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Title: Variability in neuronal expression of dopamine receptors and transporters in the substantia nigra

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Abstract:

Background: Parkinson's disease (PD) patients have increased susceptibility to impulse control disorders. Recent studies suggest that alterations in dopamine receptors in the midbrain underlie impulsive behaviours, and that more impulsive individuals, including patients with PD, exhibit an increase in occupancy of their midbrain dopamine receptors. The cellular location of dopamine receptor subtypes and transporters within the human midbrain may therefore have important implications for the development of impulse control disorders in PD.

Methods: The localisation of the dopamine receptors (D1-5) and dopamine transporter proteins in the upper brainstem of elderly adult humans (n=8) was assessed using single immunoperoxidase and double immunofluorescence (with tyrosine hydroxylase to identify dopamine neurons). The relative amount of protein expressed in dopamine neurons from different regions was assessed by comparing their relative immunofluorescent intensities.

Results: The midbrain dopamine regions associated with impulsivity (medial nigra and ventral tegmental area, VTA) expressed less dopamine transporter on their neurons than other midbrain dopamine regions. Medial nigral dopamine neurons expressed significantly greater amounts of D1 and D2 receptors, and vesicular monoamine transporter, than VTA dopamine neurons.

Conclusions: The heterogeneous pattern of dopamine receptors and transporters in the human midbrain suggests that the effects of dopamine and dopamine agonists are likely to be non-uniform. The expression of excitatory D1 receptors on nigral dopamine neurons in midbrain regions associated with impulsivity, and their variable loss as seen in PD, may be of particular interest for impulse control.

Introduction

Treated patients with Parkinson's disease (PD) have increased susceptibility to impulse control disorders with pathological gambling, compulsive shopping and hypersexuality found in approximately 14% of patients.^{1,2} Dopamine replacement therapies, especially dopamine agonist therapies, are associated with an increased risk for impulse control disorders in patients with PD, and management strategies include using the lowest effective doses of such therapies.³⁻⁵ Because of the high affinity of some dopamine agonists for autoreceptors,³⁻⁵ the regulation of intact dopamine neurons by dopamine agonists is a current focus of research. Recent studies show that the mechanism of dopamine release in the terminals and in the midbrain cell body regions is similar, relying on both diffusion and reuptake through dopamine transporters,⁶ and that impulsivity and novelty seeking traits may be mediated by D2 and D3 dopamine autoreceptors within the medial dopamine cell populations of the midbrain.⁷ These neurons innervate the ventral striatum and are located within the ventral tegmental area (VTA) and medial substantia nigra pars compacta (SNC, **Figure 1**),⁷ which we term the midbrain impulse control region. These cell groups are largely spared in patients with PD,^{8,9} and recent studies show increased dopamine receptor binding in midbrain dopamine cell groups in patients with PD and in individuals with impulse control disorders.¹⁰

*****Figure 1 about here*****

Mechanisms for dopamine release from cell bodies and dendrites have been localized in human midbrain neurons,¹¹⁻¹⁵ but it remains unclear which dopamine receptors are expressed on midbrain dopamine neurons. The excitatory D1-like receptor family (dopamine receptors D1 and D5)¹⁶ are found on at least some midbrain dopamine neurons,¹⁷ including in human brain.¹⁸⁻²² The D2-like family (D2, D3 and D4 dopamine receptors) are primarily inhibitory autoreceptors on dopamine neurons,^{17,23-26} and it is these receptors that have been the focus of research on impulse control disorders.³⁻⁵ Studies in monkeys have clearly shown different reactions of midbrain dopamine neurons to motivational signals,²⁷ and

these reactions are likely to occur via different receptor types. There have been no previous studies analyzing the cellular location of all these dopamine receptors and transporters in the impulse control region of the human midbrain. As the different responses at these dopamine receptors are considered to underlie impulse control disorders, identifying the types of dopamine receptors in intact dopamine neurons in the midbrain impulse control region is important, and the focus of this study.

Methods

Human brain samples - were obtained from the Sydney Brain Bank (tissue collection approved by the Human Research Ethics Committee of The University of New South Wales, Sydney, Australia) following approvals by their Scientific Advisory Committee (study approval number 0118) and the Human Research Ethics Advisory Committee of The University of New South Wales, Sydney, Australia (ethics approval number 090030). Formalin-fixed tissue blocks of the brainstem from eight healthy aged brain donors aged on average 81 ± 12 years (three males, five females, aged from 68 to 97 years old) were available.

Visualisation of dopamine receptors and transporters – was performed using immunohistochemistry on serially-spaced free-floating brainstem sections. Following cryoprotection in 30% buffered sucrose, brainstem tissue blocks were frozen in mounting medium and sectioned transversely either at a single medial level at $50 \mu\text{m}$ on a cryostat or through their entire rostro-caudal axis into 16 spaced series of sections (sections sampled every $800 \mu\text{m}$). Single immunoperoxidase labeling was performed on human brain sections for D1-5 dopamine receptors. Immunoperoxidase included the addition of nickel and cobalt to form a dark black reaction that could be easily distinguished from the lighter brown neuromelanin pigment present in human SNC and VTA dopamine neurons. Antigen retrieval in sodium citrate buffer (pH 8.5) was followed by quenching with 3% hydrogen peroxide (H_2O_2) and 10% methanol in 0.1M phosphate-buffered saline. Sections were then blocked in 0.25% casein with 0.05% triton X-100, washed and then incubated overnight at room

temperature in; casein with 0.05% triton X-100 and rabbit anti-D1 (1:4000; Abcam ab40653), mouse anti-D2 (1:250; Santa Cruz sc-5303), rabbit anti-D3 (1:250; Abcam ab40655), rabbit anti-D4 (at a range of dilutions; Abcam ab13318), rabbit anti-D5 (1:250; Abcam ab40656) dopamine receptor antibodies. Sections were washed and then incubated for two hours in casein containing biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse IgG (1:200; BA1000 and BA2000). Immunoreactivity was visualized by incubating for two hours in avidin-biotin-complex (Vector Labs PK-6100 at 1:100), followed by incubation in 0.5mg/ml diaminobenzidine tetrahydrochloride (Sigma D5637 DAB), with 0.02% nickel ammonium sulphate and 0.025% cobalt chloride in PBS for 3 minutes, followed by the addition of 0.001% H₂O₂, then washed, slide-mounted, dried, dehydrated and coverslipped. Specificity of the antibodies was confirmed by using only buffer instead of the primary antibodies, resulting in no positive immunoreactivity in the tissue. Bright field histological preparations were photographed at 40x using an Aperio Scanscope.

For quantitation of the level of receptor and transporter immunoreactivity in different types of midbrain dopamine neurons, double-immunofluorescent labeling was performed with tyrosine hydroxylase (TH, the rate limiting enzyme for dopamine synthesis). Antigen retrieval was performed in either sodium citrate buffer (pH 8.5) (for dopamine receptors and vesicular monoamine transporter or VMAT) or Tris buffer with 0.9% sodium chloride (pH 7.4) (for dopamine transporter or DAT). Sections then underwent 50% alcohol washes, followed by incubation in a block medium containing either 10% normal serum (VMAT and DAT) or casein with 0.05% triton X-100 (dopamine receptors) for one hour, then incubated for three nights at 4°C in the following primary antibody pairs; mouse anti-TH (1:1000; Seralab MAS424)/rabbit anti-D1 (1:3000), D3 (1:1500), D4 (at a range of dilutions), D5 (1:500), unglycosylated DAT (1:50; Santa Cruz 14002) or VMAT (1:500; Abcam ab81855); rabbit anti-TH (1:1000; Pel-Freez P40101-0)/mouse anti-D2 (1:200) or rat anti-glycosylated DAT (1:200; Millipore MAB369). Immunofluorescent labeling was visible after incubation for three hours in Alexa fluor 594 anti-mouse or anti-rabbit (for TH) and Alexa fluor 488 anti-mouse, anti-rabbit or anti-rat (each at 1:250 from Invitrogen). All sections were washed, mounted, and

coverslipped with fluorescent mounting medium (Dako S3023). To ensure specificity and non cross-reactivity of secondary fluorescent probes, a section without primary antibodies was included as a negative control. In addition, a cocktail of the secondary antibodies were applied to sections with only one primary antibody confirming antibody specificity.

Fluorescent images were captured at 40X using a Nikon Microscope ECLIPSE 90i confocal microscope with a Nikon D-ECLIPSE C1 high-resolution camera. Colour images for display purposes were converted from red-green to magenta-green using Adobe Photoshop CS4.

Quantification of the proportion of dopamine neurons in different midbrain regions containing the proteins of interest – was performed by a single investigator (SR). Four subregions containing different dopamine neuronal populations were identified (ventral SNC, dorsal SNC, medial SNC and VTA, Figure 1) at three comparable transverse levels^{28,29} for all cases (see Supplementary Figure). The proportion of neuromelanin-pigmented dopamine neurons containing the protein of interest in 2-4 captured images within each dopamine subregion at each midbrain level was determined. This procedure allowed an average 300 dopamine neurons/subregion to be sampled. Univariate analysis of variance ($P < 0.05$), cofactoring in age, time in fixation and post mortem delay, was used to determine any regional differences, with Bonferroni corrections in posthoc analyses.

Analysis of the amount of comparative protein immunofluorescence in different dopamine neuron types – was performed by a single investigator (SR) using the same sampling procedure and statistical analysis described above. The intensity of cellular immunoreactivity for each protein of interest within each dopamine neuron was assessed relative to the intensity of its cellular TH immunoreactivity, as recently described in detail.³⁰ Briefly, high-resolution grey-scale images of double-labeled neurons were captured separately for each channel and dichotomized using ImageJ-extracted intensities (0-255 scale) as being either strongly ($\geq 50\%$ of cellular TH intensity) or weakly ($< 50\%$ of cellular TH intensity) expressed using the relative intensity of the immunofluorescent signal.

Results

Cellular expression of dopamine receptor proteins in the adult human midbrain

As expected,^{31,32} D2 and D3 receptors were found in the majority (>90%) of dopamine neurons in all SNC subregions as well as in the VTA (**Figure 2B** and **Table 1**). Intense immunoreactivity for the D2 receptor was localised on the surfaces of the cell bodies and proximal dendrites of many dopamine neurons (**Figures 2B,F,J,N** and **3D**). D3 receptor immunostaining had the same neuronal localization but was less intense and was also found on surrounding small glial-like structures (**Figure 2B**). No D4 immunoreactivity was found in any of the dopamine neurons of the midbrain. We also found variably intense D1 and faint D5 receptor immunohistochemistry on dopamine neurons in the same subregions (**Figure 2A** and **Table 1**), substantiating previous studies using various other methods.^{18,20-22,33}

••Figure 2 and Table 1 about here***

As the intensity of dopamine receptor immunoreactivity was variable, further regional analysis of receptor staining intensity was performed. There were no differences between dopamine cell groups in the intensity of D3 or D5 receptor expression (**Table 1**). As expected, the majority of midbrain dopamine neurons had strong D2 receptor expression (>60%, **Figure 2J,N** and **Table 1**). The greatest regional variability was observed for the intensity of D1 receptors (**Table 1**) with an average of $44 \pm 25\%$ having strong D1 receptor expression. The impulse control region was dichotomized into two subregions by variability in the strong expression of both D1 and D2 dopamine receptors. The medial SNC had the highest D1 receptor expression in the midbrain (**Figures 2E,I,M** and **3A,B**) particularly compared with the VTA (2.7 ± 0.3 fold more strongly expressing neurons in the medial SNC, $F_{\text{region}}=11.1$, $p=0.007$, posthoc $p<0.008$, **Table 1**). The medial SNC also had 2.8 ± 0.2 fold more dopamine neurons expression strong D2-immunoreactive compared with the VTA ($F_{\text{region}}=34.8$, $p<0.0001$, posthoc $p<0.001$, **Figures 2F,J**, and **3C,D** and **Table 1**).

••Figure 3 about here***

Cellular expression of dopamine transporter proteins in the adult human SNC and VTA

As expected,³⁴⁻³⁶ most human midbrain dopamine neurons were immunoreactive for DAT (either mainly glycosylated or non-glycosylated, $93\pm4\%$) and VMAT ($96\pm1\%$) proteins (**Figure 2C,D** and **Table 1**). The proportion of dopamine neurons containing DAT was estimated by summing the proportion of neurons immunoreactive for glycosylated and non-glycosylated DAT together in further analyses. The cellular localisation of glycosylated DAT was similar to that observed for the dopamine receptors (**Figures 2C** and **3D**), while both non- glycosylated DAT (**Figures 2C** and **3D**) and VMAT (**Figure 2D**) were localised throughout the dopamine neurons.

As the intensity of dopamine transporter immunoreactivity was variable, further regional analysis of transporter staining intensity was performed. This showed variability within the dopamine subregions of the midbrain in the intensity of the functional glycosylated DAT (**Figures 2G,K,O** and **4C**), in the combined expression of the glycosylated and non-glycosylated DAT (**Figure 4C**), as well as in the intensity of VMAT immunoreactivity (**Figures 2H,L,P** and **4A,B**). The impulse control region was again dichotomized into two subregions on the basis of the strong expression of VMAT (**Table 1**). The medial SNC had a 1.5 ± 0.1 fold increase in the proportion of neurons with strong VMAT expression compared with the VTA ($F_{\text{region}}=12.7$, $p=0.001$, posthoc $p<0.004$, **Figures 2H,L** and **4A** and **Table 1**). Both impulse control subregions had low expression of intense DAT and functional DAT (**Figures 2G,K** and **4C** and **Table 1**). Strong expression of all types of DAT was greater in the ventral and dorsal SNC regions in comparison to the medial SNC and VTA subregions (1.3 fold increase, $F_{\text{region}}=12.7$, $p=0.001$, posthoc $p<0.004$, **Figure 4C** and **Table 1**). There was a strong trend ($p=0.057$) for the ventral SNC to have a 2.8 ± 1.0 fold and 8.0 ± 3.0 fold increase in the numbers of neurons strongly expressing functional DAT compared with the dorsal SNC and medial SNC/VTA respectively (**Figure 4B** and **Table 1**). This confirms previous detailed studies on this transporter in the adult human SNC.³⁶

Figure 4 about here

Discussion

We have assessed regional differences in the cellular location and expression of dopamine receptors and transporters within the normal human midbrain. These proteins are important for regulating synaptic dopamine levels and therefore may mediate risk for the development of impulse control disorders in PD. Our results show that D1-3 and D5 dopamine receptors are localised on midbrain dopamine neurons, with intense D2>D3>D1>faint D5 immunostaining. As previously described,^{35,36} DAT (glycosylated and non-glycosylated) and VMAT are also localised to these dopamine neurons. Subregional differences in expression levels were observed for D2 and D1 dopamine receptors and glycosylated and total DAT and VMAT. The subregions of interest for impulsivity are the medial SNC and VTA,^{7,37} regions relatively spared in PD,^{8,38,39} while the ventral SNC is characterized by marked early degeneration in PD. Our data show that dopamine neurons in the impulse control regions (medial SNC and VTA) express less DAT than dopamine neurons in other midbrain regions, suggesting a reduced capacity for dopamine reuptake and therefore potential for relatively greater amounts of extracellular dopamine following a similar dopamine-releasing stimulus. Our data also show a striking difference within the impulse control region, with medial SNC dopamine neurons expressing more D1 and D2 receptors and VMAT than VTA dopamine neurons. This data suggests that medial SNC neurons are likely to contain higher levels of vesicular (releasable) dopamine and to express a greater concentration of dopamine receptors and thus have the capacity to facilitate enhanced physiological effects. Overall, our data suggest that the effects of dopamine and its agonists in the impulse control region are unlikely to be uniform, confirming previous findings,²⁷ and that the expression of D1 receptors on the dopamine neurons of the medial SNC subregion could be of particular interest for activating, rather than inhibiting, dopamine pathways involved in impulse control.

The midbrain dopamine region identified as relevant for impulsivity contains two different types of dopamine neurons cytoarchitecturally. We show that dopamine neurons in these regions normally differ in their expression of dopamine receptors and transporters.

While both the medial SNC and VTA have reciprocal projections with limbic regions, and especially the ventral striatum, they provide largely separate projection systems to the ventromedial (medial olfactory tubercle and medial nucleus accumbens shell) and ventrolateral (accumbens core, lateral shell and lateral tubercle) striatum respectively.⁴⁰ These two dopamine pathways from the midbrain impulse control region are thought to mediate different aspects of reward,⁴⁰ with the medial projection system important for the regulation of arousal characterized by affect and drive, and the two systems playing different roles in goal-directed learning. Our data show that the effect of dopamine and dopamine agonists on these different reward pathways is likely to vary substantially. Disinhibition of medial SNC neurons activates dopamine release⁴¹ which would activate their D1 receptors and the ventrolateral striatal pathway directly. SNC neurons are known to differ from VTA neurons in having higher frequency pacemaker firing patterns, while VTA neurons discharge more irregularly.⁴² The reduced expression of both D1 and D2 autoreceptors on VTA compared with other midbrain dopamine neurons indicates less autoinhibition, making these neurons more responsive to direct synaptic inputs.⁴¹ The different behaviours and characteristics of the different dopamine responsive neurons within the midbrain impulse control region needs to be taken into account in further studies.

Impulse control disorders are increased in PD patients taking dopamine agonists,^{43,44} however only a minority of medicated PD patients exhibit impulse control disorders.⁴³⁻⁴⁵ Recent studies have begun to address dopaminergic mechanisms related to this phenomenon, although few studies have assessed D1 receptor activity. PD patients with impulse control disorders have reduced D2/3 autoreceptor binding in the midbrain¹⁰ and decreased ventral striatal DAT binding⁴⁶ and resting blood flow together with reduced dopamine modulation of activity during risk taking.^{47,48} They also have reduced striatal D2 autoreceptor binding⁴⁷ and a functional disconnection between the dorsal and ventral striatum.⁴⁹ As the reduction in both DAT and D2 receptor binding is more marked in the striatum in PD patients with impulse control disorders, and blood flow is reduced (marker of neuronal activity), these changes are likely to indicate a greater loss of dopamine-producing

structures and therefore dopamine regulation of impulse control. While the impulse control region of the midbrain is relatively spared in PD, the medial SNC experiences variable loss,^{38,50-52}. Variability in medial SNC neuronal loss, in association with marked neuronal loss in the ventral SNC, may underlie the development of impulse control disorders in PD, a testable hypothesis. Greater loss of the ventrolateral striatal dopamine pathway would increase the relative activity of the ventromedial striatal dopamine pathway important for the regulation of reward-related arousal. A relative increase in activity in this reward pathway with its reduced dopamine receptor autoregulation, in association with disconnection from other striatal systems, may establish a mechanism by which impulse control is reduced following appropriate stimuli.

The tendency towards impulsive behaviour of the controls analysed in this study is unknown. Thus future comparative postmortem studies on individuals with variable known levels of impulsive behaviours are required to test our hypothesis. In this regard and of relevance to PD, the first cellular change associated with increasing levels of cellular α -synuclein in midbrain dopamine neurons is reduced dopamine uptake that increases extracellular dopamine levels.^{53,54} As age is the major predisposing factor for PD, we chose to assess older controls in this study to control for age as a predisposing factor with regard to cellular protein expression levels. Previous studies have shown reductions in DAT mRNA with ageing⁵⁵ but others show there is a preservation of the proteins involved in dopamine biosynthesis and compartmentation.⁵⁶ Importantly, we found a similar distribution of neurons expressing glycosylated DAT protein to that recently published,³⁶ with higher levels of the active form of DAT in the ventral SNC dopamine neurons that are more vulnerable to PD. We have demonstrated that DAT expression and DAT glycosylation is highly variable within the midbrain dopamine cell groups. The relationship between DAT and vulnerability to cell death in PD, and the low expression of this protein in reward-related dopamine pathways warrants further study.

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Author Roles:

SR – 1A,B,C; 2A,B,C; 3A,B.

VC – 1C; 3B.

DK – 1B; 3B.

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Table and Figure Legends

Table 1: The proportion of regional dopamine neurons (expressing tyrosine hydroxylase and neuromelanin pigment) colocalising different dopamine receptors and transporters in the human SNC and VTA, with the proportion having strong intensity staining indicated (*strong*).

Figure 1: Main midbrain dopamine cell regions identified on a Nissl-stained and a nearby tyrosine hydroxylase (inset) transverse section of the midbrain. The four main regions (ventral SNC, dorsal SNC, medial SNC and VTA) containing different types of pigmented dopamine neurons are indicated in the Nissl stained section. The region corresponding to the Nissl-stained photomicrograph is identified by the box in the inset. * indicates the location of the photomicrograph in **Figure 3B**. ** indicates the location of the photomicrograph in **Figures 3D and 4B**. Aq=aqueduct, cp=cerebral peduncle, R=red nucleus, SNC=substantia nigra pars compacta, VTA=ventral tegmental area.

Figure 2: High magnification (400x) of double-labelled dopamine neurons (using tyrosine hydroxylase (TH) immunostaining, shown as magenta) and either dopamine receptors (green in A,B,E,F,I,J,M,N), glycosylated (DATG) or un-glycosylated (DATNG) dopamine transporter (green in C,G,K,O), or vesicular monoamine transporter 2 (VMAT2, green in D,H,L,P) in different midbrain dopamine cell groups. Colour images for display purposes were converted from red-green to magenta-green using Adobe Photoshop CS4. Scale in M is equivalent for all photos and colocalisation is seen as white. Panels E-H are all taken from the VTA subregion. Panels I-L are all taken from the medial SNC subregion. Panels M-P are taken from the ventral and dorsal SNC. Higher levels of D1 compared with D5, and D2 compared with D3 dopamine receptors were observed in most midbrain dopamine cell groups, with nearly all dopamine neurons containing some DAT and VMAT2. More medial SNC neurons

had strong D1 receptor expression (I) than dopamine neurons in the VTA (E) or the ventral or dorsal SNC (M). Less VTA dopamine neurons strongly expressed D2 receptors (F) or VMAT2 (H) compared with cell groups of the SNC (J,N and L,P respectively). DATG was most heavily expressed in dopamine neurons of the ventral SNC compared with the dorsal SNC (O), medial SNC (K) and VTA (G). DATG=glycosylated dopamine transporter, DATNG=non-glycosylated dopamine transporter, SNC=substantia nigra pars compacta, VMAT2=vesicular monoamine transporter 2, VTA=ventral tegmental area.

Figure 3: Graphs showing the proportion of human dopamine neurons in the ventral, dorsal and medial SNC, and VTA containing strong D1 (A) and D2 (C) immunoreactivity.

Photomicrographs showing D1 (**B**) and D2 (**D**) receptor immunoreactivity (nickel-enhanced to give a black reaction product or green Immunofluorescence) in pigmented (brown) tyrosine hydroxylase (TH, shown as magenta) immunoreactive dopamine neurons. Colour images for display purposes were converted from red-green to magenta-green using Adobe Photoshop CS4. More medial SNC dopamine neurons had strong D1 receptor expression compared with the ventral and dorsal SNC, with comparatively few VTA dopamine neurons having strong D1 receptor expression (A,B). More SNC dopamine neurons strongly expressed D2 receptors than VTA neurons (C,D). SNC=substantia nigra pars compacta, VTA=ventral tegmental area. **=significantly higher proportion of neurons strongly expressing D1 receptors. *=significantly lower proportion of neurons strongly expressing D1 or D2 receptors.

Figure 4: Graphs showing the proportion of human dopamine neurons in the ventral, dorsal and medial SNC, and VTA containing strong vesicular monoamine transporter (VMAT, **A**) and dopamine transporter (DAT, **C**) immunoreactivity. Photomicrograph in **B** shows VMAT immunoreactivity (green) in tyrosine hydroxylase (TH, shown as magenta) immunoreactive dopamine neurons (**B**). Colour image for display purposes was converted from red-green to magenta-green using Adobe Photoshop CS4. Photomicrographs in **D** show DAT immunoreactivity (nickel-enhanced to give a black reaction product) in pigmented (brown) dopamine neurons. More SNC dopamine neurons had strong VMAT expression compared with the VTA (**A**). More ventral and dorsal SNC dopamine neurons had strong total DAT

expression than either the medial SNC or VTA, with the VTA having the lowest expression

(C). Strong immunoreactivity for glycosylated DAT was more variable with the highest number of dopamine neurons strongly expressing glycosylated DAT found in the ventral SNC

(C). SNC=substantia nigra pars compacta, VTA=ventral tegmental area. *=significantly lower proportion of neurons strongly expressing D1 or D2 receptors. □=significantly higher proportion of neurons strongly expressing glycosylated DAT.

Supplementary Figure: Low magnification photomicrographs showing the cytoarchitectural distribution of the dopaminergic cell groups identified using tyrosine hydroxylase immunohistochemistry at the three rostrocaudal levels of the human midbrain. These levels

were identified in each case and adjacent section double labeled and assessed for fluorescence intensity analysis as detailed in the methods. The most caudal level (C) was at the decussation of the superior cerebellar peduncle (xscp). An intermediate level (B) where the greatest number of exiting 3rd nerve fibres occurred was assessed. The most consistent rostral level (A) was just below the opening of the aqueduct into the 3rd ventricle at the greatest cross-sectional extent of the red nucleus (R). At this rostral level there were many ascending tyrosine hydroxylase immunopositive axons in the medial aspects of the midbrain. Scale in C is equivalent for A and B. cp=cerebral peduncle, R=red nucleus, SNC=substantia nigra pars compacta, VTA=ventral tegmental area.