TITLE: α-Synuclein pathology in Parkinson disease activates homeostatic NRF2 anti-oxidant response

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S2. Curated gene expression analyses of publicly available microarray datasets in PD using Gene Expression Omnibus (GEO). **(A-D)** Anti-oxidants *GSR1* (Glutathione-Disulfide Reductase, in A), *PARK7* (Parkinsonism Associated Deglycase- DJ1, in B), *NQO1* (NAD(P)H Quinone Dehydrogenase 1, in C) and *TXN* (Thioredoxin, in D). **(E-F)** Inflammatory mediators, *TNFA* (Tumor Necrosis Factor alpha, in C) and *IL1A* (Interleukin 1, in D). The values across the datasets are expressed relative to the controls in each microarray dataset, i.e., mean value of control samples=1 (a.u., arbitrary units). Error bars represent standard deviation of the mean, s.d. In GSE7621- SN (*substantia nigra*); controls (Ctrl, n=9) and PD (Parkinson Disease cases, n=16); in GSE43490- SN (*substantia nigra*; controls, n=6 and PD, n=8), DMX (*dorsal motor nucleus of vagus*; controls, n=5 and PD, n=8), LC (*locus coeruleus*; controls, n=7 and PD, n=8); in GSE20146- GPi (*globus pallidus interna*; controls, n=10 and PD, n=10). Pair-wise comparisons were assessed by Mann-Whitney test- only significant differences (*=p≤0.05, **=p≤0.01) are highlighted. Probe IDs, microarray platforms and relevant studies are listed in Table S2. Also see Figure 2.

S3. Curated gene expression analyses of a publicly available microarray dataset (GSE26927) in selected neurodegenerative diseases using Gene Expression Omnibus (GEO). (A) *NFE2L2* (Nuclear Factor, Erythroid 2 Like 2, NRF2)**, (B)** *KEAP1* (Kelch Like ECH Associated Protein 1, NRF2 inhibitor protein), **(C-F)** Anti-oxidants *GSR1* (Glutathione-Disulfide Reductase, in C), *GCLC* (Glutamate-Cysteine Ligase Catalytic Subunit, in D), *TXN* (Thioredoxin, in E) and *PARK7* (Parkinsonism Associated Deglycase- DJ1, in F). Disease conditions include: PD (Parkinson Disease), AD (Alzheimer Disease), HD (Huntington Disease), ALS (Amyotrophic Lateral Sclerosis), MS (Multiple Sclerosis). Samples include: *substantia nigra* (controls n=7, PD n=12), *entorhinal cortex* (controls n=6, AD n=12), *cervical spinal cord* (controls n=7, ALS n=9), *nucleus caudatus* (controls n=10, HD n=10), and *grey matter lesions in frontal gyri* (controls n=10, MS n=10). Error bars represent standard deviation of the mean, s.d. Pair-wise comparisons were assessed by Mann-Whitney test- only significant differences (*=p≤0.05, **=p≤0.01, ***=p≤0.001) are highlighted. Probe IDs, microarray platform and relevant study are listed in Table S2.

S4. Immunostaining of phospho-aSyn (S129) in the brain regions of PBS injected M83$^{+/+}$ mice. (A) Representative IHC images showing phospho-aSyn (S129) immunostaining (10X low magnification views and 40X magnified views in the insets; scale bar=200 µm; Aq. in the image showing PAG, cerebral aqueduct; 4v. in the image showing vestibular nuclei image, 4th ventricle). Bregma co-ordinates for the brain regions were determined according to Paxinos and Franklin: (Bregma, -4.03 mm) midbrain at the level of superior colliculi showing PAG, red nucleus and tegmentum; (Bregma, -5.91) pontocerebellar junction showing cerebellar nuclei, cerebellar lobules (*cb1-5*), vestibular nuclei and pontine gigantocellualr nuclei (Gi); and (Bregma, 0.49 mm) forebrain showing motor cortex (M1 and M2) and corpus striatum). Also see Figure 3 (p-aSyn, S129 in brain regions of PFF aSyn injected M83$^{+/+}$ mice). Primary antibody in A: p-aSyn (S129)- 11A5.

S5. Immunostaining of phospho-NRF2 (S40) in the brain regions of PBS injected M83$^{+/+}$ mice. (A) Representative IHC images showing phospho-NRF2 (S40) immunostaining. Notice variable degrees of staining, prominently cytoplasmic, detected in red nucleus, midbrain tegmentum, motor cortex and corpus striatum (10X low magnification views and 40X magnified views in the insets; scale bar=200 µm; Aq. in the image showing PAG, cerebral aqueduct; 4v. in the image showing vestibular nuclei image, 4th ventricle). Bregma co-ordinates for the brain regions were determined, according to Paxinos and Franklin: (Bregma, -3.79 mm) midbrain at the level of superior colliculi showing PAG, red nucleus and tegmentum; (Bregma, -5.79) pontocerebellar junction showing cerebellar nuclei, cerebellar lobules (*cb1-5*), vestibular nuclei and pontine gigantocellualr nuclei (Gi); and (Bregma, 0.73 mm) forebrain showing motor cortex (M1 and M2) and corpus striatum. Also see Figure 4 (p-NRF2, S40 in brain regions of PFF aSyn injected M83$^{+/+}$ mice). Primary antibody in A: p-NRF2 (S40)- PA5-67520.

S6. Immunostaining of phospho-aSyn (S129) and phospho-NRF2 (S40) in select brain regions of M83$^{+/+}$ mice. (A-B) Representative IHC 40X magnified views showing phospho-aSyn (S129) in (A) and phospho-NRF2 (S40) immunostaining in (B), in the select brain regions of three additional animals from PFF injected cohort. Primary antibodies: p-aSyn (S129)- 11A5 and p-NRF2 (S40)- PA5-67520. **(C)** Phospho-NRF2 (S40) immunostaining in select brain regions of three additional animals from the PBS injected cohort. The animals in each cohort are designated #1, #2 and #3. Regions examined include: periaqueductal grey (PAG), red nucleus (RN), mesencephalic tegmentum (Teg.), pontine gigantocellular nuclei (Gi), pontine vestibular nuclei (VN) and deep cerebellar nuclei (DCN). Primary antibody in C: p-NRF2 (S40)- PA5-67520.   Scale bar in A-C=25 µm. Also see Fig. 3-4 and S4-5.

S7. Quantitative RT-PCR analyses of anti-oxidant gene response in the brain regions of PBS or PFF aSyn injected M83$^{+/+}$ mice. (A) *Nfe2l2* (Nuclear Factor, Erythroid 2 Like 2, NRF2), and **(B-D)** NRF2 anti-oxidant response mediators, *Txn* (Thioredoxin, in B), *Nqo1* (NAD(P)H Quinone
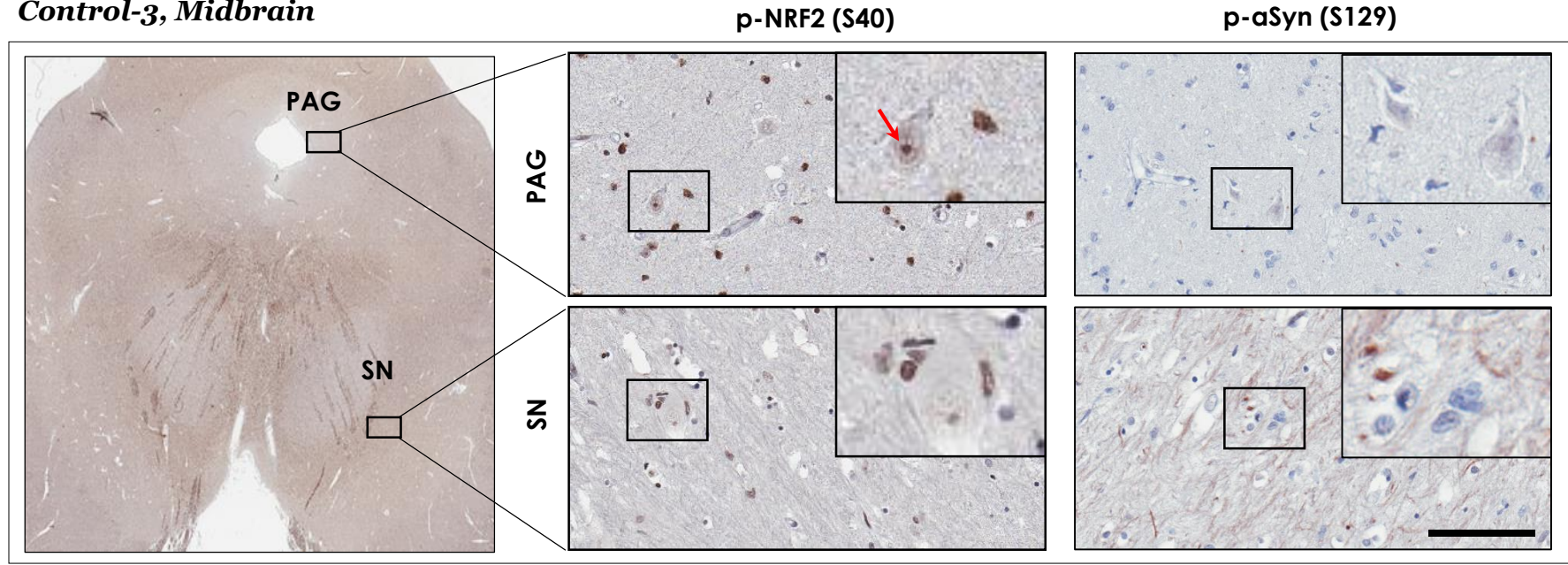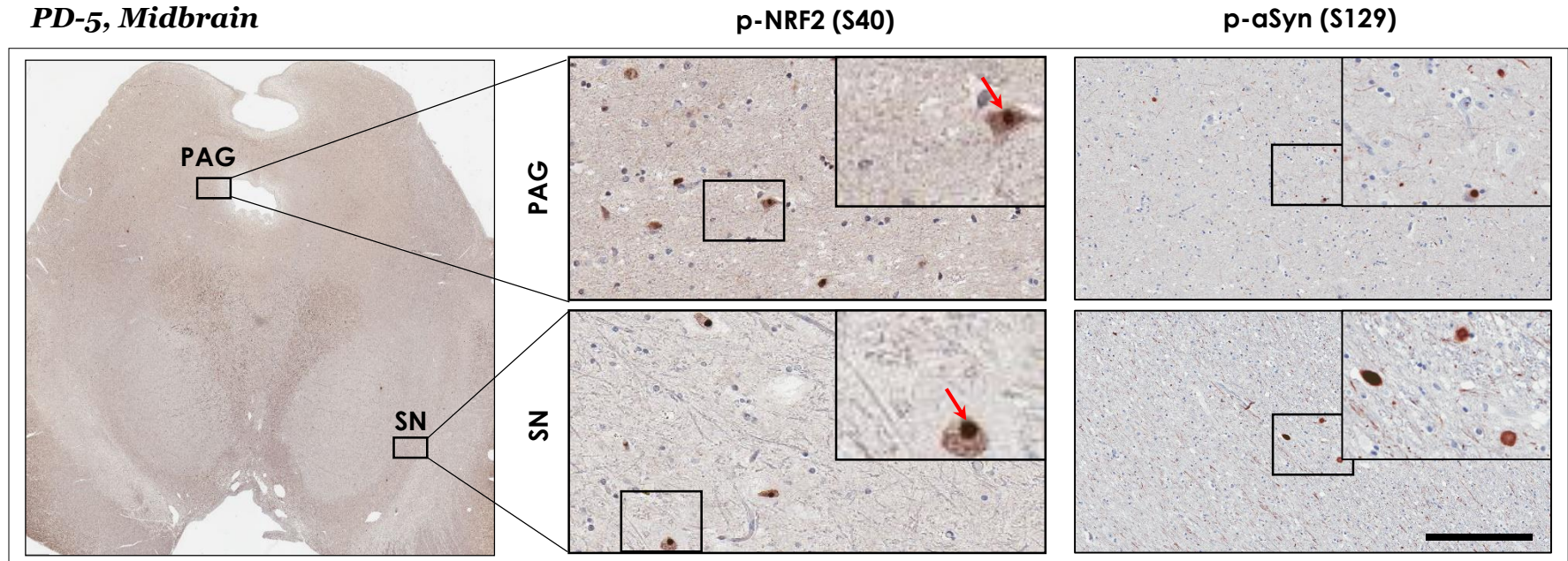
Dehydrogenase 1, in C) and *Park7* (Parkinsonism Associated Deglycase- DJ1, in D). Relative mRNA quantitation at terminal stage in the following brain regions: frontal cortex (Ctx), thalamus (Thal), corpus striatum (Str), hippocampus (Hip), midbrain (Mb) pons, medulla (Med) and cerebellum (Crb). Mouse *Gapdh* was used as a reference gene (ΔΔCT method: n=6/group, samples were run in duplicates; Error bars represent standard error of the mean, s.e.m.; Pair-wise comparisons were assessed by Mann-Whitney test- only significant differences (*=p≤0.05) are highlighted (ns= not significant); Multiple columns comparisons in One way ANOVA *post-hoc* Bonferroni test, were not significant). Also see Figure 5.

S8. Quantitative RT-PCR analyses of inflammatory gene response in the brain regions of PBS or PFF aSyn injected M83$^{+/+}$ mice. (A-B) Inflammatory mediators, *Tnfa* (Tumor Necrosis Factor alpha, in A) and *Il1a* (Interleukin 1, in B). **(C-D)** Pro-apoptotic caspases**,** *Casp3* (Caspase 3, in C) and *Casp6* (Caspase 6, in D). Relative mRNA quantitation at terminal stage in the following brain regions: frontal cortex (Ctx), thalamus (Thal), corpus striatum (Str), hippocampus (Hip), midbrain (Mb) pons, medulla (Med) and cerebellum (Crb). Mouse *Gapdh* was used as a reference gene (ΔΔCT method: n=6/group, samples were run in duplicates; Error bars represent standard error of the mean, s.e.m.; Pair-wise comparisons were assessed by Mann-Whitney test- only significant differences (*=p≤0.05) are highlighted (ns= not significant); Multiple columns comparisons in One way ANOVA *post-hoc* Bonferroni test, were not significant).
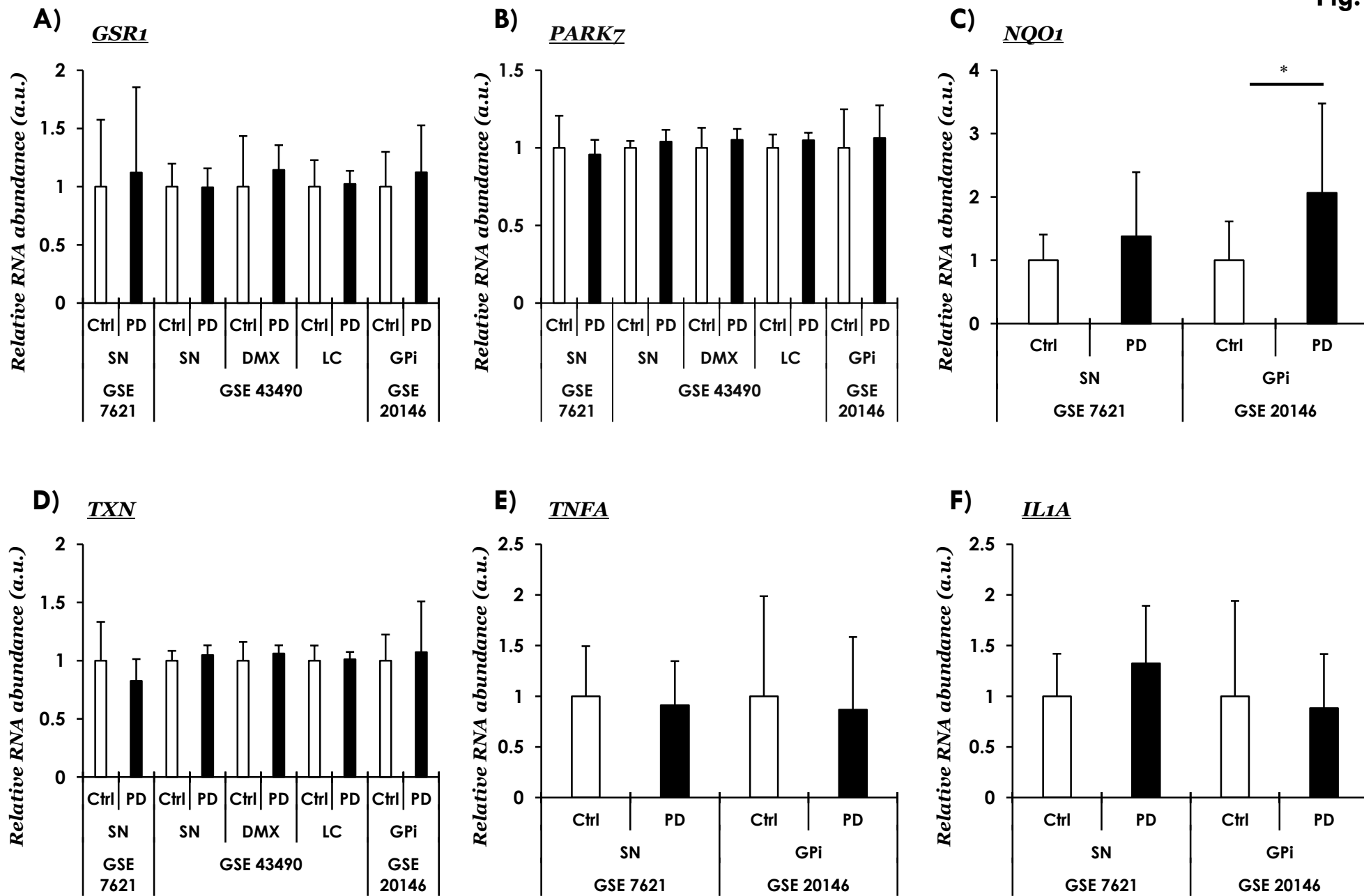
S9. Effects of transient aSyn overexpression on NRF2 anti-oxidant response in cultured N2A cells. (A-B) Western immunoblotting showing the expression of aSyn (WT-wild type and mutant A53T aSyn and Nrf2 (in A) and corresponding densitometry analyses (in B). Antibodies used in A: aSyn (BD Biosciences, clone 42 #610787) and Nrf2 (Novus Biological #NBP1-32822). Expression values in S9B were normalized using β- actin as the loading control. The error bars indicate Mean ± S.D. (n=6 biological replicates/group from 3 independent experiments). **(C)** ROS measurements by H_2-DCFDA assay in mock transfected cells or cells expressing aSyn (WT and A53T), following treatment with PBS or exposure to exogenous H_2O_2 (250 μM; 2 hours). Fluorescence values were normalized to the control cells (mock transfected, PBS treated; Mean expressed as 100%). The error bars indicate Mean ± s.e.m. (n=9-12 biological replicates/group; Pair-wise comparisons, t-test, **=0.01, ***=0.005) from 3-4 independent experiments). **(D)** Nrf2 ARE promoter activity in mock transfected cells or cells expressing aSyn (WT and A53T), following treatment with PBS or exposure to exogenous H_2O_2 (250 μM; 2 hours). Luminescence values were normalized to the control cells (mock transfected, PBS treated; Mean expressed as 1.0; a.u.=arbitrary units). The error bars indicate Mean ± s.e.m. (n=8 biological replicates/group; Pair-wise comparisons, t-test, ***=0.005 from 3 independent experiments). **(E)** Relative (ΔΔCT) expression of cytoprotective anti-oxidant genes in mock transfected cells or cells expressing aSyn (WT and A53T): *Nfe2l2, Keap1, Gclc, Gsr1 and Hmox1*. The data are representative of 3 independent experiments. The error bars
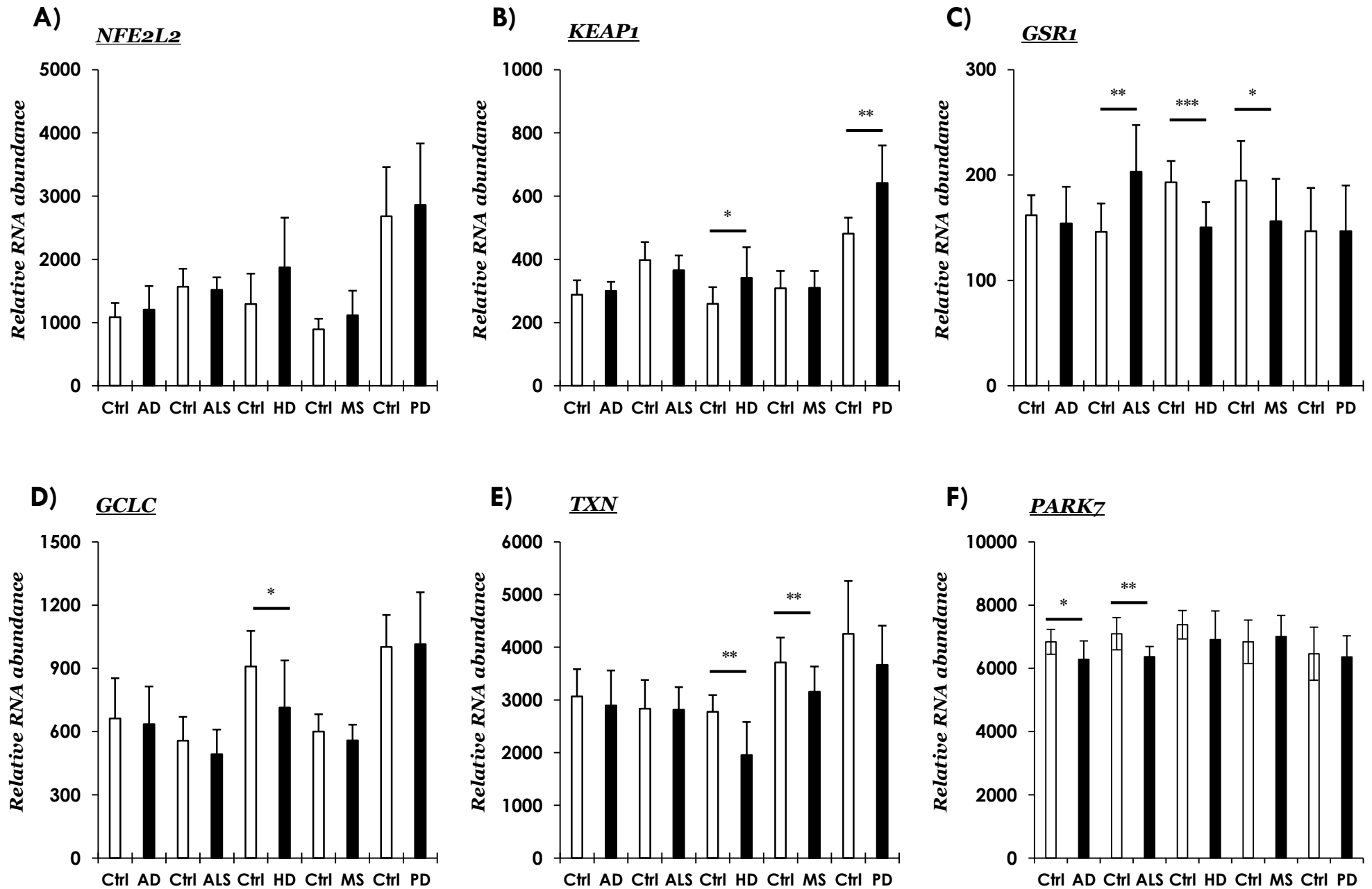
indicate Mean ± S.D. (n=2 biological replicates/group, run in duplicates in each experiment; Pair-wise comparisons in t-test were not significant). **(F-G)** Immunofluorescence (IF) microscopy analyses of Nrf2 (antibody, NBP1-32822) in mock transfected cells or cells expressing aSyn (WT and A53T), following treatment with PBS (in F) or exposure to exogenous H_2O_2 (in G; H_2O_2 treatment: 250 µM for 2 hours). DRAQ5 was used as a nuclear marker. Yellow arrows indicate select instances of Nrf2 IF detection in the cell nucleus (scale bar= 50 µm).

A) *Control-3, Midbrain*

B) *PD-5, Midbrain*

Fig. S2

A) *GSR1*

B) *PARK7*

C) *NQO1*

D) *TXN*

E) *TNFA*

F) *IL1A*

A) *NFE2L2*

B) *KEAP1*

C) *GSR1*

D) *GCLC*

E) *TXN*

F) *PARK7*

A) *PBS injected*   p-aSyn (S129)   Fig. S4

Periaqueductal grey (PAG)   *Red nucleus*   *Midbrain Tegmentum*

Aq.

Gigantocellular nuclei   *Vestibular nuclei*   *Cerebellar nuclei*

4v.

Cerebellar lobules   *Frontal Cortex (M1-M2)*   *Corpus Striatum*
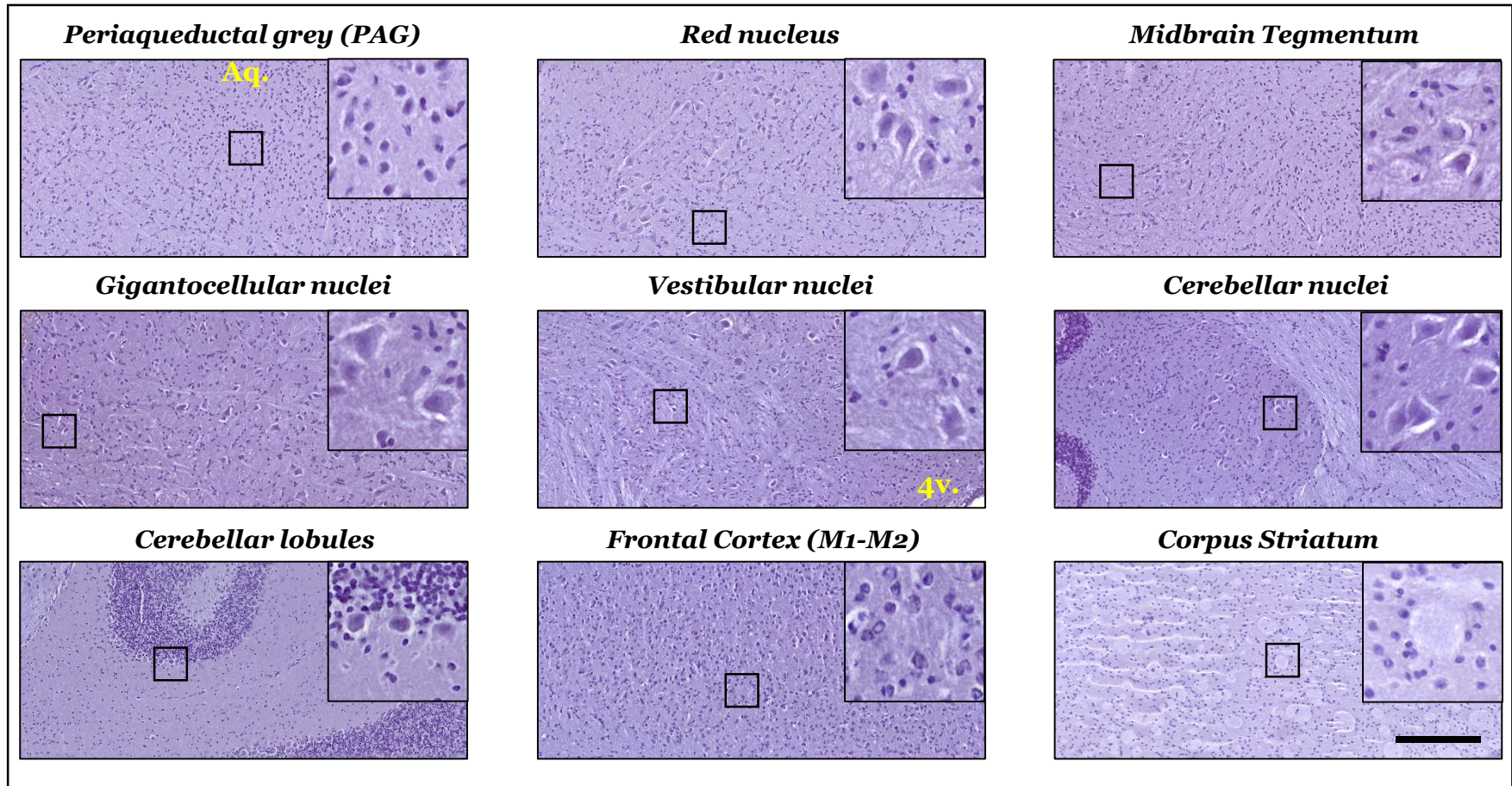
A) *PBS injected*　　　　　　　p-NRF2 (S40)　　　　　　　Fig. S5

Periaqueductal grey (PAG)　　　Red nucleus　　　Midbrain Tegmentum

Gigantocellular nuclei　　　Vestibular nuclei　　　Cerebellar nuclei

Cerebellar lobules　　　Frontal Cortex (M1-M2)　　　Corpus Striatum

Fig. S6

A) *PFF aSyn injected*

PAG RN Teg. Gi VN DCN

p-aSyn (S129): #1, #2, #3

B) p-NRF2 (S40): #1, #2, #3

C) *PBS injected*

PAG RN Teg. Gi VN DCN

p-NRF2 (S40): #1, #2, #3

A) *Nfe2l2*

B) *Txn*

C) *Nqo1*

D) *Park7*

A)

B)

C)

D)
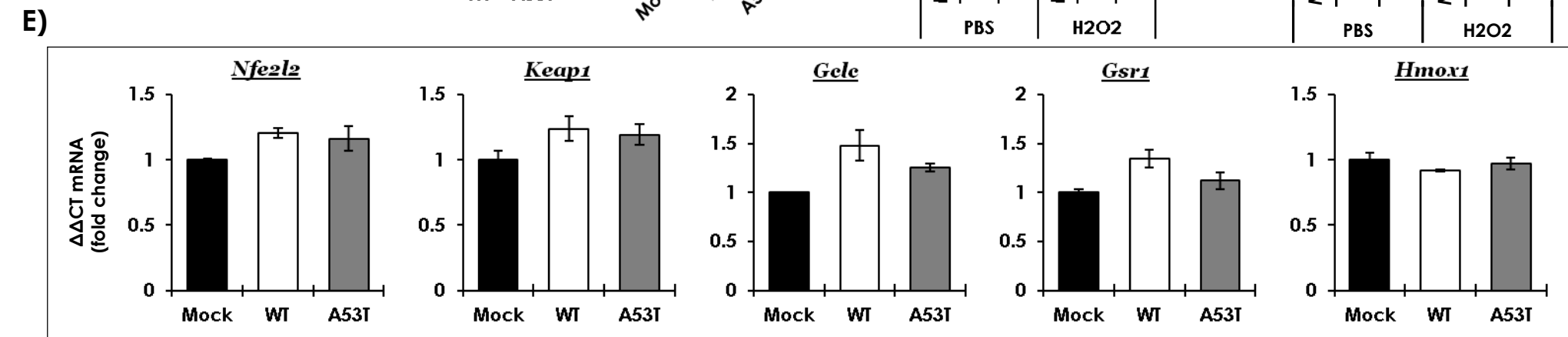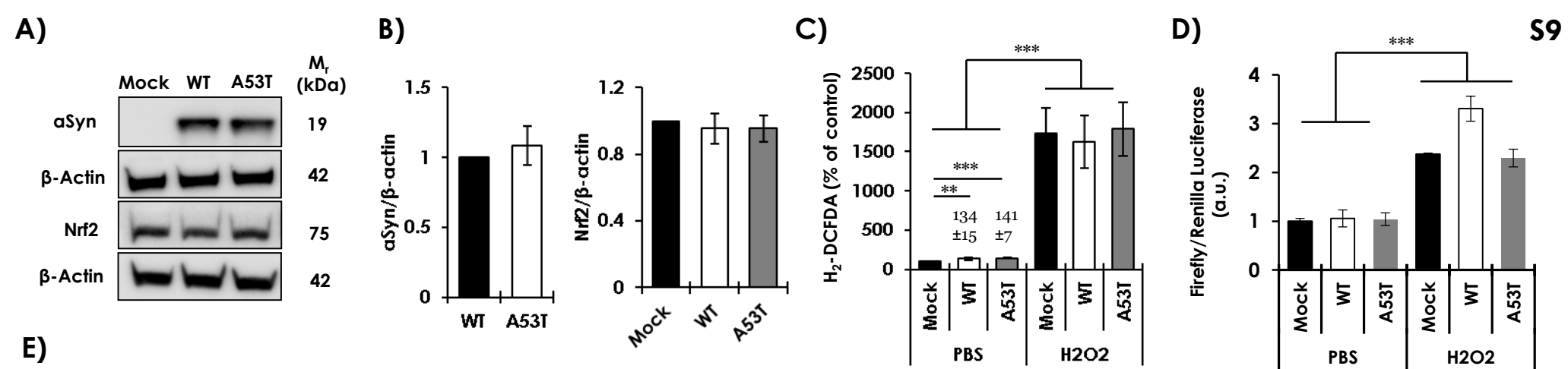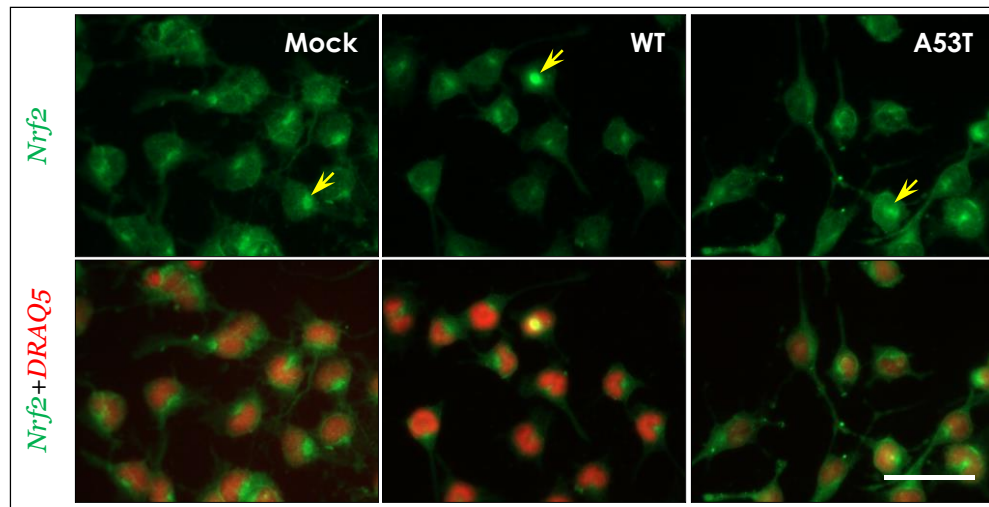
E)
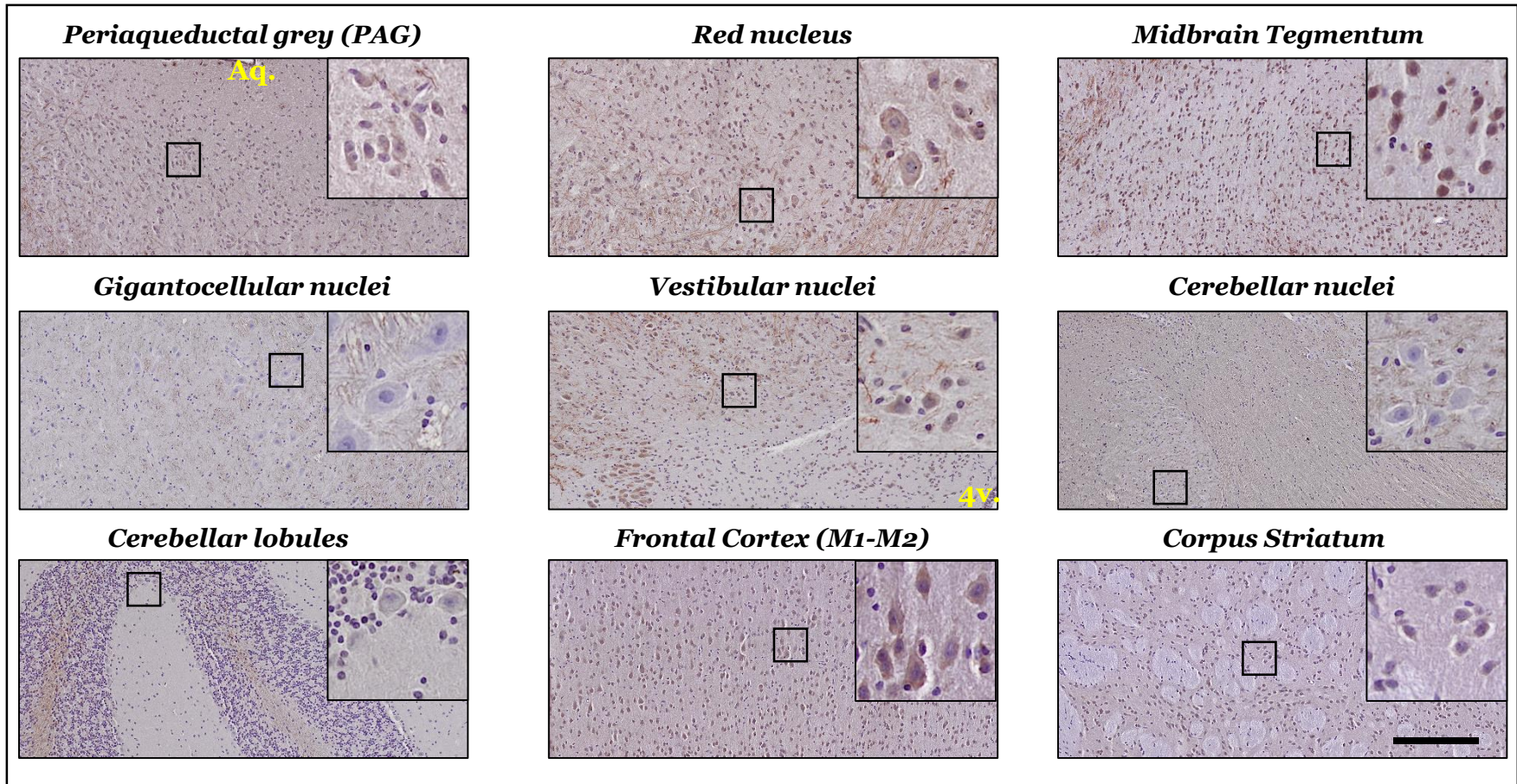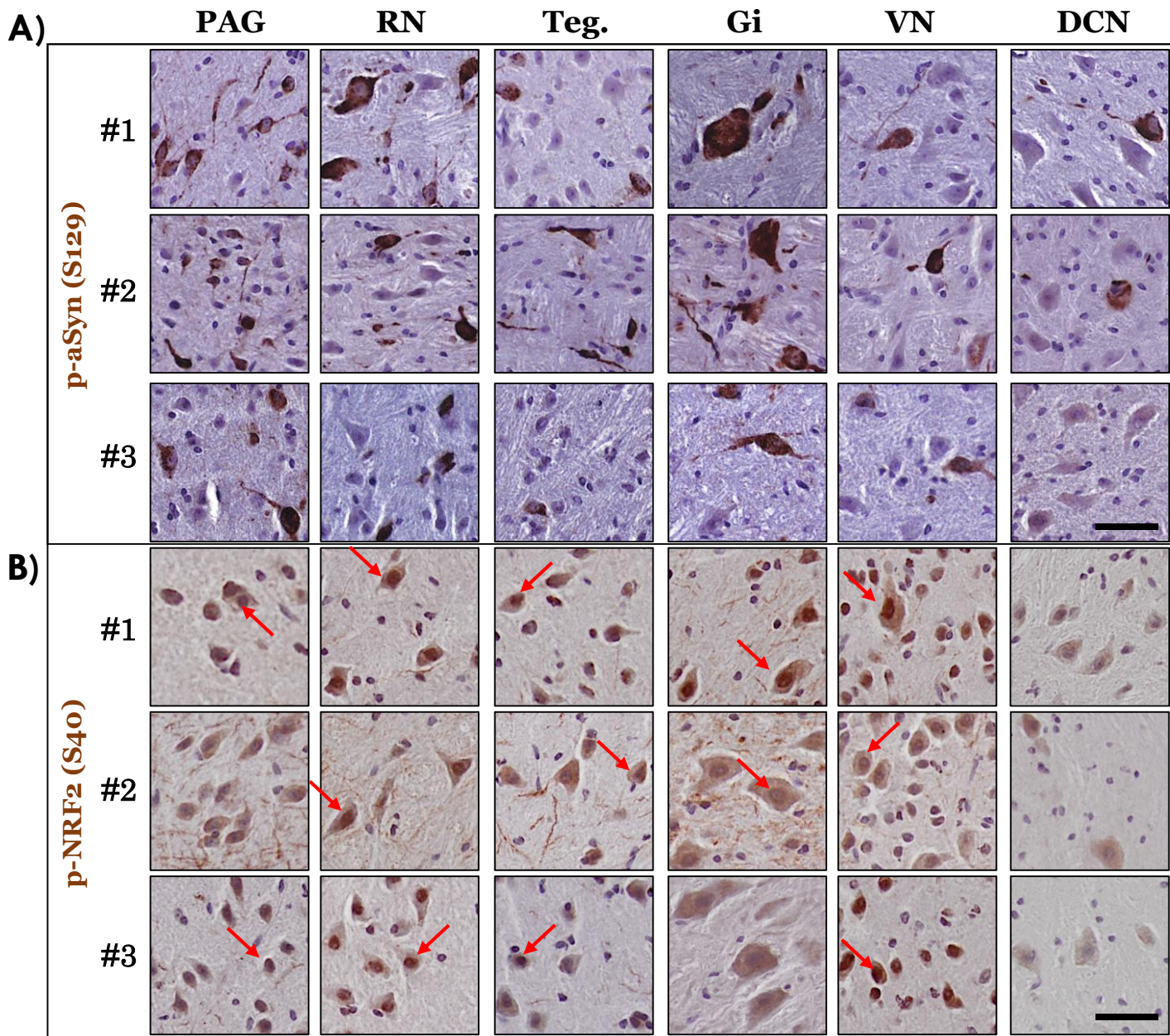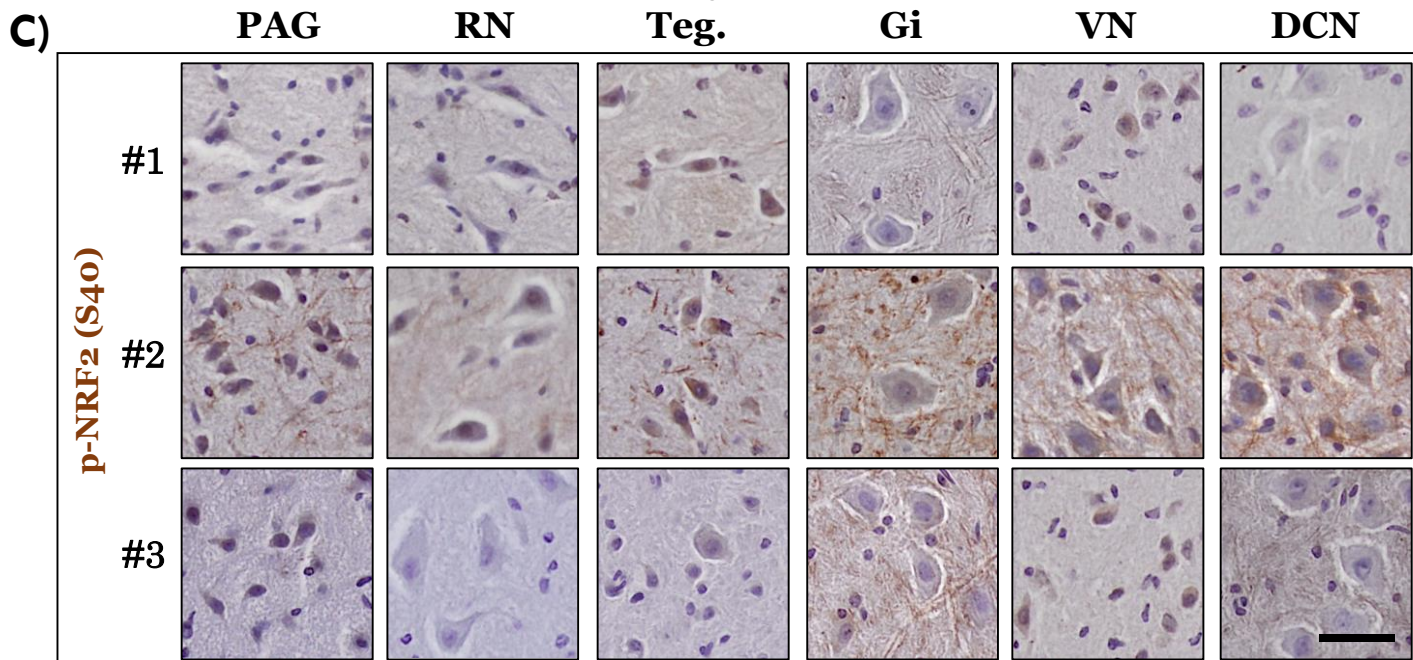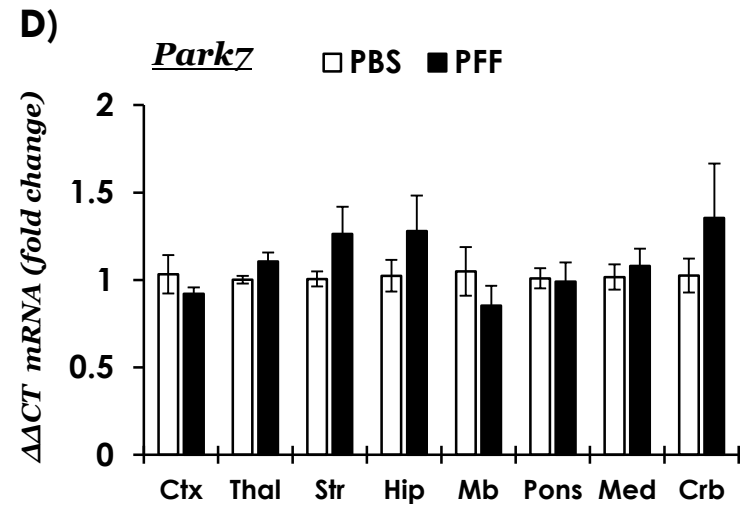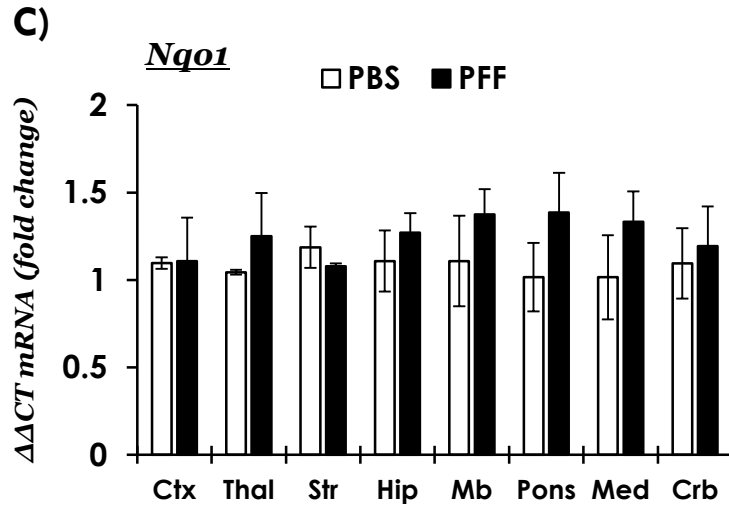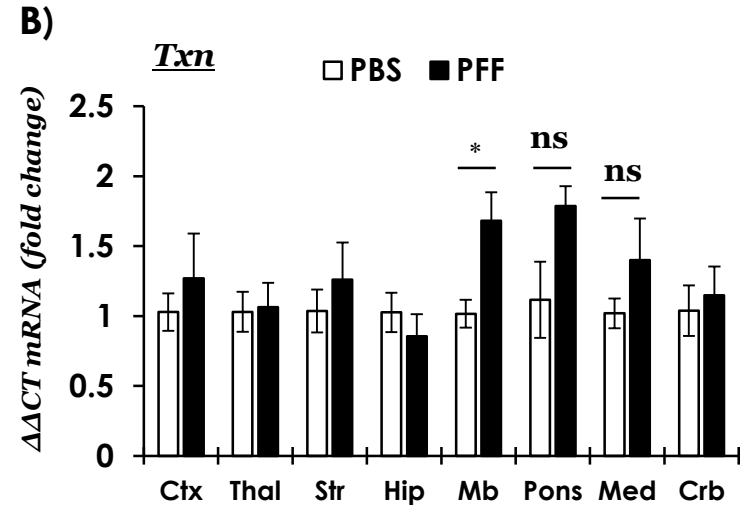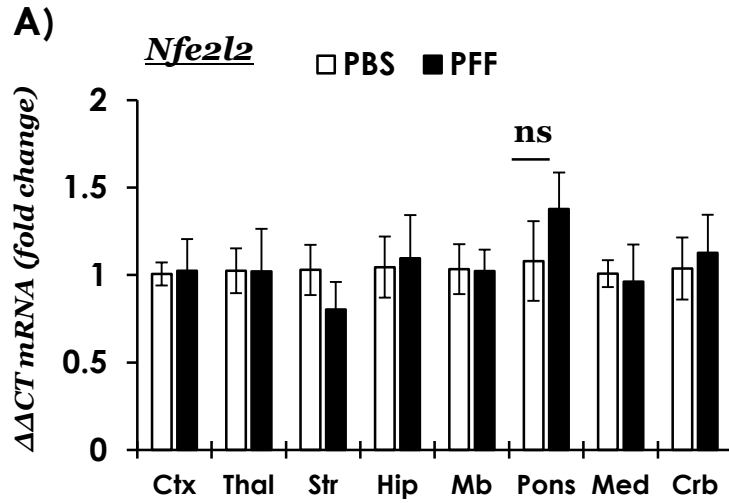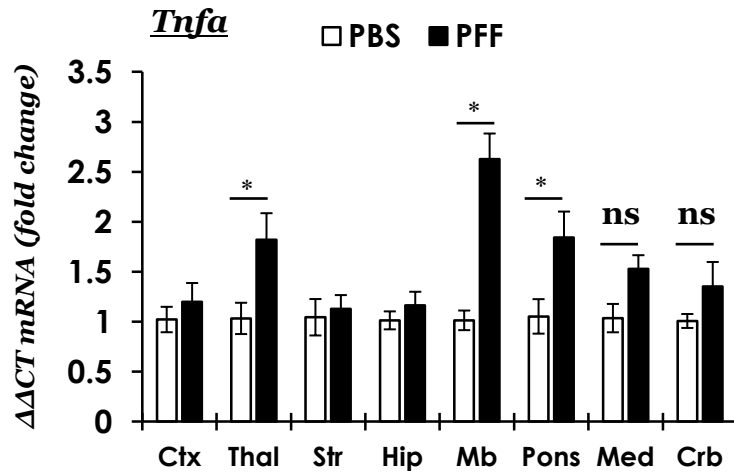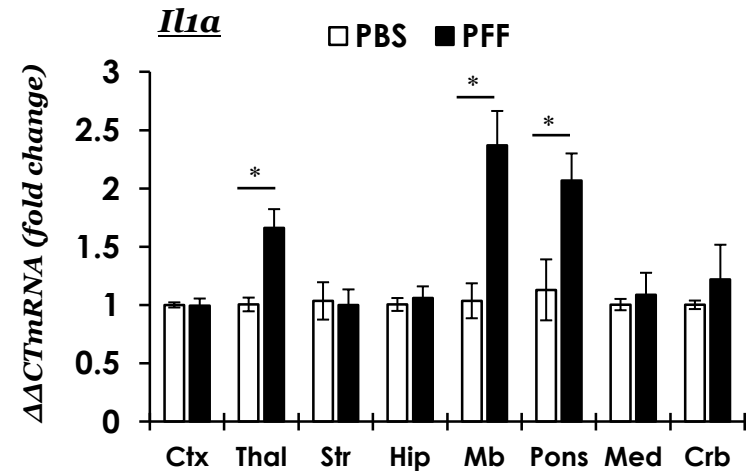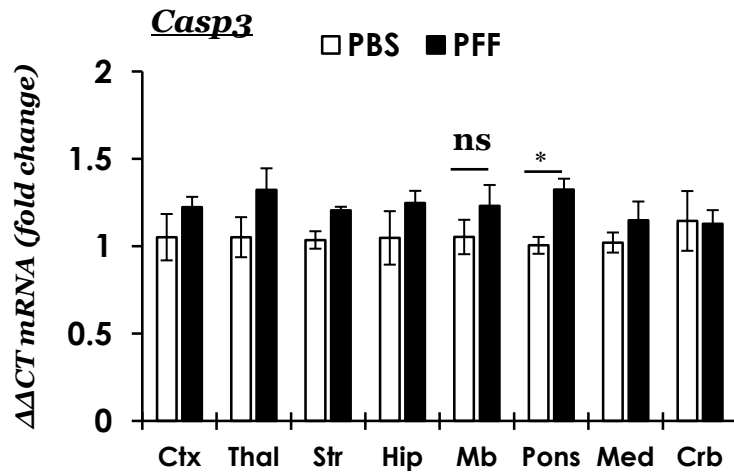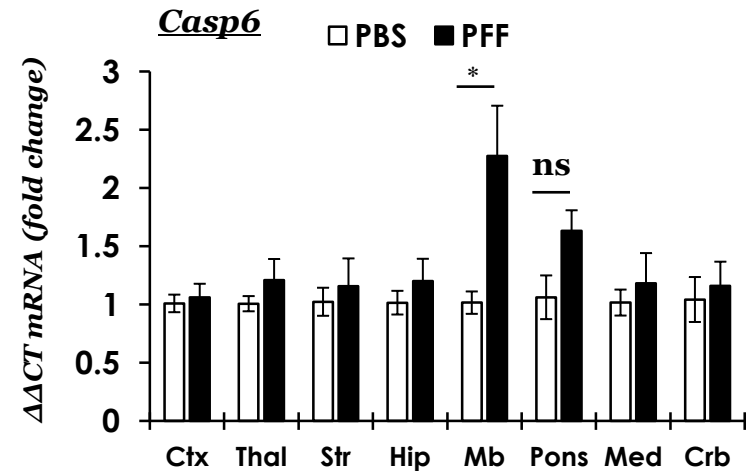
F) IF Nrf2, PBS

G) IF Nrf2, H₂O₂
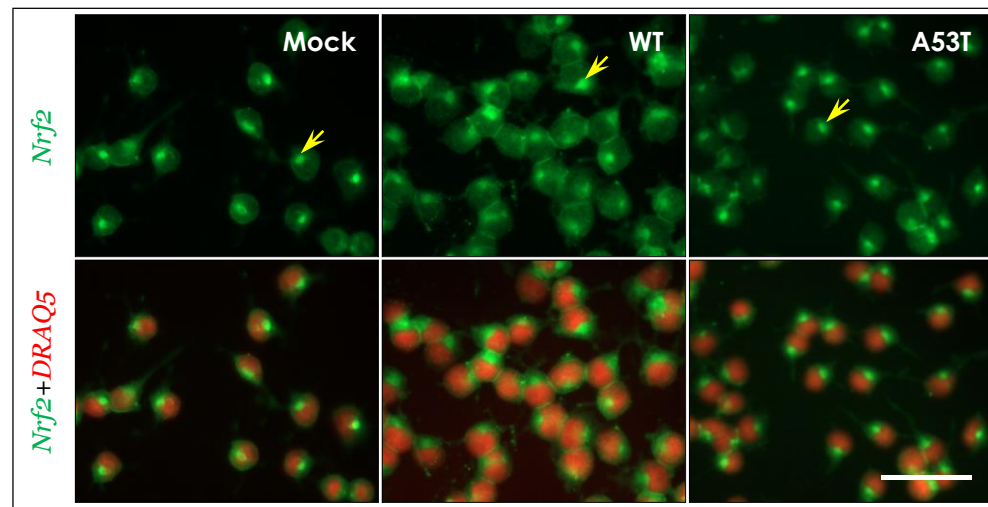
Table S1. Demographics, Control and PD cases.

Case ID	Gender	Age (years)	Additional postmortem pathology
Controls			
Control-1	Male	73	
Control-2	Male	52	
Control-3	Female	66	
PD			
PD-1	Male	65	Cortical Lewy body disease (LBD)
PD-2	Male	69	Limbic predominant LBD
PD-3	Male	81	Cortical LBD
PD-4	Male	74	Rare lewy bodies in limbic and neocortex
PD-5	Female	68	Cortical LBD

Table S2. Microarray datasets: Samples, genes and unique probe IDs				
GEO accession	**GSE7621**	**GSE43490**	**GSE20146**	**GSE26927**
Samples	SN: Ctrl (n=9), PD (n=16)	SN: Ctrl (n=6), PD (n=8) DMX: Ctrl (n=5), PD (n=8) LC: Ctrl (n=7), PD (n=8)	GPi : Ctrl (n=10), PD (n=10)	SN : Ctrl (n=7), PD (n=12) Ent. Ctx: Ctrl (n=6), AD (n=12) Cerv. SC: Ctrl (n=7), ALS (n=9) Caudate: Ctrl (n=10), HD (n=10) Front. gyri: Ctrl (n=10), MS (n=10)
Platform	GPL570 Affymetrix Human Genome U133	GPL6480 Agilent-014850 Whole Human Genome	GPL570 Affymetrix Human Genome U133 Plus	GPL6255 Illumina humanRef-8 v2.0
Reference	*Lesnick, T.G. et al.,*	*Corradini, B.R. et al.,*	*Zheng, B. et al.,*	*Durrenberger, P.F. et al.,*

Gene symbol	Unique Probe IDs			
NFE2L2	1567013_at	A_23_P5761	1567013_at	ILMN_9669
KEAP1	202417_at	A_23_P119141	202417_at	ILMN_18799
GSR	205770_at	A_23_P146084	205770_at	ILMN_14467
GCLC	1555330_at	A_23_P145114	1555330_at	ILMN_10857
HMOX1	203665_at	A_23_P120883	203665_at	ILMN_25059
TXN	208864_s_at	A_23_P60248	208864_s_at	ILMN_16294
CASP6	211464_x_at	A_23_P500799	211464_x_at	ILMN_11438
TNF	207113_s_at	Not found	207113_s_at	Not found
IL1A	208200_at	Not found	208200_at	ILMN_25320
CASP3	202763_at	A_23_P92410	202763_at	ILMN_29066
NQO1	210519_s_at	Not found	210519_s_at	ILMN_27575
PARK7	200006_at	A_23_P74740	200006_at	ILMN_6178

Abbreviations: **GSE7621**- Ctrl (control), PD (Parkinson Disease), SN (*substantia nigra*). **GSE43490-** Ctrl (control), PD (Parkinson Disease), SN (*substantia nigra*), DMX (*dorsal motor nucleus CN. X*), LC (*locus coeruleus*). **GSE20146-** Ctrl (control), PD (Parkinson Disease), GPi (*globus pallidus, interna*). **GSE26927-** Ctrl (control), PD (Parkinson Disease), AD (Alzheimer Disease), HD (Huntington Disease), ALS (Amyotrophic Lateral Sclerosis), MS (Multiple Sclerosis), SZ (Schizophrenia), SN (*substantia nigra*), Ent. Ctx (*entorhinal cortex*), Cerv. SC (*cervical spinal cord*), Front. Gyri (grey matter lesions in *frontal gyri*). Source: https://www.ncbi.nlm.nih.gov/geo/

Table S3. Primer pairs used in qRT-PCR (mouse tissue/cells)		
Gene symbol	**Forward**	**Reverse**
Nfe2l2	CACATTCCCAAACAAGATGCCT	TATCCAGGGCAAGCGACTCA
Keap1	GATCTACGTCCTCGGAGGCT	TCACTGTCCGGGTCATAGCA
Hmox1	TGCTAGCCTGGTGCAAGATAC	TGTCTGGGATGAGCTAGTGC
Txn	ATGACTGCCAGGATGTTGCT	AGAACTCCCCCACCTTTTGAC
Gsr1	GGGGCTCACTGAAGACGAAG	TCACAGCGTGATACATCGGG
Gclc	ACATCTACCACGCAGTCAAGG	CCAACATGTACTCCACCTCG
Nqo1	GGTAGCGGCTCCATGTACTC	CGCAGGATGCCACTCTGAAT
Park7	GCGGCTGCAGTCTTTAAGAAA	CAGTGACTTTGATCCCGGCT
Tnf	GTAGCCCACGTCGTAGCAAA	TTGAGATCCATGCCGTTGGC
Il1	CGCTTGAGTCGGCAAAGAAAT	TGGCAGAACTGTAGTCTTCGT
Casp6	AACCTGACTCGCAGGTTTTCA	GTTCTTCTGCTCTGAGGTCGT
Casp3	AGCTTGGAACGGTACGCTAA	GAGTCCACTGACTTGCTCCC
Gapdh	CCCTTAAGAGGGATGCTGCC	TACGGCCAAATCCGTTCACA