Distribution and quantification of immunoreactive orexin A in rat tissues

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Received 21 July 1999

Abstract A sensitive and specific radioimmunoassay for orexin A was developed. Orexin A immunoreactivity was found to be confined to the central nervous system (CNS) with the highest concentrations in the hypothalamus, inferior and superior colliculi and brainstem. Within the hypothalamus, the highest levels were found in the lateral and posterior hypothalamus. These regions had a greater orexin A content in females compared to males. The orexin A content of hypothalamic regions did not change with fasting and no difference was noted in hypothalami of rats fed a high fat diet. The hypothalamic orexin A content was not different in obese Zucker rats compared to lean controls. Thus, orexin A has a wide distribution in the CNS, but appetite regulation may not be its main function.

Key words: Orexin A; Hypothalamus; Central nervous system

1. Introduction

The understanding of the central nervous system (CNS) mechanisms that regulate the food intake and energy balance has advanced beyond the early experiments that identified regions of the hypothalamus as feeding (ventromedial hypothalamus) and satiety (lateral hypothalamus) centres. Several neuropeptides within the hypothalamus have been identified that mediate feeding and satiety [1]. Recently, a family of peptides (hypocretins/orexins) has been discovered that has been implicated in the regulation of food intake and energy balance [2,3]. Hypocretins were discovered through directional tag PCR subtraction in search of brain homologues of the gut peptide secretin [4]. Hypocretin mRNA encodes two proteins named hypocretin-1 and hypocretin-2. In search of ligands for orphan G protein-coupled receptors, two peptides, orexin A and B, were discovered. These peptides were shown to increase the food intake when injected into the lateral cerebral ventricle of rats and were therefore named orexins [5]. Furthermore, the orexin precursor (prepro-orexin) mRNA was shown to be up-regulated in the hypothalamus of fasted rats [5]. Shortly after the identification of hypocretins and orexins, it was discovered that they represent the same family of peptides with hypocretin-1 being the same as orexin A and hypocretin-2 being the same as orexin B [6].

Prepro-orexin is a 130 amino acid precursor that is processed to orexin A, a carboxy-amidated 33 amino acid peptide with two intra-chain disulfide bonds, and orexin B, a 28 amino acid carboxy-amidated linear peptide. Orexin immunoreactive fibres have been detected in the lateral hypothalamus, dorsomedial hypothalamus, posterior hypothalamus and perifornicial hypothalamus [7]. Nerve terminals immunoreactive for these peptides have been detected throughout the hypothalamus (in particular the arcuate and paraventricular nuclei), the thalamus, cerebral cortex, the limbic system and the brainstem (particularly areas that regulate the CNS monoaminergic system: the locus coeruleus and the raphe nucleus) [7]. Two G protein-coupled receptors have been identified for these peptides (OX1R and OX2R). Orexin A has a high affinity for both receptors, while orexin B has a 10-fold greater affinity for OX2R [5]. OX1R and OX2R have different distributions within the brain. OX1R mRNA has been reported to be most abundant in the ventromedial hypothalamus, but has also been detected at high levels in the tenia tecta, the hippocampal formation, dorsal raphe and locus coeruleus [8]. OX2R mRNA is predominantly expressed in the paraventricular hypothalamic nucleus, but has also been found at high levels in subthalamic and thalamic nuclei, the cerebral cortex, nucleus accumbens and anterior pretectal nucleus [8]. The widespread distribution of the orexin family of peptides and their receptors has suggested other roles for these peptides beside the regulation of feeding. For example, these peptides have been implicated in the regulation of pituitary LH release, temperature, heart rate, locomotor activity, metabolic rate, glucose homeostasis and gastric acid secretion [9–11].

The distribution of orexins has been investigated using several techniques, but quantification of these peptides throughout multiple specific regions of the CNS has so far not been carried out. In the present study, a highly sensitive and specific radioimmunoassay (RIA) for orexin A was developed. Using this assay, the content and distribution of immunoreactive orexin A (orexin A-IR) in the CNS, hypothalamic regions and peripheral tissues was quantitatively investigated in both male and female animals. The effect of fasting on the hypothalamic orexin A content and the orexin A content in the hypothalami of high fat diet (45% of calories)-fed animals and hypothalami of lean Zucker (fa/+) rats and Zucker (fa/fa) rats, with defective leptin action, was also investigated.

2. Materials and methods

2.1. Tissue preparation and peptide extraction

Adult male and female Wistar rats weighing 250–300 g ($n=4$–7; I.C.S.M., London, UK) were killed by decapitation and the brain, spinal cord, pineal gland, pituitary gland, adrenal gland, gastrointestinal tract, liver, testis, ovary, trachea, lung, heart, spleen and kidney were rapidly dissected and removed. The brain was immediately dissected into multiple regions including the hypothalamus. The hypothalamus was dissected into four regions (anterior/preoptic, ventrome-

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PII: S0014-5793(99)01030-3

dial, lateral and posterior, based on [12]) using fine dissection under magnification. The spinal cord was subdivided into cervical, thoracic and lumboscaral regions using conventional gross anatomical divisions. Adult male Wistar rats were fasted for 48 h and then decapitated with the tissues of various brain regions dissected and the hypothalamus microdissected into four regions as above. Lean fa/+ (250–300 g, $n=11$) and obese fa/fa (450–500 g, $n=12$) adult male Zucker rats (Charles River, Bicester, UK) were decapitated and their hypothalami rapidly dissected and removed. A group of male Wistar rats (250–300 g, $n=5$) was given a high fat diet (45% of calories as fat; D12451, Research Diets, New Brunswick, NJ, USA) ad libitum for 2 weeks while a control group ($n=5$) was given a low fat diet from the same supplier (10% of calories as fat; D1245OB) for the same period. At the end of 2 weeks, the animals were decapitated and their hypothalami dissected. All tissues obtained were immediately frozen in liquid nitrogen and stored at −70°C. The tissues were then extracted by boiling in 0.5 M acetic acid and stored at −20°C prior to assaying.

2.2. RIA

Antisera for orexin A were raised in New Zealand white rabbits initially inoculated with 100 μg of peptide, conjugated to bovine serum albumin (BSA) by carbodiimide, mixed with an equal volume of complete Freund's adjuvant, followed by regular booster injections. Synthetic orexin A was obtained from Peninsula laboratories (Merseyside, UK).

Orexin A antiserum showed full cross-reactivity with the synthetic peptide. The peptide used in the assays was iodinated using the iodogen method. The RIA was set up as previously described [13]. The final antibody dilution was 1:100 000. Orexin A label was used at 30 Bq/tube. The assay was performed in 0.06 M phosphate buffer (pH 7.2) containing 0.3% BSA and 0.02% Tween 20. After 3 days incubation at 4°C, antibody-bound and free fractions were separated by charcoal adsorption of the free fraction. No significant cross-reactivity was observed with orexin B or members of the secretin-glucagon family of peptides. Inter- and intra-assay variation was established to be less than 10%. The assay could detect changes of 0.2 fmol between adjacent tubes.

2.3. Chromatography

Fractionation of orexin A from extracts of Wistar rat hypothalamus, thalamus, cerebellum and cerebral cortex ($n=3$ per tissue) was carried out using Sephadex G-50 Superfine (Pharmacia, Uppsala, Sweden) on a 0.9×60 cm column and fast protein liquid chromatography (FPLC) using a high resolution reverse-phase (Pep RPC HR 5/5) C-18 column (Pharmacia, Uppsala, Sweden).

The Sephadex G-50 column was eluted at a flow rate of 3.2 ml/h at 4°C in 0.06 M phosphate buffer containing 0.2 M NaCl and 0.3% BSA and 1 ml fractions were collected. To determine the relative retention coefficient of orexin A, dextran blue (30 mg/ml), horse heart cytochrome *c* (30 mg/ml) and [^{125}I]Na (Amersham International, Buckinghamshire, UK) were added to each sample extract ($n=3$ per tissue type), which was loaded as a volume of 0.5 ml. RIA was used to observe the elution profile of orexin A from the tissue extracts and a standard of the pure synthetic peptide.

Neat tissue extracts were centrifuged and the supernatants filtered through 0.2 μm hydrophilic membranes (Sartorius AG, Goettingen, Germany). Tissue extracts and synthetic peptide were independently loaded on to the FPLC column (0.5 ml) and eluted with a 15–50% gradient of acetonitrile (ACN)/0.1% TFA over 60 min at a flow rate of 1 ml/min per fraction. 100 μl of each of the fractions collected was analysed by RIA.

2.4. Data analysis

All results are presented as mean ± S.E.M. The peptide content for brain regions and whole hypothalami is expressed as pmol/g wet weight of tissue. For discrete hypothalamic regions, data are presented as pmol/mg protein. The value of the relative elution coefficient (K_{av}) was calculated for each immunoreactive peak on G-50 column chromatography according to a modification [14] of the equation proposed by Laurent and Killander [15]. For statistical analysis, comparisons between microdissected hypothalamic regions were carried out by analysis of variance (ANOVA). If the ANOVA was found to be significant ($P<0.05$), then, further analysis was carried out using Fisher's least squares differences (LSD). The unpaired *t*-test was used for all other statistical comparisons.

Table 1
Orexin A content (mean ± S.E.M., pmol/g tissue) in brain regions of fed and 48 h-fasted male Wistar rats and spinal cord regions ($n=4$–7)

Brain/spinal cord region	Orexin A content ± S.E.M. (pmol/g wet weight)	
	Fed	48 h-fasted
Olfactory bulbs	2.4 ± 0.3	2.5 ± 0.3
Olfactory tubercles	7.3 ± 0.6	8.0 ± 0.8
Thalamus	12.5 ± 0.7	9.8 ± 1.3
Cingulate cortex	4.8 ± 0.9	4.6 ± 0.5
Frontal cortex	2.5 ± 0.3	2.7 ± 0.4
Parietal cortex	3.4 ± 0.5	5.0 ± 0.6
Occipital cortex	4.5 ± 0.4	3.8 ± 0.8
Temporal cortex	2.7 ± 0.2	3.3 ± 0.6
Amygdala	5.8 ± 0.5	8.1 ± 1.8
Septal nuclei	14.2 ± 1.7	17.2 ± 1.1*
Striatum	4.5 ± 0.4	3.8 ± 0.8
Hippocampus	1.9 ± 0.2	2.1 ± 0.2
Pons	18.5 ± 3.1	14.3 ± 3.1
Medulla	14.3 ± 1.3	16.3 ± 2.2
Midbrain	12.8 ± 1.4	16.2 ± 0.9
Cerebellum	0.7 ± 0.2	1.2 ± 0.3
Superior colliculus	23.1 ± 1.8	22.0 ± 2.0
Inferior colliculus	20.4 ± 2.8	24.8 ± 0.3
Hypothalamus (whole)	19.4 ± 1.6	20.1 ± 2.7
Lateral hypothalamus (fmol/μg protein)	2.0 ± 0.3	2.1 ± 0.2
Posterior hypothalamus (fmol/μg protein)	1.2 ± 0.2	1.1 ± 0.03
Ventromedial hypothalamus (fmol/μg protein)	2.1 ± 0.3	2.3 ± 0.2
Anterior hypothalamus (fmol/μg protein)	1.5 ± 0.1	1.6 ± 0.2
Cervical spinal cord	3.7 ± 0.8	
Thoracic spinal cord	1.3 ± 0.3	
Lumbo-sacral spinal cord	2.3 ± 0.5	

Septal nuclei is the only region showing a significant difference between fed and fasted ($P<0.05$). Spinal cord: $P<0.05$ by ANOVA, cervical versus thoracic $P<0.05$, cervical versus lumbosacral, $P<0.05$.
*$P<0.05$

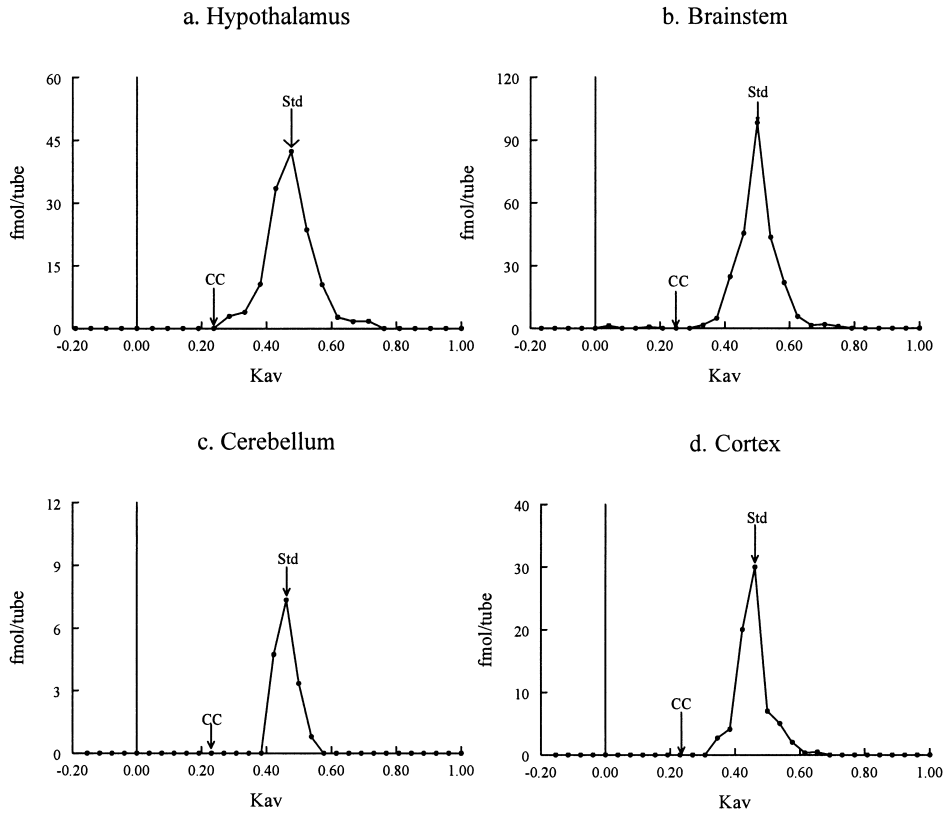

Fig. 1. Representative Sephadex G-50 column chromatography of rat. a: Hypothalamus, b: brainstem, c: cerebellum, d: cortex extracts. Std = elution of standard, CC = horse heart cytochrome *c*.

3. Results

3.1. Orexin A-IR distribution and content

No orexin A was detected in peripheral tissues or in the pineal and pituitary glands. Within the CNS, orexin A-IR was detected in all regions studied, but its content was highest in the hypothalamus, superior and inferior colliculi and brainstem (Table 1). In males, different hypothalamic regions (Table 2) showed significant differences (ANOVA $P < 0.05$, lateral versus posterior $P < 0.05$ by LSD, posterior versus ventromedial $P < 0.05$ by LSD). In females (Table 2), hypothalamic regions showed a different pattern of variation (ANOVA $P < 0.05$, lateral versus anterior $P < 0.01$ by LSD, posterior versus anterior $P < 0.01$ by LSD). Comparing the hypothalamic regions of males and females, the lateral and posterior hypothalamus of females had a greater immunoreactivity than males ($P < 0.05$ and $P < 0.01$, respectively, by a *t*-test). In the spinal cord (Table 1), the orexin A immunoreactivity of the various regions studied were found to be significantly different with the cervical spinal cord having the highest immunoreactive peptide content (ANOVA $P < 0.05$, cervical versus thoracic $P < 0.05$ by LSD, cervical versus lumbosacral $P < 0.05$ by LSD).

Table 2
Orexin A content of hypothalamic regions (mean ± S.E.M., pmol/mg protein) of male and female rats ($n = 3$–7 per region)

Hypothalamic region	Orexin A content ± S.E.M. (pmol/mg protein)	
	Male	Female
Lateral	2.0 ± 0.3	3.1 ± 0.4*
Posterior	1.2 ± 0.2	3.2 ± 0.5***
Ventromedial	2.1 ± 0.3	2.3 ± 0.2
Anterior	1.5 ± 0.1	1.3 ± 0.2

Males: $P < 0.05$ by ANOVA, lateral versus posterior $P < 0.05$, posterior versus ventromedial $P < 0.05$. Females: $P < 0.05$ by ANOVA, lateral versus anterior $P < 0.01$, posterior versus anterior $P < 0.01$.
*$P < 0.05$.
***$P < 0.001$.

3.2. G-50 column chromatography and FPLC

Sephadex G-50 chromatography showed the same elution profile and peaks for the different regions of the brain tested (Fig. 1). These immunoreactive peaks co-eluted with the synthetic orexin A, which had a K_{av} of approximately 0.49. FPLC of synthetic orexin A gave a single major immunoreactive peak eluting at 30% ACN. FPLC of tissue extracts showed major peaks corresponding to the synthetic peptide. Minor immunoreactive peaks, not detected by G-50 chromatography and indicating fragments of a lesser hydrophobicity, were observed with FPLC for the hypothalamus, brainstem and cerebral cortex, but not for the cerebellum (Fig. 2).

3.3. Orexin A-IR content in fasted rats, high fat-fed rats and Zucker rats

No significant difference in orexin A-IR content was observed for any brain or hypothalamic region (except for the septal nuclei, $P < 0.05$) when 48 h-fasted animals were compared to fed animals (Table 1). In the same tissue samples from hypothalamic regions, we have noted significant differences (up to 8-fold) with fasting in the content of other hy-

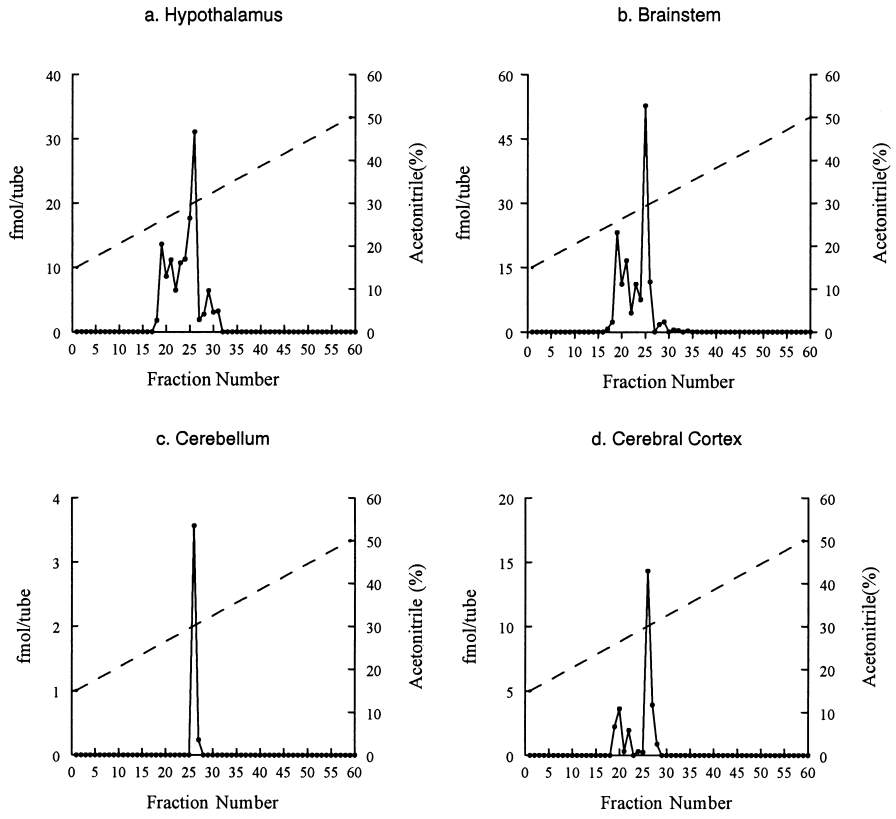

Fig. 2. Representative FPLC profiles of: a, hypothalamus; b, brainstem; c, cerebellum and d, cerebral cortex extracts. Solid line, orexin A concentration. Broken line, % ACN.

pothalamic peptides, such as cocaine and amphetamine-related transcript (CART) (unpublished data), which inhibit food intake. No significant difference in the hypothalamic orexin A-IR of high fat-fed animals was observed compared to low fat-fed controls (25.0 ± 2.0 versus 21.3 ± 2.0) despite a significantly greater average weight gain in the high fat-fed group (104 g versus 84.9 g, $P < 0.001$). No significant difference in the orexin A-IR of hypothalami from lean (fa/+) and obese Zucker (fa/fa) rats was observed (20.7 ± 1.8 pmol/g versus 19.0 ± 0.7 pmol/g).

4. Discussion

Our results demonstrate that orexin A-IR is restricted to the CNS where it has a widespread distribution. The high concentrations observed within the superior and inferior colliculi are suggestive of a role in vision and hearing while high concentrations within the brainstem are in agreement with possible regulation of the autonomic function [16]. Orexin A-IR could be detected in the spinal cord (with the cervical cord having the highest concentration), but at much smaller concentrations compared to the rest of the CNS. Recently, it has been reported that orexin immunoreactive fibres originating from the lateral hypothalamus may have an important neurotransmitter/neuromodulator function within the spinal cord, particularly in the modulation of sensation and the autonomic nervous system [16]. Within the hypothalamus, orexin A-IR was found to be most abundant in the lateral and posterior hypothalamus.

The sex differences observed in the hypothalamic regions suggest that orexin A levels may be influenced by sex steroids. It has been demonstrated that LH secretion is stimulated by intracerebroventricular orexin (A or B) in oestrogen-replaced ovariectomised rats, but these peptides are inhibitory to LH secretion in unprimed ovariectomised rats [17]. Therefore, sex steroids not only influence orexin A actions, but also affect the orexin A peptide content.

The lateral hypothalamus is an important centre for regulation of food intake with the neuropeptides melanin-concentrating hormone (MCH) and orexins being abundant there. Both centrally administered MCH [1] and orexins [5,18,19] have been reported to increase food intake, but the effects of orexin A on food intake are more consistent than orexin B. Innervation of orexin and MCH neurons in the lateral hypothalamus by NPY/AGRP neurons from the arcuate nucleus has suggested that MCH and orexins may act downstream of NPY/AGRP [20]. Orexin immunoreactive fibres, in turn, innervate NPY/AGRP neurons in the arcuate nucleus and the existence of a feedback loop has been suggested [1]. Prepro-orexin mRNA is up-regulated in the hypothalamus of fasted animals [5], but unlike a recent study of the orexin content in the brain [21], no changes in the peptide in any hypothalamic or brain region (except in the septal nuclei) has been observed in the present study. The physiological significance of changes in the orexin A-IR content in the septal nuclei with fasting is at present unclear. Unlike the orexigenic peptides NPY and galanin, no changes in the hypothalamic orexin A-IR content were observed in high fat-fed animals compared to low fat-fed controls. Although the orexin A-IR content of whole hypothalami was unchanged, it is possible

that discrete hypothalamic regions may show differences. Chronic leptin treatment has been shown to decrease the concentration of orexin A in the lateral hypothalamus [22,23]. In the present study, no change in hypothalamic orexin A-IR content was observed in Zucker rats with a defective leptin action, but discrete hypothalamic regions need to be examined for regional changes of peptide mRNA and content. The interaction of leptin with orexins is further complicated by the finding that unlike the potently orexigenic peptide NPY, prepro-orexin mRNA is lower in leptin deficient ob/ob and leptin receptor deficient db/db mice compared to control animals [24].

The widespread distribution of orexin immunoreactivity and orexin receptors within the CNS suggests that orexins are important in several CNS regulatory mechanisms. The precise physiological importance and role of orexins in the regulation of appetite (particularly its interaction with other neuropeptides and leptin) remain to be determined.

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