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Expression of tyrosine hydroxylase isoforms and phosphorylation at serine 40 in the human nigrostriatal system in Parkinson's disease

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ABSTRACT

Tyrosine hydroxylase is the key enzyme controlling the synthesis of the catecholamines including dopamine. The breakdown of dopamine into toxic compounds has been suggested to have a key role in the degeneration of the dopaminergic neurons in Parkinson's disease. Humans are unique in containing four isoforms of tyrosine hydroxylase, but understanding of the role of these isoforms under normal conditions and in disease states is limited. The aim of this work was to determine the level and distribution of the four human isoforms in tissues from healthy controls and patients with Parkinson's disease. The results show that isoform 1 and isoform 2 are the major tyrosine hydroxylase isoforms in human brain, but that tyrosine hydroxylase isoform 2 is more abundant in the substantia nigra than the tyrosine hydroxylase isoform 1. The two minor isoforms, isoform 3 and isoform 4, are expressed at a proportionally higher level in the terminal field regions (caudate and putamen) compared to the substantia nigra. There was a selective loss of tyrosine hydroxylase isoform 1 in Parkinson's disease compared to age-matched controls and a corresponding increase in the proportion of tyrosine hydroxylase isoform 2. Phosphorylation of serine 40 was significantly increased in caudate, putamen and ventral tegmental area, but not in the substantia nigra, in Parkinson's disease brain. These results show a selective sparing of tyrosine hydroxylase isoform 2 in Parkinson's disease. Isoform 2 exhibits a reduced capacity for activation compared to isoform 1, which may account for the selective sparing of cells expressing isoform 2 in Parkinson's disease. Surviving neurons in Parkinson's disease brain exhibit a substantial increase in tyrosine hydroxylase phosphorylation consistent with a compensatory mechanism of increased dopamine synthesis in the terminal field regions.

1. Introduction

Tyrosine hydroxylase (TH) [EC1.14.16.2] is the rate-limiting enzyme in the biosynthesis of the catecholamines dopamine, noradrenaline and adrenaline (Tekin et al., 2014) and is subject to a variety of regulatory mechanisms. Long-term regulation of TH is controlled by gene expression and protein levels (Tank et al., 2008). In response to acute stimulation, TH activity is primarily controlled through a combination of feedback inhibition by catecholamine binding to TH and relief of that inhibition by phosphorylation (Dunkley et al., 2004).

There are three serine (Ser) residues in TH whose level of phosphorylation increases in response to stimuli and modulates TH activity (Ser^{19}, Ser^{31}, and Ser^{40}). Only phosphorylation of Ser^{40} results in dissociation of the catecholamine from the high affinity site and subsequent activation of TH (Ramsey and Fitzpatrick, 1998). The stoichiometry of Ser^{40} phosphorylation in the brain in both cell body and terminal regions is low in vivo, varying between 3% and 7% depending on the individual brain region (Dunkley and Dickson, 2019).

Abbreviations: TH, tyrosine hydroxylase; TH1-4, human tyrosine hydroxylase isoforms 1 to 4; Ser^{19}, serine 19; Ser^{31}, serine 31; Ser^{40}, serine 40; PD, Parkinson's disease; Sn, substantia nigra; Vt, ventral tegmental area; Cd, caudate; Pu, putamen; DA, dopamine

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Interestingly, there is a subset of terminals in the striatum which connect to striatal blood vessels that appear to show significantly higher levels of Ser^{40} (and Ser^{19}) phosphorylation (Afonso-Oramas et al., 2014). Ser^{19} and Ser^{31} increase TH activity indirectly via hierarchical phosphorylation. Whereby phosphorylation of these residues increases the rate of phosphorylation of Ser^{40}, with Ser^{19} increasing the rate about 3-fold and Ser^{31} increasing the rate about 9-fold (Bevilaqua et al., 2001; Lehmann et al., 2006). Both Ser^{19} and Ser^{31} phosphorylation have been shown to potentiate TH activation in situ (Bobrovskaya et al., 2004; Lehmann et al., 2006).

TH is encoded by a single gene. Most mammals have a single form of TH, while higher primates have two (Haycock, 2002). Humans are the only species with four isoforms of TH (Lewis et al., 1993), which result from alternative splicing of TH mRNA (Grima et al., 1987; Bourdelles et al., 1988; O'Malley et al., 1987). The four human isoforms only differ by the number of amino acids N-terminal to the Ser^{31} residue in isoform 1 (TH1). TH1 is the smallest of the TH isoforms and homologous to TH in other species. Isoform 2 (TH2) contains an additional 4 amino acids inserted immediately N-terminal to Ser^{31} in TH1. Isoform 3 (TH3) has 27 additional amino acids inserted in the same position, while isoform 4 (TH4) has both the 4- and 27- amino acid inserts. N-terminal region differences characterising the four isoforms is shown diagrammatically in Fig. 1A. Threonine 8 is not shown, as there is no evidence that the phosphorylation of this site is regulated (Dunkley and Dickson, 2019). All four isoforms are expressed in human brain and adrenals. TH1 and TH2 are the two major isoforms expressed and together account for over 90% of the TH in brain. The two TH isoforms found in primates are homologous to TH1 and TH2.

As the isoforms only differ at the extreme N-terminus of the molecule, the kinetic parameters of each of the isoforms are almost identical (Daubner et al., 2011). Further, catecholamine binding and inhibition and relief of inhibition by phosphorylation of Ser^{40} (or its equivalent) is similar between the isoforms and similar to rat TH (Gordon et al., 2008; Sura et al., 2004). The key difference in the isoforms is the sequence around Ser^{31} in TH1. Of these isoforms, only phosphorylation of Ser^{31} in TH1 produces a strong hierarchical phosphorylation effect (Lehmann et al., 2006). The presence of the 4-amino acid insert in TH2 just N-terminal to this residue means that ERK cannot phosphorylate the Ser^{31} equivalent in TH2 (Lehmann et al., 2006). Further, it was shown that in situ phosphorylation of Ser^{31} contributes to the level of phosphorylation of Ser^{40} in TH1; however, this effect is absent in TH2 (Gordon et al., 2009). Therefore, hierarchical phosphorylation can occur in TH1 but not in TH2. This differential effect of hierarchical phosphorylation in TH1 and TH2 provides the first known difference in regulatory mechanism of the two major human TH isoforms.

Parkinson's disease (PD) is characterised by marked degeneration of dopaminergic substantia nigra (Sn) neurons (Lees et al., 2009), but relative preservation of the dopaminergic neurons of the ventral tegmental area (Vt). This raises the question of why differential sensitivity to degeneration of different groups of dopaminergic neurons occurs in PD. Dopamine (DA) is metabolised into toxic compounds that can produce oxidative stress in dopaminergic neurons, particularly in concert with iron (Hare and Double, 2016). Increased TH activity can lead to high levels of DA production, increasing production of dopamine-related toxic compounds and exacerbating cell death. Consistent with this, increased cytosolic DA generated through reduced activity of the vesicular dopamine transporter in mice (Caudle et al., 2007) or increased TH activity in drosophila (Stathakis et al., 1999) can produce degeneration of dopaminergic neurons similar to that seen in PD. Importantly, induced pluripotent stem cell line-derived dopaminergic neurons from idiopathic PD patients and PD patients with a homozygous loss-of-function mutation in DJ-1 show increased mitochondrial and lysosomal dysfunction and alpha-synuclein accumulation compared with controls, and this process is dependent on dopamine (Burbulla et al., 2017).

The increased sensitivity of certain groups of dopamine neurons to degeneration could result from a comparatively high dopamine content in this brain region due to the presence of particular TH isoforms, or an increase in TH phosphorylation at Ser^{40} in those cells. Information regarding the distribution and amounts of human TH isoforms is limited. Therefore, in this study, we have used immunohistochemistry and quantitative western blotting to complete the first detailed analysis of human TH isoform distribution and phosphorylation at Ser^{40} in the caudate (Cd), putamen (Pu), Sn, and Vt of healthy control and PD brains.

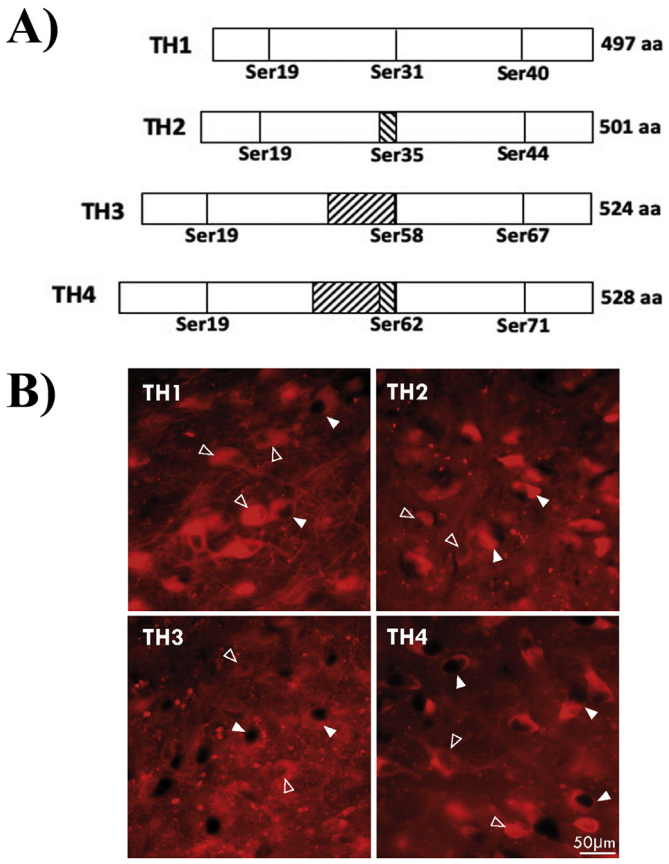

Fig. 1. Human TH isoforms and detection of the isoforms in Sn by isoform specific antibodies. (A) Diagram of human TH isoforms. Only the N-terminus this is shown as the rest of the protein is identical in all isoforms. The positioning of the three regulated phosphorylation sites are shown with the appropriate numbering for each isoform. The positioning of the 4 amino acid insert VRGQ (crosshatch down to right) and the 27 amino acid insert GAPGPSLTGSPWPGTAAPAASYTPTPR (cross hatch up to the right) is shown. The number of amino acids (aa) in each isoform is shown. (B) Representative images of Sn neurons immune-positive for each isoform-specific antibody in a single control case. Note the presence of dark-coloured neuromelanin pigment within some TH isoform-positive neurons. Solid white arrowheads indicate melanised cells while open white arrowheads indicate non-melanised cells in this brain region. Quantitative data are shown in Table 1.

2. Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. AminoLink Plus coupling gel, AminoLink Plus immobilization kit, AminoLink Plus immunoprecipitation kit, BCA protein assay kit, sodium cyanoborohydride, Sulfolink coupling gel, Sulfolink immobilization kit, and anti-rabbit secondary antibodies were all from Pierce (Rockford, IL, USA). Peptides were synthesized by Auspep (Parkville, Australia). Complete protease inhibitor cocktail tablets were from Roche (Indianapolis, IN, USA).

3. Methods

3.1. TH isoform localisation within the substantia nigra

3.1.1. Case details and preparation of brain tissue

Formalin-fixed human brain tissue was obtained with informed consent through the brain donation program of Neuroscience Research Australia (Sydney, Australia), with appropriate ethical approvals. Five control cases without neuropsychiatric, neurological or neurodegenerative disease were selected for analysis of TH isoforms (see Supplementary Table 1). Brains were immersion fixed in 15% buffered formalin for at least two weeks. Brainstems were embedded in 3% agarose and transversely sectioned at 3 mm on a rotary slicer. Three-millimetre transverse tissue blocks of right midbrain containing Sn (at the level of the exiting oculomotor nerve) were sampled and frozen serial sections cut at 50 μm thickness.

3.1.2. Immunohistochemistry

Single-labelled immunofluorescent staining for each of the four human TH isoforms (TH1-TH4) was performed on midbrain sections from each case. Polyclonal rabbit antibodies against each TH isoform were used at the following dilutions and final concentrations: TH1 1:2000, final concentration 0.25 μg/ml; TH2 1:500, final concentration 0.6 μg/ml; TH3 1:1000, final concentration 1.0 μg/ml; and TH4 1:1000, final concentration 0.8 μg/ml (Haycock, 1991; Haycock, 1993; Lewis et al., 1993). A section containing locus coeruleus was included in each staining run as a positive control, as each TH isoform is present in this nucleus (Lewis et al., 1993). Free-floating sections were pre-treated with 50% alcohol, 3% hydrogen peroxide solution, and 10% blocking normal horse serum, then incubated overnight at room temperature in primary antibody in 0.1 M Tris/1% blocking serum/0.3% Triton X-100. Subsequently, sections were washed in 0.1 M Tris buffer, incubated in fluorescent conjugated secondary antibody (goat anti-rabbit alexa 568 diluted 1:250; A11011, Molecular Probes, Eugene, OR, USA), for two hours, and finally washed in 0.1 M Tris buffer. The sections were slide mounted and cover-slipped with Vectashield mounting medium (H-1000, Vector Laboratories, Burlingame, CA, USA).

3.1.3. Microscopy and analysis

Single-labelled slides were examined using an Olympus BX51 microscope (Olympus America, Center Valley, PA, USA) with specific fluorescent filter for alexa 568 and fitted with a Spot RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) linked to a computer using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Photomicrographs were taken of 1–2 fields in the Sn medialis and lateralis, and in the medial, intermediate and lateral subregions of both the dorsal and ventral tiers. Representative immunofluorescent images of neurons immunopositive for each isoform-specific antibody within the Sn of a single control case is shown in Fig. 1B. TH isoform-positive and pigmented neurons were quantified in each field from the photomicrographs. A neuron was scored as positive if any part of the neuron was specifically stained with the TH isoform. Following visual inspection, neurons were classified as non-pigmented (containing no neuromelanin granules) or pigmented (containing neuromelanin granules). A total neuronal count for the Sn pars compacta, and each subregion, was determined in a single Nissl-stained section from each case. Data analysis was performed using SPSS software program (IL, USA). The proportion of each TH isoform was calculated as a percentage of total Sn neurons, non-pigmented neurons, and all pigmented neurons. Analysis of variance (ANOVA) demonstrated no significant differences in these values between the Sn subregions, thus the ventromedial and dorsointermediate subregions were omitted from further analysis, as they are the most cell-sparse regions within the Sn (McRitchie et al., 1995). The ventrointermediate and ventrolateral subregions were pooled into a 'ventral' group, and the dorsomedial and dorsolateral subregions pooled into a 'dorsal' group for subsequent analyses using unpaired *t*-tests. Data are expressed as the mean percentage of neurons expressing the factor of interest ± standard error of the mean.

3.2. Quantification of TH isoform levels within the basal ganglia

3.2.1. Case details and preparation of brain tissue

Fresh frozen human brain tissue from three aged controls with no evidence of neurodegenerative disease or history of neurological disorders (see Supplementary Table 2) was obtained through the brain donation program of Neuroscience Research Australia (Sydney, Australia). Fresh frozen human brain tissue from four patients with idiopathic PD were obtained from the South Australian Brain Bank (see Supplementary Table 3).

3.2.2. Expression and purification of TH1-4 and phosphorylation of TH1 at Ser^{40}

Expression and purification of human TH isoforms (TH1-4) and phosphorylation of TH1 at Ser^{40} was completed as previously described (Lehmann et al., 2006).

3.2.3. Preparation of antibodies

Total TH antibody (tTH) generation and purification was completed as previously described (Gordon et al., 2009). This antibody detects all four isoforms equally (Supplementary Fig. 1). The TH isoform-specific antibodies were prepared as previously described (Haycock, 1991) and purified as described (Gordon et al., 2009). The specificity of the antibodies was assessed by immunoblotting using purified recombinant TH isoforms (Fig. 2). Each antibody readily detected the cognate TH isoform but did not detect the three other isoforms at the highest concentrations used.

3.2.4. Homogenization of human brain tissue and immuno-isolation of TH from human brain tissue

Fresh frozen tissue was homogenised on ice in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 3 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 1 x protease inhibitor cocktail, 1 mM sodium orthovanadate, 80 μM ammonium molybdate, 5 mM β-glycerophosphate, 2 μM microcystin at a ratio of buffer (ml) to tissue (grams) of 10:1. The crude extract was aliquoted and stored at -80 °C until used for immuno-isolation. The total TH antibody was coupled to the AminoLinkPlus Coupling Resin, and spin columns were used to immuno-isolate TH according to the manufacturer's protocol. Crude extracts of post-mortem brain tissue were centrifuged at 26,000 ×*g* for 30 min at 4 °C, and 400 μl of supernatant was added to the beads and gently rocked for 24 h at 4 °C. The beads were added to spin columns and washed three times with 0.15 M NaCl, 25 mM Tris, pH 7.2. Finally, 400 μl of 50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% DTT, 2 mM EDTA was added to the beads and the sample boiled for 10 min to elute the bound protein.

3.2.5. SDS polyacrylamide electrophoresis and Western blotting

Eluted proteins were separated using 10% SDS-polyacrylamide gels, and then transferred to nitrocellulose as described (Jarvie and Dunkley, 1995). Membranes were probed with TH1-4 isoform-specific antibodies, total TH antibody, or Ser^{40} antibodies as described (Ong et al., 2014). Immunoblots were visualised on the Fujifilm Image Reader Las-3000 system after exposure to Super Signal West Femto Maximum Sensitivity Substrate. Blots were quantitated using Multi-Guage v3.0 (FujiFilm).

3.2.6. Quantitation of TH

To determine the absolute amount of each isoform in the sample, a standard curve of at least 8 concentrations of TH was run on each blot in addition to the human tissue samples. The absolute amount of TH in each sample (either total TH or a specific isoform) was calculated from the corresponding standard curve. Each tissue was probed for every

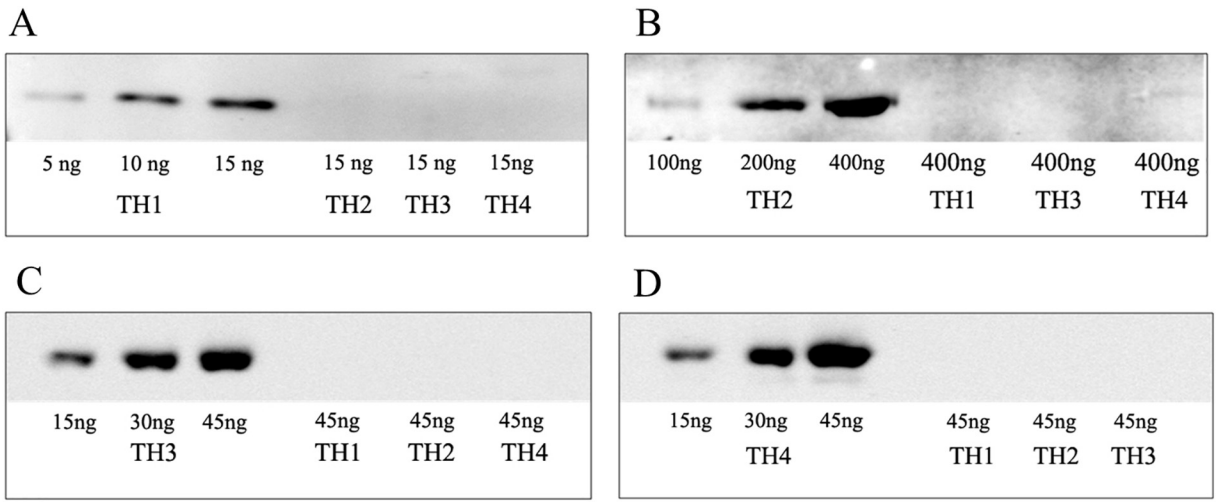

Fig. 2. Specificity of the TH isoform antibodies. The relevant recombinant TH isoform was loaded in a concentration curve, and the remaining isoforms were each loaded at the highest concentration. All blots were probed overnight at 4 °C with the purified primary antibody; these concentrations were used for the remainder of the experiments. (A) TH1 at 1:100, (B) TH2 at 1:50, (C) with TH3 at 1:100, and (D) TH4 at 1:100.

isoform between two and four times, and the average for each isoform in each tissue was taken. The amount of each isoform and total TH within a sample was determined and used to determine to proportion of each isoform in that sample. The level of phosphorylation of TH at Ser^{40} was calculated from absolute levels of TH phosphorylated at Ser^{40} and total TH levels. Isoform and phosphorylation data were analysed using the repeated measures two-way ANOVA or one-way ANOVA, respectively, each with Bonferroni post-tests for multiple comparisons. Statistical significance for the PD phosphorylation data was tested using a generalised linear mixed model because there was a different sample number in each group. Statistical significance between control and PD tissues for each tissue was tested using a student's t-test. Values $p < .05$ were considered significant.

4. Results

4.1. Distribution of neuromelanin within the Sn

Analysis of pigmentation (95% of all neurons) in the six subregions of the Sn demonstrated no differences in the percentage of either heavily or lightly pigmented neurons in any region of this nucleus (data not shown). There were no significant differences between the dorsal and ventral tiers in the percentage of non-pigmented neurons ($p = .291$), or in heavily ($p = .235$) or lightly (p = .235) pigmented neurons.

4.2. Distribution of TH isoforms within the Sn

The proportion of cells expressing each of the four TH isoforms within the entire Sn differed. The most abundant isoform was TH4, which was expressed by 74% ± 6.2% of neurons. TH1 was expressed in 50% ± 7.4% neurons, while TH2 and TH3 were expressed in 35% ± 4.6% and 32% ± 7.4% of neurons respectively. There were no significant differences between the six subregions of the Sn in the percentage of each isoform present in either the total number of neurons, the non-pigmented neurons, or the pigmented neurons (data not shown), thus the data were combined into the “ventral” and “dorsal” regions for further analyses.

Analysis of TH isoform distribution in the ventral vs. the dorsal regions demonstrated a strong trend towards a greater expression of TH2 in neurons in the dorsal tier than in the ventral tier ($p = .052$, Table 1). This differential expression of TH2 was statistically significant when pigmented neurons alone in each region were considered ($p = .047$, Table 1). In contrast, the pattern of differential expression observed for TH2 was not present for TH1, TH3 or TH4 when either total neuron number (TH1, $p = .440$; TH3, $p = .656$; TH4, $p = .314$) or only pigmented neurons were examined (Table 1). Analysis of isoform expression between the two regions demonstrated no differences for any of the four isoforms in the non-pigmented neurons (TH1, $p = .502$; TH2, $p = .593$; TH3, $p = .421$; TH4, $p = .351$; data not shown).

4.3. Analysis of human TH isoform distribution

Antibodies recognising each human TH isoform and total TH were generated by immune-isolation of each protein from Sn, Vt, Cd, and Pu of both control and PD brains. Signals from each protein of interest were compared against standard curves of the relevant TH protein, and levels of each isoform were calculated as a percent of the total sample TH. Representative blots of the total TH antibody, the four TH isoform specific antibodies, and the Ser^{40} phosphorylation-specific antibody are shown in Fig. 3. All antibodies readily detected the cognate TH in each sample, but the TH2 antibody showed a lower detection sensitivity, as shown by the increased amount of TH2 protein that needed to be loaded for detection (Fig. 3C).

4.4. Distribution of the TH1 and TH2 isoforms in control patients

The isoforms TH1 and TH2 were found in all brain regions studied (Fig. 4) and together accounted for approximately 95% of the TH in all regions. Regional levels of TH1 and TH2 in the Cd, Pu and the Vt were similar, each representing approximately 50% of total TH in the samples. In contrast, in the Sn, protein levels of TH2 were significantly higher (62%) than those of TH1 (36%, $p < .01$, $n = 3$).

4.5. Distribution of the TH3 and TH4 isoforms in control patients

The levels of TH3 and TH4 were low in all regions studied and together accounted for < 6% of all TH (Fig. 5). This was particularly apparent in the Sn and Vt, where the sum of TH3 and TH4 constituted < 2% of total TH. Levels of TH3 and TH4 were three- and four-fold proportionally higher in the terminal fields, the Cd and Pu, than in the Sn and the Vt ($p < .001$, repeated measures two-way ANOVA, n = 3). Within each region, there was no significant difference in the proportions of TH3 or TH4.

Table 1
Proportion of neurons expressing TH isoforms in the dorsal and ventral regions of the Sn. Pig: pigmented neurons only. Data are expressed as the mean percentage of neurons expressing the TH isoform of interest ± standard error of the mean.

	%TH1	%TH2	%TH3	%TH4	%pigTH1	%pigTH2	%pigTH3	%pigTH4
Ventral tier	59.1 ± 8.9	47.6 ± 9.1	38.6 ± 13.8	63.8 ± 7.5	61.0 ± 9.5	50.7 ± 9.4	41.9 ± 15.3	68.0 ± 8.4
Dorsal tier	49.3 ± 8.3	23.5 ± 5.3	31.8 ± 5.0	74.1 ± 5.9	52.9 ± 8.5	25.6 ± 5.0	36.2 ± 7.0	81.5 ± 5.2
p-value	0.440	0.052	0.656	0.314	0.543	0.047	0.746	0.210

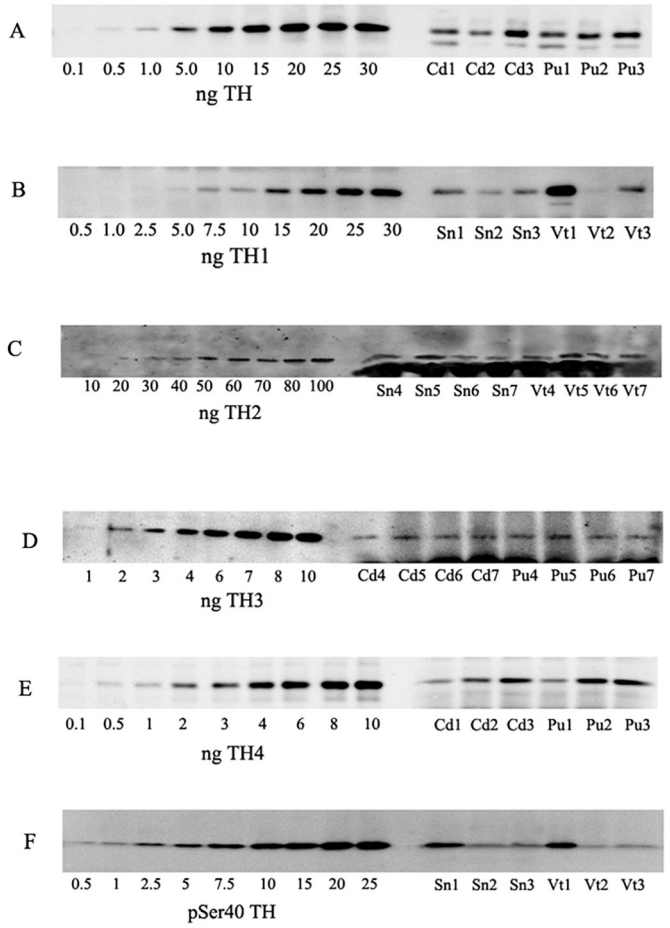

Fig. 3. Representative blots of the TH isoform and TH phosphorylation data from control and Parkinson's disease tissues. Representative Western blots are shown for total TH, the four TH isoforms, and TH phosphorylated at Ser^{40}. In each case, the purified protein standard curve is shown with samples from human tissue. Sn - substantia nigra, Vt - ventral tegmental area, Cd - caudate, Pu - putamen. The lower sensitivity of the TH2 antibody required an increased amount of the immunoprecipitation sample to be loaded. In these samples, the secondary antibody detected the small amount of the total TH antibody which leached off the beads (lower band in panel C). Nevertheless, the TH2 band was able to be separated from the antibody band and readily quantified. Samples 1, 2, and 3 are from controls, samples 4, 5, 6, and 7 are from PD patients. (A) Total TH (B) TH isoform 1 (C) TH isoform 2 (D) TH isoform 3, (E) TH isoform 4, and (F) Ser^{40} phosphorylated TH.

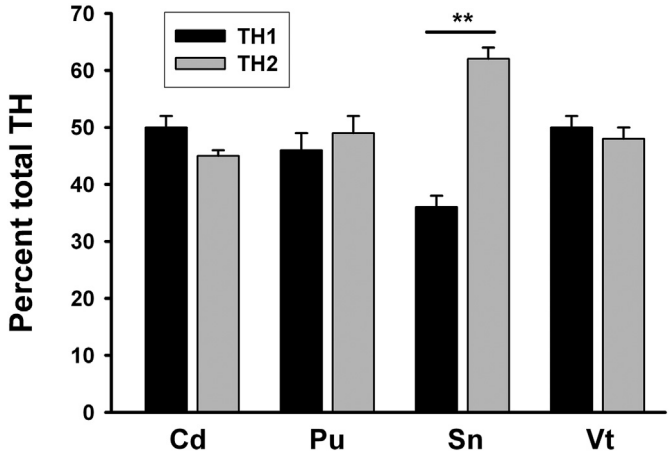

Fig. 4. TH1 and TH2 distribution in normal human brain. The level of the TH1 (black bars) and TH2 (grey bars) isoforms as a proportion of total TH was determined in four different brain regions from control patients, as described in methods. The brain regions analysed are: caudate, Cd; putamen, Pu; substantia nigra, Sn; and ventral tegmental area, Vt. Data are presented as mean ± SEM ($n = 3$). $^{**}p < .01$ for comparison of levels of TH1 vs. TH2 isoforms in Sn.

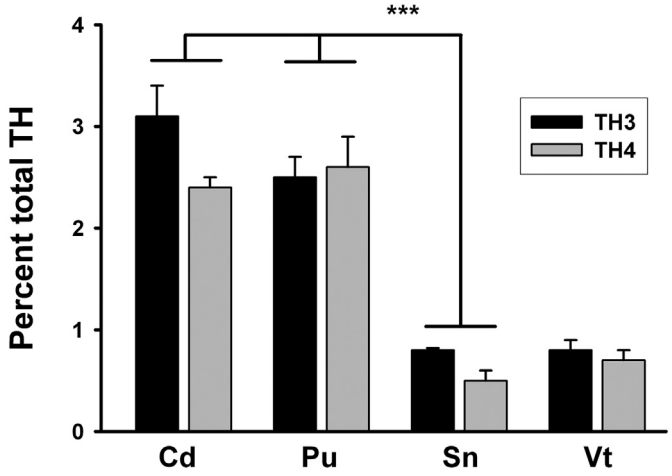

Fig. 5. TH3 and TH4 distribution in normal human brain. The level of the TH3 (black bars) and TH4 (grey bars) isoforms as a proportion of total TH was determined in four different brain regions from control patients, as described in methods. The brain regions analysed are: caudate, Cd; putamen, Pu; substantia nigra, Sn; and ventral tegmental area, Vt. Data are presented as mean ± SEM (n = 3). $^{***}p < .001$ for comparison of levels of each isoform in Cd or Pu vs. Sn.

4.6. Comparison of TH isoforms in control vs PD patients

There was a significant decrease in the relative proportion of TH1 in all four brain regions in the PD brain (Cd and Pu, $p < .05$; Sn, $p < .01$; Vt, $p < .001$), and an associated increase in the proportion of TH2 in these regions in PD (Fig. 6). These results suggest a selective loss of TH1 in PD, which is more pronounced in the cell bodies than in the terminal fields. In contrast, the proportion of TH3 was increased in the regions containing cell bodies in PD brain (Sn, $p < .01$; Vt $p < .001$), but was unchanged in the terminal fields (Fig. 7), while the proportion of TH4 was slightly but significantly decreased in the Cd of PD patients ($p < .05$).

4.7. Analysis of TH phosphorylation at Ser^{40}

To date, the location and quantity of the level of phosphorylation of TH at Ser^{40} in humans has not been reported. Tissue homogenates from PD and control tissues were immuno-isolated and quantitative western

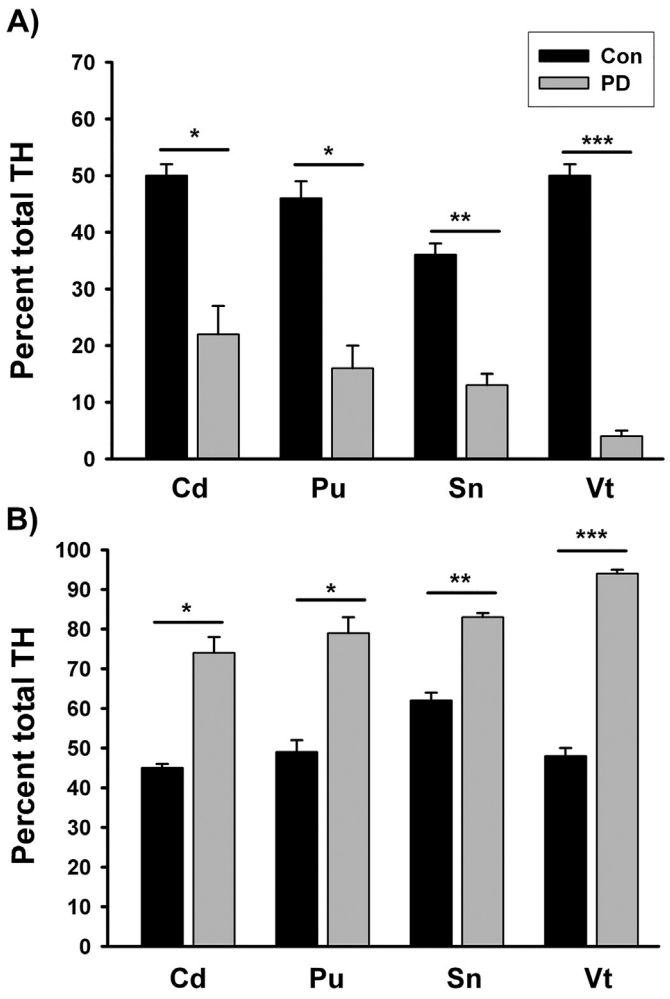

Fig. 6. Comparison of TH1 and TH2 isoforms in control and Parkinson's disease brain. The level of the TH1 (A) and TH2 (B) isoforms as a proportion of total TH was determined in four different regions from control (black bars) and PD (grey bars) brains, as described in methods. The brain regions analysed are: caudate, Cd; putamen, Pu; substantia nigra, Sn; and ventral tegmental area, Vt. Data are presented as mean ± SEM (control n = 3, PD $n = 4$). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ for comparison of control vs. PD.

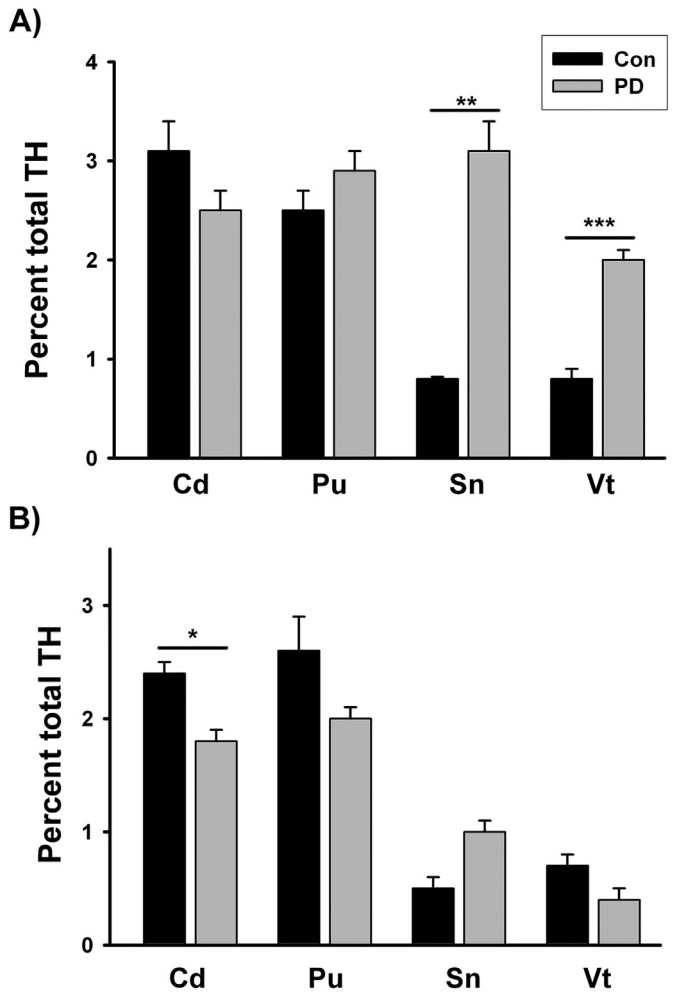

Fig. 7. Comparison of TH3 and TH4 isoforms in control and Parkinson's disease brain. The level of the TH3 (A) and TH4 (B) isoforms as a proportion of total TH was determined in four different regions from control (black bars) and PD (grey bars) brains, as described in methods. The brain regions analysed are: caudate, Cd; putamen, Pu; substantia nigra, Sn; and ventral tegmental area, Vt). Data are presented as mean ± SEM (control n = 3, PD n = 4). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ for comparison of control vs. PD.

blotting performed using a site-specific primary antibody to detect levels of Ser^{40} phosphorylation. Due to the small size difference between TH1 and TH2, it is not possible to differentiate Ser^{40} phosphorylation between the isoforms, so this data represents total phosphorylation of the two major isoforms calculated as a percentage of total TH.

Levels of Ser^{40} phosphorylation in the Cd and Pu were low, with around 2% of total TH phosphorylated at Ser^{40} (Fig. 8). Levels of Ser^{40} phosphorylation in the regions containing cell bodies were higher, with approximatively 5% of TH in the Sn and 6% of TH in the Vt was phosphorylated at Ser^{40}. Comparison of PD tissues with control tissues demonstrated a significant increase in Ser^{40} phosphorylation in PD tissues for the Cd, Pu and Vt but not the Sn (Fig. 8). The observed increase in Ser^{40} phosphorylation in PD tissue was marked, with a 5-fold increase in Ser^{40} phosphorylation in the Cd, a 6.7-fold increase in the Pu, and a 2.8-fold increase in the Vt.

5. Discussion

We present here the first quantitative analysis of TH isoform distribution and Ser^{40} phosphorylation in the healthy human brain. The number of samples analysed (three control and four PD) in this study were modest but isoform expression and post-translational modification did not vary markedly between cases in each group, thus this sample was sufficient to demonstrate significant differences in the regional distribution of TH isoforms, and differences in levels of phosphorylation at Ser40. Consistent with previous studies that determined the levels of the TH isoform-specific mRNA in post-mortem brain (Coker III et al., 1990; Ichinose et al., 1994), we show that TH1 and TH2 represent the major isoforms in all brain regions studied. However, while levels of TH1 and TH2 are equivalent in the Vt, the TH2 isoform is proportionally dominant in the Sn. This suggests that there is differential regulation of expression of the different isoforms in these two tissues. In contrast, the higher level of expression of the TH2 isoform in the Sn was not reflected in the terminal field regions, the Cd and Pu. The reason for this is unclear, but it may reflect differential transport of the two isoforms from the cell body to the terminal field in the nigrostriatal pathway.

Levels of the two minor isoforms TH3 and TH4 were found at very low levels, accounting for < 1% of the total TH in the two regions containing cell body. However, the proportion of these two isoforms in the Cd and Pu were higher, accounting for 2.5% to 3% of the total TH in these terminal field regions. This might arise from differential transport of these isoforms from the site of synthesis in the cell body to the terminal fields. TH3 and TH4 both contain the 27-amino acid insert and

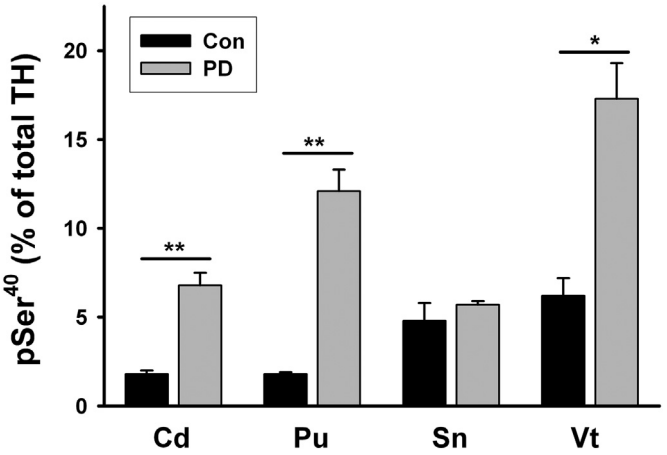

Fig. 8. Phosphorylation at Ser^{40} in control tissues and Parkinson's disease tissues. The levels of phosphorylation of Ser^{40} as a percentage of total TH was determined in four different regions from control (black bars) and PD (grey bars) brains. The brain regions analysed are: caudate, Cd; putamen, Pu; substantia nigra, Sn; and ventral tegmental area, Vt. The data is presented as the percent of total TH phosphorylated at Ser40 (mean ± SEM; PD n = 4, control n = 3). $^{*}p < .05$ and $^{**}p < .01$ for comparison of control vs. PD.

this sequence might play a role in the cellular localisation of these two minor TH isoforms. Such specific localisation suggest differences in the functional roles of these two minor isoforms in the terminal field region. For example, dopamine is stored within vesicles in the terminal region of the neuron prior to release and enrichment of these two isoforms at that site might allow these vesicles to be more readily filled with this neurotransmitter.

We also report here a relative sparing of the TH2 isoform and a relative loss of the TH1 isoform in brains of patients with PD. Our data contrast with a previous study which investigated mRNA (Ichinose et al., 1994), but the relatively large errors associated with the previous analysis may have precluded the detection of the changes seen here at the protein level, or the mRNA level may not necessarily directly reflect the level of the protein. The decrease in the TH1 isoform in PD might arise as a result of differential splicing of TH mRNA in the PD brain, so that less TH1 is synthesized. Alternatively, it is possible that cells that express a higher proportion of the TH1 isoform degenerate earlier in PD, resulting in the selective loss of this isoform. This hypothesis is consistent with the fact that the TH1 isoform is a more readily activated form, as hierarchical phosphorylation occurs in TH1 but not TH2 (Gordon et al., 2009). Thus, in response to a stimulus that increases phosphorylation of Ser^{40} (or its TH2 equivalent), the level of phosphorylation, and therefore activation, will be higher in TH1 than in TH2 (Dickson and Briggs, 2013). Consistent with this, phosphorylation of Ser^{31} is relatively high in rat brain, particularly in the striatal terminal field (Salvatore et al., 2000). In this scenario, cells expressing a greater proportion of TH1 isoform would produce increased levels of dopamine in response to an equivalent stimulus, increasing the potential for dopamine-associated toxicity, as we have previously discussed (Hare and Double, 2016).

The fact that the TH2 isoform is found at higher levels than the TH1 isoform in the Sn under normal conditions, and that there is a relative sparing of the TH2 isoform in PD, suggests that the TH2 isoform may have evolved to protect against overproduction of dopamine. Overproduction of dopamine may not pose a significant risk for animal species with relatively short life expectancies but it may be problematic for species with longer life expectancies, including humans and higher primates who both express the TH2 isoform. In this scenario the presence of the TH2 isoform may reduce the effects of dopamine-associated toxicity over a long lifespan, therefore providing a protective function in the human brain. In PD, the TH2 isoform may therefore decrease the rate of neurodegeneration and may, in part, contribute to the typical age of disease onset being late in life.

Changes in the relative proportions of the two minor isoforms TH3 and TH4 did not reflect that seen for the major isoforms. TH4 was generally unaltered in PD brain, except for a small decrease in the Cd. TH3 was unchanged in the terminal field regions, but was proportionally greater in the two regions containing cell bodies, the Sn and Vt. The differences in the terminal field vs. cell body regions suggest a possible alteration in the transport of TH3, which may be influenced by the fact that changes in the Pu occur after changes in the Sn in PD (Kordower et al., 2013).

Phosphorylation of Ser^{40} in TH is most closely associated with activation of the molecule, as it can dissociate the catecholamine from the high affinity site and activate the enzyme. Therefore, Ser^{40} phosphorylation levels are indicative of the activation state of the enzyme. The proportion of TH phosphorylated at Ser^{40} in the control brain was low and similar to that in the rat in all regions studied (Cd and Pu, 1.8% in human vs. 2% reported in rat striatum (Salvatore et al., 2000; Salvatore et al., 2009); Sn, 5% in human vs. 2%–4% in rat (Salvatore et al., 2000; Salvatore et al., 2009); Vt, 6% in human vs. 3%–5% in rat (Ong et al., 2014; Salvatore et al., 2000; Salvatore et al., 2009)).

In PD, Ser^{40} phosphorylation was increased in the Cd and Pu but unchanged in the Sn. Interestingly, there was evidence for increased phosphorylation of TH in the Vt, although the function of this is unclear. Our observation of increased phosphorylation in the Cd and Pu in PD is consistent with increased TH-specific activity reported in the Cd in PD patients (Mogi et al., 1988). Increased activation of TH in the PD brain is consistent with the long-held view that increased dopamine production within the nigrostriatal pathway represents an early compensatory response to the disease process that may delay the onset of clinical features in patients (Bernheimer et al., 1973, Ugrumov et al., 2011, Zigmond et al., 1990). While increased production of dopamine in each surviving neuron may delay the onset of clinical symptoms, this compensatory response may ultimately induce dopamine-associated toxicity within these neurons and exacerbate the degenerative process.

In summary, we have shown that there is selective sparing of hTH2, one of the two major isoforms of TH, in PD and that this isoform is characterised by a reduced potential for activation, compared to the other major isoform TH1. We have also demonstrated substantial increases in TH Ser^{40} phosphorylation, and therefore activation of TH, in both the Cd and Pu in PD brain, consistent with the hypothesis that a compensatory increase in dopamine production occurs within surviving nigrostriatal neurons in this disorder.

Ethics approval

All experiments were approved by the Human Ethics Committee of the University of Newcastle (H-158-1205) and the University of New South Wales Biomedical Human Research Ethics Advisory Panel (HC12102).

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no financial or non-financial competing interests.

Author contributions

J.S., K.E.M., L.B. and S.R. performed the experiments. J.S., K.L.D., P.R.D., G.M.H. and P.W.D. designed the experiments. J.S and P.W.D. wrote the manuscript. All co-authors have read the manuscript and agree with its content. Authors have read and approved the final

manuscript.

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Data availability

The data that support the findings of the study are available from the corresponding author upon reasonable request.

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Appendix A. Supplementary data

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