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Multiplexed Neurochemical Signaling by Neurons of the Ventral Tegmental Area

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Abstract

The ventral tegmental area (VTA) is an evolutionarily conserved structure that has roles in reward-seeking, safety-seeking, learning, motivation, and neuropsychiatric disorders such as addiction and depression. The involvement of the VTA in these various behaviors and disorders is paralleled by its diverse signaling mechanisms. Here we review recent advances in our understanding of neuronal diversity in the VTA with a focus on cell phenotypes that participate in 'multiplexed' neurotransmission involving distinct signaling mechanisms. First, we describe the cellular diversity within the VTA, including neurons capable of transmitting dopamine, glutamate or GABA as well as neurons capable of multiplexing combinations of these neurotransmitters. Next, we describe the complex synaptic architecture used by VTA neurons in order to accommodate the transmission of multiple transmitters. We specifically cover recent findings showing that VTA multiplexed neurotransmission may be mediated by either the segregation of dopamine and glutamate into distinct microdomains within a single axon or by the integration of glutamate and GABA into a single axon terminal. In addition, we discuss our current understanding of the functional role that these multiplexed signaling pathways have in the lateral habenula and the nucleus accumbens. Finally, we consider the putative roles of VTA multiplexed neurotransmission in synaptic plasticity and discuss how changes in VTA multiplexed neurons may relate to various psychopathologies including drug addiction and depression.

Keywords

reward; addiction; depression; aversion; co-transmission; dopamine; glutamate; GABA

Introduction

Midbrain dopamine neurons (DA) are most often associated with reward processing of both natural rewards (e.g., food, water, etc.) and drugs of abuse (Schultz, 2002; Wise, 2004; Sulzer, 2011). Over fifty years of intense research has led to the proposal that neurons

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belonging to the ventral tegmental area (VTA), which includes but is not limited to DA neurons, are paramount to reward processing. Many hypotheses have been put forward regarding the specific function of VTA DA neurons in reward processing, such as decision making (Salamone & Correa, 2002; Saddoris et al., 2015), flexible approach behaviors (Nicola, 2010), incentive salience (Berridge & Robinson, 1998; Berridge, 2007), and learning or the facilitation of memory formation (Adcock et al., 2006; Steinberg et al., 2013). However, several studies have also shown that VTA DA neurons are involved in the processing of aversive outcomes (Laviolette et al, 2002; Young, 2004; Pezze & Feldon, 2004; Brischoux et al., 2009; Lammel et al., 2012; Twining et al., 2014; Hennigan et al., 2015), fear (Abraham, Neve, & Lattal, 2014), aggression (Yu et al., 2014a,b), depression (Tidey & Miczek, 1996; Tye et al., 2013), and drug withdrawal (Grieder et al., 2014). Other hypotheses have proposed that VTA DA neurons play a more general role in processes such as associative learning (Brown et al., 2012), arousal (Horvitz, 2000), or general motivational salience and cognition (Bromberg-Martin et al., 2010).

The functional diversity associated with the VTA may be mediated, in part, by different VTA subpopulations of neurons. A particular advancement that may subserve the functional diversity of the VTA is the recent discovery of neurons that are capable of signaling using one or more neurotransmitters. In the present review, we cover recent literature on the diversity of VTA neuronal phenotypes as they relate to ‘multiplexed neurotransmission’. We refer the reader to recent comprehensive reviews detailing VTA cellular composition, VTA efferent and afferents, and VTA functions(Oades & Halliday, 1987; Fields et al., 2007; Ikemoto, 2007; Nair-Roberts et al., 2008; Morales & Pickel, 2012; Trudeau et al., 2014; Morales & Root 2014; Pignatelli and Bonci, 2015; Saunders et al., 2015; Luthi and Luscher, 2014). Moreover, the present review does not cover co-transmission of neurotransmitters and neuropeptides, which has long been known and recently reviewed (Morales & Pickel, 2012). Here, we use the phrase “multiplexed neurotransmission” to describe neurons that are capable of signaling using two or more neurotransmitters. In many circuits, our understanding of the specific mechanisms by which neurons utilize multiple neurotransmitters is limited. Thus, we have chosen the term multiplexed neurotransmission to encompass known and unknown mechanisms of co-release and co-transmission (e.g., Nusbaum et al., 2001; Mestikawy et al., 2011), while also allowing for the possibility of independent release of individual neurotransmitters either in time or space.

Cellular Diversity in the Ventral Tegmental Area

Following the discovery of DA as a chemical neurotransmitter in the brain (Montagu, 1957), the DAergic neurons in the “ventral tegmental area of Tsai” (Nauta, 1958) were identified by formaldehyde histofluorescence (Carlsson et al., 1962). These neurons, along with other catecholaminergic and serotonergic neurons throughout the brain were shown to comprise twelve discrete cell groups (labeled as A1-A12 groups; Dahlström & Fuxe, 1964). One feature of the A10 group, in particular, is the heterogeneous morphology among its neurons. Based on cytoarchitecture, the A10 region has been divided into two lateral nuclei [the Parabrachial Pigmented Nucleus (PBP) and Paranigral Nucleus (PN)], and three midline nuclei [the Rostral Linear Nucleus of the Raphe (RLi), Interfasicular Nucleus (IF), and Caudal Linear Nucleus (CLi)]. Traditionally, the VTA has been considered to include just

the lateral nuclei (PBP, PN) (Swanson, 1982), however, modern conceptions of VTA function have often included the midline nuclei (RLi, IF, CLi) as subnuclei of the VTA (Ikemoto, 2007; Nair-Roberts et al., 2008; Morales and Root, 2014). Thus, in this review, we use the term VTA to define the midbrain A10 structure containing lateral (PBP, PN) and midline nuclei (RLi, IF, CLi). The cellular heterogeneity within the VTA subnuclei, together with findings showing that a single A10 neuron rarely innervates multiple structures (Swanson, 1982; Takada and Hattori, 1987; Lammel et al., 2008; Hosp et al. 2015), suggests that the VTA utilizes highly specific projections from different sets of neurons.

Dopamine neurons, defined by the expression of tyrosine hydroxylase (TH) protein (Figure 1), are interspersed throughout all VTA nuclei, but are most prevalent in the lateral PBP and PN (Swanson, 1982; Ikemoto, 2007; Li et al., 2013). In addition to the co-expression of TH and aromatic decarboxylase (AADC), the majority of rat lateral PBP and lateral PN neurons co-express the dopamine transporter (DAT), D2 receptor (D2R), and vesicular monoamine transporter 2 (VMAT2) mRNA (Li et al., 2013). More medially within the rat PBP and PN, as well as within the RLi, CLi, and IF, subsets of TH-expressing neurons either express or lack different combinations of DAT, VMAT2, or D2 receptor (Li et al., 2013, reviewed in Morales and Root, 2014). Our understanding of diversity among DAergic neurons in other species than the rat is less understood. However, recent studies have shown that, while all VTA neurons in the rat VTA expressing TH mRNA co-express the TH protein, some mouse VTA neurons expressing TH mRNA lack TH protein (Yamaguchi et al., 2015). In addition, ventrally to the VTA within the interpeduncular nucleus, there is in the mouse, but not in the rat, a subpopulation of neurons expressing TH mRNA, but lacking TH protein (Yamaguchi et al., 2015; Lammel et al., 2015). So far, detailed molecular characterizations of VTA neurons of nonhuman primates or humans has not been reported.

Rat TH-expressing neurons within the lateral PBP and lateral PN have also been electrophysiologically characterized (so-called ‘primary’ neurons) based on their long-duration action potentials and hyperpolarization-activated cation currents (Grace & Onn, 1989). However, recent findings have shown that not all VTA TH-expressing neurons share these electrophysiological criteria (Margolis et al., 2006). In addition, although lack of direct electrophysiological responses to the *mu* opioid receptor agonist DAMGO has been proposed as a property shared by VTA DAergic neurons (Johnson and North, 1992), the VTA has a subpopulation of TH-expressing neurons that are directly excited or inhibited by DAMGO (Margolis et al., 2014). So far, it seems that hyperpolarization-activated cation currents, spike duration, inhibition by D2R agonist and other electrophysiological properties are unreliable predictors for the identification of all VTA DAergic neurons (Margolis et al., 2006), further supporting the heterogeneity of VTA DAergic neurons.

Along with DAergic neurons, γ-aminobutyric-acid (GABA) neurons are also present in the VTA (Nagai et al., 1983; Kosaka et al., 1987). These GABAergic neurons are relatively less prevalent than the DAergic neurons, and are identified by their expression of glutamic acid decarboxylase (GAD) 65 or 67 mRNA, isoforms of the enzyme involved in the synthesis of GABA. GABAergic VTA neurons are also identified by their expression of vesicular GABA transporter (VGaT) mRNA. Electrophysiologically, putative VTA GABAergic ‘secondary’ neurons have been characterized based on the observation that these cells are hyperpolarized

by mu opioid agonists (Johnson and North, 1992). As with VTA DAergic neurons, VTA GABAergic neurons are pharmacologically and electrophysiologically heterogeneous. For example, approximately 60% of VTA GABAergic neurons are inhibited by the *mu* opioid receptor agonist DAMGO, but all seem to be unaffected by the GABA-B agonist baclofen (Margolis et al., 2012). GABAergic neurons in the VTA are known to establish local inhibitory connections on DAergic neurons (Johnson & North, 1992; Omelchenko & Sesack, 2009), but have also been shown to project outside of the VTA to the ventral striatum (nAcc; Van Bockstaele & Pickel, 1995) basal forebrain (Taylor et al., 2014), the prefrontal cortex (Steffensen et al., 1998; Carr & Sesack, 2000), the lateral habenula (LHb; Stamatakis et al., 2013; Root et al., 2014a; Taylor et al., 2014; Lammel et al., 2015), lateral hypothalamus, preoptic area, and amygdala, as well as to structures in the thalamus, midbrain, pons and medulla (Taylor et al., 2014). GABAergic neurons are scattered throughout the A10 region, and although a detailed subregional mapping of these neurons has not been yet reported, a dense group of GABAergic neurons has been identified in an area ventro-caudal to the VTA, referred as the 'tail of the VTA' (tVTA; Kaufling et al., 2009) or the rostromedial tegmental area (RMTg; Jhou, 2005, 2009a, 2009b; Geisler et al., 2008; Lavezzi & Zahm, 2011). The GABAergic neurons of the tVTA/RMTg provide a major inhibitory control to VTA DAergic neurons (Kaufling et al., 2010; Matsui et al., 2011).

In addition to VTA DAergic and GABAergic neurons, early electrophysiological studies of the midbrain suggested the possibility of glutamatergic signaling by some VTA neurons (Wilson et al., 1982; Mercuri et al., 1985; Sulzer et al 1998; Joyce & Rapport, 2000; Chuhma et al., 2004; Ungless et al., 2004; Lavin et al., 2005; Chuhma et al., 2009). Anatomical identification of glutamatergic neurons has recently become possible due to the cloning of three distinct vesicular glutamate transporters (VGluT1, VGluT2, and VGluT3; Bellocchio et al., 1998; Bai et al., 2001; Fremeau et al., 2001, 2002; Fujiyama et al., 2001; Hayashi et al., 2001; Herzog et al., 2001; Takamori et al., 2000; Varoqui et al., 2002; Gras et al., 2002). By *in situ* hybridization, it has been demonstrated that some neurons within the VTA (Kawano et al., 2006; Yamaguchi et al., 2007; 2011), substantia nigra and retrorubral field (Yamaguchi et al., 2013) express VGluT2 mRNA, but not VGluT1 or VGluT3. The VTA-VGluT2 neurons are present in all A10 nuclei, but are particularly prevalent within midline nuclei (Yamaguchi et al., 2007, 2011). In fact, glutamatergic neurons outnumber the DAergic neurons in the rostral and medial portions of the VTA (Yamaguchi et al., 2007, 2011). Thus, these neurons represent a major subpopulation in certain parts of the VTA. Similar to VTA GABAergic neurons, VGluT2-glutamatergic neurons in the VTA establish local and extrinsic synapses (Dobi et al., 2010; Yamaguchi et al., 2011; Zhang et al., 2015; Wang et al., 2015). Specifically, glutamatergic VTA neurons establish local asymmetric synapses with both DAergic and non-DAergic neurons (Dobi et al., 2010; Wang et al., 2015). Additionally, glutamatergic VTA neurons project to other regions of the brain including the LHb (Root et al., 2014a,b), nAcc (Zhang et al., 2015), amygdala, basal forebrain, and prefrontal cortex (Hnasko et al., 2012; Taylor et al., 2014).

Moreover, increasing evidence indicates that subpopulations of VTA neurons are capable of releasing DA and GABA, or DA and glutamate (Kosaka et al., 1987; Sulzer et al., 1998; Rayport, 2001; Dal Bo et al., 2004; Trudeau, 2004; Seutin, 2005; Lapish et al, 2006; Yamaguchi et al., 2007, 2011; Hnasko et al., 2010; Tritsch et al, 2012; Li et al., 2013;

Mingote et al., 2015). Recent work from our laboratory has shown that there is a subset of VTA neurons capable of co-releasing DA and glutamate or glutamate and GABA (Zhang et al., 2015; Root et al., 2014a).

Multiplexed neurotransmission by some VTA neurons and associated circuits is a property shared by other brain structures. For instance, glutamate and GABA co-neurotransmission has been reported in epilepsy models within mossy fiber terminals (Gutiérrez et al., 2003, Gutiérrez 2003, 2005; Trudeau & Gutiérrez , 2007; Münster-Wandowski et al., 2013), developing medial trapezoid body terminals from the lateral superior olive (Gillespie et al. 2005; Noh et al., 2010), entopeduncular nucleus projection to the LHb (Shabel et al., 2014), and cortex (Fattorini et al., 2015). In addition, there is evidence for GABA and DA co-transmission in by substantia nigra pars compacta neurons as well as retinal amacrine neurons (Tritsch et al. 2012; Hirasawa et al., 2012). Other forms of neurotransmission include GABA and histamine by hypothalamic neurons (Yu et al., 2015), glutamate and acetylcholine co-transmission in striatal interneurons or medial habenula neurons (Gras et al., 2008; Ren et al., 2011; Higley et al., 2011; Nelson et al., 2014), GABA and acetylcholine co-transmission in corticopetal globus pallidus neurons (Saunders et al., 2015).

Based on the discovery that some VTA neurons exhibit multiple vesicular transporters, we have applied ultrastructural and electrophysiological approaches to determine the possible cellular mechanisms by which multiple neurotransmitters are released at the synaptic level. In the process of answering this question, we have revealed ultrastructural architectures suggesting that glutamate and GABA neuronal signaling by VTA neurons can be integrated into a single complex terminal with spatially distinct synaptic release sites for glutamate or GABA (Fig. 1) (Root et al., 2014a). We have also revealed that DA and glutamate neuronal signaling by VTA neurons can be segregated to distinct microdomains within the same axon (Fig 2), allowing for the spatially distinct release of DA or glutamate (Zhang et al., 2015).

Multiplexed Signaling by VTA-GluT2 Neurons

Following the discovery of VTA-VGluT2 neurons, further characterization of these neurons demonstrated that they are very diverse in their molecular composition, signaling properties and neuronal connectivity. Whereas many VTA-VGluT2 neurons lack both DAergic and GABAergic markers, there are subpopulations of VTA-VGluT2 neurons that co-express molecules responsible for the synthesis or vesicular transport of either DA or GABA (Li et al., 2013; Root et al., 2014a). Although the distinct targets for VTA-VGluT2 neurons remains to be determined, emerging evidence suggests preferential target sites for specific subsets of VTA-VGluT2 neurons. For instance, by a combination of retrograde tract tracing and *in situ* hybridization, it has been demonstrated that VTA VGluT2$^{(+)}$/GAD$^{(+)}$ neurons provide the major mesohabenular input to the LHb (Root et al., 2014a), and by contrast, VTA VGluT2$^{(+)}$/TH$^{(+)}$ neurons largely target the nAcc shell (Yamaguchi et al., 2011). While the molecular characterization of mesohabenular and mesoaccumbens neurons provides support for multiplexed signaling by some VTA neurons, this characterization does not provide information on the cellular mechanisms by which these neurons release more than one neurotransmitter. As discussed below, recent findings obtained by a combination of cell-type specific anterograde tract tracing and immuno-electron microscopy have demonstrated

that the VTA-VGluT2 neurons establish unique synaptic architectures for multiplexed signaling in both the LHb (Root et al., 2014a) and the nAcc (Zhang et al., 2015).

Multiplexed Signaling by Mesohabenular VGluT2 neurons

Recently available viral vectors and transgenic mice have facilitated the elucidation of the cellular mechanisms by which some VTA neurons use two distinct signaling molecules. To determine the synaptic ultrastructural features of mesohabenular axons we have taken advantage of the cell-specific viral tagging of VTA-VGluT2 neurons through the Cre-dependent expression of mCherry tethered to channelrhodopsin (ChR2) under the regulation of the VGluT2 promoter in VGluT2::Cre mice). By applying cell-specific tagging we estimated that more than 70% of mesohabenular axon terminals within the LHb co-express VGluT2 and VGaT (Root et al., 2014a). Moreover, we estimated that within the LHb both VGluT2 and VGaT are present in half of the total population of axon terminals, some of which derive from brain structures others than the VTA (e.g., from the basal ganglia; Shabel et al., 2014). While the presence of VGluT2 and VGaT within the same axon terminal has been established by immuno-electron microscopy, it remains to be determined whether each vesicular transporter is integrated into the membrane of distinct vesicles or in the same vesicular membrane. However, ultrastructural findings of the synaptic composition of individual $VGluT2^{(+)}/VGaT^{(+)}$ axon terminals show that the plasma membrane of single $VGluT2^{(+)}/VGaT^{(+)}$ axon terminals participates in the formation of both asymmetric and symmetric synapses, suggesting that glutamate-signaling is segregated to the asymmetric synapse and GABA-signaling to the symmetric (Figure 2).

In addition to the suggestion that asymmetric synapses participate in excitatory neurotransmission (Peters and Palay, 1996), GluR1-containing AMPA receptors are located in the membrane postsynaptic to the mesohabenular asymmetric synapses, but not to the symmetric synapses (Root et al., 2014a). In contrast, consistent with the suggestion that symmetric synapses participate in inhibitory neurotransmission (Peters and Palay, 1996), GABA-A receptors are located postsynaptically to the mesohabenular symmetric synapses, but not to the asymmetric synapses (Root et al., 2014a). The selective postsynaptic distribution of GluR1 to $VGluT2^{(+)}/VGaT^{(+)}$ mesohabenular terminals making asymmetric synapses and GABA-A to those making symmetric synapses indicates that $VGluT2^{(+)}/VGaT^{(+)}$ terminals release glutamate at the asymmetric synapse, and GABA at the symmetric synapses (Root et al., 2014a). Besides the formation of asymmetric and symmetric synapses by individual $VGluT2^{(+)}/VGaT^{(+)}$ axon terminals, both synapses may target separate postsynaptic dendritic spines or dendritic shafts or share a common postsynaptic dendrite. These ultrastructural findings underlie the multiplexed signaling and potential neuroplastic capacity endowed by dual $VGluT2^{(+)}/VGaT^{(+)}$ axon terminals from the VTA to the LHb.

Multiplexed Signaling by Mesoaccumbens VGluT2-DA neurons

Pioneering electrophysiological *in vitro* studies demonstrated that dopamine neurons in primary culture have the capability to release glutamate, which lead to the hypothesis that midbrain neurons co-transmit DA and glutamate (Sulzer et al., 1998; Joyce and Rayport 2000; Bourque and Trudeau, 2000). Since then, anatomical studies demonstrated that subsets

of TH-positive neurons co-express VGluT2 mRNA throughout the brain (Stornetta et al., 2002; Kawano et al., 2006), including some TH-neurons within the midline nuclei of the VTA in rats (Yamaguchi et al., 2007, 2011) and mice (Yamaguchi et al., 2015). Moreover, findings from optogenetic electrophysiological *ex vivo* recordings have shown that the VGluT2$^{(+)}$/TH$^{(+)}$ mesoaccumbens neurons use glutamate as signaling molecule (Stuber et al., 2010; Tecuapetla et al., 2010; Zhang et al., 2015; Mingote et al., 2015), and recent *in vitro* voltammetry measurements have shown that VGluT2-TH co-expressing neurons that project to nAcc release DA (Zhang et al., 2015).

So far two opposing hypotheses have been proposed to mediate the dual glutamate and DA signaling by VGluT2$^{(+)}$/TH$^{(+)}$ mesoaccumbens neurons. One of them proposes that glutamate and DA coexist (and are co-released) from the same pool of vesicles (Hnasko et al., 2010, 2012). This hypothesis has been based on the co-immunoprecipitation of VMAT2 and VGluT2 from nAcc preparations (Hnasko et al., 2010). In clear contrast, a recent study has shown lack of VMAT2 and VGluT2 co-immunoprecipitation when ultrastructurally confirmed pure nAcc synaptic vesicles were used (Zhang et al., 2015). These recent findings have led to the hypothesis that dual VGluT2$^{(+)}$/TH$^{(+)}$ mesoaccumbens neurons contain independent pools of vesicles for the accumulation of either DA or glutamate. Moreover, immuno-electron microscopy findings from intact brain tissue have shown that TH and VGluT2 do not coexist in the same axon terminal in the nAcc of either adult rats (Berube-Carriere et al., 2009; Moss et al., 2011) or mice of any age (Berube-Carriere et al., 2012). These immuno-electron microscopy findings are consistent with nAcc structural studies published over the last 40 years showing that axonal compartments engaged in excitatory signaling do not overlap with axonal compartments engaged in DA signaling (reviewed in Morales and Pickel, 2012). The lack of overlap between DA-vesicles and glutamate-vesicles may result from their segregation into two different sets of axons or segregation into micro-domains within the same axon. Although the possibility of vesicular segregation to different axons has not been discarded, recent immuno-electron microscopy findings indicate that VGluT2-vesicles from VGluT2$^{(+)}$/TH$^{(+)}$ neurons are located in axon terminals that establish asymmetric synapses, and that axonal segments adjacent to these VGluT2-axonal terminals contain TH, VMAT2 and DAT (Zhang et al., 2015). The segregation between glutamate-vesicles and DA-vesicles within the same axon appears to be highly regulated, as *in vivo* overexpression of VMAT2, in the rat, does not disrupt the segregation between these two different types of vesicles. Moreover, the vesicular segregation by VGluT2$^{(+)}$/TH$^{(+)}$ mesoaccumbens neurons is maintained in the nAcc of transgenic mice expressing ChR2 (following their viral mediated expression in VTA neurons under the regulation of either the TH-promoter or VGluT2-promoter; Zhang et al., 2015).

In summary, the characterization of mesoaccumbens and mesohabenular ultrastructural features together with the characterization of their electrophysiological and chemical properties have provided evidence for multiplexed signaling by VTA-VGluT2 neurons. These findings have demonstrated that dual rodent mesoaccumbens VGluT2$^{(+)}$/TH$^{(+)}$ neurons have adjacent cellular compartments that participate in independent glutamate-signaling and DA-signaling (Zhang et al., 2015). In contrast, the dual rodent mesohabenular VGluT2$^{(+)}$/GABA$^{(+)}$ neurons concentrate both glutamate-vesicles and GABA-vesicles within a single axon terminal that establishes both excitatory and inhibitory synapses (Root

et al., 2014a). Future studies are necessary to determine the molecular and signaling mechanisms involved in the sorting and retention of VGluT2-vesicles, GABA-vesicles (VGaT) and DA-vesicles (VMAT2) to specific microdomains within the same axon. Additional studies are also necessary to determine the extent to which the multiplexed signaling by VTA neurons is affected in brain disorders, such as addiction and depression.

Functional Diversity by VTA Neurons

The functional diversity of VTA neurons has been constantly updated (Unlgess, 2004; Stamatakis et al., 2013; Root et al., 2014b; Mejias-Aponte et al., 2015; Eddine et al., 2015; Kotecki et al., 2015; Beier et al., 2015). As detailed above, the multiplexed neurotransmission of the VTA-VGluT2 neurons is an emerging factor involved in the complexity of VTA function. Based on observations that different combinations of neurotransmitters are multiplexed throughout the brain (Trudeau 2004; Gillespie et al. 2005; Zhou et al., 2005; Gras et al., 2008; Noh et al., 2010; Higley et al., 2011; Tritsch et al. 2012; Hnasko and Edwards, 2012 Münster-Wandowski et al., 2013; Nelson et al., 2014; Root et al., 2014a; Shabel et al., 2014; Qi et al., 2014; Zhang et al., 2015; Fattorini et al., 2015; Saunders et al., 2015), we suggest that multiplexed neurotransmission conveys distinct messages depending on the neurotransmitter content of each circuit, momentary singular or multiplexed signaling, and perhaps even the time scale of neurotransmitter function. Furthermore, we speculate that changes in the influence of one or more of the multiplexed neurotransmitters, by way of either presynaptic of postsynaptic changes, may result *from* and result *in* observable changes in behavior. Recent advances in the functional diversity within the VTA neurons targeting the LHb or nAcc will be presented in the following paragraphs.

Functional Diversity by VGluT2 Mesohabenular Neurons

An example of the circuit specific nature of multiplexed neurotransmission is found in the LHb. By combination of optogenetics and electrophysiology, we have shown that activation of the mesohabenular pathway evokes release of GABA and glutamate, and that the co-transmitted GABA is capable of shunting the co-transmitted glutamate-mediated currents (Root et al., 2014a). Therefore, the simultaneous release of glutamate and GABA may be a mechanism by which the glutamatergic excitation within the LHb is autoregulated by the co-transmitted GABA. *In vivo* recordings of LHb neurons following ChR2 activation of mesohabenular fibers have shown that this activation results in GABA-induced decreases in the firing rates of most recorded LHb neurons, and in glutamate-induced increases in firing rates in fewer neurons. In addition, secondary firing patterns are often observed in which initial increases in firing rates are followed by decreased firing rates or initial decreases in firing rates are followed by increased firing rates. The *in vivo* recordings of LHb neurons suggest that stimulation on mesohabenular fibers induces predominantly GABAergic neurotransmission. Nevertheless, the observed secondary firing patterns suggest that signaling might also occur over multiple time-scales or that the contribution of each neurotransmitter might be shifted in response to specific stimuli. For instance, rat depression models reduce GABA signaling from the multiplexed glutamate-GABA inputs to LHb from entopeduncular neurons (Shabel et al., 2014).

In addition, findings from combinations of optogenetics and behavioral analysis have shown that mesohabenular stimulation of fibers from different pools of VTA neurons, including multiplexed signaling neurons, promotes different behaviors. For instance, a LHb GABA receptor-mediated reward is evoked by mesohabenular stimulation of fibers expressing ChR2 under the TH-promoter (Stamatakis et al., 2013), likely to include activation of fibers from VGluT2$^{(+)}$/GAD$^{(+)}$/TH$^{(+)}$, VGluT2$^{(+)}$/TH$^{(+)}$/GAD$^{(-)}$, and VGluT2$^{(-)}$/GAD$^{(+)}$/TH$^{(+)}$ mesohabenular neurons. However, a mild reward is evoked by mesohabenular stimulation of fibers expressing ChR2 under the GAD2-promoter (Lammel et al., 2015), likely to include activation of fiber from VGluT2$^{(+)}$/GAD$^{(+)}$/TH$^{(+)}$, VGluT2$^{(+)}$/GAD$^{(+)}$/TH$^{(-)}$, VGluT2$^{(-)}$/GAD$^{(+)}$/TH$^{(-)}$, and VGluT2$^{(-)}$/GAD$^{(+)}$/TH$^{(+)}$ mesohabenular neurons. In contrast, a LHb glutamate receptor-mediated conditioned place aversion is evoked by mesohabenular stimulation of fibers expressing ChR2 under the VGluT2-promoter (Root et al., 2014b; Lammel et al., 2015), likely to include activation of fibers from VGluT2$^{(+)}$/GAD$^{(+)}$/TH$^{(-)}$, VGluT2$^{(+)}$/GAD$^{(+)}$/TH$^{(+)}$, and VGluT2$^{(+)}$/GAD$^{(-)}$ neurons. These behavioral findings underlie the need for targeted intersectional approaches to dissect the behavioral contributions of each mesohabenular neuronal phenotype.

Multiplexed neurotransmission may affect neuronal regulation over multiple time scales, for instance “prolonged slow-actions” by monoamines (i.e., serotonin or dopamine) and “fast short actions” provided by the concomitant release of glutamate or GABA. This multiple time scale neurotransmission, by neurons endowed with the capacity for multiplexed signaling, may be found in a single DA-glutamate mesoaccumbens axon establishing segregated postsynaptic targets for DA- or glutamate-signaling (Zhang et al., 2015). Although the extent to which these mesoaccumbens DA-glutamate fibers participate in the neurobiology of drugs of abuse remains to be determined, we speculate that these axons may participate in the regulation of neuronal activity in cocaine self-administration. Specifically, electrophysiological recordings have shown that nAcc neurons exhibit rapid phasic firing patterns to related cues and actions to obtain the drug (Peoples et al., 1998; Ghitza et al., 2003, 2004, 2006; Fabbricatore et al., 2009, 2010; Coffey and Barker et al., 2015). The nAcc neurons also exhibit slow-phasic and tonic changes in firing rate that correlate with the pharmacological effects of cocaine, and do not correlate with the rapid phasic firing patterns (Fabbricatore et al., 2010). Furthermore, slow phasic pharmacologic and rapid phasic behavioral firing patterns are similarly processed in downstream accumbal targets (ventral pallidum and lateral preoptic area; Root et al., 2012, 2013; Barker et al., 2014). These dissociable fast and slow signaling patterns in the accumbens are consistent with findings suggesting that glutamate and dopamine each have specific roles in addiction-associated behaviors (Birgner et al., 2010; Alsiö et al., 2011).

Functional Diversity by TH Mesohabenular Neurons

Phenotypic characterizations of VTA-TH neurons have revealed the heterogeneous expression of several transcripts, some of which may be expressed transiently during development or may be induced in the adult brain in response to insults (e.g., drugs, stress, illness). In addition, some of these transcripts may not be translated into detectable protein levels under normal conditions, instead, this translation may depend on VTA circuit activity or be induced as a result of various brain insults (e.g., Bayer and Pickel, 1990, 1991; Garcia-

Perez et al., 2014). For instance, we have identified a subset of VTA neurons, in wild type mice, that express TH mRNA, but lack detectable levels of TH-protein in cell bodies, dendrites and axons. Some of these neurons send projections to the LHb (Yamaguchi et al., 2015). In agreement with these findings, revealing TH-mRNA$^{(+)}$/TH-protein$^{(-)}$ mesohabenular neurons in wild type mice, viral-induced expression of reporter genes (i.e., green-fluorescent-protein under the regulation of the TH-promoter) within the VTA of *TH*::cre mice has shown expression of fluorescent fibers without detectable TH-protein in the LHb (Stamatakis et al., 2013; Lammel et al., 2015; Stuber et al., 2015). These findings underlie the need to better characterize the VTA cellular composition in wild type mice, and reveal that expression of reporter genes in the mouse under the control of the TH-promoter does not guarantee the selective manipulation or mapping of DA projections.

In contrast to the mouse TH-mRNA$^{(+)}$/TH-protein$^{(-)}$ mesohabenular neurons, subsets of rat mesohabenular neurons contain detectable levels of TH-protein in the cell bodies, dendrites and axons (Root et al., 2015). However, these rat TH-protein$^{(+)}$ mesohabenular neurons rarely co-express VMAT2-mRNA in their cell bodies or VMAT2-protein in their axon terminals in LHb (Root et al., 2015). The lack of VMAT2 within mesohabenular neurons has also been documented in the mouse (Stamatakis et al., 2013; Lammel et al., 2015). Overall, these findings provide crucial information when considering the functional properties of multi-neurotransmitter neurons, as they demonstrate that specific neuronal subsets have the capacity to synthesize DA but lack the capability to package DA into synaptic vesicles for traditional vesicular release. These finding are intriguing because DA has been detected in LHb homogenates (Phillipson and Pycock, 1982; Root et al., 2015), D2 receptors have been found in a subset of LHb neurons (Aizawa et al., 2012), and exogenous DA evokes currents in LHb neurons, currents that are eliminated by D2 or D4-receptor antagonists (Jhou et al., 2013; Good et al., 2013: Root et al., 2015). However, recordings of LHb neurons from rats treated with toxins for either the elimination of VTA-TH neurons or noradrenergic fibers have demonstrated that noradrenergic habenular afferents specifically activate D4-receptors in the LHb neurons and that VTA TH-expressing neurons are not necessary for this effect (Root et al., 2015). Thus, it seems that the LHb effects on DA-receptors previously ascribed to DA release from mesohabenular fibers may be instead mediated by noradrenergic fibers.

Multiplexed transmission: Future directions and considerations for synaptic plasticity

Our ever-expanding knowledge of multiplexed signaling opens the door to new predictions about synaptic plasticity. For example, though activation of the mesohabenular projection results in glutamate and GABA release, firing patterns of LHb neurons indicate a predominant GABA-induced decrease in firing rate of LHb neurons in rodents (Root et al., 2014a). Drugs of abuse, depression, and stress alter LHb function to favor glutamatergic excitation and demote GABAergic inhibition (Meshul et al., 1998; Li et al., 2011; Shabel et al., 2014), suggesting the potential for mesohabenular plasticity in mediating part of these effects. The ability of “neurotransmitter-switching” depending on circadian and seasonal variations has also been documented (Dulcis et al., 2013; Farajnia et al., 2014), and further investigation is necessary to determine if these factors influence multiplexed signaling.

The postsynaptic signaling dominance by one neurotransmitter may also be used to balance signals from other neurotransmitters and maintain homeostasis. Indeed, it has been shown that the same neurotransmitter may elicit different responses depending on the overall extracellular environment (Laviolette et al., 2002; Twining et al., 2014). For example in the mesohabenular projection, depending on the membrane potential of the postsynaptic LHb neuron, mesohabenular stimulation results in GABA-A receptor or AMPA-receptor currents (Root et al., 2014b). With this in mind, we speculate that drugs of abuse, neurodegenerative diseases, or other circumstances that affect synapses capable of multiplexed neurotransmission may produce aberrant signaling and thus affect cognition and behavior. It is likely that the recent discoveries of unpredicted synaptic arrangements will lead to novel experimental approaches to have a better understanding of how neurotransmission shifts from homeostatic conditions, how certain neurotransmitters become amplified or silenced, and how multiplexed signals might be simultaneously sent and received.

Because many neurotransmitters have multiple postsynaptic and presynaptic effects, multiplexed neurotransmission expands the repertoire of synaptic capabilities of single neurons. In this regard, electrophysiological evidence indicates that DA is capable to affect GABAA-receptors (Tritsch et al., 2012; Kim et al., 2015; Hoerbelt et al., 2015). Thus, when glutamate and DA are multiplexed together—as they are in some mesoaccumbal projections (Zhang et al., 2015)—DA may act to modulate glutamatergic signaling, counter glutamatergic signaling, or might even behave differently depending on the postsynaptic cell (e.g., targeting of D1 receptor neurons or D2 receptor neurons). With this in mind, it is clear that novel technologies and intersectional genetic strategies will be necessary in order to decipher the unique contributions of each component of multiplexed signals (Pupe et al, 2015).

A better understanding of the presynaptic and postsynaptic elements will allow better understanding of how these specialized synapses, described above, manage multiplexed neurotransmission in the presynaptic terminal and are subsequently integrated by the postsynaptic neuron. For example, these elements may work together to facilitate spike-timing dependent mechanisms for plasticity (e.g., Watanabe et al., 2002), as it is known that neuromodulators can affect the temporal window necessary for spike timing dependent activation, or that neuromodulators can cause a switch from long-term potentiation to long term depression (Caporale & Dan 2008; Bissiere et al., 2003). Thus, the spatiotemporal relationship of segregated DAergic and glutamatergic signaling in the nAcc may act to enhance the probability of signal transduction when both transmitters are released within a short time window. A similar mechanism might apply to mesohabenular signaling, although the precise mechanism by which glutamate and GABA might work to facilitate or shunt one another is still unclear. One possibility is that the integration for either a GABAergic or a glutamate response by the post-synaptic cell would depend on the timing of other habenular afferents.

Overall, it is becoming clear that the VTA is far more complex than was initially realized. Indeed, many studies have reported heterogeneous responses of specific VTA neurons (Brischoux et al., 2009; Borgkvist et al., 2011; Margolis et al., 2014; Eddine et al., 2015; Mrejeru et al 2015; Mejias-Aponte et al., 2015), and it would seem likely that this diversity

is due (at least in part) to cells that are capable of multiplexed neurotransmission. With this in mind, it is clear that that the discovery of compound cell types has wide-reaching implications for our understanding of VTA circuit functions. Moreover, multiplexed signaling neurons are increasingly identified throughout the brain, suggesting that this unique type of signaling plays important roles in health and disease.

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Highlights

- The Ventral Tegmental Area contains neurons capable of multiplexing multiple neurotransmitters.
- Novel synaptic arrangements facilitate multiplexed neurotransmission.
- Balance shifts in multiplexed neurotransmission may accompany behavioral changes.

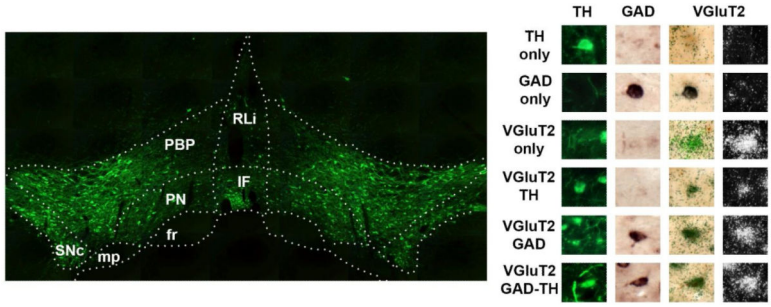

Figure 1. Neurons in the ventral tegmental area (VTA) are capable of multiplexed neurotransmission

Detection of tyrosine hydroxylase (TH) immunoreactivity within the VTA, (low magnification, left panel). VTA combined immunohistochemistry and *in situ* hybridization showing at high magnification (right panel) neurons expressing TH (green cells), glutamic acid decarboxylase mRNA (GAD 65/67; purple cells), vesicular glutamate transporter 2 mRNA (VGluT2; green or white grain aggregates) or combinations of these cell markers. *Abbreviations*. **Left:** RLi- Rostral Linear Nucleus, IF- Interfasicular Nucleus, PBP- Parabrachial Pigmented Nucleus, PN- Paranigral Nucleus, SNc- Substantia Nigra Pars Compacta, fr- fasciculus retroflexus, mp- Mammillary Peduncle, **Right:** TH- tyrosine hydroxylase, GAD- glutamic acid decarboxylase, VGluT2- vesicular glutamate transporter 2.

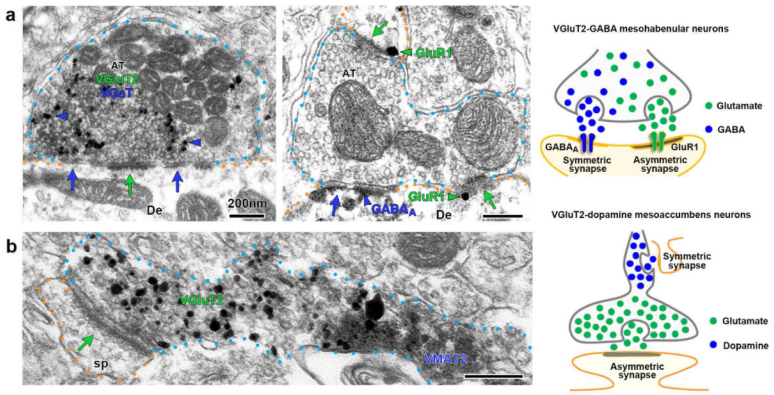

Figure 2. Ultrastructural immunolabeling reveals unpredicted mechanisms of neurotransmission within the mesohabenular and mesoaccumbal pathways

(a) Lateral habenula micrograph (left panel) showing a single mesohabenular axon terminal containing VGluT2 (scattered dark material detected by immunoperoxidase labeling) and VGaT (gold particles detected by immunogold; blue arrowheads). This single axon terminal forms both an asymmetric synapse (green arrow) and a symmetric synapse (blue arrow) with a common postsynaptic dendrite (De). Postsynaptic to a single axon terminal (middle panel), GluR1 receptors (green arrowhead) are found adjacent to asymmetric synapses (green arrows), while $GABA_A$ receptors (blue arrowhead) are found adjacent to symmetric synapses (blue arrow). (b) Nucleus Accumbens micrograph showing a messoaccumbal axon containing both VMAT2 (scattered dark material) and VGluT2 (gold particles). VMAT2 and VGluT2 are segregated within the same axon. Note that the VGluT2 microdomain corresponds to an axon terminal establishing an asymmetric synapse (arrow) with a postsynaptic dendritic spine (sp). All scale bars represent 200 nm.