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European Journal of Pharmacology 440 (2002) 199–212

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Orexins and the treatment of obesity

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Received 1 October 2001; accepted 29 October 2001

Abstract

Orexin-A and -B are two peptides derived by proteolytic cleavage from a 130-amino acid precursor, prepro-orexin, which were recently isolated from the rat hypothalamus. Orexin-A is fully conserved across mammalian species, whilst rat and human orexin-B differ by two amino acids. These peptides bind to two Gq-coupled receptors, termed orexin-1 and orexin-2. The receptors are 64% homologous and highly conserved across species. Orexin-A is equipotent at orexin-1 and orexin-2 receptors, whilst orexin-B displays moderate (~ 10 fold) selectivity for orexin-2 receptors. The distribution and pharmacology of the orexin peptides and their receptors indicate that they play a role in various regulatory systems including energy homeostasis and the regulation of feeding, the evidence for which is reviewed here.

Keywords: Orexin; Hypocretin; SB-334867; Feeding; Narcolepsy; Sleep–wake cycle

1. Pharmacology

1.1. The orexin peptides: identification and structure

Orexin-A and -B are 33- and 28-amino acid neuropeptides, respectively, which were initially isolated from the rat hypothalamus (Sakurai et al., 1998). Both peptides are derived by proteolytic cleavage from a common 130-amino acid precursor peptide, prepro-orexin, which is encoded by a single gene localised to chromosome 17Q21 in humans (Sakurai et al., 1998). Both orexin peptides are C-terminally amidated and orexin-A also undergoes N-terminal pyroglutamyl cyclisation (Lee et al., 1999). Mature orexin-A has two disulfide bridges (Cys^6-Cys^{12} and Cys^7-Cys^{14}) (Soll and Beck-Sickinger, 2000), which are essential for functional potency (Darker et al., 2001; Okumura et al., 2001), whilst orexin-B contains two α-helices linked by a flexible loop, with helix I being orientated 60–80° relative to helix II (Lee et al., 1999). Orexin-A and -B are 46% homologous with the sequence of orexin-A being fully conserved between rat, human, mouse, pig and cow (Sakurai et al., 1998; Shibahara et al., 1999; Dyer et al., 1999).

Independent of the isolation of the orexins, another group identified a hypothalamic-specific mRNA encoding a precursor protein they termed prepro-hypocretin and predicted that proteolytic processing of this would yield two peptides, hypocretin-1 (residues 28–66) and hypocretin-2 (residues 69–97) (De Lecea et al., 1998). This group also predicted that both peptides would be amidated, with the caveat that the N-terminus of hypocretin-1 was not defined (De Lecea et al., 1998). Subsequent comparisons showed that prepro-orexin and prepro-hypocretin were the same peptide, and that orexin-B and amidated hypocretin-2 were identical. Moreover, orexin-A and hypocretin-1 corresponded, allowing for the overestimation of the N-terminus of hypocretin-1 by five amino acids (Smart et al., 2000). However, it is important to note that some commercial suppliers are still selling the deamidated versions of the original predicted sequences as hypocretin-1 and hypocretin-2, and these are effectively inactive at the orexin receptors (Smart et al., 2000).

Recent studies using iodinated versions of the orexin peptides have suggested that the two peptides have quite different pharmacodynamic profiles. [^{125}I]orexin-A readily enters the brain by passive diffusion at a comparable rate to that of actively transported insulin, whilst [^{125}I]orexin-B lacks brain penetration and is rapidly metabolised in blood (Kastin and Akerstrom, 1999). The difference in the susceptibility of the orexins to metabolism in blood and, consequently, the ability to enter the brain as intact peptide, may

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0014-2999/02/$ - see front matter
PII: S0014-2999(02)01429-2

reflect the protection afforded to orexin-A by the disulphide bonds and consequent cyclisation of the peptide (Darker et al., 2001). It is important that the relative lability of these peptides is considered when attempting to interpret data from in vivo studies where systemic routes of administration have been used.

The recent development of specific radioimmunoassays (Mitsuma et al., 2000) has enabled the measurement of endogenous orexin-A in human cerebrospinal fluid (CSF) (Melberg et al., 2001) and plasma (Arihara et al., 2001), with central, but not peripheral, levels of orexin-A displaying a distinct circadian rhythm (Taheri et al., 2000; Arihara et al., 2001).

1.2. Distribution of the orexins

Prepro-orexin and the mature peptides have been identified in the central nervous system (CNS) of humans (Sakurai et al., 1998) and other mammals (Horvath et al., 1999; Yamamoto et al., 1999; Mondal et al., 1999; Nambu et al., 1999; Chen et al., 1999; Dyer et al., 1999; Sakurai, 1999), as well as amphibians (Shibahara et al., 1999; Galas et al., 2001). Prepro-orexin is abundant in adult brain but, with the exception of a low level in testis, not in a range of peripheral tissues including heart, liver, kidney, placenta and lung (Sakurai et al., 1998), although orexin-like immunoreactive neurones have been identified throughout the small intestine of the rat, mouse, guinea pig and human (Kirchgessner and Liu, 1999). Moreover, orexin-A and -B have also been identified in the rat pituitary (Date et al., 2000).

Studies have confirmed that, within the central nervous system, prepro-orexin mRNA is localised to the hypothalamus, particularly the lateral and posterior areas (Sakurai et al., 1998; Peyron et al., 1998; Taheri et al., 1999). The expression of prepro-orexin is modulated by a range of factors including nicotine (Kane et al., 2000a,b) and α-interferon (Waleh et al., 2001). However, recent immunohistochemical mapping of orexin-A and -B fibres in rat brain and spinal cord has revealed a differential distribution of the two peptides (Cutler et al., 1999). Orexin-A immunoreactive fibres are heavily expressed in the hypothalamus, thalamus and spinal cord, but are absent from the superior cervical ganglion, trigeminal ganglion and nodose ganglion (Cutler et al., 1999). Within the hypothalamus, orexin-A is most strongly expressed in the arcuate nucleus, dorsomedial hypothalamic nucleus and the paraventricular nuclei (Briski and Sylvester, 2001; Guan et al., 2001). In contrast, orexin-B immunoreactive fibres were found at fewer sites, at a much lower density, with only relatively sparse expression in the hypothalamus (Cutler et al., 1999).

Similarly, mapping of orexin immunoreactive neurones in rat tissues has identified a population of neurones localised within the hypothalamus including the perifornical nucleus, the lateral-, posterior- and dorsal medial-hypothalamic areas. (Nambu et al., 1999; Briski and Sylvester, 2001). The nerve terminals of these neurones project throughout the hypothalamus, including the arcuate-, periventricular- and perifornical-nuclei, as well as into extra hypothalamic regions including the cerebral cortex, the thalamus, circumventral organs, the limbic system, brain stem and, in particular, in the locus coeruleus and raphe nuclei (Sakurai et al., 1998; Peyron et al., 1998; Hagan et al., 1999; Nambu et al., 1999; Cutler et al., 1999). In both mouse and rat, orexin-immunoreactive axons have been detected throughout the entire length of the spinal cord, with the highest concentration of descending axons present in the dorsal region of the lateral white matter (Van den Pol, 1999; Cutler et al., 1999). A similar distribution has also been reported for human cervical cord (Van den Pol, 1999). Orexin-A immunoreactivity has also been detected in the dorsal root ganglia (Bingham et al., 2001).

Recent radioimmunoassay studies have shown that the highest levels of immunoreactive orexin-A in both the rat and human brain are found in the hypothalamus, though the peptide is also expressed in the thalamus, medulla oblongata and pons, but not in the pituitary (Arihara et al., 2000; Taheri et al., 2000). The detection of immunoreactive orexin-A throughout the brain, whilst the localisation of orexin mRNA is exclusive to the hypothalamus, suggests that orexin-A undergoes axonal transport (Arihara et al., 2000).

1.3. The orexin receptors and their pharmacology

The orexins activate two G-protein-coupled receptors, orexin-1 and orexin-2, which are 64% homologous and most closely related (26%) to the neuropeptide Y Y2 receptor. (Sakurai et al., 1998). The human and rat receptors display 94% and 95% homology for orexin-1 and orexin-2, respectively, suggesting that they are highly conserved between mammalian species (Sakurai et al., 1998). Radioligand binding studies have shown orexin-A to have equal affinity at orexin-1 and orexin-2 receptors, whilst orexin-B has approximately 10-fold greater affinity at orexin-2 than at orexin-1 (Sakurai et al., 1998). In recombinant systems, activation of either receptor causes an elevation of intracellular Ca^{2+} (Smart et al., 1999; Lund et al., 2000). In Chinese Hamster Ovary cells, this Ca^{2+} response involves a phospholipase C-mediated mobilisation of Ca^{2+} from intracellular stores and an influx of extracellular Ca^{2+}, which in turn enhances the Gq-mediated stimulation of phospholipase C (Smart et al., 1999; Lund et al., 2000). The Ca^{2+} influx is in part mediated by activation of protein kinase C (Smart et al., 1999, 2000). Consistent with this, orexin causes protein kinase C-mediated Ca^{2+} influx in hypothalamic cultures (Van den Pol et al., 1998), induces Ca^{2+} spiking in spinal neurones (Van den Pol, 1999) and increases intracellular Ca^{2+} in A10 dopaminergic neurones (Nakamura et al., 2000). There is also evidence suggesting the orexin receptors may couple to adenylate cyclase in some cells (Nanmoku et al., 2000; Mazzocchi et al., 2001). In addition, orexin-B activates Ca^{2+}-sensitive K^{+} channels in immune cells (Ichinose et al., 1998).

The agonist pharmacology of the orexin receptors determined by measurement of intracellular Ca^{2+} is comparable to that determined by radioligand binding, with orexin-A and -B being equipotent at orexin-2 receptors and orexin-B displaying moderate selectivity for orexin-2 over orexin-1 receptors (Sakurai et al., 1998; Smart et al., 1999). Neuropeptide Y and secretin have also been reported to displace [^{125}I]orexin-A binding (Kane et al., 2000a,b), but more recent studies have shown that these peptides neither bind to, nor increase intracellular Ca^{2+} in, cells expressing orexin-1 or orexin-2 (Smart et al., 2001; Holmqvist et al., 2001). This discrepancy probably arises from the specificity of the assay used in the original study where the nonspecific binding (60%) was defined using porcine secretin (Kane et al., 2000a,b; Smart et al., 2001). In addition, at least for neuropeptide Y, the apparent inhibition seen in the earlier study using a membrane preparation (Kane et al., 2000a,b) could result from a non-receptor-mediated modulation of G-protein function which does not occur in intact cells (Holmqvist et al., 2001).

More recently, orexin-1 receptor antagonists have also been described (Duxon et al., 2001; Smart et al., 2001). The most potent and selective is SB-334867 (1-(2-Methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride), which has an affinity of 40 nM at orexin-1 receptors and is at least 50-fold selective over orexin-2 receptors and a range of other G-protein-coupled receptors, as well as several ion channels (Smart et al., 2001). Furthermore, this compound is brain penetrant and attains significant brain exposure for >2 h following a 10-mg/kg dose in vivo (Haynes et al., 2000a,b; Duxon et al., 2001).

1.4. Localisation of the orexin receptors

Detailed mapping of orexin receptor mRNA distribution by in situ hybridisation has shown that the two orexin receptors are both distributed throughout the rat brain, but with differential expression patterns (Trivedi et al., 1998; Lu et al., 2000). Within the hypothalamus, the highest levels of orexin-1 receptor mRNA were found in the ventromedial hypothalamic nucleus, whilst the highest levels of orexin-2 receptor mRNA were found in the paraventricular hypothalamic nucleus (Trivedi et al., 1998). Significant levels of orexin-1 receptor mRNA were also found in locus coeruleus, tenia tecta and septohippocampal nucleus, and to a lesser extent in the hippocampus, amygdala and dorsal raphe nucleus (Trivedi et al., 1998; Lu et al., 2000). High levels of orexin-2 receptor mRNA were also detected in the hippocampus, amygdala (Lu et al., 2000) and layer VI of the cortex, but not in the locus coeruleus (Trivedi et al., 1998). Both orexin receptor mRNAs have also been identified in the gut (Kirchgessner and Liu, 1999) and the pituitary (Date et al., 2000).

Recent immunohistochemical studies have shown that the distribution of orexin-1 receptor protein is in good agreement with the mRNA data (Hervieu et al., 2001). The highest level of expression of orexin-1 receptor is in the locus coeruleus (Hervieu et al., 2001; Greco and Shiromani, 2001). The orexin-1 receptor was also densely expressed in the piriform cortex, olfactory nuclei, thalamus, dentate gyrus, anterior hypothalamus, arcuate nucleus, ventromedial hypothalamus, paraventricular nucleus, supraoptic nucleus, suprachiasmatic nucleus, rhombencephalon, dorsal tegmental nucleus and spinal cord (Hervieu et al., 2001), as well as the dorsal root ganglia (Bingham et al., 2001). The orexin-1 receptor is also expressed less strongly in a variety of other brain regions (Hervieu et al., 2001; Greco and Shiromani, 2001). Studies of the distribution of the orexin-2 receptor at the protein level have been more limited but are, again, consistent with the mRNA data in the regions examined (Greco and Shiromani, 2001). The orexin-2 receptor is strongly expressed in the subcoeruleus alpha, mesencephalic trigeminal nucleus, dorsal tegmental nucleus of Gaddens, Barrington's nucleus, pontine reticular nuclei and the ventral cochlear nucleus (Greco and Shiromani, 2001). As with the peptides, the differential distribution of the orexin receptors indicates that each may have distinct physiological distinct roles.

2. Physiological roles of the orexins

The distribution of the orexin peptides and their receptors suggests that they may play a role in energy homeostasis and the regulation of feeding (see Section 3 for details), as well as nociceptive processing, drinking, arousal and the sleep–wake cycle, cardiovascular regulation and neuroendocrine function, (Peyron et al., 1998; Smart, 1999; Hervieu et al., 2001). A range of other studies have provided further evidence linking the orexins to feeding and energy homeostasis and this is described in detail in Section 3. Similar evidence supporting the role of the orexins in the other functions outlined above has been reviewed elsewhere (Smart, 1999; Sutcliffe and DeLecea, 2000; Kilduff and Peyron, 2000; Jerman and Smart, 2001; Taheri and Bloom, 2001).

3. Evidence that orexins are involved in the regulation of feeding and metabolic rate

3.1. Location of orexins and their receptors: implications for feeding

The localisation of the orexins and their receptors has been given above (Sections 1.2 and 1.4). It was the observation (Sakurai et al., 1998) that the cell bodies of orexin neurones are found exclusively in the lateral hypothalamus (the classical ''feeding centre'') and nearby regions (perifornical nucleus; dorsal hypothalamus) that first prompted workers at SmithKline Beecham to investigate the effects of the (then unnamed) orexins on feeding. Subsequently,

numerous papers have confirmed the location of the cell bodies and described projections to regions of the brain that play a role in the regulation of feeding, for example, the area postrema, central nucleus of the amygdala, bed nucleus of the striata terminalis, lateral/medial parabrachial nucleus and nucleus of the solitary tract (Cutler et al., 1999). The last two of these represent targets for ascending vagal pathways that convey signals from the abdominal and thoracic viscera. In addition, the finding of orexin-A fibres in other hypothalamic regions, such as the paraventricular, ventromedial and arcuate nuclei (Cutler et al., 1999), is especially supportive of a role for orexins on feeding. In the arcuate nucleus, orexin fibres impinge upon cell bodies that express other peptides involved in the regulation of energy balance: neuropeptide Y, agouti-related protein, cocaine- and amphetamine-regulated transcript, and α-melanocyte-stimulating hormone; conversely, fibres from the arcuate nucleus that express these peptides impinge upon orexin cell bodies in the lateral hypothalamus (Elias et al., 1998; Broberger et al., 1998; Horvath et al., 1999; Guan et al., 2001). Leptin receptors have been identified on orexin neurones and on neurones in the arcuate and ventromedial nuclei that they innervate (Horvath et al., 1999; Funahashi et al., 2000; Håkansson et al., 1999), but as we explain below, the functional relationship between leptin and the orexins is far from clear.

Orexin receptors are also expressed in hypothalamic areas involved in the regulation of energy balance. Orexin-1 receptor mRNA is most abundant in the ventromedial and dorsomedial nuclei, and orexin-2 receptor mRNA in the paraventricular, ventromedial and arcuate nuclei, and the lateral hypothalamic area (Trivedi et al., 1998; Marcus et al., 2001; Lu et al., 2000; Yamamoto et al., 2000a). Immunohistochemistry has identified the orexin-1 receptor in many parts of the hypothalamus (Hervieu et al., 2001). Functional evidence also indicates that orexin receptors are present in regions of the brain involved in the regulation of energy balance. Thus, intracerebroventricular injection of orexins induced c-*fos* expression in, among other areas, the arcuate nucleus and the nucleus of the solitary tract (Date et al., 1999; Mullet et al., 2000); application of orexin-A to brain slices activated 85% of arcuate and 100% of nucleus of the solitary tract neurones (Brown et al., 2001; Rauch et al., 2000); both orexins evoked robust increases in synaptic activity in slices of the lateral hypothalamus (Van den Pol et al., 2001). It is important to recognise, however, that both orexins and both receptors are present in parts of the brain that have no known involvement with feeding, other than that they may help to maintain alertness and vigilance (Greco and Shiromani, 2001; Marcus et al., 2001). The evidence above is consistent with orexins playing a role in the regulation of feeding, but clearly indicates that they must play other roles as well.

Orexins and their receptors are also present in the enteric system and endocrine cells of the gut (Kirchgessner and Liu, 1999), suggesting that they may play a role in the periphery as well as centrally in the regulation of feeding.

3.2. Effects of the orexins on food intake, metabolic rate and body weight

Since the original report that intracerebroventricular (i.c.v.) injection of orexin-A or -B stimulates feeding in rats (Sakurai et al. 1998), numerous papers have confirmed the effect of i.c.v. orexin-A, and one study has shown that orexin-A stimulates feeding when given directly into the perifornical area or lateral hypothalamus (Sweet et al., 1999). The magnitude of the response varies with the time of day (Haynes et al., 1999). In addition, other workers describe very different daytime effects (e.g. contrast Edwards et al., 1999 with Shiraishi et al., 2000), leading some who find a poor response to doubt that orexin-A plays a role in the regulation of feeding (Edwards et al., 1999). However, although the effect of orexin-A is less than that of neuropeptide Y or agouti-related protein, it is as great as that of the established orexigenic peptides galanin and melanin-concentrating hormone (Arch et al., 1999), even in the hands of those who doubt its significance (Edwards et al., 1999). Moreover, the minimum dose of orexin-A that elicits a response has been as little as 0.07 nmol (0.3 μg) in our hands (Fig. 1), which is similar to threshold doses for other orexigenic peptides (Kalra et al., 1999). One group who found a poor response to orexin-A obtained a far greater response when the action of corticotropin releasing hormone was blocked. They suggested that stimulation of the release of corticotropin releasing hormone, which inhibits food intake, opposes the underlying orexigenic effect of orexin-A (Ida et al., 2000).

Many workers have found it more difficult to confirm the original report that orexin-B stimulates food intake. We failed to demonstrate this effect in six experiments in Sprague–Dawley and Wistar rats (Haynes et al., 1999), succeeding only in inhibiting the effect of orexin-A (2.3 nmol) with a high dose (23 nmol) of orexin-B (Haynes et al., 2000a). Others have also described an inhibitory effect of orexin-B on feeding (Sunter et al., 2001). However, we

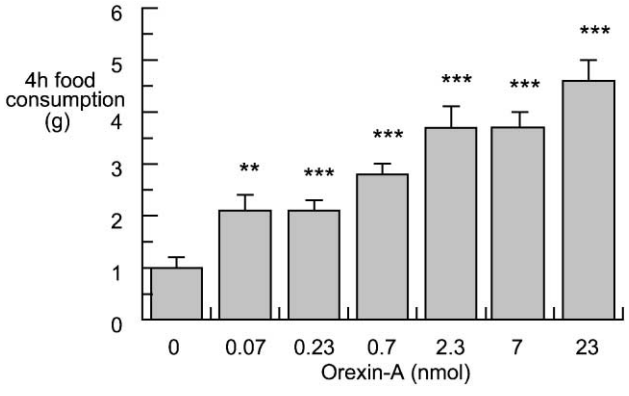

Fig. 1. Effect of a single i.c.v injection of orexin-A during the light phase on food intake over 4 h in male Sprague–Dawley rats. Each column represents the mean ± S.E.M. ($n=16$); * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared to vehicle control; 1 nmol orexin-A = 4.17 μg.

have recently seen an effect of orexin-B in Wistar–Kyoto rats (K.A. Al-Barazanji and J.R.S. Arch, unpublished observations), a strain that has low hypothalamic prepro-orexin mRNA levels (Taheri et al., 2001).

It has been reported that blockade of neuropeptide Y Y1 receptors using BW-1229U91 (Ile,Glu,Pro,DPr,Tyr,Arg,Leu,Arg,Tyr-NH_2)$_2$ cyclic(2,4′),(2′ ,4′)-diamide) totally blocks feeding stimulated by orexin-A- or -B (Ida et al., 2000; Jain et al., 2000 compare with Haynes et al., 2000a). Another neuropeptide Y Y1 receptor antagonist, BIBO3304 ((*R*)-*N*-[[4-aminocarbonyl)-phenyl]-methyl]-N2-(diphenylacetyl)-argininamide trifluoroacetate), and the neuropeptide Y Y5 receptor antagonist, CGP-71683-A ((4-[(4-amino-quinazolin-2-ylamino)-methyl]cyclohexylmethyl)-amide hydrochloride) partially blocked orexin-A-driven feeding (Yamanaka et al., 2000; Dube et al., 2001). Although prevention of the effect of orexin-A by antagonism of neuropeptide Y receptors could be due to neuropeptide Y being downstream of orexins in the feeding regulation pathway, it is also possible that neuropeptide Y plays a permissive role, operating in parallel with the orexin pathway. It must be remembered that feeding is a complex behaviour that involves both food seeking and ingestion, and it must at some point require many neuronal pathways to operate in parallel. There is in fact better evidence that orexin neurones are downstream of neuropeptide Y, since not only did i.c.v. injection of an antibody to orexin-A inhibit neuropeptide Y-driven feeding, but administration of neuropeptide Y activated Fos in orexin neurones (Niimi et al., 2001).

It is important to rule out the possibility that orexins promote feeding as a consequence of their reducing sleep and promoting arousal. This seems an unlikely explanation because orexin-B stimulates searching behaviour as or more effectively than orexin-A (Ida et al., 1999; Jones et al., 2001), but it has less effect on food intake. Similarly, neuropeptide E-I (derived from prepro-melanin-concentrating hormone) stimulates grooming rearing and locomotor activity, but it does not affect feeding (Rossi et al., 1997; Sanchez et al., 1997). Moreover, provided the dose of orexin-A is not raised so high that is evokes an initial bout of resting, it does not disrupt the normal behavioural satiety sequence. This is the process in which rats pass from a phase of predominantly feeding, through grooming to resting. Instead, the transition point from feeding to resting is delayed (Fig. 2) (Rodgers et al., 2000). This indicates that orexin-A delays the normal mechanism of satiety.

Intracerebroventricular infusion of orexin-A, but not orexin-B, promotes daytime feeding, but there is a compensatory reduction in nighttime feeding and no change in 24-h food intake or in body weight over a week (Haynes et al., 1999; Yamanaka et al., 1999). Since the suppression of nighttime feeding by orexin-A has only ever been noted following elevation of daytime feeding, it may be a counter-regulatory response to earlier overeating that occurs at a time when sensitivity to orexin-A is low. Although the failure of orexin-A to produce obesity contrasts with the obesity inducing effects of neuropeptide Y and agouti-related protein (Stanley et al., 1986; Small et al., 2001), infusion of melanin-concentrating hormone and galanin has also been reported not to induce obesity (Rossi et al., 1997; Smith et al., 1994). Thus, its failure to produce obesity does not preclude a role for orexin-A in the regulation of feeding, though it may be a rather different role from that of neuropeptide Y or agouti-related protein, for example, one not so directly linked to the action of leptin (see below).

Most hypothalamic peptides that influence food intake also influence energy expenditure, so that the two effects synergise in modulating energy balance. The effects of orexins on metabolic rate have received little attention compared to their effects on feeding. One study found that injection of orexin-A, but not orexin-B, into the third brain ventricle increased metabolic rate in mice with no associated increase in locomotor activity (Lubkin and Stricker-Krongrad, 1998). Another study showed that central infusion of orexin-A increased body temperature in rats even when they were anaesthetised, thus neither feeding nor moving (Yoshimichi et al., 2001). These findings suggest that thermogenesis and increased body temperature were due to increased sympathetic activity, an established effect of orexin-A (Shirasaka et al., 1999; Dun et al., 2000) and consistent with the increased blood glucose level observed in the rat study (Yoshimichi et al., 2001). However, sympathetic stimulation of thermogenesis usually results in increased expression of uncoupling protein-1 mRNA in brown adipose tissue, and this was not found (Yoshimichi et al., 2001). Moreover, we have been unable to detect an increase in brown adipose tissue temperature in rats in response to i.c.v. injection of orexin-A (Haynes et al., 1999). Thus, the thermogenic effect of orexin-A is perhaps not due to sympathetically mediated thermogenesis in brown adipose tissue. Thermogenesis in skeletal muscle might play a role, since uncoupling protein-3 mRNA expression was increased in this tissue following central administration of orexin-A in rats (Yoshimichi et al., 2001).

3.3. Knockout of orexins and orexin receptors

The most obvious phenotypic effect of knocking out the prepro-orexin gene is narcolepsy (Chemelli et al., 1999). In addition, such mice are hypophagic, although they grow normally (Willie et al., 2001). Surprisingly, mice overexpressing the prepro-orexin gene overeat but have a low body weight (T. Sakurai and M Yanagisawa quoted in Inui, 2000). The suggested explanation is that increased metabolic rate dominates increased food intake in these mice.

Mice have also been created in which orexin neurones are ablated by expressing a toxic transgene (ataxin-3) downstream of an upstream region of the prepro-orexin gene. These mice again have a low food intake, but it is surprising to discover that they are obese. This is apparently because they expend little energy in motor activity (Hara et al., 2001). Food consumption was decreased by 30% at 8–10

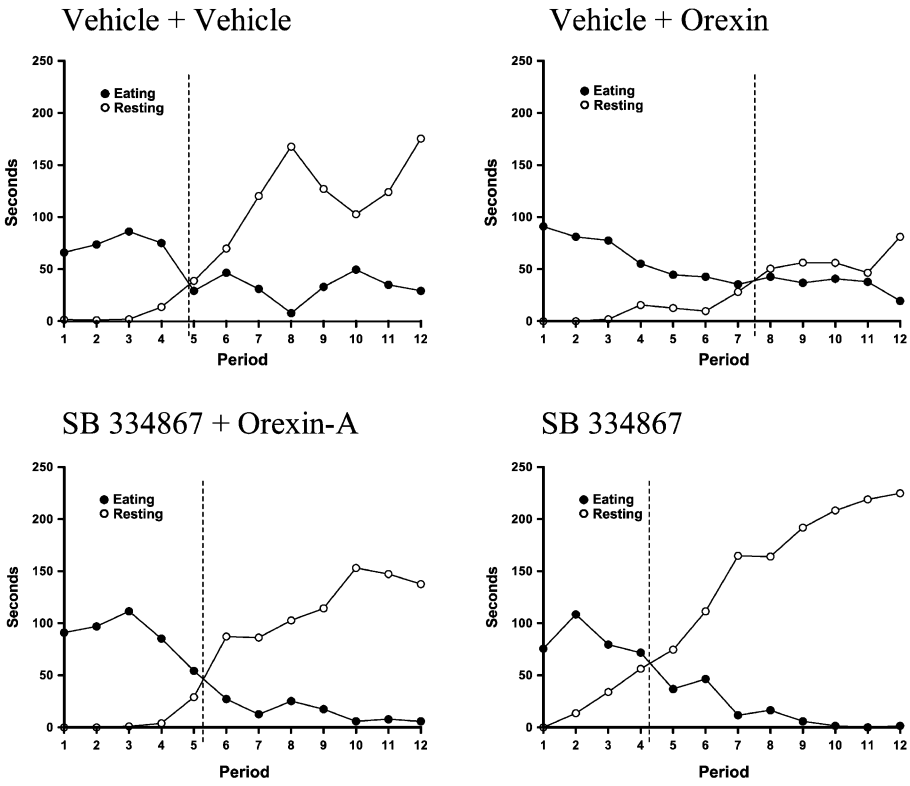

Fig. 2. Effects of orexin-A (10 μg, i.c.v.), SB-334867 (30 mg/kg, i.p.) and orexin-A (10 μg, i.c.v.) plus SB-334867 (10 mg/kg, i.p.) on the behavioural satiety sequence in male Lister hooded rats. Data are from Rodgers et al. (2001) (reproduced with permission of Blackwell Science). Rats were trained to eat a hydrated mash diet for 1 h each day during the light phase. They were videotaped during the test sessions. Mash has high palatability. Pelleted food was freely available at other times. The data show how orexin-A delays and the orexin-1 receptor antagonist advances the transition from when the rats are spending more time eating than resting to when they are spending more time resting that eating.

weeks of age before obesity became apparent, suggesting that it was not reduced to compensate for increased body fat. However, plasma leptin, which might mediate such a compensatory effect, was not measured.

Mutation of the orexin-2 receptor gene has been linked to narcolepsy (Chemelli et al., 1999; Lin et al., 1999), but at the time of writing, there have been no publications on the impact of knocking out orexin receptors on energy balance. In any event, it is advisable to be cautious in interpreting knockout studies (Sigmund, 2000): knockout of neuropeptide Y, which clearly promotes energy gain, did not reduce body weight unless it was coexpressed with the *ob* mutation, whilst knockout of neuropeptide Y Y1 or Y5 receptors tended to produce obese rather than lean animals (Inui, 2000).

3.4. Antagonism of the orexin system

It is only by antagonising the orexin system that the contribution of endogenous orexins to the normal regulation of energy balance can be fully addressed. Central injection of orexin-A antibodies reduces food intake in fasted rats (Yamada et al., 2000; Niimi et al., 2001), and there is a preliminary report that an antibody to the orexin-1 receptor also reduced feeding (Smith et al., 2000). More extensive studies have been conducted with the non-peptide orexin-1 receptor selective antagonist SB-334867, reduced orexin-A-driven feeding and feeding stimulated by an overnight fast in rats (Haynes et al., 2000b).

Just as orexin-A delayed but did not disrupt the behavioural satiety sequence, SB-334867 advanced this sequence, bringing forward the time when the rats spent more time resting than eating (Rodgers et al., 2001). By contrast, sedative agents disrupt the behavioural satiety sequence (Halford et al., 1998). SB-334867 was more potent in antagonising the delay in this transition point elicited by orexin-A than in antagonising the natural transition, as might be expected since satiety is regulated by factors additional to orexin-A (Fig. 2).

When anorectic drugs are dosed repeatedly to rodents, food intake often returns to control levels within a few days (Arch, 1981; Richard et al., 2000; Day and Bailey, 1998). This may be because as the animals become leaner, leptin levels fall, opposing the effect of the drug. The anti-obesity effects of such drugs in rodents, in fact, appear to be due to thermogenesis rather than reduced food intake. When SB-334867 (30 mg/kg, i.p.) was given once daily for 7 days and then twice daily for a further 7 days to genetically obese (*ob/ob*) mice, there was, however, no diminution in its anorectic effect and total food intake over 14 days was suppressed (Haynes et al., 2002).

Somewhat surprisingly, in view of the evidence described above that orexin-A stimulates thermogenesis, SB-334867 also appeared to stimulate thermogenesis. Thus, the brown adipose tissue became heavier and darker, and there was a trend to increased expression of uncoupling protein-1. The thermogenic effect of SB-334867 in *ob/ob* mice was confirmed by indirect calorimetry in a separate, single-dose experiment, although the effect of SB-334867 was less marked than that of a β_3-adrenoceptor agonist (Haynes et al., 2002). Since *ob/ob* mice fail to increase their metabolic rate in response to temperatures below thermoneutrality, it is possible that the thermogenic effect of SB-334867 is dependent on a low baseline level of sympathetic activity and thermogenesis. It will therefore be important to determine its effect in normal animals to establish whether there really is a paradox that both orexin-1 receptor agonists and antagonists can increase metabolic rate in the same animals. One possible explanation for such a paradox would be that orexin-A was given centrally, whereas SB-334867 was given intraperitoneally, and there are orexin-1 receptors in brown adipose tissue that may have mediated the effect of the antagonist (Haynes et al., 2002). Since SB-334867 does not appear to have any inverse agonist activity (Smart et al., 2001), such an explanation would require that orexins are normally present in brown adipose tissue in sufficient concentration to stimulate the orexin-1 receptor. Picomolar amounts of orexin-A are present in the circulation but the source of this material is not known (Arihara et al., 2001).

The combined anorectic and thermogenic effect of SB-334867 caused a reduction in body fat gain in the *ob/ob* mice. Moreover, fasting blood glucose and insulin were both suppressed, indicating that orexin-1 receptor antagonists may have potential as antidiabetic as well as anti-obesity agents.

3.5. Which orexin receptor(s) mediate the effects on energy balance?

Since orexin-A is more potent than orexin-B at the orexin-1 receptor, but not the orexin-2 receptor, it might be argued that the greater efficacy of orexin-A as a stimulant of feeding shows that the orexin-1 receptor mediates this effect. However, orexin-A is only 10-fold more potent than orexin-B at the orexin-1 receptor (Smart et al., 1999), begging the question as to why increasing the dose of orexin-B 10-fold does not elicit the same maximal effect as that of orexin-A. We have previously raised the possibility that orexin-B is more rapidly cleared than orexin-A from the area of injection (Arch, 2000), but this seems inconsistent with a recent report that some of the effects of orexin-B are greater than those of orexin-A or are not produced at all (Jones et al., 2001). At this stage, we know too little, and orexins-A and -B are not sufficiently selective to use them to dissect the roles of the two receptors, especially in vivo.

Nevertheless, the effects of the orexin-1 receptor antibody and antagonist on feeding and body weight clearly implicate the orexin-1 receptor in the regulation of energy balance. This does not preclude the role for this receptor in sleep and other aspects of behaviour and physiology. Similarly, the orexin-2 receptor is better implicated than the orexin-1 receptor in the regulation of sleep and arousal (Lin et al., 1999; Chemelli et al., 1999), but it would be premature to rule out its involvement in the regulation of energy balance until studies are conducted with a selective orexin-2 receptor antagonist.

3.6. Nutritional status and circadian rhythm

Hypothalamic levels of mRNA for other orexigenic peptides such as neuropeptide Y and agouti-related protein are increased by nutritional manipulations that promote feeding. The orexins only partly resemble other orexigenic peptides in this respect. Hypothalamic prepro-orexin mRNA levels, like those of neuropeptide Y mRNA, are increased when rats are fasted for 48 or 72 h (Sakurai et al., 1998; López et al., 2000; Cai et al., 1999). The level of orexin-1, but not orexin-2, receptor mRNA was also increased after a 72- but not a 48-h fast (López et al., 2000). No increase in the expression of prepro-orexin mRNA was detected, however, by in situ hybridisation in the lateral hypothalamus of mice that had been fasted for 60 h (Tritos et al., 2001). A possible explanation for this discrepancy comes from the observations that both hypothalamic prepro-orexin mRNA (Taheri et al., 2000) and cerebrospinal fluid levels of orexin-A in rats rise during the dark period and fall during the light period. Fasting rats for 72 h (but not 24 h) increased cerebrospinal fluid orexin-A levels measured at the end, but not at the start of the light phase when they were already high (Fujiki et al., 2001). It may be pertinent that those studies where prepro-orexin mRNA rose in response to fasting were conducted later in the light phase than the study where there was no response. Another study found no variation in the amount of prepro-orexin mRNA expression between midnight and noon (Mondal et al., 1999), but the measurements of cerebrospinal fluid orexin-A levels suggest that these are not the best times to choose to show a circadian variation.

Other studies have found no change, or at most a small change in orexin-A and -B peptide contents of the lateral hypothalamus following a 48-h fast in rats (Taheri et al., 1999; Mondal et al., 1999). Since brain levels of neuropeptides reflect the balance between synthesis and degradation (which is presumably greater when release from neurones is high), interpretation of such findings is difficult. Nevertheless, levels of neuropeptide Y do increase in various hypothalamic nuclei in response to food deprivation or restriction (Dryden et al., 1994). Levels of neuropeptide Y (and agouti-related protein) also increase during lactation, whereas levels of orexin-A and -B were unchanged in lactating rats. Strangely, the hypothalamic level of orexin-B but not orexin-A was increased by fasting the lactating animals for 48 h, despite unchanged prepro-orexin mRNA levels (Cai et al., 2001a). Orexins-A and -B are derived from

the same precursor peptide and it is probable that orexin-B cannot be formed without formation of orexin-A, so it seems that there must be selective release, degradation or clearance of the two peptides.

Hypothalamic prepro-orexin mRNA expression differs from neuropeptide Y mRNA expression by its failure to respond to chronic food restriction or streptozotocin-induced diabetes in rats (Cai et al., 1999). There was, however, a small increase in hypothalamic prepro-orexin mRNA expression in diet-restricted *ob/ob* and *db/db* mice (Yamamoto et al., 2000b).

Recently, Komaki et al. (2001) have reported that serum concentrations of orexin-A rose during a 7-day fast in obese human subjects and returned to baseline levels upon refeeding. They postulate that this orexin-A may originate from the gut rather than the central nervous system.

3.7. Glucopenia

A further difference between hypothalamic prepro-orexin mRNA and neuropeptide Y mRNA expression (Dryden et al., 1998) is that only prepro-orexin mRNA increases in response to insulin-induced hypoglycaemia (Griffond et al., 1999). This response to hypoglycaemia is found only when rats are deprived of food (Cai et al., 1999). Since the availability of food had no effect on the blood glucose level, its inhibitory effect on prepro-orexin mRNA expression was suggested to be due to a vagal signal, relayed by the nucleus of the solitary tract and parabrachial nucleus, and resulting from gastric distension or the inhibition of glucose-sensitive neurones in the hepatic portal vein (Cai et al., 1999). Further work showed that only 0.7% and 1.7% of orexin neurones immunostained for Fos in lateral hypothalamic slices of control and fed hypoglycaemic rats, respectively, but this figure increased to 13.9% in fasted hypoglycaemic rats (Cai et al., 2001b). In another study, the proportion of Fos-positive orexin neurones increased from 0% to 33% in response to insulin-induced hypoglycaemia (Moriguchi et al., 1999). As in the situation of fasted lactating rats (see above), insulin-induced hypoglycaemia increased the hypothalamic orexin-B but not the orexin-A content. It was suggested that orexin-B release is blocked by hypoglycaemia and that this contributes to the somnolence and ultimately coma induced by hypoglycaemia (Cai et al., 2001b).

Administration of 2-deoxyglucose is another way of producing glucopenia. However, despite being given at a dose that elicited the same increase in food intake as insulin, 2-deoxyglucose failed to increase the hypothalamic prepro-orexin content (Cai et al., 1999; Sergeyev et al., 2001); in one study, there was actually a decrease (Bayer et al., 2000). In an attempt to explain this paradox, one group pointed out that lesion of the zona incerta or amygdala abolishes feeding evoked by 2-deoxyglucose but not that by insulin, and suggested that modulatory inputs from these other areas may explain the divergent effects of hypoglycaemia and 2-deoxyglucose on orexin neurones (Cai et al., 1999). Another group raised the possibility that the opposing effects of insulin and 2-deoxyglucose on noradrenaline release in the hypothalamus may be relevant (Bayer et al., 2000). Neither of these explanations goes deeply enough, though, to explain the surprising finding that, despite not increasing the hypothalamic prepro-orexin mRNA concentration, injection of 2-deoxyglucose increases the number of Fos-positive orexin neurones in the lateral hypothalamus (Moriguchi et al., 1999; Briski and Sylvester, 2001). It does seem therefore that orexin neurones are activated by 2-deoxyglucose-induced glucopenia, even if this is not always reflected in the prepro-orexin mRNA level. Intriguingly, whereas insulin-induced hypoglycaemia increases prepro-orexin but not neuropeptide Y mRNA expression, 2-deoxyglucose increases neuropeptide Y, but not prepro-orexin mRNA expression (Sergeyev et al., 2001). The effect on neuropeptide Y mRNA is again only found in animals not receiving food (Giraudo et al., 1998).

The studies described above suggest that hypoglycaemia stimulates feeding, at least in part, by activating orexin neurones. Recent in vitro studies combining electrophysiology and confocal imaging of lateral hypothalamic neurones in the rat suggest that glucose-sensitive neurones (i.e. those that are activated by low glucose) and glucose-responsive neurones (activated by high glucose) are distinct from orexin neurones. However, some glucose-sensing neurones of both types appear to synapse onto orexin neurones, and vice versa (Liu et al., 2002). There is evidence that orexin-A activates glucose-sensitive neurones in the lateral hypothalamus and inhibits glucose-responsive neurones in the ventromedial hypothalamus (Shiraishi et al., 2000; Liu et al., 2002). It has long been proposed that glucose-sensitive neurones mediate hypoglycaemia-induced feeding. Thus, orexin neurones, activated by signals from peripheral or central glucose-sensitive neurones, may elicit feeding by in turn activating hypothalamic glucose-sensitive neurones (Liu et al., 2002).

Finally, it is possible that orexin and glucose-sensitive neurones also play a role in normal feeding, since this is regulated by subtle changes in glucose availability. Thus, it has been shown that small glucose falls precede spontaneous feeding in normal rats, and that administration of exogenous glucose to abolish these dips can delay feeding (Louis-Sylvestre and Le Magnen, 1980).

3.8. Orexins, leptin and obesity

We have mentioned that leptin receptors are present on some orexin neurones and on neurones that are innervated by orexin neurones. However, although there may be interactions between the orexins and leptin, the orexins do not mediate responses to leptin in the way that a number of other hypothalamic neuropeptides do. In animals with a dysfunctional leptin system (*ob/ob* and *db/db* mice, and *fa/fa* rats), mRNA levels for other orexigenic peptides (neuropeptide Y, agouti-related protein, melanin-concentrating hormone, gal-

anin) are high, consistent with their having some involvement in the increased food intake (and possibly also the decreased metabolic rate) of these animals. Conversely, mRNA levels for appetite-suppressing peptides (melanocyte stimulating hormone, neurotensin, cocaine and amphetamine-regulated transcript) are low. Fasting, which lowers endogenous leptin production, rapidly elicits changes in the same direction as those in the leptin-disrupted animals. On the other hand, administration of leptin alters these mRNA levels in the opposite direction (Kalra et al., 1999). Changes in the same direction as those elicited by administration of leptin can be achieved by giving animals a palatable or energy dense diet to raise endogenous leptin production, provided the animals have not succumbed to the phenomenon of "leptin resistance" (Bergen et al., 1999; Lin et al., 2001).

By contrast, hypothalamic prepro-orexin mRNA expression has been found to be unchanged (Tritos et al., 2001) or decreased (Yamamoto et al., 1999) in *ob/ob* and *db/db* mice. The latter result is consistent with the orexin system participating in a counter-regulatory response to obesity, rather than being its cause. This suggestion is supported by the finding that the hypothalamic prepro-orexin mRNA was suppressed in the Zucker *fa/fa* rat but not in the far less obese Zucker diabetic fatty *fa/fa* rat. Administration of the thiazolidinedione peroxisomal proliferator-activated receptor gamma agonist rosiglitazone produced marked weight gain in the Zucker diabetic fatty *fa/fa* rats and suppressed prepro-orexin mRNA expression. On the other hand, administration of rosiglitazone to normal rats produced little or no weight gain and no change in prepro-orexin mRNA expression (Cai et al., 2000). Unfortunately, this hypothesis is not supported by a report that prepro-orexin mRNA was unchanged in the hypothalamus of UCP-DTA (brown adipose tissue deficient) mice, despite their being almost twice the weight of control mice. The contrast with decreased expression of other orexigenic peptide mRNAs in UCP-DTA mice (here playing a counter-regulatory role because the leptin system is intact) nevertheless emphasises that the orexin system is not linked to leptin in the conventional fashion (Tritos et al., 2001).

As we described in Section 3.6, fasting increases the hypothalamic prepro-orexin mRNA level. It seems unlikely that this effect is mediated by a fall in plasma leptin concentration, however. The plasma leptin concentration falls rapidly in fasted rodents, whereas the rise in prepro-orexin mRNA has been reported only following a fast of at least 48 h, and not in all studies. Moreover, orexin-A levels rise throughout the dark period and fall during the light period—the opposite of what would be expected if orexin-A mediates reduced feeding in response leptin. By contrast, neuropeptide Y release peaks 20 min after lights out and then falls (Stricker-Krongrad et al., 1997).

Two studies in which leptin has been administered peripherally to rats gave results consistent with the orexin system playing a role in the reduced feeding. In one study, leptin inhibited the fast-induced rise in prepro-orexin mRNA expression (López et al., 2000); in the other, administration of leptin for a week reduced the orexin-A content of the lateral hypothalamus, although the majority of this effect was seen in animals that were pair-fed to the leptin treated animals (Beck and Richy, 1999). By contrast, leptin has been reported to increase prepro-orexin mRNA expression in the lateral hypothalamus of mice that had been fasted for 60 h. Neuropeptide Y mRNA expression was decreased in the same mice (Tritos et al., 2001). This is the study referred to previously in which fasting alone had no effect on prepro-orexin mRNA expression (Section 3.6).

There is just one (preliminary) report on prepro-orexin mRNA expression in dietary obesity (Leibowitz and Wortley, 2000). Expression was increased by feeding rats a high-fat diet, and was highest both in rats that gained most weight during the first 5 days on the diet and in those animals that became most obese over 3 weeks. These results are consistent with the hypothesis that activity of the orexin system contributes to any propensity to obesity in rats on a high-fat diet. More information is needed, however, to ascertain

Table 1
A summary of evidence that orexins are involved in the regulation of energy balance

Anatomy
- Cell bodies in the lateral hypothalamus express prepro-orexin mRNA
- Orexin neurones have reciprocal connections with neurones that express known feeding peptides in the arcuate nucleus
- Orexin receptors have been identified in the hypothalamus by mRNA expression, immunohistochemistry and neuronal responses to orexins
- Glucose sensing neurones synapse with orexin neurones

Effect of intervening in the orexin system on energy balance
- I.c.v. orexin-A potently stimulates feeding without disrupting the behavioural satiety sequence
- Antibodies to orexin-A and the orexin-1 receptor reduce feeding
- SB-334867 (orexin-1 receptor antagonist) inhibits feeding without disrupting the behavioural satiety sequence
- SB-334867 reduces food intake, stimulates metabolic rate and reduces body fat in *ob/ob* mice
- Knockout of the orexin gene, or ablation of orexin neurones by expression of a toxic gene creates mice that undereat; overexpression of the orexin gene creates mice that overeat

Effect of interventions that affect feeding on the orexin system
- Severe fasting increases hypothalamic prepro-orexin mRNA and cerebrospinal fluid orexin-A
- Insulin-induced hypoglycaemia increases prepro-orexin mRNA expression and Fos expression in orexin neurones, provided food is present in the stomach; 2-deoxyglucose also increased Fos expression in orexin neurones
- Leptin affects prepro-orexin mRNA expression (but in different directions in different situations)

Obesity and the orexin system
- Prepro-orexin mRNA expression is decreased in grossly obese animals with a defective leptin system
- Prepro-orexin mRNA expression is increased by a high-fat diet, especially in those rats that become most obese

whether the variations in prepro-orexin mRNA expression were related to reciprocal variations in leptin signalling.

4. Overview

Although the orexins clearly have other roles, notably in promoting arousal, the evidence that they (at least orexin-A and the orexin-1 receptor) play a role in the regulation of feeding seems overwhelming (Table 1). The suggestion that orexin-A stimulates feeding simply by promoting arousal has not been supported by any data. Indeed, dissociations between promotion of arousal and feeding for various agents, coupled with the differing effects of orexin-A and CNS stimulants on the behavioural satiety sequence, make this hypothesis unsustainable (Section 3.2).

Where though does the orexin system fit into the range of mechanisms that regulate feeding? In recent years, we have come to expect the release of hypothalamic peptides to be modulated by leptin and therefore be responsive to body fat stores. Feeding is regulated from meal to meal, however, by hedonic, gut and blood metabolite signals against a background of learning. It is the sensitivity of the brain to these acute stimuli that is affected by the size of the adipose mass (Woods et al., 2000; Halford and Blundell, 2000) (Fig. 3). For example, leptin and cholecystokinin have been shown to act synergistically in regulating food intake (Wang et al., 2000).

The most reasonable hypothesis in the light of current evidence is that the orexin system plays a role in mediating the response to acute energy need, in particular, low blood glucose coupled with an empty stomach. The response to orexins may be modulated by leptin—studies on the interaction between leptin and orexins have not yet been described—but orexins do not appear to directly mediate responses to leptin. On reflection, this is not surprising. It seems more likely that a system that promotes arousal should be concerned with acute energy needs, rather than with long-term energy balance.

Nevertheless, interference with the orexin system using a orexin-1 receptor antagonist does affect long-term energy balance. This is consistent with the hypothesis that the pre-meal dip in blood glucose or emptying of the stomach initiates feeding via the orexin system: it seems unlikely that blocking a system employed solely for the emergency of acute hypoglycaemia could have much influence on long-term energy balance. Moreover, this finding suggests that it is not necessary to intervene directly in the overall control of body fat content by leptin to produce a useful anti-obesity drug. Similarly, fenfluramine was an effective anti-obesity drug that acted via serotonin, although serotonin does not appear to have any direct link with leptin (Halford and Blundell, 2000). That anti-obesity drugs need not intervene directly in the action of leptin is not surprising when we consider that the increasing incidence of obesity in affluent societies is not due to some recent change in the way the leptin system operates, but to a changing environment affecting acute cues to eat (and exercise). Above all, the fact that antagonism of the orexin-1 receptor reduces food intake and body weight suggests that such agents have potential in the treatment of obesity. We have yet to see whether this potential might be compromised by other effects of orexin-1 receptor antagonism.

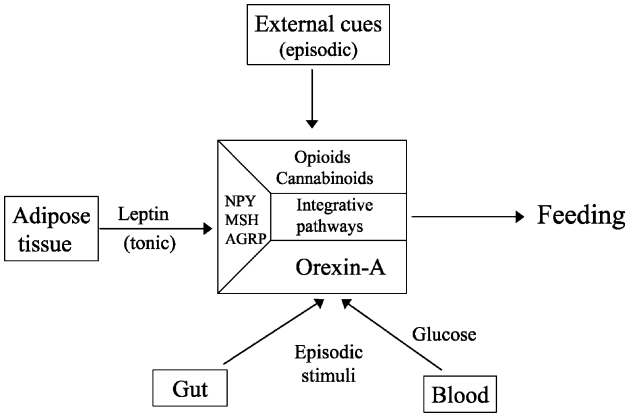

Fig. 3. The place of orexins in the regulation of feeding. Feeding is regulated by episodic external and internal stimuli, modulated by tonic stimuli that reflect the size of fat stores (Halford and Blundell, 2000). The orexins (at least orexin-A) appear to play a role in transmitting episodic stimuli—blood glucose and gastric distension—that arise internally. The segregation of hypothalamic neurotransmitters between roles is not, however, as strict as suggested by the diagram. For example, endogenous opioids may also play a role in leptin signalling (Glass et al., 1999). NPY, neuropeptide Y; MSH, α-melanocyte stimulating hormone; AGRP, agouti-related protein.

Acknowledgements

We thank Jeff Jerman for his comments on the manuscript.

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