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Summary

Residents have raised concerns regarding possible health effects of applications of pesticides in the vicinity of homes. The Health Council of the Netherlands concluded in 2014 that there were sufficient reasons to initiate an exposure assessment study among residents living close to agricultural land. The "Onderzoek Bestrijdingsmiddelen en Omwonenden" (OBO) study was initiated to clarify the extent to which agricultural use of pesticides in the vicinity of homes contributes to exposure of residents to pesticides. This report describes the results of the exposure assessment study of residents living nearby agricultural land cultivated with flower bulbs (OBO flower bulbs). It should be noted that human exposure is the focus and that health effects were not investigated in this study.

The research questions were:

i) What are concentrations of pesticides in the environment of residents living near agricultural land with the cultivation of flower bulbs compared to residents living further away?
ii) What is the personal exposure to pesticides of residents living near agricultural land with the cultivation of flower bulbs compared to residents living further away?
iii) What are the exposure sources and routes contributing to personal and environmental exposure to pesticides in areas with the cultivation of flower bulbs?

To answer the research questions, an exposure assessment strategy was developed that included environmental sampling, biomonitoring, and the collection of contextual information. Homes within 250 m of selected agricultural fields with cultivation of flower bulbs and residents living in these homes were included. Growers and their families, living in the selected area, were also eligible for participation in the study but were treated separately in the statistical analyses. Environmental samples collected from homes were analyzed for a large number of pesticides used in bulb growing and other cultures and personal samples from the residents were analyzed for five selected pesticides. Results were compared to the results from samples collected from control locations located at least 500 m away from any agricultural fields. This yielded a vast amount of data that was analyzed carefully using various methods including statistical and deterministic models to answer the research questions.

Existing deterministic exposure models for pesticides, representing different exposure routes, were coupled and verified using measurement results from OBO experimental studies on spray drift and volatilization and the field study. This provided insights into the relative importance of the different exposure routes for residential pesticide exposures.

1. Higher concentrations of several pesticides were found in environmental samples collected from inside and outside the homes of people (residents) living close to bulb fields compared to concentrations in homes further away from the fields (controls).
2. These higher concentrations of pesticides were observed in the homes of people living close to bulb fields, both in the use and non-use period.
3. Biomarkers of two out of the five analyzed pesticides were found in more than half of the urine samples from persons, including (young) children, in both residents and controls. This was observed inside as well as outside periods of pesticide use. Relationships between the concentrations of these two pesticides in urine and distance to sprayed fields or periods of pesticide use were not consistently observed. However, concentrations found in urine correlated with the concentrations of pesticides inside and outside the homes.
4. Concentrations of pesticides inside and outside the homes of growers were generally higher than those found for residents living near agricultural land.
5. Calculations showed that volatilization of pesticides from the field after spraying and pesticides in house dust are likely the most important routes for exposure to pesticides of residents living close to bulb fields in our study. Because wind during spraying was not directed towards the homes of residents, drift was not observed in the field study. From experimental studies within OBO flower bulbs we conclude that drift can reach higher altitudes and larger distances than thought before.
6. The research has generated tools for a time-resolved predictive model to estimate exposure of residents of bulb fields and other crops with downward spraying, via both air and house dust, for all pesticides, locations and moments. However, important knowledge and information gaps still remain precluding estimates on a national scale.

Some pesticides were found in urine samples among participants as well as controls, including (young) children. At the same time correlations were found between environmental and urinary concentrations of these pesticides. These outcomes need to be explored in relation to possible health implications. Such an evaluation should take into account more factors influencing pesticide concentrations, including different pesticides used, varying distances to agricultural fields, different soil types, varying weather conditions and different susceptible subgroups (e.g. unborn or young children and individuals with co-morbidities).

7. OBO flower bulbs: discussion and conclusions

7.1 The OBO bulb flower study

Approximately 30% of all Dutch homes are situated within 250 m of any agricultural field. This goes down to 18% if grass land is not considered in this calculation. Concerns have been raised about the exposures to pesticides of these residents and possible associated health effects. Several international studies on exposure of residents to pesticides have been carried out but have shown variable findings. Some observed differences in exposure levels between urban and rural populations (Courture et al, 2009) whereas others did not (Kimata et al, 2009; Koureas et al 2009). Given these differences and the lack of information on exposure levels of the Dutch (rural) population in relation to pesticide use on agricultural fields, the OBO study was initiated. This research project addressed the recommendations from the Health Council of the Netherlands (Health Council of the Netherlands, 2014) and was commissioned by the Dutch Ministries of Infrastructure & Water Management and Economic Affairs & Climate Policy.

The OBO study aimed to assess the exposure to pesticides of residents living within 250 m from an agricultural field. To address this aim, a study design was applied that combined environmental sampling (outdoor and indoor air, dust from the doormat, vacuumed floor dust, and soil from the garden), personal sampling (urine and hand wipes), and exposure models. To limit costs however, OBO was split into phases. The ministries selected flower bulbs as the crop to be studied first. This is a crop that is typically grown with extensive use of pesticides. Flower bulb growing fields are always sprayed using downward spraying techniques. Cultivations treated with side- or upward spraying techniques, such as fruit orchards, are not covered in OBO flower bulbs. The main research questions of OBO flower bulbs are:

i) What are concentrations of pesticides in the environment of residents living near agricultural land with the cultivation of flower bulbs compared to residents living further away?
ii) What is the personal exposure to pesticides of residents living near agricultural land with the cultivation of flower bulbs compared to residents living further away?
iii) What are the exposure sources and routes contributing to personal and environmental exposure to pesticides in areas with the cultivation of flower bulbs?

The OBO flower bulb study was conducted from 2016 to 2018. It included experimental measurements on spray drift and volatilization and the residents' field study. Measurements in the residents' field study, carried out in 2016 and 2017, included environmental measurements (outdoor air, indoor vacuumed floor dust, dust from indoor doormats and soil from the garden) in homes of residents within 250 m of a

target field with cultivation of flower bulbs. Growers living within 250 m of the field were also invited to participate as residents. Samples taken from this group and their environment were treated separately in the analysis of the data. A control group was recruited from areas with less than 1500 addresses per km^2, with no agricultural fields within at least 500 m but within 20 km from the target field. Residents from both location homes and control homes participated in biomonitoring and therefore collected morning urines. A pesticide application on the target field started the week-long sampling protocol. Homes and residents within 50 m of the edge of the target field participated in an additional protocol, collecting indoor air samples, first day urines and a hand wipe in the first 24h after the application. Measurements outside the period of pesticide application were also conducted during two days in both location and control homes. Selected environmental samples were analyzed on 46 different pesticides while biomarkers of five different pesticides were assessed in selected urine samples. Spray registration was collected from all fields in the area. Of the approached growers, 17% participated offering a target field and 36% shared their spray registration. Of the approached residents at the locations, 4.5% participated. The effect of the response rate for the different groups on the results is unknown.

This chapter is organized to first provide an overall summary of the main results, followed by addressing the aforementioned research questions individually with study results and discussion.

7.2 Summary of main findings

Samples were taken on many locations and under variable conditions with respect to housing, distance to fields, meteorological conditions, spraying features etc. Moreover, pesticides are not a homogeneous group of chemical compounds but cover a wide range of compounds with a large range in chemical and physical properties such as the vapor pressure of the pesticide. As a consequence, the concentrations observed show a wide range covering sometimes orders of magnitude. The summary findings describe general patterns. Results for specific pesticides can be found in chapters 4 – 6 of the report.

For environmental samples, we found higher concentrations of several pesticides inside and outside the homes of people living close to bulb fields (residents) compared to homes further away (controls). Relationships between distance to the field and pesticide concentrations as well as between periods of pesticide use/non-use and pesticide concentrations were clear for both outdoor air and indoor dust measurements, with decreasing concentrations with increasing distance. In personal samples, we detected biomarkers of pesticides in the urine samples of residents and controls, including (young) children, both during and outside periods of pesticide use. Relationships with distance or period were less evident for pesticide concentrations found in the urine of residents. However, looking at individuals, urinary concentrations correlated with the concentrations of pesticides in air and/or house dust to which they were exposed.

Drift of aerosols towards homes during actual spraying was not observed during our field measurements since the wind direction during the applications was away from the homes. The dispersion of pesticides after volatilization from the field and contact with and ingestion of house dust (possibly dragged into the homes from outside) were identified as major routes of exposure in our study.

Concentrations of pesticides in the living environment of growers and their family members were generally higher than those of residents in the same area. However, levels in urine samples from growers and their family members are in the range of those of other residents. The concentrations of measured pesticides inside and outside the homes and in the urine samples of controls indicate an exposure that is not driven by applications nearby homes. These background levels were higher during the period of pesticide use than the non-use period. From our experimental studies we concluded that the drift of pesticides downwind from downward spraying leads to measurable concentrations at greater distances (>50 m) and height (10 m) in the air than known before. Therefore, drift could still be an important route of exposure if the wind is directed towards homes during application of pesticides even at these larger distances. Predicting the total exposure of all residents near bulb fields and other crops with downward spraying, via both air and house dust, for all pesticides, all locations in the Netherlands and all moments is not yet possible. The research conducted thus far offers the components to develop models for residential pesticide exposures and thus may represent a way to upscale pesticide exposure assessment for large scale population studies.

The main findings of OBO are summarized in Box 7.1:

Box 7.1: Main findings of OBO

1. Higher concentrations of several pesticides were found in environmental samples collected from inside and outside the homes of people (residents) living close to bulb fields compared to concentrations in homes further away from the fields (controls).

2. These higher concentrations of pesticides were observed in the homes of people living close to bulb fields, both in the use and non-use period.

3. Biomarkers of two out of the five analyzed pesticides were found in more than half of the urine samples from persons, including (young) children, in both residents and controls. This was observed inside as well as outside periods of pesticide use. Relationships between the concentrations of these two pesticides in urine and distance to sprayed fields or periods of pesticide use were not consistently observed. However, concentrations found in urine correlated with the concentrations of pesticides inside and outside the homes.

4. Concentrations of pesticides inside and outside the homes of growers were generally higher than those found for residents living near agricultural land.

5. Calculations showed that volatilization of pesticides from the field after spraying and pesticides in house dust are likely the most important routes for exposure to pesticides of residents living close to bulb fields in our study. Because wind during spraying was not directed towards the homes of residents, drift was not observed in the field study. From experimental studies within OBO flower bulbs we conclude that drift can reach higher altitudes and larger distances than thought before.

6. The research has generated tools for a time-resolved predictive model to estimate exposure of residents of bulb fields and other crops with downward spraying, via both air and house dust, for all pesticides, locations and moments. However, important knowledge and information gaps still remain precluding estimates on a national scale.

The OBO study looked at exposure to pesticides of residents living near agricultural land. The study did not assess possible health effects of such exposures.

Below, the results will be discussed according to the three aims that were set for OBO flower bulbs.

7.2.1 Environmental exposure

What are concentrations of pesticides in the environment of residents living near agricultural land with the cultivation of flower bulbs compared to residents living further away?

We found elevated concentrations of pesticides in soil, outdoor and indoor air, and both vacuumed floor dust and dust from doormats of residents' homes located within 250 m of agricultural fields compared to control homes. This observation is based on the comparison of the exposed locations (residents living within 250 m of treated fields) to control locations (residents living in a non-urban area with no agricultural cultivations, defined above) and when comparing at exposed locations the use and non-use periods of studied pesticides. Pesticide concentrations in outdoor air close to the homes of residents of exposed locations were generally a factor ten or more higher than outdoor concentrations at control locations. Pesticide concentrations in both vacuumed floor dust and dust from doormats in homes of residents of exposed locations were generally a factor five higher than concentrations observed in control homes in both the use and non-use period. For some pesticides, this difference reached up to a factor 100. As expected, outdoor air concentrations tended to decline with

increasing distance to the agricultural field. The highest concentrations were found within 50 m from the treated fields and lower concentrations between 150 to 250 m. Even at the latter distances, concentrations were generally higher than the ones found at the control locations.

Elevated concentrations of pesticides in soil, outdoor and indoor air, and both types of dust samples were also found for several pesticides not applied close to the residents' homes during the measurement period. These pesticides may have been used before the measurement period started and volatilized from soil or dragged into the home, or they may have been applied further away from the homes, or used for other purposes than field spray applications. For example, elevated concentrations were found for thiophanate-methyl (as its environmental degradation product carbendazim) and pyraclostrobin. Both compounds are used in bulb disinfection. This implies that outdoor and indoor exposure levels are not only related to pesticide applications on the field but may also be related to other sources such as emissions from bulb disinfection activities or storage facilities in the neighborhood. It should also be noted that in this region pesticides have been used for many years and more persistent ones (such as carbendazim) may remain in the environment for a longer period, leading to ongoing exposures for residents.

Homes of people working in the agricultural sector ("growers") were not considered in the main analyses and the results of measurements in the growers' homes ("farm homes") were interpreted separately. Concentrations in outdoor air and both types of indoor dust samples were clearly higher in the farm homes compared to those found in the residents' homes. Concentrations of pesticides were generally a factor of two higher in air and a factor of ten higher for house dust. The higher concentrations in air were partially explained by the shorter distances to sprayed fields as compared to the homes of residents. A possible additional explanation could be bulb disinfection activities or storage facilities near farm homes. The higher concentrations in dust in comparison to residents' homes close (< 50 m) to agricultural fields may be due to (unintended) carrying pesticides to home from work through, for example, contaminated work clothing and shoes.

What are the exposure sources and routes contributing to environmental exposure to pesticides in areas with cultivation of flower bulbs?

In figure 7.1, adapted from the Health Council of the Netherlands, the different exposure routes that eventually could lead to human exposure are depicted. In this section we discuss these different routes step by step in the context of our results.

1. Spray drift
   The first step in Figure 7.1 is spray drift. During the field study, there were no applications that resulted in drift of aerosols towards the residents' homes. It is a preferred practice of the growers to apply pesticides when the wind blows away from residents' homes. However, in certain situations (not encountered in this study) homes could be situated or be build at more than one side of the field, the wind direction can shift towards the homes during an application or an application is urgently performed due to an emerging pest while the wind speed and/or direction are unfavorable. In a sensitivity analysis simulating unfavorable conditions, we found that in such cases, spray drift exposure may be more than a factor of ten higher than exposure observed in this study, which was based on the growers using 75% - 95% drift reducing techniques. The experiments carried out in this study showed that drift can reach greater heights and larger distances than reported in previous studies. Airborne spray drift is a factor ten to 100 higher than ground deposition at the same distance. In contrast to general belief, drift found behind a wind barrier is not always lower but can also be higher. This is based on experimental results from this study and depends on the porosity of the wind barrier.

2. Volatilization
   Volatilization was the dominant contributor to air concentrations on the days after an application. While the cumulative amount emitted into the air during application was about 0.2% of the dosage, the cumulative amount emitted due to volatilization can range up to several tens of percent of the dosage even for less volatile compounds. For compounds with high vapor pressure the emission from volatilization is much larger. However, the rate and extent of volatilization can be strongly affected by other processes occurring on the plant leaves, such as penetration into the plant tissue, photo-transformation, wash-off and the presence of adjuvants in the formulated product.

3. Concentrations in soil
   Concentrations of some pesticides (i.e. pendimethalin, prochloraz and pyraclostrobin) measured in soil samples collected in gardens near the residents' homes were a factor of five to ten higher than those for control homes. Clear differences in pesticide concentrations were not observed between pesticide use and non-use periods. Explanations for this observation were not investigated but slow degradation of these compounds in soil could play a role. Possibly, contaminated soil contributes to elevated levels in house dust through drag-in. The importance of this route however is not fully understood and the evidence is too limited to support strong conclusions.

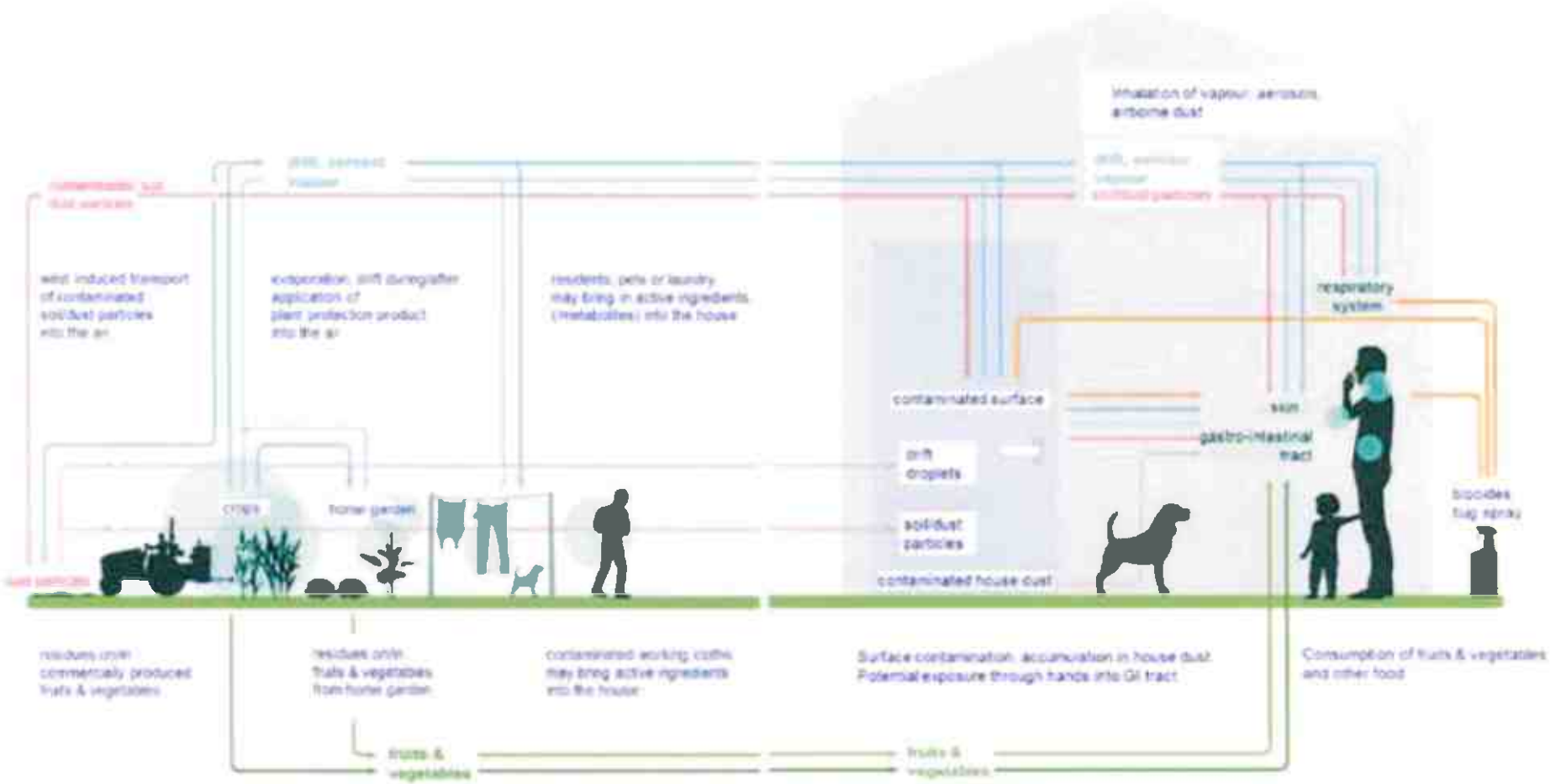

Figure 7.1: Exposure sources and exposure routes.

Different suggested sources and routes of exposure (left panel: outdoor; right panel: indoor). Colored arrows represent different type of routes: blue: direct exposure; red: indirect exposure via particles; green: indirect exposure via food; yellow: direct and indirect exposure from products used at home (Adapted from: Health Council of the Netherlands, 2014).

4. Residues on fruits and vegetables from home gardens
   A limited number of home grown fruit and vegetable samples were taken. Generally, no or only traces of pesticide residues (below 10 µg/kg, i.e., the default maximum residue limit (MRL) for pesticides not registered for use on vegetables or fruits) were observed. Overall, pesticide residues content exceeding 10 µg/kg were found in six samples (30% of tested samples). Two of these samples came from the garden of a farm home. A comparison with the situation for control residents could not be made because no fruits and vegetables samples were available from these locations.

5. Exposure related to working clothes
   The next step in figure 7.1 is exposure related to (working) clothes. This was not studied in OBO flower bulbs. Contaminated working clothes have been indicated to influence exposure of families of growers and pesticide applicators (Curwin et al, 2002). This could be related to direct contact, volatilization of pesticides from the clothes, or other routes. We did not collect information on individual habits regarding working clothes (e.g. changing of clothes outside the home, separate washing of work clothing). Therefore, the contribution of this exposure route cannot be assessed in this study.

6. Concentrations in house dust
   Elevated concentrations of pesticides were observed in house dust collected in homes of residents compared to those in control homes. The level of pesticides in house dust (i.e. vacuumed or collected from doormats) did not show a clear relationship with distance to the sprayed field. The occurrence of pesticides in house dust could be caused by several mechanisms. One is the absorption of gaseous pesticides to already present house dust. Another is the direct deposition of spray drift related particles in the house from previous applications in the vicinity of the home or from applications further away. Also, a relation with drag-in of contaminated particles is possible. Relationships between concentrations in dust and distance to fields were investigated but the results do not allow conclusions regarding the dominant mechanism and this aspect needs further research.

7. Concentrations on outdoor surfaces
   Another exposure pathway in Figure 7.1 is the contamination of surfaces. The deposition of drift, aerosols and vapor could lead to contaminated surfaces around the homes of residents. We did not measure the amount of pesticides on surfaces such as garden furniture and playground equipment. In principle, estimates could be made using the models tested in this study. We did not perform such analyses given the uncertainty regarding how this would relate to personal exposure as it would require detailed information on time-activity and use patterns. The contribution of this route therefore remains uncertain.

7.2.2 Personal exposure

What is the personal exposure to pesticides of residents living near agricultural land with cultivation of flower bulbs compared to residents living further away?

Personal exposure to pesticides in the OBO study was measured through monitoring of five biomarkers of pesticides in urine samples (asulam, thiophanate-methyl/ carbendazim, chlorpropham, prochloraz, and tebuconazole). These pesticides were chosen based on their use, feasibility of analysis, as well as representativeness of different types of pesticides, and reflect different physical-chemical properties. All, except thiophanate-methyl/carbendazim, were applied on the target fields. Thiophanate-methyl is used as a bulb disinfectant, is degraded to carbendazim in the environment, and was found as carbendazim in many of the dust samples. In an experimental study within OBO flower bulbs (the volunteer study), where volunteers were exposed via dermal or oral routes to pesticide concentrations just below the level of the Acceptable Daily Intake (ADI), the suitability of specific urinary biomarkers of exposure for each of the five pesticides was confirmed. The identified biomarkers are also the major biomarkers found in animal studies. Furthermore, the volunteer study demonstrated that urinary biomarkers were rapidly excreted after oral exposure (50% of dose excreted between three to 25 hours). Excretion rates were much lower after dermal exposure compared to oral exposure. Uptake by the skin is a comparatively slow process resulting in a slow absorption into the blood circulation, and excretion continued for at least 48 h after exposure. Conversion factors were calculated based on molar fractions for each of the individual pesticides. These factors were used for the calculation of the biomarkers excreted through urine based on the amount of the pesticide taken up via oral and dermal routes.

In the residents' field study urinary biomarker concentrations of asulam, thiophanate-methyl/carbendazim and prochloraz were generally below the limit of detection. Urinary biomarkers of chlorpropham and tebuconazole were detected in 82% and 63% of urine samples respectively, including samples from diapers. Pesticides were detected in urine samples from young and older children at levels in the same order of magnitude as found in adults. Urinary concentrations of chlorpropham were a factor two higher among residents than controls. No difference in urinary concentrations of chlorpropham was found between pesticide use and non-use periods. For tebuconazole a slight difference of a factor two in urinary concentrations was found for the residents between pesticide use and non-use periods, but not between the residents and controls. In addition, urinary concentrations of chlorpropham and carbendazim correlated with the concentrations of the pesticides in air and house dust measured in the persons' living environment. These results suggest an environmental contribution to the measured urinary pesticide concentrations of the residents for some pesticides.

Hand wipes from the day of application were collected from residents living within 50 m of the field. The five pesticides from the biomonitoring could also be identified

on the hand wipes. However, as only residents living in close proximity of the field collected hand wipes, no comparison could be made to residents living further away or controls.

Urine levels in context of the ADI

A comparison of the urine levels found in this study with a measure like the Acceptable Daily Intake (ADI) may help to place the levels in a context. There is no direct way to calculate levels in urine back towards intake. However, results from the volunteer study provided indications on how to calculate back towards intake. The observed concentrations of biomarkers in the participants' and growers' urine were in general lower compared to those in the volunteers, who received a single oral or dermal dose just below the ADI. The ADI is a measure of the amount of a specific substance that can be ingested orally on a daily basis over a lifetime without an appreciable health risk. However, this dosage comparison with the results from the volunteer study has limitations. First, the results for the five included pesticides cannot be generalized or extrapolated to other pesticides or scenarios because the ADI is different for each pesticide. Furthermore, 'worst case' scenarios for pesticide exposure were not encountered in the study. In addition, exposure of the residents is a combination of contributions through the oral, dermal, and inhalation route. As the ADI only addresses the oral intake and possible associated health effects, the applicability of the ADI as a reference for total internal exposure is uncertain. For example, respiratory pesticide intake and associated health effects are not considered by the ADI. Moreover, residents are not exposed to a single pesticide but to more pesticides at the same time. It is suggested that there might be additive or synergistic effects of being exposed to different pesticides that share a similar (biological) mode of action. Lastly, the pesticides studied here were not selected for their potential toxicity and/or health effects. Therefore, these results do not provide information regarding the presence or absence of a health risk for the residents.

It should be noted that the ADI might not be applicable for unborn and young infants until the age of 16 weeks, a phase of very rapid development (EFSA, 2017b; EFSA, 2018). However, as the youngest participant in OBO was 2 years old, this is not applicable for the presented results.

What are the exposure sources and routes contributing to personal exposure to pesticides?

Based on the modeled and measured concentrations we estimated the potential contributions of the different exposure sources and routes to personal exposure. These results indicate that when accounting for dermal and oral/inhalation uptake rates, inhalation, dermal uptake, and incidental dust ingestion could all be important contributing routes for exposure. Relative contributions differ, depending on the concentrations of the pesticide in the different compartments and on the uptake rates through the different routes.

7.3 Modelling - Integrative analysis of the exposure routes

One of the aims of the OBO-study was to develop an integrated framework of models suitable to assess exposure of residents to pesticides from nearby treated fields. As OBO flower bulbs only covered crops sprayed with downward spray techniques, the current integrated model is only field tested for these conditions. Significant progress was made in developing an integrated model framework that currently consists of a chain of inter-dependent models that predicts exposure on an hourly basis. Model verification, both through experimental studies and field observations, indicated that the developed integrated model framework could be suitable for estimating residential pesticide exposure levels on a high spatial and temporal resolution. Most of the individual models in the integrated model framework produced estimates that were within the same order of magnitude as measured levels. An exception to this were pesticide concentrations in indoor dust. These could not be explained by the existing models of gas to particle conversion. This could be because current models do not account for other sources such as the contribution of active drag-in of pesticide containing dust from outdoors to indoors. No comparisons were made between the integrated framework to dynamically model the exposure of pesticides of residents with regulatory models.

7.4 Recommendations

Based on the results of the OBO flower bulb study several recommendations can be made.

1. Estimating potential health implications of the measured environmental concentrations was not an aim of the study. However, given that measurable concentrations of some pesticides were found in urine samples among participants and controls, including children, and that correlations were found between environmental and urinary concentrations of these pesticides the current results need to be explored in relation to possible health implications. Such an evaluation should take more features into account, like other pesticides, varying layouts of locations, different soil types, more weather conditions, more routes of exposure and susceptible subgroups (e.g. unborn and young children, individuals with co-morbidities). It should be noted that detection of pesticides in urine samples of young children may cause concerns because unborn and very young children are in a phase of rapid development. Disruption of the normal development could occur at levels that are not considered hazardous for adults (Berghuis et al., 2015, Council On Environmental Health, 2012).

2. The insights obtained in this study indicate that exposure gradients were relatively modest within 250 m of fields but more pronounced when compared to homes further away (> 500 m). The recently conducted exploratory Health Survey in the Netherlands (RIVM Rapport 2018-0068) focused on presumed exposure-response functions across very short distances (0 - 50, 50 – 100, 100 – 250 and 250 – 500 m) to fields. The results from the OBO flower bulb study necessitate a re-evaluation of the Health Survey that would more specifically focus on health effects within 0-250 m of fields as compared to further away and less on increasing risk estimates with decreasing distance to fields. Such re-evaluation of the Health Survey may lead to potential additional findings above the currently reported health associations.

3. Results from this research have indicated that the exposure gradients for house dust are less clear and that routes leading to pesticides in dust (e.g. drag-in) are not well understood. As pesticides in house dust could be an important source of exposure, especially for children (due to a potentially higher intake of dust), more information on the pesticide levels in house dust and its driving factors (drag-in, absorption of gaseous pesticides to house dust) needs to be collected. We therefore recommend to carry out measurements on pesticide levels in indoor dust among residents living close to agricultural fields and compare them to controls living further away. Such a survey would need to cover a variety of crops and farming systems to understand the distribution and concentration of pesticides in house dust and to improve predictive models.

4. The current study focused on flower bulb cultivations for which downward spraying techniques were used. As indicated at the onset of the project, this does not provide insight in the exposure of residents living near crops where sideways or upward spraying techniques are used (such as fruit trees). It is known that these techniques have higher emissions due to a higher drift potential, leading to possibly higher exposure of the residents. To study these techniques and associated exposures and to improve the integrated model framework to accommodate these application techniques, it is recommended to carry out a verification study on pesticide exposure in homes surrounding orchards. This would also allow further development of models addressing other transport pathways such as drift and volatilization (e.g. further insight in the effect of obstacles).

5. The data on the residents' exposure and how the different transport pathways contribute to this exposure could be used to develop exposure scenarios for the assessment of exposure in the framework of the European authorization procedures of pesticides. The modelling framework developed in this project could be used to calculate exposure under different scenarios and could be used to improve the recently developed regulatory models (e.g. OPEX, BREAM), which now have incorporated procedures to estimate residential exposures.

6. In order to apply the integrated model framework to estimate population level exposures, the model chain will need to be further developed and computing efficiency should be increased. One of the main sources of uncertainties, however, arises from the fact that input data on applications on specific fields are difficult to obtain. In our study we were able to obtain the information related to pesticide applications (e.g. type of pesticide applied, application time) as this was asked from the growers. The timing of other applications on the target fields or other fields in the vicinity were assigned based on knowledge of used application schedules. However, to derive valid hourly/daily estimates of population level residential exposures it will be important to improve the reporting of all pesticide use by all growers. Estimates on longer time scales (such as annual averages) for indoor and outdoor air concentrations could potentially be calculated using currently available methods as exact timing of applications would be less critical. Such results could be used to highlight potentially important areas, crops, and/or pesticides for residential environmental exposure.

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Bron: https://www.theguardian.com/environment/2023/jun/01/pesticide-firms-withheld-brain-toxicity-studies-from-eu-regulators-study-finds

Pesticide firms withheld brain toxicity studies from EU regulators, study finds

Exclusive: The same studies were submitted to US regulators and some are relevant to safety levels, the researchers say

Damian Carrington *Environment editor*
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Thu 1 Jun 2023 05.00 BST

Pesticide companies failed to disclose a series of studies assessing brain toxicity to European regulators, according to new research, despite the same studies having been submitted to US regulators.

When the EU authorities were made aware of the studies, between 14 and 21 years after they were conducted, new safety limits were applied in some cases and evaluation is still under way in other cases.

The researchers described the omissions as "outrageous", concluding that "apparently non-disclosure is a problem that is not rare" and that there could be "no reliable safety evaluation of pesticides by EU authorities without full access to all performed toxicity studies".

The new research is the first systematic assessment of non-disclosure and focused only on developmental neurotoxicity (DNT) studies. The researchers found 35 DNT studies submitted to the US Environmental Protection Agency as part of the pesticide approval process but found that nine of these had not been included in dossiers sent to the EU authorities for the same pesticides.

Among the findings in the undisclosed studies were changes in brain size, delayed sexual maturation and reduced weight gain in the offspring of laboratory rats exposed to a pesticide when pregnant. The pesticides identified in the new study include the insecticides abamectin, ethoprophos and pyridaben and the fungicide fluazinam. These are, or have been, used on a range of crops including tomatoes, strawberries, potatoes and aubergines.

"Brains are unbelievably complex and so central for us being humans, and damage to brain development is immensely costly to societies," said Dr Axel Mie, at Stockholm University, Sweden, who led the new study. "So it's really

important for us to make sure that the chemical products we use are not damaging the brains of our kids and grandchildren."

Prof Christina Rudén, study co-author and also at Stockholm University, said: "Most important to me is the principle to have to tell the truth, the whole truth and nothing but the truth. It is outrageous, what they're doing."

Sarah Wiener, a Green party MEP from Austria and the European parliament rapporteur for new EU pesticide regulation proposals, said: "The analysis shows that the pesticide industry is fooling EU authorities. In the end, it is EU citizens who pay the price. Their health is jeopardised when relevant studies are withheld."

Austrian Green MP and television cook Sarah Wiener, here with Ursula von der Leyen in 2019, said that EU citizens pay with their health when relevant studies are withheld. Photograph: Patrick Seeger/EPA

"The EU therefore needs to make sure that there are harsh consequences for the withholding of data," she said. "This could mean that corporations would have to pay considerable fines."

EU regulations state that pesticide dossiers should "include a full and unbiased report of the studies conducted [unless] it is not necessary owing to the nature of the product or its proposed uses, or it is not scientifically necessary. In such a case a justification shall be provided."

A spokesperson for the European Commission said: "There is a clear obligation to submit all available adverse data as part of applications since 2013, and there is an obligation to notify adverse data when they become available since 1991."

The power to penalise companies if they unlawfully fail to disclose toxicity studies in Europe lies with national regulators. But no known penalty has

been imposed on any pesticide company to date. The UK pesticides regulator, the Health and Safety Executive, did not answer a request for comment.

In correspondence seen by the Guardian, a senior official in the European Commission's directorate for health and food safety expressed "serious concern" in September 2022 after being made aware of two of the undisclosed studies: "The fact that certain applicants have apparently not provided studies with an unfavourable outcome for certain active substances as part of the application dossiers is a serious concern."

The chemical companies said they had complied with the EU regulations, in some cases arguing they were not legally obliged to submit the studies. They also disagreed with the researchers' conclusions that some of the studies had led to tighter regulation when the EU authorities had become aware of the studies' existence, or that they could do so in the future.

Previous work estimated that exposure in the EU to organophosphate insecticides, which are now banned and were not part of the new study, caused brain damage costing €146bn a year in lost productivity. The new report said: "For some compounds, it has taken decades from the initial evidence of DNT effects in humans until such hazards became widely recognised."

The study is published in the peer-reviewed journal Environmental Health. It found nine undisclosed DNT studies produced between 2001 and 2007, up to 20 years before the submission of the most recent EU regulatory dossiers. The EU authorities became aware of the studies between 2017 and 2022, the researchers said.

Standard DNT tests expose pregnant female rats to a pesticide and assess their offspring for neuropathological and behavioural changes. The tests have been shown to identify chemicals known to cause DNT damage in humans, though in some cases humans are substantially more sensitive than the rodents.

Three of the undisclosed DNT studies have already led to regulatory changes, after subsequent evaluation by EU regulatory authorities, according to Mie and Rudén. For the pesticide abamectin, for example, new health-based safety levels for people were set, they said.

A farm worker in Bulgaria treats crops with pesticide. Photograph: valio84sl/Getty Images/iStockphoto

For ethoprophos, Mie and Rudén said the DNT study "contributed to" it being banned by EU authorities in 2019. Bayer, the company that commissioned a DNT study on ethoprophos in 2004, denied this. The EU ruling that banned the pesticide said "the risk assessment could not be finalised" for DNT or other areas of concern and also noted a "high acute risk" to birds and soil organisms. Bayer sold ethoprophos to another company in 2010.

Another four undisclosed DNT studies could have "a potential effect on toxicological reference values or hazard classification", based on Mie and Rudén's assessment of the US EPA's evaluation of the studies. One DNT did not have any regulatory impact and insufficient information was available to assess the potential regulatory impact of the ninth study. Some of the pesticides have been banned for other reasons since 2018 and overall five of the nine chemicals retain EU approval today.

A spokesperson for Syngenta, which commissioned two DNT studies on abamectin in 2005 and 2007, as well as studies on two other pesticides, said: "Syngenta has complied with all EU data requests and provided relevant study data in accordance with regulatory requirements."

after newsletter promotion

The spokesperson said the abamectin DNT studies were not submitted to EU authorities in an application for approval that was completed successfully in 2008 because the studies had been conducted for its US regulatory application and were not a requirement in the EU at the time. He said these studies were not considered to provide any new toxicological information.

Swiss agrochemicals maker Syngenta's headquarters in Basel. Photograph: Arnd Wiegmann/Reuters

However, the Efsa spokesperson said: "The [DNT] studies were used to derive health-based safety levels for consumer and operator exposure."

A spokesperson for Bayer said: "At all times, we submitted the necessary studies required by the regulations at the time. For all three active ingredients [cited in the new research], the studies would not have changed the authorities' risk assessment."

Nissan Chemical Corporation said it had submitted the DNT study for its pesticide pyridaben, completed in 2007, to EU regulatory authorities in February 2023. Mie and Rudén said the study has the potential to impact the regulation of the chemical, which is still approved in the EU.

Japanese company ISK said it had submitted a 2005 DNT study on their pesticide fluazinam to EU authorities in 2020 and said it had not been required to do so beforehand. Efsa said the study was now being evaluated as part of the assessment of whether to renew the pesticide's approval.

None of the companies said they had submitted justifications for exemption from the need to submit existing studies, though some said other DNT studies had been submitted.

Reforms proposed by Mie and Rudén to ensure all toxicity studies are submitted to EU regulatory authorities include cross-checking datasets with counterparts in other countries, such as the US EPA. "The rules should also be revised to ensure that non-disclosure of toxicity studies carries a significant legal risk for pesticide companies," they said.

Apolline Roger, a lawyer at ClientEarth, contrasted the lack of penalties for non-disclosure of toxicity studies with those imposed for breaches of EU data protection and competition laws, which can lead to fines of significant percentages of a company's annual turnover.

"You don't have [penalties] like that for this process, even though what is at stake is the dispersion of potentially very harmful substances in the environment, and therefore in our food, water and bodies," she said. "What does it say about us when we place a higher value on digital data and consumer protection than on health and the environment?"

Currently, pesticide safety studies are commissioned and paid for by the companies. Mie and Rudén suggested the studies should be commissioned by regulatory authorities, to prevent conflicts of interest, with the costs being recovered from the companies.

"[Mie and Rudén] are really finding the root of the issue when they say studies should not be made by the companies," said Roger. "It's the elephant in the room."

The Efsa spokesperson said: "In the EU regulatory system for pesticides, the burden of proof of safety lies with the company that seeks to place their product on the market." Tougher EU rules on the notification of safety studies became applicable from March 2021, meaning companies now have to notify the authorities of all studies commissioned and cannot withhold studies even if they are considered to have found no adverse findings. However, Angeliki Lysimachou, head of science and policy at Pesticide Action Network Europe, said: "That means that all the pesticides already in the market won't be examined until their reapproval comes up, which could take 10 or 15 years, sometimes more." In the meantime, the pesticides remain approved for use, she said.

Rudén said: "There's no reason we are aware of to believe that withholding evidence is limited to DNT studies, or limited to pesticides." She said the cases of tobacco and PFAS – "forever chemicals" – were previous examples of where companies withheld knowledge about toxicity from the public.

The #pesticidesecrets story was reported in collaboration with Bayerischer Rundfunk/ARD and Der Spiegel in Germany, SRF in Switzerland, and Le Monde in France.

PRODUCTIE 46

Paths from Pesticides to Parkinson's

Freya Kamel

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the ice sheet, but that the interior is drained by a network of linked cavities.

With the help of a numerical model, the authors conclude that the growth of efficient subglacial channels is limited by the surface geometry of the ice sheet. The ice sheet margins usually exhibit steep surface slopes reminiscent of mountain glaciers, but the ice sheet interior is very flat, with a small hydraulic gradient driving water flow. In other words, little potential energy is available to enlarge water pathways by melting, while creep closure of channels by ice flow is faster than in the thinner marginal areas. Both effects preclude the development of an efficient channelized system, and water flux might be constrained to a system of linked cavities at high pressure.

These results suggest that current research of subglacial processes may be too narrowly focused on the efficient drainage system that evacuates surface water. Such channelized systems, by their very nature, occupy only a small fraction of the total area. Basal hydrology often differs considerably between neighboring boreholes, and many holes show little water pressure variation. Water pressure is often not even recorded in such boreholes, or the data are discarded because of their seemingly random variations, but they might hold important information on the conditions in large parts of the under-ice environment. Moreover, drill sites are usually restricted to areas lacking crevasses for practical and security reasons. Nothing is known about processes under the crevassed zones, which are subject to an extensional flow regime, in contrast to the compressional regime at topographic lows. The focus of past investigations on fast pressure variations in easily accessible areas might skew our perception of the system under investigation.

Basal motion is a distributed process that is controlled by water pressure and conditions everywhere on the ice sheet's bed and that is undoubtedly controlled by water drainage to the ice sheet base (*8*). But it is far from obvious how water pressure variations in a spatially confined area influence the behavior of the whole system. Lacking detailed information on bed geometry and sediment properties, one might reasonably consider the boundary zone between ice and bedrock as a self-organized critical system with interacting entities that exchange water depending on pressure gradients and evolving state variables (*3*–*5*), and tightly coupled with a spring-block model of stress transfer through the surrounding ice (*9*).

Considering the under-ice environment as a self-organized critical system explains why widely different conditions are simultaneously encountered in neighboring boreholes. To obtain a meaningful sample of variations in basal conditions requires a large number of holes spread over a representative area of the ice sheet, at distances of less than one ice thickness. Such an effort is larger than any of today's small research groups can handle. Drilling and instrumenting hundreds of holes simultaneously would require a concentrated and coordinated effort.

Even after decades of theoretical and experimental progress, we are just starting to understand the variety and interrelation of processes active in the inaccessible subglacial environment. In situ observations such as those presented by Meierbachtol *et al.* (*1*) and others (*6*, *7*) are urgently needed to develop, test, and quantify predictive theories of subglacial processes. Only by understanding the dynamics at the base of the ice sheets will it be possible to predict their future evolution under a changing climate.

References

1. T. Meierbachtol, J. Harper, N. Humphrey, *Science* **341**, 777 (2013).
2. G. C. K. Clarke, *Annu. Rev. Earth Planet. Sci.* **33**, 247 (2005).
3. C. Schoof, *Nature* **468**, 803 (2010).
4. C. Schoof, I. J. Hewitt, M. A. Werder, *J. Fluid Mech.* **702**, 126 (2012).
5. I. J. Hewitt, C. Schoof, M. A. Werder, *J. Fluid Mech.* **702**, 157 (2012).
6. T. Cowton *et al.*, *J. Geophys. Res.* **118**, 29 (2013).
7. D. M. Chandler *et al.*, *Nat. Geosci.* **6**, 195 (2013).
8. I. Bartholomew *et al.*, *J. Geophys. Res.* **117**, F03002 (2012).
9. J. Faillettaz, M. Funk, D. Sornette, *Nat. Hazards Earth Syst. Sci.* **12**, 2977 (2012).

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EPIDEMIOLOGY

Paths from Pesticides to Parkinson's

Freya Kamel

High-quality studies of specific chemical pesticides are needed to determine the relationship between exposure and risk of Parkinson's disease.

Interest in the relationship between exposure to pesticides and the risk of neurodegenerative diseases including Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease (PD) is long-standing (*1*). PD, in particular, has been the subject of much debate in this context (*2*). Its symptoms typically occur later in life (at age 60 or older), with the destruction of neurons manifesting most obviously as loss of motor function. Decades of epidemiological studies have suggested that pesticide exposure is connected to the development of PD. Yet there is still much that is not clear about this relationship. The disorder likely has multiple contributing genetic and environmental factors, but how exposure to a particular chemical leads to neuronal loss and the symptoms of PD is not known. A recent meta-analysis indeed shows that epidemiologic data generally support an association between pesticides and the risk of PD (*3*). But what is needed is detailed information on the nature of exposure—which pesticides, at what dose, and for how long—to help design policies and practices that prevent the relevant exposures. Also needed is information on the cellular and molecular mechanisms that, over time, lead from pesticide exposure to neurodegeneration and ultimately to PD. Although many questions still linger, some recent studies appear to be advancing the field.

It is well known that some pesticides are toxic to humans after acute exposure to a very high amount (poisoning). However, the effects of chronic, low-dose exposure to this diverse group of chemicals are not so clear. An analysis of over 100 epidemiologic studies establishes that pesticide exposure (in the absence of poisoning) is indeed linked to PD (*3*). PD risk increased with exposure to any pesticide (1.8-fold), to herbicides (1.3-fold), or to insecticides (1.5-fold). Risk associated with exposure to any pesticide (1.6-fold) and to herbicides (1.4-fold) was elevated in high-quality studies—those with adequate size, minimal potential for bias, and good information on PD diagnosis and pesticide exposure. Although its general conclusions are not surprising, the study highlights why such a wealth of data has limited impact. Heterogeneity of study quality and lack of detailed exposure information prevent results from being definitive.

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Perhaps one of the most important unanswered questions is which pesticides are associated with PD. Of several specific pesticides or chemical classes of pesticides evaluated in the meta-analysis, only the herbicide paraquat was significantly associated with PD (2.2-fold increase in risk for ever having used the chemical). Paraquat, used to kill weeds and desiccate foliage before harvesting crops such as cotton, is one of the most widely used herbicides in the world. Thirty years ago, recreational drug users rapidly developed parkinsonism after acute exposure to a contaminant whose active agent, 1-methyl-4-phenylpyridinium (MPP+), has a chemical structure similar to that of paraquat (*2*). Epidemiological data collected since then have been considered insufficient to establish a role for paraquat in PD. Recent studies, however, have provided stronger support. Notably, risk was increased not only for farmers and pesticide applicators (*4*, *5*), but also for individuals working or living near sites where paraquat was used (*6*). Among farmers, those who had used paraquat for less than 8 days had smaller increases in risk (2.4-fold) than those who had used it for more time (3.6-fold) (*5*). The compound rotenone is also of interest because like MPP+, it disrupts mitochondrial energy production. Rotenone, which is used as an insecticide and as a piscicide (toxic to fish), is less well studied than paraquat, but a recent study of farmers suggests that it too may be associated with PD (*5*).

Exposure. Epidemiological studies suggest that pesticide exposure may increase risk of Parkinson's disease.

The strong evidence from these epidemiological studies of paraquat and rotenone is particularly important because animal models have shown that chronic, low-dose exposure of adult animals to either pesticide results in many features of PD (*7*). Long-term exposure of adult mice to a low dose of rotenone was also found to replicate the gastrointestinal dysfunction found in PD, a nonmotor feature of the disease (*8*). Further, experimental studies using these pesticides and cultured cells are providing great insights into the cellular processes involved in PD, including mitochondrial dysfunction, oxidative stress, and inflammation (*9*, *10*). These processes are affected by genetic variants found in familial PD and also play a role in sporadic PD (*9*). Paraquat increases oxidative stress whereas rotenone causes mitochondrial dysfunction, but the processes are interrelated and both pesticides ultimately affect both mechanisms (*1*, *9*).

Other specific pesticides with more recent and stronger evidence of association include organochlorine insecticides. A large study of French farmers found that these pesticides as a group are associated with PD (*11*). Although for most pesticides the amounts found in blood or urine reflect only current exposures, organochlorine insecticides are an exception, with half-lives of years. Two studies that measured the amounts of organochlorines in serum determined that dieldrin (*12*) and beta-hexachlorocyclohexane (*13*) were elevated in PD patients. This corroborates an earlier observation that the amounts of dieldrin and gamma-hexachlorocyclohexane (lindane) were increased in the brains of PD patients (*14*). Notably, dieldrin and lindane increase oxidative stress and inflammation, cellular processes involved in PD pathogenesis (*1*, *10*).

While there is good progress in examining the effects of individual pesticides, they are frequently used in combination, and the effects of mixtures need to be evaluated. Exposure to multiple pesticides may increase the risk of PD more than exposure to any one alone. For example, paraquat increased risk only 1.3-fold, but paraquat with either of the fungicides maneb or ziram increased risk up to threefold (*6*). Head injury increases risk of PD, probably by increasing inflammation. By itself, head injury increased risk twofold, whereas head injury together with paraquat exposure increased risk threefold (*15*). Smoking, by contrast, is inversely associated with PD, and effects of dieldrin were evident only in nonsmokers (*12*). These studies are intriguing, but each combination of risk factors has been evaluated in only a few studies, and replication is crucial.

Genetic susceptibility may also modify effects of pesticides on the risk of PD. Studies of gene variants related to pesticide metabolism and transport, to mitochondrial dysfunction and oxidative stress, and to familial forms of PD suggest that associations of pesticides with PD are stronger in genetically susceptible individuals (*7*). Again, most studies have assessed effects of pesticides as a group; studies of genetic susceptibility will prove most fruitful when they focus on specific pesticides.

As for other neurodegenerative diseases, recent work is also expanding our understanding of the role of pesticides. Two high-quality prospective studies—one in France and one of an agricultural community in the United States—found that chronic, low-dose exposure to any type of pesticide increased the risk of cognitive impairment, Alzheimer's disease, and other forms of dementia that arise later in life (*16*, *17*). In a meta-analysis of nine epidemiologic studies, exposure to any type of pesticide increased the risk of amyotrophic lateral sclerosis nearly twofold (*18*), and a prospective study of U.S. farmers found that organochlorine insecticides were associated with amyotrophic lateral sclerosis (*18*). These results must now be confirmed, and as with PD, more details are needed regarding the effects of specific compounds.

The recent epidemiological studies provide much needed advances in clarifying the pesticide-PD relationship. That cellular processes important to PD are affected by specific pesticides underscores the importance of the epidemiologic findings. The most pressing need is for high-quality studies with data that are sufficiently detailed to identify essential aspects of exposure. What is the important life stage and time frame for exposure? Is it duration or intensity of exposure that is important—or both? Does exposure affect the progression of PD as well as risk? Above all, information on specific pesticides is imperative, not only to create a basis for prevention but also to provide clues for experimental mechanistic studies that may suggest therapeutic strategies.

References and Notes

1. S. Mostafalou, M. Abdollahi, *Toxicol. Appl. Pharmacol.* **268**, 157 (2013).
2. K. Wirdefeldt, H. O. Adami, P. Cole, D. Trichopoulos, J. Mandel, *Eur. J. Epidemiol.* **26** (suppl. 1), S1 (2011).
3. G. Pezzoli, E. Cereda, *Neurology* **80**, 2035 (2013).
4. C. M. Tanner *et al.*, *Arch. Neurol.* **66**, 1106 (2009).
5. C. M. Tanner *et al.*, *Environ. Health Perspect.* **119**, 866 (2011).
6. A. Wang *et al.*, *Eur. J. Epidemiol.* **26**, 547 (2011).
7. J. R. Cannon, J. T. Greenamyre, *Neurobiol. Dis.* **57**, 38 (2013).
8. R. E. Drolet *et al.*, *Neurobiol. Dis.* **36**, 96 (2009).
9. D. T. Dexter, P. Jenner, *Free Radic. Biol. Med.* **62**, 132 (2013).
10. T. Taetzsch, M. L. Block, *J. Biochem. Mol. Toxicol.* **27**, 137 (2013).
11. A. Elbaz *et al.*, *Ann. Neurol.* **66**, 494 (2009).
12. M. G. Weisskopf *et al.*, *Neurology* **74**, 1055 (2010).
13. J. R. Richardson *et al.*, *Arch. Neurol.* **66**, 870 (2009).
14. F. M. Corrigan, C. L. Wienburg, R. F. Shore, S. E. Daniel, D. Mann, *J. Toxicol. Environ. Health* **59**, 229 (2000).
15. P. C. Lee *et al.*, *Neurology* **79**, 2061 (2012).
16. I. Baldi *et al.*, *Neuroepidemiology* **22**, 305 (2003).
17. K. M. Hayden *et al.*, *Neurology* **74**, 1524 (2010).
18. F. Kamel *et al.*, *Neurotoxicology* **33**, 457 (2012).

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PRODUCTIE 47

Review

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Neurotoxicity of pesticides - A link to neurodegeneration

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Free article

Abstract

Parkinson's disease (PD) is a neurodegenerative disorder which mainly targets motor symptoms such as tremor, rigidity, bradykinesia and postural instability. The physiological changes occur due to dopamine depletion in basal ganglia region of the brain. PD aetiology is not yet elucidated clearly but genetic and environmental factors play a prominent role in disease occurrence. Despite of various environmental factors, pesticides exposure has been convicted as major candidate in PD pathogenesis. Among various pesticides 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been widely investigated in PD following with paraquat (PQ), maneb (MB), organochlorines (OC) and rotenone. Effect of these pesticides has been suggested to be involved in oxidative stress, alterations in dopamine transporters, mitochondrial dysfunction, α-synuclein (αSyn) fibrillation, and neuroinflammation in PD. The present review discusses the influence of pesticides in neurodegeneration and its related epidemiological studies conducted in PD. Furthermore, we have deliberated the common pesticides involved in PD and its associated genetic alterations and the probable mechanism of them behind PD pathogenesis. Hence, we conclude that pesticides play a prominent role in PD pathogenesis and advance research is needed to investigate the alterations in genetic and mechanistic aspects of PD.

Keywords: Parkinson's disease (PD); Pesticide toxicology; Pesticides; Residual toxicant; Toxic pollutant; Toxicity.

Conflict of interest statement

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

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A pesticide and iPSC dopaminergic neuron screen identifies and classifies Parkinson-relevant pesticides

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Parkinson's disease (PD) is a complex neurodegenerative disease with etiology rooted in genetic vulnerability and environmental factors. Here we combine quantitative epidemiologic study of pesticide exposures and PD with toxicity screening in dopaminergic neurons derived from PD patient induced pluripotent stem cells (iPSCs) to identify Parkinson's-relevant pesticides. Agricultural records enable investigation of 288 specific pesticides and PD risk in a comprehensive, pesticide-wide association study. We associate long-term exposure to 53 pesticides with PD and identify co-exposure profiles. We then employ a live-cell imaging screening paradigm exposing dopaminergic neurons to 39 PD-associated pesticides. We find that 10 pesticides are directly toxic to these neurons. Further, we analyze pesticides typically used in combinations in cotton farming, demonstrating that co-exposures result in greater toxicity than any single pesticide. We find trifluralin is a driver of toxicity to dopaminergic neurons and leads to mitochondrial dysfunction. Our paradigm may prove useful to mechanistically dissect pesticide exposures implicated in PD risk and guide agricultural policy.

Parkinson's disease (PD) is a complex, multi-factorial neurodegenerative disease. The hallmark pathology of PD is aggregation of the protein α-synuclein in Lewy bodies in specific midbrain dopaminergic (mDA) neurons. Etiologic contributors include genetic, environmental factors, and aging[1]. Ample evidence links pesticides in general to PD etiology[2]. In California, which is the largest agricultural producer and exporter in the United States, there are currently 13,092 pesticide products with 1059 different active ingredients registered for use[3]. While pesticides

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are important components of modern commercial agriculture that help maximize food production, most pesticides that are applied at industrial scales have not been adequately assessed for their potential role in PD, let alone for their mechanisms of action. Aside from important work in model organisms and cellular models mostly focused on rotenone and paraquat, the specific effects of most common, widely used pesticides remain unexplored[1]. Fewer studies still have delved into the effect of co-exposures and whether pesticides may directly exert effects on human dopaminergic neurons that are particularly sensitive to environmental toxicants through oxidative stress[2–9]. A wider screen of commercially applied pesticides in relation to PD, coupled to analysis in tractable human dopaminergic neuron models, may thus identify new disease targets, provide mechanistic insights, and help revise priorities for research and public health policy.

Responding to this need, here we have developed a field-to-bench paradigm, coupling systematic epidemiologic screening with direct testing in neurons to assess and mechanistically dissect pesticide-PD relationships. First, we performed a pesticide-wide association study (PWAS). We established a record-based exposure assessment approach using agricultural pesticide application records in California to comprehensively investigate long-term ambient pesticide exposure in relation to PD risk. This enabled agnostic screening of nearly 300 specific pesticide active ingredients in an untargeted manner, without relying on self-reported exposure or pre-selection of specific pesticides. We followed this with a systematic analysis of the effects of pesticide hits on dopaminergic (mDA) neurons derived from PD patient-induced pluripotent stem cells (iPSC). This cellular platform enabled us to directly test whether pesticides identified via our PWAS exert an adverse effect on PD-patient derived mDA neurons.

We chose mDA neurons produced from iPSCs as our model-system because they represent an excellent tool for personalized in vitro disease modeling, including for central nervous system cells[10–11]. Moreover, human iPSC model systems express proteins at endogenous levels and harbor disease-relevant pathologies, including mitochondrial function, ER-to-Golgi trafficking, reduced protein synthesis, increased nitrosative stress, and deficient survival over time[6,10–17]. In this study, we used iPSCs from two different PD patients expressing wild-type α-synuclein, either at endogenous levels or increased levels (from triplication) known to drive aggressive early-onset PD. We differentiated these iPSCs into mDA neurons, a key neuron affected in PD and known to be highly sensitive to oxidative stress. Importantly, human mDA neurons exhibit fundamental differences from rodent or other human cell lines, most dramatically with the biology of dopamine oxidation[6]. While protocols for differentiation of iPSC into midbrain dopaminergic neurons have steadily improved, heterogeneity of differentiation line-to-line, clone-to-clone, and experiment-to-experiment remains a challenge. To overcome this, we recently developed a bright red fluorescent reporter engineered into the tyrosine hydroxylase locus, enabling us in this study to specifically assess the effects of pesticides on mDA neurons, free from the presence of other cell types[14].

Here we show that among the multitude of potentially PD-relevant pesticides we identified, we were able to pinpoint ten that were directly toxic to mDA neurons. Data on co-exposures, common in agricultural practices, allowed us to develop co-exposure paradigms "in the dish" to test whether combinations of pesticides lead to greater, synergistic toxicity. For example, from pesticides used in combination in cotton agriculture, we identified trifluralin together with other commonly co-applied pesticides as being significantly more toxic to mDA neurons than any of the cotton-applied pesticides alone. We attributed the trifluralin-driven neurotoxicity to mitochondrial dysfunction in those neurons. In time, this approach will enable us to further track such cellular pathologies back to epidemiologic and environmental data, to mechanistically understand the individual and combined effects of pesticides, and to hopefully help inform the judicious use of pesticides in agriculture.

Results

Population-based study overview

Since 1972, California law mandates the recording of commercial pesticide use to the pesticide use report (PUR) database, documenting nearly 50 years of agricultural application of hundreds of pesticides. We have designed a geospatial algorithm which combines this database with maps of land-use and crop cover to determine for each individual pesticide active ingredient in the PUR, the reported pounds of pesticide applied per acre within a 500 m buffer around specific locations, such as addresses, yearly since 1974[15]. We applied this system to lifetime residential and workplace address histories from participants of the Parkinson's Environment and Genes (PEG) study ($n = 829$ PD patients and $n = 824$ controls recruited as part of two independent study waves; see Methods).

PEG is a population-based Parkinson's Disease case-control study conducted in three agricultural counties in Central California[16]. Patients were enrolled early in their disease course and all were seen by UCLA movement disorder specialists for in-person neurologic exams and confirmed as having clinically-defined, idiopathic PD[17]. For each pesticide active ingredient, hereafter referred to as "pesticide", in the PUR and each PEG participant, we determined the average pounds of pesticide applied per acre per year within a 500 m buffer of each residential and workplace address over the study window (1974 to 10 years prior to index date, which was PD diagnosis for patients or interview date for controls). This approach created one summary estimate of the average pounds of pesticide applied per acre per year within the 500 m buffer for each PUR pesticide.

Description of agricultural pesticide applications in the study area, including range and location of exposure

From 1974 to 2017, there were approximately 5.9 million PUR records in the tri-county study area, documenting the application of 1355 unique pesticides. Figure 1a details the study region and all agricultural pesticide applications reported in 2000, when PEG began. The number of different PUR-reported pesticides applied per year across the three counties, which ranged from a low of 288 in 1977 through a high of 646 in 2005, and the total reported pounds applied per year aggregated across pesticides, which peaked in 1998, are shown in Fig. 1b. Of the 1355 different pesticides applied from 1974–2017, 722 were applied within the 500 m buffer of at least one PEG participant's residence or workplace.

On average, the PD patients in the study both lived and worked near commercial agricultural facilities applying more total pounds of pesticide per acre (Fig. 1c) than controls (average annual mean difference: 133 more pounds of pesticide applied per acre per year near the patients' residences versus controls' and 343 more pounds near workplaces). Of the 722 different active ingredients applied within the study participants' buffer zone, PD patients and controls on average lived near the application of 50 (SD = 44.4) and 45 (SD = 40.9) different pesticides, respectively, during the entire exposure window. The mean number near participants' workplace was 50 (SD = 45.4) for patients and 38 (SD = 39.2) for controls. The number of pesticides by year is shown in Supplementary Figure 1.

Similar differences were observed in each study wave independently, for men and women separately, and when limiting to the 288 pesticides with ≥25 exposed participants (Supplementary Data 1). Figures displaying the median values are shown in Supplementary Figure 2.

Individual pesticide associations with PD in a pesticide-wide association analysis (PWAS) described according to pesticide class, use type, and an overrepresentation analysis

We assessed each pesticide individually for PD risk in what we call a PWAS. Of the 722 pesticides described above, we included 288 in our PWAS, according to our criterion of having ≥25 exposed study

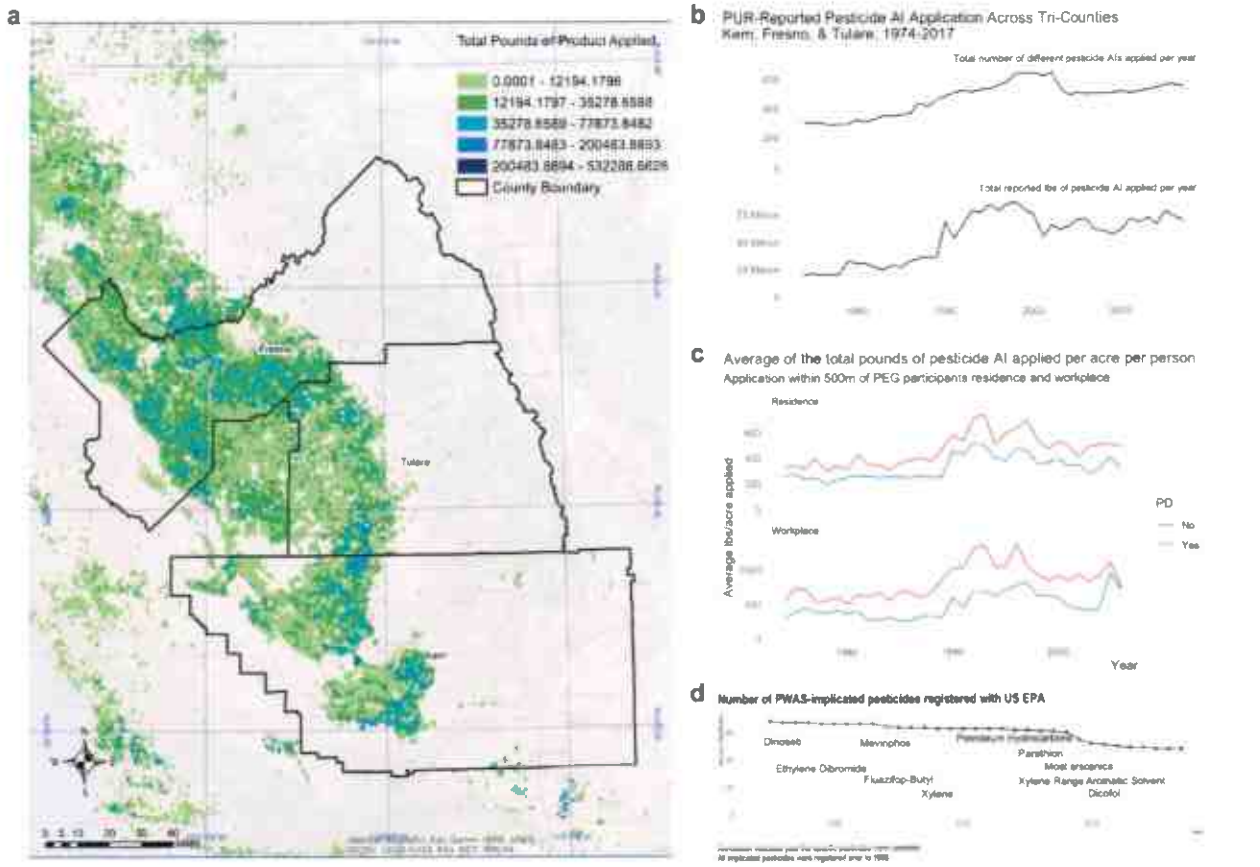

Fig. 1 | Description of agricultural pesticide use in the study area, including geography of applications, number of unique active ingredients applied by year, total pounds applied, and pesticide registration timeline. a Geography of study region for PEG cohort and total pounds of pesticides applied in the region in 2000. Total pounds of pesticides applied shown by color scale. **b** The number of different PUR-reported pesticides applied per year across the three counties and the total reported pounds of pesticide applied per year across the three counties (1974–2017). **c** The average total reported pounds of pesticide applied per acre around PEG participants' residential and workplace addresses per year from 1974–2006 (the mean index year), by PD status. Values above the 99th percentile were limited to the 99th percentile. **d** Timeline showing the number of PWAS-implicated pesticides that were registered with the US EPA by year. The annotation indicates the year the named pesticide had registration canceled or withdrawn. Source data are provided as a Source Data file.

participants. Due to special considerations for paraquat dichloride, specifically strong experimental support for the hypothesis and the interest in estimating the effects of duration and intensity of exposure, we present results from these analyses in a separate manuscript[12].

Figure 2a shows a Manhattan plot delineating the statistical significance for each pesticide, grouped by use type. Our PWAS implicated 25 pesticides as associated with PD at a meta-analysis FDR ≤ 0.01 (8.7% of all tested pesticides), another 28 at 0.01<FDR ≤ 0.05 (9.7%), and 15 at 0.05<FDR < 0.10 (5.2%) (Fig. 2b). The top five associated pesticides by FDR were sodium chlorate, dicofol, prometryn, methomyl, and xylene range aromatic solvent. These pesticides showed consistent risk profiles across location and study waves (Supplementary Fig. 3). Exposure descriptive statistics and risk estimates stratified by study wave and exposure location for pesticides associated with PD at $p < 0.10$ can be found in Supplementary Data 2 and 3.

Regulatory and toxicity information for the implicated pesticides is shown in Supplementary Data 4. Eighteen of the 25 most strongly PD-associated pesticides (FDR ≤ 0.01) are actively registered with the US EPA (43 of 68 pesticides at FDR < 0.10, Fig. 1d), while only 2 are allowed for use in the EU at time of publication. Of these 25 pesticides, 21 are considered 'bad actors' by the Pesticide Action Network (PAN)[13] as 9 have been deemed carcinogens (7 more as possible carcinogens), 6 developmental or reproductive toxins, 10 cholinesterase inhibitors, 3 known groundwater contaminants (13 more as possible groundwater contaminants), and 8 have high acute toxicity. In fact, of the 53 pesticides with an FDR ≤ 0.05, 43 have been designated 'bad actors'.

We used overrepresentation analysis (ORA) to test for enrichment of pesticide groups (toxicity groups, chemical classes, and use types) in the PD-associated pesticide set (PWAS FDR ≤ 0.05, $n = 53$ pesticides) relative to all pesticides assessed ($n = 286$ pesticides with group classifications; Supplementary Data 5). The ORA, which is commonly applied to evaluate gene-set overrepresentation, allowed us to identify classes or types of pesticides that were associated with PD more than expected and thus potentially have group characteristics important in PD etiopathogenesis.

The ORA indicated that seven of the toxicity classifications – including cholinesterase inhibitors, highly hazardous pesticides, acutely toxic pesticides, and carcinogens – were overrepresented (Fig. 2c). This indicates there were more PD-associated pesticides with these toxicity classifications than expected based on the group's distributions among all assessed pesticides. Furthermore, the odds of being among the PD-associated pesticides was also 2 to 3-fold higher for pesticides that contaminate groundwater and for those that are highly prone to drift. Figure 2d shows the percent of pesticides in each group that were associated with PD, the numbers are detailed in Supplementary Data 5.

Several pesticide chemical classes and use types were also significantly overrepresented, though small numbers limited statistical

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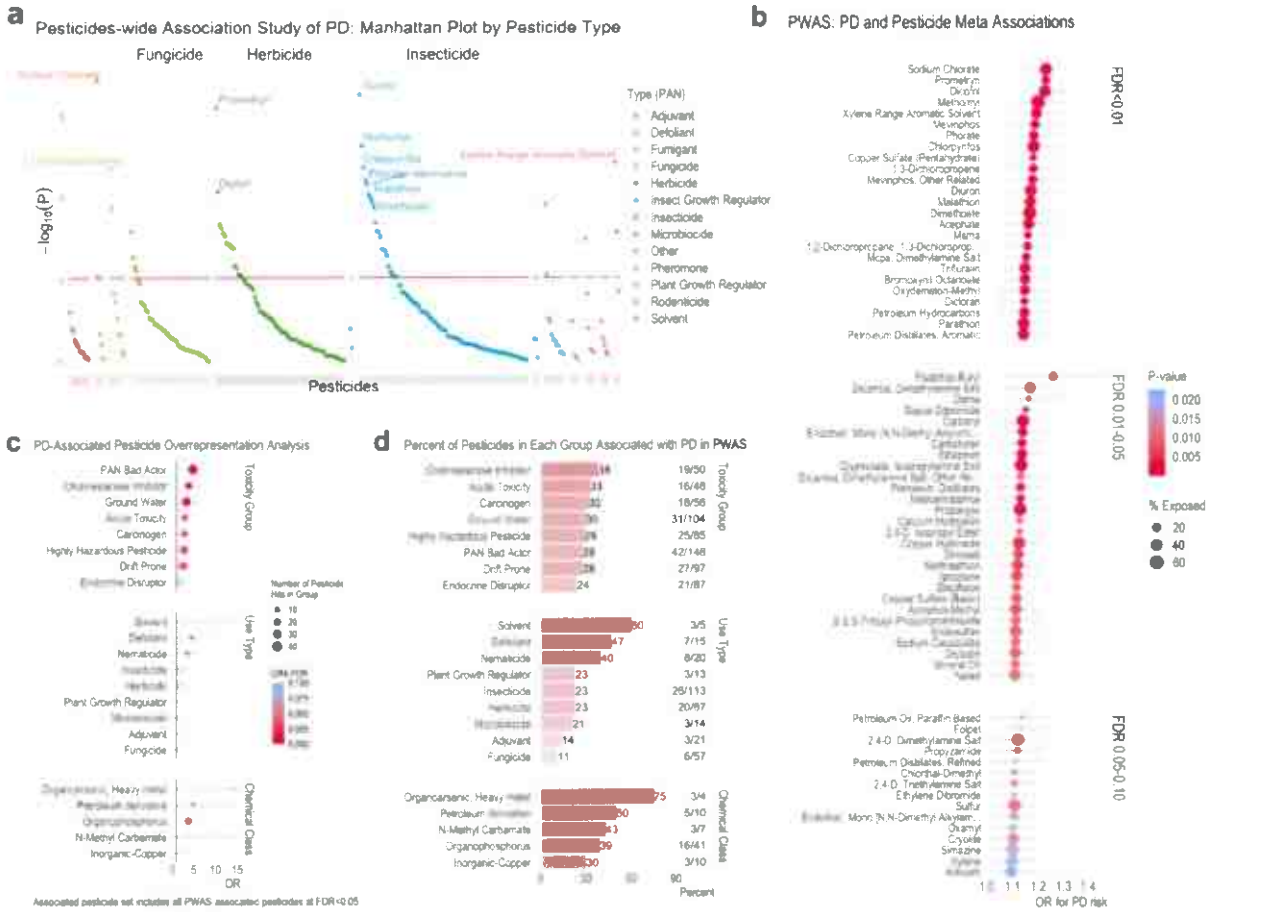

Associated pesticide set includes all PWAS associated pesticides at FDR<0.05

Fig. 2 | Pesticide-Wide Association Study analysis associated specific pesticides with PD and overrepresentation analysis implicates groups of pesticides overrepresented in the associated pesticides. a Manhattan plot detailing the -log(*p*-value) from the meta-analysis for all 288 pesticides tested for association with PD. We conducted univariate, unconditional logistic regression to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for PD with each pesticide ($n = 288$). We combined the OR estimates from each study wave and location (residential and occupational addresses) in a fixed effects meta-analysis, results shown here. *P*-values were based on a z-score statistic and two-sided interval. *P*-values were adjusted for multiple testing using an FDR and are shown in Supplementary Data 3. The red horizontal line indicates the FDR = 0.05 cut-off. **b** Dot plot displaying the odds ratio (OR; dot) and 95% CI (error bars) from the meta-analysis described above for all pesticides with an FDR < 0.10. Analysis for Figs. 2a and 2b was based on $n = 829$ PD patients and $n = 824$ controls. The log odds ratio is the center of the 95% CI on the logarithmic scale. The log odds ratio and 95% CI on the logarithmic scale were exponentiated to get the odds ratio and 95% CI. **c** Results of overrepresentation analysis to test for overrepresentation of pesticide groups (toxicity groups, chemical classes, and use types) in the set of PWAS PD-associated pesticides relative to all pesticides we assessed. Odds ratios (dot) and 95% CIs (error bars) are displayed. The log odds ratio is the center of the 95% CI on the logarithmic scale. The log odds ratio and 95% CI on the logarithmic scale were exponentiated to get the odds ratio. Given the asymmetrical nature of the resulting odds ratio, the odds ratio is no longer the center of the 95% CI. The overrepresentation analysis was based on $n = 286$ pesticide associations. The associated pesticide set includes all associated pesticides at FDR < 0.05 ($n = 53$ pesticides). **d** Bar graph indicating the percent of pesticides in each group associated with PD in the PWAS. The graph also shows the total number of pesticides tested in the PWAS from each group (denominator) and the number of pesticides in each group associated with PD (numerator) on the right. This information is used for the overrepresentation analysis. For example, there were 50 cholinesterase inhibitor pesticides assessed for association with PD, 17% of all tested pesticides (50/286). In total, 19 cholinesterase inhibitors were associated with PD at FDR < 0.05 in the PWAS (19/50, 38%). Using an odds ratio and Fisher's exact test, we found that the odds of being among the PD-associated pesticides was 3.6-fold higher for the cholinesterase inhibitors versus the non-cholinesterase inhibiting pesticides (OR = 3.62, 95% CI = 1.73, 7.50, FDR = 3.2e-03). Source data are provided as a Source Data file.

power. These included the defoliant, nematicide, and solvent use types and organophosphorus, heavy metal organoarsenic, and petroleum-derivative chemical classes. For example, while only four heavy metal organoarsenic pesticides were tested for association with PD in our PWAS, three (or 75%) were associated with PD (see Fig. 2d).

Finally, we also preformed several sensitivity analyses. The PWAS results were generally robust to including an indicator for occupational use of pesticides or fertilizer in the model (results shown in Supplementary Data 6) and including an additional set of controls (described in the supplementary materials and shown in Supplementary Data 7). Results stratified by gender are shown in Supplementary Fig. 4. The associations were mostly similar between men and women, although several pesticides showed stronger associations among men. We tested this by including an interaction term (pesticide*gender) in the models for the 68 pesticides associated with at FDR < 0.10. Five pesticides showed statistically significant interactions (interaction $p < 0.05$; Supplementary Data 8). However, further investigation is needed to validate and interpret these results, as none of the interactions were statistically significant after multiple testing correction.

Multiple PWAS-identified pesticides are toxic to iPSC-derived dopaminergic neurons from a PD patient

We tested toxicity of the PWAS-associated pesticides directly in iPSC-derived mDA neurons derived from a patient with PD who harbored

a pathologic triplication at the α-synuclein-encoding *SNCA* locus[20]. This line, from a male member of the Iowa kindred, was selected because it over-expresses wild-type α-synuclein and reflects an extreme form of the pathology associated with idiopathic PD, namely the accumulation of wild-type α-synuclein in neurons. Neurons derived from this and similar SNCA triplication iPSC lines exhibit PD-relevant phenotypes[20–23]. We thus considered it a line "sensitized" to PD-relevant stressors. We engineered these iPSCs to express a fluorescent reporter at the tyrosine hydroxylase (TH) locus to overcome the heterogeneity of mDA differentiation. This THtdTomato reporter was targeted via CRISPR-Cas9 and confirmed via PCR and Sanger sequencing (Fig. 3a–c)[14]. Endogenous THtdTomato signal colocalized with anti-TH-labelling and was consistent with the expression pattern observed in mDA neurons derived from embryonic stem cell reporter lines (Fig. 3f)[14]. This reagent enabled us to selectively evaluate the effects of pesticides on mDA neurons and exclude other contaminating cell types present in patterned iPSC-derived cultures.

Thirty-nine of the pesticides identified in the above PWAS analysis were solubilized in DMSO, water, or ethanol. A four-point dose curve protocol was used spanning a range of pesticide concentrations comparable to previously published toxicity assays performed on human cell lines including neural lineage cells[24–27]. THtdTomato-positive neurons were purified by fluorescence activated cell sorting (FACS) (Supplementary Fig. 5 shows gating strategy; Supplementary Fig. 6 provides assay overview) and used to test for sensitivity to the pesticides in a live-imaging survival assay. Quantitation of the raw number of THtdTomato-positive mDA neurons was performed at baseline prior to treatment, at seven days after treatment, and at eleven days after initial treatment (Supplementary Fig. 6 and Supplementary Fig. 7a, c). Eleven days after initial treatment was chosen as the primary end point for the assay to allow for detection of pesticides that cause rapid cell death and those that cause cell death over a more prolonged assay timeline to be detected in the same assay. At the eleven-day post-treatment endpoint, the Z-prime score for DMSO (negative control) and rotenone (positive control) was 0.549. Ziram, a known mDA neuron toxicant[28], served as an additional positive control. Rotenone produces cell death at eleven days but not seven days (Supplementary Fig. 7c). Ziram produces cell death more rapidly.

Ten PWAS pesticides led to cell death >3 standard deviations above the DMSO control mean at a concentration of 30 μM: propargite, copper sulfate (basic and pentahydrate), dicofol, folpet, naled, endothall, trifluralin, endosulfan, and diquat dibromide (Fig. 4). For example, propargite treatment produced extensive mDA neuron death and degeneration of neurites (Fig. 4b *upper* and *lower*). Dose-response curves extending below the screening concentration indicated that propargite was the most toxic, followed by diquat dibromide, folpet, and naled. All were toxic at 6 μM as well (Fig. 4c). The large standard deviation seen for dicofol was due to an outlier well. Repeat testing confirmed toxicity at concentrations of 9.5 μM and greater (Supplementary Fig. 8). Information about pesticide use type, chemical class, regulation status, and prior toxicity classifications for the 10 pesticides which produced substantial cell death is included in Supplementary Data 9. These compounds encompass a range of use types (four insecticides, three herbicides, and two fungicides), are structurally distinct, and do not have an overlapping prior toxicity classification, such as acute toxicity. Orthogonal imaging-based measurements of cell health, including neurite length, cell area, and pixel intensity of THtdT reporter were measured independent of cell count to confirm toxicity (Supplementary Fig. 7d, e).

The toxicity data determined by cell counts presented in Fig. 4 relies upon fluorescence from the THtdT knock in construct. A pesticide that dramatically reduced expression of the TH gene or greatly exacerbated degradation of the THtdTomato reporter without causing cell death could produce a false positive in this experiment. We thus performed an orthogonal viability assay using CellTitreGlo (Promega) according to the same experimental conditions as in Fig. 4 on a subset of pesticides identified as toxic in the initial screen. Viability decreased as expected with concentrations at or below the original screening concentration (Supplementary Fig. 9, $n = 1$). While we focused on the SNCA triplication line, it is possible that the increased α-synuclein levels in that line (known to cause early onset aggressive PD) may not be reflective of neurons expressing wild-type levels of α-synuclein. We thus generated an additional line with a THtdTomato reporter. This line is derived from a PD patient harboring a point mutation in the SNCA gene, which was then genetically corrected. Alpha-synuclein levels are thus endogenous with 2-copies of the wild-type gene and yet the genetic background is "permissive" to PD. All pesticides that were toxic to the SNCA triplication iPSC line also caused cell death at the screening concentration or lower in these mDA neurons (Supplementary Fig. 10).

We performed a second control to assess specificity. At our screening concentrations, our top-hit pesticides could theoretically be toxic to any cell type. One advantage of the iPSC system is that it can be differentiated to multiple cell types. Here, we selected cardiomyocytes to address this question given their established use in evaluating off-target effects of candidate drugs[29,30]. Cardiomyocytes were generated from the same SNCA triplication iPSC line. We focused on cell count at 11 days post-exposure, just as we had in mDA neurons. Cardiomyocyte beating and purity was confirmed prior to treatment with pesticides. Using the same assay timeline as the neuronal survival assay, cardiomyocytes were counted at eleven days after the first treatment with pesticides (Supplementary Fig. 11). Three of the ten pesticides (dicofol, folpet, and naled) resulted in a statistically significant reduction in cell counts ($n = 2$), while two others (diquat and propargite) trended towards reduction. A lower concentration of propargite was tested than in the original screen due to the high potency of this pesticide. Five of the ten pesticides that were toxic to mDA neurons did not demonstrate substantial cardiomyocyte toxicity by cell count at concentrations that produced significant cell death of mDA neurons: trifluralin, endothall, endosulfan, copper sulfate basic, and copper sulfate pentahydrate. These data suggest a relative selectivity for neurons over cardiomyocytes at the assayed doses.

PWAS-identified pesticides cluster based on correlation of exposures

Combinations of different pesticides are regularly applied to the same fields within the same season, year after year. We thus investigated how exposures among the PWAS-implicated pesticides were correlated. Further, we asked how the pesticides directly toxic to mDA neurons related to other PWAS-implicated pesticides, either those that did not produce significant mDA death or those that could not be tested. Finally, we used real-world exposure clusters to motivate combinatorial assessment of pesticides, and potential synergistic toxicity, in our mDA neuron assay.

Figure 5a details the correlation between all PWAS-implicated pesticides (FDR < 0.10, $n = 68$ pesticides). The heatmap, which shows broad patterns of correlation, indicates multiple groups of highly correlated pesticides. The complete pairwise correlation tables are in Supplementary Data 10 and 11. To assess how the mDA toxic pesticides correlated with the other PWAS-implicated pesticides, we used a data-driven integration and network analysis approach to assess correlations across two layers: the set of mDA toxic pesticides and the set of all other PWAS-implicated pesticides (Fig. 5b). All correlations between layers at $R > 0.45$ are shown in the circle and a detailed description of the figure is provided in the legend. An alternative layout of the network showcasing clustered pesticides is shown in Supplementary Fig. 12.

Overall, exposure to the mDA-neurotoxic pesticides was highly correlated with exposure to other PD PWAS-associated pesticides. Specifically, 75% of the PD-associated pesticides from the PWAS that

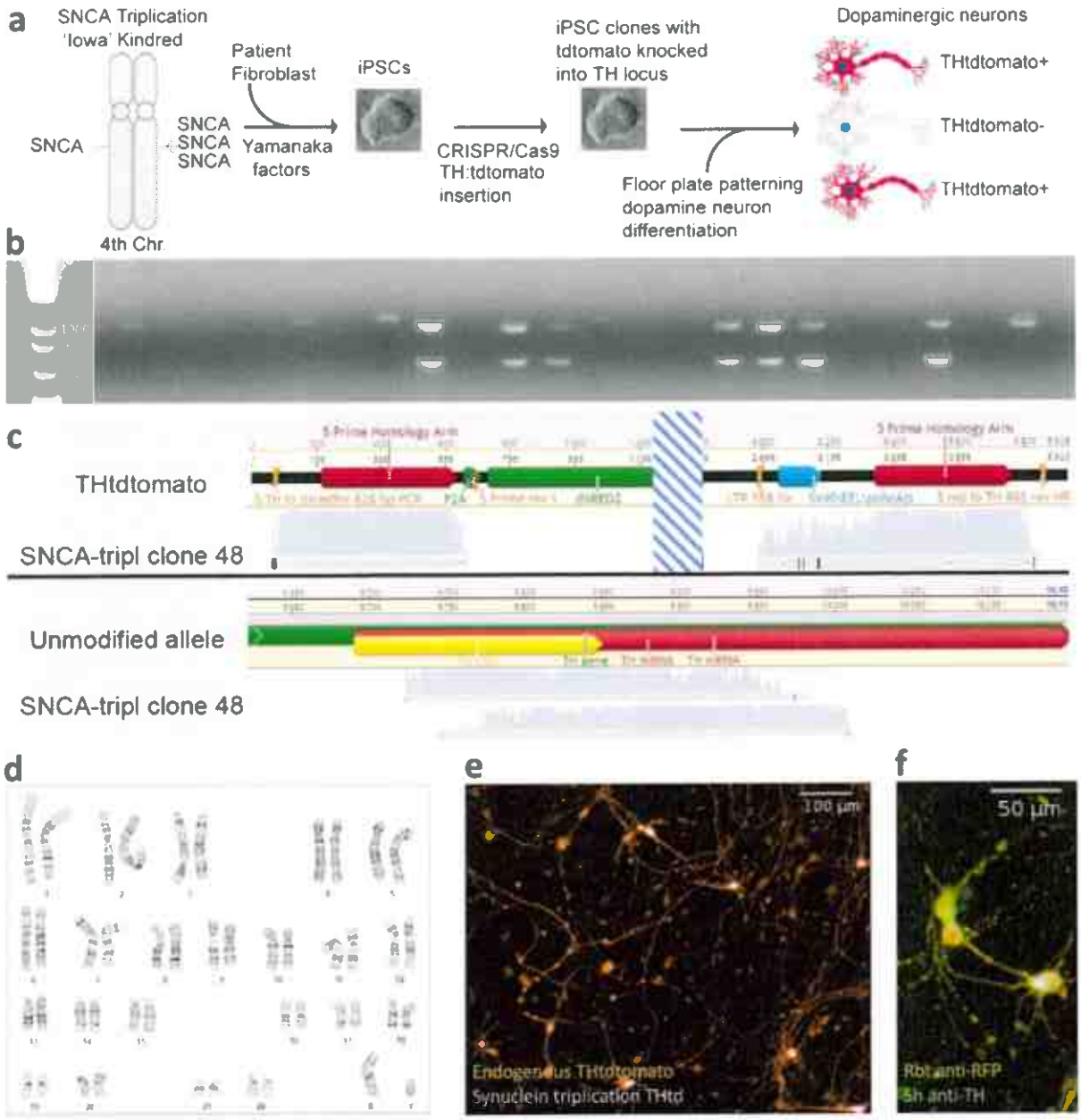

Fig. 3 | SNCA triplication THtdTomato reporter generation and quality control to facilitate purification and live-cell imaging of dopaminergic neurons. **a** Schematic demonstrating iPSC source, generation, modification, and differentiation with tdTomato reporter permitting identification and isolation of dopaminergic neurons. Previously described iPSC line derived from a patient with Parkinson's Disease caused by triplication of the SNCA locus resulting in four copies of the gene encoding α-synuclein. iPSC line was then modified with tyrosine hydroxylase:tdTomato reporter[25]. Adapted from Hallaci et al. 2022[25]. **b**–**f** Quality control and validation of SNCA triplication THtdTomato reporter line including 5' and 3' PCR products to confirm proper insertion. **b** Agarose gel stained with ethidium bromide to demonstrate examples of seven clones that contain the expected PCR products (626 bp product confirmed proper insertion of the 5' end of the reporter construct and an 878 bp product confirming proper insertion at the 3' end). PCR reactions run separately but combined into the same wells of the agarose gel for each clone to visualize clones passing and failing PCR quality control. A subset of clones have a single larger band and these are excluded from further testing. Band length was reproduced in an additional PCR from clones showing proper size to evaluate by Sanger sequencing. **c** Sanger sequencing of PCR products in (**b**) confirming correct insertion of tdTomato cassette. **d** G-banded karyotype (performed by WiCell) confirms normal karyotype in modified clone. **e** Example of live imaging of endogenous THtdTomato fluorescence at 10x. Neurons with this live imaging morphology and appearance are consistently obtained from multiple differentiations (greater than ten) from this cell line. **f** Immunofluorescence co-localization of Rabbit (Rbt) anti-RFP and Sheep (Sh) anti-tyrosine hydroxylase visualized with Alexa Fluor 546 donkey anti-rabbit and Alexa Fluor 488 donkey anti-sheep, respectively. Colocalization of anti-RFP and anti-tyrosine hydroxylase staining reproduces in greater than three differentiations in the cell line used for these experiments. Source data are provided as a Source Data file.

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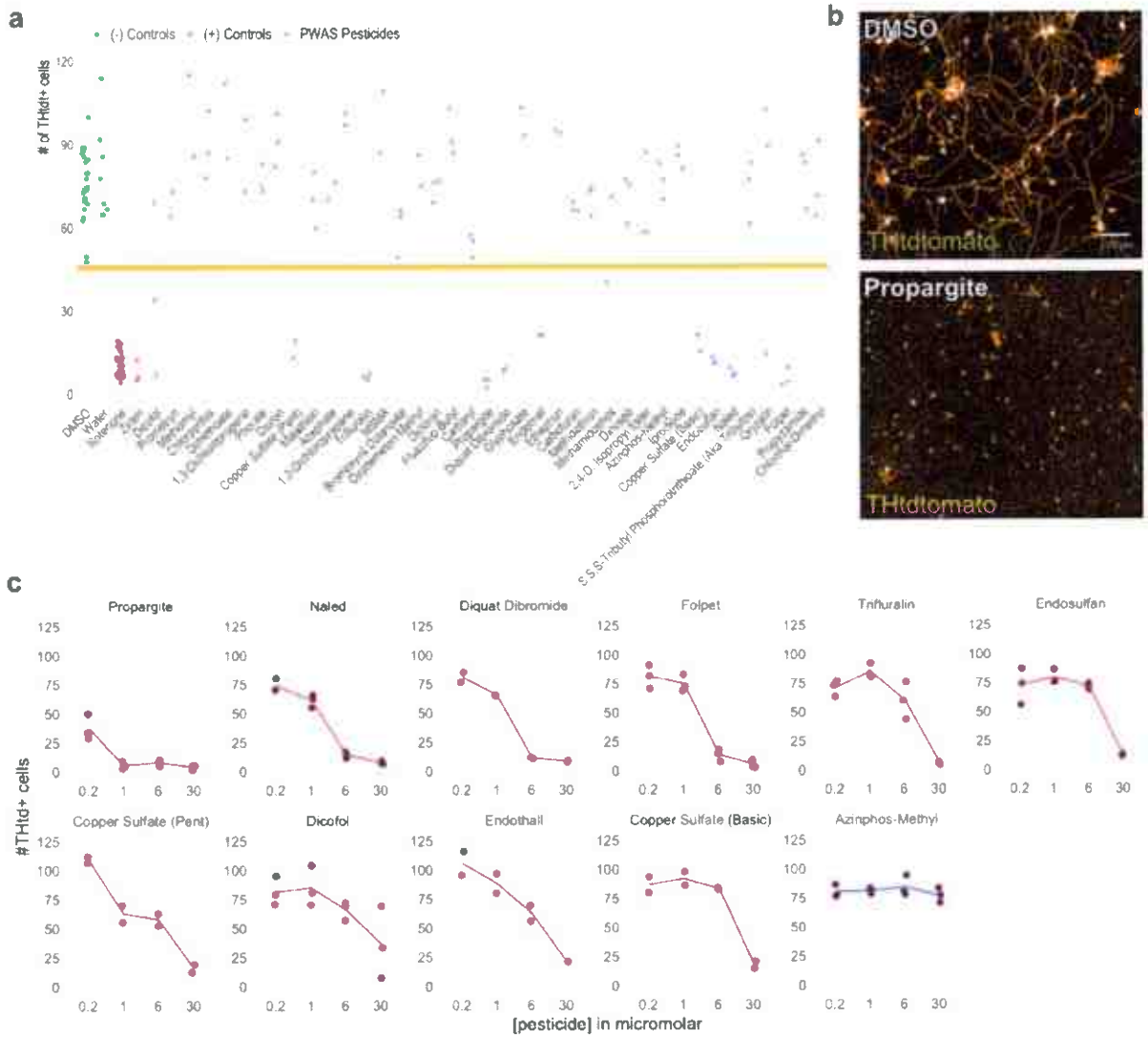
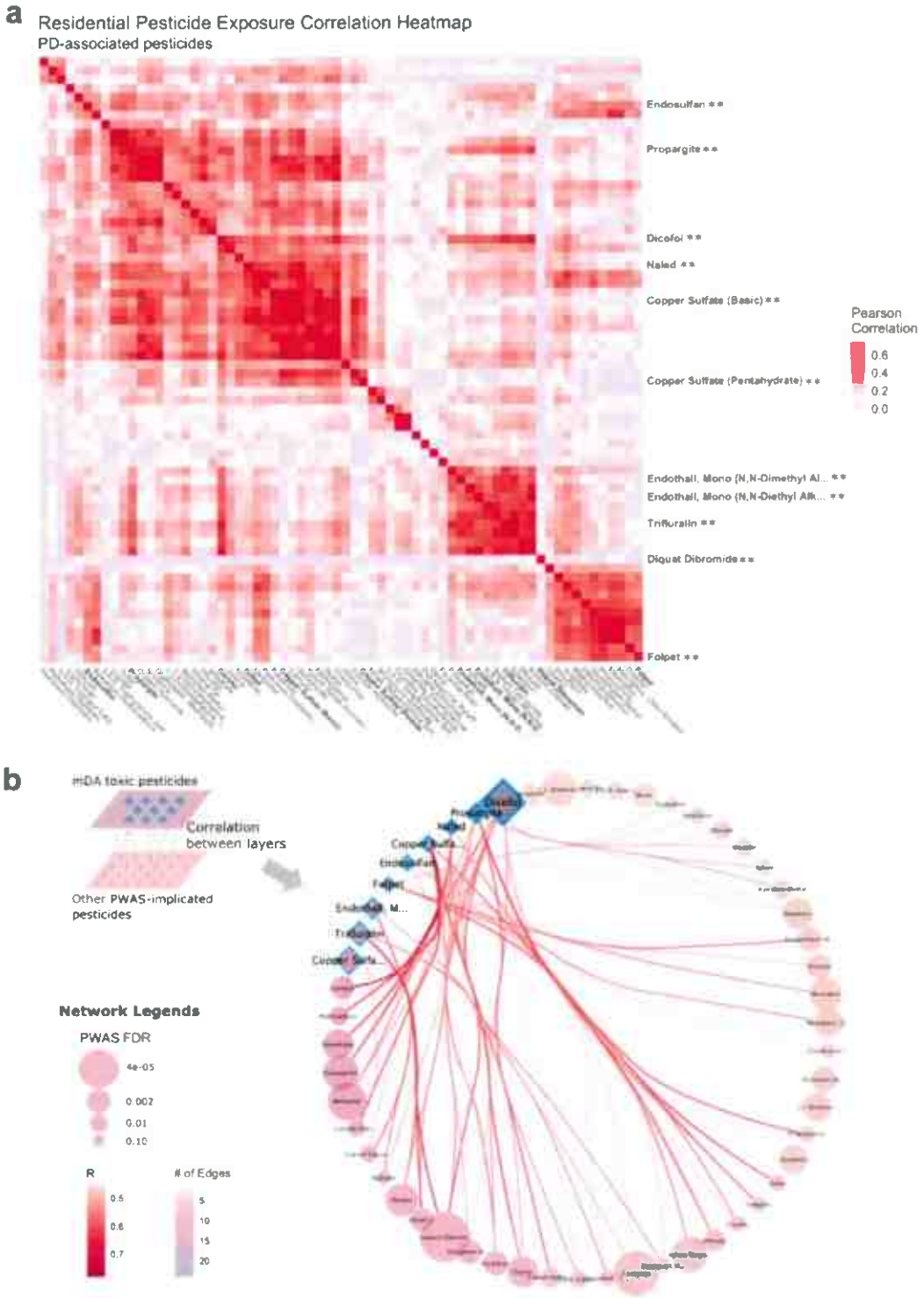


Fig. 4 | PWAS pesticides are directly toxic to PD patient-derived mDA neurons in a live imaging screen. a Scatter plot with the number of THtdTomato+ cells measured by live imaging analysis 11 days after the first treatment. DMSO controls (green data points) were present on each assay plate. Water control (green data points) was present on the assay plate containing primarily water-soluble pesticides. Rotenone (red data points) and ziram (red data points) were used as positive controls. Blue data points represent the different pesticides from the PWAS study. Horizontal line denotes three standard deviations below DMSO mean. **b** Upper image is a x10 magnification live image of a DMSO control well. Lower image is from a propargite treated well. Scale bar = 100 μM. More than five independent experiments repeated with DMSO and propargite treatment showed similar neuronal morphology with DMSO and similar extent of cell death and debris with propargite. **c** Four concentration dose curves of PWAS toxicants producing death in SNCA triplication THtdTomato sorted neurons. Cell numbers measured by high-content imaging of live cultures 11 days after first treatment. $n = 1$ with two technical replicates per water soluble pesticide and three technical replicates per DMSO and ethanol soluble pesticides per dose per pesticide for the screen dose curves. Red lines connect average cell number value for each pesticide concentration. Source data are provided as a Source Data file.

either did not result in significant mDA cell death or could not be tested were correlated at $R \geq 0.45$ with at least one pesticide that did result in significant mDA cell death. Put another way, exposure to most of the pesticides tied to PD in our PWAS, including those that did not directly lead to mDA neuron toxicity in our assay, was highly correlated with exposure to a pesticide that did exhibit such toxicity. Dicofol emerged as the strongest network hub, being the most interconnected with the highest closeness centrality and number of edges, followed by propargite and methomyl.

Next, we defined correlation clusters representing real-world co-exposure relying on an unsupervised, hierarchical clustering approach (Fig. 6a). In total, we detected 25 different pesticide clusters using residential-based exposure. These are shown as different colors in the dendrogram (Fig. 6a) and detailed in Supplementary Data 12 and 13. Some of the clusters consisted of a single pesticide, meaning these pesticides did not correlate above 0.45 with any other pesticide. The other clusters represent co-exposure profiles of interest for further in vitro testing.

We isolated one cluster of particular interest with clear co-application patterns that also contained several of the most statistically significant PD PWAS-pesticides (Fig. 6b, Cluster 7 in the Supplementary Data). Half of this cluster were defoliants, a unique type of pesticide which causes leaves to fall off plants. Defoliants are applied in agriculture almost exclusively on cotton. We confirmed this by aggregating all PUR records in the tri-county area over the study period and assessing the proportion of the applications for



each pesticide on cotton (Fig. 6b). We found 99.96% of the reported S,S,S-tributyl phosphorotrithioate (tribufos) applications were on cotton, 99.76% of sodium cacodylate, and 99.6% of sodium chlorate. Given the clear patterns of real-world co-exposure due to proximity to cotton agriculture, strength of the individual PWAS associations, and manageable cluster size for in vitro combinatorial testing, we selected members of this "cotton cluster" for further co-exposure experiments.

In vitro co-exposure testing identifies pesticides with most potent direct effects on dopaminergic neurons from the cotton cluster

Co-exposures can be modeled in vitro via concurrent treatment to directly compare the consequences of multiple direct co-exposures. We hypothesized that pesticides with the most relevance to PD risk would be likely to cause mDA neuron cell death on their own, but that toxicity could be enhanced by co-exposure to other pesticides

Fig. 5 | Pesticide exposure correlations demonstrate substantial interconnections of pesticides that produced significant mDA neuron death with many other pesticides. a Correlation heatmap indicating the pairwise Pearson correlation coefficient for 68 PWAS-implicated pesticides (FDR < 0.10), using residential address-based exposures. The pesticides which produced significant mDA neuron death in the iPSC-model are highlighted on the y-axis. No pesticides were significantly negatively correlated. Thus, the blue color represents null (R = 0) correlation to dark red representing strong correlation (R > 0.75). All pesticide labels are shown on the x-axis, the y-axis only displays labels of select pesticides, with the ** indicating that the pesticides were toxic to mDA neurons. **b** Correlation wheel showing the pesticide exposure correlations across two layers: first, the set of mDA toxic pesticides, which are designated as teal highlighted diamonds, and second, the set of all other PWAS-implicated pesticides, shown as circles. Correlations between layers at R > 0.45 are shown in the circle, correlations within layers are not shown. The size of the shapes in the correlation circle (diamonds and circles) were determined by the PWAS FDR, thus pesticides that were more strongly associated with PD in the PWAS are represented by larger sized shapes. The color of the shapes reflects the density of the connections (i.e. correlations at R > 0.45) made by that specific pesticide with others. Pesticides with a darker color are correlated with more pesticides, and arrangement around the circle is ordered from those with the most correlations (dicofol, darkest color) to the least (petroleum hydrocarbons, lightest color). Dicofol, for example, resulted in significant mDA cell death in the iPSC-model and is therefore shown as a teal highlighted diamond. It was also both (1) the most statistically significant mDA toxic pesticide in the PD PWAS (FDR = 4.2e-05) and therefore shown as the largest diamond, and (2) correlated above R > 0.45 with the most other PWAS-implicated pesticides (n = 24 pesticides), and therefore shown as the darkest color. Note, pesticides that did not correlate across layer at R > 0.45 are not shown on the wheel. Diquat dibromide, for example, was mDA toxic, however, the strongest correlation diquat displays with another pesticides was R = 0.14. Source data are provided as a Source Data file.

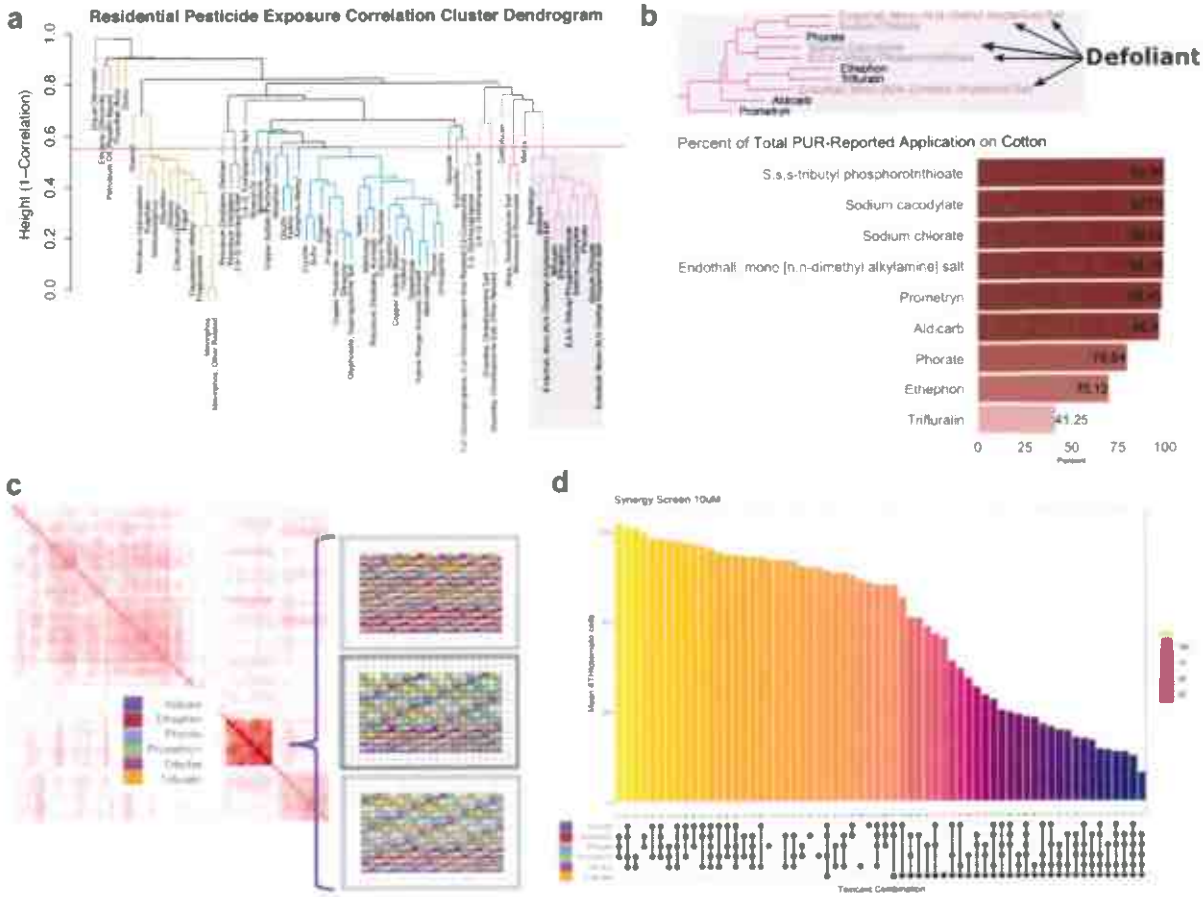


Fig. 6 | Co-exposure of pesticides in the Cotton Cluster demonstrates evidence of synergistic toxicity. a Cluster dendrogram from hierarchical clustering of the PWAS-pesticides using residential address-based exposures (clusters cut at height = 0.55), to identify groups of highly correlated pesticides for co-exposure analysis. **b** Cotton cluster: cluster identified as of interest, as it includes two of the three most significantly PD-associated pesticides in the PWAS based on FDR (sodium chlorate and prometryn) and half of the pesticides in the cluster have a use type of defoliant. The bar graph shows the proportion of all agricultural application records in the tri-county study area for each pesticide used on cotton, with the darker color representing larger proportions. For example, 99.96% of the reported S,S,S-tributyl phosphorotrithioate (aka tribufos) applications were on cotton. **c** Schematic outlining how pesticides from a single co-exposure cluster (cotton cluster) were recombined in all possible combinations of six pesticides using an HP Digital dispenser on sorted dopaminergic neurons plated into a 384 well format, similar to the survival assay described in Fig. 4. **d** An upset plot was used to sort and display the most toxic and least toxic combinations. Y-axis shows number of THtdTomato+ neurons at day 11 following treatment. Ball and stick connections along the X-axis indicate co-treatments with a ball indicating treatment with a given pesticide. Cooler (purple) colors represent lower relative cell counts while warmer (yellow) colors represent higher cell counts. DMSO control condition is depicted by the x-value lacking any ball and stick marker. N = 4 biological replicates. Source data are provided as a Source Data file.

commonly applied to the same fields (i.e., from the same exposure cluster). To test effects of co-exposure to the cotton cluster, we performed a survival assay on sorted mDA neurons from the same PD patient derived line described above using all combinations for a subset of pesticides in this cluster. The schematic in Fig. 6c illustrates how pesticides from a single co-exposure cluster (shown in more detail in Fig. 5a) were combined in all possible combinations in an exposure matrix. We used a live imaging survival assay and treatment paradigm similar to that used for data presented in Fig. 4. A digital dispenser (Hewlett Packard) was used to dispense pesticides.

A 10 μM dose was chosen for this assay as an intermediate dose, permitting detection of additive or synergistic effects. In contrast, combinations of pesticides at 30 μM proved excessively toxic during assay development. Pesticide combinations were arranged in descending order by number of surviving neurons after treatment (Fig. 6d). The average of four independent biological replicates is shown. *P*-values for individual pairwise comparison with adjustment for multiple testing are provided in Supplementary Data 14. Combinations involving trifluralin caused more mDA neuron cell death (gold bar) and there was potential synergy with tribufos (S,S,S-tributyl phosphorotrithioate) as this combination resulted in the most mDA neuron cell death of all the pairwise combinations. Trifluralin alone at 10 μM produced a 32% decrease in mDA neurons compared to DMSO, while tribufos produced an 8% decrease (neither statistically significant in this assay), but in combination they produced a 65% decrease compared to DMSO and were significantly different from the individual treatments at $p = 0.003$ for the comparison to tribufos alone and $p = 0.048$ for the comparison to trifluralin alone. Performance in this assay showed no clear correlation to the odds ratio for PD risk or FDR cutoff level described in Fig. 2b.

Trifluralin reduces spare capacity of mitochondria in mixed PD-patient-derived dopaminergic neuron cultures

Identification of trifluralin in the PWAS screen, in vitro toxicity assay, and demonstration of synergistic action prompted a deeper investigation into potential mechanisms by which this pesticide causes mDA neuron cell death. Trifluralin was previously shown to increase α-synuclein fibril formation in a cell-free system[31]. Additional biochemical and metabolic assays were utilized to examine the effects of trifluralin on mDA neurons. These assays necessitated alterations in assay timing and conditions from the original live imaging survival assay. Neurons used for biochemical assays could be matured longer in vitro prior to exposure and harvesting because the clumping that occurs frequently with prolonged two-dimensional culture does not impact biochemical or metabolic assays in the way it complicates image analysis. PD patient-derived mDA enriched cultures were treated with sublethal doses of trifluralin (6 μM) for two weeks to assess for accumulation and expression of phosphorylated α-synuclein (pS129). The timing is similar to the duration assessed in our recently described iPSC-based inclusionopathy models[32]. Western blot revealed very low levels of pS129 expression in both control and trifluralin treated conditions and no significant difference in pS129 was detected (Supplementary Fig. 13, experiment performed three times, representative blot shown). In the absence of a robust α-synuclein-related phenotype, we next examined mitochondria. While trifluralin is annotated as a mitosis inhibitor in targeted grasses and weeds, mDA neurons are known to be exquisitely sensitive to mitochondrial dysfunction, including by toxicants that target the mitochondria directly[33,34]. We thus investigated whether trifluralin could also result in mitochondrial dysfunction in mDA neurons. mDA neuron-enriched cultures were assessed for effects on mitochondrial function at 55 days in vitro at a time when more mature, arborized neurons are observed. We quantified relative mitochondrial subunit abundance, mitochondrial respiration, and function of the oxidative phosphorylation pathway in mDA neuron cultures. We measured mitochondrial subunit abundance after 24 h exposure to 30 μM trifluralin in 3 biological replicates and demonstrated a 50% reduction of Complex I (NDUFB8) and Complex IV (COX II) when compared to DMSO treated controls (Fig. 7a). There was no significant reduction or increase of the expression of other complexes in the mitochondrial respiratory chain (see original blot in Source Data file Fig. 7).

For assessment of mitochondrial function, we used the Seahorse assay, which involves sequential addition of mitochondrial complex inhibitors (Oligomycin [complex V], FCCP – uncoupler – and Rotenone [complex I]/Antimycin-A [complex III]) and measurement of media acidification and oxygen consumption rate (OCR). The effects of trifluralin were assessed in a dose-response manner in three biological replicates (Fig. 7d). We calculated mitochondrial respiration parameters (basal respiration, ATP production, maximal respiration and spare capacity) and found, with the exception of ATP production, all parameters were decreased with exposure to trifluralin at the concentrations used in the assay. The respiratory capacity of differentiated neurons was reduced to 9% when cells were treated with trifluralin at 90 μM, 26% at 60 μM, and 72% at 30 μM trifluralin compared to DMSO control, with the highest two doses being significantly different from DMSO control (Fig. 7e). The effect of trifluralin on cellular respiration was dose and time-dependent (Fig. 7d, e). Mitochondrial function was tested at early time points (6 h after treatment) with a goal of targeting more direct metabolic effects from the pesticide treatment. These effects are expected to occur at shorter time points compared to overt toxicity. We also wanted to avoid prolonged exposure that could lead to nonspecific mitochondrial damage or dysfunction to accumulate. Mitochondrial protein content, on the other hand, was assayed to measure steady state changes that could result from exposure, and thus assayed at a later time point. We followed literature precedent for these assays[35].

We also exposed mDA neurons to ziram as a control. Ziram has been implicated in PD previously and its toxicity has been tied to an inhibitory effect on the E1 ligase in mDA cultures without any described mitochondrial phenotypes[28]. Ziram exposure for 2 or 6 h at a dose greater than the LD50 from survival assays (300 nM) did not significantly alter the oxygen consumption rate (Fig. 7f) or the mitochondrial reserve capacity (14% increase in 6 h exposure compared to DMSO, Fig. 7g). This is in contrast to the effect of trifluralin at a similar ratio to its LD50 (60 μM). These data implicate mitochondrial dysfunction in trifluralin-mediated mDA neuron cell death, reinforcing that pesticides toxic to mDA neurons exhibit distinct mechanisms of toxicity.

Discussion

Pesticides have been definitively linked to Parkinson's disease etiology by prior studies[36]. However, most specific agents used in agriculture have not been assessed for potential influence on PD. Therefore, we established a field-to-bench paradigm which combined two distinct approaches: 1) broad epidemiologic screening of hundreds of pesticides for association with PD; and 2) in vitro evaluation of associated pesticides in mDA neurons. This approach permitted testing of epidemiologic hits for direct effects on dopaminergic neurons to better identify and classify PD-relevant pesticides.

The record-based exposure assessment and epidemiologic screening implicated 68 pesticides with PD. To identify potential direct effects on mDA neurons, we coupled our PWAS screen to systematic analysis of the hits in human mDA neurons derived from iPSCs of a patient with triplication of the wild type α-synuclein locus (SNCA). These cells capture human biology that may differ in critical ways from rodent or other human cell lines, most dramatically with the biology of dopamine oxidation[6].

We tested 39 of the epidemiologically implicated pesticides in vitro. Ten resulted in substantial mDA neuron cell death at 30 μM. The identified toxicity in these neurons represents a unique and

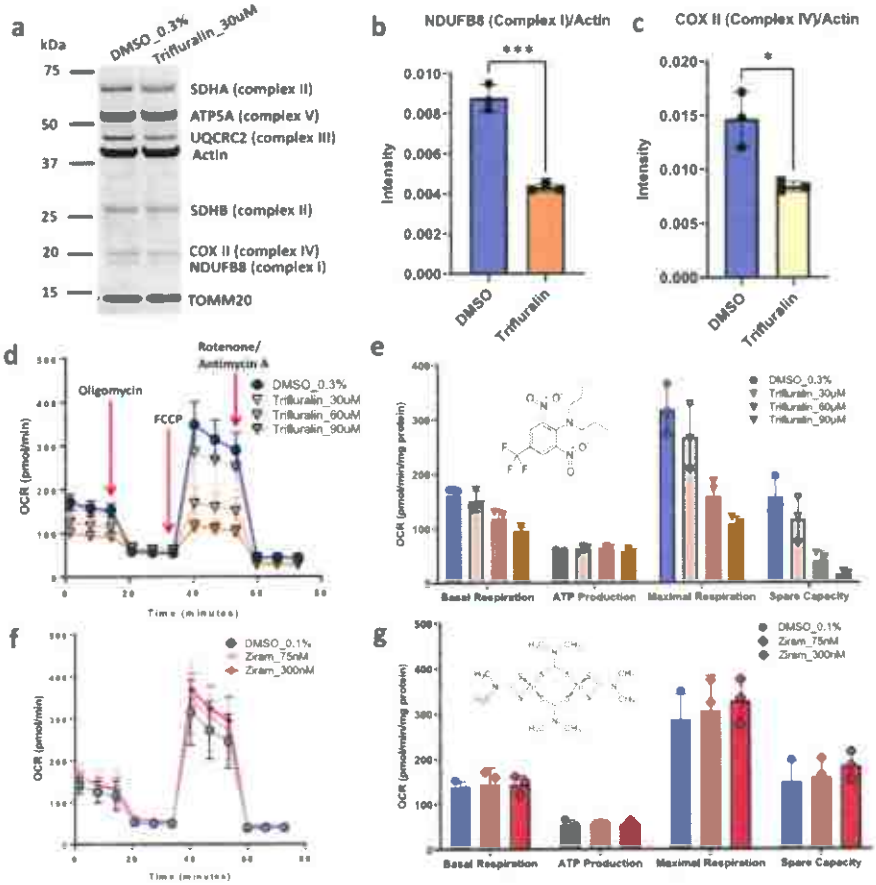


Fig. 7 | Trifluralin alters mitochondrial subunit abundance and oxygen consumption rate. Effect of Trifluralin in mitochondria subunit and effect of Trifluralin and Ziram in Mito-stress assays. **a** Western blot analysis of respiratory chain complexes for differentiated SNCA-triplication neurons at DIV 65, exposed to 0.3% DMSO and 30 μM Trifluralin for 24 h. Uncropped blots in Source Data. **b** Quantification of Complex I and **c** Complex IV from blot in **a** normalized to actin, (●) 0.3% DMSO and (■) 30 μM Trifluralin. *T*-test for mitochondria subunit, $p < 0.001$ (***), $p < 0.01$ (*), $n = 3$ biologically independent replicates. **d** Measurement of Oxygen Consumption Rate curves on Mito-stress assay for the dose-response effect of (●) DMSO 0.3% and (▼) Trifluralin (30 μM, 60 μM and 90 μM) on SNCA-triplication differentiated neurons at DIV 65 and after 6hrs exposure. **e** Metabolic parameters (Basal respiration, ATP production, maximal respiration and spare respiratory capacity) calculated from **d**. 2-way ANOVA for Mito-stress assays, $p < 0.0001$ (****); Dunnett's multiple comparisons test $p < 0.0001$ for DMSO vs. 60 μM and DMSO vs. 90 μM Trifluralin. DMSO vs. 90 μM Trifluralin not significant, $p = 0.099$. **f** Oxygen Consumption Rate curves on Mito-stress assay (●) 0.1 % DMSO and (◆) 75 nM and 300 nM Ziram exposure on SNCA-triplication differentiated neurons for 6 h. **g** Metabolic parameters for the conditions described in **f**. There were no significant differences among treatment conditions with Ziram ($p = 0.4326$). Errors bars represent standard deviation, $n = 3$ biologically independent replicates for 7d–7g. Source data are provided as a Source Data file.

previously unappreciated common characteristic of these pesticides – they do not have another previously described shared characteristic or property. Interestingly, our experiments identified propargite and confirmed previous work with this pesticide in mDA neurons from a different iPSC line[37]. Other pesticides highlighted in our studies, including diquat dibromide and the copper sulfates, also have persuasive biologic links to PD[38–43]. Notably, however, we implicated several pesticides as toxic to mDA neurons that have not previously been linked to PD or neuronal toxicity, including folpet, naled, endosulfan, and endothall. Furthermore, 8 of the 10 mDA toxic pesticides are still registered for use with the US EPA. Five of the pesticides also showed selectivity for neurons given that they did not produce substantial toxicity in cardiomyocytes. These will be the subject of future testing on other CNS cell types, co-culture systems, and 3D organoids. Given both the public health relevance due to current use and findings presented here, these agents certainly warrant additional investigation.

In the current manuscript, we focused on the differential effects of pesticides at the screening dose on mDA neurons as a first layer of specificity. We then confirmed this result in an additional cell line to assess for generalizability across dopamine neurons from different sources. Finally, we assessed cardiomyocytes to identify which pesticides are likely to be toxic to any cell rather than specific to neurons. It will be important in future investigations to develop more insight into what constitutes clinically relevant dose exposures. This is a nontrivial issue – modeling a chronic exposure "in the dish" is challenging. Developing more sensitive assays that can assess for phenotypes at low micromolar or nanomolar concentrations will be important. Functional assays, for example of neuronal activity, may be more sensitive than looking at overt toxicity. A useful indicator of a relevant toxicant may be one that is toxic to a mutant mDA neuron but not an isogenic corrected one. Finally, where possible it will be important to better model toxicant exposure in vivo in chronic exposure paradigms in animal models. Postmortem brain examination, where possible, may provide important clues to the steady state concentrations of toxicants and their metabolites in human brain as a benchmark for modeling these exposures in the laboratory setting.

Beyond individual agents, an important use of our platform is to screen pesticide co-exposures and understand synergistic

interactions. Analysis of co-treatment with components of the cotton cluster indicated that co-exposures involving trifluralin produced substantially more mDA cell death than any of the components alone. Trifluralin has been previously linked to PD in the Agricultural Health study, making this a compelling pesticide for more in depth mechanistic investigation[11].

Using functional assays in mDA neurons, we implicated mitochondrial dysfunction in trifluralin-mediated toxicity. Trifluralin is part of the dinitroaniline family of herbicides, known to cause disruption of cell division in plant cells and protozoa via de-polymerization of microtubules[43], binding to tubulin in higher plants and protozoa[44–46], but not mammalian or human tubulin[47]. We are not aware of any reports directly linking trifluralin to mitochondrial dysfunction in neurons, suggesting that the use of human mDA neurons as a model can uncover previously unrecognized toxicity pathways for pesticides. Our results support an effect of trifluralin on neuronal respiration and mitochondrial function, consistent with an extensive literature documenting mitochondrial dysfunction as fundamental to PD[50–53]. Imaging-based assays of mitochondrial potential or neuronal activity will enable assessing toxic effects of much lower doses of trifluralin. It will be interesting to understand whether the effects of pesticides are α-synuclein-dependent. Additional work is required to understand why a pesticide like trifluralin causes α-synuclein fibril formation in a cell-free system but does not cause increased phosphorylated α-synuclein[11]. This can be assessed in more tractable cell lines, with isogenic mutation-corrected, and α-synuclein knockout lines[12]. For some pesticides, like paraquat, a toxic effect through nitrosative stress has already been tied directly to α-synuclein proteotoxicity and biology[8,20]. A similar approach will be valuable to undertake for the top-hit pesticides identified in this study.

Several considerations for interpreting this wide-scale pesticide screening are noteworthy. First, we view the population-based screening as a first step to prioritize agents for more in-depth research. It does not necessarily suggest a causal role for PD in all those that screened positive, nor does it absolve those which failed to screen positive for a role in PD. As case in point, pesticide exposures were often highly correlated in the epidemiologic study. For instance, with the PWAS-implicated pesticides, we observed that even if the pesticide itself did not result in significant mDA cell death in the iPSC-model, its exposure levels correlated relatively highly with a pesticide that did. This presents some interesting interpretations for the lack of significant mDA neuron death in vitro for many of the pesticides implicated by the PWAS. First, such pesticides may in fact have no direct influence on PD and the association was simply driven by correlation with toxic co-exposures. However, the pesticides may also influence PD through other disease-relevant pathways besides mDA toxicity or be involved in key mechanisms that were not recapitulated in our cell model. Finally, the pesticides could be toxic but only in combination with other pesticides, as the cotton cluster analysis suggested.

With regard to the epidemiologic exposure assessment, while the GIS-based method is uniquely informative and has been previously validated[54], it still likely allows for some level of non-differential exposure misclassification (see Supplementary Materials). Conversely, the assessment did not rely on self-report, an advantage that cannot be overstated in population-based research. Moreover, coupling the epidemiologic screen with experimental study provides confidence in both the field and bench aspects of the paradigm.

With the in vitro modeling, limitations include relative immaturity of cultured dopaminergic neurons; a lack of a blood-brain barrier (BBB) and a greatly simplified cellular environment lacking astrocytes, microglia, endothelial cells, and circulating factors. More advanced models, such as triculture systems that reconstruct the neuroimmune axis and "BBB on a chip" methods will permit investigation of more complex inter-cellular interactions and render the platform more physiologic[55,56]. Multiplexed iPSC co-culture will even enable the investigation of whole populations of patients in a single dish[57,58].

As noted above, dosing is an important consideration in our study. The pesticide doses used in our study, while comparable to other published reports, are high[8,20,21]. We used high doses to accelerate the onset of pathologies in the dish by using higher concentrations for shorter times rather than years worth of lower-level exposure. Our in vitro models could also underestimate the toxicity of pesticides that require metabolism in the liver or by glial cells to generate harmful toxic metabolites. Additionally, with our live imaging system, we have chosen a straightforward and dramatic phenotype as the readout for pesticide toxicity–cell death. More subtle phenotypes, such as α-synuclein phosphorylation, abnormal neuronal activity, and dopamine oxidation will be important to detect effects of low doses of pesticides.

The California Pesticide Use Report system has enabled us to begin to understand the breadth of pesticide application in agricultural regions and investigate how it relates to PD in the community. Here, establishing a field-to-bench paradigm, we have combined record-based exposure assessment with a tractable and screenable iPSC-derived dopaminergic neuron model to identify and classify PD-relevant pesticides.

Our comprehensive, pesticide-wide association study has implicated both a variety of individual pesticides in PD risk and suggested relevant co-exposure profiles. Coupling this with direct testing in vitro on dopaminergic neurons, we have pinpointed pesticides that were directly toxic to human dopaminergic neurons. Further, real-world co-exposure data has allowed us to develop co-exposure paradigms "in the dish" and establish which combinations of pesticides can indeed lead to greater, synergistic mDA toxicity. Ultimately, we have identified pesticides that are both ostensibly mDA-toxic and pesticides that are not mDA-toxic, but, nonetheless, associated with increased risk of PD. In time, this field-to-bench approach will enable us to further tie cellular pathologies back to this epidemiologic and environmental data, to mechanistically understand the individual and combinatorial effects of pesticides and their interactions with genetic susceptibility. Collectively, these studies will inform the judicious use of pesticides in agriculture.

Methods

Compliance

All data presented and experiments described herein are conducted in accordance with the Institutional Review Boards of Brigham and Women's Hospital (IRB#2019P002015), Harvard University Faculty of Arts and Sciences (IRB#19-0736), and University of California, Los Angeles (IRB#21-000256 and IRB#11-001530). Informed consent was obtained from all epidemiologic study participants.

PEG study population

To assess pesticide and PD associations in the PWAS, we used the Parkinson's Environment and Genes (PEG) study ($n = 829$ PD patients; $n = 824$ controls). PEG is a population-based PD case-control study conducted in three agricultural counties in Central California (Kern, Fresno, and Tulare)[16]. Participants were recruited and enrolled in two waves: wave 1 (PEG1): 2000–2007, $n = 357$ PD patients, $n = 400$ population-based controls; wave 2 (PEG2): 2009–2015, $n = 472$ PD patients, $n = 424$ population-based controls. Patients were early in their disease course at enrollment (mean PD duration at baseline 3.0 years (SD = 2.6)), and all were seen by UCLA movement disorder specialists for in-person neurologic exams, many on multiple occasions, and confirmed as having idiopathic PD based on clinical characteristics[17]. Population-based controls for both study waves were required to be >35 years of age, have lived within one of the three counties for at least 5 years before enrollment, and not have a diagnosis of PD. More information about enrollment can be found in the Supplementary Materials. Characteristics of the PEG study subjects

are shown in Supplementary Data 15. The PD patients were on average slightly older than the controls and had a higher proportion of men, European ancestry, and never smokers.

PEG pesticide exposure assessment

We estimated ambient exposure to specific pesticide active ingredients (AIs) due to living or working near agricultural pesticide application, using record-based pesticide application data and a geographic information systems-based model[15]. We briefly describe our method but provide more detail in the Supplementary Materials.

Since 1972, California law mandates the recording of all commercial agricultural pesticide use by pest control operators and all restricted pesticide use by anyone until 1989, and then (1990-current) all commercial agricultural pesticide use by anyone, to the PUR database of the California Department of Pesticide Regulation (CA-DPR). This database records the location of applications, which can be linked to the Public Land Survey System, poundage, type of crop, and acreage a pesticide has been applied on, as well as the method and date of application. We combined this database with maps of land-use and crop cover, providing a digital representation of historic land-use, to determine the pesticide applications at specific agricultural sites[59]. PEG participants provided lifetime residential and workplace address information, which we geocoded in a multi-step process[60]. For each pesticide in the PUR and each participant, we determined the pounds of pesticide applied per acre within a 500 m buffer of each residential and workplace address each year since 1974, weighing the total poundage by the proportion of acreage treated (lbs/acre).

We were interested in long-term ambient exposures, and thus, considered the study exposure window as 1974 to 10 years prior to index date (PD diagnosis for cases or interview for controls) to account for a prodromal PD period. The exposure windows, which were on average 22 years for residential exposure and 18 years for workplace exposure, covered a very similar length and temporal period on average for patients and controls of each wave (study window comparisons shown in Supplementary Data 1 and 15). To assess exposure across the study window of interest for each pesticide, we averaged the annual lbs/acre estimates in the study window (e.g., for a participant with 22 years of exposure history, lbs/acre estimates for all 22 years were averaged), only using years for which the participants provided address information. This approach created one summary estimate of the average pounds of pesticide applied per acre per year within the 500 m buffer for each pesticide, which was estimated at residential and workplace locations separately for each participant. We log transformed the estimates offset by one, centered, and scaled the estimates to their standard deviations (SD).

Pesticide regulation and toxicity classification

We linked each of the 288 pesticides included in the PWAS to chemical, regulatory, and toxicity information using publicly available databases. We used the Pesticide Action Network (PAN) database to determine the chemical class (e.g. organophosphorus) and use type (e.g. insecticide) for each pesticide[19]. We linked each pesticide associated with PD to registration status in the United States and European Union to highlight current, active agricultural use versus historical, past use. We obtained information on pesticide regulation using the U.S. EPA pesticide label database, U.S. EPA cancellation reports, California Product Label Database, and the European Commission database, Rotterdam Notifications, and PAN. We further interrogated the PAN database to link information on known toxicity[19]. This includes whether the pesticide is drift prone based on vapor pressure, a groundwater contaminant, acutely toxic, a cholinesterase inhibitor, an endocrine disruptor, a carcinogen, or a developmental or reproductive toxicant. This information is based on public databases from government and international agencies, such as the EPA, California Prop 65 lists, World Health Organization, and International Agency for Research on Cancer. To identify a "most toxic" set of pesticides, PAN North America has designated certain pesticides as 'bad actors', if the pesticide meets any of the following criteria: known or probable carcinogen, reproductive or developmental toxicant, neurotoxic cholinesterase inhibitor, known groundwater contaminant, or a pesticide with high acute toxicity. Detailed methods on the toxicity and PAN designations can be found on the PAN website (https://www.pesticideinfo.org/).

iPSC reporter generation

Method for derivation of THtdTomato knockin clones was adapted from Ahfeldt et al. [61]. iPSC lines derived from a male patient with triplication at the α-synuclein locus (referred to as "SNCA triplication") was used for this[20,21]. A second iPSC line, generated from a distinct male donor unrelated to the SNCA triplication donor, contains two copies of wild-type α-synuclein was modified according to the same protocol summarized below. The line originally harbored an α-synuclein point mutation (E46K). We introduced the TH reporter and then genetically corrected it to create a two-copy SNCA wild-type line. THtdTomato insertion is initiated with nucleofection of two plasmids containing sequence coding for gRNAs and a third targeting plasmid were co-nucleofected using the Amaxa 4D nucleofection system on a Lonza nucleofector. Targeting plasmid contained: the targeting vector with a TH homology arm followed by tdTomato, a 2 A self-cleaving peptide sequence, a WPRE sequence, floxed puromycin selection cassette, and TH homology arm. Cells were allowed to recover for one day prior to initiation of puromycin selection. Surviving colonies were expanded and pooled for nucleofection of a pCAG-CRE:GFP vector allowing for Cre:LoxP based excision of the puromycin selection cassette and isolation by FACS of GFP + cells. Cells were plated at clonal density and resulting colonies were expanded out and subcloned. A 5' genotyping PCR producing a 626 bp product confirmed proper insertion of the 5 prime end of the reporter construct (5PrimeF: ACA TCC CCT GCT TGT TTC AA; 5PrimeR: AGC CCT CTA GCC TCA TCC TC) and a 3' genotyping PCR producing an 878 bp product (3PrimeF: TCC CTC AGA CCC TTT TAG TCA; 3PrimeR: GAG CCT CTG GAG CTG CTT G) confirmed proper insertion of the 3 prime end of the reporter into exon 14 of the TH gene. PCR products were Sanger sequenced and aligned to the sequence expected after successful targeting. A third PCR designed to produce a 285 bp product was performed to evaluate the unmodified TH allele and assess for NHEJ errors (NHEJF: CGT GAA GTT CGA CCC GTA CA; NHEJR: ACA GCT GTT GCG CTG AGA AG). Colonies passing the above quality control were differentiated into dopaminergic neurons[13,14,61] and assessed for proper expression of THtdTomato insert with both live imaging and post-fixation immunoco-localization of tdTomato reporter (rabbit anti-RFP, Rockland, 1:500) with tyrosine hydroxylase (sheep anti-TH, Pel-Freeze #960101, 1:500). Neurons were fixed with 4% paraformaldehyde for 20 min at room temperature after 35 days of differentiation as embryoid bodies and 13 days in adherent culture. Clones that pass PCR, sequencing, and differentiation quality control steps are karyotyped to assess for any abnormalities.

Dopaminergic differentiation

Dopamine neurons were generated from the THtdTomato modified iPSCs in accordance with published protocols, using minimal modification[13,14,61]. iPSC cultures were maintained in growth media (StemFlex, mTESR plus, or mTESR media). On day 0 of differentiation, confluent iPSC cultures on matrigel or geltrex were dissociated into single cells using Accutase incubated for 5–7 min at 37 degrees following a 0.5 mM EDTA in PBS wash. Accutase reaction was quenched with growth media and single cells were centrifuged, resuspended in growth media supplemented with Y-27632 at 10 μM and FGF-2 at 20 nM. Cells were plated at a density of 1 × 10^6 cells/mL in 15 cm uncoated plates (30 mL per plate) to form embryoid bodies. An additional 30 mL of growth media is added the following day. On day 2, EBs are collected into 50 mL conicals and spun at 200 x g for

3 min. Media is aspirated and EBs are replated in differentiation media (DMEM:F12 media mixed 1:1 with Neurobasal media, with PenStrep, B27 supplement without vitamin A, N2 supplement, 2-mercaptoethanol and glutamax) supplemented with 100 nM LDN-193189 and 10 μM SB431542. On day 3, 30 mL of differentiation media is added with SAg (1 μM) in addition to LDN-193189 and SB431542. Differentiation media is changed daily until day 5 at which point CHIR99021 is added (3 μM) and media changes are then performed every other day. SB431542 and SAg are withdrawn on day 9. LDN-193189 is withdrawn on day 13 and BDNF (20 ng/mL), GDNF (20 ng/mL), TGFb3 (25 ng/mL), dibutryl cAMP (0.5 mM), and DAPT (10 μM) are added. CHIR99021 is withdrawn on day 15 and TGFbeta3 is withdrawn on day 17. Spheres are maintained on uncoated plates with three times weekly media changes until dissociated for FACS and biochemical assays on day 35–42.

Dissociation, FACS, and live cell toxicant survival assay

Spheres are collected in a 15 mL conical tube from suspension culture plate, washed with PBS and resuspended in 2 mL of 0.25% Trypsin EDTA with 25 ng/mL of DNAse added prior to incubation at 37 degrees in water bath or rotating shaker for 5–7 min. 500 uL of FBS is then added to stop the reaction. Following a PBS wash, the EBs are triturated 5–7 times with a P1000 in a trituration solution (PBS with 5% FBS, 25 mm Glucose, 1x glutamax). Cells are then washed with PBS and pelleted at 300 x g for 5 min. Washes are repeated 3–5 times prior to plating or FACS sorting. Large clumps and aggregates are filtered using a 35 μM CellTrics filter. Y-27632 at 10 μM is present for sorting and collection in differentiation media. A MoFlo Astrios and MoFlo XDP (Beckman-Coulter, both equipped with 100um nozzle at 30 psi, running Summit Software V6.1.16945; analysis with FloJo 10.6.1) were used to sort single, live THtdTomato + neurons based on scatter profile, pulse width, exclusion of Sytox Red dye, and tdTomato fluorescence. The brightest 30–40% of cells are included in order to minimize non-neuronal cell types or immature/neuronal progenitors expressing a low level of the THtdTomato reporter. Sorted cells are plated onto polyornithine, poly-D-lysine, laminin, and fibronectin coated assay plates (Greiner 384 well plates) for survival assays. Sorted cells were plated at a density of 4 × 10^3 cells per well of a 384 well plate in a total volume of 45 uL on the day of plating with Y-27632. Media wash and transition to Fluorobrite (GIBCO) live imaging media was performed the following day using an Apricot Personal Pipettor (Apricot Designs) leaving 90 uL of fresh media per well. The first pesticide/toxicant treatment was performed two days after plating (Supplementary Figure 6). At five days after plating, half the media is aspirated, 45 uL of fresh media are added and treatment is repeated. At nine days after plating, half the media is aspirated, 45 uL of fresh media are added but treatment is not repeated. Live imaging is performed with a live cell chamber-equipped to maintain 5% CO2 and 37 degrees on an IXM High throughput microscope (Molecular Devices, MetaXpress imaging software version 6.5.3.427) using a x10 objective, a Texas Red filter (excitation 560/32 nm; emission 624/40) and imaging four fields per well, resulting 72% coverage per well. Images were acquired immediately prior to treatment, at 7 days after first treatment and at 11 days after first treatment.

Image analysis

Images were imported into Columbus (Perkin Elmer, version 2.9.1) analysis software. Differential brightness and roundness criteria permit the use of nuclei detection algorithms to detect the endogenous fluorescent reporter for accurate selection of cell soma with exclusion of neurites. The *Find Nuclei* script was used with method M, diameter was set at 13 μM with splitting sensitivity of 0.15 and common threshold of 0.2. The nuclei detection algorithm was further refined to eliminate debris and doublets by selecting nuclei with area >40 μM and <400 μM, and roundness >0.7. Additional brightness criteria (pixel intensity >500) were used to generate reproducible neuron cell body detection scripts that exclude debris and dead cells. Image analysis was performed in two steps to improve assay to assay reproducibility. The first step determined the average pixel intensity for detected cells that met size and roundness criteria. Average and standard deviation of fluorescence intensity was then determined for the control wells treated with DMSO. A second analysis was then designed to count all cells brighter than three standard deviations above the average fluorescence intensity in the control wells and measure neurites. These objects were counted as THtdTomato positive cells for analysis. Built in neurite detection algorithms were applied in order to identify neurites based on THtdTomato signal in these cellular processes using the Find Neurites, CSIRO neurite analysis method. For this neurite detection method, the following settings were used: smoothing window 3px, linear window 15px, contrast >1, diameter > /= 7px, gap closure distance < /= 5px, gap closure quality 0, debarb length < /= 15px, body thickening 5px and tree length < /= 20px. Neurite analysis generated a sum of total neurite length per field analyzed which was used as the primary metric for neurite analysis.

Toxicant library generation

All compounds were ordered from Sigma Aldrich as the PESTANAL analytical standard whenever possible with the following exceptions: Tribufos (S,S,S-tributyl phosphorotrithioate) obtained from Fisher/Crescent Chemical; MSMA (Monosodium acid methane arsonate sesquihydrate) obtained from Santa Cruz biotechnology: oxydemeton-methyl obtained from Santa Cruz biotechnology. Based on available solubility data, compounds were dissolved in DMSO, water, or ethanol to a working dilution of 30 mM. A subset had poor solubility that required a more dilute stock solution (15 mM or less). A limited set of compounds identified in the PWAS analysis were omitted due to high dermal/inhalation toxicity in mammals, inability to obtain a highly pure formulation, or inadequate solubility in water, DMSO, or ethanol. Working stocks of compounds were pipetted into multiwell template plates and serial dilution was performed with an Apricot Personal Pipettor. Ethanol solubilized compounds were diluted 1:1 with DMSO to improve accuracy of pipetting and permit a parallel workflow to the DMSO and water plates. Individual compound plates were then generated from template plates following serial dilution and stored at −20 °C until day of treatment. Apricot personal pipettor equipped with 125 uL volume disposable tips was used to perform dilution of compounds in culture media and treatment of dopaminergic neurons.

Viability assays

mDA neurons were sorted and pesticide treatments were performed as described. At eleven days after the first toxicant treatment, media was removed and the CellTitreGlo buffer was added directly to the assay plate using an Apricot Personal Pipettor. CellTitreGlo assay was scaled down from the manufacturer's recommended volumes to accommodate a 384 well format. Luminescence was measured on a Molecular Devices Spectramax plate reader (using SoftMax 5.4 pro software) 15 min after buffer addition.

Cardiomyocyte differentiation

The obtained hiPSC clones were cultured in StemFlex cell culture media (ThermoFisher Scientific, A3349401) in 6 well plates coated with Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (ThermoFisher Scientific, A1413202). Once confluent, the hiPSC cells were differentiated into human CMs utilizing a chemically-defined cardiomyocyte differentiation protocol[52]. Briefly, hiPSCs were treated with 6 uM CHIR99021 (Tocris, 4423) for 3 days in RPMI 1640 (Thermo Fisher Scientific, 11875119) with B27-insulin (Thermo Fisher Scientific, A1895601). Then cells were treated with the 2uM Wnt inhibitor C59 (Tocris, 5148) in RPMI/B27- for another 2 days. Between 5–10 days of differentiation, RPMI/B27- media was used and changed

every other day, on day 11 cells were switched to RPMI/B27 + insulin and beating was observed. To improve the CM purity, cells were cultured in RPMI/B27 + insulin without glucose, for 3 days. After purification, the iPSC-CMs were dissociated with TrypLE 10x (Thermo Fisher Scientific, A1217703) and replated in 6-well Matrigel coated plates at a density of 3×10^6 per well in RPMI/B27 + containing 10% KOSR and ROCK inhibitor Y-27632 (Tocris, 1253). After 2 days, cells were cultured again in RPMI/B27 + without glucose for 3 days prior to switch to 3 ml of RPMI/B27 + insulin. Cells where cultured for an additional week with media changes every 2–3 days prior to subsequent imaging studies.

Cardiomyocyte cell count assays

hiPSC-CMs were plated on Geltrex at 40,000 cells per well of a 384-well plate (Greiner Bio-One) in 75 µl of RPMI/B27 + insulin containing 10% KOSR and ROCK inhibitor Y-27632. The next day media was changed to RPMI/B27 + insulin final volume 90 µl and cells were maintained for a minimum of 5 days prior to drug treatment and imaging. For the analysis, 45 µl of media was removed and hiPSC-CMs were loaded with 45 µl 2x TMRM (T668, ThermoFisher Scientific) with Hoechst 33258 (ThermoFisher Scientific). After 10 min incubation in a 37 °C 5% CO2 incubator, cells were washed by removing 90 µL of media and replacing it with 90 µL RPMI/B27 + insulin. Hoechst 33258 signal was acquired across 4 tiled fields per well at 20x, using an EVOS M7000 (ThermoFisher Scientific). Nuclei were counted using onboard software.

Western blot

For western blots, protocol was performed as described with minimal modifications[32]. Briefly, 20–30 µg of protein per sample was subjected to SDS-PAGE using NuPAGE 4–12% Bis-Tris protein gels in NuPAGE MES SDS Running Buffer, electrophoresed at 150 V for 1 h. Dry transfer from polyacrylamide gel to PVDF membrane was conducted with the iBlot 2 Gel Transfer Device (Thermo Fisher) using preset P0 program (20 V 1 min; 23 V 4 min; 25 V 2 min). The membrane was fixed in 4% paraformaldehyde in PBS for 30 min at room temperature with orbital shaking and washed three times for 5 min with PBS. Membranes were blocked in 5% nonfat milk in PBS 1 h with orbital shaking and subsequently incubated overnight in 5% block with 0.1% Tween20 and the respective primary antibodies at the desired dilution (see below for antibody information and dilutions), at 4 degrees C with orbital shaking. After four washes for 5 min in 0.05% Tween20/PBS, membranes were incubated with the HRP-conjugated secondary antibody at 1:10,000 in blocking solution at room temperature with orbital shaking. After four washes for 5 min in 0.05% Tween20/PBS, the blot was incubated for 5 min in chemiluminescence detection solution (Bio-Rad Clarity Western ECL Cat# 1705060). Signal detection was performed on an iBright imaging system (Invitrogen). Primary antibodies utilized include rabbit anti-alpha synuclein phospho S129 (Abcam Cat # ab51253; 1:1000), mouse anti-Alpha Synuclein (Clone: 42, BD Transduction Laboratories Cat. # 610787, 1:1,000), and mouse anti-GAPDH (Clone 6C5, Millipore Cat# MAB 374; 1:15,000).

Combinatorial treatments

The combinatorial treatment plate maps were designed and performed using a D300e Digital Dispenser (Hewlett Packard) equipped with T8 and D4 dispenseheads. HP software was used to generate plate maps using the "synergy" function for 384 well format that produced all possible combinations of 6 different compounds at 10 µM concentration. Compounds dissolved in ethanol were further diluted 1:1 with DMSO for dispensing accuracy. DMSO concentration was normalized to 0.3% DMSO to maintain accurate dispensing and account for higher order combinations of multiple compounds. DMSO controls were present on each plate to assess for extent of plate-to-plate variability within a given biological replicate. Upset plots were generated in R (Bioconductor) to visualize pesticide combinations on the x-axis (https://jokergoo.github.io/ComplexHeatmap-reference/book/upset-plot.html#upset-making-the-plot). Treatment timeline was identical to that described above for toxicant live imaging assays. Toxicants were combined as described. Synergy plots were created from an upset plot of each toxicant combination (ggupset(0.3.0)) and a bar plot of the day 11 neurons' mean THtdTomato brightness (ggplot2 (3.3.5) with a viridis plasma palette (0.5.1). Each combination of two toxicant's THtdTomato count values was compared via Student's *t*-test, and *p*-values were adjusted for multiple testing with Benjamini-Hochberg false discovery rate to *q*-values (reported in Supplementary Data 14).

Agilent seahorse XF cell Mito stress assay

Cellular respiration of SNCA triplication THtd differentiated neurons in presence of ziram (45708 Milipore Sigma, CAS No 137-30-4) and trifluralin (111020171, MiliporeSigma, CAS No 1582-09-8) was accessed using the Seahorse XF 96 Extracellular Flux Analyzer and the XF Cell Mito stress test kit (Agilent Technologies) following Agilent Technologies guidelines. Mixed dissociated THtd neuronal cultures at day 35 of differentiation were seeded at a volume of 100 µL and a density of 1×10^5 cells per well in the inner 60 wells of a Polyethylenimine (PEI)-laminin pre-coated Seahorse 96 well plate. The day of the experiment, 20 days after seeding (55 days of total differentiation), cells were treated with 0.3% DMSO (control wells) and differentiation medium containing trifluralin at 90 µM, 60 µM and 30 µM (treatment wells) for 6 h. After this treatment period, medium was removed and 100 µL of the seahorse assay medium was added to each well of the entire plate to start the Mito stress assay, where Oligomycin (1 µM), FCCP (0.5 µM) and Rotenone/Antimycin-A (1 µM) are added at specific time points of the assay to measure metabolic outcome for each condition. After the assay finished, the assay medium was removed from the wells and cells were flash-frozen for subsequent measurement of total protein from each well using Pierce BCA assay kit (23225, Thermo Fisher). Total protein content was used to normalize the data. Data analysis performed with Wave software (Agilent Technologies, version 2.6.1.53). At least three biological replicates (each with its own corresponding set of six technical replicates) were performed for the assay. Distinct and separate differentiations from the same parental iPSC lines were assessed as biological replicates.

Mitochondrial subunit assays

The mitochondria subunit assay is based on published methodology[33]. Cell pellet generation: SNCA triplication differentiated neurons were dissociated from EBs at day 35 of differentiation. Mixed dissociated neuronal cultures were seeded at a density of 2×10^6 cells per well in the inner 8 wells of 24 well plates coated with polyethylenimine-Laminin (PEI-Lam). Cells were treated with trifluralin 30 days after seeding (65 days of total differentiation). Differentiation medium containing trifluralin at 90 µM, 60 µM 30 µM, and 10 µM and matched 0.3% DMSO control were prepared. The existing differentiation medium for each well to be treated was replaced with 1 mL of freshly prepared trifluralin-containing differentiation medium. Cells under those conditions were incubated for 6 h. After the incubation period, cells were harvested by pipetting, transferred to 1.5 mL Eppendorf tubes and centrifuged at 4 °C for 5 min at 300 x *g*, medium was removed, and cell pellets were stored at −80 °C for biochemical analysis. Protein Extraction: Aqueous extraction of proteins from cell pellets was done by resuspending the pellets in 100 µL of 1X dilution from 4X blue LDS buffer (B0007, life technologies) plus protease (11697498001, Sigma) and phosphatase (4906837001, Sigma) inhibitors cocktail, sonication for 2x using a tip sonicator at 40% power for 15 s on ice. Samples were boiled for 5 min at 100 °C and then centrifuged for 10 min at 850 x *g* at 4 °C. Proteins contained in supernatant were quantified using the Pierce BCA assay kit (23225, Thermo Fisher). Western blot: Protein

samples (30 μg) were prepared using 4x Bolt LDS sample buffer (B0007, Thermo Fisher) and 10X Bolt Sample reducing agent (B0009, Thermo Fisher) and boiled for 5 min and then loaded into precast Bolt 12-well mini Bis-Tris 4–12% gels and run in MES/SDS buffer for 30 min at 200 V. iBlot dry blotting system (Thermo Fisher) was used to transfer into iBlot nitrocellulose membrane (Thermo Fisher). Licor Odyssey Buffer PBS (Cat. no. 927–40000, Licor) was used to block the membranes at room temperature for 1 h, followed by overnight incubation with agitation at 4 °C with the following primary antibodies: mitoProfile total OXPHOS Human WB antibody cocktail (1:1,000, ab110411), SDHA monoclonal antibody (1:10,000, 459200), anti-TOMM20 (1:1,000, HPA011562) and actin antibody (1:1,200, Sigma A2066). Four 5 min washes with PBS-T (0.05% tween) were performed followed by incubation for 1 h at room temperature with secondary antibodies 680-anti-rabbit, 800-anti-mouse at 1:10,000 dilution in Licor Odyssey plus 0.1% Tween. Membranes were scanned using Licor Clx scanner. Analysis of protein bands was performed using Image Studio software. Three independent differentiations were used for three biological replicates.

Statistics and reproducibility

A detailed description of analytic methods for the epidemiologic study, described in four parts, can be found in the Supplement Materials, including methods used 1) to describe the extent of agricultural pesticide application in the study area; 2) for the pesticide-wide association analysis, which made use of meta-analyses and logistic regression; 3) for pesticide group overrepresentation analysis; and 4) for PD-associated pesticide clustering. All epidemiologic analyses were done in R version 4.1.0. Statistical analysis for in vitro assays was performed in Graph Pad PRISM software. Power calculations were used to assess feasibility of the pesticide-wide association study, based on 80% power, given the epidemiologic study sample size, and alpha=1.7e-4 to account for multiple testing with a Bonferroni correction. With respect to in vitro assays, no statistical method was used to predetermine sample size. Previous experience with live imaging assays and neuronal survival assays was used to guide approximate numbers of cells needed and fields imaged for each condition. No data were excluded from the analyses. While the in vitro experiments were not randomized, an image analysis algorithm was applied equally to all wells following optimization of parameters on negative control and positive control wells. The investigators were not blinded to allocation during epidemiologic analysis, in vitro experiments, and outcome assessment.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Further data that was analyzed during the current study cannot be made publicly available due to participant privacy as they provide information about residential location. Deidentified data that support the findings of this study are available on request from the corresponding author BR. Source data are provided with this paper.

Code availability

Processing code for the source data is provided on github (https://github.com/KCPaul-lab/NatComms_PWAS).

References

1. Ascherio, A. & Schwarzschild, M. A. The epidemiology of Parkinson's disease: risk factors and prevention. *Lancet Neurol.* **15**, 1257–1272 (2016).
2. Goldman SM, Musgrove RE, Jewell SA, Di Monte DA. Chapter Three - Pesticides and Parkinson's Disease: Current Experimental and Epidemiological Evidence. In: *Advances in Neurotoxicology* (eds Aschner M, Costa LG). Academic Press (2017).
3. California Department of Pesticide Regulation. Actively Registered Active Ingredients (AI) by Common Name.).
4. Liu, C. et al. A Scientometric Analysis and Visualization of Research on Parkinson's Disease Associated With Pesticide Exposure. *Front Pub. Health.* **8**, 91 (2020).
5. Minakaki, G., Krainc, D. & Burbulla, L. F. The Convergence of Alpha-Synuclein, Mitochondrial, and Lysosomal Pathways in Vulnerability of Midbrain Dopaminergic Neurons in Parkinson's Disease. *Front. Cell Dev. Biol.* **8**, 580634 (2020).
6. Burbulla, L. F. et al. Dopamine oxidation mediates mitochondrial and lysosomal dysfunction in Parkinson's disease. *Science* **357**, 1255–1261 (2017).
7. Gonzalez-Rodriguez, P. et al. Disruption of mitochondrial complex I induces progressive parkinsonism. *Nature* **599**, 650–656 (2021).
8. Ryan, S. D. et al. Isogenic human iPSC Parkinson's model shows nitrosative stress-induced dysfunction in MEF2-PGC1alpha transcription. *Cell* **155**, 1351–1364 (2013).
9. Cooper, O. et al. Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease. *Sci. Transl. Med.* **4**, 141ra190 (2012).
10. Khurana, V., Tardiff, D. F., Chung, C. Y. & Lindquist, S. Toward stem cell-based phenotypic screens for neurodegenerative diseases. *Nat. Rev. Neurol.* **11**, 339–350 (2015).
11. Kim, T. W. et al. Biphasic Activation of WNT Signaling Facilitates the Derivation of Midbrain Dopamine Neurons from hESCs for Translational Use. *Cell Stem Cell.* **28**, 343–355.e345 (2021).
12. Kriks, S. et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* **480**, 547–551 (2011).
13. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872 (2007).
14. Ahfeldt, T. et al. Pathogenic Pathways in Early-Onset Autosomal Recessive Parkinson's Disease Discovered Using Isogenic Human Dopaminergic Neurons. *Stem Cell Rep.* **14**, 75–90 (2020).
15. Cockburn, M. et al. Prostate cancer and ambient pesticide exposure in agriculturally intensive areas in California. *Am. J. Epidemiol.* **173**, 1280–1288 (2011).
16. Ritz, B. R., Paul, K. C. & Bronstein, J. M. Of Pesticides and Men: a California Story of Genes and Environment in Parkinson's Disease. *Curr. Environ. Health Rep.* **3**, 40–52 (2016).
17. Hughes, A. J., Ben-Shlomo, Y., Daniel, S. E. & Lees, A. J. What features improve the accuracy of clinical diagnosis in Parkinson's disease: a clinicopathologic study. *Neurology* **42**, 1142–1146 (1992).
18. Paul, K. C., Cockburn, M., Gong, Y., Bronstein, J. & Ritz, B. Agricultural paraquat dichloride use and Parkinson's disease in California Central Valley. *medRxiv* https://doi.org/10.1101/2022.12.28.22284022 (2022).
19. A.H. Ksehbrosc. PAN Pesticide Database. (ed Network PA) (2014).
20. Chung, C. Y. et al. Identification and rescue of alpha-synuclein toxicity in Parkinson patient-derived neurons. *Science* **342**, 983–987 (2013).
21. Byers, B. et al. SNCA triplication Parkinson's patient's iPSC-derived DA neurons accumulate alpha-synuclein and are susceptible to oxidative stress. *PLoS One.* **6**, e26159 (2011).
22. Ludtmann, M. H. R. et al. α-synuclein oligomers interact with ATP synthase and open the permeability transition pore in Parkinson's disease. *Nat. Commun.* **9**, 2293 (2018).
23. Mittal, S. et al. beta2-Adrenoreceptor is a regulator of the alpha-synuclein gene driving risk of Parkinson's disease. *Science* **357**, 891–898 (2017).
24. Singleton, A. B. et al. alpha-Synuclein locus triplication causes Parkinson's disease. *Science* **302**, 841 (2003).

25. Hallacli, E. et al. The Parkinson's disease protein alpha-synuclein is a modulator of processing bodies and mRNA stability. *Cell* **185**, 2035–2056.e2033 (2022).
26. Hsieh, J. H. et al. Identifying Compounds with Genotoxicity Potential Using Tox21 High-Throughput Screening Assays. *Chem. Res. Toxicol.* **32**, 1384–1401 (2019).
27. Tong, Z. B. et al. Characterization of three human cell line models for high-throughput neuronal cytotoxicity screening. *J. Appl. Toxicol.* **37**, 167–180 (2017).
28. Chou, A. P. et al. Ziram causes dopaminergic cell damage by inhibiting E1 ligase of the proteasome. *J. Biol. Chem.* **283**, 34696–34703 (2008).
29. Liang, P. et al. Drug Screening Using a Library of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Reveals Disease-Specific Patterns of Cardiotoxicity. *Circulation* **127**, 1677–1691 (2013).
30. Burnett, S. D. et al. Population-based toxicity screening in human induced pluripotent stem cell-derived cardiomyocytes. *Toxicol. Appl. Pharmacol.* **381**, 114711 (2019).
31. Uversky, V. N., Li, J., Bower, K. & Fink, A. L. Synergistic Effects of Pesticides and Metals on the Fibrillation of α-Synuclein: Implications for Parkinson's Disease. *NeuroToxicol.* **23**, 527–536 (2002).
32. Lam, L. et al. Rapid iPSC inclusionopathy models shed light on formation, consequence and molecular subtype of α-synuclein inclusions. *bioRxiv* https://doi.org/10.1101/2022.11.08.515615 (2022).
33. Nisticò, R., Mehdawy, B., Piccirilli, S. & Mercuri, N. Paraquat- and rotenone-induced models of Parkinson's disease. *Int. J. Immunopathol. Pharmacol.* **24**, 313–322 (2011).
34. McLaughlin, B. A., Nelson, D., Erecińska, M. & Chesselet, M. F. Toxicity of dopamine to striatal neurons in vitro and potentiation of cell death by a mitochondrial inhibitor. *J. Neurochem.* **70**, 2406–2415 (1998).
35. Monzio Compagnoni, G. et al. Mitochondrial Dysregulation and Impaired Autophagy in iPSC-Derived Dopaminergic Neurons of Multiple System Atrophy. *Stem Cell Rep.* **11**, 1185–1198 (2018).
36. Goldman, S. M. Environmental Toxins and Parkinson's Disease. *Ann. Rev. Pharmacol. Toxicol.* **54**, 141–164 (2014).
37. Zhou, T. et al. A hPSC-based platform to discover gene-environment interactions that impact human beta-cell and dopamine neuron survival. *Nat. Commun.* **9**, 4815 (2018).
38. Bisaglia, M. & Bubacco, L. Copper Ions and Parkinson's Disease: Why Is Homeostasis So Relevant? *Biomolecules* **10**, 195 (2020).
39. Bonneh-Barkay, D., Reaney, S. H., Langston, W. J. & Di Monte, D. A. Redox cycling of the herbicide paraquat in microglial cultures. *Mol. Brain Res.* **134**, 52–56 (2005).
40. Franco, R., Li, S., Rodriguez-Rocha, H., Burns, M. & Panayiotidis, M. I. Molecular mechanisms of pesticide-induced neurotoxicity: Relevance to Parkinson's disease. *Chemico-Biol. Interact.* **188**, 289–300 (2010).
41. Gorell, J. M. et al. Occupational exposure to manganese, copper, lead, iron, mercury and zinc and the risk of Parkinson's disease. *Neurotoxicology* **20**, 239–247 (1999).
42. Lee, J. C., Gray, H. B. & Winkler, J. R. Copper(II) Binding to α-Synuclein, the Parkinson's Protein. *J. Am. Chemical Society.* **130**, 6898–6899 (2008).
43. Lindquist, N. G., Larsson, B. S. & Lydén-Sokołowski, A. Autoradiography of [14C]paraquat or [14C]diquat in frogs and mice: Accumulation in neuromelanin. *Neurosci. Lett.* **93**, 1–6 (1988).
44. Shrestha, S. et al. Pesticide use and incident Parkinson's disease in a cohort of farmers and their spouses. *Environ. Res.* **191**, 110186 (2020).
45. Chen, J., Yu, Q., Patterson, E., Sayer, C. & Powles, S. Dinitroaniline Herbicide Resistance and Mechanisms in Weeds. *Front Plant Sci.* **12**, 634018 (2021).
46. Carvalheiro, M. et al. Trifluralin liposomal formulations active against Leishmania donovani infections. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV* **71**, 292–296 (2009).
47. Fennell, B. J., Naughton, J. A., Dempsey, E. & Bell, A. Cellular and molecular actions of dinitroaniline and phosphorothioamidate herbicides on Plasmodium falciparum: tubulin as a specific antimalarial target. *Mol. Biochem. Parasitol.* **145**, 226–238 (2006).
48. Giles, N. L., Armson, A. & Reid, S. A. Characterization of trifluralin binding with recombinant tubulin from Trypanosoma brucei. *Parasitol Res.* **104**, 893–903 (2009).
49. Fernandes, T. C. C., Pizano M. A. & Marin-Morales, M A. *Characterization, Modes of Action and Effects of Trifluralin: A Review*. Ch. 19. https://doi.org/10.5772/55169 (2013).
50. Devi, L., Raghavendran, V., Prabhu, B. M., Avadhani, N. G. & Anandatheerthavarada, H. K. Mitochondrial import and accumulation of α-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. *J. Biol. Chem.* **283**, 9089–9100 (2008).
51. Greene, J. C. et al. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. *Proc. Natl. Acad. Sci.* **100**, 4078–4083 (2003).
52. Park, J. et al. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature* **441**, 1157–1161 (2006).
53. Roy, S. & Wolman, L. Ultrastructural observations in Parkinsonism. *J. Pathology.* **99**, 39–44 (1969).
54. Ritz, B. & Costello, S. Geographic model and biomarker-derived measures of pesticide exposure and Parkinson's disease. *Ann. N. Y. Acad. Sci.* **1076**, 378–387 (2006).
55. Guttikonda, S. R. et al. Fully defined human pluripotent stem cell-derived microglia and tri-culture system model C3 production in Alzheimer's disease. *Nat. Neurosci.* **24**, 343–354 (2021).
56. Vatine, G. D. et al. Human iPSC-Derived Blood-Brain Barrier Chips Enable Disease Modeling and Personalized Medicine Applications. *Cell Stem Cell.* **24**, 995–1005.e1006 (2019).
57. Mitchell, J. M. et al. Mapping genetic effects on cellular phenotypes with "cell villages". *bioRxiv* https://doi.org/10.1101/2020.06.29.174383 (2020).
58. Cederquist, G. Y. et al. A Multiplex Human Pluripotent Stem Cell Platform Defines Molecular and Functional Subclasses of Autism-Related Genes. *Cell Stem Cell.* **27**, 35–49.e36 (2020).
59. California Department of Water Resources Land Use Surveys. In: *CDWR*) (2013).
60. McElroy, J. A., Remington, P. L., Trentham-Dietz, A., Robert, S. A. & Newcomb, P. A. Geocoding addresses from a large population-based study: lessons learned. *Epidemiology* **14**, 399–407 (2003).
61. Tomishima M. Midbrain dopamine neurons from hESCs. In: *StemBook*) (2008).
62. Lian, X. et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proc. Natl. Acad. Sci.* **109**, E1848–E1857 (2012).

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Author contributions

PWAS and epidemiologic co-exposure conception, design, and analysis: K.C.P. SNCA triplication knock-in line generation, dopamine neuron experiments: R.K. Mitochondrial assays, Seahorse assays: E.L.M. Transgenic reporter constructs: T.A. Dopamine neuron experiments: J.B. Cardiomyocyte differentiation, contractility analysis, pesticide treatment, toxicity analysis: A.K., E.R., H.P., Y.J.L., R.L., R.K. Statistics, graphing of cotton cluster upset plots: K.H. PWAS analysis interpretation and PUR map: M.F., Y.Y. Ambient pesticide exposure assessment: M.C., L.K.T. Neurology exams, interpretation, and data collection for PEG study: J.B., B.R. Experimental design, data analysis, manuscript writing: K.C.P., R.K., E.L.M., L.R., V.K., B.R.

Competing interests

L.L.R. is a founder of and a member of the Scientific Advisory Board of Vesalius Therapeutics, a private biotechnology company, and an owner of stock options. He is a member of the Scientific Advisory Board of Yumanity Therapeutics and a shareholder. Both companies study Parkinson's disease. B.R., M.C., and R.C.K. have been retained as expert consultants for plaintiffs in a lawsuit on the role of paraquat in Parkinson's disease causation. V.K. is a co-founder of and senior advisor to DaCapo Brainscience and Yumanity Therapeutics, companies focused on central nervous system diseases. The remaining authors declare no competing interests.

Additional information

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Agricultural activities and the incidence of Parkinson's disease in the general French population

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Abstract

Most studies on pesticides and Parkinson's disease (PD) focused on occupational exposure in farmers. Whether non-occupational exposure is associated with PD has been little explored. We investigated the association between agricultural characteristics and PD incidence in a French nationwide ecologic study. We hypothesized that persons living in regions with agricultural activities involving more intensive pesticide use would be at higher risk. We identified incident PD cases from French National Health Insurance databases (2010-2012). The proportion of land dedicated to 18 types of agricultural activities was defined at the canton of residence level. We examined the association between agricultural activities and PD age/sex-standardized incidence ratios using multivariable multilevel Poisson regression adjusted for smoking, deprivation index, density of neurologists, and rurality (proportion of agricultural land); we used a false discovery rate approach to correct for multiple comparisons and compute q-values. We also compared incidence in clusters of cantons with similar agricultural characteristics (k-means algorithm). We identified 69,010 incident PD cases. Rurality was associated with higher PD incidence ($p < 0.001$). Cantons with higher density of vineyards displayed the strongest association ($RR_{top/bottom\ quartile} = 1.102$, 95% CI = 1.049-1.158; q-trend = 0.040). This association was similar in men, women, and non-farmers, stronger in older than younger persons, and present in all French regions. Persons living in the cluster with greatest vineyards density had 8.5% (4.4-12.6%) higher PD incidence ($p < 0.001$). In France, vineyards rank among the crops that require most intense pesticide use. Regions with greater presence of vineyards are characterized by higher PD risk; non-professional pesticides exposure is a possible explanation.

Keywords: Agriculture; Epidemiology; Incidence; Parkinson's disease; Pesticid

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Review

Is Pesticide Use Related to Parkinson Disease? Some Clues to Heterogeneity in Study Results

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Background: Previous systematic reviews have indicated that pesticide exposure is possibly associated with Parkinson disease (PD). However, considerable heterogeneity has been observed in study results.

Objective: We aimed at providing an update of the literature published on PD and exposure to pesticides by performing a systematic review and meta-analysis. In addition, we investigated whether methodological differences between studies could explain the heterogeneity in study results.

Methods: We identified studies through a systematic literature search. We calculated summary risk ratios (sRRs) for pesticide exposure and subcategories using random effects meta-analyses and investigated sources of heterogeneity by meta-regression and stratified analyses.

Results: Thirty-nine case–control studies, four cohort studies, and three cross-sectional studies were identified. An sRR of 1.62 [95% confidence interval (CI): 1.40, 1.88] for pesticide exposure (ever vs. never) was found. Summary estimates for subclasses of pesticides indicated a positive association with herbicides and insecticides, but not with fungicides. Heterogeneity in individual study results was not related to study design, source of control population, adjustment of results for potential confounders, or geographical area. However, results were suggestive for heterogeneity related to differences in the exposure assessment. Job title–based exposure assignment resulted in a higher sRR (2.5; 95% CI: 1.5, 4.1) than did assignment based on self-reported exposure (e.g., for self-reported ever/never exposure, sRR = 1.5; 95% CI: 1.3, 1.8).

Conclusions: This review affirms the evidence that exposure to herbicides and insecticides increase the risk of PD. Future studies should focus on more objective and improved methods of pesticide exposure assessment.

Key words: exposure assessment, fungicides, herbicides, insecticides, meta-analysis, Parkinson disease, pesticides, systematic review. *Environ Health Perspect* 120:340–347 (2012). http://dx.doi.org/10.1289/ehp.1103881 [Online 21 October 2011]

Parkinson disease (PD) is an idiopathic degenerative disorder of the central nervous system that impairs motor skills, cognitive processes, and other functions. The etiology of PD is largely unknown, although some genetic factors have been identified (Bekris et al. 2010; Shulman et al. 2011). Based on published epidemiological and toxicological studies, pesticides may be involved in the etiology of PD (Brown et al. 2006). However, epidemiological evidence is far from conclusive, as considerable heterogeneity has been observed in study results (Brown et al. 2006; Li et al. 2005; Priyadarshi et al. 2000). Possible methodological causes of heterogeneity in study results have been suggested and include differences in study design, control selection, diagnosis of patients, and statistical analysis (Brown et al. 2006). Differences in exposure assessment methods could contribute to heterogeneity as well. Most previous studies relied almost exclusively on self-reported exposures, a process that is prone to recall bias, especially in case–control studies, and could lead to false-positive associations. Alternatively, one could speculate that PD patients might underreport pesticide exposure because of cognitive deficits, leading to false-negative associations. Furthermore, differences in the definition of exposure to pesticides (occupational vs. nonoccupational use, ever/never vs. regular use) could also result in heterogeneous study results. Lastly, the regions where the studies have been conducted could be of importance as regulation, types, and use of pesticides may differ from region to region.

Several recent studies have been published on pesticide exposure and PD risk, including some prospective (cohort) studies. In the present analysis, we aimed at providing an update of the literature published since the last systematic review on PD (Brown et al. 2006) and exposure to pesticides and pesticide subcategories by performing a systematic review and meta-analysis. We specifically set out to address the question of whether the previously described heterogeneity in study findings could be explained by differences in study design and exposure assessment methods.

Methods

Data source. We searched the databases Embase (http://www.Embase.com/), starting with 1974, and Medline (http://www.ncbi.nlm.nih.gov/pubmed/), starting with 1950, through November 2010 using the search term "Parkinson" in combination with "pesticide*," "insecticide*," "fungicide*," "herbicide*," "rodenticide*," "organochlorine*," "organophosphate*," "carbamate*," "glyphosate*," "paraquat," "maneb," "lindane," "dieldrin," "rotenone," "DDT," or "environmental factors." The search was limited to publications in English, French, German, or Dutch; to human studies; and to original publications. We also searched the reference lists of the retrieved publications.

Study selection. We included studies that specifically investigated PD or parkinsonism. We included cohort studies, case–control studies, and cross-sectional studies. No reviews, case reports, or conference abstracts were included. We excluded studies that summarized results of pesticide exposure only within a broad category of "chemical exposure." Exposure to pesticides was defined as use of pesticides by the subject, thus excluding environmental studies.

Data extraction. Two reviewers (M.M., M.B.) independently extracted reported risk estimates [i.e., odds ratios (ORs), risk ratios (RRs), or prevalence ratios], study designs, exposure assessment methods, and types of source population for the controls. We also evaluated subcategories of pesticides and extracted data about exposure–response relations and individual pesticides. Two other researchers (A.H., R.V.) acted as referees in cases of any differences. If authors reported adjustment for potential confounders, we preferred adjusted risk estimates over crude risk estimates. In cases where no risk estimate or 95% confidence interval (CI) was reported, we calculated crude risk estimates and 95% CIs with the reported numbers. Where risk estimates were reported separately for men and women, we pooled the risk estimates with a within-study meta-analysis (Vlaanderen et al. 2011). Of studies with more than one control group, the results of population controls were preferred above the results of hospital controls because population controls are generally considered to be a more representative comparison group than hospital controls.

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Statistical analysis. Because of the observed heterogeneity in study results, we conducted a DerSimonian and Laird (1986) random effects meta-analysis to pool the results of the separate studies for risk for pesticide exposure and the subgroups of herbicides, insecticides, and fungicides. We also stratified by whether or not nonoccupational exposure (e.g., gardening) was included in the exposed group. This was because of potential differences between occupational and nonoccupational exposures in intensity and frequency of exposures. In one publication, results both for occupational and for occupational and/or nonoccupational exposure were reported (Frigerio et al. 2006). We chose to include risk estimates of the more inclusive exposure definition, although final results did not differ when we included the risk estimates based on only occupational exposure (data not shown).

Subsequently, we explored whether heterogeneity in observed risk estimates could be explained by study and exposure assessment characteristics. We did so by stratification and used meta-regression to explore statistical significance of these characteristics. Given the limited number of studies, we only explored one characteristic at a time. Characteristics explored were the type of exposure assessment (self-reported ever/never pesticide exposure, self-reported regular pesticide exposure, or exposure assessment based on reported job titles by expert judgment and/or applying a job-exposure matrix), source of control population [hospital, general population, or other (studies using family members or case acquaintances as controls, or studies that used a combination of different sources)], geographical area (North America, Europe, or other), and whether adjustments were made for potential confounders. The I^2 measure was used to quantify the heterogeneity between studies; I^2 can be interpreted as a measure of the percentage of the total variation that cannot be explained by chance (Higgins et al. 2003). *p*-Values for heterogeneity are based on the *Q*-statistic. Small study effects were tested with funnel plots and Egger's test (Egger et al. 1997). All analyses were performed with Stata (version 10; StataCorp, College Station, TX, USA) with the metan, metareg, metafunnel, and metabias commands. All statistical tests were two sided, and a *p*-value of < 0.05 was considered statistically significant.

Results

The search in Embase and Medline yielded 883 publications, of which 52 publications met the inclusion criteria. We excluded 3 publications (Fong et al. 2005; Menegon et al. 1998; Smargiassi et al. 1998) where the study population had been included in subsequent publications (De Palma et al. 1998; Fong et al. 2007; McCann et al. 1998). Lastly, one study (Taylor et al. 1999) was excluded because the reported data showed risk per year of pesticide exposure, which was not comparable with reported risk ratios of other studies. Among the remaining 48 publications, there were two studies for which the relevant results were reported in two separate publications each (Firestone et al. 2005, 2010; Semchuk et al. 1992, 1993). Thus, results of a total of 46 studies were used in the meta-analysis.

An overview of the study characteristics of the included studies can be found in Table 1. There were 39 case–control studies, 4 cohort studies, and 3 cross-sectional studies;

Table 1. Overview of the studies included in the meta-analyses.

Study	Study design	Location	Cases	Controls	Exposure assessment	Adjustments	Remarks
Ho et al. 1989	CC_o	Hong Kong	35 PD patients Age range, 65–87 years	105 age/sex matched	SR-E/N Occ/Non-Occ P	—	—
Koller et al. 1990	CC_h	USA	150 PD patients Age range, 39–87 years Mean age, 66 years	150 age/sex matched	SR-E/N Occ only P	—	OR calculated from reported numbers
Golbe et al. 1990	CC_o	USA	106 PD patients No age information	106 spouses	SR-R Occ/Non-Occ P	—	OR calculated from reported numbers
Zayed et al. 1990	CC_p	Canada	42 PD patients No age information	84 age/sex matched	SR-R Occ/Non-Occ P	Age, sex	—
Wong et al. 1991	CC_h	USA	38 PD patients Mean age, 70 years	38 age/sex matched	SR-E/N Occ/Non-Occ P	—	OR calculated from reported numbers
Stern et al. 1991	CC_o	USA	80 PD patients, diagnosed after 60 years of age No age information	80 age/sex/race/ participating center matched	SR-E/N Non-Occ only H, I	—	—
Jiménez-Jiménez et al. 1992	CC_h	Spain	128 PD patients Mean age, 66.8 years	256 age/sex matched	SR-R Occ/Non-Occ P	—	OR calculated from reported numbers
Semchuk et al. 1992, 1993	CC_p	Canada	130 PD patients Age range, 36–97 years Mean age, 68.5 years Participation, 88%	260 age/sex matched Participation, 76%	SR-E/N Occ only P, H, I, F	—	Herbicides OR adjusted for PD family history and head trauma
Hubble et al. 1993	CC_o	USA	63 PD patients Mean age: urban patients, 69.3 years; rural patients, 69.0 years	75 with similar mean age	SR-R Occ/Non-Occ P	Age < 65 years; male; lifestyle, ethnicity; family history; fresh produce consumption; history of head trauma, depression or CNS infection	—
Butterfield et al. 1993	CC_o	USA	63 PD patients, diagnosed before 51 years of age Age range, 35–72 years Mean age, 49 years Participation, 69%	68 age/sex/diagnosis year frequency matched Participation, 41%	SR-R Occ/Non-Occ H, I, F	Age, sex, race, age at diagnosis, education, family history	95% CIs calculated from ORs and *p*-values F-OR is not adjusted
Morano et al. 1994	CC_h	Spain	74 PD patients Mean age, 68.4 years	148 age/sex matched	SR-R Occ/Non-Occ P	—	OR calculated from reported numbers
Hertzman et al. 1994	CC_p	Canada	142 PD patients Mean age, 70.4 years	124 controls 45–80 years of age Participation, 61%	SR-E/N Occ only P, H, I, F	—	Reported results were pooled for men and women A second control group consisting of hospital controls was not used in this meta-analysis

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40 publications reported on pesticides, 15 on herbicides, 15 on insecticides, and 9 on fungicides. Three studies included all parkinsonism (Duzcan et al. 2003; Engel et al. 2001a; Tanner et al. 2009); the rest studied idiopathic PD. Four studies showed only results in men (Engel et al. 2001a; Fall et al. 1999; Petersen et al. 2008; Petrovitch et al. 2002). One study included only cases with a disease diagnosis before 51 years of age (Butterfield et al. 1993), which is much lower than the average age of disease onset in all other studies (generally ~ 60 years of age). Information about participation rates was provided for only 13 of the 39 case–control studies. Studies that reported

Table 1. continued

Study	Study design	Location	Cases	Controls	Exposure assessment	Adjustments	Remarks
Chaturvedi et al. 1995	CS	Canada	87 PD patients No age information	2,070 controls from cross-sectional study among elderly	SR-R Non-Occ only P	—	—
Seidler et al. 1996	CC_p	Germany	379 PD patients < 66 years of age Mean age, 56.2 years Participation, 71%	379 age/sex matched	SR-E/N Occ/Non-Occ H, I	Smoking, education	The reported results for exposure categories were pooled A second control group consisting of neighborhood was not used in this meta-analysis
Liou et al. 1997	CC_h	Taiwan	120 PD patients Age range, 37–91 years Mean age, 63.1 years	240 age/sex matched	SR-R Occ/Non-Occ P	—	—
De Palma et al. 1998	CC_h	Italy	100 PD patients Mean age, 66.6 years	200 controls, similar in age and sex	JT Occ/Non-Occ P	—	Substantial leisure activities were also classified for exposure
Chan et al. 1998	CC_h	Hong Kong	215 PD patients Age < 60 years, 13.5% Age 60–69 years, 33.5% Age 70–79 years, 33.5% Age ≥ 80 years, 19.5%	313 age/sex/hospital matched	SR-E/N Occ only P	Smoking, family history, rural living, well-water drinking, farming, consumption of tea, fruit vegetables and vitamins/ liver oil supplements	Substantial difference between OR from unadjusted and adjusted analysis. Unadjusted OR = 1.80 (95% CI: 0.90, 3.58)
McCann et al. 1998	CC_o	Australia	224 PD patients Mean age, 70.3 years	310 age/sex/ ethnicity/residential area/site of collection matched	SR-R Occ only P	—	—
Gorell et al. 1998	CC_p	USA	144 PD patients 50 years or older Age 50–59 years, 9.0% Age 60–69 years, 30.6% Age 70–79 years, 46.5% Age ≥ 80 years, 13.9% Participation, 81%	464 ages/sex/race frequency matched Participation, 65%	SR-E/N Occ only, and Non-Occ only H, I, F	Age, sex, race, smoking	—
Werneck and Alvarenga 1999	CC_h	Brasil	92 PD patients Age range, 55–78 years Mean age, 70.6 years	110 age/sex matched	SR-R Occ/Non-Occ P	—	—
Fall et al. 1999	CC_p	Sweden	113 PD patients Age range, 40–75 years Mean age, 63.9 years Participation, 90%	263 from same age category Participation, 82%	SR-E/N Occ only P, I	Smoking, alcohol, coffee, and fried/ broiled meat consumption, carpenters, cabinetmakers	Only results for men are shown I-OR is not adjusted
Kuopio et al. 1999	CC_p	Finland	123 PD patients Mean age, 69.3 years	246 age/sex/ municipality matched Participation, 68%	SR-E/N Occ only H	—	The reported results for "pesticides" do not contain herbicides and are not included in this review
Preux et al. 2000	CC_h	France	140 PD patients Mean age, 71.1 years	280 age/sex matched	SR-E/N Occ/Non-Occ P	—	OR calculated from reported numbers
Herishanu et al. 2001	CC_h	Israel	93 PD patients No age information	93 age/sex matched	SR-E/N Occ/Non-Occ P	Smoking, birth country, peptic disease, work in construction or in mechanical factory	—
Engel et al. 2001a	CS	USA	65 parkinsonism patients No age information	310 of original 1,300 men who previously participated in a cohort study	SR-E/N Occ only P, H, I, F	Age, smoking	The study was among men only
Behari et al. 2001	CC_h	India	377 PD patients Age range, 24–86 years Mean age, 56.8 years Participation, 100%	377 age matched Participation, 100%	SR-E/N Occ/Non-Occ H, I	—	ORs calculated from reported numbers
Zorzon et al. 2002	CC_h	Italy	136 PD patients Mean age, 70.0 years	272 age/sex matched	SR-E/N Occ/Non-Occ P	Smoking	
Petrovitch et al. 2002	Co	Hawaii	99 PD patients after 30 years of follow-up Median age at diagnosis, 73.7 years Range, 54–89 years	Baseline, 7,986 Japanese men in Hawaii	SR-R Occ/Non-Occ P	—	RR calculated from reported incidence numbers
Duzcan et al. 2003	CC_p	Turkey	36 parkinsonism patients, > 50 years of age Age 50–59 years, 11.1% Age 60–69 years, 30.6% Age 70–79 years, 47.2% Age ≥ 80 years, 11.1%	108 age/sex matched	SR-R Occ/Non-Occ P	—	—

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participation rates had rates between 69% and 100% for cases and between 41% and 100% for controls.

Figure 1 shows PD relative risk estimates for any pesticide exposure based on studies of occupational and/or nonoccupational exposures, and studies of occupational exposures only. The summary risk ratios (sRRs) between these two groups were very similar, with sRRs of 1.69 (95% CI: 1.38, 2.06) and 1.52 (95% CI: 1.23, 1.89), respectively, and an overall sRR for all studies combined of 1.62 (95% CI: 1.40, 1.88). The I^2 for all studies combined was 63.7%. Only three studies estimated effects of nonoccupational exposure only (Chaturvedi et al. 1995; Elbaz et al. 2009; Firestone et al. 2005), with an sRR of 1.18 (95% CI: 0.86, 1.63).

Meta-analyses by herbicide, insecticide, and fungicide exposure are shown in Figure 2. In line with the results for any pesticide exposure, we did not observe noticeable differences between studies of occupational exposures only and studies of nonocccupational and

Table 1. continued

Study	Study design	Location	Cases	Controls	Exposure assessment	Adjustments	Remarks
Baldereschi et al. 2003	CS	Italy	113 PD patients Mean age , 78.1 years	Study among 4,496 randomly selected elderly	SR-E/N Occ only P	Age, sex, education, smoking	Having a pesticide use license was used as a proxy for pesticide use
Baldi et al. 2003a	CC_p	France	84 PD patients, > 69 years of age Mean age, 75.6 years	252 age/sex matched	JT Occ only P	Age, sex, smoking, education	—
Baldi et al. 2003b	Co	France	24 PD patients after 5-year follow-up No age information	Baseline, 1,507 persons who were ≥ 65 years of age in specific area	JT Occ only P	Smoking, education	Reported results for men and women were pooled
Nuti et al. 2004	CC_p	Italy	190 PD patients Mean age, 63.9 years	190 age/sex/ sociocultural factors matched	SR-E/N Occ/Non-Occ P	—	OR calculated from reported numbers
Frigerio et al. 2006	CC_p	USA	149 PD patients Age range, 41–97 years Mean age, 70.0 years Participation, 76%	129 age/sex matched Participation, 66%	SR-E/N Occ/Non-Occ P, H, I	Age, sex	Also results occupational only (farming)
Ascherio et al. 2006	Co	USA	413 PD patients after 9-year follow-up Mean onset age, 70 years	Baseline: 184,190 persons	SR-R Occ/Non-Occ P	Age, sex, smoking, coffee, NSAIDs, education, physical activity	—
Kamel et al. 2007	Co	USA	78 PD patients after 5-year follow-up Age ≤ 50 years, 9% Age 51–60 years, 40% Age 61–70 years, 41% Age > 70 years, 10%	Baseline: 84,738 persons (applicants for pesticide use certification and their spouses)	SR-E/N Occ/Non-Occ P	Age, state, applicator or spouse	—
Dick et al. 2007	CC_o	Scotland, Sweden, Romania, Italy, Malta	767 PD patients Mean age, 69.8 years Participation, 77%	1,989 age/sex/country frequency matched Participation, 59%	SR-E/N (+ JT) Occ/Non-Occ P	Age, sex, country, smoking, family history, ever knocked unconscious	—
Fong et al. 2007	CC_h	Taiwan	153 PD patients Mean age, 71.7 years	155 age/sex/ birthplace matched	SR-R Occ only P	Age, sex, smoking	—
Brighina et al. 2008	CC_o	USA	833 PD patients, Age range, 32–91 years Median age, 67.7 years	361 age/sex/region matched and 472 siblings	SR-R Occ/Non-Occ P, H, I, F	Age, sex	—
Hancock et al. 2008	CC_o	USA	319 PD patients Age range, 29–94 years Mean age, 65.6 years	296 relatives and spouses	SR-E/N Occ/Non-Occ P, H, I	Age, sex, smoking, caffeine consumption	—
Petersen et al. 2008	CC_p	Faroe islands	79 PD patients Mean age, 74.4 years	154 age/sex matched	SR-E/N Occ only P	Smoking	Only OR in men is shown because no exposed women in study
Elbaz et al. 2009	CC_p	France	224 PD patients < 76 years of age Median age, 69.0 years Participation, 83%	557 age/sex/region matched Participation, 75%	SR-E/N Occ only, and Non-Occ only P, H, I, F	Smoking, Mini Mental State Examination score	Reported I-OR, H-OR, and F-OR for men and women were pooled The OR for Non-Occ only is unadjusted
Tanner et al. 2009	CC_o	USA	519 parkinsonism patients Age range, 30–88 years Median age, 65 years	511 age/sex/ location frequency matched	SR-E/N Occ only P	Age, sex, ethnicity, smoking, alcohol, caffeine, head injury	—
Vlajinac et al. 2010	CC_h	Serbia	110 PD patients Mean age, 60.8 years Participation, 100%	220 age/sex/urban or rural living matched Participation, 100%	SR-E/N Occ/Non-Occ P, H, I, F	I-OR is adjusted for gardening, rural living, well and spring water drinking, dyes or naphtha exposure, service-sector work	OR, H-OR, and F-OR calculated from reported numbers
Firestone et al. 2005, 2010	CC_h	USA	404 PD patients Age range, 29–88 years Median age, 69 years Participation, 70%	526 age/sex frequency matched Participation, 60%	SR-E/N Occ only, and Non-Occ only P, H, I, F	Age, ethnicity, smoking	Reported results for all pesticides were pooled for men and women Only results for men are shown for the subgroups for Occ only
Manthripragada et al. 2010	CC_p	USA	351 PD patients Age ≤ 60 years, 22% Age > 60 years, 78%	363 controls from same region	SR-E/N (+ JT) Occ only P	Age, sex, ethnicity, smoking, education, county	—

Abbreviations: CC_h, case–control study with hospital controls; CC_o, case–control study with controls from other sources or a combination of sources; CC_p, case–control study with population controls; CNS, central nervous system; Co, cohort study; CS, cross-sectional study; F, fungicides; H, herbicides; I, insecticides; JT, job titles; Non-Occ only, only nonoccupational exposure included in the exposed group; NSAIDs, nonsteroidal anti-inflammatory drugs; Occ only, only occupational exposure included in the exposed group; Occ/Non-Occ, nonoccupational exposure included in the exposed group; P, pesticides; SR-E/N, self-report ever/never; SR-R, self-report regular.

occupational exposures combined. The sRR for exposure to fungicides did not indicate an association with PD (overall sRR = 0.99; 95% CI: 0.71, 1.40; Figure 2C), in contrast with positive sRRs for exposure to herbicides (overall sRR = 1.40; 95% CI: 1.08, 1.81; Figure 2A) and insecticides (overall sRR = 1.50; 95% CI: 1.07, 2.11; Figure 2B).

Funnel plots of effect estimates for exposure to pesticides and pesticide subcategories were suggestive of small study effects, with a tendency for smaller studies to report higher relative risks compared with larger studies (Figure 3), with Egger's test p-values of 0.057, 0.338, 0.208, and 0.680 for pesticide, herbicide, insecticide, and fungicide effect estimates, respectively.

Figure 4 presents subgroup sRR estimates for those factors *a priori* hypothesized to be related to the observed heterogeneity in study results. The only study characteristic that was suggestive of contributing to heterogeneity was the exposure assessment method, with the lowest summary estimates observed for self-reported exposures (n = 36) and highest sRR for studies with exposures estimated based on reported job titles (n = 3). However, these differences were not statistically significant (p = 0.30). There was no evidence for a difference in summary estimates by adjustment of results for potential confounders, type of control population source, geographical area, or by study design. We also investigated whether adjustment for smoking had an effect on the summary risk estimate. Almost identical results were found for studies that did or did not correct for smoking (data not shown). Similar analyses for the subcategories herbicides and insecticides rendered similar results as for all pesticides (data not shown).

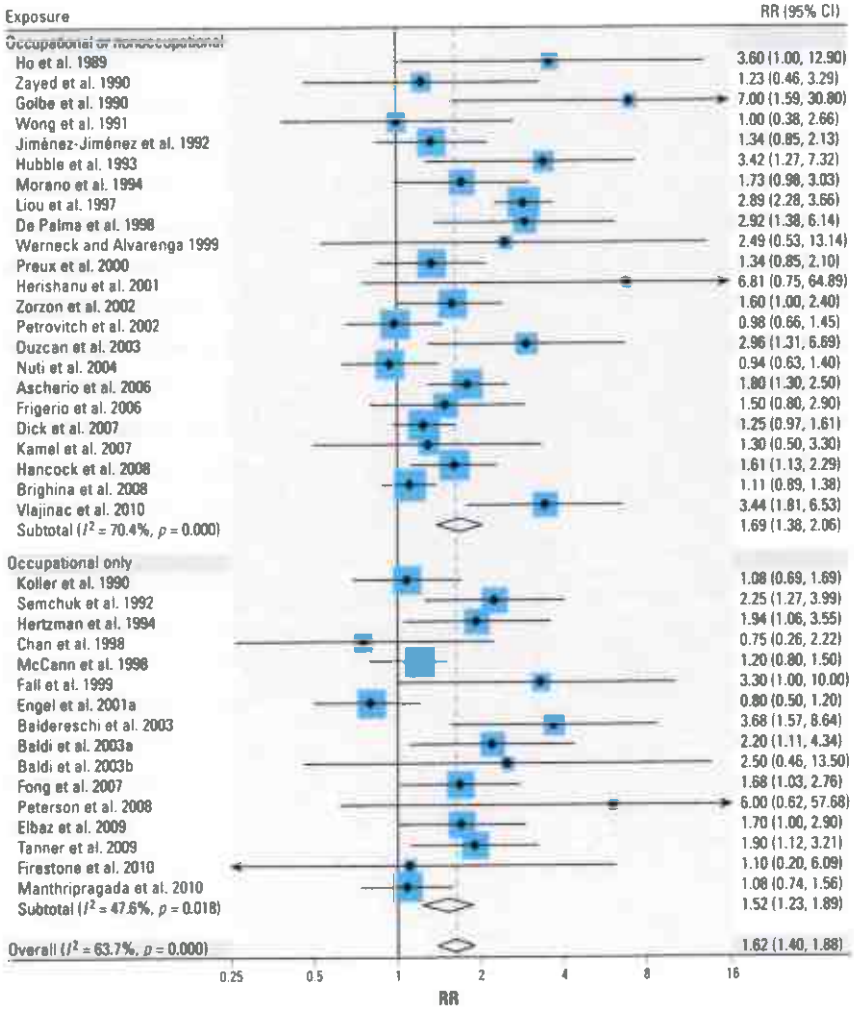

Figure 1. Forest plot for study-specific RRs and sRRs (95% CIs) of PD associated with the use of pesticides. The studies are ordered by publication year and stratified by studies that did or did not include nonoccupational exposure in the exposed group. Studies were pooled with the random effects method. The size of the squares reflects the statistical weight of the study in the meta-analyses.

Discussion

Our systematic review indicated that PD is related to pesticide exposure with an sRR of 1.62 (95% CI: 1.40, 1.88). However, there was substantial heterogeneity among individual study estimates (I^2 = 63.7%). Summary estimates also indicated positive associations of PD with herbicides and insecticides, but not with fungicides. We systematically investigated several factors that could explain heterogeneity in study results, but none appeared to be related to the observed heterogeneity, with the possible exception of the method of exposure assessment. Studies that based their exposure assessment on job titles reported somewhat higher risk estimates than studies that used self-reported exposures, but the difference did not reach statistical significance, in part because of low numbers of studies relying on job title and expert judgment.

Including persons who were nonoccupationally exposed to pesticides together with those occupationally exposed resulted in a very similar sRR. Given that occupational pesticide applications are in general more frequent and on larger areas than are nonoccupational exposures, one would have anticipated higher RRs for studies focusing only on occupational exposures. On the other hand, use of protective equipment during nonoccupational applications may be less. The fact that summary results were similar for both types of studies could indicate that nonoccupational and occupational pesticide exposures carry similar risks, or that most of the exposures in the combined studies were occupational. In the three studies that exclusively reported on nonoccupational pesticide exposures, only a small increase in relative risk was observed (sRR = 1.18; 95% CI: 0.86, 1.63), suggesting that risks associated with nonoccupational pesticide exposures are lower than from occupational exposures. Nevertheless, nonoccupational pesticide exposure cannot be ruled out as a risk factor for PD based on these analyses.

Studies used different methods for exposure assessment and assignment. Most studies (36 of 39) were based on self-reported exposure to pesticides, defined as ever versus never use or as regular versus nonregular use. No difference in sRR was seen between these two definitions of self-reported exposure, although it could have been expected that using a more stringent definition of exposure would have resulted in stronger associations. Studies that used reported job titles and expert judgment, and/or that used a job-exposure matrix to estimate exposures, resulted in a higher sRR compared with studies using self-reported pesticide exposures. This difference cannot be explained by recall bias, because in that case, higher risk ratios would have been expected for studies relying on self-reported exposures. A more likely explanation is that subjects are

not able to reliably report exposures to pesticides, resulting in nondifferential exposure misclassification and bias toward the null (Daniels et al. 2001; Engel et al. 2001b). The fact that some heterogeneity is observed in study results by exposure assessment method indicates that this may be an important factor that should be taken into consideration when designing or interpreting studies.

A broad range of different pesticides exist with different chemical compositions and working mechanisms. In line with the conclusions of Brown et al. (2006), we found that both herbicides and insecticides, but not fungicides, were associated with PD. However, it is difficult to disentangle the effect of herbicides and insecticides given that the use of these two pesticide groups is often highly correlated. This is illustrated by the fact that we observed a correlation coefficient of 0.79 between the study-specific RRs of herbicides and insecticides. Few studies have focused on specific pesticides precluding any meaningful meta-analyses (Brighina et al. 2008; Elbaz et al. 2009; Engel et al. 2001a; Firestone et al. 2010; Hancock et al. 2008; Hertzman et al. 1994; Kamel et al. 2007; Liou et al. 1997; Seidler et al. 1996; Semchuk et al. 1992; Tanner et al. 2009; Vlajinac et al. 2010). However, it is interesting to note that the subgroup of organochlorines was significantly associated with PD in three studies (Elbaz et al. 2009; Hancock et al. 2008; Seidler et al. 1996). This is also in line with studies on biomarkers in serum (Richardson et al. 2009; Weisskopf et al. 2010) and in the brains of deceased patients (Corrigan et al. 2000; Fleming et al. 1994). Organochlorines are mainly insecticides, including DDT (dichlorodiphenyltrichloroethane), dieldrin, and heptachlor.

Funnel plots gave some indication for a small-study effect, such that larger effect estimates appeared to be associated with smaller studies, which suggests that the sRR might be slightly overestimated. In addition, the studies included were generally small, resulting in imprecise effect estimates that could have contributed to the substantial heterogeneity in study results. Meta-regression analyses provided no evidence for a difference in sRRs based on study design, geographical area, adjustment for potential confounders, or type of control population. As such, factors explaining the heterogeneity observed remain largely elusive. We were not able to investigate the effect of differences in criteria used for the diagnosis of PD because there was substantial variation in the exact inclusion criteria among the studies that reported on the criteria used. However, in most of the studies, the diagnosis was made by a physician and included the presence of two or three of the cardinal symptoms of PD, often with some

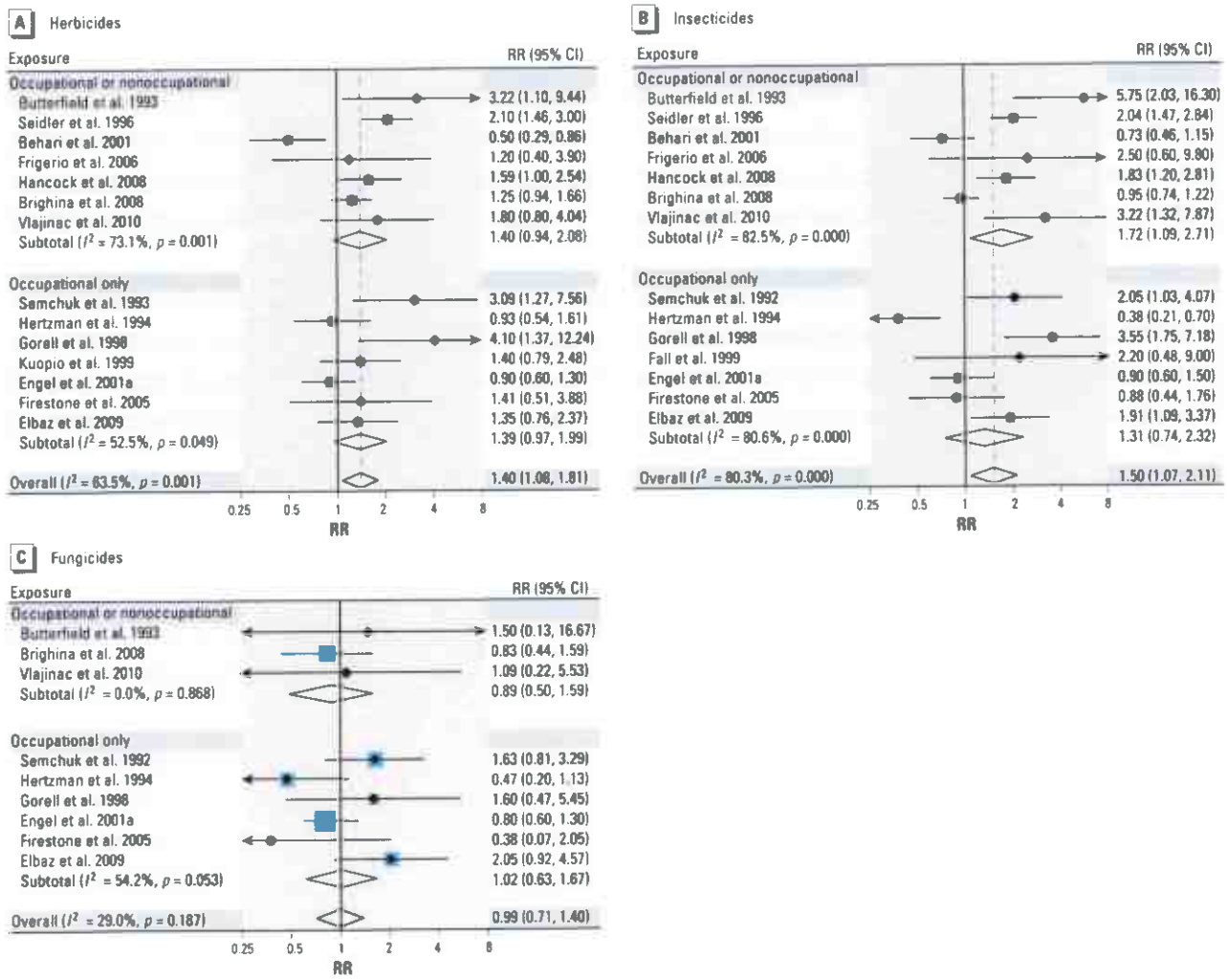

Figure 2. Forest plots for study-specific RRs and sRRs (95% CIs) of PD associated with the use of herbicides (*A*), insecticides (*B*), and fungicides (*C*). The studies are ordered by publication year and stratified by studies that did or did not include nonoccupational exposure in the exposed group. Studies were pooled with the random effects method. The size of the squares reflects the statistical weight of the study in the meta-analyses.

additional inclusion and exclusion criteria. Variations in participation rates could also contribute to study heterogeneity. The ability to investigate this factor was limited because only 13 of the case–control studies reported participation rates. The same is true for differences across sex. Only 8 studies showed separate results for men and women, but the results were not conclusive: RRs were higher for men than for women in 3 studies (Baldi et al. 2003b; Frigerio et al. 2006; Hertzman et al. 1994), higher for women than men in 3 other studies (Chan et al. 1998; Elbaz et al. 2009; Firestone et al. 2010), and comparable between men and women in the remaining 2 studies (Ascherio et al. 2006; Brighina et al. 2008). Heterogeneity in the results could also arise from both quantitative and qualitative differences in types of agriculture in the study areas. Although we compared large regions (i.e., North America, Europe, and other), this analysis would not have captured regional differences in the types of agriculture and pesticides used. Analyses by time periods might provide some clues because pesticide use has changed over the decades, but data were insufficient to perform a meaningful analysis of changes over time.

Conclusion

Our overall summary risk estimates strongly suggest that exposure to pesticides, and to herbicides and/or insecticides in particular, increases the risk of developing PD. Heterogeneity among study-specific RRs could not easily be explained by methodological differences, except for a suggestive effect of exposure assessment characteristics. Future studies should therefore focus on using more objective semiquantitative methods for exposure assessment such as job- or crop-exposure matrices, rather than relying solely on self-report. Although classes of pesticides have been linked to PD, it remains important to identify the specific chemicals responsible for this association. Therefore, in new, preferably prospective studies, attention should be given to collecting detailed information on specific pesticide use.

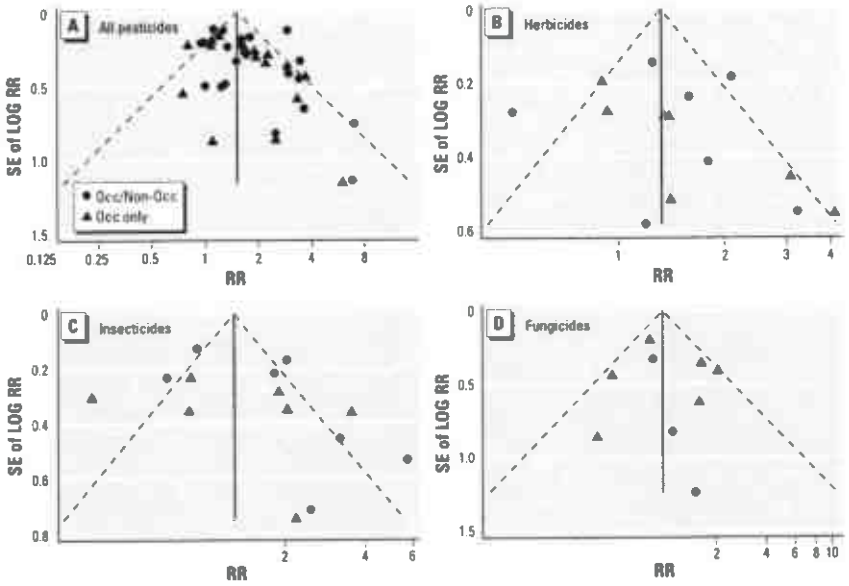

Figure 3. Funnel plots of studies included in the meta-analysis for the risk of PD associated with the use of pesticides (*A*), herbicides (*B*), insecticides (*C*), and fungicides (*D*). Circles represent studies that included nonoccupational exposure in the exposed group, and triangles represent studies that were based on occupational exposure only. Egger's test *p*-values were 0.057, 0.338, 0.208, and 0.680 for pesticide, herbicide, insecticide, and fungicide effect estimates, respectively.

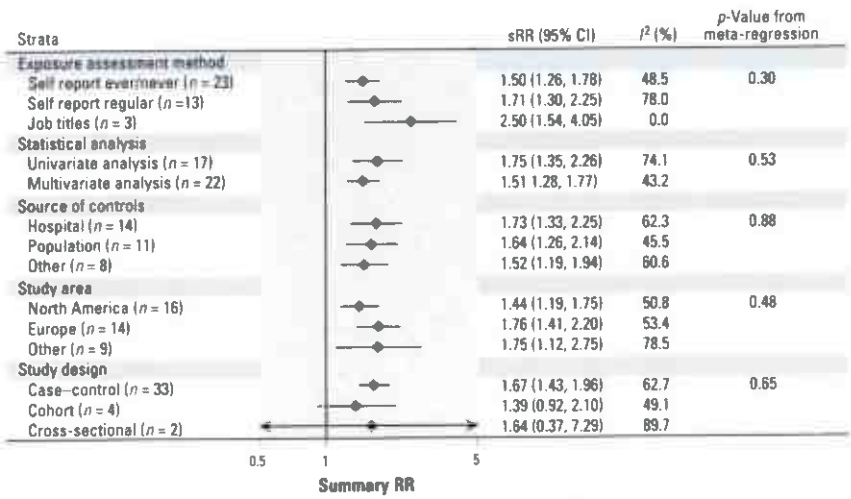

Figure 4. sRRs (95% CIs) for strata of exposure assessment method, statistical analysis, source of controls, study area, and study design. The *p*-value from meta-regression represents the *p*-value of the *F*-test in case of more than two categories, whereas it represents the *p*-value for the *t*-test in the case of the two statistical analysis strata.

References

Ascherio A, Chen H, Weisskopf MG, O'Reilly E, McCullough ML, Calle EE, et al. 2006. Pesticide exposure and risk for Parkinson's disease. Ann Neurol 60(2):197–203.

Baldereschi M, Di Carlo A, Vanni P, Ghetti A, Carbonin P, Amaducci L, et al. 2003. Lifestyle-related risk factors for Parkinson's disease: a population-based study. Acta Neurol Scand 108(4):239–244.

Baldi I, Cantagrel A, Lebailly P, Tison F, Dubroca B, Chrysostome V, et al. 2003a. Association between Parkinson's disease and exposure to pesticides in southwestern France. Neuroepidemiology 22(5):305–310.

Baldi I, Lebailly P, Mohammed-Brahim B, Letenneur L, Dartigues JF, Brochard P. 2003b. Neurodegenerative diseases and exposure to pesticides in the elderly. Am J Epidemiol 157(5):409–414.

Behari M, Srivastava AK, Das RR, Pandey RM. 2001. Risk factors of Parkinson's disease in Indian patients. J Neurol Sci 190(1–2):49–55.

Bekris LM, Mata IF, Zabetian CP. 2010. The genetics of Parkinson disease. J Geriatr Psychiatry Neurol 23(4):228–242.; doi:10.1177/0891988710383572 [Online 11 October 2010].

Brighina L, Frigerio R, Schneider NK, Lesnick TG, de Andrade M, Cunningham JM, et al. 2008. α-synuclein, pesticides, and Parkinson disease: a case-control study. Neurology 70(16 pt 2):1461–1469.

Brown TP, Rumsby PC, Capleton AC, Rushton L, Levy LS. 2006. Pesticides and Parkinson's disease—is there a link? Environ Health Perspect 114:156–164.

Butterfield PG, Valanis BG, Spencer PS, Lindeman CA, Nutt JG. 1993. Environmental antecedents of young-onset Parkinson's disease. Neurology 43(6):1150–1158.

Chan DK, Woo J, Ho SC, Pang CP, Law LK, Ng PW, et al. 1998. Genetic and environmental risk factors for Parkinson's disease in a Chinese population. J Neurol Neurosurg Psychiatry 65(5):781–784.

Chaturvedi S, Ostbye T, Stoessl AJ, Merskey H, Hachinski V. 1995. Environmental exposures in elderly Canadians with Parkinson's disease. Can J Neurol Sci 22(3):232–234.

Corrigan FM, Wienburg CL, Shore RF, Daniel SE, Mann D. 2000. Organochlorine insecticides in substantia nigra in Parkinson's disease. J Toxicol Environ Health A 59(4):229–234.

Daniels JL, Olshan AF, Teschke K, Hertz-Picciotto I, Savitz DA, Blatt J. 2001. Comparison of assessment methods for pesticide exposure in a case-control interview study. Am J Epidemiol 153(12):1227–1232.

De Palma G, Mozzoni P, Mutti A, Calzetti S, Negrotti A. 1998. Case-control study of interactions between genetic and environmental factors in Parkinson's disease. Lancet 352(9145):1986–1987.

DerSimonian R, Laird N. 1986. Meta-analysis in clinical trials. Control Clin Trials 7(3):177–188.

Dick FD, De Palma G, Ahmadi A, Scott NW, Prescott GJ, Bennett J, et al. 2007. Environmental risk factors for Parkinson's disease and parkinsonism: the Geoparkinson study. Occup Environ Med 64(10):666–672.

Duzcan F, Zencir M, Ozdemir F, Catin GO, Bagci H, Heutink P, et al. 2003. Familial influence on parkinsonism in a rural area of Turkey (kizilcaboluk-denizli): a community-based case-control study. Mov Disord 18(7):799–804.

Egger M, Davey Smith G, Schneider M, Minder C. 1997. Bias in meta-analysis detected by a simple, graphical test. BMJ 315(7109):629–634.

Elbaz A, Clavel J, Rathouz PJ, Moisan F, Galanaud JP, Delemotte B, et al. 2009. Professional exposure to pesticides and Parkinson disease. Ann Neurol 66(4):494–504.

Engel LS, Checkoway H, Keifer MC, Seixas NS, Longstreth WT Jr, Scott KC, et al. 2001a. Parkinsonism and occupational exposure to pesticides. Occup Environ Med 58(9):582–589.

Engel LS, Seixas NS, Keifer MC, Longstreth WT Jr, Checkoway H. 2001b. Validity study of self-reported pesticide exposure among orchardists. J Expo Anal Environ Epidemiol 11(5):359–368; doi:10.1038/sj.jea.7500176.

Fall PA, Fredrikson M, Axelson O, Granerus AK. 1999. Nutritional and occupational factors influencing the risk of Parkinson's disease: a case-control study in southeastern Sweden. Mov Disord 14(1):28–37.

Firestone JA, Lundin JI, Powers KM, Smith-Weller T, Franklin GM, Swanson PD, et al. 2010. Occupational factors and risk of Parkinson's disease: a population-based case-control study. Am J Ind Med 53(3):217–223; doi:10.1002/ajim.20788 [Online 18 December 2009].

Firestone JA, Smith-Weller T, Franklin G, Swanson P, Longstreth WT Jr, Checkoway H. 2005. Pesticides and risk of Parkinson disease: a population-based case-control study. Arch Neurol 62(1):91–95.

Fleming L, Mann JB, Bean J, Briggle T, Sanchez-Ramos JR. 1994. Parkinson's disease and brain levels of organochlorine pesticides. Ann Neurol 36(1):100–103; doi:10.1002/ana.410360119 [Online 8 October 2004].

Fong CS, Cheng CW, Wu RM. 2005. Pesticides exposure and genetic polymorphism of paraoxonase in the susceptibility of Parkinson's disease. Acta Neurol Taiwan 14(2):55–60.

Fong CS, Wu RM, Shieh JC, Chao YT, Fu YP, Kuao CL, et al. 2007. Pesticide exposure on southwestern Taiwanese with *MnSOD* and *NQO1* polymorphisms is associated with increased risk of Parkinson's disease. Clin Chim Acta 378(1–2):136–141; doi:10.1016/j.cca.2006.11.006 [Online 17 November 2006].

Frigerio R, Sanft KR, Grossardt BR, Peterson BJ, Elbaz A, Bower JH, et al. 2006. Chemical exposures and Parkinson's disease: a population-based case-control study. Mov Disord 21(10):1688–1692.

Golbe LI, Farrell TM, Davis PH. 1990. Follow-up study of early-life protective and risk factors in Parkinson's disease. Mov Disord 5(1):66–70.

Gorell JM, Johnson CC, Rybicki BA, Peterson EL, Richardson RJ. 1998. The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. Neurology 50(5):1346–1350.

Hancock DB, Martin ER, Mayhew GM, Stajich JM, Jewett R, Stacy MA, et al. 2008. Pesticide exposure and risk of Parkinson's disease: a family-based case-control study. BMC Neurol 8:6; doi:10.1186/1471-2377-8-6 [Online 28 March 2008].

Herishanu YO, Medvedovski M, Goldsmith JR, Kordysh E. 2001. A case-control study of Parkinson's disease in urban population of southern Israel. Can J Neurol Sci 28(2):144–147.

Hertzman C, Wiens M, Snow B, Kelly S, Calne D. 1994. A case-control study of Parkinson's disease in a horticultural region of British Columbia. Mov Disord 9(1):69–75.

Higgins JP, Thompson SG, Deeks JJ, Altman DG. 2003. Measuring inconsistency in meta-analyses. BMJ 327(7414):557–560; doi:10.1136/bmj.327.7414.557 [Online 4 September 2003].

Ho SC, Woo J, Lee CM. 1989. Epidemiologic study of Parkinson's disease in Hong Kong. Neurology 39(10):1314–1318.

Hubble JP, Cao T, Hassanein RE, Neuberger JS, Koller WC. 1993. Risk factors for Parkinson's disease. Neurology 43(9):1693–1697.

Jiménez-Jiménez FJ, Mateo D, Gimenez-Roldan S. 1992. Exposure to well water and pesticides in Parkinson's disease: a case-control study in the Madrid area. Mov Disord 7(2):149–152.

Kamel F, Tanner C, Umbach D, Hoppin J, Alavanja M, Blair A, et al. 2007. Pesticide exposure and self-reported Parkinson's disease in the agricultural health study. Am J Epidemiol 165(4):364–374.

Koller W, Vetere-Overfield B, Gray C, Alexander C, Chin T, Dolezal J, et al. 1990. Environmental risk factors in Parkinson's disease. Neurology 40(8):1218–1221.

Kuopio AM, Marttila RJ, Helenius H, Rinne UK. 1999. Environmental risk factors in Parkinson's disease. Mov Disord 14(6):928–939.

Li AA, Mink PJ, McIntosh LJ, Teta MJ, Finley B. 2005. Evaluation of epidemiologic and animal data associating pesticides with Parkinson's disease. J Occup Environ Med 47(10):1059–1087.

Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, et al. 1997. Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. Neurology 48(6):1583–1588.

Manthripragada AD, Costello S, Cockburn MG, Bronstein JM, Ritz B. 2010. Paraoxonase 1, agricultural organophosphate exposure, and Parkinson disease. Epidemiology 21(1):87–94; doi:10.1097/EDE.0b013e3181c15ec6.

McCann SJ, LeCouteur DG, Green AC, Brayne C, Johnson AG, Chan D, et al. 1998. The epidemiology of Parkinson's disease in an Australian population. Neuroepidemiology 17(6):310–317.

Menegon A, Board PG, Blackburn AC, Mellick GD, Le Couteur DG. 1998. Parkinson's disease, pesticides, and glutathione transferase polymorphisms. Lancet 352(9137):1344–1346.

Morano A, Jiménez-Jiménez FJ, Molina JA, Antolín MA. 1994. Risk-factors for Parkinson's disease: case-control study in the province of Caceres, Spain. Acta Neurol Scand 89(3):164–170.

Nuti A, Ceravolo R, Dell'Agnello G, Gambaccini G, Ballini G, Kiferle L, et al. 2004. Environmental factors and Parkinson's disease: a case-control study in the Tuscany region of Italy. Parkinsonism Relat Disord 10(8):481–485.

Petersen MS, Halling J, Bech S, Wermuth L, Weihe P, Nielsen F, et al. 2008. Impact of dietary exposure to food contaminants on the risk of Parkinson's disease. Neurotoxicology 29(4):584–590; doi:10.1016/j.neuro.2008.03.001.

Petrovitch H, Ross GW, Abbott RD, Sanderson WT, Sharp DS, Tanner CM, et al. 2002. Plantation work and risk of Parkinson disease in a population-based longitudinal study. Arch Neurol 59(11):1787–1792.

Preux PM, Condet A, Anglade C, Druet-Cabanac M, Debrock C, Macharia W, et al. 2000. Parkinson's disease and environmental factors. Matched case-control study in the Limousin region, France. Neuroepidemiology 19(6):333–337.

Priyadarshi A, Khuder SA, Schaub EA, Shrivastava S. 2000. A meta-analysis of Parkinson's disease and exposure to pesticides. Neurotoxicology 21(4):435–440.

Richardson JR, Shalat SL, Buckley B, Winnik B, O'Suilleabhain P, Diaz-Arrastia R, et al. 2009. Elevated serum pesticide levels and risk of Parkinson disease. Arch Neurol 66(7):870–875; doi:10.1001/archneurol.2009.89.

Seidler A, Hellenbrand W, Robra BP, Vieregge P, Nischan P, Joerg J, et al. 1996. Possible environmental, occupational, and other etiologic factors for Parkinson's disease: a case-control study in Germany. Neurology 46(5):1275–1284.

Semchuk KM, Love EJ, Lee RG. 1992. Parkinson's disease and exposure to agricultural work and pesticide chemicals. Neurology 42(7):1328–1335.

Semchuk KM, Love EJ, Lee RG. 1993. Parkinson's disease: a test of the multifactorial etiologic hypothesis. Neurology 43(6):1173–1180.

Shulman JM, De Jager PL, Feany MB. 2011. Parkinson's disease: genetics and pathogenesis. Annu Rev Pathol 6:193–222; doi:10.1146/annurev-pathol-011110–130242.

Smargiassi A, Mutti A, De Rosa A, De Palma G, Negrotti A, Calzetti S. 1998. A case-control study of occupational and environmental risk factors for Parkinson's disease in the Emilia-Romagna region of Italy. Neurotoxicology 19(4–5):709–712.

Stern M, Dulaney E, Gruber SB, Golbe L, Bergen M, Hurtig H, et al. 1991. The epidemiology of Parkinson's disease. A case-control study of young-onset and old-onset patients. Arch Neurol 48(9):903–907.

Tanner CM, Ross GW, Jewell SA, Hauser RA, Jankovic J, Factor SA, et al. 2009. Occupation and risk of parkinsonism: a multicenter case-control study. Arch Neurol 66(9):1106–1113.

Taylor CA, Saint-Hilaire MH, Cupples LA, Thomas CA, Burchard AE, Feldman RG, et al. 1999. Environmental, medical, and family history risk factors for Parkinson's disease: a New England-based case control study. Am J Med Genet 88(6):742–749.

Vlaanderen J, Lan Q, Kromhout H, Rothman N, Vermeulen R. 2011. Occupational benzene exposure and the risk of lymphoma subtypes: a meta-analysis of cohort studies incorporating three study quality dimensions. Environ Health Perspect 119:159–167.

Vlajinac HD, Sipetic SB, Maksimovic JM, Marinkovic JM, Dzoljic ED, Ratkov IS, et al. 2010. Environmental factors and Parkinson's disease: a case-control study in Belgrade, Serbia. Int J Neurosci 120(5):361–367; doi:10.3109/00207451003668274.

Weisskopf MG, Knekt P, O'Reilly EJ, Lyytinen J, Reunanen A, Laden F, et al. 2010. Persistent organochlorine pesticides in serum and risk of Parkinson disease. Neurology 74(13):1055–1061; doi:10.1212/WNL.0b013e3181d76a93 [Online 30 March 2010].

Werneck AL, Alvarenga H. 1999. Genetics, drugs and environmental factors in Parkinson's disease. A case-control study. Arq Neuropsiquiatr 57(2B):347–355.

Wong GF, Gray CS, Hassanein RS, Koller WC. 1991. Environmental risk factors in siblings with Parkinson's disease. Arch Neurol 48(3):287–289.

Zayed J, Ducic S, Campanella G, Panisset JC, Andre P, Masson H, et al. 1990. Environmental factors in the etiology of Parkinson's disease [in French]. Can J Neurol Sci 17(3):286–291.

Zorzon M, Capus L, Pellegrino A, Cazzato G, Zivadinov R. 2002. Familial and environmental risk factors in Parkinson's disease: a case-control study in north-east Italy. Acta Neurol Scand 105(2):77–82.

PRODUCTIE 51

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College voor de toelating van
gewasbeschermingsmiddelen
en biociden

Via E-mail
To:
European Food Safety Authority, Executive Director, dr. B. Url

c.c.:
DG SANTE, Head Of Unit Pesticides and Biocides, Dr. Klaus Berend
European Chemicals Agency, Executive Director, Dr. Björn Hansen
RIVM, Head of Department for Food Safety, Dr. Anton Rietveld
Radboud University Nijmegen, Prof. B. Bloem

Datum: 9th of March 2021

Behandeld door
Ms. Dr. I.T.M. Becks-Vermeer

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202103090024

Subject: possible relation between the use of specific pesticides and the development of Parkinson's disease

Dear dr. Url,

We would like to express our concerns with regard to a possible relation between the use of specific pesticides and the development of Parkinson's disease. This issue has generated a lot of attention in the Netherlands in recent years, including the medical and research field, the media and the political arena. On behalf of the Dutch Ministry of Agriculture, Nature and Food Quality the RIVM is currently conducting an exploratory study on data requirements for neurotoxicity in relation to the development of neurodegenerative diseases.

In November 2020, professor Bas Bloem[1], a neurologist with special interest in this area, gave a presentation to the Ctgb board. He explained that it is difficult to establish an unambiguous causal relationship between active substances in our regulatory framework and the development of Parkinson's disease since there is a lag time of up to 10-15 years before the first symptoms of the disease become manifest. Despite these difficulties, he pointed out that more and more evidence is emerging substantiating a possible relationship between the use of pesticides and the development of Parkinson's disease. Specifically, there is growing experimental and epidemiological evidence supporting this possible relation between exposure to pesticides and the risk of Parkinson's disease.

[1] Radboud Univ Nijmegen, Med Ctr, Dept Human Genet, NL-6525 ED Nijmegen, Netherlands; https://www.linkedin.com/in/bas-bloem-45307777/

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gewasbeschermingsmiddelen
en biociden

A particular concern is the recent recognition that Parkinson's disease is the fastest growing neurological condition in the world, further fueling concerns that environmental toxins such as pesticides may be contributing to this growth.
On February 22 we had a brief discussion with EFSA [redacted] on this subject. He explained the current activities EFSA is involved in, specifically the EFSA Pilot Project on New Approach Methodologies (NAMs) for tebufenpyrad risk assessment, in collaboration with Anses. The overall outcome is intended to be used in the renewal process for tebufenpyrad.

However, there are many more currently approved active substances for which we do not know whether exposure could be associated with Parkinson's disease.
We therefore request EFSA to specifically screen the currently approved active substances for a possible association with Parkinson's disease (e.g. based on chemical structure and toxicological profile). Furthermore, it is well known that brain lesions affecting the substantia nigra can cause a number of movement disorders including Parkinson's disease. The next step could be to perform dedicated tests on active substances earmarked on the basis of the initial screening for their specific toxic effects on the substantia nigra. In our view, such experiments would have to be performed in ageing animals that are known to be more vulnerable to the toxic effects of pesticides. These experiments should also consider the possibility that combined exposure to multiple pesticides (that are often used in combination in agriculture) lead to greater neurotoxicity and a further enhanced risk of developing Parkinson's disease. Would this research fit into the EFSA Pilot Project on NAMs?

We hope that EFSA will be open to the suggestions above in order to gain more insight into a possible relationship of currently approved active substances in pesticides and the development of Parkinson's disease.

Sincerely yours,

The Board for the Authorisation of Plant Protection Products and Biocides,
for this organisation:

ir. J.T. de Leeuw
Chairman of the Board

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PRODUCTIE 52

efsa
European Food Safety Authority

EXECUTIVE DIRECTOR

Ref. BU/GdS/MT/AT/ss (2021) OC-2021-24570142

Ir. J.F. de Leeuw
Chairman of the Board
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Plant Protection Products
and Biocides – ctgb
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6717 LL Ede
The Netherlands

Subject/Re.: possible relation between the use of specific pesticides and the development of Parkinson's disease

Ref. IC-2021-24570142 – your email dated 09/03/2021

Dear Dr de Leeuw,

Thank you for approaching EFSA on this important topic.

The area of environmental neuroscience and the impact of chemical exposure on brain health are a growing field, which includes the assessment of plant protection products as a potential risk factor.

EFSA is engaged in this area, recognising the complexity of the different environmental contributors to the several neurodegeneration processes.

EFSA and its PPR Panel published two Scientific Opinions in 2015 as a follow up of a meta-analysis contracted by EFSA to the University of Ioannina, where a positive relationship was highlighted between exposure to pesticides and Parkinson's disease. In agreement with your position, the Panel concluded that a causality link cannot be established with the available information, and that a different approach for hazard identification and characterization should be undertaken.

One of the activities undertaken by the PPR Panel culminated in the development of adverse outcome pathways (AOP) for Parkinsonian disorders. The measurable key events (KEs) included in the AOP can be used to test and identify chemicals that might contribute to the disease via the identified pathway. An AOP approach is considered important in the study of mechanisms of toxicity to enable linking those mechanisms through epidemiological studies to neurological disorders. Therefore, data on biological processes occurring in the context of adverse outcomes is essential to show their relevance in the disease aetiology. This mechanistic understanding enables the development of tools for a predictive toxicology approach, which can support regulatory decisions.

This approach was applied and implemented in a work package of the EU-ToxRisk[1] project, and for the ongoing assessment of the active substance Tebufenpyrad; while the two activities have different regulatory problem formulations, they are both based on New Approach Methodologies (NAMs). Similar methodologies are used by EFSA for the developmental neurotoxicity (DNT) assessment of chemicals. In this case, an Integrated Approach for Testing and Assessment (IATA), informed by AOP, was used to answer different regulatory problem formulations (e.g. screening of many chemicals or

[1] https://www.eu-toxrisk.eu/

single substance hazard characterization), and NAMs were developed to measure selected KEs in DNT adverse outcome pathways. This project is in an advanced state, and can serve as a valid experience also for other environmental neuroscience endpoints. A key lesson learned from these parallel projects is that they require the interaction of specialists from different fields to be successful. This is therefore an opportunity for a multidisciplinary collaboration between regulators, academics, laboratory experts and stakeholders. The preliminary results of the DNT project are promising, and indicate that the approach could be used to support regulators in identifying substances of potential concern, and to change the current testing paradigm for the assessment of the potential link between neurodegenerative adverse outcomes and chronic low-level exposure of environmental contaminants, including pesticides.

We therefore believe that the acquired expertise can also be useful to tackle the problem of the Parkinsonian syndromes which, however, requires an even greater multidisciplinary approach, given that genetic predispositions and epidemiological data are essential for a better definition of the problem. In addition, a more elaborated plan is necessary to move to the screening of large chemical classes, to define a tiered approach for the identification of neurodegenerative hazards, and for the assessment of mixtures.

For this purpose, we believe it is necessary to take stock of the situation from a scientific and multidisciplinary point of view. Our aim is to identify the gaps in research, collect feedback on the availability of suitable test methods and test systems to be used in the screening programme, and estimate the policy implications for environmental neuroscience. We are therefore considering the organisation of a scientific event that would have the aim of exploring the current knowledge landscape and future opportunities in neurotoxicology, and would create the basis for a series of scientific projects with the collaboration of the Member States. The debate should also focus on the need to prioritize chemicals to be tested, and to develop higher throughput methods using molecular or cellular approaches. Testing pesticides through these tools would represent a first step towards understanding the complexity of the mechanisms that regulate neurological functions, and, subsequently, towards developing regulatory action.

We aim at defining a plan to be shared with you in the upcoming weeks, and remain available for further discussions. EFSA looks forward to working with CTGB and MSs on this topic.

Yours sincerely,

efsa
European Food Safety Authority

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