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HETEROGENEOUS INTRASTRIATAL PATTERN OF PROTEINS REGULATING AXON GROWTH IN NORMAL ADULT HUMAN BRAIN

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Abstract

There is much controversy regarding the extent of axon regeneration/sprouting ability in adult human brain. However, intrinsic differences in axon/neurite growth capability amongst striatal (caudate, putamen, nucleus accumbens) subdivisions could conceivably underlie, in part, their differential vulnerability in degenerative human brain disorders. To establish whether the distribution of axon growth markers in mature human striatum might be uniform or heterogeneous, we measured the intra-striatal pattern, in autopsied brain of normal subjects (n=40, age 18-99), of proteins involved in regulating axon growth. These proteins included polysialylated neural cell adhesion molecule (PSA-NCAM), microtubule-associated proteins TUC-4 (TOAD/Ulip/CRAMP-4) and doublecortin (DCX), and Bcl-2. The distribution of the marker proteins within the striatum was heterogeneous and inversely related to the pattern of dopamine loss previously characterized in Parkinson's disease (PD), with levels in nucleus accumbens>caudate>putamen, ventral>dorsal, and rostral putamen>caudal. In contrast, distribution of glial markers including glial fibrillary acidic protein (GFAP) and human leukocyte antigens (HLA-DR α and HLA-DR/DQ/DP β), other Bcl-2 family proteins, and control proteins neuron-specific enolase and α -tubulin in the striatum was either homogeneous or had a pattern unmatched to dopamine loss in PD. The putamen also showed more marked age-dependent decreases in concentrations of PSA-NCAM, TUC-4, and DCX and increases in GFAP levels than caudate. We conclude that the intrastriatal pattern of several key axon growth proteins is heterogeneous in adult human brain. Further investigation will be required to establish whether

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this pattern, which was inversely correlated with the pattern of dopamine loss in PD, is involved to any extent in the pathophysiology of this degenerative disorder.

Keywords

Parkinson's disease; axon growth; PSA-NCAM; TUC-4; doublecortin; Bcl-2; glial fibrillary acidic protein; human leukocyte antigen; striatum; aging

The cardinal feature of Parkinson's disease (PD) is the loss of dopaminergic innervation of the striatum (caudate, putamen, nucleus accumbens; Ehringer and Hornykiewicz, 1960). Striatal dopamine loss follows a specific pattern in PD (see Fig. 2), with the putamen, as a whole, losing more dopamine than the caudate, and the nucleus accumbens (NACS) being the least affected. Within caudate/putamen, the dorsal portion is more affected whereas rostrocaudally, posterior putamen loss > anterior and anterior caudate loss > posterior; this pattern is not observed or only less so in other degenerative parkinsonisms including postencephalitic parkinsonism, progressive supranuclear palsy, and multiple system atrophy (MSA) (Bernheimer et al., 1973; Kish et al., 1988; Morrish et al., 1996; Tong et al., 2006b; see Hornykiewicz, 1998 for review).

Previous efforts to explain sub-regionally specific vulnerability of dopamine neurones in PD have focused largely on nigral cell bodies with little attention paid to the axon terminal region, possibly because of the assumption that pathological changes likely begin in the cell body, rich in concentration of Lewy Body, the neuropathological hallmark of PD. Thus, studies of different characteristics amongst dopamine cell bodies such as neuromelanin (Gibb and Lees, 1991; Hirsch et al., 1988; Kastner et al., 1992), calcium stress (Chan et al., 2007; Damier et al., 1999; Gibb, 1992; Yamada et al., 1990), the dopamine transporter (Gonzalez-Hernandez et al., 2004; Uhl et al., 1994), and interactions between calcium, cytosolic dopamine and α -synuclein (Mosharov et al., 2009) have disclosed some association between levels of the markers and extent of cell loss, but still of uncertain significance (Obeso et al., 2010).

The relatively unexplored retrograde dying-back hypothesis (Appel, 1981; Hornykiewicz, 1998; Kish et al., 1992; Lach et al., 1992) suggests that dopamine neuron degeneration in PD could originate in axons and their terminals in striatum. Recently, the dying-back concept has received a new practical emphasis: restoration of damaged axon growth or sprouting from intact neurons might provide a more successful neuroprotective strategy for PD than restoration of a whole neuron (Cheng et al., 2010) should the prohibitive factors for axon/neurite regeneration in adult brain be overcome. In this respect, a relevant question is whether there exist intrinsic differences in axon growth capability among striatal subdivisions that might partly underlie their differential vulnerability in PD and could influence future neuroprotective/neurorestorative strategies.

The present study was undertaken to examine the distribution pattern and aging changes in normal human striatum of a panel of marker proteins involved in regulating axon/neurite outgrowth, including PSA-NCAM (Rutishauser, 2008), TUC-4 (Quinn et al., 1999), and DCX (Francis et al., 1999; Gleeson et al., 1999) to establish first, whether the pattern is, or is not, heterogeneous and secondly whether the subregional distribution might correlate with that previously reported for dopamine loss in PD. The markers selected are all highly expressed during neonatal brain development and much down-regulated in adulthood and are considered to serve as markers of axon/neurite growth capacity (Franz et al., 2008; Nacher et al., 2001). For comparison, we examined the anti- and pro-death Bcl-2 family proteins (Adams and Cory, 1998) and the glial markers including GFAP, HLA-DR α , and

HLA-DR/DQ/DP β as apoptosis (Levy et al., 2009) and inflammation/microgliosis (Hirsch and Hunot, 2009) have also been implicated in PD pathogenesis.

Materials and Methods

This study was approved by the Research Ethics Board of the Centre for Addiction and Mental Health at Toronto. A total of 40 (male/female: 28/12) autopsied brains were obtained from neuropsychiatrically normal subjects [age: 48 ± 3 (18-99) years; postmortem (death to freezing) interval (PMI): 14 ± 1 (4-25) hours; mean $\pm$ SEM (range)]. The causes of death were cardiovascular disease (24), trauma (10), bronchopneumonia (1), cancer (3), drowning (1), and natural death (1). One half-brain was used for neuropathological examination (no significant abnormalities reported), whereas the other half was frozen for neurochemical analyses. Twenty subjects were used for detailed intra-striatal distribution study, including a young [33 ± 4 (18-50) years, PMI: 14.1 ± 1.9 hours] and an aged [71 ± 4 (55-99) years, PMI: 14.7 ± 2.0 hours] group ($n = 10$ each); however, since the two groups were not different in intra-striatal distribution of the proteins of interest, data were pooled together and analyzed as one single group. In addition, autopsied brains from an eight-day old infant (female, PMI=9 hours, suspected of having had sudden infant death syndrome), three patients with MSA (Tong et al., 2010) (68 ± 3 years; PMI: 10 ± 1 hours) and biopsied human temporal cortical samples (nonepileptic, $n=5$, 24 ± 4 years) following operations for the treatment of intractable temporal lobe epilepsy (see Kish et al., 2009 for details) were also used to help characterize the antibodies employed with respect to gross neonatal vs. adult brain differences, influence of gliosis (putamen of patients with MSA; Probst-Cousin et al., 1998), and postmortem time (biopsy vs. autopsied tissue), respectively. Cultured cell lysate was also used as positive controls, including SK-N-MC cell (human neuroepithelioma) for DCX and THP-1 cell (human monocytic leukemia) for HLA-D (from Santa Cruz Biotechnology Inc., Santa Cruz, CA), and Jurkat cell (human T-cell leukemia; from BD Biosciences Canada, Mississauga, ON) and A431 cell (human epidermoid carcinoma; from Upstate Biotechnology, Lake Placid, NY) for Bcl-2 family proteins.

Brain dissection was carried out according to published procedures (Kish et al., 1988; Tong et al., 2006a). The caudate (head) samples were taken from coronal slices #2 (rostral), #5 (middle), and #8 (caudal) and putamen from slices #5 (rostral), #8 (middle), and #11 (caudal) and, in case of missing samples, from the adjacent slice available. Slices #2, #5, #8, and #11 correspond approximately to plates #T4-2432 (at the rostral limit of putamen), -2152 (section rostral to the crossing of the anterior commissure and at the caudal half of NACS; see Tong et al., 2006a), -1954 (section caudal to the crossing of the anterior commissure), and -1765 (section at the rostral hippocampus) of Riley, 1943, respectively. In each slice, caudate and putamen were divided horizontally into dorsal, intermediate, and ventral subdivisions. NACS samples of the lateral portion (Tong et al., 2006a) were available from 10 of the subjects. For the aging study, caudate and putamen samples of the intermediate subdivision of the middle portion of both nuclei from another 20 subjects and frontal cortical (Brodmann area 9) samples from a total of 35 subjects were used.

Aliquoted tissue homogenates were used throughout. Tissue samples were homogenized in ice-cold 50 mM Tris-HCl, 2 mM EGTA, pH 7.4, containing 1% (vol/vol) protease inhibitors cocktail (cat# P8340, Sigma-Aldrich, St-Louis, MO). Protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine plasma albumin as the standard. Levels of GFAP were determined by sandwiched ELISA (O'Callaghan, 1991) using purified porcine GFAP (cat# AG230, Chemicon International, Temecula, CA) as the standard. Protein levels (in μ g protein tissue standard/ μ g protein sample) of PSA-NCAM, TUC-4, DCX, Bcl-2 family proteins, HLA-DR α and the control proteins neuron-specific enolase (NSE) and α -tubulin in tissue homogenates were determined by quantitative

immunoblotting according to published procedures (Tong et al., 2007), using a pooled human frontal cortical sample as the tissue standard, except that a pooled human cerebellar cortical sample was used as the standard for HLA-DR α . The antibodies employed and the conditions established are provided in Table 1. The coefficients of variation were <10% for all the proteins examined within and between blots. For simplicity only, “immunoreactivity” of the proteins examined will be referred to as “levels”.

Statistical analyses were performed by using StatSoft STATISTICA 7.1 (Tulsa, Oklahoma, USA). One-way repeated measures ANOVA (among subdivisions of putamen/caudate) or one-way ANCOVA with age as the covariate (among putamen, caudate, and NACS) followed by post hoc Bonferroni tests were performed ($\alpha=0.05$). Correlations between age and levels of the proteins of interest were examined by linear or non-linear (quadratic) least square regression using GraphPad Prism 4.0 (La Jolla, CA, USA). The criterion of statistical significance was adjusted for multiple comparisons by using the Benjamini and Liu False Discovery Rate controlling procedure (FDR_{BL}, Benjamini et al., 2001). The total number of P values for adjustment was 37 [3 (brain regions) x 12 proteins (PSA-NCAM, TUC-4, DCX, bcl-2 family proteins, and GFAP) and one brain region for HLA-DR α]. Unless specified in *Results*, no significant correlation (Pearson) was observed between PMI and levels of the protein markers.

Results

Specificity of the antibodies against PSA-NCAM, TUC-4, and DCX in neonatal vs. adult human brain

As expected (Fig. 1), the monoclonal (IgM, clone 2-2B; Bonfanti et al., 1992; Rougon et al., 1986) antibody against polysialic acid (PSA), which is linked mainly to neural cell adhesion molecules (NCAM) in vertebrates, detected in a neonatal human brain sample one major broad band with a MW of ~140-220 kDa. In adult brain, the intensity was markedly decreased and centered at about 200 kDa.

The polyclonal antibody raised against a 13 amino acids peptide (YDGPVFDLTTTPK) at the C-terminus of human TUC-4 detected in neonatal brain two closely located protein bands at about 64 and 62 kDa (with the upper band of higher intensity; Fig. 1), respectively, consistent with observations in neonatal rat brain (Rosslenbroich et al., 2003). In adult brain, the intensity of the upper band was markedly decreased whereas the lower band was barely detectable. With longer exposure, a faint protein band at ~75 kDa was also observed, which might correspond to the N-terminal extended variant of TUC-4, i.e., TUC-4b (Quinn et al., 2003). However, due to its low levels of expression relative to the 64 kDa TUC-4 species (i.e., TUC-4a of Quinn et al., 2003), the 75 kDa band was not examined in this study.

The polyclonal antibody raised against a C-terminus peptide of human DCX detected in neonatal brain one major band centered at ~45 kDa (Fig. 1), which is slightly larger than the predicted MW of 40 kDa from the amino acid sequence of DCX but is consistent with the literature showing abnormal electrophoretic mobility of DCX (Francis et al., 1999; Gleeson et al., 1999) and previous reports using the same antibody (Fahrner et al., 2007; Knoth et al., 2010). A positive control of SK-N-MC human neuroepithelioma cell lysate showed the same major protein band at 45 kDa. The intensity of this band was markedly decreased in adult vs. neonatal brain. The antibody also detected in neonatal brain two minor protein bands of much lower intensity at about 40 kDa and 100 kDa, respectively, with the former barely detectable in autopsied adult brain. The 40 kDa band possibly represented a postmortem proteolytic product as biopsied tissue did not show this band (Knoth et al., 2010) and the 100 kDa band likely a non-specific product as its intensity was higher in adult than in

neonatal brain (Fig. 1). No significant correlation was observed between PMI of the subjects examined and levels of DCX (45 kDa).

Intra-striatal distribution of PSA-NCAM, TUC-4, and DCX

As shown in Fig. 2 (see Fig. 3 for representative immunoblots), NACS contained highest levels of PSA-NCAM, TUC-4, and DCX amongst the striatal subdivisions, followed by the caudate and the putamen. Levels of the three proteins in NACS were 189%, 142%, and 50%, respectively, higher than those of putamen, and 150%, 80%, and 17%, respectively, higher than those of caudate. Dorsoventral gradients in caudate and putamen were also observed for PSA-NCAM and TUC-4, but not for DCX, with higher levels of the proteins in ventral vs. dorsal portions of caudate/putamen (range: +31-110% for PSA-NCAM and +16-60% for TUC-4).

Overall, the rostral portions of putamen had higher levels of the marker proteins than did the middle and caudal subdivisions, in particular for PSA-NCAM and TUC-4 in rostral-ventral putamen where levels of the two proteins were 25-156% and 65-136%, respectively, higher than those in middle and caudal putamen. The rostrocaudal gradient for DCX in putamen was shallow, with levels in the rostral portions 15-27% higher than those in the caudal. There was no significant rostrocaudal gradient for DCX or TUC-4 in the caudate, but the caudal-ventral and caudal-intermediate caudate had significantly higher levels of PSA-NCAM than the dorsal and intermediate portions of the rostral and middle caudate (+35-83%).

Comparing caudate with putamen, although the overall differences in levels of PSA-NCAM (+15%), TUC-4 (+34%), and DCX (+28%) did not survive Bonferroni adjustment when levels in all the subdivisions were averaged, most of the caudate subdivisions had significantly higher levels of TUC-4 (+12-136%) and DCX (+21-51%) than the middle and caudal putamen subdivisions. The caudal-ventral and caudal-intermediate caudate also had significantly higher levels of PSA-NCAM than the dorsal putamen subdivisions (+52-152%). However, the rostral-ventral putamen had significantly higher levels of PSA-NCAM (+34-86%) and TUC-4 (+41-48%) than the dorsal caudate subdivisions, corresponding to its relatively lower dopamine loss in PD.

As control proteins, NSE and α -tubulin (Fig. 2 and 3) and the major PSA carrier NCAM (NCAM-180, -140, -120; data not shown) were, in contrast to the axon growth markers, homogeneously distributed among the striatal subdivisions.

Immunoblotting assay of the Bcl-2 family proteins

As shown in Fig. 4, the antibodies for the anti- (Bcl-2 and Bcl-x_L) and pro- (Bcl-x_S, Bax, Bak, Bad, Bid, and Bim_{EL}) apoptotic Bcl-2 family proteins detected in Jurkat (human T-cell leukemia) or A431 (human epidermoid carcinoma, for Bax, as Jurkat cell does not express detectable amount of Bax; Miyashita et al., 1995) cell lysate positive controls and autopsied human brain usually only one major protein band at the expected molecular weight position with the exception of Bcl-x_L/x_S and Bad. The antibody for Bcl-x detected both Bcl-x_L and Bcl-x_S, with the former as a doublet of 29/30 kDa, consistent with the literature (Krajewski et al., 1994) and the latter as a much weaker 19 kDa band. The antibody for Bad detected in some autopsied brains another slightly smaller protein band (21 kDa) than the intact (23 kDa). Detection of the 21 kDa band, presumably a partially truncated form of Bad, appeared to be influenced by PMI as biopsied tissue samples did not show this lower band. The 21 kDa Bad band was also observed more frequently in aged (>50 years; in 13 of 15 subjects) than in younger subjects (in 8 of 25 subjects; $\chi^2=11$, $P=0.001$). Significant negative correlations were observed between levels of the 23 kDa intact Bad (Bad_{intact}) with age,

although not with PMI, of the subjects in brain regions examined (caudate, putamen, and frontal cortex; $r = -0.51$ to -0.64 , $n = 34$ to 40 , $P < 0.001$; significant after FDR_{BL} procedure). However, the extent of Bad truncation (7-64% of total Bad) was consistent amongst brain regions examined in individual subjects and was not significantly correlated with PMI or age of the subjects. Moreover, levels of total Bad (Bad_{total} , intact and truncated) in autopsied brains were not significantly different from those in biopsied samples and were not significantly correlated with either age or PMI (data not shown), suggesting that the partially truncated Bad was not significantly further degraded and that the addition of the 23 and 21 kDa Bad bands could reasonably provide a measure of total Bad in autopsied brains with partial digestion of Bad. In further studies, both Bad_{intact} and Bad_{total} are reported (see Fig. 5). Levels of other Bcl-2 family proteins in autopsied frontal cortex were not significantly different from those in biopsied tissue samples (data not shown).

Intra-striatal distribution of the Bcl-2 family proteins

Similar to the pattern of PSA-NCAM, TUC-4, and DCX, levels of Bcl-2 were significantly higher in NACS than in caudate and putamen (+86% and +57%, respectively; Fig. 5). Within caudate/putamen, there was a dorsoventrally increasing, albeit shallow, gradient of Bcl-2 that was significant in rostral putamen (+25%) and in middle (+20%) and caudal caudate (+17%). Bcl-2 levels were significantly higher in caudal-ventral than rostral subdivisions of caudate (+28-40%) and in rostral-ventral than caudal-dorsal (+27%) and caudal-intermediate (+22%) portions of putamen. Comparing caudate with putamen, the overall 18% increase in Bcl-2 levels was mainly explained by differences between ventral-middle/ventral-caudal caudate and subdivisions of putamen (except rostral-ventral). The intra-striatal distribution of other Bcl-2 family proteins was also examined. With the exception of higher levels of Bid in caudal-ventral caudate and of Bim_{EL} in caudal-ventral caudate/putamen than in other striatal subdivisions, these bcl-2 family proteins were generally evenly distributed in striatum (see Fig. 3 for representative immunoblots).

Intra-striatal distribution of GFAP

Levels of the astroglial marker GFAP were determined in subdivisions of caudate and putamen ($n=8$) by sandwiched ELISA and the specificity of the antibody used was first confirmed by Western blot. Consistent with the literature (Dahl and Bignami, 1975; David et al., 1997), four closely located protein bands with approximate MW of 50, 48, 45, and 43 kDa, respectively, were detected in autopsied human brain (Fig. 3 and 6). However, the multiple bands were unlikely to be explained *in toto* by postmortem degradation (David et al., 1997) since biopsied human temporal cortical samples also showed similar multiple bands, although with the 50 kDa and 45 kDa bands predominating. Further, there was no significant correlation between PMI and levels of the 50 kDa band or other protein bands, which was also confirmed by ELISA assays of total GFAP (data not shown). As expected, the putamen of patients with MSA, in which gliosis is a characteristic (Probst-Cousin et al., 1998), had markedly increased levels of GFAP and protein aggregates and fragments immunoreactive for GFAP (Fig. 6).

As shown in Fig. 3 and 7, GFAP distribution in the striatum was heterogeneous. There was a significant rostrocaudally increasing gradient of GFAP levels in both caudate (3.6 fold increase) and putamen (2.5 fold increase), with that in caudal caudate significantly higher than that in rostral and middle caudate/putamen. Overall, the caudate had higher levels of GFAP than the putamen (2.36 ± 0.58 vs. 1.40 ± 0.53 $\mu\text{g}/\text{mg}$ protein; $P < 0.05$, paired Student's *t*-test).

Intra-striatal distribution of HLA-DR α and HLA-DR/DQ/DP β

The monoclonal antibody (clone TAL.1B5) against the α -chain of HLA-DR (Adams et al., 1983; Styren et al., 1990) detected in human brain one major band at the expected 33 kDa position and, in some brain samples, another minor protein band of slightly smaller MW (Fig. 6). The clone CR3/43 antibody (Graeber et al., 1994) against the highly polymorphic (Finn and Levy, 1982) β -chain of HLA-DR/DQ/DP detected multiple closely located protein bands of MW 26-32 kDa, with relative intensity of the bands highly variable but specific to different subjects since the banding nature did not change among different brain regions examined in individual subjects (data not shown). As a positive control, THP-1 cell lysate (human monocytic leukemia) showed closely located multiple proteins bands centered at the expected MW by both TAL.1B5 and CR3/43. Similar to the findings with GFAP, the putamen of MSA patients had markedly increased levels of both HLA-D subunits, which could be explained by massive microgliosis in this degenerating brain region (Probst-Cousin et al., 1998). Because of the complex and variable banding feature of CR3/43 immunoreactivity between subjects, levels of HLA-DR/DQ/DP β were not quantified and were only examined qualitatively.

Autopsied striatum from six subjects disclosed no significant heterogeneity in subdivisional distribution of HLA-DR α and HLA-DR/DQ/DP β (Fig. 3 and 7). Although not quantified, the relative intensity of the protein bands for HLA-DR/DQ/DP β (CR3/43) matched closely to that of HLA-DR α (TAL.1B5).

Aging effects

As shown in Fig. 8, there were non-linear age effects on levels of PSA-NCAM, TUC-4, and DCX in frontal cortex and putamen with the data best fitted with quadratic (U-shaped) functions ($P = 0.003$, significant after FDR_{BL} procedure). The decrease in levels of the marker proteins from 18 to 50 years old (linear correlation, dotted lines in Fig. 8; $r = -0.47$ to -0.77) was significant with the exception of TUC-4 in the frontal cortex in which a trend ($P=0.02$) was observed before adjustment. After 50 to 60 years of age, concentrations of the proteins became more variable and even tended to increase with age (e.g., TUC-4). In contrast, in caudate, no significant influence of age on levels of the marker proteins were observed except for a trend for decreased PSA-NCAM levels (linear correlation, 18-99 years; $r = -0.39$, $P=0.01$) and a trend for decreased DCX levels (linear correlation, 18-50 years; $r = -0.48$, $P=0.02$) that did not survive adjustment for multiple comparisons. Comparing caudate with putamen, the percentage decreases in levels of the markers proteins from 18 to 50 years old were -24% vs -61% for PSA-NCAM, +2% vs. -58% for TUC-4, and -39% vs. -51% for DCX.

Significant or trends for significant correlations between age and levels of the Bcl-2 family proteins were limited to positive correlations between age and levels of Bcl-2 (Fig. 8) in the three brain regions ($r = 0.42$ to 0.48 , $P = 0.01$ to 0.002 ; significant in caudate after FDR_{BL} procedure), a finding consistent with a previous report in frontal cortex (Jarskog and Gilmore, 2000), and negative correlations between age and levels of Bid ($r = -0.45$ to -0.79 , $P = 0.004$ to $P < 0.0001$; significant in putamen and frontal cortex after FDR_{BL} procedure) and Bad_{intact} (see above).

There were significant positive correlations (Fig. 8) between age and levels of the glial markers in putamen (GFAP and HLA-DR α) and frontal cortex (GFAP). Except for several outliers, levels of GFAP in putamen remained low until 50 to 60 years of age and then increased markedly, similar to findings reported in human hippocampus (David et al., 1997). In contrast, levels of GFAP in caudate were not significantly correlated with age although

after excluding one outlier there was a trend for a positive correlation ($P=0.04$ before adjustment).

Discussion

This is the first comprehensive quantitative study in human brain of the detailed intrastriatal distribution of several categories of proteins (axon growth, apoptosis, gliosis) implicated in the pathogenesis of PD. Our major finding is that, in normal human striatum, the distribution of the axon growth-regulating proteins PSA-NCAM, TUC-4, and DCX, and the anti-death protein Bcl-2, which also promotes axon/neurite growth (Chen et al., 1997; Oh et al., 1996; Zhang et al., 1996), is not uniform, but heterogeneous. Further, this pattern is inversely related to the pattern of striatal dopamine loss reported in PD (Kish et al., 1988; see Fig. 2). In contrast, we found no such association with the apoptosis and gliosis markers, with the exception, as mentioned above, of the apoptosis marker Bcl-2. We also observed a differential aging effect on levels of the axon growth markers (reduction) and GFAP (increase), with the putamen showing a more marked age-related change of protein levels than the caudate.

Limitations

A generic concern is that our postmortem brain findings might not represent those in living human brain. Although we did not find any significant influence of PMI on levels of the protein outcome measures (except for GFAP and the pro-apoptotic protein Bad), and the characteristics of the proteins in our Western blot assays in human brain were similar to those derived from experimental animal brain studies (Bernier and Parent, 1998; Szele et al., 1994; Yang et al., 2004), post- and pre-mortem (e.g., drug exposure, agonal status) factors cannot be excluded, in particular for the late age (after 50-60 years) variation in levels of PSA-NCAM, TUC-4, and DCX (Fig. 8). However, it can be argued that such influences would reasonably have affected the striatum as a whole (vs. intrastriatal differences) as well as levels of the control proteins, which did not show the subregional pattern of the axon growth-regulating proteins. Finally, although the three axonal growth proteins are routinely employed as markers in developing brain and in those rare areas of adult brain that can undergo some neurogenesis (e.g., subventricular zone lining the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus) (von Bohlen Und Halbach, 2007), their functional significance in mature brain parenchyma, as discussed below, is controversial.

Axonal growth markers and PD

Recently, Cheng and colleagues (Cheng et al., 2010) put forward the argument that degeneration of dopamine axons/terminals, not cell bodies, may be the critical pathology in PD and should therefore be the primary target for neuroregenerative therapies. However, support for this speculation is largely derived from the demonstration in PD that estimates of dopamine nerve terminal markers appear to be more severely affected than those of the cell body (Hornykiewicz, 1998; see also Kish et al., 1992), a finding that does not exclude possibility of a primary cell body-based pathology. There is also the difficulty that, outside of a few extra-striatal areas of adult human brain (von Bohlen Und Halbach, 2007), neuronal regeneration is still considered to be not possible and controversial (Di Giovanni, 2009; Silver, 2010). Further, clear evidence using a “validated” marker is still lacking in demonstrating axonal regeneration (or even an abortive attempt) of injured dopamine neurones in human brain. This difficulty is exemplified in a postmortem human brain study purporting to provide neuropathological evidence that infusion of a growth factor into striatum of a patient with PD causes “sprouting of dopaminergic fibers” but in which authors acknowledged that increased level of the outcome measure (a dopamine nerve terminal marker) could not actually distinguish between actual sprouting vs. upregulation of the

marker in spared neurons (Love et al., 2005). Some data using animal models of PD suggest the possibility of sprouting of injured dopamine neurons (Lee et al., 2008; Song and Haber, 2000), but the findings are somewhat uncertain because of reliance on dopamine nerve terminal markers (see Liberatore et al., 1999 for discussion).

Notwithstanding the above uncertainties, a case can be made that levels of PSA-NCAM, TUC-4, and DCX reflect, to some extent, the capability of brain regions to undergo axon/neurite growth/regeneration. Thus, all proteins are highly expressed in early development but much down-regulated in mature brain (e.g., see Fig. 1). The further slow but steady decreases in levels of the marker proteins during adult aging until the 5th-6th decades are consistent with the observations of diminished capability of axonal regeneration and functional recovery after brain injury in aged as compared to younger subjects (Popa-Wagner et al., 2009) and the timing is coincidental with recent MRI findings of brain grey matter density/volume and cortical thickness losses during human aging (Fjell et al., 2009a; 2009b; Sowell et al., 2007; Sowell et al., 2003; Walhovd et al., 2009). In animal studies, NCAM and DCX were among those over-expressed during *in vitro* neurite outgrowth of dissected superior cervical ganglia and dorsal root ganglia tissues (Szpara et al., 2007). PSA overexpression can promote regeneration of severed corticospinal axon processes (El Maarouf et al., 2006) whereas overexpression of DCX promotes neurite growth in cultured CNS neurons (Blackmore et al., 2010). In addition, both TUC-4 (Alabed et al., 2010) and DCX (Tint et al., 2009) also promote axon branching, which might be important in axon collateral sprouting in case of partial axonal damage.

We found that levels of the axon growth markers were somewhat lower in normal human putamen than in the other striatal subdivisions. This raises the possibility that the putamen, which bears the brunt of dopamine loss in PD, might possibly have an environment during adulthood less conducive to axonal regeneration and more vulnerable to age-related axonal damage of dopamine neurones than the adjacent caudate and NACS. This might be related to the late age surge in levels of GFAP in putamen as reactive astrogliosis is generally considered to be inhibitory to axon regeneration and experimental evidence suggests that aged subjects have more rapid and stronger glial reactions detrimental to recovery (Popa-Wagner et al., 2009). Our observations are also consistent with recent MRI findings that the putamen has more grey matter loss than the caudate during human brain development and aging (Fjell et al., 2009b; Greenberg et al., 2008; Ostby et al., 2009; Walhovd et al., 2009) and that the putamen, in contrast to the caudate, has increased fractional anisotropy derived from diffusion tensor imaging during human aging (Abe et al., 2008; Wang et al., 2010).

Notwithstanding the above considerations, however, it must be strongly emphasized that the significance of our neurochemical observations is uncertain and highly speculative with respect to the pathophysiology of PD and to the susceptibility, in this disorder, of different striatal subdivisions (e.g., putamen vs. caudate) to pharmacological axonal regenerative therapy. In particular, as mentioned above, levels of the markers are very low in mature (vs. developing) human brain and therefore might well have no functional consequence, whatsoever, in brain of the adult. It is also possible that the neurodegenerative process in PD could lead to marked changes in the expression and distribution of the proteins not disclosed in our analysis of normal brain. Nevertheless, the results of our study, although providing only correlative data, suggest at the very least increased attention to the possible involvement of intra-regional differences in axon growth capability in disorders of the human striatum.

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Abbreviations

DCX	doublecortin
GFAP	glial fibrillary acidic protein
HLA	human leukocyte antigen
MSA	multiple system atrophy
NACS	nucleus accumbens
NSE	neuron-specific enolase
PD	Parkinson's disease
PMI	postmortem interval
PSA-NCAM	polysialylated neural cell adhesion molecule
TUC	TOAD (Turned on after division)/Ulip (Unc-33-like phosphoprotein)/CRAMP (Collapsin response mediator protein)

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Figure 1.

Western blot of PSA-NCAM, TUC-4, and doublecortin (DCX) in human brain. Lane 1, caudate sample from an eight days old female infant (PMI = 9 hours); lane 2 and 3, caudate and putamen samples, respectively, from a 28 years old female adult (PMI = 7 hours); lane 4, SK-N-MC human neuroepithelioma cell lysate as a positive control for DCX. * indicates a non-specific band. Note low levels of the marker proteins in brain of adults as compared to infants and lower protein levels in putamen than in caudate.

Figure 2.

The intra-striatal pattern of dopamine loss in PD and the distribution of PSA-NCAM, TUC-4, doublecortin (DCX), and the control proteins NSE and α -tubulin in normal human striatum. Data for dopamine loss were from 10 patients with PD and 11 normal control subjects and were previously published (Kish et al., 1988; Tong et al., 2006b; reproduced with permission from Massachusetts Medical Society and American Medical Association). Levels of the marker proteins (mean $\pm$ SEM) were from brains of 20 (putamen and caudate) or 10 [nucleus accumbens (NACS)] normal subjects. The data for putamen and caudate in the right panels were the mean of all subdivisions shown in the left panels. For clarity, the results of statistics [one-way repeated measures ANOVA (among subdivisions of putamen

and caudate) or one-way ANCOVA with age as the covariate (among putamen, caudate, and NACS) followed by post hoc Bonferroni tests] were not shown but were described in the text. Note the opposite pattern of intra-striatal dopamine loss in PD vs. the distribution of the proteins regulating axon outgrowth in normal brain.

Figure 3. Representative Western blots of PSA-NCAM, TUC-4, doublecortin (DCX), Bcl-2 family proteins, glial markers, and the control proteins in subdivisions of putamen and caudate of normal human subjects. D, dorsal; I, intermediate; V, ventral.

Figure 4.

Western blots of the Bcl-2 family proteins in human brain. Lanes 1, Jurkat (human T-cell leukemia) cell lysate (20 μ g); lanes 2 and 3, frontal cortical sample (30 μ g) from a 41 years old male subject (PMI = 5 hours) and a 36 years old male subject (PMI = 23 hours), respectively; lane 4, A431 (human epidermoid carcinoma) cell lysate (25 μ g). Because of the absence of Bax in Jurkat cells, A431 cell was used instead as a positive control for Bax. *indicates a non-specific band due to reaction with the 2nd antibody. The relative molecular weight of the Bcl-2 family proteins was confirmed by strip and reprobe of the blots. Note a slightly smaller band for Bad in some autopsied human brains (lane 4).

Figure 5.

The intra-striatal distribution of the Bcl-2 family proteins in normal human brain. Shown are mean $\pm$ SEM in brain of 20 (putamen and caudate) or 10 [nucleus accumbens (NACS)] normal subjects. The data for putamen and caudate in the right panels were the mean of all subdivisions shown in the left panels. For clarity, the results of statistics [one-way repeated measures ANOVA (among subdivisions of putamen and caudate) or one-way ANCOVA with age as the covariate (among putamen, caudate, and NACS) followed by post hoc Bonferroni tests] were not shown but were described in the text. Note the dorsoventral (NACS vs. caudate/putamen), mediolateral (caudate vs. putamen), and rostrocaudal gradients of the distribution of Bcl-2.

Figure 6. Western blot of the glial markers for astrocyte (GFAP) and microglia (HLA-DR α , clone TAL.1B5 and HLA-DR/DQ/DP β , clone CR3/43) in putamen of normal human subjects (C1-C4) and patients with multiple system atrophy (MSA, M1-M3). Biopsied human temporal cortical samples were also used for the detection of GFAP. THP1 (human monocytic leukemia) cell lysate was used as a positive control for the detection of human leukocyte antigens. *indicates aggregates and fragments of GFAP in MSA brains. Note the multiple bands nature of GFAP and HLA-DR/DQ/DP β (over-exposure for the last lane of THP-1) and markedly increased levels of the glial markers in putamen of MSA patients.

Figure 7.

The intra-striatal distribution of the glial markers for astrocyte (GFAP) and microglia (HLA-DRα) in normal human brain. Shown are mean $\pm$ SEM in brain of eight subjects for GFAP and six subjects for HLA-DRα. For clarity, the results of statistics (one-way repeated measures ANOVA followed by post hoc Bonferroni tests) were not shown but were described in the text. Note the rostrocaudal increasing gradient of GFAP in putamen and caudate.

Figure 8.

Age-related changes of PSA-NCAM, TUC-4, doublecortin (DCX), Bcl-2, and the glial markers (GFAP and HLA-DR α) in normal human brain. The solid lines are least square regression using either quadratic or linear functions as indicated (shown are P values before multiple comparisons adjustment using the Benjamini and Liu False Discovery Rate controlling procedure, Benjamini et al., 2001; in bold for those significant after adjustment). The dotted lines are linear correlations ($P < 0.05$ before adjustment) for PSA-NCAM, TUC-4 and DCX in the age range of the lines. Note the difference between putamen and caudate in the influence of age on levels of the axon growth-regulating proteins and GFAP.

Table 1

Antibody sources and conditions used.

Antigen (MW observed)	Supplier	Catalogue#	Isotype	Working dilution
<u>Axon growth markers</u>				
PSA-NCAM (>200 kDa)	Chemicon	MAB5324	Mouse IgM (2-2B)	1:12,000
TUC-4 (64 kDa)	Chemicon	AB5454	Rabbit antiserum	1:15,000
DCX (45 kDa)	Santa Cruz Biotech.	sc-8066	Goat IgG (C-18)	1:2,000
<u>Bcl-2 family proteins</u>				
Bcl-2 (26 kDa)	Santa Cruz Biotech.	sc-7382	Mouse IgG ₁	1:1,000
Bcl-x _L /x _S (29/30, 19 kDa)	BD Biosciences	556361	Rabbit antiserum	1:13,000
Bim _{EL} (25.5 kDa)	Chemicon	AB17003	Rabbit IgG	1:12,000
Bak (25 kDa)	Upstate Biotech.	06-536	Rabbit IgG	1:8,000
Bad (23 kDa)	Santa Cruz Biotech.	sc-8044	Mouse IgG ₁	1:2,500
Bid (22 kDa)	BD Biosciences	550365	Rabbit antiserum	1:12,000
Bax (21 kDa)	Santa Cruz Biotech.	sc-493	Rabbit IgG	1:3,000
<u>Glial markers</u>				
GFAP	DAKO	Z0334	Rabbit IgG	1 µg/well (ELISA)
(50, 48, 45, and 43 kDa)	Chemicon	MAB360	Mouse IgG ₁	1:10,000 (ELISA) 1:80,000 (WB)
HLA-DRα (TAL.1B5, 33 kDa)	DAKO	M0746	Mouse IgG ₁	1:300
HLA-DR/DQ/DPβ (CR3/43, 26-32 kDa)	DAKO	M0775	Mouse IgG ₁	1:1,000
<u>Control proteins</u>				
NSE (47 kDa)	Santa Cruz Biotech.	sc-21737	Mouse IgG ₁	1:60,000
α-tubulin (55 kDa)	Zymed	13-8000	Mouse IgG ₁	1:7,500
<u>Secondary antibodies</u>				
anti-mouse IgG ₁ -HRP	Southern Biotech.	1070-05	Goat IgG	1:5,000 (ELISA) 1:10,000 (WB)
anti-mouse IgM-HRP	Calbiochem	401225	Goat IgG	1:10,000
anti-rabbit IgG-HRP	Pierce	31460	Goat IgG	1:10,000
anti-goat IgG-HRP	Southern Biotech.	6165-05	Rabbit IgG	1:10,000