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Publication Date

2013-10-01

DOI

10.1016/j.envres.2013.08.001

Peer reviewed

Environmental Research 126 (2013) 1–8

Contents lists available at ScienceDirect

Environmental Research

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

Pesticides that inhibit the ubiquitin–proteasome system: Effect measure modification by genetic variation in *SKP1* in Parkinson's disease

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ARTICLE INFO

Article history:
Received 16 April 2013
Received in revised form
7 July 2013
Accepted 2 August 2013
Available online 27 August 2013

Keywords:
Epidemiology
Gene–environment interaction
Parkinson's disease
Pesticides
Ubiquitin–proteasome system

ABSTRACT

Cytoplasmic inclusions known as Lewy bodies, a hallmark of Parkinson's disease (PD) pathology, may protect against cytotoxic proteins. Since the ubiquitin–proteasome system (UPS) degrades cytotoxic proteins, dysfunction in the UPS may contribute to PD etiology. Our goal in this study was to screen pesticides for proteasome inhibition and investigate (i) whether ambient exposures to pesticides that inhibit the UPS increase PD risk and (ii) whether genetic variation in candidate genes of the UPS pathway modify those increased risks. We assessed 26S UPS activity in SK-N-MC^{u} cells by fluorescence. We recruited idiopathic PD cases (n=360) and population-based controls (n=816) from three counties in California with considerable commercial agriculture. We determined ambient pesticide exposure by our validated GIS-based model utilizing residential and workplace address histories. We limited effect measure modification assessment to Caucasians (287 cases, 453 controls). Eleven of 28 pesticides we screened inhibited 26S UPS activity at 10 μM. Benomyl, cyanazine, dieldrin, endosulfan, metam, propargite, triflumizole, and ziram were associated with increased PD risk. We estimated an odds ratio of 2.14 (95% CI: 1.42, 3.22) for subjects with ambient exposure to any UPS-inhibiting pesticide at both residential and workplace addresses; this association was modified by genetic variation in the *s-phase kinase-associated protein 1* gene (*SKP1*; interaction p-value=0.005). Our results provide evidence that UPS-inhibiting pesticides play a role in the etiology of PD and suggest that genetic variation in candidate genes involved in the UPS pathway might exacerbate the toxic effects of pesticide exposures.

1. Introduction

Parkinson's disease (PD) is characterized by both motor deficits and non-motor symptoms that significantly impact quality of life for those affected and their caregivers. The pathology of PD is considered to affect multiple organ systems (Jellinger, 2012) but its characteristic motor symptoms result from dopaminergic dysfunction following the loss of neurons in the substantia nigra. A hallmark of PD pathology is the cytoplasmic inclusions known as Lewy bodies predominantly composed of alpha-synuclein protein (Spillantini et al., 1997) but also frequently containing ubiquitin, ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1), synphilin, and parkin proteins among others (Licker et al., 2009). It remains unclear whether Lewy bodies themselves are toxic or whether their accumulation may be a protective mechanism against cytotoxic proteins that have not been removed by other means (Wakabayashi et al., 2012).

Degradation of potentially cytotoxic proteins is the responsibility of two cellular mechanisms: the ubiquitin–proteasome system (UPS) and autophagy. The UPS breaks down proteins in a five-step process: (1) activation of ubiquitin by an E1 enzyme, (2) conjugation of activated ubiquitin to an E2 enzyme, (3) ubiquitination of a protein by an E3 ligase, (4) breakdown of the polyubiquitinated protein in the 26S proteasome, and (5) recycling of the ubiquitin by deubiquitinating enzymes such as UCH-L1

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http://dx.doi.org/10.1016/j.envres.2013.08.001

(Betarbet et al., 2005). The UPS has been a candidate pathway for PD etiology since linkage of rare familial PD to genetic defects in *parkin* (*PARK2*), an E3 ubiquitin ligase involved in both UPS degradation of cellular proteins and lysosome-dependent degradation of mitochondrial proteins (Yoshii et al., 2011), and to defects in *ubiquitin carboxyl-terminal hydrolase L1* (*ubiquitin thiolesterase*) (*UCHL1*). Studies in cell culture, rodents (although these remain controversial), and human PD brain tissue add further support to this hypothesis (Bove et al., 2006; Chou et al., 2008, 2010; Kordower et al., 2006; Manning-Bog et al., 2006; Mcnaught and Jenner, 2001; Mcnaught and Olanow, 2006; Schapira et al., 2006; Zeng et al., 2006). However, whether UPS dysfunction is a cause, as opposed to a consequence, of altered upstream pathological processes in PD remains a question.

Previous studies have linked individual pesticides to proteasome inhibition in model systems (Chou et al., 2008, 2010; Wang et al., 2006; Wills et al., 2012) and, while pesticide exposure has regularly been associated with increased risk of PD (Van Der Mark et al., 2012), epidemiologic studies rarely investigate individual pesticides identified to have specific biologic mechanism of action. We conducted a population-based case-control study of PD based in a highly agricultural region of Central California that provides the unique opportunity to estimate ambient exposure to individual pesticides by combining address data from our subjects and state records of pesticide usage in commercial agriculture. Additionally, recognizing that PD occurrence is likely a convergence of environmental exposures, genetic susceptibility, and aging (Vance et al., 2010), we have genetic material available for the investigation of gene–environment interaction. Therefore, to elucidate the role of UPS dysfunction in PD etiology we screened a number of pesticides for 26S UPS inhibition and then we investigated the hypotheses that (i) pesticides shown to alter UPS function in our model system are associated with an increased risk of PD and (ii) the impact of UPS-inhibiting pesticides is modified by variation in candidate genes related to the UPS.

2. Methods

2.1. Pesticide screen for 26S proteasome inhibition

From the list of pesticides to which the study population was potentially exposed (i.e., at least one subject was assigned any exposure between 1974 and 1999 according to our geographical information system computer model described below), we selected 28 compounds (see Supplementary materials, Table S1) and screened for their ability to inhibit 26S UPS activity in a cellular model. These compounds were selected to reflect the range of chemical structures of the 106 active ingredients to which subjects were potentially exposed. A concentration of 10 μM was chosen to ensure relevance to environmental exposures given that higher concentrations are used to kill target species. Pesticides were dissolved in DMSO to a final concentration of 0.025%, with the exception of sulfur which was dissolved in carbon disulfide. Untreated control cells were exposed only to 0.025% DMSO; carbon disulfide did not alter UPS activity.

An amount of 26S UPS activity was determined by FACS as previously described (Wang et al., 2006). Briefly, neuroblastoma SK-N-MC cells transfected with an EGFP-degron fusion protein and passaged multiple times were exposed to test compounds (2 mL/well) for 48 h prior to FACS analysis (Beckman XL-MCL). UPS inhibition was inferred from high fluorescence (FL) corresponding to the level of EGFP-degron fusion protein that was not selectively degraded by the UPS (Bence et al., 2001). UPS inhibition is reported as the percentage of inhibition as compared to the positive control, as previously described (Wang et al., 2006):

$$\%\ \text{UPS Inhibition} = (\text{FL\_pesticide} - \text{FL\_vehicle})/(\text{FL\_lactacystin} - \text{FL\_vehicle}) \times 100\%$$

2.2. Subject recruitment for population-based study

Case definitions (Jacob et al., 2010) and subject recruitment (Wang et al., 2011) are described elsewhere. Briefly, we enrolled idiopathic PD patients (2001–2007) and population-based controls (2002–2011) from three predominantly rural counties in California with considerable commercial agriculture. Of 1167 PD patients identified, 604 did not meet eligibility criteria; 90 could not be examined by our movement disorder specialists; 94 did not meet published criteria for idiopathic PD (Hughes et al., 1992); and 6 withdrew between examination and interview. Among the 373 PD cases identified within 3 years of initial PD diagnosis, we reclassified 13 as not idiopathic PD after follow-up (Ritz et al., 2012) resulting in 360 enrolled cases.

We recruited population-based controls under two strategies: (1) from 2002 to 2007 we contacted randomly selected controls via mail/phone and (2) from 2008 to 2011 we contacted potential subjects through in-person visits to randomly selected clusters of five neighboring households. Under strategy #1, we contacted 1212 subjects of whom 457 were ineligible (89% due to age); 409 declined participation, became too ill, or moved away; and 346 enrolled. Additionally, we included 62 "restricted controls" who enrolled through an early mailing for which the number of eligible subjects who declined is not known. Under strategy #2, we contacted 4756 subjects of whom 3515 were ineligible (88% due to age); 634 declined participation; and 607 enrolled.

All subjects completed a telephone interview for collection of demographics, risk factor data, and residential/workplace address histories used in geocoding. On average, cases completed the interview 2 years (s.d. 1.4 years) after initial PD diagnosis. Subjects also provided either blood or saliva for DNA. Written informed consent was obtained from all enrolled subjects or their proxies; all procedures were approved by the UCLA Institutional Review Board.

2.3. Pesticide exposure assessment

Ambient exposures to commercially applied pesticides were estimated by a geographic-information-system-based (GIS) computer model previously described in detail (Goldberg et al., 2008; Wang et al., 2011). Briefly, self-reported historical residential and workplace addresses were geocoded and manually resolved. By combining geocoded data for pesticide applications, as determined from California Department of Pesticide Regulation Pesticide Use Reports (collected since 1974) and California Department of Water Resources land use maps, with the subject's geocoded addresses, a subject's average annual exposure was estimated and assumed to be proportional to the poundage of active ingredient applied to crop acreage within a 500-meter radius of each address. A 26-year average exposure was calculated for the years 1974–1999, restricting our exposure window to the time prior to initial PD diagnosis. Subjects were considered to have "high" ambient exposure to a pesticide if their 26-year average was equal to or greater than the median 26-year average in exposed controls and to have no/low exposure to that pesticide otherwise. All cases and 816 controls provided sufficient historical address data to generate pesticide exposure estimates; 183 controls who completed an abbreviated questionnaire and 16 controls who completed the full questionnaire had insufficient address histories and do not contribute to these analyses.

While our prior work has suggested that ambient exposure to a pesticide at residential addresses is associated with a lower odds ratio than ambient exposure to that same pesticide at workplace addresses, the estimates are rarely statistically different (Wang et al., 2011). Therefore, for the individual pesticide main effects analyses we have categorized subjects according to a three-level ordinal variable for each pesticide: (1) no/low exposure to all UPS-inhibiting pesticides (common reference group for all comparisons), (2) high exposure to the index pesticide at residence alone or workplace alone, and (3) high exposure to the index pesticide at both residence and workplace. We excluded from analysis of the index pesticide subjects with high exposure to any non-index UPS-inhibiting pesticide as well as no/low exposure to the index pesticide (e.g., for analysis of benomyl as the index pesticides, subjects with high exposure to endosulfan but no/low exposure to benomyl were excluded from the analysis of benomyl). Exclusion of subjects with high exposure to any non-index pesticide from analysis of the index pesticide enables the common reference group (subjects with no/low exposure to all UPS-inhibiting pesticides) (i) to be consistent across all analyses of all individual UPS inhibiting pesticides and (ii) to contain less exposure misclassification.

2.4. Genetic variant assessment

In addition to *PARK2* and *UCHL1*, we considered as candidate genes relevant to the UPS pathway: (i) *ubiquitin-like modifier activating enzyme 1* (*UBA1*, GeneID: 7317), which encodes an E1 enzyme and has been observed to be down-regulated in PD compared to control brains (Hauser et al., 2005); (ii) *ubiquitin-like modifier activating enzyme 6* (*UBA6*, GeneID: 55236) due to its similarity to *UBA1*; (iii) *ubiquitin-conjugating enzyme E2D 1* (*UBE2D1*, GeneID: 7321), which encodes an E2 enzyme; and (iv) *s-phase kinase-associated protein 1* (*SKP1*, GeneID: 6500), which encodes an E3 enzyme and has been observed to be down-regulated in PD compared to controls brains (Grunblatt et al., 2004).

Selection of single nucleotide polymorphism (SNPs) and genotyping methods are described in the Supplementary materials. Twenty-five SNPs (see Supplementary materials, Table S2) met quality control standards for call rate, concordance between duplicates, and Hardy–Weinberg equilibrium (HWE) in controls; four SNPs, rs5906352 and rs6611347 in *UBA1* and rs11131714 and rs2262366 in *UBA6*, were excluded from analysis due to low call rates; no SNPs were excluded due to deviation from HWE. Five of the 25 SNPs investigated met our screening criteria of allele frequency chi square p-value ≤ 0.05 for difference in distribution

between cases and controls: *SKP1* (rs2284312), *PARK2* (rs9365292), *UBA6* (rs354872), *UBE2D1* (rs11593650), and *UBA1* (rs4529579). We selected these five SNPs for effect measure modification analyses as well as *UCHL1* (rs5030732) because of prior support for its role in PD (Ragland et al., 2009).

2.5. Effect measure modification

For the effect measure modification (statistical gene–environment interaction) analyses, we combined across all UPS-inhibiting pesticides previously considered in the main effects analyses described above such that subjects were assigned: (1) no/low exposure to all UPS-inhibiting pesticide (reference group), (2) high exposure to any one UPS-inhibiting pesticide at residence alone or workplace alone, and (3) high exposure to at least one UPS-inhibiting pesticide at both residence and workplace. Because of potential genetic heterogeneity among different racial/ethnic groups and too small a sample size for separate analyses of non-Caucasians, only Caucasian subjects were considered for effect measure modification analyses. Three of 290 Caucasian cases lacked DNA samples for genotyping and were excluded from these analyses. Additionally, at the time of genotyping not all controls had been enrolled and interviewed, thus only 453 of 563 Caucasian controls contribute to the effect measure modification analyses.

2.6. Statistical analyses

For pesticide marginal effects we estimated odds ratios (OR) and 95% confidence intervals (95%CI) using unconditional logistic regression. We evaluated effect measure modification of the combined pesticide variable by each SNP by including a product term (pesticide*SNP) in the regression model. We utilized a dominant genetic model for effect measure modification analyses due to small numbers of variant homozygotes for some SNPs; the dominant genetic model will typically produce effect estimates similar to those of the heterozygotes in the log-additive genetic model when the variant homozygotes are rare. All regression models were adjusted for age as a continuous variable (defined as age at PD diagnosis for cases and age at enrollment for controls), sex (male/female), and smoking status (ever/never). *P*-values presented are unadjusted for the number of tests performed; for multiple testing considerations, we performed 14 tests.

We assessed potential confounding of the pesticide-specific marginal effects by other pesticide exposures by adjusting for propensity scores (Robins et al., 1992) incorporating variables for (1) any high exposure to the other UPS-inhibiting pesticides and (2) any high exposure to organophosphates, organochlorines, dithiocarbamates, or paraquat/maneb, as we have seen an association between ambient exposure to these pesticides/classes and PD (Costello et al., 2009; Wang et al., 2011). Additionally, we assessed potential confounding by (3) self-reported regular use of any home pesticides at least twice per year at some time between 18 and 45 years of age and (4) medium or high likelihood of occupational exposure to pesticides based on job title as defined by our job exposure matrix (Liew et al., In press). Moreover, we assessed the robustness of the pesticide-specific marginal effects by performing sensitivity analyses as described in Supplementary materials. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

3. Results

From among the 28 pesticides screened (see Supplementary materials, Table S1), 26S UPS activity was significantly inhibited by 10 μM exposure to propargite, cyanazine, and both of the tested organochlorines (dieldrin, endosulfan) in addition to the imidazoles benomyl, carbendazim, and triflumizole, but not the imidazole precursor thiophanate-methyl. Only three dithiocarbamates (ferbam, metam, ziram) were inhibitors at 10 μM. Neither paraquat nor maneb inhibited 26S UPS activity at the tested concentration, but did inhibit at 100 and 50 μM, respectively (data not shown). The screened carbamates (aldicarb, methomyl), dicarboxymides (captan, folpet, vinclozolin), organophosphates (diazinon, dimethoate, methidathion, parathion, phorate), sulfur, and the solvent carbon disulfide did not inhibit UPS activity. Thus, we considered for our epidemiologic analyses the 11 pesticides that inhibited 26S UPS activity at 10 μM ($p<0.0001$, Table 1).

Of the 11 pesticides that inhibited the UPS in our screen, no subjects in our study population (see Supplementary materials, Table S3) were exposed to any level of ambient carbendazim, only 2 subjects were exposed to any level of ferbam, and 12 subjects were exposed to any level of rotenone. Therefore, these pesticides had too low a prevalence to be evaluated individually. For each of the remaining eight UPS-inhibiting pesticides, we consistently observed a trend of increasing risk of PD across our ordinal exposure variable (all *p*-values for trend <0.05; Table 2 and Supplementary materials, Table S3) with ambient exposure to benomyl, endosulfan, metam, and propargite each with *p*-values for trend ≤ 0.003.

Table 1
Pesticides that inhibit 26S proteasomal activity.

Compound	Class	Relative inhibition[a] (% ± SEM)
Benomyl	Imidazole	6.5 ± 0.0
Carbendazim	Imidazole	5.4 ± 1.1
Cyanazine	Triazine	2.5 ± 0.3
Dieldrin	Organochlorine	2.7 ± 0.9
Endosulfan	Organochlorine	3.2 ± 0.8
Ferbam	Dithiocarbamate	6.5 ± 1.1
Metam	Dithiocarbamate	3.2 ± 0.0
Propargite	Unclassified	16.8 ± 1.2
Rotenone	Botanical	6.6 ± 1.2
Triflumizole	Imidazole	4.2 ± 0.4
Ziram	Dithiocarbamate	5.9 ± 0.8

[a] Relative inhibition is expressed as the percentage of fluorescence induced by 5 μM lactacystin in SK-N-MC[u] cells; all *p*-values less than 0.0001; $n=3$–6 for each compound.

With the exception of cyanazine, in Caucasian subjects with genotype data (Table 2) we observed odds ratios (ORs) ranging from 2.31 (95% CI: 1.45, 3.70) to 5.74 (95% CI: 1.12, 29.5) between PD and UPS-inhibiting pesticides when comparing subjects with high ambient exposure at both residential and workplace addresses to those with no/low exposure at both locations. For subjects with high ambient exposure at either residential or workplace address, we observed ORs ranging from 1.21 to 2.21 with some confidence intervals excluding the null. When we combined UPS-inhibiting pesticides, we observed a marginal OR of 2.14 (95% CI: 1.42, 3.22) for subjects with high ambient exposure to any one pesticide at both residential and workplace addresses; but no more than a possible weak association for subjects with high ambient exposure to any one pesticide at either residential or workplace addresses alone (OR=1.29, 95% CI: 0.92, 1.82). Trends in effect estimates were similar when considering all subjects with pesticide exposure assessment, regardless of self-reported ancestry or the availability of genotypes (see Supplementary materials, Table S4). Analyses assessing potential confounding by other pesticide exposures and robustness of the models (see Section 2 and Supplementary materials) did not materially change the estimates.

We observed effect measure modification (statistical gene–environment interaction) of the pesticide-PD association by the T allele of *SKP1* rs2284312 (Fig. 1, interaction OR=4.63, *p*-value=0.005); specifically, among subjects with high ambient exposure at both residence and workplace the OR for subjects with at least one T allele (i.e., subjects with the CT or TT genotype) is 7.57 (95% CI: 3.14, 18.3) whereas the OR for subjects with no T allele (i.e., subjects with the CC genotype) is 1.62 (95% CI: 0.98, 2.66). We observed possible effect measure modification by the A allele of *PARK2* rs9365292 in subjects exposed at either location (interaction OR=1.87, $p=0.086$), although we observed nearly identical ORs for subjects exposed at both residence and workplace regardless of *PARK2* rs9365292 genotype. We observed no effect measure modification of the pesticide association by *UCHL1* rs5030732, *UBA6* rs354872, *UBE2D1* rs11593650, or *UBA1* rs4529579 (Table 3).

4. Discussion

We observed an increase in PD risk with high ambient exposures at both residential and workplace addresses for almost all of the UPS-inhibiting pesticides investigated in this study.

Table 2
Associations between PD and ambient exposure to UPS-inhibiting pesticides in the Parkinson's, Environment & Genes (PEG) Study[a].

Index pesticide and exposure level[b]	Cases/controls	OR[c] (95%CI)	Trend *p*-value
Common reference group			
No/low exposure to all UPS-inhibiting pesticides[d]	111/223	1.00 (Reference)	
Benomyl			
High exposure to benomyl at residence or workplace	67/93	1.46 (0.99, 2.16)	
High exposure to benomyl at residence and workplace	34/28	2.32 (1.32, 4.06)	0.002
High exposure to other pesticides and no/low exposure to benomyl[e]	75/109	n.c.	
Cyanazine			
High exposure to cyanazine at residence or workplace	23/25	1.97 (1.06, 3.68)	
High exposure to cyanazine at residence and workplace	4/5	1.63 (0.42, 6.27)	0.030
High exposure to other pesticides and no/low exposure to cyanazine[e]	149/200	n.c.	
Dieldrin			
High exposure to dieldrin at residence or workplace	16/13	2.18 (1.01, 4.73)	
High exposure to dieldrin at residence and workplace	6/2	5.74 (1.12, 29.5)	0.005
High exposure to other pesticides and no/low exposure to dieldrin[e]	154/215	n.c.	
Endosulfan			
High exposure to endosulfan at residence or workplace	63/92	1.40 (0.94, 2.08)	
High exposure to endosulfan at residence and workplace	32/23	2.58 (1.43, 4.66)	0.002
High exposure to other pesticides and no/low exposure to endosulfan[e]	81/115	n.c.	
Metam			
High exposure to metam at residence or workplace	28/26	2.21 (1.23, 3.99)	
High exposure to metam at residence and workplace	5/3	3.21 (0.74, 13.8)	0.002
High exposure to other pesticides and no/low exposure to metam[e]	143/201	n.c.	
Propargite			
High exposure to propargite at residence or workplace	69/114	1.25 (0.85, 1.84)	
High exposure to propargite at residence and workplace	52/45	2.31 (1.45, 3.70)	0.003
High exposure to other pesticides and no/low exposure to propargite[e]	55/71	n.c.	
Triflumizole			
High exposure to triflumizole at residence or workplace	13/12	1.96 (0.86, 4.48)	
High exposure to triflumizole at residence and workplace	4/2	3.50 (0.62, 19.6)	0.031
High exposure to other pesticides and no/low exposure to triflumizole[e]	159/216	n.c.	
Ziram			
High exposure to ziram at residence or workplace	36/58	1.21 (0.75, 1.96)	
High exposure to ziram at residence and workplace	19/11	3.30 (1.50, 7.26)	0.012
High exposure to other pesticides and no/low exposure to ziram[e]	121/161	n.c.	

Abbreviations: PD, Parkinson's disease; UPS, ubiquitin–proteasome system; OR, odds ratio; CI, confidence interval; n.c., not calculated.

[a] Limited to Caucasian subjects with exposure and genotype assessment. Results for subjects with exposure assessment, regardless of self-reported ancestry or genotype assessment, are presented in Supplementary materials, Table S4.

[b] Subjects were classified to have high exposure if the study period (1974–1999) average annual exposure for the index pesticide was greater than or equal to the pesticide-specific median in exposed controls.

[c] Odds ratio adjusted for age (continuous), gender (male/female), and smoking status (ever/never).

[d] Subjects in the reference group have no/low exposure to ambient benomyl, cyanazine, dieldrin, endosulfan, ferbam, metam, propargite, rotenone, triflumizole, and ziram at both residences and workplaces.

[e] Subjects with high exposure to any other (non-index) UPS-inhibiting pesticide and no/low exposure to the index pesticide were excluded from analysis of the index pesticide; they do not contribute to trend test.

Approximately 70% of subjects designated as highly exposed to any one UPS-inhibiting pesticide were actually exposed to two or more pesticides (40% were exposed to three or more), thus we had limited ability to investigate any single pesticide to the exclusion of others, and our findings cannot attribute causation or even strength of association to any specific pesticide. However, these results provided evidence that UPS-inhibiting pesticides play a role in the etiology of PD.

We recently reported that benomyl also inhibits aldehyde dehydrogenase activity which might contribute to PD etiology (Fitzmaurice et al., 2013), endosulfan induces apoptosis and contributes to oxidative stress (Kannan et al., 2000; Song et al., 2012), and rotenone is a known mitochondrial complex I inhibitor (Martinez and Greenamyre, 2012). Therefore, it is highly likely that some of the pesticides we identified as UPS inhibitors also have toxic mechanisms of action in one or more alternate biologic processes hypothesized as contributing to PD etiology. Further complicating this picture, we previously reported an association between PD and well-water consumption (Gatto et al., 2009), a possible route of exposure to propargite along with other ground water pollutants. This highlights the possibility that our subjects are exposed to pesticides through other routes in addition to ambient environmental exposures from pesticide drift. Still, our sensitivity analyses suggest that the UPS-inhibiting pesticide associations we report are independent of exposure to other pesticides, such as organophosphates, and independent of household or active occupational pesticide use.

At first glance, the small levels of UPS inhibition observed here might not appear significant, but we previously found that this level of inhibition results in cell death (Chou et al., 2010; Wang et al., 2006), suggesting that the UPS is critical for cell survival and even a relatively small amount of inhibition results in significant detrimental effects. Furthermore, in translating these models to the development of a chronic disease, even low levels of inhibition could be responsible for PD pathogenesis over the decades of exposure considered in the human population. Prior studies in model systems have observed alterations in UPS activity, either by assessing 20S activity in cell lysates or 26S activity in live cells. The latter, which we assessed in this study, has the added benefit of determining activity for intact UPS function. Previously we demonstrated that administration of benomyl,

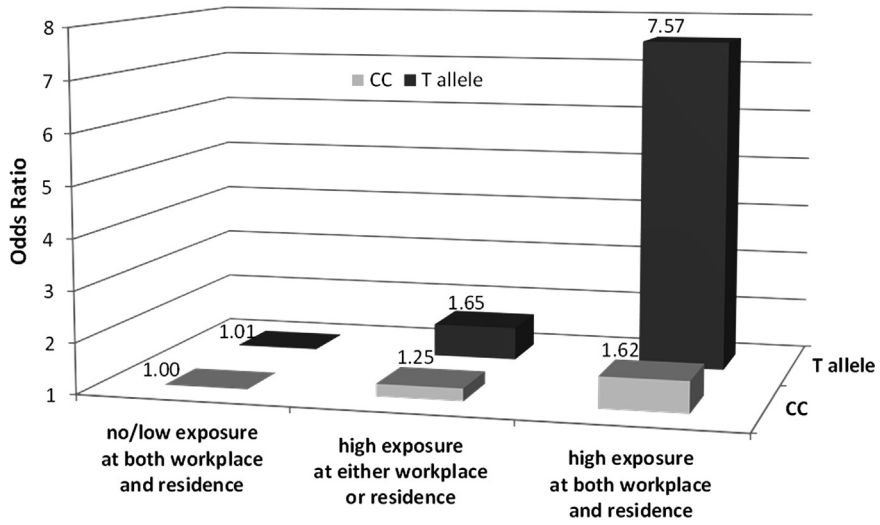

Fig. 1. Subjects with at least one T allele (i.e., subjects with the CT or TT genotype; dark gray) who have ambient exposure to UPS-inhibiting pesticides at both residential and workplace addresses have a significantly stronger association with PD compared to subjects with no T allele (i.e., subjects with the CC genotype; light gray) and a similar level of exposure; there is evidence for effect measure modification by genotype [OR (95%CI) for interaction = 4.63 (1.59–13.5), *p*-value for interaction = 0.005]. There is no effect measure modification by genotype for subjects with ambient exposure at either residence or workplace [OR (95%CI) for interaction = 1.30 (0.60–2.83), *p*-value for interaction = 0.506].

dieldrin, diethyldithiocarbamate (DETC), endosulfan, rotenone, and ziram decreased 26S activity but did not alter 20S activity (Wang et al., 2006). Others have reported decreased 20S activity with manganese ethylene-bis-dithiocarbamate, the major active component of maneb (Zhou et al., 2004) or paraquat (Yang and Tiffany-Castiglioni, 2007) but these pesticides did not impact 26S activity at environmentally-relevant concentrations (Wang et al., 2006). There are no subjects exposed to DETC in our study population, therefore it was not investigated. While we have reported a positive association between paraquat/maneb and PD (Costello et al., 2009) adjustment for exposure to paraquat/maneb did not alter our results, suggesting that the observed effect of ambient exposure to UPS-inhibiting pesticides is independent of paraquat/maneb exposures and their effects on 20S activity.

PD etiology likely involves the complex interplay of aging, behavioral factors, environmental exposures, and genetic susceptibility (Gao and Hong, 2011). Therefore, we considered possible effect measure modification of the pesticide association and observed a statistical interaction between high UPS-inhibiting pesticide exposure and a genetic variant in the *s-phase kinase-associated protein 1* (*SKP1*) gene, albeit we cannot exclude the possibility that this is a chance finding. The combination of high ambient exposure at both residential and workplace addresses and at least one T allele (CT or TT genotype) in *SKP1* rs2284312 resulted in a considerably larger odds ratio compared to high exposure at both locations and the CC genotype (ORs 7.57 and 1.60, respectively); the combination of no/low exposure to pesticides and at least one the T allele resulted in no association (OR = 1.01) suggesting no effect of the T allele in the absence of UPS-inhibiting pesticide exposure.

While the function of rs2284312 is unknown, prior research suggests that *SKP1* may participate in the complex etiology of PD. The SKP1 protein is a component of the SKP1-cullin1-fbox (SCF) protein complexes involved in target identification for ubiquitination and cell cycle regulation (Bai et al., 1996), as well as presynaptic differentiation (Liao et al., 2004). A microarray study has demonstrated decreased expression of *SKP1* in PD brains compared to age matched controls (Grunblatt et al., 2004) and a murine substantia-nigra cellular model reflected a decrease in *Skp1a* after treatment with 1-methly-4-pyenyl-1,2,3,6-tetrhydropyridine (MPTP) (Fishman-Jacob et al., 2009). This study observed a down-regulation of key dopaminergic regulators, *solute carrier family 6* (*neurotransmitter transporter, dopamine*), *member 3* (i.e., *DAT*) and *solute carrier family 18 (vesicular monoamine), member 2* (i.e., *VMAT2*) suggesting a role for *Skp1a* in dopaminergic cell viability. We previously reported an association between a gain-of-function VMAT2 haplotype and decreased risk of PD (Glatt et al., 2006) as well as an association between DAT, paraquat/maneb exposure, and PD (Ritz et al., 2009). Inclusion of these genetic factors in the regression model did not decrease the observed effect measure modification by *SKP1*.

The null findings for *UCHL1* are unexpected as rs5030732 (S18Y) has strong prior support for a role in PD. However, a prior study (Elbaz et al., 2003) that considered S18Y also observed no modification by or interaction with pesticides. While the S18Y variant modifies the hydrolase activity of the resultant protein (Nishikawa et al., 2003), this modification might not impact the UPS function of UCH-L1. In fact, neurons derived from an *Uchl1* S18Y mutant show no change in UPS function (Kyratzi et al., 2008). Our finding of possible effect measure modification by *PARK2* rs9365292, a SNP of unknown function, for one but not both of our exposure categories needs further investigation. We speculate that our finding might suggest a protective role of the gene in subjects with less pesticide exposure, but that the protection provided might be overwhelmed by higher levels of pesticide exposure. *PARK2* is an intriguing candidate and target for increased susceptibility to UPS-inhibiting pesticides in PD as down-regulation resulted in increased vulnerability to UPS inhibition (Yang et al., 2007) and UPS inhibition reduced mRNA expression (Koch et al., 2009) and prevented parkin-mediated mitophagy (Chan et al., 2011), suggesting a link between UPS and mitochondrial dysfunction.

Our study, designed specifically to investigate gene–environment interaction in PD, is one of the largest study populations with genetic material, detailed exposure assessment focused particularly on pesticides, and movement disorder specialist-confirmed idiopathic PD cases examined repeatedly over time. Because of the *a priori* focus on pesticides, our study assessed multiple data sources for pesticide exposure including self-reported use, occupational history, and the record-based GIS model that generated the exposure assignments for this analysis. Despite these advantages our results are still vulnerable to biases of observational research in humans. We have attempted to minimize the possible effect of

Table 3
Effect measure modification: UPS-inhibiting pesticide exposure and UPS-related genetic variants in PD.

Genetic variant pesticide exposure level	Homozygous wildtype		Variant carriers		Interaction	
	Cases/Controls[a]	OR[b] (95%CI)	Cases/Controls[a]	OR[b] (95%CI)	OR (95%CI)	*p*-value
SKP1 rs2284312						
No/low exposure to all UPS-inhibiting pesticide[c]	79/148	1.00 (Reference)	29/52	1.01 (0.59, 1.73)	n.c.	n.c.
High exposure at either residence or workplace	68/103	1.25 (0.83, 1.90)	33/37	1.65 (0.95, 2.85)	1.30 (0.60, 2.83)	0.506
High exposure at both residence and workplace	43/50	1.62 (0.98, 2.66)	29/7	7.57 (3.14, 18.3)	4.63 (1.59, 13.5)	0.005
PARK2 rs9365292						
No/low exposure to all UPS-inhibiting pesticide[c]	64/118	1.00 (Reference)	44/82	0.98 (0.61, 1.59)	n.c.	n.c.
High exposure at either residence or workplace	51/90	1.05 (0.66, 1.67)	50/48	1.93 (1.17, 3.20)	1.87 (0.92, 3.81)	0.086
High exposure at both residence and workplace	40/31	2.40 (1.36, 4.22)	32/25	2.30 (1.25, 4.24)	0.98 (0.42, 2.29)	0.956
UCHL1 rs5030732						
No/low exposure to all UPS-inhibiting pesticide[c]	67/93	1.00 (Reference)	37/40	1.28 (0.74, 2.24)	n.c.	n.c.
High exposure at either residence or workplace	65/63	1.43 (0.89, 2.30)	32/31	1.48 (0.81, 2.68)	0.81 (0.35, 1.84)	0.608
High exposure at both residence and workplace	47/28	2.32 (1.30, 4.13)	23/16	2.09 (1.01, 4.33)	0.70 (0.27, 1.86)	0.479
UBA6 rs354872						
No/low exposure to all UPS-inhibiting pesticide[c]	92/124	1.00 (Reference)	15/19	1.09 (0.52, 2.27)	n.c.	n.c.
High exposure at either residence or workplace	75/97	1.06 (0.70, 1.59)	21/11	2.71 (1.23, 5.94)	2.36 (0.80, 6.97)	0.120
High exposure at both residence and workplace	56/44	1.72 (1.06, 2.81)	12/3	5.33 (1.46, 19.5)	2.85 (0.62, 13.0)	0.177
UBE2D1 rs11593650						
No/low exposure to all UPS-inhibiting pesticide[c]	84/99	1.00 (Reference)	25/41	0.74 (0.41, 1.32)	n.c.	n.c.
High exposure at either residence or workplace	83/75	1.31 (0.85, 2.02)	19/27	0.92 (0.47, 1.78)	0.95 (0.39, 2.31)	0.908
High exposure at both residence and workplace	55/35	1.80 (1.06, 3.06)	16/12	1.81 (0.80, 4.11)	1.36 (0.47, 3.91)	0.567
UBA1 rs4529579						
No/low exposure to all UPS-inhibiting pesticide[c]	63/69	1.00 (Reference)	41/72	0.62 (0.37, 1.05)	n.c.	n.c.
High exposure at either residence or workplace	54/50	1.21 (0.72, 2.03)	38/57	0.75 (0.44, 1.29)	1.00 (0.46, 2.14)	0.992
High exposure at both residence and workplace	39/21	2.09 (1.11, 3.96)	26/26	1.12 (0.59, 2.15)	0.86 (0.34, 2.17)	0.753

Abbreviations: UPS, ubiquitin–proteasome system; PD, Parkinson's disease; OR, odds ratio; CI, confidence interval; *PARK2, Parkinson protein 2, E3 ubiquitin protein ligase (parkin)*; *UCHL1, ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)*; *SKP1, S-phase kinase-associated protein 1*; *UBA6, ubiquitin-like modifier activating enzyme 6*; *UBE2D1, ubiquitin-conjugating enzyme E2D 1*; *UBA1, ubiquitin-like modifier activating enzyme 1*.

[a] The number of subjects per exposure group changes across genetic variants because genotyping occurred at different times during the recruitment and enrollment process therefore not all subjects contributed to each analysis.

[b] Dominant genetic model odds ratio adjusted for age (continuous), gender (male/female), and smoking status (ever/never).

[c] Subjects in the reference group have no/low exposure to ambient benomyl, cyanazine, dieldrin, endosulfan, ferbam, metam, propargite, rotenone, triflumizole, and ziram at both residences and workplaces.

exposure misclassification and increase specificity by focusing on pesticide associations for subjects most highly exposed. Even though our study is one of the largest with such specific pesticide data, it is likely under-powered to detect associations for pesticides of low prevalence (such as rotenone in our study) or smaller size interactions. Notwithstanding these limitations, we observe associations with PD for the pesticides identified in our screen as UPS inhibitors and modification of that pesticide effect by a candidate gene in the UPS pathway.

A growing body of research has investigated the possibility that associations between pesticide exposures and PD may vary dependent upon a susceptibility genotype (Dutheil et al., 2010; Elbaz et al., 2004; Fong et al., 2007; Goldman et al., 2012; Hancock et al., 2008; Lin et al., 2011) and some results have been replicated in independent study populations including the gene–environment interactions for *solute carrier family 6* (*neurotransmitter transporter, dopamine*), *member 3* (Kelada et al., 2005; Ritz et al., 2009), and *paraoxonase 1* (Dick et al., 2007; Manthripragada et al., 2010). Our results contribute to the growing body of literature attempting to parse the combined effects of environment and genetic susceptibility on the occurrence of PD. This study strongly suggests a role for UPS-inhibiting pesticides in the etiology of PD and the possibility that genetic susceptibility may exacerbate the effects of exposure to these pesticides.

Funding sources

This work was funded in part by NIEHS Grants R01-ES010544, U54-ES012078, P01-ES016732; the Michael J. Fox Foundation; The

Parkinson Alliance, and the American Parkinson Disease Association. The funding organizations had no role in the design, conduct, interpretation, or publication of this work.

Human subjects protections

Written informed consent was obtained from all enrolled subjects or their proxies. All procedures were approved by the UCLA Institutional Review Board: IRB #11-001530. The UCLA IRB's Federalwide Assurance (FWA) with Department of Health and Human Services is FWA00004642 (IRB00004473).

Acknowledgments

We are exceptionally grateful to all study participants, whose generosity made this research possible. We thank Dr. Papp (UCLA); Drs. Checkoway and Farin (University of Washington); and Drs. Rotter and Taylor (Cedars-Sinai Medical Center) for genotyping. We thank Sharon Li for assistance in growth of cell lines.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2013.08.001.

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