Review

Recent progress in orexin/hypocretin physiology and pharmacology

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Abstract
Orexin peptides and their cognate receptors were discovered 14 years ago. They soon took a very central position in the regulation of sleep and wakefulness. Active studies have further elucidated these functions as well as the role of orexins in, for instance, appetite, metabolism, analgesia, addiction, and stress response. This review summarizes all the important fields but especially aims at focusing on novel findings and future directions.

Keywords: G-protein-coupled receptor; hypocretin; neuropeptide; orexin; orexin receptor.

Introduction
It is now 14 years since the first publications describing orexins/hypocretins and orexin receptors (1, 2), and 13 years since the first indications of the dramatic role orexins play in the regulation of the wakefulness and sleep pattern, discovered in connection with narcolepsy (3, 4). The first time I started writing a review about orexins in 2001 (5), somewhat <300 published papers were available on orexins. Currently, the number is almost 10-fold, and there has been significant progress in many fields. Thus, summarizing all the aspects would now be a much more demanding task and require a vast space, even though only recent progress was mainly to be covered – certainly progress cannot be described without linking it to the past. In this review, I thus aim at presenting a complete picture of orexin functions but many aspects still have to be dealt with very briefly because of spatial limitations. Special focus will be on the developments in the field and possible pitfalls. For a more thorough presentation of specific functions, readers are in each part directed to specialist reviews.

Orexin/hypocretin peptides and receptors

Peptides
Native functional orexin peptides are orexin-A and orexin-B (also known as hypocretin-1 and hypocretin-2) (Figure 1A), produced from the precursor preproorexin (PPO, also known as preprohypocretin). PPO contains one of each peptides, both originating from the second exon of the PPO gene. Orexin peptides show high sequence conservation over the mammalian species, while the sequence divergence becomes higher in the lower vertebrates (Figure 1B). Only orexin-A is readily measured in the cerebrospinal fluid (CSF), probably due to its higher stability (see ‘Human narcolepsy’).

The dual nomenclature (orexin vs. hypocretin) has its origin in the concurrent discovery of the peptides by two research groups (1, 2). While hypocretin refers to the hypothalamus and the putative relation to secretin of the incretin peptide family (1), orexin comes from the increased appetite (oreksis [Greek] → orexis[Latin]) upon intracerebroventricular (i.c.v.) injection of orexins (2). No consensus has been reached in the matter and thus both names are used in parallel. A compromise presented by the International Union of Pharmacology is to name the peptides and receptor proteins on the basis of the orexin nomenclature, and the genes on the basis of the hypocretin nomenclature (http://www.iuphar-db.org/); however, this does not seem to fully apply in practice.

Receptors
Orexin receptors belong to the G-protein-coupled receptors (GPCRs), where they make their own subgroup, and the closest homology to other GPCRs is ~30% while the homology between the OX₁ and OX₂ subtypes is >60% (5); the homology is higher, as expected, in the transmembrane (TM) helix domains (5). There are no known pharmacological differences between the orthologues.

Orexin receptor structure is not known as neither of the receptor isoforms has been crystallized. This is not surprising as GPCR crystallization is altogether a demanding task requiring radical receptor modification and tremendous amount of trial and error, and most GPCR models are constructed by homology modeling on the basis of the existing crystal structures. Availability of a larger number of crystal structures (6) increases the reliability of homology modeling, but not fully realistic models can be made yet. Concerning orexin
determining the high affinity for SB-674042 and SB-334867, respectively. However, in contrast to SB-334867, SB-674042 binding is also affected by the extreme N-terminus including TM1. Also, the agonist selectivity between orexin-A, orexin-B, and Ala$_{11}$, d-Leu$_{15}$-orexin-B seems to be mostly determined by the region around TM3 and TM4 (8, 10).

No other physiological ligands except orexin-A and -B are known to bind to orexin receptors nor are orexins known to act on other targets (2, 11).

Agonist pharmacology

The original publication on orexin receptors showed that orexin-A and -B were equal ligands for the OX$_2$ receptor, which concerns both binding and activation of Ca$^{2+}$ elevation, whereas OX$_1$ receptor showed 10-fold preference for orexin-A (2). The data were obtained in recombinant Chinese hamster ovary (CHO) cells, and the same has since been shown in other recombinant systems with respect to Ca$^{2+}$ elevation or phospholipase C (PLC) activation. This concept has been eagerly used to distinguish between OX$_1$ and OX$_2$ receptors in native systems [see, e.g., ref. (12)]. However, even in recombinant systems, deviations from this ligand profile can be seen. For OX$_1$ receptors, different responses show different ligand profile and for some responses, orexin-A and -B are essentially indistinguishable at OX$_1$ [see discussion in ref. (12)]. Calcium elevation through each receptor also shows different agonist profiles in CHO and human embryonic kidney (HEK)-293 cells (12). An obvious suggestion for the explanation would be biased agonism (also known as ‘signal trafficking’ or ‘agonist trafficking of receptor responses’) (13, 14). Thus, when we see a difference in Ca$^{2+}$ response between two cell types (12), the explanation may be found in different expression of G-protein subspecies or different components of the response (Ca$^{2+}$ influx vs. release).

Orexin peptide mutagenesis has been made in a few studies, probably to discover pharmacophore motifs for OX$_1$ and OX$_2$ receptors. The studies, in short, show that the N-terminus
can be truncated with successive loss of activity, whereas the C-terminus does not allow any truncation or addition whatsoever (see discussion in refs. 9, 10, and 15; also Kukkonen et al., unpublished finding). Alanine and other scans have allowed identification of more or less important amino acids; for instance, Ala^{11}, d-Leu^{15}-orexin-B was suggested to show high selectivity for OX_{1}, although we have recently shown this not to be the case (12). Altogether, the peptide mutagenesis data strongly suggest that no very suitable pharmacophore for small molecular ligand development can be extracted from the orexin peptides.

Agonist profiles, using most often orexin-A or orexin-B or sometimes even Ala^{11}, d-Leu^{15}-orexin-B, have been sometimes used as an indication of involvement of a particular orexin receptor subtype, or a ‘selective agonist’ used to ‘selectively’ activate one of the receptor subtypes. In most cases, apparently reasonable-looking results have been obtained, whereas in others the results have been most unexpected. For instance, in human adrenal cortical cells, which express OX_{1} mRNA in great dominance over OX_{2} mRNA, orexin-A stimulates cortisol secretion with an EC_{50} around 0.2 nM, while orexin-B lacks any activity (16). On the other side, orexin-B has sometimes been found to be the only active orexin or at least significantly more potent or efficacious than orexin-A [see ref. (5)]. Needless to say, on the basis of the variable pharmacological results obtained in the heterologous expression systems (12) and the entire concept of biased agonism, this type of pharmacology cannot be used.

No small molecular agonists are known (or at least published) for orexin receptors. The lack of agonistic ligands with desirable properties imposes problems on the investigations of the physiological orexin functions and hinders exploration of orexinergic therapy in some disease states like narcolepsy and cancer (see ‘Human narcolepsy’ and ‘Narcolepsy’).

Antagonist pharmacology Orexin receptor antagonists have been developed by several pharmaceutical companies, including Actelion, Banyu, Biovitrum, GlaxoSmithKline, Hoffmann-La Roche, Jansen, Johnson & Johnson, Merck, and Sanoft-Aventis, with the first obvious aim in appetite suppression but later rather sleep induction in insomnia. The current ligands have recently been thoroughly reviewed (17) and I will thus only present a brief overview. The first OX_{1} receptor-selective antagonist to arrive was SB-334867 from GlaxoSmithKline, soon followed by other OX_{1}-selective antagonists SB-408124, SB-410220, and SB-674042; of the last one, even a [^3H]-labeled radioligand was presented (18). The first OX_{2}-selective antagonist, with at least 250-fold selectivity, was the tetrahydroisoquinoline amide, commercially known as ‘TCS-OX2-29’, from Banyu Pharmaceutical Co. (19).

It is unclear to the author of this review whether there was an original intent to develop subtype-selective ligands, which has later shifted toward non-selective ones (dual orexin receptor antagonists – DORAs), or whether the discoveries have been more coincidental. The rationale of associating mainly OX_{1} receptors with wakefulness and sleep pattern regulation comes from the genetically narcoleptic canines; however, OX_{1} and OX_{2} knockout mice could not fully confirm this, and knockout of both receptors was required for a strong narcoleptic phenotype (20). The situation in man is not known. SB-334867 (or other GSK OX_{1} antagonists) has been used in a number of studies with only minor effects on wakefulness or sleep architecture [see, e.g., ref. (21)]. In contrast, the dual antagonist almorexant has been shown to be an effective sleep inducer (22). However, another study suggests that selective block of OX_{1} receptors with JNJ-10397049 would be more effective and that OX_{1} block would actually counteract the hypnotic effect of OX_{2} block (21). Unfortunately, some promising dual antagonists have failed in phase III tests or earlier, including almorexant; the reasons for the failures have not always been published. Fortunately, there are numerous patented compounds, some of which have already reached phase II or III (SB-649868, MK-4305) (17).

Most of the antagonists are not publically available; however, at least the OX_{1}-selective SB-334867 and SB-408124, OX_{2}-selective TCS-OX2-29, and the non-selective TCS 1102 can be purchased (Tocris and Sigma-Aldrich).

Fluorescent and radiolabeled ligands The only generally available non-selective radioligand for orexin receptors is [^125I]-orexin-A [see, e.g., ref. (11), [^125I]-orexin-A is, however, not well suited for binding experiments, as it shows high glass fiber filter binding, which is difficult to block. Thus, most experiments have been performed on intact attached cells, where the non-bound ligand is simply removed by washing. However, the agonistic effect of orexin-A may make the results difficult to interpret. Additional disadvantages include the heavy radiation of [^125I]. Agonist binding (to the so-called high-affinity site) requires receptor-G-protein interaction and is also dependent on the type of G-protein (13). [^125I]-orexin-B (human, mouse/rat) is also available, at least from Bachem and Phoenix Pharmaceuticals.

[^3H]-SB-674042 was reported as a high-affinity OX_{1}-selective radioligand (18). This compound behaves nicely in intact CHO cells, but it shows some problems in cell homogenates/membrane preparations, where it seems to mainly bind to an orexin-A-insensitive site (own observation, communication with others). In addition, this ligand is not commercially available. Researchers at Roche have also published results using custom-synthesized [^3H]-almorexant, [^3H]-EMPA, and [^3H]-SB-674042 [e.g., ref. (7)].

Fluorescently labeled orexin-A is commercially available and has also been used in some studies. However, neither affinity nor agonistic potency/efficacy of the labeled peptide has been reported.

Receptor mutants and polymorphisms The most well-known orexin receptor mutants are those present in narcoleptic canines (canarc mutants), which prematurely truncate the OX_{1} receptor or otherwise interfere with orexin binding (Glu54Lys) [reviewed in ref. (5)]. There also is significant receptor sequence diversity reported in man, which may rather represent polymorphisms than mutations (although the frequencies of different variants in populations are not known) [reviewed in ref. (5)]. Correlation with some
disorders has been found for some of the genotypes (23–26); however, there is thus far no evidence for causality.

**Progress** The major advances in the research of orexin peptides and receptors as such seem to be in the development of receptor antagonists, and I feel we are facing very interesting years with regard to physiological and clinical studies with these ligands. Hopefully, some end up being licensed as drugs as well. Unfortunately, there does not seem to be corresponding development of orexin receptor agonists. There has not been any major breakthrough in orexin peptide mutagenesis, but the few receptor mutagenesis studies are interesting and may turn the focus toward computer-aided antagonist and agonist development on orexin receptors.

**Tools for orexin research**

**Transgenic animal models**

Conventional (constitutive) knockout techniques have been used to ablate both the orexin peptides [PPO-knockout (PPO-ko)] (3, 27, 28) and each orexin receptor [OX₁ and OX₂ knockouts and the double-knockout (OX₁-ko, OX₂-ko, and DKO, respectively)] (20, 29, 30). Hereditarily narcoleptic canines represent a native OX₂-ablation phenotype.

The proximal (~3.2 kb) PPO promoter is enough to direct expression to orexinergic neurons only (31). This has been used in a number of transgenes or viral constructs to express proteins selectively in orexinergic neurons. These proteins include, for instance, green fluorescent protein (GFP) [to allow identification of the orexinergic neurons (32)], a GFP fusion construct with a C-terminal tetanus toxin fragment (TTCT-GFP) [for mapping of the upstream neuronal pathways to the orexinergic neurons (33)], as well as channelrhodopsin-2 and halorhodopsin [see below; refs. (34) and (35)]. Ataxin-3 is a polyglutamine repeat protein, which, when mutated toward a higher Gln repeat number, causes spinocerebellar ataxia type 3 by inducing cell death in neurons expressing this protein. Direction of expression of ataxin-3 to a specific neuron population can be used to ablate these neurons. This is used in the PPO-pro-ataxin-3 mouse and rat, where a toxic fragment of ataxin-3 is expressed under the PPO promoter (36, 37). This induces a selective postnatal loss of orexinergic neurons – the proximal PPO promoter activity apparently only commences postnatally (see ‘Metabolic rate and thermogenesis’) (27, 36, 37). Also, a mouse with ectopic PPO overexpression has been produced (38).

Optogenetic control implies heterologous expression of photoactivatable proteins [reviewed in ref. (39)]. Often, light-activated ion channels, GPCRs, or pumps, like channelrhodopsin-1 and -2 or halorhodopsin, have been used. Both channelrhodopsin-2 and halorhodopsin have been expressed in orexinergic neurons, allowing their activation or inhibition, respectively, on demand through an implanted optical fiber (34, 35).

A number of other mutants, not directly affecting orexin- or orexin receptor-expressing neurons, native or transgenic, have been used in orexin receptor studies [see, e.g., refs. (29) and (40–42)]. Short-term genetic manipulation with transgene expression (35, 43) or siRNA (44) has been applied even in intact animals, often by viral delivery.

The phenotypes and other findings made with the transgenic and other animal models are described in detail under the relevant section, especially under ‘Central nervous system’.

**Receptor ligands**

There are plenty of small molecular antagonists synthesized against orexin receptors but only a small minority of these are commercially available. The only known orexin receptor agonists are based on the native orexin peptides; both the native orexin-A and orexin-B and some synthetic variants have been reported. The only commercially available radioligands are [125I]-labeled orexin-A and -B. For details on orexin receptor ligands, see ‘Agonist pharmacology’ and ‘Antagonist pharmacology’.

**Antibodies**

There are plenty of commercially available antibodies against all five obvious orexin targets (PPO, orexin-A and -B, OX₁ and OX₂). My own experience is only from antibodies against the receptors and that is several years back. In an immunohistochemistry (IHC) test of a number of antibodies against human OX₁ and OX₂ receptors, not one of these recognized the receptors overexpressed in CHO cells. Similar reports have been received from other critical colleagues. On the other hand, we have reason to believe that some anti-orexin receptor antibodies may recognize receptors where there are none. This is not surprising, as anti-GPCR antibodies often end up bad with both false-positive and false-negative results. To my knowledge, the reason for this is not known. Antibodies against orexin peptides constitute a similar question. Although I do not have any personal experience of this, it seems that radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) determinations of the plasma (or peripheral tissue) orexins are easily prone to high cross-reactivity with other antigens [ref. (45); also symposium presentations and personal communications with other researchers]. Determinations in the CSF have been suggested to be somewhat more accurate (45); if so, this is probably because some cross-reactants are lacking in the CSF. Even in the CSF, absolute values are not consistent between different antibodies (45). It is possible that a proper purification process like high-performance liquid chromatography (HPLC) together with RIA could give reliable results, but such procedure has not been clearly established and verified.

It would also be possible that some of the antibodies used in RIAs would really be specific; however, as these do not seem to have been verified, this is uncertain. Concerning the PPO antibodies, I have no information either for or against.

On the basis of these experiences and other pieces of information, I would not suggest using any anti-orexin or anti-orexin receptor antibody as a primary source of information unless the expression could be verified by mRNA and also,
rather, functional data, or the antibodies could be somehow validated. An excellent test for mice would be to verify the data in parallel in samples of PPO-, OX1-, or OX2-ko mice, or, with any species, knock down the targets using siRNA to eliminate false-positives. Heterologous expression could be used to eliminate false-negatives, although this may be a less certain test. The problems described above do not mean that the antibodies would not work — and, for instance, the receptor antibody data often seem to nicely fit together with other expression data in the central nervous system (CNS) or functional data — but that this cannot be taken for granted, and that studies should, in my opinion, not be published without careful controls as suggested above, and without own sound judgment. It should be noted that for the antibody determination, preabsorption with excess exogenous antigen, etc., cannot be used as a proof for antibody specificity.

Progress

There has been a consequent development of animal models and virus vectors, cross-breeding with other animal models, and wider and more critical analyses of the results. It seems that the conditional transgenic techniques combined with virus vectors are the way to go also in orexin research. Hopefully, the antagonists developed will become available as a complementary approach to genetic manipulations. It is also important to realize that the knockout models are thus far restricted to mice, and it seems that orexin physiology is not completely similar in all species. The antibody issue is very serious and it does not seem as if there had been any progress at all; we have simply not been critical enough.

Physiological functions

Most of the physiological functions are rather superficially reviewed as there are expert reviews on more or less distinct aspects of orexins [see, e.g., refs. (46–49) and also under the specific subjects below]. In general, it can be concluded that the CNS orexin functions and pathways have been actively mapped, whereas the possible peripheral orexin functions are thus far much more unclear.

Central nervous system

Orexinergic neurons Orexinergic neurons reside, in all the investigated mammalian species (actually, for man there is rather little information), in the lateral hypothalamic area or its vicinity (like the perifornical area) in a mixed population with, e.g., melanin-concentrating hormone-expressing neurons. Essentially, the entire orexinergic neuron population also expresses prodynorphin, secretogranin II, and neuronal activity-regulated pentraxin, and as also more recently suggested, nociceptin/orphanin FQ [see, e.g., refs. (50–52) and the review in ref. (5)]. It should be mentioned that these peptides/proteins are also expressed in other brain areas independent of orexin. Subpopulations of the orexinergic neurons have been suggested to express galanin and glutamate (5, 53).

Orexinergic neurons are suggested to be directly regulated by glutamate, γ-aminobutyric acid (GABA), acetylcholine, glycine, different monoamines, purine nucleotides and nucleosides, and many peptide transmitters, on the basis of conclusions made using IHC, GFP labeling of orexinergic neurons, upstream and downstream tracing from orexinergic neurons, and functional responses in orexinergic neurons. Owing to limited space, I shall here not review these aspects in detail; the readers are suggested to consult the original publications of especially tracing studies (33, 54) and reviews of these (5, 48, 55). Some of the regulation may originate from long-range projections and some from local hypothalamic connections or interneurons. While many of the regulators have been properly proven, we should nevertheless adopt a critical view on some other ones, until verified. Some of the mediators have been suggested by IHC or functional responses only. In the former case, the actual regulators may be other than those detected by IHC and the antibodies may not always be fully specific. In the latter case, the fact that receptors for a particular transmitter are present on orexinergic neurons does not necessarily mean this regulation occurs in the physiological context. Also, to pinpoint the responses to orexinergic neurons and not to on a putative presynaptic site, the synaptic activity has to be properly blocked (and rather also the synaptic potentials investigated); this has not been done in all studies. Finally, pharmacology used to determine receptor types may not always necessarily be valid.

Putative circulating 'mediators' or otherwise diffusible ones directly active on orexinergic neurons would include leptin (below), glucose (56), and dietary amino acids (57). A (sub)population of the orexinergic neurons was originally suggested to express leptin receptor Ob-Rb + the associated transcription factor STAT3, although Ob-Rb expression and leptin response has been placed on neurons upstream of orexinergic neurons instead in recent studies by the Myers group [see, e.g., ref. (42)].

Downstream projections of orexinergic neurons were originally mapped using PPO-IHC and also by in situ hybridization of the orexin receptor mRNA, a more indirect but possibly more selective method (reviewed in refs. 5 and 46). Owing to putatively serious shortcomings (see ‘Antibodies’), I would not use studies using orexin receptor or orexin-A or orexin-B IHC as a primary source of information. Despite their small number, orexinergic neurons project to many but distinct CNS areas of the brain stem and forebrain (reviewed in refs. 5 and 46). In some cases, the cells affected are not those having their cell bodies in the target tissues, but orexinergic neurites may also synapse on axon terminals.

More recent studies have also used anterograde and retrograde tracing to and from orexin neurons, revealing upstream and downstream connections. Anterograde tracing has been obtained with biotinylated dextran (54) or transgenic expression of TTC-GFP (33), while, for instance, diamine yellow (58), cholela toxin subunit b (54, 59, 60), or latex microspheres (59, 60) have been used for retrograde tracing. It should be noted that each of these techniques has its own advantages and disadvantages. Thus, although the techniques are very powerful, because of some limitations and possible artifacts,
the results are best analyzed in parallel to peptide IHC and in situ hybridization data. Altogether, the results stand well in that comparison (see discussion in refs. 33 and 54).

The anterograde and retrograde tracing studies, together with IHC and in situ hybridization studies, have also identified interesting reciprocal connections for orexinergic neurons. Such reciprocal ‘long-range’ connections may be found between orexinergic neurons and, for instance, dorsal raphé nucleus (33, 54, 61, 62). Shorter reciprocal connections are also abundant within the hypothalamus between orexinergic neurons and, e.g., arcuate and paraventricular nuclei (33, 53, 54, 61, 62). Orexinergic neurons project to other orexinergic neurons, suggestive of axon collateral-mediated positive or negative autoregulation (53, 63, 64) either directly or through presynaptic effects in the lateral hypothalamus (32, 63, 64).

Orexin neuron activity (as measured by c-fos expression) and PPO mRNA production have been suggested to increase in fasting or glucopenia [reviewed in ref. (5)]. Neurons of different CNS nuclei, as well as some peripheral cells, most notably pancreatic α- and β-cells, are regulated by glucose in a positive or a negative manner. Hypothalamic orexinergic neurons are directly inhibited by glucose [reviewed in ref. (56)]. Exposure to glucose (or some other monosaccharides, including 2-deoxyglucose) activates a $K^+$ conductance hyperpolarizing the orexinergic neurons, which is thought to mediate the inhibition observed. The conductance has not been molecularly identified to date [reviewed in ref. (56)]. In contrast, dietary amino acids depolarize orexinergic neurons in a manner dependent partially on tolbutamided-sensitive $K_{xtp}$ channels and partially on Na$^+$-coupled amino acid uptake (57). Dietary amino acids also induce increased locomotion possibly related to activation of orexinergic neurons. Could part of the higher vigilance supposed to relate to high-protein/low-carbohydrate diet as compared with low-protein/high-carbohydrate diet be caused by direct glucose and amino acid effects on orexinergic neurons?

### Regulation of wakefulness and sleep

Expert reviews on the role of orexins in the regulation of arousal and sleep pattern have been recently published (see below). In short, orexin receptor stimulation by i.c.v. orexin-A increases wakefulness and reduces REM and deep sleep stages. Similar is seen with direct optogenetic stimulation of orexinergic neurons [PPO-pro-channelrhodopsin-2 lentivirus-transduced mice (35)]. The role of the orexinergic system in the regulation of sleep and wakefulness is much more profound yet, as indicated by transgenic animal studies. PPO-ko mice show narcoleptic phenotype with only a mild cataplexy; only DKO mice show the strong narcoleptic phenotype of the PPO-ko (20, 30). Knockout of OX1- and OX2-mice do not show any behavioral abnormalities and only wakefulness cycle fragmentation, while OX2-ko show a narcoleptic phenotype with only a mild cataplexy; only DKO mice show the strong narcoleptic phenotype of the PPO-ko (20, 30). Hereditary, narcoleptic canines represent a native OX2-ablation phenotype. These animals, as discussed below, show a strong narcoleptic phenotype, and thus the role of the orexin receptor subtypes may be somewhat different in different species. Direct evidence is not available for man, but DORAs are mainly developed as hypnotics (see ‘Antagonist pharmacology’). On the other hand, selective OX2 block has been suggested to be more beneficial for sleep induction than dual block (21). The selective postnatal loss of orexinergic neurons in PPO-pro-ataxin-3 mice and rats causes a strong narcoleptic phenotype (36, 37), which can be ameliorated by ectopic PPO overexpression (38, 43). Restoration of OX2 receptor expression in the posterior hypothalamus (including tuberomamillary nucleus) reverses the sleepiness in conditionally reversible OX2-ko mice (65).

Optogenetic inhibition of orexinergic neurons in PPO-pro-halorhodopsin mice is only effective in inducing slow-wave-sleep during the inactive period but not during the active phase (34). This is interesting as functional inactivation of orexinergic neurons would be expected to lead to similar phenotype as in PPO-pro-ataxin-3 mice. However, it is possible that halorhodopsin activation in orexinergic neurons only inhibits the neurons in some degree, and does not lead to complete abolishment of their electrical activity. In contrast, optogenetic stimulation of the lateral hypothalamus of mice transduced with lentivirus-harboring channelrhodopsin-2 under PPO promoter induces awaking (35).

For presentation and discussions on the exact neuronal circuitry in REM and non-REM sleep and wakefulness, please see, e.g., refs. (46) and (66–68).

### Human narcolepsy

The major investigated form of canine narcolepsy is the hereditary variant, caused by inactivating mutations in the OX2 receptor gene (see above). In contrast, there is only one known human, genetically determined case of narcolepsy, and the rest of the occurrences of this rare disease are sporadic, and thus thought not to be determined by mutations in orexin or orexin receptor genes. Idiopathic narcolepsy, however, has for long been known to display a pronounced genetic component, as this disease is strongly associated with some major histocompatibility complex/human leukocyte antigen (MHC/HLA) type II haplotypes. Strongest association is seen with HLA DQB1*0602 [reviewed in refs. (69) and (70)]. This association would indicate autoimmune reaction; however, no clear evidence for such has been demonstrated thus far, despite extensive searches (71).

Human narcoleptics with the classic cataplectic phenotype show radically reduced orexin-A levels in the CSF samples, where orexin-A can be measured with some certainty (45) (please also see ‘Antibodies’). This indicates that orexinergic neurons are reduced in number or signaling. The autoimmune hypothesis would point at the orexinergic neuron death with support from postmortem studies with human narcoleptics (72–75). Reversely, narcoleptic phenotype is obtained in many transgenic rodent models upon elimination of orexins or orexinergic neurons (see ‘Transgenic animal models’ and ‘Regulation of wakefulness and sleep’).

Assuming autoimmune death of orexinergic neurons in human narcolepsy, autoantibodies have been looked for in a number of projects with some published and non-published...
results. Any conclusive evidence has been hard to find (71), but one of the hits has been antibodies against Tribbles homolog 2. It should be kept in mind that MHC type II molecules interact with T-helper cells and thus cell death could be triggered not only through B-cell-mediated antibody production but also by antibody production-independent activation of microglia; also, T-cell-independent MHC type II molecule signaling has been suggested (71). Orexinergic neurons are not known to express any other unique antigens except PPO and the mature orexin peptides, which are intracellular antigens, and thus unlikely to trigger cell death. However, orexinergic neurons might be particularly sensitive to some assault or more accessible to antibodies or cytotoxic cells. It is also feasible that orexinergic neurons are regulated by some other neurons, death of which might leave orexinergic neurons without maintenance support, and thus the actual target neurons of the immunological assault could be found somewhere else. Bystander killing of these neurons would be another option (71). Furthermore, immunologically triggered elimination of the orexinergic neurons is not the only possibility; the apparent genetic association with HLA DQB1*0602 could instead involve another co-segregating gene of the same region.

In summary, while significant co-segregation of some molecular markers with narcolepsy can be seen in man, the conclusions are far from straightforward. One of the reasons for the difficulties is the slow disease process and lack of postmortem samples from patients with active disease process. Recently, epidemic-like occurrence of juvenile narcolepsy was observed in many Western countries. This took place in the wake of the H1N1 influenza (pig flu) or vaccinations against H1N1, and thus the narcoleptic neuron death could have been generated by, e.g., bystander immunogenicity. Especially strong epidemic was observed in Finland and Sweden. Correlation with inoculation with a vaccine, which contains a strong adjuvant, rather than the H1N1 infection, has been suggested by the Finnish and Swedish governmental agencies investigating the epidemic. However, the number of cases is still rather low, and as the knowledge of the disease mechanism is nil, any conclusions are difficult to draw. It should also be pointed out that at least the Finnish victims of the epidemic, although showing the classic genotypes and phenotypes – HLA DQB1*0602-positivity and low orexin levels in the CSF – seem to display additional symptoms besides the classic narcoleptic symptoms. It is thus possible that the diseases may be different from the classic narcolepsy, e.g., by involving a larger region of the brain or non-complete elimination of the orexinergic neurons.

Feeding The name orexin was coined after the original discovery of increased feeding in rats after cerebroventricular injection of orexins during the rest period (2). Later, injection of orexins directly to a number of nuclei has been shown to have the same effect in the short-term [reviewed in ref. (49)]. The OX1 antagonist SB-334867 reduces food intake in rats in the short run and enhances behavioral satiety of rats on palatable food diet [reviewed in ref. (5)]. Mapping of orexinergic pathways indicates multiple reciprocal connections with feeding-regulating hypothalamic nuclei (like paraventricular and arcuate nuclei), and orexinergic neurons respond to glucose and possibly, directly or indirectly, to leptin (reviewed in ref. (56); see also ‘Orexinergic neurons’). Interestingly, ectopic overexpression of PPO in mice does not lead to increased body weight, and the mice do not even gain more body weight on high-fat diet as they adjust their energy expenditure accordingly (29). However, PPO overexpression is not able to compensate for the obesity induced by leptin deficiency of ob/ob mice (29).

Food deprivation or acute hypoglycemia has been reported to induce PPO mRNA expression and activate orexinergic neurons as indicated by c-fos expression [reviewed in ref. (5)]. mRNA for orexin receptors has been reported to increase in fasting in the hypothalamus and to decrease in the adrenal cortex, and the signaling profile of the receptors is changed (76). OX1 and OX2 receptor mRNA as well as luminal orexin-A-stimulated bicarbonate secretion are reduced in fasting in rat (77).

PPO-ko and PPO-pro-ataxin-3 mice are more sensitive to weight gain on high-fat diet than wild-type mice (27, 28). OX1- and OX2-ko mice (DKO not tested) show essentially the same total weight gain and fat mass as the wild-type (wt) mice when in normal or high-fat chow (29). PPO-pro-ataxin-3 mice were observed to be hypophagic and obese (36), in contrast to the regularly lean PPO-ko mice (27), which might suggest reduced metabolic rate in both animal models. However, the phenotype of the PPO-pro-ataxin-3 mice is significantly different in different mouse backgrounds while that of the PPO-ko mice is not (27). Also, possible differences in the physical activity of wt and genetically manipulated animals have not been consequently examined, and later studies have also questioned some of the findings (28, 78). No gross metabolic abnormalities have been reported for the hereditarily narcoleptic canines lacking functional OX2 receptors to the knowledge of the writer of this review.

The current view on appetite regulation is that it may not be as major a function of orexin peptides as wakefulness/sleep pattern control, and the wakefulness- and activity-stimulating effects may, at least in part, underlie the increased feeding seen with orexin injections into the brain. The conclusions based on the different transgenic models (above) are complicated, and contradictory findings even in the principally same system are seen. My conclusion is that the gross manipulations of the orexinergic system may cause too many overlapping and apparently opposite responses and adjustments (see also under ‘Metabolic rate and thermogenesis’) and may therefore constitute too blunt an instrument.

Metabolic rate and thermogenesis

Already, the early studies on orexins could report that central injection of orexins elevated metabolic rate, likely induced by the concomitantly observed increase in sympathetic activation [reviewed in ref. (5)]. Both human narcoleptics and genetically narcoleptic mice have been suggested to be unable to elevate their metabolic rate to compensate for the calorie intake (27, 28, 79). Orexins are also known to elevate locomotor activity [see, e.g., ref. (40)], and thus the caloric expenditure could take place indirectly.
Injection of orexins in the rat brain ventricular space induces thermogenesis and activation of sympathetic output to brown adipose tissue (BAT) (60, 80–82). Orexergic neurons project to raphé pallidus and further, through second-order neurons, to BAT. Orexin injection in the fourth cerebral ventricle increases c-fos expression in raphé pallidus and BAT thermogenesis, and elevates body temperature (59, 60). Central orexin-A injection in rats or ectopic PPO overexpression in mice does not increase uncoupling protein-1 (UCP-1; also known as thermogenin) expression in BAT (29, 83); however, UCP-1 expression is not the only determinant of caloric expenditure of BAT. As an apparently contradictory finding, SB-334867 has been reported to increase BAT thermogenesis and UCP-1 expression in rat (84, 85).

Two recent studies have assessed the role of orexins in BAT development and regulation using transgenic mouse models. Zhang et al. (86) showed that when mice were exposed to handling stress (temperature probe insertion in the rectum), wt mice as well as PPO-ko mice responded by stronger thermogenesis than PPO-pro-ataxin-3 mice, in a β3-adrenoceptor-dependent manner, suggesting BAT involvement. UCP-1 was induced in BAT in the former mouse genotypes but not in the latter. Direct stimulation of BAT activity with β3-adrenoceptor agonist was similar in all mouse genotypes. c-fos expression was seen to increase in the orexinergic neurons of the hypothalamus upon stress (86). Thus, handling stress was suggested to activate orexergic neurons, which would polysynaptically, through sympathetic neurons, project to BAT. However, the crucial transmitter of orexinergic neurons required for signaling to BAT would not be orexin but something else, in apparent contradiction to the studies described above. In another study, PPO-ko mice were observed to develop serious obesity on a high-fat diet, ascribed to the inability to increase the metabolic rate (28). BAT was poorly differentiated and harbored little lipid in PPO-ko mice. Orexin was suggested to be required for prenatal or perinatal BAT development as intraperitoneal injections of orexin-A in the mother during pregnancy were able to rescue some BAT differentiation in PPO-ko mice. In cellular model systems, orexin-A supported differentiation of precursor cells to brown adipocytes through the OX1 receptor, suggesting that orexins are needed at the level of BAT and not in the beginning of the polysynaptic neuronal pathway. The signaling cascades are not fully explored and some crucial evidence is still missing; however, it is suggested that orexin signaling requires p38 mitogen-activated protein kinase and bone morphogenic protein receptor 1A signaling (28). Earlier studies suggested that PPO expression (in the rat) in the lateral hypothalamus would be nil or very low during the embryonic phase, while some other later studies have somewhat revised this [see, e.g., refs. (87) and (88)]. The proximal 3.2-kb PPO promoter (89), used to express heterologous constructs selectively in the orexergic neurons, only seems to show significant postnatal expression (36, 37); however, this truncated promoter may not contain all the native regulatory elements. BAT development takes place in utero and during the first few neonatal days (28). The rescue of PPO-ko mice was made by orexin injections during the pregnancy. PPO mRNA is detected in the placenta (28, 90), and Sellayah et al. suggest that this could be the source of orexin for the fetus, whose own hypothalamic orexin production may not yet have started. However, this would suggest that the genotype of the mother would decide the BAT phenotype of the pups while the genotype of the pups would bear no significance. The study, although an important breakthrough, raises many new questions to be answered in the future, not least for what possible role orexins may have for human BAT development and activation; BAT was recently found also in adult humans (91). Another problem to be solved are the apparent contradictions between the findings in refs. (86) and (28), for instance the very central issue of differentiation status and function of BAT in PPO-ko mice.

**Stress and sympathetic activation** Orexin neurites project to the thoracic and lumbar spinal cord and excite sympathetic neurons of intermediolateral cell column (58, 92), in addition to caudal raphé (see above). I.c.v. injection of orexin or injection in specific brain stem sites induces sympathetic activation and release of norepinephrine/epinephrine in the circulation [reviewed in ref. (5); see also refs. (93–98) and ‘Metabolic rate and thermogenesis’]. Neuronal activity from brain stem nuclei to BAT has in particular been observed (‘Metabolic rate and thermogenesis’). Recently, also orexin-triggered sympathetic signaling to skeletal muscle was seen (97). I.c.v. orexins can induce stereotypic behavior related to stress response [reviewed in ref. (5)]. PPO-ko and PPO-pro-ataxin-3 mice show failures in sympathetic regulation (99).

Orexins have also been suggested to activate the hypothalamo-pituitary-adrenal axis to glucocorticoid release [reviewed in ref. (100)]. Most results support possible orexin actions to both adrenocorticotropic hormone release and direct stimulation of adrenal cortex. The CNS orexin responses, increased wakefulness, increased metabolic rate, stress response, stimulated locomotion, etc., have not been consequently correlated. It would not be far-fetched to assume that these responses may go hand in hand and affect each other.

**Reward, motivation and addiction** Aston-Jones and coworkers have shown that morphine conditioning activates orexergic neurons (101). Morphine seeking was reinstated by activation of orexergic signaling and blocked by SB-334867. Similar was seen by de Lecea and coworkers with respect to cocaine-seeking and stress-induced relapse (102). However, orexin-A itself was suggested to reduce reward. A number of studies have followed since. While the simplified view is that orexins are involved in the reward from food and a number of drugs probably through dopaminergic systems, the results and conclusions are more complicated than that, and it would be wisest for the reader to consult the expert reviews on this subject (103–105).

**Other implications** Central delivery of orexin-A enhances anxiety-like behavior in mice and rats (106, 107). Nicotine-induced anxiogenic affect was abolished by SB-334867 or in PPO-ko mice (108), while footshock-induced anxiety was
abolished by injection of TCS-OX2-29 but not by SB-334867 in the thalamic paraventricular nucleus (107). In OX2-ko mice as well as in SB-334867-treated wt mice, an antidepressive effect was suggested (on the basis of a forced swim test), whereas in OX1-ko mice an opposite (depressant) effect was seen (109). In a rat panic anxiety model (Na-lactate-precipitated panic in rats with inhibited GABA synthesis), block of OX1 receptor with SB-334867 was enough to largely eliminate the panic response (44).

A number of studies on mice and rats identify orexins as analgetic when administered intravenously, i.e., or intrathetically but not intraperitoneally. Analgesia is observed in the models for acute thermal, mechanical, chemical, or inflammatory pain (110–112). When tested, the responses were sensitive to SB-334867 but not the opioid antagonist naloxone (110, 113).

Orexinergic neurons are involved in native analgesia. Orexinergic neurons are activated in mice upon long-lasting stress and pain. PPO-KO mice show more hyperalgesia and less antinociception than the wt mice (114). PPO-pro-ataxin-3 mice lack stress-induced antinociception, but this can be reinstated by orexin-A administration.

**Progress** Tracing studies represent a major advance in mapping of the orexin pathway. The synthetic tracers and genetically encoded ones have their own advantages and disadvantages, but in combination of competent studies have revealed important information of upstream and downstream ‘connectomics’ of orexinergic neurons. However, we still do not have direct proof of subdivision of orexinergic neurons to functional (and anatomical?) groups – or is it so that such do not exist? Feeding as such has been fading as a major function of orexins, probably at the cost of metabolic rate. However, we also here strongly depend on conclusions based on the rodent models.

Regulation of sleep and wakefulness are not novel functions, but some studies have shed new light on these. For instance, optogenetic techniques allow assessment of the function of orexinergic neurons in physiological context. Other important findings have come from significant recovery of wakefulness/sleep regulation in some transgenic animal models. Human narcolepsy has been investigated in many studies but it does not seem as though we are much closer to a solution. Highly interesting are the more recently discovered functions of orexins in reward/addiction, analgesia, anxiety, and mood.

**Periphery of the body**

Orexin and orexin receptor expression has been investigated throughout the peripheral organs of mainly rat, using both mRNA detection [reverse transcriptase polymerase chain reaction (RT-PCR), *in situ* hybridization] and IHC. For the reasons discussed under ‘Antibodies’, I would personally trust orexin peptide or receptor expression data obtained with antibody techniques only when verified by mRNA and also, rather, functional data. Reports on peripheral orexin peptide and receptor expression and functions have been reviewed (5, 47, 77, 100, 115, 116) and I shall thus here, owing to space limitations, only very briefly discuss the issues.

The sites where orexin receptor expression and functional responses are seen without any doubt are few, essentially the gastrointestinal tract, adrenal cortex, male reproductive tract, adipose tissue (especially BAT, see under ‘Metabolic rate and thermogenesis’), and pituitary gland. The major questions still is whether the responses to exogenous orexins anyhow reflect physiological signaling, which would require access to endogenous orexin. Theoretically, peripheral orexin receptors could be activated by circulating orexin coming from the CNS or some peripheral endocrine source, or by locally produced (paracrine) orexin. It is unclear whether any circulating produced orexin-A could be found in the plasma in high enough levels to stimulate orexin receptors (see ‘Antibodies’ and ‘Orexin peptide and orexin receptor determinations’). Studies have investigated orexin penetration from the blood to the rat, mouse or dog brain, and obtained contradictory results (110, 117); in two studies, the exit of orexin-A from the CNS to the plasma has been assessed (117, 118).

Testis is the only rat peripheral location where both PPO mRNA and protein expression have been verified by Northern blotting, RT-PCR, and IHC (5, 47, 115, 116); in contrast, no expression has been seen in the ovaries. In contrast, PPO mRNA was detected in human kidney, placenta, stomach, ileum, colon, adrenal gland, and pancreas (90), and in the epididymis and penis only of the male genital tract (119). PPO mRNA has also been detected in mouse placenta (28) and rat small intestine (longitudinal muscle or myenteric plexi) (120). Concerning the pituitary gland, orexinergic projections have only been observed in the posterior part (62). Whether orexinergic neurites project to median eminence from where orexins could be directly released to the pituitary portal circulation is a matter of debate (121, 122). Thus, in the lack of consequent approaches to reveal the existence of peripheral orexin production and release, no major approaches have been made in this field, except what probably concerns BAT.

**Cellular signaling**

Orexins receptors show very significant versatility in their signaling (see Figure 3 for a summary). Although the OX1 receptor has been more often studied, at least in recombinant systems, there is no evidence to indicate that the signal coupling of the receptor subtypes differs. Orexin receptor activate members of at least three families of G-proteins, namely of the Gq, Gi, and Gs families (76, 123, 124). For most orexin receptor responses seen in native or recombinant cells, G-protein-coupling is not known – determination of G-protein-coupling is not a trivial task (13) – but most often orexin receptors are thought to signal through Gq-proteins because the response cascades often would seem to fit in the Gi profile [Ca2+ elevation, PLC and protein kinase C (PKC) activation, insensitivity to pertussis toxin]. Orexin receptors have also been suggested to interact with other proteins than the heterotrimeric G-proteins, in agreement with many GPCRs. The interaction of orexin receptors with dynein light chain Tctex-types 1 and 3 was recently
Ca\(^{2+}\) influx may be stimulated independent of the K\(^+\) channels, depending on the cell type. However, despite constant and significant progress in research, we still do not have a nearly complete picture of orexin signaling. For instance, most of the findings made in cell lines have not been linked to the native tissues. Orexin receptor signaling in its multitude is interesting in many respects. It offers clues to the functional effect seems to be less significant, at least in the long run, plastic changes are imminent. In some other immediate time frame in response to orexin stimulation, and in recombinant cells, these have been suggested to belong to the TRPC subfamily of TRP channels (135). In some cell types, Na\(^+\) influx is the primary response, followed by Ca\(^{2+}\) influx through the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). This is the so-called reverse mode of the NCX, where three Na\(^+\) are extruded using the driving force of one Ca\(^{2+}\). NCX might also act in this signaling in the normal mode to extrude Ca\(^{2+}\) while depolarizing the cells with Na\(^+\) [see, e.g., refs. (136) and (137)]. However, the pharmacology of the inhibitors KB-R7943 and Ni\(^{2+}\) used to determine NCX involvement is not always clear. Ca\(^{2+}\) elevations have also been observed in primary neuronal and endocrine cells, mainly as Ca\(^{2+}\) influx [see, e.g., ref. (58)].

Orexin receptors were early on shown to activate PLC. We have recently shown, in recombinant CHO cells, that they also activate other phospholipases, namely PLD and cytosolic PLA\(_2\) (138–141) and also diacylglycerol lipase (141). Lipid-derived messengers, such as diacylglycerol, phosphatidic acid, arachidonic acid, 2-arachidonoylglycerol, and inositol-1,4,5-trisphosphate, are thus likely mediators or orexin responses. However, for most of these signal substances, the physiological significance remains to be shown.

In the long run, orexin receptors can affect cell plasticity and fate. Plastic changes are seen in the nervous system as well as in recombinant cells (142–145). After a longer exposure, orexins can induce programmed cell death of recombinant cells as well as some cancer cell types (146–148). Several mechanisms for this have been suggested (126, 147).

Thus, in the CNS, strong neuroexcitation is seen in the immediate time frame in response to orexin stimulation, and in the long run, plastic changes are imminent. In some other (pathogenic?) tissues, programmed cell death is induced. Because of the lack of space, I cannot here review these aspects; interested readers are referred to my old review (128) as the only expert review on these aspects, although even that one is much outdated in many respects. Nevertheless, despite constant and significant progress in research, we still do not have a nearly complete picture of orexin signaling. For instance, most of the findings made in cell lines have not been linked to the native tissues. Orexin receptor signaling in its multitude is interesting in many respects. It offers clues to the

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**Figure 3** Proposed orexin receptor signaling pathways.

Orexin receptors primarily interact with at least three G-protein families, the protein phosphatase SHP-2, dynamin light chain Tetex-types 1 and 3 (Dynlt), and β-arrestin (β-ar). These, or some of the downstream cascades of these, regulate ion channels and second messenger-producing enzymes, which release second messengers, which may act on ion channels or kinases. Final outcomes of the cascades may, in the different time frames, be excitation, plastic effects, or even cell death. The signaling cascades, naturally, also act laterally and in ‘retrograde’ direction. 2-AG, 2-arachidonoylglycerol; DAG, diacylglycerol; LPC, lysophosphatidic acid; PA, phosphatidic acid; PKA and PKD, protein kinase A and D, respectively; PUFAs, polyunsaturated fatty acids (e.g., arachidonic acid); VGCC, voltage-gated Ca\(^{2+}\) channels.

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Ca\(^{2+}\) elevation is a prominent response for orexin receptors, seen in essentially all cell types where measurements have been made [reviewed in ref. (128)]. This Ca\(^{2+}\) elevation takes place, in part, through PLC-mediated inositol-1,4,5-trisphosphate production and intracellular Ca\(^{2+}\) release followed by a secondary Ca\(^{2+}\) influx (store-operated influx). In addition to this, Ca\(^{2+}\) influx may be stimulated independent of the PLC cascade [see, e.g., refs. (129) and (130); reviewed in ref. (128)]. This may take place through depolarization-activated voltage-gated Ca\(^{2+}\) channels or through non-selective cation channels, depending on the cell type.

Orexin receptors depolarize neurons by two means, inhibition of K\(^+\) channels and activation on non-selective cation channels. Both mechanisms may even be seen in the same cells [see, e.g., ref. (131)]. ‘Leak-type’ voltage-gated K\(^+\) channels [probably of the inward-rectifier (Kir) type; see, e.g., refs. (131) and (132)] are likely the K\(^+\) channel targets of orexin receptor signaling. Kir channels are typical GPCRs, and orexin receptors have been shown to inhibit some of these channels also in heterologous expression systems (133). Non-selective cation channels targeted by orexin receptors have not been molecularly identified, but likely candidates would be transient receptor potential (TRP) channels. TRP channels constitute a heterogeneous family of channels with more than 20 members, and they are expressed in every cell type (134). Different TRP channels show different permeabilities for K\(^+\), Na\(^+\), and Ca\(^{2+}\), and thus they may act in different degrees either as Na\(^+\) channels (depolarizing) and Ca\(^{2+}\) channels (Ca\(^{2+}\) influx). Non-selective cation channels have indeed been suggested to mediate both types of responses in orexin receptor signaling; in some recombinant cells, these have been suggested to belong to the TRPC subfamily of TRP channels (135). In some cell types, Na\(^+\) influx is the primary response, followed by Ca\(^{2+}\) influx through the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). This is the so-called reverse mode of the NCX, where three Na\(^+\) are extruded using the driving force of one Ca\(^{2+}\). NCX might also act in this signaling in the normal mode to extrude Ca\(^{2+}\) while depolarizing the cells with Na\(^+\) [see, e.g., refs. (136) and (137)]. However, the pharmacology of the inhibitors KB-R7943 and Ni\(^{2+}\) used to determine NCX involvement is not always clear. Ca\(^{2+}\) elevations have also been observed in primary neuronal and endocrine cells, mainly as Ca\(^{2+}\) influx [see, e.g., ref. (58)].

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orexin physiology; could it also be involved in some pathogenic processes? Second, it can be used as a model system for investigations of GPCR signaling and regulation of this toward particular outputs.

**Expert opinion**

During the first years after their discovery, there was an explosion of studies on orexin physiology. Orexin rapidly took a central position in the regulation of sleep; this concept has been strengthened and deepened by further studies. In contrast, stimulation of appetite, described already in 1998, has rather shifted to the regulation of metabolism. More recently discovered physiological functions identified in the CNS, worth considering, are analgesia, anxiety and depression, and reward/addiction. One interesting aspect, thus far little investigated, is in which degree the apparently different responses reflect the same processes, for instance connections between wakefulness, motor activity, appetite, anxiety, and stress response. State-of-the-art technology has been used in many of the recent discoveries. For instance, genetic constructs (e.g., expression of GFP under the PPO promoter) and other techniques have allowed investigation of the properties of orexinergic neurons and their upstream and downstream projections. The projection nuclei responsible for specific orexin responses have been identified. Development of orexin receptor antagonists has been very active. The investigations of orexin receptor signaling have made significant advances, also in the field of cell plasticity (not reviewed here).

While the knowledge of many processes regulated by orexins and pharmacotherapeutic opportunities has been ever-increasing, knowledge in many fields is poor or, at best, fragmented. Orexin peptide measurements may not have improved (see ‘Antibodies’). We still do not know whether there really is any physiological role for orexins in the periphery. Lack of understanding of basics of the systems studied (e.g., GPCR behavior) has led to significant misconceptions, for instance on the signaling profiles of OX₁ and OX₂ receptors. Despite significant approaches, the etiology of narcolepsy has remained elusive.

**Outlook**

**Receptor (subtype) involvement**

An interesting issue involved with orexin receptor subtypes is the pharmacological determination of involvement of either subtype in a particular response. SB-334867 was a very important tool when it arrived, as it, for years, represented the sole way of assessing OX₁ receptor involvement. However, we may feel a bit skeptical to all the responses mediated by OX₁ receptors according to these studies. OX₁- and OX₂ receptor expression in the CNS is often seen in the same nuclei, and they should also be involved in the regulation of the same processes through different nuclei [see, e.g., ref. (51)]. If both receptor subtypes take part in a response, blocking of one of these may be enough to ‘cut the chain’ or reduce the signal strength below a critical threshold. SB-334867 is not extremely selective for OX₁ [in recombinant CHO and HEK-293 cells 50–100-fold over OX₂ (9, 149)], and it also inhibits at least serotonin 5-HT₁₆ and 5-HT₂₅ receptor (149). A regular dose of SB-334867 (10 mg/kg weight) gives a concentration of 3.8 µM in the (rat?) brain at 2 h (149); this concentration is likely to partially inhibit also the other receptor targets. On the other hand, even genetic elimination of one of the receptor subtypes produces dramatic effects: OX₁ is more centrally involved in the regulation of sleep and wakefulness than is OX₂ in dogs and mice. Thus, the data obtained with SB-334867 are not necessarily misleading but may have to be re-evaluated using the more recent OX₂-selective antagonists. We are altogether heading for a very exciting time when not only the physiological functions but also the therapeutic regimens planned (especially in insomnia) are evaluated. However, only very few of the tens of compounds known are commercially available. Making these compounds available should be advantageous not only for the independent scientist working on the field but also for the companies themselves. There also seems to be a lack of development of agonist ligands as research tools and drug candidates; I sincerely hope that some company would take up this challenge.

The pharmacological and biochemical features of (the apparent) orexin receptor subforms are enough to give the scientist gray hair. Sometimes, orexin-B seems to be more potent or efficacious than orexin-A, which cannot be explained by any data from ‘pure’ (heterologous) expression systems. Some studies suggest distinct physiological signaling features for each subtype. Would the mutations/polymorphisms known for human orexin receptors or the alternative splicing of mouse OX₁ (150) have any correlate in the receptor function, expression, or pharmacology? Are orexins the only native ligands for these receptors or, alternatively, are OX₁ and OX₂ receptors the only receptors for orexins? How would the apparently multiple signaling pathways regulated by OX₁ and OX₂ receptors be chosen from to suit the needs of a particular cell type? Currently, we have no firm evidence to make any conclusions of these issues.

**Orexin peptide and orexin receptor determinations**

A number of problems to solve are associated with studies on orexin peptide and orexin receptor expression. As discussed above, the antibody-based determination of receptor expression has to be considered with utmost care, and I do not recommend trusting in antibody data only (see ‘Antibodies’). A major challenge for orexin studies would be to establish proper antibodies for all the targets and make these accessible for all interested researchers. If this is not possible – the targets may indeed be problematic – a collaborative effort should be started to establish an alternative analysis method for orexin peptide levels in tissues including CSF and plasma. The mature peptides are more crucial as PPO and the receptor expression has to be considered with utmost care, and I do not recommend trusting in antibody data only (see ‘Antibodies’). The technique would likely be based on HPLC followed by mass spectrometry or RIA. One of the reasons why such technique does not already exist is likely the good access.
to commercial RIA kits, the quality of which is usually not scrutinized by the users. Development of a reliable determination method would be of outmost importance for objective diagnosis of narcolepsy and for the studies on involvement of orexins in other conditions and disease states. Most acutely, we do not know whether there are any peripheral circulating orexins, and thus the physiology of the peripheral orexin responses remains elusive.

Orexin signaling

Orexin receptors are capable of mediating multiple cellular responses in the immediate level and also in the long run (e.g., plastic effects). However, for most response types and many tissues, the physiological significance is not known. Clearly, it is reasonable to assume that orexins only induce cell death in some targets but not in others (for instance, in the CNS). Investigations of orexin signaling have major importance for identification of possible novel pathological and therapeutic principles. For instance, could we kill cancer cells through these pathways even in cancer types where orexin receptors are not expressed? On a more basic level, research on orexin receptor signaling serves to elucidate novel signal pathways for GPCRs as well as regulation of coupling to different signaling mechanisms in different tissues.

Narcolepsy

Narcolepsy is currently the only disease known to connect to orexins. It is quite possible that the pathogenesis of narcolepsy is related to immunological responses unrelated to orexin physiology, but, nevertheless, without a firm knowledge, this issue is difficult to avoid for orexin researchers. It is difficult to predict whether we will be able to solve this mystery, but I sincerely hope so. The issue is also very important for reinstatement of trust in the vaccination campaigns. Independent of resolution of the pathogenic processes behind narcolepsy, narcoleptic individuals might gain advantage of exogenous orexin receptor stimulation (as suggested on the basis of the results of refs. (38) and (43)). This could take place by orexin replacement therapy or by synthetic orexin receptor agonists. It is, however, questionable, whether any pharmaceutical company would find it economically feasible to start developing agonist ligands for such a rare disease, and of course such treatment regimens might imply expected and unexpected risks (for instance, unwanted cell death).

Highlights

• Skillful use of antibodies, mRNA detection, tracing, and genetic constructs have allowed rather comprehensive mapping of the pathways upstream and downstream from the orexinergic neurons.
• Much is known about the stimuli regulating the activity of orexinergic neurons, but still some central issues remain elusive, including the glucose-regulatory mechanism on orexinergic neurons, the anatomical and functional subpopulations of orexinergic neurons, and the entire peripheral orexin signaling.
  • The major physiological orexin response seems to lie in the regulation of wakefulness (and sleep pattern), while other known significant functions include metabolism, stress response, reward, and analgesia, with the possible ‘rising stars’ of anxiety and depression.
  • Studies suffer from lack of rigorously verified antibodies for orexin peptide and receptor determinations. A collaborative effort should be started to establish a ‘gold standard’ assay for quantitative orexin peptide determination.
  • The pathogenesis of narcolepsy continues to evade the researchers.
  • A significant number of novel orexin receptor antagonists are in the pipeline, and we can hope to soon have more ligands for research use as well as the first licensed drugs. What may be the side effects and possible novel indications?
  • Small molecular orexin receptor agonists have not been reported; might some company start developing those for use in narcolepsy or other diseases (like cancer)?
  • Orexin receptors appear to be able to couple to a multitude of different signaling pathways in different cell types; how are these pathways determined and what is relevant in which tissue?

Acknowledgments

I gratefully acknowledge support from the Academy of Finland, the Magnus Ehrnrooth Foundation, and the University of Helsinki Research Funds.

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Received April 2, 2012; accepted June 1, 2012

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