The mesocorticolimbic pathway originating in the ventral midbrain is classically viewed as a dopaminergic (DAergic) projection, it is in many ways distinct from the nigrostriatal pathway originating from the more laterally placed substantia nigra (SN). Unlike the nigrostriatal pathway that is almost entirely DAergic, only ~30–40% of the mesofrontal projection neurons are DAergic (Swanson 1982). Cells sending projections to prefrontal cortex (PFC) are found in many subregions of ventral midbrain with the highest density of mesocortical neurons originating in rostromedial regions of ventral tegmental area (VTA), including the rostral VTA, the rostralmedial portions of the parabrachial pigmented (PBP) and paranigral (PN) nuclei, the rostral linear nucleus of the raphe (RLi), and the interfascicular nucleus (IF; Deniau et al. 1980; Fallon 1981; Swanson 1982). These rostral-medial regions of ventral midbrain happen to also be those that possess a high density of neurons expressing messenger RNA (mRNA) for the vesicular glutamate transporter 2 (VGLUT2; Fremeau et al. 2001, 2002; Fujiyama et al. 2001; Yamaguchi et al. 2004), in contrast to more caudal and lateral subregions where the proportion of VGLUT2-positive neurons is rather small (Kawano et al. 2006; Descarries et al. 2008; Nair-Roberts et al. 2008). For example, in regions such as the rostral VTA and anterior medial PN nuclei, 20% to over 50% of the neurons express mRNA for VGLUT2. Likewise, injection of retrograde tracer into the PFC revealed that approximately half of the retrogradely labeled ventral midbrain neurons were positive for VGLUT2 (Hur and Zaborszky 2005).

Although the ventral midbrain contains a population of dopamine (DA) and glutamatergic neurons, a controversial issue is what portion of these neurons releases both neurotransmitters. It has been known for some time that stimulation of VTA axons can rapidly depolarize PFC neurons or evoke an excitatory postsynaptic potential (EPSP) (Bernardi et al. 1982; Lewis and O’Donnell 2000; Lavin et al. 2005) that is eliminated by glutamate but not DA antagonists (Lavin et al. 2005). It was also observed that the VTA-evoked EPSPs in the PFC were eliminated by a catecholamine-selective neurotoxin 6-hydroxy-dopamine (6-OHDA) lesion of the VTA, suggesting that they were generated by the release of glutamate from DA neurons (Lavin et al. 2005; Lapish et al. 2007). Likewise, Chuhma et al. (2004) showed that the EPSP in the mesoaccumbal pathway was also the result of glutamate released from DA neurons. Very recently, even more direct and conclusive support for the release of glutamate from VTA DA neurons was provided by Hnasko et al. (2010) who showed that selectively removing VGLUT2 expression in DA neurons eliminated the EPSP in the mesoaccumbal pathway, while 2 groups (Stuber et al. 2010; Tecuapetla et al. 2010) showed that selective optogenetic activation of DA fibers produced excitatory postsynaptic currents (EPSCs) in the vast majority of medial nucleus accumbens (NAC) neurons.

While these electrophysiological data suggest that glutamate is readily released from VTA neurons, the extent of the glutamatergic projection to the PFC in particular has not been quantified. It is difficult to address this issue at the level of the VTA because there is no simple correlation between cell number and axon terminal density. The density of DAergic varicosities in the rat PFC was estimated to be ~1 × 10^5/mm^3 (Descarries et al. 1987), yet the VTA contains ~40 000 DAergic cells (Nair-Roberts et al. 2008) and only a small portion of them project to PFC (Swanson 1982). Therefore, even if only a few VTA neurons contain VGLUT2, they could potentially exert a rather large influence on the PFC if they ramify as extensively as DA axons. Since we were interested specifically in the extent of the glutamate-versus-DA projection at the level of the PFC, we injected the anterograde tracer Phaseolus vulgaris leucoagglutinin or biotinylated dextran amine (BDA) into 4 ventral midbrain subregions and then analyzed the chemical identity of filled fibers in the PFC. We found a remarkably strong and regionally heterogeneous VGLUT2 projection to PFC, with a smaller population of fibers containing both VGLUT2 and tyrosine hydroxylase (TH). Thus, in view of previous characterization of a prominent
γ-aminobutyric acidergic (GABAergic) projection of the VTA-PFC pathway (Carr and Sesack 2000), the present data suggest that is more appropriate to view the VTA-PFC pathway as predominately an amino acid projection with a DA component.

Materials and Methods

Materials

Ibotenic acid was purchased from Tocris (Ellisville, MI, USA), 6-OHDA from Sigma Chemical Company (St Louis, MO, USA), and BDA from Molecular Probes/Invitrogen (Carlsbad, CA, USA). Phasusolus vulgaris leucoagglutinin (PHA-L), goat anti-PHA-L, and FITC-conjugated streptavidin were purchased from Vector Laboratories (Burlingame, CA, USA). Fluoromount was purchased from SouthernBiotech (Birmingham, AL, USA). Mouse anti-GAD65 (MAB351R), guinea pig anti-VGLUT2 (AB 5907), the Control Peptide for VGLUT2 (C176), and rabbit anti-TH (AB 152) were purchased from Millipore/Chemicon (Temecula, CA, USA). Normal donkey serum (NDS) and AMCA-, FITC-, CY3-, and CY5-conjugated donkey secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Animals

Adult male Sprague-Dawley rats weighing 290–340 g were used. Experiments were conducted in accordance with the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. All efforts were made to minimize the suffering and the number of animals used in the present study.

Iontophoretic Injection of Tracers

The anterograde tracer PHA-L (5% in 10 mM phosphate-buffered saline [PBS], pH 7.4) or BDA (10% in 10 mM phosphate buffer [PB], pH 7.4) was iontophoretically injected into the VTA of adult Sprague-Dawley male rats anaesthetized with isoflurane (5% v/v) or chloral hydrate (400 mg/kg). Fluoromount was injected into the VTA with PHA-L, each rat received 2 injections—separated by 15 min. The coordinates for injections in rostral, medial, and caudal VTA were 5.0, 5.2, and 5.5 mm posterior to Bregma, respectively, 1.6–1.8 mm lateral to the midline, and 7.8–8.0 mm ventral to the scull surface according to the atlas of Paxinos and Watson (2005). Electrodes were lowered at a 10° angle in the coronal plane. To increase coverage area of the VTA with PHA-L, each rat received 2 injections—separated by 200 μm ventrally. Following a 14- to 17-day survival period, animals were transcardially perfused under deep pentobarbital anesthesia (100 mg/kg) with 50 mL of isotonic NaCl followed by ice-cold mixture of 4% paraformaldehyde (PFA) and 0.05% glutaraldehyde in PB (0.1 M, pH 7.4). The brains were removed, postfixed in 4% of PFA in 0.1 M PB overnight at 4 °C, and then cryoprotected by immersion in 30% sucrose (w/v) in 0.1 M PB for 48 h at 4 °C. The brains were then divided into fore- and hindbrain regions, and 40-μm-thick coronal sections containing the entire frontal cortex and the region of brainstem containing the VTA were cut on a cryostat.

Ibotenic Acid and 6-OHDA VTA Lesions

To confirm the VTA origin of cortical PHA-L-labeled fibers, we performed lesions of VTA cell bodies using ibotenic acid (a nonselective neurotoxic agent) or 6-OHDA (a putatively selective DA neurotoxin) prior to injection of the tracer. Under anesthesia with a mixture of ketamine (80 mg/kg, ip) and xylazine (15 mg/kg, intramuscular), 0.6 μL ibotenic acid (5 mg/mg/mL in 0.9% NaCl) or 3 μL of 6-OHDA (4 μg/μL in 0.9% NaCl and 0.025% ascorbic acid) was slowly pressure injected over 10 min into the VTA (5.2 mm posterior to Bregma, 1.5 mm lateral, 7.6 mm ventral, at a 10° angle in the coronal plane) through glass electrodes with a tip diameter of ~100 μm. Electrodes were left in place for an additional 10 min after injections. Approximately 4 weeks later, PHA-L was iontophoretically injected into the VTA using the same coordinates but approaching the VTA from the contralateral hemisphere.

Multiple Immunofluorescence Labeling

All immunostaining procedures were performed on free-floating sections. Slices were rinsed in PB and preincubated with 5% NDS and 0.5% Triton X-100 in 50 mM PBS overnight at 4 °C. For double, triple, and quadruple labeling, sections were incubated with selected combinations of primary antibodies in PBS with 2% NDS and 0.3% Triton X-100 for 48 h at 4 °C. The primary antibodies were used at the following dilutions: goat anti-PHA-L (1:1000), rabbit anti-TH (1:1000), guinea pig anti-VGLUT2 (1:2500), and mouse anti-GAD65 (1:1000). Subsequently, sections were rinsed in PBS and incubated with AMCA-, FITC-, CY3-, and CY5-conjugated donkey secondary antibodies diluted (1:200) in PBS containing 0.3% Triton X-100 for 2 h at room temperature. For visualization of BDA, fluorescein-conjugated streptavidin (1:100) was included in the mixture of secondary antibodies. Finally, slices were rinsed in PBS and mounted and coverslipped with Fluoromount. Control experiments examining nonspecific staining of secondary antibodies were performed by omission of primary antibodies. To verify the specificity of guinea pig anti-VGLUT2 antibody, immunolabeling was also performed with the antibody preincubated with corresponding Control Peptide diluted 1:100 for 1 h at 4 °C.

Data Acquisition and Image Analysis

Images were acquired using an Olympus FV10-ASW laser-scanning confocal microscope. PHA-L/BDA-filled fibers were randomly chosen under wide field illumination with excitation and emission filters for FITC and a consecutive series of images of FITC, CY3, and CY5 signals from the chosen fibers were acquired using a x60 objective. Images were captured in 0.3-μm steps along the x-axis with a 0.088-μm pixel size for x- and y-axis. A 90-μm pinhole size in combination with x2 or x3 optical zoom resulted in optical slices of 0.727 and 0.205 μm for the x- and y-axis, respectively.

We quantified the number of PHA-L varicosities containing VGLUT2, TH, or GAD65 that were at least 2 times above background using Imaris 3D image analysis software (Version x64 6.2.1, Bitplane AG, Zurich, Switzerland). To increase the accuracy of identification of the surfaces, images were first deconvolved to reduce point spread functions using the adaptive blind 3D deconvolution method (AutoQuant X, Version X2.1.3; Media Cybernetics, Inc., Bethesda, MD) prior to analysis. We then identified PHA-L varicosities using the surface module of Imaris with a 0.55 micron diameter filter. These surfaces were used to mask VGLUT2, TH, and GAD65 clusters that were localized inside the varicosities. Following this, we created surfaces for VGLUT2, TH, and GAD65 using a 0.27-μm-diameter filter and determined the number of varicosities that were positive for each of these markers. The values that were used for filtering represent the minimum diameter necessary to accurately identify PHA-L varicosities and clusters for VGLUT2, TH, and GAD65 after images were deconvolved. An example of surfaces for PHA-L varicosities and the masked signals for VGLUT2 are shown in Supplementary Figure 1.

Results

We injected anterograde tracer PHA-L or BDA into 4 rostromedial subregions of the ventral midbrain near the midline as illustrated in Figure 1. The sites were chosen because they project to the PFC and/or had previously been shown to have VGLUT2-containing cells. The injection sites were as follows: 2 rats received PHA-L injections in the medial-to-caudal parabrachial pigmented nucleus (PBP) close to the midline, while 1 rat received a BDA injection into the medial PBP, 2 rats received PHA-L injections into the medial PN nucleus and 2 rats received PHA-L injections into the medial rostral linear nucleus (RLi); 1 rat received a PHA-L injection into the interfascicular subnucleus (IF) and 1 rat received an injection of BDA into the rostral VTA. In each animal, the iontophoretic injection of PHA-L or BDA resulted in a localized deposit of tracer with loaded cells spanning ~200–300 μm from the injection site. No obvious differences were observed between the 2 tracers at the level of the PFC or VTA. Examples of PHA-L and BDA
injection sites in the VTA are shown in Figures 2A and 4A. Double staining of slices for PHA-L or BDA and TH revealed that DAergic as well as non-DAergic cells in the VTA contained tracer (Fig. 2A). Since only a small fraction of VTA neurons were loaded with tracer in each case, only a modest number of fibers were filled with tracer in PFC sections. We never observed cell bodies filled with tracer in either the PFC or the NAC, indicating that little or no retrograde transport occurred. However, when tracer injections were made into the medial PN nucleus of the VTA, we did find filled cells outside the VTA, indicative of retrograde transport, and hence this tissue was not analyzed further.

Fiber Characteristics Following Injections into the PBP
Following PHA-L injections into the PBP, fibers and varicosities filled with tracer were observed within the ipsilateral PFC and adjacent medial shell of NAC with very few fibers in the contralateral hemisphere. Within the PFC, the highest density of fibers was observed in layers 5 and 6 of infralimbic and prelimbic cortices. Within these regions, we observed 2 major types of PHA-L-labeled fibers: 1) long, thick, and smooth nonbranching fibers that lacked obvious terminal specializations and were mostly found near the corpus callosum in deep layer 6 and 2) fibers with bulbous varicosities of variable sizes that were commonly found throughout the PFC. On the same fiber, we could observe varicosities that varied significantly in size.

Of 43 cortical fibers filled with tracer following PBP injections, 190 varicosities were analyzed for the presence of TH and VGLUT2 (Table 1). Only fibers with at least one distinguishable varicosity and only varicosities that were part of a fiber were analyzed. TH labeling was present in both fibers and varicosities and for a given TH-positive fiber, virtually all varicosities were TH positive. Yet, of the 43 fibers analyzed, only 9 had TH in their axons and varicosities. In contrast, while VGLUT2 labeling was completely confined to varicosities, on the same PHA-L-loaded fiber we often observed VGLUT2-
positive as well as VGLUT2-negative varicosities (Fig. 2B1). From 190 analyzed varicosities, 84 were positive for VGLUT2 and 23 were positive for both VGLUT2 and TH. We did not observe any obvious differences in the sizes of varicosities that were positive for TH, VGLUT2, or both markers.

As a control, we performed an identical analysis on tissue from the NAC. In the NAC, following PBP injections, most of the tracer-filled fibers were very thin, with small and barely distinguishable varicosities, although thicker fibers with large bulbous varicosities were also observed. Within the NAC, we analyzed 57 varicosities in 28 fibers for the presence of TH and VGLUT2. While 51 of the 57 varicosities were positive for TH, none of the 51 were also positive for VGLUT2 (Fig. 3). Both small TH-positive varicosities on thin fibers as well as large TH-positive varicosities resembling those in medial PFC were negative for VGLUT2. Only 2 fibers filled with tracer were negative for TH and possessed varicosities positive for VGLUT2. Given that identical techniques were used for NAC and PFC slices, the differences between the fiber characteristics in the 2 regions following tracer injection into the PBP illustrate that the large number of VGLUT2 fibers in PFC were not an artifact of the staining, tissue preparation, or image analysis. On the other hand, these data should not be used as an indication of the extent of glutamatergic transmission in the mesoaccumbens pathway in general (see Discussion). In fact, while injections into the rostral-medial VTA produced only a few...
labeled fibers in the NAC, all 9 tracer-filled fibers were immunopositive for VGLUT2, while 2 were also immunopositive for TH. Therefore, as in the mesofrontal pathway, the distribution of the VGLUT2 projection to the NAC appears to be very heterogeneous.

**Fiber Characteristics Following Injections into the RLi**

Although it may be inappropriate to include the RLi as part of the VTA, it was included in the present study because it possesses DA neurons and sends projections to the PFC. PHA-L tracer deposits were confined to the medial RLi with only a few scattered tracer-filled neurons in the adjacent PBP (Fig. 4A). At the level of the PFC, sparsely labeled fibers and varicosities were observed bilaterally in layers 5 and 6. We analyzed 41 fibers and 107 varicosities and found that only 14 of the 41 fibers were positive for TH (Table 1). Of the 107 varicosities analyzed, 47 were positive for VGLUT2 and 32 were positive for both TH and VGLUT2. Therefore, consistent with the observation of Kawano et al. (2006) that a large number of cells in the RLi express mRNA for VGLUT2 and TH, our data suggest that many of these neurons send a strong VGLUT2/TH projection to the PFC.

**Fiber Characteristics Following Injections into the Rostral VTA**

BDA injections targeting the rostral VTA covered a significant portion of the rostral VTA with a small minority of cells loaded with tracer more caudally in medial RLi (Fig. 4). At the level of the PFC, modest numbers of BDA-containing fibers were observed in the ipsilateral hemisphere and a few BDA-containing fibers in the contralateral medial PFC. Of 34 fibers filled with BDA in the PFC (Table 1), 9 were positive for TH. Of the 136 varicosities identified, 70 were found to be immunopositive for VGLUT2 only, 14 for TH only, and 30 for both VGLUT2 and TH. Of the 15 fibers analyzed, 13 were positive for TH and again, all the varicosities in these fibers contained TH (data not shown). Of the 89 varicosities analyzed, only 5 were positive for VGLUT2 alone and 25 were positive for both TH and VGLUT2 (Table 1). Therefore, while injections into the rostral VTA, RLi, and PBP yielded a significant proportion of VGLUT2 relative to TH varicosities, this was not the case for the IF that was more similar to the mesoaccumbens projection in that it had a very large TH component.

**Fiber Characteristics Following Injections into the IF**

Tracer injection into the IF resulted in small deposits that were confined to its medial portion (Fig. 1). Tracer-stained fibers and varicosities were found bilaterally in layers 5 and 6 of the medial PFC. Of the 15 fibers analyzed, 13 were positive for TH and again, all the varicosities in these fibers contained TH (data not shown). Of the 89 varicosities analyzed, only 5 were positive for VGLUT2 alone and 25 were positive for both TH and VGLUT2 (Table 1). Therefore, while injections into the rostral VTA, RLi, and PBP yielded a significant proportion of VGLUT2 relative to TH varicosities, this was not the case for the IF that was more similar to the mesoaccumbens projection in that it had a very large TH component.
projection to the PFC, and this explains why it was so effective. Lesions appeared to destroy most of the DA and VGLUT2 varicosities (56%) found in nonlesioned animals. Thus, 6-OHDA provided evidence that the mesocortical pathway has a very large but regionally heterogeneous VGLUT2 component.

One potential confound in the present study was that tracers injected into VTA subregions may be taken up by fibers of passage resulting in fibers labeled in the PFC that did not arise from the site of injection within the VTA. To address this issue, fiber-sparing ibotenic acid lesions were performed in the PBP 4 weeks prior to injection of the tracer into the same area. These lesions eliminated all fibers in the PFC (data not shown), confirming that the projections arose from cell bodies within VTA subregions and not fibers of passage that would have been spared.

Previously, we found that 6-OHDA lesions of the VTA eliminated the EPSP recorded in the PFC (Lavin et al. 2005). Based on this result, we argued for the existence of corelease in the pathway (Lavin et al. 2005; Lapish et al. 2007). To test this hypothesis, we again made 6-OHDA lesions but in this case confirmed that the projections arose from cell bodies within VTA subregions and not fibers of passage that had been spared.

Fiber Characteristics Following Injections of Ibotenic Acid or 6-OHDA into the PBP

After 6-OHDA lesions of the PBP, there was a marked disappearance of TH-positive cell bodies and axons in the VTA (Fig. 5B). There was also almost a total disappearance of TH axons and axon terminals in the NAC, and only a few TH-positive axons remained in the medial PFC that likely arose from the more lateral SN that was spared by our 6-OHDA lesions. Of the 33 varicosities on the remaining 19 fibers, half possessed GAD65-positive varicosities indicating they were GABAergic fibers. Yet to our surprise, only 5 of the 33 (15%) remaining varicosities were positive for VGLUT2. This is a very small amount compared with the large proportion of VGLUT2 varicosities (56%) found in nonlesioned animals. Thus, 6-OHDA lesions appeared to destroy most of the DA and VGLUT2 projection to the PFC, and this explains why it was so effective in eliminating the EPSP evoked in PFC by VTA stimulation in our past study (Lavin et al. 2005).

Finally, to control for nonspecific staining, we performed additional experiments where we preincubated the anti-VGLUT2 antibody with its Control Peptide (1:50) for 1 h at 4°C. Subsequent staining resulted in an almost complete absence of signal (although some slight residual staining was sometimes observed at the slice surface). We compared 2 sets of PHA-L-filled fibers from the same rat with tracer injections in RL1. One group was taken from slices stained with anti-VGLUT2 antibody and another from slices stained with anti-VGLUT2 antibody preincubated with the Control Peptide (Fig. 2C, D). In the absence of the blocking peptide, of 37 varicosities from 14 fibers analyzed, 31 (83%) were found to be positive for VGLUT2. In contrast, in the presence of the blocking peptide, of 42 varicosities from 17 fibers analyzed, only 1 varicosity was stained and only very weakly. This confirms that our staining protocol specifically targeted actual VGLUT2-containing fibers.

Discussion

The present data showed that for 4 ventral midbrain subregions situated close to the midline, ~60% of their varicosities in the PFC contained VGLUT2. ~41% contained TH, and ~32% were immunopositive for both. However, there were vast differences in the strength of the glutamate component emanating from these different subregions, with a maximum of ~74% of varicosities being VGLUT2 positive in fibers from the rostral VTA and a minimum of ~33% for fibers from the IF region. Fibers from the IF in contrast had the largest relative population of TH-positive varicosities. In general, these results provide evidence that the mesocortical pathway has a very large but regionally heterogeneous VGLUT2 component.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** The presence of TH and VGLUT2 in fibers in the PFC after a BDA injection into the rostral VTA. (A) Left: a deposit of BDA (green) centered in the rostral VTA (Case 25); middle: same slice of midbrain showing the staining for TH (white); right: merging of images at left and middle. (B1) Triple-immunofluorescence staining of a BDA-positive fiber (green) in the PFC, which is also TH positive (white) and contains VGLUT2-positive varicosities (red). (B2) Triple-immunofluorescence staining of BDA-positive fibers (green) that possess VGLUT2-positive varicosities (red) that are not TH positive (white). In (B1) and (B2), the regions in the dotted white box in the top left image are shown at higher magnification in the smaller flanking images. These smaller images show the individual fluorescent channels for BDA (green), TH (white), and VGLUT2 (red). In (A), the scale bar is 10 μm for the larger image and 2 μm for the smaller surrounding images.
**Release of Glutamate from VTA Neurons**

It is safe to say that the extent of glutamatergic communication by VTA neurons has until recently been greatly underestimated. However, detailed analysis of the mRNA content of VTA neurons is beginning to change this perception. Dobi et al. (2010) recently reported that within the VTA itself, a surprisingly large number of the local fibers contain VGLUT2. Yamaguchi et al. (2007) reported that in the rostral VTA, more neurons expressed VGLUT2 mRNA than TH mRNA (~338 neurons/mm² versus ~280 neurons/mm², respectively). Within the medial PN region, they observed that ~282 neurons/mm² expressed VGLUT2 mRNA compared with only ~183 neurons/mm² that expressed TH mRNA. Consistent with these observations, the present data indicated that for many subregions of the VTA, the glutamatergic component was larger than the DA component.

A lingering question remains as to whether the glutamate projection from the VTA comes from a separate population of glutamate neurons or from glutamate-containing DA neurons. Anatomical evidence for possibility of glutamate release from DA neurons was first provided by Kaneko et al. (1990) who showed that DA neurons were immunopositive for a glutamate-synthesizing enzyme. However, more conclusive evidence has been provided by the finding that a subset of midbrain DA neurons contain mRNA for VGLUT2 and therefore have at least the potential to package glutamate into vesicles for release. Overall, the number of midbrain DA neurons containing VGLUT2 mRNA is rather small compared with the total number of midbrain DA cells (Kawano et al. 2006; Yamaguchi et al. 2007; Nair-Roberts et al. 2008). Furthermore, VGLUT2 possessing DA neurons were mostly found in A10 cell group and are practically absent in the A8 and A9 cell groups. Within the A10 group, there is a further subregion-specific pattern with significantly higher densities of VGLUT2-containing DA neurons in rostromedial subregions of the VTA and adjacent RLi where the mesocortical projection takes its origin. According to Kawano et al. (2006), 52.7% of DA cells in RLi express mRNA for VGLUT2, while in the present study, following RLi tracer injections, 30% of varicosities in the PFC were positive for both VGLUT2 and TH. In the more DA-rich regions such as the PBP, rostral VTA, and IF, on average, 20.7% of varicosities were both VGLUT2 and TH positive, a number that is consistent with the data of Seguela et al. (1988) who reported that between 16% and 22% of DAergic terminals in the rat PFC made asymmetric synapses, which are classically viewed as excitatory or glutamatergic. Taken together, these data indicate that a significant proportion of DA neurons have the machinery available to release glutamate in mesocortical pathway.

Although VGLUT2 may be present on synaptic vesicles for the purposes of promoting DA storage (Hnasko et al. 2010), there is also electrophysiological evidence for functional corelease of glutamate from DA neurons in the mesocortical pathway. An early study by Bernardi et al. (1982) as well as a more recent study (Lavin et al. 2005) have shown that VTA stimulation depolarizes or evokes a clear EPSP in PFC neurons recorded intracellularly in vivo. This EPSP can be eliminated by catecholamine-selective neurotoxin 6-OHDA lesions of VTA, suggesting that it requires the integrity of DA neurons (Lavin et al. 2005; Lapish et al. 2007). In the present study, when we injected 6-OHDA into the PBP 4 weeks prior to tracer injections, we found not only a marked disappearance of TH-positive cell bodies and axons in the PFC but also a significant decrease in VGLUT2 fibers as well. This finding now explains the loss of EPSPs in mesocortical pathway in VTA-lesioned rats observed by Lavin et al. (2005). This finding was initially taken as evidence that most of the glutamate in the mesocortical pathway comes from DA neurons. Yet in light of the present data, it would seem that this conclusion is somewhat premature given that non-DAergic neurons also die as a secondary consequence of the destruction of DAergic cells by 6-OHDA. Therefore, although it is clear that the glutamatergic component of the mesocortical pathway is very large, it is not possible...
using our data to determine whether it arises mainly from purely glutamatergic VTA neurons or from DA neurons.

On the other hand, there is now good support for corelease from recent electrophysiological studies. Cultured DA neurons contain VGLUT2 and can produce glutamatergic EPSPs (Sulzer et al. 1998; Joyce and Rayport 2000; Dal Bo et al. 2004; Descaries et al. 2008). Likewise, glutamatergic EPSPs can be evoked in NAC neurons by VTA stimulation in a novel VTA-NAC acute slice preparation (Chuhma et al. 2004). Furthermore, elimination of VGLUT2 selectively in DA neurons in these slices using dopamine transporter (DAT)-Cre/Vglut2lox knockout mice removed this EPSP (Hnasko et al. 2010). Yet perhaps the most conclusive electrophysiological evidence for corelease has been provided recently by both Stuber et al. (2010) and Técupetla et al. (2010) who used optogenetic techniques to selectively activate DA neurons and found that doing so produced EPSCs in the vast majority of medial NAC neurons recorded in brain slices. Collectively, these data build a compelling case for corelease of glutamate from DA neurons in the mesoaccumbens pathway.

At first glance, these data might seem at odds with the present data where we found little evidence for TH/VGLUT2 coexpression in the NAC, as well as the known anatomical characteristics of the mesoaccumbens pathway. For instance, even though ~2% of neurons contain mRNA for both TH and VGLUT2 within the VTA, at the level of the NAC, electron microscopic studies failed to reveal VGLUT2 content in TH-positive varicosities in adult tissue (Descaries et al. 2008; Berube-Carriere et al. 2009). This negative result may be due to the potentially low detection threshold of the preembedding immunogold technique used in these studies. On the other hand, considering absolute number of varicosities analyzed in that study was ~500, if the VGLUT2 protein was present in just 0.5% or less of TH varicosities it would have escaped detection. Yet, even such a low percentage of VGLUT2-containing DA varicosities could be physiologically significant solely because of the massive density of DA varicosities in the rat striatum, which is on the order of ~1 x 10⁶/µm³ (Doust et al. 1986). As a result, if VGLUT2 was present in just 0.5% of varicosities, it would translate into 500 000 varicosities per cubic millimeter. Given the estimate of 10 000-20 000 neurons/µm³ in the adult rat striatum (Doust et al. 1986), this means that there would be about 25-50 DA terminals expressing VGLUT2 per striatum neuron (assuming all cells receive an equal input). Therefore, one would actually predict that the glutamatergic component should be detected electrophysiologically yet escape immunohistochemical detection if only a few hundred varicosities were analyzed.

The situation in the mesocortical pathway is somewhat different. The density of DAergic varicosities in rat PFC is ~100-fold lower than in striatum (Doust et al. 1986; Descaries et al. 1987), while the density of neurons is much higher at ~100 000/mm³. Given this and that the synaptic incidence of DAergic terminals in rat PFC was reported to be as high as 93%, it means that a given pyramidal neuron would receive about 10 contacts from DA-containing fibers. In the present study, we reported that upwards of 30% of mesocortical fibers expressed immunoreactivity for both VGLUT2 and TH. This means that a given pyramidal neuron would receive on average input from ~3 TH varicosities that also contain VGLUT2. Given this small number, if the experiments with optogenetic activation of DA fibers (Stuber et al. 2010) were performed on PFC neurons, it would be much less likely that they would ever detect an EPSC at the soma. On the other hand, the most notable feature of the mesocortical pathway is the high density of VGLUT2-only fibers. Therefore, in order to get a large EPSC in this projection, one would need to somehow optogenetically activate DA neurons and the population of glutamate neurons in the VTA.

**Functional Implications**

As noted above, while regions such as the RLi, rostral VTA, and medial PN nuclei are very rich in glutamate, the number of glutamatergic neurons in the general population of all VTA neurons is relatively small (Nair-Roberts et al. 2008). However, for the most part, the regions sending projections to the PFC, such as the medial PBP, rostral VTA, and RLi (Fallon 1981; Swanson 1982), appear to also contain the highest proportion of neurons expressing VGLUT2. Accordingly, following injection of retrograde tracer into the PFC, almost half of the labeled VTA neurons were positive for the VGLUT2 (Hur and Zaborszky 2005). One could justifiably ask why this would be the case?

DA neurons in the midbrain encode prediction error signals by emitting brief bursts in response to events that are better than predicted or a brief cessation of firing to events that are worse than predicted (Schultz et al. 1997). It has been suggested that this signal is communicated to the forebrain via DA, which is certainly a likely scenario for regions such as the striatum. Yet, there are many reasons to believe that DA may not be ideal to transmit this type of fast signal to the PFC, as reviewed previously (Lapish et al. 2007). VTA neurons signal prediction errors by alterations in firing on the order of ~500 ms or less (Schultz et al. 1997). In the PFC, there are fewer DA transporters (Sesack et al. 1998) and increases in DA release to even very phasic stimulation lasts on the order of many seconds (Garris et al. 1993; Lavine et al. 2005). Furthermore, in response to stressful events that are presumably worse than expected, PFC DA levels measured electrochemically increase for tens of minutes past the stressful event (Doherty and Gratton 1996; Brake et al. 2000; Zhang et al. 2005). Therefore, even though events change firing bidirectionally in the VTA on the order of milliseconds, within the PFC the DA signal is neither temporally precise nor bidirectional in its response to events of positive and negative valence. However, the release of glutamate is tightly coupled to neuronal firing and as we have argued could provide a highly temporally precise prediction error signal in the PFC, while the slower DA signal might modulate the state of the cortex on more protracted time scales (Seamans and Yang 2004; Lavine et al. 2005; Lapish et al. 2007). The present data buttress this claim by showing that there are a larger number of glutamatergic than DAergic fibers projecting from the rostromedial midbrain to the PFC. These fibers could provide a plausible substrate for fast and perhaps localized transmission of brief prediction error signals from the midbrain. In this way, the lack of DAT and fast DA signaling as one moves medially in the midbrain may have been compensated by a progressive increase in the amount of VGLUT2.

Recently, DA neuron-specific VGLUT2 knockout mice were shown to exhibit a blunted response to psychostimulants and altered risk-taking behavior, suggesting that corelease is functionally important for behaviors that depend on the mesoaccumbens DA system (Birgner et al. 2010; Hnasko et al. 2010). However, given the apparent size of the glutamatergic component of the mesocortical pathway shown here,
glutamate neurons and glutamate-containing DA neurons may have an even greater role in PFC-dependent processes, such as working memory, behavioral flexibility, and decision making.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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