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Dual-transmitter neurons: Functional implications of co-release and co-transmission

Christopher E Vaaga^{a,b}, Maria Borisovska^b, and Gary L Westbrook^b

Christopher E Vaaga: vaaga@ohsu.edu; Maria Borisovska: borisovs@ohsu.edu

^aNeuroscience Graduate Program, Oregon Health and Science University, Portland, Oregon, USA

^bVollum Institute, Oregon Health and Science University, Portland, Oregon, USA

Abstract

Co-transmission, the ability of a neuron to release multiple transmitters, has long been recognized in selected circuits. However, the release of multiple primary neurotransmitters from a single neuron is only beginning to be appreciated. Here we consider recent examples of co-transmission as well as co-release – the packaging of multiple neurotransmitters into a single vesicle. The properties associated with each mode of release greatly enhance the possible action of such neurons within circuits. The functional importance of dual- (or multi-) transmitter neurons extends beyond actions on postsynaptic receptors, due in part to differential spatial and temporal profiles of each neurotransmitter. Recent evidence also suggests that the dual-transmitter phenotype can be dynamically regulated during development and following injury or disease.

Keywords

co-release; co-transmission; dual-transmitter neurons; synaptic transmission

Introduction

Neurotransmitter phenotype has long been recognized as a hallmark of neuronal identity. The classical view was that each neuron releases a single neurotransmitter, leading to the “one neuron, one transmitter” hypothesis [1], formalized by John Eccles as Dale’s Principle [2]. However, we now know that many neurons throughout the brain are capable of releasing two or more neurotransmitters [3–5]. For simplicity, in this review we refer to these cells as “dual” transmitter neurons. Some of the first evidence for such dual- (or multi-) transmitter neurons was noted in 1982, when Jan *et al.* reported a very slow synaptic potential in a subset of sympathetic neurons that accompanied the well-established

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Corresponding Author: Gary L Westbrook, Vollum Institute, L474, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239 USA, westbroo@ohsu.edu, (503) 494-5429.

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cholinergic transmission (Figure 1) [6]. This slow potential was mediated by a peptide, LHRH (luteinizing hormone-releasing hormone), indicating that the presynaptic neuron released a neuroactive peptide as well as acetylcholine [6,7]. Such co-transmission, defined broadly as the release of multiple neurotransmitters from a single neuron, has been reported for many neuromodulators including ATP, neuroactive peptides, neurotrophic factors and even ions such as Zn^{2+} [8–15]. Recent evidence, however, suggests that neurons can co-transmit not only neuromodulators but also multiple primary neurotransmitters including fast-acting neurotransmitters, monoamines and acetylcholine [16–20].

Although dual-transmitter neurons are found throughout the brain, the functional significance of co-transmission on neuronal circuits has been difficult to dissect. This difficulty arises, in part, because in addition to activating postsynaptic receptors, co-released neurotransmitters can modulate pre- and postsynaptic responses and even modulate the packaging of other neurotransmitters into synaptic vesicles [4]. Additionally, each neurotransmitter may be differentially released in time and space, thereby complicating analysis. A consideration of all these parameters is necessary to understand how dual-transmitter neurons alter the computational capabilities of neuronal circuits. Of note, the functional importance of co-transmission has been better described in select invertebrate systems, where each transmitter can differentially enhance the ability of the circuit to participate in multiple computational tasks [21–24]. Here we focus on recent studies of dual-transmitter neurons, including the mechanisms governing the release of multiple neurotransmitters, and the functional importance of co-transmission and co-release on circuit function in the mammalian CNS.

Co-release vs. Co-transmission

The release of multiple neurotransmitters from a single neuron does not necessarily imply co-release, i.e. that two or more neurotransmitters are packaged into a single population of synaptic vesicles (Figure 2a). Co-transmission can be more broadly defined as the release of multiple neurotransmitters from non-overlapping pools of synaptic vesicles (Figure 2b). The distinction between co-release and co-transmission is important because each mode of release can have different potential impacts on circuit function. For example, release from different sets of vesicles can be differentially regulated by differential Ca^{2+} sensitivity or the spatial segregation of vesicles (Figure 2b).

Recent work by Tritsch and colleagues (2012) provides an elegant example of *co-release* in the ventral tegmental area (VTA) [25**]. Their work indicates that dopaminergic neurons targeting striatal spiny neurons co-release GABA. Surprisingly, the conditional knockout of the vesicular GABA transporter (VGAT) [26–28], failed to eliminate GABA release [25**]. Instead, inhibition or conditional knockout of the vesicular monoamine transporter (VMAT2), which was thought to only package monoamines [29], completely eliminated GABA release [25**]. Although this work did not examine changes at the single vesicle level, the results indicate that GABA is a non-canonical substrate for VMAT2. Although the function of co-released GABA within the VTA circuitry has not yet been well characterized, the ability of VMAT2 to package a non-canonical substrate, such as GABA, into synaptic

vesicles suggests that monoaminergic neurons expressing VMAT2 are capable of co-releasing GABA.

Co-transmission, on the other hand, has been demonstrated in a handful of circuits, including the retina where a subpopulation of starburst amacrine cells release acetylcholine (ACh) and GABA [30–32]. Consistent with a co-transmission phenotype, cholinergic synapses are uniformly distributed on the postsynaptic neuron, whereas GABAergic synapses are non-uniformly distributed [32]. Furthermore, the release of GABA and ACh have different Ca^{2+} sensitivities, suggesting that co-transmission in this circuit involves spatial segregation of vesicles and differential release [32]. Ostensibly, these features allow single starburst amacrine cells to participate in distinct, yet related circuit functions.

Interestingly, the co-transmission phenotype can be confined to a subset of synaptic boutons in an individual neuron. For example, in cultured dopaminergic neurons, immunohistochemical studies suggest that most boutons contain vesicular glutamate and monoamine transporters, but only a single vesicular transporter type could be detected in a significant fraction of terminals [33]. However, these studies could not distinguish whether the transporters were colocalized in the same vesicles, a necessary condition for co-release. Similarly, neurons in the dorsal raphe, the nucleus that provides serotonergic innervation to many brain regions, can also release glutamate [18,34–35]. In this case, the proportion of terminal varicosities that co-express vesicular glutamate transporter and the vesicular monoamine transporter varies across target regions, and such co-expression is completely lacking in recurrent axon collaterals projecting back to the raphe nucleus [34,35]. Selectively targeting of the co-transmission phenotype to some boutons, but not others, may allow neurons to convey different information to distinct downstream targets.

Functional implications of co-release in neuronal circuits

Broadly speaking, there are three classes of dual-transmitter neurons: neurons that release two fast-acting neurotransmitters; neurons that release a fast-acting neurotransmitter and a slow-acting monoamine; and neurons that release a fast-acting neurotransmitter and a neuromodulator, defined here as peptides, ions or other small molecules (Figure 3). Each class can have different effects on pre- and postsynaptic neurons, thus altering the functional impact of dual-transmitter neurons within circuits. The effect of dual-transmitter neurons that release two fast acting neurotransmitters (such as glutamate and GABA or GABA and glycine) is primarily determined by postsynaptic factors (Figure 3a). These factors include the kinetics and polarity of postsynaptic currents, the specific receptor types expressed, and the synaptic vs. extrasynaptic distribution of the receptors (Figure 3a). For example, the net effect of neurons that co-release GABA and glycine appears not to depend on the quantity of either neurotransmitter in individual vesicles, but rather by the GABA and/or glycine receptors expressed postsynaptically [36–37]. Of course, fast acting neurotransmitters can also bind to presynaptic G-protein coupled receptors, such as metabotropic glutamate receptors or GABA_B receptors, thereby modulating presynaptic release dynamics. The effect of dual-transmitter neurons releasing a fast-acting neurotransmitter with a slower-acting monoamine is more complex (Figure 3b). This complexity arises from different temporal profiles as well as modulation by presynaptic receptors. Most co-released monoamines act

via G-protein coupled receptors, making the response intrinsically slower. Furthermore, activation of G-protein coupled receptors can have multiple postsynaptic effects with different time courses. The list of neuromodulators that can be co-released is extensive, including ions such as Zn^{2+} , neurotrophins, peptides and ATP. Neuromodulators can modulate the presynaptic terminal, elicit postsynaptic currents, provide trophic support to the postsynaptic neuron, directly modulate receptor dynamics, and alter gene expression (Figure 3c). For example, Zn^{2+} can bind to NMDA receptors and prolong the EPSC, thereby altering the computational capabilities afforded by NMDA receptors [38]. Despite the increasing attention to “dual”-transmitter neurons, recent evidence suggests that neurons can release more than two chemical messengers, such as a subset of ventral tegmental neurons that can release dopamine, GABA and glutamate [25^{**}, 39^{*}].

The Spectrum of Co-release and Co-transmission

Co-release of GABA and glycine

Release of GABA and glycine from some inhibitory neurons represents a robust example of co-release [16,37^{**},40–41]. The first physiological evidence of glycine/GABA co-release was reported in the spinal cord, where miniature IPSCs had both GABA and glycine receptor components [16], suggesting that both neurotransmitters were present in single vesicles. Co-release of GABA and glycine is perhaps not surprising given that the vesicular inhibitory amino acid transporter (VIAAT, also known as VGAT) is expressed in GABAergic and glycinergic neurons [26] and can package both neurotransmitters into synaptic vesicles [27–28,42–43]. Given the low selectivity of VIAAT, it is not immediately clear which factors control the differential release of GABA and glycine in different neuronal populations. However, the availability of neurotransmitter in the cytosol should affect vesicular packaging [37^{**},41,44]. Consistent with this hypothesis, GABA and glycine compete for vesicular loading, as altering their cytoplasmic concentration changes the GABA/glycine ratio in synaptic vesicles [37^{**},41,43,45]. In fact, acute changes in cytosolic GABA or glycine concentrations can result in dynamic modulation of IPSC amplitude, independent of vesicle turnover [37^{**}]. These results suggest that the packaging of neurotransmitters into synaptic vesicles, and therefore, the amplitude of IPSCs are dynamically modulated on fast timescales [37^{**},41].

Co-release of glutamate and dopamine in the VTA

The dopaminergic midbrain has emerged as a surprisingly heterogeneous population of neurons, which is partly reflected in the co-transmission phenotype of individual neurons. The first evidence of co-transmission was noted in cultured dopaminergic neurons, which express the vesicular glutamate transporter (VGLUT2) and upon stimulation elicit fast glutamatergic EPSCs both in autaptic cultures and co-cultures [46–48]. The co-transmitter phenotype was later verified *in vivo*, as VTA afferents to the nucleus accumbens co-express tyrosine hydroxylase (TH) and VGLUT2 [49–51], however VGLUT2 expression declines with age [52–54]. Although it is difficult to determine the protein composition of a single vesicle, biochemical studies suggest that VMAT2-positive vesicles can co-express VGLUT2 [55^{**}]. However, recent work suggests that while dopamine and glutamate share similar release properties (such as sensitivity to D_2 receptor agonists and paired-pulse ratio) they are

differentially affected by cocaine, suggesting the two transmitters may be in non-overlapping pools of vesicles [56**].

The transmitter phenotype of neurons within the VTA is heterogeneous, highlighting the ability of these neurons to convey distinct messages to each of their postsynaptic targets. For example, optogenetic stimulation of afferents from the VTA elicits glutamatergic EPSCs in the nucleus accumbens, but not the dorsal striatum [50–51,57]. As mentioned, VTA afferents that target striatal spiny neurons are capable of co-releasing GABA and dopamine through the non-canonical packaging of GABA by VMAT2 [25**]. Interestingly, a presumably separate population of VTA neurons that target cholinergic interneurons release dopamine alone in the dorsal striatum, whereas in the medial shell of the nucleus accumbens this population co-releases dopamine and GABA, and yet in the core they co-release dopamine, GABA and glutamate [39*]. Further evidence suggests that co-released dopamine can modulate the glutamatergic response by actions on pre- and post-synaptic receptors [49]. Taken together, these results indicate that the transmitter phenotype of different populations of “dopaminergic neurons” have widely different transmitter phenotypes depending on the postsynaptic target.

The importance of glutamate co-release from monoaminergic neurons extends beyond the effects of each transmitter on pre- and postsynaptic receptors. If vesicular glutamate transporters and vesicular monoamine transporters are expressed on an individual synaptic vesicle, the amount of monoamine packaged into the vesicle can be enhanced by vesicular synergy, defined as the enhanced packaging of one neurotransmitter as a result of packaging another neurotransmitter into the same vesicle [4,55**]. Mechanistically, expression of the vesicular glutamate transporter is thought to dissipate the vesicle potential while maximizing the pH gradient used to package monoamines, thereby increasing the concentration of monoamine in the vesicle and enhancing monoaminergic transmission [4,55**]. For example, conditional knockout of the vesicular glutamate transporter in dopaminergic neurons caused behavioral deficits associated with dopaminergic transmission, as well as a reduction in the electrochemical detection of dopamine in the VTA [55**,58–60]. Vesicular synergy also occurs in cholinergic neurons, as VGLUT conditional knockouts have a hypocholinergic phenotype [61].

Co-transmission of dopamine and GABA in the olfactory bulb

In the olfactory bulb, a subset of periglomerular interneurons express both tyrosine hydroxylase and GAD70, which suggests they can release both dopamine and GABA [62,63]. Recently, Borisovska and colleagues (2013), using dual patch clamp/amperometric recordings from autaptically cultured dopaminergic periglomerular neurons, reported that stimulation with a single action potential resulted in a fast, GABAergic IPSC but failed to elicit an immediate barrage of amperometric spikes [64**]. Instead, the frequency of amperometric events that detect dopamine release increased slowly over seconds [64**]. The temporal disparity of dopamine and GABA release suggests that these two neurotransmitters are not only packaged into different vesicles, a feature of co-transmission, but also differentially released. In brain slices, optogenetic stimulation of periglomerular neurons

produces a temporally biphasic inhibition-excitation, providing further support for of the temporal disparity of dopamine and GABA release [64^{**},65^{*}].

The neurotransmitter phenotype is plastic

Co-release during development

Emerging evidence suggests that the transmitter phenotype of a neuron is plastic. Even in single transmitter neurons, the neurotransmitter phenotype can be dynamically modulated in response to sensory stimuli. For example, in the hypothalamus, neurons can switch from releasing dopamine to somatostatin with changes in photoperiod [•65]. Similarly in dual-transmitter neurons, there is evidence that the transmitter phenotype can change during normal development. In many circuits co-release is restricted to early periods of development. For example, in the developing auditory brainstem, GABAergic/glycinergic neurons of the medial nucleus of the trapezoid body (MNTB) transiently co-release (or co-transmit) glutamate [67]. Although the role of this co-released glutamate during development is still under examination, the Ca²⁺ transients elicited by NMDA receptor activation could refine the synaptic map between the MNTB and its target [67–69].

One surprising example of co-release has been reported in the developing hippocampus, in which mossy fibers, long considered to be exclusively glutamatergic, express the GABA synthesis enzyme, GAD70 [70]. In young animals, electrical stimulation of mossy fibers can elicit GABAergic IPSCs in CA3 neurons [17,71^{**}–72]. Although this response could result from the disynaptic activation of inhibitory interneurons, the IPSCs persist in the presence of glutamate receptor antagonists, ostensibly required to activate local interneurons, and persist in conditions of minimal stimulation [17,71^{**}–72]. Furthermore, mossy fiber release of GABA is subject to the same regulation as glutamate release in this pathway [73–75], suggesting that mossy fibers co-release or co-transmit glutamate and GABA [17,71^{**}]. Although GABAergic co-release in this pathway remains contentious [76,77^{**}], stimulation of mechanically dissociated mossy fiber boutons attached to CA3 neurons also elicited GABAergic IPSCs, eliminating possible disynaptic recruitment of GABAergic interneurons [71^{**}]. The developmental pattern of co-release suggests a role in initial circuit formation or synapse stabilization [17,71^{**}–72]. Granule cells, however, undergo continuous adult neurogenesis [78]; therefore it remains possible that co-release occurs from immature granule cells that are generated throughout the life of the animal. Interestingly, the dual-transmitter phenotype can be detected in adult animals following hippocampal seizures [79–81]. Given that seizures dramatically increase granule cell neurogenesis and mossy fiber sprouting [82,83], re-expression of the co-transmission phenotype could result from the increase in immature neurons, or a change in the phenotype of existing, mature granule cells. Interestingly, GAD70 is also unregulated in mossy fibers of human patients with temporal lobe epilepsy [84]. Ostensibly, the increased GABAergic tone within the hippocampus could be neuroprotective.

Dynamic changes in dual-transmitter phenotype with injury and disease

Given the plasticity of the neurotransmitter phenotype, it is perhaps not surprising that co-release may be affected by injury or disease. In the VTA, for example, lesioning with the

dopaminergic neurotoxin 6-OHDA causes a rapid increase in the fraction of VTA neurons that co-express vesicular glutamate transporters [52,54]. Furthermore, co-transmission of dopamine and GABA in the olfactory bulb may be modulated in Parkinson's disease. Prior to the onset of motor symptoms, 73% of sporadic Parkinson's disease patients have a reduced sense of smell [85,86]. Surprisingly, these olfactory deficits are accompanied by a *doubling* of tyrosine hydroxylase positive interneurons in the olfactory bulb [87,88]. The increase in TH expression may represent increased neurogenesis of dual-transmitter neurons [89]. However, it is also possible that the existing population of GABA-only periglomerular interneurons upregulate TH expression to become dual-transmitter neurons. Although the contribution of the putative increase in dopamine tone has not been functionally examined, dopamine decreases the release probability of incoming olfactory receptor neurons [63,90], thereby likely decreasing olfactory sensory input. The role of co-transmission in disease has only recently been described and thus further examples are expected in the future.

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Highlights

- Co-release and co-transmission are distinct, separate modes of release
- There are multiple classes of dual-transmitter neurons
- The consequence of co-release extends beyond effects on postsynaptic receptors
- The transmitter phenotype of a neuron is plastic, in development and disease

Figure 1. Co-transmission in the sympathetic nervous system

Stimulation of a sympathetic ganglion neuron revealed (a) a fast, nicotinic EPSP (left) and higher stimulation evoked an action potential (right). (b and c) Such stimuli can also elicit a slow muscarinic IPSP (b) or EPSP (c) depending on the stimulation conditions. (d) Strong stimulation generated a late slow EPSP consistent with co-transmission by the neuromodulator LHRH. Note timescale difference in each panel. Adapted from Figure 1 of Jan et al., 1979.

Figure 2. Co-release and Co-transmission are distinct modes of release

(a) With co-release, both neurotransmitters (mixed red and blue) are packaged into the same set of synaptic vesicles. Upon an action potential invading the presynaptic terminal, vesicles containing both neurotransmitters are released into the synaptic cleft. **(b)** In contrast, co-transmission requires neurotransmitters be sequestered into distinct populations of synaptic vesicles with differential release mediated by differential Ca^{2+} sensitivities (left panel). For example, a single action potential might release one set of vesicles (red), but multiple action potentials might be required to release both sets of vesicles (red and blue). Alternatively, co-transmission can rely on spatial segregation of vesicle populations to different boutons (right panel) in which case, unique information is transmitted to different postsynaptic targets.

Figure 3. Functional considerations of different classes of co-released transmitters

Different classes of dual-transmitter neurons have distinct functional implications for circuit dynamics. **(a)** The effect of neurons that release two fast-acting neurotransmitters (such as GABA and Glycine) is primarily determined by the postsynaptic receptors; including (1) the kinetics and amplitude of the postsynaptic current, (2) the specific receptor types expressed in the postsynaptic neuron and (3) the synaptic or extrasynaptic location of postsynaptic receptors. **(b)** For neurons that release a fast-acting neurotransmitter and a monoamine, additional factors that determine the functional output include (4) second messenger cascades and intrinsically slower currents, such as GIRK and (5) modulation of the presynaptic terminal. **(c)** The action of neurons that release a fast-acting neurotransmitter and a neuromodulator include (6) direct receptor modulation, (7) postsynaptic changes in gene expression and (8) trophic support.