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Trophic factors differentiate dopamine neurons vulnerable to Parkinson's disease

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Abstract

Recent studies suggest a variety of factors characterize substantia nigra neurons vulnerable to Parkinson's disease, including the transcription factors Pitx3 and Otx2 and the trophic factor receptor DCC, but there is limited information on their expression and localisation in adult humans. Pitx3, Otx2 and DCC were immunohistochemically localised in the upper brainstem of adult humans and mice and protein expression assessed using relative intensity measures and online microarray data. Pitx3 was present and highly expressed in most dopamine neurons. Surprisingly, in our elderly subjects no Otx2 immunoreactivity was detected in dopamine neurons, although Otx2 gene expression was found in younger cases. Enhanced DCC gene expression occurred in the substantia nigra, and higher amounts of DCC protein characterised vulnerable ventral nigral dopamine neurons. Our data show that, at the age when Parkinson's disease typically occurs, there are no significant differences in the expression of transcription factors in brainstem dopamine neurons, but those most vulnerable to Parkinson's disease rely more on the trophic factor receptor DCC than other brainstem dopamine neurons.

Key words: deleted in colorectal cancer; dopamine neurons; orthodenticle homeobox 2; Pitx3; substantia nigra

Abbreviations: DCC= deleted in colorectal cancer; Otx2= orthodenticle homeobox 2; PD= Parkinson's disease; SNC= substantia nigra pars compacta; TH= tyrosine hydroxylase; VTA= ventral tegmental area

1. Introduction

By the onset of the motor symptoms of Parkinson's disease (PD) neuronal degeneration is still largely confined to a very restricted region of the substantia nigra, the ventral tier (Dickson, et al., 2009). This is despite a more widespread deposition of α -synuclein in Lewy bodies and neurites at this early disease stage (Dickson, et al., 2009). Identifying the cellular proteins and mechanisms that underlie this vulnerability is a priority. A number of studies on laboratory animals have identified candidate cellular proteins that appear to be expressed in restricted populations of brainstem dopamine neurons (Double, et al., 2010) but confirmation of their expression in adult humans is generally lacking.

Dopamine neurons in the brainstem are not confined to the substantia nigra pars compacta (SNC or A9 cell group) but are also located in the ventral tegmental area (VTA or A10 cell group)(**Figure 1**). Approximately two-thirds are found in the SNC and one-third in the VTA (Halliday, et al., 2012, Smidt and Burbach, 2007) with these two types of dopamine neurons known to have molecular and genetic differences (Chung, et al., 2005, Greene, et al., 2005). Within the SNC only the ventral subset of neurons degenerates by the onset of motor symptoms (Fearnley and Lees, 1991, Gibb and Lees, 1991), the dorsal tier neurons being resistant to PD at this stage (Dickson, et al., 2009).

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

The two main types of brainstem dopamine neurons (A9 and A10) are regulated by important transcription factors that determine their phenotype, with several of these transcription factors maintaining their expression into adulthood (Orme, et al., 2009). These include Nurr1 (Backman, et al., 1999, Saucedo-Cardenas, et al., 1998), Pitx3 (Smidt, et al., 1997), the engrailed genes (Simon, et al., 2001) and orthodenticle homeobox 2 (Otx2). Nurr1 and the engrailed genes are ubiquitously expressed in brainstem dopamine neurons (Simon, et al., 2001), whilst Pitx3 and Otx2 have been implicated in establishing the two main dopamine cell phenotypes, the A9 SNC and the A10 VTA neurons. Pitx3 expression continues to be expressed in adult mice but only in ventral SNC neurons (van den Munckhof, et al., 2003). There have been no descriptions of Pitx3 expression in the adult human brainstem. In detailed work in mice with some confirmation in primates, Otx2 expression has been found to be restricted to VTA neurons (Simeone, et al., 2011) with animal studies supporting the concept that its expression contributes to the differential vulnerability between the SNC and VTA in PD

(Di Salvio, et al., 2010a). For cell phenotype maintenance, trophic factors are also vital, with factors like GDNF which supports all dopamine neurons (Sullivan and Toulouse, 2011) currently undergoing therapeutic trials in PD (Rangasamy, et al., 2010). A trophic factor receptor with restricted expression to the ventral part of the SNC has been identified in adult mice (Osborne, et al., 2005) and may be important for the maintenance of these most vulnerable neurons. This receptor, known as deleted in colorectal cancer (DCC), recognises the guidance and neurite outgrowth factor netrin-1 (Bradford, et al., 2009). Confirmation of the restricted adult expression of transcription and trophic factors in human dopamine neurons vulnerable to PD may provide better targets for early therapeutic interventions.

2. MATERIALS AND METHODS

2.1 Human and mouse brain tissue

Formalin-fixed human brain tissue was 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 blocks of the entire upper part of the brainstem from five aged (85 ± 3 years, three males, two females without neuropsychiatric, neurological or neurodegenerative disease) and three middle aged (58 ± 16 years, two males, one female without brainstem neurodegenerative disease) brain donors were obtained. In addition, the on-line public resources of the Allen Institute for Brain Science was used to assess regional gene expression in the public datasets for a young (24 years, male) and a middle aged (39 years, male) human. C57BL/6J mice at different ages (male at 4 weeks, 3 males at 8-10 weeks, male at 10 months, female at 2 years) were obtained from the Animal Resource Centre, Perth, Australia and the study protocols approved by the Animal Care and Ethics Committee of The University of New South Wales, Sydney, Australia (ACEC 11/75A). Following transcardial perfusion of the mice with 4% paraformaldehyde (4°C , pH7.4), the brains were post-fixed in the same fixative for four hours and then cryoprotected in 30% sucrose-buffer.

2. 2 Tissue processing

Human tissue blocks were cryoprotected in 30% buffered sucrose, frozen in mounting medium, and sectioned transversely. The brainstems were sectioned through their entire rostro-caudal axis at 50µm on a cryostat and 16 spaced series of sections were obtained (sections sampled every 800µm). Each mouse brain was cut into 40 µm sections on a freezing microtome in the coronal plane and consecutive sections of the SNC were harvested from 2.30-4.60mm posterior to bregma.

Single immunoperoxidase labeling was performed on human brain sections, with 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 dopamine neurons. Antigen retrieval in either sodium citrate buffer (pH 8.5) (for Pitx3 and DCC) or citrate buffer (pH 6) (for Otx2), was followed by quenching with 3% hydrogen peroxide (H₂O₂) and 10% methanol in 0.1M phosphate-buffered saline (PBS). Sections were washed and then incubated overnight at room temperature in 2% normal serum in PBS with 0.3% Triton X-100 and rabbit anti-Pitx3 (1:100; ZYMED laboratories, 38-2850), 0.25% casein with 0.2% Triton X-100 and rabbit anti-DCC (1:1000; rabbit polyclonal antiserum, 2744, was raised against a purified preparation of the mouse DCC extracellular domain, comprising the four immunoglobulin domains and the first two fibronectin type III repeats (Gad, et al., 2000)), or 2% normal serum in PBS with 0.3% Triton X-100 and goat anti-Otx2 (1:40; R&D systems, AF1979). Sections were washed and then incubated for two hours in 2% normal serum (for Pitx3 and Otx2) or 0.25% casein (for DCC) containing biotinylated goat anti-rabbit IgG or biotinylated horse anti-goat IgG (BA2000 and BA9500, at 1:200). 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. After rinsing in PBS, all sections were mounted and dried overnight, dehydrated, delipidated, and coverslipped. Specificity of the Pitx3, Otx2 and DCC 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 using an Aperio Scanscope.

For double-immunofluorescent labeling, human sections underwent the same antigen retrieval as above, while mice sections were processed in citrate buffer (pH 8.0). Sections were washed in 50% alcohol washes (human only), then incubated in a block medium (for both species) containing 1%

bovine serum (mice only), 10% normal serum, 0.25% casein (human DCC only) and Triton-X-100 (mice: 0.25% for Pitx3 and Otx2, 0.05% for DCC, human: 0.2% for Pitx3 and Otx2, 0.3% for DCC) for one hour, then incubated for two nights in the following primary antibodies; anti-TH (human; Pel-Freez P40101-0 and mice; Sigma T2928) /anti-Pitx3 (human; goat/rabbit at 1:500/1:80, mice; mouse/rabbit at 1:1000/1:100), anti-TH/anti-DCC (human; goat/rabbit at 1:500 for both; Mice; mouse/rabbit at 1:2000/1:1000) and anti-TH/anti-Otx2 (human; rabbit/goat at 1:500/1:25, Mice; mouse/goat at 1:1000/1:50). Immunofluorescent labeling was visible after incubation for three hours in Alexa fluor 488 anti-mouse, Alexa fluor 594 anti-rabbit, Alexa fluor 594 anti-mouse, Alexa fluor 488 anti-rabbit, Alexa fluor 488 anti-goat (Invitrogen A-11001 and A-11012, Invitrogen A-11032, A-11034 and A-11055; each at 1:250). The mouse tissue sections were washed and counterstained with 4',6-diamidino-2-phenylindole, dilactate (Invitrogen D3571). All sections were washed, mounted, and coverslipped with fluorescent mounting medium (Dako S3023). To test the specificity for all immunohistochemical reactions, and to ensure non cross-reactivity of secondary fluorescent probes, sections without primary antibodies were included as negative controls. In addition, a cocktail of the secondary antibodies were applied to sections with only one primary antibody incubated on each section, confirming antibody specificity. Fluorescent images were captured using a Nikon Microscope ECLIPSE 90i confocal microscope with the Nikon D-ECLIPSE C1 high-resolution camera. The images were converted from red-green to magenta-green using Adobe Photoshop CS4.

2.3 Analysis

2.3.1 Identification of the main dopamine subregions in the upper brainstem

There are multiple ways of subdividing the brainstem dopamine neurons (McRitchie, et al., 1996), but for this analysis a simplified scheme based on the following three broad regions was applied: SNC ventral tier, SNC dorsal tier, and VTA (see **Figure 1**). For the human sections, the atlas of the human brainstem (Paxinos and Huang, 1995) was used, and for the mice sections, the atlas of the mouse brain (Franklin and Paxinos, 2008) was used to determine anatomical landmarks and the cytoarchitecture of these three main upper brainstem dopamine cell groups. Three comparable transverse levels containing the main dopamine cell groups were chosen in each case for more detailed analyses (see below). The dopamine cell groups were similarly identified in the online public

resources (magnetic resonance imaging scans, MNI co-ordinates, Nissl stains of coronal brainstem sections, gene expression maps) provided by the Allen Institute for Brain Science.

2.3.2 Quantification of the proportion of brainstem dopamine neurons containing the proteins of interest

To determine the proportion of dopamine neurons containing Pitx3, Otx2 and DCC immunoreactivity, 40x images of the peroxidase-labelled human brainstem sections were captured using an Aperio Scanscope, and neuromelanin pigment was used to identify human dopamine neurons, and 40x images of the double-labelled mouse brainstem sections were captured using a Nikon ECLIPSE 90i confocal microscope and the proportion of TH-immunoreactive neurons with Pitx3, Otx2 or DCC counted. Images were randomly captured from 2-4 areas within each of the ventral SNC, dorsal SNC, and VTA in the three transverse brainstem sections for all humans and mice. This sampling procedure allowed an average 300 neurons in each human and 90 neurons in each mouse to be used to calculate the proportion of double-labelled dopamine neurons.

2.3.3 Analysis of the amount of protein expression in different dopamine neuron types using comparative fluorescent intensities

DCC immunoreactivity was noted to be variable within the cell bodies, so further assessment of the neuronal intensity of DCC compared with TH immunoreactivity was performed. 40x images of the double-labelled human brainstem sections were captured using a Nikon ECLIPSE 90i confocal microscope and the neuronal intensity of proteins assessed as recently published (Reyes, et al., 2012). Briefly, high-resolution grey-scale images of neurons were dichotomized using ImageJ-extracted intensities (0-255 scale, threshold of $\pm 50\%$ of cellular TH intensity) as being either of strong or weak DCC immunofluorescence using the same sampling procedure described above.

2.3.4 Analysis of the amount of gene expression in A9 versus A10 human dopamine neurons using online microarray data

The *Allen Human Brain Atlases* are a publicly available online resource of gene expression information in human brains provided by the Allen Institute of Brain Science. Quantitative genome-wide microarray-based gene expression profiles in a young and middle-aged human brain with

accompanying anatomic and histology data are provided. The relative levels of expression of the Pitx3, Otx2 and DCC genes were available at the same three brainstem levels we assessed histologically. For each gene, quantitative data was available from two to three SNP probes within each gene. Using the provided navigable magnetic resonance (MR) images to identify the SNC and VTA in each brain section, we obtained a readout of the level of gene expression for each probe in the SNC and VTA as a z-score of the average expression across all brain pixels for each case. As no immunoreactivity was observed for Otx2 in the human SNC and VTA, gene expression levels in the superior colliculi in the same sections (an area with obvious Otx2 immunoreactivity) were also obtained for comparison. Because quantitative gene expression data is available for every brain pixel, a 0 z score may reflect an area of limited or no real gene expression. We identified above average or selective gene expression as regions with levels above one standard deviation z score or, for those genes that had more variable high and low expression, by creating an average deviation using a ratio of the highest over the lowest z scores. IBM SPSS software was used to determine differences between regional gene expression levels with age. Univariate analysis of variance ($P < 0.05$) using the multiple samples of each region and the multiple probes for each gene of interest was determined using Bonferroni corrections in posthoc analyses and cofactoring for age. Data are expressed as the mean expression scores \pm standard error for all probes for the gene of interest in all samples of the SNC and VTA.

3. RESULTS

3.1 Expression of Pitx3 protein in the adult human and mouse SNC and VTA

Pitx3 transcription factor Immunoreactivity was found in the majority of dopamine neurons in both the ventral and dorsal tiers of the SNC as well as in the VTA in both species (**Figure 2 and Table 1**). Compared to middle aged humans and mice at many ages, aged humans had some Pitx3 protein in the cytoplasm as well as in the nucleus (**Figure 3B**). Only rare non-dopamine neurons in these regions had Pitx3 immunoreactivity.

*****Figures 2 and 3 and Table 1 about here*****

To determine if gene expression for Pitx3 varied between SNC and VTA neurons, analysis of online microarray data was undertaken. Assessment of the overall distribution of Pitx3 mRNA in the brain (**Supplementary Figure**) shows only restricted regions of high expression with the highest z-

score being 2.7 and the lowest z-score -3.6. Using these scores, we interpreted selective expression as revealed by z-scores above 0.75 (2.7/3.6). Pitx3 mRNA was selectively expressed in SNC and VTA neurons in both human cases (no significant difference between cases)(**Figure 3C**), however, there was more SNC Pitx3 expression compared with the VTA ($p=0.049$)(**Figure 3C, Table 2 and Supplementary Figure**).

*****Table 2 about here*****

3.2 Expression of Otx2 protein in the adult human and mouse SNC and VTA

Surprisingly, no Otx2 immunoreactivity was found in the dopamine neurons in the SNC or VTA in the human brains we studied (**Figure 4A-C and Table 1**), although strong Otx2 immunoreactivity was observed in the nearby substantia nigra pars reticulata and interpeduncular nucleus as well as in the superior colliculi (**Figure 5D,E**). In mice, reproducible Otx2 immunoreactivity was observed largely in VTA dopamine neurons (**Figure 4D-F and Table 1**), as previously described for this transcription factor (Chung, et al., 2010, Di Salvio, et al., 2010a), as well as in the regions of high expression noted in humans.

*****Figures 4 and 5 about here*****

To investigate if mRNA expression occurred to any extent in either the SNC or VTA of humans and whether age was a potential factor in reducing Otx2 gene expression, analysis of microarray data on Otx2 mRNA provided by the Allen Institute of Brain Science was undertaken (**Table 2 and Supplementary Figure**). Assessment of the overall distribution of Otx2 mRNA in the brain (**Supplementary Figure**) shows only restricted high expression in certain regions (ie. thalamus and cerebellum), with more expression in the younger versus the middle-aged case (**Supplementary Figure**). The highest z-score was 2.5 and the lowest z-score -2.1, and using these z-scores we interpreted selective expression as being above 1.2 (2.5/2.1). We also assessed expression in the superior colliculi, an area distinct from the SN and VTA in which we could definitely observe Otx2 protein expression in humans (see above). Univariate analysis for regional differences covarying for age revealed both differences between regions and with age for Otx2 mRNA expression ($F_3=20.8$, $p<0.0001$, **Figure 5F**). For both cases, Otx2 mRNA expression was highest in the superior colliculi, followed by the VTA and lowest in the SNC ($F_{\text{region}}=24.8$, $p<0.0001$, **Table 2**) with post-hoc analysis revealing that expression differed between all three regions ($p\leq 0.04$, **Figure 5F**). However, only the superior colliculi had strong Otx2 mRNA expression, even if the z-score for strong expression was

considered above 1 (**Figure 5F**). There was a significant reduction in expression between the younger and middle-aged case ($F_{\text{age}}=12.7$, $p=0.003$, adjusted $R^2=0.78$, **Figure 5F**) with the SNC being well below average Otx2 mRNA expression levels. This suggests that with increasing age there is likely to be a further reduction in Otx2 mRNA expression in these regions.

To further assess the change in Otx2 expression in brainstem dopamine neurons with age, the proportion of Otx2-immunopositive dopamine neurons was evaluated in brainstem sections from mice of different adult ages (4, 8-10 weeks, 10 months and 2 years) and in the brains of three middle-aged humans 25 years younger than the aged control cohort originally assessed. Consistent with the lack of Otx2 mRNA in middle age (**Figure 5F**), there was no Otx2 immunoreactivity in any the human SNC or VTA dopamine neurons in these cases (**Figure 5A-C**). It should be noted that appropriate Otx2 immunoreactivity was observed in other regions (eg. superior colliculi, **Figure 5D**) known to have high expression in humans. In the adult mice assessed, the proportion of Otx2-immunoreactive VTA dopamine neurons remained relatively constant over the ages assessed (average= $60\pm 7\%$, **Supplementary Table**), suggesting a potentially important species difference in the adult expression of the Otx2 transcription factor in brainstem dopamine neurons.

3.3 Expression of DCC protein in the adult human and mouse SNC and VTA

DCC is the receptor for the trophic factor netrin and is differentially expressed in brainstem dopamine neurons in adult mice (Osborne, et al., 2005). In both human and mouse brainstems, DCC immunoreactivity was localised as granular foci on the surface as well as internally in the cytoplasm of the dopamine neurons (**Figure 6**). DCC immunoreactivity was also observed in the non-dopaminergic neurons of the substantia nigra pars reticulata in both humans and mice. Over 90% of human dopamine neurons in the ventral and dorsal SNC and VTA contained DCC immunoreactivity with little variation between regions (**Table 1**). While the majority of brainstem dopamine neurons in mice contained DCC immunoreactivity (**Table 1**), there was variability between the dopamine regions. All ventral SNC dopamine neurons but only 85% of dorsal SNC and 62% of VTA dopamine neurons contained DCC immunoreactivity in mice (**Table 1**).

*****Figure 6 about here*****

Within the dopamine cell regions, variable intensity of neuronal DCC immunoreactivity was observed (**Figure 7A**). Assessment of the proportion of dopamine neurons in each region containing

strong DCC immunoreactivity showed that the human ventral SNC had a 2.0 ± 0.3 fold increase in the proportion compared with the dorsal SNC ($F_{\text{region}}=9.3$, $p=0.004$, posthoc $p=0.003$), with most ventral SNC dopamine neurons containing strong DCC immunoreactivity (**Figure 7A**). The same assessment in mice found a 6.7 ± 2.1 fold increase in strong DCC immunoreactivity in ventral SNC neurons compared with dorsal SNC and VTA neurons ($F_{\text{region}}=19.8$, $p<0.0001$, posthoc $p<0.0001$). Again most ventral SNC neurons contained strong DCC immunoreactivity in mice.

*****Figure 7 about here*****

To determine if gene expression for DCC varied between human SNC and VTA neurons, analysis of the online microarray data provided by the Allen Institute of Brain Science was performed. Assessment of the overall distribution of DCC mRNA in the brain (**Supplementary Figure**) shows only restricted regions of high expression with the highest z-score being 2.6 and the lowest z-score -3.0. Using these scores, we interpreted selective expression as revealed by z-scores above 0.87 (2.6/3.0). Similar to Pitx3 mRNA, DCC mRNA was selectively expressed in the both SNC and VTA neurons independent of age (no significant difference between the cases)(**Figure 7B**), with more SNC DCC expression compared with the VTA ($p=0.017$) (**Figure 7B, Table 2 and Supplementary Figure**).

4. Discussion

In the present human tissue study we assessed two transcription factors (Pitx and Otx2) and a trophic factor receptor (DCC) previously suggested as potentially important for the maintenance of different dopamine neuronal cell types in non-human species. In particular, we chose factors that were thought to concentrate either within dopamine neurons known to be vulnerable to PD or within neurons that are spared by PD. Our basic findings are that the transcription factors do not distinguish between vulnerable and less vulnerable dopamine neurons in the SNC, but that SNC neurons vulnerable to PD usually maintain higher amounts of DCC, suggesting they are more reliant on trophic factor maintenance.

4.1 On a cell-by-cell basis, adult human dopamine neurons appear to express the same transcription factors

The majority of human studies on transcription factors important for dopamine neurons deal with their role in the development from stem cells into their different phenotypes, due largely to their

potential in advancing cell replacement therapies (Andersson, et al., 2006, Kim, 2011, Thomas, 2010). In these studies (and in many other animal model studies) two main dopamine cell phenotypes are identified in the upper brainstem, SNC and VTA dopamine neurons, and their differentiation rather than maintenance has been studied in detail. The focus of the present study is on the long-term maintenance of brainstem dopamine neurons into aged adulthood, and from the literature four main transcription factors were identified (Nurr1, engrailed genes, Pitx3 and Otx2), with two of these suggested to be differentially expressed (Pitx3 and Otx2). There have been no previous human tissue studies on these transcription factors in the SNC and VTA. Our study confirms the strong expression of Pitx3 in all adult human dopamine neurons. More surprisingly, our study could not identify Otx2 expression in adult human dopamine neurons.

A number of recent genetic studies show that polymorphisms in the *Pitx3* gene associates with early onset and familial forms of PD (Bergman, et al., 2010, Fuchs, et al., 2009, Le, et al., 2011) and *Pitx3* knock-out mice show specific loss of SNC dopamine neurons (Hwang, et al., 2003, Nunes, et al., 2003, Smidt, et al., 2004). In our study Pitx3 immunoreactivity was not restricted to SNC neurons, and certainly was not restricted to those in the ventral SNC that are most vulnerable to PD. This is in line with other gene and protein expression studies in rodents (Korotkova, et al., 2005, Smidt, et al., 2004, Zhao, et al., 2004). We next assessed online microarray data to see if mRNA expression differed between SNC and VTA dopamine neurons, because the resolution of the data did not differentiate ventral and dorsal SNC. This analysis showed unexpectedly that the intensity of staining was regionally different, however the density of dopamine neurons in these regions in humans is also significantly different. On a cell-by-cell basis, Pitx3 immunoreactivity was found in the vast majority of all brainstem dopamine neurons in aged humans, with the intensity of regional mRNA data reflecting the dopamine cell density observed in these regions. Because Pitx3 has been shown to be important for the regulation of the rate-limiting enzyme for dopamine synthesis (Lebel, et al., 2001) and the regulation of neurotrophic factors (including BDNF and GDNF (Yang, et al., 2008) both of which have been trialed as PD therapeutics (Nagahara and Tuszynski, 2011, Sullivan and Toulouse, 2011)), and of essential microRNAs for neuronal maintenance (Kim, et al., 2007), its continued expression in adult human dopamine neurons is not surprising.

4.2 Otx2 does not appear critical for the maintenance of human brainstem dopamine neurons

There have been a number of exciting recent studies suggesting that Otx2 is at least partially responsible for the differential vulnerability of the SNC to PD (Chung, et al., 2010, Di Salvio, et al., 2010a). This concept has not been followed up with significant data from human brain tissue. While there is no doubt that Otx2 is important in establishing the VTA dopamine neuron phenotype (Simeone, et al., 2011), most tissue based data has been generated in mice with only limited data from adult primate tissue on either its expression or localization in the brainstem (Di Salvio, et al., 2010b). To look at mRNA expression in adult humans, we compared a region of known high expression (the superior colliculi) with expression levels in the SNC and VTA of a young and middle-aged human. There was a significant decline with age in the intensity of Otx2 mRNA expression across all regions examined, and in the SNC and VTA expression was reduced to below average by early middle age. This was not observed for either Pitx3 or DCC mRNA expression using the same analysis techniques, and also differed from the age dependent reduction in NURR1 protein that has been identified in the SNC of humans (Chu, et al., 2002). It is the intensity of NURR1 protein that declines within the SNC neurons in aged humans, not an absence of the protein, as we observed with Otx2 in all types of human brainstem dopamine neurons. As previously identified in a single adult human study (Chung, et al., 2010), the expression of Otx2 mRNA was higher in the VTA compared with the SNC, but our protein localization study could not identify significant Otx2 protein in aged humans compared with clear Otx2 immunoreactivity in aged-mice VTA neurons. Of note, although Otx2 expression in human brainstem dopamine neurons appears to decline significantly in early age, high levels of Otx2 protein are maintained in the superior colliculi of aged humans. Overall these data suggest that Otx2 may be important for maintaining certain dopamine neuronal phenotype in adult mice but not in aged humans.

4.3 DCC is found in higher amounts in the ventral SNC neurons vulnerable to PD

DCC is best known as a receptor for the guidance factor netrin, which enhances the growth and stability of dopamine axon terminal arborization (Lai Wing Sun, et al., 2011, Xu, et al., 2010). Adult mice with a loss of function mutation in the *DCC* gene have significant changes in dopamine functioning with DCC protein levels influencing the extent of neuronal branching and synaptic differentiation (Manitt, et al., 2011). DCC is also important in neurons for remote protein synthesis and

membrane plasticity. DCC forms a protein complex with SNARE proteins to regulate membrane turnover and exocytosis (Cotrufo, et al., 2011, Prensa and Parent, 2001). In both axons and dendrites, DCC also anchors components of the protein synthetic machinery to the plasma membrane and when activated by netrin releases this machinery for local protein synthesis (Tcherkezian, et al., 2010). These studies suggest that DCC is important for the regulation and maintenance of complex, remote axonal and dendritic neural structures, including synaptic machinery.

There have been no previous human tissue studies on DCC in the SNC or VTA. Our study did not find selective expression of DCC on a single cell basis, although in mice we can confirm the pattern of differential expression previously observed in rodents (Osborne, et al., 2005). The assessment of online human microarray data revealed that the intensity of DCC mRNA expression was increased in the SNC compared with the VTA, although this is likely to be attributed to the aforementioned neuronal density differences between these dopamine cell groups. Considering the function of DCC and the greater size of both neurons and brain structures human dopamine cells innervate compared with mice, it may not be surprising that DCC is found in a greater percentage of dopamine neurons in humans. To determine if more DCC protein is required by ventral SNC dopamine neurons in humans, we assessed the intensity of immunoreactivity of the protein within individual cells using a standardized published technique (Reyes, et al., 2012). We observed significantly more DCC immunoreactivity in ventral SNC neurons that are more vulnerable to PD compared with less vulnerable dopamine neurons. In adult mice there is a gradient of netrin-1 protein expression that concentrates in striatal regions thought to be innervated by ventral SNC dopamine neurons (Shatzmiller, et al., 2008), consistent with an increased concentration of DCC protein in these neurons. Serial section reconstructions of the axonal arborizations of dopamine neurons in rats show that they are some of the most highly branching neurons found in the brain, with some covering as much as 6% of the entire striatum (Matsuda, et al., 2009). Different patterns of axonal arborization are found in the ventral SNC (dopamine neurons located in the pars reticulata in rats) compared to the dorsal SNC (known as the ventral tier overlying the pars reticulata in rats) (Prensa and Parent, 2001). Ventral SNC dopamine neurons innervate nearby dopamine neurons as well as branching to a number of other basal ganglia structures before terminating in the striatum. This contrasts with the dorsal SNC neurons, which mainly terminate in the striatum. If such differences exist in humans, then

increased DCC in the ventral SNC neurons may facilitate the maintenance and plasticity of these exuberant remote axonal arborizations.

Polymorphisms in the genes encoding netrin-1 and DCC are associated with a loss of function and increased susceptibility to develop PD (Lesnick, et al., 2007, Lin, et al., 2009). A loss of DCC function is likely to have a more significant impact in neurons requiring high maintenance of axonal and dendritic structural integrity, like ventral SNC neurons. In mice expressing mutant human α -synuclein, there is an age-dependent redistribution of synaptic SNARE proteins and a reduction in dopamine release (Garcia-Reitböck, et al., 2010) that may either be linked to or cause a loss of DCC function. α -Synuclein has been shown to block the activation of the SNARE complex (Darios, et al., 2010), potentially also blocking DCC function (Cotrufo, et al., 2011). α -Synuclein has also been shown to increase intracellularly in human SNC neurons with age (Chu and Kordower). Such an increase would not only affect SNARE function, but also potentially DCC function. Our study showing that ventral SNC neurons rely more heavily on DCC would predispose these rather than other brainstem dopamine neurons to loss of neurotrophic support in the presence of such molecular changes with age, potentially initiating the selective neurodegeneration of these neurons early in the pathogenesis of PD.

4.4 Conclusions

Our study shows that the expression of Pitx3, Otx2 or DCC is not restricted to particular sub-populations of brainstem dopamine neurons in aged humans. However, more DCC receptor is expressed within the ventral SNC neurons in aged humans, neurons that are the most vulnerable to PD. This highlights a potential role for DCC in the pattern of susceptibility to degeneration found in PD.

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6. Disclosure statement

a) The authors declare the following potential conflicts of interest.

Stock ownership: **Others?** GMH owns stock in Chochlear (2004-present) and NIB Holdings (2007-present).

Honoraria: **Others?** GMH has received travel expenses from the National Health & Medical Research Council of Australia (2010 & 2011 Research Fellowships Panels, 2011 Academy), International Conference on Alzheimer's Disease (2010 organising committee and meeting), Shanghai Movement Disorders Meeting (4/2010), NIH (4/2010 Workshop on Gaucher Disease & Parkinsonism), Elan Pharmaceuticals (4/2010 & 6/2011 San Francisco seminars and 9/2011 Tokyo Parkinson's disease conference), World Parkinson's Congress (2010), International Frontotemporal Dementia Conference (2010), International Society for Neurochemistry (2010 Asia Pacific Conference), International Synuclein Meeting (2010), International Movement Disorders Meeting (2011 organising committee and meeting), NeuraSyn Summer School (2011), and WFN Congress on Parkinson's disease and related disorders (2011).

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b) Human brain tissue samples were received from the Sydney Brain Bank which has approval to collect and distribute human brain tissue for research studies by the Human Research Ethics

Committee of the University of New South Wales under the Human Tissue Act of New South Wales.

The research studies on human brain tissue were approved by the Human Research Ethics Advisory Biomedicine Panel of the University of New South Wales and the Scientific Advisory Committee of the Sydney Brain Bank.

Mice were obtained from the Animal Resource Centre, Perth, Australia and the study protocols approved by the Animal Care and Ethics Committee of The University of New South Wales, Sydney, Australia (ACEC 11/75A).

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Table 1 – Colocalization of Pitx3, Otx2 or DCC immunoreactivity in dopamine neurons (see methods) in the ventral SNC, dorsal SNC and VTA (% \pm SD).

<i>Region</i>	<i>Ventral SNC</i>		<i>Dorsal SNC</i>		<i>VTA</i>	
	Human	Mouse	Human	Mouse	Human	Mouse
% with PITX3	94 \pm 8	94 \pm 8	96 \pm 1	95 \pm 15	96 \pm 7	80 \pm 15
% with Otx2	0	8 \pm 0	0	2 \pm 0	0	53 \pm 24
% with DCC	95 \pm 2	100 \pm 0	96 \pm 3	85 \pm 17	92 \pm 3	62 \pm 8

Table 2 – Average z-scores (\pm SD) of Pitx3, Otx2, and DCC gene expression in the SNC and VTA.

	SNC	VTA	P-value
Pitx3	1.4 \pm 0.1	0.7 \pm 0.3	*0.05
Otx2	-0.4 \pm 0.4	0.5 \pm 0.3	*0.04
DCC	1.7 \pm 0.1	0.8 \pm 0.2	*0.02

*Indicates statistical significance as $P \leq 0.05$

Supplementary Table – The proportion (\pm SD) of dopamine neurons with Otx2 immunoreactivity in the ventral SNC, dorsal SNC, and VTA of different aged mice.

	<i>Ventral SNC</i>	<i>Dorsal SNC</i>	<i>VTA</i>
4 weeks	0 \pm 0%	1 \pm 2%	57 \pm 13%
10 weeks	0 \pm 0%	0 \pm 0%	55 \pm 13%
10 months	0 \pm 0%	4 \pm 5%	71 \pm 6%
2 years	0 \pm 0%	3 \pm 3%	58 \pm 4%

Figure legends

Figure 1: Photomicrographs showing the comparative location of dopamine neurons in the ventral and dorsal SNC and VTA in the human (**A**) and mouse (**B**) brainstem. The representative sections are immunohistochemically stained for tyrosine hydroxylase (TH), the rate limiting enzyme for dopamine, and the mouse sections were counterstained with cresyl violet. Surrounding landmarks including the periaqueductal gray (PAG), red nucleus (R), cerebral peduncle (cp), and the exiting third nerve (3n) are shown.

Figure 2: Representative photomicrographs showing Pitx3 immunoreactivity (green) in most dopamine neurons (TH; magenta converted from red) located in the ventral (**A,D**) and dorsal SNC (**B,E**), as well as the VTA (**C,F**) in humans (**A-C**) and mice (**D-F**). Sections are 40-50 μ m thick and there is some overlap of the cytoplasm on the nuclei of some cells. Asterisks mark neuromelanin pigment in the human dopaminergic neurons (**A,B**). Scale in **C** is equivalent for **A-B**, and scale in **F** is equivalent for **D-E**.

Figure 3: Assessment of variability in neuronal Pitx3 immunoreactivity (**A,B**) and regional gene expression (**C**).

A,B Photomicrographs comparing Pitx immunoreactivity in sections from middle-aged versus old humans. Neurons were labeled with an antibody to Pitx3 (green) and tyrosine hydroxylase (TH; magenta converted from red). In middle-age, nuclear Pitx3 expression was found in many dopamine neurons (**A**; nucleus marked by asterisks), while with aging some Pitx3 protein was also located in the neuronal cytoplasm of the dopamine neurons (**B**; as marked by arrow).

C Assessment of Pitx3 mRNA in the SNC and VTA in a 24 and 39 year old human brain expressed as an increase (\pm SD) above the average z score for the entire brain. The selective expression of Pitx3 was determined to be at z-scores above 0.75 (as indicated by dashed line in **C**). Pitx3 mRNA was expressed above background in both regions independent of age with a significant increase in SNC Pitx3 expression compared with the VTA.

Figure 4: Representative photomicrographs showing Otx2 immunoreactivity in the regions containing dopamine neurons. Neurons were labeled with an antibody to Otx2 (Green) and tyrosine hydroxylase (TH; magenta converted from red). In humans, Otx2 immunoreactivity was not observed in dopamine neurons in the ventral SNC (**A**), dorsal SNC (**B**) or VTA (**C**). In mice nuclear expression was restricted to the VTA (**F**) and was not observed in the ventral (**D**) or dorsal (**E**) SNC. Strong Otx2 immunoreactivity was observed in non-dopaminergic cells in the nearby substantia nigra pars reticulata (**D**). Scale in **C** is equivalent for **A-B**, and scale in **F** is equivalent for **D-E**.

Figure 5: Assessment of regional variability in neuronal Otx2 immunoreactivity (**A-E**) and gene expression (**F**).

A-E High magnification photomicrographs of sections immunohistochemically stained for Otx2. Otx2 immunoreactivity was not observed in neuromelanin-pigmented (light brown) dopamine neurons in the human ventral SNC (**A**), dorsal SNC (**B**) or VTA (**C**), however surrounding regions including the superior colliculi (**D**) and the interpeduncular nucleus (**E**) had obvious Otx2 immunopositive nuclei. Scale in **C** is equivalent for **A-E**.

F Assessment of Otx2 mRNA in the SNC, VTA and superior colliculi in a 24 and 39 year old human brain expressed as an increase (\pm SD) above the average z score for the entire brain. The selective expression of Otx2 was determined to be at z-scores above 1 (as indicated by dashed line in **F**). Only in the superior colliculi was Otx2 mRNA selectively expressed, with neither the SNC or VTA having expression levels above background. In the regions with dopamine neurons, Otx2 mRNA expression was higher in the VTA. Across all regions there was a significant reduction in Otx2 expression from the younger to the older case, with the older case having Otx2 mRNA expression levels in the SNC well below average.

Figure 6: Representative photomicrographs showing DCC immunoreactivity in the regions containing dopamine neurons. Neurons were labeled with an antibody to DCC (black nickel-cobalt-enhanced chromagen in **A-C** or green fluorescence in **D-F**) and tyrosine hydroxylase (TH; magenta converted from red in **D-F**), with the dopamine neurons in humans containing light brown neuromelanin pigment (**A-C**). In humans, the majority of pigmented dopamine neurons had DCC immunoreactivity (**A-C**), although some VTA pigmented dopamine neurons were DCC immunonegative (asterisk in the inset in **C**). In mice, the majority of SNC dopamine neurons colocalised DCC and TH (shown in white in **D-E**), whereas fewer VTA dopamine neurons colocalised DCC (shown in white in **F**). Scale in **C** is equivalent for **A-B**, and scale in **F** is equivalent for **D-E**.

Figure 7: Assessment of the variability in DCC immunoreactivity within the SNC (**A**) and DCC mRNA expression in the SNC and VTA (**B**).

A Graph showing the proportion of human dopamine neurons in the ventral and dorsal SNC containing strong DCC immunoreactivity. The human ventral SNC had a significant 2.0 fold increase in the proportion of neurons with strong intensity DCC immunoreactivity compared with the dorsal SNC.

B Assessment of DCC mRNA in the SNC and VTA in a 24 and 39 year old human brain expressed as an increase (\pm SD) above the average z score for the entire brain. The selective expression of DCC was determined to be at z-scores above 0.87 (as indicated by dashed line in **F**). DCC mRNA was selectively expressed in both the SNC and VTA independent of age, with significantly higher DCC expression in the SNC compared with the VTA.

Supplementary figure: Genome-wide microarray based gene expression profiles obtained from the *Allen Human Brain Atlases* online resource provided by the Allen Institute for Brain Science. For each gene (Pitx3, Otx2 and DCC) the expression profiles of the entire brain in a young (24 year old) and middle-aged (39 year old) human case are provided. Data from only the midbrain region are magnified for each gene and case, with the SNC, VTA and SC labeled. The information from two gene probes was available for Pitx3 and DCC, whilst three probes were available for Otx2. In the profiles green indicates a low expression and red a strong expression, as shown on the provided scale.