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MAPPING DOPAMINERGIC DEFICIENCIES IN THE SUBSTANTIA NIGRA/VENTRAL TEGMENTAL AREA IN SCHIZOPHRENIA

Matthew W Rice¹, Rosalinda C Roberts², Miguel Melendez-Ferro^{1,2,◆}, and Emma Perez-Costas^{1,2,*},◆

¹Department of Psychology, University of Alabama at Birmingham

²Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham

Abstract

Previous work from our laboratory showed deficits in tyrosine hydroxylase protein expression within the substantia nigra/ventral tegmental area (SN/VTA) in schizophrenia. However, little is known about the nature and specific location of these deficits within the SN/VTA.

The present study had two aims: 1) test if tyrosine hydroxylase deficits could be explained as the result of neuronal loss; 2) assess if deficits in tyrosine hydroxylase are subregion specific within the SN/VTA, and thus, could affect specific dopaminergic pathways.

To achieve these objectives: 1) we obtained estimates of the number of dopaminergic neurons, total number of neurons and their ratio in matched SN/VTA schizophrenia and control samples; 2) we performed a qualitative assessment in SN/VTA schizophrenia and control matched samples that were processed simultaneously for tyrosine hydroxylase immunohistochemistry.

We did not find any significant differences in the total number of neurons, dopaminergic neurons, or their ratio. Our qualitative study of TH expression showed a conspicuous decrease in labeling of neuronal processes and cell bodies within the SN/VTA, which was sub-region specific. Dorsal diencephalic dopaminergic populations of the SN/VTA presented the most conspicuous decrease in TH labeling. These data support the existence of pathway-specific dopaminergic deficits that would affect the dopamine input to the cortex without significant neuronal loss. Interestingly, these findings support earlier reports of decreases in tyrosine hydroxylase labeling in the target areas for this dopaminergic input in the prefrontal and entorhinal cortex. Finally, our findings support that tyrosine hydroxylase deficits could contribute to the hypodopaminergic state observed in cortical areas in schizophrenia.

Correspondence: Emma Perez-Costas, PhD, Campbell Hall 415, 1720 2nd Avenue South, Birmingham, AL 35294, USA, Phone: +1 205 934 3850, epcostas@gmail.com.

◆Equal contributors

Ethical Standards

The manuscript does not contain clinical studies or patient data.

Conflict of Interest

The authors do not have any conflict of interest to report.

Keywords

Tyrosine hydroxylase; immunohistochemistry; mesocortical; mesolimbic; neuropathology; neuropsychiatric disorders

INTRODUCTION

Anomalies in the dopaminergic system are an intrinsic pathological feature of schizophrenia (see as reviews van Os and Kapur 2009; Abi-Dargham et al. 2010; Perez-Costas et al. 2010; Tost et al. 2010). In addition, the effectiveness of all currently available antipsychotic medications is directly linked to the action on these drugs on the dopaminergic system (Carlsson et al. 1957; Carlsson and Lindqvist 1963; Seeman and Lee 1975; Seeman et al. 1976; Creese et al. 1976; see also as reviews Frankle and Laruelle 2002; Howes and Kapur 2009; Perez-Costas et al. 2010). Despite the importance of dopaminergic pathologies in schizophrenia, only a few studies have examined the substantia nigra/ventral tegmental area in schizophrenic brains (Bogerts et al. 1983; Ichinose et al. 1994; Mueller et al. 2004; Perez-Costas et al. 2012; Howes et al. 2013; Williams et al. 2013).

The substantia nigra/ventral tegmental area (SN/VTA) complex is a heterogeneous collection of dopaminergic cell groups that extends from diencephalic to mesencephalic territories in many vertebrates, including rodents, non-human primates, and humans (Puelles and Verney 1998; see as reviews Smits et al. 2006; Smidt and Burbach 2007; Smits et al. 2013). The SN/VTA contains the largest number of dopaminergic neurons in the brain (see as reviews van Domburg and ten Donkelaar 1991; Haber and Fudge 1997; Nieuwenhuys et al. 2008), with average estimates ranging in most studies from approximately 250,000–440,000 dopaminergic neurons within the complex (Bogerts et al. 1983; Hirsch et al. 1988; van Domburg and ten Donkelaar 1991; Damier et al. 1999a; Kubis et al. 2000).

The human SN/VTA presents high complexity and is composed of several dopaminergic neuronal subpopulations that differ in their neurodevelopmental origin (Zecevic and Verney 1995; Puelles and Verney 1998; Verney et al. 2001), genetic and neurochemical profile (Haber et al. 1995; Grimm et al. 2004; Thuret et al. 2004; Chung et al. 2005; Luk et al. 2013), projection sites (van Domburg and ten Donkelaar 1991; Damier et al. 1999a; Nieuwenhuys et al. 2008), and susceptibility to disease (Gibb and Lees 1991; Damier et al. 1999b; Hauser et al. 2005; Fuchs et al. 2009; Bergman et al. 2010). In fact, developmental studies of the human brain have clearly demonstrated the existence of well-defined rostro-caudal (i.e. anterior-posterior) subregions within the SN/VTA, which contain subgroups of dopaminergic cells with different genetic profiles and developmental schedule (Puelles and Verney 1998; Verney et al. 2001; van den Heuvel and Pasterkamp 2008; Nelander et al. 2009). For example, TH-expressing dopaminergic neurons located in the caudal sub-region (i.e. immature mesencephalon) of the SN/VTA are already detected by the 6th gestational week, while in the rostral SN/VTA sub-region (i.e. immature diencephalon or prosomeres 1–3 of the neural tube) these cells are not clearly observed until the 10th gestational week (Zecevic and Verney, 1995; Verney, 1999; Puelles and Verney 1998; Verney et al. 2001). In the adult brain, this diencephalic sub-region include approximately all SN/VTA neuronal

populations located rostral to the exit of the 3rd cranial nerve and to the transition between the parvocellular and magnocellular parts of the red nucleus (see for anatomical details van Domburg and ten Donkelaar 1991; Damier et al. 1999a; Nieuwenhuys et al. 2008). In addition, detailed neuropathological studies in Parkinson's disease have shown that diencephalic SN/VTA dopaminergic neurons are significantly more resilient to neuronal death than the mesencephalic ones (Damier et al. 1999b), thus supporting the validity of using developmental criteria to define SN/VTA sub-regions for neuropathological studies in the adult brain.

Detailed studies on the projection targets of the dopaminergic neurons of the SN/VTA have also demonstrated the existence of neuronal subgroups within the complex that preferentially project to cortical or subcortical regions (see as reviews van Domburg and ten Donkelaar 1991; Haber and Gdowski 2004; Nieuwenhuys et al. 2008). For example, dorsal neuronal groups of the SN/VTA in primates preferentially provide dopaminergic input to cortical areas through the mesocortical pathway, although they also partially contribute to the mesolimbic pathway (Gaspar et al. 1992; Haber and Fudge 1997; see also as reviews Haber and Godowski 2004; Nieuwenhuys et al. 2008). Interestingly, dopaminergic deficits in these pathways have been associated with the hypodopaminergic state observed in individuals diagnosed with schizophrenia (Akil et al. 1999, 2000; Finlay 2001; Seamans and Yang 2004; Toda and Abi-Dargham 2007), and are most closely related to the cognitive deficits that are a core feature of the illness (Finlay 2001; Seamans and Yang 2004; Tanaka 2006; Toda and Abi-Dargham 2007). Ventrally-located neurons of the SN/VTA complex preferentially project to subcortical regions such as the dorsal and ventral striatum through the nigrostriatal, and in part, the mesolimbic pathway (see as reviews Haber and Godowski 2004; Nieuwenhuys et al. 2008). Disturbances of dopamine neurotransmission within the nigrostriatal pathway in schizophrenia are associated with a hyperdopaminergic state, and are most closely linked to the positive symptoms (e.g. psychosis) seen in this illness (Seeman 1987; Davis et al. 1991; Toda and Abi-Dargham 2007; Howes and Kapur 2009; Meyer-Lindenberg 2010).

Dopamine plays a key role in the modulation of cognitive processes in the prefrontal cortex, and it is known that low dopamine levels reduce working memory performance in humans (Seamans and Yang 2004; Floresco and Magyar 2006; Tanaka 2006). Several different molecular pathologies that produce as a net result a deficit of dopaminergic neurotransmission in the prefrontal cortex have been identified in schizophrenia subjects (Akil et al. 1999; Seamans and Yang 2004; Guillin et al. 2007; Tan et al. 2007; Raznahan et al. 2011; Perez-Costas et al. 2012a; Kaalund et al. 2013; Lopez-Garcia et al. 2013). Among these pathologies, work from our laboratory has revealed deficits in TH protein expression in the SN/VTA in schizophrenia subjects, although these deficits were only observed in cases that contained the rostral (diencephalic) region of the SN/VTA complex (Perez-Costas et al. 2012a), which indicates a possible sub-region specific deficit within the complex. In this study we sought to elucidate if these TH deficits could be related to overall and/or subregion-specific reductions in neuronal number, by examining both total and dopaminergic neuronal counts in schizophrenia subjects compared to non-psychiatric controls. In addition, we performed a qualitative assessment on the specific location of these TH expression deficits.

METHODS

Ethics statement

All human brain samples were obtained from the Maryland Brain Collection (Maryland Psychiatric Research Center, University of Maryland School of Medicine) with approval from the Maryland Brain Collection Steering Committee. All experimental procedures were approved by the University of Alabama at Birmingham Institutional Review Board (protocols #N110505002 and #N131015007) and were in accordance with The Code of Ethics of the World Medical Association.

Postmortem human brain samples

For all postmortem human cases, two independent psychiatrists established DSM-IV diagnoses based on the review of available medical records and data obtained from the Structured Clinical Interview for DSM-III-R (Spitzer et al., 1992) and DSM-IV Axis I disorders with the next-of-kin. Non-psychiatric control cases had enough information from medical records and the next-of-kin to discard any major neurological or neuropsychiatric disorders. Table 1 contains demographic data, as well as information on the use of antipsychotic treatment, cause of death, and other relevant parameters for the samples used in this study.

All postmortem SN/MTA samples were dissected as described by Damier et al (1999a) and preserved in 4% paraformaldehyde prepared in 0.1M phosphate buffer pH 7.4 (PB), at 4°C. Prior to sectioning, the tissue was thoroughly rinsed in PB (8 rinses, 1 hour each), and immersed in a solution containing 30% sucrose in PB at 4°C for two weeks. Tissue samples were frozen using dry ice and sectioned on a freezing microtome. Eight adjacent (i.e. parallel) series were obtained perpendicular to the longitudinal axis of the brainstem at a thickness of 50µm. All samples were sectioned in this manner to allow for replication of the experiments, and to maintain backup series for future studies. All series were equivalent and contained sections of the entire rostro-caudal extent of the SN/MTA.

Series #1 and 2 were collected in PB containing 0.02% sodium azide and stored at 4°C until use. Series #3 through 8 were collected in a cryoprotectant solution prepared as described in Watson et al. (1986) and stored at -20°C. For all cases, series #1 was stained using thionin (FD Neurotechnologies, MD, USA; PS101-01) to assess for any morphological anomalies related to poor preservation of the tissue, and only those cases that presented good preservation of the SN/MTA were included in the study. Exclusion criteria were used in the same manner for schizophrenia and control samples as previously described (Perez-Costas et al, 2012a).

Immunohistochemistry

Complete series preserved in cryoprotectant solution were used for immunohistochemistry. Sections were rinsed ten times (5 minute each) in 0.01M phosphate buffered saline (PBS) at room temperature (RT) to remove the cryoprotectant solution. Sections were then transferred to citrate buffer (Vector Laboratories, Burlingame, CA, USA; H-3300) for ten minutes at RT, prior to a 30 minute citrate pretreatment at 80°C. Following pretreatment,

sections were rinsed in citrate buffer at RT and transferred to PBS. After that, endogenous peroxidase was blocked with 5% H₂O₂ in PBS for 30 minutes at RT, followed by 6 rinses (5 minute each) in PBS. Sections were then incubated with 10% normal horse serum (NHS; Vector Laboratories, S2000) in PBS containing 0.5% Triton X-100 (PBS-T) for 1 hour at RT. Immediately after, sections were incubated for 66 hours at 4°C with an anti-TH antibody (Millipore, Billerica, MA, USA; MAB318, diluted 1:5000) prepared in a solution containing 3% NHS in PBS-T. Next, sections were rinsed in PBS (6 rinses, 5 minutes each) and incubated with a biotinylated horse anti-mouse secondary antibody (Vector Laboratories, BA-2001, diluted 1:400) in a solution containing 3% NHS in PBS-T for 45 minutes at RT. After this, sections were rinsed 6 times (5 minute each) in PBS and incubated with a peroxidase-coupled avidin-biotin-complex (Vector Laboratories PK6100, diluted 1:100) for 45 minutes at RT. Finally, sections were rinsed 6 times (5 minute each) in PBS and developed using a 3,3'-diaminobenzidine peroxidase kit (Vector Laboratories, SK4100). A negative control, incubated in a solution lacking the primary antibody and consisting of 3% NHS in PBS-T, was also performed to ensure specificity of the secondary antibody. In addition, the specificity of the primary antibody has been previously determined by the manufacturer.

For each case, a minimum of two complete series representing the entire rostro-caudal extent of the SN/VTA were immunolabeled for TH. One of these series was immunolabeled for TH without counterstaining, while the other series was counterstained using thionin (FD Neurotechnologies) after immunolabeling. In all cases, sections were dehydrated in ethanol, cleared in xylene and coverslipped using Eukitt (Electron Microscopy Sciences, Hatfield, PA, USA; 15322).

Schizophrenia and matched control cases were processed together for immunohistochemistry, carefully maintaining the exact same incubation and developing conditions across cases.

Photography and image processing

Images were obtained using a Nikon Eclipse 50i microscope equipped with a Nikon DS-Fi1 color digital camera ((Nikon, Tokyo, Japan). Images were saved as uncompressed TIFF files at a 2560×1920 resolution. Adjustments of brightness/contrast were done using the same parameters for all images. These adjustments as well as photomontage and lettering of figure plates were done using Corel DRAW X5 (Corel Corporation, Ottawa, Canada).

Neuronal counts and mapping of TH deficits

Oversampling test for study design—A pilot study was conducted to determine the optimal number of sections, and the number of counting frames to be analyzed from each section. A complete series (approximately 30–32 sections) from each of two control cases (C3 and C6) were used to perform this preliminary assessment. Each section was photographed using a 10X objective and setting randomly a fixed point in the Z axis within the 50µm thickness of the section. Ten non-overlapping images were taken from each section to cover the entire SN/VTA complex within the section. Each image was considered a “counting frame” with the bottom and right margins of the image as the forbidden lines of

the counting frame. Neurons were selected for counting only if the entire cell body and the nucleus were clearly defined in the image. Neurons touching the right and bottom borders of the frame were excluded from counting. A “complete series estimate” (CSE) for the number of total and dopaminergic neurons was obtained as the mean of the counts of all the neurons (dopaminergic or total) for one entire series for the two cases. The neuronal counts accuracy was calculated as the “percentage of variation” from the CSE using the following formula:

$$\% \text{ of variation} = [(\text{Partial count estimate} - \text{CSE}) / \text{CSE}] \times 100$$

A summary of the results of this oversampling test is reported in Table 2 (see also results).

Neuronal counts in schizophrenia and control cases—Following the oversampling test, the optimal counting scheme was set as follows: **1)** For each case, using a complete SN/VTA series labeled for TH and counterstained with thionin, the rostral and caudal boundaries of the SN/VTA were determined using the Paxinos and Huang (1995) atlas of the human brainstem as well as the neuroanatomical study of van Domburg and ten Donkelaar (1991). The boundary between the diencephalic (i.e. rostral) and mesencephalic (i.e. mid-caudal) sub-regions of the SN/VTA was defined as the transition between the parvocellular and magnocellular parts of the red nucleus, at the level of the exit of the oculomotor nerve (see e.g. Damier et al. 1999a). **2)** The first section to be counted was selected randomly within 1200µm of the rostral boundary of the SN/VTA using a random number generator (i.e. random.org). **3)** From this randomly chosen rostral starting point, every third section of the series, and up to the caudal limit of the SN/VTA, was selected for neuronal counts (i.e. approximately 10–11 sections per case). **4)** A random focal point in the Z axis was chosen within the 50µm thickness of the section. **5)** Using the 10X objective, five images representing 5 randomly placed and non-overlapping “counting frames” were taken for each section within the SN/VTA. **6)** The “cell counter” plug-in of NIH Image J software version 1.46r (<http://rsbweb.nih.gov/ij/>) was used for the manual labeling of dopaminergic and total neurons in the images. This plug-in immediately records the manually-identified neurons in a spreadsheet according to categories previously defined by the experimenter (e.g. “neuron”, “dopaminergic neuron”).

To avoid investigator bias on data collection, all cases were coded, and the researcher performing the neuronal counts was blind for the case diagnoses. In addition, our experimental design prevented any risk of double-counting neurons since each section selected for neuronal counts was 1200µm apart (i.e. one series was used for counts out of 8 series collected, and from this series, 1 out of every 3 sections was counted). Dopaminergic neurons were identified by the presence of TH immunolabeling, and non-dopaminergic neurons identified by their purple-blue thionin staining and lack of TH immunostaining.

Qualitative mapping of dopaminergic deficits—Three schizophrenia cases and their most closely matched control cases were used for this qualitative analysis. Two entire SN/VTA series per case labeled for TH were used for this assessment. For this part of the study we selected schizophrenia cases SZ1, SZ3 and SZ6, for which we previously assessed TH protein expression by western-blot in the opposite hemisphere (Perez-Costas et al. 2012;

cases SZ1, SZ4, SZ6). The matched controls cases were C2, C5 and C6, from which C6 was also included in the previous protein expression study (i.e. case C4 in Perez-Costas et al. 2012).

Statistical analysis of neuronal counts

Effect of demographic and sample quality variables on outcome measures—

Schizophrenia and non-psychiatric control cases were matched as much as possible for demographic (i.e. age, gender, race), and sample (i.e. postmortem interval, brain pH) variables. We used multiple regression to assess the possible influence of demographic and sample variables on our outcome measures (i.e. cell counts) independent of disease status. Demographic and sample variables (i.e. postmortem interval and pH) were tested in separated analyses. For the analysis of demographic variables a “dummy coding” was used to numerically interpret gender and race. The effect of demographic and sample variables was assessed independently for each brain region/sub-region (i.e. entire SN/NTA, diencephalic region, mesencephalic region). However, the effect of these variables on all cell counts of a given region/sub-region was assessed in the same analysis, correcting for multiple comparisons, and the adjusted R^2 is reported to eliminate the add-on effect of testing for multiple outcome variables.

Neuronal counts in schizophrenia versus controls—All data collected on neuronal counts were assessed for the presence of significant deviations from normality. In addition, the presence of outliers was determined using the “*Robust regression and Outlier Removal*” (ROUT) test for outlier detection (Motulski and Brown 2006), with the coefficient Q set to 1%. Unpaired t-tests were corrected for multiple comparisons using the False Discovery Rate (FDR) with Q set at 1% (Motulsky 2010). Multiple comparisons corrections were applied for all parameters tested in the same specific region/sub-region (e.g. total number of neurons, dopamine neurons and ratio in the diencephalic SN/NTA were treated as multiple comparisons). Statistical analysis and graphical representation of the results obtained were done using GraphPad Prism 6.0 software (Graphpad Software Inc., La Jolla, CA, USA).

RESULTS

Oversampling test for study design

In order to obtain accurate estimates of the number of dopaminergic and total neurons, we performed an oversampling study using two control cases (C3 and C6), in which neuronal counts were performed for the entire rostro-caudal extent of the SN/NTA. A summary of the results obtained is shown in Table 2. From this assessment we concluded that we can obtain highly accurate cell count estimates by starting at a random section within the most rostral 1200µm of the SN/NTA, and counting cells on every 3rd section. We also showed that reducing the number of counting frames to one-half (i.e. 5 randomly selected frames counted per section) did not have a significant impact on the accuracy of the counts.

Demographic and sample features

The effect of demographic (i.e. age, gender, race) and sample (i.e. brain pH and postmortem interval) variables on neuronal counts (i.e. total neurons and dopamine neurons) was

assessed by performing independent multiple regression analyses for these two types of variables. Data obtained are summarized in Tables 3 and 4. None of these variables had any significant effect on cell counts (total or dopamine neurons) for any of the regions/sub-regions (i.e. rostro-caudal, rostral, mid-caudal SN/VTA) analyzed.

Dopaminergic and total neuronal counts

Rostral and caudal limits of the SN/VTA were defined as described in previous neuroanatomical studies and atlases that used the same sectioning orientation used in the present study (van Domburg and ten Donkelaar 1991; Paxinos and Huang 1995; Damier et al. 1999a; Nieuwenhuys et al. 2008). The rostral sub-region was defined as the diencephalic territories of the SN/VTA, while mid-caudal sub-region consisted in the mesencephalic territories of the complex. These subdivisions were included in the study design (see methods), and were based on previous developmental studies showing clearly demarcated diencephalic and mesencephalic SN/VTA sub-regions in the human (Zecevic and Verney 1995; Puellas and Verney 1998; Verney et al. 2001), and conspicuous differences in the response to pathological processes of these two sub-regions (Gibb and Lees 1991; Damier et al. 1999b; Hauser et al. 2005).

Neuronal counts in the entire SN/VTA—We obtained estimates of the total number of neurons and dopaminergic neurons for the entire SN/VTA. Unpaired t-test corrected for multiple comparisons were used to compare these measurements in schizophrenia versus controls. There were no significant differences in the total number of neurons (schizophrenia=603,160±165,990; control=656,537±128,964; $p=0.5272$, $t=0.6529$, $df=11$), number of dopaminergic neurons (schizophrenia=218,528±70,563; control=249,161±44,353; $p=0.3997$, $t=0.8760$, $df=11$), or the ratio of dopaminergic/total neurons (schizophrenia=0.3610±0.0735; control=0.3819±0.0328; $p=0.5099$, $t=0.6811$, $df=11$) [Figure 1].

Neuronal counts in the diencephalic SN/VTA—Unpaired t-test corrected for multiple comparisons yielded non-significant differences in the total number of neurons (schizophrenia=224,064±28,903; control=202,519±61,180; $p=0.4479$, $t=0.787$, $df=11$), number of dopaminergic neurons (schizophrenia=70,640±25,495; control=70,512±22,552; $p=0.9925$, $t=0.0096$, $df=11$), or the ratio of dopaminergic/total neurons (schizophrenia=0.3198±0.1168; control=0.3554±0.0748; $p=0.5189$, $t=0.6664$, $df=11$) in the diencephalic (rostral) sub-region of the SN/VTA [Figure 2].

Neuronal counts in the mesencephalic SN/VTA—Unpaired t-test corrected for multiple comparisons for the mesencephalic (mid-caudal) sub-region also yielded non-significant differences in the total number of neurons (schizophrenia=379,096±174,111; control=454,018±93,937; $p=0.3445$, $t=0.9876$, $df=11$), number of dopaminergic neurons (schizophrenia=147,888±52,813; control=178,649±50,190; $p=0.3051$, $t=1.0757$, $df=11$), or the ratio of dopaminergic/total neurons (schizophrenia=0.4165±0.1389; control=0.3898±0.0410; $p=0.6354$, $t=0.4876$, $df=11$) [Figure 3].

Qualitative mapping of dopaminergic deficits in the SN/VTA

There is great variability in the sectioning orientation and nomenclature of subdivisions of the human SN/VTA in anatomical studies, creating great complexity for comparative assessments of neuropathology. In the present study all samples were sectioned in a plane perpendicular to the main axis of the brainstem, which is the same sectioning orientation previously used by van Domburg and ten Donkelaar (1991), Damier et al (1999a) and Nieuwenhuys et al (2008) detailed anatomical studies of the human SN/VTA. For the qualitative assessment of TH expression we also followed the major sub-regions within the SN/VTA (i.e. diencephalic versus mesencephalic sub-regions) previously used for the neuronal counts. In addition, we also took into account the dorso-ventral and medio-lateral location of the dopaminergic cell groups, defining three sub-areas for our qualitative assessment: dorsal SN/VTA, ventro-lateral SN/VTA, and ventro-medial SN/VTA [Figures 4–6].

Diencephalic SN/VTA—The dorsal area of the diencephalic SN/VTA included the nuclei identified by van Domburg and ten Donkelaar (1991) as the pars lateralis of the substantia nigra, the anterolateral nucleus of the substantia nigra compacta, and the diencephalic part of the parabrachial nucleus pigmentosus. The ventrolateral area corresponded with the anterointermediate nucleus of the substantia nigra compacta, while the ventro-medial area included the anteromedial nucleus of the substantia nigra compacta and the diencephalic part of the paranigral nucleus of van Domburg and ten Donkelaar (1991) [Figures 4A–B].

The diencephalic sub-region of the SN/VTA presented an overall conspicuous reduction of TH labeling in schizophrenia compared to matched controls [Figure 4]. Interestingly, there was also a clear dorso-ventral pattern of TH deficits in schizophrenia compared to controls [Figures 4–5]. In schizophrenia, dorsal areas of the SN/VTA showed the lowest TH labeling, while ventral areas (ventro-lateral and ventro-medial), although also presenting clearly decreased labeling, consistently showed stronger TH expression than dorsal SN/VTA dopaminergic neuronal groups [Figures 4 and 5]. Immunolabeling for TH was clearly decreased in cell bodies and also in neuronal processes [Figure 5].

Mesencephalic SN/VTA—The dorsal area of the mesencephalic SN/VTA included the postero-superior and postero-lateral subnuclei of the substantia nigra compacta, as well as the mesencephalic part of the parabrachial nucleus pigmentosus of van Domburg and Donkelaar (1991), while the ventrolateral and ventromedial areas corresponded with the posteromedial nucleus of the substantia nigra compacta and the mesencephalic part of the paranigral nucleus, respectively.

In the mesencephalic region of the SN/VTA differences in TH labeling between schizophrenia and controls were subtle [Figure 6]. Dorsal SN/VTA presented moderate to subtle reductions in overall labeling [Figures 6C–D, F–G]. Ventrolateral and ventromedial SN/VTA areas did not present clear differences in TH immunolabeling between schizophrenia and their matched control cases [Figures 6C, 6E, 6F, 6H].

DISCUSSION

The present study shows evidence of conspicuous sub-region specific deficits in TH protein expression within the diencephalic sub-region of the SN/VTA, without neuronal loss.

Neuropathological changes in the SN/VTA, and more specifically, in the dopaminergic cell groups of the SN/VTA in schizophrenia subjects have been scarcely studied. An early report by Bogerts et al. (1983) reported deficits in volume and neuronal size in specific subnuclei of the SN/VTA, while a recent work has reported increases in the volume of dopaminergic cells as well as glial anomalies in the mesencephalic portion of the SN/VTA (Williams et al. 2013). However, most of the few postmortem studies performed have focused on the expression of tyrosine hydroxylase (Ichinose et al. 1994; Mueller et al. 2004; Perez-Costas et al. 2012a; Howes et al. 2013), which is the rate-limiting enzyme for the production of dopamine. Tyrosine hydroxylase expression studies have reported different results, from increases in TH mRNA (Mueller et al. 2004) and TH protein immunolabeling (Howes et al. 2013), to no changes in TH mRNA (Ichinose et al. 1994; Perez-Costas et al. 2012a), and deficits in TH protein expression (Perez-Costas et al. 2012a). The differences in the data obtained in these studies could be due to several factors including the heterogeneity of the disease (see as reviews Keshavan et al. 2011; Nasrallah et al. 2011), methodological differences in the detection of TH mRNA and protein expression, and of special importance, differences in the sampling area within the SN/VTA. Our results will be discussed taking into account developmental and neuroanatomical considerations and addressing potential functional implications.

Neuronal counts

Neuronal count estimates obtained in the present study for total neurons as well as dopaminergic neurons, in schizophrenia and control samples, were comparable to previous reports in which estimates were obtained from the entire SN/VTA (Bogerts et al. 1983; Hirsch et al. 1988; van Domburg and ten Donkelaar 1991; Damier et al. 1999a; Rudow et al. 2008). Our analysis of the effect of demographic variables on the number of dopaminergic neurons showed no significant effect of any of these variables, including age. While progressive cell loss related to normal aging has been reported in the SN/VTA (Fearnley and Lees 1991; Kubis et al. 2000; Rudow et al. 2008), most of the cases included in the present study were in the “middle age” group, ranging from 40–65 years of age, and only two cases were above this age (see Table 1). Previous studies assessing total and dopaminergic neuronal cell number in the entire SN/VTA have shown similar results for dopaminergic neuronal estimates in their “middle age” groups (Kubis et al. 2000; Rudow et al. 2008). It is worth noting that although Rudow et al. (2008) reported significant reductions in neuronal number with age in their study, their differences were reported by comparing individuals with an average age for their groups of 19.9 ± 1.21 (young) vs 50.1 ± 5.01 (middle age) vs 87.4 ± 7.87 (elderly), thus large gaps of age (e.g. > 17 years between the “young” and “middle age”, and >20 years between the “middle age” and “elderly”) were present among these groups.

Our comparison of neuronal counts in the schizophrenia and control cases did not reveal significant differences in the total number of neurons or dopaminergic neurons, both, when

the entire SN/VTA was analyzed as a whole, and when the diencephalic and mesencephalic subregions were assessed independently. These results confirm the early report by Bogerts et al. (1983) who also found a lack of significant differences in neuronal number in schizophrenia subjects compared to controls in the SN/VTA.

Qualitative mapping of TH immunolabeling

The qualitative mapping of TH labeling in the SN/VTA was done in three of the schizophrenia cases and their most closely matched control cases (see methods). In this qualitative assessment we found conspicuous deficits in TH immunolabeling in diencephalic (rostral) sub-region, while TH deficits in the mesencephalic sub-region were weak or not found (see Figure 4–5 versus Figure 6). These data are in accordance with our previous western-blot study, in which we found that TH protein deficits in schizophrenia cases were only present in protein homogenates that contained the rostral sub-region of the SN/VTA and could not be explained as an effect of antipsychotic medication (Perez-Costas et al. 2012a). In addition, in the present study we found that TH deficits affected very conspicuously not only the cell bodies, but also the neuronal processes of these dopaminergic neurons (see detail images in Figure 5), which suggests that these deficits should also be present in the target areas of these projections. Interestingly, Akil et al. (1999, 2000) have reported layer-specific deficits in dopaminergic processes in the prefrontal and entorhinal cortex of schizophrenia subjects. In earlier neuroanatomical studies of dopaminergic afferents of the human SN/VTA, it was already described that approximately the rostral third of the SN/VTA preferentially provided dopaminergic input to cortical areas (see as a review van Domburg and ten Donkelaar 1991). Supporting this idea, detailed developmental studies have shown that in the human embryonic brain, the rostral (i.e. diencephalic) dopaminergic neurons of the SN/VTA differentiate later, and that there is a caudo-rostral gradient of maturation for these cells and their projections (Zecevic and Verney 1995; Verney 1999; Verney et al. 2001). The dopaminergic SN/VTA neurons of the mesencephalic sub-region are clearly observed at gestational week 6, and their projections reach subcortical areas such as the dorsal striatum by the 7th–8th week of embryonic development. Meanwhile the dopaminergic neurons of the diencephalic subregion are not clearly observed until the 10–11th week (Zecevic and Verney 1995; Verney 1999; Puelles and Verney 1998; Verney et al. 2001). Dopaminergic projections reach cortical regions by week 13th (Zecevic and Verney 1995; Verney 1999; Verney et al. 2001), correlating with the maturation of the diencephalic SN/VTA neurons.

Developmental and neuropathological studies on the primate SN/VTA support the existence of fundamental differences between the diencephalic and mesencephalic SN/VTA dopaminergic neurons. In the adult brain, dopaminergic neurons of these two sub-regions present different neurochemical profiles (Haber et al. 1995; Grimm et al. 2004; Thuret et al. 2004; Chung et al. 2005; Luk et al. 2013) and susceptibility to pathology (Gibb and Lees 1991; Damier et al. 1999b; Hauser et al. 2005; Fuchs et al. 2009; Bergman et al. 2010; Surmeier et al. 2010). It has been shown that these neuronal populations are segregated from early on in the development of the embryonic human brain (Zecevic and Verney 1995; Verney 1999; Puelles and Verney 1998; Verney et al. 2001). Gene expression boundaries between the diencephalic and mesencephalic sub-regions are developed very early on, thus

separating the immature dopaminergic cell precursors of both sub-regions, which are exposed to different neurochemical factors (Smits et al. 2006; Smidt and Burbach 2007; Smits et al. 2013). Also supporting the existence of fundamental differences between diencephalic and mesencephalic sub-regions, the detailed study by Damier et al (1999b) showed that dopaminergic SN/VTA neurons located in the mesencephalic sub-region degenerate at much earlier stages in Parkinson's disease than diencephalic SN/VTA neurons (see also as a review, Surmeier et al. 2010). These findings strongly support the lack of homogeneity among mesodiencephalic dopaminergic neurons, and make plausible that diencephalic vs mesencephalic dopaminergic neurons present different susceptibilities to pathological processes, not only in Parkinson's disease, but also in other illnesses such as schizophrenia.

Taking into account all the evidence presented above, we hypothesize that TH deficits in the SN/VTA of schizophrenia subjects will preferentially (or perhaps exclusively) affect dopaminergic cortical projections of the mesocortical and mesolimbic pathways, thus connecting the present findings with the hypodopaminergic state observed in the cortex in schizophrenia patients (Finlay 2001; Remington et al. 2011), and with postmortem studies showing dopaminergic deficits in the cortex (Akil et al. 1999; 2000). Although Howes et al. (2013) have reported increases in TH labeling, a careful review of their study shows that there are fundamental differences in their sampling methods and postmortem interval, as well as in the area sampled. While in our study we have mapped the entire SN/VTA, Howes et al. (2013) performed an analysis of an area located at the level of the superior colliculus, limiting their study to a small portion of the mesencephalic SN/VTA sub-region. In addition, in the supplementary data section of Howes et al. (2013) it is reported that their time from death to sample preservation ranged from 24 to 126 hours for the schizophrenia group, and between 24 and 68 hours for the control group, while in the present study all cases were below 32 hours from death to preservation (see Table 1). In our qualitative assessment of the mesencephalic SN/VTA, we only found subtle differences in TH immunolabeling restricted to the dorsal portion of the mesencephalic SN/VTA between schizophrenia and control subjects, while ventrolateral and ventromedial areas did not present any differences (see Figure 6). The lack of conspicuous differences in mesencephalic SN/VTA is also in agreement with our previous quantitative study showing no significant differences in TH protein expression in this sub-region (Perez-Costas et al. 2012a).

Another feature clearly observed in our qualitative assessment of the rostral SN/VTA of the schizophrenia samples was the presence of a dorso-ventral pattern of TH deficits, in which the dorsal region was the most affected (see Figure 4). It has been consistently reported that dopaminergic neurons located in the "dorsal tier" of the SN/VTA (see as a review Haber and Godowski 2004), which approximately correspond with the dorsal sub-area of the present study, preferentially project to cortical areas (Fallon and Loughlin 1987; Gaspar et al. 1992; Haber et al. 1995; Haber and Godowski 2004; Nieuwenhuys et al. 2008), further supporting a direct link between the tyrosine hydroxylase deficits reported here and the reports by Akil et al. (1999, 2000) on TH deficits in specific layers of the entorhinal and prefrontal cortex.

It is important to note that for most of the schizophrenia cases included in this study we have previously assessed TH mRNA and protein expression in the opposite hemisphere (i.e. SZ2-

SZ6 of the present study were cases SZ1 and SZ3-SZ6 of Perez-Costas et al. 2012a), in which we found TH protein deficits without a decrease in TH mRNA expression. Those data suggested that TH deficits in schizophrenia must occur after mRNA transcripts are successfully produced. This is also supported by a preliminary study in which we found that deficits in TH protein expression in schizophrenia significantly correlate with deficits in poly-c-binding protein 4, which is involved in the stabilization of TH mRNA for its translation into protein (Perez-Costas et al. 2012b). In addition, we have found deficits in the expression of specific subunits of cytochrome c oxidase that are also linked to the diencephalic sub-region of the SN/VTA in schizophrenia (Rice et al. 2014), which may also contribute to a lack of proper TH protein synthesis.

In summary, the present study confirms our previous findings of dopaminergic deficits in the SN/VTA in schizophrenia subjects (Perez-Costas et al. 2012a), mapping these deficits to the rostral SN/VTA, and supporting the existence of pathway-specific deficits in dopaminergic neurotransmission that would preferentially or exclusively affect cortical areas. In addition, we show here that these deficits cannot be attributed to significant neuronal loss but rather to a deficit in TH protein synthesis. Dopamine is essential for the proper performance of tasks controlled by the prefrontal cortex, which include working memory, behavioral flexibility and attention (Goldman-Rakic 1996; Mizoguchi et al. 2009; Winter et al. 2009). Future studies should address the mechanisms involved in these dopaminergic deficits, the relation of subregion-specific TH deficits in mesodiencephalic dopaminergic neurons with anomalies in target areas such as the prefrontal cortex, and the relation of these deficits with specific symptoms observed in the clinic in schizophrenia.

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Figure 1. Neuronal counts for the entire SN/VTA

A–B) Number of total and dopaminergic neurons in the schizophrenia and control groups. In (A), data are presented as the mean neuronal count for each group with their standard deviation. In (B), individual data for each subject is plotted, with the horizontal bar indicating the mean for the group. **C–D)** Ratio of dopaminergic/total neurons. The bar graph in (C) represents the mean ratio for each group and their standard deviations. The scatter plot in (D) represents the ratio values for each individual subject, with the horizontal bar indicating the mean ratio for each group. No significant differences were observed for any of these measures between the two groups for the SN/VTA as a whole. Abbreviations: CTRL: control; SZ: schizophrenia

Figure 2. Neuronal counts in the diencephalic (rostral) SN/VTA

A–B) Number of total and dopaminergic neurons in the schizophrenia and control groups for the diencephalic sub-region of the SN/VTA. In (A), data are presented as the mean neuronal count for each group with their standard deviation. In (B), individual data for each subject is plotted, with the horizontal bar indicating the mean for the group. **C–D)** Ratio of dopaminergic/total neurons. The bar graph in (C) represents the mean ratio for each group and their standard deviations. The scatter plot in (D) represents the ratio values for each individual subject, with the horizontal bar indicating the mean ratio for each group. No significant differences were observed for any of these measures between the two groups in the diencephalic SN/VTA. Abbreviations: CTRL: control; SZ: schizophrenia

Figure 3. Neuronal counts in the mesencephalic (mid-caudal) SN/VTA

A–B) Number of total and dopaminergic neurons in the schizophrenia and control groups for the mesencephalic sub-region of the SN/VTA. In (A), data are presented as the mean neuronal count for each group with their standard deviation. In (B), individual data for each subject is plotted, with the horizontal bar indicating the mean for the group. **C–D)** Ratio of dopaminergic/total neurons. The bar graph in (C) represents the mean ratio for each group and their standard deviations. The scatter plot in (D) represents the ratio values for each individual subject, with the horizontal bar indicating the mean ratio for each group. No significant differences were observed for any of these measures between the two groups in the mesencephalic SN/VTA. Abbreviations: CTRL: control; SZ: schizophrenia

Figure 4. Tyrosine hydroxylase labeling in the diencephalic SN/VTA

A) Sagittal schematic drawing indicating the rostro-caudal level of sectioning (red line) of the images shown in this figure. **B)** Representative schematic drawing of a transverse section at the level shown in A. Three SN/VTA subareas defined by the dorso-ventral and medio-lateral location of the dopaminergic neurons are indicated (dashed lines in gray-shaded area). There was a conspicuous qualitative difference in overall immunolabeling between matched schizophrenia (**C–E**) and control (**F–H**) samples in the diencephalic sub-region of the SN/VTA. Note also that within this sub-region, the dorsal area of the SN/VTA presented the weakest immunolabeling in the schizophrenia samples (**C**), while was very intensely labeled in the controls (**F**). Ventro-lateral areas of the SN/VTA presented also notably weaker immunolabeling in schizophrenia samples (**D**) compared to the controls (**G**). Differences in labeling were less apparent when comparing the ventro-medial area of matched schizophrenia (**E**) and control samples (**H**).

The white asterisks indicate a similar level within the section for comparison of TH labeling between the schizophrenia and control samples. Coordinates indicate the orientation of the sections: **C**: caudal; **D**: dorsal; **L**: lateral; **R**: rostral. Dashed lines in **D**, **G** indicate the approximate transition between the dorsal (**d**) and ventro-lateral (**vl**) regions of the SN/VTA. **Abbreviations**: **cp**: cerebral peduncle; **CTRL**: control; **d**: dorsal SN/VTA; **III**: oculomotor nerve exit; **m**: mammillary body; **pg**: periaqueductal grey; **RN**: red nucleus; **SN/VTA**:

Substantia nigra/ventral tegmental area; **SZ**: schizophrenia; **Thal**: thalamus; **vl**: ventro-lateral SN/VTa; **vm**: ventro-medial SN/VTa. Calibration bar: 1 mm in C–H.

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Figure 5. Tyrosine hydroxylase labeling of cell and processes in the diencephalic SN/VTA

A, D) *Dorsal subarea of the diencephalic SN/VTA:* In schizophrenia (A, A') dopaminergic cell bodies presented the typical neuromelanin pigmentation (n), but TH labeling was very weak. Dopaminergic processes also presented weak labeling for TH (white arrowheads). The matched control (D, D') displayed very intense immunostaining for TH both in cell bodies and processes. **B, E)** *The ventro-lateral subarea of the diencephalic SN/VTA* also presented conspicuous deficits in TH immunolabeling in schizophrenia (B, B') compared with a matched control (E, E'). Note also that there is a gradient of TH immunolabeling deficit, with the areas close to the transition between the ventro-lateral (vl) and dorsal (d) subareas presenting even weaker TH labeling in the schizophrenia (B) but not in the control (E). Boxed areas in **A, D, B, E** are shown at higher magnification in **A', D', B', E'**, respectively.

C, F) *The ventro-medial subarea of the SN/VTA* presented scarce dopaminergic neurons (arrows) sparsely distributed among a dense net of thin TH labeled processes both in the schizophrenia (C) and control (F) samples. The differences in TH immunolabeling between schizophrenia and matched controls for this ventro-medial subarea were more subtle, displaying overall moderate qualitative deficits of TH labeling in their dense net of thin dopaminergic processes, and no clear qualitative reductions of labeling in dopaminergic cell bodies.

The white asterisks indicate a similar level within the section for comparison of TH labeling between the schizophrenia and control samples. Coordinates indicate the orientation of the sections: D: dorsal; L: lateral. Abbreviations: **CTRL**: control; **SZ**: schizophrenia. Calibration bar: 250 μm in A–F.

Figure 6. Tyrosine hydroxylase labeling in the mesencephalic SN/VTA

A) Sagittal schematic drawing indicating the rostro-caudal level of sectioning (red line) of the images shown in this figure. **B)** Representative schematic drawing of a transverse section at the level shown in A. Three SN/VTA subareas defined by the dorso-ventral and medio-lateral location of the dopaminergic neurons are indicated (dashed lines in gray-shaded area). **C, F)** These images show the overall pattern of TH labeling in the mesencephalic sub-region of matched schizophrenia (C) and control (F) samples. In these low magnification images the differences in labeling between schizophrenia and controls were very subtle. **D, G)** At higher magnification, in the dorsal (d) subarea there were moderate-to-subtle deficits in labeling in schizophrenia (D) compared with matched control (G) samples. **E, H)** In the ventro-lateral (vl) subarea there were no obvious differences between schizophrenia (E) and matched control (H) samples.

Coordinates indicate the orientation of the sections: C: caudal; D: dorsal; L: lateral; R: rostral. The white asterisks in C and F indicate a similar level within the section for comparison of TH labeling between the schizophrenia and control samples. Dashed lines in C, F indicate the approximate transition between the dorsal (d) and ventro-lateral (vl) regions of the SN/VTA. The areas marked with dashed boxes in C and F are shown at high magnification in D, E, G, H. Arrowheads in D–E and G–H indicate some examples of TH immunolabeling in thin processes.

Abbreviations: **cp**: cerebral peduncle; **CTRL**: control; **d**: dorsal SN/VTA; **III**: oculomotor nerve exit; **m**: mammillary body; **pg**: periaqueductal grey; **RN**: red nucleus; **SN/VTA**: Substantia nigra/ventral tegmental area; **SZ**: schizophrenia; **Thal**: thalamus; **vl**: ventro-lateral SN/VTA; **vm**: ventro-medial SN/VTA. Calibration bars: 1 mm in C, F; 500 μ m in D, E, G, H.

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Table 1

Demographic and clinical data of the cases used for the study.

Schizophrenia samples (n=6)							
Case #	Age (years)	race	gender	COD	PMI (hours)	pH	SZ subtype
SZ1	45	W	M	ASCVD	31	6.78	CUT
SZ2	53	W	M	ASCVD	14	6.01	Undifferentiated
SZ3	46	W	F	DVT	25	6.18	NOS
SZ4	54	W	M	Suicide (bleeding)	19	6.58	NOS
SZ5	83	W	M	Electrocution	18	6.98	NOS
SZ6	42	AA	F	ASCVD	13	5.86	Chronic paranoid
Mean	53.80				19.83	6.40	Fluphenazine + Quetiapine (T + A)
SD	15.04				6.74	0.45	
Control samples (n=7)							
Case #	Age (years)	race	gender	COD	PMI (hours)	pH	APD type
C1	49	AA	M	Cardiac arrest	19	6.76	Chlorpromazine + Clozapine + Fluphenazine + Quetiapine (T + A)
C2	45	W	M	Cardiac arrest	18	6.97	Quetiapine (A)
C3	51	AA	M	ASCVD	19	7.18	Clozapine (A)
C4	63	W	M	ASCVD	11	6.71	Quetiapine (A)
C5	37	W	F	DVT	10	6.49	Quetiapine (A)
C6	62	W	M	Cardiac arrest	19	6.48	
C7	67	AA	M	ASCVD	17	6.68	
Mean	53.43				16.14	6.75	
SD	10.92				3.93	0.25	

Abbreviations: A, atypical antipsychotic; AA, African American; APD, antipsychotic drug; ASCVD, arteriosclerotic cardiovascular disease; COD, cause of death; CUT, chronic undifferentiated type; DVT, deep vein thrombosis; F, female; M, male; NOS, not otherwise specified; PMI, postmortem interval; SD, standard deviation; SZ subtype, schizophrenia subtype; T, typical antipsychotic; W, white.

Table 2

Oversampling data summary

Neuronal counts obtained as the average of the neuronal counts of two different control cases. Neuronal count estimates of the SN/VTA (both hemispheres) were performed by counting neurons in a entire series. Each counting frame corresponds to the area of the SN/VTA fitting in an image taken with the 10X magnification objective.

Number of sections per series used for counts	Number of counting frames per section	Estimated total neurons	% variation from CSE	Estimated dopamine neurons	% variation from CSE
All sections	10	635,240	0	361,464	0
All sections	5	635,568	0.05%	357,248	1.17%
1/3 rd	10	651,624	2.58%	370,416	2.48%
1/3 rd	5	638,832	0.56%	354,048	2.05%

Abbreviations: CSE, complete series estimate; 1/3rd, one out of each 3 sections.

Table 3

Effect of demographic variables (i.e. age, gender, race) on neuronal counts. These analyses were done testing the effect of these variables on cell counts regardless of disease status. For all analyses $n=13$, $df=9$. None of the analyses yielded significant results.

OUTCOME MEASURES	ENTIRE SN/VTA	DIENCEPHALIC SN/VTA	MESENCEPHALIC SN/VTA
Total neurons	$p=0.2141 / R^2_a=0.1697$	$p=0.5490 / R^2_a=-0.0665$	$p=0.4145 / R^2_a=0.0138$
Dopaminergic neurons	$p=0.2283 / R^2_a=0.1561$	$p=0.1520 / R^2_a=0.2377$	$p=0.4597 / R^2_a=-0.0145$

Abbreviations: R^2_a = adjusted R2 for multiple comparisons.

Table 4

Effect of sample quality variables (i.e. postmortem interval, brain pH) on neuronal counts. These analyses were done testing the effect of these variables on cell counts regardless of disease status. For all analyses $n=13$, $df=10$. None of the analyses yielded significant results.

OUTCOME MEASURES	ENTIRE SN/VTA	DIENCEPHALIC SN/VTA	MESENCEPHALIC SN/VTA
Total neurons	$p=0.2055 / R^2_a=0.1256$	$p=0.8659 / R^2_a=-0.1659$	$p=0.1947 / R^2_a=0.1349$
Dopaminergic neurons	$p=0.1710 / R^2_a=0.1571$	$p=0.9619 / R^2_a=-0.1907$	$p=0.1423 / R^2_a=-0.1875$

Abbreviations: R^2_a = adjusted R2 for multiple comparisons.