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What optogenetic stimulation is telling us (and failing to tell us) about fast neurotransmitters and neuromodulators in brain circuits for wake-sleep regulation

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Abstract

In the last eight years optogenetic tools have been widely used to identify functional synaptic connectivity between specific neuronal populations. Most of our knowledge comes from the photo-activation of channelrhodopsin-2 (ChR2) expressing inputs that release glutamate and GABA. More recent studies have been reporting releases of acetylcholine and biogenic amines but direct evidence for photo-evoked release of neuropeptides is still limited particularly in brain slice studies. The high fidelity in the responses with photo-evoked amino-acid transmission is ideal for ChR2-assisted circuit mapping and this approach has been successfully used in different fields of neuroscience. Conversely, neuropeptides employ a slow mode of communication and might require higher frequency and prolonged stimulations to be released. These factors may have contributed to the apparent lack of success for optogenetic release of neuropeptides. In addition, once released, neuropeptides often act on multiple sites and at various distances from the site of release resulting in a greater complexity of postsynaptic responses. Here, we focus on what optogenetics is telling us – and failing to tell us – about fast neurotransmitters and neuropeptides.

Optogenetic activation of specific neurons or projections has been rapidly adopted in many areas of neuroscience to study circuits of specific behaviors and to establish functional synaptic connectivity between specific neuronal populations [1,2]. Light pulses directed on the cell bodies of neurons expressing channelrhodopsin-2 (ChR2) produce a photocurrent that depolarizes and evokes neuronal firing [3] and trains of short light pulses can entrain neuronal firing at different ranges of frequencies depending on the membrane properties of the target cells [4]. Photostimulation directed at axons/terminals activates specific inputs and evokes neurotransmitter release. This approach has been used in brain slices to activate short and long-range projections even when the cell bodies that give rise to the targeted input are not contained in the recorded slices. Combining optogenetic targeting of the presynaptic

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elements with patch-clamp recordings of the postsynaptic neurons is a powerful approach to demonstrate functional synaptic connectivity between selective nodes of the neuronal circuit [5]. In whole-animal experiments fiber optics can be implanted to stimulate specific afferent inputs and to deconstruct neuronal circuits controlling specific behaviors [6]. The modalities of synaptic communication of amino-acid neurotransmitters and neuropeptides are very different [7-9] and probably respond differently to optogenetic activation. Other neurotransmitters such as of acetylcholine (through muscarinic receptors) and biogenic amines share some properties with amino-acids and some with neuropeptides [10-12] and are not discussed in this review. We focus on the comparison between fast-acting amino-acid and slow-acting neuropeptide transmissions and on the electrophysiological outcomes when they are optogenetically activated. We review here the stimulation patterns required for release, then compare the electrophysiological responses of amino-acids and neuropeptides, and finally review studies in which combined whole-animal and brain slice optogenetic approaches were used to map the neuronal circuitry for sleep and wake regulation.

Release of neuropeptides might require a different pattern of photostimulation than that used to release fast neurotransmitters

Although whole animal behavioral studies have reported that optogenetic stimulation can also evoke responses consistent with release of peptides [13-19], the dynamic of their release and the postsynaptic neurons on which they act remain unclear. These questions could be answered in a brain slice preparation. However, in brain slices, where photo-evoked release of amino-acid neurotransmitters has been demonstrated in many studies, there has been little evidence of release of the accompanied neuropeptides [17,20,21]. This might be due to the type of photostimulation paradigms that has been used in the whole-animal and the brain slice studies. While single brief (2-5 ms) light pulses are sufficient to evoke release of glutamate or GABA these protocols might be insufficient to release neuropeptides. Generally, the release of neuropeptides requires a higher firing frequency and longer duration of firing than the release of glutamate or other fast neurotransmitters [22-24]. This might be related to the location of the synaptic vesicles. While the clear vesicles containing glutamate or GABA are closely related to the voltage dependent Ca^{2+} channels in the active zone [25,26], the large dense core vesicles containing neuropeptides are located in the synaptic terminal more diffusely and away from the active zone [9,27]. Hence the release of neuropeptides requires intracellular Ca^{2+} to rise to higher levels to allow Ca^{2+} to reach the site where the neuropeptide-containing vesicles are stored. Overall the release of neuropeptides is less efficient and slower (occurring over 10 ms to seconds after stimulation) than the release of amino-acid transmitters (which occurs in less than one millisecond) [23,28]. Single action potentials can release fast neurotransmitters but can rarely release neuropeptides [27,29]. It is interesting that studies using electrical stimulation found that the ideal pattern for neuropeptide release is an initial brief high-frequency stimulation to quickly raise Ca^{2+} levels, followed by a tonic low frequency stimulation between 3 to 10 Hz [30]. This suggests that in addition to the frequency, the pattern and duration of the photostimulation might be important factors for releasing neuropeptides.

Amino-acids and neuropeptides are most often co-expressed and co-released [7,9]. Researchers have selectively blocked the release or the action of individual transmitters in order to isolate the functions of the different neurotransmitters involved. This can be achieved using specific antagonists [14,31] or knock-out mice [13,14]. In addition, because amino-acids and neuropeptides are probably released at different firing frequencies, one could use low frequency stimulation to drive the release of glutamate or GABA without neuropeptides and use higher frequency stimulations to drive the co-release of amino-acids and neuropeptides [13,32]. Furthermore, in behavioral studies, the inhibition of neuronal activity or specific synaptic inputs is perhaps even more informative than the activation of synaptic release. Indeed the inhibitory optogenetic tools such as the halorhodopsins (NpHRs), the archaerhodopsins (Arch or ArchT) and the chloride-conducting channelrhodopsin (ChloCs) are starting to be more widely used [33-36]. These tools are employed to silence neurons and their synaptic outputs in freely-behaving animals and they allow the testing of whether selective neuronal populations and synaptic inputs are necessary for specific behavioral functions [13,37-41].

Diversity in the electrophysiological responses to the release of fast neurotransmitters and neuropeptides

A second problem in recording peptidergic responses to optogenetic stimulation is the complexity and time scale of the responses that are seen. Fast neurotransmitters are mostly released from synaptic terminals whereas neuropeptides are released from terminals and from somato-dendritic varicosities [7,8]. Once released, amino-acid transmitters rapidly cross the synaptic cleft to activate receptors on the postsynaptic membrane (point-to-point transmission) whereas neuropeptides diffuse in the extracellular space and they can act on multiple sites. Neuropeptides can activate G-protein coupled receptors (GPCRs) on postsynaptic target neurons or on synaptic terminals to modulate synaptic activity. The G-protein receptors can have myriad effects in addition, including modifying the kinetics of channels and intracellular signaling cascade responses to later incoming stimuli. Such changes can affect the membrane potential of the target neuron or its response to other stimuli subtly, but for a prolonged period of time. Neuropeptides have also been shown to act as retrograde signals regulating their own release (homosynaptic modulation) or the activity of other afferent inputs to their target neurons (heterosynaptic modulation) [23,29]. Technical aspects of brain slice recordings should also be taken into consideration. For example, the responses through G-protein receptors may be diminished by the wash-out of the cytoplasm as the patch electrode contents diffuse into the neuron. Additionally, in slice preparations, neurons are not receiving their normal patterned inputs, so it is impossible to determine how the neuropeptide might modulate the response to those inputs. Photo-evoked release of neuropeptides from ChR2 expressing neurons might therefore result in a complex array of electrophysiological responses, in many cases quite different from the responses mediated by optogenetic release of glutamate or GABA.

Another important difference between amino-acid and peptidergic transmission is the kinetics of the synaptic responses. For example, neuropeptide release is slower than the release of amino-acid, and peptides, neuropeptides diffuse to activate receptors that may be

many microns away from the release site (volume transmission). In addition neuropeptides act on the postsynaptic target neurons by activating GPCRs that leads to a cascade of intracellular second messengers. This response is much slower than the response through the ionotropic receptors employed by amino-acid neurotransmitters. As a result the onset of the neuropeptide-mediated responses is much longer than for glutamate or GABA mediated postsynaptic events. Also the rise-time of amino-acid mediated postsynaptic currents is short allowing for precise measurement of postsynaptic response onset delay whereas peptide mediated currents have slow onset kinetics and it is often difficult to segregate single synaptic events. In addition, while for amino-acid-mediated postsynaptic responses, the delay and the dependence on action potentials (responses blocked by tetrodotoxin) are reliable parameters to distinguish between monosynaptic versus polysynaptic connections (4-6 ms delay is considered the threshold for monosynaptic responses) [42,43], these same properties cannot be used for neuropeptide transmission. In fact, because neuropeptides have such a long-range of diffusion that can vary among circuits, neuropeptide-mediated postsynaptic responses have long delays and low fidelity, impairing point to point mapping.

Another potential problem in studying optogenetic-mediated release of neuropeptides is the detection of the synaptic response. The current evoked from the activation of GPCRs can be small and can go undetected. On the other hand, the duration of neuropeptide transmission is often much longer than amino-acid signals. Therefore optogenetic stimulation of terminals expressing glutamate or GABA together with neuropeptides is likely to produce an initial response mediated by the fast neurotransmitters, immediately followed by a longer lasting response mediated by the release of the neuropeptides (see Fig. 1).

Optogenetic studies reveal neuronal circuits for sleep and wake regulation

Circuits that govern sleep and wake regulation were among the very first to be studied with optogenetic methods [14]. In addition, a combination of *in vivo* and *in vitro* measurements has given us important information regarding the sleep-wake neuronal circuitry, the conditions for the release of fast neurotransmitters and neuropeptides and the functional consequences of their release [32,44-46].

One example is represented by the optogenetic stimulation of the orexin (also called hypocretin) system. The orexin neurons form a cluster in the lateral hypothalamus [47,48] that promotes and maintains wakefulness; they are also involved in feeding behavior and reward processes [49-52]. In addition to orexin-A and -B, orexin neurons make and release glutamate [20,53,54], and they produce the inhibitory peptide dynorphin [55,56]. Orexin and glutamate can be localized at the same terminals but in different vesicles [53] and if co-released they should act synergistically to excite target neurons. Little is known about the conditions of release of dynorphin, although a recent electron microscopy study has reported that orexin and dynorphin are colocalized within the same synaptic vesicles [57]. Different targets of the orexin neurons may express orexin, glutamate, or κ -opioid receptors, or some combination of these. For example while orexin directly excites tuberomammillary nucleus (TMN) neurons and NPY neurons of the arcuate nucleus [58-60] dynorphin has no post-synaptic effects but reduces GABAergic synaptic input to these neurons [61]. Therefore in these cases co-release of orexin, glutamate and dynorphin would produce synergistic effects

that increase activity in the target cells. A further level of complexity is that orexin, glutamate and dynorphin have effects that differ over time [62]. In addition, dynorphin could also be locally released from somato-dendritic varicosities and thus act as a retrograde signal to inhibit afferent inputs to the orexin neurons [63]. Experiments using optogenetic stimulation of orexin neurons and terminals are starting to unravel the dynamics of orexin and glutamate releases [32] and may soon reveal the functional role of dynorphin in these neurons.

Optogenetic activation of orexin neurons or their projections to the locus coeruleus (LC) using ChR2 in whole-animal studies has been shown to promote awakening [14,31]. This response was significantly reduced but not occluded in orexinligand knockout mice or when an orexin antagonist was given, suggesting that in addition to the orexin signaling another neurotransmitter, most likely glutamate, contributes to the arousal response [14,18]. The dynamics of co-release of orexin and glutamate are starting to be understood. We know for example that the optogenetic stimulation that promotes transitions to wakefulness is time- and frequency-dependent and requires trains of light pulses (5 Hz or greater) that last at least 10 seconds. These conditions might be more relevant for the release of orexin than for the release of glutamate. In fact single light pulses to orexin terminals expressing ChR2 were sufficient to release glutamate onto tuberomammillary nucleus (TMN) neurons in brain slices, but failed to evoke an orexin-mediated postsynaptic response [20]. TMN neurons express orexin receptor 2 and are excited by orexin [58,64]. Thus the lack of orexin-mediated response during *in vitro* optogenetic stimulation could be due to (i.) lack of orexin exocytosis; (ii.) release of orexin at levels not sufficient to evoke a detectable electrophysiological postsynaptic response; or (iii.) the stimulation parameters being inadequate to cause orexin release. The latter possibility was underscored by the recent demonstration that either long (> 30 seconds) trains of light pulses at 5 Hz or shorter trains (10 s) but at higher frequency (20 Hz) could evoke an orexin-mediated slow-onset inward postsynaptic current in TMN neurons [32]. Similar results were also reported with photostimulation of orexin axons and terminals in the LC where short trains (1 s) of light pulses at 10 Hz elicited a small orexin-mediated slow-onset depolarization of LC neurons [65]. Therefore it appears that particularly high frequency photostimulation is not a requirement for the release of orexin, which is consistent with evidence that orexin neurons fire at maximum frequency not higher than 10 Hz during attentive wakefulness [66] and up to 20 Hz during sensory stimulation [67], but hints that prolonged firing is perhaps key for orexin release.

This requirement may be physiologically relevant if brief firing of the orexin neurons during brief awakenings or during postural changes is insufficient to evoke the release of orexin, thus permitting an easy return to sleep. However if the orexin neurons remain active the release of orexin would begin promoting full arousal. In support of this hypothesis, research in orexin knockout mice suggests that orexin signaling is essential for sustaining long wake bouts but has little impact on brief arousals from sleep [68]. These findings are consistent with those showing that optogenetic release of glutamate and orexin in brain slices evokes a rapid glutamate-mediated postsynaptic current that rapidly wanes due to the depletion of the readily-releasable pool of glutamate containing vesicles [32]. Therefore while the orexin-

mediated postsynaptic response has a delayed onset and slow kinetics compatible with the slow dynamics of neuropeptide release and the kinetics of GPCR-mediated currents, the orexin outlasts the glutamate-mediated response consistent with the long lasting effects of neuropeptides [32].

Three recent studies have shown that optogenetic activation of the melanin-concentrating hormone (MCH) neurons promotes sleep – both REM and non-REM sleeps in one study [69] and only REM sleep in the others [13,70]. MCH neurons are a cluster of neurons in the lateral hypothalamus that are involved in energy homeostasis [71]. MCH neurons are also active during REM sleep [72] but the direct evidence for their involvement in REM sleep regulation only came with the results from these optogenetic studies. In both studies 1 Hz stimulation was insufficient but 10-20 Hz was effective in prolonging REM sleep. This effect is maintained in MCH receptor knock-out mice suggesting that there is an as yet unknown receptor for MCH, or that the effect is due to release of another neurotransmitter, most likely GABA [13]. In addition optogenetic stimulation of MCH input to the TMN and to the medial septum but not to the dorsal raphe increased REM sleep as well [13]. In brain slices single brief light pulses directed to MCH cell bodies, axons and terminals evoked GABA release and short latency GABA_A mediated inhibitory postsynaptic currents (IPSCs) in TMN neurons, but 10 second trains of stimulation at 20 Hz appeared to release MCH that had no direct effect on TMN neurons but increased GABAergic IPSC frequency in TMN neurons suggesting a presynaptic facilitation of the GABAergic input to the TMN [13].

We recently found in brain slices that photostimulation of TMN neurons and their axons and terminals in which we selectively expressed ChR2, evokes the release of histamine locally and distally in the ventrolateral preoptic nucleus (VLPO), which contains sleep-active neurons [73,74]. Within the TMN region photo-release of histamine, possibly from somato-dendritic vesicles, suppresses GABAergic inputs onto TMN neurons via H3 receptors. In the VLPO, photostimulation of the TMN input increased inhibitory synaptic input to the VLPO neurons. This response is indirect; it is mediated via H1 receptors through the activation of GABAergic interneurons that project to VLPO neurons. Both these effects were only observed when we used very long, slow trains of light pulse stimulation (1 Hz, for 2-8 minutes) whereas single light pulses failed to evoke histamine-mediated responses. The onset of the effects of photo-released histamine was slow (1-3 min) and persisted long after the stimulation was completed (10-15 min). Based on the onset delay, kinetics, and duration of the responses to photostimulation of the TMN neurons, histamine appears to act via volume transmission [75-77]. In addition, the action on synaptic inputs would suggest that once released, histamine might diffuse a short distance to activate H3 receptors in the TMN region and H1 receptors in VLPO, acting on presynaptic terminals of afferent inputs to TMN and VLPO neurons. Overall the release of histamine has more in common with the mechanisms of transmission of neuropeptides than with amino-acid neurotransmission. Importantly, this *in vitro* optogenetic study demonstrated synergistic mechanisms through which histamine may promote arousal by disinhibiting TMN neurons while inhibiting VLPO neurons and perhaps their projections to TMN neurons.

Conclusions

Studies employing ChR2-assisted circuit mapping in brain slices provide strong evidence for functional synaptic connectivity between selective neuronal populations. The majority of our current knowledge still comes from photo-evoked release of fast neurotransmitters whereas direct evidence for the release of neuropeptides is lagging. This scenario however might rapidly change, particularly since new promising optogenetic tools with large photocurrents and faster kinetics have been recently developed [34]. The advantage of these new opsins is their fast-off kinetics that prevent sustained depolarization between pulses allowing membrane repolarization in a similar fashion as during the firing of a burst of high frequency action potentials. These suggest great potential for investigating synaptic co-release. However, in the case of neuropeptide transmission, slow release, variability in the time delay of the postsynaptic responses and the complexity of the actions might still be a limitation for circuit mapping. The development of fast-kinetics optogenetic tools combined with the growing understanding of the dynamics of release and the actions of classic neurotransmitters and neuromodulators is bound to broaden our functional mapping of the brain circuits involved in sleep and wakefulness as well as many other areas of neuroscience.

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Highlights

- Optogenetic tools release fast neurotransmitters but maybe not peptides.
- Neuropeptides use a slow mode of communication with complex postsynaptic responses.
- ChR2-assisted circuit mapping for neuropeptide transmission have some limitations.
- Optogenetic tools have been used to map the circuitry for sleep and wake regulation.

Figure 1. Comparison of optogenetic releases of amino-acid neurotransmitters and neuropeptides

Photostimulation at low frequency of ChR2 expressing axons/terminals releases only amino-acid neurotransmitters (left) but a higher frequency and prolonged photostimulation evokes release of both amino-acid and neuropeptides (right). Fast acting amino-acid transmitters activate ionotropic receptors on the postsynaptic neurons (1). Neuropeptides act slowly to activate postsynaptic G-protein-coupled receptors (GPCRs) on the postsynaptic neurons (2), on synaptic terminals to modulate synaptic activity (3, homosynaptic and 4, heterosynaptic modulations) and they can diffuse from the release site to act on distant postsynaptic targets

(5). Neuropeptides can also be released from somato-dendritic varicosites and they can act as retrograde signals (6).