Figure 5. Licopyranocoumarin and glycyrurol protected cells against MPP^+-induced disappearance of mitochondrial membrane potential. (**A**) NGF-differentiated PC12D cells were treated with various concentrations of licopyranocoumarin or glycyrurol in the presence of 0.3 mM MPP^+ for 48 h. Collected cells were stained with JC-1 and analyzed by flow cytometry. (**B**) The ratio of cells exhibiting disappearance of mitochondrial membrane potential was analyzed. Values are the means of three independent experiments; bars, s.d. $^*p<0.05$, $^{**}p<0.01$ compared with MPP^+ group cells.
doi:10.1371/journal.pone.0100395.g005

system. Membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is known to be a neurotoxin-related oxidase enzyme system [64,65], and enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), thioredoxin reductase (TPx) and catalase [66]. Therefore, it is likely that LPC and GCR might induce the imbalance by inhibiting oxidase activity directly or neurotoxin-induced activation of oxidase system. Furthermore, we can't exclude the possibility that LPC and GCR could induce the expression or activation of antioxidant enzymes.

In summary, we identified *choi-joki-to* and *daio-kanzo-to* as neuroprotective herbal medicines, and both LPC and GCR were identified as neuroprotective substances from *Glycyrrhiza* contained in *choi-joki-to* and *daio-kanzo-to*. LPC or GCR exert their neuroprotective effects by inhibiting MPP^+-induced ROS production and thus limiting JNK activation, and causing a subsequent decrease in $\Delta\Psi_{mit}$. Our proposed mechanism is illustrated in Figure 9. Further studies are required to elucidate the molecular mechanisms for the suppression of ROS generation by LPC and GCR in PC12D cells. Our findings enliven the prospect of using LPC, GCR, *choi-joki-to* and *daio-kanzo-to* as effective and safe natural therapeutic agents in PD; *in vivo* trials in MPTP animal models are needed.

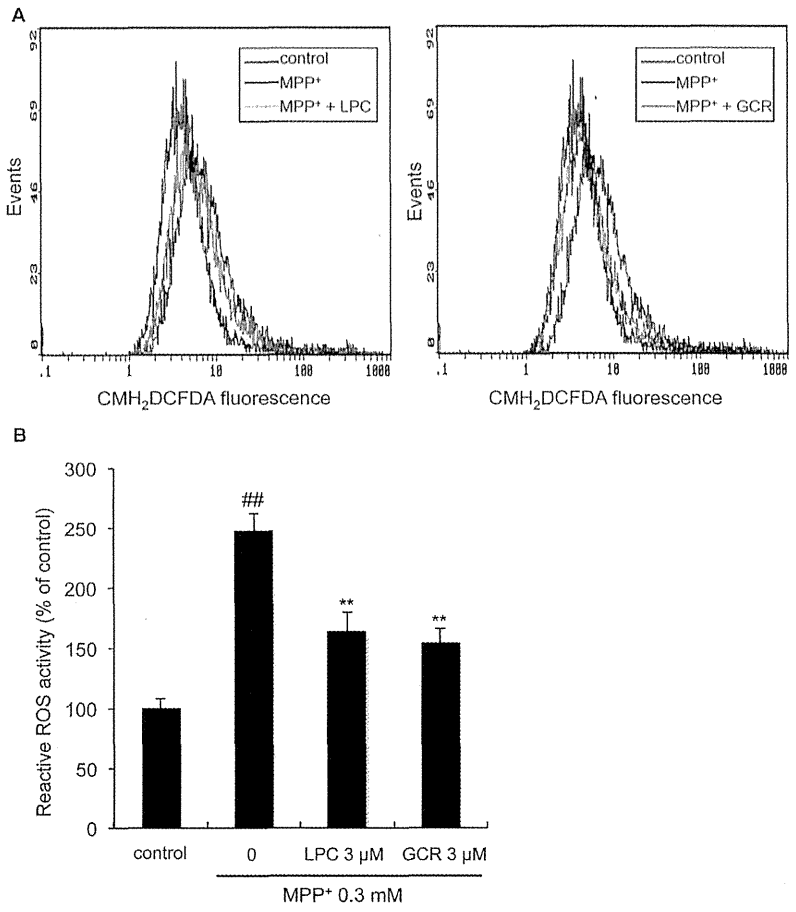

Figure 6. Licopyranocoumarin and glycyrurol decreased MPP^+-induced intracellular ROS generation. (**A**) NGF-differentiated PC12D cells were pre-incubated for 1 h with 3 μM licopyranocoumarin (LPC) or 3 μM glycyrurol (GCR), then treated with 0.3 mM MPP^+ for 12 h. Then, the samples were loaded with 2.5 μM CM-H_2DCFDA and the fluorescence intensities were measured by flow cytometry. (**B**) The ratio of cells exhibiting ROS production was analyzed. Values are the means of four independent experiments; bars, s.d. $^{\#\#}p<0.01$ compared with control cells. $^{**}p<0.01$ compared with MPP^+ group cells.
doi:10.1371/journal.pone.0100395.g006

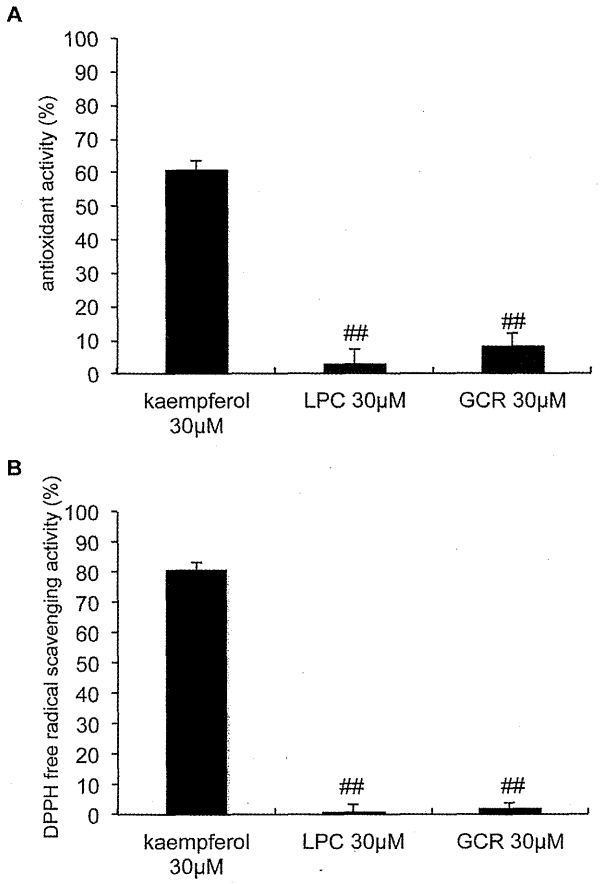

Figure 7. Licopyranocoumarin and glycyrurol lacked potency for scavenging free radicals. Antioxidant activities of licopyranocoumarin (LPC) and glycyrurol (GCR) were measured by (**A**) a β-carotene bleaching assay system and (**B**) a DPPH radical scavenging assay. Kaempferol served as the positive control. Values are the means of three independent experiments; bars, s.d. $^{\#\#}p<0.01$ compared with antioxidant activity of kaempferol.
doi:10.1371/journal.pone.0100395.g007

Materials and Methods

Reagents

MPP^+, Rotenone, linoleic acid, 2,2-Diphenyl-1-pocrylhydrazyl (DPPH), SP600125 and mouse monoclonal anti-β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Taxol, cisplatin, JC-1 and pyridinium iodide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nerve growth factors, CM-H_2DCFDA, and β-carotene standard were purchased from Alomone Labs (Jerusalem, Israel), Life Technologies (Carlsbad, CA) and Kanto Chemical Co. (Tokyo, Japan), respectively. Rabbit polyclonal anti-JNK antibody and rabbit monoclonal anti-phospho-JNK antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling (Beverly, MA), respectively. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG used as a secondary antibodies were from GE Healthcare (Little Chalfont, UK).

Cell cultures

PC12D was identified a new subline of PC12 pheochromocytoma cells (PC12D cells) in which neurites are extended within 24 h in response to cAMP-enhancing reagents as well as in response to nerve growth factor (NGF) [30]. PC12D cells were cultured in Dulbecco's modified Eagle medium supplemented with 5% (v/v) inactivated fetal bovine serum, 10% (v/v) inactivated horse serum, 100 U/mL penicillin G, 0.6 mg/mL L-glutamine, and 0.1 mg/mL kanamycin at 37°C with 5% CO_2. PC12D cells were differentiated by 100 ng/mL NGF treatment for 48 h.

Cell viability assays

For the trypan blue dye exclusion assay, differentiated PC12D cells were cultured in 48-well dishes. Drug-treated or untreated cells were stained with trypan blue (Sigma Chemical Co.), and the ratio of viable cells was determined using a hemocytometer. Cell viability (%) means the ratio of the number of trypan blue-impermeable cells to total cell count. IC_{50} values were calculated by linear regression analysis from the inhibition of MPP^+-induced cell death at different concentrations of the drug.

Cell cycle analysis

To examine apoptosis, differentiated PC12D cells were harvested after drug treatment. The cells were washed with PBS and fixed with 70% ethanol at 4°C for more than 1 h. The cells were then stained with propidium iodide (PI) solution according to a previously reported protocol [67]. The labeled nuclei were subjected to flow cytometry (FCM, Beckman-Coulter, Miami, FL).

Measurements of mitochondrial membrane potential

Changes in mitochondrial membrane potentials were assessed JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraehylbenzimidazolylcarbocyanineiodide) (Wako) was used according to the manufacturer's protocol. Briefly, treated cells were collected by pipetting and removing medium. Next, the cells were incubated in medium containing 2.5 μg/ml JC-1 for 20 min at 37°C. Cells were then washed with PBS. JC-1 fluorescence was measured by a flow cytometer.

Measurement of intracellular ROS

Intracellular ROS production was measured using CM-H_2DCFDA. The cells were plated at a density of 12×10^4 cells per 12-well dish. The cells were treated with MPP^+ and test compounds for 12 h, and then trypsinized and collected. After the cells were washed with PBS, incubated with 2.5 μM CM-H_2DCFDA in HBSS at 37°C for 30 min, and then washed again with PBS three times. The relative levels of fluorescence were quantified by using a flow cytometer.

β-carotene bleaching assay

This assay was carried out according to the β-carotene bleaching method [68]. A mixture of β-carotene and linoleic acid was prepared by adding a mixture of 0.3 mg of β-carotene in 3 mL chloroform, 40 mg linoleic acid and 400 mg Tween 20. Chloroform was removed and 100 mL of distilled water was added to form an emulsion with continuous shaking. Aliquots (0.1 mL) of the β-carotene/linoleic acid emulsion were mixed with 1 μL of sample solution and incubated in a water bath at 50°C. The oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60-min period. Control samples contained 1 μL of methanol. Antioxidant activity is expressed as percent inhibition relative to control after 60 min incubation using the following equation:

$$AA(\%) = 100(DR_c - DR_s)/DR_c,$$

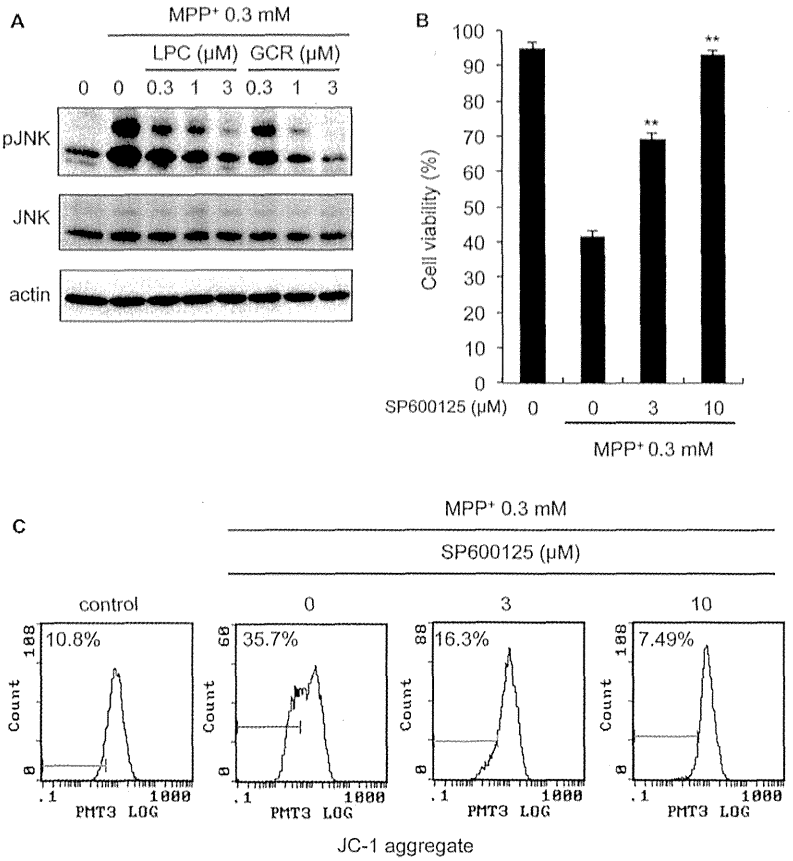

Figure 8. Licopyranocoumarin and glycyrurol attenuated MPP$^+$-induced JNK activation. (**A**) NGF-differentiated PC12D cells were treated with various concentrations of licopyranocoumarin (LPC) or glycyrurol (GCR) and 0.3 mM MPP$^+$ for 36 h, and JNK and phosphor-JNK level were detected by Western blot. NGF-differentiated PC12D cells were treated with SP600125 and 0.3 mM MPP$^+$ for 48 h. Thereafter (**B**) cell viability was measured by trypan blue dye exclusion assay and (**C**) mitochondrial membrane potentials were assessed by JC-1 assay. Values of (B) are the means of three independent experiments; $^{**}p<0.01$ compared with MPP$^+$ group cells.
doi:10.1371/journal.pone.0100395.g008

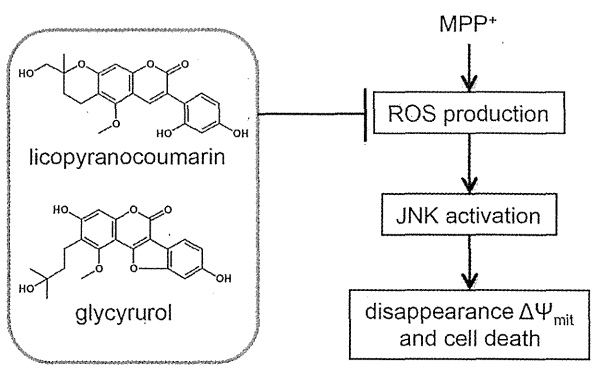

Figure 9. Suggested model for neuroprotection of licopyranocoumarin and glycyrurol against MPP$^+$-induced toxicity in PC12D cells. Both licopyranocoumarin and glycyrurol exert neuroprotective effects against MPP$^+$-induced toxicity via suppression of ROS generation and of JNK activation.
doi:10.1371/journal.pone.0100395.g009

where AA = antioxidant activity; DR_c = degradation rate of the control = $[\ln(a/b)/60]$; DR_s = degradation rate in presence of the sample = $[\ln(a/b)/60]$; a = absorbance at time 0; b = absorbance at 60 min.

DPPH radical scavenging assay

The DPPH radical scavenging effect of test compounds was determined according to the previously described method [68]. The reaction mixtures contained 100 μL ethanol, 125 μM DPPH, and test compounds. After 2 min of incubation at room temperature, the absorbance was recorded at 517 nm.

Extraction and isolation of licopyranocoumarin and glycyrurol from *Glycyrrhiza*

Compounds were extracted from dried and pulverized *Glycyrrhiza* (50 g) with 90% EtOH, then filtrated and concentrated *in vacuo*. This suspension was adjusted to pH 7.0, followed by extraction with EtOAc (5 L) twice; the organic layer was concentrated to yield residue (3.76 g). The EtOAc extract was fractionated by centrifugal partition chromatography (CPC) with $CHCl_3$:MeOH:H_2O (5:6:4). The obtained crude active extract

was applied on Sephadex LH20 column chromatography (Sephadex LH-20, 70 μM; GE Healthcare, NJ, USA), and eluted with MeOH. The active fraction (250.6 mg) was further purified by preparative octadecyl silyl (ODS) HPLC (YMC-Pack ODS-AQ, YMC Co. Ltd., Japan) with 40% aqueous CH_3CN to give pure licopyranocoumarin (10.8 mg) and glycyrurol (4 mg), respectively.

Western blotting

Cells were lysed in RIPA buffer (25 mM HEPES (pH 7.2), 1.5% Triton X-100 (Wako), 1% sodium deoxycholate (Wako), 0.1% SDS, 0.5 M NaCl (Wako), 5 mM EDTA, 50 mM NaF (Sigma), 0.1 mM sodium vanadate (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF) with sonication. The lysates were centrifuged at 13,000 rpm for 15 min to yield the soluble cell lysates. For immunoblotting, cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a polyvinylidene fluoride membrane (Millipore) by electroblotting and then incubated with appropriate antibodies. Immune complexes were detected with an Immobilon Western kit (Millipore), and luminescence was detected with a LAS-1000 mini (Fujifilm Co., Tokyo, Japan).

Statistical analysis

All statistical analyses in bar plots were performed with a two-tailed paired Student's *t*-test.

Supporting Information

Figure S1 The correlation between contents of *Glycyrrhiza* and neuroprotective activity in herbal medicines.
(TIF)

Figure S2 Toxicity of EtOAc extract of *Glycyrrhiza*. NGF-differentiated PC12D cells were treated with various concentrations of EtOAc extract of *Glycyrrhiza* for 48 h. Cell viability was evaluated by trypan blue dye exclusion assay.
(TIF)

Figure S3 Licopyranocoumarin and glycyrurol preferentially showed cytoprotective effects in neuronal cells. HeLa cells or NGF-differentiated PC12D cells were treated with various concentrations of licopyranocoumarin (LPC) or glycyrurol (GCR) in the presence of 0.3 μM Rotenone for 48 h. Cell viability was evaluated by trypan blue dye exclusion assay.
(TIF)

Author Contributions

Conceived and designed the experiments: NH MI. Performed the experiments: TF DY. Analyzed the data: TF MK. Contributed reagents/materials/analysis tools: TF SS ET MI. Wrote the paper: TF SS ET NH MI.

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Parkinsonism and Related Disorders 20S1 (2014) S128–S131

Contents lists available at SciVerse ScienceDirect

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The promises of stem cells: stem cell therapy for movement disorders

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ARTICLE INFO

Keywords:
Stem cell
Movement disorders
Parkinson's disease
Induced pluripotent stem cell

SUMMARY

Despite the multitude of intensive research, the exact pathophysiological mechanisms underlying movement disorders including Parkinson's disease, multiple system atrophy and Huntington's disease remain more or less elusive. Treatments to halt these disease progressions are currently unavailable. With the recent induced pluripotent stem cells breakthrough and accomplishment, stem cell research, as the vast majority of scientists agree, holds great promise for relieving and treating debilitating movement disorders. As stem cells are the precursors of all cells in the human body, an understanding of the molecular mechanisms that govern how they develop and work would provide us many fundamental insights into human biology of health and disease. Moreover, stem-cell-derived neurons may be a renewable source of replacement cells for damaged neurons in movement disorders. While stem cells show potential for regenerative medicine, their use as tools for research and drug testing is thought to have more immediate impact. The use of stem-cell-based drug screening technology could be a big boost in drug discovery for these movement disorders. Particular attention should also be given to the involvement of neural stem cells in adult neurogenesis so as to encourage its development as a therapeutic option.

1. Introduction

Scientists have known of the existence of stem cells, the unspecialized cells found in all multicellular organisms that can self-renew through self-division and differentiate into diverse specialized cell types, for over a century. Yet it has been only since the late 1990s, when human embryonic stem cells were first cultured in the laboratory, that the field of stem cell research has become the focus of intense scientific interest.

There are essentially three kinds of stem cells: embryonic stem (ES) cells, which are isolated from the inner cell mass of blastocysts; adult stem cells, which are found in various developed tissues such as bone marrow cells; and induced pluripotent stem (iPS) cells, which are artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing a "forced" expression of specific genes.

One of the most astounding applications of stem cells is in the treatment and cure of a wide variety of movement disorders including Parkinson's disease (PD), multiple system atrophy (MSA) and Huntington's disease (HD). Several ways of how stem cells are being explored and used in both basic and clinical applications of current movement disorders research include disease modeling, drug toxicity screening/drug discovery, gene therapy and cell replacement therapy.

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1353-8020/$ – see front matter

In most cases, it is difficult to obtain the damaged cells in a disease and to study them in detail. Stem cells, either carrying the disease gene or engineered to carry disease genes, offer an alternative for laboratory studies. Researchers are able to model disease processes *in vitro* and perform more relevant and informative biological assays, thereby better understanding the mechanisms underlying the disease. Stem cells have also been used in the laboratory to screen for new drugs. It has been revealed that very few drugs have been tested on human-diseased cells before human testing. Liver and heart toxicity problems account for about 30% of drugs that fail in early-stage clinical trials, indicating a need for more efficient means of drug toxicity testing before clinical trials. The use of stem cells with specific diseases may correct this situation. Furthermore, given their unique regenerative abilities, stem cells offer the possibility of a renewable source of cell replacement therapies for neurological diseases.

However, stem cell research has been controversial and has raised ethical dilemmas primarily concerning the creation, treatment, and destruction of human embryos inherent to research involving ES cells. The recent discovery of iPS cells, hailed as a potential alternative to ES cells, provides researchers with a unique tool to derive neurons from patient-specific iPS cells for the study of neurological diseases. More importantly, iPS cell research obviates many ethical and resource-related concerns posed by ES cells while prospectively matching their potential for scientific use.

In recent years, the discovery of constitutive ongoing neurogenesis in the adult human brain has challenged the traditional view of a fixed circuitry in functionally normal brains, and has raised high hopes that the adult brain may have the capacity for self-renewal

after injury, thereby sidestepping the need for transplantation. Primary neural precursor cells reside in specialized zones called "neurogenic niches". A population of neural stem cells (NSCs) preserves enough germinal character to maintain neurogenesis throughout life and, once differentiated, their daughter cells integrate into already existing neuronal networks. Whether adult neurogenesis can be induced under certain circumstances in regions that lack constitutive adult neurogenesis remains controversial, but several studies have reported the isolation of NSCs from different regions of the adult brain, including the substantia nigra pars compacta (SNc). Therefore, there has been considerable interest within the scientific community to gain understanding of the possible correlation between neurogenesis and pathogenesis of movement disorders, which could help the future development of novel therapeutic intervention.

2. Stem cell therapy for PD and MSA

There has been a long history of fetal tissue transplantation for the treatment of patients with advanced PD. Despite the wake of a long series of encouraging open-label studies, initial enthusiasm for cell replacement therapy by grafting fetal neuronal precursor cells into the striatum has vanished after two double-blind placebo-controlled clinical trials showing only moderate symptomatic improvement and the occurrence of severe disabling dyskinesia. These problems should be solved before fetal tissue transplantation can be considered a therapeutic option for PD [1].

Studies have shown ES cell transplanted into the brains of PD rat model differentiated into dopaminergic neurons, restoring partial neural function [2]. PD rodent models subjected to engraftment of dopaminergic neurons derived from human ES cells demonstrated complete behavioral restoration and motor function improvement. Similarly, parkinsonian monkeys receiving transplantation showed excellent DA neuron survival, function and lack of neural overgrowth, indicating potential for the development of cell-based therapies in PD [3].

It was recently shown that reprogramming mouse embryonic fibroblasts with four transcription factors Oct4, Sox2, Klf4, and c-Myc induces pluripotency [4], enabling generation of iPS cells from patients with a variety of diseases [5]. iPSC-derived midbrain dopaminergic neurons from a patient with a triplication in the α-synuclein gene (SNCA) showed accumulation of α-synuclein, inherent overexpression of markers of oxidative stress, and sensitivity to peroxide-induced oxidative stress, precisely recapitulating the cause of disease in the patients [6,7]. Comparably, PARK2 iPSC-derived neurons exhibited mitochondrial dysfunction associated with increased oxidative stress and α-synuclein accumulation, resembling pathogenic changes in patient brains [8]. Neurons derived from mutant PINK1 iPS cells displayed impaired recruitment of lentivirally expressed Parkin to mitochondria, increased mitochondrial copy number and upregulation of PGC-1α, an important regulator of mitochondrial biogenesis, upon mitochondrial depolarization [9]. LRRK2 mutant iPSC-derived DA neurons demonstrated increased susceptibility to oxidative stress, consistent with existing understanding of early PD phenotypes [10]. Such disease-specific iPS cells offer an unprecedented opportunity to recapitulate both normal and pathologic human tissue formation *in vitro*, thereby facilitating disease investigation and drug development.

Furthermore, generation of iPS cells provides a new avenue for transplantation therapy as it can avoid immunorejection, a major complication in current transplantation medicine. Wernig et al. [11] reported upon transplantation into the fetal mouse brain, iPSC-derived neural precursor cells extensively differentiate into glia and neurons. Functional recovery was observed after transplantation of iPSC-derived midbrain dopamine neurons into the adult brain of Parkinsonian rats. Risk of tumor formation from grafted cells was minimized by the separation of contaminating pluripotent cells and committed neural cells using fluorescence-activated cell sorting. Encouraging data from rodent studies then prompted subsequent assessment in a primate model. Kikuchi et al. [12] observed that human iPSC-derived neural progenitor cells grafted in the brain of a primate PD model survived as dopaminergic neurons for as long as six months, implying the therapeutic potential of human iPS cells. Direct reprogramming of mouse and human fibroblasts into induced neural stem cells (iNSCs) has been proven feasible with a single factor, Sox2. iNSCs express NSC markers and resemble wild-type NSCs in their morphology, self-renewal, ability to form neurospheres and differentiate into several types of mature neurons as well as astrocytes and oligodendrocytes, indicating multipotency. Importantly, implanted iNSCs can survive and integrate in mouse brains without tumorigenic potential. As an additional merit, this method allows shortening of the duration for neuronal cell creation from fibroblasts [13].

Adult stem cells comprise mesenchymal stem cells, hematopoietic stem cells, ectodermal stem cells and so on. Scientific interest in adult stem cells is spotlighted on their ability to divide or self-renew indefinitely, and generate all the cell types of the organ from which they originate, potentially regenerating the entire organ from a few cells. Numerous studies using expanded and/or induced bone marrow-derived mesenchymal stem cells have been reported for animal models and yet only three clinical studies with intracerebral or intravasal application of these cells have been reported for PD and MSA patients. In two open-label studies, subventricular application of both allogenic and autologous bone marrow-derived mesenchymal stem cells showed improvement of motor behavior as reflected by reduction of UPDRS ON and OFF scores in most but not all PD patients [14,15]. In a randomized placebo-controlled trial involving a small number of cognitively intact MSA-C patients, mesenchymal stem cell therapy was safe and was able to delay the progression of neurological deficits with functional improvement in the follow-up period in some of the patients [16].

3. Adult neurogenesis in Parkinson's disease

Increasing evidence points to the presence of adult neural stem cells in many areas of the mammalian brain, mainly in the hippocampus and subventricular zone (SVZ) near the lateral ventricle. It is well known that changes occurring in the SVZ depend upon the pathological condition. Dopamine is an important molecule in neurogenesis. Therefore many investigators now focus on neurogenesis in PD. Höglinger et al. [17] reported reduction in the numbers of proliferating cells in the SVZ of postmortem brains of PD patients, implying that generation of neural precursor cells is impaired in PD as a consequence of dopaminergic denervation. However, controversy regarding neurogenesis in the SVZ in PD models persists. Some groups reported decreased neural precursor proliferation while some reported increased neural precursor proliferation in the SVZ of PD models.

Likewise, whether dopaminergic neurogenesis occurs in the adult substantia nigra (SN) in PD brains or in PD animal models remains a matter of debate. So we evaluated nigral neurogenesis in animal models and PD autopsy brains. We first performed retroviral labeling in a PD rodent model and observed efficient labeling of proliferating cells in SN with retroviral transduction of green fluorescent protein. But many of these labeled cells became microglia and none had differentiated into tyrosine-hydroxylase (TH)-positive neurons. Second, staining for intrinsic markers of neurogenesis showed that there were no proliferating cells in the SN of PD patients but a large number of polysialylated neural cell

adhesion molecule (PSA-NCAM)-positive cells were detected in SN pars reticulata (SNr) of some PD patients. In rat and primate models, dopamine-depleted hemispheres showed more PSA-NCAM staining than the intact side. A small number of TH and PSA-NCAM double positive cells, indicative of newly differentiated dopaminergic neurons, were detected [18]. However, no TH and PSA-NCAM double positive cells in PD patients were detected. Despite not being conclusive enough, these results suggest enhanced neural reconstruction in PD, which may be important in the design of new therapies against the progression of PD.

4. Stem cell therapy for Huntington's disease

Huntington's disease (HD) is characterized by a loss of brain striatal neurons that occurs as a consequence of an expansion of a cytosine adenine guanine (CAG) trinucleotide repeat encoding polyglutamine (polyQ) in the first exon of the Huntingtin gene. Therapeutic strategies are largely based on the amelioration of mutant huntingtin-related metabolic impairment and cellular toxicity. Yet cell replacement may be a potential therapy when cell death has become prominent in later stages of the disease. Numerous preclinical studies reported the efficiency of human fetal striatal tissue in providing functional recovery in various rodent and non-human primate models of striatal neuronal loss. On this basis, several clinical trials have assessed fetal cell transplantation for treatment in HD patients. Delivery of fetal striatal primordium into the caudate putamen of patient's brain was done via surgical stereotactic method. Yet due to heterogeneity in experimental design and small sample size, these clinical trials provided divergent data and reported modest improvements even in the best of cases. Some patients showed symptomatic improvement following the transplant but disease progression ensued with no greater survival. To address this issue, three ongoing randomized controlled clinical trials are reassessing fetal graft efficacy.

The ethical and immunological concerns associated with fetal allografts, along with the practical need to obtain tissue that is precisely staged, accurately dissected and freshly collected, imply that availability of fetal tissues for cell transplantation in the brain is likely to be extremely limited. Thus there is an urgent and active search for alternative sources. Human ES serve as a readily renewable source of potential medium spiny neurons for cell replacement therapy in HD patients. *In vivo* differentiation of striatal progenitor derived from human ES cells into striatal neurons following xenotransplantation into adult rats has first been described by Aubry et al. [19], opening an avenue of human ES cell therapy for HD. However, long-term proliferation of human neural progenitors leads to xenograft overgrowth in the rat brain, hindering its clinical use. HD-specific iPS cells have also been generated and reproduced CAG-repeat-expansion-associated gene expression phenotypes upon differentiation into neural cells, representing a well-characterized resource to elucidate the disease mechanism in HD and providing a human stem cell platform for screening new candidate therapeutics [20]. Also, An et al. [21] reported that iPS cells derived from the HD patient could be corrected by the replacement of the expanded CAG repeat with a normal repeat using homologous recombination, and the correction persists upon differentiation into striatal neurons *in vitro* and *in vivo*. Notably, correction of the HD-iPSCs normalized pathogenic HD signaling pathways and reversed disease phenotypes in neural stem cells. The ability to make patient-specific, genetically corrected iPS cells from HD patients is crucial for the eventual use of these cells in cell replacement therapy.

As mentioned earlier, neurogenesis has recently been observed in the adult human brain, suggesting the possibility of endogenous neural repair. Curtis et al. [22] first reported augmentation of neurogenesis as reflected by increased progenitor cell proliferation in the subependymal layer adjacent to the caudate nucleus, in response to neuronal cell loss in the caudate nucleus in HD. Degree of cell proliferation increased with pathological severity and increasing CAG repeats in the HD gene. Most importantly, proliferating cells were shown to express neuronal markers, indicating the generation of neurons and glial cells in diseased human brain. These results provide evidence for the regenerative potential of the human brain. Further, on the basis that ependymal overexpression of brain-derived neurotrophic factor (BDNF) stimulates neuronal addition to the adult striatum from subependymal progenitor cells while Noggin potentiates this process by suppressing subependymal gliogenesis and increasing progenitor availability, Cho et al. [23] found that BDNF and Noggin induced striatal neuronal regeneration, delayed motor impairment, and extended survival in R6/2 huntingtin transgenic mice, suggesting a new therapeutic strategy for HD.

5. Challenges of stem cell research

Before stem cells can be used to treat a myriad of disorders, many technical obstacles that hinder the clinical use must be overcome. The first major concern is that ES- and iPS-derived grafts have been reported to induce formation of teratomas. The tumor formation depends on the extent to which the cells are selectively enriched and differentiated prior to transplantation. Contamination with undifferentiated multipotent cells permits teratogenesis in the host. There are a number of successful engraftments of human ES cells-derived cells within the brain as treatment for PD and HD without tumor formation. But these studies were conducted in rodents and did not include long assessment periods. This problem may be solved with the establishment of safe stem cells incorporated with an anti-tumorigenic system by virus-mediated suicidal gene introduction [24]. These suicidal genes can serve as cell death switches that halt potentially deadly reactions.

Second, human ES cells express low levels of human leukocyte antigen class I molecules in both undifferentiated and differentiated states and might elicit immune responses. To address this issue, researchers found that short-term immune-dampening treatment enables human embryonic stem cells to avoid rejection after transplantation. Breakthrough of iPS cells also potentially allows generation of patient-specific donor cells that would likely, although not certainly, evade rejection as autograft. However, some researchers opined that iPSC-derived neurons will not be suitable for transplantation until the oncogenes and retroviruses used are replaced with more controlled methods of reprogramming. The problems that remain would likely be overcome through years of intensive research.

Recently a critical issue regarding clinical use of unapproved stem cell treatment in many clinics in some countries has been revealed. Those clinics claimed success in treating patients, including PD patients, but none has published data from controlled clinical trials. PD experts expressed concerns that these treatments might provide anecdotal, poorly controlled and transient improvement in patients and were dubious if the infused cells would survive for more than a few days in patients because so far there are neither scientific nor clinical data to support long-term benefits of hematopoietic or neural stem cell therapies for PD patients. Leading researchers now emphasize the need to strictly regulate stem cell therapy by requiring the organizations using stem cells to register their research and clinical activities, source of stem cells and ethical procedures.

6. Conclusion

In summary, stem cell research has made tremendous progress to date, offering new and promising potentials for the use of

these cells as therapeutic agents. However, it has also been the subject of much debate, and a great deal of research is required to overcome the existing technical hurdles including tumorigenesis and immune response so as to enable development of novel approaches that could be translated into effective and well-tolerated clinical application. Though in its infancy, generation of iPS cells is a breakthrough in stem cell research that, in the long term, may lessen the need to use human ES cells that is always at the crux of ethical concerns.

Conflict of interests

The authors have no conflicts of interest to declare.

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Advance in PD Research Explored a New Field on Ubiquitin Biology

Since the genetic interaction between parkin and PINK1[1,2] and the involvement of these genes in mitochondrial autophagy[3] (mitophagy) has been identified, mitophagy has emerged as one of the hottest topics in PD research. Recent studies show that PINK1 acts as an upstream factor for parkin and is essential for both phosphorylation and activation of parkin that is consequently recruited to damaged mitochondria. However, because a phosphomimetic parkin mutation cannot fully rescue the phenotype of PINK1 deficiency, it was suspected that PINK1 has substrates other than parkin.

In this paper,[4] Koyano et al. address this question. By using phosphate-affinity (Phos-tag) polyacrylamide gel electrophoresis, they found that ubiquitin is phosphorylated at Ser65 in HeLa cells treated with CCCP, a mitochondrial uncoupler, in a PINK1-dependent manner. They next showed that the combination of phosphomimetic ubiquitin and parkin are sufficient for full activation of parkin even in the absence of PINK1. Finally, they proposed a hypothesis for the mechanism underlying parkin activation, namely that phosphorylated ubiquitin unlocks the autoinhibition of the catalytic domain of parkin allosterically. Two other recent articles have also reported on PINK1-mediated phosphorylation of ubiquitin.[5,6]

Ubiquitin, a widely used posttranslational modifier, is posttranslationally modified by PINK1. The significance of phosphorylation of ubiquitin are not known.

The discovery of modified ubiquitin will certainly open up new horizons in ubiquitin biology. From a clinical perspective, phosphorylated ubiquitin may represent a novel and specific biomarker for mitochondrial damage in neurodegenerative disorders, including PD. ■

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Relevant conflicts of interest/financial disclosures: Nothing to report.

Received: 11 June 2014; Accepted: 16 June 2014
Published online 18 August 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/mds.25977

Neurobiology of Aging 35 (2014) 266.e5–266.e14

Contents lists available at ScienceDirect

Neurobiology of Aging

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

The protective effect of LRRK2 p.R1398H on risk of Parkinson's disease is independent of *MAPT* and *SNCA* variants

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http://dx.doi.org/10.1016/j.neurobiolaging.2013.07.013

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ARTICLE INFO

Article history:
Received 9 April 2013
Received in revised form 10 July 2013
Accepted 15 July 2013
Available online 17 August 2013

Keywords:
Parkinson's disease
LRRK2
SNCA
MAPT
Interaction
Genetics

ABSTRACT

The best validated susceptibility variants for Parkinson's disease are located in the α-synuclein (*SNCA*) and microtubule-associated protein tau (*MAPT*) genes. Recently, a protective p.N551K-R1398H-K1423K haplotype in the leucine-rich repeat kinase 2 (*LRRK2*) gene was identified, with p.R1398H appearing to be the most likely functional variant. To date, the consistency of the protective effect of LRRK2 p.R1398H across *MAPT* and *SNCA* variant genotypes has not been assessed. To address this, we examined 4 *SNCA* variants (rs181489, rs356219, rs11931074, and rs2583988), the *MAPT* H1-haplotype–defining variant rs1052553, and LRRK2 p.R1398H (rs7133914) in Caucasian (n = 10,322) and Asian (n = 2289) series. There was no evidence of an interaction of LRRK2 p.R1398H with *MAPT* or *SNCA* variants (all $p \geq 0.10$); the protective effect of p.R1398H was observed at similar magnitude across *MAPT* and *SNCA* genotypes, and the risk effects of *MAPT* and *SNCA* variants were observed consistently for LRRK2 p.R1398H genotypes. Our results indicate that the association of LRRK2 p.R1398H with Parkinson's disease is independent of *SNCA* and *MAPT* variants, and vice versa, in Caucasian and Asian populations.

1. Introduction

With an estimated prevalence of between 1% and 2% in individuals more than 65 years of age, Parkinson's disease (PD) is one of the most common age-related neurodegenerative disorders (de Lau and Breteler, 2006; Postuma and Montplaisir, 2009). Long thought of as a sporadic disease, PD now has a well-established genetic component that includes both disease-causing mutations as well as risk-modifying susceptibility variants (Gasser et al., 2011). Of the PD susceptibility variants that have been identified thus far, the best validated have involved those located in the α-synuclein (*SNCA*) gene, which also contains several pathogenic mutations that are linked to familial PD, and in the microtubule-associated protein tau (*MAPT*) gene (Gasser et al., 2011). More specifically, associations with PD have been identified in both Caucasian and Asian populations at the 3′ and 5′ ends of the *SNCA* gene (Mizuta et al., 2006; Mueller et al., 2005; Pankratz et al., 2009; Ross et al., 2007; Satake et al., 2009; Simón-Sánchez et al., 2009; Winkler et al., 2007), whereas the H1 haplotype in *MAPT* is associated with PD in Caucasians but not in Asians, owing to the almost complete absence of the H2 haplotype in the latter group (Evans et al., 2004; Healy et al., 2004; Skipper et al., 2004; Tobin et al., 2008; Wider et al., 2010).

Variation in the leucine-rich repeat kinase 2 (*LRRK2*) gene, which like *SNCA* harbors disease-causing mutations of its own has also been associated with susceptibility to PD in both Caucasian and Asian populations. The majority of proposed *LRRK2* PD risk variants have been relatively rare (minor allele frequencies [MAFs] between 1% and 5%) and have included p.G2385R and p.R1628P in Asian populations as well as the more recently identified p.A419V (in Asians), and p.M1646T (in Caucasians) (Di Fonzo et al., 2006; Farrer et al., 2007; Ross et al., 2008, 2011; Tan et al., 2010). The most common *LRRK2* PD risk factor to date, identified by several groups including our own, has involved a 3-variant (p.N551K-R1398H-K1423K) protective haplotype in both populations (Ross et al., 2011; Tan et al., 2010). It has been shown that the p.1398H variant has reduced kinase activity in comparison to the wild-type p.R1398 (Tan et al. 2010). Given these data, the p.R1398H (rs7133914) substitution, which occurs with a MAF of approximately 7% in Caucasians and 10% in Asians (Heckman et al., in press; Tan et al., 2010), is the most likely functional variant on the haplotype. The protective effect of p.R1398H appears to be strongest in Asians, in whom consistent odds ratios of 0.75 and 0.73 have been observed in studies by Tan et al. (2010) and Ross et al. (2011), with a similar odds ratio of 0.79 observed in a smaller study by Chen et al. (2011). In Caucasians, the odds ratio for p.R1398H observed in the aforementioned study by Ross et al. in a series of 6995 patients and 5595 control subjects was 0.89. This is very similar to the findings of a large meta-analysis of genome-wide association studies, in which, albeit not nominally significant, LRRK2 p.R1398H (MAF ~ 6.7%) had a protective odds ratio of 0.92 and 95% confidence limits ranging from 0.83 to 1.02 in regard to susceptibility to PD (Nalls et al., 2011; personal communication).

To best determine risk of PD for a given individual and to elucidate potential future therapeutic implications, it is important not only to identify individual genetic risk factors but also to understand how these risk factors interact with one another. However, sample sizes needed to reasonably evaluate evidence of such gene–gene interactions are usually fairly large and can be difficult to achieve. This is because the risk factor of interest in an interaction study (presence of the genotype of interest for both variants) occurs much less frequently than the genotype for the individual variants, which can result in a lack of precision in estimated interaction effects. Collaboration between members of the Genetic Epidemiology of Parkinson's Disease (GEO-PD) Consortium and the resulting large number of patients with PD and controls offers the opportunity to effectively examine how recognized susceptibility variants for PD may or may not interact with one another. Such a study was previously undertaken by the GEO-PD Consortium, in which *SNCA* and *MAPT* variants were

examined in relation to risk of PD and found to have independent effects (Elbaz et al., 2011). The identification of PD susceptibility variants in *LRRK2* raises the question of whether the effects of these variants may be modified by those in *SNCA* or *MAPT*, or vice versa. The aim of this study was to evaluate the interaction of the common *LRRK2* susceptibility variant p.R1398H with *SNCA* and *MAPT* variants in relation to risk of PD using Caucasian and Asian patient–control subject series obtained through the GEO-PD Consortium.

2. Methods

2.1. Subjects

As of 2013, the GEO-PD Consortium includes 57 sites from 29 countries and 6 continents that have agreed to share DNA and data for 38,686 patients with PD and 34,871 control subjects (http://www.geopd.org/). A total of 20 sites participating in the GEO-PD Consortium provided data to be used in the current study as part of a project initiated in 2009. The majority of the Caucasian subjects used in this study were also included in the previously mentioned GEO-PD *SNCA-MAPT* interaction study (Elbaz et al., 2011), and the subjects included in this study are a subset of those included in the previously referred to investigation of *LRRK2* exonic variants in relation to PD (Ross et al., 2011). To be consistent with the association analysis in the latter study involving *LRRK2* exonic variants, carriers of *LRRK2* pathogenic variants (n = 64) were excluded. Subjects were not genotyped for known pathogenic *SNCA* mutations and therefore this was not part of our exclusion criteria. In total, 7342 patients with PD and 5269 control subjects from 13 different countries on 4 continents were studied. These subjects were divided into a Caucasian series (5991 patients with PD, 4331 controls, 16 sites, 10 countries) and an Asian series (1351 patients with PD, 938 controls, 4 sites, 3 countries). Table 1 provides demographic information for the Caucasian and Asians series, whereas site-specific information is displayed in Supplementary Table 1.

Patients were diagnosed with PD using standard criteria (Bower et al., 1999; Gelb et al., 1999; Hughes et al., 1992). Controls were individuals free of PD or a related movement disorder at the time of examination. All subjects were unrelated within and between diagnosis groups. The Mayo Clinic Institutional Review Board approved the study; each individual site received local institutional review board approval, and all subjects provided informed consent.

2.2. Genetic analysis

Four *SNCA* variants (3′ end of gene: rs181489, rs356219, rs11931074; 5′ end of gene: rs2583988) as well as the *MAPT* H1-haplotype defining variant rs1052553 were genotyped because of consistently replicated associations with PD (Healy et al., 2004; Mueller et al., 2005; Mizuta et al., 2006; Pankratz et al., 2009; Ross et al., 2007; Satake et al., 2009; Skipper et al., 2004; Simón-Sánchez et al., 2009; Tobin et al., 2008; Wider et al., 2010; Winkler et al., 2007). These 5 variants were chosen for the aforementioned GEO-PD *SNCA-MAPT* interaction study (Elbaz et al., 2011). The REP1 polymorphism located in the *SNCA* promoter has also been associated with PD (Krüger et al., 1999; Maraganore et al., 2006); however, because the 263-bp allele (which has shown the strongest association with PD) is relatively rare, we did not evaluate REP1 in the current study. The *LRRK2* variant rs7133914 (p.R1398H) was also selected for inclusion because of the aforementioned findings demonstrating that is the most likely functional variant on a 3-variant haplotype (all 3 variants in strong linkage disequilibrium with $r^2 > 0.84$ in controls) that affects risk of PD in a protective manner (Ross et al., 2011; Tan et al., 2010).

Table 1
Subject characteristics for the Caucasian and Asian series

Variable	Patients with PD	Controls
Caucasian series	n = 5991	n = 4331
Age, y	69 ± 11 (18–106)	65 ± 15 (21–107)
Gender		
Male	3453 (58%)	2045 (47%)
Female	2538 (42%)	2286 (53%)
Age at onset, y	59 ± 12 (18–96)	NA
Asian series	n = 1351	n = 938
Age, y	61 ± 12 (20–91)	60 ± 11 (23–89)
Gender		
Male	672 (50%)	322 (34%)
Female	679 (50%)	616 (66%)
Age at onset, y	54 ± 12 (20–89)	NA

Sample mean ± SD (minimum–maximum) is given for age of subjects and age at onset. Information was unavailable regarding age in the Caucasian series (147 patients with PD, 21 controls) and Asian series (371 patients with PD, 298 controls). Information was unavailable regarding age at onset in the Caucasian series (723 patients) and Asian series (8 patients).
Key: NA, not applicable; PD, Parkinson's disease; SD, standard deviation.

DNA was sourced from blood and was stored in a freezer at −80 °C. All samples were de-identified with an anonymous code from each site and only a minimal clinical dataset. All *LRRK2* and *SNCA* genotyping was done using MassArray iPLEX chemistry and analyzed using Typer 4.0 (Sequenom, San Diego, CA). *MAPT* rs1052553 was genotyped using an ABI Taqman genotyping assay on an ABI 7900HT Fast Real-Time PCR system and analyzed using SDS 2.2.2 software (Applied Biosystems, Foster City, CA). All genotyping was performed at the Mayo Clinic Florida neurogenetics laboratory (Jacksonville, FL). Primer sequences are provided in Supplementary Table 2 for all variants except for *MAPT* rs1052553. Positive control DNA was run for each variant. Call rates in each series were >95%. There was no evidence of departure from Hardy–Weinberg equilibrium in controls for any of the sites (all $p > 0.05$ after Bonferroni correction).

2.3. Statistical analysis

All analysis was performed separately for the Caucasian and Asian series. Associations of individual *SNCA* variants, *MAPT* rs1052553, and LRRK2 p.R1398H with PD, and pairwise interactions of LRRK2 p.R1398H with *SNCA* and *MAPT* variants in relation to PD, were evaluated using odds ratios (ORs) and 95% confidence intervals (CIs) from fixed-effects logistic regression models adjusted for site. Interactions were evaluated on a multiplicative scale only because it has been shown that when at least one of the interacting factors is protective, biological interactions are expected to result in departure from multiplicative effects (Weinberg, 1986).

We considered LRRK2 p.R1398H under a dominant model (presence vs. absence of the minor allele) in all analyses owing to the very small number of homozygotes of the minor allele, whereas *SNCA* variants were evaluated under an additive model (effect of each additional minor allele), dominant model, recessive model (presence of 2 copies vs. 0 or 1 copy of the minor allele) and genotype model (general comparison across genotypes). *MAPT* rs1052553 was also evaluated under additive, dominant, recessive, and genotype models, but with effects corresponding to the major allele to be consistent with previous reports in which ORs correspond to the H1 risk allele. In Caucasians, 3-gene interactions were also examined. Sensitivity of results to model adjustment for age and gender and to the use of random-effects models (DerSimonian and Laird, 1986) were also assessed when evaluating interactions.

Between-site heterogeneity in interaction ORs was examined using χ^2 tests based on the Q statistic, and also by estimating the I^2 statistic, which measures the proportion of variation in interaction ORs between sites due to heterogeneity beyond chance (Higgins and Thompson, 2002).

A relatively large number of statistical tests of gene–gene interaction were performed in our analyses (24 in the Caucasian series and 8 in the Asian series). To adjust for multiple testing and to control the family-wise error rate at 5%, we used a Bonferroni correction separately for each series, after which p values ≤ 0.0021 (Caucasian series) and ≤ 0.00625 (Asian series) were considered as statistically significant. All statistical analyses were performed using R Statistical Software (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria).

3. Results

A summary of allele and genotype frequencies for *SNCA* variants, *MAPT* rs1052553, and LRRK2 p.R1398H in our Caucasian and Asian patient-control series is provided in Supplementary Table 3, along with country-specific frequencies. The *SNCA* variants rs181489 and rs2583988 as well as *MAPT* rs1052553 were observed extremely rarely in Asian patients and controls and, as such, were not assessed in association analysis. *SNCA* variants were in relatively weak linkage disequilibrium in controls ($r^2 \leq 0.32$) with the exception of rs181489 and rs356219 in the Caucasian series ($r^2 = 0.58$), rs181489 and rs2583988 in the Caucasian series ($r^2 = 0.53$), and rs356219 and rs11931074 in the Asian series ($r^2 = 0.97$).

To best interpret the results of gene–gene interaction analysis, it is helpful to first understand the effects of individual variants on risk of PD, and therefore single-variant associations with PD for the *SNCA*, *MAPT*, and *LRRK2* variants, which have largely been reported before in the aforementioned GEO-PD studies (Elbaz et al., 2011; Ross et al., 2011), are displayed in Supplementary Table 4. As has been previously shown, all variants were significantly associated with PD.

Evaluations of pairwise interactions of LRRK2 p.R1398H with *SNCA* variants and *MAPT* rs1052553 in relation to PD for the Caucasian series are shown in Table 2. To simplify our presentation of interaction results, we have focused on additive and genotype models for *SNCA* and *MAPT* variants in Table 2, because all of these variants had the strongest association with PD under an additive model except *SNCA* rs11931074 (which was also strongly associated with PD under an additive model), and because genotype models allow for the most general test of interaction. Gene–gene interactions under dominant and

Table 2
Interactions of LRRK2 p.R1398H with *SNCA* and *MAPT* variants in regard to susceptibility to Parkinson's disease (PD) in the Caucasian series under additive and genotype models

Variant/genotype	LRRK2 p.R1398H	Sample genotype count and frequency	Test of association		Test of interaction
			OR (95% CI)	p value	
SNCA rs181489					
CC	GG	3908 (39.9%)	1.00 (reference)	NA	Additive model
CC	GA or AA	599 (6.1%)	0.82 (0.69–0.98)	0.030	OR = 1.06
CT	GG	3636 (37.1%)	1.14 (1.04–1.25)	0.0070	95% CI = 0.88–1.28
CT	GA or AA	542 (5.5%)	1.08 (0.90–1.30)	0.42	$p = 0.52$
TT	GG	967 (9.9%)	1.65 (1.42–1.92)	1.4E-10	Genotype model[a]
TT	GA or AA	136 (1.4%)	1.40 (0.98–2.00)	0.066	$p = 0.14$
SNCA rs356219					
AA	GG	3087 (30.9%)	1.00 (reference)	NA	Additive model
AA	GA or AA	440 (4.4%)	0.82 (0.67–1.01)	0.060	OR = 0.98
AG	GG	4142 (41.5%)	1.15 (1.04–1.26)	0.0060	95% CI = 0.82–1.17
AG	GA or AA	628 (6.3%)	1.11 (0.93–1.32)	0.27	$p = 0.81$
GG	GG	1476 (14.8%)	1.51 (1.33–1.73)	7.2E-10	Genotype model[a]
GG	GA or AA	219 (2.2%)	1.10 (0.83–1.46)	0.51	$p = 0.32$
SNCA rs11931074					
GG	GG	7443 (74.6%)	1.00 (reference)	NA	Additive model
GG	GA or AA	1061 (10.5%)	0.85 (0.74–0.97)	0.017	OR = 1.06
GT	GG	1300 (12.9%)	1.34 (1.18–1.51)	6.8E-6	95% CI = 0.79–1.43
GT	GA or AA	232 (2.3%)	1.32 (1.00–1.74)	0.052	$p = 0.69$
TT	GG	59 (0.6%)	1.46 (0.84–2.62)	0.19	Genotype model[a]
TT	GA or AA	12 (0.1%)	0.67 (0.20–2.24)	0.51	$p = 0.61$
SNCA rs2583988					
CC	GG	4495 (44.6%)	1.00 (reference)	NA	Additive model
CC	GA or AA	677 (6.7%)	0.82 (0.69–0.97)	0.019	OR = 1.07
CT	GG	3480 (34.6%)	1.20 (1.09–1.31)	0.0001	95% CI = 0.89–1.29
CT	GA or AA	500 (5.0%)	1.13 (0.93–1.37)	0.23	$p = 0.47$
TT	GG	800 (7.9%)	1.42 (1.21–1.67)	1.9E-5	Genotype model[a]
TT	GA or AA	117 (1.2%)	1.22 (0.84–1.80)	0.30	$p = 0.56$
MAPT rs1052553[b]					
GG	GG	364 (3.6%)	1.00 (reference)	NA	Additive model
GG	GA or AA	58 (0.6%)	0.54 (0.30–0.97)	0.041	OR = 1.05
GA	GG	2617 (25.7%)	1.10 (0.88–1.38)	0.41	95% CI = 0.85–1.30
GA	GA or AA	398 (3.9%)	1.05 (0.78–1.41)	0.75	$p = 0.65$
AA	GG	5881 (58.0%)	1.36 (1.10–1.70)	0.0055	Genotype model[a]
AA	GA or AA	858 (8.4%)	1.19 (0.92–1.53)	0.19	$p = 0.29$

ORs and P values result from fixed-effects logistic regression models. For tests of association, the 2 given variants were combined into 1 variable, and the model was adjusted for site. For tests of interaction, models included each of the 2 variants, their interaction, and site. Additive models and genotype models refer to the characterization of *SNCA* and *MAPT* variants; only dominant models were considered for LRRK2 p.R1398H because of the small number of rare homozygotes for this variant. Interaction ORs under an additive model are interpreted as the multiplicative increase in the effect of the minor allele for LRRK2 p.R1398H on PD corresponding to each additional risk allele for *SNCA* and *MAPT* variants, or alternatively as the multiplicative increase in the effect of each additional risk allele for *SNCA* and *MAPT* variants on PD corresponding to presence of the minor allele for LRRK2 p.R1398H.

Key: CI, confidence interval; OR, odds ratio.

[a] Tests of interaction under a genotype model do not produce a single interaction OR, and therefore only a P value is given.

[b] The A allele for *MAPT* rs1052553 corresponds to the H1 haplotype.

recessive models for *SNCA* and *MAPT* variants are shown in Supplementary Tables 5 and 6. In site-adjusted analyses, no interactions of LRRK2 p.R1398H with *SNCA* and *MAPT* variants approached significance after multiple testing adjustment under any statistical model (all interaction $p \geq 0.10$); the protective effect of p.R1398H on risk of PD observed in similar magnitude for different genotypes of *SNCA* and *MAPT* variants, whereas the risk effects of *SNCA* and *MAPT* variants were seen similarly for subjects with and without a copy of the minor allele for p.R1398H. All interaction ORs were close to 1.0 in magnitude indicating lack of any interaction with LRRK2 p.R1398H, the only exceptions involving rare genotypes for *MAPT* rs1052553 under a dominant model (Supplementary Table 5) and *SNCA* rs11931074 under a recessive model (Supplementary Table 6), which are best interpreted with caution owing to the non-significant interactions and very low genotype frequencies. The lack of interaction of LRRK2 p.R1398H with *MAPT* and *SNCA* variants was also observed when adjusting for age and gender (Supplementary Table 7) in those subjects with that information available (98%) and also when using a random effects model (Supplementary Table 8). Results of country-specific interaction analysis are shown in Supplementary Table 9. Between-site heterogeneity regarding interactions with LRRK2 p.R1398H was low for *SNCA* rs356219, rs11931074, and rs2583988 ($I^2 = 0\%$, $p \geq 0.45$) and moderate for *SNCA* rs181489 and *MAPT* rs1052553 ($I^2 = 25\%$–36%, $p \geq 0.075$) (Supplementary Table 8).

More detailed analysis combining genotypes across all 3 genes for *SNCA* variants, *MAPT* rs1052553, and LRRK2 p.R1398H in the Caucasian series is displayed in Supplementary Table 10 and Fig. 1, where rare homozygotes were collapsed with heterozygotes for each variant to avoid extremely rare 3-variant genotype combinations. There was no evidence of any interaction in these 3-gene analyses (all, $p \geq 0.63$).

Interactions of LRRK2 p.R1398H with *SNCA* variants rs356219 and rs11931074 in the Asian series are examined in Table 3 in analyses adjusted for site. Individual effects of LRRK2 p.R1398H and *SNCA* variants on risk of PD were observed consistently across variants in the other gene, with no statistically significant evidence of gene–gene interaction (all interaction, $p \geq 0.14$). All interaction ORs were between 1.17 and 1.39, indicating a slight but nonsignificant reduction of the protective effect of LRRK2 p.R1398H on risk of PD when the risk allele for *SNCA* variants was present, and a similar small and nonsignificant enhancement of the *SNCA* risk effects, given the protective genotype for p.R1398H (Fig. 2). Results were similar when adjusting for age and gender (Supplementary Table 7) in the

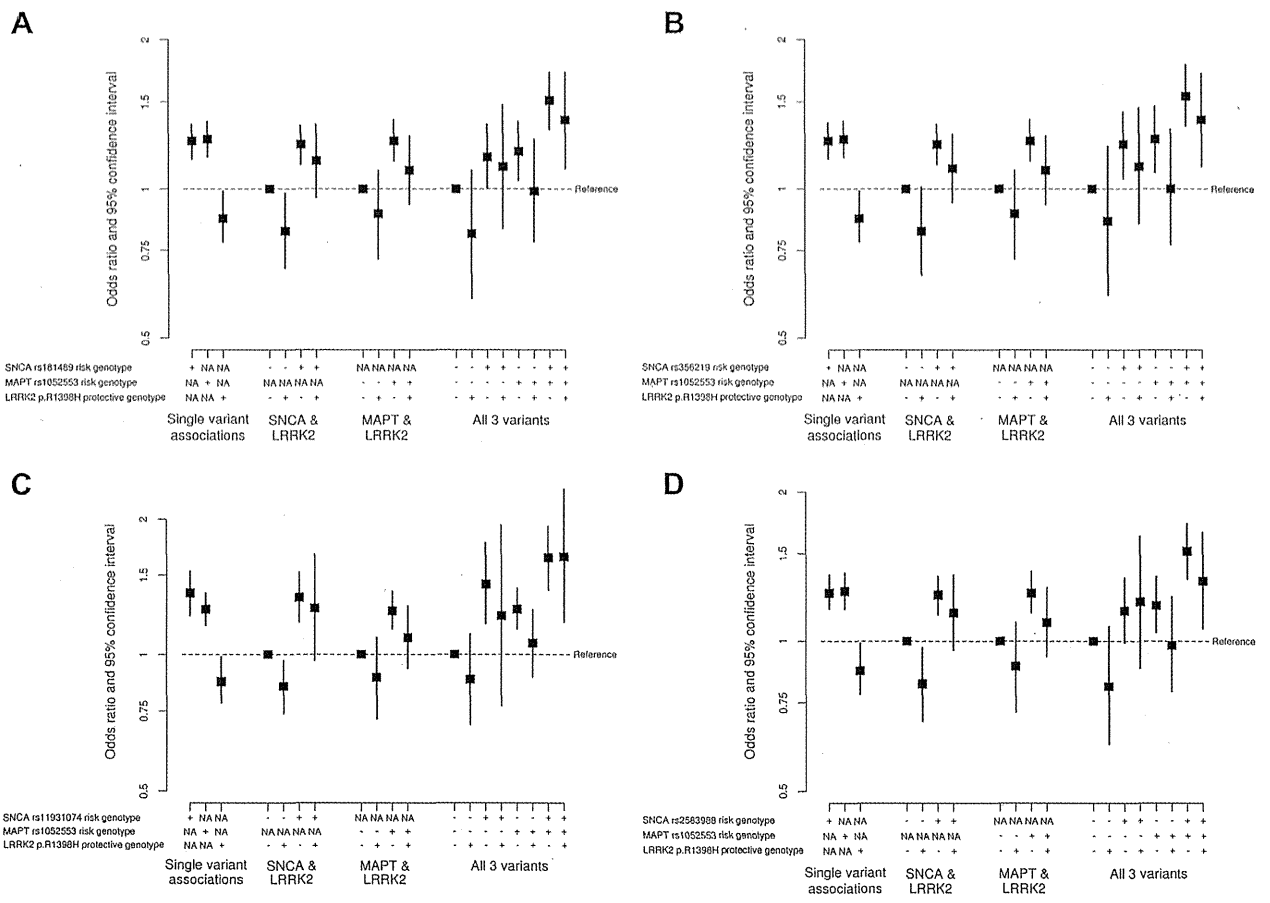

Fig. 1. (A) Individual and combined effects of *SNCA* rs181489, *MAPT* rs1052553, and LRRK2 p.R1398H on risk of Parkinson's disease (PD) in the Caucasian series. For *SNCA* rs181489, the risk genotype was CT or TT (i.e., presence of the minor allele). (B) Individual and combined effects of *SNCA* rs356129, *MAPT* rs1052553, and LRRK2 p.R1398H on risk of PD in the Caucasian series. For *SNCA* rs356129, the risk genotype was AG or GG (i.e., presence of the minor allele). (C) Individual and combined effects of *SNCA* rs11931074, *MAPT* rs1052553, and LRRK2 p.R1398H on risk of PD in the Caucasian series. For *SNCA* rs11931074, the risk genotype was GT or TT (i.e., presence of the minor allele). (D) Individual and combined effects of *SNCA* rs2583988, *MAPT* rs1052553, and LRRK2 p.R1398H on risk of PD in the Caucasian series. For *SNCA* rs2583988, the risk genotype was CT or TT (i.e., presence of the minor allele). (A–D) For *MAPT* rs1052553, the risk genotype was AA (i.e., presence of 2 copies of the major allele); for LRRK2 p.R1398H, the protective genotype was GA or AA (i.e., presence of the minor allele). NA indicates that a given SNP was not involved in the particular portion of the analysis.

Table 3
Interactions of LRRK2 p.R1398H with *SNCA* variants in regard to susceptibility to Parkinson's disease (PD) in the Asian series

Variant/genotype	LRRK2 p.R1398H	Sample genotype count and frequency	Test of association		Test of interaction
			OR (95% CI)	*p* value	
Additive/genotype models[a]					
SNCA rs356219					
AA	GG	282 (12.9%)	1.00 (reference)	N/A	Additive model
AA	GA or AA	83 (3.8%)	0.64 (0.39–1.06)	0.087	OR = 1.17
AG	GG	808 (37.0%)	1.59 (1.21–2.09)	0.0009	95% CI = 0.87–1.59
AG	GA or AA	232 (10.6%)	1.19 (0.84–1.69)	0.33	$p = 0.30$
GG	GG	623 (28.5%)	2.09 (1.56–2.79)	6E-7	Genotype model[d]
GG	GA or AA	156 (7.1%)	1.84 (1.23–2.77)	0.0031	$p = 0.59$
SNCA rs11931074					
GG	GG	302 (13.3%)	1.00 (reference)	N/A	Additive model
GG	GA or AA	89 (3.9%)	0.61 (0.37–0.98)	0.044	OR = 1.25
GT	GG	843 (37.2%)	1.55 (1.19–2.02)	0.0012	95% CI = 0.93–1.69
GT	GA or AA	243 (10.7%)	1.06 (0.75–1.49)	0.75	$p = 0.14$
TT	GG	630 (27.8%)	1.90 (1.43–2.51)	7.8E-6	Genotype model[d]
TT	GA or AA	158 (7.0%)	1.75 (1.18–2.61)	0.0059	$p = 0.31$
Dominant model[b]					
SNCA rs356219					
AA	GG	282 (12.9%)	1.00 (reference)	N/A	OR = 1.23
AA	GA or AA	83 (3.8%)	0.64 (0.39–1.06)	0.087	95% CI = 0.71–2.14
AG or GG	GG	1431 (65.5%)	1.78 (1.38–2.31)	1.10E-5	$p = 0.47$
AG or GG	GA or AA	388 (17.8%)	1.41 (1.03–1.92)	0.030	
SNCA rs11931074					
GG	GG	302 (13.3%)	1.00 (reference)	N/A	OR = 1.25
GG	GA or AA	89 (3.9%)	0.61 (0.37–0.98)	0.043	95% CI = 0.74–2.15
GT or TT	GG	1473 (65.0%)	1.69 (1.31–2.17)	4.3E-5	$p = 0.41$
GT or TT	GA or AA	401 (17.7%)	1.28 (0.95–1.73)	0.11	
Recessive model[c]					
SNCA rs356219					
AA or AG	GG	1090 (49.9%)	1.00 (reference)	N/A	OR = 1.22
AA or AG	GA or AA	315 (14.4%)	0.72 (0.56–0.93)	0.011	95% CI = 0.78–1.92
GG	GG	623 (28.5%)	1.48 (1.21–1.83)	0.0002	$p = 0.38$
GG	GA or AA	156 (7.1%)	1.31 (0.93–1.87)	0.13	
SNCA rs11931074					
GG or GT	GG	1145 (50.6%)	1.00 (reference)	N/A	OR = 1.39
GG or GT	GA or AA	332 (14.7%)	0.66 (0.52–0.85)	0.0011	95% CI = 0.90–2.17
TT	GG	630 (27.8%)	1.38 (1.12–1.69)	0.0020	$p = 0.14$
TT	GA or AA	158 (7.0%)	1.27 (0.90–1.80)	0.18	

ORs and *p* values result from fixed-effects logistic regression models. For tests of association, the 2 given variants were combined into 1 variable, and the model was adjusted for site. For tests of interaction, models included each of the 2 variants, their interaction, and site. Additive models, genotype models, dominant models, and recessive models refer to the characterization of *SNCA* variants; only dominant models were considered for LRRK2 p.R1398H because of the small number of rare homozygotes for this variant. Key: CI, confidence interval; OR, odds ratio.

[a] Interaction ORs under an additive model are interpreted as the multiplicative increase in the effect of the minor allele for LRRK2 p.R1398H on PD corresponding to each additional risk allele for *SNCA* variants, or alternatively as the as the multiplicative increase in the effect of each additional risk allele for *SNCA* variants on PD corresponding to presence of the minor allele for LRRK2 p.R1398H.

[b] Interaction ORs under a dominant model are interpreted as the multiplicative increase in the effect of the minor allele for LRRK2 p.R1398H on PD corresponding to presence of the risk allele for *SNCA* variants, or alternatively as the as the multiplicative increase in the effect of presence of the risk allele for *SNCA* variants on PD corresponding to presence of the minor allele for LRRK2 p.R1398H.

[c] Interaction ORs under a recessive model are interpreted as the multiplicative increase in the effect of the minor allele for LRRK2 p.R1398H on PD corresponding to presence of 2 risk alleles for *SNCA* variants, or alternatively as the as the multiplicative increase in the effect of presence of 2 risk alleles for *SNCA* variants on PD corresponding to presence of the minor allele for LRRK2 p.R1398H.

[d] Tests of interaction under a genotype model do not produce a single interaction OR, and therefore only a p value is given.

subgroup of Asian individuals for whom that information was available (71%) and also under a random effects model (Supplementary Table 8). Interactions between LRRK2 p.R1398H and *SNCA* variants under additive and recessive models are shown in Supplementary Table 11 separately for each Asian country; between-site heterogeneity in interactions with *LRRK2* p.R1398H was moderate for both *SNCA* rs356219 and rs11931074 in the Asian series (I^2 = 46%–55%, $p \geq 0.084$, Supplementary Table 8).

4. Discussion

Recently, a 3-variant (p.N551K-R1398H-K1423K) haplotype in the *LRRK2* gene was shown to affect susceptibility to PD in a protective manner in both Caucasian and Asian populations (Ross et al., 2011; Tan et al., 2010). The p.R1398H substitution appears to be the most likely functional variant, as it is located in the conserved Roc domain, and there is supporting evidence of reduced kinase activity (Tan et al., 2010). Although a number of previous investigations have examined interactions between the well-validated PD susceptibility variants located in the *SNCA* and *MAPT* genes (Biernacka et al., 2011; Elbaz et al., 2011; Goris et al., 2007; Mamah et al., 2005; McCulloch et al., 2008; Simón-Sánchez et al., 2009; Trotta et al., 2012; Wider et al., 2011), no study reported to date has examined interactions of LRRK2 p.R1398H with *SNCA* and *MAPT* variants. The results of our large case-control study involving both Caucasian and Asian individuals indicate that the protective effect of LRRK2 p.R1398H is observed consistently for different *SNCA* and *MAPT* genotypes, whereas, similarly, the *SNCA* and *MAPT* risk effects are observed for individuals with and without the protective p.R1398H allele.

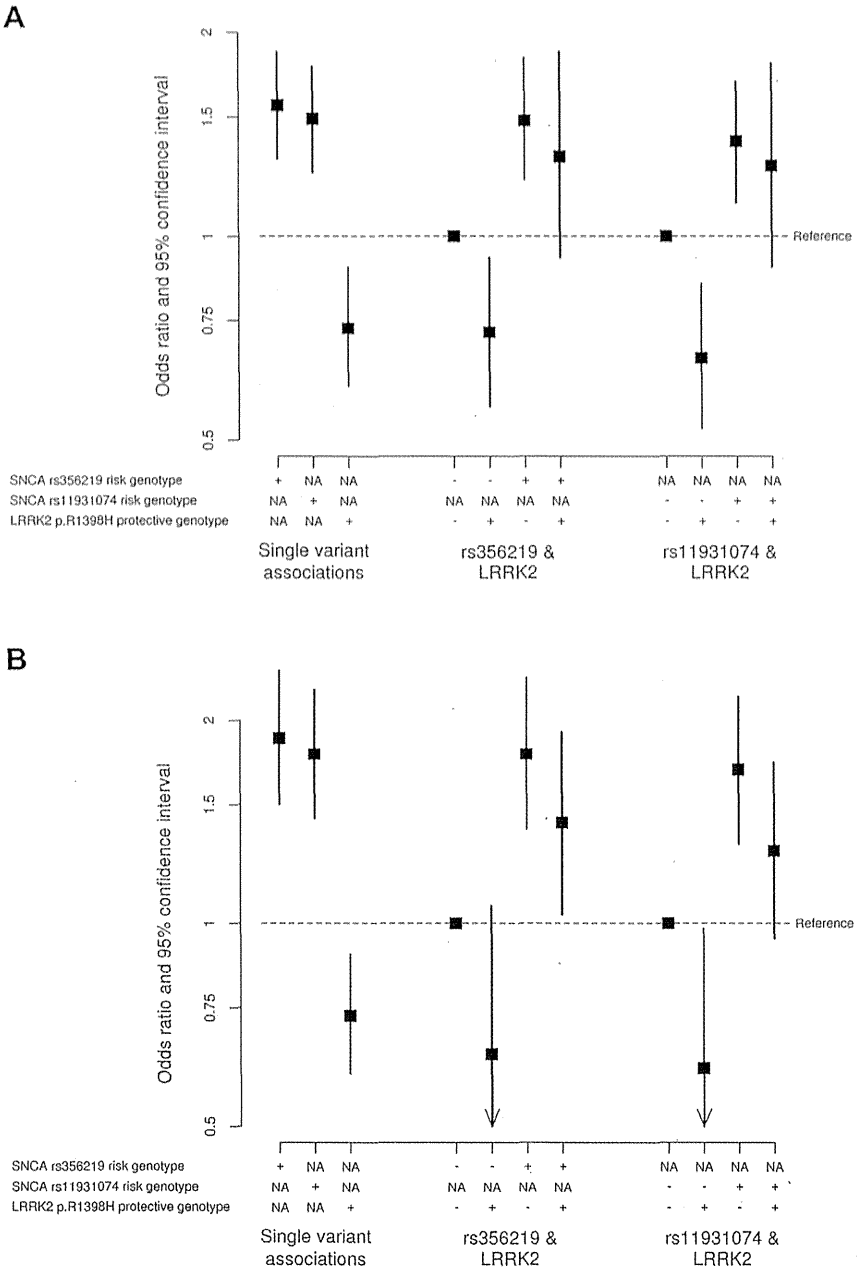

Fig. 2. (A) Individual and combined effects of *SNCA* rs356219, *SNCA* rs11931074, and LRRK2 p.R1398H on risk of Parkinson's disease (PD) in the Asian series. *SNCA* rs356219 and rs11931074 were considered under a recessive model (i.e., presence vs. absence of 2 copies of the minor allele). For *SNCA* rs356219, the risk genotype was GG. For *SNCA* rs11931074 , the risk genotype was TT. (B) Individual and combined effects of *SNCA* rs356219, *SNCA* rs11931074, and LRRK2 p.R1398H on risk of PD in the Asian series. *SNCA* rs356219 and rs11931074 were considered under a dominant model (i.e., presence vs. absence of the minor allele). For *SNCA* rs356219, the risk genotype was AG or GG. For *SNCA* rs11931074 , the risk genotype was GT or TT. (A and B) For LRRK2 p.R1398H, the protective genotype was GA or AA (i.e., presence of the minor allele). NA indicates that a given SNP was not involved in the particular portion of the analysis.

Despite the relatively large number of interactions and statistical models considered, the independent effects on PD risk for LRRK2 p.R1398H, *MAPT* rs1052553, and *SNCA* variants were observed with a very high level of consistency in our study. This was most apparent in the large Caucasian series, for which all interaction ORs were between 0.80 and 1.13, with the exception of the 2 aforementioned instances involving rare genotypes for *MAPT* rs1052553 and *SNCA* rs11931074. In addition, between-site heterogeneity in interaction effects was low to moderate in Caucasians. Although the protective effect of LRRK2 p.R1398H on risk of PD was observed consistently across *SNCA* variant genotypes in Asians, perhaps the least convincing evidence of lack of gene–gene interaction was observed in this series. Albeit not approaching significance even before adjustment for multiple testing, the magnitude of this observed protective effect was slightly smaller when the risk genotype for *SNCA* variants was present, whereas, conversely, the observed risk effects of *SNCA* variants were marginally stronger in individuals with the protective p.R1398H genotypes. In addition,

heterogeneity in interaction effects between sites was highest in the Asian series. However, it is important to highlight that it would be very unusual to observe a complete lack of gene–gene interaction (i.e., interaction OR = 1) in all scenarios simply because of natural sampling variability, particularly given the number of possible interactions that were examined. Nonetheless, given the smaller size of our Asian series in comparison to the Caucasian series, it will be important to validate our findings in larger series of Asian individuals.

Recent studies have supported our earlier work indicating that the effects of *SNCA* and *MAPT* variants on PD risk are independent of one another (Biernacka et al., 2011; Trotta et al., 2012; Wider et al., 2011). Although our current study is the first to date to examine the potential interaction of the protective LRRK2 p.R1398H substitution with *MAPT* and *SNCA* variants in regard to risk of PD, previous studies have evaluated interactions with, or combined effects of, *LRRK2* variants and those in *SNCA* and *MAPT*. In their analysis of 1098 patients with PD and 1098 matched controls from the United States (a subset of which were also used in the current study), Biernacka et al. (2011) found no statistically significant evidence of gene–gene interaction when considering 8 intronic *LRRK2* variants, 10 *SNCA* variants (8 intronic, 1 3′ downstream and 1 5′ Rep1), and 8 *MAPT* variants (6 intronic, 1 3′ UTR, and 1 H1/H2). Wang et al. (2012) concluded that other genes, including *MAPT* and *SNCA*, modified *LRRK2*-related risk for PD in a Chinese cohort of 2013 sporadic PD patients and 1971 controls. This was based on findings that, in comparison to individuals harboring only the LRRK2 p.G2385R or p.R1628P risk variants, the risk of PD is increased in individuals with these and other PD risk variants. However, it is unclear whether this represents independent or interactive effects, and the sample sizes of the combined risk-variant groups examined were quite small. The results of these studies are consistent with those of our own, with the effect of *LRRK2* variants on PD susceptibility appearing to be independent of *SNCA* and *MAPT* risk factors for PD.

The strengths of our study, including the large sample size and inclusion of subjects from a variety of different populations, are important to highlight; however, several limitations should also be acknowledged. A key question is whether the lack of interaction of LRRK2 p.R1398H with *SNCA* and *MAPT* variants is a consequence of sample size or the frequencies of the examined variants. To assess the possibility of a false-negative association, it is most helpful to examine 95% confidence limits for observed interaction odds ratio estimates (Goodman and Berlin, 1994). These confidence limits were generally relatively tight in the larger Caucasian series, indicating a lack of a biologically significant interaction in this population, but were wider in the Asian series, further highlighting the need for validation of our findings in that series. In addition, as is generally the case for large-scale collaborative studies attempting to address a focused research question that involves a small number of genetic variants, without available genome-wide population control markers, population stratification could potentially have had an impact on our results. However, this potential limitation is lessened by the fact that our logistic regression models were adjusted by site, which makes any possible population stratification a site-specific issue. Other limitations of our study include the different diagnostic criteria across the different sites and the lack of a standardized inclusion/exclusion criteria for patients with PD and controls.

In conclusion, our study provides evidence that the effect of LRRK2 p.R1398H on risk of PD is independent of the *MAPT* H1-haplotype defining variant rs1052553 and *SNCA* variants, and vice versa. This lack of gene–gene interaction was apparent in both our large Caucasian patient-control series and our smaller Asian series. Evaluation of interactions involving individuals of other ethnic backgrounds, other rarer *LRRK2* susceptibility variants, and PD susceptibility variants at other loci (Lill et al., 2012) is needed in order to move toward a fuller understanding of the genetic architecture of PD susceptibility.

Disclosure statement

J.O.A., M.J.F., and Z.K.W. report holding a patent on *LRRK2* genetic variability. M.J.F. has received royalties for licensing of genetically modified *LRRK2* mouse models. D.M.M. declares a patent pending entitled "Methods to Treat PD." C.K. and R.K. declare receiving payment in their role as consultants for Centogene and Takeda Pharmaceutical, respectively. All other authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by a grant from The Michael J. Fox Foundation for Parkinson's Research (O.A.R., M.J.F.). Original funding for the GEO-PD was supported by a grant from The Michael J. Fox Foundation for Parkinson's Research Edmond J. Safra Global Genetics Consortia program (D.M.M.). The Mayo Clinic Jacksonville is a Morris K. Udall Center of Excellence in Parkinson's Disease Research (grant no. P50 NS072187) and was supported by a the gift from the family of Carl Edward Bolch, Jr, and Susan Bass Bolch (R.J.U., Z.K.W., O.A.R.). O.A.R. acknowledges funding support from the National Institutes of Health (grant no. R01 NS078086). This research was undertaken, in part, thanks to funding from the Canada Excellence Research Chairs program (M.J.F., C.V.G.). Leading Edge Endowment Funds, provided by the Province of British Columbia, LifeLabs, and Genome BC, support the Dr Donald Rix BC Leadership Chair (M.J.F.). D.M.M. acknowledges the National Institutes of Health for funding support (grant no. R01ES10751). Studies at individual sites were supported by a number of different funding agencies world-wide including; Italian Ministry of Health (Ricerca Corrente, Ricerca Finalizzata); the Swedish Parkinson Academy; the Swedish Parkinson Foundation; the Federal Ministry for Education and Research (BMBF, NGFNplus; 01GS08134) (R.K.); the NGFNplus (Neuron-Parkinson-subproject 7) (S.G.); CHRU de Lille, University of Lille 2, INSERM; French Ministry PHRCs (1994/, 2002/1918, 2005/1914); Association France Parkinson (2005); Fondation de France 2004-013306; Fondation de la Recherche Médicale (2006); PPF (synucléothèque 2005–2009); the 2 Centres de Ressources Biologiques (IPL-Lille, CHRU-Lille) and its scientific committee (A.D., M.C.C.H., Philippe Amouyel, Florence Pasquier, Régis Bordet); funding from France-Parkinson Association and the program "Investissement d'avenir" ANR-10-IAIHU-06; the Swedish Research Council; the Swedish Society for Medical Research; the Swedish Society of Medicine; funds from the Karolinska Institutet and the Parkinson Foundation in Sweden (K.W.); the National Institutes of Health and National Institute of Neurological Disorders and Stroke (grant nos. 1RC2NS070276, NS057567, and P50NS072187); Mayo Clinic Florida Research Committee CR programs (M.J.F., Z.K.W.); the Geriatric Medical Foundation of Queensland (G.D.M.); a career development award from the Volkswagen Foundation and from the Hermann and Lilly Schilling Foundation (C.K.); the Research Committee of University of Thessaly (code 2845); and Laboratory of Neurogenetics, Biomedicine Department, CERETETH, Larissa, Greece (code 01-04-207) (G.H., E.D.).

A number of individuals must be acknowledged for their contributions to make this work possible; Ferdinanda Annesi, PhD; Patrizia Tarantino, PhD (Institute of Neurological Sciences, National Research Council); Monica Gagliardi, PhD, (Institute of Neurological Sciences, National Research Council, Cosenza Italy), Chiara Riva,

PhD (Department of Neuroscience and Biomedical Technologies, University of Milano-Bicocca, Monza, Italy); Roberto Piolti, MD (Department of Neurology, Ospedale San Gerardo, Monza, Italy); Alessandro Ferraris MD, PhD (IRCCS Casa Sollievo della Sofferenza Hospital, Mendel Laboratory, San Giovanni Rotondo, Italy); Aurélie Duflot, (UMR837 Inserm-Univ Lille 2, CHRU de Lille), Jean-Philippe Legendre, Nawal Waucquier (Neurologie et Pathologie du Mouvement, Clinique de Neurologie du CHU de Lille). Anna Rita Bentivoglio, MD, PhD, Tamara Ialongo, MD, PhD, Arianna Guidubaldi, MD, Carla Piano, MD (Institute of Neurology, Catholic University, Rome, Italy); Phil Hyu Lee, MD, PhD (Department of Neurology, Yonsei University College of Medicine, Seoul, Korea); Jan Reimer (Department of Neurology, Skåne University Hospital, Sweden); Hiroyo Yoshino, PhD, Manabu Funayama, PhD, Yuanzhe Li, MD, PhD (Juntendo University School of Medicine, Tokyo, Japan). From the Queensland Parkinson's Project: R.S. Boyle and A. Sellbach (Princess Alexandra Hospital, Brisbane), J. D. O'Sullivan (Royal Brisbane and Women's Hospital, Brisbane), G.T. Sutherland, G.A. Siebert and N.N.W. Dissanayaka (Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan, QLD).

Finally, we acknowledge all of the patients and control subjects who kindly donated DNA to make collaborative studies like these possible.

A full list of GEO-PD consortium member sites is provided in the Supplementary Text.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2013.07.013.

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