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Characterisation of a novel *NR4A2* mutation in Parkinson's disease brain

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Abstract

Objective—We performed a mutation screen of *NR4A2* (also known as NURR1) in 409 Parkinson's disease (PD) patients. We identified a novel single base substitution in the 5'UTR of the *NR4A2* (also known as NURR1) gene (c.-309C > T).

Results—We have performed expression studies in neuronal cell lines showing that the c.-309C > T mutation reduces *NR4A2* mRNA expression *in vitro*. We have confirmed this finding *in vivo* by performing allele specific real-time PCR from brain tissue harbouring the 309C > T mutation and show a 3.48 ± 1.62 fold reduction in mRNA expression of the mutant allele compared to wild-type. In addition we have undertaken genome wide expression analysis of the mutant *NR4A2* brain and shown underexpressed genes were significantly enriched for gene ontology categories in nervous system development and synaptic transmission and overexpressed genes were enriched for unfolded protein response and morphogenesis. Lastly we have shown that the c.-309C > T mutation abrogates the protective effect of wild-type *NR4A2* against apoptotic stress.

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Conclusions—Our findings indicate the c.-309C > T mutation reduces *NR4A2* expression resulting in the downregulation of genes involved in the development and maintenance of the nervous system and synaptic transmission. These downregulated pathways contained genes known to be transactivated by *NR4A2* and were not disrupted in idiopathic PD brain suggesting causality of the mutation.

Keywords

NR4A2; Parkinsonism; Whole genome expression analysis; Allele specific expression analysis

Parkinson's disease (PD) is a common neurodegenerative disorder. The aetiology of PD is unknown; however, mechanistic insight has been gained through the study of rare monogenic forms [1,6].

NR4A2 (*NURRI*) encodes a transcription factor regulating the expression of genes including tyrosine hydroxylase and is required for the differentiation/maintenance of nigral dopaminergic neurons [2,13,19,20]. Homozygous *NR4A2* knockout mice fail to develop dopaminergic neurons whereas heterozygotes have reduced brain dopamine and develop age-dependent locomotor deficits including impaired horizontal and vertical movement and performance on rotarod testing compared to age-matched wild-type mice. [4,11,24]. A reduction of *NR4A2* in the adult brain may increase the vulnerability of dopaminergic neurons to stress, and play a role in the pathogenesis of PD [14].

Le et al. reported two mutations (c.-291 delinsT and c.-245T > G) in the 5'-untranslated region (UTR) of *NR4A2* in autosomal dominant PD [15]. These mutations decreased *NR4A2* expression *in vitro* and in lymphocytes of *NR4A2* PD patients. Over ten follow-up studies have identified only three additional mutations (c.-253C > T, c.-223C > T, and Ser125Cys), indicating that *NR4A2* mutations are rare [8,9,10,12,16,22,23,25]. Moreover, the lack of *in vivo* functional data has raised questions about the pathogenicity of *NR4A2* in PD.

We report a novel 5'-UTR *NR4A2* mutation (c.-309C > T). We also provide evidence, including *in vivo* expression data from an *NR4A2* mutant PD brain and an *in vitro* viability assay, that this mutation adversely affects *NR4A2* function.

The Institute of Neurology Ethics Committee approved this study. PD patients were examined by a movement neurologist ($n = 122$) or by pathological confirmation at the Queen Square Brain Bank (QSBB) ($n = 287$) [7]. 118 patients had a family history of PD, 71 compatible with autosomal dominant inheritance (66 Caucasian, 5 Asian). The remaining 47 familial index cases had at least one PD affected sibling but it was not clear whether the inheritance pattern was autosomal dominant or recessive (32 Caucasian, 13 Asian, two African). Controls were Caucasian and sourced from the southeast of England.

DNA was extracted from peripheral lymphocytes or frozen brain tissue using standard protocols. The entire *NR4A2* open reading frame (eight exons) was sequenced in all 409 patients using Big Dye Terminator version 3.1 (Applied Biosystems®). Primers and conditions available on request.

For the c.-309C > T mutated brain, histological sections were stained using haematoxylin and eosin and Bielschowsky's silver impregnation. The immunohistochemical primary antibodies used were as follows: tau (Rabbit polyclonal, 1:200, no pre-treatment) DAKO, UK; Ab (monoclonal, 1:100, formic acid and pressure cooking pre-treatment) DAKO, UK; α -synuclein (N-19, goat polyclonal, 1:1000, formic acid pre-treatment) Autogen Bioclear, UK.

Wild-type (wt) *NR4A2* and mutant c.-245T > G *NR4A2* cloned into the pCMX vector were gifts from Dr Weidong Le (Houston, USA), with permission from Dr Hiroshi Ichinose (Tokyo, Japan). The c.-309C > T *NR4A2* mutation was introduced by site-directed mutagenesis (QuikChange, Stratagene). The NuRE-POMC-Luc Plasmid was gifted by Dr Jacques Drouin (Montreal, Canada). pCMX plasmid was gifted by Dr John Achermann (London, UK).

SH-SY5Y cells were co-transfected, in triplicate wells, with SV40 renilla plasmid, wt or mutant *NR4A2* expression vector and POMC luciferase reporter vector. Cells were assayed for firefly and renilla luciferase levels using the Dual-Luciferase[®] Reporter Assay System according to the manufacturer's protocol. Values are normalised to the pCMX empty vector.

SH-SY5Y cells were co-transfected with wt or mutant *NR4A2* and GFP cDNA (*NR4A2*:GFP DNA ratio of 2:3). Cells were harvested and resuspended in 1X Annexin V binding buffer (BD Biosciences, Oxford, UK) and incubated with Annexin V conjugated with phycoerythrin (Annexin V-PE) before being analyzed on an Epics XL flow cytometer. 30,000 cells were analyzed per sample (in triplicate) and Annexin V-PE fluorescence was determined in the FL2 channel gated for GFP positive events. There was no significant difference in transfection efficiency between constructs as assessed by GFP measurement by FACS (data not shown). Statistical analysis was performed using one-way ANOVA with post hoc Bonferroni correction and Scheffé's multiple comparison *t*-test.

SH-SY5Y cells were transfected with *NR4A2* constructs as above. Total RNA was extracted using TRIZOL[®] Reagent (Invitrogen) and then treated with DNase I (Amersham-Pharmacia) for 1 h. Real-time PCR was performed on the DNA Engine Opticon system (MJ Research) using SYBR I Green technology as previously described [3]. Primer sequences and conditions are available on request. The data obtained from real-time PCR were adjusted by the 2^{-C_t} method [17]. We confirmed the PCR efficiencies of the *NR4A2* and β -Actin primers were comparable by assaying serial cDNA dilutions, plotting the resultant curves and measuring their slopes (data not shown).

RNA was extracted from flash frozen frontal cortex brain tissue and reverse transcribed. The following experiments were performed on an ABI PRISM 7500 Sequence Detection System using a TaqMan assay for the c.-309C > T mutation. To generate a standard curve for the experiment, wt and c.-309C > T *NR4A2* plasmid DNA was mixed to the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8. A linear regression line was fitted to the standard curve generated by plotting the log of the mixing ratios against the log of the C_t values of each allele. The allele ratio of the brain sample was then extrapolated by intercepting the linear

regression line of the standard curve. Three experiments were performed in duplicate with two separate cDNA samples from the affected brain.

Statistical significance was determined using one-way analysis of variance (ANOVA) and post hoc analysis with Bonferroni correction.

Differential gene expression was ascertained on the Illumina Sentrix HumanRef8 gene expression chip (Illumina inc., San Diego, USA). RNA was extracted from striatal sections of all samples and reverse transcribed. The cDNA was hybridised to Illumina human refseq-8 chips following manufacturer's protocol. Data normalisation and outlier detection was performed using the lumi package for R.

Hierarchical cluster analysis of the normalised log2 transformed expression values with multiscale bootstrap ($n = 10,000$) using the average method and correlation based dissimilarity matrix was performed using the pvclust R package [21]. Pvclust also calculates the probability values for each cluster using multiscale bootstrap resampling techniques, the bootstrap probability (BP) and an approximately unbiased p -value.

Differential gene expression between disease groups was calculated using the limma package in R. The gene lists obtained from limma were tested for association with gene ontology (GO) terms using the GOstats R package. Gene lists were first filtered to exclude genes that were unexpressed in striatum and those which did not significantly differ in expression ($p > 0.001$) between the test sample and controls. The remaining genes were then sorted by either over- or underexpression. Hypergeometric testing of the filtered and sorted lists against all known genes on the Sentrix chip identified GO terms either over- or under-represented in the test samples [5].

From 409 PD patients, a single patient with apparently sporadic late-onset PD was identified with a c.-309C > T mutation in exon 1 of *NR4A2*. This novel mutation affected a highly conserved base situated 18 base pairs and 64 base pairs upstream of the pathogenic c.-291delinsT and c.-245T > G mutations reported by Le et al. [15]. The c.-309C > T mutation was not observed in 868 control chromosomes.

The patient had typical features of idiopathic PD (iPD), presenting with asymmetrical right hand resting tremor and rigidity aged 55yrs. At diagnosis the Hoehn and Yahr score was 1 and Mini-mental test score 30/30. Response to L-dopa was excellent and disease progression was slow. At the age of 67, a formal "on state" assessment revealed no bradykinesia or rigidity and a mild right resting hand tremor. The Mini-mental score remained at 30 and Beck depression score was 15. Two years later, examination revealed mild bilateral bradykinesia and rigidity, no tremor, and peak-dose dyskinesia of the tongue and head. The Mini-mental and Beck scores decreased to 26 and 8, respectively. In 1998, a year before the patient's death aged 74 (19 years after onset), it was commented that the parkinsonism was mild. No family history of parkinsonism was recorded, however we were unable to contact surviving relatives due to the protocols of our ethical approval.

The brain weighed 1130g (normal range 1300–1400g). There was pallor of the substantia nigra and locus coeruleus. Histological examination showed moderate to severe neuronal

loss in the substantia nigra, most severe in the ventro-lateral region. Lewy bodies were present in residual neurones of the substantia nigra. α -Synuclein immunohistochemistry demonstrated the presence of Lewy bodies and neurites in the substantia nigra, locus coeruleus, pontine tegmentum tegmentum of the medulla including the Xth nerve nucleus and cortex. α -Synuclein-positive neurites were seen in the CA2/3 region of the hippocampus. The neocortical areas and hippocampal formation contained sparse neuritic A β plaques and tau-immunoreactive neurofibrillary tangles respectively consistent with pathological ageing.

The mRNA expression level of the c.-245T > G *NR4A2* mutant was significantly ($p < 0.001$) reduced by approximately 80–90% compared to wt, in agreement with a previous study [15]. We observed an approximate 50% reduction in mRNA expression of the c.-309C > T *NR4A2* mutant that was significantly different from both the wt and the c.-245T > G *NR4A2* mutant respectively (Fig. 1A). Co-transfection of a GFP reporter with *NR4A2* constructs, in parallel wells, revealed no difference in transfection efficiency between constructs (data not shown).

We next co-transfected *NR4A2* constructs with a construct containing the proopiomelanocortin gene (POMC) promoter that contains tandem NuRE sites fused to luciferase. A SV40 renilla Plasmid was also co-transfected to control for variation in transfection efficiency. The luciferase activities of the c.-309C > T and c.-245T > G *NR4A2* mutants were both significantly lower than that of wt *NR4A2*, but not significantly different from each other (ANOVA, overall $p < 0.0001$, Fig. 1B). These *in vitro* data confirmed that the c.-309C > T mutant was sufficient to affect the transactivating role of *NR4A2*.

SH-SY5Y cells were transiently co-transfected with wild-type and mutant (c.-309C > T and c.-245T > G) *NR4A2* cDNA and GFP and then stressed with MG-132, which inhibits proteasome function and induces apoptosis [18]. Measuring Annexin V by FACS, we found that overexpression of wild-type *NR4A2* but not mutant *NR4A2* significantly reduced the level of apoptotic cell death induced by MG-132 in GFP-positive cells (vector, 36.4% $\pm$ 5.5; wild-type *NR4A2*, 27.9% $\pm$ 5.3; 309C > T, 33.2% $\pm$ 2.7; 245T > G, 32.8% $\pm$ 5.3; $p < 0.05$; ANOVA $n = 6$) (Fig. 2).

We next evaluated the effect of the c.-309C > T mutation on *NR4A2* mRNA levels using an allele specific real-time quantitative assay in flash frozen brain tissue. The 95% confidence interval from a total of 12 data points indicated a 3.48 ± 1.62 fold decrease in c.-309C > T allele compared to wt.

To compare genome wide expression profiles of the mutant *NR4A2* brain and iPD, striatal RNA was extracted from the c.-309C > T brain, five iPD patients and five neurologically normal controls. Global gene expression differences were initially examined by multiscale hierarchical bootstrap clustering. The *NR4A2* mutant brain robustly clustered independently of the iPD samples (see Fig. 3) indicating significant differences in gene expression patterns. We next generated lists of the genes uniquely up- or downregulated in *NR4A2* and iPD. We then investigated whether the differentially expressed genes were enriched for one or more functional categories as defined by the gene ontology (GO) project using hypergeometric

testing. The results indicate that (i) genes under-expressed in *NR4A2* were significantly enriched for GO categories involved in nervous system development and synaptic transmission (Table 1) and (ii) genes overexpressed in the *NR4A2* brain were enriched for the unfolded protein response and morphogenesis (Table 2).

We have identified a novel PD-associated mutation in the 5'UTR of the *NR4A2* gene. Using an allele specific quantitative PCR assay, we directly demonstrate a 3-fold reduction in expression from the *NR4A2* -309T allele in brain tissue compared to the wt C allele. The mechanism of how this 5'UTR mutation reduces mRNA remains unclear but may result in nonsense mediated decay.

In vitro studies further show that the -309T allele caused a significant decrease in *NR4A2* expression. Although this mutation resulted in a smaller reduction in *NR4A2* mRNA compared with the c.-245T > G mutant, it caused an equivalent reduction in the transactivation of a downstream target gene which raises the possibility of a dominant negative gain of function as previously suggested by Le et al. [15]. The c.-309C > T mutation also reduced the protective effect of wild-type *NR4A2* in cells stressed with the proteasome inhibitor MG-132.

We hypothesised that a mutation that affects transactivation properties may be reflected in the genome wide expression profile in brain. We tested this hypothesis on RNA from flash frozen striatal sections of the *NR4A2* c.-309C > T mutant, 5 idiopathic PD samples and 5 matched neurologically normal controls. Hierarchical clustering of the expression profiles suggested divergent expression profiles for the *NR4A2* c.-309C > T mutant compared to both controls and iPD. By analysing the GO term associations of the genes uniquely up- and downregulated in the *NR4A2* mutant brain it became apparent that the expression difference identified by the hierarchical clustering was being driven by an under-representation of genes involved in nervous system development and synaptic transmission and an over-representation of genes involved in protein misfolding and morphogenesis. These results must be interpreted with caution as they are based on a single *NR4A2* mutant. However, by using differentially expressed lists that are unique to the *NR4A2* sample we have controlled against secondary effects that may arise during the progression of the disease. The under-representation of genes involved in nervous system development could be attributed directly to the *NR4A2* mutation. *NR4A2* is known to be critical to midbrain development, from where our test tissue is derived. The overexpression of genes involved in the protein misfolding response pathway is an interesting result which correlates with previous findings of increased neuronal stress in heterozygous *Nr4a2* knockout mice [14] and the results of our *in vitro* viability assay which showed increased vulnerability of neuroblastoma cells expressing the *NR4A2* mutants to a proteasome inhibitor.

In conclusion we demonstrate a mutation in exon1 of *NR4A2* that results in an *in vivo* allele specific reduction in *NR4A2* mRNA levels and a distinct striatal gene expression profile compared to 'idiopathic' PD. Together with *in vitro* studies, these results suggest that this mutation adversely affects *NR4A2* function and may be sufficient to cause PD.

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Fig 1.

Panel A. Mean mRNA expression and Panel B, mean luciferase activities shown as a percentage of wild-type (wt) NR4A2 construct (100%) transfected in SH-SY5Y cells, with POMC promoter luciferase fusion construct 309 and 245 are c.-309C > T and -245T/G mutants of NR4A2, and pCMX indicates the empty vector. The error bars indicate the standard error of the mean of 3 experiments in triplicates each. The significance of differences was analyzed by ANOVA and Bonferroni post hoc test. Panel A: *wt vs c.-309C > T vs or -245T/G ($p < 0.001$) and #c.-309C > T vs -245T/G ($p < 0.001$) and Panel B, *c.-309C > T vs wt and c.-245T > G vs wt are both significantly different ($p < 0.001$).

Fig 2.

Mean difference in rate apoptosis between stressed and basal conditions in SH-SY5Y cells transiently co-transfected with GFP and either mutant *NR4A2*, wild-type *NR4A2* or empty vector following exposure to MG-132. 309 and 245 are c.-309C > T and -245T/G mutants of *NR4A2*, and pCMX indicates the empty vector. The error bars indicate the standard error of the mean of 2 experiments in triplicate. The significance of differences was analyzed by ANOVA using 6 data points from 2 independent experiments and Bonferroni post hoc test. *wt vs c.-309C > T or c.-245T > G or pCMX ($p < 0.05$).

Fig 3.

Dendrogram of the hierarchical clustering of differentially expressed genes with significant p -values between *NR4A2* c.-309C > T striatum, 5 iPD samples and 5 controls. Au, approximately unbiased p -value. BP, bootstrap probability.

Table 1

Enrichment of GO terms (significance @ $p < 0.0001$) in the list of genes which were significantly underexpressed uniquely in *NR4A2* brain compared to iPD.

<i>p</i> -Value	GO ID	GO description
2.22E-09	GO:0007399	Nervous system development
1.11E-08	GO:0019226	Transmission of nerve impulse
1.34E-08	GO:0007268	Synaptic transmission
7.63E-07	GO:0051179	Localisation
7.86E-07	GO:0045055	Regulated secretory pathway
1.37E-06	GO:0001505	Regulation of neurotransmitter levels
2.95E-06	GO:0007267	Cell-cell signaling
3.20E-06	GO:0007269	Neurotransmitter secretion
3.74E-06	GO:0006810	Transport
3.80E-06	GO:0051234	Establishment of localisation
1.07E-05	GO:0045104	Intermediate filament cytoskeleton organisation and biogenesis
2.28E-05	GO:0003001	Generation of a signal involved in cell-cell signaling
2.99E-05	GO:0030182	Neuron differentiation
3.70E-05	GO:0045103	Intermediate filament-based process
4.10E-05	GO:0048699	Generation of neurons
5.32E-05	GO:0030030	Cell projection organisation and biogenesis
5.32E-05	GO:0048858	Cell projection morphogenesis
6.20E-05	GO:0048812	Neurite morphogenesis
6.20E-05	GO:0048667	Neuron morphogenesis during differentiation
9.49E-05	GO:0022008	Neurogenesis

Table 2

Enrichment of GO terms (significance @ $p < 0.0001$) in the list of genes which were significantly overexpressed uniquely in *NR4A2* brain compared to iPD.

<i>p</i> -Value	GO ID	GO description
4.68E-06	GO:0006986	Response to unfolded protein
4.68E-06	GO:0051789	Response to protein stimulus
4.89E-06	GO:0009887	Organ morphogenesis
2.91E-05	GO:0048598	Embryonic morphogenesis
5.85E-05	GO:0001539	Ciliary or flagellar motility