Supplementary Information

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Supplementary Methods

Sample Preparation

For both brain banks one cerebral hemisphere had been fixed for approximately 3 weeks in buffered formalin for paraffin histology, the other sliced coronally at approximately 1-cm intervals and the slices frozen at -80°C. Frozen tissue was dissected from the ventral and dorsal BA18 and BA19 for each individual. Paraffin sections of occipital cortex from blocks including both BA18 and BA19 were cut at 7-µM thickness.

Homogenate Preparation

Fresh frozen tissue was dissected from ventral BA18 and BA19 and dorsal BA18 and BA19. To produce the SDS homogenates used for most of the assays in this project, 200 mg of tissue was homogenised in 1 ml of chilled (at 4°C) 1% SDS lysis buffer in a Precellys tissue homogeniser (Bertin Instruments, Labtech International, UK) for 2 x 15 s at 6000 g, with 6-10 zirconia beads in a 2-ml homogenate tube.

The homogenates were then centrifuged for 15 min at 13,000 g at 4°C. The supernatant was aliquoted into non-binding 96-well storage plates (Thermo Scientific) and kept frozen at -80°C until required.

Choline acetyltransferase (ChAT) homogenates were prepared using a method adapted from Peng et al.(32) 50-100 mg of fresh frozen tissue was placed in a 2-ml homogenate tube with 6-10 zirconia beads. The volume of all buffers in µl added was calculated as 15 x the tissue weight in mg. Buffer A was 50 mM potassium phosphate, 2 mM EDTA, pH7.4. Buffer B was 50 mM potassium phosphate, 2 mM EDTA, 500 mM NaCl, pH7.4. Buffer C was 50 mM potassium phosphate, 2 mM EDTA, 0.6% triton X-100, pH7.4. After the addition of each buffer the tissue was homogenised for 2 x 15 s at 6000 g in a Precellys tissue homogeniser prior to centrifugation at 4°C for 30 min at 28,000 x g. Buffers A, B and C were added sequentially with the supernatant being removed after each homogenisation, prior to the addition of the next buffer. The fractions extracted were therefore soluble ChAT (buffer A), ionic membrane-bound ChAT (buffer B) and membrane-bound ChAT (buffer C). The total ChAT homogenates used in the assays described below were prepared by adding equal volumes of each supernatant. As no protease inhibitors were used in the sample preparation, the whole operation was carried out with the samples either on ice or at 4°C. The samples were aliquoted and frozen at -80°C until required. The reason for omitting protease inhibitors cocktail in the buffers is that one or more of these inhibitors also irreversibly inhibit the cholinergic enzymes and thereby would interfere with the downstream enzyme activity assessments.(33)

The soluble and insoluble extracts used for the α-synuclein assays were prepared in a third buffer. 200 mg of tissue was homogenised in 1 ml of chilled TBS extraction buffer with 1% NP-40 (pH7.4) in a Precellys tissue homogeniser (2 x 15 s at 6000 g) with 6-10 zirconia beads in a 2 ml homogenate tube. The homogenates (the soluble or membrane bound fraction) were then centrifuged for 15 min at 13,000 g at 4°C. The supernatants were removed and frozen at -80°C until required. The pellet was re-

suspended in 400 µl of guanidine HCl buffer (3 parts guanidine HCl to 1 part 50 mM Tris, pH8), homogenised again in a Precellys tissue homogeniser (2 x 15 s at 6000 g) and then spun for 15 min at 13,000 g at 4°C. The supernatant was removed, aliquoted into non-binding 96 well storage plates (Thermo Scientific) and frozen at -80°C until required. Total protein was measured for all samples with a Coomassie Protein Plus kit (Thermo Scientific).

vWF Dot Blot

This was performed as previously described.(34) Prior to assembling the dot blot manifold the membrane was soaked in TBS for at least 10 min. Samples were centrifuged again at 13,000g for 15 min at 4°C to ensure that no particulate matter remained. They were then diluted 1:400 in TBS and 100 µl of each sample was loaded in duplicate onto a nitrocellulose membrane in a dot blot vacuum manifold. In addition, seven serial dilutions of a reference homogenate (starting at 1:100) were loaded in duplicate for each blot. Blanks consisted of 100 µl TBS. After 75 min the blots were removed from the manifold and washed for 3 x 10 min in TTBS before being blocked at room temperature in 5% milk/TBS for 1 h with agitation. After a further 3 x 10 min washes in TTBS the primary antibody (Dako A0082) diluted 1:3000 in 5% milk/TTBS was added and the membranes were incubated overnight with agitation at 4°C. Following 3 x 30-min washes the membrane was incubated with goat anti-rabbit antibody (Vector) diluted 1:5000 in 5% milk/TTBS for 1 h with agitation at room temperature. After a further 3 x 30 min washes 6 ml of substrate was added to each membrane and the membrane imaged in a BioRad imager (chemiluminescence protocol) after 4 min. All samples were assayed in duplicate on each membrane and on two different membranes.

Immunohistochemistry for α-Synuclein

FFPE sections were de-waxed in clearene (2 x 5 min) and then dehydrated in 100% alcohol (2 x 3 min) prior to incubation in 3% hydrogen peroxide in methanol for 45 min. Following this, the sections were washed in running water for 10 min before being placed in formic acid for 3 min. They were then

microwaved in EDTA, pH8, for 10 min and washed in running water again for 10 min. The primary antibody (Vector VP-A106) diluted 1:800 was applied at room temperature overnight. The next day sections were drained and blocking serum (Vector labs elite ABC kit) was added for 20 min. After 2 x 3 min washes in PBS ABC solution (Vector labs elite ABC kit) was added for 20 min, and the sections were washed for 2 x 3 min in PBS. DAB (Vector labs) was added for 10 min, after which sections were washed in running water for 10 min. DAB staining was enhanced by incubation in copper sulphate solution for 4 min prior to a further 5-min wash in running water. The sections were then placed in Gill II Haematoxylin for 20 sec then washed for 10 min in running water. Finally the sections were cleared by 2 x 2 min immersion in 100 % alcohol and 2 x 5 min in clearene prior to their being mounted in clearium.

Supplementary Tables & Figures

Table S1: Results of the ELISAs performed in this study

	Controls n=32		PD no VH n=11		PD + VH n=26		Statistical Evidence
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
AChE dorsal BA18 ng/mg	28.305	30.764	49.968	14.66	57.603	14.625	Kwallis X^2=15.744, p<0.001
AChE ventral BA18 ng/mg	1.530	0.708	1.493	0.262	1.181	0.208	Kwallis X^2=7.565, p=0.022
AChE dorsal BA19 ng/mg	31.964	41.587	46.633	10.29	54.6	16.996	Kwallis X^2=16.946, p<0.001
AChE ventral BA19 ng/mg	33.542	35.108	56.134	19.637	59.451	17.324	Kwallis X^2=10.472, p=0.005
BChE dorsal BA18 ng/mg	5.014	1.648	4.059	0.977	4.478	1.311	ANOVA p=0.299
BChE ventral BA18 ng/mg	6.075	3.038	9.04	1.731	7.758	1.927	Kwallis X^2=10.509, p=0.005
BChE dorsal BA19 ng/mg	4.859	1.740	3.807	1.178	4.311	1.186	Kwallis X^2=4.436, p=0.109
BChE ventral BA19 ng/mg	36.856	35.473	59.57	19.99	63.201	17.32	Kwallis X^2=10.219, p=0.006
MAG:PLP ratio dorsal BA18	1.31	0.211	1.22	0.326	1.371	0.297	ANOVA p=0.260
MAG:PLP ratio ventral BA18	18.645	9.611	24.211	9.498	29.396	14.748	Kwallis X^2=14.674, p<0.001
MAG:PLP ratio dorsal BA19	1.818	0.883	2.15	0.591	2.583	0.632	Kwallis X^2=12.921, p=0.002
MAG:PLP ratio ventral BA19	23	15.141	14.669	5.358	16.768	3.442	Kwallis X^2=20.216, p<0.001
VEGF dorsal BA18 ng/mg	0.12	0.044	0.111	0.028	0.175	0.211	ANOVA p=0.608
VEGF ventral BA18 ng/mg	0.153	0.154	0.109	0.126	0.157	0.148	Kwallis X^2=2.033, p=0.362
VEGF dorsal BA19 ng/mg	0.521	1.788	0.826	1.553	0.262	0.477	Kwallis X^2=2.373, p=0.305
VEGF ventral BA19 ng/mg	0.233	0.193	0.219	0.211	0.205	0.196	Kwallis X^2=0.928, p=0.629
von Willebrand factor dorsal BA18 arbitrary units/mg	1.289	0.426	1.282	0.378	1.614	1.857	Kwallis X^2=0.027, p=0.987
von Willebrand factor ventral BA18 arbitrary units/mg	1.589	0.698	1.147	0.467	1.077	0.478	Kwallis X^2=10.397, p=0.006
von Willebrand factor dorsal BA19 arbitrary units/mg	6.41	22.799	13.811	27.551	2.911	5.01	Kwallis X^2=0.986, p=0.611
von Willebrand factor ventral BA19 arbitrary units/mg	2.091	1.536	1.355	0.567	1.242	1.007	Kwallis X^2=12.158, p=0.002

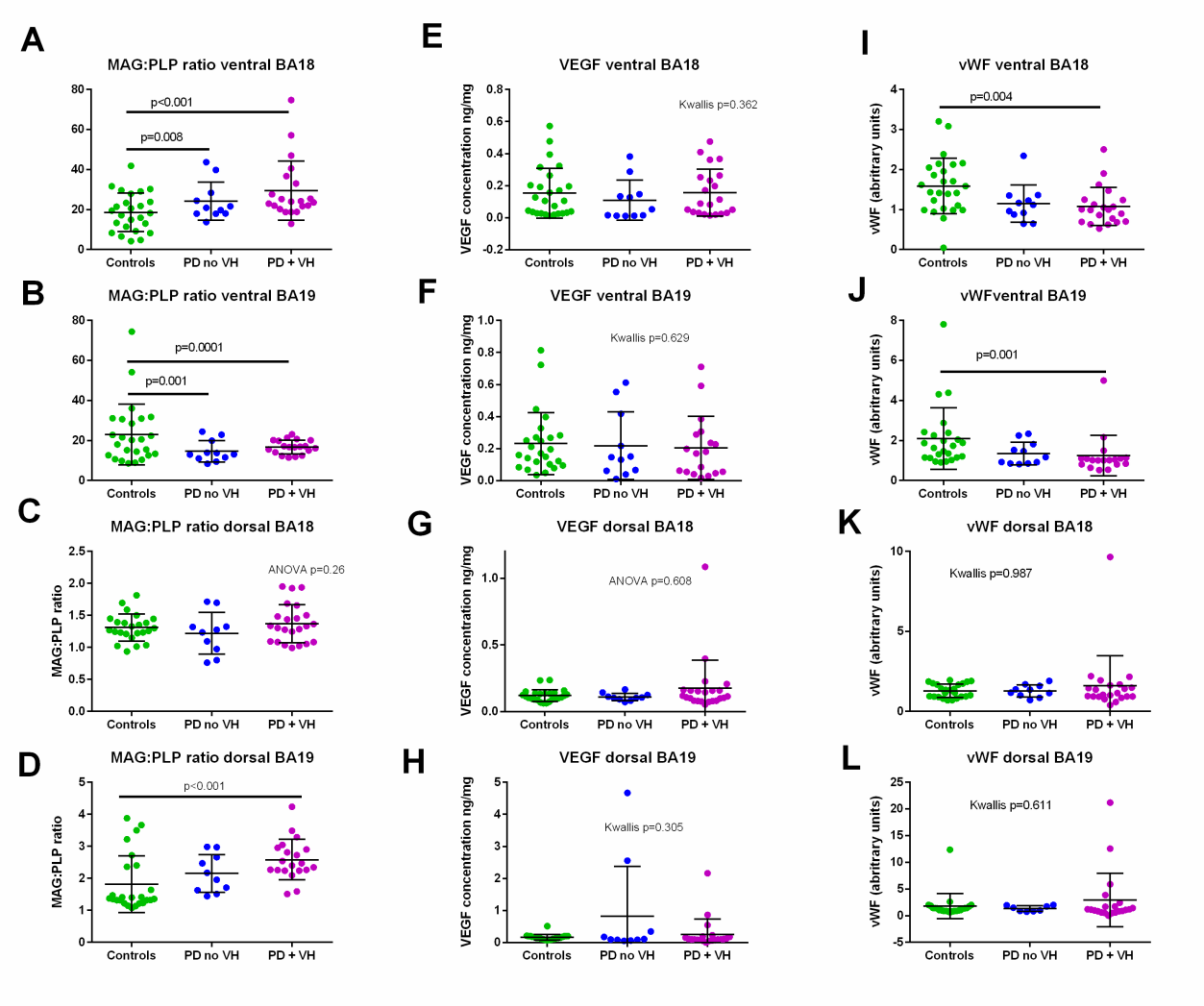

Figure S1: Biochemical markers of chronic hypoperfusion in ventral BA18 & 19.

Perfusion markers were increased in ventral BA18 in the PD + VH group, as shown by the increased MAG:PLP1 ratio (A). Conversely MAG:PLP1 was reduced in the ventral BA19 in both PD groups (B). There was a more consistent direction of effect on vWF (I and J). There was no between-group difference in VEGF in any of the brain areas (E -H).There was no evidence of a between-group difference in any of the proteins measured in dorsal BA18 (C,G,K). MAG:PLP1 was increased in the PD+VH group in dorsal BA19 (D) but this was not associated with changes in either VEGF (E) or vWF (F).

	Controls n=32		PD no VH n=11		PD + VH n=26		Statistical Evidence
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
Insoluble α-synuclein dorsal BA18 ng/mg	1.026	0.59	2.186	1.474	1.955	2.244	Kwallis X^2=10.502, p=0.024
Insoluble α-synuclein ventral BA18 ng/mg	2.116	0.907	3.314	0.38	3.203	0.829	Kwallis X^2=21.162, p<0.001
Insoluble α-synuclein dorsal BA19 ng/mg	0.666	0.253	1.09	0.178	1.06	0.218	Kwallis X^2=17.445, p<0.001
Insoluble α-synuclein ventral BA19 ng/mg	0.393	0.240	0.687	0.263	0.601	0.279	ANOVA p=0.006
Average Lewy body count per field (dorsal)	0.413	0.505	0.829	0.715	0.610	0.591	Kwallis X^2=3.431, p=0.180

Table S2: Alpha synuclein concentrations in ventral and dorsal BA18 &19.

As expected, insoluble α-synuclein was higher in the PD groups in all brain areas tested.

	Controls n=32		PD no VH n=11		PD + VH n=26		Statistical Evidence
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
ChAT concentration dorsal BA18 (arbitrary units/mg)	0.575	0.137	0.612	0.075	0.756	0.188	ANOVA p=0.001
ChAT concentration dorsal BA19 (arbitrary units/mg)	0.978	0.194	0.687	0.283	0.839	0.163	ANOVA p=0.103
ChAT activity ventral BA18 (nmol/min/ml/mg)	401.9	169.0	324.7	114.6	279.3	155.7	Kwallis X^2=7.132 p=0.028
ChAT activity ventral BA19 (nmol/min/ml/mg)	324.0	403.7	91.9	93.1	112.1	134.9	Kwallis X^2=5.343 p=0.069
ChAT activity dorsal BA18 (nmol/min/ml/mg)	173.4	64.1	159.1	51.1	168.8	35.6	Kwallis X^2=0.274 p=0.872
ChAT activity dorsal BA19 (nmol/min/ml/mg)	158.5	73.2	135.2	80.1	235.0	339.1	Kwallis X^2=0.665 p=0.717

Table S3: Results of the ChAT activity and concentration assays for this study.

ChAT concentration was elevated in the PD+VH group in dorsal BA18 and ChAT activity was reduced in the PD+VH group in ventral BA18. A similar but non-significant trend was seen in ventral BA19. There was no evidence of a between-group difference in dorsal ChAT activity.

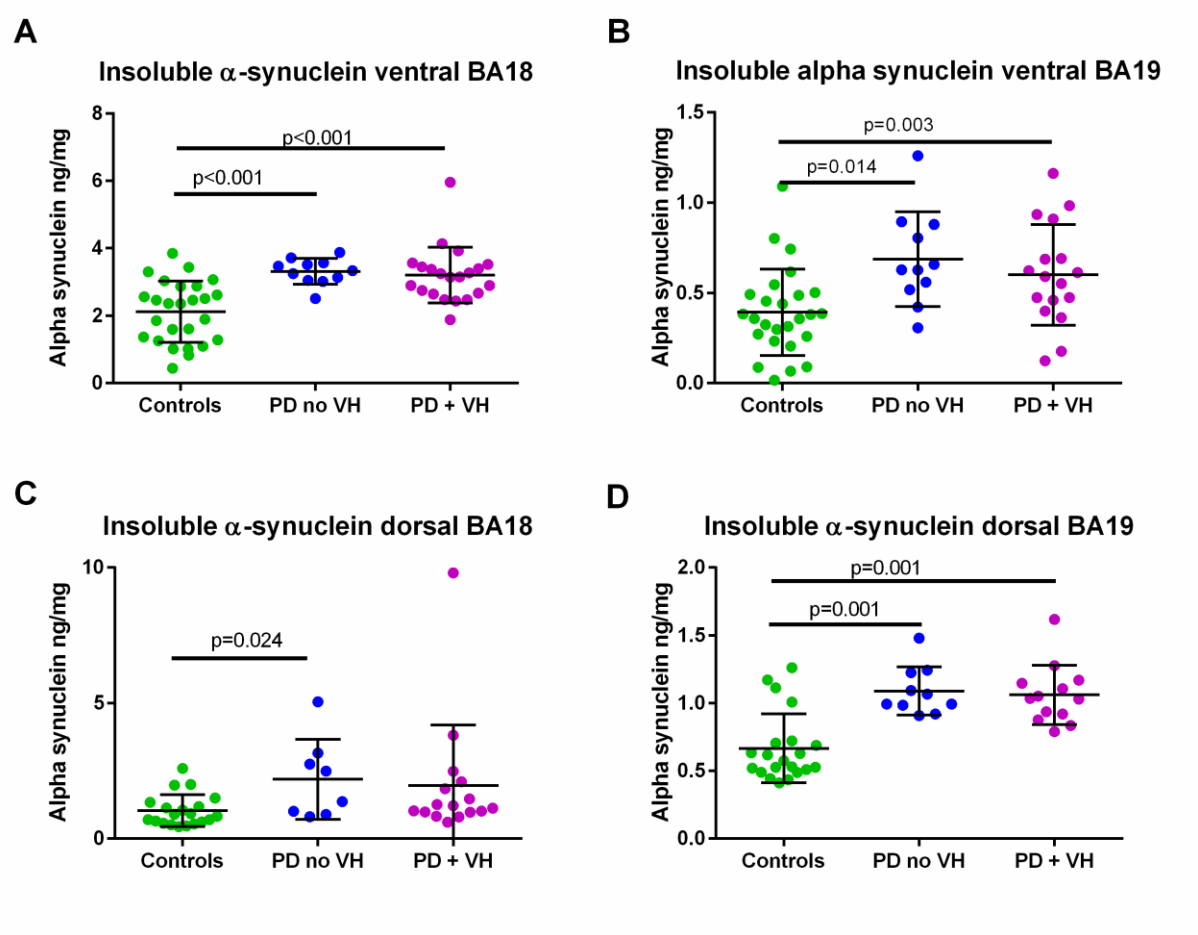

Figure S2: Insoluble alpha synuclein as measured by ELISA.

As expected insoluble, α-synuclein was increased in both PD groups in all 4 brain areas under investigation (A=D). There was no evidence of a difference between the PD-VH and PD+VH groups.

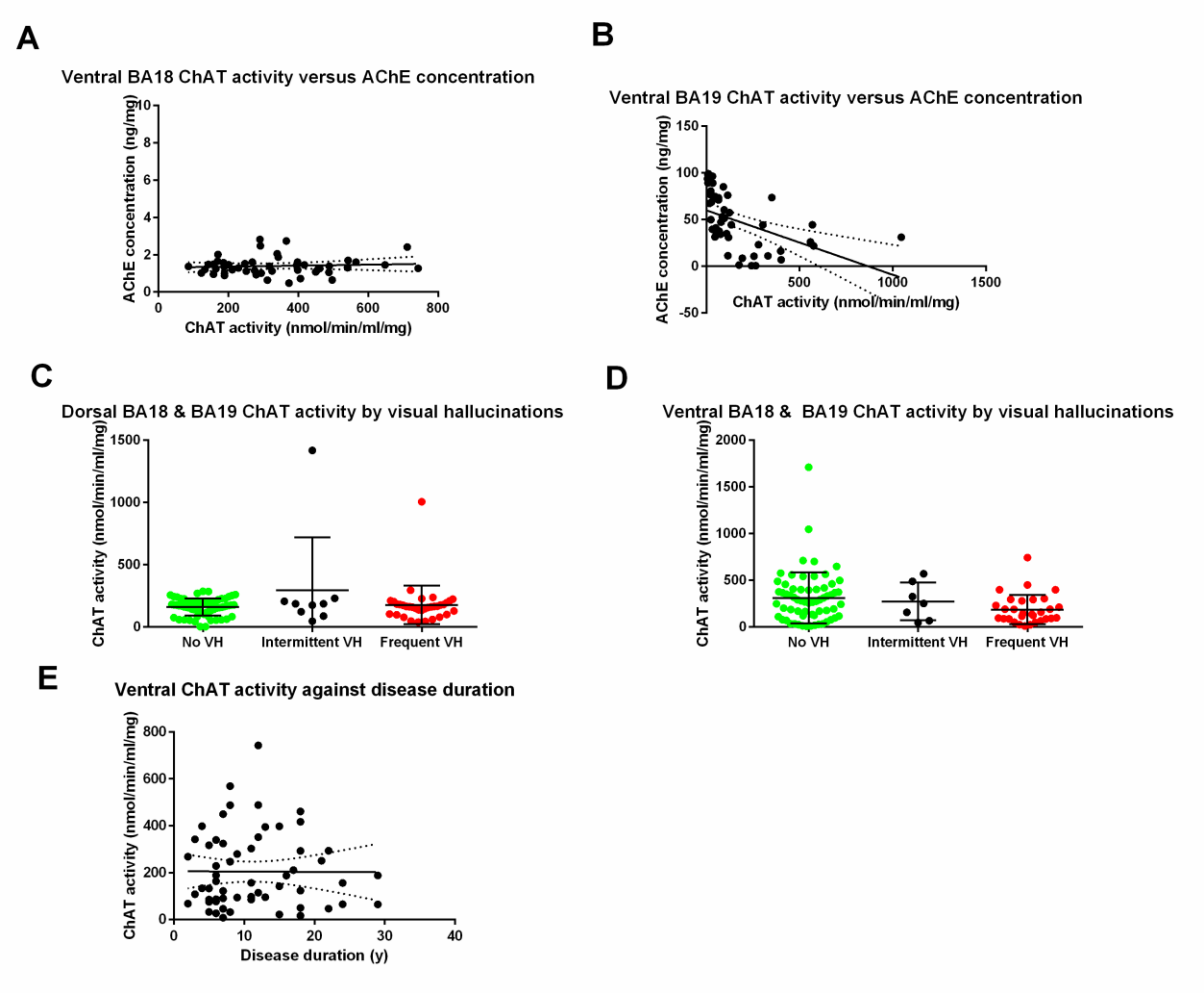

Figure S3: ChAT activity and AChE concentration.

There was no evidence of a relationship between ventral BA18 ChAT activity and AChE concentration (A) but ChAT activity declined in ventral BA19 (B) with increasing AChE concentration. There was no evidence that ventral ChAT activity altered with disease duration.

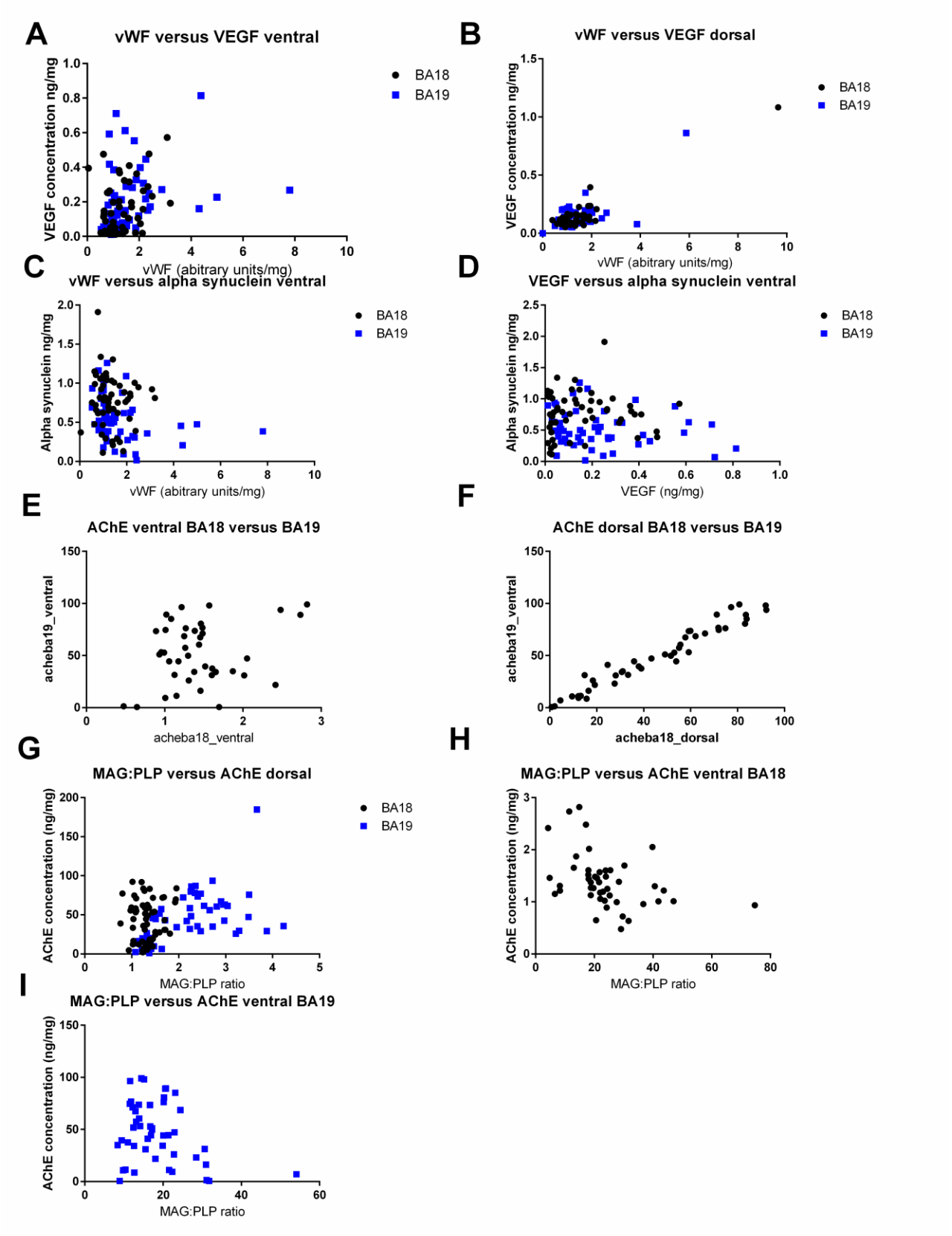

Figure S4: vWF correlated with VEGF in both ventral and dorsal brain areas.

Spearmans's rho for ventral brain areas (A) = 0.27, p=0.042 and for dorsal brain areas (B) Spearman's rho = 0.39, p=0.004. There appeared to be a negative correlation between vWF and α-synuclein (C) although the evidence for this was stronger in ventral BA19 (Spearman's rho = -0.40, p=0.003) than

in ventral BA18 (Spearman's rho = -0.15, p=0.25). Likewise there appeared to be a stronger negative correlation between VEGF and α-synuclein (D) in ventral BA19 (Spearman's rho = -0.22, p=0.011) than in ventral BA18 (Spearman's rho = -0.07, p=0.59). There was a good correlation between AChE in the two dorsal brain areas (F) but not in the two ventral brain areas (E, Spearman's rho = 0.11, p=0.49). There was a clear negative correlation between the MAG:PLP1 ratio and AChE concentration in ventral BA18 (H, Spearman's rho = -0.50, p=0.0003) but not in ventral BA19 (I, Spearman's rho = -0.17, p=0.24). There was a positive correlation (G) between AChE and the MAG:PLP ratio in dorsal BA19 (Spearman's rho = 0.65, p<0.001) but not in dorsal BA18, where there was no clear relationship (Spearman's rho = 0.012, p=0.93)