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Multi-transmitter neurons in the mammalian central nervous system

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It is firmly established that many mammalian neurons release various combinations of amino acids, their derivatives, and other small molecules from presynaptic terminals in order to signal to their postsynaptic targets. Here we discuss recent findings about four types of multi-transmitter neurons—those that release GABA and acetylcholine (Ach); dopamine (DA) and GABA or glutamate; and glutamate and GABA. The mechanisms of co-release in each class differ and highlight the complex and dynamic nature of neurotransmitter release. Furthermore, identifying the neurotransmitter signature of each neuron and the post-synaptic targets of each neurotransmitter remain challenging. The existence of multi-transmitter neurons complicates the interpretation of connectomic wiring diagrams and poses interesting challenges for our understanding of circuit function in the brain.

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Introduction

Our conception of how a particular presynaptic neuron influences downstream cells is determined by which small molecule-based neurotransmitter – for example, 5-HT, Ach, GABA, glutamate, and monoamines – it releases. However, many additional classes of molecules – for example, peptides (substance P, opioids, neuropeptide Y), gases (nitric oxide), and lipophilic molecules (prostaglandins, endocannibinoids) – are released in an activity-dependent manner and used to signal to neighboring neurons. In addition, many neurons release multiple small-molecule neurotransmitters. Thus, the designation of one molecule as a principal neurotransmitter obscures the true diversity of synaptic signaling.

Rather than repeat recent comprehensive reviews [1–3], here we discuss four examples of multi-transmitter neurons for which new results have recently been published. These are neurons in the entopeduncular nucleus (EP) that release GABA and glutamate, neurons in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) that release DA and GABA or glutamate, and vasoactive intestinal peptide (VIP)-expressing cortical interneurons that release GABA and Ach. Although we focus on results from the analysis of mouse brain, some of these neurotransmitter combinations are conserved across brain areas and species. In many species that are not genetically tractable and in studies from the pre-optogenetics era, the functional demonstration of co-transmission is often lacking. Nevertheless, neurons with markers or functional evidence suggesting a dual GABAergic/cholinergic phenotype are found, for example, in *C. elegans*, birds, and the mammalian retina [4–8]. Furthermore, in zebrafish, catecholamine-producing neurons likely release GABA or glutamate [9], and DANs in mammalian retina and olfactory bulb also appear to be GABAergic [10,11]. The examples presented here therefore highlight common motifs shared across species and brain areas.

GABA and glutamate release from entopeduncular nucleus neurons

The entopeduncular nucleus (EP; analogous to the globus pallidus internus (GPi) in primates) is a major output nucleus of the basal ganglia and sends a large projection to lateral habenula (LHb). In monkeys [12] and in mice [13••], rewarding outcomes reduce and aversive ones increase firing in EP → LHb projecting neurons. This is consistent with the LHb evaluating the outcome of an action and biasing future behavior to maximize positive outcomes and minimize negative ones. Optogenetic studies in mice confirm a causal role for EP → LHb projections in reward motivated behaviors [13••] and are consistent with a net inhibitory effect of LHb activity on dopaminergic neurons of the VTA [15].

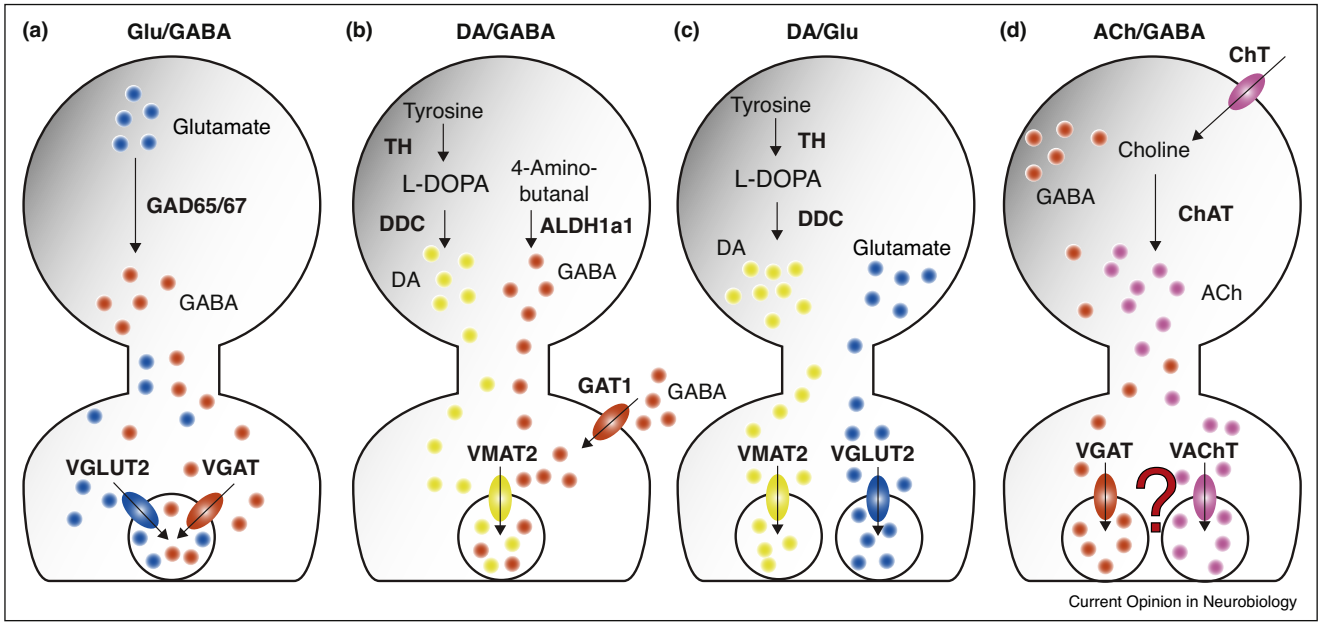
A neuron subtype in the EP projects only to the LHb [16,17] and releases both GABA and glutamate from the same axon onto individual LHb neurons [18••]. These neurons contain GABA synthesizing enzymes (*Gad1*/GAD67 and *Gad2*/GAD65) as well as vesicular transporters for GABA (*Slc32a1*/VGAT) and glutamate (*Slc17a6*/VGLUT2), suggesting that both neurotransmitters are packaged into synaptic vesicles via canonical mechanisms [18••,19]. Interestingly, GABA and glutamate appear to be

packaged into the same synaptic vesicles, resulting in simultaneous release of both neurotransmitters (Figure 1a). This conclusion is based on: (1) the electrophysiological observation of biphasic miniature and spontaneous post-synaptic currents at holding potentials between the reversal potentials of GABA and glutamate; and (2) ultrastructural observation by immunoelectron microscopy of GABA and VGLUT2 in close proximity (<30 nm) to each other in EP axon terminals within the LHb [18••]. Interestingly, inputs to LHb from the ventral tegmental area also release both glutamate and GABA [20•,21], but, in this case, apparently from distinct sites [20•].

In vitro, optogenetic activation of EP axons leads to time-locked spiking of neurons in the LHb [18••], suggesting a net excitatory effect despite the release of GABA. In this case, simultaneous release of both GABA and glutamate may serve to sharpen the time course of excitation by accelerating the decay of the post-synaptic potential. Alternatively, pre- or post-synaptic mechanisms may dynamically alter the glutamate/GABA current ratios evoked by activity of individual EP → LHb neurons. Indeed, synaptic transmission within the LHb is highly plastic and GABA and glutamate release from EP axons are modulated independently [18••,22]. Induction of depressive-like states in rodents and their treatment with selective serotonin reuptake inhibitors (SSRIs) or exposure of EP axons to serotonin *in vitro*, appears to act on the presynaptic terminal to modulate GABA and glutamate release [18••,23]. On the postsynaptic side, potential independent regulation of GABA and glutamate receptor trafficking to the synapse would allow each LHb neuron to set the relative strength of each input in a continuous fashion from strongly inhibitory to strongly excitatory. Since the LHb appears to lack GABAergic interneurons, such regulation may be necessary for projection neurons to implement gain control.

Alternatively, the graded regulation of synaptic weights of EP inputs could be used by LHb neurons to learn to encode 'reward prediction errors (RPE)'—that is, to modulate activity in proportion to the degree of unexpectedly bad or good outcomes in specific contexts. Some inputs might predict an aversive outcome and thus need to contribute positively to activity in the LHb whereas others might predict reward and contribute negatively. The sign and strength of each synapse from GABA/glutamate multitransmitter EP neurons, which carry information about sensory state, motor action, and other environmental variables, may be regulated to assign such negative and positive contributions to the activity of individual LHb neurons.

Figure 1



Diverse mechanisms of neurotransmitter transport, synthesis and synaptic vesicle packaging in four types of coreleasing neurons. **(a)** GABA is synthesized by GAD65/67 in LHb projecting EP neurons and both GABA and glutamate may be packaged into the same synaptic vesicle (via VGAT and VGLUT2, respectively) at axon terminals. Recent evidence suggests LHb projecting VTA neurons release GABA and glutamate from distinct axonal sites [20]. **(b)** DANs accumulate DA via synthesis from Tyrosine, but accumulate GABA though plasma membrane transport (GAT1) or via non-canonical ALDH1a1 dependent synthesis in the SNc. Both neurotransmitters are packaged into synaptic vesicles via VMAT2. **(c)** DANs synthesize and package DA via canonical mechanisms, but are also capable of releasing glutamate which is packaged by VGLUT2. DANs may release DA and glutamate at distinct axonal microdomains. It is currently unclear if DA/Glu neurons also release GABA as in (b). **(d)** VIP/Chat cortical interneurons synthesize and package Ach and GABA via canonical mechanisms, however it is unknown if these transmitters have distinct postsynaptic targets or if they are coreleased at the same axon terminal.

More studies are needed to determine if GABA/glutamate co-transmission serves any of these proposed functions.

Dopamine and GABA or glutamate

Axons of midbrain dopamine neurons (DANs) release GABA in the striatum and nucleus accumbens (NAC) [24]. This is difficult to deduce based on transcriptional and immunohistochemical analyses since DANs in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNC) do not express classical synthesizing enzymes (*Gad1*/GAD67 and *Gad2*/GAD65) or vesicular transporters (*Slc32a1*/VGAT) for GABA and these genes are not essential for GABA release from DANs onto striatal projection neurons (SPNs) [24,25,26••]. Instead, the brain vesicular monoamine transporter (*Slc18a2*/VMAT2) is necessary in DANs for GABA release and can substitute for VGAT to sustain GABA release in classical GABAergic neurons, suggesting that VMAT2 is a vesicular GABA transporter [24]. Nevertheless, biochemical demonstration of GABA transport by VMAT2 across reconstituted membranes is lacking. The GABA packaged and released by DANs is acquired from the extracellular space via action of a plasma membrane GABA transporter (*Slc6a1*/GAT1) or produced autonomously via *Aldh1a1*-dependent GABA synthesis [25•,26••]. We propose that essentially all SNC DANs release GABA, given the near ubiquitous expression of either *Aldh1a1* or *GAT1* [25•,26••,27]. Furthermore, as is true of DA, GABA can be released from DAN axons in an action potential independent manner, for example, following opening of presynaptic nicotinic cholinergic receptors [28,29].

It is difficult to prove that the molecule released from DA axons that activates $GABA_A$ receptors in SPNs is GABA as opposed to another agonist of the notoriously promiscuous $GABA_A$ receptors. Evidence in favor of GABA is that $GABA_A$ receptor mediated currents in SPNs evoked by optogenetic activation of DAN axons are abolished by antagonists of the plasma-membrane GABA transporter GAT1, reduced by blocking the GABA-producing enzyme *Aldh1a1*, and sustained by exogenous expression of VGAT in DANs when VMAT2 is inhibited [24,25•]. Furthermore, given the apparent function of VMAT2 as a vesicular GABA transporter, DA and GABA are likely packaged into and released from the same vesicles (Figure 1b). Many other central monoaminergic neurons, which universally express VMAT2, may be GABAergic depending on the expression of GABA synthetic enzymes or uptake mechanisms.

Recently, the function of GABA release from DA neurons has been probed. As is true of other aldehyde dehydrogenases, Aldh1a1 metabolizes alcohols, which displace and antagonize processing of endogenous substrates; thus ethanol decreases Aldh1a1-dependent GABA production and reduces GABA release from DANs [26••]. Conversely, via unclear mechanisms, deletion of *Aldh1a1* increases ethanol intake in mice. A separate study demonstrated that deletion of *Ube3A*, the gene underlying Angelman's syndrome, from tyrosine hydroxylase-expressing neurons decreases GABA release in NAC from VTA DANs without altering DA release [30••]. Furthermore, this manipulation enhances operant self-stimulation of VTA neurons, an effect that is reversed by restoring GABA release with exogenous over-expression of VGAT in DANs.

These studies provide the first hints that release of GABA from DANs is an important regulator of striatal circuitry. DA increases the spiking rate of active Type 1 DA receptor-expressing direct pathway SPNs (dSPNs) [31]. Therefore, simultaneous release of DA and GABA, even via volume transmission, could enhance spiking in active dSPNs related to ongoing behavior while inhibiting action potential generation of inactive neurons. Depending on one's theory of DA and basal ganglia function, this would be predicted to favor completion of the current action sequence, increase the vigor with which the action is carried out, or promote repetition of the action in the future [32–36]. How such potential effects relate to alcohol consumption or self-stimulation of VTA remains unclear.

A subset of DANs in the VTA, but likely not the SNC, express *Slc17a6*, the gene encoding the vesicular glutamate transporter VGLUT2 and release glutamate in an action-potential-dependent manner (reviewed in Ref. [37]). Early optogenetic studies reported glutamate release from DANs in NAC brain slices [38,39], and subsequent analyses revealed similar glutamate release in dorsal striatum [24,25•]. It is possible that DAN-mediated glutamate release in both NAC and striatum originates from VTA axons, since these also enter the dorsal striatum but reflect different aspects of behavior than do SNC DA axons [40]. The presynaptic regulation of glutamate release from DANs is different than that of GABA/DA, suggesting that the release sites are likely distinct, consistent with ultrastructural analyses (Figure 1c) [25•,41,42•]. The function of glutamate releasing DA neurons is unknown and whether glutamate/DA neurons also release GABA has not been determined electrophysiologically.

VIP/ChAT cortical interneurons

We previously reported the widespread potential for cholinergic neurons of the forebrain, including the major basal forebrain projection to cortex, to co-release GABA [1,2,43••,44]. Another potential source for cortical Ach comes from relatively less studied cholinergic interneurons that are intrinsic to cortex. Immunohistochemical analysis indicates that these cortical cholinergic interneurons are a subset of VIP-expressing interneurons, as all choline acetyltransferase (ChAT)-expressing

interneurons label for VIP, and ~1/3 of all cortical VIP interneurons are positive for ChAT [45–50]. Several studies reported variable expression of the GABAergic markers GAD65 and GAD67 in cholinergic cortical interneurons [46,49,51] indicating they may not be GABAergic. However, to our knowledge, a non-GABAergic population of cortical VIP interneurons has never been described, and this variability may reflect difficulty in reliably immunostaining for GABAergic markers at the cell body rather than the lack of a GABAergic phenotype.

Cortical interneurons that co-express ChAT and VIP have also been identified by single-cell transcriptional analysis of cortical neurons. A study of 3000 single-cell transcriptomes from cortex and hippocampus identified 16 interneuron subclasses, one of which (their #9) co-expresses transcripts for VIP and cholinergic markers [52•]. Further analysis of their data (http://linnarssonlab.org/cortex/) reveals that this subclass also expresses the vesicular acetylcholine transporter (*Slc18a3*/VAChT), Ach synthetic enzyme (*Chat*/ChAT), and membrane choline transporter (*Slc5a7*/ChT). Independent transcriptional analysis of 1,679 cells from visual cortex of adult male mice with greater read-depth [53•] identified 23 distinct GABAergic cell populations, with one (cluster 46, consisting of six neurons) distinguished primarily by co-expression of VIP and ChAT. Both of these studies show robust expression of the GABAergic transcripts *Gad1*/GAD67 and *Slc32a1*/VGAT, in contrast to the variable GABAergic identity reported by immunostaining. Combined with the previous analyses of protein expression, these results indicate that ChAT interneurons are a distinct subclass of VIP GABAergic interneurons.

Relatively little is known about the functional properties of these VIP/ChAT interneurons. One study explicitly probed their synaptic properties using paired recordings between ChAT interneurons and nearby pyramidal cells [51]. This approach did not reveal a specific post-synaptic effect of Ach release, but instead identified presynaptic modulation of excitatory inputs onto pyramidal neurons via nicotinic Ach receptors (nAchRs). However, the use of a GFP-expressing BAC transgenic line to identify ChAT interneurons may have altered the properties of synaptic release through overexpression of VAChT from the BAC or introduced unclear biases in the selection of presynaptic neurons [54]. The potentially important contributions of GABA release from these cells as well as effects of Ach on non-pyramidal targets were not examined. An independent study, focused on cooperative activity between interneuron classes [55], showed that blocking nAChRs slightly decreases the degree to which firing in VIP interneurons stimulates neighboring VIP interneurons, suggesting that Ach release from VIP/ChAT cells normally excites other VIP neurons. Given previous evidence that GABA release from VIP interneurons inhibits somatostatin-expressing interneurons but not other VIP interneurons [56], this suggests that VIP/ChAT interneurons may release Ach and GABA onto different post-synaptic targets, perhaps from separate synaptic vesicle populations (Figure 1d). However, further experiments are required to explicitly test this possibility.

Speculation on the future of research into multi-transmitter neurons

The recognition of neurotransmitter corelease as a widespread phenomenon further complicates the study of the brain and the understanding of circuits within it. Not only must we consider the possibility of synaptic effects from multiple transmitters, but also the possibility that neurotransmitter content is variable over time, regulated by activity, or dependent on the specific context or post-synaptic target [57].

A neuron might signal via one neurotransmitter during development to aid in establishing proper circuit connectivity and switch to a second to provide a particular circuit function later in life. Such developmental neurotransmitter switching occurs in the auditory system, where projections from the medial nucleus of the trapezoid body to the lateral superior olive switch from releasing glutamate early in development, to GABA and then glycine in adulthood [58,59]. Early cotransmission of glutamate is necessary for establishing appropriate tonotopy in this inhibitory auditory pathway [60]. The neurotransmitter content of a neuron could also be acutely activity-dependent, such that the released neurotransmitters differ based on the experience of the animal or activity of the circuit. For example, suppression of Ca^{2+} spiking in *Xenopus* spinal neurons increases the proportion that are glutamatergic or cholinergic, whereas increasing spiking promotes GABAergic and glycinergic transmission [61,62]. Environmental factors can also influence neurotransmitter specification, as greater light exposure increases the number of DANs in the ventral suprachiasmatic nucleus of *Xenopus laevis* [63] and decreases their number in rat hypothalamus [64]. At a circuit level, since striatal GABA release from DANs is dependent on uptake from the extracellular space, the GABA content of DAN synaptic vesicles is predicted to be determined by recent activity of SPNs, which release GABA within striatum via collateral axons. Similarly, release of 'borrowed' neurotransmitters via uptake from plasma membrane transporters has been suggested for several neurons classes [8,65,66].

The analysis of synaptic transmission and of neurotransmitter co-transmission will benefit from technical advances. In our own group, we often struggle to identify the postsynaptic partners of multiple neurotransmitter neurons and to discover which targets sense each released molecule. This is particularly challenging in cerebral cortex, in which a dizzying array of cell classes and high-specificity of wiring renders the potential set of

post-synaptic partners too large to screen by whole-cell recordings. Thus, high-throughput synapse mapping techniques, such as non-toxic anterograde trans-synaptic viruses with channelrhodopsin expression in presynaptic neurons, or quantitative analysis of putative synapses with array tomography, are needed to direct functional analyses to potentially connected neurons. In addition, dynamic optical reporters of extracellular neurotransmitter levels, such as now used to detect synaptic glutamate [67], are needed to detect where and when a neurotransmitter is released independent of a specific postsynaptic electrophysiological response.

In conjunction with such technical advances, we need to exploit our advancing cell-biological understanding of the mechanisms of co-transmission to determine the function and regulation of each released neurotransmitter. The presence of neurotransmitter-specific release sites in multi-transmitter neurons indicates the existence of poorly understood presynaptic mechanisms governing the differential formation of terminals of each class. Furthermore, potential non-neuronal targets of neurotransmitters, such as microglia, astrocytes, and vascular endothelial cells must be examined. Similarly, further study of non-electrical consequences of neurotransmitter release is needed to understand the full nature of synaptic transmission.

Lastly, considering the richness of multiple transmitter synaptic transmission that has been discovered using candidate analyses, novel transcriptional, biochemical, and metabolic approaches are needed to identify the chemical identity and function of unknown neurotransmitters. Undoubtedly the studies made possible by these technical advances will expand the menagerie of multi-transmitter neurons by discovering more bizarre cellular beasts specialized to fill unique functional circuit niches.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Conflict of interest statement

Nothing declared.

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