Cell type- and pathway-specific synaptic regulation of orexin neurocircuitry

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Abstract

Orexin-expressing neurons are located exclusively in the lateral hypothalamic and perifornical areas and exhibit complex connectivity. The intricate wiring pattern is evident from a diverse function for orexin neurons in regulating many diverse physiological processes and behaviors including sleep, metabolism, circadian cycles, anxiety, and reward. Nevertheless, the precise synaptic and circuitry-level mechanisms mediating these processes remain enigmatic, partially due to the wide connectivity of the orexin system, complex neurochemistry of orexin neurons, and previous lack of suitable tools to address its complexity. Here we summarize recent advances, focusing on synaptic regulatory mechanisms in the orexin neurocircuitry, including both the synaptic inputs to orexin neurons as well as their downstream targets in the brain. A clear and detailed elucidation of these mechanisms will likely provide novel insight into how dysfunction in orexin-mediated signaling leads to human disease and may ultimately be treated with more precise strategies.

1. Introduction

Synaptic transmission mediates information flow in the brain and governs many behavioral and physiological processes. Synaptic communication is tightly regulated by neuromodulators, including a large number of neuropeptides that are released in response to specific sensory, physiologic, and behavioral cues. Orexins/hypocretins are neuromodulatory neuropeptides that were discovered in parallel by two independent groups in 1998 (de Lecea et al., 1998; Sakurai et al., 1998b) for their role in regulating feeding and metabolism. Two isoforms of orexin have been identified, namely orexin-A and orexin-B (also known as hypocretin-1/2), which are both derived from prepro-orexin, a 130 amino acids polypeptide precursor encoded by chromosome 12 in humans. Orexin-A and -B bind to two G-protein-coupled receptors (GPCRs) called OX₁R and OX₂R (Kukkonen and Leonard, 2014; Sakurai et al., 1998a) with differential affinity (orexin-A and -B bind to OX₂R with similar affinities, whereas orexin-A has much higher affinity for the OX₁R compared to orexin-B) (Sakurai et al., 1998a). In the brain, orexins are expressed by a small group of neurons located within

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the lateral hypothalamic area (LHA)/perifornical area (PFA), and posterior hypothalamus (Nambu et al., 1999). Initially, orexins were believed to mainly function within the hypothalamus to promote food intake (Sakurai et al., 1998b), though it was soon later discovered that orexins are also critical for maintaining wakefulness, since loss of orexin signaling results in a severe sleep disorder termed narcolepsy in animal models (Chemelli et al., 1999) and in humans (Nishino et al., 2000). Interestingly, orexin-deficient narcoleptic patients exhibit increased body weight (Tsujino and Sakurai, 2013; Yamanaka et al., 2003), suggesting that the physiologic and behavioral functions regulated by orexin are much more complex than initially conceived. Indeed, over the past few decades, the orexin system has been intensively studied and many regulatory roles for orexins have been defined. These include metabolism, reproduction, wakefulness, motivation, stress, reward, as well as addiction to alcohol and drugs of abuse (Aston-Jones et al., 2010; Mahler et al., 2014; Tsujino and Sakurai, 2013). These findings raise questions concerning how orexin neurons regulate such diverse and seemingly unrelated physiologic and behavioral functions. The functional heterogeneity among orexin neurons may contribute to their varied regulatory roles, and many anatomical studies demonstrate that orexin neurons receive a wide number of synaptic inputs and project broadly to numerous brain regions (Gonzalez et al., 2016; Mochizuki and Yoshida-Court, 2015; Peyron et al., 1998; Sakurai et al., 2005). Moreover, in contrast to the restricted localization of orexin neurons, OX₁R and OX₂R are expressed widely in the CNS. Some brain regions exclusively express the OX₁R isoform, for example, the cingulate cortex, septohippocampal nucleus, and medial preoptic area (Chen et al., 2017b; Trivedi et al., 1998), whereas other brain regions, including the neocortex, ventral tegmental area, preoptic area, and subthalamic nucleus, express both OX₁R and OX₂R (Marcus et al., 2001; Sheng et al., 2018; Trivedi et al., 1998). Thus, the diverse physiologic and behavioral processes regulated by orexin neurons are reflected by the rich and heterogeneous connectivity of the orexin system within the brain.

In line with the diverse and important functions, orexin receptor antagonists have been targeted to treat insomnia, substance abuse, and eating disorders such as bulimia and nocturnal eating (Scammell and Winrow, 2011), while orexin agonists could be immensely helpful for patients with narcolepsy (Takenoshita et al., 2018). Nevertheless, these strategies still mostly lack receptor subtype specificity, as well as brain region or neurocircuitry-level specificity. In order to fully harness the potential therapeutic benefit of targeting the orexin system, we propose that a comprehensive elucidation of the synaptic mechanisms associated with the orexin system at the pathway- and cell type-defined circuitry level is urgently needed.

2. Connectivity of Orexin neurons

2.1 Synaptic inputs to orexin neurons

It has been estimated that only ~ 3000 orexin/hypocretin neurons in rodent brain (Nambu et al., 1999; Peyron et al., 1998). Although this group of neurons is small in number, it is clear that they receive extensive axonal inputs from many diverse brain regions. Indeed, classical tracing experiments demonstrated the rich afferent innervation of putative orexin neurons in the rat brain. Specifically, by injecting the retrograde tracer cholera toxin B (CTB) to the

orexin-expressing region of the LHA, it was shown that abundant retrograde labeled neurons were found to be localized in the allocortex, claustrum, lateral septum (LS), bed nucleus of the *stria terminalis* (BNST), as well as many hypothalamic regions including the preoptic area, dorsomedial nucleus, posterior hypothalamus, other regions within the LHA and brain stem (Yoshida et al., 2006). These data suggest that orexin neurons potentially receive diverse afferent inputs, a notion supported by anterograde labeling using biotinylated dextrans (Yoshida et al., 2006). While these tracing experiments were informative, they do not formally prove that orexin neurons indeed receive synaptic inputs from these brain regions because these tracers do not specifically target orexin neurons and do not trans-synaptically label upstream/downstream neurons. The synaptic inputs to orexin neurons were also attempted using genetically-encoded trans-synaptic tracers (Sakurai et al., 2005). In this study, a transgenic animal expressing nontoxic C-terminal fragment of tetanus toxin (TTC) and green fluorescent protein (GFP) under control of the human *prepro-orexin* promoter was constructed. TTC mediated retrograde *trans-synaptic* transport of the GFP was used to identify upstream neurons forming synapses onto orexin neurons. This method revealed synaptic inputs from the amygdala, basal forebrain cholinergic neurons, GABAergic neurons in the preoptic area, and serotonergic neurons in the median/paramedian raphe nuclei (Sakurai et al., 2005). More recently, advances in retrograde tracing technology that employ pseudorabies viruses together with mouse genetics (Larsen et al., 2007; Wickersham et al., 2007) have provided further definitive information regarding the monosynaptic inputs to orexin neurons (Gonzalez et al., 2016).

Thus, the consensus remains that the inputs to LHA orexin neurons are widely distributed and arise in large part from limbic structures important for motivation/reward behaviors as well as preoptic regions that regulate circadian rhythms and sleep, however direct synaptic inputs from the suprachiasmatic nucleus to orexin neurons seems to be rare (Inutsuka and Yamanaka, 2013; Sakurai et al., 2005). Collectively, these studies suggest that orexin neurons integrate a variety of interceptive and homeostatic signals including hunger, stress, circadian signals, and autonomic challenges.

2.2. The outputs of orexin neurons.

In situ hybridization experiments showed that the receptors for orexin peptides (i.e., OX₁R and OX₂R) are expressed complementarily throughout the brain including cortical and subcortical structures (Marcus et al., 2001), while orexin neurons project from the LHA to many hypothalamic and subcortical nuclei including the ventral tegmental area (VTA), Nucleus accumbens (NAc) and locus coeruleus (Mochizuki and Yoshida-Court, 2015; Peyron et al., 1998). Studies utilizing optogenetics identified that orexin neurons form functional synapses with tuberomammillary histaminergic neurons (Eriksson et al., 2004; Schone et al., 2012; Schone et al., 2014); and genetically-specified retrograde tracing experiments using Cre-dependent driver lines and conditional pseudorabies viruses indicated that at least some orexin neurons do project to the VTA and form synapses with dopaminergic neurons (Beier et al., 2015). Whether and how axonal outputs from the orexin neurons form functional synapses with other neurons warrants further investigation.

2.3 Anatomical heterogeneity and its possible relationship with diverse functions

A number of studies report that orexin neurons may exhibit different physiologic functions depending on their specific anatomical distribution within the LHA. Laterally located orexin neurons preferentially regulate motivation, reward, and addiction, while orexin neurons derived from the PFA are more likely to be involved in control of metabolism, wakefulness, attention, and stress (Acuna-Goycolea and van den Pol, 2004; Baldo et al., 2004; Dergacheva et al., 2016; Estabrooke et al., 2001; Gompf and Aston-Jones, 2008; Harris and Aston-Jones, 2006; Harris et al., 2007; Lee et al., 2005; Moorman et al., 2016). For example, it has been shown that cocaine and morphine both induce Fos-activation within only the laterally-located orexin neurons and that chemical suppression of this group of orexin neurons impairs cocaine induced conditioned place preference (Baldo et al., 2004; Harris and Aston-Jones, 2006; Harris et al., 2007; Moorman et al., 2016; Richardson and Aston-Jones, 2012). In contrast, footshock and restraint stress mainly activate the perifornical orexin neurons (Estabrooke et al., 2001). This functional dichotomy is supported by the notion that orexin neurons within a defined sub-region of the hypothalamus likely receive distinct inputs and project to specific downstream targets (Floyd et al., 2001; Yoshida et al., 2006). Also, it is likely that these orexin neurons have complex gene expression profiles. Indeed, dynorphin (Chou et al., 2001), nociceptin/orphanin FQ (Maolood and Meister, 2010) as well as neurotensin (Furutani et al., 2013) are also expressed, in addition to orexin peptide. Thus, although orexin neurons are mainly glutamatergic and co-release glutamate (Schone et al., 2012; Schone et al., 2014), the full neurochemistry of orexin neurons is likely complex, raising the possibility that orexin cells could exhibit functional plasticity. Indeed, the numbers of orexin neurons can vary under different physiological or pathological conditions (Allard et al., 2007; Mikrouli et al., 2011). Recent development in single cell transcriptomics in hypothalamic neurons (Chen et al., 2017a; Mickelsen et al., 2017) can likely provide valuable insight into these questions and the diversity of orexin neurons. Moreover, it will be important to establish whether these functionally distinct subgroups of orexin neurons project through convergent or divergent circuits.

3. Synaptic mechanisms of orexin neurons

Orexin neuronal activity is modulated by various nutritional cues (including the peptide hormones leptin and ghrelin), metabolites (e.g., glucose, amino acids), and circadian rhythms (Horvath and Gao, 2005; Rao et al., 2007). Since synaptic transmission transduces fast information flow within the brain via release of excitatory or inhibitory neurotransmitters to downstream neurons, the activity of orexin is also regulated by synaptic transmission. Given the diverse connectivity of orexin neurons as described above, we argue that a full understanding of the synaptic mechanisms that influence orexin neuronal activity requires that the specific synaptic inputs and outputs be considered. Recent development in mouse genetics, pathway-tracing methods, as well as optogenetic and chemogenetic tools have provided new opportunities to address this question (Sternson et al., 2016). Indeed, we and others have recently applied this integrative cell-type and circuit specific strategy to identify the detailed functionality of LHA orexin neurons (Gonzalez et al., 2016; Liu et al., 2017). Although these studies are informative, it needs to be recognized that the synaptic regulation and connectivity of orexin neurons might have plasticity and undergo

reorganization under different physiological (e.g. circadian and hunger) or pathological conditions (Horvath and Gao, 2005; Laperchia et al., 2017), highlighting the need for careful documentation of experimental methods and interpretation of results.

3.1 Regulation of excitatory input to orexin neurons:

Like many other neurons in the brain, orexin neurons receive prominent excitatory inputs (Acuna-Goycolea and van den Pol, 2004; Horvath and Gao, 2005; Liu et al., 2017), however direct functional evidence regarding the precise origins and neuronal identities associated with these inputs is limited. It has been reported that orexin excites orexin neurons via local glutamatergic neurons (Li et al., 2002). Melanin concentrating hormones (MCH) released by neighboring neurons in the LHA attenuates activity of orexin neurons likely by suppressing their presynaptic excitatory inputs (Rao et al., 2008). Therefore, local neuronal networks within the LHA appear to play a crucially important role in regulating excitability of orexin neurons via synaptic communication (For review, see Burt et al. 2011).

Given the involvement of orexin neurons in regulating body weight homeostasis, locomotion, and sleep, it is conceivable that excitatory synaptic inputs to orexin neurons are tightly controlled by signals related to these behaviors. For example adenosine, a sleep-promoting factor, depresses the amplitude of evoked excitatory postsynaptic potentials and the frequency of spontaneous and miniature excitatory postsynaptic currents in LHA orexin neurons, and as a result, orexin neurons fire fewer action potentials. This mechanism might be related to the apparent involvement of the LHA in regulating sleep (Liu and Gao, 2007). Orexigenic 'hunger' signals likely potentiate excitatory transmission onto LHA orexin neurons because fasting reversibly potentiates excitatory transmission onto orexin neurons due to synaptic remodeling of excitatory synapses (Horvath and Gao, 2005). Recently, combined with neural tracing, we also found that acute fasting increases excitatory drive onto LHA orexin neurons that project to VTA (Liu et al., 2017). Moreover, we also found that leptin, an adipocyte derived hormone that conveys energy state related signals from the periphery to the brain, inhibits LHA orexin neurons. We examined the effects of leptin on LHA-to-VTA projecting orexin neuronal function, and found that leptin suppresses excitatory synaptic strength of these synapses via a presynaptic mechanism (Liu et al., 2017). In support of this observation, leptin receptor expression was not observed in LHA orexin neurons (Leininger et al., 2009). Moreover, it has also been suggested that neurotensin and galanin-expressing neurons within the hypothalamus, which express leptin receptor, likely contribute to the suppressive effect of leptin on orexin-expressing neuron's activity. However, we find that the excitatory input to VTA-projecting orexin neurons persists when the signaling of either neurotensin or galanin receptors are blocked (Fig. 1). Therefore, leptin likely inhibits orexin neurons by both a reduction in excitatory synaptic drive and also by postsynaptically activating potassium channels in leptin receptor-expressing neurotensin/galanin neurons (Goforth et al., 2014). Interestingly, we also showed that the regulatory effect of leptin in LHA-to-VTA projecting orexin neurons is highly dependent on whole-body energy states: both energy deprivation (fasting) and excessive energy storage (obesity) abolish presynaptic regulation by leptin. Fasting presumably causes hypoglycemia and hypoleptinemia, and, remarkably, we found that the diminished leptin sensitivity present under fasting conditions could be reversed by increasing the extracellular

glucose concentration (Liu et al., 2017), suggesting that these complex peptidergic signaling mechanisms are further influenced by metabolic and nutritional cues.

3.2 Regulation of inhibitory transmission onto orexin neurons

Orexin neurons are innervated by far fewer inhibitory synapses than excitatory synapses. Nevertheless, inhibitory input is critical for regulating the excitability of orexin neurons, and sensitivity to changes in neuroendocrine or physiological conditions of experimental animals (Horvath and Gao, 2005). Indeed, orexin neurons fire routinely during waking behavior (Lee et al., 2005) and may be less excitable during sleep possibly due to GABAergic inhibition (Alam et al., 2005). Sleep deprivation significantly enhances inhibitory synaptic drive onto orexin neurons, likely by promoting inhibitory synapses formation (Matsuki et al., 2015). Using optogenetic activation of serotonergic terminals in the hypothalamus, it was shown that serotonin likely facilitates GABAergic inhibitory inputs to orexin neurons as well as directly inhibits orexin neurons via 5HT_{1A} receptor-mediated signaling (Chowdhury and Yamanaka, 2016). Also, it was shown that adenosine suppresses inhibitory transmission onto orexin neurons via a presynaptic mechanism (Xia et al., 2012).

The specific origins of inhibitory inputs in these studies are unclear, though local inhibitory innervation is likely (Agostinelli et al., 2017; Hassani et al., 2010; Laperchia et al., 2017). Using targeted optogenetics in *Vgat-IREs-Cre mice*, a recent study (Ferrari et al., 2018) showed strong evidence that orexin neuronal activity is directly influenced by local inhibitory neurons in the LHA and PFA regions. Moreover, this local inhibitory synaptic transmission can be suppressed by carbachol application through muscarinic receptors. Interestingly, application of dynorphin, which is co-expressed by orexin neurons, also produces a suppressive effect on this local inhibitory transmission through κ -opioid receptors, whereas orexin has no impact. It has also been suggested that GABAergic neurons located within the *zona incerta* promote sleep by direct inhibition on orexin neurons (Liu et al., 2017). GABAergic neurons in the preoptic area amygdala also form functional synapses onto orexin neurons (Nakamura et al., 2009; Saito et al., 2013). Finally, the lateral septum is another important brain region that may provide significant inhibitory input to orexin neurons (Mahler and Aston-Jones, 2012; Sartor and Aston-Jones, 2012), though this needs further functional confirmation.

3.3 Synaptic regulations in the target regions by orexin neurons

3.3.1 The functional and neurochemical complexities of orexin neurons—As mentioned earlier, orexin neurons in the LHA are capable of co-releasing glutamate and dynorphin (Schone et al., 2012; Torrealba et al., 2003) (Apergis-Schoute et al., 2015; Chou et al., 2001; Muschamp et al., 2014; Schone et al., 2014). Additionally, these neurons also express nociceptin/orphanin FQ (Maolood and Meister, 2010), amylin (Li et al., 2015), and neurotensin (Furutani et al., 2013). Therefore, changes in orexin neuronal activity and synaptic signaling will likely convey a plethora of diverse signals to downstream targets including both fast synaptic signals and relatively slower neuromodulatory information relayed by peptides or other neuroactive substances (Fig. 2). It is proposed that neuropeptide release requires prolonged and higher levels of neuronal activation (Arrigoni and Saper, 2014; Jackson et al., 1991; Muschol and Salzberg, 2000). Furthermore, it is not conclusively

established whether different neuropeptides are released together or as a function of whether distinct circumstances or physiological states. Therefore, effects mediated by classical neurotransmitters and neuropeptides released by orexin neurons likely arise from distinct release mechanisms or release machineries that could differentially regulate how, and under what circumstance(s), either signal is transduced.

Classical neurotransmitter release requires the synaptic active zone and neuropeptide release has been suggested to occur outside of these specializations to allow diffusion into a larger target region (van den Pol, 2012). Therefore, orexin-containing neuronal fibers may control synaptic transmission onto neurochemically distinct cell subtypes located some distance from the putative peptidergic releasing sites. This notion is supported by the fact that orexin neurons heavily innervate the paraventricular nucleus of the hypothalamus (PVN), but only form classical synapses onto spinally projecting neurons (SPNs). Nevertheless, activation of orexin neurons inhibits PVN oxytocin neurons likely via release of neuropeptides that suppress their presynaptic excitatory input. This orexin function possibly maintains the circadian rhythm of oxytocin secretion to preserve homeostasis, while the synaptic pathway between orexin neurons and SPNs may be important for wakefulness and attention (Dergacheva et al., 2017).

Moreover, as mentioned earlier, classical neurotransmitter release typically occurs on the millisecond timescale, whereas high-frequency action potential firing and prolonged depolarization may be required for neuropeptides release (Arrigoni and Saper, 2014; Jackson et al., 1991; Muschol and Salzberg, 2000), which could be slower or delayed. OX₁R, OX₂R, and opioid receptors are all GPCRs which, in general, transduce multiple signaling responses on slower timescales. Therefore, if orexin neurons co-release glutamate and peptides, each will likely induce signals with different temporal characteristics and in response to different physiologic cues. Finally, when we consider the distinct and sometimes opposing signaling responses mediated by different neuromodulatory signals, further complication can occur. For example, the orexin receptor primarily couples with excitatory Gq-proteins (Sakurai et al., 1998b), while dynorphin is a ligand for the κ -opioid receptor which transduces inhibitory Gi/Go-mediated second messenger signals that activate GIRK channels (Chavkin et al., 1982). Thus, it is difficult to predict how these opposing signaling mechanisms might work together to coordinate physiological and behavioral outcomes (Chou et al., 2001; Li et al., 2014; Li and van den Pol, 2006; Muschamp et al., 2014).

3.3.2 Modulatory function of Orexin in the VTA—The VTA has been considered a major target for orexin-mediated regulation of reward-associated behaviors (Harris et al., 2005; Harris and Aston-Jones, 2006). In our recent study, by injecting retrograde beads that can be endocytosed by axonal terminals, presumably at synapses (Brown and Hestrin, 2009; Katz et al., 1984; Kobbert et al., 2000; Lammel et al., 2008), we found that orexin neurons send axonal fibers to the VTA with most residing in the more lateral part and extending to the fornix (Liu et al., 2017). Moreover, it is worth noting that limited numbers of orexin-containing synaptic formations are found in the VTA, implying that orexin neurons unlikely impact VTA cells via directly releasing glutamate onto them (Beier et al., 2015; Dehkordi et al., 2017). Nevertheless, application of orexins in VTA slices increases action potential firing of VTA dopaminergic (DA) neurons (Korotkova et al., 2003; Liu et al.,

2017). This effect might be mediated by augmentation of excitatory synaptic transmission as it has been demonstrated that orexin-A and orexin-B synergistically facilitate excitatory transmission onto VTA DA neurons by distinct synaptic mechanisms. Specifically, orexin-A activates postsynaptic OX₁R to quickly potentiate NMDAR-mediated neurotransmission, which is followed by a delayed increase in AMPAR-mediated currents. On the other hand, while orexin-B presynaptically facilitates glutamate release and concurrently activates postsynaptic OX₂R to selectively potentiate NMDAR-mediated, but not AMPAR-mediated, excitatory postsynaptic currents (Borgland et al., 2008). This result is supported by behavioral studies showing the necessity of concurrent orexin and AMPAR-mediated signaling for cues to elicit cocaine seeking (Mahler et al., 2013), whereas VTA NMDAR-mediated synaptic transmission is more important for inducing burst firing of DA neurons and the acquisition of reward learning. These effects may be related to mechanisms associated with other drugs of abuse since blockade of orexin signaling in the VTA abolishes the increased excitatory transmission that can be induced by morphine (Baimel and Borgland, 2015). It is worth noting that, although orexin-positive fibers are abundant in the VTA (Fadel and Deutch, 2002), they infrequently form synapses with VTA neurons (Balcita-Pedicino and Sesack, 2007). This suggests, as mentioned before, that these effects are likely mediated by peptidergic signals that are transduced via diffusion over some distance, rather than being confined to synaptic release sites (van den Pol, 2012).

Summary

Synaptic transmission enables fast information flow in the brain and is subject to regulation by neuromodulatory neuropeptides, such as orexin. Orexin neurons in the hypothalamus exhibit complex connectivity and participate in a range of behaviors including sleep, food intake, metabolism, and reward. In order to fully understand the synaptic mechanisms through which the orexin system accomplishes these complex regulations (Fig. 2), we suggest that the following aspects should be considered:

1. Pathway- and cell type-specific analyses should be conducted because the mechanisms associated with various synaptic inputs and outputs can vary. This requires a combination of approaches including optogenetics, mouse genetics, and neural tracing and recent work using these methods has begun to reveal the functional connectivity and regulation of the orexin system (Agostinelli et al., 2017; Apergis-Schoute et al., 2015; Chowdhury and Yamanaka, 2016; Ferrari et al., 2018; Saito et al., 2013) (Liu et al., 2017). Additionally, single cell genomics (Chen et al., 2017a) will likely be helpful for further defining hypothalamic neuronal subtypes and for understanding their respective functions.
2. Since orexin neurons are regulated by many signals and cues related to sleep, reward, feeding, and metabolism, the preconditioning of the animals used in experiments, as well as the experimental conditions, must be carefully controlled and considered when designing experiments interpreting results. For example, our recent effort in understanding the LHA-to-VTA projecting orexin neurons revealed that the peptide hormone leptin regulates excitatory synaptic drive onto orexin neurons, but that this effect is highly-dependent on energy state (Liu et al.,

2017). These findings are consistent with the literature showing that synaptic inputs to orexin neurons can undergo rapid remodeling under varied energy states (Horvath and Gao, 2005).

3. Orexin neurons also release glutamate and other neuropeptides including dynorphin, nociceptin/orphanin FQ, amylin, and neurotensin. The effects of these co-released substances should be considered in addition to orexin (Apergis-Schoute et al., 2015; Chou et al., 2001; Furutani et al., 2013; Li et al., 2015; Maolood and Meister, 2010; Muschamp et al., 2014; Schone et al., 2012; Schone et al., 2014; Torrealba et al., 2003). Whether these diverse neuroactive substances provide synergistic, opposing, or divergent effects is unclear (Li et al., 2014; Li and van den Pol, 2006). Moreover, as stated earlier, the release kinetics and stimulus requirements for classical neurotransmitter release are likely different from neuropeptides, and it is also unclear the extent to which these diverse neuroactive substances are secreted from the same populations of vesicle pools and subcellular localizations.

Although many studies have addressed important aspects of the orexin system, they have also clearly shown its vast complexity. Thus, there remain outstanding questions and exciting opportunities to delineate the cellular, circuit, and synaptic mechanisms which will likely reveal new principles for understanding modulation of brain function by neuropeptides and inform targeted strategies for therapeutic intervention

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Highlights:

- The connectivity of Orexin system is complex
- Synaptic transmissions in orexin neurons are regulated by neuromodulators
- Pathway- and cell type-specificity need to be considered for understanding of the synaptic transmission in orexin system

Figure 1, Involvement of local neurocircuitry in the regulatory effects of leptin on synaptic transmission in LHA orexin neurons.

A. Schematic drawing of possible involvement of local neurocircuitry on excitatory inputs to orexin neurons in response to leptin receptor (LeR) activation. **B.** Galanin receptor activation is not required for leptin's inhibitory effect on excitatory effect on excitatory synaptic transmission on orexin neurons that project to the VTA. **C.** GABA_B receptor activation is not required. **D.** Neurotensin receptor activation is not required as well. PTX, TTX, and AP5 are presented in the bath ACSF to isolate mEPSCs. All antagonists were applied to achieve stable baseline before leptin (100nM) administration. Data are presented as Mean+S.E.M.

Numbers of neurons recorded were indicated in the bars. Student t-tests were used for statistical analyses. ** $p < 0.01$ and *** $p < 0.001$.

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Figure 2, Diagram showing the major cell-type and pathway specific synaptic regulation in orexin neurocircuitry.

Amy: amygdala nucleus; BNST: bed nucleus of the stria terminalis; CeA: central nucleus of amygdala nucleus; dRph: dorsal raphe nucleus; HP: hippocampus; LHA: lateral hypothalamic area; LC: Locus coeruleus; LS: lateral septum; NAc: nucleus accumbens; PAG: periaqueductal gray; POA: preoptic area; PVN: paraventricular nucleus of the hypothalamus; SUBv: ventral subiculum; VTA: ventral tegmental area; SCN: suprachiasmatic nucleus.