From glutamate co-release to vesicular synergy: vesicular glutamate transporters

Salah El Mestikawy[*‡], *Åsa Wallén-Mackenzie*[§], *Guillaume M. Fortin*[||], *Laurent Descarries*[¶] *and Louis-Eric Trudeau*[||]

Abstract | Recent data indicate that 'classical' neurotransmitters can also act as co-transmitters. This notion has been strengthened by the demonstration that three vesicular glutamate transporters (vesicular glutamate transporter 1 (VGLUT1), VGLUT2 and VGLUT3) are present in central monoamine, acetylcholine and GABA neurons, as well as in primarily glutamatergic neurons. Thus, intriguing questions are raised about the morphological and functional organization of neuronal systems endowed with such a dual signalling capacity. In addition to glutamate co-release, vesicular synergy — a process leading to enhanced packaging of the 'primary' transmitter — is increasingly recognized as a major property of the glutamatergic co-phenotype. The behavioural relevance of this co-phenotype is presently the focus of considerable interest.

*Institut National de la Santé et de la Recherche Médicale (INSERM), U952; Centre National de la Recherche Scientifique (CNRS) UMR 7224; Université Pierre et Marie Curie (UPMC), Paris 06, Pathophysiology of Central Nervous System Disorders, 9 quai Saint Bernard, 75005 Paris, France.
‡Douglas Hospital Research Center, Department of Psychiatry, McGill University, 6875 Boulevard LaSalle Verdun, Quebec, Canada, H4H 1R3.
§Department of Neuroscience, Unit of Developmental Genetics, Uppsala University, BOX 593, S-751 24, Sweden.
||Department of Pharmacology, Groupe de Recherche sur le Système Nerveux Central, Faculty of Medicine, Université de Montréal.
¶Departments of Pathology and Cell Biology and of Physiology, Groupe de Recherche sur le Système Nerveux Central, Faculty of Medicine, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Quebec, Canada, H3C 3J7.
Correspondence to L-E.T.
e-mail: louis-eric.trudeau@umontreal.ca
doi:10.1038/nrn2969
Corrected online 16 May 2011

Almost 80 years after the formulation of the hypothesis epitomized as Dale's principle (BOX 1), there are many reasons to think that the complexity of the chemical and anatomical organization of the mammalian nervous system is much greater than was previously imagined. It is becoming increasingly clear that most if not all neurons of the central and the peripheral nervous systems do not use a single chemical transmitter. Neuropeptides were long considered to be the most frequently co-localized transmitter in monoamine-, acetylcholine- (ACh) and GABA-containing neurons of the CNS. However, there is mounting evidence that many neuronal populations in the brain and spinal cord — hitherto defined as using a single, 'classical' transmitter plus or minus a neuropeptide — in fact use more than a single non-peptide transmitter.

The recent identification of proton-dependent carrier molecules, which transport glutamate from the cytosol into synaptic vesicles and thereby allow for the exocytotic release of glutamate, has renewed interest in the concept of neurons using multiple transmitters. The expression of a vesicular glutamate transporter (VGLUT) is not only used to formally identify neurons that are primarily glutamatergic but also suffices to assign a glutamatergic phenotype to neurons already known to use another transmitter. In this Review, we provide a brief historical overview of the discovery and functions of VGLUTs and discuss the increasing evidence for the presence of VGLUTs in 'non-glutamatergic' CNS neurons. We then consider cellular, physiological and behavioural implications of such a phenomenon, including glutamate co-release and enhanced packaging of the primary transmitter through a process called vesicular synergy.

Discovery of the VGLUTs

In 1994, Ni and colleagues isolated brain-specific Na^+-dependent inorganic phosphate co-transporter (BNPI), a 'brain-specific' protein that exhibited weak similarities with Na^+-dependent inorganic phosphate transporters[1]. It came as a surprise when 6 years later, two independent studies elegantly unravelled that BNPI was in fact a vesicular glutamate transporter[2,3]. Indeed, the heterologous transfection of this protein sufficed to endow GABA neurons with a glutamatergic phenotype[3]. BNPI was subsequently renamed vesicular glutamate transporter 1 (VGLUT1) and was shown to share all of the basic characteristics previously reported for glutamate accumulation by brain vesicles, including: first, millimolar affinity for glutamate; second, an inability to transport aspartate, glutamine or GABA; third, a dependence on the proton gradient; fourth, a dependence on the $\Delta\Psi$ component (vesicular transmembrane potential) of the pH gradient (and not the ΔpH); and fifth, biphasic chloride dependence (allosteric activation between 1 and 4 mM, inhibition above 10 mM)[4,5].

Box 1 | **Dale's principle**

Dale's principle is commonly quoted as stating that, "Neurons release only a single type of neurotransmitter at all of their synapses"[92,93]. Notwithstanding the fact that we know today that this is not the case and that many, if not most, neurons use more than a single transmitter, it is important to set the record straight and acknowledge what Sir Henry Hallet Dale (1875–1968), the famous Nobel laureate pharmacologist, actually said. In a lecture published in 1934, entitled 'Pharmacology and nerve endings'[94], Dale wrote:

"When we are dealing with two different endings of the same sensory neurone, the one peripheral and concerned with vaso-dilatation and the other at a central synapse, can we suppose that the discovery and identification of a chemical transmitter of axon-reflex vasodilatation would furnish a hint as to the nature of the transmission process at a central synapse? The possibility has at least some value as a stimulus to further experiment."

In reality, the statement was rather conservative and open-minded, and simply suggested that before considering a complicated hypothesis, one should start with the simplest one: that neurons perhaps release the same neurotransmitters from all of their axon terminals.

Interestingly, VGLUT1 was found to be strongly expressed by a subpopulation of neurons in the cerebral cortex, hippocampus and cerebellar cortex (FIG. 1a), with very little expression in subcortical regions; this suggested that in these subcortical regions, glutamate vesicular packaging is mediated by a second subtype of the transporter[6].

In 2000, another member of the Na^+-dependent inorganic phosphate transporters (named differentiation-associated Na^+-dependent inorganic phosphate co-transporter (DNPI)) was discovered by Aihara *et al.*[7]. DNPI shared a high degree of homology with BNPI (VGLUT1), and was strongly enriched in subcortical regions[7,8] (FIG. 1a). In 2002, five research groups reported that DNPI was also a vesicular glutamate transporter and renamed it VGLUT2 (REFS 9–13). Similar to VGLUT1, VGLUT2 translocates glutamate inside vesicles using an electrochemical proton gradient as the driving force[11] and is distributed in excitatory axon terminals that form asymmetrical synapses[11,14]. It was concluded from these early observations that VGLUTs are genuine markers of glutamatergic synapses. Moreover, the presence of a single type of VGLUT seemed to be sufficient to fill synaptic vesicles with glutamate[15].

In 2002, a third subtype of VGLUT — VGLUT3 — was discovered, which shared all structural and functional characteristics of the other two VGLUTs[16–19]. Unlike *Vglut1* and *Vglut2* transcripts, which were widely expressed in cortical and subcortical regions, respectively, the distribution of *Vglut3* mRNA was restricted to neuronal populations not previously considered as primarily glutamatergic: VGLUT3 was found in serotonin (5-hydroxytryptamine (5-HT)) neurons in raphe nuclei, ACh neurons in the dorsal and ventral striatum, and subclasses of GABA interneurons (basket cells) in the cerebral cortex and hippocampus[17,18,20,21] (FIG. 1a). In addition, VGLUT3 is present in subgroups of primarily glutamatergic neurons in the raphe, habenula, hypothalamus, olfactory tubercles and sensory inner hair cells of the cochlea[17,20,22–25].

Three main conclusions resulted from this initial phase of discovery and characterization. First, three VGLUTs ensure the vesicular uptake of glutamate in CNS neurons and thus represent unambiguous markers of glutamatergic transmission; second, VGLUT1 and VGLUT2 are expressed in neuronal populations already known to be glutamatergic; and third, VGLUT3, by contrast, is mostly expressed in neurons that were not initially identified as glutamatergic, indicating that glutamate may act as a co-transmitter in these cells. As described below, a broader examination of the distribution and functions of VGLUTs subsequently revealed that VGLUT1 and VGLUT2 can also be expressed in neurons not initially characterized as glutamatergic, thus raising the hypothesis that glutamate acts as a co-transmitter in many types of neurons (see BOXES 2,3 for a discussion of the role of VGLUTs as transporters of inorganic phosphate and chloride).

VGLUTs in 'non-glutamatergic' neurons

Abundant cytochemical data have demonstrated the expression of vesicular glutamate transporters in CNS neurons whose identity is defined by another 'primary' neurotransmitter (TABLE 1). Thus far, each of the three VGLUTs has been shown to be present in some ACh and GABA neurons of the CNS, but only VGLUT2 is expressed in some noradrenaline, adrenaline or dopamine neurons, and only VGLUT3 seems to be expressed in some 5-HT neurons (FIG. 1b).

VGLUT2 in dopamine neurons. Soon after the identification of VGLUT2, early studies combining *in situ* hybridization and immunocytochemistry revealed the existence of *Vglut2* mRNA in noradrenergic neurons of the A1 and A2 groups and in adrenergic neurons of the C1, C2 and C3 groups of the rat medulla, raising the possibility that glutamate might act as a co-transmitter in catecholamine neurons[26,27].

The first evidence that both *Vglut2* mRNA and VGLUT2 protein could be present in dopamine neurons came from a study combining single-cell reverse transcription PCR (RT-PCR) with tyrosine hydroxylase (TH) and VGLUT2 immunocytochemistry on isolated mesencephalic dopamine neurons from postnatal rat in microculture[28]. A subsequent single-cell RT-PCR study showed *Vglut2* mRNA to be present in mesencephalic dopamine neurons of both newborn (postnatal day 0 (P0)) and P45 mice, with a much higher yield at P0 (REF. 29), suggesting that *Vglut2* expression might be developmentally regulated. Subsequent analyses revealed that *Vglut2* mRNA is expressed in TH-expressing subpopulations of hypothalamic and ventral tegmental area (VTA) dopamine neurons, whereas those in the substantia nigra show barely detectable levels of *Vglut2* mRNA[30–32]. A dynamic regulation of *Vglut2* expression in these neurons has also been observed in a study that examined *Vglut2* and TH mRNA expression in the ventral mesencephalon of rats treated with 6-hydroxydopamine (6-OHDA)[33]. The abundance of *Vglut2* mRNA was increased in surviving dopamine neurons of these rats, suggesting an induction of *Vglut2* expression under pathological conditions or a negative regulation by dopamine[34]. Both in culture and *in vivo*, there are indications that *Vglut2*

Asymmetrical synapse
An asymmetrical synapse (or Gray type I synapse) contains predominantly round or spherical small synaptic vesicles and are characterized by a thickened postsynaptic density. Asymmetrical synapses are thought to be excitatory.

Tyrosine hydroxylase
The enzyme that converts tyrosine to dihydroxyphenylalanine (DOPA). This reaction is the rate-limiting step in the biosynthesis of catecholamines (dopamine, noradrenaline and adrenaline).

Microculture
A primary culture system that allows single-neuron cultures by growing neurons on microdroplets of growth substrate.

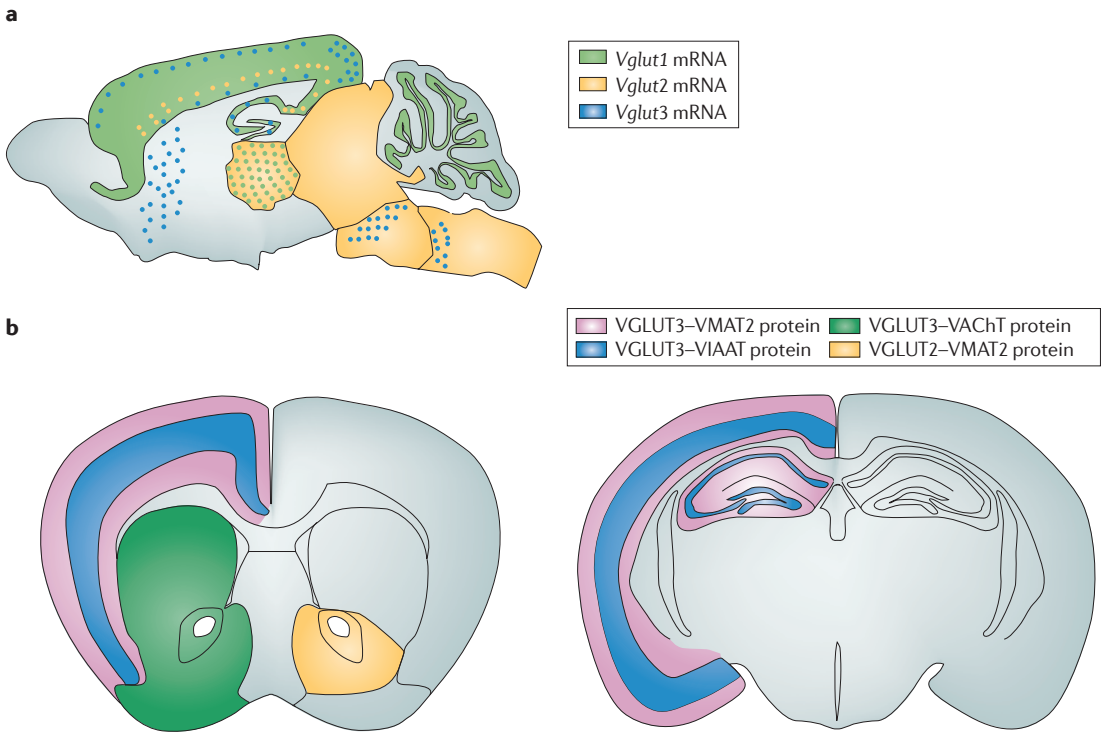


Figure 1 | **Distribution of vesicular glutamate transporters in the brain. a** | Schematic representation in a sagittal rat brain section of the anatomical distribution of mRNAs transcribed from the genes encoding the three vesicular glutamate transporters (VGLUTs). Vesicular glutamate transporter 1 (*Vglut1*) mRNA is found mostly in the cerebral cortex and hippocampus, and *Vglut2* mRNA is found mostly in subcortical structures, including the thalamus and the brain stem. *Vglut3* mRNA has a more restricted distribution in serotonin neurons of the midbrain raphe nuclei, acetylcholine neurons of the striatum and basal forebrain and a subset of GABA interneurons in the cerebral cortex and hippocampus. **b** | Schematic representation in coronal rat brain sections of the anatomical regions in which VGLUT2 or VGLUT3 proteins have been shown to be present in axon terminals containing dopamine (shown in yellow), serotonin (shown in pink), acetylcholine (shown in dark green) or GABA (shown in blue). Vesicular monoamine transporter 2 (VMAT2), vesicular acetylcholine transporter (VAChT) and vesicular inhibitory amino acid transporter (VIAAT) may also be used to visualize dopamine and serotonin, acetylcholine and GABA axon terminals, respectively. Vesicular synergy can theoretically occur in all brain regions indicated.

expression by mesencephalic dopamine neurons might be regulated through a contact-dependent interaction with GABA neurons and with other dopamine neurons[29]; in particular, contact with GABA neurons seems to act as a strong repressor of *Vglut2* expression in dopamine neurons, and lesioning striatal GABA neurons — which project to dopamine neurons — leads to an increase in the proportion of dopamine neurons expressing *Vglut2*.

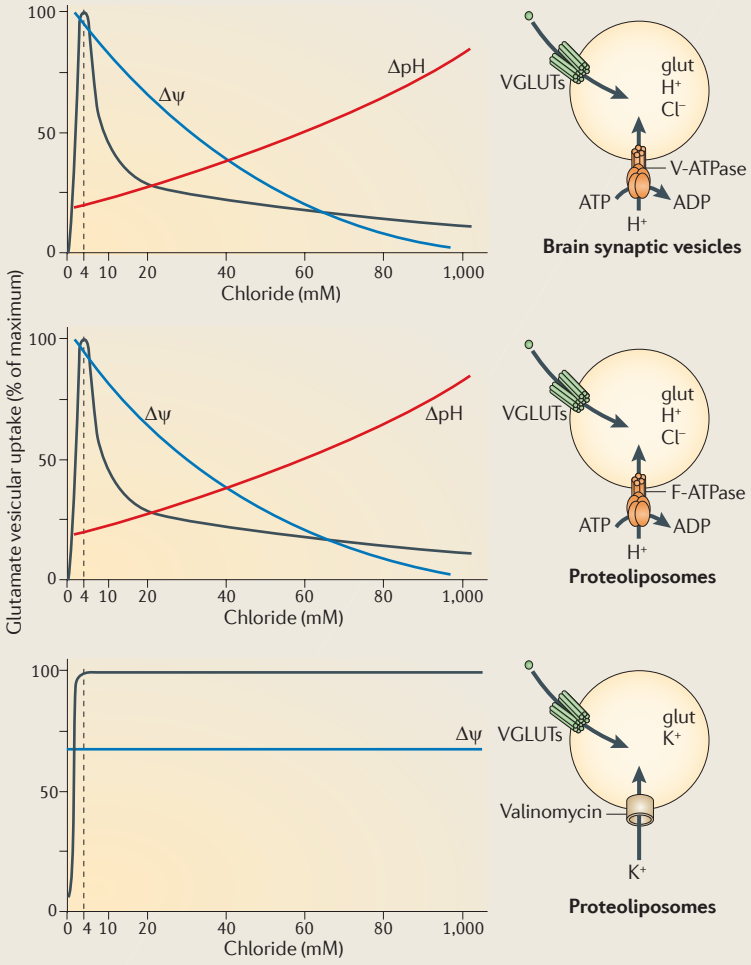
Recent immunoelectron microscopic studies in P15 rats showed that 28% of all TH immunopositive axon terminals in the nucleus accumbens, and 17% of those in the neostriatum, contain VGLUT2 (REF. 33). Interestingly, such double labelling was no longer found in adult rats (P90), which suggested an age-dependent regulation of VGLUT2 and TH co-expression[34,35], although it is also possible that VGLUT2 and TH are segregated in different branches and/or axon terminals at later ages.

VGLUT3 in serotonin neurons. In most parts of adult rat or mouse dorsal and median raphe nuclei, the vast majority (~80%) of 5-HT neurons express *Vglut3* mRNA[18,36], and VGLUT3 protein is present in the cell bodies and dendrites of these neurons[17,22,23,37–40]. Although midbrain 5-HT neurons project widely and diffusely to most parts of the forebrain (for example, REF. 41), VGLUT3 has thus far only been detected in subsets of 5-HT axon terminals, notably in the cerebral cortex[37,38,40] and hippocampus[18,37,40,42]. Results from triple immunofluorescence confocal microscopy studies suggested that terminals that are positive for both 5-HT and VGLUT3 rarely contain the 5-HT transporter (SERT), in contrast to those that apparently lack VGLUT3 (REFS 37,39). 5-HT axon terminals of the olfactory bulb[40], amygdala[40], ventral tegmental area[43], supra-ependymal plexus[37] and intermediolateral cell column of the rat spinal cord[44] also show VGLUT3 immunoreactivity.

VGLUTs in acetylcholine neurons. *Vglut1* and *Vglut2* mRNA are expressed in ACh (that is, choline-acetyltransferase (ChAT) immunopositive) motor neurons of the rat spinal cord[45], but VGLUT1 and VGLUT2 protein seem to be absent from the motor endplates in skeletal muscles[45–47]. VGLUT1 protein is, however, present in

Choline-acetyltransferase (ChAT). The enzyme that catalyses the synthesis of acetylcholine from acetyl-CoA and choline. One isoform of ChAT has been identified — this is a specific marker of cholinergic neurons.

Box 2 | **VGLUTs and chloride transport**



Chloride plays an intricate part in synaptic vesicle homeostasis and its implication in vesicle acidification has long been known[95]. The impact of Cl^- on vesicular glutamate (glut) accumulation was first established with crude preparations of brain vesicles. Naito and Ueda reported that the permeant anion Cl^- sharply stimulated vesicular glutamate uptake at low concentrations, whereas it had an inhibitory effect at higher concentrations[5]. How Cl^- regulates glutamate uptake is not totally clear. In brain synaptic vesicles, the steep stimulation of vesicular glutamate accumulation at low vesicular Cl^- concentrations (below 4 mM) has been proposed to be related to the presence of a positive allosteric regulatory binding site on the vesicular glutamate transporters (VGLUTs) (with a highly cooperative Hill coefficient of ≈3 (REFS 96,97)). The inhibitory effect of high vesicular Cl^- levels on glutamate accumulation could be due to a buffering role. Specifically, VGLUT bioenergetics depends mostly on the $\Delta\psi$ component of the $\Delta\mu H^+$ (see REF. 98 for a review), and in the absence of an intravesicular buffering anion the vacuolar-type proton ATPase (V-ATPase) will generate a strong positive potential ($\Delta\psi$; see the figure, shown by a blue line) and a small pH difference between the vesicular lumen and the cytoplasm (ΔpH; see the figure, shown by a red line). A high luminal concentration of Cl^- will buffer H^+ ions (generating HCl), allowing V-ATPase to accumulate more protons inside the vesicle. Consequently, a high Cl^- concentration decreases $\Delta\psi$ and increases ΔpH (for example, REF. 99). As $\Delta\psi$ is the main driving force for glutamate accumulation, high Cl^- concentrations would inhibit vesicular glutamate accumulation (see the figure, left panels).

The role of Cl^- in regulating glutamate accumulation raises the question of whether vesicles bear a Cl^- channel or transporter. The proteome of synaptic vesicles does not seem to include any Cl^- channels[100]. However, two independent groups reconstituted vesicular glutamate transporter 1 (VGLUT1) or VGLUT2 in proteoliposomes containing the bacterial F-ATPase (also known as ATP synthase holoenzyme (TFOF1)) as an energy donor[99,101] and quite convincingly established that in this *in vitro* system, VGLUTs indeed behave as Cl^- channels[99,101]. By contrast, a recent study using liposomes containing VGLUT2 and valynomicin (used to generate a K^+ driving force) exclusively, reported that VGLUT2 activity plateaued and did not decrease at high Cl^- concentrations[97], a finding that is incompatible with the hypothesis that VGLUT2 acts as a Cl^- channel. This conclusion was confirmed through the use of isotope tracing methods ([^{36}Cl] uptake) and Cl^- fluorescence probes (6-methoxy-*N*-(3-sulphopropyl)-quinolinum monohydrate) to directly measure Cl^- transport[97]. Thus, the data seem to indicate that VGLUTs do not function as chloride channels; the vesicular chloride channel therefore remains to be identified. Given these most recent data, a provocative and controversial suggestion emerges: perhaps ATP-dependent chloride transport (REF. 95) could be driven by the V-ATPase itself. One way to assess this new hypothesis would be to reconstitute highly purified V-ATPase in artificial liposomes and to assay them for [^{36}Cl] uptake. Indeed, when ATPases are absent (see the figure, right panels), the $\Delta\psi$ remains stable and VGLUT activity is no longer inhibited by high chloride concentrations.

What is the relevance of these considerations for vesicular synergy (BOX 4) and co-transmission? Concerning vesicular synergy, the buffering role of the anion could very well be played by chloride and/or glutamate. If this is the case, then chloride should be able to accelerate the vesicular accumulation of serotonin, acetylcholine, dopamine or GABA. However, recent evidence suggests that vesicular synergy is most probably operated by glutamate and not by chloride[79]. Thus, as far as we can judge today, glutamate acts both as a neurotransmitter and as a buffering anion in subpopulations of serotonin, acetylcholine, dopamine and GABA terminals.

Box 3 | **VGLUTs and Pi transport**

Studies in *Xenopus laevis* oocytes transfected with mRNA encoding vesicular glutamate transporters (VGLUTs) suggested that these proteins might also transport inorganic phosphate (Pi)[1,7]. Pi uptake via VGLUTs was reinvestigated in 2006, when Juge *et al.* reconstituted proteoliposomes with purified vesicular glutamate transporter 2 (VGLUT2) and bacterial F-ATPase (also known as ATP synthase holoenzyme (TFOF1)) (as the energy source)[99]. They detected — in addition to a $\Delta\psi$–driven glutamate vesicular accumulation — uptake of Pi when a Na^+ gradient was imposed to this simplified system. Surprisingly, the Pi transport in proteoliposomes was not affected by the presence of glutamate, VGLUT inhibitors (including Evans blue), the chloride concentration or mutagenesis of amino acids that are key to VGLUT2 function[99]. These results suggest either that the compact putative three-dimensional structure of VGLUTs bear two independent transport machineries (one for glutamate and one for Pi)[99,102] or that Na^+-dependent Pi transport is not an intrinsic property of the VGLUTs.

Of the three proposed functions of VGLUTs — glutamate transport, Cl^- transport and Pi transport — Pi transport is the least studied and the least firmly established. Additional experiments demonstrating that Na^+-dependent Pi uptake is an intrinsic property of VGLUTs are still awaited; reconstitution of VGLUTs alone in proteoliposomes may be required (REF. 97).

ACh terminals that innervate striated oesophageal muscle[46], and VGLUT2 in non-cholinergic axons that originate from spinal motor neurons and contact Renshaw cells[45]. Renshaw cells are also contacted by cholinergic collaterals from spinal motor neurons, a pathway involved in feedback regulation of motor circuitry. The absence of VGLUT protein in all axon terminals established by motor neurons that do express *Vglut1* or *Vglut2* transcripts has led to the suggestion that spinal motor neurons might give rise to separate sets of cholinergic and glutamatergic axon terminals, with the latter possibly involved in the regulation of motor functions[47] (however, see REF. 48 for conflicting results). VGLUT1 is also expressed in ACh axon terminals of the interpeduncular nucleus in mice, which may account for the co-release of glutamate and ACh upon optogenetic stimulation of medial habenula neurons projecting to this nucleus[49].

In adult mouse and rat brain, *Vglut3* mRNA is expressed in ACh interneurons of dorsal and ventral striatum[17,18], and some of the nucleus basalis ACh neurons projecting to the basolateral amygdala[50]. In ACh interneurons, VGLUT3 protein seems to be targeted to the soma and proximal dendrites[16,51], as well as to the majority of, if not all, axon terminals[17,18,51]. The role of VGLUT3 in the somatodendritic compartment of these neurons remains to be elucidated.

VGLUTs in GABA neurons. Neurons in several brain regions have been shown to express, seemingly paradoxically, both the inhibitory transmitter GABA and VGLUTs. For example, in cat retina, a subset of cone bipolar cells (and their terminals) contains VGLUT1 as well as GABA, its synthetic enzyme glutamate decarboxylase 2 (GAD65) and the vesicular inhibitory amino acid transporter (VIAAT)[52]. There are also GABAergic axon terminals containing VGLUT1, VGLUT2 or VGLUT3 in developing and/or adult rat cerebral cortex and/or hippocampus[20,38,51,53–56]. *Vglut2* is expressed by some GABA neurons in the ventral tegmental area[31], and in adult female rats *Vglut2* mRNA and VGLUT2 protein have been detected in GABA neurons of the hypothalamic anteroventral periventricular nucleus and in their terminals (in the rostral preoptic area), respectively[57]. During early postnatal development, *Vglut3* mRNA and VGLUT3 protein are present in Purkinje cells and terminals around their cell body, respectively[58], as well as in neurons of the nucleus of the trapezoid body and their immature synapses that are both GABAergic and glycinergic (GABA/glycine synapses), in the lateral superior olive (LSO)[59]. In adult rat brain, VGLUT3 is found in cholecystokinin-positive GABAergic basket cells in the cerebral cortex and hippocampus[21], and preprotachykinin B-producing GABAergic interneurons in the neocortex[38].

The glutamatergic co-phenotype

The growing evidence for the coexistence of VGLUTs in monoamine, ACh and GABA neurons raises fundamental issues regarding the structural and functional properties of neurons endowed with such a dual phenotype. A first and obvious question is whether glutamate is actually released by these neurons *in vivo*. Then, assuming it is, a second question is whether such glutamate release occurs at the same axon terminals that release the primary neurotransmitter (FIG. 2). Thirdly, if both glutamate and the primary transmitter are released from the same terminals, are the vesicular transporters for both transmitters present in the same or in different synaptic vesicles (FIG. 2)? Moreover, what are the mechanisms of VGLUT sorting and trafficking that are required to explain the fact that axon terminals can contain heterogeneous pools of synaptic vesicles with and without VGLUTs, as well as heterogeneous populations of axon terminals with and without VGLUT-containing vesicles? Lastly, when a VGLUT is present in synaptic vesicles that also contain a vesicular transporter for another transmitter, does this have any impact on the vesicular loading of this transmitter? These questions are discussed below.

Functional properties of the glutamatergic co-phenotype of dopamine neurons. Patch-clamp recordings of isolated rat mesencephalic dopamine neurons in microculture provided the first evidence that CNS dopamine neurons could form axon terminals capable of releasing glutamate[60,61]. The short latency and rapid rise time of the recorded responses were strongly suggestive of synaptic contacts, even though in adult rats *in vivo*, these neurons release dopamine mainly from axon terminals that do not form morphologically-defined synaptic membrane specializations[62]. Interestingly, in the nucleus accumbens of P15 rats, all dopamine terminals doubly immunolabelled for TH and VGLUT2 were indeed found to make a synaptic junction, as opposed to those labelled for TH only[33,34]. Recent studies in adult mice have provided unequivocal evidence for the release of glutamate by VTA dopamine neurons *in vivo*[63,64]. These studies demonstrated that optogenetic activation of axon terminals originating from VTA dopamine neurons induced AMPA receptor-mediated glutamatergic fast synaptic responses in nucleus accumbens neurons. Furthermore, targeted deletion of *Vglut2* in the mesencephalic dopamine neurons completely abolished such light-dependent excitatory postsynaptic potentials[64].

Renshaw cell
A GABAergic interneuron found in the ventral horn of the spinal cord. Renshaw cells form and receive excitatory recurrent collaterals from, and send inhibitory synapses on to, spinal motor neurons.

Optogenetics
The use of genetically encoded light-activated proteins (for example, ion channels) to control functional parameters (for example, the membrane potential) of targeted neuronal populations.

Vesicular inhibitory amino acid transporter
(VIAAT; also known as VGAT). A proton-dependent vesicular transporter that accumulates the inhibitory transmitters GABA and glycine into synaptic vesicles.

Table 1 | **Anatomical distribution of transmitter-defined neurons containing a VGLUT in the CNS**

Neuronal type	mRNA expression	Protein localization	
		Cell bodies	Terminals
***Vglut1*/VGLUT1**			
Acetylcholine	Spinal cord motor neurons[45]		Nucleus interpeduncularis[49]
GABA		Retina[52]	Cerebral cortex[54], hippocampus[56], cerebellar cortex[35,56], retina[52]
***Vglut2*/VGLUT2**			
Noradrenaline	Medulla A1, A2 cell groups[26], area prostrema[26]		
Adrenaline	Medulla C1 cell group[26,27], medulla C2, C3 cell group[26]		
Dopamine	Medulla A9, A10 cell groups[29–31,33,34], medulla A11 cell group[30]		Nucleus accumbens[33–35], neostriatum[33–35]
Acetylcholine	Spinal cord motor neurons[45,47]		Spinal cord[47]
GABA	AVPV[57]		rPOA[57], hippocampus[53,55,56]
***Vglut3*/VGLUT3**			
Serotonin	DRN, MRN[18,36]	DRN[17,22,23,37,39,40], MRN[17,23,37,39,40]	Cerebral cortex[37,38,40], hippocampus[21,37,40,42], olfactory bulb[40] amygdala[40], VTA[43], supra-ependymal plexus[37], spinal cord (IML)[44]
Acetylcholine	Striatum[17,18], basal forebrain[50]	Striatum[16,51], basal forebrain[50]	Striatum[17,18,51], amygdala[50]
GABA	Hippocampus[45]	Cerebral cortex[38], hippocampus[16,21], Purkinje cells[58], nucleus of the trapezoid body[59]	Cerebral cortex[38], hippocampus[20,51], Purkinje cell layer[58], superior olive[59]

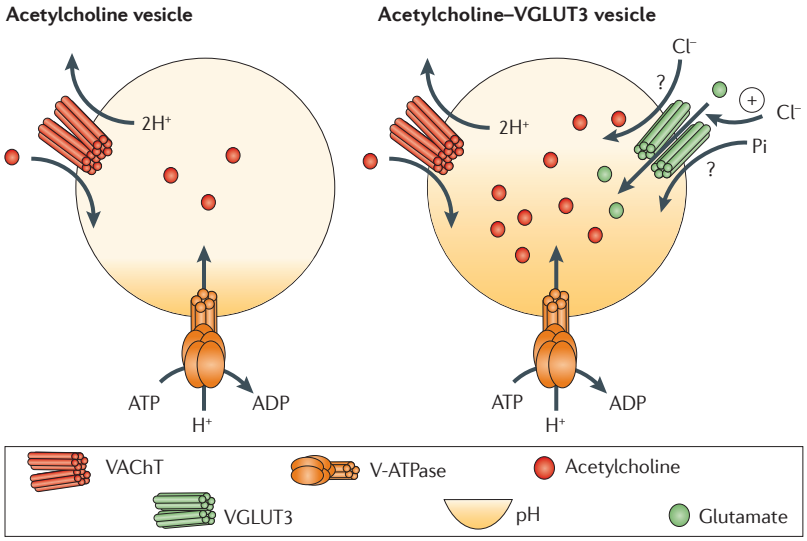
Data are mostly from rat or mouse. Transmitter identity of CNS neurons defined by double labelling of a vesicular glutamate receptor (*Vglut*) mRNA or VGLUT protein together with an mRNA or protein for transmitter, biosynthetic enzyme, plasma membrane transporter or specific vesicular transporter. AVPV, anteroventral periventricular nucleus; DRN, dorsal raphe nucleus; IML, intermediolateral cell column; MRN, median raphe nucleus; rPOA, rostral preoptic area; VTA, ventral tegmental area.

The question arises as to whether such glutamate release from dopamine neurons occurs at the same axon terminals that release dopamine. Immunolabelling studies performed in cultured rat dopamine neurons before the identification of VGLUTs showed that the vast majority of axon varicosities formed by dopamine neurons in single neuron microculture were double labelled for TH and glutamate, suggesting that most terminals have the capacity to release both transmitters[60] (see also REFS 62,65 for early ultrastructural data suggesting that dopamine neurons establish two morphologically distinct subsets of axon terminals). Close examination of VGLUT2 immunoreactivity *in vitro*[28] and *in vivo*[66], however, suggests that dopamine neurons possess different subsets of axon terminals — perhaps as many as three: a first that contains only TH; a second, perhaps smaller, subset containing both TH and VGLUT2; and a third subset containing VGLUT2 but not TH (FIG. 2). The possibility that TH might be absent from some axonal branches and/or axon terminals of dopamine neurons may come as a surprise in light of the fact that such biosynthetic enzymes are known to be mostly cytosolic. However, there is some evidence that TH may be membrane bound even to synaptic vesicles, notably in the striatum[67], and it may therefore be unevenly distributed among axon terminals. Indeed, isolated dopamine neurons in culture have a large number of VGLUT2 immunopositive, TH immunonegative varicosities, confirmed as axon terminals by the presence of synaptic vesicle 2-related protein (SV2)[28]. A recent study has suggested that VGLUT2 is only found in a small subset of TH- and synaptic vesicular amine transporter (VMAT2)-containing axon terminals in cultured dopamine neurons[68]. The notion of a phenotypical heterogeneity among axon terminals of dopamine neurons might also explain why in adult rats (as opposed to P15 rats), axon terminals double labelled for TH and VGLUT2 are no longer observed in the ventral or dorsal striatum[34]. This is even the case after a partial 6-OHDA lesioning of the mesencephalic dopamine neurons, which is known to activate expression of *Vglut2* and increase the co-localization of TH and VGLUT2 in the dopamine axon terminals of postnatal rats[34]. These results now need to be re-evaluated by quantifying the co-localization of VGLUT2 with other dopaminergic markers such as the sodium-dependent dopamine transporter (DAT) and VMAT2.

Functional properties of the glutamatergic co-phenotype of 5-HT neurons. An early electrophysiological study demonstrated that isolated 5-HT neurons in culture can show fast AMPA receptor-mediated synaptic currents[69].

Dopamine transporter
A plasma membrane protein from the family of Na^+- and Cl^--dependent transporters. It efficiently takes dopamine up from the extracellular space into neurons (affinity 10^{-7} M) using energy based on the Na^+ gradient generated by the Na^+/K^+ ATPase.

Box 4 | **Molecular mechanisms of vesicular synergy**



Neurotransmitter vesicular uptake is driven by an electrochemical gradient of protons ($\Delta\mu H^+$) across the membrane, which is established by the vacuolar type proton ATPase (V-ATPase; see the figure)[103]. Vesicular packaging of cationic transmitters such as acetylcholine and dopamine through the vesicular acetylcholine transporter (VAChT; see the figure, shown in red) and the vesicular monoamine transporter (VMAT2; not shown), respectively, largely depends on the pH gradient across the vesicular membrane (ΔpH). By contrast, the function of vesicular glutamate transporters (VGLUTs) is largely driven by the vesicular transmembrane potential ($\Delta\Psi$) component of the proton gradient[98]. GABA and glycine accumulation by the vesicular inhibitory amino acid transporter (VIAAT) depends on both ΔpH and $\Delta\Psi$[98].

The kinetic properties of VMAT2 and VAChT are such that to accumulate one molecule of monoamine, 2 protons are extruded from the vesicle (see the figure, left panel). This has the consequence that VMAT2 and VAChT rapidly dissipate the ΔpH. Studies using the pH-sensitive dye acridine orange have shown that glutamate and chloride acidify synaptic vesicles through distinct and additive mechanisms[3,80,104]. It can thus be proposed that the presence of a VGLUT results in more acidified vesicles, and this enables VMAT2 and VAChT to accumulate higher amounts of their respective neurotransmitter in these vesicles[79]. Interestingly, despite its partial dependence on ΔpH, vesicular accumulation of GABA by VIAAT is also accelerated by glutamate[56]. The VGLUT-dependent increased vesicular accumulation of serotonin, acetylcholine, dopamine or GABA is known as vesicular synergy.

We suggest that vesicular synergy is fulfilled by glutamate itself[79] (BOX 2). However, a possible role for other anions putatively transported by VGLUTs — Cl^- or Pi — may be involved (BOXES 2,3). In addition, low concentrations of Cl^- could have an indirect effect on vesicular synergy through its allosteric regulation of VGLUTs.

Vesicular monoamine transporters
(VMATs). Synaptic vesicle proteins that translocate monoamines (dopamine, noradrenaline, 5-HT and histamine) from the cytoplasm into vesicles. The driving force is the proton gradient generated by the vacuolar-type proton ATPase (V-ATPase). Two isoforms have been cloned, *VMAT1* in the periphery and *VMAT2* in the CNS. VMATs belong to a large family of sugar transporters that also includes the vesicular acetylcholine transporter (VAChT).

Tryptophan hydroxylase
(TPH). The rate-limiting enzyme for the biosynthesis of serotonin (5-hydroxytryptamine (5-HT)). TPHs convert tryptophan to 5-hydroxytryptophan. Two *TPH* genes have been identified in mammals: *TPH1* is expressed in the periphery and *TPH2* in raphe nuclei.

In a recent study, optogenetic stimulation of neuron axonal fibres arising from mouse raphe nuclei[42] triggered short-latency AMPA receptor-mediated synaptic responses in GABAergic interneurons in the hippocampus. Interestingly, in many neurons, the fast synaptic response was partially reduced by a 5-HT_3 ionotropic receptor antagonist (albeit at a relatively high dose)[42], suggesting that glutamate and 5-HT mediate synaptic excitation in the same time frame. By contrast, optical stimulation evoked slow postsynaptic-like responses (rise time slower than 100 ms) in a subset of CA1 hippocampal pyramidal neurons, and these were reduced by a metabotropic 5-HT_{1A} receptor antagonist[42] (see also REF. 69 for a similar and earlier finding *in vitro*). A caveat of this study was that channelrhodopsin 2 was not expressed in raphe neurons under the control of a 5-HT neuron-selective promoter, thus making it possible that VGLUT3-expressing non-5-HT neurons[23,36,38] were also optically stimulated. Nonetheless, the finding strongly suggests that 5-HT neurons can co-release glutamate and suggests the intriguing possibility that such co-release can lead to the encoding of parallel signals that operate along different timescales. Such dual synaptic responses — with a fast, ionotropic component and a slower, metabotropic component — have not yet been described in response to activation of dopamine or ACh neurons.

In isolated cultured postnatal rat 5-HT neurons, a majority of 5-HT immunoreactive axon varicosities contain VGLUT3, a substantial subset contains only 5-HT and a small subset contains VGLUT3 but not 5-HT[16]. Similarly, in embryonic rat midbrain raphe cultures, some tryptophan hydroxylase (TPH) immunopositive varicosities are VGLUT3 immunopositive[18]. Axon terminals that are immunopositive for both VGLUT3 and 5-HT have also been reported in the VTA and substantia nigra (pars compacta)[67,70]; however, the vast majority of 5-HT varicosities in these areas seem to be VGLUT3 immunonegative[67], suggesting segregation of the co-transmitters to different axonal branches (FIG. 2). By contrast, in the limbic cortex, hippocampus and lateral septum, 30–50% of 5-HT immunoreactive terminals contain VGLUT3 and VMAT2, the rest being VGLUT3 immunonegative[37] (FIG. 2). Taken together, these findings are consistent with axon terminal heterogeneity, as already suggested for dopamine neurons.

Functional properties of the glutamatergic co-phenotype of ACh neurons. Cholinergic neurons of the medial septum in microculture can release glutamate

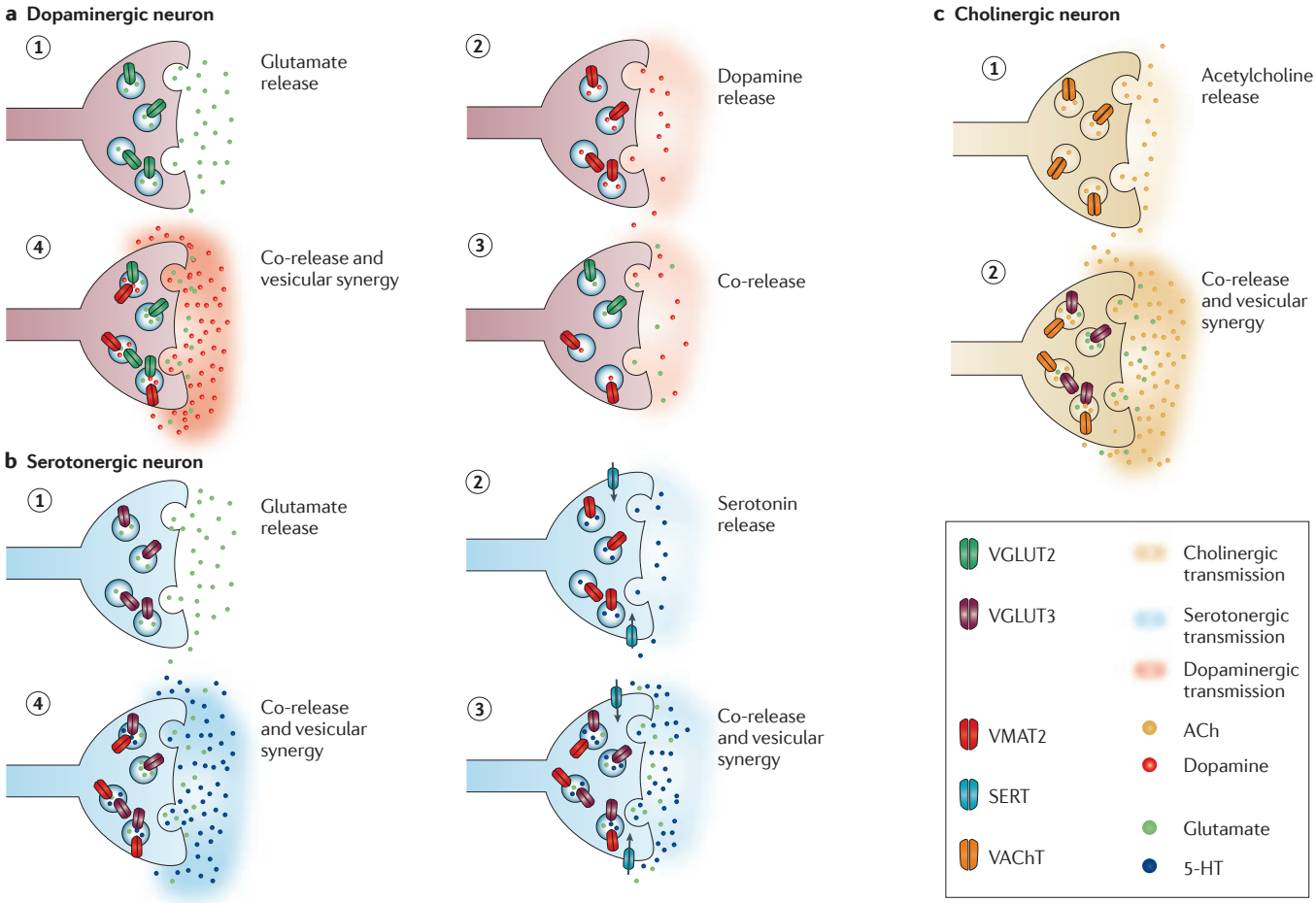


Figure 2 | **Heterogeneity of terminals co-expressing VGLUTs and other vesicular neurotransmitter carriers.** Schematic representation of the possible modes of co-transmission in dopamine, serotonin (5-hydroxytryptamine (5-HT)) and acetylcholine (Ach) neurons. **a** | For dopamine neurons, terminals that contain only vesicular glutamate transporter 2 (VGLUT2) and that only release glutamate (**1**), terminals expressing vesicular monoamine transporter 2 (VMAT2) alone (**2**), terminals with both types of vesicular transporter, expressed in separate pools of synaptic vesicles (**3**) and terminals with synaptic vesicles that contain both VMAT2 and VGLUT2 (**4**) are shown. We propose that vesicular synergy only occurs at type **4** terminals, leading to enhanced extracellular accumulation of dopamine following its exocytotic release. **b** | For 5-HT neurons, terminals that contain only VGLUT3 and that only release glutamate (**1**), terminals expressing VMAT2 and 5-HT transporter (SERT) (**2**), terminals with synaptic vesicles that contain both VMAT2 and VGLUT3, in addition to the plasma membrane transporter SERT (**3**) and terminals with synaptic vesicles that contain both VMAT2 and VGLUT3, but without a plasma membrane transporter (**4**) are shown. We propose that vesicular synergy only occurs at type **3** and **4** terminals, and that the absence of SERT leads to further enhancement of extracellular 5-HT following its exocytotic release. **c** | For ACh neurons, terminals expressing VAChT alone (**1**) and terminals with synaptic vesicles that contain both vesicular ACh transporter (VAChT) and VGLUT3 (**2**) are shown. We propose that vesicular synergy only occurs at type **2** terminals, leading to enhanced extracellular accumulation of ACh following its exocytotic release. Thus, the various combinations of vesicular and plasma membrane transporters could determine the strength of dopamine, 5-HT and ACh neurotransmission. Figure is modified, with permission, from REF. 37 © (2010) Society for Neuroscience.

Autaptic connection
A synaptic contact established by a neuron onto its own dendrites or cell body.

Non-synaptic axon terminal
An axon terminal (varicosity) that displays no morphologically identifiable synaptic membrane specialization (junctional complex). Also referred to as an asynaptic terminal or free nerve ending.

Vesicular acetylcholine transporters
(VAChTs). Synaptic vesicle proteins mediating the accumulation of acetylcholine into secretory vesicles. VAChTs use the proton gradient generated by the vacuolar-type proton ATPase (V-ATPase) as the driving force.

from autaptic connections[71], in keeping with the previous demonstration of *Vglut1* or *Vglut2* expression in a subset of these neurons[72]. Interestingly, numerous neurons displayed mixed synaptic responses, with both AMPA receptor-mediated and nicotinic receptor-mediated components, which is suggestive of co-release of the two transmitters[71]. Optogenetic stimulation of ACh neurons of the medial habenula projecting to the interpeduncular nucleus evoked fast, glutamate-mediated synaptic responses in neurons of this region[49]. Interestingly, stimulus trains at 20–50 Hz evoked slow nicotinic receptor-mediated postsynaptic inward current (15 s rise time) in target neurons[49]. This finding provides strong evidence for the hypothesis that glutamate co-release from cholinergic neurons mediates fast signals to target neurons, whereas release of ACh — presumably from non-synaptic axon terminals[73,74] — mediates excitatory signals on a much slower timescale.

Glutamate release from striatal ACh interneurons has not yet been demonstrated. However, dual immunolabelling for both the vesicular acetylcholine transporter

(VAChT) and VGLUT3 has demonstrated that the vast majority of neostriatal ACh axon varicosities (terminals) do contain VGLUT3 (REF. 17). Thus, in contrast to dopamine and 5-HT neurons, striatal ACh interneurons seem to show little, if any, segregation of their two vesicular transporters in distinct terminals (FIG. 2).

Functional properties of the glutamatergic co-phenotype of GABA neurons. Although a recent study provided strong evidence for *in vivo* release of GABA from glutamatergic hippocampal granule neurons[75], glutamate release by hippocampal GABA neurons remains to be demonstrated. Glutamate release from immature VGLUT3-positive GABA/glycine synapses has, however, been reported in the auditory system[59,76]. The early developmental expression of *Vglut3* in some GABA neurons and the fact that VGLUT3 endows GABA/glycine synapses with the ability to release glutamate and to stimulate NMDA receptors[59,76] suggests that glutamate release might have a role in the functional maturation of the GABAergic/glycinergic circuitry. Indeed, VGLUT3-mediated glutamate release in GABA/glycine neurons contributes to a refinement of the synaptic connections in the auditory system[76].

Sorting of VGLUTs. Co-transmission in the strictest sense implies that two neurotransmitters are released at the same time from a common pool of synaptic vesicles within one axon terminal. A few examples of such co-release have already been documented, such as the release of glycine and GABA at synapses in the spinal cord[77]. Co-release of two transmitters from the same vesicle is quite difficult to demonstrate conclusively and would imply that some vesicles contain vesicular transporters for two different neurotransmitters. A number of recent studies have used synaptic vesicle immunopurification (using paramagnetic beads coupled to vesicular transporter antibodies) to show that this can indeed occur (FIG. 2). For example, VGLUT1 and VGLUT2 are co-expressed in the same vesicles in the hippocampus[78], as are VIAAT and VGLUT1 in cortical neurons[54], VAChT and VGLUT3 in striatal ACh interneurons[79], VAChT and VGLUT1 in the interpeduncular nucleus[49], and VMAT2 and VGLUT2 in striatal axon terminals[80]. Furthermore, VIAAT and VGLUT2 can be found on the same vesicle subset in vesicles purified from whole brain[56]. By contrast, an immunoelectron microscopy study showed that in terminals of the dentate gyrus of the rat hippocampus, VIAAT and VGLUT2 are co-expressed but localized to different subsets of vesicles[53]. It is thus possible that transporter co-localization differs regionally. Whether it is also developmentally regulated is a question that remains to be explored.

How vesicular transporters are segregated in different populations of vesicles and axon terminals is an important area for future research. Neuronal synaptic proteins are thought to be synthesized in the membrane of the rough endoplasmic reticulum, followed by trafficking through the Golgi complex. Newly synthesized vesicular proteins such as VGLUTs can then be shipped to the appropriate location — mainly axon terminals — by trafficking either through constitutive or regulated secretory vesicles (see REFS 81,82 for recent reviews). A specific synaptic vesicle trafficking motif has been identified in a VMAT orthologue in *Drosophila* spp., however, corresponding sorting domains in VGLUTs have been more elusive[83]. Although direct data are presently lacking, it has been proposed that — similar to VMAT2 — VGLUT3 might be sorted to regulated secretory vesicles to reach both the somatodentritic and axonal compartments of neurons[81].

Most studies documenting the presence of VGLUTs and another vesicular transporter on the same vesicles used immunopurification coupled to western blot analyses. However, the lack of resolution of these biochemical methods does not allow a quantification of the percentage of co-localization or the identification of differences in expression between vesicles. Being able to answer such questions would increase our understanding of the nature and functions of co-transmission. For example, axon terminals are known to contain two functionally distinct pools of vesicles, known as the reserve pool and the readily releasable pool[84]; could vesicles from these two pools be molecularly heterogeneous, with only one of two pools containing vesicles with two types of vesicular transporters? A better understanding of the sorting of VGLUTs and other vesicular carriers to various vesicular pools, as well as of the dynamics of vesicular protein content, may come from the use in coming years of high-resolution microscopy techniques such as stimulated emission depletion (STED) microscopy and total internal reflection fluorescence (TIRF) microscopy[85].

Vesicular synergy. In addition to enabling the co-release of glutamate, there is increasing evidence that the presence of a VGLUT in axon terminals may have other important functional consequences. For example, it may result in increased filling of synaptic vesicles with the primary transmitter, and hence, increased release of this transmitter. This has been demonstrated for VGLUT3 in ACh and 5-HT neurons[37,79], and has been suggested for VGLUT2 in dopamine neurons[80] and GABA neurons[56], but remains unexplored in other systems.

One study showed that in wild-type mice, VGLUT3 and VAChT were located in a common pool of vesicles and that vesicular co-accumulation of glutamate resulted in increased ACh filling of vesicles[79]. The functional significance of this was indicated by the hypocholinergic phenotype of *Vglut3* knockout mice (*Vglut3*$^{-/-}$ mice)[79] as well as their increased basal and cocaine-stimulated locomotor activity[79]. Such cooperation between two vesicular transporters was termed vesicular synergy[37,79] (BOX 4).

Experiments in *Vglut3*$^{-/-}$ mice also demonstrated that vesicular synergy between VGLUT3 and VMAT2 occurs in 5-HT terminals of the hippocampus and prelimbic cortex[40]. Here, glutamate promoted the packaging of 5-HT in synaptic vesicles of VGLUT3 immunoreactive terminals[40]. Interestingly, the 5-HT terminals that also contained VGLUT3 were immunonegative for the 5-HT reuptake transporter SERT[40], suggesting that these terminals might have the capacity to locally

Stimulated emission depletion (STED) microscopy
A high-resolution fluorescence microscopy technique that takes advantage of de-excitation of fluorescent dyes to partly overcome the resolution limit imposed by diffraction.

Total internal reflection fluorescence (TIRF) microscopy
A high-resolution fluorescence microscopy technique that takes advantage of a laser-induced evanescent wave of fluorescence emission very close to the interface of two media that have different refractive indices.

deliver a strong and prolonged 5-HT signal through both enhanced release (through vesicular synergy) and reduced reuptake (FIG. 2). These results suggest that the strength of 5-HT transmission might vary greatly, both regionally and temporally, according to the combination of vesicular and membrane transporters present in a given 5-HT terminal. It is noteworthy that *Vglut3*$^{-/-}$ mice also displayed increased anxiety-like behaviour[37], whereas other behaviours regulated by 5-HT transmission, such as aggression and depression-like responses, were unaltered[40], suggesting a specific role for glutamate in limbic areas and in anxiety-related behaviours.

The mechanism underlying vesicular synergy between glutamate and other co-transmitters probably involves VGLUT-dependent acidification of the vesicular lumen[79,80]. However, which one of the 3 substrates of VGLUTs (glutamate, chloride or Pi) is the actual buffering anion used to increase vesicular accumulation of 5-HT, ACh, dopamine or GABA is not yet clearly established (BOXES 2,3,4).

A role for VGLUTs in reward?

Little is presently known about the behavioural effects of glutamate co-transmission or of expression of a VGLUT in monoamine and ACh neurons. Conditional deletion of a *Vglut* in specific neuron subsets would enable one to gain insight into this important question, but so far this has only been achieved in dopamine neurons.

Based on the previously mentioned expression of *Vglut2* in dopamine neurons of the medial A10 area and the rostral linear nucleus[30] — regions shown to project to the nucleus accumbens[86] — and on electrophysiological recordings showing glutamatergic transmission in mesoaccumbal slice preparations[87], it has been suggested that VGLUT2-mediated glutamate co-transmission might have a role in reward-relevant pathways. A first evaluation of this question has been performed in mice in which *Vglut2* was specifically deleted in dopamine neurons (*Vglut2*$^{f/f;DAT\text{–}Cre}$ mice). There are two *Vglut2*$^{f/f;DAT\text{–}Cre}$ lines, which differ both with regard to the floxed *Vglut2* allele and to the DAT-Cre line used[80,88].

The first *Vglut2*$^{f/f;DAT\text{–}Cre}$ mice showed normal basal cognitive and locomotor functions[88]. In response to an acute challenge with low dose amphetamine (which triggers dopamine release), the conditional knockout mice showed reduced locomotor response compared to littermate controls. However, with increasing doses of amphetamine, they displayed increased locomotion and rearing[88]. In the other line of *Vglut2*$^{f/f;DAT\text{–}Cre}$ mice, cocaine (which blocks dopamine reuptake) induced a smaller increase in locomotion compared with controls[80]. As these studies administered different psychostimulants, a unified picture of how the absence of VGLUT2 influences psychostimulant-induced locomotion has not yet emerged.

Does VGLUT expression in dopamine neurons have a role in reward learning? A first attempt to address this question used a conditioned place preference paradigm[80]. This study did not detect any differences between *Vglut2*$^{f/f;DAT\text{–}Cre}$ and control mice, which led the authors to suggest that at least some forms of reward and associative learning remain intact in the absence of VGLUT2. Further analyses are required to evaluate other aspects of the reward system — for example, drug self-administration paradigms could provide a measure of goal-directed responding and be utilized to discriminate between the reinforcing effects of a substance and the motivation to consume it. Thus, it is clear that more research is needed to address the possible role of VGLUT2-mediated co-transmission in the reward circuitry and in reward-related behaviour.

Conclusions and future directions

The time is now ripe to envisage new models of transmitter signalling in the CNS, taking into account that subpopulations of neurons use glutamate as a co-transmitter and/or as a synergistic enhancer of vesicular packaging and release. It may even be time to revisit the concept that neurons use a particular transmitter as a 'primary' transmitter and another as a 'secondary' transmitter, especially if the co-transmitter phenotype varies during development or in response to physiological signals and injury. At the very least, four key issues still need to be tackled.

The first issue involves the fate and role of glutamate released from the axon terminals of dopamine, 5-HT, ACh or GABA neurons. Optogenetic studies have already demonstrated that glutamate released from such terminals can activate ionotropic glutamate receptors, but it remains to be examined whether a majority of these terminals make direct contact with postsynaptic cells or exert their effect presynaptically through axonal ionotropic or metabotropic glutamate receptors. In addition, it is not known whether glutamate co-transmission has the potential to regulate synaptic transmission and mechanisms of synaptic plasticity such as long-term potentiation, and whether glutamate-releasing terminals release only glutamate or co-release it along with the neuron's 'primary' transmitter. With currently available techniques, co-release of two transmitters from the same vesicle can only be conclusively demonstrated by the presence of mixed miniature synaptic currents, mediated by both ionotropic glutamate receptors and ionotropic receptors for ACh (nicotinic receptors), 5-HT (5-HT_3 receptors) or GABA (GABA subtype A ($GABA_A$) receptors).

Even if co-release is difficult to prove, the demonstration of vesicular synergy between two transmitters — such as has already been shown for ACh and glutamate, 5-HT and glutamate, and dopamine and glutamate — speaks in favour of the presence of two transporters in a common pool of vesicles. A second issue to be addressed, therefore, is the physiological significance of such synergy. This will require experiments in which the functional effects of a loss of glutamate release *per se* can be discriminated from the effects of a loss of vesicular synergy. The impact of vesicular synergy on quantum size also needs to be clarified, as the influx of cytoplasmic glutamate into synaptic vesicles may considerably increase vesicular storage of the coexistent transmitter, by as much as 300% in the case of striatal ACh[79]. Vesicular synergy operates in the physiological range of glutamate concentrations (between 0.1 mM

Conditioned place preference paradigm
A behavioural test commonly used with rodents, in which drug administration is paired with specific environmental cues. On the test day, the proportion of time spent in the chamber previously associated with the drug provides an estimate of the positive subjective properties of the drug, as well as of its addictive potential.

Miniature synaptic current
A synaptic current that is due to the simultaneous activation of ionotropic receptors following the release of a quantum of neurotransmitter. A mixed miniature synaptic current is possible if two different types of neurotransmitters are present in a given synaptic vesicle and the corresponding receptors are present postsynaptically.

and 10 mM[79]), which implies that, in the presence of a VGLUT, the ACh or monoamine quantal size could vary markedly depending on the cytoplasmic concentration of glutamate. Continued research on this topic should determine whether — and at which intracytoplasmic concentrations — glutamate is able to regulate the amount of ACh, 5-HT or dopamine stored in synaptic vesicles. Experiments that directly test whether glutamate transport by the VGLUTs, as opposed to Cl^- or Pi transport (BOXES 2,3), is required for vesicular synergy are also needed to clarify the molecular mechanism leading to increased vesicular accumulation of 5-HT, ACh, dopamine or GABA.

Third, additional experiments are required to further document the morphological and functional heterogeneity of axon terminals in dual phenotype neurons expressing a VGLUT. If VGLUTs are found in only a subset of axon terminals established by a given neuron, as seems to be the case for dopamine neurons[68], this requires a finely regulated sorting mechanism that has not yet been defined. Identification of such a mechanism will probably benefit from analysing the proteome of axon terminals and the specific proteins that associate with VGLUTs.

Finally, considering the expression of VGLUT2 and VGLUT3 early in development[10,11,13,16–18,25,51,58], it will be important to investigate whether VGLUTs in dopamine, 5-HT or ACh neurons could have a developmental role, such as promoting neuronal survival or synapse formation. A detailed examination of the impact of conditional deletion of *Vgluts* on neuronal survival and morphology should be a first step towards answering this question.

Knowledge emerging from studies of VGLUT-mediated co-transmission may also provide new insights into normal and pathological CNS function. Very little is known about the role of dual phenotype neurons and glutamate co-transmission in the regulation of behaviour. Progress in this direction will undoubtedly continue to come from the study of constitutive and conditional knockout mice, which have already shown that the deletion of *Vgluts* in monoamine and ACh neurons leads to altered anxiety-related behaviours, sensitivity to psychostimulants and/or locomotion[37,80,88]. The pathophysiological implications of glutamate co-transmission are also of great potential interest. Recent studies suggest that expression of *Vgluts* — and thus the transmitter phenotype of neurons — is highly plastic and can be altered in pathological contexts, such as in response to brain lesions or to psychotropic medications[29,33,89–91]. Further studies are now required to evaluate whether and how up- or downregulation of *Vgluts* in monoamine, ACh or GABA neurons might be pathogenic.

1. Ni, B., Rosteck, P. R. Jr, Nadi, N. S. & Paul, S. M. Cloning and expression of a cDNA encoding a brain-specific Na^+-dependent inorganic phosphate cotransporter. *Proc. Natl Acad. Sci. USA* **91**, 5607–5611 (1994).
This is the seminal paper describing the molecular cloning of the first vesicular glutamate transporter. Subsequently renamed VGLUT1, this protein was initially characterized as a brain-specific plasma membrane sodium-dependent inorganic phosphate transporter.
2. Bellocchio, E. E., Reimer, R. J., Fremeau, R. T. Jr & Edwards, R. H. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* **289**, 957–960 (2000).
3. Takamori, S., Rhee, J. S., Rosenmund, C. & Jahn, R. Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* **407**, 189–194 (2000).
In this pioneering report, the authors elegantly established that BNPI was in fact a vesicular glutamate transporter (VGLUT1). See also reference 2, which was published shortly after this and reached the same conclusion.
4. Disbrow, J. K., Gershten, M. J. & Ruth, J. A. Uptake of L-[^{3}H] glutamic acid by crude and purified synaptic vesicles from rat brain. *Biochem. Biophys. Res. Commun.* **108**, 1221–1227 (1982).
5. Naito, S. & Ueda, T. Characterization of glutamate uptake into synaptic vesicles. *J. Neurochem.* **44**, 99–109 (1985).
6. Ni, B., Wu, X., Yan, G. M., Wang, J. & Paul, S. M. Regional expression and cellular localization of the Na^+-dependent inorganic phosphate cotransporter of rat brain. *J. Neurosci.* **15**, 5789–5799 (1995).
7. Aihara, Y. *et al.* Molecular cloning of a novel brain-type Na^+-dependent inorganic phosphate cotransporter. *J. Neurochem.* **74**, 2622–2625 (2000).
8. Hisano, S. *et al.* Regional expression of a gene encoding a neuron-specific Na^+-dependent inorganic phosphate cotransporter (DNPI) in the rat forebrain. *Brain Res. Mol. Brain Res.* **83**, 34–43 (2000).
9. Bai, L., Xu, H., Collins, J. F. & Ghishan, F. K. Molecular and functional analysis of a novel neuronal vesicular glutamate transporter. *J. Biol. Chem.* **276**, 36764–36769 (2001).
10. Fremeau, R. T. Jr *et al.* The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron* **31**, 247–260 (2001).
11. Herzog, E. *et al.* The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *J. Neurosci.* **21**, RC181 (2001).
12. Takamori, S., Rhee, J. S., Rosenmund, C. & Jahn, R. Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter (VGLUT2). *J. Neurosci.* **21**, RC182 (2001).
13. Varoqui, H., Schafer, M. K., Zhu, H., Weihe, E. & Erickson, J. D. Identification of the differentiation-associated Na^+/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. *J. Neurosci.* **22**, 142–155 (2002).
14. Bellocchio, E. E. *et al.* The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission. *J. Neurosci.* **18**, 8648–8659 (1998).
15. Daniels, R. W. *et al.* A single vesicular glutamate transporter is sufficient to fill a synaptic vesicle. *Neuron* **49**, 11–16 (2006).
16. Fremeau, R. T. Jr. *et al.* The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc. Natl Acad. Sci. USA* **99**, 14488–14493 (2002).
17. Gras, C. *et al.* A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. *J. Neurosci.* **22**, 5442–5451 (2002).
Vesicular glutamate transporters were initially believed to be genuine markers of primarily glutamatergic neurons. This was the first paper to report the presence of a VGLUT in neurons previously thought to be 'non-glutamatergic'.
18. Schafer, M. K., Varoqui, H., Defamie, N., Weihe, E. & Erickson, J. D. Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *J. Biol. Chem.* **277**, 50734–50748 (2002).
19. Takamori, S., Malherbe, P., Broger, C. & Jahn, R. Molecular cloning and functional characterization of human vesicular glutamate transporter 3. *EMBO Rep.* **3**, 798–803 (2002).
20. Herzog, E. *et al.* Localization of VGLUT3, the vesicular glutamate transporter type 3, in the rat brain. *Neuroscience* **123**, 983–1002 (2004).
21. Somogyi, J. *et al.* GABAergic basket cells expressing cholecystokinin contain vesicular glutamate transporter type 3 (VGLUT3) in their synaptic terminals in hippocampus and isocortex of the rat. *Eur. J. Neurosci.* **19**, 552–569 (2004).
22. Commons, K. G. Locally collateralizing glutamate neurons in the dorsal raphe nucleus responsive to substance P contain vesicular glutamate transporter 3 (VGLUT3). *J. Chem. Neuroanat.* **38**, 273–281 (2009).
23. Jackson, J., Bland, B. H. & Antle, M. C. Nonserotonergic projection neurons in the midbrain raphe nuclei contain the vesicular glutamate transporter VGLUT3. *Synapse* **63**, 31–41 (2009).
24. Ruel, J. *et al.* Impairment of SLC17A8 encoding vesicular glutamate transporter-3, VGLUT3, underlies nonsyndromic deafness DFNA25 and inner hair cell dysfunction in null mice. *Am. J. Hum. Genet.* **83**, 278–292 (2008).
25. Seal, R. P. *et al.* Sensorineural deafness and seizures in mice lacking vesicular glutamate transporter 3. *Neuron* **57**, 263–275 (2008).
26. Stornetta, R. L., Sevigny, C. P. & Guyenet, P. G. Vesicular glutamate transporter DNPI/VGLUT2 mRNA is present in C1 and several other groups of brainstem catecholaminergic neurons. *J. Comp. Neurol.* **444**, 191–206 (2002).
27. Stornetta, R. L., Sevigny, C. P., Schreihofer, A. M., Rosin, D. L. & Guyenet, P. G. Vesicular glutamate transporter DNPI/VGLUT2 is expressed by both C1 adrenergic and nonaminergic presympathetic vasomotor neurons of the rat medulla. *J. Comp. Neurol.* **444**, 207–220 (2002).
28. Dal Bo, G. *et al.* Dopamine neurons in culture express VGLUT2 explaining their capacity to release glutamate at synapses in addition to dopamine. *J. Neurochem.* **88**, 1398–1405 (2004).
This was the first report demonstrating the presence of a vesicular glutamate transporter in dopamine neurons, thus providing a molecular explanation for their ability to release glutamate — at least *in vitro*.
29. Mendez, J. A. *et al.* Developmental and target-dependent regulation of vesicular glutamate transporter expression by dopamine neurons. *J. Neurosci.* **28**, 6309–6318 (2008).
30. Kawano, M. *et al.* Particular subpopulations of midbrain and hypothalamic dopamine neurons express vesicular glutamate transporter 2 in the rat brain. *J. Comp. Neurol.* **498**, 581–592 (2006).
31. Yamaguchi, T., Sheen, W. & Morales, M. Glutamatergic neurons are present in the rat ventral tegmental area. *Eur. J. Neurosci.* **25**, 106–118 (2007).

32. Yamaguchi, T. *et al.* Differential distribution of the two subtypes of glutamatergic neurons within the midbrain dopamine system. *Soc. Neurosci. Abstr. 366.3* (San Diego, California, 13–17 November 2010).
33. Dal Bo, G. *et al.* Enhanced glutamatergic phenotype of mesencephalic dopamine neurons after neonatal 6-hydroxydopamine lesion. *Neuroscience* **156**, 59–70 (2008).
This was the first study to show the presence of VGLUT2 protein in axon terminals of mesencephalic dopamine neurons *in vivo*, as well as an induction of *VGLUT2* expression in these neurons under pathological conditions. See also reference 34 for a follow-up report suggesting regression of the VGLUT2 co-phenotype of dopamine neurons with age, in normal development, and following lesions or sprouting after injury.
34. Bérubé-Carrière, N. *et al.* The dual dopamine-glutamate phenotype of growing mesencephalic neurons regresses in mature rat brain. *J. Comp. Neurol.* **517**, 873–891 (2009).
35. Descarries, L. *et al.* Glutamate in dopamine neurons: synaptic versus diffuse transmission. *Brain Res. Rev.* **58**, 290–302 (2008).
36. Hioki, H. *et al.* Vesicular glutamate transporter 3-expressing nonserotonergic projection neurons constitute a subregion in the rat midbrain raphe nuclei. *J. Comp. Neurol.* **518**, 668–686 (2010).
37. Amilhon, B. *et al.* VGLUT3 (vesicular glutamate transporter type 3) contribution to the regulation of serotonergic transmission and anxiety. *J. Neurosci.* **30**, 2198–2210 (2010).
38. Hioki, H. *et al.* Chemically specific circuit composed of vesicular glutamate transporter 3- and preprotachykinin B-producing interneurons in the rat neocortex. *Cereb. Cortex* **14**, 1266–1275 (2004).
39. Mintz, E. M. & Scott, T. J. Colocalization of serotonin and vesicular glutamate transporter 3-like immunoreactivity in the midbrain raphe of Syrian hamsters (*Mesocricetus auratus*). *Neurosci. Lett.* **394**, 97–100 (2006).
40. Shutoh, F., Ina, A., Yoshida, S., Konno, J. & Hisano, S. Two distinct subtypes of serotonergic fibers classified by co-expression with vesicular glutamate transporter 3 in rat forebrain. *Neurosci. Lett.* **432**, 132–136 (2008).
41. Hornung, J. P. The human raphe nuclei and the serotonergic system. *J. Chem. Neuroanat.* **26**, 331–343 (2003).
42. Varga, V. *et al.* Fast synaptic subcortical control of hippocampal circuits. *Science* **326**, 449–453 (2009).
This was the first paper providing direct evidence of *in vivo* glutamate release by CNS monoamine neurons, through the use of optogenetics.
43. Zhang, S. & Morales, M. Serotonergic axons terminals with glutamatergic phenotype make synapses on both dopaminergic and nondopaminergic neurons in the ventral tegmental area. *Soc. Neurosci. Abstr. 488.6* (San Diego, California, 13–17 November 2010).
44. Oliveira, A. L. *et al.* Cellular localization of three vesicular glutamate transporter mRNAs and proteins in rat spinal cord and dorsal root ganglia. *Synapse* **50**, 117–129 (2003).
45. Herzog, E. *et al.* Expression of vesicular glutamate transporters, VGLUT1 and VGLUT2, in cholinergic spinal motoneurons. *Eur. J. Neurosci.* **20**, 1752–1760 (2004).
46. Kraus, T., Neuhuber, W. L. & Raab, M. Vesicular glutamate transporter 1 immunoreactivity in motor endplates of striated esophageal but not skeletal muscles in the mouse. *Neurosci. Lett.* **360**, 53–56 (2004).
47. Nishimaru, H., Restrepo, C. E., Ryge, J., Yanagawa, Y. & Kiehn, O. Mammalian motor neurons corelease glutamate and acetylcholine at central synapses. *Proc. Natl Acad. Sci. USA* **102**, 5245–5249 (2005).
48. Gezelius, H., Wallen-Mackenzie, A., Enjin, A., Lagerstrom, M. & Kullander, K. Role of glutamate in locomotor rhythm generating neuronal circuitry. *J. Physiol. Paris* **100**, 297–303 (2006).
49. Ren, J. *et al.* Habenula "cholinergic" neurons corelease glutamate and acetylcholine and activate postsynaptic neurons via distinct transmission modes. *Neuron* **69**, 445–452 (2011).
These authors used an optogenetic approach to establish that habenulo-interpeduncular cholinergic projections co-release glutamate and acetylcholine to activate postsynaptic signals with different kinetics.
50. Nickerson Poulin, A., Guerci, A., El Mestikawy, S. & Semba, K. Vesicular glutamate transporter 3 immunoreactivity is present in cholinergic basal forebrain neurons projecting to the basolateral amygdala in rat. *J. Comp. Neurol.* **498**, 690–711 (2006).
51. Boulland, J. L. *et al.* Expression of the vesicular glutamate transporters during development indicates the widespread corelease of multiple neurotransmitters. *J. Comp. Neurol.* **480**, 264–280 (2004).
52. Kao, Y. H. *et al.* Evidence that certain retinal bipolar cells use both glutamate and GABA. *J. Comp. Neurol.* **478**, 207–218 (2004).
53. Boulland, J. L. *et al.* Vesicular glutamate and GABA transporters sort to distinct sets of vesicles in a population of presynaptic terminals. *Cereb. Cortex* **19**, 241–248 (2009).
54. Fattorini, G. *et al.* VGLUT1 and VGAT are sorted to the same population of synaptic vesicles in subsets of cortical axon terminals. *J. Neurochem.* **110**, 1538–1546 (2009).
55. Soussi, R., Zhang, N., Tahtakran, S., Houser, C. R. & Esclapez, M. Heterogeneity of the supramammillary-hippocampal pathways: evidence for a unique GABAergic neurotransmitter phenotype and regional differences. *Eur. J. Neurosci.* **32**, 771–785 (2010).
56. Zander, J. F. *et al.* Synaptic and vesicular coexistence of VGLUT and VGAT in selected excitatory and inhibitory synapses. *J. Neurosci.* **30**, 7634–7645 (2010).
57. Ottem, E. N., Godwin, J. G., Krishnan, S. & Petersen, S. L. Dual-phenotype GABA/glutamate neurons in adult preoptic area: sexual dimorphism and function. *J. Neurosci.* **24**, 8097–8105 (2004).
58. Gras, C. *et al.* Developmentally regulated expression of VGLUT3 during early post-natal life. *Neuropharmacology* **49**, 901–911 (2005).
59. Gillespie, D. C., Kim, G. & Kandler, K. Inhibitory synapses in the developing auditory system are glutamatergic. *Nature Neurosci.* **8**, 332–338 (2005).
60. Sulzer, D. *et al.* Dopamine neurons make glutamatergic synapses *in vitro*. *J. Neurosci.* **18**, 4588–4602 (1998).
61. Bourque, M. J. & Trudeau, L. E. GDNF enhances the synaptic efficacy of dopaminergic neurons in culture. *Eur. J. Neurosci.* **12**, 3172–3180 (2000).
62. Descarries, L., Watkins, K. C., Garcia, S., Bosler, O. & Doucet, G. Dual character, asynaptic and synaptic, of the dopamine innervation in adult rat neostriatum: a quantitative autoradiographic and immunocytochemical analysis. *J. Comp. Neurol.* **375**, 167–186 (1996).
63. Tecuapetla, F. *et al.* Glutamatergic signaling by mesolimbic dopamine neurons in the nucleus accumbens. *J. Neurosci.* **30**, 7105–7110 (2010).
This paper used optogenetics to provide the first conclusive demonstration that mouse mesostriatal dopamine neurons *in vivo* release glutamate and give rise to excitatory postsynaptic responses in neurons of the nucleus accumbens. See also reference 64, which was published shortly after this and reached the same conclusion. Importantly, this later study also showed the disappearance of glutamate release in conditional VGLUT2 knockout mice.
64. Stuber, G. D., Hnasko, T. S., Britt, J. P., Edwards, R. H. & Bonci, A. Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. *J. Neurosci.* **30**, 8229–8233 (2010).
65. Hattori, T., Takada, M., Moriizumi, T. & Van der Kooy, D. Single dopaminergic nigrostriatal neurons form two chemically distinct synaptic types: possible transmitter segregation within neurons. *J. Comp. Neurol.* **309**, 391–401 (1991).
66. Forlano, P. M. & Woolley, C. S. Quantitative analysis of pre- and postsynaptic sex differences in the nucleus accumbens. *J. Comp. Neurol.* **518**, 1330–1348 (2010).
67. Tsudzuki, T. & Tsujita, M. Isoosmotic isolation of rat brain synaptic vesicles, some of which contain tyrosine hydroxylase. *J. Biochem.* **136**, 239–243 (2004).
68. Fortin, G., Mendez, J. A., Bourque, M. J. & Trudeau, L. E. Dopamine neurons establish heterogeneous subtypes of axon terminals only a subset of which contain VMAT2 and VGLUT2. *Soc. Neurosci. Abstr. 815.6* (Chicago, Ilinois, 17–21 October 2009).
69. Johnson, M. D. Synaptic glutamate release by postnatal rat serotonergic neurons in microculture. *Neuron* **12**, 433–442 (1994).
This paper used an elegant microculture system to demonstrate co-release of glutamate by a monoamine neuron (in this case, 5-HT) for the first time. See also reference 60 for similar findings in dopamine neurons.
70. Martin-Ibanez, R. *et al.* Vesicular glutamate transporter 3 (VGLUT3) identifies spatially segregated excitatory terminals in the rat substantia nigra. *Eur. J. Neurosci.* **23**, 1063–1070 (2006).
71. Huh, C. Y., Danik, M., Manseau, F., Trudeau, L. E. & Williams, S. Chronic exposure to nerve growth factor increases acetylcholine and glutamate release from cholinergic neurons of the rat medial septum and diagonal band of Broca via mechanisms mediated by p75NTR. *J. Neurosci.* **28**, 1404–1409 (2008).
72. Sotty, F. *et al.* Distinct electrophysiological properties of glutamatergic, cholinergic and GABAergic rat septohippocampal neurons: novel implications for hippocampal rhythmicity. *J. Physiol.* **551**, 927–943 (2003).
73. Descarries, L., Gisiger, V. & Steriade, M. Diffuse transmission by acetylcholine in the CNS. *Prog. Neurobiol.* **53**, 603–625 (1997).
74. Mechawar, N., Watkins, K. C. & Descarries, L. Ultrastructural features of the acetylcholine innervation in the developing parietal cortex of rat. *J. Comp. Neurol.* **443**, 250–258 (2002).
75. Gutierrez, R. *et al.* Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. *J. Neurosci.* **23**, 5594–5598 (2003).
76. Noh, J., Seal, R. P., Garver, J. A., Edwards, R. H. & Kandler, K. Glutamate co-release at GABA/glycinergic synapses is crucial for the refinement of an inhibitory map. *Nature Neurosci.* **13**, 232–238 (2010).
77. Chery, N. & de Koninck, Y. Junctional versus extrajunctional glycine and $GABA_A$ receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. *J. Neurosci.* **19**, 7342–7355 (1999).
78. Herzog, E., Takamori, S., Jahn, R., Brose, N. & Wojcik, S. M. Synaptic and vesicular co-localization of the glutamate transporters VGLUT1 and VGLUT2 in the mouse hippocampus. *J. Neurochem.* **99**, 1011–1018 (2006).
79. Gras, C. *et al.* The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone. *Nature Neurosci.* **11**, 292–300 (2008).
Using a *Vglut3* knockout mouse model, this study was the first to describe vesicular synergy for acetylcholine and glutamate in striatal cholinergic interneurons. This was also the first time that the concept of vesicular synergy was proposed as a functional role of VGLUTs in neurons that are not classically known to be glutamatergic.
80. Hnasko, T. S. *et al.* Vesicular glutamate transport promotes dopamine storage and glutamate corelease *in vivo*. *Neuron* **65**, 643–656 (2010).
This was the first study to examine the putative role of VGLUT2-mediated glutamate co-entry in promoting vesicular monoamine storage by use of an *in vitro* system. This paper also addressed reward learning in a conditional VGLUT2 knockout mouse, and found it to be normal.
81. Santos, M. S., Li, H. & Voglmaier, S. M. Synaptic vesicle protein trafficking at the glutamate synapse. *Neuroscience* **158**, 189–203 (2009).
82. Fei, H., Grygoruk, A., Brooks, E. S., Chen, A. & Krantz, D. E. Trafficking of vesicular neurotransmitter transporters. *Traffic* **9**, 1425–1436 (2008).
83. Grygoruk, A. *et al.* A tyrosine-based motif localizes a *Drosophila* vesicular transporter to synaptic vesicles *in vivo*. *J. Biol. Chem.* **285**, 6867–6878 (2010).
84. Voglmaier, S. M. & Edwards, R. H. Do different endocytic pathways make different synaptic vesicles? *Curr. Opin. Neurobiol.* **17**, 374–380 (2007).
85. Mutch, S. A. *et al.* Protein quantification at the single vesicle level reveals that a subset of synaptic vesicle proteins are trafficked with high precision. *J. Neurosci.* **31**, 1461–1470 (2011).
86. Ikemoto, S. Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res. Rev.* **56**, 27–78 (2007).
87. Chuhma, N. *et al.* Dopamine neurons mediate a fast excitatory signal via their glutamatergic synapses. *J. Neurosci.* **24**, 972–981 (2004).
88. Birgner, C. *et al.* VGLUT2 in dopamine neurons is required for psychostimulant-induced behavioral activation. *Proc. Natl Acad. Sci. USA* **107**, 389–394 (2010).
This was the first report describing the behavioural consequences of the conditional deletion of a VGLUT in dopamine neurons. The knockout mice showed a blunted behavioural activation in response to amphetamine compared to controls.

89. Trudeau, L. E. & Gutierrez, R. On cotransmission & neurotransmitter phenotype plasticity. *Mol. Interv.* **7**, 138–146 (2007).
90. Moutsimilli, L. *et al.* Selective cortical VGLUT1 increase as a marker for antidepressant activity. *Neuropharmacology* **49**, 890–900 (2005).
91. Moutsimilli, L. *et al.* Antipsychotics increase vesicular glutamate transporter 2 (VGLUT2) expression in thalamolimbic pathways. *Neuropharmacology* **54**, 497–508 (2008).
92. Strata, P. & Harvey, R. Dale's principle. *Brain Res. Bull.* **50**, 349–350 (1999).
93. Eccles, J. C., Fatt, P. & Koketsu, K. Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. *J. Physiol.* **126**, 524–562 (1954).
94. Dale, H. H. Pharmacology and nerve endings. *Proc. R. Soc. Med.* **28**, 319–330 (1934).
95. Xie, X. S., Stone, D. K. & Racker, E. Determinants of clathrin-coated vesicle acidification. *J. Biol. Chem.* **258**, 14834–14838 (1983).
96. Hartinger, J. & Jahn, R. An anion binding site that regulates the glutamate transporter of synaptic vesicles. *J. Biol. Chem.* **268**, 23122–23127 (1993).
97. Juge, N. *et al.* Metabolic control of vesicular glutamate transport and release. *Neuron* **68**, 99–112 (2010).
98. Edwards, R. H. The neurotransmitter cycle and quantal size. *Neuron* **55**, 835–858 (2007).
99. Juge, N., Yoshida, Y., Yatsushiro, S., Omote, H. & Moriyama, Y. Vesicular glutamate transporter contains two independent transport machineries. *J. Biol. Chem.* **281**, 39499–39506 (2006).
100. Takamori, S. *et al.* Molecular anatomy of a trafficking organelle. *Cell* **127**, 831–846 (2006).
101. Schenck, S., Wojcik, S. M., Brose, N. & Takamori, S. A chloride conductance in VGLUT1 underlies maximal glutamate loading into synaptic vesicles. *Nature Neurosci.* **12**, 156–162 (2009).
102. Almqvist, J., Huang, Y., Laaksonen, A., Wang, D. N. & Hovmoller, S. Docking and homology modeling explain inhibition of the human vesicular glutamate transporters. *Protein Sci.* **16**, 1819–1829 (2007).
103. Moriyama, Y. & Yamamoto, A. Glutamatergic chemical transmission: look! Here, there, and anywhere. *J. Biochem.* **135**, 155–163 (2004).
104. Maycox, P. R., Deckwerth, T., Hell, J. W. & Jahn, R. Glutamate uptake by brain synaptic vesicles. Energy dependence of transport and functional reconstitution in proteoliposomes. *J. Biol. Chem.* **263**, 15423–15428 (1988).

Acknowledgements
Research in the El Mestikawy Laboratory was supported by grants from the Institut National de la Santé et de la Recherche Médicale, Agence Nationale pour la Recherche, Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Canadian Research chair, Douglas Mental Health University Institute and Canadian Foundation for Innovation. Research in the Mackenzie Laboratory was supported by the Swedish Research Council, the Swedish Brain Foundation, the Åhlén and Wiberg Foundations, the National Board of Health and Welfare and Uppsala University, Sweden. Research in the Descarries Laboratory was supported by grant NRF-3,544 from the Canadian Institutes of Health Research (CIHR). Research in the Trudeau Laboratory was also supported by grants from the CIHR, the National Alliance for Research on Schizophrenia and Depression, Neuroscience Canada and the Natural Sciences and Engineering Research Council of Canada. L.-E.T. and A.W.-M. share a grant from the Swedish Foundation for International Cooperation in Research and Higher Education. The authors thank B. Amilhon for help in the design of Figure. 2, N. Bérubé-Carrière for help in the preparation of Table 1 and B. Gasnier for fruitful discussions.

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION

Louis-Eric Trudeau's homepage: http://www.mapageweb.umontreal.ca/trudeal
Salah El Mestikawy's Douglas Mental Health University Institute homepage: http://pmsnc.snv.jussieu.fr/index.php/fr/elmestikawy
Salah El Mestikawy's Pathophysiology of Central Nervous System Disorders homepage: http://www.douglas.qc.ca/page/salah-el-mestikawy-lab
Åsa Wallén-Mackenzie's homepage: http://www.neuro.uu.se/forskning/forskargrupp.html&id=41
Laurent Descarries's homepage: http://www.patho.umontreal.ca/recherche/fiches_chercheurs/ldescarries_fr.htm

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

Author Biographies

Salah El Mestikawy received his Ph.D. in neuroscience in the laboratory of J. Glowinski at the Collège de France, Paris, France, in 1982. He then worked as a researcher, with a permanent position from the Centre National pour la Recherche Scientifique in the laboratory of M. Hamon in Paris and in the laboratory of M. Caron at Duke University, Durham, North Carolina, USA, between 1983 and 1998. He presently holds the position of Directeur de Recherche at the Université Pierre et Marie Curie, Paris, France and a Canada Research Chair at McGill University, Montreal, Canada. He directs two research groups working in Paris and Montreal. His research interests are in neurotransmission, dopamine, serotonin (5-HT), glutamate, biosynthetic enzymes, receptors, transporters, and normal and pathological brain.

Åsa Wallén-Mackenzie received her Ph.D. from the Karolinska Institute, Stockholm, Sweden, in 2002, defending her thesis on the role of nuclear hormone receptors in midbrain dopamine neurons. She went on to a first postdoctoral position at AstraZeneca plc and a second at Uppsala University, Sweden, working on the then recently identified vesicular glutamate transporters. In 2008 she received an assistant professorship from the Swedish Research Council and started her own laboratory. Her group focuses on the study of glutamate–dopamine interactions in neuronal circuits of the reward system and basal ganglia. In 2010 she was appointed Associate Professor in neuroscience at Uppsala University.

Laurent Descarries is renowned for his pioneering descriptions of the ultrastructural features of central monoamine and acetylcholine neurons. These studies were the origin of the concept of diffuse (volume) transmission, which complements that of synaptic transmission as a functional paradigm. More recently, his laboratory demonstrated the internalization of serotonin 5-HT_{1A} autoreceptors *in vivo* upon treatment with a selective serotonin reuptake inhibitor (SSRI), a phenomenon subsequently shown amenable to positron emission tomography (PET) imaging in humans. Further immunoelectron microscopic investigations of serotoninergic mechanisms involved in depression and its SSRI treatment, as well as of the glutamatergic co-phenotype of transmitter-defined neurons, are in progress.

Louis-Eric Trudeau obtained an M.Sc. in neuroscience at the Université Pierre et Marie Curie and a Ph.D. in neuroscience from the Université de Montréal, Quebec, Canada (1994), for a thesis on synaptic plasticity in *Aplysia*. After a postdoctoral fellowship at Iowa State University, USA, working on synaptic transmission in hippocampal neurons, he was recruited back to the Université de Montréal in 1997 and established a research group focusing on dopamine neuron physiology and pharmacology. In recent years, he has been exploring the functions of the dopamine D2 autoreceptor and glutamate and dopamine co-transmission. His discovery of the expression of vesicular glutamate transporter 2 by dopamine neurons has triggered renewed interest in understanding the importance of glutamate co-transmission by these neurons. Guillaume Fortin is a pharmacology Ph.D. student in the Trudeau Laboratory. The focus of his thesis work is on elucidating the functional roles of glutamate co-transmission in dopamine neurons.

Online at-a-glance summary

- Vesicular transporters accumulate neurotransmitters in synaptic vesicles before their regulated release. They are key functional markers as they define the 'transmitter phenotype' of a given neuron.
- Vesicular glutamate transporters (VGLUTs) are found not only in neurons previously known to use glutamate as their primary transmitter but also in 'non-glutamatergic' neurons, including some that release a monoamine, acetylcholine or GABA.
- The role of VGLUTs in these non-glutamatergic neurons is the subject of intense research. Two major roles have thus far been proposed: co-release of glutamate as a co-transmitter and enhanced packaging of the primary transmitter through a mechanism called 'vesicular synergy'.
- The co-release of glutamate by serotonin (5-HT), dopamine and acetylcholine neurons was initially demonstrated *in vitro*, in isolated neuron microcultures. These initial discoveries were recently validated *in vivo* in the mouse, using optogenetics and patch-clamp electrophysiology.
- Vesicular synergy is emerging as an important function of VGLUTs in acetylcholine, serotonin and dopamine neurons. Its molecular mechanisms are still incompletely defined.
- The behavioural consequences of glutamate co-release and/or vesicular synergy by dopamine, serotonin, acetylcholine or GABA neurons have only recently begun to be explored.
- Recent work in knockout mice suggests: first, that vesicular glutamate transporter 2 (VGLUT2) in dopamine neurons regulates behavioural activation induced by psychostimulant drugs; second, that VGLUT3 in cholinergic interneurons regulates basal and cocaine-stimulated locomotor activity; and third, that VGLUT3 in 5-HT neurons regulates anxiety-related behaviours.

00

From glutamate co-release to vesicular synergy: new perspectives on the functions of vesicular glutamate transporters

Salah El Mestikawy, Åsa Wallén-Mackenzie, Guillaume M. Fortin, Laurent Descarries and Louis-Eric Trudeau

Vesicular glutamate transporters are expressed not only in glutamate neurons but also in monoamine, acetylcholine and, intriguingly, GABA neurons. Trudeau and colleagues discuss the role of these transporters in glutamate co-release and vesicular synergy — a process leading to enhanced packaging of the 'primary' transmitter.

Subject categories
Synaptic transmission, cellular neuroscience