

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, endocannabinoids) – 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

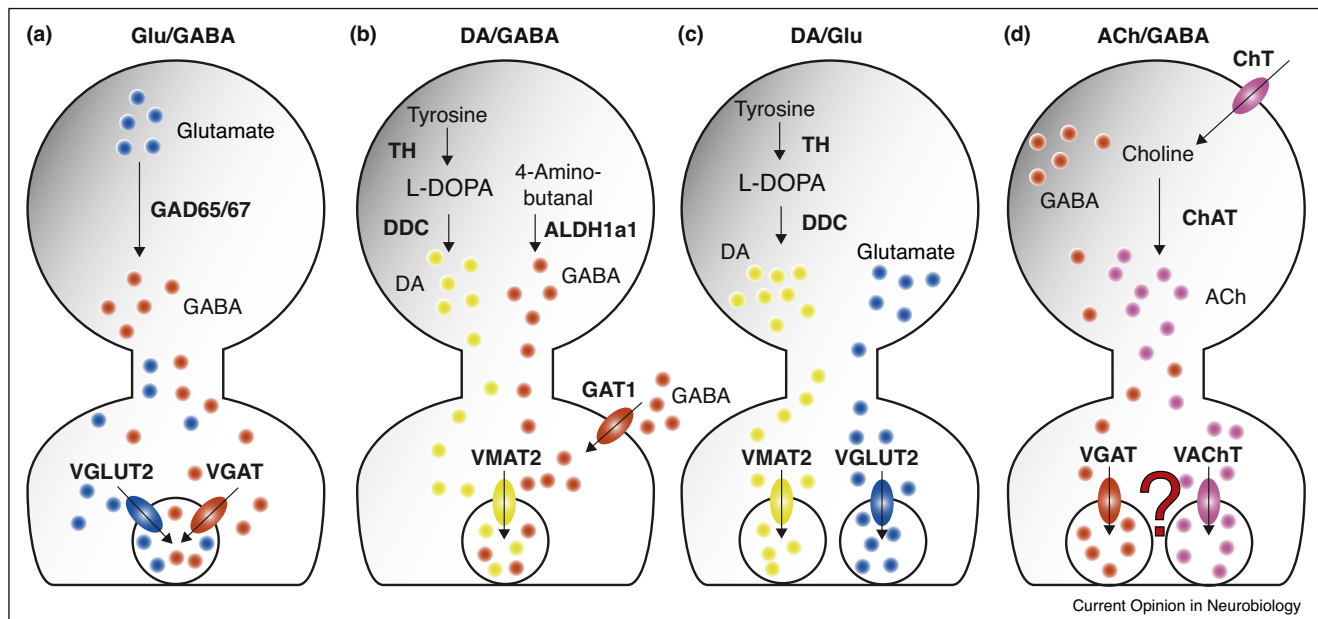
packaged into the same synaptic vesicles, resulting in simultaneous release of both neurotransmitters (Figure 1a). This conclusion is based on: (1) the electrophysiological observation of biphasic miniature and spontaneous post-synaptic currents at holding potentials between the reversal potentials of 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 multi-transmitter 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 through 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.

References and recommended reading

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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References

1. Tritsch NX, Granger AJ, Sabatini BL: **Mechanisms and functions of GABA co-release.** *Nat. Rev. Neurosci.* 2016, **17**:139-145.
2. Granger AJ, Mulder N, Saunders A, Sabatini BL: **Cotransmission of acetylcholine and GABA.** *Neuropharmacology* 2016, **100**: 40-46.
3. Hnasko TS, Edwards RH: **Neurotransmitter corelease: mechanism and physiological role.** *Annu. Rev. Physiol.* 2012, **74**:225-243.
4. McEachern AE, Margiotta JF, Berg DK: **gamma-Aminobutyric acid receptors on chick ciliary ganglion neurons in vivo and in cell culture.** *J. Neurosci.* 1985, **5**:2690-2695.
5. Engisch KL, Fischbach GD: **The development of ACh- and GABA-activated currents in normal and target-deprived embryonic chick ciliary ganglia.** *Dev. Biol.* 1990, **139**:417-426.
6. Engisch KL, Fischbach GD: **The development of ACh- and GABA-activated currents in embryonic chick ciliary ganglion neurons in the absence of innervation in vivo.** *J. Neurosci.* 1992, **12**:1115-1125.
7. O'Malley DM, Masland RH: **Co-release of acetylcholine and gamma-aminobutyric acid by a retinal neuron.** *Proc. Natl. Acad. Sci. U. S. A.* 1989, **86**:3414-3418.
8. Gendrel M, Atlas EG, Hobert O: **A cellular and regulatory map of the GABAergic nervous system of *C. elegans*.** *eLife* 2016, **5**.
9. Filippi A, Mueller T, Driever W: **vglut2 and gad expression reveal distinct patterns of dual GABAergic versus glutamatergic cotransmitter phenotypes of dopaminergic and noradrenergic neurons in the zebrafish brain.** *J. Comp. Neurol.* 2014, **522**: 2019-2037.
10. Hirasawa H, Betensky RA, Raviola E: **Corelease of dopamine and GABA by a retinal dopaminergic neuron.** *J. Neurosci.* 2012, **32**:13281-13291.
11. Borisovska M, Bensen AL, Chong G, Westbrook GL: **Distinct modes of dopamine and GABA release in a dual transmitter neuron.** *J. Neurosci.* 2013, **33**:1790-1796.
12. Hong S, Hikosaka O: **The globus pallidus sends reward-related signals to the lateral habenula.** *Neuron* 2008, **60**:720-729.
13. Stephenson-Jones M, Yu K, Ahrens S, Tucciarone JM, van Huijstee AN, Mejia LA, Penzo MA, Tai L-H, Willbrecht L, Li B: **A basal ganglia circuit for evaluating action outcomes.** *Nature* 2016, **539**:289-293.
- This study shows that increasing or decreasing activity of LHB-projecting EP neurons in mice can influence behavior by effecting negative or positive reinforcement, respectively. The authors demonstrate that manipulations of this circuit element can bidirectionally impact future choices, in a probabilistic switching task.
15. Ji H, Shepard PD: **Lateral habenula stimulation inhibits rat midbrain dopamine neurons through a GABA(A) receptor-mediated mechanism.** *J. Neurosci.* 2007, **27**:6923-6930.
16. Parent M, Lévesque M, Parent A: **Two types of projection neurons in the internal pallidum of primates: single-axon tracing and three-dimensional reconstruction.** *J. Comp. Neurol.* 2001, **439**:162-175.
17. Parent A, De Bellefeuille L: **Organization of efferent projections from the internal segment of globus pallidus in primate as revealed by fluorescence retrograde labeling method.** *Brain Res.* 1982, **245**:201-213.
18. Shabel SJ, Proulx CD, Piriz J, Malinow R: **GABA/glutamate co-release controls habenula output and is modified by antidepressant treatment.** *Science* 2014, **345**:1494-1498.
- The is the first study to show GABA/glutamate corelease from EP axons. The authors provide evidence that GABA and glutamate are packaged into the same synaptic vesicle and regulated by depressive states.
19. Barroso-Chinea P, Rico AJ, Pérez-Manso M, Roda E, López IP, Luis-Ravelo D, Lanciego JL: **Glutamatergic pallidothalamic projections and their implications in the pathophysiology of Parkinson's disease.** *Neurobiol. Dis.* 2008, **31**:422-432.
20. Root DH, Mejias-Aponte CA, Zhang S, Wang H-L, Hoffman AF, Lupica CR, Morales M: **Single rodent mesohabenular axons release glutamate and GABA.** *Nat. Neurosci.* 2014, **17**: 1543-1551 <http://dx.doi.org/10.1038/nn.3823>.

The authors show that inputs from the VTA to the Lhb corelease GABA and glutamate. *In vivo* recordings demonstrate that this input can either excite or inhibit individual Lhb neurons.

21. Yoo JH, Zell V, Gutierrez-Reed N, Wu J, Ressler R, Shenasa MA, Johnson AB, Fife KH, Faget L, Hnasko TS: **Ventral tegmental area glutamate neurons co-release GABA and promote positive reinforcement.** *Nat. Commun.* 2016, **7**:13697.
 22. Meye FJ, Soiza-Reilly M, Smit T, Diana MA, Schwarz MK, Mamelì M: **Shifted pallidal co-release of GABA and glutamate in habenula drives cocaine withdrawal and relapse.** *Nat. Neurosci.* 2016, **19**:1019-1024.
 23. Shabel SJ, Proulx CD, Trias A, Murphy RT, Malinow R: **Input to the lateral habenula from the basal ganglia is excitatory, aversive, and suppressed by serotonin.** *Neuron* 2012, **74**:475-481.
 24. Tritsch NX, Ding JB, Sabatini BL: **Dopaminergic neurons inhibit striatal output through non-canonical release of GABA.** *Nature* 2012, **490**:262-266.
 25. Tritsch NX, Oh W-J, Gu C, Sabatini BL: **Midbrain dopamine neurons sustain inhibitory transmission using plasma membrane uptake of GABA, not synthesis.** *eLife* 2014, **3**:e01936.
- Using electrophysiology and *in situ* hybridization, the authors show that GABA release from midbrain DA neurons requires the plasma membrane GABA transporter *mGat1* rather than canonical GABA synthetic enzymes GAD65/67.
26. Kim J-I, Ganesan S, Luo SX, Wu Y-W, Park E, Huang EJ, Chen L, Ding JB: **Aldehyde dehydrogenase 1a1 mediates a GABA synthesis pathway in midbrain dopaminergic neurons.** *Science* (80-) 2015, **350**:102-106.
- The authors dopaminergic neurons synthesize GABA using an alternative *Aldh1a1*-dependent pathway. Deletion of *Aldh1a1* from midbrain DA neurons reduces GABA release and the remaining component is eliminated by blockade of plasma membrane GABA transporters.
27. Poulin J-F, Zou J, Drouin-Ouellet J, K-YA Kim, Cicchetti F, Awatramani B: **Defining midbrain dopaminergic neuron diversity by single-cell gene expression profiling.** *Cell Rep.* 2014, **9**:930-943.
 28. Threlfell S, Lalic T, Platt NJ, Jennings KA, Deisseroth K, Cragg SJ: **Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons.** *Neuron* 2012, **75**:58-64.
 29. Nelson AB, Hammack N, Yang CF, Shah NM, Seal RP, Kreitzer AC: **Striatal cholinergic interneurons drive GABA release from dopamine terminals.** *Neuron* 2014, **82**:63-70.
 30. Berrios J, Stamatakis AM, Kantak PA, McElligott ZA, Judson MC, Aita M, Rougie M, Stuber GD, Philpot BD: **Loss of UBE3A from TH-expressing neurons suppresses GABA co-release and enhances VTA-NAc optical self-stimulation.** *Nat. Commun.* 2016, **7**:10702.
- This study investigates the role of GABA release from mesoaccumbal TH-expressing neurons in reward-seeking behaviors. The authors show that Angelman syndrome associated gene *Ube3a* is required for normal levels of GABA release from TH-neurons, and restoring typical levels of GABA release from TH neurons can ameliorate dysfunctional reward-seeking behaviors in mice lacking *Ube3a*.
31. Gerfen CR, Surmeier DJ: **Modulation of striatal projection systems by dopamine.** *Annu. Rev. Neurosci.* 2011, **34**:441-466.
 32. Dudman JT, Krakauer JW: **The basal ganglia: from motor commands to the control of vigor.** *Curr. Opin. Neurobiol.* 2016, **37**:158-166.
 33. Hikosaka O: **Neural systems for control of voluntary action—a hypothesis.** *Adv. Biophys.* 1998, **35**:81-102.
 34. Nelson AB, Kreitzer AC: **Reassessing models of basal ganglia function and dysfunction.** *Annu. Rev. Neurosci.* 2014, **37**:117-135.
 35. Hamid AA, Pettibone JR, Mabrouk OS, Hetrick VL, Schmidt R, Vander Weele CM, Kennedy RT, Aragona BJ, Berke JD: **Mesolimbic dopamine signals the value of work.** *Nat. Neurosci.* 2016, **19**:117-126.
 36. Schultz W: **Dopamine reward prediction-error signalling: a two-component response.** *Nat. Rev. Neurosci.* 2016, **17**:183-195.
 37. Morales M, Margolis EB: **Ventral tegmental area: cellular heterogeneity, connectivity and behaviour.** *Nat. Rev. Neurosci.* 2017, **18**:73-85 <http://dx.doi.org/10.1038/nrn.2016.165>.
 38. Stuber GD, Hnasko TS, Britt JP, Edwards RH, Bonci A: **Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate.** *J. Neurosci.* 2010, **30**:8229-8233.
 39. Tecuapetla F, Patel JC, Xenias H, English D, Tadros I, Shah F, Berlin J, Deisseroth K, Rice ME, Tepper JM *et al.*: **Glutamatergic signaling by mesolimbic dopamine neurons in the nucleus accumbens.** *J. Neurosci.* 2010, **30**:7105-7110.
 40. Howe MW, Dombeck DA: **Rapid signalling in distinct dopaminergic axons during locomotion and reward.** *Nature* 2016, **535**:505-510.
 41. Barker DJ, Root DH, Zhang S, Morales M: **Multiplexed neurochemical signaling by neurons of the ventral tegmental area.** *J. Chem. Neuroanat.* 2016, **73**:33-42.
 42. Zhang S, Qi J, Li X, Wang H-L, Britt JP, Hoffman AF, Bonci A, Lupica CR, Morales M: **Dopaminergic and glutamatergic microdomains in a subset of rodent mesoaccumbens axons.** *Nat. Neurosci.* 2015, **18**:386-392.
- This study uses electron microscopy and immunohistochemistry to show that dopaminergic terminals in the nucleus accumbens have distinct microdomains for the release of glutamate marked by VGLUT2 or dopamine marked by VMAT.
43. Saunders A, Granger AJ, Sabatini BL: **Corelease of acetylcholine and GABA from cholinergic forebrain neurons.** *eLife* 2015, **4**:AD65.
- This study shows that ChAT-expressing neurons of the forebrain colocalize with genetically-labeled GABAergic markers VGAT and GAD65 and that optogenetic activation of ChAT-expressing fibers in cortex results in monosynaptic release of not only ACh, but also GABA.
44. Saunders A, Oldenburg IA, Berezovskii VK, Johnson CA, Kingery ND, Elliot HL, Tiao X, Gerfen CR, Sabatini BL: **A direct GABAergic output from the basal ganglia to frontal cortex.** *Nature* 2015, **521**:85-89.
 45. Eckenstein F, Baughman RW: **Two types of cholinergic innervation in cortex, one co-localized with vasoactive intestinal polypeptide.** *Nature* 1984, **309**:153-155.
 46. Kosaka T, Tauchi M, Dahl L: **Cholinergic neurons containing GABA-like and/or glutamic acid decarboxylase-like immunoreactivities in various brain regions of the rat.** *Exp. Brain Res.* 1988, **70**:605-617.
 47. Bayraktar T, Staiger J, Acasady L, Cozzari C, Freund T, Zilles K: **Co-localization of vasoactive intestinal polypeptide, γ -aminobutyric acid and choline acetyltransferase in neocortical interneurons of the adult rat.** *Brain Res.* 1997, **757**:209-217.
 48. Gonchar Y, Wang Q, Burkhalter A: **Multiple distinct subtypes of GABAergic neurons in mouse visual cortex identified by triple immunostaining.** *Front. Neuroanat.* 2008, **1**:3.
 49. Consonni S, Leone S, Becchetti A, Amadeo A: **Developmental and neurochemical features of cholinergic neurons in the murine cerebral cortex.** *BMC Neurosci.* 2009, **10**:18.
 50. Asmus SE, Cocanougher BT, Allen DL, Boone JB, Brooks EA, Hawkins SM, Hench LA, Ijaz T, Mayfield MN: **Increasing proportions of tyrosine hydroxylase-immunoreactive interneurons colocalize with choline acetyltransferase or vasoactive intestinal peptide in the developing rat cerebral cortex.** *Brain Res.* 2011, **1383**:108-119.
 51. von Engelhardt J, Eliava M, Meyer AH, Rozov A, Monyer H: **Functional characterization of intrinsic cholinergic interneurons in the cortex.** *J. Neurosci.* 2007, **27**:5633-5642.
 52. Zeisel A, Munoz-Manchado AB, Codeluppi S, Lonnerberg P, La Manno G, Jureus A, Marques S, Munguba H, He L, Betsholtz C *et al.*: **Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq.** *Science* 2015, **347**:1138-1142.
- This was the first study to apply recently developed high-throughput single-cell RNA sequencing to classify neurons in the adult mouse brain. This transcriptional profiling confirms the presence of dual VIP/ChAT

cortical interneurons, and is a valuable resource for identifying other potential multi-transmitter neurons.

53. Tasic B, Menon V, Nguyen TN, Kim TK, Jarsky T, Yao Z, Levi B, Gray LT, Sorensen SA, Dolbeare T *et al.*: **Adult mouse cortical cell taxonomy revealed by single cell transcriptomics.** *Nat. Neurosci.* 2016, **19**:335-346.
- This study also uses single-cell RNA sequencing to classify cortical cells in adult mouse according to transcriptional profiling, and confirms the presence of VIP/ChAT cortical interneurons. Together with Zeisel *et al.* [52], these studies demonstrate the promise in large-scale, freely available transcriptional data-sets for guiding experiments in synaptic function.
54. Kolisnyk B, Guzman MS, Raulic S, Fan J, Magalhães AC, Feng G, Gros R, Prado VF, Prado MAM: **ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains.** *J. Neurosci.* 2013, **33**:10427-10438.
55. Karnani MM, Jackson J, Ayzenshtat I, Tucciarone J, Manoocheri K, Snider WG, Yuste R: **Cooperative subnetworks of molecularly similar interneurons in mouse neocortex.** *Neuron* 2016, **90**:86-100.
56. Pfeffer CK, Xue M, He M, Huang ZJ, Scanziani M: **Inhibition of inhibition in visual cortex: the logic of connections between molecularly distinct interneurons.** *Nat. Neurosci.* 2013, **16**:1068-1076.
57. Spitzer NC: **Neurotransmitter switching? No surprise.** *Neuron* 2015, **86**:1131-1144.
58. Nabekura J, Katsurabayashi S, Kakazu Y, Shibata S, Matsubara A, Jinno S, Mizoguchi Y, Sasaki A, Ishibashi H: **Developmental switch from GABA to glycine release in single central synaptic terminals.** *Nat. Neurosci.* 2004, **7**:17-23.
59. Gillespie DC, Kim G, Kandler K: **Inhibitory synapses in the developing auditory system are glutamatergic.** *Nat. Neurosci.* 2005, **8**:332-338.
60. Noh J, Seal RP, Garver JA, Edwards RH, Kandler K: **Glutamate co-release at GABA/glycinergic synapses is crucial for the refinement of an inhibitory map.** *Nat. Neurosci.* 2010, **13**:232-238.
61. Borodinsky LN, Root CM, Cronin JA, Sann SB, Gu X, Spitzer NC: **Activity-dependent homeostatic specification of transmitter expression in embryonic neurons.** *Nature* 2004, **429**:523-530.
62. Borodinsky LN, Spitzer NC: **Activity-dependent neurotransmitter-receptor matching at the neuromuscular junction.** *Proc. Natl. Acad. Sci.* 2007, **104**:335-340.
63. Dulcis D, Spitzer NC: **Illumination controls differentiation of dopamine neurons regulating behaviour.** *Nature* 2008, **456**:195-201.
64. Dulcis D, Jamshidi P, Leutgeb S, Spitzer NC: **Neurotransmitter switching in the adult brain regulates behavior.** *Science (80-)* 2013, **340**:449-453.
65. Snow H, Lowrie MB, Bennett JP: **A postsynaptic GABA transporter in rat spinal motor neurones.** *Neurosci. Lett.* 1992, **143**:119-122.
66. Lebrand C, Cases O, Adelbrecht C, Doye A, Alvarez C, El Mestikawy S, Seif I, Gaspar P: **Transient uptake and storage of serotonin in developing thalamic neurons.** *Neuron* 1996, **17**:823-835.
67. Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, Gordus A, Renninger SL, Chen T-W, Bargmann CI *et al.*: **An optimized fluorescent probe for visualizing glutamate neurotransmission.** *Nat. Methods* 2013, **10**:162-170.