2-1-2018

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Sherie Ma

Balázs Hangya

Christopher S. Leonard
New York Medical College

William Wisden

Andrew L. Gundlach

Recommended Citation

Ma, S., Hangya, B., Leonard, C. S., Wisden, W., & Gundlach, A. L. (2018). Dual-Transmitter Systems Regulating Arousal, Attention, Learning and Memory. *Neuroscience and Biobehavioral Reviews, 85*, 21-33. https://doi.org/10.1016/j.neubiorev.2017.07.009

HHS Public Access

Author manuscript

Neurosci Biobehav Rev. Author manuscript; available in PMC 2019 February 01.

Published in final edited form as:

Neurosci Biobehav Rev. 2018 February ; 85: 21–33. doi:10.1016/j.neubiorev.2017.07.009.

Dual-transmitter systems regulating arousal, attention, learning and memory

Sherie Ma[a,b,*], **Balázs Hangya**[c], **Christopher S. Leonard**[d], **William Wisden**[e], and **Andrew L. Gundlach**[a,b,f,*]

[a]The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria, Australia

[b]Florey Department of Neuroscience and Mental Health, The University of Melbourne, Victoria, Australia

[c]'Lendület' Laboratory of Systems Neuroscience, Department of Cellular and Network Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary

[d]Department of Physiology, New York Medical College, Valhalla, New York, USA

[e]Department of Life Sciences, Imperial College London, London, UK

[f]Department of Anatomy and Neuroscience, The University of Melbourne, Victoria, Australia

Abstract

An array of neuromodulators, including monoamines and neuropeptides, regulate most behavioural and physiological traits. In the past decade, dramatic progress has been made in mapping neuromodulatory circuits, in analysing circuit dynamics, and interrogating circuit function using pharmacogenetic, optogenetic and imaging methods. This review will focus on several distinct neural networks (acetylcholine/GABA/glutamate; histamine/GABA; orexin/glutamate; and relaxin-3/GABA) that originate from neural hubs that regulate wakefulness and related attentional and cognitive processes, and highlight approaches that have identified dual transmitter roles in these behavioural functions. Modulation of these different neural networks might be effective treatments of diseases related to arousal/sleep dysfunction and of cognitive dysfunction in psychiatric and neurodegenerative disorders.

Keywords

Acetylcholine; Arousal and attention; GABA; Glutamate; Histamine; Learning and memory; Orexin; Hypocretin; Relaxin-3

[*]Corresponding authors at: The Florey Institute of Neuroscience and Mental Health, 30 Royal Parade, Parkville, Victoria 3052, Australia, sherie.ma@florey.edu.au (S. Ma), andrew.gundlach@florey.edu.au (A.L. Gundlach).

CONFLICT OF INTEREST

The authors claim no conflict of interest.

1. INTRODUCTION

Neuromodulators, including acetylcholine, monoamines and neuropeptides regulate most if not all behavioural and physiological traits, including arousal, sleep, motivation, emotion and memory. We define arousal as a response to counteract actual or potential dangers, such as potentially threatening sensory stimuli, or increased attention demand, such as during learning processes or reward seeking, in addition to waking from sleep. A critical but understudied feature of these systems is that they contain more than a single transmitter or modulator. Neuromodulation by, for example amines and neuropeptides, occurs simultaneously with classical neurotransmission of small molecule neurotransmitters, which is thought to provide neural network flexibility and enhanced complexity in the synaptic integration of relayed information (Nusbaum et al., 2001). This is perhaps not surprising since most, if not all neurons, appear to release one or more classical transmitters and one or more neuropeptides (Hnasko and Edwards, 2012; Vaaga et al., 2014) and this has been reported for the vast majority of neuropeptide systems (see Table 1 for several key examples).

While co-transmitters have long been recognised to expand the spatial and temporal signalling repertoires of neurons across phyla (Nusbaum et al., 2001), approaches to understand the functional significance and interactions of co-transmission/release in systems regulating arousal, learning and memory have only recently been developed. This review will focus on four dual transmitter systems that uniquely originate from centralised ‘hubs’ of neurons with long-range, highly connected projections to diverse brain regions regulating arousal and memory, where some of these systems have clearly identified functions in these processes (e.g. cholinergic/GABA, histaminergic/GABA and orexinergic/glutamate) while the functions of others, like the relaxin-3/GABA system, are emerging (Figure 1). In addition, these systems display a substantial degree of interaction, including between orexin/glutamate and monoamine neurons (Kohlmeier et al., 2013), between orexin/glutamate and histamine/GABA neurons in driving wakefulness (Mieda et al., 2011; Yu et al., 2015), and between multiple peptide systems and cholinergic/GABA networks (Ishibashi et al., 2015; Damborsky et al., 2016), essential for attention and learning processes (Hasselmo and Sarter, 2011; Hangya et al., 2015). There are also less familiar interactions between relaxin-3/GABA and orexin/glutamate (Blasiak et al., 2015), corticotropin-releasing factor (CRF) (Ma et al., 2013) and serotonin systems (Lawther et al., 2015). We will highlight new data on the function of these systems and the known or putative roles of their co-transmitters.

2. Basal forebrain neurons that release acetylcholine and GABA may control cortical plasticity and learning

The basal forebrain (BF) gives rise to one of the best characterised neuromodulatory hubs, the cholinergic projection system. The unusually long axon trees of cholinergic neurons (Wu et al., 2014) innervate virtually all cortical areas and many subcortical centres (Figure 1A; Saper, 1984; Zaborszky et al., 2015). This wide-ranging network has been associated with many cognitive functions, most notably sustained attention, and learning and memory (Everitt and Robbins, 1997; Hasselmo and Sarter, 2011). Recently, evidence supporting the

co-release of the neurotransmitter GABA from cholinergic terminals has been reported (Saunders et al., 2015a,b; Tritsch et al., 2016) and here we discuss possible implications of this finding in light of recent advances regarding cholinergic function.

2.1. Acetylcholine co-transmitters

Recent studies have demonstrated that, in addition to acetylcholine (ACh), forebrain cholinergic neurons can release other neurotransmitters. It has been shown that two separate cholinergic systems, the tonically-active interneurons of the striatum (Gras et al., 2002; Guzman et al., 2011; Higley et al., 2011) and the cholinergic cells of the medial habenula (Ren et al., 2011) can release both ACh and glutamate. Co-localisation of the vesicular glutamate transporter VGLUT3 with cholinergic markers was also demonstrated in the basal forebrain (Manns et al., 2001; Gritti et al., 2006; Nickerson Poulin et al., 2006) and cultured BF neurons were shown to release both acetylcholine and glutamate (Allen et al., 2006; Huh et al., 2008). However, basal forebrain neurons expressing both choline acetyltransferase (ChAT) and VGLUT3 appear to, more or less, specifically project to the amygdala, and co-release of glutamate in the cortex by cholinergic neurons has not been detected (Nickerson Poulin et al., 2006).

While cortical co-release of ACh and glutamate hitherto lacks solid support, a recent study demonstrated that cholinergic cells can also release the inhibitory transmitter, GABA (Saunders et al., 2015b). This is in line with previous reports of the co-localisation of ChAT and the vesicular GABA transporter (VGAT) (Kosaka et al., 1988; Fisher and Levine, 1989; Beaulieu and Somogyi, 1991). Saunders and colleagues (2015b) employed optogenetic stimulation of cholinergic axons and applied pharmacological blockade of postsynaptic currents to demonstrate the dual nature of transmitter release onto layer 1 interneurons in the cortex. Therefore, the question remains whether ACh and GABA are released from the same vesicles (‘co-release’), different vesicles of the same terminals (‘co-transmission’) or even different terminals (Figure 2). Saunders *et al.* (2015b) performed array tomography to quantify co-localisation at the terminal level and found that both single-transmitter and double-transmitter terminals can be identified in the cortex. It is worth noting that BF cholinergic neurons may not be unique regarding a capability of ACh-GABA co-transmission, as cortical (GABA) interneurons immunopositive for vasoactive intestinal peptide (VIP) co-express ChAT. Whether this co-expression is a reflection of functional co-transmission is not known. Nevertheless, this should be taken into account when investigating co-release from BF cholinergic neurons, since global ChAT-ChR2 transgenic lines as well as staining at terminal levels do not distinguish local cortical and BF cholinergic/GABAergic terminals.

2.2. Inputs that stimulate basal forebrain acetylcholine/GABA neurons and downstream effects

There is a wealth of anatomical evidence for diverse inputs to BF cholinergic neurons, including from the striatum, frontal cortex, brainstem, lateral hypothalamus and other neuromodulatory centres (Zaborszky et al., 2012). Recently, two elegant studies using rabies viruses for monosynaptic retrograde tract tracing reached similar conclusions regarding the proportional inputs to cholinergic neurons (Do et al., 2016; Hu et al., 2016). However, little

is known about the firing of the input neurons to cholinergic cells during behaviour and how these inputs are integrated by cholinergic dendrites and cell bodies to drive activity.

In contrast, many studies have probed the downstream effects of the cholinergic system on the activity of target neurons, cortical and subcortical networks and behaviour. At the cellular level, both principal neurons and interneurons express a diverse array of muscarinic (metabotropic) and nicotinic (ionotropic) cholinergic receptors, both pre- and postsynaptically. ACh is capable of depolarising and/or hyperpolarising specific target neurons through these receptors on multiple time scales, and can exert an intricate influence on the release of multiple neurotransmitters (Gu, 2002; Demeter et al., 2013; Lin et al., 2015). At the circuit level, ACh has been shown to desynchronise cortical networks, decrease pairwise correlations among neurons, increase the processing of bottom-up versus top-down inputs, and increase cortical activation in general (Buzsaki et al., 1988; Metherate et al., 1992; Pinto et al., 2013; Avery et al., 2014; Sugihara et al., 2016). Importantly, ACh has a strong influence on synaptic plasticity both in terms of controlling sensory receptive fields and tuning curves, and controlling spike timing-dependent plasticity on a fine temporal scale (Kilgard and Merzenich, 1998a; Disney et al., 2007; Froemke et al., 2007; Seol et al., 2007; Berg, 2011; Gu and Yakel, 2011). Finally, at the behavioural level, the ability of central cholinergic neurons to increase cortical activation and fine-tune synaptic plasticity may underlie their widely observed effects on attention, and learning and memory (Everitt and Robbins, 1997; Shuler and Bear, 2006; Hasselmo and Sarter, 2011; Letzkus et al., 2011; Pinto et al., 2013).

Since ACh/GABA co-release has only been demonstrated recently, we can only speculate on the interactions between these co-transmitters. Given the widespread involvement of the cholinergic system in plasticity, one candidate function of the GABA co-transmission may be the fine control of cortical plasticity (Seol et al., 2007; Gu and Yakel, 2011; Hasselmo and Sarter, 2011; Lin et al., 2015; Tritsch et al., 2016). Thus, GABA signalling at postsynaptic sites may influence how the cholinergic system tunes synaptic weights to modify receptive fields, tuning curves, and its contribution to reinforcement learning processes (Kilgard and Merzenich, 1998b; Yu and Dayan, 2005; Disney et al., 2007; Froemke et al., 2007, 2013). Alternatively, GABA release may also be a source of plasticity in the sense that differential control of presynaptic GABA release, both temporally and spatially, including during bursts of cholinergic neurons, may influence the strength of cholinergic modulation in a spatiotemporally and cell type specific manner. Therefore pre- and postsynaptic GABA signalling may provide rapid regulation of cholinergic control of cortical plasticity. Additionally, by inhibiting interneurons that target principal cells, GABA released from cholinergic terminals may add to the disinhibitory effects mediated by cholinergic activation, including disinhibition via cortical layer 1 and VIP interneurons (Letzkus et al., 2011; Lee et al., 2013; Pi et al., 2013; Fu et al., 2014; Granger et al., 2016; Tritsch et al., 2016). This may lead to enhancement of the signal to noise ratio in processing sensory signals and contribute to the cholinergic enhancement of ‘bottom-up’ information transfer (Sarter et al., 2001).

2.3. Temporal release of acetylcholine and its co-transmitters

Understanding the behavioural contingencies that determine the precise timing of acetylcholine release would inform how cholinergic neurons mediate different cognitive processes. By measuring ACh release with choline sensitive voltammetry probes, it has been revealed that cholinergic transients in the medial prefrontal cortex are related to successful detection of visual cues in a sustained attention task and are considerably faster than previously appreciated (Parikh et al., 2007; Sarter et al., 2009). Moreover, these cholinergic transients were absent after consecutive detections, and were therefore associated with attentional shifts (Howe et al., 2013).

Nevertheless, there is little direct data on the activity of cholinergic neurons in behaving animals. However, a recent study probed the activity of optogenetically identified cholinergic neurons of the mouse nucleus basalis and horizontal nucleus of the diagonal band of Broca, two distinct parts of the BF cholinergic system with non-overlapping efferent projections (Hangya et al., 2015). Mice were trained on an auditory detection task that required sustained attention and incorporated reinforcement learning. Surprisingly, while some non-cholinergic neurons exhibited strong attentional correlates, BF cholinergic neurons were instead activated with short latency and high precision after punishment and reward. This is in agreement with two calcium imaging studies in which cholinergic fibres in the hippocampus (Lovett-Barron et al., 2014) and cholinergic cell bodies in the BF (Harrison et al., 2016) were found to report airpuff punishments. Moreover, cholinergic responses scaled with the unexpectedness of the reinforcer, i.e., ‘reinforcement surprise’ (Hangya et al., 2015), or more simply, cholinergic neurons responded when reward was unexpected, but displayed no or very little response to expected reward (Hangya et al., 2015). This is the likely reason for a failure to detect strong reward responses in a related study using imaging techniques, where reward delivery was predictable and thus behaviourally expected (Harrison et al., 2016).

These results suggest cholinergic neurons may be strongly involved in learning through reinforcement (Everitt and Robbins, 1997; Yu and Dayan, 2005; Hasselmo and Sarter, 2011; Letzkus et al., 2011), and are capable of precisely timing cortical activation with respect to reinforcement delivery (Buzsaki et al., 1988; Disney et al., 2007; Pinto et al., 2013). Whether there is a one-to-one coupling between acetylcholine release and firing of cholinergic neurons (Sarter et al., 2009, 2014), and whether ACh and GABA are released with similar temporal dynamics after reward and punishment, are important open questions.

3. Histaminergic neurons that promote wakefulness co-transmit GABA

Histamine is well known as a neurotransmitter involved in peripheral immune system signalling, as upon noxious stimulation, mast cells co-release large quantities of histamine and other chemicals which cause vasodilation. A common side-effect of antihistamines (specifically histamine H1 receptor antagonists) is drowsiness, providing direct support for the wake-promoting role for histamine (Monnier et al., 1967; Nicholson et al., 1991). Its presence in brain was discovered in 1943 (Kwiatkowski, 1943), and histamine neurons are exclusively located in the posterior hypothalamic tuberomammillary nucleus (TMN) (Watanabe et al., 1983; Panula et al., 1984; Takeda et al., 1984), as these neurons express

histidine decarboxylase (HDC), the enzyme which synthesises histamine by decarboxylating the amino acid, histidine (Haas et al., 2008). Although TMN neurons are the only neuronal source of brain histamine, microglia have been reported to also express *hdc* mRNA (Katoh et al., 2001). In this regard, central histamine also regulates microglial migration and cytokine release underlying brain immune responses (Rocha et al., 2014).

3.1. Histamine co-transmitters

It has been known for many years that histamine neurons express glutamic acid decarboxylase (GAD) enzymes and GABA (Takeda et al., 1984; Airaksinen et al., 1992; Trottier et al., 2002; Kukko-Lukjanov and Panula, 2003; Sundvik and Panula, 2012), and in fact, TMN neurons were identified as GABAergic before they were identified to be histaminergic (Vincent et al., 1983). TMN histamine neurons also express the vesicular GABA transporter, VGAT (Yu et al., 2015), which is consistent with the mRNA distributions of GABAergic markers *gad1*, *gad2* and *vgat* mRNA in the mouse TMN (www.brain-map.org; Lein et al., 2007). In addition to GABA, a subpopulation of histaminergic TMN neurons in the rat co-express thyrotropin releasing hormone and galanin (Airaksinen et al., 1992; Chotard et al., 2002), but the latter expression was not observed in human (Airaksinen et al., 1992; Trottier et al., 2002).

Histamine acts as a classical neuromodulator in a similar manner to noradrenaline, dopamine or ACh (Bolam and Ellender, 2016). It regulates, enables and adjusts the activity of various neural circuits by binding to metabotropic H1, H2 or H3 receptors (Haas et al., 2008; Panula et al., 2015). For example, histamine inhibits ACh, glutamate or GABA release by binding to presynaptic hetero-H3 receptors, it can depolarise neurons by binding to H1 or H2 receptors, or generate changes in phosphorylation of ion channels which influence the rate at which neurons fire action potentials (Atzori et al., 2000).

3.2. Inputs that stimulate TMN histamine/GABA neurons and downstream effects to promote wakefulness

TMN histaminergic neurons are inhibited by GABAergic and galaninergic inputs from the ventrolateral preoptic area, which promote sleep (Sherin et al., 1998). Other neural inputs include hypothalamic orexin/glutamate neurons that provide some excitatory input to histamine neurons (Schone et al., 2014). Similar to the other neural projection hubs discussed in this review (ACh, orexin, relaxin-3), TMN histamine neurons send long-range projections throughout the brain, for example, to the neocortex, striatum and other hypothalamic areas (Figure 1B; Panula et al., 1989; Wada et al., 1991) and vesicular histamine is transported via the monoamine transporter, VMAT2. Notably, histaminergic axons do not form synapses, as assessed by electron microscopy (Takagi et al., 1986), consistent with volume or paracrine, rather than synaptic transmission (Bolam and Ellender, 2016).

In vivo unit recordings in mice have demonstrated a correlation between a population of TMN neurons and wakefulness, whereby ~5 Hz action potentials were observed after transition from slow-wave sleep to wake, continued firing occurred during wakefulness, and a cessation of firing occurred before the onset of electroencephalogram (EEG)

synchronisation and sleep (Sakai et al., 2010). Interestingly, these firing characteristics were unaltered in TMN neurons of *hdc*-knockout mice (Sakai et al., 2010), suggesting that endogenous histamine is not necessary for the expression of the firing properties of these neurons. Consistent with a wealth of earlier findings (Monnier et al., 1967; Lin et al., 1988, 1989; Nicholson et al., 1991; Parmentier et al., 2002; Haas et al., 2008; Schone et al., 2014), direct and selective pharmacogenetic activation of histamine neurons causes hyperactivity and wakefulness (Yu et al., 2015). Histamine neuron activation also promotes feeding, sexual appetite and locomotion, and motivated behaviour in general, all diverse aspects of wakefulness (Torrealba et al., 2012; Riveros et al., 2015).

3.3. Dissecting the paracrine contributions of histamine and GABA functions co-release

Despite the long-standing suspicion that histamine and GABA were putative co-transmitters, the specific function of GABA was unclear, even though the presence of GABA in histaminergic neurons of all vertebrates examined, suggested an essential function. Considering the paracrine fashion of histamine release (Takagi et al., 1986), GABA likely functions via similar volume transmission. In this regard, GABA is an electroneutral zwitterion at physiological pH, with reduced binding to the extracellular matrix (Roberts and Sherman, 1993). Thus, it can diffuse widely, and would only be limited by GABA reuptake transporters that limit long-range diffusion. Ambient, non-synaptic GABA has been demonstrated to produce sustained inhibitory currents (known as G_{tonic}) by activating high-affinity extrasynaptic ionotropic $GABA_A$ receptors that contain delta subunits (Brickley and Mody, 2012; Yu et al., 2015).

Prior to current genetic manipulation technology, there was no clear way to selectively test the GABAergic role of histamine neurons. More recently, their expression of the *hdc* gene allowed a way to selectively manipulate these neurons through generation of HDC-Cre recombinase mice (Zecharia et al., 2012). This mouse line was used to ‘knock down’ the *vgat* gene, and thus GABA release, selectively in histamine neurons (Yu et al., 2015). The resultant mice exhibited increased locomotion, and significantly enhanced wakefulness during the dark (active) phase (Yu et al., 2015). Selective optogenetic stimulation of histaminergic projections to the neocortex and caudate-putamen increased G_{tonic} onto pyramidal neurons and medium spiny neurons, respectively, and *vgat* knock-down abolished these evoked tonic inhibitory currents, but they still occurred in the presence of H1 and H2 receptor antagonists (Yu et al., 2015). Thus, during wakefulness, GABA is delivered widely in neocortical and striatal circuitry by volume transmission and is co-transmitted with histamine to regulate and constrain promotion of wakefulness by histamine. However, there appears to be heterogeneity in TMN histamine neurons, whereby not all of them co-transmit GABA. Selective optogenetic stimulation of histamine neuron projections to the preoptic hypothalamus induces only histamine release which, however, stimulates local GABAergic neurons to produce an overall net inhibitory effect (Williams et al., 2014). Thus, histamine/GABA co-transmission is circuit specific.

The precise role for co-release of GABA and histamine, and its apparent stop-go nature, with GABA being inhibitory and histamine being excitatory, is not known. The GABA component may be neuroprotective, as excessive stimulation of circuits for wakefulness

could be excitotoxic. In this regard, mice with *vgat* knock down selectively in histaminergic neurons are extremely hyperactive during their circadian wake period (Yu et al., 2015). Alternatively, tonic GABA inhibition could mediate precision of, for example, pyramidal cell firing (Wlodarczyk et al., 2013), whereby the G_{tonic} component of histamine signalling could serve to strengthen and complement the enhancement of cognitive and attentional processes produced by histamine. Studies on GABA co-transmission or co-release, with peptides or glutamate, observed fast monosynaptic $GABA_A$ receptor responses, and not a slow G_{tonic} response (Tong et al., 2008; Tritsch et al., 2012; Jego et al., 2013; Root et al., 2014; Shabel et al., 2014; Tritsch et al., 2014, 2016). An intriguing example is GABA co-release with dopamine, where GABA is not synthesised by dopamine neurons, but is instead transported into axons by mGAT1 and mGAT4 for packaging into dopamine vesicles by VMAT2 (Tritsch et al., 2012, 2014, 2016). For histamine neurons, no compensatory function of VMAT2 was observed (Yu et al., 2015). Furthermore, GABA containing vesicles are distinct from those containing histamine in TMN neurons, suggestive that these neurotransmitters are co-transmitted and not co-released (Kukko-Lukjanov and Panula, 2003). Future studies would be needed to determine if different firing patterns or frequencies differentially promote GABA and/or histamine release.

4. Orexin and glutamate co-transmission in promoting arousal

In 1998, two laboratories independently discovered the neuropeptides orexin-A/hypocretin-1 and orexin-B/hypocretin-2 peptides (de Lecea et al., 1998; Sakurai et al., 1998). These peptides were shown to have excitatory activity in cultured hypothalamic neurons (van den Pol et al., 1998) and to be synthesised exclusively in the CNS by a neural hub in the perifornical lateral hypothalamus with widespread projections (Figure 1C; Peyron et al., 1998). Among many target areas, these projections conspicuously terminated within regions associated with feeding, metabolism, arousal and reward (Kilduff and Peyron, 2000), including the histaminergic TMN and other hypothalamic structures, the noradrenergic locus coeruleus (LC), the serotonergic dorsal raphe (DR), the dopaminergic ventral tegmental area (VTA), and the cholinergic BF and mesopontine laterodorsal tegmental nucleus (LDT) and pedunculopontine nucleus (PPT). Through a convergence of compelling genetic studies, a key function of these neurons was revealed to be the stabilisation of waking and sleep states. Indeed genetic deletion of orexin peptides or their two known receptors in mice resulted in a phenotype strikingly similar to the human sleep-disorder, narcolepsy (Chemelli et al., 1999; Lin et al., 1999; Kinsanuki et al., 2001; Mochizuki et al., 2004; Kalogiannis et al., 2011). Moreover, the *canarc-1* gene, which confers heritable narcolepsy in dogs was discovered to be a null mutation of the orexin-2 receptor gene (Lin et al., 1999). These animal models all displayed key features of narcolepsy in humans, including fragmentation of wake and sleep periods and epochs of sudden postural atonia during waking, termed cataplexy. Shortly thereafter, it was shown that human narcoleptics with cataplexy were orexin-peptide deficient (Peyron et al., 2000) and that the orexin neurons themselves are largely absent in brains from adult narcoleptic patients (Thannickal et al., 2000). Thus, loss of orexin peptides or receptors in animals produces a phenotype very similar to human narcolepsy, which itself results from a loss of orexin neurons.

4.1. Orexin co-transmitters

Orexin neurons express a number of other neuropeptides, including galanin (Håkansson et al., 1999), dynorphin (Chou et al., 2001) and neuronal activity-regulated pentraxin (Reti et al., 2002). There is also strong evidence for co-expression of vesicular glutamate transporters, VGLUT1 and VGLUT2 (VGLUT2>VGLUT1) (Rosin et al., 2003; Henny et al., 2010), but not GAD67, suggesting that glutamate and orexin and perhaps other peptides, are co-transmitters. Indeed, electron microscopy studies in the TMN and other hypothalamic areas, and in VTA, LC, LDT and DR, indicate orexin-immunoreactive neurons form asymmetric and some symmetric synapses, having a typical synaptic ultrastructure with small synaptic vesicles clustered near active zones, and larger, dense-core orexin-immunoreactive vesicles further away (Horvath et al., 1999; Diano et al., 2003; Torrealba et al., 2003; Balcita-Pedicino and Sesack, 2007; Cid-Pellitero and Garzon, 2011; Del Cid-Pellitero and Garzón, 2011).

Physiological evidence for glutamate co-transmission in orexin projections has been obtained in the TMN and LC, using optogenetic stimulation of orexin axons (Figure 1C) (Schöne et al., 2012, 2014; Sears et al., 2013). In the TMN, brief light pulses that evoked presynaptic action potentials, also evoked short latency EPSPs that were abolished by AMPA receptor antagonism with CNQX (Schöne et al., 2012, 2014). Moreover, these EPSPs rapidly increased the firing of TMN neurons with trains of light pulses. These light-evoked fast EPSPs also displayed paired-pulse depression, which caused the evoked EPSPs to substantially diminish in size across pulse trains of 10 s. Interestingly, it was only during this time course of synaptic depression that orexin was released to act on TMN neurons and promote firing by means of a slow onset and long lasting depolarising current (Schone et al., 2014). Thus, as observed in other peptide-releasing terminals, orexin release requires sustained high-frequency firing (> 10 Hz) (for review, see van den Pol, 2012).

One putative function of glutamate and orexin co-transmission is to provide complementary signals related to orexin neuron firing, with the fast EPSPs conveying a signal proportional to the *time derivative* of input firing, and orexin conveying a signal proportional to the *time integral* of input firing (Schöne et al., 2014). This type of control, if deployed in a feedback circuit can effectively promote circuit stability and may help maintain consolidated states of waking, depending on circuit structure (Schöne et al., 2014).

4.2. Inputs that stimulate hypothalamic orexin/glutamate neurons and downstream effects to promote wakefulness

Neural inputs regulating orexin neurons arise from BF cholinergic neurons, preoptic area GABAergic neurons, serotonergic raphe neurons, lateral septum, bed nucleus of the stria terminalis, dorsomedial nucleus, periaqueductal grey, amygdala, and many hypothalamic regions (Sakurai et al., 2005; Yoshida et al., 2006). The orexin/glutamate system has been proposed to receive negative feedback from wake-promoting modules in order to provide dynamic behavioural control of arousal and wakefulness (Schöne and Burdakov, 2016). Evidence for this negative feedback schema is strongest for the serotonergic system since orexin neurons are inhibited by serotonergic inputs from the dorsal and median raphe. Optogenetic activation of serotonergic terminals in the lateral hypothalamus directly

inhibited orexin neurons via $5HT_{1A}$ receptors, and also indirectly inhibited orexin neurons by facilitating GABAergic inhibitory inputs, without affecting glutamatergic inputs (Chowdhury and Yamanaka, 2016). Moreover, orexin neurons receive dense glutamatergic and GABAergic inputs from the substantia innominata and magnocellular preoptic area, but no innervation from BF cholinergic neurons (Agostinelli et al., 2017).

In addition to discovering orexin peptides, Sakurai et al. (1998) identified two (previously orphan) G-protein-coupled receptors that bind and are activated by orexins, which they termed the orexin-1 and -2 receptors (OX1, OX2). These receptors have distinct but partially overlapping expression patterns throughout the CNS (Marcus et al., 2001). Many studies have investigated the excitatory/depolarising effects of orexin/glutamate neurons on target brain nodes regulating sleep/wakefulness (via BF, LDT/PPT, preoptic area, raphe nucleus, TMN and LC), feeding (amygdala, nucleus accumbens, hypothalamic paraventricular and arcuate nuclei, and nucleus of the solitary tract) and reward/emotion (lateral septum, amygdala, nucleus accumbens, hypothalamic paraventricular nucleus, VTA, and nucleus incertus) (Kohlmeier et al., 2013; Schöne et al., 2014; Kastman et al., 2016; for reviews see Leonard and Kukkonen, 2014; Schöne and Burdakov, 2016). Given its relatively slow time course, orexin signalling is well suited to modulate the postsynaptic integration of fast synaptic inputs, including glutamatergic EPSPs arising from co-transmission, as suggested by two recent studies. The first examined whether the unusually large membrane current noise associated with orexin depolarisation in cholinergic LDT and PPT neurons and serotonergic DR neurons could provide high frequency input, in addition to a slow depolarisation (Ishibashi et al., 2015). The ‘noisy’ orexin input doubled the spectral amplitude of membrane current at gamma band frequencies in cholinergic neurons and more than tripled it in serotonergic neurons. Using a dynamic clamp to add a virtual orexin current further revealed that this noisy input activates an intrinsic, Ca^{2+}-dependent resonance centred on theta and alpha frequencies in these neurons. These findings suggest the noisy orexin input can function to boost the effectiveness of EPSPs in the theta and alpha frequency range by stochastic resonance and by engaging intrinsic postsynaptic resonances within these target neurons. The second study revealed that activation of OX1 and OX2 strongly enhanced the post-spike afterhyperpolarisation in serotonergic DR neurons (Ishibashi et al., 2016). This orexin-enhanced AHP strongly altered the firing properties of DR neurons by increasing spike-frequency adaptation. In signal-processing terms, enhanced spike-frequency adaptation would increase the high-pass filter characteristics of these neurons by attenuating firing to steady inputs, without attenuating firing to rapidly varying inputs like those encoding behavioural events.

In the DR, this may be particularly important for reward signal processing, since orexin neurons increase firing during reward (Hassani et al., 2016) and the firing of most serotonergic DR neurons transition from tonic to burst firing during reward acquisition (Li et al., 2016). Thus, orexin receptor signalling during active waking, when orexin neurons are firing, would enhance encoding of rapidly varying signals from glutamate inputs, including those mediated by co-transmission, and promote phasic outflow from raphe neurons. In the absence of orexin, this signal-processing would be impaired and the effectiveness of fast synaptic inputs including those mediated by co-transmission would be attenuated. Indeed, perhaps the loss of these postsynaptic tuning actions in the absence of orexin, limit the

behavioural impact of signals conveyed by glutamate co-transmission (see next section). Further experiments will be necessary to directly test this idea.

4.3. Implications of orexin and glutamate co-transmission for behaviour

To date, the possible behavioural implications of glutamate/orexin co-transmission have been explored indirectly, via three lines of experimentation. First, optogenetic stimulation of orexin neurons in mice produced sleep-to-wake transitions at an average latency of ~30 s, which was shorter than the average latency following stimulation in control mice (~60 s) (Adamantidis et al., 2007). If glutamate co-transmission was an important contributor to this latency shortening, waking latencies would be expected to remain shorter than control following disrupted orexin signalling. However, optical stimulation of orexin neurons did not produce significantly shorter latency waking when OX1 was blocked by an antagonist or in orexin peptide null mice. This indicates orexin signalling is necessary and suggests that glutamate co-transmission is insufficient to drive short latency waking.

Secondly, while genetic ablation of orexin neurons would be expected to result in more severe phenotypes than just the knockout of the orexin peptides, total ablation of orexin neurons, produced by expressing a toxic ataxin-3 gene product in them, produced a narcolepsy phenotype with only subtle differences to that produced by orexin peptide deletion (Hara et al., 2001). These findings suggest orexin signalling is required for stabilising behavioural states and suppressing cataplexy, rather than potential co-transmitters. However, these experiments need to be interpreted with caution since phenotype severity is often sensitive to genetic background and environmental factors, which was clearly illustrated by the late-onset obesity which is much more severe in orexin/ataxin-3 mice than orexin knockout mice with mixed genetic backgrounds than when both transgenes are on a pure C57BL6 genetic background. Despite this, mice lacking orexin neurons still become more obese than orexin null mice when they are both on a C57BL6 background and fed high-fat chow, suggesting that one or more co-transmitter plays a discernible role in regulating metabolism (Hara et al., 2005).

Finally, the narcolepsy phenotype in orexin neuron-ablated mice was effectively rescued by either ICV orexin or non-specific central re-expression of the prepro-orexin peptide (Mieda et al., 2004), suggesting that orexin is also sufficient to stabilise waking and suppress cataplexy in the absence of orexin neurons and their co-transmitters. In this regard, it is noteworthy that in addition to making morphological synapses, orexin-immunoreactive processes containing dense-core vesicles also make membrane appositions and free varicosities lacking synaptic structure, suggesting the non-synaptic release of orexin without glutamate release may also occur (Balcita-Pedicino and Sesack, 2007; Cid-Pellitero and Garzon, 2011; Del Cid-Pellitero and Garzón, 2011). These studies do not rule out a behavioural role for glutamate co-transmission, but suggest that a role in these behaviours depends on the integrity of orexin signalling.

5. Relaxin-3/GABA and nucleus incertus networks in promoting arousal

In comparison to the ACh/GABA, histamine/GABA and orexin/glutamate systems, the relaxin-3/GABA system is currently not as well characterised. Relaxin-3 is still considered a

relatively ‘novel’ neuropeptide, although it was discovered quite soon after the orexins (Bathgate et al., 2002). It is synthesised in neuronal populations of the midbrain and pons, and the distribution of these neurons and relaxin-3-immunoreactive projections have been mapped in rat (Burazin et al., 2002; Tanaka et al., 2005; Ma et al., 2007), mouse (Smith et al., 2010), and *Cynomolgus* monkey (Ma et al., 2007). Although it the most recently discovered member of the relaxin peptide family, bioinformatics analysis revealed that the relaxin-3 gene is highly conserved and relaxin-3 is the ancestral peptide of the relaxin and insulin-like peptide superfamily (Wilkinson et al., 2005). Similarly, analysis of relaxin-3 immunoreactivity in neural projections of rodent and primate brain reveals the neuroanatomical distribution of relaxin-3 and its projection targets are highly conserved (Ma et al., 2007, 2009b; Smith et al., 2010).

Relaxin-3 is synthesised in four pontine/midbrain regions: nucleus incertus (NI), pontine raphe (PnR), periaqueductal grey (PAG) and an area dorsal to the substantia nigra (dSN) (Figure 1D). The receptor for relaxin-3 is known as RXFP3 (relaxin family peptide 3 receptor) (Liu et al., 2003; Ma et al., 2017b), which is similarly distributed to relaxin-3-containing nerve fibres. RXFP3 is generally associated with inhibitory actions on intracellular cAMP signalling via $G_{i/o}$-protein coupling (Liu et al., 2003), but in brain, RXFP3 activation is capable of both hyperpolarisation and depolarisation of neurons (Blasiak et al., 2013).

5.1. Relaxin-3 co-transmitters

The NI contains the major population of relaxin-3 neurons (Ma et al., 2017b), and is a compact GABAergic nucleus located caudal to DR, below the fourth ventricle and dorsal tegmental nucleus of Gudden, midline of the LC and LDT, and anterior to the prepositus hypoglossal nucleus (Goto et al., 2001; Olucha-Bordonau et al., 2003). It is positioned among other well characterised brainstem neural hubs important for arousal and sleep (Brown and McKenna, 2015; Weber and Dan, 2016). While largely GABAergic (Ford et al., 1995), NI neurons are neurochemically and functionally heterogeneous. In this regard, all relaxin-3 neurons are GABAergic, reflected by colocalisation with GAD65/7 and the calcium-binding protein, calbindin (Ma et al., 2007). However, the nature of, and interaction between, relaxin-3 and GABA transmission in these neurons remains to be examined, using similar electrophysiological and genetic technology to that applied to the histamine/GABA and orexin/glutamate systems.

5.2. Inputs that stimulate brainstem relaxin-3/GABA neurons and downstream effects on behaviour and arousal

In the rat brain, the NI receives primary neural inputs from pyramidal neurons of the prelimbic and anterior cingulate cortices, lateral habenula, interpeduncular nucleus, median raphe, medial septum, preoptic area and lateral hypothalamus (Goto et al., 2001; Olucha-Bordonau et al., 2003). These inputs suggest the NI integrates information largely related to behavioural planning and hippocampal function, however the excitatory or inhibitory nature of these inputs, and inputs specific to relaxin-3 neurons, remain to be characterised. Approximately half of all NI neurons express CRF receptor-1 (CRF_1), which includes all relaxin-3 neurons; and in urethane-anesthetised rats, all relaxin-3 neurons (and some non-

relaxin-3 neurons) exhibited increased spontaneous firing in response to CRF, whereas a population of non-relaxin-3 neurons exhibited decreased firing (Ma et al., 2013). This CRF input appears to originate from the preoptic area (Ma et al., 2013). More recent studies demonstrated that intra-NI infusion of an CRF_1 antagonist blocked yohimbine-induced reinstatement of alcohol seeking in alcohol-preferring (iP) rats (Walker et al., 2016), whereas intra-NI infusion of a dopamine D2/3 agonist, resulted in hypolocomotion via inhibitory D2 receptors expressed by CRF_1/relaxin-3 neurons (Kumar et al., 2015).

Orexins also regulate the activity of NI and relaxin-3 neurons via OX2 (Blasiak et al., 2015; Kastman et al., 2016). NI relaxin-3 neurons receive an orexinergic innervation from neurons in the lateral hypothalamus *and* perifornical area, and orexin-A produced depolarisation and action potential firing of NI neurons *in vitro* via OX2 (Blasiak et al., 2015). Furthermore, intra-NI injection of an OX2 antagonist attenuated yohimbine-induced reinstatement of alcohol seeking in iP rats (Kastman et al., 2016) and intra-NI injections of orexin-A enhanced locomotor activity and food intake in Sprague-Dawley rats.

Similar to the aforementioned systems, neural tract-tracing of NI neurons revealed broad, long-range projections to cortical and sub-cortical regions (Figure 1D). These connections suggest that the NI is positioned to modulate prefrontal and hippocampal cortical activity, locomotor behaviour, attentive states, and learning processes, in part via dense connections with components of the septohippocampal system and cortex (Goto et al., 2001; Olucha-Bordonau et al., 2003), and there is accumulating experimental evidence that supports these functions (for review see Ma and Gundlach, 2015). The projection pattern of NI neurons corresponds well with the distribution of relaxin-3-immunoreactive axons/terminals and RXFP3 mRNA (Ma et al., 2007), suggesting that relaxin-3 is largely produced and transported by NI neurons.

Early functional studies demonstrated that electrical stimulation or lesion of the NI induced or attenuated hippocampal theta rhythm (4–12 Hz oscillations in neural activity), respectively (Nunez et al., 2006); and hippocampal theta rhythm is important for memory processes (Vertes, 2005) and mood (Gray and McNaughton, 2000). Inactivation of the NI also impaired spatial learning and memory in rats trained in the Morris water maze task (Nategh et al., 2015). A recent study aimed at better understanding the function of NI neurons used a chemogenetic approach to stimulate the NI network in awake rats and assessed the effects on behaviour and cortical activity, via electroencephalogram (EEG) recordings. Clozapine-N-oxide (CNO)-induced activation of the hM3Dq DREADD (i.e. designer receptor activated by designer drug), expressed in NI neurons and throughout their projections resulted in increased wakefulness and locomotor activity in the homecage, detected in EEG and telemetry activity recordings, during the normal inactive phase (Ma et al., 2017a). Detailed spectral analyses identified enhanced cortical desynchronisation, particularly during periods of rest, such that cortical activity was similar that observed during active movement (Ma et al., 2017a). Furthermore, in rats conditioned to associate an audible tone with footshock, NI stimulation was associated with increased ‘active’, vigilant behaviours, such as head-scanning and darting, as opposed to passive behaviours, such as freezing, in response to impending threat (Ma et al., 2017a). Increased locomotor activity

and enhanced emotional reactivity is consistent with the 'operational definition' of general arousal (Quinkert et al., 2011).

Further studies are required to determine the sites of action and neuronal target populations that underlie the different effects of NI stimulation, as well as the contribution of relaxin-3 vs GABA transmission or relaxin-3/GABA co-transmission to these effects, perhaps using the approach adopted to characterise the histamine/GABA system (Yu et al., 2015). Nonetheless, data consistent with these findings was reported, with conventional electrical stimulation of the NI producing forward locomotion (Farooq et al., 2016). Taken together, these findings strongly suggest the NI receives multiple neurochemical inputs that are important for behavioural arousal and motivated behaviours, actions that are likely mediated by NI GABA transmission and, in some part, by relaxin-3/RXFP3 signalling.

5.3. Putative target nodes underlying relaxin-3/GABA signalling effects on arousal and behaviour

Likely downstream targets of the NI that mediate these behavioural effects include the medial septum, which is densely innervated by NI neurons (Goto et al., 2001; Olucha-Bordonau et al., 2003) and which, via pacemaker cell connections with the hippocampus, contributes to spatial navigation and learning and memory (Hangya et al., 2009; Pang et al., 2011; Roland et al., 2014). Moreover, recent studies have demonstrated that glutamatergic neurons in the medial septum control initiation and velocity of locomotion and associated entrainment of hippocampal theta oscillations (Bender et al., 2015; Fuhrmann et al., 2015; Robinson et al., 2016). In this regard, the medial septum expresses high levels of RXFP3 and infusion of an RXFP3-specific agonist into the region enhanced hippocampal theta oscillations, whereas an antagonist attenuated hippocampal theta oscillations and resulted in dose-dependent impairment of spatial working memory, assessed by the spontaneous alternation task in rats (Ma et al., 2009a).

Any interaction between the cholinergic and relaxin-3/NI systems is currently unclear, although high levels of acetylcholinesterase have been detected in the NI (Olucha-Bordonau et al., 2003), suggesting that cholinergic regulation of NI neurons may occur. Furthermore, NI neurons innervate the broad lateral preoptic area and BF (Goto et al., 2001; Olucha-Bordonau et al., 2003), which receives a dense relaxin-3 innervation and expresses high levels of RXFP3 (Ma et al., 2007). Therefore, it will be of interest to examine the possible interaction of NI/relaxin-3 afferents with cortically-projecting cholinergic and GABAergic neurons in the basal forebrain and the impact on cortical activity, attention and arousal.

It has been established that NI relaxin-3 neurons interact with neighbouring serotonergic neurons in the DR, whereby relaxin-3 neurons express 5-HT_{1A} receptors and central serotonin depletion by p-chlorophenylalanine administration resulted in elevated relaxin-3 mRNA expression (Miyamoto et al., 2008). A more recent study demonstrated that rats treated with the anxiogenic benzodiazepine, FG-7142, displayed enhanced anxiety-like behaviour that was associated with activated populations of relaxin-3 NI neurons and serotonergic DR neurons (Lawther et al., 2015), suggesting a functional association between these signalling systems in anxiety, associated with co-activation of serotonergic and relaxin-3 systems. Conversely, relaxin-3 fibres from the NI course through the DR, which

contains high levels of RXFP3 mRNA (Ma et al., 2007), although the effects of relaxin-3/RXFP3 signalling on serotonin neuron activity remains to be investigated.

The function of the smaller relaxin-3 neuron populations located in the PnR and dSN has not been investigated. The relaxin-3 neurons in the PAG have been shown to strongly innervate the thalamic intergeniculate leaflet (IGL) (Blasiak et al., 2013), which contains neuropeptide-Y (NPY) neurons that are known to modulate suprachiasmatic nucleus function and associated circadian rhythms (Muscat and Morin, 2006). The IGL is also a site of orexin action and modulation of sleep-wake balance (Nixon and Smale, 2005; Palus et al., 2015), but interactions between orexin and relaxin-3 signalling within the IGL have not yet been examined (Ma et al., 2007).

6. Conclusions

Although it was established several decades ago that exocytosis of one or more neuropeptides accompanies the release of classical neurotransmitters in most neurons (Burnstock, 2004), recent evidence is identifying the physiological importance of co-transmission of multiple neuromodulators with classical small-molecule neurotransmitters. The precise functions of neuromodulator/neurotransmitter co-release are still unclear, but a recent review on GABA co-transmission by Tritsch et al. (2016) speculated that it may contribute to (*i*) the fine-tuning of the magnitude and duration of synaptic effects, as is the case for histamine-GABA neurons, whereby GABA serves to provide a brake on overactivation by histamine (Yu et al., 2015), (*ii*) fine-tuning of the membrane potential of target neurons to optimise the actions of cotransmitters, and/or (*iii*) complementary modulation of the plasticity of synapses and neuronal circuits. Here we reviewed four co-transmitter systems that originate from hubs of neurons with long-range, highly connected projections - ACh/GABA, histamine/GABA, orexin/glutamate, and relaxin-3/GABA, all of which have been demonstrated to regulate arousal, wakefulness, and attention, as well as associated cognitive functions including learning and memory. Therefore, it is not surprising that these networks have identified anatomical and functional interactions. However, understanding how these multiple dual transmission systems are coordinated to cooperate or compete in facilitating similar cognitive functions, and how co-transmission shapes these network level interactions and behavioural functions, requires further research.

Acknowledgments

We thank Katalin Sviatkó for help with the figures. SM was supported by a National Health and Medical Research Council of Australia project grant (1067522). BH was supported by the ‘Lendület’ Program of the Hungarian Academy of Sciences (LP2015-2/2015) and he is a member of the FENS-Kavli Network of Excellence. CSL was funded by PHS grant NS027881. WW holds a joint Wellcome Trust Investigator Award (together with Nicholas P Franks; 107841/Z/15/Z). ALG was supported by a National Health and Medical Research Council of Australia Research Fellowship (1005985) and project grant (1067522), and a Brain & Behavior Research Foundation (USA) NARSAD Independent Investigator Award.

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Highlights

- Acetylcholine and GABA co-transmission may control cortical plasticity and learning.
- Histamine and GABA co-transmission are necessary for appropriate wakefulness.
- Orexin and glutamate co-transmission may function to stabilize wakefulness.
- Relaxin-3 and GABA co-transmission influence stress-related arousal and behaviours.

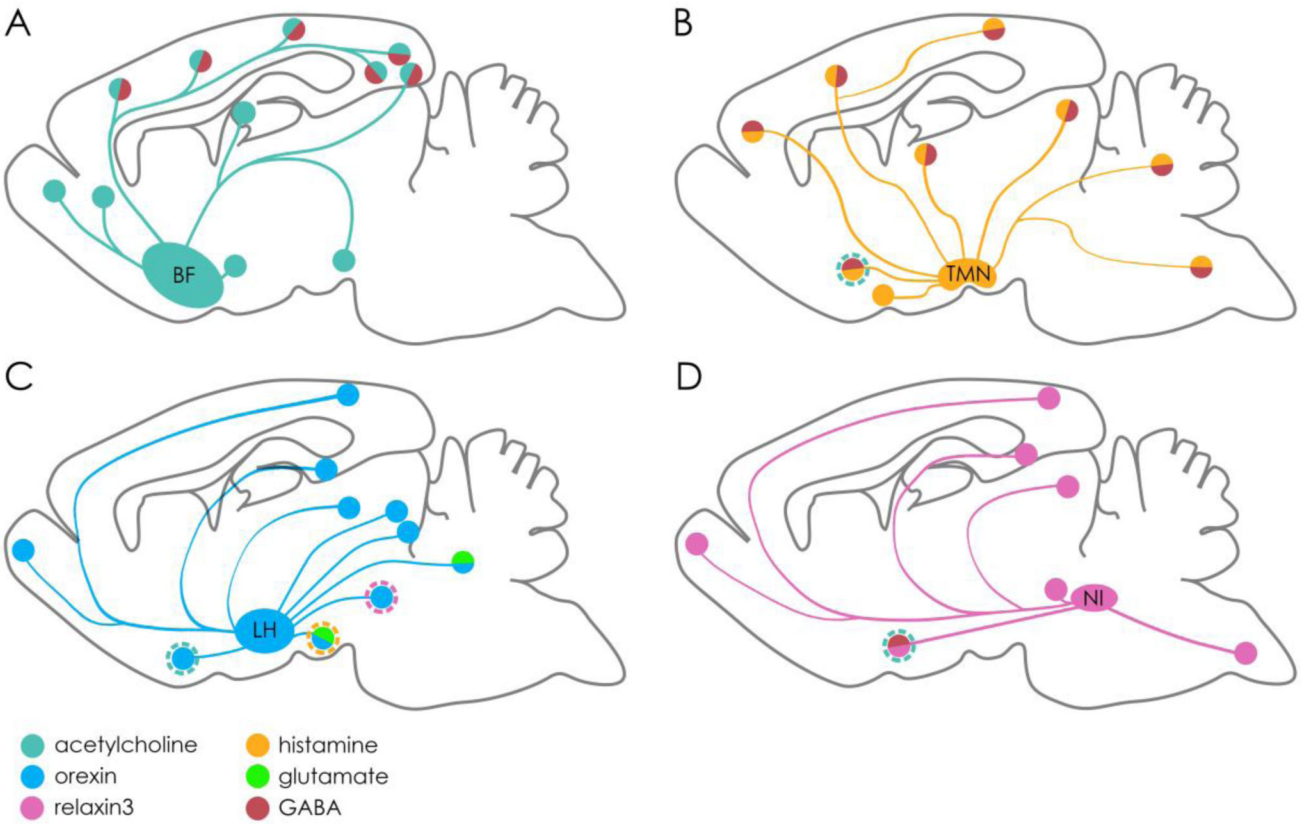

Figure 1. Dual neurotransmitter neural hubs regulating arousal, attention, learning and memory

(**A**) Basal forebrain (BF) cholinergic projections. Known sites of co-transmission are labelled by dual colour. (**B**) Histaminergic projections from the tuberomammillary nucleus (TMN). (**C**) Orexin projections from the lateral hypothalamus (LH). (**D**) Relaxin-3 projections from the nucleus incertus (NI). Known interactions between these systems are marked by dashed outlines.

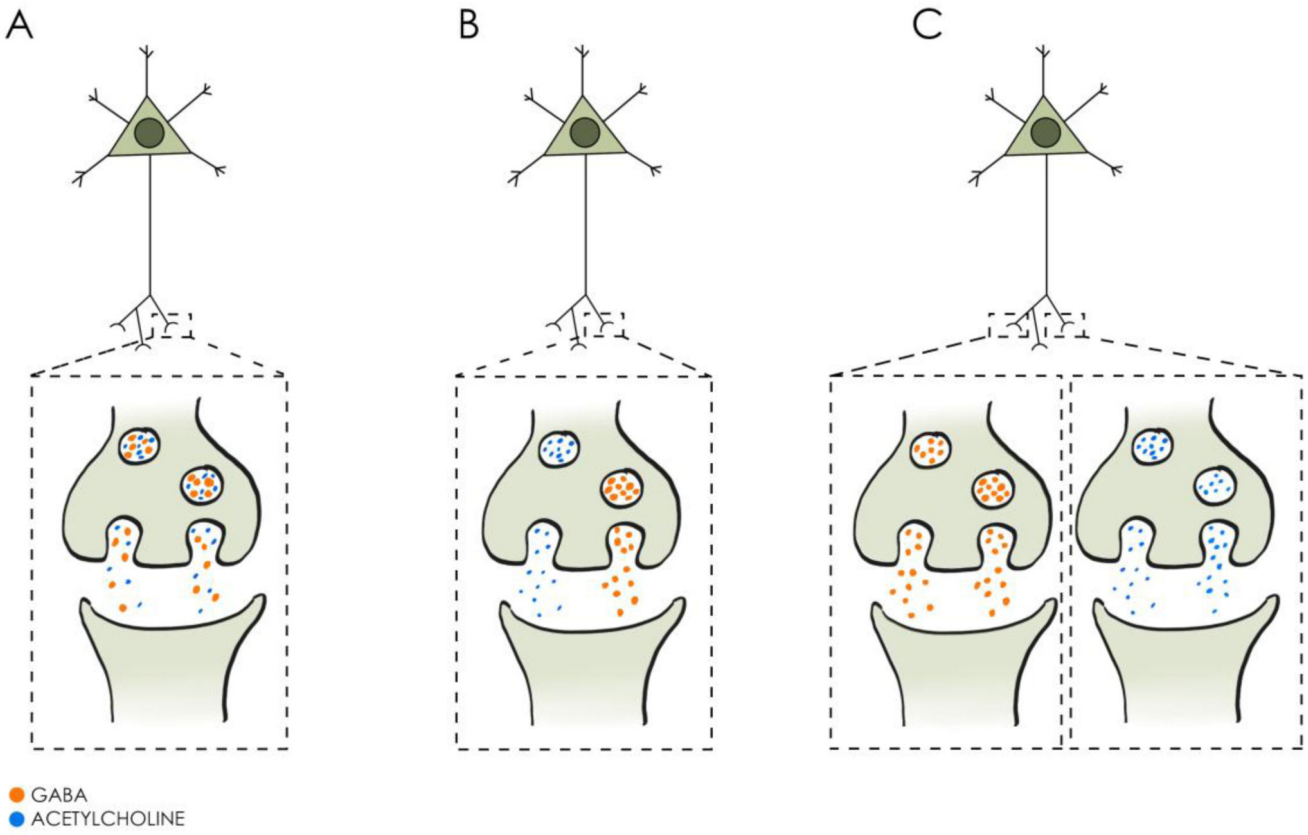

Figure 2. Co-release vs co-transmission of acetylcholine and GABA

ACh and GABA may be released from: (**A**) the same vesicles, (**B**) separate vesicles in the same terminals, or (**C**) separate terminals of cholinergic neurons.

Table 1

General overview of dual neurotransmitter systems.

Neuropeptide	Co-transmitter	Neuroanatomical location	References
AGRP	GABA	hypothalamic arcuate nucleus	(Dicken et al., 2015); (Wu and Palmiter, 2011); (Wu et al., 2009); (Krashes et al., 2013)
CART	GABA	hypothalamus	(Elias et al., 2001)
		lateral septum	(Janzso et al., 2010)
CCK	GABA	hippocampus	(Lubkemann et al., 2015); (Whissell et al., 2015)
		olfactory bulb	(Gutierrez-Mecinas et al., 2005)
		cortex	(Whissell et al., 2015)
		amygdala	(Sosulina et al., 2010)
	Glutamate + GABA	cortex	(Somogyi et al., 2004)
		hippocampus	(Somogyi et al., 2004)
CRF	GABA	cortex	(Kubota et al., 2011); (Kono et al., 2017)
		hippocampus	(Hooper and Maguire, 2016); (Kono et al., 2017)
		hypothalamus	(Romanov et al., 2017)
		bed nucleus of the stria terminalis	(Kono et al., 2017); (Partridge et al., 2016)
		amygdala	(Kono et al., 2017); (Partridge et al., 2016)
	Glutamate	hypothalamus	(Romanov et al., 2017)
		piriform cortex	(Kono et al., 2017)
		inferior olivary nucleus	(Kono et al., 2017)
Galanin	GABA	spinal cord	(Tiong et al., 2011)
		TMN	(Kukko-Lukjanov and Panula, 2003); (Melander et al., 1986)
		vlPOA	(Sherin et al., 1998)
		hypothalamic arcuate nucleus	(Melander et al., 1986)
MCH	GABA	medial preoptic area	(Rondini et al., 2010)
		lateral hypothalamus	(Harthoorn et al., 2005)
	Glutamate	lateral hypothalamus	(Chee et al., 2015); (Harthoorn et al., 2005); (Abrahamson et al., 2001)
Neurotensin	GABA	amygdala, BNST	(Day et al., 1999)
NPS	Glutamate	peri-coerulear area	(Xu et al., 2007)
NPY	GABA	striatum	(Faust et al., 2016)
		amygdala	(Wood et al., 2016)
		hypothalamic arcuate	(Sun et al., 2016); (Dicken et al., 2015)
		cortex	(Perrenoud et al., 2013); (Kubota et al., 2011)
		intergeniculate leaflet	(Takatsuji and Tohyama, 1989)
		hippocampus	(Fu and van den Pol, 2007); (Lubkemann et al., 2015)
		spinal cord	(Polgar et al., 2011); (Polgar et al., 1999)
POMC	GABA	hypothalamic arcuate nucleus	(Hentges et al., 2004); (Wittmann et al., 2013); (Jarvie and Hentges, 2012); (Jarvie and Hentges, 2012); (Dicken et al., 2012)

Neuropeptide	Co-transmitter	Neuroanatomical location	References
	Glutamate	hypothalamic arcuate nucleus	(Wittmann et al., 2013); (Jarvie and Hentges, 2012); (Dicken et al., 2012)
SOM	GABA	amygdala	(McDonald and Zaric, 2015)
		cortex	(Fuchs et al., 2016); (Urban-Ciecko and Barth, 2016)
		hippocampus	(Fuchs et al., 2016); (Lubkemann et al., 2015)
		dorsal motor nucleus of vagus	(Lewin et al., 2016)
		spinal cord	(Dougherty et al., 2009)
		olfactory bulb	(Gutierrez-Mecinas et al., 2005)
Substance P	GABA	intergeniculate leaflet	(Takatsuji and Tohyama, 1989)
		striatum	(Bolam et al., 1983); (Tan and Bullock, 2008)
		cortex	(Jakab et al., 1997); (Foley et al., 1992)
VIP	GABA	cortex	(Jackson et al., 2016); (Pronneke et al., 2015); (Xu et al., 2010)
		SCN	(Fan et al., 2015)
		hippocampus	(Jinno and Kosaka, 2003); (Hajos et al., 1996)
		posterior piriform cortex	(Young and Sun, 2009)
		spinal cord	(Dougherty et al., 2009)
		olfactory bulb	(Gracia-Llanes et al., 2003)