Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2017 | 142 (Suppl. 2) | 90–102 doi: 10.1111/jnc.14003

REVIEW

Cholinergic/glutamatergic co-transmission in striatal cholinergic interneurons: new mechanisms regulating striatal computation

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Abstract

It is well established that neurons secrete neuropeptides and ATP with classical neurotransmitters; however, certain neuronal populations are also capable of releasing two classical neurotransmitters by a process named co-transmission. Although there has been progress in our understanding of the molecular mechanism underlying co-transmission, the individual regulation of neurotransmitter secretion and the functional significance of this neuronal 'bilingualism' is still unknown. Striatal cholinergic interneurons (CINs) have been shown to secrete glutamate (Glu) in addition to acetylcholine (ACh) and are recognized for their role in the regulation of striatal circuits and behavior. Our review highlights the recent research into identifying mechanisms that regulate the secretion and function of Glu and ACh released by CINs and the roles these neurons play in regulating dopamine secretion and striatal activity. In particular, we focus on how the transporters for ACh (VAChT) and Glu (VGLUT3) influence the storage of neurotransmitters in CINs. We further discuss how these individual neurotransmitters regulate striatal computation and distinct aspects of behavior that are regulated by the striatum. We suggest that understanding the distinct and complementary functional roles of these two neurotransmitters may prove beneficial in the development of therapies for Parkinson's disease and addiction. Overall, understanding how Glu and ACh secreted by CINs impacts striatal activity may provide insight into how different populations of 'bilingual' neurons are able to develop sophisticated regulation of their targets by interacting with multiple receptors but also by regulating each other's vesicular storage.

Keywords: addiction, cocaine, dopamine, Parkinson's disease, vesicular acetylcholine transporter, vesicular glutamate transporter 3.

J. Neurochem. (2017) **142** (Suppl. 2), 90–102.

This is an article for the special issue XVth International Symposium on Cholinergic Mechanisms.

The notion that a neuron releases only one neurotransmitter is known as Dale's principle, although it is not clear that Sir Henry Dale actually proposed this as it stands now (Burnstock 1976). However, we owe to Sir Dale the idea of describing neurons based on their chemical messengers, which has been extremely helpful for our understanding of

Received November 16, 2016; revised manuscript received February 28, 2017; accepted March 1, 2017.

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Abbreviations used: ΔΨ, membrane potential; ΔpH, chemical gradient; ACh, acetylcholine; AChE, acetylcholinesterase; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ChAT, choline acetyltransferase; CHT, choline transporter; CIN, cholinergic interneuron; CNS, central nervous system; D1, dopamine receptor 1; D2, dopamine receptor 2; $GABA_AR$, GABA-A receptor; GABA, gamma-aminobutyric acid; Glu, glutamate; LID, levodopa-induced dyskinesia; mAChR, muscarinic acetylcholine receptor; mGLUR, metabotropic glutamate receptor; MSN, medium spiny neuron; nAChR, nicotinic acetylcholine receptor; NMDAR, *N*-methyl-D-aspartate receptor; PD, Parkinson's disease; PNS, peripheral nervous system; VAChT, vesicular acetylcholine transporter; VGLUT3, vesicular glutamate transporter; VTA, ventral tegmental area.

chemical communication in the brain and peripheral tissues. For many years, we have known that neurons that release classical neurotransmitters can also secrete neuropeptides and other bioactive molecules, such as ATP (Hökfelt *et al.* 1977). More recently, it has become clear that two classical neurotransmitters can exist and be secreted by the same neuron (for review, see El Mestikawy *et al.* 2011; Hnasko and Edwards 2012), challenging the dogma that all synapses of a given neuron can release only one neurotransmitter. Although more understanding has been gained in recent years about the molecular mechanisms underlying this chemical communication 'bilingualism', how the secretion of two classical neurotransmitters from the same neuronal population is regulated and its functional significance are still largely unknown.

Classical neurotransmitters present in the same neuron have been suggested to modulate each other's packaging into synaptic vesicles (Bankston and Guidotti 1996; Gras *et al.* 2008; Amilhon *et al.* 2010; Hnasko *et al.* 2010; Frahm *et al.* 2015) and they may provide reciprocal regulation of their secretion via pre-synaptic actions in the nerve terminal. Most classical neurotransmitters can interact with multiple receptors and co-transmission may allow them to differentially activate specific groups of neighboring neurons based on the different populations of receptors they express. All these factors complicate our understanding of the functional consequences of co-transmission. Chemical synapses, compared to electrical synapses, offer the possibility of increased levels of regulation in neuronal communication (Ovsepian and Vesselkin 2014). Co-transmission provides the ultimate level of sophistication for chemical synapses, adding many new ways for regulation within circuits. This increased signaling complexity may help to strengthen a given physiological action, but it may also generate opposite or complementary signals through the concerted activity of only one neuronal population.

Cholinergic interneurons (CINs) in the striatum represent one of several neuronal populations where the significance of co-transmission of two different classical neurotransmitters has just started to be revealed (Gras *et al.* 2008; Guzman *et al.* 2011; Higley *et al.* 2011; Divito *et al.* 2015; Sakae *et al.* 2015). These neurons have been shown to release the excitatory neurotransmitter glutamate (Glu) in addition to acetylcholine (ACh) (Gras *et al.* 2002, 2008; Higley *et al.* 2011; Nelson *et al.* 2014). Cholinergic neurons are recognized for their important role in the control of striatal circuits and striatum-based behavior (for review, see Lim *et al.* 2014; Gonzales and Smith 2015; Prado *et al.* 2017), but how exactly they contribute to striatal functions in physiological and pathological conditions is still not completely understood. Their ability to secrete these two chemical messengers complicates the picture even more. In this review, we will discuss the molecular and cellular properties that allow CINs to secrete these two classical transmitters and we will try as much as possible to disentangle their actions. We will further discuss how manipulations of neurotransmitter secretion in CINs may provide new pharmacological targets in the treatment of striatum-related diseases.

Populations of cholinergic neurons in the brain

ACh is an ancient signaling molecule that has existed for billions of years (Wessler *et al.* 2003; also, see review by Wessler & Kirkpatrick (2017) in this issue). Cholinergic neurons are abundant in both the central (CNS) and peripheral nervous system (PNS), where they act to regulate a plethora of physiological functions. In the PNS, ACh influences both involuntary and voluntary actions through the autonomic and somatic nervous systems, respectively. In the CNS, ACh rapidly transmits its information across the synapse via ionotropic, nicotinic receptors. In addition, ACh can function as a neuromodulator, acting through metabotropic, muscarinic receptors to regulate a variety of circuits (see reviews by Picciotto *et al.* 2012; Dineley *et al.* 2015 and Soreq 2015, for a detailed discussion on general aspects of central cholinergic neurotransmission).

Most brain regions receive cholinergic innervation through axonal fibers that originate from projection neurons in relatively distant nuclei. The main nuclei include clusters of cholinergic somata in the brainstem, basal forebrain and medial habenula (Mesulam *et al.* 1983b). In the brainstem, cholinergic neurons are found within the pedunculopontine tegmental nucleus and the laterodorsal complex (Mesulam *et al.* 1983b; Satoh *et al.* 1983; Vincent *et al.* 1986; Wang and Morales 2009). These cholinergic cell groups innervate several brain areas including the midbrain, thalamus, cerebellum and basal ganglia, and recent work has shown that the absence of cholinergic signaling from these neurons in mice causes dysfunctional gait (Janickova *et al.* 2017). Moreover, it has also been suggested that these cholinergic neurons play a role in the control of the sleep–wake cycle (Datta and Siwek 1997; Van Dort *et al.* 2015) and in reward signaling (Xiao *et al.* 2016).

In the basal forebrain, cholinergic neurons are found in the medial septum, nuclei of the diagonal band nucleus, substantia innominata and nucleus basalis of Meynert (Mesulam *et al.* 1983a). These cholinergic neurons innervate the hippocampus, thalamus, amygdala and neocortex (Mesulam *et al.* 1983a). Basal forebrain cholinergic signaling regulates several aspects of cognition including attention (Sarter *et al.* 1999; Gill *et al.* 2000; Dalley *et al.* 2001; Kolisnyk *et al.* 2013a), cognitive flexibility (Roberts *et al.* 1990, 1992; Ridley *et al.* 1994; Cabrera *et al.* 2006; Kolisnyk *et al.* 2013b), working memory, spatial learning as well as associative learning (Al-Onaizi *et al.* 2017). Cholinergic neurons located in the medial habenula, which are also important for the modulation of cognition-dependent executive functions (Kobayashi *et al.* 2013), innervate the

interpeduncular nucleus (Sastry *et al.* 1979; Kimura *et al.* 1981).

In the striatum, most cholinergic innervation comes from interneurons (Woolf and Butcher 1981). Cholinergic interneurons (CINs) compose < 3% of all cells in the striatum. Morphologically, they can be easily distinguished from other striatal neurons as they are significantly larger (somata measure 20–50 microns) and feature rich axonal and dendritic arborizations (Bolam *et al.* 1984; Contant *et al.* 1996). These anatomical features allow widespread cholinergic innervation within the striatum, despite the relatively small number of CINs. The significance of CINs for the control of striatal circuits and functions has long been recognized (Doshay and Constable 1957). Recently, it has been demonstrated that the striatum also receives innervation from brainstem cholinergic neurons (Dautan *et al.* 2014).

The striatum is the main input structure of the basal ganglia and is commonly divided into the dorsal (caudate and putamen in primates) and ventral striatum (also called nucleus accumbens). These two regions differ in terms of their afferent and efferent connections (Holland and Rescorla 1975; Gerfen and Young 1988; Haynes and Haber 2013). The dorsal striatum receives glutamatergic input from the thalamus and cortex, and dopaminergic input from the substantia nigra pars compacta. The ventral striatum receives glutamatergic input from the thalamus, cortex, ventral tegmental area (VTA) and from the hippocampus and basolateral amygdala, while dopaminergic input also comes from the VTA (Gerfen 1984; Gerfen and Young 1988; Berendse and Groenewegen 1990; Ragsdale and Graybiel 1991; Lynd-Balta and Haber 1994; Stuber *et al.* 2010; Tecuapetla *et al.* 2010; Hnasko *et al.* 2012).

The electrophysiological properties of CINs have been well studied in primates (Kimura *et al.* 1984; Aosaki *et al.* 1994a). In the primate striatum, CINs correspond to tonically active neurons, which spontaneously fire with a frequency between 2 and 12 Hz (Wilson *et al.* 1990). The tonic activity of CINs is endogenously generated, mainly by the action of a persistent sodium current through NaV1.6 channels (Bennett and Wilson 1999; Bennett *et al.* 2000; Wilson 2005). Thus, basal neurotransmitter release by striatal CINs originates mainly from this autonomous activity. In reaction to salient external stimuli, CINs respond with a pause (lasting approximately 200 ms) in their tonic activity usually followed by a rebound phase of excitation (Aosaki *et al.* 1994b, 1995; Apicella *et al.* 1997). At times, the excitation can also precede the pause (Aosaki *et al.* 1995). CINs firing activity is usually synchronized, so given the rich connectivity of each CIN cell, it can influence vast regions of the striatum at the same time (Raz *et al.* 1996; Goldberg *et al.* 2004). Pathological depletion of dopamine in the striatum was shown to lead to hypersynchronization of CINs (Raz *et al.* 1996); however, the exact mechanism and source of the synchronization has not yet been uncovered. Notably, thalamostriatal glutamatergic input was suggested to regulate CIN synchronization (Lim *et al.* 2014).

Control of CINs excitability and firing is modulated by different neurotransmitters. CINs express dopamine receptors of the D1 and D2 subfamily with a higher expression of the inhibitory D2 receptor (Yan and Surmeier 1997; Yan *et al.* 1997); thus, dopaminergic input to CINs is mainly inhibitory, resulting in reduced firing and decreased neurotransmitter release (MacKenzie *et al.* 1989; Aosaki *et al.* 1994a; DeBoer and Abercrombie 1996; DeBoer *et al.* 1996; Straub *et al.* 2014). Gamma-aminobutyric acid (GABA), released from striatal GABAergic interneurons (Martone *et al.* 1992; Sullivan *et al.* 2008), can also inhibit CINs activity through its action on $GABA_A$ receptors (Sato *et al.* 2014). CINs are also subject to autoinhibition as they express two inhibiting types of muscarinic acetylcholine receptors, M2 and M4 (Smiley *et al.* 1999; Ding *et al.* 2006).

Regulation of ACh synthesis and release

In cholinergic nerve terminals, maintenance of ACh synthesis and secretion relies heavily on three cholinergic proteins for its precise regulation (see review Prado *et al.* 2002). The high-affinity choline transporter, which is responsible for the uptake of choline used for ACh synthesis in cholinergic nerve endings (Ribeiro *et al.* 2006). The enzyme choline acetyltransferase (ChAT), which synthesizes ACh using choline and acetyl-coA (Dobransky *et al.* 2000; Prado *et al.* 2002) and the vesicular acetylcholine transporter (VAChT), which packages ACh into synaptic vesicles for secretion (Nguyen *et al.* 1998; Prado *et al.* 2013).

As noted above, ACh can bind to either nicotinic (nAChR) or muscarinic (mAChR) receptors on effector cells. nAChRs are ligand gated ion channels that move sodium, potassium and calcium across membranes (see review Dineley *et al.* 2015). Neuronal nAChRs can be homomeric or heteromeric with any combination of α2–α10 and β2–β4 subunits. The striatum primarily expresses the α4β2, α6β2 and, to lesser extent, α7 subtypes (Clarke *et al.* 1985). mAChRs are also expressed throughout the CNS and PNS, and they are divided into two classes depending on the coupling to a G protein. The first class consists of M1, M3 and M5 subtypes that are coupled to a Gq protein (Wess 2012). Conversely, the second class consists of M2 and M4 subtypes that use Gi and Go heterotrimeric proteins. In the striatum, ACh primarily affects intrinsic excitability and synaptic inputs through M1, M2 and M4 mAChRs subtypes, which are expressed at the highest level in the striatum (Bernard *et al.* 1992). Figure 1 illustrates receptor localization in the striatum.

Termination of synaptic transmission is achieved within milliseconds by acetylcholinesterase, which clears ACh from the synaptic cleft (see review Soreq and Seidman 2001). Acetylcholinesterase hydrolyses ACh, thereby breaking it

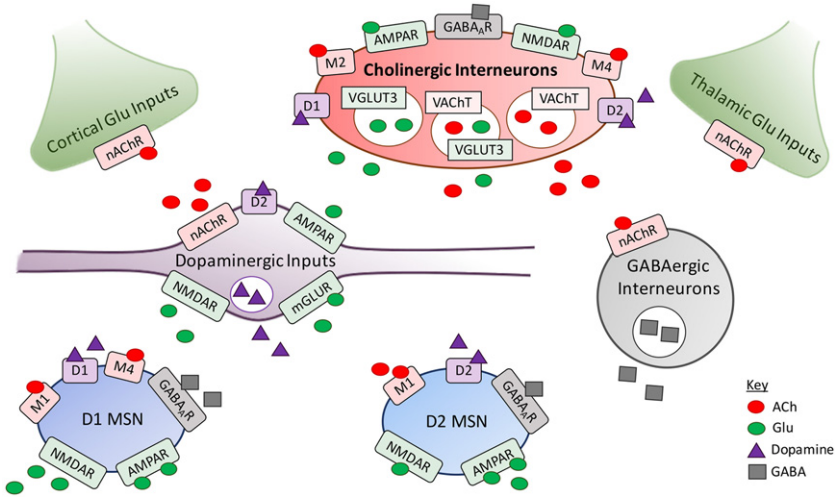

Fig. 1 Cholinergic and glutamatergic signaling by cholinergic interneuron (CIN)s: Schematic drawing of cholinergic, dopaminergic and glutamatergic inputs as well as co-transmission in the striatum.

down into choline and acetate. Free acetate diffuses into the surrounding medium, whereas choline is transported into the pre-synaptic neuron by the choline transporter where it is conjugated to acetate by ChAT to regenerate ACh.

Synaptic loading of ACh and Glu in CINs

In the striatum, synaptic vesicles of CINs express the vesicular glutamate transporter 3 (VGLUT3), in addition to VAChT (Gras *et al.* 2002). These two transporters are responsible for the synaptic loading of Glu and ACh, respectively. VGLUT3 is the third member of the SLC17 family of vesicular transporters for excitatory amino acids which is comprised of VGLUT1-3 (Bellocchio *et al.* 2000; Takamori *et al.* 2000; Fremeau *et al.* 2002; Schäfer *et al.* 2002; Ruel *et al.* 2008). In adults, VGLUT1 is expressed in the cortex, hippocampus and cerebellum, whereas VGLUT2 is expressed in the thalamus and brainstem (Fremeau *et al.* 2004); albeit, the expression pattern differs during development. Moreover, the complete expression pattern of VGLUT1 and VGLUT2 has yet to be established. VGLUT3 is commonly found in neurons that traditionally use neurotransmitters other than Glu as a signaling molecule. In particular, VGLUT3 is expressed in the caudate/putamen, olfactory tubercle, nucleus accumbens, hippocampus, dorsal raphe, scattered in the cortex and even within astrocytes (Fremeau *et al.* 2002; Gras *et al.* 2002; Schäfer *et al.* 2002).

At a single synaptic vesicle, the exact number of VAChT molecules present is unknown; however, studies on synaptic vesicles in the Torpedo electric organ have suggested an average of four VAChT molecules which was shown to pack more than 40 000 ACh molecules per synaptic vesicle (Anderson *et al.* 1986; Van der Kloot 2003). In mammals, the vesicles are much smaller with the number estimated to be between 2000 and 10 000 ACh molecules per cholinergic vesicle (Whittaker and Sheridan 1965), albeit the precise numbers are unknown. VAChT expression is a limiting factor in ACh release from cholinergic neurons (Prado *et al.* 2013). Over-expression of VAChT in cultured developing *Xenopus* spinal neurons leads to an increase in ACh loading and consequently an increase in excitatory post-synaptic currents (Song *et al.* 1997). Correspondingly, an increase in VAChT expression in mice is associated with an increase in ACh loading in synaptic vesicles and release (Kolisnyk *et al.* 2013b; Sugita *et al.* 2016). Furthermore, our studies performed on heterozygous and homozygous VAChT knockdown mice or conditional VAChT knockout mice have indicated that ACh release decreases proportionally to the decrease in VAChT expression (Prado *et al.* 2006; Lima *et al.*, 2010; Guzman *et al.* 2011; Martins-Silva *et al.* 2011). Vesicular transporters have slow rates of ACh turnover; VAChT is thought to transport one molecule of ACh per second (Varoqui and Erickson 1996). Hence, unless there are mechanisms *in vivo* that accelerate the vesicular transport of ACh, it might take several minutes to fill a vesicle with the neurotransmitter if there are only few transporters per vesicle.

It was estimated for VGLUT1 and VGLUT2 that there are approximately 4–14 copies of transporter per vesicle (Takamori *et al.* 2006; Mutch *et al.* 2011). Yet, it is unclear if the rate of vesicle filling is influenced by the number of VGLUT copies. Decreased VGLUT1 expression in mice and dVGLUT in *Drosophila* had little effect on vesicle filling (Fremeau *et al.* 2004; Daniels *et al.* 2006). On the other hand, recent experiments using Glu uncaging showed that the time required for the refilling of vesicles with Glu, after washing out vesicular Glu, is approximately 15 s allowing an estimation for the number of VGLUT molecules on a single vesicle to be 10 (Hori and Takahashi 2012). In addition, Glu uptake by vesicles expressing VGLUT3 may be slower when compared to the VGLUT2 isoform (Gras *et al.* 2002). Therefore, decreased VGLUT3 expression has the potential to compromise Glu transmission if the refilling rate is not able to maintain optimal Glu concentration in synaptic vesicles during recycling.

Transport of both ACh by VAChT and Glu by VGLUT3 requires a H^+ electrochemical gradient that is created by the vesicular H^+-ATPase. The difference in H^+ concentration inside and outside the vesicle gives rise to the chemical gradient (ΔpH) and membrane potential ($\Delta\Psi$). Both gradients are used by vesicular transporters as a driving force for the movement of neurotransmitter across the vesicular membrane and each transporter varies in their reliance on the chemical and electrical component of the gradient (Tabb *et al.* 1992; Nguyen *et al.* 1998; Bellocchio *et al.* 2000; Fremeau *et al.* 2002). Specifically, the activity of VAChT depends more on the ΔpH than on the $\Delta\Psi$. This could be due to the fact that for each ACh molecule transported into the vesicle two H^+ protons must be moved in the opposite direction (Nguyen *et al.* 1998). Thus, because ACh itself is protonated, each ACh that goes inside the vesicle drives the loss of two H^+ protons but only one positive charge. In contrast to VAChT, all three VGLUT transporters rely mostly on $\Delta\Psi$ (Maycox *et al.* 1988; Juge *et al.* 2006). Glu bears a negative charge in physiological pH. Hence, each molecule of Glu transported intraluminally is accompanied by the loss of a certain number of nH^+ protons (the exact stoichiometry remains unknown) and the loss of n + 1 number of positive charges. In other words, while ACh dissipates ΔpH faster than $\Delta\Psi$, the opposite is true for the transport of Glu.

Vesicular synergy between VAChT and VGLUT3 in CINs

Vesicular synergy for VAChT and VGLUT has been proposed in striatal CINs and in cholinergic neurons of the medial habenula (Gras *et al.* 2008; Frahm *et al.* 2015). Given that VAChT and VGLUT3 rely differently on pH as their driving force for vesicular filling, these two transporters may work synergistically if present on the same synaptic vesicle. Because the ΔpH dissipates twice as fast as the $\Delta\Psi$ during ACh transport (loss of two H^+ and only one charge per each ACh molecule), additional transport of anions intraluminally is needed to restore the balance. It was suggested that, among other mechanisms, it is the transport of negatively charged Glu into the same vesicle that can play this balancing role (Gras *et al.* 2008). Of course, the opposite view on the relationship between VAChT and VGLUT (VAChT facilitates the function of VGLUT by compensating the faster dissipation of $\Delta\Psi$ VGLUT) is also possible (Frahm *et al.* 2015).

Vesicular synergy between VAChT and VGLUT requires both transporters to be located on the same synaptic vesicles, and it is still debatable whether this is the case in CINs. Initial data in the striatum suggested that at least one population of vesicles can express both VGLUT3 and VAChT in the same vesicles (Gras *et al.* 2008; Nelson *et al.* 2014). In particular, Nelson *et al.* (2014) show that elimination of VGLUT3 diminishes post-synaptic responses activated either by Glu and ACh. It is not yet clear how knockout of VGLUT3 can reduce cholinergic signaling. In the medial habenula, vesicular synergy between VAChT and VGLUT1 has been suggested and co-localization of these two transporters on the same synaptic vesicles was documented using electron microscopy and immunoprecipitation (Ren *et al.* 2011; Frahm *et al.* 2015). However, optogenetic activation of ChAT expressing neurons in the medial habenula elicited either a slow cholinergic or fast glutamatergic post-synaptic response depending on the frequency of stimulation (Ren *et al.* 2011). This may indicate that ACh and Glu can be released separately from different pools of synaptic vesicles. Alternatively, it can mean that high frequency stimulation is required to release a sufficient number of vesicles necessary for slow volume transmission by ACh. In contrast, perhaps a single pulse stimulation is adequate to release enough Glu to elicit synaptic transmission. However, currently, we do not have sufficient evidence to confirm or exclude the possibility that VAChT and VGLUT3 are expressed only at the same synaptic vesicles in CINs, or that there are different pools of vesicles that express predominantly one or the other transporter. It is important to note that there are other examples in which multiple neurotransmitters released by the same neurons can be effectively separated in time and space, including neurons from the VTA releasing GABA and Glu in the lateral habenula and VTA neurons releasing dopamine and Glu in the nucleus accumbens (Root *et al.* 2014; Zhang *et al.* 2015).

VAChT trafficking to synaptic vesicles has been extensively studied and a C-terminal di-leucine motif has been shown to be a predominant signal for endocytosis and trafficking (Liu and Edwards 1997; Tan *et al.* 1998; Krantz *et al.* 2000; Barbosa *et al.* 2002; Ferreira *et al.* 2005). Furthermore, some trafficking signals for VGLUT1 and VGLUT2 are known (Fremeau *et al.* 2004; Vinatier *et al.* 2006; Voglmaier *et al.* 2006). In contrast, we know little about specific mechanisms of VGLUT3 trafficking (Fremeau *et al.* 2002; Gras *et al.* 2002; Voglmaier *et al.* 2006; Gagnon and Parent 2014). If VAChT and VGLUT3 were to use the same sets of mechanisms for their trafficking, it is possible that they could compete for the synaptic protein interactions required for endocytosis and vesicular targeting. In contrast, if these proteins use a distinct set of signals for their trafficking they may be directed to distinct populations of vesicles. It is therefore likely that there may be populations of vesicles containing both transporters and also pools of vesicles that express one or the other transporter. Defining the contributions of these vesicle pools and the rules that guide VAChT and VGLUT3 trafficking will be critical for understanding these processes.

Although the precise mechanisms regulating ACh/Glu co-transmission are still unknown, the striatal output neurons,

medium spiny neurons (MSNs), can be modulated by the two neurotransmitters released by CINs (see Fig. 1) (Witten *et al.* 2010; Higley *et al.* 2011). MSNs are commonly divided into two groups. The first consists of D1 expressing MSNs that send projections directly to output nuclei of the basal ganglia including the substantia nigra pars reticulata and the internal portion of the globus pallidus. The second consists of D2 expressing MSNs that reach the output nuclei indirectly (multisynaptically) through the external portion of the globus pallidus and the subthalamic nucleus (Gerfen *et al.* 1990; Wu *et al.* 2000). Both types of MSNs can be modulated by ACh through mAChRs. Both D1 and D2 MSNs express Gq-coupled (activating) M1 and Gi-coupled (inhibiting) M4 mAChRs, with M4 being more abundant on the D1 MSNs (Bernard *et al.* 1992; Yan *et al.* 2001). In addition, the excitability of both types of MSNs can be influenced by CINs indirectly, through the action of GABAergic interneurons that express nAChRs (English *et al.* 2011; Faust *et al.* 2016). Also, all striatal glutamatergic inputs (namely from the cortex and thalamus) express nAChR which help facilitate Glu release (Girasole and Nelson 2015). Figure 1 summarizes all of these potential mechanisms.

Another significant role striatal CINs play is to regulate dopamine release. Dopaminergic nerve terminals from the VTA and substantia nigra pars compacta express nAChRs, which allow ACh to positively modulate dopamine release (Exley and Cragg 2008; Threlfell *et al.* 2012). *In vivo* studies confirmed the role ACh plays in dopamine release, as mice that cannot secrete ACh from CINs exhibit a significant decrease in dopamine efflux in the nucleus accumbens (Sakae *et al.* 2015). In addition, CIN-secreted Glu also modulates accumbal dopamine release (Cachope *et al.* 2012; Sakae *et al.* 2015). Mice with a global disruption of VGLUT3 show a significant increase in striatal dopamine release and the same transient increase occurs if metabotropic Glu receptor (mGluR) antagonists are applied to the nucleus accumbens of control mice (Sakae *et al.* 2015). Thus, ACh and Glu released from CINs seem to exert opposing effects on dopamine release, whereas ACh (through nAChRs) stimulates dopamine release, Glu (through mGluRs) inhibits dopamine release (Sakae *et al.* 2015).

Physiological role of VAChT and VGLUT3

CINs have been shown to be key modulators of striatal circuits and striatum-dependent behaviors. Studies targeting CINs through ablation or optogenetics have suggested that ACh regulates spontaneous motor activity and reward-related behaviors (Hikida *et al.* 2001; Kitabatake *et al.* 2003; Witten *et al.* 2010). However, these studies did not separate the effect of VAChT and VGLUT3 mediated co-transmission, and consequently, there is a gap in our knowledge about the functional role of CINs ACh/Glu co-transmission. Recently, the development of novel genetic tools has started to reveal the significance of CINs co-transmission.

Global elimination of VGLUT3 in mice induces hyperlocomotion (Gras *et al.* 2008; Sakae *et al.* 2015). Given that VGLUT3-KO mice showed decreased ACh synaptic loading in CINs, this behavior was originally attributed to VGLUT3 influencing ACh release (Gras *et al.* 2008). However, it must be considered that VGLUT3-KO animals suffer from deficits including seizures, deafness and pain sensations that may contribute to compensatory changes in dopamine and other brain systems and could also affect behavior (Seal *et al.* 2008, 2009).

Studies in mice that cannot release ACh from CINs show no alterations in locomotor activity thereby suggesting that VGLUT3-mediated Glu release from CINs may play a role in locomotion (Guzman *et al.* 2011). In fact, hyperactivity induced by loss of VGLUT3 may be influenced by the role CINs-secreted Glu has in MSN excitability and/or in dopaminergic signaling (Higley *et al.* 2011; Sakae *et al.* 2015). Notably, elimination of VGLUT3 causes an increase in dopamine secretion and increased activation of MSNs that express D1 receptors as well as an increase in dendritic spine density and corticostriatal activity in the nucleus accumbens. In contrast to MSNs that express D1 receptors, those that express D2 receptors are less affected (Sakae *et al.* 2015). Interestingly, even though mice with elimination of VAChT from CINs show a decrease in dopamine secretion, they do not show any alteration in locomotion (Guzman *et al.* 2011; Sakae *et al.* 2015). This result might be related to the fact that these mutants show increased responses to direct activation of both D1 and D2 dopamine receptors, suggesting compensatory mechanisms (Guzman *et al.* 2011).

The reinforcing properties of cocaine rely on dopaminergic transmission and consequently bidirectional regulation of dopamine release by CINs may influence cocaine responses. Consistent with this hypothesis, global elimination of VGLUT3 changes the response of mice to the rewarding properties of cocaine (Gras *et al.* 2008; Sakae *et al.* 2015). VGLUT3-KO mice have an increased initial reaction to cocaine and do not further sensitize to the drug. Furthermore, VGLUT3-KO mice have increased cocaine self-administration and are more vulnerable to relapse. Interestingly, a higher rate of non-synonymous mutations in the VGLUT3 gene (SLC17A8) has been found in individuals suffering from severe addiction (Sakae *et al.* 2015). Consequently, it is possible that drug seeking behavior in certain human populations may be correlated with allelic variation in VGLUT3. Together, these results suggest that Glu released from VGLUT3-expressing neurons normally blunts the reinforcing properties of cocaine and therefore cocaine-induced behaviors (Sakae *et al.* 2015). In contrast, ACh released from CINs does not extensively influence cocaine related endophenotypes (Guzman *et al.* 2011). Striatal VAChT-KO mice exhibit behavioral sensitization and

cocaine preference to the same degree as control mice. This unexpected result might also be related to the fact that striatal VAChT-KO mice have an increased response to D1 and D2 agonists, which suggests that decreased dopamine release in these mutants was compensated by increased dopamine receptor responses. It is also possible that the relative ratios of striatal cholinergic and glutamatergic tone can mediate output signaling; hence, changing the ratio between ACh/Glu may itself change circuit function.

Co-transmission by cholinergic neurons beyond the striatum

Co-transmission also occurs in other cholinergic neurons. For instance, optogenetic studies have shown that some basal forebrain cholinergic neurons present co-transmission of GABA and ACh (Saunders *et al.* 2015) and retrograde tracing, and *in situ* hybridization has indicated that VAChT and VGLUT3 are co-expressed in basal forebrain cholinergic neurons that project to the basolateral amygdala (Nickerson Poulin *et al.* 2006). However, the physiological role for this co-transmission is still unknown. It has also been shown that ACh and GABA are co-released from cholinergic starburst amacrine cells of the retina (Lee *et al.* 2010). Co-transmission is also evident in the medial habenula where VAChT is co-expressed with VGLUT1 (Ren *et al.* 2011; Frahm *et al.* 2015). VAChT and VGLUT1 demonstrate vesicular synergy and in conjunction regulate nicotine dependence, specifically desensitization to nicotine reward and withdrawal symptoms (Frahm *et al.* 2015). These results suggest that co-transmission is likely a common feature of cholinergic neurons, and therefore, it is possible that most cholinergic neurons in the brain can modulate circuits by secreting another neurotransmitter in addition to ACh.

VGLUT3 and VAChT in motor diseases and addiction

Changes in the striatum are involved in a number of motor diseases, including Parkinson's disease (PD), Huntington's disease and dystonia. PD is commonly associated with changes in the brain's dopaminergic system, however, recent research has focused on how the cholinergic system also plays a role in disease progression (see review Perez-Lloret *et al.* 2016). Notably, degeneration of cholinergic neurons in the pedunculopontine tegmental nucleus is associated with mobility/gait impairments and degeneration of basal forebrain cholinergic neurons is associated with cognitive decline in PD patients (Bohnen *et al.* 2006; Müller *et al.* 2015). Additionally, it has been shown that cognitive decline in PD is correlated with lower cortical levels of ChAT and nAChRs (Aubert *et al.* 1992; Mattila *et al.* 2001).

In PD, dopaminergic neurons that project to the dorsal striatum degenerate leading to an imbalance of dopaminergic inputs and cholinergic innervation in the striatum. CINs are key to attenuating the imbalance, and correspondingly, optogenetic inhibition of CINs alleviates motor deficits in a PD mouse model (Maurice *et al.* 2015; Pienaar *et al.* 2015; Ztaou *et al.* 2016). Pharmacological approaches have also been used to influence CINs activity. For instance, preclinical studies showed that M4 mAChR antagonists can restore altered dopamine-ACh balance in PD (Mayorga *et al.* 1999; Karasawa *et al.* 2003). In addition, intrastriatal administration of antagonists for M1 and M4 mAChRs alleviated motor deficits in PD mouse models, further confirming that altered cholinergic transmission is key in the development of PD motor symptoms (Ztaou *et al.* 2016). More recently, research has focused on altering Glu transmission in the striatum. For instance, it has been shown that *N*-methyl-D-aspartate receptor antagonists targeting the striatum can improve PD symptoms in animal models (Carroll *et al.* 1995; Mitchell *et al.* 1995; Nash and Brotchie 2002). Similarly, targeting striatal metabotropic mGLURs has also been shown to ameliorate rigidity in PD (Konieczny *et al.* 1998; Dawson *et al.* 2000). In particular, antagonists for mGLUR5 (expressed by CINs) improve motor performance and restore reaction time in PD mouse models by influencing ACh release from CINs (Breysse *et al.* 2002).

Altering VAChT and VGLUT3 expression/function in CINs may prove beneficial in the treatment of PD. A PD mouse model with a global knockout of VGLUT3 showed improved motor functioning, likely, because the inhibitory effect of Glu on dopamine release was diminished (Cachope *et al.* 2012; Divito *et al.* 2015; Sakae *et al.* 2015). Likewise, sensorimotor deficits in PD are often associated with a decrease in striatal dopamine release and subsequent over-activation of CINs (Fink-Jensen *et al.* 2011). Over-activation of CINs can be modeled by haloperidol administration, which, in mice, induces immobility that closely resembles the muscular rigidity and postural instability associated with PD. Sakae *et al.* (2015) have shown that catalepsy induced by haloperidol is attenuated in VGLUT3-KO mice and unpublished data from our laboratory shows that striatal VAChT-KO mice also show decreased haloperidol-induced catalepsy. It remains unknown if this phenotype is as a result of decreased ACh or Glu, as the deletion of each neurotransmitter's transporter may decrease the release of the other neurotransmitter (Gras *et al.* 2008; Frahm *et al.* 2015). Furthermore, mice with fewer cholinergic neurons in the striatum are less responsive to haloperidol (Hitzemann *et al.* 1993). Thus, minimizing CINs over-activation by targeting VAChT and/or VGLUT3 could potentially reduce sensorimotor symptoms of PD.

Accumulating evidence suggests that CINs play a role in the development of levodopa-induced dyskinesia (LID) (Ding *et al.* 2011; Won *et al.* 2014; Divito *et al.* 2015; Gangarossa *et al.* 2016). Levodopa significantly alleviates motor deficits associated with PD, but prolonged therapy

leads to LID, characterized by abnormal and involuntary movements such as dystonia and chorea. It has been shown that after decreased striatal dopaminergic innervation, as observed in PD, CINs lose autoreceptor control of firing (Ding *et al.* 2006). In PD mouse models, repeated treatment with levodopa enhances basal firing of CINs and also increases the sensitivity of CINs to dopamine. It has been suggested that these changes, known to be mediated by extracellular signal-regulated kinase activation within CINs, contribute to the expression of LID (Ding *et al.* 2011). Additionally, there is evidence that antagonizing cholinergic tone may reduce LID. For instance, ablation of CINs attenuates LID in Parkinsonian mice (Won *et al.* 2014). Muscarinic receptor antagonists reduce LID (Ding *et al.* 2011). Likewise, α4β2 and α6β2 KO mice have decreased baseline LID and nicotinic antagonists also decrease LID (Quik *et al.* 2013; Quik *et al.*, 2014). Furthermore, chronic application of nicotine can attenuate LID in monkey, rat and mouse models of PD, as repeated exposure to nicotine desensitizes nicotinic receptors thereby decreasing cholinergic tone (Quik *et al.*, 2007; Bordia *et al.* 2008, 2010).

Interestingly, Glu released from CINs seem to have a major influence on the development of LID as VGLUT3-KO mice (Gangarossa *et al.* 2016) as well as striatal selective VGLUT3-KO mice (Divito *et al.* 2015), showed attenuated LID. In contrast, LID is not attenuated in mice with selective elimination of striatal VAChT (Gangarossa *et al.* 2016). These results suggest that striatal VAChT is not involved in the development of LID, whereas VGLUT3 may play an essential role. Additionally, these data suggest that modulation of VGLUT3 expression/function in CINs could benefit levodopa therapy, making the VGLUT3 transporter a promising therapeutic target for prevention of LID.

Altering VAChT and/or VGLUT3 expression/function in CINs may also be a beneficial therapeutic target for addiction. In drug addiction there is elevated dopaminergic transmission in the striatum, which may be in part regulated by CINs (Cachope *et al.* 2012; Threlfell *et al.* 2012; Sakae *et al.* 2015; see review by Volkow *et al.* 2011). For instance, in rats, there is a correlation between the percent of CINs activated in the nucleus accumbens and the amount of cocaine self-administered (Berlanga *et al.* 2003). Furthermore, ablation of CINs in the nucleus accumbens increases the sensitivity to the rewarding properties of cocaine in mice (Hikida *et al.* 2001). Clinical research has focused on investigating the role the cholinergic system plays in addiction and many pre-clinical studies have shown promising results with the use of acetylcholinesterase inhibitors (de la Garza and Johanson 1982; Andersen *et al.* 2007). Notably, acetylcholinesterase inhibitors were shown to decrease cocaine self-administration in monkeys (de la Garza and Johanson 1982). However, if cholinergic signaling in the striatum is selectively eliminated (striatal VAChT-KO) it does not extensively influence cocaine endophenotypes in mice (Guzman *et al.* 2011).

Conclusion

VGLUT3 and VAChT co-expression confers CINs the ability to release ACh and Glu, thus increasing the potential mechanisms by which CINs may regulate striatal function. CINs-secreted ACh and Glu appear to have distinct and complementary functional roles and understanding their unique functions in striatal activity may prove beneficial in the development of disease therapies. It is also possible that the ratios of Glu/ACh released by CINs, which may depend on patterns of stimulation that favor the secretion of one or the other neurotransmitter, might also function as relevant signals to activate different striatal functions. Understanding how these two neurotransmitters may be secreted by CINs may aid in the development of new pharmacological approaches for a variety of diseases that affect striatal functions, including motor diseases and addiction.

Acknowledgments and conflict of interest disclosure

Marco A.M. Prado is an handling editor for the Journal of Neurochemistry. The other authors have no conflict of interest to declare. This work was supported by the Canadian Institutes of Health Research (MOP 93651, MOP 136930, MOP 126000, MOP 89919), NSERC (402524-2013), Brain Canada, Weston Brain Institute, Canadian Foundation for Innovation, and Ontario research fund (M.A.M.P. and V.F.P.). O.K. received a scholarship from CIHR.

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