Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

Neuron

CellPress

Review

Synaptic and circuit functions of multitransmitter neurons in the mammalian brain

Michael L. Wallace[1,*] and Bernardo L. Sabatini[2]
[1]Department of Anatomy and Neurobiology, Boston University Chobanian & Avedisian School of Medicine, Boston, MA, USA
[2]Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, Boston, MA, USA
*Correspondence: mlwall12@bu.edu
https://doi.org/10.1016/j.neuron.2023.06.003

SUMMARY

Neurons in the mammalian brain are not limited to releasing a single neurotransmitter but often release multiple neurotransmitters onto postsynaptic cells. Here, we review recent findings of multitransmitter neurons found throughout the mammalian central nervous system. We highlight recent technological innovations that have made the identification of new multitransmitter neurons and the study of their synaptic properties possible. We also focus on mechanisms and molecular constituents required for neurotransmitter corelease at the axon terminal and synaptic vesicle, as well as some possible functions of multitransmitter neurons in diverse brain circuits. We expect that these approaches will lead to new insights into the mechanism and function of multitransmitter neurons, their role in circuits, and their contribution to normal and pathological brain function.

INTRODUCTION

A neuron is often defined by the identity of the neurotransmitter it releases (i.e., glutamatergic, GABAergic, cholinergic, etc.), with this label specifying the function of a neuron within a circuit. However, the designation of a molecule as the ''principal'' neurotransmitter of a neuron obscures the genuine diversity of synaptic signaling and hampers further inquiries into neuronal and circuit function, as many, if not all, neurons release more than one neurotransmitter.

The many recent discoveries of multitransmitter neurons in mammalian systems were preceded by a long history of study in other organisms,[1] such as the amphibians,[2] mollusks,[3] and the crustacean somatogastric system.[4] Nevertheless, a review of multitransmitter neurons in the mammalian brain is timely, as exciting new studies exemplify the diverse array of neuronal subtypes in the brain that have the molecular machinery necessary to release more than one neurotransmitter.[5–9] Furthermore, new ''intersectional'' genetic and viral tools permit manipulation of specific neuronal populations suspected of neurotransmitter corelease[10] and enable functional analysis of cotransmitting neurons *in vitro* and *in vivo*.[11,12]

We expect emerging *in vivo* studies to lend new insight into how each neurotransmitter released from a multitransmitter neuron(s) contributes to the dynamics and function of interconnected circuits during behavior. As many of the multitransmitter neurons we review here have historically been viewed as releasing a single neurotransmitter, studies in this field have the potential to assign functions previously ascribed to dopamine (DA), acetylcholine (Ach), or serotonin (5-HT) to different neurotransmitters released from those same neuronal populations. We conclude that neuroscience is now at a stage where we can confidently identify when and where cotransmission occurs in the brain, as well as reveal its contribution to normal and pathological brain function.

Neurotransmitters take many forms, including small molecules (Ach, glutamate, GABA, and monoamines), peptides (enkephalin, dynorphin, somatostatin [*Sst*]), purines (ATP), lipophilic esters (endocannabinoids), and gases (nitric oxide). Here, we will focus on activity-dependent neurotransmission of multiple small molecule neurotransmitters (i.e., cotransmitting or multitransmitter neurons) within the mammalian central nervous system (Tables 1 and 2). Furthermore, we will not discuss changes in neurotransmitter release during development, neurotransmitter switching, and corelease of neurotransmitters in other model organisms, as recent reviews can be found elsewhere.[13–15] We close by speculating on some possible cellular and circuit functions for release of two or more transmitters in different brain regions that we highlight throughout the review.

MECHANISMS/DETERMINANTS FOR VESICULAR CORELEASE

Vesicular release of a neurotransmitter requires a neuron to accumulate the signaling molecule in the presynaptic terminal to a concentration that permits it to be packaged into a vesicle and released. Accumulation can occur by neurotransmitter synthesis and/or reuptake, and packaging is performed by the vesicular transporters. Therefore, concluding that a neuron can only release one or more neurotransmitters (i.e., that it can corelease), requires confirmation that both steps occur. Each of these components can also be independently modulated or modified to restrict or enhance release of a transmitter, underscoring their importance to neurotransmission. Finally, knowledge of these components allows precise genetic perturbation of corelease by manipulating each neurotransmitter individually.

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

Table 1. Neurotransmitters and associated vesicular transporters

Neurotransmitter (abbr.)	Vesicular transporter (protein)	Vesicular transporter (*gene*)
GABA	vGAT/vMAT2	*Slc32a1/Slc18a2*
Glutamate (Glu)	vGluT1/2/3	*Slc17a7/Slc17a6/Slc17a8*
Acetylcholine (Ach)	vAchT	*Slc18a3*
Glycine (Gly)	vGAT	*Slc32a1*
Dopamine (DA)	vMAT2	*Slc18a2*
Serotonin (5-HT)	vMAT2	*Slc18a2*

Table 2. Definitions

Corelease: process by which two (or more) neurotransmitters are released by a single neuron following an action potential.
Cotransmission: process by which two (or more) neurotransmitters are synaptically released in response to an action potential and detected by receptors on the postsynaptic cell.
Multitransmitter neuron: a neuron that is capable of releasing more than one neurotransmitter.
Co-packaging: process by which a coreleasing neuron transports two different neurotransmitters into the same synaptic vesicle.

In this context, we will review the examples of multitransmitter neurons from the mammalian central nervous system and identify, if possible, when the molecular requirements for synthesis, accumulation, and packaging are determined. Importantly, neurons can uptake and package neurotransmitters using atypical pathways, making this process sometimes difficult.

VESICULAR TRANSPORTER SYNERGY AND ANTAGONISM

In multitransmitter neurons, where vesicular packaging of more than one neurotransmitter occurs, it is important to consider how vesicular transporters are differentially sensitive to intravesicular factors such as pH or membrane potential.[16] This becomes particularly important when considering multitransmitter neurons that exhibit co-packaging of two neurotransmitters within the same vesicle (examples discussed below).

Synaptic vesicles are acidified by vacuolar H+-dependent adenosine triphosphatases (V-ATPases) that pump protons into the vesicle lumen.[17] This creates a pH gradient (ΔpH) and a vesicular membrane potential ($\Delta\Psi$), both of which can power the uptake of neurotransmitters by vesicular transporters (Table 1).[16] Vesicular transporters for monoamines (vMAT1/2) and Ach (VAChT) transport positively charged neurotransmitters in exchange for 2H+ and are more dependent on ΔpH than $\Delta\Psi$.[18,19] Conversely, the transport of glutamate by vGluT1/2/3 depends primarily on $\Delta\Psi$. Glutamate is an anion at neutral pH, and loading of glutamate into vesicles produces a change in charge and H+ opposite to vMAT and VAChT.[20] These complementary dependencies may explain the observed synergies between vGluT and VAChT or vGluT and the vesicular zinc transporter (ZnT3) when these are localized to the same synaptic vesicle.[5,21,22]

In contrast, the vesicular GABA/glycine transporter (vGAT) transports neutral zwitterions. Studies conclude that vGAT acts as a GABA/H+ antiporter rather than a GABA/Cl− cotransporter and depends on both ΔpH and $\Delta\Psi$.[23,24] Therefore, the presence of vGAT on synaptic vesicles may have little impact on the activity of other transporters on the same vesicle, with little effect on their function due to its codependence on ΔpH and $\Delta\Psi$ and its minimal effect on either gradient. Functional studies on vesicles carrying both vGAT and another vesicular transporter, such as vGluT2, are needed to test whether vGAT may alter the co-packaging of other transmitters into the same vesicle.[25,26] Below, we take these details into account in multitransmitter neurons where there is evidence for co-packaging and discuss how it may affect signaling.

MULTITRANSMITTER NEURONS WITH CO-PACKAGING

GABA/glutamate

Typically, neurotransmitter release, at least through actions on ionotropic receptors, is thought to have either an inhibitory or excitatory effect on the postsynaptic cell. Perhaps, then, the most confounding example of multitransmitter neurons arises from GABA/glutamate cotransmitting neurons. Recently, using a combination of genetic crosses and *in situ* hybridization, a screen for GABA/glutamate coreleasing neurons revealed that 30 different brain regions have neurons that express the genes required for synaptic vesicle packaging of both GABA (vGAT; *Slc32a1*) and glutamate (vGluT2/3; *Slc17a6*/*Slc17a8*).[8] These findings indicated that GABA/glutamate cotransmission may be more widespread than previously thought. However, cotransmission by most of these recently identified cell types has yet to be physiologically confirmed; therefore, here we will focus on three regions where the existence of GABA/glutamate coreleasing neurons has been most convincingly demonstrated: supramammilary nucleus (SuM), ventral tegmental area (VTA), and entopeduncular nucleus (EP).

Electron microscopy (EM) studies of axon terminals in the dentate gyrus (DG) have demonstrated anatomical evidence for the existence of GABAergic and glutamatergic vesicular transporters in the same axon.[27] These studies found that these terminals arose from long-range projections from the SuM and provided evidence that the GABAergic and glutamatergic vesicles segregated into distinct pools and occupied distinct presynaptic terminals.[28] Furthermore, optogenetic activation of SuM terminals in the DG produces monosynaptic, short-latency release of both GABA and glutamate onto postsynaptic granule cells and interneurons.[29] Studies using chemogenetic manipulation of projections from the SuM to DG reveal that this circuit is involved in the regulation of arousal.[30]

Neuronal diversity within the VTA has been studied in depth and excellent detailed reviews exist elsewhere.[31] One of the VTA neuronal subtypes is distinguished by coexpression of the genes encoding the vesicular transporters for GABA (*Slc32a1*) and glutamate (*Slc17a6*).[32] These neurons are distributed mostly in the medial subregions of the VTA, including the interfascicular nucleus, paranigral nucleus, and rostral linear nucleus of the raphe.[33] They monosynaptically connect to neurons within the lateral habenula (mostly medial subregions) and cotransmit

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

GABA and glutamate onto individual lateral habenula (LHb) neurons.[32] *In vivo* optogenetic excitation of VTA input to the LHb (using either *Slc17a6-IRES-Cre* or *Slc32a1-IRES-Cre* mice) results in either excitation, inhibition, or both of extracellularly recorded LHb neurons, suggesting that the postsynaptic LHb neuron, presumably by differential insertion of ionotropic GABA and glutamate receptors, may determine whether the input is primarily excitatory or inhibitory. Immunogold EM indicates that >60% of the axon terminals in LHb from VTA are positive for both vGAT and vGluT2, while smaller subsets label for one or the other transporter. Interestingly, immunogold EM also indicated that single axon terminals make both symmetric (GABAergic) and asymmetric (glutamatergic) synapses (see Figure 5 in Root et al.[33]), suggesting segregation of synaptic vesicles into glutamatergic and GABAergic synapses within the same axon and bouton. Finally, immunogold EM and immunoprecipitation of synaptic vesicles from LHb found that vesicles were largely vGluT2 or vGAT positive, but rarely copositive, suggesting that GABA and glutamate are typically packaged into separate vesicles in the LHb.[33] Together, these studies suggest that even though individual axons from the VTA express both vGluT2 and vGAT, synaptic vesicles and vesicular transporters are largely segregated into distinct pools for release, rather than released together at the same synapse. How a neuron traffics each transporter to a separate vesicle pool and successfully forms and maintains both a symmetric and an asymmetric synapse from a single bouton to a common postsynaptic dendrite is unknown and indicates that a great deal remains to be discovered about the molecular processes of synapse specification and maintenance.

The EP (globus pallidus internus [GPi] in primates) is a major output nucleus of the basal ganglia and is typically discussed as a GABAergic region that inhibits targets in the thalamus as part of cortical-basal ganglia-thalamocortical loops.[34] However, EP/GPi has diverse neuronal populations and functions, the latter primarily via its dense projections targeting the LHb.[35–37] Due to immunohistochemical and behavioral findings, LHb-projecting EP/GPi neurons were first suggested to be glutamatergic, purely excitatory, and signal aversive/negative outcomes.[38,39] Later, they were found to simultaneously transmit both glutamate and GABA and to be capable of both exciting and inhibiting LHb neurons[40,26] (Figure 1A). GABA/glutamate coreleasing neurons in the EP of mice and GPi in humans were found to be marked by expression of *Sst*, facilitating specific targeting of these neurons in functional studies.[40] Similar to LHb-projecting VTA neurons, LHb-projecting EP neurons have single axon terminals that make both symmetric (GABAergic) and asymmetric (glutamatergic) synapses, as determined by EM.[33]

Recent studies using electrophysiological recordings and minimal optical stimulation of synapses of EP *Sst* neurons in LHb examined whether GABA and glutamate are co-packaged in the same synaptic vesicle or segregated into independent vesicular pools. Optical quantal analysis determined that the statistical properties of unitary responses resulting from activation of cotransmitting EP release sites were consistent only with co-packaging of GABA and glutamate into the same synaptic vesicle[25] (Figure 2). These results support a model in which individual synaptic vesicles express both vGAT and vGluT2, and GABA/glutamate are released simultaneously to activate postsynaptic receptors across the synaptic cleft. The excitatory/inhibitory ratio at each synapse can be adjusted independently by regulating the activity of the vesicular transporters[26,41] or adjusting the composition of postsynaptic receptors. The structure, organization, and regulation of the postsynaptic receptors and related molecular constituents of GABA/glutamate synapses remain unknown but appear specialized within LHb due to low expression of the glutamatergic postsynaptic-terminal-associated MAGUK PSD-95.[25] Further studies are needed to understand the interactions between vGAT and vGluT2 transport into single vesicles, as each transmitter relies on distinct chemical and electrical gradients for vesicular transport, which may alter their efficacy when present on the same vesicle.[24,42,43]

GABA/glycine

Studies from the 1990s demonstrated cotransmission of GABA and glycine from inhibitory interneurons in the spinal cord.[44] These studies were some of the first to describe the ''co-packaging'' phenomenon, where two different neurotransmitters are loaded into individual synaptic vesicles. GABA/glycine cotransmission has since been observed in other areas, including the auditory brainstem and cerebellum.[45–47] By adulthood, both transmitters are inhibitory, activating their cognate chloride permeable ligand-gated ion channels, and hyperpolarizing their postsynaptic targets.[48] The kinetics of glycine receptors are faster than those of GABA receptors, resulting in an inhibitory post synaptic current (IPSC) with fast and slow components.[44,45,49] Notably, GABA and glycine compete for packaging into synaptic vesicles as both are transported by the same vesicular transporter vGAT[50] (Figure 1A). Therefore, packaging GABA and/or glycine into vesicles is largely dependent on their intracellular (intraaxonal) concentration, which are controlled by either intracellular synthesis (glutamate decarboxylase [GAD] 1/2) or plasma membrane transporters (GlyT1/2), respectively.[51] These results suggest that the potency of a synapse depends not only on the number of postsynaptic receptors but also on the concentrations of presynaptic transmitter and how much transmitter is loaded into the vesicle. Therefore, assumptions regarding saturation of postsynaptic receptors following single vesicle fusion may not hold at these or other synapses, where competition for vesicular transport is high. This may be a general feature of multitransmitter neurons that exhibit co-packaging, as it likely puts a lower limit on the amount of each neurotransmitter loaded into a single vesicle when compared with one vesicle with one transmitter. Together, these studies frame an interesting role for cotransmission, where two transmitters provide hyperpolarization of the postsynaptic cell with different kinetics, allowing the presynaptic neuron tight control over postsynaptic firing rates.

ACh/glutamate

Cholinergic neurons have an essential role in many circuit and cognitive functions within the brain, from learning and memory,[52] sensory perception,[53] synaptic plasticity,[54] and arousal.[55] However, many of these neurons also contain the vesicular glutamate transporters (vGluT1/2/3) that are coexpressed with molecular machinery for Ach synthesis, vesicular packaging, and release in multiple brain regions, including the striatum, basal forebrain,

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

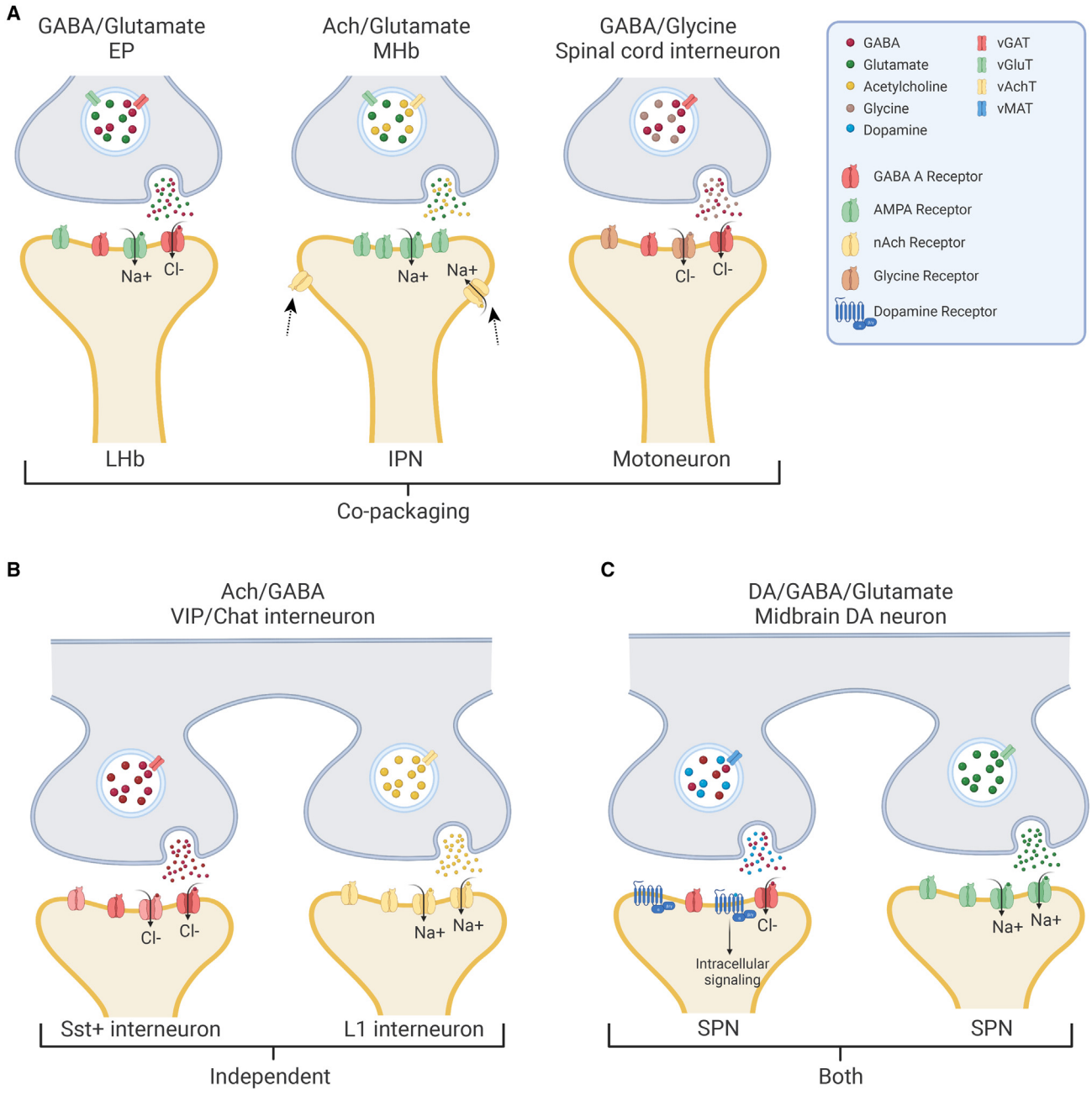

Figure 1. Synaptic diversity of cotransmitting neurons

(A) Three examples of cotransmitting neurons that employ vesicular co-packaging of two neurotransmitters at the presynaptic terminal.

(B) Ach/GABA cotransmitting neurons found in cortex (VIP/Chat+) release Ach and GABA at different presynaptic terminals and independently package these neurotransmitters into separate vesicle pools.

(C) Many midbrain DA neurons release three neurotransmitters; DA and GABA are co-packaged in the same vesicle, whereas glutamate is independently packaged and released at separate presynaptic sites.

and medial habenula (mHb).[22,56,57] Axon terminals from mHb to the interpeduncular nucleus (IPN) contain both vGluT1/2 and VAChT and cotransmit both glutamate and Ach onto individual neurons in the IPN.[56] Single-cell sequencing and *in-situ* hybridization (ISH) studies confirmed that these neurons reside in the ventral and ventrolateral subregion of the mHb and form a genetically distinct subtype.[58,59] Although postsynaptic AMPA and NMDA receptors are activated by single action potentials (APs) in the presynaptic axon, nicotinic Ach receptors (nAchRs) were only activated slowly following prolonged (50 Hz, 5 s) stimulation of inputs, indicating that glutamate and Ach have different transmission modes.[56]

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

CellPress

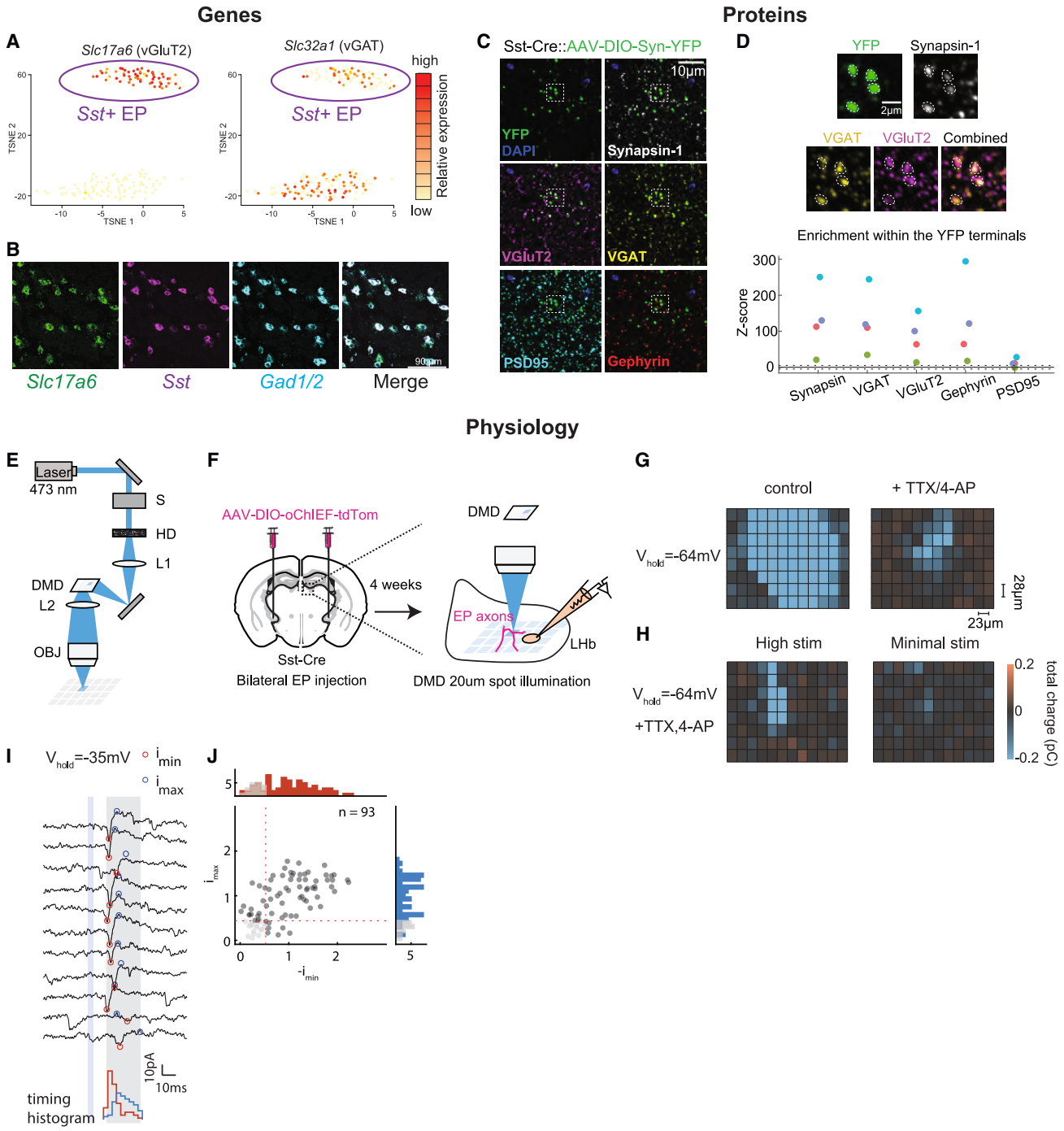

Figure 2. Methods for investigating individual cotransmitting neurons and synapses

(A) Methods to evaluate gene expression in single neurons, such as single-cell whole transcriptome sequencing (shown), can examine expression levels of many genes in single neurons to determine whether the genetic constituents required for neurotransmitter corelease are present (dots circled in purple represent individual *Sst*+ GABA/glutamate coreleasing neurons isolated from EP, color represents gene expression level for vGluT2 [left] or VGAT [right]).

(B) Fluorescence *in situ* hybridization (FISH) allows for confirmation of single-cell sequencing (Sc-seq) results in tissue without losing spatial patterns of expression.

(C) Confocal image of tissue section from the LHb, containing axons labeled from *Sst*+ EP neurons (YFP) and stained for synaptic proteins. Examining protein expression in synaptic terminals using high resolution methods, such as array tomography (shown), electron microscopy, and super-resolution imaging, is critical for examining the distribution/localization of pre- and postsynaptic vesicular transporters, receptors, and synaptic organizers.

(D) (Top) Zoomed image of area highlighted in (C) showing overlapping expression of VGAT and VGlut2 in synaptic terminals. (Bottom) Enrichment of each protein within a terminal over scrambled expression patterns demonstrates high concentrations of VGAT and VGluT2 in presynaptic terminals.

(E) Diagram of optical components required for stimulation of individual synapses in acute brain slices.

(legend continued on next page)

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

Striatal cholinergic interneurons (CINs) cotransmit Ach and glutamate locally onto spiny projection neurons (SPNs)[60] and dopaminergic axonal terminals.[61–63] Glutamate release onto SPNs activates postsynaptic ionotropic glutamate receptors with distinct AMPA/NMDA ratios compared with cortico-striatal synapses, suggesting distinct plasticity rules from these disparate inputs.[60] Heroic electrophysiological experiments that intracellularly recorded directly from DA axon terminals in the striatum unambiguously determined that synaptic release of Ach from CINs onto dopamine axons primarily activates nAchRs and is even capable of locally inducing APs.[62] These axonally induced APs are capable of driving DA release *in vitro*[61] and have been hypothesized to act as a mechanism for local control of DA release within the striatum.[64–68]

Immunogold EM and immunoprecipitation experiments suggest that vGluT and VAChT co-package glutamate and ACh into the same synaptic vesicle in both mHb to IPN synapses and striatal cholinergic to medium spiny neuron synapses[21,22,56] (Figure 1A). Interestingly, several groups have found that vGluT and VAChT function synergistically and that depletion of one transporter or neurotransmitter also depletes loading and release of the other.[21,22,63,69] These findings lie in contrast to those described above for neurons that co-package GABA and glycine, which show an antagonistic relationship with regard to co-packaging. Together, these examples illustrate the importance of vesicular transporter synergism/antagonism for neurons that co-package two neurotransmitters into synaptic vesicles.

5-HT/glutamate

Serotonin neurons from the mid and dorsal raphe nuclei innervate the entire forebrain and act through a wide variety of receptors (14 distinct subtypes), eliciting differential physiological responses.[70,71] It then may come as no surprise that a consensus on the primary functions of the serotonin system is lacking, ranging from reinforcement,[72] to suppression of locomotion,[73] to promotion of anxiety-like behaviors.[74] To add to this complexity, it is now clear that a subset of serotonergic neurons in the dorsal and median raphe nuclei express vGluT3 (*Slc17a8*).[75–77] Those in the dorsal raphe project widely throughout the cortex and increase or decrease their activity to reward and punish, respectively.[76,78] 5-HT/vGluT3+ neurons from the dorsal raphe also target the VTA, where they make asymmetric synapses and monosynaptically release glutamate to excite accumbal-projecting dopamine neurons.[79] Although the membrane serotonin transporter (SERT) is coexpressed at axonal sites with vGluT3, it is unknown whether serotonin and glutamate are co-packaged and released from the same presynaptic synapse or whether they are packaged into different vesicles and/or released at different sites.[79] In support of co-packaging, synergism has been reported between vGluT3 and vMAT2, by which vGluT3 promotes vesicular loading of 5-HT via vMAT2.[80] In addition, deletion of either serotonin synthetic enzyme tryptophan hydroxylase 1 (*Tph1*) or vGluT3 (*Slc18a8*) from 5-HT/vGluT3+ neurons results in increased anxiety-like behaviors, suggesting that disrupting signaling from either transmitter has similar consequences.[78,80] Compounding the diverse and extensive functions of 5-HT neurons, median raphe 5-HT/vGluT3+ neurons also activate CA1 inhibitory interneurons to disynaptically inhibit CA1 pyramidal neurons.[81] Together, these data suggest a complementary or synergistic relationship between glutamate and 5-HT release from 5-HT/vGluT3+ neurons, with direct effects on cortical and midbrain structures. The detailed mechanisms by which 5-HT and glutamate release affects circuit or synaptic function in these areas are yet to be revealed.

MULTITRANSMITTER NEURONS WITH INDEPENDENT RELEASE

Ach/GABA

In contrast to neurons that cotransmit Ach and glutamate, separate neuronal subpopulations are capable of cotransmitting Ach and GABA. These two types of Ach-releasing neurons form non-overlapping neuronal populations, reside in different brain regions, and have distinct synaptic mechanisms for corelease of neurotransmitters. Here, we review Ach/GABA-releasing neurons from the retina, basal forebrain/globus pallidus, and cortex, where evidence points to distinct pools of synaptic vesicles containing either Ach or GABA, which may be released at separate sites.

Functional and immunohistochemical studies identified starburst amacrine cells (SACs) as the sole source of Ach in the retina.[82,83] Further studies suggested that these cells also express GABA synthetic enzymes and are capable of releasing GABA.[84,85] More recently, an elegant study employing paired recordings from neighboring retinal neurons demonstrated monosynaptic transmission of both Ach and GABA from SACs to direction-sensitive retinal ganglion cells (DSGCs).[86] Release of GABA from SACs is restricted to the dendrites of the postsynaptic DSGCs' null direction, whereas release of Ach is not spatially restricted. Ach and GABA release are differentially sensitive to calcium concentration and calcium channel antagonists, suggesting separate vesicle populations.[86] These studies suggest that Ach and GABA are segregated into different synaptic vesicles and/or axonal boutons to endow single SACs with spatial selectivity over cotransmission of neurotransmitters.

Elsewhere in the CNS, neurons expressing genes for both Ach and GABA synthesis and vesicular transport were

(F) Illustration of viral targeting of optogenetic activators to specific genetically defined cotransmitting neurons in the entopeduncular nucleus (EP) and activation of their axons using light guided by a DMD (digital micromirror device) while performing whole-cell recordings in LHb.
(G) Optical stimulation can be targeted to a grid of many small spots that overlay the recorded neuron and allow stimulation of single axons. Action potentials are blocked (TTX/4-AP) to restrict spreading of optical axonal stimulation to multiple synapses on the same axon.
(H) Careful calibration of optical stimulus parameters is required to enter into a minimal stimulation regime, where stimulation of individual synapses is ensured and quantal analysis can be performed.
(I) When the neuron is voltage clamped at an intermediate potential, both GABAergic (blue dot) and glutamatergic (red dot) postsynaptic currents can be observed simultaneously on single trials.
(J) Scatterplot of the peak amplitudes for all trials shown in (I) to highlight strong correlation between GABAergic and glutamatergic responses, and providing strong evidence for co-packaging of the two neurotransmitters into the same synaptic vesicle (figure modified from Kim et al. [25] and Wallace et al.[40]).

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

detected in the basal forebrain and later in the globus pallidus.[87–90] Optogenetic activation of cortically projecting Ach/GABA-releasing neurons evoked monosynaptic GABA-mediated inhibitory currents and Ach-mediated nicotinic excitatory currents in layer 1 interneurons. The GABA-mediated IPSC could be ablated by selective genetic deletion of *Slc32a1* (vGAT) in *Chat-Cre*+ neurons, while the nicotinic excitatory postsynaptic current (EPSC) persisted, indicating that the loading of Ach did not depend on GABA vesicular transport.[89] The proportion of postsynaptic layer 1 interneurons receiving an EPSC or an IPSC differed significantly, and most received only one type of input. Additional array tomography studies of cortical axons projecting from pallidal Ach/GABA neurons revealed physically separate sites of vGAT and vAChT labeling, suggesting distinct vesicular pools for the two neurotransmitters.[90] More recently, olfactory-bulb-projecting cholinergic neurons from the basal forebrain were also shown to cotransmit GABA and Ach onto deep, short axon cells within the internal plexiform layer of the bulb, reinforcing the pervasive nature of GABA cotransmission from this population of cholinergic neurons throughout the brain.[91]

Bipolar cortical interneurons expressing vasoactive intestinal polypeptide (VIP) and ChAT were first identified using immunohistochemistry in rodents and account for ~1/3 of all VIP+ cortical neurons.[92,93] Transcriptomically similar neuronal subpopulations (VIP/ChAT+) have also been identified with large-scale single-cell sequencing of the motor and visual cortex in mouse, marmosets, and humans.[6,94–96] These studies also confirmed that this cortical subtype expresses all the required genes for synthesis and release of both GABA and Ach. Systematic circuit mapping of the synaptic output of VIP/ChAT+ interneurons was recently undertaken to define how these neurons are integrated into cortical microcircuits.[97,98] Optogenetic excitation of VIP/Chat+ neurons in the motor and visual cortex revealed GABAergic output primarily to other interneuron subtypes, with a bias toward the *Sst*+ cortical interneurons. Cholinergic EPSCs were sparse, but primarily detected in layer 1 interneurons and other VIP cells.[97] Immunohistochemical analysis also supported independent trafficking of vGAT and vAChT in VIP/Chat+ axon terminals. This suggests, much like the cholinergic neurons in the basal forebrain and globus pallidus, that there are distinct vesicle populations for Ach and GABA release (Figure 1B). Interestingly, studies have revealed significant regional differences in the strength and connectivity of cholinergic output from VIP/ChAT+ neurons, as cholinergic output from VIP/ChAT+ neurons to layer 1 interneurons in the mouse and rat medial prefrontal cortex occurs at a higher probability and produces a larger postsynaptic response.[97,98] However, these differences may be due to the existence of a unique population of VIP− CINs in this region.[97] Some evidence has begun to point toward a function for VIP/ChAT+ neurons in medial prefrontal cortex (mPFC), as optogenetically inhibiting this population following the cue in a 5-choice serial reaction time task reduced correct responses.[98] Additional studies are needed to dissect how this neuronal population may differentially modulate local circuitry in distinct brain regions.

Glutamate/glycine

Highlighting the diversity of neurotransmitter combinations and mechanisms of release, a class of vGluT3+ retinal amacrine cells was recently found to release both the inhibitory neurotransmitter glycine and excitatory neurotransmitter glutamate onto distinct populations of retinal ganglion cells (RGCs).[99,100] Glycinergic synapses are highly selective and shape contrast and size selectivity of postsynaptic "suppressed by contrast" RGCs by depressing tonic firing.[100] Glutamatergic synapses from vGluT3+ retinal amacrine cells are more promiscuous and target OFF alpha ganglion cells and other retinal cell types but avoid suppressed by contrast RGCs.[99] Therefore, vGluT3+ retinal amacrine cells simultaneously excite cells activated by contrast and inhibit cells suppressed by contrast, performing a dual role in retinal circuits. Although transmission of glycine (and absence of glutamate) appears specific to a particular postsynaptic cell type, it is unknown whether glycine and glutamate are targeted to segregated pools of synaptic vesicles or whether specificity for one neurotransmitter or the other is determined by the presence or absence of postsynaptic receptors. Although EM studies have shown amacrine cell processes forming symmetric and asymmetric synapses,[101] vGAT is not expressed in vGluT3+ retinal amacrine cells or axon terminals,[102] leaving the substrate for release of glycine a mystery.

MULTITRANSMITTER NEURONS WITH MIXED CO-PACKAGING AND INDEPENDENT RELEASE

DA/glutamate

Dopamine neurons of the ventral midbrain (DANs) comprise a diverse population of neurons targeting different regions and capable of releasing multiple neurotransmitters in addition to dopamine.[31,103,104] Following initial findings of glutamate cotransmission *in vitro*,[105–107] dopamine neurons in the ventral midbrain projecting to ventral and dorsal striatum expressing the vGluT2 were confirmed, using optogenetic stimulation in brain slices, to release glutamate onto striatal SPNs and CINs.[108–110] Immunogold EM studies and immunoprecipitation experiments suggest that vGluT2+ and vMAT2+ vesicles segregate into distinct vesicle populations and even separate axonal boutons/microdomains,[111] raising the possibility that release of these two transmitters may be controlled independently (Figure 1C). Functional studies have also demonstrated differential synaptic release properties and coupling to calcium channels for dopaminergic and glutamatergic transmission from DANs.[112] In contrast, selective deletion of *Slc17a6* from DA neurons *in vivo* abolished glutamate release, decreased DA release in the ventral striatum, reduced locomotor responses to cocaine administration in mice,[113] and enhanced sucrose and cocaine self-administration.[114] These studies also found that vGluT2 was capable of stimulating monoamine uptake into synaptic vesicles by vMAT2 by lowering the intravesicular pH, providing a mechanism for reduced DA release in the absence of vGluT2.[113] Differences in vesicular colocalization findings in these two studies[111,113] could be explained by the relative abundance of vGluT2/vMAT2 vesicles, compared with vMAT2 and vGluT2 only vesicles. The abundance of the latter would be expected to be in much greater as only a subset of DA terminals contains vGluT2, while the ventral striatum contains many axonal terminals from thalamus with only vGluT2 or from DANs that contain only vMAT2.

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

DA/GABA

Unlike glutamate release from DANs, which requires vGluT2, striatal GABA release from DANs does not require its typical cognate vesicular transporter vGAT.[115] Indeed, in DANs, neither GABA synthetic enzymes nor vesicular transporters are required for GABA release; instead, vMAT2 is required for GABA packaging into vesicles[115,116] (Figure 1C). Thus, the traditional markers used to identify GABAergic neurons cannot be used to determine which DANs release GABA. In support of the function of vMAT2 as a bonafide GABA vesicular transporter, it can substitute for vGAT in classical GABAergic neurons to sustain GABA release.[115] Furthermore, DANs do not synthesize GABA using canonical GABA synthesis (i.e., using GAD65/67); instead, they appear to accumulate GABA in two ways, (1) through an aldehyde dehydrogenase 1a1 (Aldha1a)-dependent pathway[116] and/or (2) by scavenging extracellular striatal GABA via the membrane GABA transporter GAT1.[117,118] However, only the GABA transporter, GAT1, is both necessary and sufficient to sustain GABA release in DANs, as knockout of *Aldha1a* reduces GABA release from DANs by about 50%.[116,118] Furthermore, although Aldha1a was proposed to act within DANs to produce GABA via an atypical biosynthetic pathway,[116] cell-type-specific control of *Aldha1a* expression indicates that its effects on GABA release from DANs occur through a still-mysterious action in non-dopaminergic cells.[118] Therefore, it is likely that all DANs that express GAT1 are capable of GABA release, in addition to DA, throughout striatum.

Despite being packaged by the same vesicular transporter, net DA and GABA transmission can have differential sensitivity to extracellular calcium concentration and presynaptic modulation by GPCRs. These findings suggest that DA and GABA may be segregated into not fully overlapping and potentially separate vesicle populations, despite both depending on vMAT2 for vesicular loading.[119] Some evidence has begun to point toward a function for GABA corelease from DANs. Decreased GABA release from TH-positive neurons, resulting from genetic deletion of E3 ubiquitin ligase *Ube3a*, enhances positive reinforcement, leading to increased optogenetic self-stimulation.[120] Additionally, reducing GABA cotransmission from DANs by genetic deletion of *Aldh1a1* increases ethanol intake and preference in mice, suggesting that GABA cotransmission from DANs plays a role in reward-/ethanol-seeking behaviors.[116] Together, these studies illustrate the complexity of neurotransmitter release from DANs. They have the capability to signal at multiple timescales (see below on hypothesized functions) and spatially separable presynaptic sites, depending on the neurotransmitter released (DA, GABA, or glutamate), postsynaptic neuron, or striatal subregion.[121] Teasing out the contribution of each cotransmitted signal to behavioral functions typically ascribed to DA alone would be a major contribution to the fields of motivated behavior, reinforcement learning, and associated diseases.

CIRCUIT FUNCTIONS OF COTRANSMISSION

Possible cellular and circuit functions for the release of two or more transmitters include diverse temporal control over postsynaptic firing, increased ranges of synaptic plasticity, and frequency-dependent regulation of neurotransmission. Here, we highlight several examples of observed and potential cellular or circuit functions of cotransmission.

Multiple levels of temporal control of postsynaptic activity

Real-time monitoring of the kinase activity in behaving animals indicates that DA release, via regulation of intracellular cAMP production, controls the activity of protein kinase A (PKA) in SPNs over the timescale of tens of seconds, with differential effects on neurons that express type 1 or type 2 dopamine receptors.[122] Elegant experiments demonstrating the postsynaptic effects of optogenetically evoked DA on type 1 dopamine receptors expressing SPN firing indicate that DA release has long-lasting (minutes) effects on spiking output of striatal neurons.[123] DA release affects the postsynaptic cell considerably more slowly (~500-ms delay) than the synaptic currents generated by either cotransmitted GABA or glutamate (2–4 ms).[115,123] These significant differences in the latency of the response to the released transmitter impart each with different functions. GABA or glutamate can precisely activate or inhibit single APs from SPNs (demonstrated in Tritsch et al.[115]). In contrast, following a delay, DA could push the neuron into a long-lasting state of increased excitability in which activity of other excitatory synapses (e.g., incoming cortical or thalamic input) could be readily paired with postsynaptic firing and engage mechanisms for spike-timing-dependent plasticity (Figure 3A).

Frequency-dependent regulation of neurotransmitter release

Investigations into Ach/glutamate release from the mHb to the IPN revealed frequency-dependent effects of cotransmission on postsynaptic cells. Single optogenetic stimuli of axon terminals from mHb to IPN reliably evoke monosynaptic glutamatergic synaptic currents with no contribution from Ach receptors.[56] However, following prolonged stimulation of axon terminals at higher frequencies (20–50 Hz), a slow depolarizing current was observed that was blocked by nAch antagonists.[56] Several groups have found evidence for Ach and glutamate being copackaged into the same synaptic vesicles at this synapse[21,22,56,124]; therefore, glutamate acts as the temporally precise, point-to-point transmitter, while Ach acts as a volume transmitter, only accumulating enough to activate postsynaptic receptors at high presynaptic firing rates. Furthermore, whereas glutamate release may only activate a few postsynaptic receptors within the synapse and occasionally elicit a spike, Ach release can activate many more extrasynaptic receptors and result in high-frequency AP firing in multiple neurons within the IPN simultaneously (Figure 3B).

Target-specific transmission of distinct neurotransmitters

Ach/GABA release from cortical VIP/Chat+ neurons provides an example of how a single neuron type can have differential effects dependent on the postsynaptic neuronal subtype. Ach is the primary transmitter when the postsynaptic neuron is a layer 1 interneuron or another VIP/Chat+ neuron and the postsynaptic receptors are nAchRs. But GABA is the primary transmitter when

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

CellPress

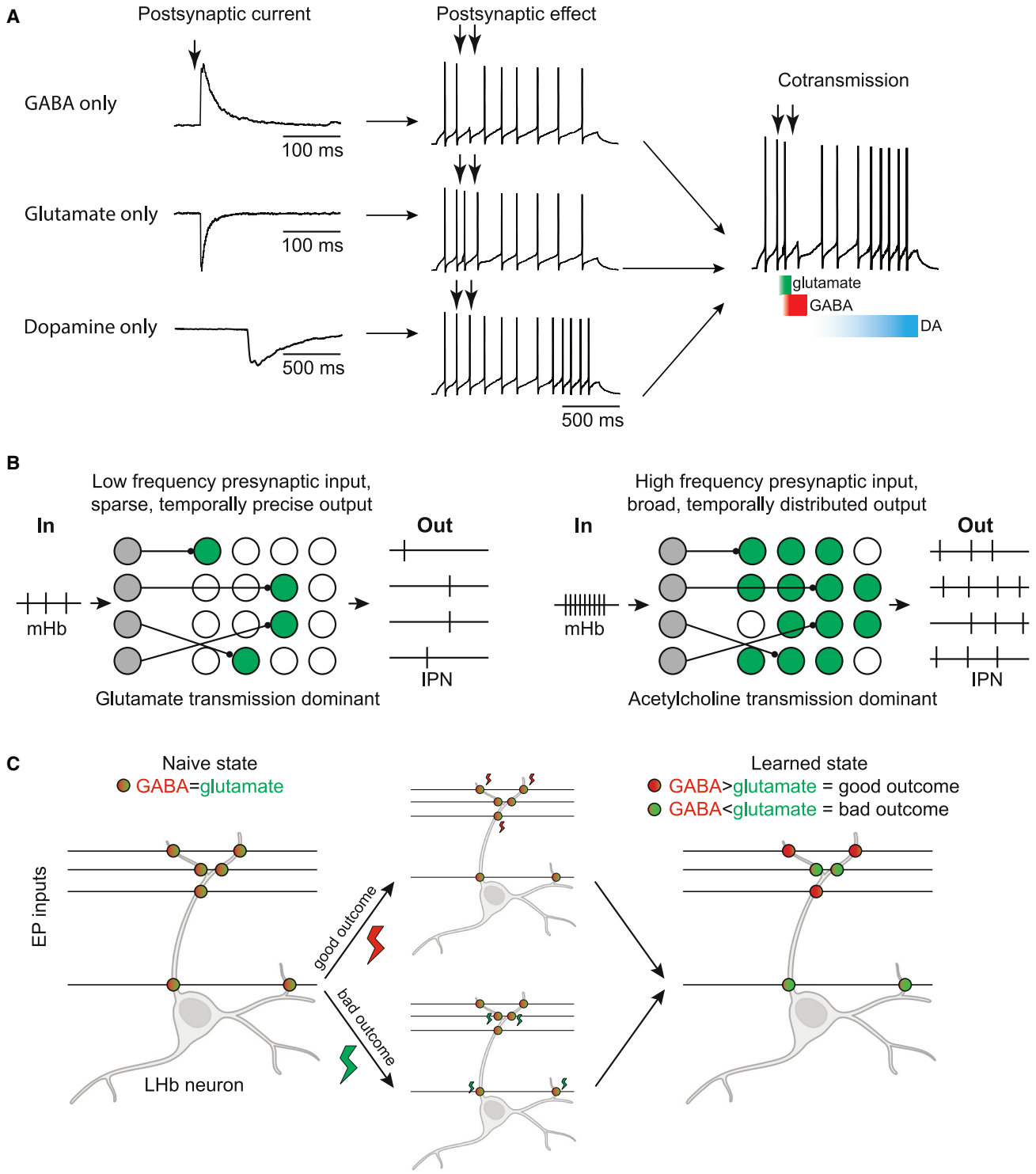

Figure 3. Possible cellular and circuit mechanism of multitransmitter neurons

(A) GABA and glutamate release from midbrain DANs cause fast excitation and slightly longer inhibition of postsynaptic cells direct-pathway spiny projection neurons (dSPNs) due to the kinetics of each ionotropic receptor. The physiological effects following activation of type 1 DA receptors is delayed by ~500 ms and increases firing rate toward the end of the spike train. Arrowheads mark timing of presynaptic action potential.

(legend continued on next page)

Please cite this article in press as: Wallace and Sabatini, Synaptic and circuit functions of multitransmitter neurons in the mammalian brain, Neuron (2023), https://doi.org/10.1016/j.neuron.2023.06.003

the postsynaptic neuron is a Sst+, PV+, or 5HT3aR+ interneuron. The net effect of VIP/Chat+ activity on the cortical microcircuit is disinhibition of excitatory pyramidal neurons by engaging multiple cell types across the cortical column.[97,98] Accumulating evidence in VIP/Chat+ cells, and other Chat+ neurons throughout the brain, suggests that this is accomplished by specialized presynaptic terminals that target different neurotransmitters to specific synaptic sites, allowing synapse-specific control of postsynaptic firing.[90,97,124]

GABA/glutamate cotransmission as a substrate for learning in a biological neural network

The finding that individual synaptic vesicles from EP *Sst* neurons in the LHb contain both GABA and glutamate opens an interesting possibility for a form of synaptic plasticity whereby the sign and weight of a synapse can be dynamically set in an activity-dependent manner.[25] Unlike other brain areas that separate GABA and glutamate into separate signaling channels, this feature renders this synapse similar to those found in artificial neural networks in which each synapse can take a signed and graded value. Furthermore, GABA/glutamate cotransmitting neurons are rare in the brain, but the LHb receives many projections from such neurons,[8] suggesting a specialized function of cotransmission for the computation performed by this circuit.

LHb neurons increase their activity when adverse outcomes occur or are predicted and provide a disynaptic inhibitory (-GABAergic) input to VTA DANs.[125,126] Thus, the neurons of LHb, during reward-reinforced behaviors, learn to calculate the negative expected value of specific actions or contexts. In contrast, VTA DAN neurons calculate reward-prediction error (RPE)—the difference between the experienced value of an activity and that which had been predicted. As a recent study reported that non-DA neurons within VTA encode experienced value,[127] VTA DA neurons could simply calculate RPE by subtracting the predicted value (provided by LHb) from the experienced value (provided by non-DA VTA neurons).

The activity-dependent insertion and removal of GABA and glutamate ionotropic receptors from postsynaptic terminals opposed to GABA/glutamate coreleasing synapses provides a mechanism for LHb neurons to learn to calculate expected value from information about sensory state, past experience, motor action, and other variables received from diverse brain areas, including EP. Simple activity-dependent learning rules, such as "insert GABA receptors into synapses that are activated when something good happens," and, conversely, "glutamate receptors in those active when something bad happens," provide the equivalent of perceptron-like learning rules that classify contexts as good or bad[128] (Figure 3C). Going further, if the VTA, or another brain structure, provides a graded RPE-like signal to the LHb during learning (RPE is the error signal for value), then a modification of this learning rule performs signed gradient descent to settle on a linear combination of signed and graded synaptic weights that calculate value directly. Such a mechanism is biologically plausible, as the strength at many classes of synapses is regulated by the postsynaptic neuron via activity-dependent and dynamic insertion or removal of ionotropic receptors from the postsynaptic terminal. Although RPE-encoding VTA DA release in the LHb is a natural error signal for such plasticity, VTA projections do not appear to release DA in the LHb,[129,130] such that the identity of this hypothetical error signal is still unknown. It might be carried by another neuromodulator, such as serotonin or norepinephrine, or by the activity of one of the many other GABA/glutamate coreleasing projections to the LHb.

CAVEATS AND STEPS FORWARD

Single-cell sequencing is an important new tool for genetically defining cell types in tissues throughout an organism. Studies performed in the brain have led to discoveries of new neuronal classes, new features of known neuron types, and—perhaps most importantly—an initial unbiased framework for further investigation into neuronal diversity in the brain. These studies have also shown the ubiquity of neuron types that have the genetic constituents required for the release of more than one neurotransmitter.[6,7,9,40,131] An important caveat to these findings is that most analyses performed in these studies are performed on populations of neurons. Therefore, it is important to confirm that individual neurons express all the genes necessary for the release of more than one neurotransmitter, as analysis of the population alone may lead to incorrect conclusions regarding cotransmission. Careful characterization of mouse lines used to target genetically defined neurons is critical, as off-target Cre expression can easily lead to misinterpretation of cotransmission when, in fact, two distinct neuronal populations are labeled. Additionally, *in situ* transcriptional and electrophysiological analysis of suspected coreleasing neurons are critical confirmatory steps in identifying bona fide coreleasing neurons.

If we are to eventually understand the function that coreleasing neurons play in neural circuits and behavior, then the mechanism by which two or more neurotransmitters are released by a single axon or single synapse must be determined. Functionally determining whether both transmitters are simultaneously released by an axon/synapse and whether they are packaged into the same or distinct synaptic vesicles constrains models and hypotheses surrounding the function of cotransmission in a given circuit. To determine these properties at coreleasing axons/synapses, minimal stimulation experiments are often necessary to examine the quantal content at an individual release site (Figure 2). Unfortunately, classical minimal stimulation approaches using extracellular stimulation are confounded when axons from many different types of presynaptic neurons are overlapping, making it impossible to determine whether one axon is being stimulated when

(B) Ach/glutamate release from mHb to the IPN may be sparse but temporally precise when mHb firing rates are low, with synaptic transmission dominated by glutamate and minimal activation of extrasynaptic Ach receptors. During high-frequency activity, spillover of Ach reaches and activates extrasynaptic Ach receptors on many postsynaptic cells, leading to broad, but temporally imprecise, activation of IPN.
(C) LHb neurons assigning "value" to each presynaptic input from EP, based on good or bad outcomes. Because EP inputs cotransmit GABA and glutamate, LHb can tune each synapse positive or negative by insertion of glutamate or GABA receptors, respectively. This process is the equivalent of perceptron-like learning rules that classify contexts as good or bad in artificial neural networks.

looking for two types of postsynaptic response. Fortunately, approaches employing optogenetics permit genetic targeting of stimulation exclusively to a coreleasing neuron class, but careful spatiotemporal calibration of optical stimulation is required to convincingly stimulate individual axons/synapses.[11,25] These approaches, combined with super-resolution/expansion microscopy and imaging of pre- and postsynaptic proteins,[132,133] will lead to new insights into the mechanism and function of coreleasing neurons and begin to illuminate mysterious facets of their cell biology. This includes understanding how cells with multiple classes of synaptic vesicles can target them to different terminals, as well as what factors determine whether each synaptic vesicle harbors one or multiple classes of neurotransmitter transporters. It is even more mysterious how these properties are maintained through the complex synaptic vesicle life cycle, although a preponderance of kiss-and-run-type release events[134] might lessen the challenge. Presumably, protein domains present on the vesicular transporter, which have previously been implicated in vesicle assembly, sorting, or recycling, as well as on other vesicular proteins, must contribute to establishing the unique biology of multitransmitter neurons.[112,135–137]

Separately, a combination of experimental and theoretical work is necessary to understand the diversity of functions of multitransmitter neurons. For example, the dopamine, GABA, and glutamate released by DANs may elicit prolonged waves of postsynaptic effects, cascading from the millisecond changes in excitability triggered by ionotropic receptors, to the tens of seconds on biochemical signaling and excitability[109,115,122,123] (Figure 3A). These temporal waves could be different for each postsynaptic target, which is sensitized to different subsets of transmitters by the complement and type of receptors it expressed.[109,138,139] Conversely, GABA and Ach coreleased in the cortex may act in parallel on different targets to achieve a single common function, such as placing the cortex in a more excitable and pro-plasticity state by simultaneously inhibiting *Sst*+ interneurons with GABA while using Ach release to excite disinhibitory interneurons and activate muscarinic receptors on pyramidal cells.[90,97,98,140,141] Lastly, as discussed above, GABA and glutamate corelease might serve to sharpen postsynaptic potentials but might also create signed and graded synapses that provide an ideal substrate for plasticity (Figure 3C). Much remains to be discovered about both the peculiar cell biology of these cells and their function in circuits and, ultimately, behavior.

ACKNOWLEDGMENTS

This work was supported by funding to M.L.W. (Whitehall Foundation, R00NS105883 NIH, and NARSAD Young Investigator Award) and B.L.S. (R01NS103226 NIH). We thank Dr. Janet B. Wallace and Dr. Joseph M. Martinez for their comments and critical reading of the manuscript. Figure 1 was created with BioRender.com (agreement # IG256AX89O).

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Kupfermann, I. (1991). Functional studies of cotransmission. Physiol. Rev. *71*, 683–732. https://doi.org/10.1152/PHYSREV.1991.71.3.683.

2. Jan, Y.N., Jan, L.Y., and Kuffler, S.W. (1979). A peptide as a possible transmitter in sympathetic ganglia of the frog. Proc. Natl. Acad. Sci. USA *76*, 1501–1505. https://doi.org/10.1073/PNAS.76.3.1501.

3. Sossin, W.S., Sweet-Cordero, A., and Scheller, R.H. (1990). Dale's hypothesis revisited: different neuropeptides derived from a common prohormone are targeted to different processes. Proc. Natl. Acad. Sci. USA *87*, 4845–4848. https://doi.org/10.1073/PNAS.87.12.4845.

4. Katz, P.S., and Harris-Warrick, R.M. (1990). Neuromodulation of the crab pyloric central pattern generator by serotonergic/cholinergic proprioceptive afferents. J. Neurosci. *10*, 1495–1512. https://doi.org/10.1523/JNEUROSCI.10-05-01495.1990.

5. Upmanyu, N., Jin, J., von der Emde, H.V., Ganzella, M., Bösche, L., Malviya, V.N., Zhuleku, E., Politi, A.Z., Ninov, M., Silbern, I., et al. (2022). Co-localization of different neurotransmitter transporters on synaptic vesicles is sparse except for VGLUT1 and ZnT3. Neuron *110*, 1483–1497.e7. https://doi.org/10.1016/J.NEURON.2022.02.008.

6. Saunders, A., Macosko, E.Z., Wysoker, A., Goldman, M., Krienen, F.M., de Rivera, H., Bien, E., Baum, M., Bortolin, L., Wang, S., et al. (2018). Molecular diversity and specializations among the cells of the adult mouse brain. Cell *174*, 1015–1030.e16. https://doi.org/10.1016/j.cell.2018.07.028.

7. Smith, S.J., Sümbül, U., Graybuck, L.T., Collman, F., Seshamani, S., Gala, R., Gliko, O., Elabbady, L., Miller, J.A., Bakken, T.E., et al. (2019). Single-cell transcriptomic evidence for dense intracortical neuropeptide networks. eLife *8*. https://doi.org/10.7554/eLife.47889.

8. Xu, J., Jo, A., DeVries, R.P., Deniz, S., Cherian, S., Sunmola, I., Song, X., Marshall, J.J., Gruner, K.A., Daigle, T.L., et al. (2022). Intersectional mapping of multi-transmitter neurons and other cell types in the brain. Cell Rep. *40*, 111036. https://doi.org/10.1016/J.CELREP.2022.111036.

9. Yao, Z., van Velthoven, C.T.J., Kunst, M., Zhang, M., McMillen, D., Lee, C., Jung, W., Goldy, J., Abdelhak, A., Baker, P., et al. (2023). A high-resolution transcriptomic and spatial atlas of cell types in the whole mouse brain. Preprint at bioRxiv. https://doi.org/10.1101/2023.03.06.531121.

10. Poulin, J.F., Zou, J., Drouin-Ouellet, J., Kim, K.Y., Cicchetti, F., and Awatramani, R.B. (2014). Defining midbrain dopaminergic neuron diversity by single-cell gene expression profiling. Cell Rep. *9*, 930–943. https://doi.org/10.1016/j.celrep.2014.10.008.

11. Kim, S.A., and Sabatini, B.L. (2022). Analytical approaches to examine gamma-aminobutyric acid and glutamate vesicular co-packaging. Front. Synaptic Neurosci. *14*, 1076616. https://doi.org/10.3389/FNSYN.2022.1076616.

12. Sabatini, B.L., and Tian, L. (2020). Imaging neurotransmitter and neuromodulator dynamics in vivo with genetically encoded indicators. Neuron *108*, 17–32. https://doi.org/10.1016/J.NEURON.2020.09.036.

13. Spitzer, N.C. (2017). Neurotransmitter switching in the developing and adult brain. Annu. Rev. Neurosci. *40*, 1–19. https://doi.org/10.1146/ANNUREV-NEURO-072116-031204.

14. Nusbaum, M.P., Blitz, D.M., and Marder, E. (2017). Functional consequences of neuropeptide and small-molecule co-transmission. Nat. Rev. Neurosci. *18*, 389–403. https://doi.org/10.1038/nrn.2017.56.

15. Jékely, G., Melzer, S., Beets, I., Kadow, I.C.G., Koene, J., Haddad, S., and Holden-Dye, L. (2018). The long and the short of it - a perspective on peptidergic regulation of circuits and behaviour. J. Exp. Biol. *221*. https://doi.org/10.1242/JEB.166710.

16. Hnasko, T.S., and Edwards, R.H. (2012). Neurotransmitter corelease: mechanism and physiological role. Annu. Rev. Physiol. *74*, 225–243. https://doi.org/10.1146/annurev-physiol-020911-153315.

17. Nakanishi-Matsui, M., Sekiya, M., Nakamoto, R.K., and Futai, M. (2010). The mechanism of rotating proton pumping ATPases. Biochim. Biophys. Acta *1797*, 1343–1352. https://doi.org/10.1016/J.BBABIO.2010.02.014.

18. Johnson, R.G., Carty, S.E., and Scarpa, A. (1981). Proton: substrate stoichiometries during active transport of biogenic amines in chromaffin ghosts. J. Biol. Chem. *256*, 5773–5780. https://doi.org/10.1016/S0021-9258(19)69274-4.

19. Nguyen, M.L., Cox, G.D., and Parsons, S.M. (1998). Kinetic parameters for the vesicular acetylcholine transporter: two protons are exchanged for one acetylcholine. Biochemistry *37*, 13400–13410. https://doi.org/10.1021/BI9802263.

20. Tabb, J.S., Kish, P.E., Van Dyke, R., and Ueda, T. (1992). Glutamate transport into synaptic vesicles. Roles of membrane potential, pH gradient, and intravesicular pH. J. Biol. Chem. *267*, 15412–15418. https://doi.org/10.1016/S0021-9258(19)49549-5.

21. Frahm, S., Antolin-Fontes, B., Görlich, A., Zander, J.F., Ahnert-Hilger, G., and Ibañez-Tallon, I. (2015). An essential role of acetylcholine-glutamate synergy at habenular synapses in nicotine dependence. eLife *4*, e11396. https://doi.org/10.7554/eLife.11396.

22. Gras, C., Amilhon, B., Lepicard, E.M., Poirel, O., Vinatier, J., Herbin, M., Dumas, S., Tzavara, E.T., Wade, M.R., Nomikos, G.G., et al. (2008). The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone. Nat. Neurosci. *11*, 292–300. https://doi.org/10.1038/nn2052.

23. Kish, P.E., Fischer-Bovenkerk, C., and Ueda, T. (1989). Active transport of gamma-aminobutyric acid and glycine into synaptic vesicles. Proc. Natl. Acad. Sci. USA *86*, 3877–3881. https://doi.org/10.1073/PNAS.86.10.3877.

24. Farsi, Z., Preobraschenski, J., Van Den Bogaart, G., Riedel, D., Jahn, R., and Woehler, A. (2016). Single-vesicle imaging reveals different transport mechanisms between glutamatergic and GABAergic vesicles. Science *351*, 981–984. https://doi.org/10.1126/SCIENCE.AAD8142.

25. Kim, S.A., Wallace, M.L., El-Rifai, M., Knudsen, A.R., and Sabatini, B.L. (2022). Co-packaging of opposing neurotransmitters in individual synaptic vesicles in the central nervous system. Neuron *110*, 1371–1384.e7. https://doi.org/10.1016/J.NEURON.2022.01.007.

26. Shabel, S.J., Proulx, C.D., Piriz, J., and Malinow, R. (2014). Mood regulation. GABA/glutamate co-release controls habenula output and is modified by antidepressant treatment. Science *345*, 1494–1498. https://doi.org/10.1126/science.1250469.

27. Boulland, J.L., Qureshi, T., Seal, R.P., Rafiki, A., Gundersen, V., Bergersen, L.H., Fremeau, R.T., Edwards, R.H., Storm-Mathisen, J., and Chaudhry, F.A. (2004). Expression of the vesicular glutamate transporters during development indicates the widespread corelease of multiple neurotransmitters. J. Comp. Neurol. *480*, 264–280. https://doi.org/10.1002/cne.20354.

28. Boulland, J.L., Jenstad, M., Boekel, A.J., Wouterlood, F.G., Edwards, R.H., Storm-Mathisen, J., and Chaudhry, F.A. (2009). Vesicular glutamate and GABA transporters sort to distinct sets of vesicles in a population of presynaptic terminals. Cereb. Cortex *19*, 241–248. https://doi.org/10.1093/cercor/bhn077.

29. Hashimotodani, Y., Karube, F., Yanagawa, Y., Fujiyama, F., and Kano, M. (2018). Supramammillary Nucleus Afferents to the Dentate Gyrus Co-release Glutamate and GABA and Potentiate Granule Cell Output. Cell Rep *25*, 2704–2715. https://doi.org/10.1016/j.celrep.2018.11.016.

30. Pedersen, N.P., Ferrari, L., Venner, A., Wang, J.L., Abbott, S.B.G., Vujovic, N., Arrigoni, E., Saper, C.B., and Fuller, P.M. (2017). Supramammillary glutamate neurons are a key node of the arousal system. Nat. Commun. *8*, 1405. https://doi.org/10.1038/s41467-017-01004-6.

31. Morales, M., and Margolis, E.B. (2017). Ventral tegmental area: cellular heterogeneity, connectivity and behaviour. Nat. Rev. Neurosci. *18*, 73–85. https://doi.org/10.1038/nrn.2016.165.

32. Root, D.H., Mejias-Aponte, C.A., Zhang, S., Wang, H.L., Hoffman, A.F., Lupica, C.R., and Morales, M. (2014). Single rodent mesohabenular axons release glutamate and GABA. Nat. Neurosci. *17*, 1543–1551. https://doi.org/10.1038/nn.3823.

33. Root, D.H., Zhang, S., Barker, D.J., Miranda-Barrientos, J., Liu, B., Wang, H.L., and Morales, M. (2018). Selective brain distribution and distinctive synaptic architecture of dual glutamatergic-GABAergic neurons. Cell Rep. *23*, 3465–3479. https://doi.org/10.1016/j.celrep.2018.05.063.

34. Nelson, A.B., and Kreitzer, A.C. (2014). Reassessing models of Basal Ganglia function and dysfunction. Annu. Rev. Neurosci. *37*, 117–135. https://doi.org/10.1146/annurev-neuro-071013-013916.

35. Nauta, H.J.W. (1974). Evidence of a pallidohabenular pathway in the cat. J. Comp. Neurol. *156*, 19–27. https://doi.org/10.1002/CNE.901560103.

36. Herkenham, M., and Nauta, W.J. (1977). Afferent connections of the habenular nuclei in the rat. A horseradish peroxidase study, with a note on the fiber-of-passage problem. J. Comp. Neurol. *173*, 123–146. https://doi.org/10.1002/cne.901730107.

37. Parent, A., and De Bellefeuille, L. (1982). Organization of efferent projections from the internal segment of globus pallidus in primate as revealed by fluorescence retrograde labeling method. Brain Res. *245*, 201–213.

38. Hong, S., and Hikosaka, O. (2008). The globus pallidus sends reward-related signals to the lateral habenula. Neuron *60*, 720–729. https://doi.org/10.1016/j.neuron.2008.09.035.

39. Shabel, S.J., Proulx, C.D., Trias, A., Murphy, R.T., and Malinow, R. (2012). Input to the lateral habenula from the basal ganglia is excitatory, aversive, and suppressed by serotonin. Neuron *74*, 475–481. https://doi.org/10.1016/j.neuron.2012.02.037.

40. Wallace, M.L., Saunders, A., Huang, K.W., Philson, A.C., Goldman, M., Macosko, E.Z., McCarroll, S.A., and Sabatini, B.L. (2017). Genetically distinct parallel pathways in the entopeduncular nucleus for limbic and sensorimotor output of the basal ganglia. Neuron *94*, 138–152.e5. https://doi.org/10.1016/j.neuron.2017.03.017.

41. Meye, F.J., Soiza-Reilly, M., Smit, T., Diana, M.A., Schwarz, M.K., and Mameli, M. (2016). Shifted pallidal co-release of GABA and glutamate in habenula drives cocaine withdrawal and relapse. Nat. Neurosci. *19*, 1019–1024. https://doi.org/10.1038/nn.4334.

42. Egashira, Y., Takase, M., Watanabe, S., Ishida, J., Fukamizu, A., Kaneko, R., Yanagawa, Y., and Takamori, S. (2016). Unique pH dynamics in GABAergic synaptic vesicles illuminates the mechanism and kinetics of GABA loading. Proc. Natl. Acad. Sci. USA *113*, 10702–10707. https://doi.org/10.1073/PNAS.1604527113.

43. Zimmermann, J., Herman, M.A., and Rosenmund, C. (2015). Co-release of glutamate and GABA from single vesicles in GABAergic neurons exogenously expressing VGLUT3. Front. Synaptic Neurosci. *7*, 16. https://doi.org/10.3389/fnsyn.2015.00016.

44. Jonas, P., Bischofberger, J., and Sandkühler, J. (1998). Corelease of two fast neurotransmitters at a central synapse. Science *281*, 419–424.

45. Turecek, J., and Regehr, W.G. (2020). Cerebellar and vestibular nuclear synapses in the inferior olive have distinct release kinetics and neurotransmitters. eLife *9*, 1–22. https://doi.org/10.7554/eLife.61672.

46. O'Brien, J.A., and Berger, A.J. (1999). Cotransmission of GABA and glycine to brain stem motoneurons. J. Neurophysiol. *82*, 1638–1641. https://doi.org/10.1152/jn.1999.82.3.1638.

47. Dugué, G.P., Dumoulin, A., Triller, A., and Dieudonné, S. (2005). Target-dependent use of co-released inhibitory transmitters at central synapses. J. Neurosci. *25*, 6490–6498. https://doi.org/10.1523/JNEUROSCI.1500-05.2005.

48. Awatramani, G.B., Turecek, R., and Trussell, L.O. (2005). Staggered development of GABAergic and glycinergic transmission in the MNTB. J. Neurophysiol. *93*, 819–828. https://doi.org/10.1152/jn.00798.2004.

49. Nabekura, J., Katsurabayashi, S., Kakazu, Y., Shibata, S., Matsubara, A., Jinno, S., Mizoguchi, Y., Sasaki, A., and Ishibashi, H. (2004). Developmental switch from GABA to glycine release in single central synaptic terminals. Nat. Neurosci. *7*, 17–23. https://doi.org/10.1038/nn1170.

50. Wojcik, S.M., Katsurabayashi, S., Guillemin, I., Friauf, E., Rosenmund, C., Brose, N., and Rhee, J.S. (2006). A shared vesicular carrier allows synaptic corelease of GABA and glycine. Neuron *50*, 575–587. https://doi.org/10.1016/J.NEURON.2006.04.016.

51. Apostolides, P.F., and Trussell, L.O. (2013). Rapid, activity-independent turnover of vesicular transmitter content at a mixed glycine/GABA synapse. J. Neurosci. *33*, 4768–4781. https://doi.org/10.1523/JNEUROSCI.5555-12.2013.

52. Hasselmo, M.E. (2006). The role of acetylcholine in learning and memory. Curr. Opin. Neurobiol. *16*, 710–715. https://doi.org/10.1016/J.CONB.2006.09.002.

53. Pinto, L., Goard, M.J., Estandian, D., Xu, M., Kwan, A.C., Lee, S.H., Harrison, T.C., Feng, G., and Dan, Y. (2013). Fast modulation of visual perception by basal forebrain cholinergic neurons. Nat. Neurosci. *16*, 1857–1863. https://doi.org/10.1038/nn.3552.

54. Morishita, H., Miwa, J.M., Heintz, N., and Hensch, T.K. (2010). Lynx1, a cholinergic brake, limits plasticity in adult visual cortex. Science *330*, 1238–1240. https://doi.org/10.1126/science.1195320.

55. Teles-Grilo Ruivo, L.M., Baker, K.L., Conway, M.W., Kinsley, P.J., Gilmour, G., Phillips, K.G., Isaac, J.T.R., Lowry, J.P., and Mellor, J.R. (2017). Coordinated acetylcholine release in prefrontal cortex and hippocampus is associated with arousal and reward on distinct timescales. Cell Rep. *18*, 905–917. https://doi.org/10.1016/J.CELREP.2016.12.085.

56. Ren, J., Qin, C., Hu, F., Tan, J., Qiu, L., Zhao, S., Feng, G., and Luo, M. (2011). Habenula "cholinergic" neurons co-release glutamate and acetylcholine and activate postsynaptic neurons via distinct transmission modes. Neuron *69*, 445–452. https://doi.org/10.1016/J.NEURON.2010.12.038.

57. Allen, T.G.J., Abogadie, F.C., and Brown, D.A. (2006). Simultaneous release of glutamate and acetylcholine from single magnocellular "cholinergic" basal forebrain neurons. J. Neurosci. *26*, 1588–1595. https://doi.org/10.1523/JNEUROSCI.3979-05.2006.

58. Wallace, M.L., Huang, K.W., Hochbaum, D., Hyun, M., Radeljic, G., and Sabatini, B.L. (2020). Anatomical and single-cell transcriptional profiling of the murine habenular complex. eLife *9*. https://doi.org/10.7554/eLife.51271.

59. Hashikawa, Y., Hashikawa, K., Rossi, M.A., Basiri, M.L., Liu, Y., Johnston, N.L., Ahmad, O.R., and Stuber, G.D. (2020). Transcriptional and spatial resolution of cell types in the mammalian habenula. Neuron *106*, 743–758.e5. https://doi.org/10.1016/J.NEURON.2020.03.011.

60. Higley, M.J., Gittis, A.H., Oldenburg, I.A., Balthasar, N., Seal, R.P., Edwards, R.H., Lowell, B.B., Kreitzer, A.C., and Sabatini, B.L. (2011). Cholinergic interneurons mediate fast VGluT3-dependent glutamatergic transmission in the striatum. PLoS One *6*, e19155. https://doi.org/10.1371/JOURNAL.PONE.0019155.

61. Liu, C., Cai, X., Ritzau-Jost, A., Kramer, P.F., Li, Y., Khaliq, Z.M., Hallermann, S., and Kaeser, P.S. (2022). An action potential initiation mechanism in distal axons for the control of dopamine release. Science *375*, 1378–1385. https://doi.org/10.1126/science.abn0532.

62. Kramer, P.F., Brill-Weil, S.G., Cummins, A.C., Zhang, R., Camacho-Hernandez, G.A., Newman, A.H., Eldridge, M.A.G., Averbeck, B.B., and Khaliq, Z.M. (2022). Synaptic-like axo-axonal transmission from striatal cholinergic interneurons onto dopaminergic fibers. Neuron *110*, 2949–2960.e4. https://doi.org/10.1016/J.NEURON.2022.07.011.

63. Nelson, A.B., Hammack, N., Yang, C.F., Shah, N.M., Seal, R.P., and Kreitzer, A.C. (2014). Striatal cholinergic interneurons drive GABA release from dopamine terminals. Neuron *82*, 63–70. https://doi.org/10.1016/j.neuron.2014.01.023.

64. Mohebi, A., Pettibone, J.R., Hamid, A.A., Wong, J.T., Vinson, L.T., Patriarchi, T., Tian, L., Kennedy, R.T., and Berke, J.D. (2019). Dissociable dopamine dynamics for learning and motivation. Nature *570*, 65–70. https://doi.org/10.1038/s41586-019-1235-y.

65. Threlfell, S., Lalic, T., Platt, N.J., Jennings, K.A., Deisseroth, K., and Cragg, S.J. (2012). Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons. Neuron *75*, 58–64. https://doi.org/10.1016/j.neuron.2012.04.038.

66. Pfeiffer, B.E., Zang, T., Wilkerson, J.R., Taniguchi, M., Maksimova, M.A., Smith, L.N., Cowan, C.W., and Huber, K.M. (2010). Fragile X mental retardation protein is required for synapse elimination by the activity-dependent transcription factor MEF2. Neuron *66*, 191–197. https://doi.org/10.1016/j.neuron.2010.03.017.

67. Zhou, F.M., Liang, Y., and Dani, J.A. (2001). Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. Nat. Neurosci. *4*, 1224–1229. https://doi.org/10.1038/nn769.

68. Cachope, R., Mateo, Y., Mathur, B.N., Irving, J., Wang, H.L., Morales, M., Lovinger, D.M., and Cheer, J.F. (2012). Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. Cell Rep. *2*, 33–41. https://doi.org/10.1016/J.CELREP.2012.05.011.

69. Nelson, A.B., Bussert, T.G., Kreitzer, A.C., and Seal, R.P. (2014). Striatal cholinergic neurotransmission requires VGLUT3. J. Neurosci. *34*, 8772–8777. https://doi.org/10.1523/JNEUROSCI.0901-14.2014.

70. McCorvy, J.D., and Roth, B.L. (2015). Structure and function of serotonin G protein-coupled receptors. Pharmacol. Ther. *150*, 129–142. https://doi.org/10.1016/J.PHARMTHERA.2015.01.009.

71. Liu, Z., Lin, R., and Luo, M. (2020). Reward contributions to serotonergic functions. Annu. Rev. Neurosci. *43*, 141–162. https://doi.org/10.1146/annurev-neuro-093019-112252.

72. Liu, Z., Zhou, J., Li, Y., Hu, F., Lu, Y., Ma, M., Feng, Q., en Zhang, J.E., Wang, D., Zeng, J., et al. (2014). Dorsal raphe neurons signal reward through 5-HT and glutamate. Neuron *81*, 1360–1374. https://doi.org/10.1016/J.NEURON.2014.02.010.

73. Teissier, A., Chemiakine, A., Inbar, B., Bagchi, S., Ray, R.S., Palmiter, R.D., Dymecki, S.M., Moore, H., and Ansorge, M.S. (2015). Activity of raphé serotonergic neurons controls emotional behaviors. Cell Rep. *13*, 1965–1976. https://doi.org/10.1016/J.CELREP.2015.10.061.

74. Urban, D.J., Zhu, H., Marcinkiewcz, C.A., Michaelides, M., Oshibuchi, H., Rhea, D., Aryal, D.K., Farrell, M.S., Lowery-Gionta, E., Olsen, R.H.J., et al. (2016). Elucidation of the behavioral program and neuronal network encoded by dorsal raphe serotonergic neurons. Neuropsychopharmacol. *41*, 1404–1415. https://doi.org/10.1038/npp.2015.293.

75. Gras, C., Herzog, E., Bellenchi, G.C., Bernard, V., Ravassard, P., Pohl, M., Gasnier, B., Giros, B., and El Mestikawy, S. (2002). A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. J. Neurosci. *22*, 5442–5451. https://doi.org/10.1523/JNEUROSCI.22-13-05442.2002.

76. Ren, J., Isakova, A., Friedmann, D., Zeng, J., Grutzner, S.M., Pun, A., Zhao, G.Q., Kolluru, S.S., Wang, R., Lin, R., et al. (2019). Single-cell transcriptomes and whole-brain projections of serotonin neurons in the mouse dorsal and median raphe nuclei. eLife *8*. https://doi.org/10.7554/eLife.49424.

77. Huang, K.W., Ochandarena, N.E., Philson, A.C., Hyun, M., Birnbaum, J.E., Cicconet, M., and Sabatini, B.L. (2019). Molecular and anatomical organization of the dorsal raphe nucleus. eLife *8*. https://doi.org/10.7554/eLife.46464.

78. Ren, J., Friedmann, D., Xiong, J., Liu, C.D., Ferguson, B.R., Weerakkody, T., DeLoach, K.E., Ran, C., Pun, A., Sun, Y., et al. (2018). Anatomically defined and functionally distinct dorsal raphe serotonin sub-systems. Cell *175*, 472–487.e20. https://doi.org/10.1016/j.cell.2018.07.043.

79. Wang, H.L., Zhang, S., Qi, J., Wang, H.L., Cachope, R., Mejias-Aponte, C.A., Gomez, J.A., Mateo-Semidey, G.E., Beaudoin, G.M.J., Paladini, C.A., et al. (2019). Dorsal raphe dual serotonin-glutamate neurons drive reward by establishing excitatory synapses on VTA mesoaccumbens dopamine neurons. Cell Rep. *26*, 1128–1142.e7. https://doi.org/10.1016/J.CELREP.2019.01.014.

80. Amilhon, B., Lepicard, E., Renoir, T., Mongeau, R., Popa, D., Poirel, O., Miot, S., Gras, C., Gardier, A.M., Gallego, J., et al. (2010). VGLUT3 (vesicular glutamate transporter type 3) contribution to the regulation of serotonergic transmission and anxiety. J. Neurosci. *30*, 2198–2210. https://doi.org/10.1523/JNEUROSCI.5196-09.2010.

81. Varga, V., Losonczy, A., Zemelman, B.V., Borhegyi, Z., Nyiri, G., Domonkos, A., Hangya, B., Holderith, N., Magee, J.C., and Freund, T.F. (2009). Fast synaptic subcortical control of hippocampal circuits. Science *326*, 449–453. https://doi.org/10.1126/science.1178307.

82. Masland, R.H., Mills, J.W., and Cassidy, C. (1984). The functions of acetylcholine in the rabbit retina. Proc. R. Soc. Lond. B Biol. Sci. *223*, 121–139. https://doi.org/10.1098/RSPB.1984.0086.

83. Voigt, T. (1986). Cholinergic amacrine cells in the rat retina. J. Comp. Neurol. *248*, 19–35. https://doi.org/10.1002/CNE.902480103.

84. O'Malley, D.M., and Masland, R.H. (1989). Co-release of acetylcholine and gamma-aminobutyric acid by a retinal neuron. Proc. Natl. Acad. Sci. USA *86*, 3414–3418.

85. Brecha, N., Johnson, D., Peichl, L., and Wässle, H. (1988). Cholinergic amacrine cells of the rabbit retina contain glutamate decarboxylase and gamma-aminobutyrate immunoreactivity. Proc. Natl. Acad. Sci. USA *85*, 6187–6191. https://doi.org/10.1073/PNAS.85.16.6187.

86. Lee, S., Kim, K., and Zhou, Z.J. (2010). Role of ACh-GABA cotransmission in detecting image motion and motion direction. Neuron *68*, 1159–1172. https://doi.org/10.1016/J.NEURON.2010.11.031.

87. Tkatch, T., Baranauskas, G., and Surmeier, D.J. (1998). Basal forebrain neurons adjacent to the globus pallidus co-express GABAergic and cholinergic marker mRNAs. NeuroReport *9*, 1935–1939. https://doi.org/10.1097/00001756-199806220-00004.

88. Kosaka, T., Tauchi, M., and Dahl, J.L. (1988). Cholinergic neurons containing GABA-like and/or glutamic acid decarboxylase-like immunoreactivities in various brain regions of the rat. Exp. Brain Res. *70*, 605–617.

89. Saunders, A., Granger, A.J., and Sabatini, B.L. (2015). Corelease of acetylcholine and GABA from cholinergic forebrain neurons. eLife *4*. https://doi.org/10.7554/eLife.06412.

90. Saunders, A., Oldenburg, I.A., Berezovskii, V.K., Johnson, C.A., Kingery, N.D., Elliott, H.L., Xie, T., Gerfen, C.R., and Sabatini, B.L. (2015). A direct GABAergic output from the basal ganglia to frontal cortex. Nature *521*, 85–89. https://doi.org/10.1038/nature14179.

91. Case, D.T., Burton, S.D., Gedeon, J.Y., Williams, S.P.G., Urban, N.N., and Seal, R.P. (2017). Layer- and cell type-selective co-transmission by a basal forebrain cholinergic projection to the olfactory bulb. Nat. Commun. *8*, 652. https://doi.org/10.1038/s41467-017-00765-4.

92. Bayraktar, T., Staiger, J.F., Acsady, L., Cozzari, C., Freund, T.F., and Zilles, K. (1997). Co-localization of vasoactive intestinal polypeptide, γ-aminobutyric acid and choline acetyltransferase in neocortical interneurons of the adult rat. Brain Res. *757*, 209–217. https://doi.org/10.1016/S0006-8993(97)00218-7.

93. Eckenstein, F., and Baughman, R.W. (1984). Two types of cholinergic innervation in cortex, one co-localized with vasoactive intestinal polypeptide. Nature *309*, 153–155. https://doi.org/10.1038/309153a0.

94. Bakken, T.E., Jorstad, N.L., Hu, Q., Lake, B.B., Tian, W., Kalmbach, B.E., Crow, M., Hodge, R.D., Krienen, F.M., Sorensen, S.A., et al. (2021). Comparative cellular analysis of motor cortex in human, marmoset and mouse. Nature *598*, 111–119. https://doi.org/10.1038/s41586-021-03465-8.

95. Zeisel, A., Muñoz-Manchado, A.B., Codeluppi, S., Lönnerberg, P., La Manno, G., Juréus, A., Marques, S., Munguba, H., He, L., Betsholtz, C., et al. (2015). Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. Science *347*, 1138–1142. https://doi.org/10.1126/science.aaa1934.

96. Tasic, B., Menon, V., Nguyen, T.N., Kim, T.K., Jarsky, T., Yao, Z., Levi, B., Gray, L.T., Sorensen, S.A., Dolbeare, T., et al. (2016). Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. Nat. Neurosci. *19*, 335–346. https://doi.org/10.1038/nn.4216.

97. Granger, A.J., Wang, W., Robertson, K., El-Rifai, M., Zanello, A.F., Bistrong, K., Saunders, A., Chow, B.W., Nuñez, V., Turrero García, M.T., et al. (2020). Cortical ChAT+ neurons co-transmit acetylcholine and GABA in a target- and brain-region-specific manner. eLife *9*, 1–29. https://doi.org/10.7554/eLife.57749.

98. Obermayer, J., Luchicchi, A., Heistek, T.S., de Kloet, S.F., Terra, H., Bruinsma, B., Mnie-Filali, O., Kortleven, C., Galakhova, A.A., Khalil, A.J., et al. (2019). Prefrontal cortical ChAT-VIP interneurons provide local excitation by cholinergic synaptic transmission and control attention. Nat. Commun. *10*, 5280. https://doi.org/10.1038/s41467-019-13244-9.

99. Lee, S., Zhang, Y., Chen, M., and Zhou, Z.J. (2016). Segregated glycine-glutamate co-transmission from vGluT3 amacrine cells to contrast-suppressed and contrast-enhanced retinal circuits. Neuron *90*, 27–34. https://doi.org/10.1016/J.NEURON.2016.02.023.

100. Tien, N.W., Kim, T., and Kerschensteiner, D. (2016). Target-specific glycinergic transmission from VGluT3-expressing amacrine cells shapes suppressive contrast responses in the retina. Cell Rep. *15*, 1369–1375. https://doi.org/10.1016/J.CELREP.2016.04.025.

101. Marshak, D.W., Chuang, A.Z., Dolino, D.M., Jacoby, R.A., Liu, W.S., Long, Y.E., Sherman, M.B., Suh, J.M., Vila, A., and Mills, S.L. (2015). Synaptic connections of amacrine cells containing vesicular glutamate transporter 3 in baboon retinas. Vis. Neurosci. *32*, E006. https://doi.org/10.1017/S0952523815000036.

102. Haverkamp, S., and Wässle, H. (2004). Characterization of an amacrine cell type of the mammalian retina immunoreactive for vesicular glutamate transporter 3. J. Comp. Neurol. *468*, 251–263. https://doi.org/10.1002/CNE.10962.

103. Garritsen, O., van Battum, E.Y., Grossouw, L.M., and Pasterkamp, R.J. (2023). Development, wiring and function of dopamine neuron subtypes. Nat. Rev. Neurosci. *24*, 134–152. https://doi.org/10.1038/s41583-022-00669-3.

104. Poulin, J.F., Gaertner, Z., Moreno-Ramos, O.A., and Awatramani, R. (2020). Classification of midbrain dopamine neurons using single-cell gene expression profiling approaches. Trends Neurosci. *43*, 155–169. https://doi.org/10.1016/J.TINS.2020.01.004.

105. Chuhma, N., Zhang, H., Masson, J., Zhuang, X., Sulzer, D., Hen, R., and Rayport, S. (2004). Dopamine neurons mediate a fast excitatory signal via their glutamatergic synapses. J. Neurosci. *24*, 972–981. https://doi.org/10.1523/JNEUROSCI.4317-03.2004.

106. Joyce, M.P., and Rayport, S. (2000). Mesoaccumbens dopamine neuron synapses reconstructed in vitro are glutamatergic. Neuroscience *99*, 445–456. https://doi.org/10.1016/S0306-4522(00)00219-0.

107. Dal Bo, G., St-Gelais, F., Danik, M., Williams, S., Cotton, M., and Trudeau, L.E. (2004). Dopamine neurons in culture express VGLUT2 explaining their capacity to release glutamate at synapses in addition to dopamine. J. Neurochem. *88*, 1398–1405. https://doi.org/10.1046/J.1471-4159.2003.02277.X.

108. Stuber, G.D., Hnasko, T.S., Britt, J.P., Edwards, R.H., and Bonci, A. (2010). Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. J. Neurosci. *30*, 8229–8233. https://doi.org/10.1523/JNEUROSCI.1754-10.2010.

109. Straub, C., Tritsch, N.X., Hagan, N.A., Gu, C., and Sabatini, B.L. (2014). Multiphasic modulation of cholinergic interneurons by nigrostriatal afferents. J. Neurosci. *34*, 8557–8569. https://doi.org/10.1523/JNEUROSCI.0589-14.2014.

110. Tecuapetla, F., Patel, J.C., Xenias, H., English, D., Tadros, I., Shah, F., Berlin, J., Deisseroth, K., Rice, M.E., Tepper, J.M., et al. (2010). Glutamatergic signaling by mesolimbic dopamine neurons in the nucleus accumbens. J. Neurosci. *30*, 7105–7110. https://doi.org/10.1523/JNEUROSCI.0265-10.2010.

111. Zhang, S., Qi, J., Li, X., Wang, H.L., Britt, J.P., Hoffman, A.F., Bonci, A., Lupica, C.R., and Morales, M. (2015). Dopaminergic and glutamatergic microdomains in a subset of rodent mesoaccumbens axons. Nat. Neurosci. *18*, 386–392. https://doi.org/10.1038/nn.3945.

112. Silm, K., Yang, J., Marcott, P.F., Asensio, C.S., Eriksen, J., Guthrie, D.A., Newman, A.H., Ford, C.P., and Edwards, R.H. (2019). Synaptic vesicle recycling pathway determines neurotransmitter content and release properties. Neuron *102*, 786–800.e5. https://doi.org/10.1016/J.NEURON.2019.03.031.

113. Hnasko, T.S., Chuhma, N., Zhang, H., Goh, G.Y., Sulzer, D., Palmiter, R.D., Rayport, S., and Edwards, R.H. (2010). Vesicular glutamate transport promotes dopamine storage and glutamate corelease in vivo. Neuron *65*, 643–656. https://doi.org/10.1016/J.NEURON.2010.02.012.

114. Alsiö, J., Nordenankar, K., Arvidsson, E., Birgner, C., Mahmoudi, S., Halbout, B., Smith, C., Fortin, G.M., Olson, L., Descarries, L., et al. (2011). Enhanced sucrose and cocaine self-administration and cue-induced drug seeking after loss of VGLUT2 in midbrain dopamine neurons in mice. J. Neurosci. *31*, 12593–12603. https://doi.org/10.1523/JNEUROSCI.2397-11.2011.

115. Tritsch, N.X., Ding, J.B., and Sabatini, B.L. (2012). Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. Nature *490*, 262–266. https://doi.org/10.1038/nature11466.

116. Kim, J.I., Ganesan, S., Luo, S.X., Wu, Y.W., Park, E., Huang, E.J., Chen, L., and Ding, J.B. (2015). Aldehyde dehydrogenase 1a1 mediates a

GABA synthesis pathway in midbrain dopaminergic neurons. Science *350*, 102–106. https://doi.org/10.1126/science.aac4690.

117. Tritsch, N.X., Oh, W.J., Gu, C., and Sabatini, B.L. (2014). Midbrain dopamine neurons sustain inhibitory transmission using plasma membrane uptake of GABA, not synthesis. eLife *3*, e01936.

118. Melani, R., and Tritsch, N.X. (2022). Inhibitory co-transmission from midbrain dopamine neurons relies on presynaptic GABA uptake. Cell Rep. *39*, 110716. https://doi.org/10.1016/J.CELREP.2022.110716.

119. Zych, S.M., and Ford, C.P. (2022). Divergent properties and independent regulation of striatal dopamine and GABA co-transmission. Cell Rep. *39*, 110823. https://doi.org/10.1016/J.CELREP.2022.110823.

120. Berrios, J., Stamatakis, A.M., Kantak, P.A., McElligott, Z.A., Judson, M.C., Aita, M., Rougie, M., Stuber, G.D., and Philpot, B.D. (2016). Loss of UBE3A from TH-expressing neurons suppresses GABA co-release and enhances VTA-NAc optical self-stimulation. Nat. Commun. *7*, 10702. https://doi.org/10.1038/ncomms10702.

121. Chuhma, N., Oh, S.J., and Rayport, S. (2023). The dopamine neuron synaptic map in the striatum. Cell Rep. *42*, 112204. https://doi.org/10.1016/J.CELREP.2023.112204.

122. Lee, S.J., Lodder, B., Chen, Y., Patriarchi, T., Tian, L., and Sabatini, B.L. (2021). Cell-type-specific asynchronous modulation of PKA by dopamine in learning. Nature *590*, 451–456. https://doi.org/10.1038/s41586-020-03050-5.

123. Lahiri, A.K., and Bevan, M.D. (2020). Dopaminergic transmission rapidly and persistently enhances excitability of D1 receptor-expressing striatal projection neurons. Neuron *106*, 277–290.e6. https://doi.org/10.1016/j.neuron.2020.01.028.

124. Takács, V.T., Cserép, C., Schlingloff, D., Pósfai, B., Szőnyi, A., Sos, K.E., Környei, Z., Dénes, Á., Gulyás, A.I., Freund, T.F., et al. (2018). Co-transmission of acetylcholine and GABA regulates hippocampal states. Nat. Commun. *91*, 2848. https://doi.org/10.1038/s41467-018-05136-1.

125. Proulx, C.D., Hikosaka, O., and Malinow, R. (2014). Reward processing by the lateral habenula in normal and depressive behaviors. Nat. Neurosci. *17*, 1146–1152. https://doi.org/10.1038/nn.3779.

126. Hikosaka, O., Sesack, S.R., Lecourtier, L., and Shepard, P.D. (2008). Habenula: crossroad between the basal ganglia and the limbic system. J. Neurosci. *28*, 11825–11829. https://doi.org/10.1523/JNEUROSCI.3463-08.2008.

127. Zhou, W.L., Kim, K., Ali, F., Pittenger, S.T., Calarco, C.A., Mineur, Y.S., Ramakrishnan, C., Deisseroth, K., Kwan, A.C., and Picciotto, M.R. (2022). Activity of a direct VTA to ventral pallidum GABA pathway encodes unconditioned reward value and sustains motivation for reward. Sci. Adv. *8*, eabm5217. https://doi.org/10.1126/SCIADV.ABM5217.

128. Rosenblatt, F. (1958). The perceptron: a probabilistic model for information storage and organization in the brain. Psychol. Rev. *65*, 386–408. https://doi.org/10.1037/H0042519.

129. Morales, M., and Root, D.H. (2014). Glutamate neurons within the midbrain dopamine regions. Neuroscience *282*, 60–68. https://doi.org/10.1016/J.NEUROSCIENCE.2014.05.032.

130. Stamatakis, A.M., Jennings, J.H., Ung, R.L., Blair, G.A., Weinberg, R.J., Neve, R.L., Boyce, F., Mattis, J., Ramakrishnan, C., Deisseroth, K., et al. (2013). A unique population of ventral tegmental area neurons inhibits the lateral habenula to promote reward. Neuron *80*, 1039–1053. https://doi.org/10.1016/J.NEURON.2013.08.023.

131. Tasic, B., Yao, Z., Graybuck, L.T., Smith, K.A., Nguyen, T.N., Bertagnolli, D., Goldy, J., Garren, E., Economo, M.N., Viswanathan, S., et al. (2018). Shared and distinct transcriptomic cell types across neocortical areas. Nature *563*, 72–78. https://doi.org/10.1038/s41586-018-0654-5.

132. Chang, J.B., Chen, F., Yoon, Y.G., Jung, E.E., Babcock, H., Kang, J.S., Asano, S., Suk, H.J., Pak, N., Tillberg, P.W., et al. (2017). Iterative expansion microscopy. Nat. Methods *14*, 593–599. https://doi.org/10.1038/nmeth.4261.

133. Dani, A., Huang, B., Bergan, J., Dulac, C., and Zhuang, X. (2010). Superresolution imaging of chemical synapses in the brain. Neuron *68*, 843–856. https://doi.org/10.1016/J.NEURON.2010.11.021.

134. Watanabe, S., Rost, B.R., Camacho-Pérez, M., Davis, M.W., Söhl-Kielczynski, B., Rosenmund, C., and Jorgensen, E.M. (2013). Ultrafast endocytosis at mouse hippocampal synapses. Nature *504*, 242–247. https://doi.org/10.1038/nature12809.

135. Li, H., Santos, M.S., Park, C.K., Dobry, Y., and Voglmaier, S.M. (2017). VGLUT2 trafficking is differentially regulated by adaptor proteins AP-1 and AP-3. Front. Cell. Neurosci. *11*, 324. https://doi.org/10.3389/FNCEL.2017.00324.

136. Fei, H., Grygoruk, A., Brooks, E.S., Chen, A., and Krantz, D.E. (2008). Trafficking of vesicular neurotransmitter transporters. Traffic *9*, 1425–1436. https://doi.org/10.1111/J.1600-0854.2008.00771.X.

137. Colgan, L., Liu, H., Huang, S.Y., and Liu, Y.J. (2007). Dileucine motif is sufficient for internalization and synaptic vesicle targeting of vesicular acetylcholine transporter. Traffic *8*, 512–522. https://doi.org/10.1111/J.1600-0854.2007.00555.X.

138. Zucca, S., Zucca, A., Nakano, T., Aoki, S., and Wickens, J. (2018). Pauses in cholinergic interneuron firing exert an inhibitory control on striatal output in vivo. eLife *7*. https://doi.org/10.7554/eLife.32510.

139. Chuhma, N., Mingote, S., Moore, H., and Rayport, S. (2014). Dopamine neurons control striatal cholinergic neurons via regionally heterogeneous dopamine and glutamate signaling. Neuron *81*, 901–912. https://doi.org/10.1016/J.NEURON.2013.12.027.

140. Karnani, M.M.M., Jackson, J., Ayzenshtat, I., Tucciarone, J., Manoocheri, K., Snider, W.G.G., and Yuste, R. (2016). Cooperative subnetworks of molecularly similar interneurons in mouse neocortex. Neuron *90*, 86–100. https://doi.org/10.1016/J.NEURON.2016.02.037.

141. Chen, Y., Granger, A.J., Tran, T., Saulnier, J.L., Kirkwood, A., and Sabatini, B.L. (2017). Endogenous Gαq-coupled neuromodulator receptors activate protein kinase A. Neuron *96*, 1070–1083.e5. https://doi.org/10.1016/J.NEURON.2017.10.023.