Neurotransmitter Corelease: Mechanism and Physiological Role

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Abstract

Neurotransmitter identity is a defining feature of all neurons because it constrains the type of information they convey, but many neurons release multiple transmitters. Although the physiological role for corelease has remained poorly understood, the vesicular uptake of one transmitter can regulate filling with the other by influencing expression of the H⁺ electrochemical driving force. In addition, the sorting of vesicular neurotransmitter transporters and other synaptic vesicle proteins into different vesicle pools suggests the potential for distinct modes of release. Corelease thus serves multiple roles in synaptic transmission.

INTRODUCTION TO THE NEUROTRANSMITTER CYCLE

Exocytosis: the fusion of a vesicle with the plasma membrane

LDCV: large dense-core vesicle

Classical

neurotransmitters:

small molecules synthesized or recycled locally, transported into vesicles, and released to convey an extracellular signal

GABA:

γ-aminobutyric acid

ACh: acetylcholine

VGAT: vesicular GABA (and glycine) transporter

VMAT: vesicular monoamine transporter

Plasma membrane neurotransmitter

transporter: active at the plasma membrane, this protein transports neurotransmitter from the extracellular space back into the presynaptic neuron or glia

SERT: serotonin transporter

Chemical neurotransmission depends on the regulated synthesis and release of a range of soluble mediators. In the case of lipophilic or gaseous molecules such as endocannabinoids and nitric oxide, which readily penetrate biological membranes, release is regulated at the level of synthesis. However, the hydrophilic compounds that mediate most forms of both synaptic transmission and neuromodulation are packaged into vesicles that undergo regulated release by exocytosis. For neural peptides, synthesis and translocation into the secretory pathway occur at the endoplasmic reticulum, with subsequent packaging into large dense-core vesicles (LDCVs) at the trans-Golgi network. LDCVs then translocate to release sites in the axon or dendrites and undergo regulated release in response to the appropriate physiological stimulus. However, the time required for passage through the secretory pathway and along neuronal processes limits the capacity for sustained release and hence high-frequency transmission. Fast synaptic transmission is thus mediated by classical neurotransmitters that undergo local synthesis and recycling. Indeed, synaptic vesicles recycle locally, at the nerve terminal, through a carefully orchestrated process of exo- and endocytosis known as the synaptic vesicle cycle (1). In addition, release from rapidly recycling synaptic vesicles depends on their capacity to refill with transmitter at the nerve terminal, and presynaptic boutons have developed mechanisms to recapture released transmitter as well as to synthesize it de novo as part of a parallel, integrated process known as the neurotransmitter cycle. The expression of specialized biosynthetic enzymes and transporters required for the neurotransmitter cycle thus defines transmitter phenotype. A recent proteomic analysis indeed shows that glutamatergic and GABAergic synaptic vesicles differ primarily in the expression of vesicular transporters for glutamate and GABA (γ -aminobutyric acid) (2).

NEUROTRANSMITTER CORELEASE

Although it has generally been assumed that neurons release only one classical neurotransmitter, exceptions continue to accumulate. The first demonstration of corelease involved ATP and acetylcholine (ACh) in the electric organ of Torpedo californica (3, 4). Subsequent work showed that ATP is frequently stored and released with other, often cationic classical transmitters in the central and peripheral nervous systems of both invertebrates and vertebrates (for review see Reference 5). Because the vesicular GABA transporter (VGAT, also known as vesicular inhibitory amino acid transporter) also transports glycine (6), that some neurons release both inhibitory transmitters is not surprising (7–9). Similarly, the vesicular monoamine transporter VMAT2 recognizes serotonin and histamine as well as catecholamines and is expressed by essentially all monoamine neurons. The biosynthetic enzymes for different monoamines are expressed by specific subpopulations, but the plasma membrane monoamine transporters show only modest substrate selectivity, indicating the potential for uptake, storage, and release of one monoamine by a neuron that does not produce that particular transmitter. For example, the antidepressant drug fluoxetine, which selectively inhibits the plasma membrane serotonin transporter (SERT), redistributes serotonin from serotonergic to dopaminergic terminals, where serotonin also undergoes release, and this redistribution may contribute to the antidepressant action of fluoxetine (10). In addition, glutamate-releasing thalamocortical neurons (as well as some retinal ganglion cells) express SERT and VMAT2 transiently during development, conferring the ability to take up and release serotonin during the critical period for maturation of this projection (11). Conversely, many monoamine neurons corelease glutamate when grown in culture (12, 13), and dopaminergic periglomerular cells in the olfactory bulb also corelease GABA (14). Even motor neurons thought to release only ACh may corelease glutamate from collateral synapses within the spinal cord (15).

Although the evidence for the corelease of classical neurotransmitters in vivo is clear, and the occurrence more widespread than originally anticipated, the physiological significance remains largely unknown. In this review, we therefore focus on the consequences of corelease for vesicle filling, neurotransmission, synaptic plasticity, and behavior.

VESICLE FILLING

Proton Electrochemical Driving Force

The filling of synaptic vesicles with neurotransmitter depends on the energy stored in a H⁺ electrochemical gradient ($\Delta\mu_{H+}$) produced by the vacuolar-type H⁺-ATPase. The vacuolar H⁺ pump resembles the F0/F1 ATPase (ATP synthase) of mitochondria in structure and function. However, rather than using H⁺ flux to produce ATP, the vacuolar H⁺ pump uses ATP hydrolysis to drive H⁺ transport into membranes of the secretory pathway, including endosomes, lysosomes, synaptic vesicles, and LDCVs (16). $\Delta\mu_{H+}$ in turn comprises both a chemical gradient (Δ pH) and a membrane potential ($\Delta\psi$), and the transport of all classical transmitters into synaptic vesicles depends on both components (**Figure 1**). However, classical studies have shown that the different transport activities depend to differing extents on Δ pH and $\Delta\psi$ due to the charge on the substrate and the stoichiometry of coupling to H⁺.

Vesicular Transporters

The vesicular transporters for monoamines (VMAT) and ACh (VAChT) exchange two luminal H^+ for each molecule of cytosolic transmitter (17–19). However, only the charged monoamine is recognized, and ACh is permanently protonated, so each transport cycle results in a net loss from the lumen of 2 H^+ but only +1 charge, accounting for the greater dependence of these activities on ΔpH than on $\Delta \psi$. The greater consumption of ΔpH than of $\Delta \psi$ in turn requires the replacement of more H^+ than charge by the H^+ pump. Because the number of charges pumped by the H^+ -ATPase must equal the number of H^+ , the regeneration of the gradients dissipated by vesicular monoamine and ACh transport thus requires an additional mechanism that can restore the necessary balance.

VGAT recognizes glycine as well as GABA. GABA and glycine exchange for an unknown number of H⁺, and as zwitterions, their uptake depends equally on ΔpH and on $\Delta \psi$ (20, 21). Despite the clear role for ΔpH in vesicular GABA transport, recent work using functional reconstitution of purified mammalian VGAT has suggested that the activity requires cotransport of 2 Cl⁻ and hence relies predominantly if not exclusively on $\Delta \psi$ (22). Previous work had not identified a requirement for Cl⁻, but the apparent affinity of VGAT for Cl⁻ appears high, suggesting that only low concentrations may be required (22). However, the assays used may reflect only kinetics, and determining the stoichiometry using thermodynamic measurements at equilibrium will be important.

In contrast to VMAT and VAChT, the vesicular glutamate transporters (VGLUTs) depend primarily on $\Delta\psi$. The three isoforms (VGLUT1–3) exhibit generally complementary patterns of expression in the brain but very similar transport activity (reviewed in References 23–25). Despite the primary reliance on $\Delta\psi$, VGLUT activity retains some dependence on Δ pH even after dissipation of $\Delta\psi$ (26, 27), suggesting that the mechanism involves H⁺ exchange. Independently of H⁺ coupling, however, glutamate uptake depends more on $\Delta\psi$ because at neutral pH, glutamate is anionic. If exchanged for *n*H⁺ (and the stoichiometry of coupling remains unknown), glutamate influx results in an efflux of *n* + 1 charge. The VGLUTs thus produce an imbalance between $\Delta \mu_{H+}$: H⁺ electrochemical gradient

Vacuolar-type H⁺-ATPase (or H⁺

pump): a complex of V0 and V1 subunits homologous to the F0 and F1 subunits of mitochondrial ATP synthase; uses ATP hydrolysis to pump H⁺ into organelles

∆pH: pH gradient

 $\Delta \psi$: organelle membrane potential

VAChT: vesicular ACh transporter

VGLUT: vesicular glutamate transporter



Figure 1

Vesicular neurotransmitter transporters depend differentially on the chemical and electrical components of the H⁺ electrochemical gradient ($\Delta \mu_{H+}$). The vacuolar-type H⁺-ATPase generates the $\Delta \mu_{H+}$ required for transport of all classical neurotransmitters into synaptic vesicles. However, different vesicular neurotransmitter transporters rely to differing extents on the two components of $\Delta \mu_{H+}$: the chemical gradient (ΔpH) and the electrical gradient ($\Delta \psi$). (a) The vesicular accumulation of monoamines and acetylcholine (ACh) involves the exchange of protonated cytosolic transmitter for two luminal H⁺. The resulting movement of more H⁺ than charge dictates a greater dependence on ΔpH than on $\Delta \psi$ for both vesicular ACh transport and vesicular monoamine transport. (b) Vesicular glutamate transport may not involve H⁺ translocation. In the absence of $\Delta \psi$, however, disruption of ΔpH inhibits uptake, suggesting that the transport of anionic glutamate involves exchange for nH^+ , resulting in the movement of n + 1charge and hence greater dependence on $\Delta \psi$ than on ΔpH . Transport of the neutral zwitterion GABA (and glycine) involves the movement of an equal number of H⁺ and charge, consistent with the similar dependence of vesicular GABA transporter on ΔpH and $\Delta \psi$. These differences suggest that vesicles storing monoamines or ACh may have mechanisms to favor the accumulation of ΔpH at the expense of $\Delta \psi$, whereas those storing glutamate may promote a larger $\Delta \psi$. The extent to which vesicles differ in their expression of these two components remains unknown, but intracellular Cl- carriers such as the synaptic vesicle-associated ClC-3 promote vesicle acidification by dissipating the positive $\Delta \psi$ developed by the vacuolar H⁺ pump, thereby disinhibiting the pump to make larger ΔpH . The vesicular glutamate transporters (VGLUTs) can also contribute to ΔpH formation because entry of glutamate as an anion similarly dissipates $\Delta \psi$ to promote ΔpH . Interestingly, a Cl⁻ conductance associated with the VGLUTs may also promote acidification by Cl- (41).

charge and H^+ similar to but opposite that created by VMAT and VAChT, implicating additional mechanisms to balance the two components of $\Delta\mu_{H+}$ so that the H^+ pump can continue to function.

It is widely assumed that the expression of a vesicular neurotransmitter transporter confers the potential for regulated release of available substrate. Indeed, all known transporters contain signals that target them to endocytic vesicles, even in non-neural cells (28), and the expression of $\Delta \mu_{H+}$ by endosomes should in principle drive their activity. Heterologous expression of the VMATs by a range of cell lines indeed confers robust monoamine uptake by endosomes. However, measuring the activity of other vesicular transporters after heterologous expression has been extremely difficult, perhaps because they have a much lower apparent affinity (low millimolar K_m) for substrate than do the VMATs ($K_m < 1 \mu M$), but at least in some cases perhaps because the endosomes of non-neural cells lack essential components such as factors that regulate the expression of $\Delta \mu_{H+}$ as ΔpH or $\Delta \psi$.

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THE REGULATION OF ΔpH BY ANION FLUX

More attention has focused on the factors that promote the formation of ΔpH than on those promoting $\Delta \psi$ because organelle ΔpH is easier to measure than $\Delta \psi$ and because it is presumed to have a more important biological role: in ligand dissociation from receptors within the endocytic pathway, in the processing of propeptides within the biosynthetic pathway, and in proteolytic degradation within lysosomes, as well as in vesicular neurotransmitter transport. Importantly, in vitro studies have repeatedly shown that the simple addition of ATP to activate the H⁺ pump does not suffice to produce substantial ΔpH . With activation of the H⁺ pump, $\Delta \psi$ accumulates before the bulk concentration of H⁺ increases, arresting the activity of the pump before the development of ΔpH . Dissipation of $\Delta \psi$, generally considered to involve anion entry, allows the ATPase to continue pumping H⁺ and to produce ΔpH .

Chloride

The principal anion involved in vesicle acidification is presumed to be Cl⁻. In the absence of Cl⁻, synaptic vesicles and other isolated organelles show only a small acidification upon the addition of ATP. The addition of Cl⁻ then leads to a concentration-dependent increase in Δ pH, presumably by dissipating $\Delta\psi$ (26, 27, 29). Intracellular members of the ClC Cl⁻ channel family are considered to mediate the Cl⁻ permeability of acidic vesicles, with ClC-3 the predominant but probably not the only isoform on synaptic vesicles (30). Interestingly, work on the related ClCs 4–7 as well as on a bacterial homolog shows that these proteins do not function as channels but rather as Cl⁻/H⁺ exchangers with a stoichiometry of 2Cl⁻:1H⁺ (31–35). In this case, Cl⁻ entry is coupled to H⁺ efflux, which seems counterproductive because Cl⁻ entry acts primarily to increase Δ pH. In the case of ClCs, however, the loss of 1 H⁺ is accompanied by the loss of +3 charge, dissipating $\Delta\psi$ more than Δ pH and thus stimulating the H⁺-ATPase to replenish these gradients. For an equivalent [Cl⁻] gradient, 2Cl⁻:1H⁺ exchange would thus produce a larger Δ pH than a simple Cl⁻ channel would (29, 36, 37). For 2Cl⁻:1H⁺ exchange, the concentration gradient of Cl⁻ at equilibrium is predicted by the equation

$$2\log_{10}([\text{Cl}^-]_i/[\text{Cl}^-]_o) = \log_{10}([\text{H}^+]_i/[\text{H}^+]_o) + 3\Delta\psi/(2.3RT/F),$$
1

where *R* is the gas constant, *T* is the absolute temperature, *F* is Faraday's constant, and the vATPase determines ΔpH and $\Delta \psi$. Estimating that the proton pump can generate a total $\Delta \mu_{H+} \sim 3$ (i.e., $\Delta pH \sim 3$ pH units, $\Delta \psi \sim 180$ mV, or a combination of both) (38, 39),

$$3 = \log_{10}([\mathrm{H^+}]_i/[\mathrm{H^+}]_o) + \Delta \psi/(2.3RT/F).$$

Replacing $\Delta \psi$ in Equation 1 with $(2.3RT/F)(3 - \log_{10}([H^+]_i/[H^+]_o))$ predicts

$$\log_{10}([\mathrm{H^+}]_i/[\mathrm{H^+}]_o) = 4.5 - \log_{10}([\mathrm{Cl^-}]_i/[\mathrm{Cl^-}]_o).$$

In contrast, if the ClC or another protein present on synaptic vesicles functioned as a simple Cl⁻ channel, the concentration gradient of Cl⁻ at equilibrium would be predicted by the Nernst equation:

$$\log_{10}([\text{Cl}^-]_{\rm i}/[\text{Cl}^-]_{\rm o}) = \Delta \psi/(2.3RT/F).$$

Replacing $\Delta \psi$ with $(2.3RT/F)(3 - \log_{10}([H^+]_i/[H^+]_o))$, as above,

$$\log_{10}([\mathrm{H^+}]_i/[\mathrm{H^+}]_o) = 3 - \log_{10}([\mathrm{Cl^-}]_i/[\mathrm{Cl^-}]_o).$$

For an equivalent concentration gradient of anion, the H^+ exchange mechanism thus counterintuitively produces a substantially larger ΔpH (by 1.5 pH units) than does a simple ion channel.

KO: knockout

Conversion of two ClCs into Cl⁻ channels in knockin mice indeed impaired the function of the endocytic pathway (36, 37). However, no change in acidification was observed, raising the possibility that the two mechanisms differ primarily in the luminal concentration of Cl⁻. It is unclear why changes in luminal Cl⁻ would affect the function of the endocytic pathway if not through a change in ΔpH , but the anion gradients likely differ between the two mechanisms.

Considering the established role of ClCs in endosome/lysosome acidification, it is surprising that recent work has suggested a primary role for the VGLUTs in Cl⁻ flux by synaptic vesicles. Originally, the analysis of ClC-3 knockout (KO) mice had suggested a role for that isoform in the acidification of synaptic vesicles, but the analysis was complicated by severe degeneration of the hippocampus and the retina (30). In younger ClC-3 KO mice, the defect appeared much less significant (40, 41). In contrast, synaptic vesicles from VGLUT1 KO mice showed a more profound defect in acidification due to Cl⁻ (41), suggesting that the VGLUTs mediate Cl⁻ flux by synaptic vesicles. Indeed, the expression of other so-called type I phosphate transporters of the VGLUT family confers a Cl⁻ conductance (42), and the VGLUTs also promote acidification of synaptic vesicles by Cl⁻ (41, 43). In addition to their essential role in packaging glutamate, the VGLUTs may thus exhibit Cl⁻ channel activity, and vesicular glutamate transport shows a clear biphasic dependence on Cl⁻ (26, 27). In addition, the Cl⁻ dependence of glutamate transport may reflect allosteric activation rather than effects on the driving force (44).

However, it remains unclear how a Cl⁻ conductance might contribute to the kinetic properties of glutamate transport. Recent work has indeed failed to detect any Cl⁻ flux after functional reconstitution of purified VGLUT2 (45), and the analysis involved direct measurement of flux rather than indirect effects on acidification. Thus, whether the VGLUTs and/or ClCs mediate Cl⁻ entry into glutamatergic synaptic vesicles remains uncertain. Taken together, however, the data suggest that synaptic vesicles storing glutamate, which are the most abundant in brain, express more VGLUT than ClC; Cl⁻ entry would indeed dissipate the $\Delta\psi$ required for vesicular glutamate transport, and previous work has suggested that substrates can inhibit the Cl⁻ conductance associated with VGLUTs and related proteins (42, 43).

What then would be the role for a Cl⁻ conductance associated with glutamatergic vesicles? Recent work in reconstituted proteoliposomes has suggested that Cl⁻ efflux can promote glutamate uptake (41). Immediately after endocytosis, synaptic vesicles should contain large amounts of Cl⁻ captured from the extracellular space. Although luminal Cl⁻ may exchange directly for cytosolic glutamate, Cl⁻ efflux more likely generates the $\Delta \psi$ required for vesicular glutamate transport, and this possibility requires direct testing. A high priority is to determine whether luminal Cl⁻ influences the filling of native synaptic vesicles, rather than simply the filling of artificial membranes whose much larger size may confer new properties. In any case, the acidification of nonglutamatergic synaptic vesicles presumably depends on ClCs, and ClC-3 may be only one of several isoforms involved. Indeed, recent work using ClC-3 KO mice has shown major defects in GABA release, apparently due to the impaired acidification of GABAergic synaptic vesicles (40).

Previous work has also demonstrated the synergistic effect of ATP (also an anion) on serotonin uptake by chromaffin granules (46). Although ATP is present in all cells, this effect presumably requires vesicular nucleotide transport, which may occur only in cells that release ATP. Manipulation of the recently described vesicular nucleotide transporter VNUT (47) will therefore be required to assess the physiological role of ATP.

Glutamate

Independently of the Cl⁻ flux that may be mediated by VGLUTs, vesicular glutamate transport itself has profound effects on ΔpH (26, 27). As an anion, glutamate, like Cl⁻, dissipates $\Delta \psi$

and hence promotes ΔpH . Indeed, glutamate alone acidifies synaptic vesicles in the presence of ATP to activate the H⁺ pump, presumably reflecting the abundance of glutamatergic vesicles in the mammalian brain. We do not know the stoichiometry of ionic coupling by the VGLUTs, but the sensitivity to ΔpH (27, 43, 48) supports a H⁺ exchange mechanism despite the primary dependence of VGLUTs on $\Delta \psi$. Assuming the exchange of 1 H⁺ for 1 glutamate and hence the movement of +2 charge,

$$\log_{10}([glu^{-}]_i/[glu^{-}]_o) = \log_{10}([H^{+}]_i/[H^{+}]_o) + 2\Delta\psi/(2.3RT/F).$$

5.

VTA: ventral

tegmental area

Again replacing $\Delta \psi$ with $(2.3RT/F)(3 - \log_{10}([H^+]_i/[H^+]_o))$,

$$\log_{10}([\mathrm{H^+}]_{\mathrm{i}}/[\mathrm{H^+}]_{\mathrm{o}}) = 6 - \log_{10}([\mathrm{glu^-}]_{\mathrm{i}}/[\mathrm{glu^-}]_{\mathrm{o}}). \tag{6}$$

For a given anion gradient, glutamate flux through the VGLUTs (Equation 6) is therefore predicted to generate ΔpH 1.5 units greater than Cl⁻ flux does through even an intracellular ClC (Equation 3) and 3 units greater than Cl⁻ flux does through a channel (Equation 4).

Consistent with these predictions, we found that different anions have nonredundant effects on vesicle filling with transmitter (29), presumably by producing different ΔpH . Although Cl⁻ suffices to promote ΔpH and to stimulate the ΔpH -dependent storage of cationic transmitters (29, 49), we and others found that glutamate can also increase the packaging of monoamines (29, 50, 51) and ACh (52) into isolated synaptic vesicles. Indeed, a subset of monoamine and cholinergic neurons express VGLUTs: A number of catecholamine populations including midbrain dopamine neurons in the ventral tegmental area (VTA) express VGLUT2 (53, 54), whereas serotonergic neurons in the dorsal raphe and cholinergic interneurons in the striatum express VGLUT3 (55-57; reviewed in Reference 25). However, it is unclear how glutamate promotes vesicle filling in the presence of substantially higher cytosolic Cl⁻ concentrations, and most previous work showing stimulation of vesicle filling by glutamate has relied on very low Cl⁻ (50–52). We recently found that the effects of glutamate on monoamine filling persist even at physiological Cl⁻ (20 mM) (29), indicating that the two anions do not have redundant roles. Surprisingly, glutamate produces larger synaptic vesicle pH gradients than does Cl^- at concentrations up to ~ 12 mM. The acidification by glutamate saturates at concentrations greater than 2–4 mM, consistent with the known VGLUT K_m (1–3 mM). In addition, the acidification produced by glutamate is more stable than that produced by Cl⁻: After inhibition of the H⁺ pump, ΔpH collapses immediately in vesicles acidified with Cl⁻, but much more slowly in those acidified with glutamate (29). Although glutamate has a much higher pK_a than does Cl⁻ and can thus serve as a better buffer, the increased stability of ΔpH is in large part attributable to the mechanism of anion flux. In the absence of an electrical shunt, synaptic vesicle ΔpH is quite stable because the efflux of H⁺ will create a negative $\Delta \psi$ that opposes further efflux. In the case of vesicles acidified with Cl⁻, however, H⁺ can leave the vesicle because Cl⁻ efflux through a channel-like mechanism dissipates $\Delta \psi$. In the case of vesicles acidified with glutamate, glutamate cannot leave the vesicle because the H⁺ exchange mechanism opposes coupled H⁺ influx into acidic vesicles. In contrast to a channel, the H⁺ exchange mechanism thus serves to lock H^+ inside synaptic vesicles, stabilizing ΔpH and promoting vesicular uptake of monoamines and ACh (Figure 2) (29).

Using KO mice, recent work has demonstrated the physiological significance of VGLUT coexpression with VMAT2 or VAChT on synaptic vesicles in vivo. Originally, there was some concern that adult dopamine neurons did not express VGLUT2 (58, 59), and expression does appear to be highest early in development or after injury (60–63). However, mature conditional knockout (cKO) mice lacking VGLUT2 selectively in dopamine neurons clearly show a reduction in both dopamine storage and evoked dopamine release (29) that presumably accounts for their reduced response to psychostimulants (29, 64). The reduction is anatomically restricted to the ventral

a Cl⁻ channel

b Glutamate:H⁺ exchange



Figure 2

Glutamate flux produces larger and more stable changes in vesicular ΔpH than does Cl⁻. Changes in ΔpH of isolated synaptic vesicles were monitored using acridine orange (5 µM) in 140 mM choline gluconate, 10 mM K⁺ gluconate, 10 mM HEPES, pH 7.4. Acidification was triggered by the sequential addition of 1 mM ATP and 2 mM Cl⁻ followed by either (a) 14 mM Cl⁻ or (b) 4 mM glutamate; more Cl⁻ is required to produce an equivalent initial change in ΔpH . The traces in black indicate vesicles without any further addition. Where indicated by the arrows, the K⁺ ionophore valinomycin (val) (50 nM; gray), the proton pump inhibitor bafilomycin (baf) (250 nM; dark blue/red), or both (light blue/pink) were added. The rate of alkalinization immediately after bafilomycin addition (dark blue/red) is much faster in the vesicles acidified with Cl⁻, indicating that vesicles acidified with glutamate maintain a more stable ΔpH . Although increased buffering may contribute to the stabilization of ΔpH by glutamate, valinomycin accelerates the bafilomycininduced collapse in ΔpH across membranes acidified with glutamate (*pink*), but not across membranes acidified with Cl⁻ (*light blue*), indicating an important role for negative $\Delta \psi$ in the stability of ΔpH in glutamate-acidified vesicles. We hypothesize that the negative $\Delta \psi$ developing upon H⁺ efflux impedes further dissipation of ΔpH . In the case of vesicles acidified with Cl⁻, anion efflux through a channel (c) would shunt the developing negative $\Delta \psi$, allowing the continued efflux of H⁺ and rapid collapse of Δp H. In the case of vesicles acidified with glutamate, a H⁺/anion exchange mechanism (d) would impede glutamate efflux because it would be coupled to the uphill movement of H^+ into acidic vesicles. Because glutamate efflux is disfavored, H^+ efflux is slow and ΔpH more stable. Thus, the differences in mechanism of anion flux (channel versus H⁺ exchange) confer differences in the stability of Δp H. Glutamate thus serves to lock H⁺, and hence cationic transmitters such as acetylcholine and monoamines, inside secretory vesicles. Panels *a* and *b* reproduced from Reference 29 with permission from Elsevier.

striatum, consistent with the expression of VGLUT2 by VTA dopamine neurons projecting to the ventral striatum but not by their neighbors in the substantia nigra pars compacta (SNc) that innervate the dorsal striatum (53, 54). These data are also consistent with the presence of TH^+ asymmetric (presumably excitatory) synapses in the ventral but not the dorsal striatum (65, 66).

Because VGLUT proteins usually localize exclusively to axon terminals, identification of VGLUT⁺ cell populations has generally required quantitative polymerase chain reaction, in situ hybridization, or alternatively immunoelectron or confocal microscopy to examine nerve terminals directly. However, the low levels of VGLUT2 in mature dopamine neurons have sometimes eluded detection with the less sensitive of these methods, leading to conflicting conclusions about the expression of VGLUT2 by midbrain dopamine neurons (53, 54, 58–64, 67, 68). Using transgenic mice expressing GFP (green fluorescent protein) under the control of VGLUT2-regulatory elements, we observed clear colocalization of GFP with tyrosine hydroxylase in a medial subset of VTA neurons (29), consistent with a recent comprehensive report using in situ hybridization (54). Because the coexpressing neurons compose only a fraction of all dopamine neurons in the VTA, the effect of the KO on dopamine stores in vivo may greatly underestimate the effect on this subset. Thus, midbrain dopamine neurons may differ dramatically in the storage and release of dopamine, due to the heterogeneous expression of VGLUT2.

A KO of VGLUT3 has also been used to assess the role of glutamate storage and release by cholinergic interneurons of the striatum, which along with serotonin neurons in the raphe express high levels of VGLUT3. Constitutive disruption of VGLUT3 produces increased locomotor activity that can be reversed by the inhibition of acetylcholinesterase, and the animals show a reduction in vesicular ACh (and serotonin) uptake and release (50, 52). In contrast to wild-type animals, these animals also show no stimulation of vesicular ACh or serotonin transport by glutamate. However, the expression of VGLUT3 by a number of neuronal populations and the unconditional inactivation of VGLUT3 in these animals make it difficult to conclude that the behavioral abnormalities reflect a specific alteration in ACh release by striatal interneurons. The biochemical effect of glutamate on ACh and monoamine costorage thus seems clear, but the conditional inactivation of VGLUT3 or even of VAChT in genetically defined cell populations will be required to address the role of this phenomenon in behavior.

Although the dissipation of $\Delta \psi$ required for vesicle acidification has generally been attributed to anion entry, recent observations from non-neural cells suggest a role for cation efflux in lysosome ΔpH (69). Cl⁻ clearly promotes lysosome acidification in vitro, but this report suggests a smaller role in intact (or at least permeabilized) cells, with the efflux of luminal cation (apparently K⁺) responsible in vivo. Nonetheless, the considerable data from ClC KO mice documenting effects on acidification within the endosome/lysosome pathway make it very difficult to exclude a role for Cl⁻ and these proteins in ΔpH formation.

THE REGULATION OF $\Delta \psi$ BY CATION FLUX

Do endocytic vesicles have a specific mechanism to promote formation of $\Delta \psi$? Or does $\Delta \psi$ result simply from the absence of a counterion such as Cl⁻ or glutamate? In general, $\Delta \psi$ has received little attention for an independent role in the secretory pathway, but vesicular glutamate transport clearly depends on $\Delta \psi$. Although recent attention has focused on the expression of VGLUTs as a presynaptic determinant of quantal size (70–72), the number of transporters per vesicle will change primarily the kinetics of transport, not the thermodynamic equilibrium reached at steady state (1). However, changes in the driving force should have dramatic effects on the extent as well as on the rate of vesicle filling, so the regulation of $\Delta \psi$ has important implications for transmitter release. **SNc:** substantia nigra pars compacta

Quantal size: the postsynaptic response to the release of a single secretory vesicle

NHE: Na⁺/H⁺ exchanger

SAC: starburst amacrine cell

DSGC: direction-selective ganglion cell

LSO: lateral superior olive

Although very little is known about the factors that promote the formation of $\Delta \psi$, recent work has identified intracellular members of the Na⁺/H⁺ exchanger (NHE) family that could serve this function. NHEs catalyze the electroneutral exchange of monovalent cation for H⁺, and plasma membrane isoforms have an important role in the regulation of cytosolic pH (73). Intracellular isoforms recognize K⁺ as well as Na⁺, and several isoforms localize to endosomes (74), where they should dissipate ΔpH and thus enable the H⁺ pump to increase $\Delta \psi$. Interestingly, recent human genetic studies have implicated intracellular isoform NHE6 in Angelman syndrome (75) and NHE9 in autism (76).

Cation channels may also influence the formation of $\Delta\psi$. In this case, K⁺ entry would promote the formation of $\Delta\psi$ independently of the H⁺ pump. Interestingly, the TRPM7 (transient receptor potential cation channel, subfamily M, member 7) channel localizes to synaptic vesicles and influences quantal size, although it also interacts with proteins involved in fusion and affects the frequency of release (77, 78). However, the work on TRPM7 has involved cholinergic neurons, whereas the presence of an active K⁺ conductance on synaptic vesicles may shunt the $\Delta\psi$ required for vesicular glutamate transport.

INDEPENDENT ROLES FOR CORELEASED NEUROTRANSMITTERS

In addition to the presynaptic consequences for vesicle filling, corelease has implications for the activation of postsynaptic receptors. Both coreleased transmitters may activate receptors, with the potential for distinct modes of signaling, and recent work has begun to elucidate the physiological role of corelease.

Corelease of GABA and Acetylcholine from Starburst Amacrine Cells

Starburst amacrine cells (SACs) contribute to direction-selective motion sensing by the vertebrate retina. SACs have a radially symmetric dendritic morphology that overlaps with dendrites from neighboring SACs as well as direction-selective ganglion cells (DSGCs) in the inner plexiform layer. Dual recordings show that SACs release more GABA onto DSGCs in response to light moving in the nonpreferred direction than in response to light moving in the preferred direction. Indeed, GABA release, presumably from SACs, appears to be essential for direction selectivity (79).

In addition to inhibitory GABA, SACs release ACh, activating nicotinic (nACh) receptors on DSGCs. However, the activation of nACh receptors is not required for direction selectivity (79). To characterize the release of both transmitters, a recent study using paired recordings demonstrated that, whereas GABA release by SACs is selective for movement in the null direction, the cholinergic response is greater with movement in the preferred direction (80). Both GABA and ACh currents depend on external Ca^{2+} , supporting a vesicular release mechanism, but ACh release shows much less sensitivity to Ca^{2+} than does GABA release, providing physiological evidence that different vesicle populations mediate release of the two transmitters. These observations are consistent with a proposed dual role for SACs as encoding direction selectivity through GABA release and encoding motion sensitivity through ACh release.

GABA and Glutamate Corelease from the Medial Nucleus of the Trapezoid Body

Neurons in the lateral superior olive (LSO) function as interaural coincidence detectors essential for sound localization. They accomplish this by integrating tonotopically precise excitatory input

from the ipsilateral cochlear nucleus with inhibitory GABAergic and glycinergic inputs from the contralateral medial nucleus of the trapezoid body (MNTB). During development, however, MNTB neurons transiently express VGLUT3 and corelease glutamate between postnatal day (P)0 and P12 (81). In VGLUT3 KO mice, MNTB cells still form synapses onto LSO neurons that are indistinguishable from those in control animals at P1–2; however, the strengthening of these inhibitory synapses that normally occurs by P10–12 fails to occur in VGLUT3-null mice (82). Furthermore, tonotopic projections from the MNTB that project diffusely within the LSO at P1 fail to sharpen normally in the absence of VGLUT3. But why is glutamate release important when GABA is excitatory [due to a shift in E_{Cl} (the equilibrium potential for Cl⁻)] during the same time frame? Presumably, the specific activation of NMDA receptors confers the plasticity required for normal development (81). The results thus support a role for glutamate corelease in synapse refinement that underlies sound localization in the auditory system.

GABA and Glutamate Corelease from Hippocampal Mossy Fibers

In the hippocampus, mossy fibers derived from granule cells in the dentate gyrus form glutamatergic synapses onto CA3 pyramidal neurons, where they also corelease GABA. Early in development, pyramidal neurons express VGAT and glutamic acid decarboxylase, the enzyme responsible for GABA biosynthesis, but the genes involved subsequently downregulate (83–85). For the first 3 weeks after birth, stimulation of mossy fiber inputs produces GABA-mediated currents in pyramidal neurons (86). However, the significance of this transient GABA corelease remains unknown, and at this time, GABA currents are still excitatory due to the shift in Cl⁻ reversal potential.

Interestingly, epileptic activity rekindles expression of the GABAergic phenotype in adult granule cells (87–90). At this point, GABA transmission is inhibitory and may thus serve a distinct, homeostatic role to restrain the excitability responsible for epilepsy.

Monoamine and Glutamate Corelease

The first clear evidence that monoamine neurons corelease glutamate derived from dissociated neurons grown in isolation so that they could form synapses onto only themselves. Stimulation of both serotonin (12) and dopamine (13) neurons produced fast excitatory currents blocked by glutamate receptor antagonists, indicating the potential for glutamate corelease to activate postsynaptic receptors. However, the postnatal decline in VGLUT2 expression by midbrain dopamine neurons (60, 63) raised the possibility that VGLUT2 expression in vitro (67) might simply reflect dedifferentiation. The low level of VGLUT2 expression by midbrain dopamine neurons in the adult raised further questions about the physiological relevance of these in vitro observations. The phenotype of mice lacking VGLUT2 specifically in dopamine neurons and the anatomical evidence for VGLUT2 expression by a medial subset of VTA neurons have provided clear evidence for the effects of glutamate on costored dopamine but have not directly addressed the role of glutamate as an independent signal.

In 2004, the Rayport laboratory (91) published a landmark study that used an acute, horizontal slice preparation to demonstrate the presence of a monosynaptic glutamatergic projection from VTA to nucleus accumbens at both P10 and P21. The next year, the Seamans laboratory (92) showed that VTA stimulation in vivo rapidly leads to glutamate release in the prefrontal cortex (PFC). Although both of these studies supported an independent role for the glutamate released by dopamine neurons, questions remained about the specificity of stimulation, particularly after the identification of purely glutamatergic neurons in the ventral midbrain (53, 59) that we now know also project to both the ventral striatum and the PFC (54, 93).

MNTB: medial nucleus of the trapezoid body

In contrast, genetic approaches have recently provided definitive physiological evidence that glutamate released by at least a subset of dopamine neurons in adult mice activates ionotropic glutamate receptors on postsynaptic medium spiny neurons in the striatum. Using cre recombinase selectively expressed by dopamine neurons to activate a conditional allele of the light-activated cation channel channelrhodopsin-2, we and others observed glutamate responses evoked by direct illumination of the striatum (94, 95). In addition to the increased specificity, the ability to stimulate glutamate release directly at presynaptic boutons circumvented the unavoidable transection of mesolimbic projections in horizontal slices, resulting in larger postsynaptic responses. Robust glutamate-mediated AMPA receptor currents were observed in the ventral striatum but not in the dorsal striatum, even though light evoked dopamine release at both sites (94), consistent with the restricted expression of VGLUT2 by dopamine neurons in the VTA but not in the SNc (53, 54). Furthermore, the cKO of VGLUT2 in dopamine neurons completely abolished these responses (94).

What then is the role of this glutamate signal? The most robust phenotype observed in cKO mice that lack glutamate corelease from dopamine neurons is a reduction in psychostimulantinduced locomotion (29, 64). This may be most easily explained by the reduction in dopamine release that we attribute to a reduction in vesicular dopamine storage (29). However, the activation of postsynaptic ionotropic receptors by the glutamate released from dopamine neurons likely encodes distinct information.

One possibility is that the glutamate released by dopamine terminals contributes to the prediction-error signal encoded in the firing rates of dopamine neurons (96, 97). A subset of tonically active midbrain (presumably dopamine) neurons burst fire in response to unexpected rewards or to rewards better than predicted by a conditioned cue. Conversely, they slow or pause firing in response to rewards worse than predicted (98). Consistent with these changes in firing, extracellular dopamine measured by fast-scan cyclic voltammetry changes as predicted in rodents performing goal-directed tasks (99). However, we do not know how dopamine signaling by metabotropic G protein-coupled dopamine receptors can maintain the fidelity of synaptic transmission required for learning tasks dependent on subsecond cue discrimination. As a neuromodulator activating G protein-coupled receptors, dopamine presumably acts on slower timescales (i.e., seconds to minutes). In contrast, the glutamate coreleased by dopamine neurons produces a rapid, transient postsynaptic response more tightly coupled to dopamine neuron firing and is thus well positioned to convey temporally precise information about reward (for excellent reviews see References 100 and 101). This hypothesis predicts deficits in reward learning by cKO mice lacking VGLUT2 in dopamine neurons, but initial assessment using conditioned place preference (CPP) showed no such deficits (29). However, mice can also learn CPP in the absence of dopamine (102, 103), and the cue-reward pairing involved in CPP occurs continuously over the course of 20 min and may therefore not depend on transient subsecond bursts in dopamine neuron firing.

The expression of channelrhodopsin in raphe nuclei has also revealed an optically evoked glutamate-mediated response in the hippocampus, presumably from the population of serotonergic neurons expressing VGLUT3 (104). However, these experiments did not use genetic manipulation to limit channelrhodopsin expression to serotonergic neurons, so the responses may derive from neighboring nonserotonergic neurons in the raphe. Indeed, despite the strong expression of VGLUT3 mRNA in raphe nuclei, to what extent VGLUT3 and serotonergic markers are coexpressed or compose separate neuronal populations, similar to the nondopaminergic VGLUT2⁺ population of neurons in the medial midbrain, remains unclear. However, the anatomical evidence supports VGLUT3 expression by at least a subset of serotonergic neurons (105–109).

Acetylcholine and Glutamate Corelease

Channelrhodopsin was also used recently to demonstrate that, in addition to the role of glutamate costorage in promoting vesicular ACh filling in striatal interneurons (52), the released glutamate activates ionotropic receptors on medium spiny neurons. Consistent with VGLUT3 expression by these cells, the response was abolished in VGLUT3 KO mice (110). Recent work has also identified corelease of ACh and glutamate by neurons of the medial habenula. Expressed in cholinergic neurons, channelrhodopsin confers light-evoked release of glutamate as well as of ACh within the interpeduncular nucleus of the midbrain (111). However, brief illumination evokes primarily the glutamate response, with the ACh response requiring more sustained stimulation. Released from the same neuron, the two transmitters may thus subserve distinct roles in signaling, perhaps due to differences in the distance between release site and postsynaptic receptors (i.e., between synaptic and volume transmission) or perhaps as a function of release from different vesicle populations.

DISTINCT AND OVERLAPPING POOLS OF SYNAPTIC VESICLES

The ability of one transmitter to affect the storage of another through changes in the H⁺ electrochemical driving force requires localization of the two vesicular transporters to the same secretory vesicle, but several recent observations suggest that release can also occur from distinct vesicle populations. In retinal SACs, GABA release and ACh release respond differently to Ca²⁺ (80), providing unequivocal evidence for release from different vesicles. Immunolabeling for endogenously expressed proteins also suggests that dopaminergic release sites are heterogeneous in their capacity to store glutamate (13, 53, 60-62, 67, 68). In midbrain dopamine neurons, heterologous expression of differentially tagged vesicular glutamate and monoamine transporters shows colocalization at most boutons, but a significant fraction express only one or the other (112), consistent with the original suggestion that catecholamine and glutamate markers may segregate to distinct synapses both in vitro and in vivo (13, 65). However, in contrast to the VGLUTs, which generally reside only at presynaptic boutons, VMAT2 localizes to dendrites as well as to axons, but the segregation occurs even with the analysis restricted to axonal sites. The segregation of monoamine and glutamate markers to different release sites may indeed contribute to the failure to detect VGLUT expression in tyrosine hydroxylase-positive striatal projections by immunoelectron microscopy (60, 68). Hippocampal neurons show no evidence of such segregation, indicating mechanisms specific to dopamine neurons. In addition, optical imaging with a pHluorin-based reporter shows that field stimulation evokes release of a greater proportion of VGLUT1 than VMAT2 at boutons (112), suggesting that the two proteins exhibit overlapping but differential localization to synaptic vesicle pools.

Considerable previous work has shown that only a fraction of the synaptic vesicles in a presynaptic bouton are available for evoked release, even after prolonged stimulation (113). This so-called recycling pool can be only a small fraction of all the vesicles present, with the remaining, so-called resting pool of uncertain physiological role. Because the proportion of several synaptic vesicle proteins in this recycling pool is generally the same (\sim 50–60%), it has been assumed that they will all exhibit the same distribution between recycling and resting (unresponsive) pools. However, the relatively small recycling pool size of VMAT2 (20–30%) indicates that in addition to the segregation of dopamine and glutamate vesicles at different boutons, dopamine and glutamate vesicles also segregate to at least some extent within individual boutons where they both reside. Interestingly, the differential exocytosis of VMAT2 and VGLUT occurs in hippocampal as well as in midbrain dopamine neurons, indicating the potential for differential corelease of classical transmitters by many if not all neuronal populations.

Recent work has suggested that the VGLUTs may control the probability of transmitter release, perhaps accounting for the differential release of two transmitters by the same neuron. The distribution of VGLUT1 and -2 originally suggested a correlation of VGLUT1 with synapses having a low probability of release (such as hippocampal synapses and parallel fiber synapses in the cerebellum) and VGLUT2 with synapses having a high probability of release (114). Although it has been difficult to understand how the transporter might control fusion, recent work has indeed suggested that the known interaction of VGLUT1 with the endocytic protein endophilin (115) may also influence exocytosis (116). Alternatively, the two transporters may simply recycle through slightly different mechanisms, consistent with the role of endophilin in endocytosis, and these mechanisms may generate vesicles with different release probability. Rather than influencing the release machinery, the transporter may thus simply target to vesicles with different properties. The difference between VMAT2 and the VGLUTs in overall recycling pool size supports this possibility, but it may be more difficult to assess directly the targeting of VGLUT1 and -2 to distinct subsets within the recycling pool. Because synaptic vesicles have generally been considered homogeneous in terms of biochemical composition, considerable basic work will be required to characterize the properties of these subsets and to identify the proteins responsible for their properties, as well as the mechanisms responsible for sorting these proteins into functionally distinct vesicle pools.

SUMMARY POINTS

- 1. The filling of synaptic vesicles with different transmitters relies on different components of $\Delta\mu_{H+}.$
- 2. $\Delta \mu_{H+}$ can be expressed as ΔpH , $\Delta \psi$, or a combination of both.
- 3. The entry of Cl⁻ and other anions promotes the formation of ΔpH by dissipating $\Delta \psi$, thereby disinhibiting the H⁺ pump.
- 4. Cation flux may promote the formation of $\Delta \psi$.
- 5. Many neuronal populations corelease two classical transmitters.
- 6. Costorage with glutamate promotes the vesicular transport of monoamines and ACh.
- 7. Coreleased neurotransmitters can activate their cognate postsynaptic receptors.
- 8. Corelease of two transmitters can also occur from independent vesicle populations.

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104. Uses channelrhodopsin to demonstrate the release of glutamate from hippocampal projections of serotonergic raphe nuclei.

111. Relies on optogenetics to demonstrate a glutamatergic response in the interpeduncular nucleus elicited by optical stimulation of cholinergic projections from the medial habenula.

112. Using

heterologous expression in primary dissociated culture, this study shows that VGLUT2 and VMAT2 traffic to overlapping but distinct synaptic boutons and respond differently to stimulation when expressed in midbrain dopamine neurons.