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Review

Orexin/hypocretin modulation of the basal forebrain cholinergic system: Insights from in vivo microdialysis studies

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Abstract

Since its discovery less than a decade ago, interest in the hypothalamic orexin/hypocretin system has blossomed due to the diversity and importance of the roles played by these neuropeptides. Orexin neurons have widespread projections throughout the central nervous system and intense research has focused on elucidating the pathways and mechanisms by which orexins exert their diverse array of functions. Our group has recently focused on orexin inputs to the basal forebrain cholinergic system, which plays a crucial role in cognitive – particularly attentional – function. Orexin cells provide a robust input to cholinergic neurons in the basal forebrain and act here to modulate cortical acetylcholine release. Orexin A also increases local glutamate release within the basal forebrain, suggesting an additional, indirect effect of orexins on basal forebrain cholinergic activity. Orexin activation of the basal forebrain cholinergic system appears to be especially relevant in the context of homeostatic challenges, such as food deprivation. Thus, orexins can stimulate cortical cholinergic transmission which, in turn, may promote the detection and selection of stimuli related to physiological needs. In this manner, orexin interactions with the basal forebrain cholinergic system are likely to form a link between arousal and attention in support of the cognitive components of motivated behavior.

Keywords: Acetycholine; Arousal; Attention; Basal forebrain; Glutamate; Hypothalamus

Contents

The orexin/hypocretin system has generated a tremendous amount of interest in both the scientific and lay literature since the discovery of these fascinating neuropeptides was first reported less than a decade ago. Indeed, a PubMed search for orexin or hypocretin yields over 1300 unique references in the scientific literature alone — an astonishing figure considering the relatively brief period of time since the discovery of these peptides. Prominent among the diverse physiological roles of these peptides is a profound effect on arousal and modulation of state-dependent behavior (Estabrooke et al., 2001; Mochizuki et al., 2004). Not surprisingly, an extensive body of work has already accumulated documenting a rich anatomical and pharmacological basis for orexin interactions with other cortically-projecting neuromodulatory systems with prominent roles in these same phenomena, including brainstem dopamine and

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norepinephrine nuclei (Fadel and Deutch, 2002; Horvath et al., 1999). Our lab has focused primarily on orexin interactions with the basal forebrain cholinergic system, the principle extrinsic source of the neurotransmitter acetylcholine (ACh) in the mammalian neocortex and a crucial mediator of several aspects of attentional function. We propose that orexin inputs to the basal forebrain cholinergic system form an anatomical substrate for links between arousal and attention, and that these interactions might be particularly important as a means by which interoceptive cues bias allocation of attentional resource toward related exteroceptive stimuli.

1. A brief overview of the orexin/hypocretin system

The first reports of the discovery of the orexin/hypocretin peptides were published by two groups working independently in the late 1990's. A group at the University of Texas Southwestern Medical Center, led by Masashi Yanagisawa, used a cell-based reporter system to screen orphan G-protein coupled receptors for endogenous ligands and identified a family of peptides that bound to 2 related GPCR's (Sakurai et al., 1998). This group named these novel neuropeptides orexins – from the Greek orexis, meaning appetite – because they promoted feeding behavior upon intracerebroventricular administration and were found to be expressed exclusively in neurons located in and around the classical "feeding center" of the mammalian brain, the lateral hypothalamus. Concurrently, a separate group working primarily at the Scripps Research Institute under the direction of Gregor Sutcliffe termed these peptides hypocretins due to their hypothalamus-restricted pattern of expression and because these peptides share significant sequence homology with the incretin family of gut hormones (de Lecea et al., 1998). These initial reports demonstrated that a single prepro–orexin gene, via alternative splicing, gives rise to two functional peptides, orexin A (OxA; hypocretin 1) and orexin B (OxB; hypocretin 2). Furthermore, it was shown that these peptides (hereafter referred to simply as orexins) act on two separate G-protein coupled receptors, the orexin 1 receptor (Ox1R), which binds OxA with substantially higher affinity than OxB, and the orexin 2 receptor (Ox2R), which binds both OxA and OxB with roughly equal affinity. Within a few years after their discovery, convergent data from human, canine and murine analyses demonstrated a clear association between disruptions in orexin signaling and narcolepsy. Specifically, post-mortem analysis showed that orexin-synthesizing neurons were largely lost in brains from narcoleptic patients (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000) and a spontaneously-occurring form of canine narcolepsy was found to be associated with a loss-of-function mutation in Ox2R (Lin et al., 1999). Consistent with a causative role for disrupted orexin signaling in these naturally-occurring forms of sleep disorders, deletion of the prepro–orexin gene in mice resulted in a narcoleptic phenotype (Chemelli et al., 1999). The orexin system has subsequently been implicated in phenomena beyond those explicitly related to sleep disorders and ingestive behavior, including antipsychotic drug responses (Fadel et al., 2002), psychostimulant addiction (Borgland et al., 2006; Boutrel et al., 2005) and reward and motivation (Harris et al., 2005; Scammell and Saper, 2005). The central role played by orexin neurons in these widely-divergent phenomena has led to their description as physiological integrators (de Lecea et al., 2002) whose activity is crucial for sensing peripheral cues related to interoceptive status and coordinating appropriate autonomic, behavioral and cognitive responses (Harris and Aston-Jones, 2006; Sakurai, 2007).

We have taken a particular interest in interaction of orexin peptides with the basal forebrain cholinergic system, which is comprised of a loose continuum of magnocellular, acetylcholine (ACh)-utilizing neurons distributed among contiguous, but heterogeneous, ventral forebrain structures. We have focused on the corticopetal component of the basal forebrain cholinergic system (the Ch4 subgroup) which arises from magnocellular, choline acetyltransferase (ChAT)-positive neurons distributed in the substantia innominata/ventral pallidum and nucleus basalis magnocellularis, and provides the principal cholinergic innervation of the entire mammalian neocortex (Mesulam et al., 1983). These neurons project diffusely to virtually all layers and areas of the neocortical mantle (Bigl et al., 1982), where the primary physiological effect of ACh is to modulate the response of pyramidal cells to other – particularly glutamatergic – cortical input (McCormick, 1993; Metherate and Ashe, 1993). This widespread innervation of the neocortex by basal forebrain cholinergic neurons is an important mediator of cortical activation supporting of cognitive function, especially in the realm of attention (Sarter and Bruno, 1999). To the extent that attention is dependent on arousal, or a general physiological state of readiness for action, interactions between the orexin system and other neuromodulatory systems – including the cholinergic and noradrenergic systems – that mediate various aspects of attention are likely to be of substantial functional significance.

2. Orexins as a component of hypothalamic inputs to the basal forebrain

There is a clear anatomical substrate for interactions between orexin neurons and the basal forebrain cholinergic system. Even before the discovery of the orexin/hypocretin system it was recognized that there is a fairly robust projection from the lateral hypothalamus to cholinergic parts of the ventral forebrain and that these inputs might be important for relaying interoceptive information (Cullinan and Zaborszky, 1991; Zaborszky and Cullinan, 1989). While the lateral hypothalamus has been classically conceptualized as a "feeding center", it is also part of the posterior hypothalamus, a major component of the mammalian arousal system (Jones, 2003; Lin et al., 1989; Robinson and Whishaw, 1974; Swett and Hobson, 1968). This suggests that this hypothalamic area is involved in supporting the autonomic and behavioral responses necessary for homeostatic regulation. More recent phenotypic descriptions of hypothalamic-basal forebrain projections indicate a substantial contribution of orexin neurons to this pathway. Orexin immunoreactive fibers in the rat distribute widely to various basal forebrain structures (Cutler et al., 1999; Date et al., 1999; Peyron et al., 1998); included among these basal forebrain targets is the substantia

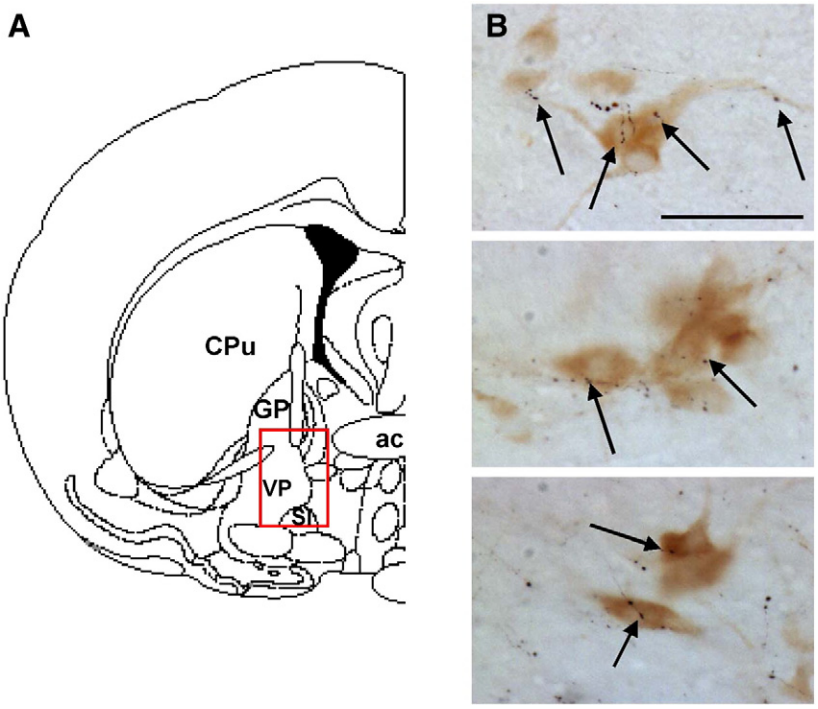

Fig. 1. Orexin fibers innervate cholinergic neurons in the basal forebrain. **A.** Rostral parts of the corticopetal cholinergic basal forebrain include clusters of choline acetyltransferase (ChAT)-positive neurons in the ventral pallidum (VP) and contiguous substantia innominata (SI), outlined by the red rectangle. Figure modified after Paxinos and Watson (Paxinos and Watson, 1998). **B.** Photomicrographs of double-label immunoperoxidase histochemistry showing three instances of orexin fibers (black) around clusters of cholinergic neurons (light brown). Numerous points of putative appositional contact (arrows) between orexin-positive fibers or varicosities and ChAT-positive somata or dendrites are observed within the VP/SI. While the antibody used to reveal orexin immunoreactivity is specific for orexin A, it is presumed that these projections colocalize both orexin A and orexin B. CPu, caudate–putamen; GP, globus pallidus; ac, anterior commissure. Scale bar in top panel of B indicates approximately 50 μm.

innominata, which receives a predominantly ipsilateral orexin input (Espana et al., 2005). Orexin-immunoreactive fibers in the substantia innominata and contiguous ventral pallidum make apparent appositional contacts on ChAT-positive cells [Fig. 1; see also (Fadel et al., 2005)], suggesting the potential for a direct influence of orexins over corticopetal cholinergic projections. While the existence of these postulated monosynaptic connections between orexin fibers and cholinergic neurons awaits ultrastructural confirmation, a combined electron and light microscopic study has shown that orexin immunoreactive varicosities observed at the light microscopic level have the ultrastructural appearance of presynaptic axon terminals, with numerous dense core vesicles (Wang et al., 2003). Thus, these varicosities likely represent functional orexin synapses on and around cholinergic somata and perikarya as has already been described for septohippocampal cholinergic neurons (Wu et al., 2004). This suggests that orexin neurons contribute substantially to the previously-recognized projection from the lateral hypothalamus to the cholinergic basal forebrain (Cullinan and Zaborszky, 1991) and implicates the basal forebrain as an integral component of a distributed network that underlies orexin effects on arousal (Espana et al., 2005).

3. Neurochemical and behavioral effects of orexins in the basal forebrain

Microdialysis across the sleep-wake cycle reveals significant increases in basal forebrain release of OxA during paradoxical, or rapid eye movement (REM) sleep (Kiyashchenko et al., 2002) although electrophysiological data suggest that this may reflect burst discharge of orexin neurons during phasic REM as these cells are largely silent during tonic REM (Mileykovskiy et al., 2005). REM sleep is also associated with activation of corticopetal cholinergic neurons (Jones, 2003; Szyjmusiak, 1995), suggesting a potential role for orexins in this phenomenon.

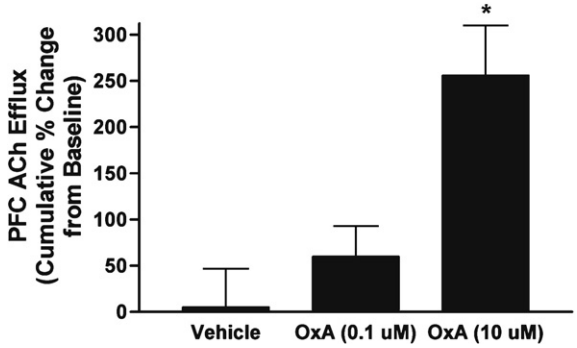

Fig. 2. Acetylcholine efflux measured by in vivo microdialysis in the prefrontal cortex of unanesthetized rats during intrabasalis administration of vehicle or OxA. ACh efflux is represented as cumulative percent change from baseline efflux (area under the curve) during a one-hour period in which vehicle (normal artificial cerebrospinal fluid) or OxA (0.1 or 10.0 μM) was continuously administered into the ventral pallidum/substantia innominata portion of the basal forebrain via a second microdialysis probe. Dialysates were analyzed by liquid chromatography with electrochemical detection as described previously (Fadel et al., 2005; Fadel et al., 2001). *$P<0.05$ vs. vehicle for $N=6$ rats. Modified after Fadel et al., 2005.

Clearly, however, Fos expression and in vivo electrophysiological data both indicate a high level of orexin neuron activity during transitions to wakefulness (Estabrooke et al., 2001; Lee et al., 2005). Similarly, intrabasalis administration of OxA via reverse microdialysis or direct intracranial infusion increases behavioral indices of wakefulness (Espana et al., 2001; Thakkar et al., 2001). Intrabasalis administration of orexin A produces robust increases in ACh release within the PFC [Fig. 2; see also (Fadel et al., 2005)] and amplifies the effects of pedunculopontine tegmentum stimulation on electroenephalograph desynchrony (Dong et al., 2006), suggesting that orexins may increase arousal via complementary and synergistic effects on both basal forebrain and brainstem cholinergic systems (Bernard et al., 2006; Vazquez and Baghdoyan, 2001).

4. How do orexins activate basal forebrain cholinergic neurons?

Orexin A activates both types of orexin receptors with roughly equal affinity; hence, effects of this peptide on the basal forebrain cholinergic system do not point to a specific receptor subtype. Furthermore, both Ox1R and Ox2R appear to be expressed in parts of the basal forebrain that include corticopetal cholinergic neurons (Hervieu et al., 2001; Kilduff and de Lecea, 2001; Marcus et al., 2001) and electrophysiological and neurochemical data are consistent with a role for both Ox1R and Ox2R in activation of the basal forebrain cholinergic system. In vitro electrophysiological data indicate that OxB is at least as potent as OxA at exciting basal forebrain cholinergic cells, suggesting primarily an Ox2R-mediated effect (Eggermann et al., 2001). These observations place the basal forebrain cholinergic system within the distributed network underlying the effects of orexins on arousal and wakefulness, as the narcoleptic phenotype associated with loss of orexin neurons or peptides in humans and mice is largely recapitulated in narcoleptic canines with a spontaneously-occurring loss of function mutation in Ox2R (Lin et al., 1999). However, other studies have suggested that the effects of basal forebrain orexin administration on wakefulness are largely Ox1R-mediated. Lateral ventricular administration of OxA, for example, is more effective than OxB at increasing electroencephalographic, electromyographic and behavioral indices of wakefulness, and these effects are recapitulated with direct intrabasalis administration of OxA (Espana et al., 2001). Also, in anesthetized rats, intrabasalis administration of OxA is more effective than OxB at increasing somatosensory cortical ACh release and inducing an arousal-like electroencephalograph pattern (Dong et al., 2006). Our preliminary studies on the effects of orexins on cortical cholinergic transmission are also consistent with an Ox1R mechanism, as stimulated cortical ACh release under conditions tied to feeding-related arousal is largely blocked by the Ox1R antagonist, SB-334867 (Frederick-Duus et al., 2007). Due to the lack of a commercially available Ox2R antagonist, the current data do not allow for definitive conclusions regarding which of the orexin receptor subtypes is most heavily involved in activation of the basal forebrain cholinergic system. Indeed, the two receptors may play different, but complementary roles in response to varying types of homeostatic challenges. Ultrastructural studies document-

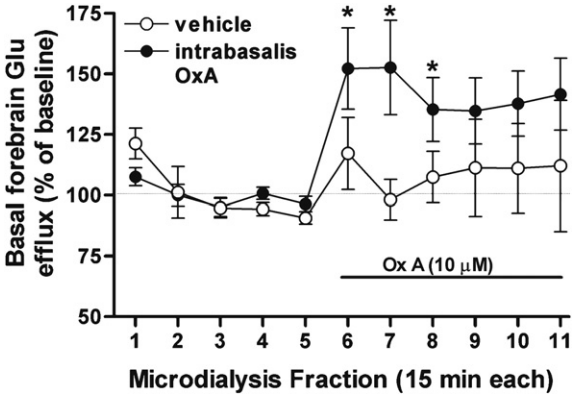

Fig. 3. Orexin A effects on glutamate release in the basal forebrain. Orexin A (10 μM) was infused continuously into the ventral pallidum/substantia innominata for 90 min beginning with the sixth collection interval. As a control a separate microdialysis session was conducted on the same animals in which the inlet of the basal forebrain microdialysis probe was switched to a second syringe containing identical artificial cerebrospinal fluid (vehicle) at the onset of the sixth collection. Dialysates were subjected to pre-column *o*-phthaldialdehyde derivatization and analyzed by liquid chromatography with electrochemical detection as previously described (Reznikov et al., 2007). Local glutamate efflux was significantly elevated (*$P<0.05$ vs. vehicle; $N=4$ rats) during the first three collections following onset of basal forebrain OxA administration. No significant effect on local GABA was observed. As further evidence that the glutamate effect was not artifactual, no glutamate peak was recorded upon HPLC analysis of the 10 μM OxA solution itself, indicating that the increases in glutamate were reflective of endogenous changes rather than detection of an amino acid product derived from the exogenous neuropeptide.

ing the precise pre- and post-synaptic localization of Ox1R and Ox2R within the basal forebrain as well as commercial availability of additional selective agonists and antagonists of these receptors will provide much-needed anatomical and pharmacological data concerning the mechanisms and functional contexts underlying orexin effects on basal forebrain cholinergic neurons.

While the receptor mechanisms underlying the direct effects of orexin on basal forebrain cholinergic activity remain to be fully elucidated, it is also possible that local orexin release modulates cholinergic neuron activity indirectly, via local glutamate release. Indeed, we now have evidence that administration of OxA (via reverse microdialysis) in the basal forebrain – at concentrations that stimulate cortical ACh release – increases local glutamate efflux (Fig. 3). The source of this glutamate is not clear. Orexin receptors are expressed in several brain regions that supply putative glutamatergic inputs to the basal forebrain, including the prefrontal cortex, midline nuclei of the thalamus and pedunculopontine tegmentum (Carnes et al., 1990; Grove, 1988; Zaborszky et al., 1997). Although it has not been shown that orexin receptors are necessarily located on terminals of these projections, presynaptic facilitation of neurotransmitter – particularly glutamate – release seems to be a widespread mechanism by which orexins excite neurons. Such a phenomenon has been described for orexin effects on presynaptic facilitation of paraventricular thalamocortical synapses within the PFC (Lambe et al., 2005) and within the perifornical hypothalamus, where both OxA and OxB can stimulate orexin neurons via a mechanism that appears to depend on presynaptic facilitation of glutamate release from local circuit neurons (Li et al., 2002; van den Pol et al., 1998). Although

speculative, it is possible that such a mechanism may also operate within the basal forebrain, as greater than 80% of calretinin-immunoreactive neurons in this area – at least some of which are likely to be local circuit neurons – are positive for the glutamate transmitter synthetic enzyme, phosphate-activated glutaminase (Gritti et al., 2003). Furthermore, colocalization studies demonstrate that at least some orexin neurons are also glutamatergic (Torrealba et al., 2003), suggesting that OxA might increase basal forebrain glutamate release by an excitatory autoreceptor mechanism.

While the synaptic circuitry underlying orexin effects on basal forebrain glutamatergic transmission still must be fully defined, these findings have clear functional significance for the mechansims and contexts underlying orexin effects on cholinergic neurotransmission. For example, ionotropic glutamatergic transmission within the basal forebrain is required for stimulated cortical ACh release in response to a complex, food-paired stimulus (Fadel et al., 2001). Expression of orexins and their receptors is increased by food deprivation (Cai et al., 1999; Karteris et al., 2005; Lu et al., 2000) and food anticipatory arousal appears to be dependent on the orexin system (Akiyama et al., 2004; Mieda et al., 2004; Mistlberger et al., 2003). Collectively, these data support a role for basal forebrain orexin transmission in activating cortical cholinergic transmission in response to motivationally salient stimuli.

5. Orexin–ACh interactions in the context of homeostatic function

Orexin neurons are sensitive to chemical correlates – such as glucose and leptin – of interoceptive cues (e.g., hunger) that signal the physiological status of the body (Burdakov et al., 2005; Burdakov et al., 2006; Hakansson et al., 1999). In turn, these neurons are thought to regulate arousal level according to energy balance (Yamanaka et al., 2003a). Collectively, then, the available evidence supports a model in which orexin activation of the basal forebrain cholinergic system is important for biasing the allocation of attentional resources toward exteroceptive cues related to physiological status. This hypothesis is based in part on the heuristic observation that proper behavioral responses to homeostatic challenges, such as hunger, entail a cognitive component — namely an awareness of the interoceptive state and enhanced attentional processing of stimuli related to the underlying homeostatic challenge. But this hypothesis has an additional foundation based on anatomical and functional studies over the last several years: hypothalamic regions, cell populations and neuroactive substances that have traditionally been viewed in terms of neuroendocrine and descending visceral function have clear connections with more rostral brain regions and transmitter systems that play crucial roles in cognition. Thus, while we are only beginning to elucidate the phenotypic and topographical organization of these hypothalamic pathways, orexins are clearly part of the anatomical substrates allowing interoceptive cues to influence cognitive function.

Several lines of converging evidence from both the clinical and animal literature suggest a role for the orexin system in attentional function. Direct infusions of OxB into the prefrontal cortex of rats improves performance in an attentional task (Lambe et al., 2005). Additional, albeit indirect, evidence also suggests a potential role for orexins in attentional function. Orexins appear to play a prominent role in the reinstatement of drug-seeking behavior in rats (Borgland et al., 2006; Boutrel et al., 2005; Harris et al., 2005), a phenomenon that clearly entails a substantial attentional component. Certain atypical antipsychotic drugs that improve cognitive – especially attentional – function appear to preferentially activate orexin neurons that project to rostral forebrain targets, providing a potential mechanistic link between therapeutic outcome and metabolic changes in these patients (Fadel et al., 2002). Narcoleptic patients – who presumably lack orexin neurons – demonstrate attentional deficits even during periods of normal wakefulness (Rieger et al., 2003). Interestingly, canine narcolepsy is also associated with neurodegeneration in parts of the basal forebrain — suggesting that a primary deficit in orexin signaling might contribute to postsynaptic degeneration (Siegel et al., 1999) and impaired ACh-dependent cognitive function. In addition, several studies have shown a decline in markers of orexin function in aged animals (Downs et al., 2006; Porkka-Heiskanen et al., 2004; Terao et al., 2002; Zhang et al., 2002) suggesting a potential role for orexins in age-related deficits cholinergic-dependent cognitive function. Finally, recent anatomical and pharmacological studies show that orexin neurons themselves are innervated by basal forebrain cholinergic neurons and are activated by ACh (Bayer et al., 2005; Sakurai et al., 2005; Yamanaka et al., 2003b). Elegant genetically-encoded tracer studies in mice, for example, demonstrate substantial monosynaptic inputs to orexin neurons arising from multiple cholinergic regions of the basal forebrain, including the nucleus basalis of Meynert, medial septum and diagonal band of Broca (Sakurai et al., 2005). Collectively, these data suggest that orexin neurons and the basal forebrain cholinergic system may reciprocally regulate each other's activity in a ongoing manner that integrates physiological status and exteroceptive cues. The receptor mechanisms underlying the excitatory effect of descending cholinergic projections on orexin neurons remain to be elucidated as most electrophysiological studies have used non-selective cholinergic agonists such as carbachol or ACh itself. However, a potential nicotinic contribution is suggested by the fact that systemic nicotine elicits Fos expression in orexin neurons (in an $\alpha 4\beta 2$-dependent manner) and locally applied nicotine increases both ACh and glutamate release in the LH/PFA (Pasumarthi and Fadel, 2006; Pasumarthi et al., 2006). It is tempting to speculate that cholinergic inputs to orexin neurons may activate orexins in a "top-down" fashion, supporting increased arousal in response to attentional processing of salient stimuli, whereas orexin inputs to the basal forebrain cholinergic system may support the activation of attentional systems governed by interoceptive states. These speculations await rigorous empirical testing.

6. Conclusions

Consistent with their widespread pattern of projections, orexin neurons have been implicated in a dizzying array of

physiological, neuroendocrine and behavioral phenomena. Delineating the precise role played by these peptides in these varied functions will require that our understanding of orexin anatomy and function be properly integrated the functions of target regions implicated in more precise aspects of these phenomena. Orexins provide robust inputs to corticopetal cholinergic neurons of the basal forebrain, which are crucial for several aspects of attentional function. The available data support a model in which orexin projections to the basal forebrain cholinergic system support the cognitive component of behavior motivated by homeostatic challenges. Alterations in these interactions may represent a common neural substrate of the attentional dysfunction that accompanies such diverse conditions as drug relapse and age-related cognitive decline.

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