explant; P < 0.05, Fig. 3] and NPY [basal 46 ± 7 fmol/explant versus orexin-A (100 nM) 75 ± 13 fmol/explant; P < 0.05, Fig. 4] release from hypothalamic explants *in vitro*. Orexin-A had no effect on AVP [basal 231 ± 35 fmol/explant versus orexin-A (100 nM) 226 ± 57 fmol/explant; P = NS, Fig. 5] or noradrenaline release [basal 3 ± 0.7 nmol/l versus orexin-A (100 nM) 3 ± 0.91 nmol/l; P = NS, Fig. 6]. 56 mM KCl solution significantly stimulated release of CRF (Fig. 3), NPY (Fig. 4), AVP (Fig. 5) and noradrenaline (Fig. 6) relative to basal. This depolarizing dose of KCL stimulates peptide release from vesicular storage pools. The differing degrees of KCl-stimulated release, for different peptides over basal, may reflect differing amounts stored within vesicles for different peptides.

Effect of orexin-A and BIBP3226 on CRF release

Orexin-A did not stimulate CRF release in the presence of the NPY Y1 selective receptor antagonist BIBP3226 [basal 1.4 ± 0.2 pmol/explant versus orexin-A (100 nM) + BIBP3226 (1 μM) 2 ± 0.3 pmol/explant; P = NS, Fig. 3] and BIBP3226 alone did not alter CRF release [basal 1.4 ± 0.2 pmol/explant

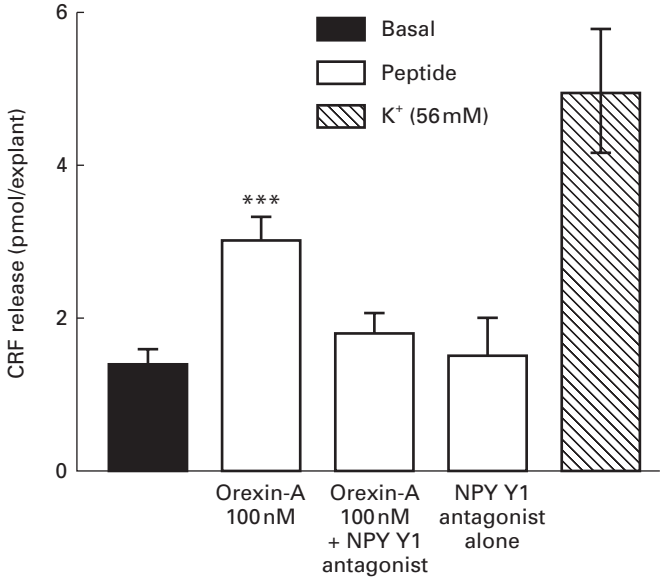

FIG. 3. The effect of orexin-A with and without BIBP3226 [neuropeptide Y (NPY) Y1 antagonist], and BIBP3226 alone on corticotropin releasing factor (CRF) release in hypothalamic slices harvested from male rats (n = 90 hypothalami per experiment). ***P < 0.0005 versus basal CRF release.

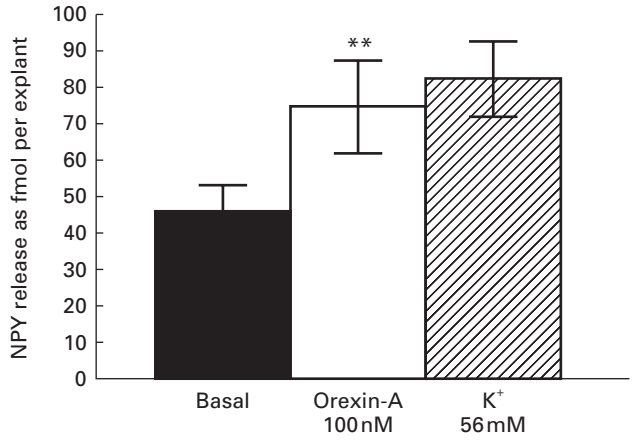

FIG. 4. The effect of orexin-A on neuropeptide Y (NPY) release in hypothalamic slices harvested from male rats (n = 90 hypothalami). **P < 0.005 versus basal NPY release.

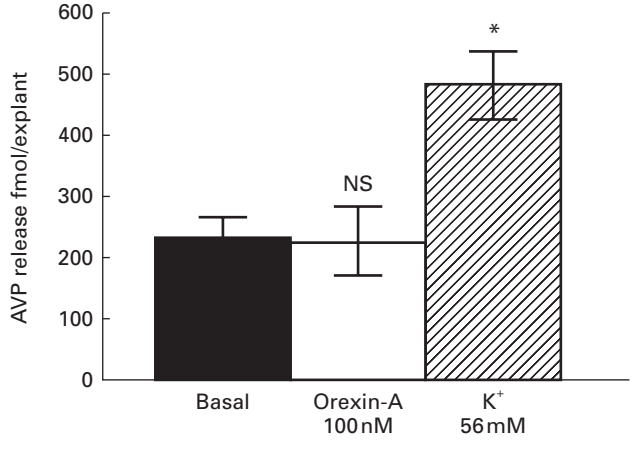

FIG. 5. The effect of orexin-A on arginine vasopressin (AVP) release in hypothalamic slices harvested from male rats (n = 90 hypothalami). P = ns versus basal AVP release. *P < 0.05 versus basal AVP release.

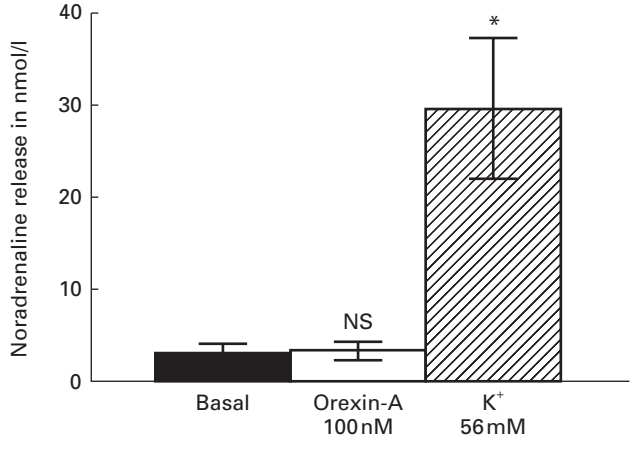

FIG. 6. The effect of orexin-A on noradrenaline release in hypothalamic slices harvested from male rats. P = NS orexin-A versus basal noradrenaline release. *P < 0.05 K^+ versus basal noradrenaline release.

versus BIBP3226 (1 μM) 1.5 ± 0.5 pmol/explant; P = NS, Fig. 3].

Discussion

We have shown that i.c.v. injection of orexin-A stimulates ACTH and corticosterone in male rats *in vivo*, and CRF but not AVP in hypothalamic explants *in vitro*. Kuru *et al.* (11) have recently shown a significant stimulation in ACTH at 30 min following i.c.v. orexin-A, which is consistent with the sustained elevation in ACTH at 90 min following i.c.v. orexin-A that we observed in our study. Although the stimulatory effect of orexin-A on plasma corticosterone (9) and ACTH (11) is consistently observed, the mechanism mediating this effects remains to be established. Selective stimulation of either CRF or AVP release, or stimulation of both CRF and AVP, has been shown to be specific to different stressors, all resulting ultimately in activation of the HPA axis (29, 30). Published studies suggest that CRF may be the dominant factor mediating responses to acute stress and AVP to chronic or repeated stress (31). Our *in vitro* findings suggest orexin stimulates the HPA axis selectively via CRF and not AVP release. Although CRF and AVP are colocalized in areas of the PVN, they are generally differentially distributed within discrete clusters of neurones within the PVN, and it could be that orexin-A fibres innervate subdivisions of the PVN containing primarily CRF neurones (32). The presence of different transcription binding sites in CRF and AVP genes and their modulation by different transcription factors (33, 34) may also partly underlie the differential response of CRF and AVP neurones to orexin-A *in vitro*.

Grooming behaviour in rats is closely associated with the stress reaction and CRF release (35, 36). Investigators have shown that orexin-A stimulates grooming behaviour following lateral ventricular administration in rats (37), and that this orexin-A induced behaviour is attenuated by prior administration of the CRF antagonist, α-helical CRF (37). This is consistent with our finding that orexin-A stimulates CRF release from hypothalamic explants *in vitro*. Thus, orexin-A may act as an acute activator of the HPA axis primarily through CRF release.

The parvocellular division of the PVN receives noradrenergic innervation predominantly from A_2 cells of the brainstem (18, 19, 38), and there is evidence that central catecholamines stimulate the HPA axis (32). We have shown that orexin-A has no effect on noradrenaline release *in vitro*. This suggests that, in the isolated hypothalamus, orexin-A does not influence CRF release via stimulation of noradrenergic pathways. Orexin-A significantly stimulates NPY release *in vitro* and the stimulation of CRF by orexin-A is completely attenuated in the presence of the selective NPY Y1 receptor antagonist, BIBP3226. These data may suggest that orexin-A stimulates CRF release in part via NPY, and that this effect may involve the NPY Y1 receptor.

We have previously reported that orexin-A inhibits prolactin release *in vivo* and stimulates luteinizing hormone releasing hormone (LHRH) release *in vitro* (39). Others have demonstrated *in vivo* stimulation of LH release in ovarectomized, steroid replaced female rats (25). NPY stimulates LHRH and LH release acutely, and this effect is dependent on

the steroid mileu (40, 41). NPY has been shown to inhibit prolactin release in studies where injection of NPY Y1 receptor antisense mRNA into the medial-preoptic area stimulates prolactin release (42). The apparently discrepant actions of orexin-A in stimulating the HPA and HPG axes, whilst at the same time inhibiting prolactin release, could be explained by an increase in NPY, stimulated by orexin-A, mediating these effects.

Orexin-A administration i.c.v. in rats not only stimulates the HPA axis, but also is associated with increased arousal (9). Conversely, defects of orexin signalling in murine and canine models and orexin deficiency in humans are associated with disordered sleep/wake cycles and narcolepsy (43–46). Interestingly, the hormonal changes characteristic of sleep onset include a prolactin and growth hormone surge, with a nadir in levels of cortisol/corticosterone (47, 48), and these are directly opposite to hormonal changes observed following i.c.v. administration of orexin-A *in vivo*. It could be that orexin-A has a physiological role mediating/regulating the endocrine changes associated with different arousal states. It would be interesting to measure the anterior pituitary hormones in models of orexin deficiency, including human narcolepsy.

We have demonstrated a robust effect of orexin-A administered i.c.v. on ACTH and corticosterone release *in vivo* and on CRF and NPY release *in vitro*. Orexin-A may be important in activation of the HPA axis and may mediate its effects via NPY. The orexin-A receptor subtype mediating these effects remains to be identified.

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