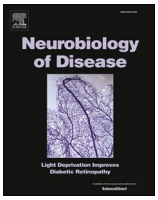
Neurobiology of Disease 180 (2023) 106067

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi



NF-κB/c-Rel DNA-binding is reduced in substantia nigra and peripheral blood mononuclear cells of Parkinson's disease patients

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ARTICLE INFO

Keywords:
c-Rel
NF-κB
Parkinson's disease
Peripheral blood mononuclear cells

ABSTRACT

Although Parkinson's disease (PD) key neuropathological hallmarks are well known, the underlying pathogenic mechanisms of the disease still need to be elucidated to identify innovative disease-modifying drugs and specific biomarkers. NF-κB transcription factors are involved in regulating several processes associated with neurodegeneration, such as neuroinflammation and cell death, that could be related to PD pathology. NF-κB/c-Rel deficient (c-rel$^{-/-}$) mice develop a progressive PD-like phenotype. The c-rel$^{-/-}$ mice present both prodromal and motor symptoms as well as key neuropathological features, including nigrostriatal dopaminergic neurons degeneration, accumulation of pro-apoptotic NF-κB/RelA acetylated at the lysine 310 residue (Ac-RelA(lys310)) and progressive caudo-rostral brain deposition of alpha-synuclein. c-Rel inhibition can exacerbate MPTP-induced neurotoxicity in mice. These findings support the claim that misregulation of c-Rel protein may be implicated in PD pathophysiology. In this study, we aimed at evaluating c-Rel levels and DNA-binding activity in human brains and peripheral blood mononuclear cells (PBMCs) of sporadic PD patients. We analyzed c-Rel protein content and activity in frozen substantia nigra (SN) samples from post-mortem brains of 10 PD patients and 9 age-matched controls as well as in PBMCs from 72 PD patients and 40 age-matched controls. c-Rel DNA-binding was significantly lower and inversely correlated with Ac-RelA(lys310) content in post-mortem SN of sporadic PD cases, when compared to healthy controls. c-Rel DNA-binding activity was also reduced in PBMCs of followed-up PD subjects. The decrease of c-Rel activity in PBMCs from PD patients appeared to be independent from dopaminergic medication or disease progression, as it was evident even in early stage, drug-naïve patients. Remarkably, the levels of c-Rel protein were comparable in PD and control subjects, pointing out a putative role for post-translational modifications of the protein in c-Rel dysfunctions.

These findings support that PD is characterized by the loss of NF-κB/c-Rel activity that potentially has a role in PD pathophysiology. Future studies will be aimed at addressing whether the reduction of c-Rel DNA-binding could constitute a novel biomarker for PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide. The key neuropathological hallmarks of PD are the loss of dopaminergic nigrostriatal neurons and the accumulation of Lewy bodies and Lewy neurites, proteinaceous inclusions mainly composed of alpha-synuclein (α-syn) (Poewe et al., 2017).

Available treatments for PD are essentially symptomatic and aim to replace dopamine (DA) in the nigrostriatal pathway, i.e. DA precursor L-DOPA, dopamine agonists and enzyme inhibitors (Connolly and Lang,

Abbreviations: α-syn, alpha-synuclein; Bcl-xL, B-cell lymphoma-extra-large; DA, dopamine; MnSOD, manganese superoxide dismutase; PBMCs, peripheral blood mononuclear cells; PD, Parkinson's disease; SN, substantia nigra; ROS, reactive oxygen species; UCP4, uncoupling protein 4.

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https://doi.org/10.1016/j.nbd.2023.106067
Received 17 October 2022; Received in revised form 20 February 2023; Accepted 3 March 2023
Available online 7 March 2023

2014). At present, a number of biomarkers and tests have been proposed for the early detection of PD but in many cases they lack sufficient specificity and sensitivity and cannot be implemented as routing clinical procedures. The lack of biomarkers has also affected studies targeting disease-modification, which would benefit of a more extensive knowledge of PD underlying pathogenic mechanism (Espay et al., 2020), (Masato et al., 2021), (Colombo et al., 2020), (Engelender et al., 2022).

Several mechanisms, such as mitochondrial impairment, oxidative stress, excitotoxicity, or dysfunction of the ubiquitin proteasomal and autophagy lysosomal pathways have been related to PD pathology. Still, neuroinflammation, with the engagement of the innate and adaptive immunity, has been suggested to play a role in PD neurodegeneration (Tansey and Goldberg, 2010), (Mattson and Arumugam, 2018), (Ganguly et al., 2017), (Wang et al., 2020a), (Parnetti et al., 2019), (Picca et al., 2021), (Lim and Tan, 2007), (Gelders et al., 2018), (Murphy et al., 2015), (Alvarez-Erviti et al., 2010).

Diverse members of NF-κB transcription factor family are cardinally involved in regulating inflammatory pathways, apoptosis and neurodegeneration (Pizzi et al., 2002), (Pizzi and Spano, 2006), (Camandola and Mattson, 2007). NF-κB family is composed by five proteins: RelA (also named p65), RelB, c-Rel, p105/p50 and p100/52 that can diversely combine with each other to form distinct, transcriptionally active homo- or heterodimers. NF-κB dimer composition determines the pro- or anti-apoptotic activity of the transcription factor (Sarnico et al., 2009), (Sarnico et al., 2012), (Lanzillotta et al., 2015). Also, the acetylation state of RelA subunit can promote both neuroprotective and neurotoxic actions (Lanzillotta et al., 2010), (Mincheva-Tasheva and Soler, 2013). We previously showed that a specific aberrant acetylation pattern of RelA, characterized by site-specific increase at the lysine 310 residue (Ac-RelA (lys310)), triggers the expression of pro-apoptotic genes in brain ischemia (Lanzillotta et al., 2013), (Mota et al., 2020). On the contrary, the presence of c-Rel in the activated NF-κB dimers attenuates brain damage induced by ischemic injury (Sarnico et al., 2009) and is responsible for neuroprotection prompted by IL-1β (Pizzi et al., 2002) and mGlu5 agonists (Pizzi et al., 2005) in a cell-based model of neurotoxicity or by leptin in cell-based and murine model of brain ischemia (Valerio et al., 2009). Recent evidences have also confirmed a rapid activation of c-Rel in MPTP-treated mouse brain, carrying a pro-survival and anti-inflammatory effect (Wang et al., 2020b). c-Rel mediates its neuroprotective, anti-apoptotic activity by inducing the expression of proteins contributing to mitochondrial homeostasis, such as B-cell lymphoma-extra-large (*Bcl-xL*), and reactive oxygen species (ROS) scavenging, i.e. manganese superoxide dismutase (*MnSOD*) (Chen et al., 2000), (Bernard et al., 2001), (Pizzi et al., 2005), (Sarnico et al., 2009). c-Rel can also reduce the production of ROS by increasing the transcription of mitochondrial uncoupling protein 4 (*UCP4*) gene (Ho et al., 2012).

We previously showed that male mice deficient for NF-κB/c-Rel protein (c-rel$^{-/-}$ mice) develop a sporadic PD-like phenotype (Baiguera et al., 2012), (Porrini et al., 2017), (Parrella et al., 2019), (Parrella et al., 2022). Young c-rel$^{-/-}$ mice (2 months of age) exhibit prodromal symptoms such as hyposmia and constipation. Since 5 months of age, the c-rel$^{-/-}$ mice exhibit a progressive Braak-like caudo-rostral pattern of brain α-syn deposition, starting from the lower brainstem (dorsal motor nucleus of the vagus and locus coeruleus) and olfactory bulbs, which at 12 months of age also involves the substantia nigra (SN) (Parrella et al., 2019). Interestingly, 12-month-old c-rel$^{-/-}$ mice SN exhibits a mild neuroinflammatory state with lack of astrogliosis (Porrini et al., 2017) that is paralleled by increased oxidative/nitrosative stress and decreased levels of DA transporter in the striatum (Parrella et al., 2019). At this premotor age, c-rel$^{-/-}$ mice display anxiety and depressive-like behavior (Parrella et al., 2022). Finally, 18-month-old c-rel$^{-/-}$ mice, that show 40% loss of nigral dopaminergic neurons, accumulation of iron and divalent metal transporter 1, with reduced striatal dopaminergic fibers and DA content, exhibit hypomotility and gait-related deficits reversed by L-DOPA treatment (Baiguera et al., 2012). Also, 18-month-old c-rel$^{-/-}$ mice display increased levels of pro-apoptotic Ac-RelA (lys310) in the striatum (Lanzillotta et al., 2015). Of note, SN neurodegeneration in 18-month-old c-rel$^{-/-}$ mice is accompanied by reduced transcription of factors involved in mitochondrial homeostasis and antioxidant system (Bcl-xL, UCP4, UCP5, MnSOD) (Parrella et al., 2019).

These observations suggest that NF-κB/c-Rel dysregulation might contribute to PD pathophysiology. Thus, to check on possible c-Rel defects in PD biological specimens, we evaluated the c-Rel protein level and activity in post-mortem brain and peripheral blood mononuclear cells (PBMCs) of sporadic PD and control subjects. Our results show that the DNA-binding activity of c-Rel is reduced in both post-mortem SN and PBMCs of PD cases. The reduction of c-Rel activity in peripheral cells is evident even in early stage, drug naïve patients. The present findings, corroborated by data on the progressive parkinsonian phenotype of c-rel$^{-/-}$ mice, highlight the possible involvement of c-Rel dysfunction in PD pathophysiology. In addition, they hint that c-Rel activity may be taken into account as a potential biomarker for PD.

2. Material and methods

2.1. Human tissues

Fresh frozen tissue of the SN from 10 PD patients and 9 age-matched control subjects were kindly supplied by the Parkinson's UK Brain Bank, a Charity funded by Parkinson's UK (Imperial College London, London, UK). The study on human brain samples was performed in accordance of the local clinical research regulations and obtained approval from the Ethics Committee of Brescia District (NP no. 1537, 3 December 2013).

2.2. Participants

Patients with a clinical diagnosis of PD were recruited at Neurology Unit of ASST Spedali Civili Hospital, University of Brescia, Italy. Caregivers of patients were asked to participate as healthy controls (HC) and were matched for age and prevalence of autoimmune diseases to PD patients. Each subject underwent a standardized interview for concomitant diseases and medications and a neurological examination including the motor assessment with the Unified Parkinson's disease Rating Scale (UPDRS)-III score (Pilotto et al., 2016). PD patients under dopaminergic treatment were evaluated during ON periods of the therapy. Autoimmune disorders and vascular risk factor were considered as possible variables correlated to c-Rel modifications (Gilmore and Gerondakis, 2011), (Gaspar-Pereira et al., 2012). Each patient underwent brain structural imaging in order to exclude prominent cortical/subcortical infarcts or atypical parkinsonism. Total L-DOPA equivalent Daily Dose (LEDD) was calculated according to standard conversion as previously reported (Pilotto et al., 2018). The following exclusion criteria were applied: (1) atypical Parkinsonism including corticobasal syndrome, progressive supranuclear palsy, multiple system atrophy, dementia with Lewy bodies at baseline; (2) other neurological disorders or significant medical problems (i.e., hepatic or renal failure, chronic respiratory insufficiency) potentially responsible for encephalopathy; (3) deep brain stimulation; (4) bipolar disorder, schizophrenia, history of drug or alcohol abuse; (5) dementia according to current PD dementia criteria (Emre et al., 2007); (6) negative nigrostriatal dopaminergic imaging; (7) recent traumatic events or acute fever/inflammation at the time of blood sample (Pilotto et al., 2021). This study was approved by Ethics Committee of Brescia District (NP no. 2209, 12 February 2016) and in conformity with the Helsinki Declaration. Informed consent was obtained from all participants.

2.3. Isolation of PBMCs from blood samples

PBMCs were isolated from the venous blood of 72 PD patients and 40 age-matched healthy subjects of both sexes and between 40 and 81 years

of age, recruited for postmeridian follow-up at the Neurology Divisions of the University of Brescia. The subjects enrolled in the study were not fasted. The PBMCs preparation was done on ACCUSPIN System-HISTOPAQUE-1077 (polysaccharose and sodium diatrizoate, density 1.077 ± 0.001 g/ml; Sigma Aldrich), according to the following procedures.

The ACCUSPIN System-HISTOPAQUE-1077 and phosphate buffer (PBS) solutions (NaCl 8 g/l; KCl 0.2 g/l; $Na_2HPO_4•2H_2O$ 1.44 g/l; KH_2PO_4 0.24 g/l; pH 7.4) were adjusted to room temperature. Ten ml of blood were collected from the subjects into vacutainer tubes containing EDTA as anticoagulant (Grossi et al., 2021). All the collected blood was combined in a single 50-ml tube and centrifuged at 1200 x *g* for 10 min at room temperature. The blood cell pellet was adjusted to a volume of 20 ml with PBS. The diluted blood was layered onto the porous high-density polyethylene barrier over the HISTOPAQUE-1077 stratum in each tube, and then centrifuged at 800 x *g* for 15 min without break. The PBMCs ring that separates above the barrier was collected and transferred into a new 50-ml tube. The volume was adjusted to 30 ml with PBS, followed by centrifugation at 250 x *g* for 10 min. The supernatant was discarded, and the cell pellet was resuspended. The volume was adjusted to 25 ml with PBS. This was again centrifuged at 400 x *g* for 7 min, the supernatant was discarded and the pellet containing PBMCs was stored at −80 °C until analysis.

2.4. DNA-based ELISA analysis of c-Rel activity

The binding activity of NF-κB/c-Rel subunit was measured in total protein extracts from human brain tissues or PBMCs by a DNA-based ELISA technique, using the TransAM®-NF-κB kit (Active Motif) with some modifications.

Frozen human SN tissues or PBMCs frozen pellets were lysed by sonication in 100–140 μl of cold Lysis buffer consisting of 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 2 mM EDTA, 0.5% NP40, 1 mM PMSF and 1× protease inhibitor cocktail (Sigma Aldrich), and then centrifuged at 15000 x *g* for 20 min at 4 °C. The supernatant-containing proteins was collected and the protein extracts were quantified, aliquoted and stored at −80 °C.

ELISA conditions were set in a first series of PBMCs collected from 23 PD patients and 17 age-matched HC recruited at San Camillo Hospital (Venice). The c-Rel activity was evaluated by TransAM®-NF-κB kit following kit manufacturer's instructions, except for the primary antibody that was the anti-c-Rel antibody (C-terminal) (# sc-71, Santa Cruz Biotechnology). Data shown in Supplementary Fig. 1 revealed a significant decrease of c-Rel DNA-binding activity in PD patients compared to HC subjects, expressed as optical density (median: 12 for PD patients, 180 for HC subjects; Wilcoxon *p*-value = 0.006).

To achieve the quantification of DNA-bound c-Rel in post-mortem SN as well as in PBMCs collected at the Neurology Divisions of University of Brescia, the first antibody was replaced with the anti-c-Rel antibody (N-terminal) (# sc-70, Santa Cruz Biotechnology) allowing the recognition of the N-terminal c-Rel peptide used to obtain a standard curve.

Aliquots of human SN (50 μg) or PBMCs (80 μg) total proteins extracts were transferred to 96-well plates containing high-density immobilized κB oligonucleotides. In parallel, increasing amounts of a c-Rel peptide (# sc-4030, Santa Cruz Biotechnology), corresponding to aminoacids 1–300 of the transcription factor and containing both the DNA-binding and dimerization sequences, were analyzed. Either the c-Rel peptide or the active form of c-Rel subunit, bound to the target DNA sequence, were detected by primary anti-c-Rel antibody (N-terminal, # sc-70, 1:1500), followed by the secondary HRP-conjugated antibody (1:1000) provided by the kit. The developing solution was added for 10 min and the absorbance of the samples was measured with a spectrophotometer at a wavelength of 450 nm. The data obtained are expressed as ng of c-Rel protein over μg of total protein extracts.

2.5. Western blot analysis

The levels of c-Rel protein content in SN and PBMCs extracts were evaluated by western blot technique (Faggi et al., 2019). Briefly, total protein extracts (60 μg proteins/sample) were resolved by 4%–12% SDS PAGE gel and transferred to a nitrocellulose membrane (Amersham). Membranes were then incubated with either anti-c-Rel (1:200, # sc-71, Santa Cruz Biotechnology), anti-Ac-RelA (lys310) (1:500, # ab19870, Abcam) or anti-GAPDH (1:2000, #MAB374, Merck Millipore) primary antibody and secondary antibodies coupled to alkaline phosphatase (AP) (1:2000, Promega). Immunopositive bands were visualized by AP chemiluminescent substrate (Novex®, ThermoFisher). Gel analysis was performed using the Gel Pro.3 analysis software (MediaCybernetics).

2.6. Statistical analyses

Descriptive statistics (stratified respect HC/PD or HC/PD/PD naïve) were computed for the datasets analyzed. Specifically: a) mean, standard deviation (sd), median and range (min-max) for quantitative variables; b) absolute (n) and percentage (%) frequencies for qualitative variables (nominal and ordinal). Differences in subgroups HC/PD (or HC/PD/PD naïve) were evaluated with Wilcoxon test (or Kruskal-Wallis when more than two groups are compared) for quantitative variables, or Fisher exact test for qualitative variables (in all cases two-tailed test with significance level equals to 0.05) (Marziano et al., 2019). In case of multiple comparison (HC/PD/PD naïve), the pairwise tests were adjusted with Hommel correction.

Spearman coefficient (ρ_s) and corresponding test (Dancelli et al., 2013), (Abate et al., 2020), (Salvi et al., 2019), (Meroni et al., 2021) were used for evaluating the correlation between c-Rel activity and relative protein content.

Differences in peripheral c-Rel DNA-binding activity have been evaluated by means of a linear model adjusted for the effect of age, sex, autoimmune disease and hypercholesterolemia. This analysis was repeated on: (*i*) HC and (*ii*) PD.

3. Results

3.1. Reduced DNA-binding activity of NF-κB/c-Rel in post-mortem SN of PD patients

To test possible alterations of c-Rel function in PD brain, we analyzed the DNA-binding activity of the transcription factor in protein extracts of

Table 1
Clinical characteristics of PD patients and age-matched HC cases from the UK PD Brain Bank.

Variables	HC ($n = 9$)	PD ($n = 10$)	p-value
Age (years)			
mean ± sd	78.67 ± 8.08	78.90 ± 5.69	0.947 (a)
median	78.00	80.50	
range (min – max)	68.00–90.00	69.00–87.00	
Sex (female)			
n (%)	5 (55.56%)	4 (40.00%)	0.656 (b)
Age at disease onset (years)			
mean ± sd	–	63.30 ± 10.21	–
median		66.50	
range (min – max)		42.00–76.00	
Disease duration (years)			
mean ± sd	–	16.00 ± 8.56	–
median		14.00	
range (min – max)		4.00–34.00	
PMI (years)			
mean ± sd	23.22 ± 12.73	13.89 ± 6.07	0.102 (a)
median	22.00	15.00	
range (min – max)	10.00–48.00	2.00–22.00	

(a) Wilcoxon test; (b) Fisher exact test.

the SN from post-mortem brains of 10 PD patients and 9 age-matched HC. Table 1 reports descriptive statistics on their clinical characteristics (age, sex, and Post-mortem Interval - PMI), stratified for HC subjects and PD patients. No significant differences were observed in the two groups (all p-values >0.05).

c-Rel activity was measured by DNA-based ELISA estimating the amount of c-Rel-containing dimers capable to bind the κB-DNA oligonucleotide. Of note, we observed a significant reduction in the level of c-Rel DNA-binding in PD patients compared to HC subjects (median: 0.02 and 0.14 ng/μg of protein, respectively, Wilcoxon test p-value = 0.004, Fig. 1A). Moreover, age, age at disease onset, disease duration, PMI and sex do not influence the c-Rel activity (correlation test p-value and Wilcoxon-test p-value are all greater than 0.05).

To correlate the c-Rel DNA-binding activity with the relative protein content of the transcription factor, c-Rel expression was also evaluated by western blot in the protein extracts from the SN. No significant difference in c-Rel expression was detected between PD and HC groups (p-value = 0.437, Fig. 1B and C). The lack of correlation between the activity and the protein content of c-Rel was confirmed by computing the Spearman correlation coefficients and corresponding test either considering all the subjects analyzed (ρ_s = 0.22, p-value = 0.374, Fig. 1D) or separated groups (ρ_s = 0.16, p-value = 0.657 for PD patients; ρ_s = 0.46, p-value = 0.213 for HC subjects).

As observed in 18-month-of-age c-rel$^{-/-}$ mice, we detected an increase of Ac-RelA (lys310) in the SN of PD patients, compared to HC subjects (Wilcoxon test p-value = 0.003, Fig. 2A and B). An inverse correlation was found between the acetylation state of RelA in lysine 310 and c-Rel DNA-binding activity, as confirmed by computing the Spearman correlation coefficients and corresponding test considering all the subjects analyzed (ρ_s = 0.505, p-value = 0.0345, Fig. 2C).

3.2. Reduced DNA-binding activity of NF-κB/c-Rel in PBMCs of PD patients

To understand whether c-Rel dysfunction is a trait of PD pathology detectable also in peripheral tissues, we analyzed c-Rel activity in

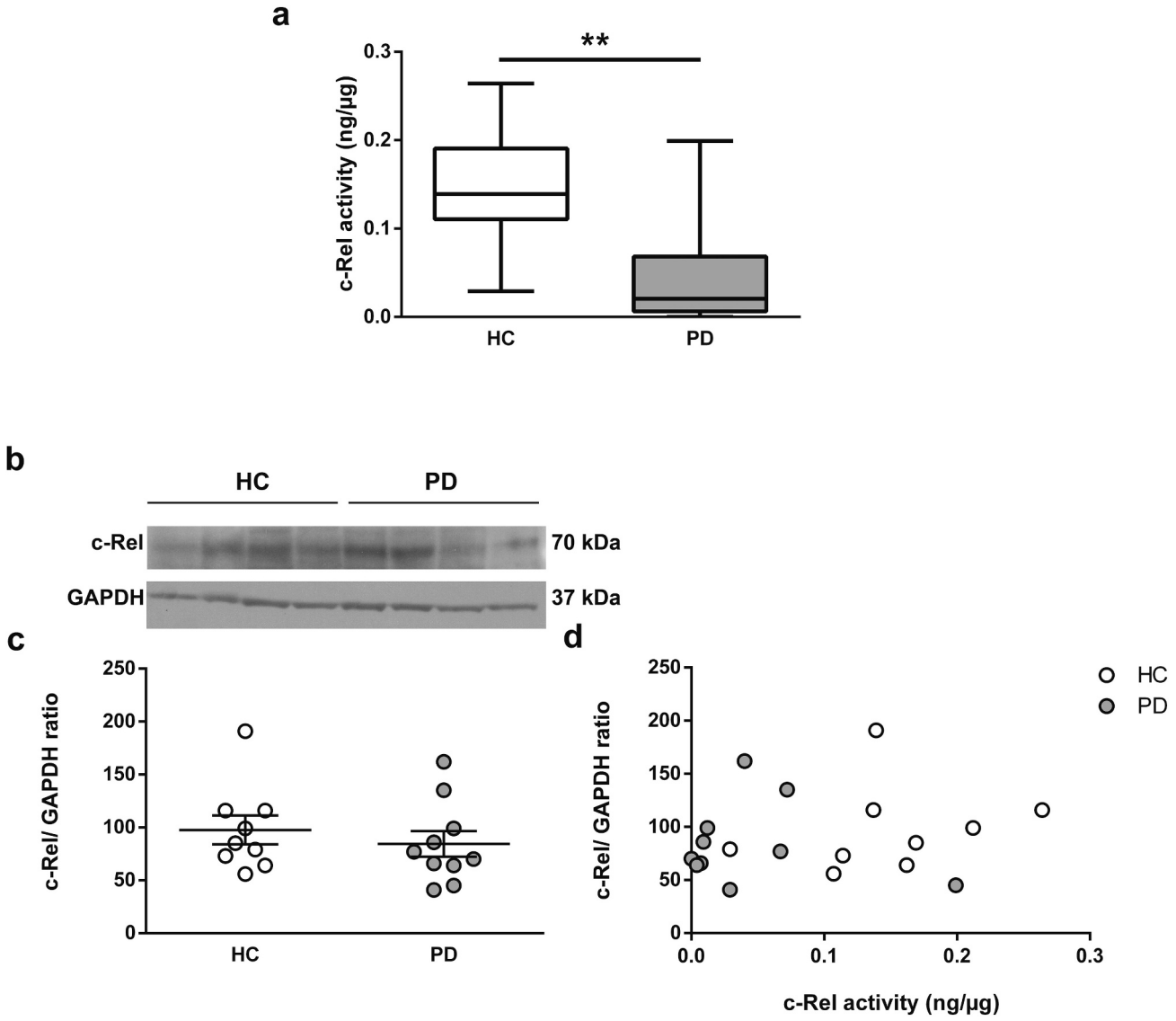

Fig. 1. NF-κB/c-Rel DNA-binding activity and protein expression in the SN protein extracts from post-mortem brains of PD patients and age-matched HC. (a) Decrease of NF-κB/c-Rel DNA-binding activity in SN of PD patients (represented by Tukey boxplot where the central lines of the boxes correspond to median values). Data were expressed as ng of c-Rel protein over μg of total protein extracts. ** $p < 0.01$, Wilcoxon test. (b) Representative immunoblotting and (c) densitometric analysis revealed no difference in c-Rel protein levels between PD patients and HC subjects. Data are expressed as mean ± sd. (d) Scatterplot shows no correlation (evaluated by means of Spearman correlation coefficient) between c-Rel activity and protein content. n = 9 for HC subjects, n = 10 for PD patients.

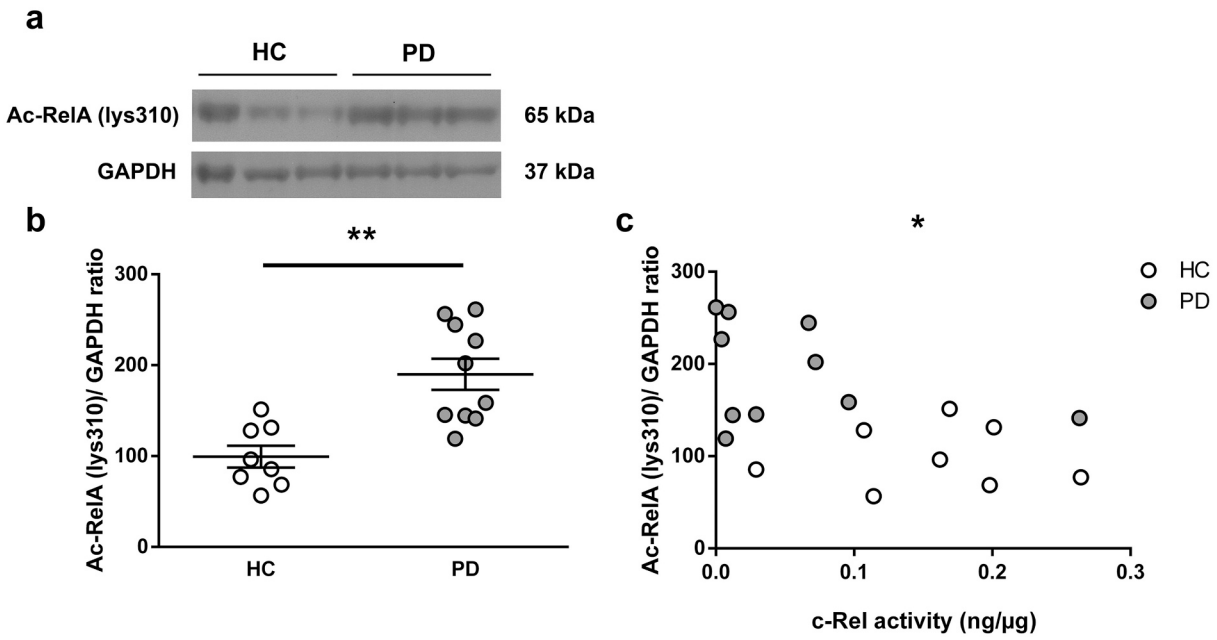

Fig. 2. NF-κB/RelA acetylation of lysine in the 310 position in the SN protein extracts from post-mortem brains of PD patients and age-matched HC. (a) Representative immunoblotting and (b) densitometric analysis revealed increase of Ac-RelA (lys310) levels in SN of PD patients. Data are expressed as mean ± sd. ** $p < 0.01$, Wilcoxon test. (c) Scatterplot shows an inverse correlation (evaluated by means of Spearman correlation coefficient) between c-Rel activity and RelA acetylation state in lysine 310. $n = 8$ for HC subjects, $n = 10$ for PD patients.

PBMCs from a cohort of 72 PD patients and 40 age-matched controls (Table 2).

PD patients were either drug-naïve (21 PD naïve) or under dopaminergic therapy (51 PD subjects). When compared to the PD under therapy (evaluated during ON periods), the PD naïve patients had a lower disease duration (median: 5.50 vs 1.00, *p*-value <0.01) and higher UPDRS-III score (median: 17.00 vs 25.00, p-value = 0.029). No association between PD, naïve or under therapy, and autoimmune disease, diabetes, or hypertension was detected (Table 2, *p*-values >0.05). Hypercholesterolemia was more frequent in PD and PD naïve patients (29.41% and 47.62% respectively, while for HC patients 12.50%, *p*-value = 0.019. Using the pairwise comparison HC vs PD: *p*-value = 0.148; HC vs PD naïve: p-value = 0.013; PD vs PD naïve: p-value = 0.177). This was in line with other studies (Hu et al., 2008), (Miyake et al., 2010), even if the occurrence and the role of hypercholesterolemia in PD patients is still debated (Paul et al., 2015), (Jin et al., 2019).

Table 2
Clinical characteristics of PD patients (drug naïve or in follow-up with dopaminergic drugs) and HC.

Variables	HC ($n = 40$)	PD ($n = 51$)	PD naïve ($n = 21$)	p-value
Age at evaluation (years)				
mean ± sd	66.16 ± 8.32	68.35 ± 8.43	66.10 ± 9.03	0.418 (c)
median	69.00	70.00	69.00	
range (min – max)	40.00–80.00	44.00–81.00	47–77	
Sex (female)				
n (%)	26 (65.00%)	23 (45.10%)	7 (33.33%)	***0.039*** (b)
Disease duration (years)				
mean ± sd	–	6.04 ± 3.86	1.33 ± 1.28	***<0.01*** (a)
median		5.50	1.00	
Range (min – max)		0.00–16.00	0.00–4.00	
UPDRS-III (total score)				
mean ± sd	–	17.62 ± 9.77	25.10 ± 12.27	***0.029*** (a)
median		17.00	25.00	
range (min – max)		2.00–40.00	10.00–53.00	
LEDD (mg/die)				
mean ± sd	–	417.28 ± 263.15		–
median		344.50	-	
range (min – max)		0.00–1035.00		
Autoimmune diseases (Yes)				
n (%)	8 (20.00%)	9 (17.65%)	0 (0.00%)	0.053 (b)
Diabetes (Yes)				
n (%)	3 (7.50%)	3 (5.88%)	2 (9.52%)	0.847 (b)
Hypertension (Yes)				
n (%)	11.00 (27.50%)	20.00 (39.22%)	10.00 (47.62%)	0.389 (b)
Hypercholesterolemia (Yes)				
n (%)	5.00 (12.50%)	15.00 (29.41%)	10.00 (47.62%)	***0.019*** (c)

(a) Wilcoxon test; (b) Fisher exact test; (c) Kruskal-Wallis test. Pairwise comparison with Hommel corrections is reported in the main text. In bold and italics: *p*-values <0.05.

DNA-based ELISA analysis on PBMCs protein extracts revealed a decreased c-Rel DNA-binding activity in both PD patients under medication and PD drug-naïve compared to HC (median: 0.087, 0.084, and 0.124 ng/μg of protein, respectively; Kruskal-Wallis p-value = 0.006). In detail, the pairwise comparison highlighted differences between HC and PD patients while the two different groups of PD patients were homogeneous (HC vs PD: p-value = 0.016; HC vs PD naïve: p-value = 0.014; PD vs PD naïve: p-value = 0.511, Fig. 3A).

It is noteworthy that c-Rel DNA-binding activity did not correlate with age at the time of recruitment or at the disease onset, with the disease duration, with UPDRS-III score in drug-naïve or under therapy PD patients and, in the latter, with the LEDD (all correlation test *p*-values >0.05), suggesting that c-Rel alterations were present from the first stages of the disease. Moreover, the median values of c-Rel activity were not significant different in subjects (PD or HC) with autoimmune diseases, diabetes, hypertension, or in males respect to females (all Wilcoxon p-values >0.05). On the contrary, the median level of c-Rel activity decreased in subjects with hypercholesterolemia compared to those with normal level of cholesterol (0.08 versus 0.11 ng/μg of protein, Wilcoxon *p*-value = 0.005).

To better understand the relationship between the c-Rel activity (outcome) and the hypercholesterolemia (covariate), two linear models (the first on all PD patients and the second on HC subjects), adjusted for the effect of age, sex, and the autoimmune disease (additional covariates), were estimated. The choice of estimating two models was taken following the result of the Bartlett's test, which confirms that the subpopulations of PD patients and HC subjects are not homogeneous with respect to the covariates used in the models (K-square statistic = 1149.6, with a corresponding *p*-value <0.001). Only PD patients showed a hypercholesterolemia inversely related to c-Rel activity (coefficient of the model = −0.036 with a p-value = 0.026; HC subjects showed a coefficient = −0.017 with a *p*-value = 0.593). This result supports that c-Rel activity defect and hypercholesterolemia are distinct traits in PD patients analyzed in this study. The coefficients of the remaining covariates (sex, age, and autoimmune disease) are not significant for both models (all *p*-values are >0.05, Supplementary Table 1).

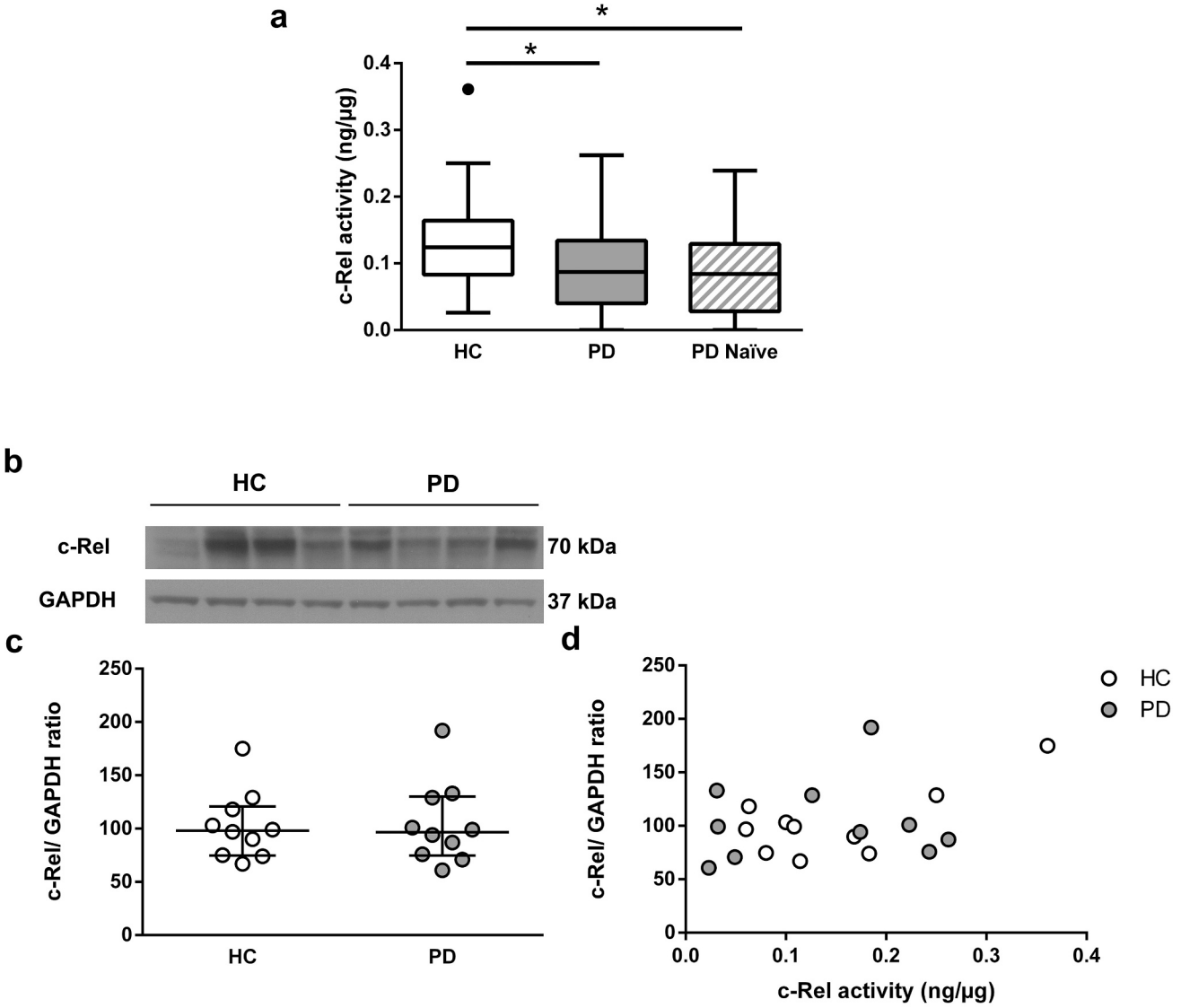

Fig. 3. NF-κB/c-Rel DNA-binding activity and protein expression in PBMCs protein extracts from PD patients and age-matched HC. (a) Decrease of NF-κB/c-Rel DNA-binding activity in PBMCs of drug-naïve ($n = 21$) or treated ($n = 51$) PD patients compared to HC subjects ($n = 40$) (represented by Tukey boxplot where the central lines of the boxes correspond to median values). Data were expressed as ng of c-Rel protein over μg of total protein extracts. * $p < 0.05$. (b) Representative immunoblotting and (c) densitometric analysis revealed no difference in c-Rel protein levels among two selected groups of PD patients and HC subjects (n = 10). Data are expressed as mean ± sd. (d) Scatterplot shows no correlation (evaluated by means of Spearman correlation coefficient) between c-Rel activity and protein content.

Finally, the PBMCs protein extracts of two small representative groups composed of 10 PD patients and 10 HC from the latter cohort, both of which including subjects with low or high levels of c-Rel DNA-binding activity, were selected to evaluate the expression levels of the transcription factor. In line with the results on the protein lysates from the post-mortem SN, no difference in c-Rel protein content was detected among HC subjects and PD patients (p-value = 1.000, Fig. 3B and C). Also, Spearman correlation coefficients and corresponding test confirmed the lack of correlation between the activity and the protein content of c-Rel, either considering all the subjects analyzed ($\rho_s = 0.24$, p-value = 0.309, Fig. 3D) or separated groups ($\rho_s = 0.067$, p-value = 0.865 for PD patients; $\rho_s = 0.20$ p-value = 0.584 for HC subjects), supporting that the defect in c-Rel activity is not associated with a reduction in the expression of the protein.

4. Discussion

The results of this study indicate that the DNA-binding activity of NF-κB/c-Rel factor is reduced in the SN of post-mortem PD brains as well as in the PBMCs of PD patients under therapy or drug-naïve. This observation suggests a possible role of c-Rel deficit in PD pathophysiology that fully collimates with the manifestation of a PD-like phenotype with prodromal symptoms, Braak-like stereotyped diffusion of synucleinopathy, mild inflammation without gliosis and late-onset L-DOPA-responsive parkinsonism in c-rel$^{-/-}$ mice. (Baiguera et al., 2012), (Porrini et al., 2017), (Parrella et al., 2019), (Parrella et al., 2022). Likewise the brain of c-rel$^{-/-}$ mice, the SN of PD cases displayed increased levels of the pro-apoptotic NF-κB/RelA acetylated at the lysine 310 residue. The Ac-RelA (lys310) amounts inversely correlated with c-Rel DNA-binding activity.

Consistently with the demonstration of c-Rel contribution in preserving SN health in mice, a recent study carried out in a MPTP-induced model of PD showed that c-Rel inhibition exacerbates the noxious effect of the neurotoxin by promoting nigrostriatal dopaminergic neuron damage and microglial activation (Wang et al., 2020b).

Here we show that the defect in DNA-binding activity of c-Rel in the post-mortem SN of sporadic PD cases did not correlate with a reduction of protein levels. c-Rel activity was also impaired in PBMCs protein extracts from a cohort of PD patients, compared to HC. Interestingly, c-Rel DNA-binding was reduced both in the PBMCs from drug-naïve PD subjects and in those from PD patients under dopaminergic therapy, indicating that c-Rel activity deficit is not secondary to drug treatment. Moreover, since drug-naïve PD patients, compared to PD subjects under therapy, had lower disease duration, the c-Rel activity deficit appears independent from the progression of PD pathology.

Our data also support that, as observed in the protein extracts from the SN, the defect of c-Rel DNA-binding in PBMCs was not due to a reduction in the content of c-Rel protein. Although the lack of changes in c-Rel protein appeared in contrast with the reduction of c-Rel mRNA observed in whole blood of PD patients reported by Wang and co-authors (Wang et al., 2020b), such a discrepancy could rely on the fact that these authors analyzed “whole blood” rather than PBMCs. In agreement with our evidence, a recent RNA-seq analysis showed that the *REL* gene is not differentially expressed in monocytes, a subpopulation of PBMCs, from PD patients and HC subjects (Schlachetzki et al., 2018). Therefore, the reduction of c-Rel DNA-binding activity in PBMCs of PD subjects most likely results from post-translational modification of c-Rel protein occurring, or even anticipating, PD pathology. Future studies are warranted to probe this hypothesis.

Despite the multiple evidence suggesting a potential involvement of c-Rel deficiency in PD pathogenesis, the molecular underpinnings still need to be elucidated. c-Rel-containing NF-κB dimers promote the transcription of *UCP4*, *MnSOD* and *Bcl-xL* genes preserving oxygen/nitrogen free radicals balance and mitochondrial health (Lanzillotta et al., 2015), (Ramsden et al., 2012), (Chen et al., 2011), (Berman et al., 2009), (Ho et al., 2012), (Flynn and Melov, 2013), (Halliwell, 2012), (Bernard et al., 2001), (Chen et al., 2000), (Sarnico et al., 2009), (Pizzi et al., 2005). A body of evidence shows that during aging, or in the early stage of PD, loss of mitochondrial redox homeostasis became evident in the brain (Jenner and Olanow, 1996), (Cassarino et al., 1997), (Mattson and Arumugam, 2018) (Cardozo-Pelaez et al., 1999). The consequent accumulation of oxygen/nitrogen free radicals has been suggested to contribute to α-syn aggregation and neuronal death (Schildknecht et al., 2013), (Ganguly et al., 2017) (Hodara et al., 2004), (Yu et al., 2010). Notably, the c-Rel target genes regulating mitochondrial homeostasis in PD, *Bcl-xL* and *MnSOD* transcripts were reduced in the SN of 18-month-old c-rel$^{-/-}$ mice (Parrella et al., 2019) as in post-mortem PD brains (Simunovic et al., 2009). Collectively, these findings support that c-Rel dysfunction could affect PD pathogenesis by impinging mitochondrial homeostasis.

Moreover, it is worth considering that c-Rel is highly expressed in mature hematopoietic cells (Liou and Hsia, 2003), (Liou et al., 1994), (Carrasco et al., 1994), (Weih et al., 1994) and is involved in the adaptive immunity, in particular in T and B lymphocyte differentiation and function (Gilmore and Gerondakis, 2011). c-Rel-mediated transcription plays an important role in regulatory T cells (Treg) development, through the induction of Foxp3 expression that is fundamental for Treg function (Fulford et al., 2015). In addition, c-Rel alterations have been linked to human diseases involving immune system, in particular the *REL* gene is a susceptibility locus for a plethora of autoimmune diseases, i.e. celiac disease (Trynka et al., 2009) and rheumatoid arthritis (Gregersen et al., 2009), (Eyre et al., 2010), (Varadé et al., 2011), and lymphoid malignancies, i.e. B cell lymphoma (Hodgkin’s and non-Hodgkin’s), or chronic lymphocytic leukemia (Gilmore and Gerondakis, 2011).

Interestingly, peripheral adaptive immunity has been reported to be also involved in PD (Varadé et al., 2011), (Mosley et al., 2012), (Cappellano et al., 2013). T lymphocytes (both CD8+ and CD4+ subtypes) have been found in post-mortem brain specimens from PD subjects and in PD animal models (Brochard et al., 2009), (Fuzzati-Armentero et al., 2019). Circulating CD4+ T lymphocytes are decreased in PD patients (Jiang et al., 2017), (Kustrimovic et al., 2018), especially T helper (Th) 17, Th2 and Treg subsets, and appear more prone to acquire the pro-inflammatory Th1 phenotype (Kustrimovic et al., 2018). The acquisition of Th1 profile by CD4+ T lymphocytes in PD subjects could also be strengthened by Treg impairment in controlling pro-inflammatory cytokines release by effector T cells (Kustrimovic et al., 2018), (Saunders et al., 2012). Likewise PD patients, c-rel$^{-/-}$ mice display a defect in T and B lymphocytes proliferation, survival and differentiation, with important reduction of Treg number (Gilmore and Gerondakis, 2011). These observations highlight that beside increasing susceptibility of brain dopaminergic neuron to aging, reduced c-Rel activity in PBMCs may have a role in alterations of adaptive immunity associated with systemic inflammation occurring in PD brain (Przedborski, 2010), (Mosley et al., 2012), (Cappellano et al., 2013), (Brochard et al., 2009), (Fuzzati-Armentero et al., 2019).

We acknowledge some limitations of the study, mainly related to cross-sectional design and limited sample size, not allowing a proper evaluation of different progression patterns of patients according to the individual c-Rel baseline levels. Furthermore, the study tested c-Rel activity and expression in PBMCs and further analyses on cerebrospinal fluid are thus warranted to extend the findings. Furthermore, the study did not include other neurodegenerative conditions including Alzheimer’s or Lewy bodies diseases, that definitively need to be tested in the next future. Limitations notwithstanding, this is one of first studies claiming for an early involvement of NF-κB/c-Rel pathways in PD supported by both neuropathology and in vivo peripheral findings. Additionally, results on PBMCs from early stage, drug-naïve patients strongly suggest that peripheral c-Rel activity in PD subjects is defective independently from dopaminergic medication or disease progression.

5. Conclusions

In conclusion, we here reported that NF-κB/c-Rel dysfunction is present in both brain and circulating cells of PD patients. These observations, when coupled with the parkinsonian phenotype reported for c-rel$^{-/-}$ mice, suggest that c-Rel alterations can be crucially involved in the pathophysiology of PD. Finally, our findings highlight that the deficit of c-Rel DNA-binding activity in PBMCs deserves further investigation as a potential biomarker of PD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2023.106067.

Ethics approval and consent to participate

The study on human brain samples was performed in accordance of the local clinical research regulations and obtained approval from the Ethics Committee of Brescia District (NP no. 1537, 3 December 2013). The study on PBMCs samples was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Brescia District (protocol code NP no. 2209, 12 February 2016).

Informed consent was obtained from all subjects involved in the study.

Funding

This research was funded by Fondazione Cariplo (Italy), grant n° 2014-0769, by MIUR PON Ricerca e Innovazione 2014–2020 PerMedNet project n° ARS01_01226 and by grants (ex 60%) from the University of Brescia, Italy. VP receives researcher fellowship from SIF-MSD, Italy, and from Fondazione Cariplo, grant n° 2018-0391. The funders had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

CRediT authorship contribution statement

Vanessa Porrini: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Andrea Pilotto:** Investigation, Resources, Data curation, Formal analysis, Writing – review & editing. **Marika Vezzoli:** Formal analysis, Data curation, Writing – review & editing. **Annamaria Lanzillotta:** Validation, Investigation. **Michele M. Gennari:** Validation, Investigation. **Sonia Bonacina:** Resources, Data curation. **Antonella Alberici:** Resources, Data curation. **Rosanna Turrone:** Investigation, Resources. **Arianna Bellucci:** Conceptualization, Supervision, Writing – review & editing. **Angelo Antonini:** Investigation, Resources, Writing – review & editing. **Alessandro Padovani:** Supervision, Writing – review & editing. **Marina Pizzi:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

Tissue samples and associated clinical and neuropathological data were supplied by the Parkinson's UK Brain Bank, funded by Parkinson's UK, a charity registered in England and Wales (258197) and in Scotland (SC037554).

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