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Glutamate neurons within the midbrain dopamine regions

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Abstract

Midbrain dopamine systems play important roles in Parkinson's disease, schizophrenia, addiction, and depression. The participation of midbrain dopamine systems in diverse clinical contexts suggests these systems are highly complex. Midbrain dopamine regions contain at least three neuronal phenotypes: dopaminergic, GABAergic, and glutamatergic. Here, we review the locations, subtypes, and functions of glutamatergic neurons within midbrain dopamine regions. Vesicular glutamate transporter 2 (VGluT2) mRNA-expressing neurons are observed within each midbrain dopamine system. Within rat retrorubral field (RRF), large populations of VGluT2 neurons are observed throughout its anteroposterior extent. Within rat substantia nigra pars compacta (SNC), VGluT2 neurons are observed centrally and caudally, and are most dense within the laterodorsal subdivision. RRF and SNC rat VGluT2 neurons lack tyrosine hydroxylase (TH), making them an entirely distinct population of neurons from dopaminergic neurons. The rat ventral tegmental area (VTA) contains the most heterogeneous populations of VGluT2 neurons. VGluT2 neurons are found in each VTA subnucleus but are most dense within the anterior midline subnuclei. Some subpopulations of rat VGluT2 neurons co-express TH or glutamic acid decarboxylase (GAD), but most of the VGluT2 neurons lack TH or GAD. Different subsets of rat VGluT2-TH neurons exist based on the presence or absence of vesicular monoamine transporter 2, dopamine transporter, or D2 dopamine receptor. Thus, the capacity by which VGluT2-TH neurons may release dopamine will differ based on their capacity to accumulate vesicular dopamine, uptake extracellular dopamine, or be autoregulated by dopamine. Rat VTA VGluT2 neurons exhibit intrinsic VTA projections and extrinsic projections to the accumbens and to the prefrontal cortex. Mouse VTA VGluT2 neurons project to accumbens shell, prefrontal cortex, ventral pallidum, amygdala, and lateral habenula. Given their molecular diversity and participation in circuits involved in addiction, we hypothesize that individual VGluT2 subpopulations of neurons play unique roles in addiction and other disorders.

Midbrain dopamine (DA) neurons are hypothesized to play roles in reward-based behavior and addiction (Wise, 1978, 2008), reward prediction and learning by error detection (Schultz and Dickinson, 2000), effort-based decision making (Salamone and Correa, 2002), flexible

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reward-directed behaviors (Ikemoto and Panksepp, 1999; Nicola, 2010), incentive salience (Berridge, 2007), stimulus salience (e. g., prediction of rewarding and aversive events; Young et al., 2005), aversion (Lammel et al., 2014; Volman et al., 2014), depression (Nestler and Carlezon Jr, 2006; Yadid and Friedman, 2008), and fear (Pezze and Feldon, 2004). The extensive, divergent behavioral roles of midbrain dopamine neurons, predominantly from the VTA, indicate that this system is highly heterogeneous. This heterogeneity may be reflected in part by the diverse phenotypic characteristics among DAergic neurons and their interactive brain structures (Yetnikoff et al. 2014; Ford et al. 2014; Overton et al. 2014; Lammel *et al.*, 2014; Morales and Pickel, 2012: Li *et al.*, 2013; Margolis *et al.*, 2006a,b; 2008; Ford *et al.*, 2006).

The midbrain DAergic neurons are interspersed with GABAergic neurons and with glutamatergic neurons (Kawano *et al.*, 2006; Yamaguchi *et al.*, 2007; 2011; 2013; Nair-Roberts *et al.*, 2008). Based on electrophysiological and pharmacological properties, *ex vivo* electrophysiological recordings from midbrain neurons have provided evidence for three subpopulations of neurons (primary, secondary and tertiary neurons) (Grace & Onn, 1989; Johnson & North, 1992; Cameron et al., 1997; Ungless et al., 2004). The subpopulation of primary neurons has been recognized as DAergic neurons that in their majority have long duration action potentials and hyperpolarization-activated cation current (Ih) (Grace & Onn, 1989). In contrast, the subpopulation of secondary neurons has been recognized as GABAergic neurons with short action potential durations and without Ih (Johnson & North, 1992). The third subpopulation of neurons lacks the electrophysiological properties associated with DAergic or GABAergic neurons, and it has been suggested to use glutamate as a signaling molecule (Ungless *et al.*, 2004).

The presence of glutamatergic neurons within the midbrain DA regions was initially suggested from *in vivo* (Wilson *et al.*, 1982; Mercuri *et al.*, 1985; Ungless et al., 2004; Lavin *et al.*, 2005; Chuhma *et al.*, 2009), and *in vitro* electrophysiological findings (Sulzer et al., 1998; Joyce and Rayport, 2000; Chuhma et al., 2004). *In vivo* studies have shown that electrical stimulation of the substantia nigra pars compacta (SNC) evokes excitatory postsynaptic currents (EPSCs) in dorsal striatal neurons (Wilson *et al.*, 1982). The presence of a nigrostrial glutamatergic pathway has been further supported by ultrastructural analysis of anterograde labeled material showing axon terminals originating from the SNC lacking Tyrosine Hydroxylase (TH, marker of midbrain DA neurons), and making putative excitatory asymmetric synapses in the dorsal striatum (Hattori *et al.*, 1991). Electrical stimulation of the neighboring VTA also evokes EPSCs in neurons within the medial prefrontal cortex (mPFC, Mercuri *et al.*, 1985; Lavin *et al.*, 2005), and within the nucleus accumbens (nAcc, Chuhma *et al.*, 2009).

The earliest electrophysiological *in vivo* studies reporting EPSCs evoked by midbrain electrical stimulation did not ascribe these excitatory responses to the release of glutamate from DA neurons (Wilson *et al.*, 1982; Mercuri *et al.*, 1985). However, later studies proposed release of glutamate from DA neurons as a mechanism to evoke EPSCs from VTA efferents (Lavin *et al.*, 2005; Chuhma *et al.*, 2009). The idea that glutamate is released by DAergic neurons was initially proposed by Kaneko *et al.*, (1990) based on the observation that antibodies against glutaminase were able to immunolabel all catecholaminergic neurons.

However, glutaminase is an enzyme necessary for the production of metabolic glutamate, as such is present in many non-glutamatergic neurons (Laake *et al.*, 1999), thus glutaminase is not a selective marker for glutamate signaling neurons. Nevertheless, *in vitro* electrophysiological studies have shown glutamatergic signaling by midbrain cultured DA neurons (Sulzer *et al.*, 1998; Joyce and Rayport, 2000; Bourque and Trudeau, 2000; Sulzer and Rayport, 2000) and midbrain slices (Chuhma *et al.*, 2004, 2009).

Anatomical identification of midbrain glutamatergic neurons

The analysis of glutamatergic neurons has been greatly advanced in the last decade due to the cloning of three distinct vesicular glutamate transporters (VGluT1, VGluT2 and VGluT3), which accumulate glutamate into vesicles for its synaptic release (Bellocchio *et al.*, 1998; Takamori *et al.*, 2000; Bai *et al.*, 2001; Fremeau *et al.*, 2001; Fujiyama *et al.*, 2001; Hayashi *et al.*, 2001; Herzog *et al.*, 2001; Varoqui *et al.*, 2002). VGluT1 and VGluT2 are restricted to known glutamatergic neurons, and their presence has become a reliable molecular marker to identify the distribution and synaptic connectivity of glutamatergic neurons within different brain regions. While VGluT1 and VGluT2 are highly concentrated in synaptic vesicles in axonal terminals of glutamatergic neurons (Fremeau *et al.*, 2001; Fujiyama *et al.*, 2001; Herzog et al., 2001), they are undetectable in other neuronal compartments, such as cell bodies, dendrites, and axons. Thus, cellular detection of mRNA transcripts encoding VGluT1 or VGluT2 is so far the only available and reliable method to identify cell bodies of glutamatergic neurons in non-transgenic animals. To detect glutamatergic neurons within the rat DA midbrain regions, *in situ* hybridization methods have been applied with the use of radioactive and non-radioactive probes (Kawano *et al.*, 2006; Yamaguchi *et al.*, 2007; 2011; 2013; Nair-Roberts *et al.*, 2008; Berube-Carriere *et al.*, 2009). These *in situ* hybridization studies have shown the following major findings: first, that in the adult rat there are neurons expressing VGluT2 mRNA, but not VGluT1 nor VGluT3, in the VTA (Kawano *et al.*, 2006; Yamaguchi *et al.*, 2007), in the SNC (Yamaguchi *et al.*, 2013), and in the retrorubral field (RRF; Yamaguchi *et al.*, 2013). Second, that different experimental conditions used for detection of VGluT2 mRNA in the adult rat may explain discrepancies in the number of VGluT2-expressing neurons detected in the VTA (Kawano *et al.*, 2006; Berube-Carriere *et al.*, 2009; Yamaguchi *et al.*, 2007, 2011), SNC and RFF (Nair-Roberts *et al.*, 2008;Yamaguchi *et al.*, 2013). Third, that the VGluT2 neurons within the RRF, SNC and neighboring lateral aspects of the VTA [lateral aspects of parabrachial pigmental (PBP) and paranigral (PN) nuclei] appear to be similar to each other, but different from those present in the midline nuclei of the A10 region (medial aspects of both PBP and PN; rostral linear nucleus, RLi; interfascicular nucleus, IF and caudal linear nucleus, CLi). Four, that although some VGluT2 neurons in the midline nuclei of the VTA co-express TH, the vast majority of VGluT2 neurons lack TH within the mature rat RRF, SNc, and lateral VTA (Yamaguchi *et al.*, 2011, 2013; Li *et al.*, 2013). These four major findings will be further detailed in this review.

Glutamatergic neurons within the RRF and the SNC

By applying radioactive *in situ* hybridization in combination with TH immunolabeling, we have found that the vast majority of VGluT2-expressing neurons do not co-express TH

within the RRF, SNC (Yamaguchi *et al.*, 2013), or lateral PBP and lateral PN of the VTA (Yamaguchi *et al.*, 2007, 2011; Li *et al.*, 2013) (Figures 1-2). In contrast, some of the VGluT2 neurons located in the midline nuclei of the VTA (medial PBP, medial PN, RLi, IF, and CLi) co-express TH (VGluT2-TH neurons; Figures 1-2). Quantitative analysis of the relative frequency of VGluT2, VGluT2-TH and TH neurons indicates that their prevalence is not constant throughout the RRF, SNC and lateral and medial nuclei of the VTA. VGluT2-TH neurons are not found in the RRF or the SNC. Within the RRF, the number of VGluT2 neurons increases from rostral to caudal levels, but the proportion of VGluT2 neurons in relation to those expressing TH is constant throughout its rostro-caudal levels. On average, the RRF neurons are in a ratio of 1 VGluT2 cell per every 1.7 TH cells. Within the SNC, VGluT2 neurons are often observed within its caudal laterodorsal portion and rarely observed in the rostral SNC. On average, the SNC VGluT2 cells are in a ratio of 1 VGluT2 cell per every 4.4 TH cells. Because the rat VGluT2 neurons located in the SNC lack TH, we concluded that glutamatergic neurons and DAergic neurons are two distinct subpopulations of neurons in the SNC and proposed that a single nigrostriatal dopaminergic neuron is unlikely to form two chemically distinct synaptic classes, a dopaminergic and an excitatory (Yamaguchi *et al.*, 2013), as previously proposed by Hattori *et al.*, (1991).

VGluT2 neurons within the VTA

In contrast to the apparent uniformity among the VGluT2 neurons within the RRF and the SNC, the VGluT2 neurons within the VTA are heterogeneous in their concentration, distribution and composition (Yamaguchi *et al.*, 2007, 2011; Li *et al.*, 2013; Root et al., 2013). Although some VGluT2 neurons expressing either TH or GABAergic markers are present within the medial aspects of the VTA (Yamaguchi *et al.*, 2007; Root et al, 2013), most VGluT2 neurons lack both TH and GABAergic markers. These findings lead us to propose that the VGluT2 neurons constitute a third type of neuron within the VTA (Yamaguchi *et al.*, 2007). The VGluT2-TH neurons are present in a zone initiated at the border between the PBP and the RLi and expanding 400 μm laterally (medial PBP) (Figures 3-4), and in a zone initiated at the border between the PN and the IF and expanding 200 μm laterally (medial PN) (Figures 3-4). While the majority of VGluT2 neurons lack TH (72–79%) within these medial aspects of the PBP and PN, the subpopulation of dual VGluT2-TH neurons makes about half of the total population of TH neurons. Within the midline nuclei (RLi, IF, and CLi) of the VTA, most of the VGluT2 neurons lack TH (63–89%; Figure 1), and a few co-express TH (12–37%). These VGluT2-TH neurons account for roughly half of the neurons containing TH in the rostral aspects of both the RLi and the IF nuclei, and less than 17% of those neurons containing TH within the caudal levels of the IF and CLi. The differential distributions of VGluT2-only, VGluT2-TH, and TH-only neurons within each of the subdivisions of the VTA (Figure 1) underscore the cellular difference between the lateral (PBP and PN) and medial nuclei of the VTA.

Dual VGluT2-TH neurons

Evidence for the expression of VGluT2 mRNA in a subset of TH neurons observed by radioactive *in situ* hybridization procedures is supported by quantitative RT-PCR of individual laser micro-dissected VTA neurons (Yamaguchi *et al.*, 2011; Li *et al.*, 2013).

These VGluT2-TH neurons express aromatic L-amino acid decarboxylase, and as such have the capability to synthesize DA. However, only a subset of VGluT2-TH neurons express vesicular monoamine transporter (VMAT2), dopamine transporter (DAT), or D2 receptor, indicating that although all VGluT2-TH neurons can produce DA, only some of them are capable of accumulating vesicular DA, capable of capturing extrasynaptic DA, or be autoregulated by dopamine (Figure 5). Interestingly, the relative amount of TH mRNA is similar between the TH-only and TH-VGluT2 neurons that contain both VMAT2 and DAT. By comparison, the amounts of both TH mRNA and DAT mRNA are lower in the TH-only and the TH-VGluT2 neurons that lack VMAT2. These findings indicate that the concentration of TH mRNA is not influenced by its coexistence with VGluT2, but rather by the absence of VMAT2.

Corelease of glutamate and DA has been suggested from *in vitro* findings showing EPSCs in midbrain cultured DA neurons (Sulzer *et al.*, 1998; Bourque and Trudeau, 2000; Joyce and Rayport, 2000; Sulzer and Rayport, 2000; Chuhma *et al.*, 2004, 2009), and EPSCs in nAcc slices evoked by local light stimulation of VTA fibers expressing channelrhodopsin2 under the regulation of the TH- or DAT-promoters (Stuber *et al.*, 2010; Tecuapetla *et al.*, 2010). While these optogenetic studies provide evidence for the release of glutamate by mesoaccumbens putative DA neurons, the release of DA by midbrain glutamate neurons has not been directly demonstrated. Nevertheless, co-release of glutamate and DA from the same pool of vesicles has been suggested as a mechanism of neurotransmission by dual glutamate-DA neurons (Hnasko *et al.*, 2010). This suggestion has been based on co-immunoprecipitation of VMAT2 and VGluT2 from ~6-week old rat ventral striatal (nAcc and olfactory tubercle) preparations as well as electrochemical findings from 3-week old rat ventral striatal preparations showing glutamate-mediated vesicular acidification that increases monoamine storage (Hnasko *et al.*, 2010). It is unclear whether or not VGluT2 and VMAT2 are present within the same pool of vesicles after 6 weeks of age, and the extent to which VGluT2 participates in DA vesicular filling *in vivo*. However, ultrastructural analysis of the nAcc indicates that while VGluT2 and TH coexist in some nAcc axon terminals within 15 day old rats (Berube-Carrere *et al.*, 2009), they do not co-exist in the adult rat (Berube-Carrere *et al.*, 2009; Moss *et al.*, 2011) or at any age in mice (Berube-Carrere *et al.*, 2012). Despite the lack of support for subcellular co-existence of VGluT2 and TH in adult rodents, it has been suggested that VGluT2 facilitates vesicular DA filling in the nAcc of adult mice based on studies with conditioned knock-out mice depleted of VGluT2 in DAT neurons (cKO mice). In these studies, the cKO mice showed an attenuated drug-induced locomotor response following a single injection of cocaine or methamphetamine in comparison to the wild type (WT) mice (Birgner *et al.*, 2010; Hnasko *et al.*, 2010; Fortin *et al.*, 2012). In addition, although cocaine conditioned place preference by cKO and WT mice are similar (Hnasko *et al.*, 2010), cKO mice consume higher levels of both sugar and cocaine, and display a significant elevation in cue-induced drug seeking compared to WT mice (Alsiö *et al.*, 2011). It has been suggested that these behavioral effects are due to the hypothesized lack of VGluT2 on DA vesicles, which would reduce vesicular DA levels (Alsiö *et al.*, 2011). However, it has also been suggested that functional differences may result indirectly from developmental alterations within the DA system due to embryonic deletion of the VGluT2 gene, which has been shown to result in many morphological and

functional alterations (Alsiö *et al.*, 2011; Berube-Carriere *et al.*, 2012). Further research is necessary to determine how VGluT2-TH neurons may corelease glutamate and dopamine.

Intrinsic and extrinsic inputs by VTA glutamatergic neurons

It is well known that VTA neurons receive extensive glutamatergic innervation (Geisler *et al.*, 2007). A role for local VTA VGluT2 neurons in VTA neurotransmission has been suggested based on electrophysiological and anatomical findings showing that some VTA VGluT2 neurons establish local glutamatergic synapses on VTA DA and non-DA neurons (Dobi *et al.*, 2010). These studies indicate that VTA VGluT2 neurons provide local glutamatergic neurotransmission. This novel model of local communication in the VTA expands the previously reported local GABAergic and DAergic signaling in this brain region (Johnson and North, 1992a,b; Cameron and Williams, 1993), raising the possibility that local glutamatergic neurons play a role in regulating VTA neuronal activity. Because functional μ-opioid receptors are present in VTA GABA neurons and μ-opioid receptor transcripts are detected in VTA VGluT2 neurons (Kudo *et al.*, 2014), opioids may play an important role in the regulation of the local VTA neuronal network (Johnson and North, 1992 a,b).

In addition to the local projections of VTA VGluT2 neurons, subsets of VGluT2 neurons innervate the mPFC (Lavin *et al.*, 2005; Hur and Zaborszky, 2005; Yamaguchi *et al.*, 2011; Gorelova *et al.*, 2012; Taylor et al. 2014), nAcc (Stuber *et al.*, 2010; Tecuapetla *et al.*, 2010; Yamaguchi *et al.*, 2011; Adrover *et al.*, 2014; Hnasko *et al.*, 2012; Chuhma *et al.*, 2009; 2014; Taylor et al. 2014), lateral habenula, amygdala, or ventral pallidum (Hnasko *et al.*, 2012; Taylor et al. 2014). The degree to which VGluT2-only or dual VGluT2-TH neurons innervate the mPFC or nAcc has been determined by the implementation of a multistep procedure that combines *in vivo* injections of the retrograde tract tracer Fluoro-Gold in the mPFC or nAcc, and postmortem phenotypic characterization of the retrogradely labeled neurons by a combination of immunolabeling and radioactive *in situ* hybridization (Yamaguchi *et al.*, 2011). By applying this experimental approach, it has been determined that near to 40% of the mesocortical neurons are VGluT2-only neurons, and about 10% have an unidentified phenotype (not VGluT2 or TH). Approximately 25% of mesocortical neurons are TH-only neurons, and the remaining 25% are VGluT2-TH neurons. These findings indicate that there is a major glutamatergic pathway from the midbrain to the mPFC (Yamaguchi *et al.*, 2011). The identification of this mesocortical glutamatergic pathway is consistent with electrophysiological findings showing that stimulation of the VTA evokes EPSCs in mPFC neurons, which is eliminated by glutamate antagonists but not by DA antagonists (Lavin *et al.*, 2005). In contrast to a major mesocortical glutamatergic pathway, TH is present in the vast majority (80%) of the VTA neurons that project to the nAcc. However, about one-third of these TH neurons co-express VGluT2 mRNA, despite the fact that VTA VGluT2-TH neurons represent only a small fraction of both the TH neurons and a small fraction of the VGluT2 neurons (Yamaguchi *et al.*, 2011). Because VTA projections rarely collateralize (Fallon, 1981; Swanson, 1982; Albanese and Minciacchi, 1983), we suggest that the nAcc is a preferential target of the VGluT2-TH neurons. The identification of a mesoaccumbens pathway from VGluT2-TH neurons is consistent with electrophysiological results showing that optical stimulation of efferents from dopaminergic

neurons expressing channelrhodopsin produces EPSCs in medium spiny neurons (Stuber *et al.*, 2010; Tecuapetla *et al.*, 2010; Adrover *et al.*, 2014), and in cholinergic interneurons (Chuhma *et al.*, 2014) of the nAcc.

Conclusions and future directions

All glutamatergic neurons within the major midbrain DA subdivisions (RRF, SNc, and VTA) contain VGluT2 mRNA, and they may provide fast non-DA excitatory signaling. The VGluT2 neurons within the RRF and SNc do not co-express TH, and as such they are incapable of DA co-release. The VTA contains two major classes of VGluT2 neurons: VGluT2 neurons lacking TH (VGluT2-only neurons), which are present in all subdivisions of the VTA, and VGluT2 neurons co-expressing TH (VGluT2-TH neurons) that are restricted to the medial portions. Dual VGluT2-TH neurons have the capability to produce DA, but not all have the ability to accumulate DA into synaptic vesicles or the capacity to take DA up from the extracellular space. While the release of glutamate from dual VGluT2-TH neurons has been established, it remains to be determined whether these neurons release DA. Both VGluT2-only and VGluT2-TH neurons innervate the mPFC and the nAcc; thus, in addition to the well-recognized mesocorticolimbic DA and GABA pathways, there exists a parallel mesocorticolimbic glutamatergic pathway from which some fibers may co-release glutamate and DA. The mechanism underlying this potential glutamate and DA co-release remains to be determined.

Even though the vast majority of VGluT2 neurons within the midbrain DA regions do not have the capacity to produce DA, studies of their role in brain function have been limited until recently in part due to the lack of reliable methods for targeting these neurons. Borgius *et al.*, (2010) have recently developed VGluT2::cre transgenic mice that can be used for cre-dependent expression of selective molecules (opsins, neuronal tracers, etc.). However, the selective expression of cre within midbrain endogenous VGluT2 neurons remains to be determined in these mice. In addition, although great advances have been achieved in the analysis of the different neuronal phenotypes within the VTA, especially within its midline nuclei, several fundamental questions remain to be addressed. As we covered in this review, midbrain VGluT2 neurons, especially those within the VTA have considerable molecular heterogeneity. Most of midbrain VGluT2 neurons lack the capability of producing DA. Of the subset of VGluT2 neurons capable of producing DA, some may release DA by traditional vesicular mechanisms and others may either not release DA at all or may release it by a mechanism independent of vesicular accumulation of DA by VMAT2. Some VGluT2 neurons synthesize GABA and therefore may be capable of co-transmitting glutamate and GABA. The target areas and functions of each VGluT2 subtype remain to be determined. Given their molecular diversity and participation in circuits involved in addiction and depression, we hypothesize that individual VGluT2 subpopulations of neurons play unique roles in drug addiction and several motivated behaviors.

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- VGluT2-expressing glutamate neurons reside within RRF, SNC, and VTA
- Some VTA VGluT2 neurons, but not RRF or SNC VGluT2 neurons, co-express tyrosine hydroxylase
- Subsets of VGluT2-TH neurons co-express or lack VMAT2, DAT, or D2 receptor
- VTA VGluT2 neurons exhibit local VTA projections as well as and extrinsic projections
- Accumbens medial shell is a preferential target of VTA VGluT2-TH neurons

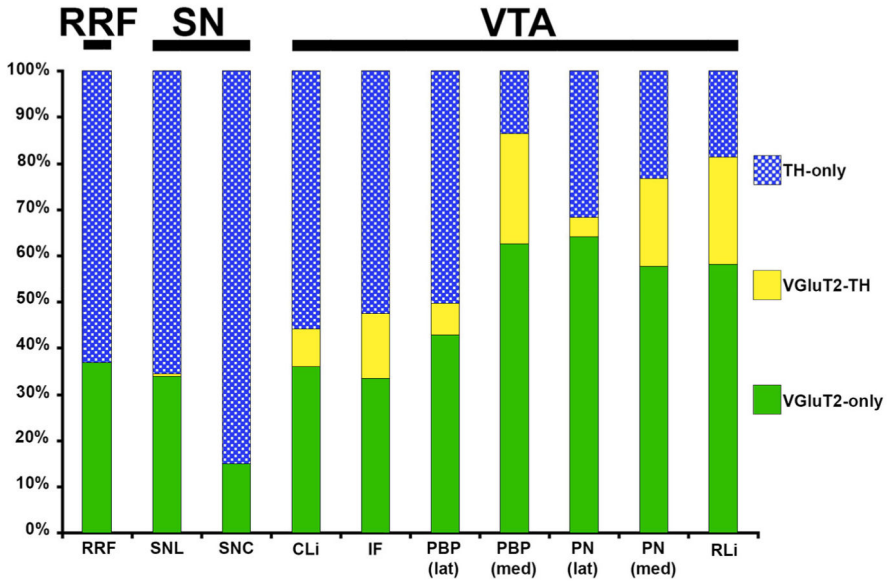

Figure 1.

Distribution of glutamatergic and dopaminergic neurons of the rat RRF, SNc, and VTA. VGluT2-only neurons express VGluT2 mRNA and lack TH-immunoreactivity. VGluT2-TH neurons co-express VGluT2 mRNA and TH-immunoreactivity. TH-only neurons lack VGluT2 mRNA but express TH-immunoreactivity. RRF - retrorubral field; SNL - lateral division of the substantia nigra pars compacta; SNC - substantia nigra pars compacta; CLi - caudal linear nucleus; PBP(lat) - lateral parabrachial pigmentosis nucleus; PBP(med) - medial parabrachial pigmentosis nucleus; PN(lat) - lateral paranigral nucleus; PN(med) - medial paranigral nucleus; RLi - rostral linear nucleus. Data from Yamaguchi *et al.*, 2011.

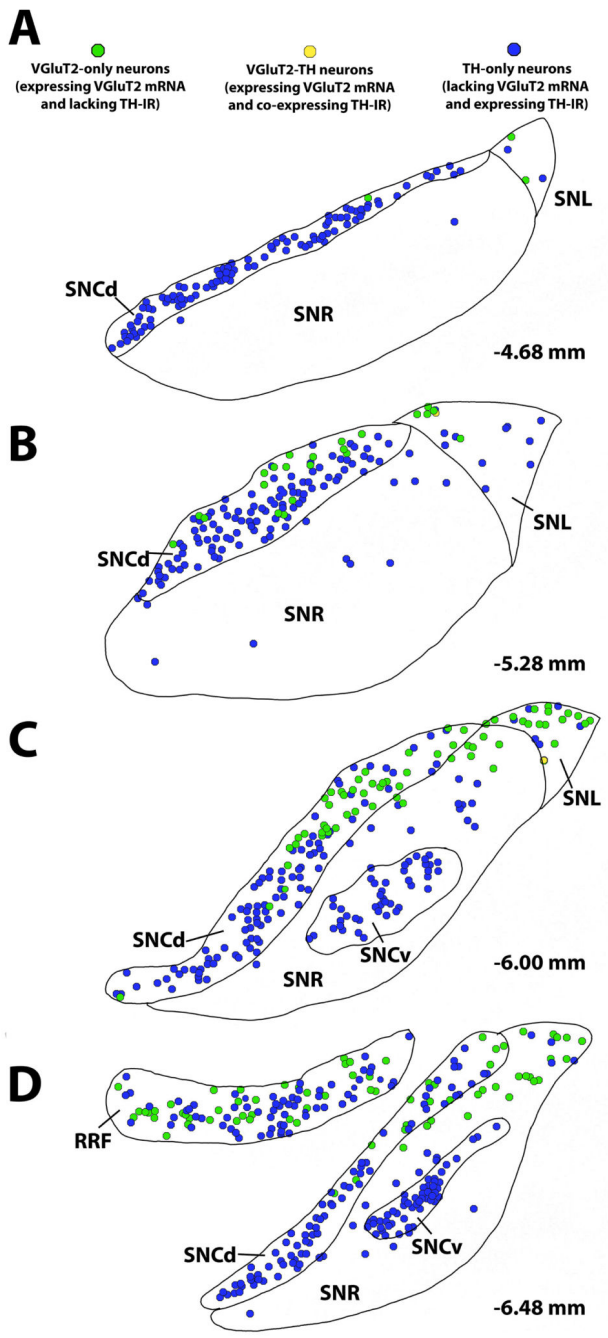

Figure 2.

Distribution map of glutamatergic and dopaminergic neurons of the rat SNC across four anteroposterior planes and rat RRF in a single plane. SNCd – substantia nigra pars compacta dorsal tier; SNCv – substantia nigra pars compacta ventral tier; SNL – substantia nigra pars lateralis; SNR – substantia nigra pars reticulate; RRF – retrorubral field. Data from Yamaguchi *et al.*, 2013.

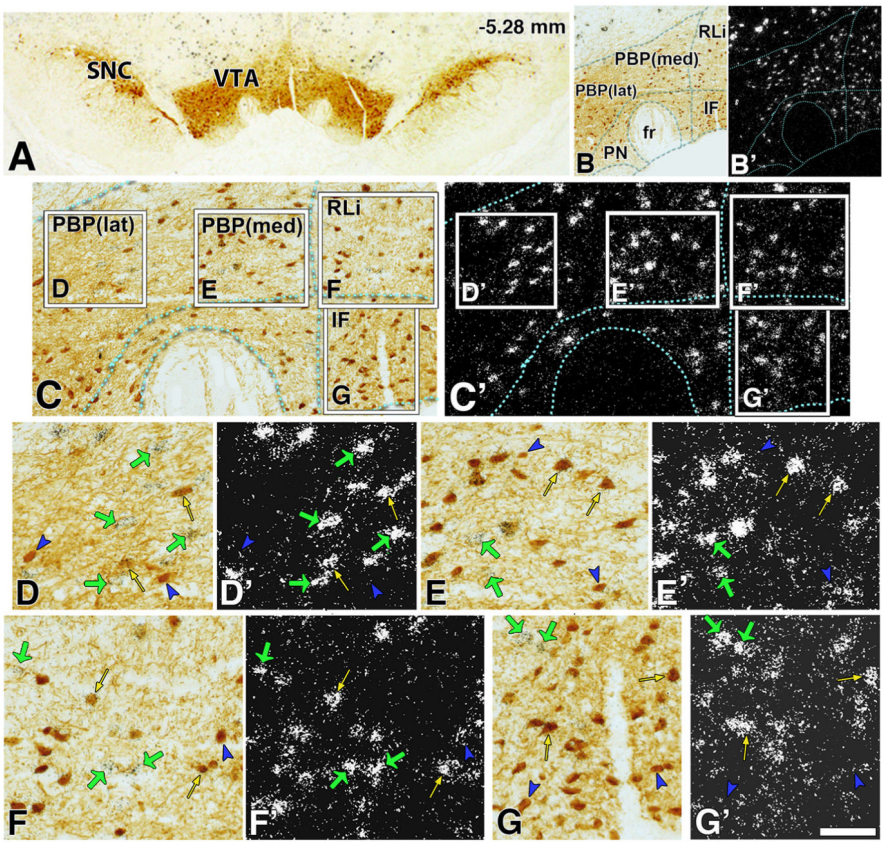

Figure 3.

Glutamatergic and dopaminergic neurons of the rat VTA. A. TH-immunoreactivity (brown) showing the SNc (left) and VTA (middle). B-C. Higher power images of VTA. TH-immunoreactivity (B,C) and VGluT2 mRNA (B',C') are displayed as brown reaction product (TH-immunoreactivity under bright field) and aggregates of silver grains (white grains under dark field or black grains under darkfield). D. High power image of selected PBP(lat) region in C. E. High power image of selected PBP(med) region in C. F. High power image of selected RLi region in C. High power image of selected IF region in C. Green arrows indicate VGluT2-only neuron. Yellow arrows indicate VGluT2-TH neuron. Blue arrowheads indicate TH-only neuron. fr - fasciculus retroflexus. Figure modified from Yamaguchi *et al.*, 2011.

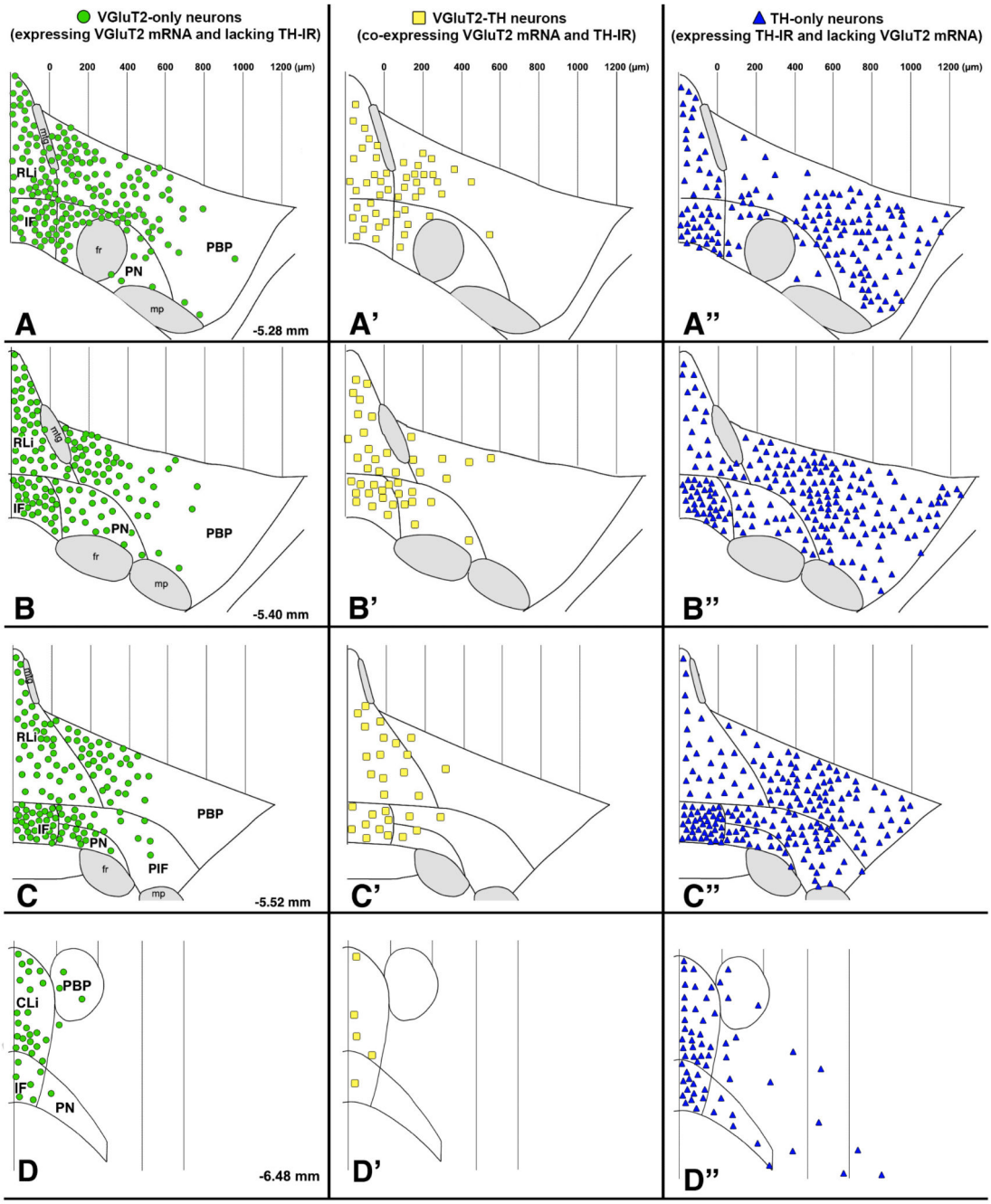

Figure 4.

Distribution map of glutamatergic and dopaminergic neurons of the rat VTA across four anteroposterior planes. Figure modified from Yamaguchi *et al.*, 2011.

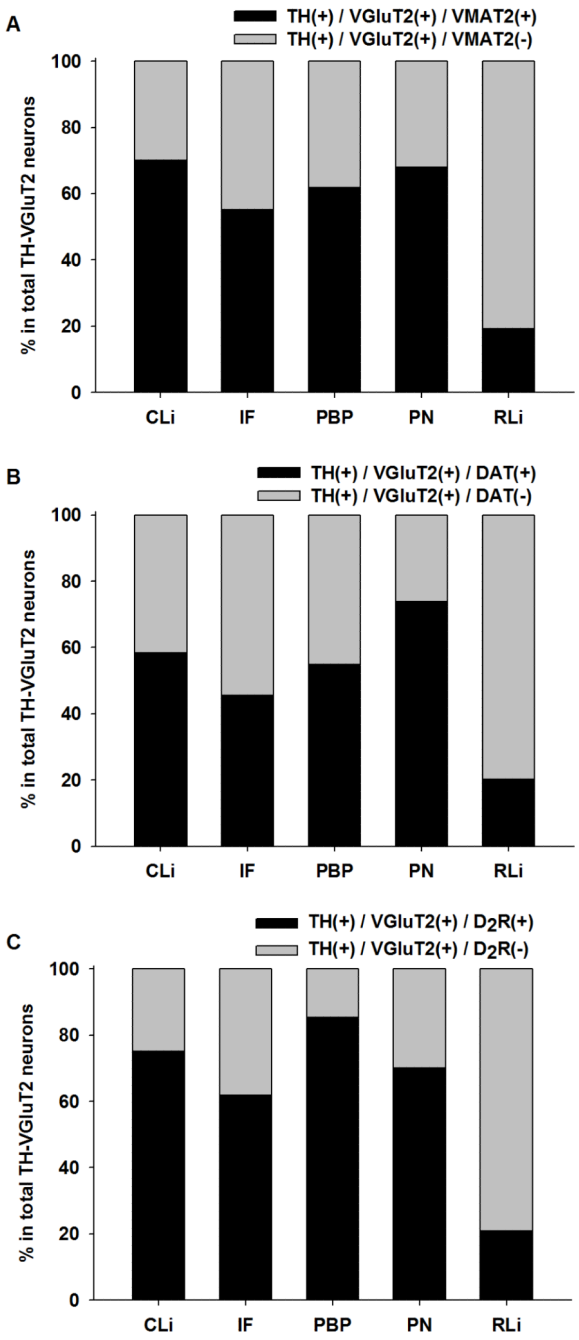

Figure 5.

Subregional distribution of rat VTA VGluT2-TH neurons co-expressing VMAT2 mRNA, DAT mRNA, or D2 receptor mRNA (mean and SEM). Black bars in A, B, and C indicate VGluT2-TH neurons coexpressing VMAT2 mRNA, DAT mRNA, or D2 receptor mRNA respectively. Gray bars in A, B, or C indicate VGluT2-TH neurons lacking VMAT2 mRNA, DAT mRNA, or D2 receptor mRNA, respectively. Figure modified from Li *et al.*, 2013