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Neurotransmitter Co-release: 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 it has become clear that many neurons in fact release multiple transmitters. Although the physiological role for co-release 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. Co-release thus serves multiple roles in synaptic transmission.

Keywords

neurotransmitter co-release; neurotransmitter co-storage; synaptic vesicle pools; vesicular neurotransmitter transporters

INTRODUCTION TO THE NEUROTRANSMITTER CYCLE

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 which 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 define transmitter phenotype. A

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recent proteomic analysis indeed shows that glutamatergic and GABAergic synaptic vesicles differ primarily in the expression of vesicular transporters for glutamate and GABA (2).

NEUROTRANSMITTER CO-RELEASE

Although it has generally been assumed that neurons release only one classical neurotransmitter, exceptions continue to accumulate. The first demonstration of co-release involved adenosine triphosphate (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 (5)]. Since the vesicular GABA transporter (VGAT) also transports glycine (6), it is not surprising that some neurons have been shown to release both inhibitory transmitters (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 which does not produce that particular transmitter. For example, the anti-depressant drug fluoxetine, which selectively inhibits the plasma membrane serotonin transporter (SERT), redistributes serotonin from serotonergic to dopaminergic terminals, where it also undergoes release, and this redistribution may contribute to its antidepressant action (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 co-release glutamate when grown in culture (12, 13), and dopaminergic periglomerular cells in the olfactory bulb also co-release GABA (14). Even motor neurons thought to release only ACh may co-release glutamate from collateral synapses within the spinal cord (15).

Although the evidence for the co-release 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 co-release for vesicle filling and for 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 (μ_{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, but 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). μ_{H+} in turn comprises both a chemical gradient (pH) and membrane potential (ψ), and the transport of all classical transmitters into synaptic vesicles depends on both components (Figure 1). However,

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classical studies have shown that the different transport activities depend to differing extents on pH and ψ 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 2 lumenal 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 pH than ψ. The greater consumption of pH than ψ in turn requires the replacement of more H^+ than charge by the H^+ pump. Since the number of charges pumped by the H^+-ATPase must equal the number of H^+, regeneration of the gradients dissipated by vesicular monoamine and ACh transport thus requires an additional mechanism that can restore the necessary balance.

The vesicular GABA transporter (VGAT, also known as vesicular inhibitory amino acid transporter, or VIAAT) recognizes glycine as well as GABA. GABA and glycine exchange for an unknown number of H^+ and as zwitterions, their uptake depends more equally on pH and ψ (20, 21). Despite the clear role for pH 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 ψ (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, it remains possible that the assays used reflect only kinetics, and it will be important to determine the stoichioimetry using thermodynamic measurements at equilibrium.

In contrast to VMAT and VAChT, the vesicular glutamate transporters (VGLUTs) depend primarily on ψ. The three isoforms (VGLUT1-3) exhibit generally complementary patterns of expression in the brain but very similar transport activity, and this topic has recently been reviewed (23–25). Despite the primary reliance on ψ, VGLUT activity retains some dependence on pH even after dissipation of ψ (26, 27), suggesting that the mechanism involves H^+ exchange. Independent of H^+ coupling, however, glutamate uptake depends more on ψ because at neutral pH, glutamate is anionic. If exchanged for nH^+ (and the stoichiometry of coupling remains unknown), glutamate influx results in the efflux of $n + 1$ charge. The VGLUTs thus produce an imbalance between charge and H^+ similar to but opposite that created by VMAT and VAChT, implicating additional mechanisms to balance the two components of μ_{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 of the known transporters contain signals which target them to endocytic vesicles even in non-neural cells (28), and the expression of μ_{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, it has been extremely difficult to measure the activity of other vesicular transporters after heterologous expression, perhaps because they have a much lower apparent affinity (low millimolar Km) for substrate than the VMATs

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(Km <1 μM), 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 μ_{H+} as pH or ψ.

THE REGULATION OF pH BY ANION FLUX

More attention has focused on the factors that promote formation of pH than on those promoting ψ because organelle pH is easier to measure than ψ 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 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 pH. With activation of the H^+ pump, ψ accumulates before the bulk concentration of H^+ increases, arresting the activity of the pump before development of pH. Dissipation of ψ, generally considered to involve the entry of anion, allows the ATPase to continue pumping H^+ and produce pH.

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 addition of ATP. The addition of Cl^- then leads to a concentration-dependent increase in pH, presumably by dissipating ψ (26, 27, 29). Intracellular members of the ClC chloride 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 a bacterial homologue show that these proteins do not function as channels but rather as Cl^-/H^+ exchangers with a stoichiometry of 2 Cl^- : 1 H^+ (31–35). In this case, Cl^- entry is coupled to H^+ efflux, which seems counterproductive since Cl^- entry acts primarily to increase pH. In the case of ClCs, however, the loss of 1 H^+ is accompanied by the loss of +3 charge, dissipating ψ more than pH, and thus stimulating the H^+-ATPase to replenish these gradients. For an equivalent $[Cl^-]$ gradient, $2Cl^-$: $1H^+$ exchange would thus produce a larger pH than a simple Cl^- channel (29, 36, 37): for $2Cl^-$: $1H^+$ exchange, the concentration gradient of Cl^- at equilibrium is predicted by the equation

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

where R is the gas constant, T the absolute temperature, F Faraday's constant, and the vATPase determines pH and ψ. Estimating that the proton pump can generate a total μ_{H+} ~3 (i.e., pH ~3 pH units, ψ ~180 mV or a combination of both) (38, 39):

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

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

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$$\log_{10}([\mathrm{H}^+]_\mathrm{i}/[\mathrm{H}^+]_\mathrm{o})=4.5-\log_{10}([\mathrm{Cl}^-]_\mathrm{i}/[\mathrm{Cl}^-]_\mathrm{o}) \quad (3)$$

On the other hand, if the ClC or another protein present on synaptic vesicles functions as a simple Cl^- channel, the concentration gradient of Cl^- at equilibrium would be predicted by the Nernst equation:

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

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

$$\log_{10}([\mathrm{H}^+]_\mathrm{i}/[\mathrm{H}^+]_\mathrm{o})3-\log_{10}([\mathrm{Cl}^-]_\mathrm{i}/[\mathrm{Cl}^-]_\mathrm{o}) \quad (4)$$

For an equivalent concentration gradient of anion, the H^+ exchange mechanism thus counter-intuitively produces a substantially larger pH (by 1.5 pH units) than a simple ion channel. Conversion of two ClCs into Cl^- channels in knock-in mice indeed impairs 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 pH, but it does seem likely that the anion gradients differ between the two mechanisms.

Considering the established role of ClCs in endosome/lysosome acidification, it is surprising to note 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 hippocampus and retina (30). Using 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 the acidification due to Cl^- (41), suggesting that they mediate Cl^- flux by synaptic vesicles. Indeed, the expression of other so-called type I phosphate transporters of the VGLUT family have been shown to confer 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 has been suggested to reflect allosteric activation rather than effects on the driving force (44).

On the other hand, 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. It thus remains uncertain whether the VGLUTs and/or ClCs mediate Cl^- entry into glutamatergic synaptic vesicles. 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 ψ 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 it was suggested that lumenal Cl^- exchange directly for cytosolic glutamate, it seems more likely that Cl^- efflux generates the $\Delta\psi$ required for vesicular glutamate transport, and this possibility requires direct testing. It will also be very important to determine whether luminal Cl^- influences the filling of native synaptic vesicles, rather than simply artificial membranes whose much larger size may confer new properties. In any case, the acidification of non-glutamatergic 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 only occur in cells that actually release ATP. Manipulation of the recently described vesicular nucleotide transporter VNUT (47) will therefore be required to assess its physiological role.

Glutamate

Independent 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 will, like Cl^-, dissipate $\Delta\psi$ and hence promote 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 their primary dependence on $\Delta\psi$. Assuming the exchange of 1 H^+ for 1 glutamate and hence the movement of +2 charge,

$$\log_{10}([\mathrm{glu}^-]_i/[\mathrm{glu}^-]_o)=\log_{10}([\mathrm{H}^+]_i/[\mathrm{H}^+]_o)+2\Delta\psi/(2.3\,\mathrm{RT/F}). \quad (5)$$

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

$$\log_{10}([\mathrm{H}^+]_i/[\mathrm{H}^+]_o)=6-\log_{10}([\mathrm{glu}^-]_i/[\mathrm{glu}^-]_o) \quad (6)$$

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 through even an intracellular ClC (equation 3) and 3 units greater than Cl^- flux through a channel (equation 4).

Consistent with these predictions, we have found that different anions have non-redundant effects on vesicle filling with transmitter (29), presumably by producing different ΔpH.

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Although Cl^- suffices to promote pH and stimulate the pH-dependent storage of cationic transmitters (29, 49), we and others have 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) while serotonergic neurons in the dorsal raphe and cholinergic interneurons in the striatum express VGLUT3 (55–57) [reviewed in (25)]. However, it has remained 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 have 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 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, pH 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 Cl^- and can thus serve as a better buffer, the increased stability of pH is in large part attributable to the mechanism of anion flux. In the absence of an electrical shunt, synaptic vesicle pH is quite stable because the efflux of H^+ will create a negative ψ 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 ψ. 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 pH and promoting vesicular uptake of monoamines and ACh (Figure 2) (29).

Using knockout mice, recent work has demonstrated the physiological significance of VGLUT co-expression with VMAT2 or VAChT on synaptic vesicles *in vivo*. Originally, there was some concern that adult dopamine neurons did not in fact 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 striatum, consistent with the expression of VGLUT2 by VTA dopamine neurons projecting to the ventral striatum but not 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 ventral but not dorsal striatum (65, 66).

Since VGLUT proteins usually localize exclusively to axon terminals, identification of $VGLUT^+$ cell populations has generally required quantitative PCR, *in situ* hybridization, or alternatively, immuno- electron or confocal microscopy to examine nerve terminals directly. However, the low levels of VGLUT2 in mature dopamine neurons have sometimes eluded

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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 VGLUT2-GFP BAC transgenic mice, we have 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). Since the co-expressing neurons comprise only a fraction of all dopamine neurons in the VTA, the effect of the knockout on dopamine stores *in vivo* may in fact 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 knockout 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 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, they 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 co-storage thus seems clear, but the conditional inactivation of VGLUT3 or even VAChT in genetically defined cell populations will be required to address the role of this phenomenon in behavior.

Although the dissipation of ψ 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 pH (69). Chloride clearly promotes lysosome acidification *in vitro*, but this report suggests a smaller role in intact (or at least permeabilized) cells, with the efflux of lumenal 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 the formation of pH.

THE REGULATION OF ψ BY CATION FLUX

Do endocytic vesicles have a specific mechanism to promote formation of ψ? Or does ψ result simply from the absence of a counterion such as Cl^- or glutamate? In general, ψ has received little attention for an independent role in the secretory pathway, but vesicular glutamate transport clearly depends on ψ. 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 rate of vesicle filling, so the regulation of ψ has important implications for transmitter release.

Although very little is known about the factors that promote formation of ψ, recent work has identified intracellular members of the Na^+/H^+ exchanger (NHE) family that could serve

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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 localize to endosomes (74) where they should dissipate pH and thus enable the H^+ pump to increase ψ. Interestingly, recent human genetic studies have implicated intracellular isoform NHE6 in Angelman syndrome (75), and NHE9 in autism (76).

Cation channels may also influence formation of ψ. In this case, K^+ entry would promote formation of ψ independent of the H^+ pump. Interestingly, the TRPM7 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, it is important to note that the work on TRPM7 has involved cholinergic neurons, whereas the presence of an active K^+ conductance on synaptic vesicles might shunt the ψ required for vesicular glutamate transport.

INDEPENDENT ROLES FOR CO-RELEASED NEUROTRANSMITTER

In addition to the presynaptic consequences for vesicle filling, co-release has implications for the activation of postsynaptic receptors. The two transmitters may both activate receptors, with the potential for distinct modes of signaling, and recent work has begun to elucidate the physiological role of co-release.

Co-release of GABA and ACh 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 ON-OFF 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 non-preferred than the preferred direction. Indeed, GABA release, presumably from SACs, appears to be essential for direction selectivity (79).

In addition to the 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^{++}, supporting a vesicular release mechanism, but release of ACh shows much less sensitivity to Ca^{++} than GABA release, requiring stronger stimulation, and providing physiological evidence that different vesicle populations mediate release of the two transmitters. These observations are consistent with a proposed dual role for SACs, encoding direction selectivity through the release of GABA, and motion sensitivity through the release of ACh.

GABA/glutamate co- release from MNTB

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

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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 co-release glutamate between P0 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). Further, tonotopic projections from 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 itself excitatory (due to a shift in E_{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 co-release inrefinement of the synapse that underlies sound localization in the auditory system.

GABA/glutamate co-release 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 co-release GABA. Early in development, pyramidal neurons express VGAT and glutamic acid decarboxylase, the enzyme responsible for GABA biosynthesis, but these genes subsequently downregulate (83–85). For the first 3 weeks after birth, stimulation of mossy fiber inputs indeed produces GABA-mediated currents in pyramidal neurons (86). However, the significance of this transient GABA co-release remains unknown, and it is important to note that 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 the adult granule cells (87–90). At this point, GABA transmission is inhibitory and may thus serve a distinct, possibly homeostatic role to restrain the excitability responsible for epilepsy.

Monoamine/glutamate co-release

The first clear evidence that monoamine neurons co-release glutamate derived from dissociated neurons grown in isolation so that they can only form synapses onto themselves. Stimulation of both serotonin (12) and dopamine (13) neurons produced fast excitatory currents blocked by glutamate receptor antagonists, indicating the potential for glutamate co-release 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 co-stored dopamine, but not directly addressed the role of glutamate as an independent signal.

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In 2004, the laboratory of Steve Rayport 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 (NAc) at both P10 and P21 (91). The next year, the laboratory of Jeremy Seamans showed that VTA stimulation *in vivo* rapidly leads to glutamate release in the prefrontal cortex (PFC) (92). 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 ventral striatum and PFC (54, 93).

On the other hand, genetic approaches have recently provided definitive physiological evidence that glutamate released by at least a subset of DA 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 have 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 but not 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 SNc (53, 54). Further, the conditional knockout 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 co-release from dopamine neurons is a reduction in psychostimulant-induced 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 (presumaby 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, it is not clear how dopamine signaling by metabotropic G protein-coupled dopamine receptors could 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 time scales (i.e., seconds to minutes). In contrast, the glutamate co-released by dopamine neurons produces a rapid, transient postsynaptic response more tightly coupled to dopamine neuron firing and thus well-positioned to convey temporally precise information about reward [for excellent reviews see (100, 101)]. This hypothesis predicts deficits in reward-learning by cKO mice lacking VGLUT2 in dopamine

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neurons, but initial assessment using conditioned place preference 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 minutes and may therefore not depend on transient sub-second bursts in dopamine neuron firing.

The expression of channelrhodopsin in raphe nuclei has also revealed an optically evoked glutamate-mediated response in 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 it remains possible that the responses derived from neighboring non-serotonergic neurons in the raphe. Indeed, despite the strong expression of VGLUT3 mRNA in raphe nuclei, it has remained unclear to what extent VGLUT3 and serotoninergic markers are coexpressed or comprise separate neuronal populations, similar to the non-dopaminergic VGLUT2$^+$ population of neurons in the medial midbrain. On the other hand, the anatomical evidence supports expression of VGLUT3 by at least a subset of serotonergic neurons (105–109).

ACh/glutamate co-release

Channelrhodopsin was also used recently to demonstrate that in addition to the role of glutamate co-storage in promoting vesicular ACh filling in striatal interneurons (52), the released glutamate activates ionotropic receptors on medium spiny neurons. Consistent with the expression of VGLUT3 by these cells, the response was abolished in VGLUT3 KO mice (110). Recent work has also identified co-release of ACh and glutamate by neurons of the medial habenula. Expressed in cholinergic neurons, channelrhodopsin confers light-evoked release of glutamate as well as 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, the release of GABA and ACh respond differently to Ca^{++} (80), providing unequivocal evidence for release from different vesicles. Immunolabeling for endogenously expressed proteins also suggest 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 might 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 axons, but the segregation occurs even with the analysis restricted to axonal sites. The segregation of

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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 immuno-electron 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 the VGLUT1 at boutons than VMAT2 (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. Since the proportion of several synaptic vesicle proteins in this recycling pool is generally the same (~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 midbrain dopamine neurons, indicating the potential for differential co-release of classical transmitters by many if not all neuronal populations.

Recent work has suggested that the VGLUTs may in fact 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. Since synaptic vesicles have generally been considered homogeneous in terms of biochemical composition, considerable basic work will now be required to characterize the properties of these subsets and identify the proteins responsible for these properties, as well as those responsible for sorting them into functionally distinct vesicle pools.

Acronyms

μ_{H+}	H+ electrochemical gradient

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pH	pH gradient
ψ	Organelle membrane potential
VMAT	Vesicular monoamine transporter
LDCV	Large dense core vesicles
SERT	Serotonin transporter
ACh	Acetylcholine
VAChT	Vesicular ACh transporter
VGLUT	Vesicular glutamate transporter
GABA	γ-aminobutyric acid
VGAT	Vesicular GABA (and glycine) transporter
VNUT	Vesicular nucleotide transporter
ATP	Adenosine triphosphate
NHE	Na^+/H^+ exchanger
VTA	Ventral tegmental area
SNc	Substantia nigra, pars compacta
NAc	Nucleus accumbens
MNTB	Medial nucleus of the trapezoid body
LSO	Lateral superior olive
SAC	Starburst amacrine cell
DSGC	Direction-selective ganglion cell
nAChR	Nicotinic acetylcholine receptors
ClC	Chloride channel
KO	Knockout

Definitions list (20 max)

Exocytosis	The fusion of a vesicle with the plasma membrane
Classical neurotransmitters	Small molecules synthesized or recycled locally, transported into vesicles and released to convey an extracellular signal
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, that uses ATP hydrolysis to pump H^+ into organelles

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Vesicular neurotransmitter transporter	Located on secretory vesicles, these transporters use the electrochemical gradient produced by the vacuolar proton pump to fill vesicles with neurotransmitter
Plasma membrane neurotransmitter transporter	Active at the plasma membrane, these proteins transport neurotransmitter from the extracellular space back into the presynaptic neuron or glia
Quantal size	The postsynaptic response to release of a single secretory vesicle

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Summary Points

1. The filling of synaptic vesicles with different transmitters relies on different components of μ_{H+}.
2. μ_{H+} can be expressed as either pH, ψ or a combination of both.
3. The entry of Cl^- and other anions promote the formation of pH by dissipating ψ, thereby disinhibiting the H^+ pump.
4. Cation flux may promote the formation of ψ.
5. Many neuronal populations co-release two classical transmitters.
6. Co-storage with glutamate promotes the vesicular transport of monoamines and ACh.
7. Co-released neurotransmitters can activate their cognate postsynaptic receptors.
8. Co-release of two transmitters can also occur from independent vesicle populations.

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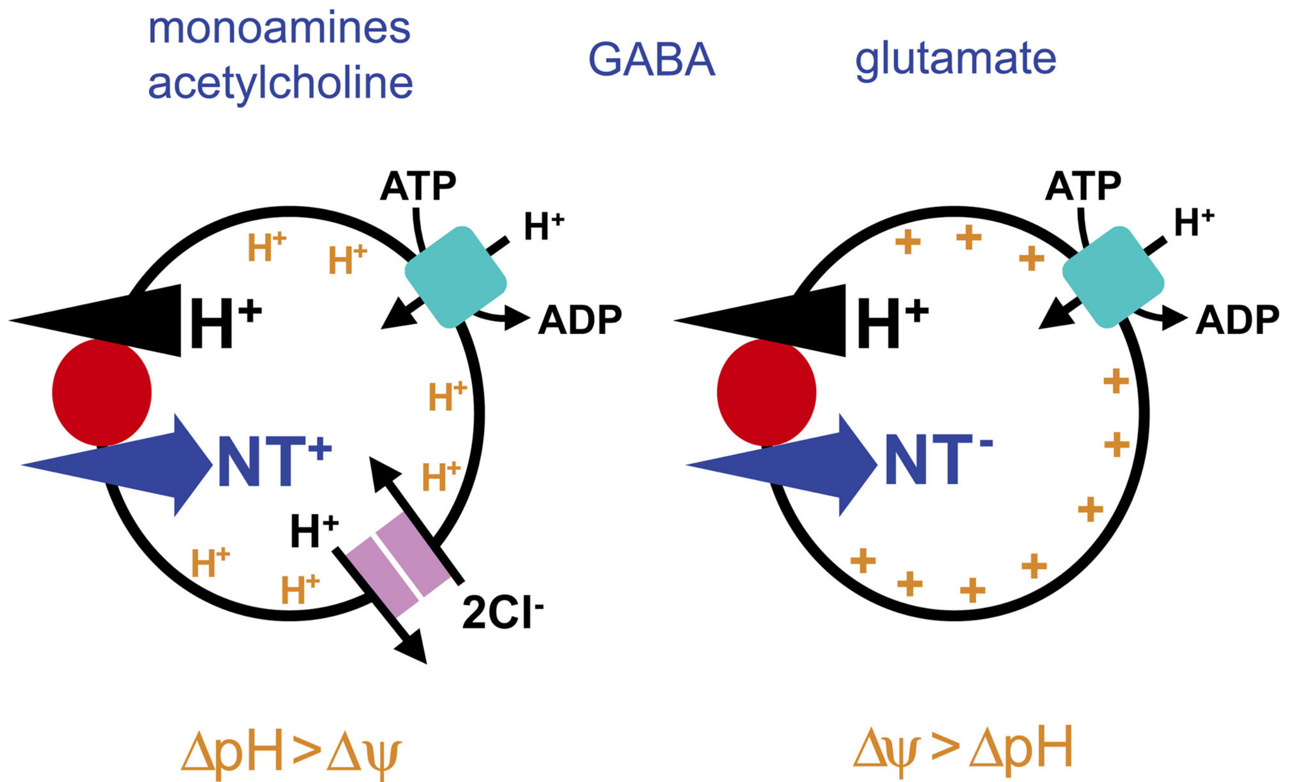

Figure 1. Vesicular neurotransmitter transporters depend differentially on the chemical and electrical components of the H^+ electrochemical gradient

The vacuolar-type H^+-ATPase generates the H^+ electrochemical gradient ($\mu_H{}^+$) required for transport of all classical neurotransmitters into synaptic vesicles. However, different vesicular neurotransmitter transporters rely to differing extents on the two components of $\mu_H{}^+$, the chemical gradient (pH) and the electrical gradient (ψ). The vesicular accumulation of monoamines and ACh (left) involves the exchange of protonated cytosolic transmitter for two lumenal H^+. The resulting movement of more H^+ than charge dictates a greater dependence on pH than ψ for both VAChT and VMAT. Vesicular glutamate transport (right) may not involve H^+ translocation. In the absence of ψ, however, disruption of pH inhibits uptake, suggesting that the transport of anionic glutamate involves exchange for nH^+, resulting in the movement of n + 1 charge and hence greater dependence on ψ than pH. 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 VGAT on pH and ψ. These differences suggest that vesicles storing monoamines or ACh may have mechanisms to favor the accumulation of pH at the expense of ψ, whereas those storing glutamate may promote a larger ψ. The extent to which vesicles differ in their expression of these two components remains unknown, but intracellular chloride carriers such as the synaptic vesicle-associated ClC-3 promote vesicle acidification by dissipating the positive ψ developed by the vacuolar H^+ pump, thereby disinhibiting the pump to make larger pH. The VGLUTs can also contribute to formation of pH because as an anion, glutamate entry similarly dissipates ψ to promote pH.

Interestingly, a Cl^- conductance associated with the VGLUTs may also promote acidification by Cl^-.

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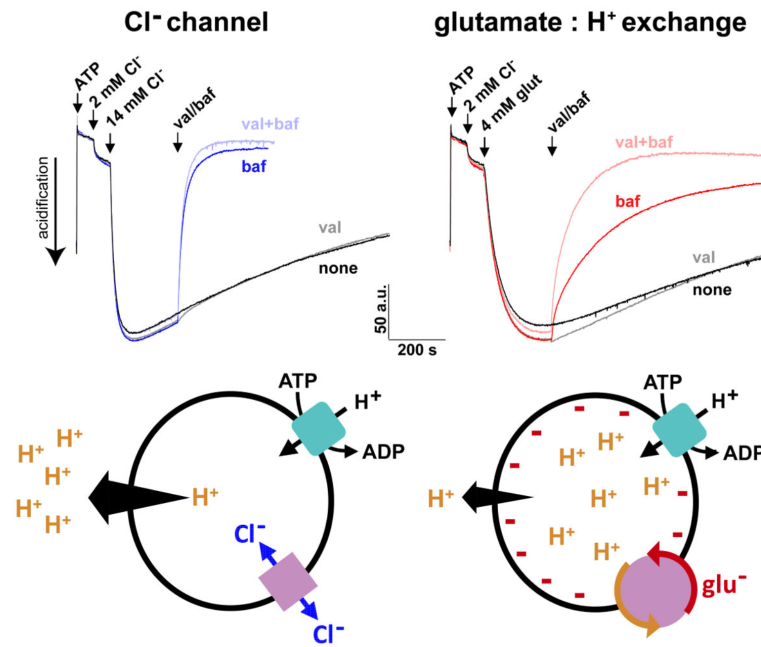

Figure 2. Glutamate flux produces larger and more stable changes in vesicular pH than chloride

Changes in pH 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 14 mM Cl^- (top left) or 4 mM glutamate (top right); more Cl^- is required to produce an equivalent initial change in pH. The traces in black indicate vesicles without any further addition. At the arrow, the K^+ ionophore valinomycin (50 nM, gray), the proton pump inhibitor bafilomycin (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 pH. Although increased buffering may contribute to the stabilization of pH by glutamate, valinomycin accelerates the bafilomycin-induced collapse in pH across membranes acidified with glutamate (pink) but not with Cl^- (light blue), indicating an important role for negative ψ in the stability of pH in glutamate-acidified vesicles. We hypothesize that the negative ψ developing upon H^+ efflux impedes further dissipation of pH. In the case of vesicles acidified with Cl^-, anion efflux through a channel (bottom left) would shunt the developing negative ψ, allowing the continued efflux of H^+ and rapid collapse of pH. In the case of vesicles acidified with glutamate, a H^+ : anion exchange mechanism (bottom right) would impede anion efflux because it would be coupled to the uphill movement of H^+ into acidic vesicles. Since glutamate efflux is disfavored, H^+ efflux is slow and pH more stable. Thus, the differences in mechanism of anion flux (channel versus H^+ exchange) confer differences in the stability of pH. Glutamate thus serves to ‘lock’ H^+, and hence cationic transmitters such as ACh and monoamines, inside secretory vesicles.