History of Orexin Research

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Abstract Orexin A and orexin B (also known as hypocretin 1 and hypocretin 2) are hypothalamic neuropeptides that were discovered as endogenous cognate ligands for two orphan G-protein coupled receptors in 1998. Initially, these peptides were reported as regulators of feeding behavior (Sakurai et al. in Cell 92:573–585, 1998). Thereafter, several studies suggested that orexin deficiency causes narcolepsy in several mammalian species including humans, highlighting roles of this hypothalamic neuropeptide in the regulation of sleep and wakefulness (Sakurai in Nat Rev Neurosci 8:171–181, 2007). Studies of efferent and afferent systems of orexin-producing neurons have revealed that orexin neurons has close interactions with systems that regulate emotion, energy homeostasis, the reward system, and arousal (Boutrel et al. in Proc Natl Acad Sci USA 102:19168–19173, 2005; Yamanaka et al. in Neuron 38:701–713, 2003a; Akiyama et al. in Eur J Neurosci 20:3054–3062, 2004; Mieda et al. in J Neurosci 24:10493–10501, 2004; Sakurai et al. in Neuron 46:297–308, 2005; Yoshida et al. in J Comp Neurol 494:845–861, 2006; Harris et al. in Nature 437:556–559, 2005; Narita et al. in J Neurosci 26:398–405, 2006). Subsequent studies suggested that emotionally salient cues and contexts excite orexin neurons to promote arousal, and to support behavior. This system seems to be important to maintain the vigilance and arousal during doing various motivated and adaptive behaviors. Recently, suvorexant, a dual orexin receptor antagonist, has become clinically available for treatment of insomnia. In this chapter, I will overview the history of orexin research, highlighting some of the physiological roles of orexins.

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1 Orexin and Orexin Receptors

1.1 Identification of Orexin (Hypocretin)

In 1998, we identified and purified novel neuropeptides, orexin A and orexin B, from rat brain extracts as two endogenous peptide ligands for an orphan G-protein-coupled receptor (GPCR), called HFGAN-72 by means of a method called "reverse pharmacology" (Sakurai et al. 1998). We expressed numbers of GPCRs in HEK293 or CHO cells, and used them as then assay system to identify the cognitive ligands for each receptor. We successfully identified and purified two peptide ligands for HFGAN-72 from rat brains. Subsequent structural analysis and molecular cloning studies showed that both rat orexin A and orexin B are derived from a common precursor peptide, *prepro-orexin* (Fig. 1a).

Our structural analysis of purified peptides showed that orexin A is a 33-amino-acid peptide with an N-terminal pyroglutamyl residue, two intra-chain disulfide bonds, and C-terminal amidation. This structure is completely conserved among several mammalian species identified so far (human, rat, mouse, cow, sheep, dog and pig). Orexin B is a 28-amino-acid, C-terminally amidated linear peptide. There are several species differences in the structure of orexin B, although also highly conserved. The C-terminal half of orexin B is very similar to that of orexin A, whereas the N-terminal half is more variable.

An mRNA encoding the same precursor peptide was independently identified by de Lecea et al. as a hypothalamus-specific transcript (de Lecea et al. 1998), as described in detail in the separate chapter (de Lecea). They predicted that the transcript encoded a polypeptide precursor that is cleaved to form two neuropeptides, termed hypocretin-1 and hypocretin-2 (corresponding to orexin A and orexin B, respectively).

1.2 Orexin Receptors

Because an in vitro study suggested that orexin B has a much lower affinity to HFGAN72 than orexin A did, we had searched several EST data bases, and identified another subtype of orexin receptor, which we term orexin-2 receptor (OX2R), which appeared to have similar affinities to both orexin A and orexin B, and we re-named HFGAN72 as orexn-1 receptor (OX1R) (Fig. 1a). The actions of orexins are mediated via these two G-protein coupled receptors (GPCRs). OX1R has one-order-of-magnitude greater affinity for orexin A over orexin B. In contrast, OX2R binds both ligands with similar affinities (Sakurai et al. 1998) (Fig. 1b). *OX1R* and *OX2R* mRNAs exhibit a markedly different and basically complementary distributions, suggesting that these receptors have distinct physiological roles through different neuronal pathways (Marcus et al. 2001; Mieda et al. 2011). Recently, the structure of the human OX_2R bound to suvorexant was solved using

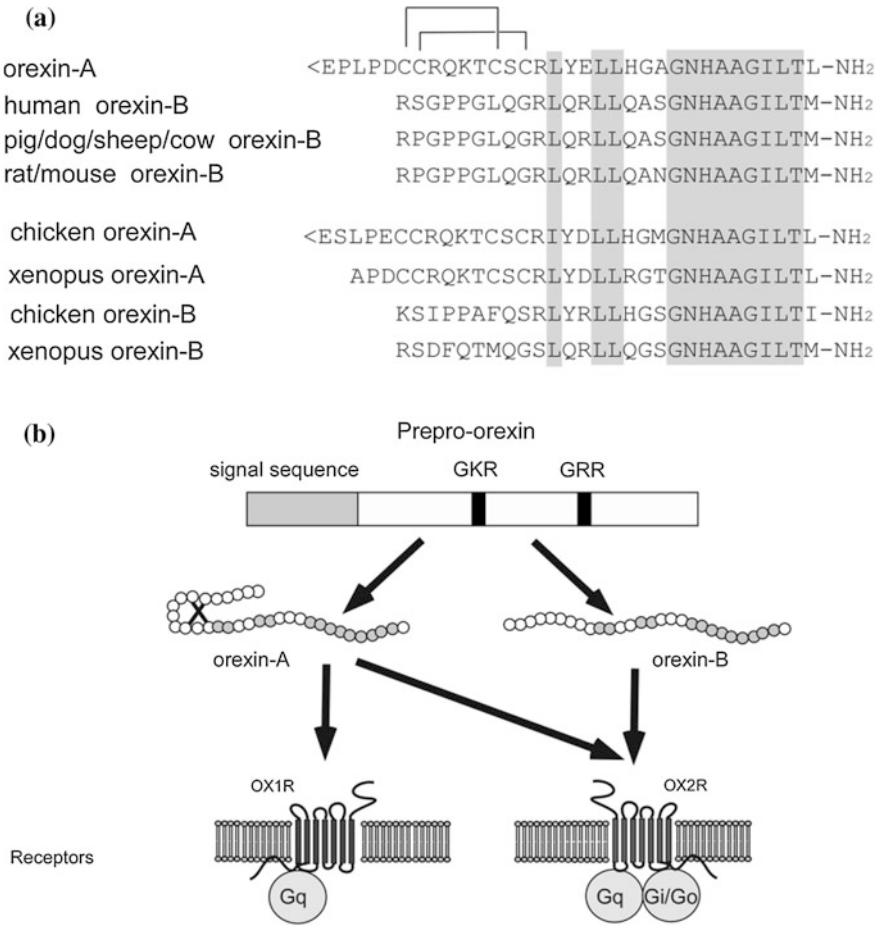

Fig. 1 Orexin and orexin receptors. **a** Structures of various species of orexin A and orexin B. The topology of the two intrachain disulfide bonds of orexin A is indicated above the sequence. *Shadows* indicate amino acid identity. Mammalian orexin A sequences thus far identified (human, rat, mouse, pig, dog, sheep, cow) are all identical. **b** Orexin A and orexin B are derived from a common precursor peptide, prepro-orexin. The actions of orexins are mediated via two G protein-coupled receptors named orexin-1 (OX1R) and orexin-2 (OX2R) receptors. OX1R is relatively selective for orexin A, whereas OX2R shows similar affinities for both orexin A and orexin B. OX1R is coupled to the Gq subclass of heterotrimeric G proteins, whereas OX2R couples to Gi/o and/or Gq in neuronal cell lines

lipid-mediated crystallization (Yin et al. 2014). The structure revealed that suvorexant adopts a π-stacked horseshoe-like conformation and binds to the receptor deep in the orthosteric pocket, stabilizing extracellular salt bridges and blocking transmembrane helix motions necessary for activation. Signal transduction system and physiological roles of these receptors are discussed in a separate chapter by Mieda et al.

2 Orexin-Producing Neurons

2.1 *Anatomical Feature of Orexin-Producing Neurons*

We and others raised antisera for orexin, and examined histological characteristics of orexin-producing neurons. Numbers of orexin neurons are estimated to be around 3,000 in rat or mouse brains, or 70,000 in human brains (Peyron et al. 1998; Nambu et al. 1999). These neurons are found exclusively in the hypothalamic regions, including the lateral hypothalamic area (LHA), perifornical area, and posterior hypothalamus (PH) (Peyron et al. 1998; Nambu et al. 1999; Date et al. 1999) (Fig. 2). However, orexin-imunoreactive fibers were observed in the almost entire neuroaxis excluding the cerebellum (Peyron et al. 1998; Nambu et al. 1999; Date et al. 1999). Especially dens staining of fibers were found in the paraventricular nucleus of the thalamus, several regions of the hypothalamus, including

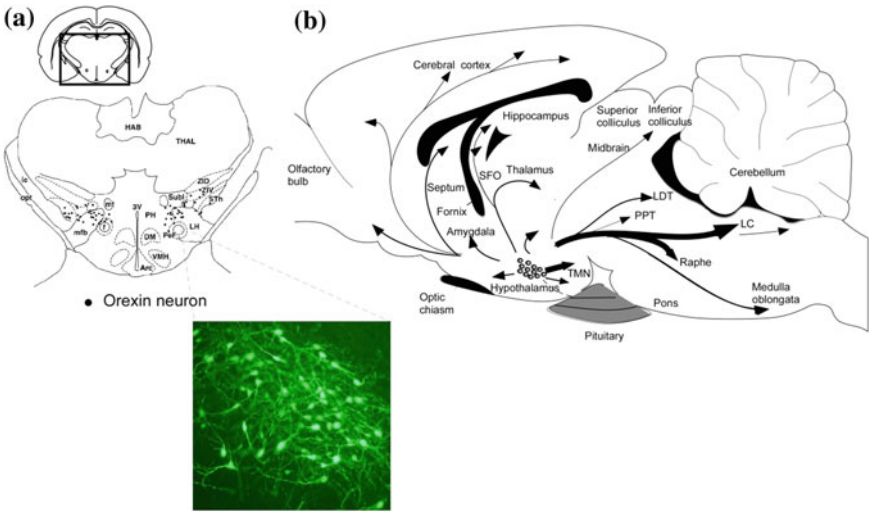

Fig. 2 Schematic drawing of coronal section and sagittal section of rat brain, summarizing the orexin neuronal system. **a** *Prepro-orexin* mRNA-containing neurons are shown in *black* superimposed upon anatomical structures of the hypo- and subthalamic areas. The *rectangle* designates the area schematized in the figure. Abbreviations: lateral hypothalamic area (*LH*), perifornical nucleus (*PeF*), posterior hypothalamic area (*PH*), subthalamic nucleus (*Sth*), subincertal nucleus (*SubI*), ventral zona incerta (*ZIV*). Additional landmarks include: thalamus (*THAL*), habenular complex (*HAB*), internal capsule (*ic*), optic tract (*opt*), mammillothalamic tract (mt), fornix (*f*), medial forebrain bundle (*mfb*), third ventricle (*3V*), arcuate hypothalamic nucleus (*Arc*), dorsomedial hypothalamic nucleus (*DM*), and ventromedial hypothalamic nucleus (*VMH*). *Inset* shows immunostaining image of orexin neurons. **b** Orexin neurons are found only in the lateral hypothalamic area and project to the entire central nervous system. The *thickness of arrows* represents relative abundance of projections. Abbreviations: third ventricle (*3V*), fourth ventricular (*4V*), tuberomammillary nucleus (*TMN*), locus coeruleus (*LC*), laterodorsal tegmental nucleus (*LDT*), pedunculopontine nucleus (*PPT*)

arcuate nucleus, ventromedial hypothalamus, and posterior hypothalamus, and most notably, monoaminergic nuclei in the hypothalamic/brain stem regions, such as the locus coeruleus (LC) (containing noradrenergic neurons), raphe nuclei (containing serotonergic neurons), tuberomammillary nucleus (TMN) (containing histaminergic neurons), and laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT) (containing cholinergic neurons) (Nambu et al. 1999; Date et al. 1999; Peyron et al. 2000). The distribution of the orexin receptor mRNA was consistent with these projection sites, with differential expression of each subtype; within the brain, *OX1R* is most abundantly expressed in the LC, while *OX2R* is highly expressed in the TMN (Marcus et al. 2001). Both regions are important for the maintenance of arousal (Marcus et al. 2001). The raphe nuclei, LDT/PPT, and ventral tegmental area (VTA) contain both *OX1R* and *OX2R* (Marcus et al. 2001), although they are expressed in distinct neuronal populations in each region (Mieda et al. 2011). These observations suggest that these monoaminergic regions, implicated in the regulation of wakefulness, are major effector sites of orexins.

In vivo recording studies revealed changes of orexin neuronal activity across the sleep-wake cycle in rats or mice (Mileykovskiy et al. 2005; Lee et al. 2005; Takahashi et al. 2008). Basically, orexin neurons fire during active waking, decrease discharge during quiet waking, and virtually cease firing during both rapid eye movement (REM) and non-rapid eye movement (NREM) sleep. This firing pattern was consistent with earlier studies showing that Fos expression (a marker of neuronal activity) in orexin neurons in rats or mice is increased during the dark, active period in which the awake state is dominant (Estabrooke et al. 2001), and orexin levels in CSF peak during the dark period and decrease during the light period in which the sleep state is dominant (Yoshida et al. 2001).

2.2 Regulation of Orexin Neurons

2.2.1 Input and Output of Orexin Neurons

Orexin system is involved in the diverse functions. Knowledge about the regulatory mechanisms of orexin neurons is important for understanding the roles of orexin neurons in these functions (Fig. 3). Studies using anterograde and retrograde tracers suggest that orexin neurons receive abundant projections from the lateral septum, preoptic area, amygdala, bed nucleus of the stria terminalis (BNST), posterior/dorsomedial hypothalamus, and the raphe nuclei (Sakurai et al. 2005; Yoshida et al. 2006). Input and output of orexin neurons are described in detail in a chapter by Mochizuki.

Abundant input from the limbic system suggests it plays a part in the regulation of the firing rate of orexin neurons by conveying emotive factors to maintain arousal. The limbic input to orexin neurons might also be involved in the regulation of feeding behavior, because some of the affective content of the perception of food

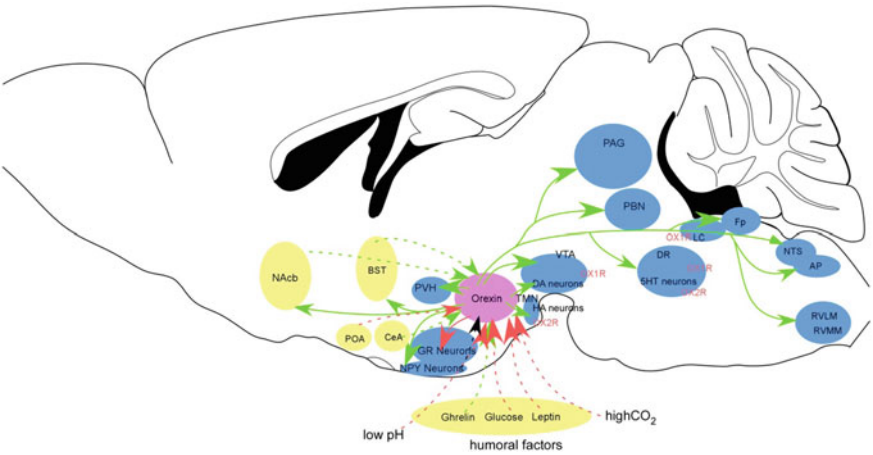

Fig. 3 Connections of orexin neurons with other regions. Orexin neurons in the lateral hypothalamic area (*LHA*) provide a link between the limbic system, energy homeostasis and the brain stem nuclei. Modified from Sakurai (2007). *Circles* show major target sites for orexins. Included in these are the locus coeruleus (LC, containing noradrenaline, NA), tuberomammillary nucleus (TMN, containing histamine, HA), raphe nuclei (Raphe, containing 5-HT), ventral tegmental area (VTA, containing dopamine, DA), and laterodorsal/pedunclopontine tegmental nuclei (PPT/LDT, containing acetylcholine, Ach). Orexin neurons promote wakefulness through the monoaminergic/cholinergic nuclei that are wake-active. Connection between dopaminergic centers and orexin neurons plays to modulate the reward systems. Input from the limbic system might be important to regulate the activity of orexin neurons upon emotional stimuli to evoke emotional arousal or fear-related responses. Sleep-active neurons in the POA send inhibitory influences to monoaminergic/cholinergic neurons and orexin neurons. Orexin neurons send both direct excitatory input to cholinergic neurons in the LDT/PPT and indirect inhibitory input to these cells through GABAergic local interneurons and GABAergic neurons in the substantia nigra pars reticulata (Takakusaki et al. 2005). Noradrenergic neurons in the LC and serotonergic neurons in the RN also send inhibitory influences to these cholinergic neurons. Blood glucose levels also affect the activity of orexin neurons through fluctuations of glucose levels in the CSF and vagal afferent. *NAc* nucleus accumbens; *PVH* paraventricular hypothalamic nucleus; *TMN* tuberomammilary nucleus; *LHA* lateral hypothalamic area; *DMH* dorsomedial hypothalamus; *ARC* arcuate nucleus; *VTA* ventral tegmental area; *SN* substantia nigra; *SCN* suprachiasmatic nucleus; *RN* raphe nucleus; *LC* locus coeruleus; *PPT* pedunculopontine tegmental nucleus; *LDT* laterodorsal tegmental nucleus

is thought to be processed in the amygdala and limbic system (Berthoud 2004). Interestingly, it is well known that food perception often evokes cataplexy in narcoleptic dogs (Reid et al. 1998), suggesting that orexin signaling is activated upon perception of food, and that this system is necessary to evoke normal feeding behavior.

Orexin neurons also receive abundant input from the preoptic area (POA), which is thought to play an important role in initiation and maintenance of sleep. This connection seems to be important for silencing orexin neurons during sleep (Saito et al. 2013).

2.2.2 Factors that Influence Firing of Orexin Neurons

In vitro electrophysilogical studies identified a number of factors that affect activity of orexin neurons (Table 1). In addition to the classical aminoacid neurotransmitters, glutamate and GABA, several factors were shown to influence the activity of orexin neurons. Both noradrenaline and serotonin inhibited orexin neurons through the activation of G protein-regulated inwardly rectifying K^+ (GIRK or Kir3) channels via α_2-adrenoceptors and $5HT_{1A}$-receptors, respectively (Li et al. 2002; Yamanaka et al. 2003b, 2006). The cholinergic agonist carbachol activated 27 %

Table 1 Factors that influence activity of orexin neurons

Excitation	Receptor involved
Glutamate	AMPAR, NMDAR mGluRs
Acetylcholine (muscarinic) (27 %)	M3
Orexin	OX_2R
Ghrelin	GHSR
Cholecystokinin	CCKA
Neurotensin	NTSR2 (unpublished data)
Vasopressin	V1a
Oxytocin	V1a
Glucagon-like peptide 1	ND
Corticotropin-releasing factor	CRFR1
Thyrotropin-releasing hormone	TRH1
BRS3 agonist	BRS3
ATP	P2X
H^+	ASIC1a
CO_2	ND
Mixture of amino acids	System-A amino acid transporters
Inhibition	
Glucose	Unknown
GABA	$GABA_A$, $GABA_B$
Glycine	Glycine receptor
Serotonin	$5HT_{1A}$
Noradrenaline	α_2
Dopamine	α_2
Acetylcholine (muscarinic) (6 %)	ND
Neuropeptide Y	Y_1
Enkephalin	μ opioid-R
Nociceptin	NOPR
Leptin	Ob-R
Adenosine	A_1
BRS3 agonist	BRS3

and inhibits 6 % of orexin neurons (Sakurai et al. 2005; Yamanaka et al. 2003b), whereas histamine had little effect on orexin neurons. Although orexin neurons do not express functional dopamine receptors, dopamine inhibited orexin neurons by acting through α_2-adrenoceptors (Yamanaka et al. 2003b, 2006). Several neuropeptides, including cholecystokinin (CCK-8S), neurotensin, oxytocin, and vasopressin, induced depolarization and excitation of orexin neurons (Tsujino et al. 2005). A synthetic surrogate ligand for an orphan receptor, BRS-3, also directly activated these neurons, although it also inhibits orexin neurons through activation of GABAergic interneurons (Furutani et al. 2010). Orexin itself was also shown to activate orexin neurons through OX2R, suggesting a positive feedback mechanism that maintain orexin neuronal activity (Yamanaka et al. 2010). Similarly, neurotensin was shown to co-localize with orexin neurons and excites orexin neurons (Furutani et al. 2013).

Interestingly, metabolic signals also seem to contribute to the regulation of orexin neurons. Decreasing the extracellular glucose concentration produced depolarization and increased the frequency of firing of orexin neurons, whereas increasing glucose concentration induced hyperpolarization and cessation of firing (Yamanaka et al. 2003a; Burdakov et al. 2005). Importantly, this mechanism is sensitive enough to monitor variations in glucose levels in cerebrospinal fluid reflecting those occurring physiologically between normal meals (Burdakov et al. 2005). These responses were shown to be mediated by tandem-pore K^+ (K_{2P}) channels (Burdakov et al. 2006).

In addition, a gut/stomach-derived hormone, ghrelin activated 60 % of dispersed orexin neurons with depolarization and an increase in firing frequency (Yamanaka et al. 2003a). By contrast, bath-application of leptin, an anorexigenic protein hormone secreted by adipocytes, was found to robustly inhibit most of the orexin neurons examined, causing hyperpolarization and a decrease in firing rate (Yamanaka et al. 2003a). These findings show that peripheral humoral factors that are related to energy metabolism influence the activity of orexin neurons (Sakurai et al. 1998; Yamanaka et al. 2003a; Willie et al. 2001). The ability of orexin neurons of sensing metabolic signals might play an important physiological role in the regulation of feeding behavior. This suggests that negative energy balance activates orexin neurons to increase arousal, thereby reinforcing food-seeking/feeding pathways. Consistently, orexin neurons-ablated mice failed to exhibit this fasting-induced arousal (Yamanaka et al. 2003a), suggesting that orexin neurons are necessary for evoking adaptive behavioral arousal during fasting. The mechanism that helps to ensure survival in nature clearly involves orexins. As discussed later, motivation toward food might also activate orexin neurons when animals are fasted.

3 Physiological Functions of Orexins

3.1 Orexin and Feeding

We initially reported orexins as factors that is involved in the regulation of feeding behavior (Sakurai et al. 1998). Orexin neurons are bilaterally and synmetorically distributed within the LHA and adjacent regions (Sakurai et al. 1998). The LHA has been thought to be the "feeding center", because lesions in this region caused anorexia, whereas electrical stimulation resulted in overeating and obesity in rats (Anand and Brobeck 1951). Indeed, we found an orexigenic effect of intracerebroventricular (icv) administration of orexin A and orexin B in rats (Sakurai et al. 1998), and this effect was subsequently confirmed in several species (Sakurai 2007b). Furthermore, central administration of an orexin antibody or an OX1R antagonist have been shown to decrease food intake (Yamada et al. 2000; Haynes et al. 2000a).

Intraperitoneal administration of the selective OX1R antagonist (1-SORA) SB-334867 or RNAi-mediated knockdown of the orexin gene reduced food intake in mice exposed to mild food restriction (Sharf et al. 2010a), and orexin-deficient mice showed decreased food intake (Willie et al. 2001; Hara et al. 2005). However, importantly, orexin signaling increases not only food intake but also energy expenditure, and a decrease in the overall orexin tone generally results in obesity (Funato et al. 2009; Hara et al. 2001). This is consistent with the findings that human narcolepsy patients show increased incidence of obesity (Hara et al. 2001). The role of orexins in body weight regulation is discussed in a separate chapter of this volume (Funato).

The orexin system may contribute to the regulation of energy homeostasis by integrating information regarding metabolic state and regulating wakefulness to support feeding behavior (Yamanaka et al. 2003a; Mieda and Sakurai 2012; Sakurai and Mieda 2011). Indeed, mice lacking orexin neurons do not show an increase in wakefulness or locomotor activity in response to starvation, unlike wild-type mice (Yamanaka et al. 2003a). Moreover, *prepro-orexin* mRNA is upregulated in fasted animals (Sakurai et al. 1998) and several studies reported that the firing rates of orexin neurons are influenced by glucose, triglycerides and amino acids (Yamanaka et al. 2003a; Burdakov et al. 2005b; Venner et al. 2011; Chang et al. 2004; Karnani et al. 2011). Orexin neurons were also shown to be innervated by neurons in the arcuate nucleus (which are primary sensors for plasma leptin levels) (Elias et al. 1998), and they are directly inhibited by leptin and excited by ghrelin (Yamanaka et al. 2003a). Together, these observations suggest that orexin neurons sense the animal's metabolic and nutritional status through both direct and indirect pathways, and integrate it in order to evoke a level of arousal necessary to promote food-seeking behavior in response to negative energy balance. In addition, motivation toward food might also contribute to activate orexin neurons.

As already mentioned, one of the possible mechanisms by which orexins promote feeding is that these factors increase arousal to secure feeding behavior. However, although the OX2R is thought to be a major player in the regulation of wakefulness, the studies using 1-SORA SB-334867 pointed to the importance of OX1R in the regulation of food seeking (Sharf et al. 2010a; Haynes et al. 2000b). This suggests that the orexin system influences food intake and wakefulness through at least partially different receptors and pathways.

Orexins might also directly affect the neuronal circuits in the hypothalamus that are implicated in the regulation of feeding behavior. They inhibit glucoreceptor neurons in the ventromedial hypothalamus (VMH), and excite neuropeptide Y (NPY) neurons in the arcuate nucleus and melanin-concentrating hormone (MCH) neurons in the LHA (van den Pol et al. 2004; Shiraishi et al. 2000; Yamanaka et al. 2000). Local injection of orexin in the paraventricular nucleus of the hypothalamus (PVN), DMH or LHA increased food intake in rats (Dube et al. 1999; Thorpe et al. 2003; Sweet et al. 1999). The area postrema and nucleus of the solitary tract (NTS) were also shown to be involved in orexin-mediated feeding (Baird et al. 2009; Thorpe and Kotz 2005). Together, orexin is likely to promote feeding by influencing multiple elements of the feeding circuitry.

Orexin-mediated feeding seems to be closely related with the reward system, in which OX1R has been also shown to be involved (Sharf et al. 2010a; Harris et al. 2005b; Choi et al. 2010). In rats, orexin increased the motivation to food-seeking when administered icv, especially for palatable food (Borgland et al. 2009, 2010; Thorpe et al. 2005). Furthermore, feeding behavior induced by administration of the mu-opioid receptor agonist DAMGO (D-Ala(2)-N-MePhe(4)-Gly-ol(5)-enkephalin) into the shell of the nucleus accumbens (NAc) was dependent on OX1R activation (Zheng et al. 2007), and intraperitoneal injection of the 1-SORA SB-334867 reduced high-fat food intake in food-restricted rats (Choi et al. 2010; Borgland et al. 2009; Nair et al. 2008).

A recent study showed that the number of Fos-immunoreactive orexin neurons in the hypothalamus increased in response to a chow-predictive (that is, conditioned) cue in rats (Petrovich et al. 2012). Similarly, the expectation of receiving a palatable food like chocolate increased numbers of Fos-positive hypothalamic orexin neurons (Choi et al. 2010). Numbers of Fos-positive orexin neurons in the LHA were increased after conditioned place preference training for a sweet cereal reward in rats (Harris et al. 2005a). Together, these findings indicate that orexin plays a role in food pursuit, especially when motivation towards food is high (e.g., when an animal is food-deprived or when foods are palatable), or when reward-conditioned cues are present. Input from the limbic system and NAc, which are thought to process the affective content of the perception of food (Berthoud 2004), might be involved in this function. Notably, food-related cues often evoke cataplexy in narcoleptic dogs (which are defective in orexin signaling) (Reid et al. 1998), suggesting that the perception of food normally induces orexin signaling, and that this signaling is necessary to elicit feeding behavior, including the

maintenance of motor activity and wakefulness. Collectively, orexin neurons are likely to be excited by food-related cues and/or a low energy balance through neuronal connections with the limbic system and by factors that indicate a low energy balance to ensure feeding behavior.

3.2 *Orexin and Wakefulness*

The importance of orexins in the maintenance of wakefulness was highlighted by the findings that showed the involvement of the dysfunction of orexin signaling in a sleep disorder, narcolepsy. Clues that revealed the dysfunction of orexins are involved in narcolepsy initially came from animal models (Chemelli et al. 1999; Lin et al. 1999) (see other chapters). The link between orexin signaling and narcolepsy was subsequently supported by studies with human patients (Peyron et al. 2000; Thannickal et al. 2000).

Narcolepsy is a debilitating neurological disorder that affects approximately 1 in 2,000 individuals in the United States (Mignot 1998). A cardinal symptom of the disorder is excessive daytime sleepiness (an insurmountable urge to sleep), which often results in falling asleep at inappropriate times and situations ('sleep attack'). The latency for rapid eye movement (REM) sleep is notably reduced in narcolepsy patients, and the existence of 'sleep-onset REM periods' (i.e. REM-sleep is directly preceded by an awake period) is one of the diagnostic criteria for narcolepsy. Nocturnal sleep is also often disturbed by nocturnal wakening combined with the occurrence of hypnagogic hallucinations, vivid dreaming, and sleep paralysis. Narcolepsy patients often suffer from a condition called "cataplexy", which is a sudden weakening of muscle tone, ranging from jaw dropping and speech slurring to complete bilateral collapse of the postural muscles. Cataplexy is usually triggered by emotional stimuli. Unlike the sleep attack, consciousness is preserved during cataplexy.

A postmortem study of human narcoleptic brains showed no detectable levels of orexin peptides in the cortex and pons, in which normally orexinergic projections are found. In the hypothalamus, an 80–100 % reduction in the number of neurons containing detectable *prepro-orexin* mRNA or orexin-like immunoreactivity were found (Peyron et al. 2000; Thannickal et al. 2000). More than 90 % of patients with narcolepsy are shown to have decreased orexin A levels in the cerebrospinal fluid (Mignot et al. 2002). Relationship between orexin deficiency and narcolepsy is described in detail in other chapters (Nishino, Kanbayashi).

As already discussed, the projection pattern of orexin neurons and distributions of orexin receptor mRNAs suggested that main effector sites for orexin are monoaminergic/cholinergic neurons in the brainstem, and electrophysiological experiments also showed that firing rates of monoaminergic cells in these nuclei are increased by orexins. For instance, noradrenergic cells of the LC (Hagan et al. 1999; Horvath et al. 1999), dopaminergic cells of the VTA (Nakamura et al. 2000), serotonergic cells of the dirsal raphe (DR) (Liu et al. 2002; Brown et al. 2002), and

histaminergic cells in the TMN (Yamanaka et al. 2002) were all shown to increase their firing rates by orexins. The firing rates of these monoaminergic neurons are well known to be associated with sleep/wakefulness states. They fire tonically during awake period, less during NREM sleep, and cease firing during REM sleep (Vanni-Mercier et al. 1984), displaying similar firing patterns with orexin neurons. These observations suggest that firing of these wake-active monoaminergic neurons mediates arousal are supported by orexins. Orexin neurons also project directly to the LDT/PPT, which contain cholinergic neurons. Some populations of these cholinergic neurons are implicated in the maintenance of wakefulness and REM sleep (W/REM-on neurons) (Shouse and Siegel 1992), whereas other populations are implicated in desynchronization of cerebral cortex and muscle atonia during REM sleep (REM-on neurons) (Shouse and Siegel 1992). Pharmacologically, a direct injection of orexin A into the LDT of cats results in an increased awake time and a decreased REM sleep time (Xi et al. 2001). In addition, several reports have shown that orexin induces long-lasting excitation of cholinergic neurons in the LDT (Takahashi et al. 2002). However, recent work also showed that orexin A inhibits cholinergic neurons in the PPT via activation of GABAergic local interneurons and GABAergic neurons in the substantia nigra pars reticulate (SNr), which send inhibitory projections to the PPT (Takakusaki et al. 2005). Since orexins show strong inhibitory effects on REM sleep, the indirect inhibition of cholinergic neurons of orexin might play an important role during REM sleep. In fact, we found robust expression of *OX1R* in the cholinergic neurons, and both receptor expressions in GABAergic neurons in these regions (Mieda et al. 2011). Collectively, these results indicate that in the LDT/PPT, orexin may directly activate W/REM-on cholinergic neurons through OX1R to facilitate wakefulness. Simultaneously, orexin is likely to activate GABAergic interneurons through both receptors to inhibit REM-on cholinergic neurons. Additionally, orexinergic activations of wake-active noradrenergic and serotonergic neurons in the LC and raphe nuclei, respectively, are likely to counteract activation of REM-on cholinergic neurons in the LDT/PPT during wakefulness (Sakurai 2007a; Pace-Schott and Hobson 2002). This is consistent with the fact that tricyclic antidepressants and serotonin-specific reuptake inhibitors are effective for treating cataplexy in narcoleptic patients.

Recent optogenetic studies revealed that orexin neurons are also glutamatergic (Schone et al. 2014). Glutamate-mediated fast and orexin-mediated slow neurotorasmission make it possible for orexin neurons to convert signals into transient and sustained signals in the same postsynaptic target neurons. The downstream pathways of orexin receptor-expressing neurons are described in more detail in a separate chapter (Mieda).

3.3 Orexin and Reward/Addiction

Besides feeding and arousal, orexin system also plays an important role in the reward system. The reward system is closely related to both feeding and

wakefulness. Cues and contexts associated with rewards, including food, sex and drugs, increase the number of Fos-positive orexin neurons and *prepro-orexin* mRNA levels (Sakurai et al. 1998; Harris et al. 2005a; Di Sebastiano et al. 2011; Cason et al. 2010). Orexin neurons send dense projections to the ventral tegmental area (VTA), in which dopaminergic neurons that send innervations to the NAc are localized (Yoshida et al. 2006) (Fig. 2). The NAc in turn sends projections to orexin neurons, constituting a reciprocal link. Intracerebroventricular injection of orexins or local administration into the VTA can reinstate previously-extinguished drug-seeking or food-seeking behaviour in rodents (Boutrel et al. 2005; Harris et al. 2005a), and orexin neurons are activated during the behavioural expression of preferences for cues associated with reward (Harris et al. 2005a). The VTA expresses both OX1R and OX2R (Marcus et al. 2001), with dopaminergic neurons predominantly expressing OX1R, and orexin signaling in the VTA has been implicated in reinforcement and reward-related processes via actions on VTA dopamine neurons (Balcita-Pedicino and Sesack 2007) (Fig. 3).

An increasing body of work shows that orexin neurons also play a part in the behavioural presentation of addiction to drugs including cocaine, amphetamine, morphine, heroin, nicotine, ethanol and cannabinoids (Espana et al. 2011; Mahler et al. 2012; Martin-Fardon and Boutrel 2012). Generally, orexin seems to be involved in the modulation of highly-motivated reward seeking, especially when this seeking is triggered by external cues that are conditioned with the rewards. Although the 1-SORA SB-334867 did not affect the expression of cocaine and amphetamine-sensitization in animals tested immediately after training (Borgland et al. 2006), it blocked the expression of sensitization after a period of abstinence following amphetamine sensitization training, as did the DORA almorexant (Quarta et al. 2010; Winrow et al. 2010). This suggests that the OX1R is involved in the acquisition of sensitization. In another example, the 1-SORAs SB-334867 and GSK-1059865 attenuated the expression of cocaine and amphetamine-induced conditioned place preference in rats (Gozzi et al. 2011; Hutcheson et al. 2011; Sartor and Aston-Jones 2012).

Importantly, neither orexins nor their receptor antagonists affect self-adminstration of addictive drugs such as cocaine in rodents (Espana et al. 2010, 2011; Hutcheson et al. 2011), suggesting that orexins have no major role in the reinforcing or the priming effects of cocaine. However, orexin A has been shown to promote motivation in a study using rats in which high levels of effort were required for seeking addictive drugs in self-administration paradigms (Borgland et al. 2009). These results suggested that orexins plays an essential role in reward-seeking not by influencing the primary reinforcing or priming effects of rewards, but by supporting motivated behavior. Intraperitoneal injections or intra-VTA administration of the 1-SORA SB-334867 blocked reinstatement of cocaine seeking elicited by either discrete cues or contextual stimuli in rats (Smith et al. 2009, 2010; James et al. 2011), whereas intraperitoneal injection of the 2-SORA 4-PT did not affect cue-induced reinstatement (Smith et al. 2009). These observations suggest that orexin neurons might be activated by external reward-related stimuli and then send information to the VTA to induce reinstatement through the activation of OX1R.

Orexins also seem to play a role in addiction to drugs other than cocaine and amphetamine. For example, Fos expression was increased in orexin neurons in rats following acute nicotine administration or nicotine withdrawal (Pasumarthi et al. 2006; Plaza-Zabala et al. 2013), and nicotine withdrawal was decreased in orexin knockout mice (Plaza-Zabala et al. 2013). Prior intraperitoneal administrations of the 1-SORA SB-334867, but not the 2-SORA TCSOX229, attenuated nicotine withdrawal (Plaza-Zabala et al. 2013), as did intra-PVN infusion of the 1-SORA SB-334867 (Plaza-Zabala et al. 2013). Moreover, systemic administration of the DORA almorexant or the 1-SORA SB-334867 reduced nicotine self-administration (Hollander et al. 2008; LeSage et al. 2010).

Orexin knockout mice and wild-type mice that received the 1-SORA SB-334867 show reduced morphine withdrawal responses (Sharf et al. 2010a, b; Georgescu et al. 2003), suggesting that orexin may also be involved in opiate addiction. Furthermore, intraperitoneal injection of the 1-SORA SB-334867 reduced the expression of morphine-induced conditioned place preference in rats and mice (Harris et al. 2005a; Sharf et al. 2010b). Similarly, a report showed that orexin-deficient mice did not show morphine-induced conditioned-place preference and hyperlocomotion (Narita et al. 2006), although this finding is argued in another study (Sharf et al. 2010b). Action of orexin in the VTA is important for the expression of morphine-induced conditioned place preference (Richardson and Aston-Jones 2012). Moreover, in contrast with the lack of effect of the 1-SORA SB-334867 on cocaine self-administration, intraperitoneal delivery of the 1-SORA SB-334867 reduced heroin self-administration (Smith and Aston-Jones 2012).

3.4 Stress, Emotion and Emotional Memory

Emotional stimuli are known to evoke autonomic responses and arousal through neural connections between the amygdala and LHA, the region orexin neurons locate. Historically, the LHA has been recognized as the ‘defence area’, as electrical stimulation here can evoke aggressive behavior and the accompanying sympathetic activation (Hilton 1982). The orexin system has close functional relationships with systems that regulate emotion and stress response, including so-called “fight or flight” response.

Orexin system has been shown to be involved in emotion-induced changes in autonomic and neuroendocrine functions. Orexin neurons in the LHA receive innervations from limbic regions including the lateral septum, bed nucleus of the stria terminalis (BNST) and the amygdala (Sakurai et al. 2005; Yoshida et al. 2006) and send projections to monoaminergic and cholinergic regions in the brain stem, periaqueductal grey (PAG), parabrachial nucleus, NST, PVN, and the rostral ventrolateral and ventromedial medulla (RVLM and RVMM) (Peyron et al. 1998; Nambu et al. 1999). Thus, orexin neurons may link limbic structures with arousal and premotor autonomic centres (Fig. 2). Indeed, several studies showed that excitation of orexin neurons increases arousal along with increased sympathetic

outflow in response to various physiological and emotional stimuli (Kayaba et al. 2003a; Zhang et al. 2006, 2009, 2010).

Notably, oral administration of a DORA, almorexant, inhibited cardiovascular responses to novelty and contextual fear (Furlong et al. 2009) without affecting responses to cold or restraint stress. Similarly, orexin knock-out mice have a decreased cardiovascular response to social stress but a normal response to a tail pinch (Kayaba et al. 2003a). Together with the finding that oral administration of the 1-SORA ACT335827 reduced the tachycardic response to social stress (Steiner et al. 2013), these data suggest that orexin more profoundly contributes to autonomic responses to psychological stressors than to physical stressors. Similarly, narcoleptic patients show reduced autonomic responses to emotional stimuli, especially aversive ones (Tucci et al. 2003), whereas they have a normal cardiovascular response to basic homeostatic challenges (such as head-up tilt, Valsalva manoeuver and cold pressor test). This suggests that orexin regulates the sympathetic nervous system primarily in response to salient emotional cues or contexts, rather than physical stress.

Several papers have suggested that dysregulation of the orexin system has been implicated in anxiety and panic-like behaviour in humans and rats (Johnson et al. 2010). For example, humans with panic anxiety have higher orexin levels in the cerebrospinal fluid compared to subjects without panic anxiety (Johnson et al. 2010), as do patients with post-traumatic stress disorder (PTSD) (Strawn and Geracioti 2008).

The limbic input to orexin neurons (Sakurai et al. 2005; Yoshida et al. 2006; Winsky-Sommerer et al. 2004) might regulate or modulate physiological responses to emotional and stressful stimuli. Indeed, the cardiovascular and locomotor responses that wild-type mice show after exposure to an intruder mouse are diminished in orexin-deficient mice (Kayaba et al. 2003b). Similarly, cardiovascular responses to air-jet stress were reduced in mice in which orexin neurons were genetically ablated (Zhang et al. 2006). Disinhibition of the amygdala or BNST through microinjections of a $GABA_A$ receptor antagonist increased Fos immunoreactivity in nuclei of orexin neurons and induced cardiorespiratory excitation in wild-type mice but not in mice lacking orexin neurons (Zhang et al. 2009). Together, these observations indicate that orexin neurons are excited by input from the amygdala and BNST (Zhang et al. 2009). Thus, it is possible that activation of orexin neurons by the limbic system maintains wakefulness during emotional arousal by conveying various emotional stimuli to orexin neurons.

The regulation of orexin neurons by the limbic system is also implicated in pathophysiology of cataplexy, as strong, generally positive emotional stimuli are well known to trigger this phenomenon in patients. Cholinergic neurons in the PPT have a role in REM sleep-related atonia (Shiromani et al. 1988) and are therefore likely to be implicated in cataplexy as well. Indeed, local injections of orexin into the PPT strongly inhibited REM-related atonia in cats (Takakusaki et al. 2005). Thus, excitatory input from the limbic system might increase orexin release in the PPT in order to sustain muscle tone that may be required to respond to salient situations.

Orexin neurons send abundant projections to the LC, and noradrenergic neurons strongly express OX1R (Marcus et al. 2001; Mieda et al. 2011). The orexin–LC pathway was shown to be involved in the formation of emotional memory in mice (Soya et al. 2013). Mice lacking OX1R displayed reduced freezing (a behavioral expression of fear) and reduced lateral amygdala activation (as measured by expression of the immediate-early gene *Zif268*) in response to cued- and contextual fear stimuli (Soya et al. 2013). Interestingly, this study showed that re-expression of OX1R in noradrenergic neurons in the LC by an AAV-mediated gene transfer in these mice restored both freezing time and lateral amygdala activation in the test phase of the cued fear conditioning procedure, but not in the contextual fear procedure. Mice lacking the OX2R ($Ox2r^{-/-}$ mice) also showed reduced freezing in the contextual fear test, but normal freezing in the cued fear test (Soya et al. 2013). This study thus suggested that OX1R, but not OX2R, plays a major role in the establishment of explicit cue-dependent emotional memory.

Icv infusion of the 1-SORA SB 334867 blocked the establishment of long-term fear memory, whereas infusion of the 2-SORA TCS-OX2-29 did not (Sears et al. 2013). Furthermore, blockade of OX1R signalling in the LC before conditioning, but not immediately after conditioning inhibited threat-memory formation. These findings suggest that OX1R signalling is important during the learning phase of fear memory formation. In human, individuals with narcolepsy showed reduced amygdala activity during aversive conditioning (Ponz et al. 2010), suggesting that orexin deficiency may result in impaired emotional learning.

A recent study using pharmacological MRI in rats found that amphetamine-induced activation in the extended amygdala, BNST and NAc, regions involved in emotion processing and emotional memory formation, was attenuated by administration of the 1-SORA GSK1059865, whereas activation of the frontal cortex and thalamus, regions that are involved in regulating arousal, was attenuated by the 2-SORA JNJ-1037049 (Gozzi et al. 2011). These findings suggest that OX2R plays a major role in maintenance of arousal, while OX1R is predominantly involved in processing emotive or reward information.

Together, these observations suggest that the orexins are important in the formation of cued fear memory. This is consistent with the observation that human narcolepsy patients show impairments in fear response acquisition as well as reduced amygdala activity relative to controls when exposed to aversively conditioned stimuli (Ponz et al. 2010; Khatami et al. 2007).

Stress response is also closely related with emotion, and orexin is also involved in this response. Early studies showed that icv administration of orexin results in increased CRH levels in the hypothalamus and in activation of the hypothalamus–pituitary–adrenal (HPA) axis (Al-Barazanji et al. 2001; Sakamoto et al. 2004) and, conversely, that orexin neurons are activated by CRH (Winsky-Sommerer et al. 2004, 2005). Indeed, an in vitro study, application of CRH depolarized the membrane potential and increased the firing rate in a subpopulation of orexin neurons by activating CRHR1 on these neurons (Winsky-Sommerer et al. 2004). These

findings are in accordance with the reciprocal connections between CRH neurons in the PVN and orexin neurons in the LHA (Winsky-Sommerer et al. 2004) and suggest that orexin might play a role in activating the HPA axis in response to stress. Indeed, forced swim stress caused orexin neuron activation and increased plasma levels of adrenocorticotropic hormone (ACTH) (Chang et al. 2007), and icv administration of a 2-SORA reduced this ACTH response. Considering that the PVN expresses abundant OX2R mRNA (Marcus et al. 2001), these results suggest that stress increases orexin neuron firing to stimulate CRH neurons in the PVN through OX2R.

Numbers of Fos-positive orexin neurons were reported to be increased by a fear-conditioned cue, but not by restraint stress (Furlong et al. 2009). These observations again suggest that orexin neurons are activated by psychological stressors. This response seems to be an appropriate response to which requires proper vigilance level and attention to environmental cues.

4 Therapeutic Potential of Orexin Agonists/Antagonists

Several orexin receptor antagonists (DORAs and SORAs) are expected to become next-generation drugs for insomnia. To date, several orexin receptor antagonists with different pharmacological characteristics have been developed (Table 2), and a DORA suvorexant has been already clinically available for insomnia disorder in Japan and the U.S. OX2R selective antagonists were also shown to be effective in animal studies for inducing sleep (Dugovic et al. 2009; Etori et al. 2014). The clinical features of orexin receptor antagonists for insomnia treatment are discussed in detail in a separate chapter (Uslaner).

On the other hand, orexin agonists a promising candidate for narcolepsy treatment in the future. Given the broad range of function of the orexin system, these drugs might be also beneficial for treating a variety of conditions other than sleep disorders.

As discussed above, it has been shown that orexin mediates many behaviors associated with drug addiction in rodents due to its effects on the VTA (Harris et al. 2007). It is reasonable to speculate that orexin receptor antagonists might be effective for treating drug addiction. Recent report showed that orexin-1 receptor antagonist SB-334867 reduces the acquisition and expression of cocaine-conditioned reinforcement and the expression of amphetamine-conditioned reward, suggesting that OXR1-selective antagonists (1-SORA) have the potential to be a treatment for individuals struggling with drug relapse and dependency (Hutcheson et al. 2011). 1-SORA might also be effective for panic disorders, because they were shown to inhibit elevations of mean arterial pressure, heart rates and freezing responses in rat models of panic disorder (Johnson et al. 2010).

Table 2 Orexin receptor antagonists

	Compound	Affinity		Units	Reference
		OX1R	OX2R		
DORA	ACT-078573 (almorexant)	7.9 (human), 7.8 (rat)	8.1 (human), 7.8 (rat)	pIC_{50}	Brisbare-Roch et al. (2007)
DORA	MK-4305	9.26	9.46	pK_i	Cox et al. (2010)
OX1R SORA	SB-410220	7.7	nd	pK_i	Langmead et al. (2004)
OX1R SORA	SB-334867	7.2	nd	pK_i	Langmead et al. (2004)
OX1R SORA	SB-408124	7	nd	pK_i	Langmead et al. (2004)
OX1R SORA	[3H]SB-674042	8.3	nd	pK_d	Langmead et al. (2004)
OX1R SORA	SB-410220	8.1	6.3	pK_b	Langmead et al. (2004)
OX1R SORA	SB-334867	7.4	5.7	pK_b	Porter et al. (2001)
OX1R SORA	SB-408124	7.7	5.9	pK_b	Langmead et al. (2004)
OX1R SORA	SB-674042	9	6.9	pK_b	Langmead et al. (2004)
OX2R SORA	1-(2-bromo-phenyl)-3-((4S,5S)-2,2-dimethyl-4-phenyl-[1,3]dioxan-5-yl)-urea	5.3–6.1	6.8–7.1	pK_i	McAtee et al. (2004)
OX2R SORA	1-(2,4-dibromo-phenyl)-3-((4S,5S)-2,2-dimethyl-4-phenyl-[1,3]dioxan-5-yl)-urea (JNJ-10397049)	5.3–5.8	8.0–8.6	pK_i	McAtee et al. (2004)

5 Conclusion and Future Perspective

Proper arousal levels are necessary for executing any purposeful behavior that requires high motivation. These behaviors are usually triggered by external cues. These functions are closely related and are interconnected through the orexin system.

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