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14. ABSTRACT

Gene environment interactions (GxE) are key to better understanding PD. We are testing pesticides for their effects "in the dish", and in the context of mutations in the synuclein and GBA genes that are also being analyzed in Dr Ritz's epidemiologic cohort. Our experiments will enable us to answer whether key genetic risk factors create sensitivities in patients to particular toxicants and we will ascertain whether gene-toxicant interactions play out specifically at the level of the dopamine neuron. To date, we have generated a unique set of reagents from patients with PD caused by synuclein and GBA mutations and observed differential effects to PD-linked pesticides and toxicants with respect to survival, neurite outgrowth and calcium signaling. From the PEG cohort data and leveraging agricultural pesticide application records (CA-PUR database (discussed below), 1974-2018), we have established long-term exposure profiles for over 200 widely used agricultural pesticides for 1,870 PD patients and population-based controls. Using this data for analysis, we have generated a list of 33 pesticide toxicants that are both significantly associated with PD (FDR<0.01) and have exposure in both α-syn SNPs and GBA variant carriers. This epidemiologic analysis agnostically highlighted, out of pesticides widely used agriculturally in California over the study period, the most significantly associated with PD. In synergy, the lab-based teams (Rubin and Khurana) will in the next phase of research test these epidemiologically derived pesticides in in vitro cell lines in the context of mutations in the synuclein and GBA mechanisms. Validated hits from these screens will then be used in conjunction with SNCA and GBA genetic data to assess GxE interactions with pathway relevant SNPs in the PEG study.

15. SUBJECT TERMS
Parkinson's disease, neurodegeneration, induced pluripotent stem cells, gene-environment interactions (GxE), pesticides, dopaminergic neurons, high throughput screening, familial Parkinson's disease

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TABLE OF CONTENTS

1. INTRODUCTION:

Gene environment interactions (GxE) are key to better understanding PD. We are testing epidemiologically identified pesticides for their effects "in the dish" on dopaminergic neurons and in the context of mutations in the synuclein and GBA genes that are also being analyzed in Dr Ritz's epidemiologic cohort. Our experiments will enable us to answer whether key genetic risk factors create sensitivities in patients to particular toxicants and we will ascertain whether gene-toxin interactions play out specifically at the level of the dopamine neuron. We are constructing exposure profiles for patients harboring common SNPs in α-syn and GBA in the existing epidemiologic data. We are systematically identifying environmental risk factors and pathways that cause selective degeneration of human DA neurons using a screening approach. This project will provide an extensive phenotypic and functional description of the interaction between variably penetrant genetic forms of PD-causing genes, and environmental toxicants in well-controlled human DA neurons.

2. KEYWORDS:

Parkinson's disease, neurodegeneration, induced pluripotent stem cells, gene-environment interactions (GxE), pesticides, dopaminergic neurons, high throughput screening, familial Parkinson's disease.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine whether synuclein triplication and GBA mutations increase susceptibility of DA neurons to known environmental toxicants
Major Task 1: Modify cell lines with THtd reporter (85% complete, delayed by COVID-19 labwork hiatus)
Major Task 2: Establish baseline responses to toxicants in the SNCA triplication line and compare exposure phenotypes across other mutations (50% complete)
Major Task 3: Establish exposure profiles for α-syn and GBA SNPs from PEG database (subtask 1 and subtask 2, 100% completed)
Major Task 4: Test toxicant lists from PEG data in corresponding in vitro cell lines (not applicable to this reporting period)

Specific Aim 2: Systematically identify environmental risk factors and pathways that cause selective degeneration of human DA neurons.
Major Task 5: Assay development to optimize timing of Experiment 1+2 (toxin and custom library screens) (33% complete, delayed by COVID-19 labwork hiatus)
Major Task 6: Perform Screens on DA neurons (not applicable to this reporting period)
Major Task 7: Validated hits from screen used to identify pathway-relevant SNPs for evaluation in the PEG cohort (not applicable to this reporting period)

What was accomplished under these goals?

1) Major activities undertaken during the annual reporting period were primarily the acquisition, modification, and characterization of multiple patient iPSC lines used for insertion of the tyrosine hydroxylase::tdtomato (THtd) reporter construct, toxicant sensitivity primary assays, optimization of secondary assays, and screening assay development. Major activities with the PEG epidemiologic cohort included developing pesticide exposure profiles for all pesticides agriculturally used in Central California based on historical agricultural pesticide use reports and occupational and home pesticide use based on self-report, establishing exposure profiles SNCA and GBA variant carriers, and comparing exposure profiles between patients and controls to select toxicants to be used in future corresponding in vitro cell line experiments.
2) Specific objectives included:
    a. Modify cell lines with THtd reporter (Major Task 1).
    b. Perform validation and quality control of reporter lines produced (Major Task 1).
    c. Establish baseline responses to toxicants in GBA mutant lines (part of Major Task 2).
    d. Establish calcium imaging assay for characterization of toxicant responses
    e. Develop pesticide profiles and specific pesticide quantitative measures from GIS models at occupational and residential addresses, home and garden and occupational pesticide use reporting for all risk variant carriers and controls. Analyses comparing these profiles for PD risk have been carried out in the second half of the year. Separate exposure profiles by SNCA and GBA SNPs have been established (Major Task 3).
3) Significant results from the Rubin lab primarily relate to objective 2a/b/c. Upon acquiring the GBA mutant and corrected lines from the NINDS repository, we performed a genotyping PCR using published primers. Sequencing of the product to confirm the A to G mutation which results in the N370S mutation. Following the CRISPR-Cas9 mediated knock in of the THtdtomato reporter construct, we recovered multiple clones from both the GBA mutant iPSC line and the GBA corrected iPSC line that passed the necessary PCR quality controls. This includes detecting appropriately sized PCR products with primers designed to flank the 5' and 3' insertion sites, followed by Sanger sequencing of these bands to confirm proper insertion without frameshift mutations or other recombination errors (Figure 1). One clone per genotype was then sent to WiCell for karyotyping. Both cell lines have normal karyotypes. These cell lines were then differentiated according to our standard dopamine neuron protocol confirming proper expression of the reporter (Figure 2). The SNCA-triplication line was validated and subjected to the same quality control workflow as described above for the generation of the GBA lines. We added an additional quality control step to assess for non-homologous end joining errors by Sanger sequencing of the CRISPR site at the unmodified TH locus. This additional step was added because we discovered occasional NHEJ errors occur at the non-modified locus in other cell lines. Three clones passed all aspects of quality control but only one clone (clone #48, Figure 1) was sent for karyotyping to reduce costs. This line has a normal karyotype, demonstrates bright, appropriate expression of the THtdtomato reporter and will therefore be used for subsequent experiments and for the screens in major task 6 and 7 (Figure 2).

There were not any dramatic morphological or differentiation phenotypes observed when comparing the GBA mutant and corrected lines. THtdtomato(+) neurons from both lines are similar in morphology to one another and their appearance is consistent with other published knock-in lines generated with this reporter (Ahfeldt et al 2020, PMID 31902706). Multiple clones from both lines generated typical percentages of dopamine neurons (50-70%) as demonstrated by FACS analysis and imaging following our published floor plate patterning differentiation protocol. The SNCA-knockdown reporter line is the only remaining line described in the SOW which needs to be generated. The COVID-19 wet lab shutdown has put generation of this line slightly behind schedule. The initial batch of nucleofected clones had insufficient numbers of clones that passed all QC steps, so another round of modification has been initiated. This will not slow other aspects of the project and we expect to have the line generated and completed quality control in time for the experiments in Major Task 2, Subtask 3 (months 18-24 of the project timeline).

Following generation and validation of the SNCA-triplication line described above, screening assay development and toxicant response profiles were initiated. FACS purified THtdtomato dopaminergic neurons were compared to mixed cultures containing other non-THtdtomato expressing cells. The primary readouts of the assay were survival and neurite length. Comparison of the sorted and mixed cultures indicated that sensitivity (to both cell death and neurite loss) to a subset of toxicants was either very similar in both assay conditions, or the sorted cells showed increased sensitivity compared to mixed (Table 1). This result suggests to us that using FACS sorted THtdtomato dopaminergic neurons for the compound and toxicant screens will provide the most sensitive and easily quantified readout of cell death and neurite loss. The unprecedented need to suspend wet lab work for an extended period of time during the first few months of the COVID-19 pandemic afforded us the opportunity to assess whether cryopreserved dopaminergic neurons could be used for the survival and neurite growth assays. A limited assessment of these cryopreserved neurons suggested that they are not optimal for the assays due to variable survival after thawing. However, batches that showed good survival after thawing did demonstrate comparable sensitivity to toxicants with respect to neurite length when compared to freshly assayed cells from the same cell line (data not shown). After establishing baseline responses to a subset of toxicants with freshly differentiated dopaminergic neurons from the SNCA-triplication line, we expanded the analysis to the GBA mutant and GBA corrected lines, again focusing on sorted THtdtomato dopaminergic neurons. Initial replicates have been analyzed at the time of reporting with additional replicates in-process/pending. These experiments suggest that the SNCA-triplication line may be more sensitive to paraquat and rotenone while having similar sensitivity to permethrin (Figure 3). Prior to the generation of the SNCA-triplication reporter line and the GBA reporter lines, we began to characterize the response of the E46K synuclein mutant line to a subset of the toxicants described in Table 3 of the Project Narrative. For all toxicant experiments, live images were acquired 11 days after treating sorted dopaminergic neurons with toxicants and image analysis revealed different sensitivities to toxicants, with ziram showing the most potent toxicity and cyanazine showing the least toxicity (Figure 4). These doses and the experience with this assay allowed us to have useful starting points for dose ranges in the SNCA-triplication experiments described above.

Significant results from the Khurana lab primarily relate to objective 2d. We further refined the biochemical assay to measure calcium dynamics in spontaneously active differentiated neurons, based on the use of the fluorescent dyes Fluo-4 and Fura-2. As reported previously, we have established the use of the Calcium Signaling Analyzer (CaSiAn) software tool, an analysis method to quantify calcium dynamics in electrically active differentiated dopaminergic neurons harboring a GBA mutation and exposed to toxicants, e.g. rotenone. The CaSiAn software tool offers a semi-automatic solution for the analysis of calcium imaging data. Therefore, we decided to implement an established, but unpublished, calcium image analysis pipeline that runs in MatLab. This pipeline combines the versatility of the CaSiAn software tool, with automatic preprocessing, cellular segmentation, fluorescent traces generation and spike train inference for a given set of time-lapse calcium images. This pipeline allows the processing of large number of images automatically. Figure 5 describes the set of steps for processing images using this calcium imaging pipeline. By using this image analysis pipeline, we can automatically identify active neurons in selected regions of interest, segment the active neurons, assign identifiers (numbering), obtain the fluorescent traces for each neuron and their spike trains (which represent a probability of action potentials). Following these automated steps, we use the functionality of CaSiAn tool to quantify various features of the fluorescent trace during the acquisition time. These features are correlated to different aspects of calcium physiology and allow us to describe and compare these parameters between cell lines and treatment conditions. Additional efforts have been focused on developing an assay setup that allows us to utilize the ratiometric properties of the Fura-2 dye for more quantitative analysis of calcium flux. For this assay, we are developing a method that allows us to perfuse toxicants and compounds of interest.

Significant results for the Ritz lab relate to Specific Aim 1, Major Task 3, subtasks 1 and 2. We have established pesticide exposure profiles and quantitative exposure measures for future GxE analysis (Major Task 7), created separate exposure profiles by SNCA and GBA variant status (variants identified via ClinVar) to identify toxicants that patients harboring common α-syn SNPs were exposed to in the PEG cohort, and generated a list of 33 pesticide toxicants that are both significantly associated with PD (FDR<0.01) and have exposure in both α-syn SNPs and GBA variant carriers/non-carriers. This list will provide the foundation for Specific Aim 1, Major Task 4: Test toxicant lists from PEG data in corresponding in vitro cell lines.

PEG is a population-based case-control PD study from three agricultural counties in Central California, comprised of two, independent study waves, recruited some ten years apart (wave 1: 2001-2007 & wave 2: 2010-2016; n=849 PD patients, n=1021 population-based controls). For the 1,870 PEG participants, we have generated pesticide exposure profiles based on self-report of household pesticides use, occupational pesticide use established through a Job Exposure Matrix (JEM), and a GIS model based on historical agricultural pesticide use reports and occupational and residential addresses. As per our statement of work, we have established exposure profiles for PEG study participants using all three measures. The majority of our work over the reporting period has focused on the GIS-based model, which allows us to assess the associations with specific pesticides that will be targeted by the lab-based teams (Rubin and Khurana). To assess exposure to specific pesticides in PEG, we have used a geospatial algorithm we previously developed to estimate ambient pesticide exposure due to living or working near agricultural pesticide applications called *GRAPES*: a GIS model for ambient pesticide exposures that uses a sophisticated computer algorithm to combine California state mandated commercial pesticide use reports, land use, and

participant address histories for exposure assessment.

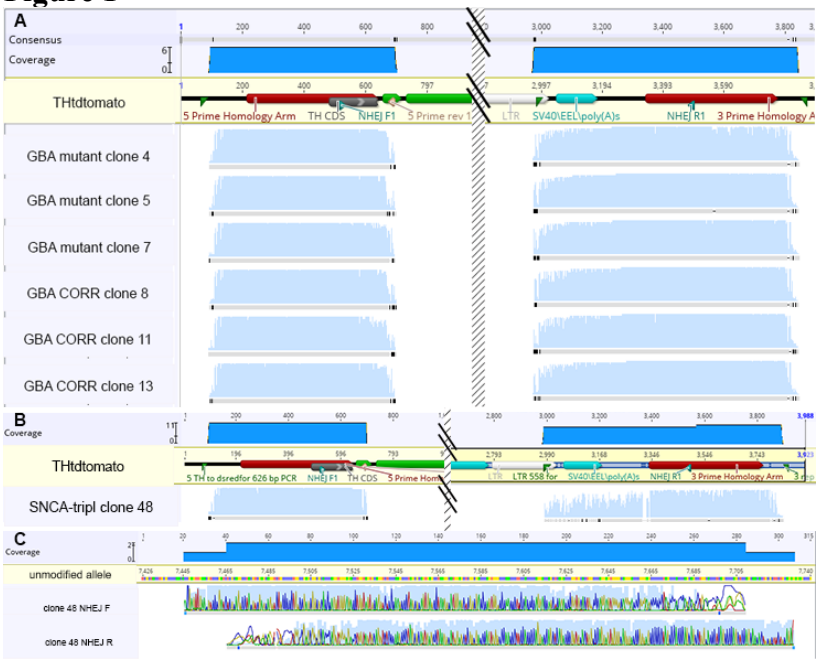
Since 1972, California law mandates the recording of all agricultural pesticide use to the pesticide use reports (PUR) database of the CA Dept. of Pesticide Regulations. For more than 700 pesticide active ingredients applied in the PEG study area, the PUR records locations of applications, poundage, type of crop and acreage a pesticide has been applied on, as well as the method and date of application, documenting over 40 years of agricultural pesticide applications in California. We combined this database with maps of land use and crop cover, providing a digital representation of historical land-use. Our GIS system pinpoints pesticide applications at a precise agricultural site for each month. PEG participants provided lifetime address information which we previously geocoded in a multi-step process. Based on this unique system, we estimated pesticide exposures for all PUR pesticides and study participants based on residential or occupational proximity to pesticide application since 1974. The output of our model, is a yearly average of lbs/pesticide applied within a 500m buffer of the participants residence or occupation for all pesticides included in the PUR database. To generate long-term exposure profiles to each pesticide, we averaged the yearly exposure data from 1974-10 years prior to PD diagnosis or interview (controls). From our GIS model, we have now established real-world exposure profiles for all PEG participants for 700 agriculturally used pesticides, averaged over a 40-year time period.

Of these 700 pesticides, to select those to assess for PD association, we then excluded all pesticides that had less than 25 participants with exposure, required exposure in both common SNCA and GBA variant carriers and non-carriers which will allow for future GxE analysis (Major Task 7), and required exposure in both study waves of the PEG, to select pesticides that showed PD association in both intendent study waves (see above) for replication. This left 288 pesticides with exposure profiles in PEG, which we then analyzed for PD risk. The aim here was to both provide epidemiologic evidence for novel pesticide-PD associations and to provide the toxicant list for Specific Aim 1, Major Task 4.

For PD-pesticide analysis, we used 1,870 unrelated, study participants and the yearly averaged pesticide exposure profiles described above to the 288 pesticides. We analyzed all of the pesticide associations with PD using logistic regression, controlling for age, sex, race/ethnicity, smoking, and education, and we combined our results from the two study waves in a meta-analysis. This provides confidence in selected pesticides, as we are able to select only pesticides that are independently associated in both study populations (replicated from PEG wave 1 to PEG wave 2, different study participants, ~10 years later). From this analysis, we identified 33 pesticides associated with PD at a meta FDR<0.01 (see Figures 6 and 7). While the cost of this type of analysis is the multiple-testing correction, our analysis is able to handle this due to both the relatively large sample size (n=1,870) and level of associations between pesticides and PD ($p<0.5e-5$ for the top associations). Furthermore, the pay-off from this agnostic analysis, is highlighting out of all pesticides the most strongly associated and suggest novel associations. We have provided the list of 33 pesticides most strongly associated with PD (meta FDR<0.01), the associations, and exposure profiles in Table 2.

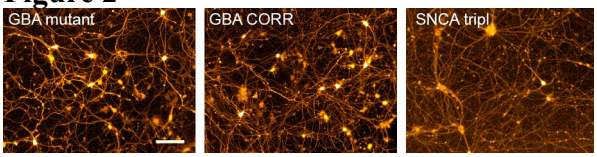
This list of 33 pesticides will be used in the next phase of research in the in vitro cell lines. The exposures will be selected according to the strength of their association with PD in our PEG study; we will then prioritize testing those of most common use and exposure in the population (see Table 2 exposure numbers) and also those most relevant to neuroprotective regulatory action as well as testing agents from different chemical classes. Validated hits from these screens will then be used in conjunction with SNCA and GBA genetic data to assess GxE interactions with pathway relevant SNPs in the PEG study as part of Major Task 7 (not applicable for this reporting period).

Figure 1



A. GBA sequence alignment of each clone at 5 prime and 3 prime insertion sites mapped to expected sequence of TH:tdtomato knock in sequence. Crosshatched region represents cropped sequence within the reporter for ease of visualization. **B.** Sequence alignment of SNCA-tripl THtdtomato knock in clone 48. Sequence alignment of additional clones omitted for simplicity. Upper panel shows a cropped version of the expected THtdtomato knock in allele with Sanger sequencing of PCR products from clone 48 demonstrating proper insertion at the 5 prime and 3 prime ends of the reporter construct. **C.** Integrity of the unmodified allele of TH in the region of the CRISPR-targeted sequence.

Figure 2



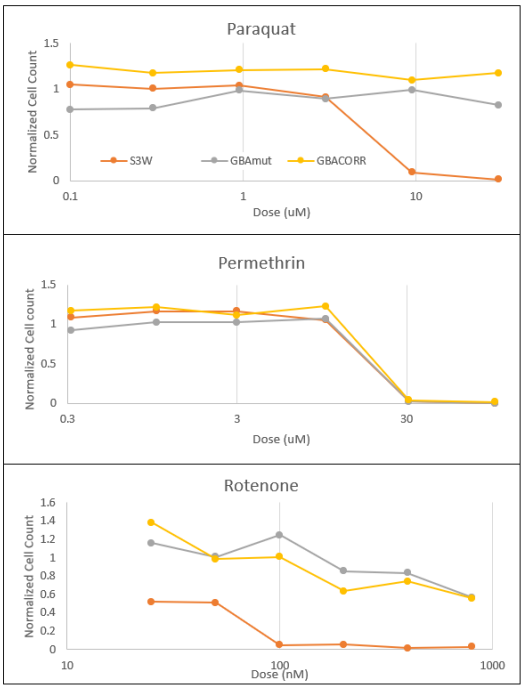
Live cell imaging of endogenous reporter fluorescence for GBA mutant TH:tdtomato knock in (left panel), isogenic corrected control GBA line with TH:tdtomato knock in (center panel), and SNCA triplication TH:tdtomato knock in (right panel). Images acquired with Molecular devices IXM high content imaging system with 10x objective. Scale bar 100uM.

Table 1

SNCA-triplication	EC50 values in µM **condition**	
Toxicant	sorted	mixed
Rotenone	0.025	0.043
Cyanazine	696	574
Permethrin	21.4	22
Paraquat	5	ND (>30)

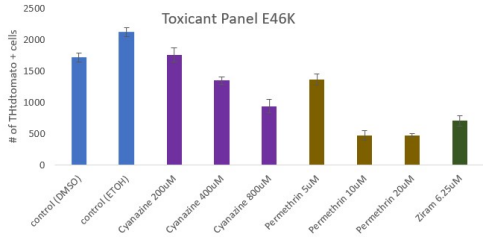
SNCA triplication toxicant sensitivity assay comparison: sorted vs mixed samples. EC50 represents the value at which 50% of the maximum cell death occurred. EC50 values were calculated using a Smart Fit algorithm in GeneData software. Experiment was performed as a 6 concentration dose curve in triplicate.

Figure 3



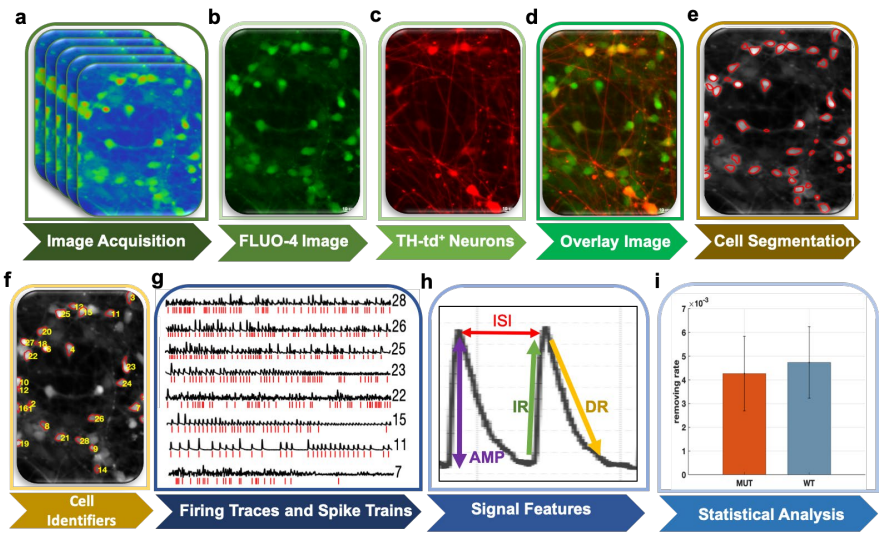
FACS purified THtd+ neurons from the SNCA-tripl ("S3W"), GBA mutant ("GBAmut") and GBA corrected ("GBACORR") cell lines treated with multiple doses of toxicants linked to PD risk. Cell numbers measured by high content imaging of live cultures 11 days after first treatment and normalized to DMSO control for each line

Figure 4



FACS purified THtd+ neurons from the E46K synuclein mutant treated with multiple doses of toxicants linked to PD risk. Cell numbers measured by high content imaging of live cultures 11 days after first treatment. Error bars = 2 SD

Figure 5



Automated image analysis pipeline for calcium imaging data analysis to quantify neuronal activity.

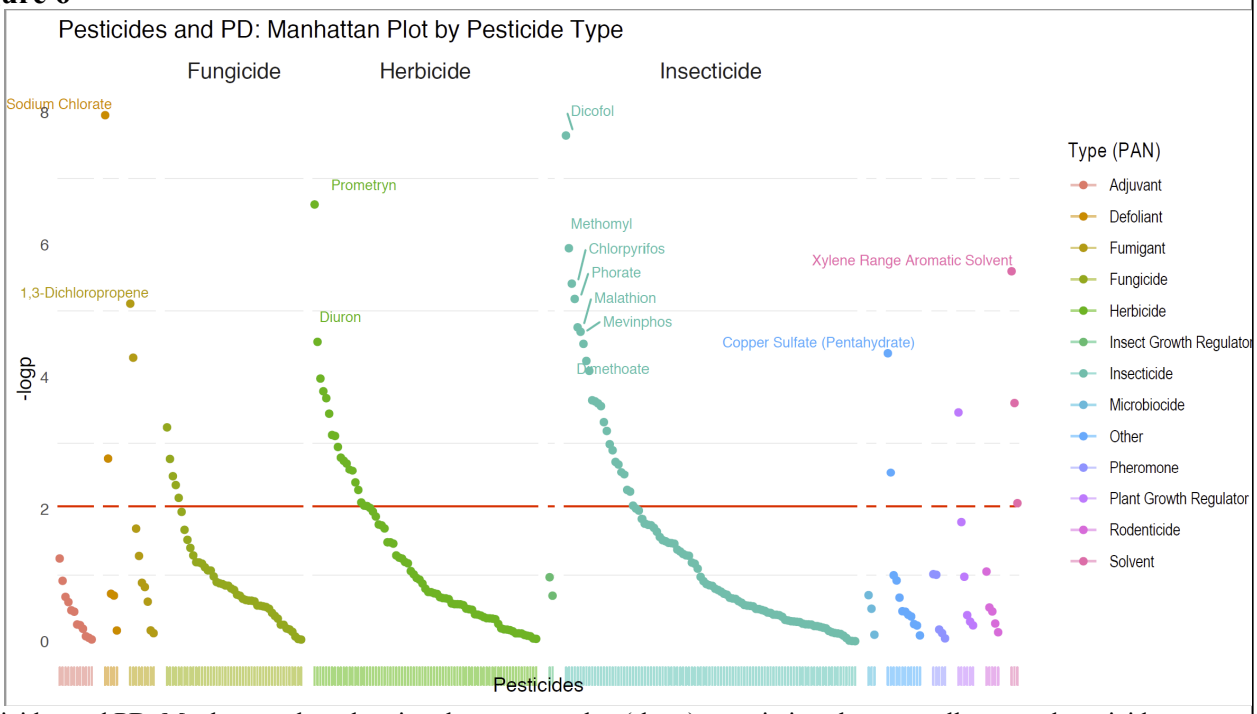
(a) Time lapse Calcium imaging data of a neuronal population are acquired.
(b) Fluo-4 is used as calcium indicator
(c) Representative picture of TH-td$^+$ neurons
(d) Overlay of Fluo-4 and TH-td$^+$ neurons.
(e) Region of interest (ROIs) corresponding to individual neurons are identified
(f) Identity numbers for each segmented neurons are assigned
(g) Firing traces and spike trains (red lines) are measured for each signal in (f).
(h) Calcium transients are detected and their features (Inter Spike Interval (ISI), Amplitude (AMP), Increasing Rate (IR), Decreasing Rate (DR)) are estimated.
(i) Statistical analysis is performed to estimate differences in calcium transient parameters between mutant (MUT) and gene corrected (WT) neuronal populations.

Table 2. PEG identified Pesticide-PD associations and exposure information for PD related pesticides

Common Name	Type	Class	meta OR	meta p-vaule	meta FDR	R: Any Exposure		C: Any Exposure	
						PD n (%)	Control n (%)	PD n (%)	Control n (%)
Sodium Chlorate	Defoliant, Herbicide, Microbiocide	Inorganic	1.26	1.11E-08	3.20E-06	441 (49.4)	476 (43.4)	440 (50.9)	420 (42.4)
Dicofol	Insecticide	Organochlorine	1.25	2.25E-08	3.24E-06	425 (47.6)	492 (44.8)	395 (45.7)	371 (37.4)
Prometryn	Herbicide	Triazine	1.24	2.48E-07	2.38E-05	209 (23.4)	199 (18.1)	173 (20)	161 (16.2)
Methomyl	Insecticide	N-Methyl Carbamate	1.22	1.13E-06	8.17E-05	592 (66.4)	689 (62.8)	565 (65.3)	584 (58.9)
Xylene Range Aromatic Solvent	Solvent, Insecticide	Petroleum derivative	1.21	2.53E-06	1.45E-04	352 (39.5)	398 (36.3)	313 (36.2)	282 (28.5)
Chlorpyrifos	Insecticide, Nematicide	Organophosphorus	1.20	3.89E-06	1.87E-04	474 (53.1)	559 (51)	409 (47.3)	398 (40.2)
Phorate	Insecticide, Nematicide	Organophosphorus	1.20	6.61E-06	2.72E-04	263 (29.5)	266 (24.2)	239 (27.6)	217 (21.9)
1,3-Dichloropropene	Fumigant, Nematicide	Halogenated organic	1.19	7.79E-06	2.81E-04	211 (23.7)	217 (19.8)	190 (22)	162 (16.3)
Malathion	Insecticide	Organophosphorus	1.19	1.78E-05	5.68E-04	362 (40.6)	405 (36.9)	390 (45.1)	315 (31.8)
Mevinphos	Insecticide	Organophosphorus	1.20	2.07E-05	5.96E-04	242 (27.1)	262 (23.9)	245 (28.3)	200 (20.2)
Diuron	Herbicide	Urea	1.18	2.94E-05	7.57E-04	418 (46.9)	475 (43.3)	373 (43.1)	349 (35.2)
Dimethoate	Insecticide	Organophosphorus	1.18	3.15E-05	7.57E-04	551 (61.8)	625 (57)	506 (58.5)	501 (50.6)
Copper Sulfate (Pentahydrate)	Algaecide, Fungicide, Insecticide, Water Treatment, Molluscicide	Inorganic-Copper	1.20	4.37E-05	9.69E-04	107 (12)	119 (10.8)	101 (11.7)	78 (7.9)
1,2-Dichloropropane, 1,3-Dichloropropene And Related C3 Compounds	Fumigant, Nematicide	Halogenated organic	1.18	5.10E-05	1.05E-03	189 (21.2)	210 (19.1)	179 (20.7)	144 (14.5)
Mevinphos, Other Related	Insecticide	Organophosphorus	1.19	5.70E-05	1.09E-03	242 (27.1)	262 (23.9)	245 (28.3)	200 (20.2)
Acephate	Insecticide	Organophosphorus	1.17	8.06E-05	1.45E-03	372 (41.7)	408 (37.2)	369 (42.7)	294 (29.7)
Trifluralin	Herbicide	2,6-Dinitroaniline	1.17	1.05E-04	1.79E-03	376 (42.2)	429 (39.1)	350 (40.5)	343 (34.6)
Msma	Herbicide, Defoliant	Organoarsenic, Heavy metal	1.18	1.64E-04	2.62E-03	151 (16.9)	168 (15.3)	152 (17.6)	125 (12.6)
Bromoxynil Octanoate	Herbicide	Hydroxybenzonitrile	1.16	2.08E-04	3.13E-03	321 (36)	356 (32.5)	263 (30.4)	253 (25.5)
Carbofuran	Insecticide, Nematicide	N-Methyl Carbamate	1.17	2.24E-04	3.13E-03	254 (28.5)	249 (22.7)	255 (29.5)	189 (19.1)
Parathion	Insecticide	Organophosphorus	1.16	2.33E-04	3.13E-03	426 (47.8)	511 (46.6)	423 (48.9)	429 (43.3)
Petroleum Distillates, Aromatic	Solvent, Herbicide, Insecticide	Petroleum derivative	1.16	2.47E-04	3.13E-03	428 (48)	480 (43.8)	386 (44.6)	364 (36.7)
Carbaryl	Insecticide, Plant Growth Regulator, Nematicide	N-Methyl Carbamate	1.16	2.50E-04	3.13E-03	478 (53.6)	566 (51.6)	433 (50.1)	436 (44)

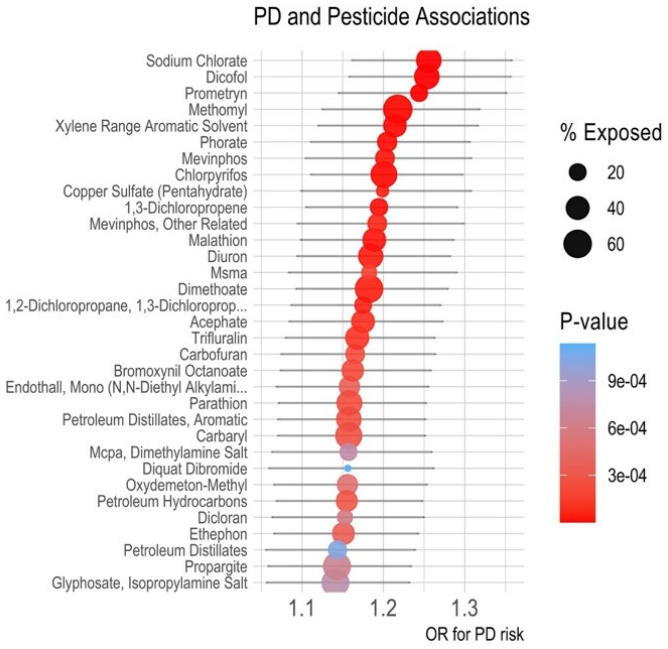
Petroleum Hydrocarbons	Insecticide, Adjuvant	Petroleum derivative	1.16	2.75E-04	3.30E-03	302 (33.9)	334 (30.4)	306 (35.4)	238 (24)
Ethephon	Plant Growth Regulator	Organophosphorus	1.15	3.42E-04	3.94E-03	322 (36.1)	372 (33.9)	299 (34.6)	282 (28.5)
Endothall, Mono (N,N-Diethyl Alkylamine) Salt	Herbicide, Defoliant	Unclassified	1.16	3.57E-04	3.96E-03	300 (33.6)	298 (27.2)	264 (30.5)	238 (24)
Oxydemeton-Methyl	Insecticide	Organophosphorus	1.16	4.79E-04	5.11E-03	295 (33.1)	292 (26.6)	289 (33.4)	241 (24.3)
Dicloran	Fungicide	Substituted Benzene	1.15	5.75E-04	5.91E-03	151 (16.9)	172 (15.7)	211 (24.4)	182 (18.4)
Propargite	Insecticide	Unclassified	1.14	6.51E-04	6.46E-03	523 (58.6)	620 (56.5)	501 (57.9)	519 (52.4)
Mcpa, Dimethylamine Salt	Herbicide	Chlorophenoxy acid or ester	1.16	7.49E-04	7.19E-03	194 (21.7)	215 (19.6)	171 (19.8)	150 (15.1)
Glyphosate, Isopropylamine Salt	Herbicide	Phosphonoglycine	1.14	7.75E-04	7.20E-03	515 (57.7)	612 (55.8)	463 (53.5)	454 (45.8)
Petroleum Distillates	Insecticide, Adjuvant, Solvent	Petroleum derivative	1.14	1.03E-03	9.01E-03	221 (24.8)	245 (22.3)	212 (24.5)	173 (17.5)
Diquat Dibromide	Herbicide, Dessicant	Bipyridylium	1.16	1.14E-03	9.62E-03	70 (7.8)	76 (6.9)	98 (11.3)	61 (6.2)

Figure 6



Pesticides and PD. Manhattan plots showing the meta p-value (-logp) associations between all assessed pesticides (288) and PD, separated by pesticide type. Results from PEG PD-pesticide association (Specific Aim 1, Major Task 3), to identify toxicant lists from PEG data to test in corresponding *in vitro* cell lines (Specific Aim 1, Major Task 4).

Figure 7



Top pesticides associated with PD from PEG PD-pesticide association (Specific Aim 1, Major Task 3) (FDR<0.01; n=33 pesticides). These pesticides will now be selected from to test in in vitro cell lines.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Rubin Lab: During the next reporting period work will focus on a number of tasks outlined in the Statement of Work. The SNCA-triplication knockdown reporter line will be made and characterized (final part of Major Task 1). Survival responses and neurite outgrowth after toxicant treatment will be measured with adequate replicates for the SNCA-tripl THtdtomato line (final part of Major Task 2, Subtask 1). We will complete assay development for the screens on the SNCA-tripl THtdtomato line and the toxicant and custom library screens will be performed (Major Task 5 subtask 1+2, Major Task 6, Subtask 1).

Khurana lab: For the next reporting period, we plan to accomplish subtask 2, specifically working on secondary assays (autophagic flux, ER-Golgi trafficking, mitochondrial subunit and dopamine oxidation assays) on SNCA-triplication THtdtomato, GBA mutant THtdtomato and GBA gene corrected THtdtomato differentiated neurons. We will also finish the implementation of the calcium imaging assay based on Fura2 AM using an experimental set up with a perfusion chamber to expose $THtd^{+}$ differentiated neurons to selected toxicants, acquire calcium imaging data and perform the analysis using the image analysis pipeline we have implemented.

Ritz lab: During the next reporting period the Ritz lab will focus on Milestone #2, we will prepare a manuscript describing our PD-pesticide associations discovered as part of Major Task 3 and submit for peer-reviewed publication. We will also aim to present our findings at a national meeting. Next, we will begin Major Task 7, starting specifically with data/literature mining for SNPs in relevant pathway components as we await the findings from Major Task 4, which aims to test the epidemiology identified pesticides in the in vitro models, to proceed to GxE pathway analysis of hits and evaluation of SNPs in PEG cohort.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The reagents developed by the Rubin lab (specifically the GBA reporter lines and the triplicated SNCA reporter line) will extend our ability to evaluate the effects of toxicants and compounds directly on dopaminergic neurons. These reagents will allow for clear evaluation of the sensitivity of dopaminergic neurons to various toxicants that people have been exposed to in the environment. Toxicants that cause cell death at lower doses will provide evidence to the field of gene/environment interactions and Parkinson's disease epidemiology.

In the PEG study, by analyzing all pesticides widely used in Central California, we implicate over 30 real-world pesticides most strongly associated with PD. This research provides epidemiologic evidence related to specific pesticides which can now be used to inform future lab-based experiments with novel pesticide targets and drive preventative policy around these patterns or agents.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Although preliminary, these data have potential to influence the recommendations regarding the widespread use of multiple agricultural or consumer products based on how likely they are to cause dopaminergic cell death. The work has potential to increase awareness concerning these products and safety protocols that should be put in place. Furthermore, these data might lead to the identification of still unknown environmental toxicants that would then limit their use in agricultural or industrial processes.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

No major changes in approach were undertaken in the dopamine neuron experiments. More focus was placed on the sorted neuron experiments because these neurons are more sensitive to most toxicants and the assays have proven more reproducible.

Actual or anticipated problems or delays and actions or plans to resolve them

Delays occurred as a result of the COVID-19 suspension of wet lab work. These were anticipated in the previous technical report. Since resumption of experiments, the lab remains at 50% capacity. We have been able to maximize our productivity during these capacity restrictions by reducing the amount of time that individuals working on the project overlap and therefore allowing us to maintain a consistent amount of effort on the hands on/wet lab experiments. The delays we experienced are discussed in section 3 above and put us a few months behind schedule on a limited set of tasks. We are prioritizing the cell culture experiments which will generate data needed by our collaborating labs in order to prevent the delay from impacting the overall project timeline. For example, we are attempting to perform the screening assay development and screens as soon as possible because the toxicants and compounds identified in this task will be utilized in Major Task 7 by the Ritz lab. In the Rubin lab, we anticipate that we will utilize the no cost extension at the end of the project to make up for time lost as a result of the COVID-19 wet lab shutdown. We have also encountered difficulties obtaining an ideal toxicant library. The Enzo toxicity library initially proposed has a high percentage of drugs with end organ toxicity. A library with primarily environmental or industrial toxicants would be a more ideal screening library. We have contacted NCATS to request access to a portion of their tox21 library, but have not yet been successful in obtaining a commitment to utilize the library. Another possibility we are considering as an alternative to publicly available libraries is to compile a small custom library from the list of PD-associated pesticides from the PEG data set described by our collaborators from the Ritz lab (~79 toxicants). If we can procure a majority of these toxicants within the next 3 months and are not provided with a commitment for access to the NCATS library in that timeframe, we would plan to switch the approach to this PEG-derived list.
In the Khurana lab, we experienced a delay in the development and implementation of imaging and biochemical assays due to the COVID-19 suspension of non-essential laboratory activities. All of this notwithstanding, we have made substantial progress on assay development as noted above. However, thus far we are not yet at the point of being able to distinguish mutant from isogenic mutation-corrected controls with the assays we have developed. We hope to catch up on this, but this has undoubtedly been a follow-on effect of the shutdown.

Changes that had a significant impact on expenditures

Expenditures on supplies, materials and services was temporarily reduced during the suspension of wet lab activities as a result of the COVID-19 pandemic.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Nothing to report.

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations. .

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Lee Rubin, PhD
Project Role: PI
Researcher Identifier:
Nearest Person Month worked: 2
Contribution to Project: Experiment planning, data analysis, coordination with co-PIs, supervision of Rich Krolewski, Gizem Rizki, Jack Blank

Name: Gizem Rizki, PhD
Project Role: Scientist
Research Identifier: N/A
Nearest person month worked: 2
Contribution to Project: assay development, dopaminergic neuron experiments, screen planning

Name: Richard Krolewski, MD, PhD
Project Role: Scientist
Research Identifier: N/A
Nearest person month worked: 3
Contribution to project: Experiment planning, data analysis, coordination/project management, generation of transgenic lines, dopaminergic neuron experiments, supervision of Jack Blank.
*Dual appointment-Paid through a professional services billing agreement with Brigham and Women's Hospital

Name: Jack Blank
Project Role: Research Assistant
Research Identifier: N/A
Nearest person month worked: 4
Contribution to project: Dopamine neuron experiments, generation of transgenic lines, data analysis.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

See attached addendum for Rubin lab other support.

What other organizations were involved as partners?

Due to the nature of this partnering PI award, individuals working on the project from each of the three institutions (Rubin lab- Harvard/FAS; Khurana lab-BWH/Harvard; Ritz lab- UCLA) via video conference meetings and exchange of data. Other than this partnership, there is nothing to report from the Rubin, Khurana and Ritz labs.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: "Rubin- Changes to Other Support 10.13.2020"

Awards CLOSED since 7/3/2019

Title: **SPARC- Iterative Screen-Seq: a means to reprogram a cell into any somatic cell type;** Sponsor: The Broad Institute; Period of Performance: 07/01/16-12/31/2019

Title: **Enhancing Maturation of In Vitro Derived Human Neurons;** Sponsor: The Broad Institute**;** Period of Performance: 8/1/16-7/31/19

Title: **AstraZeneca-Melton beta-stem cell project;** Sponsor: Astra Zeneca AB**;** Period of Performance: 7/1/17-2/19/20 (Project ongoing, Rubin portion complete)

Title: **Elevian Research Collaboration;** Sponsor: Elevian, Inc.; Period of Performance: 2/26/18-2/25/20 (Project in NCE, Rubin portion complete)

Title: **Establishing a screening platform to chemically reprogram human cells for cell therapy and drug discovery;** Sponsor: Harvard Stem Cell Institute; Period of Performance: 9/1/17-8/31/20

Title: **Establishing a Platform to Chemically Reprogram Human Cells into any Somatic Cell Type;** Sponsor: Harvard University- Dean's Competitive Fund for Promising Scholarship; Period of Performance: 7/1/17-9/2020

Awards MODIFIED since 7/3/2019 ***(Modifications in Italics)***

Title: **Modeling of psychiatric disease in human brain organoids;** Sponsor: The Broad Institute; *Modifications: Period of Performance: 4/1/15-6/30/2021; Total Awarded: Increased to ; Effort Reduced to .72 CM*

Title: **Designing screening assays for complex neurological disorders;** Sponsor: The Broad Institute; *Modifications: Period of Performance: 2/1/16-6/30/2021; Total Awarded: Increased to ; Effort Reduced to .72 CM*

Title: **In vitro Production of Satellite Cells for Cell Therapy and Drug Discovery;** Sponsor: Blavatnik Biomedical Accelerator at Harvard University; *Modifications: Period of Performance: 7/1/17-12/31/2020; Total Awarded: Increased to*

New Awards since 7/3/2019

Title: **Targeting Brain Vasculature to Improve Aging-Associated Decline of CNS Function** Sponsor: RejuverVas AG; Period of Performance: 12/20/19-9/30/2023; Total Awarded: ; Effort: 1.2 CM; Role: PI

Title: **Integrating models of the brain with the rest of the body: a collaboration with Emulate to develop an improved BBB-Chip;** Sponsor: Harvard Stem Cell Institute; Award # DP-0184-20-00; Period of Performance: 2/1/20-1/31/21; Total Awarded: Total Costs; No measurable effort; Role: PI

Title: **Direct and Indirect Effects of GDF11 in the Aging Central Nervous System;** Award # 1R01AG072086-01; Sponsor: NIH/NIA; Period of Performance: 9/1/20-5/31/2025; Total Awarded : (Total available to Dr. Rubin: $1,974,765) Effort: 2.4 CM; Role: PI

Title: **Identifying and Correcting Dementia-Associated Changes in the Blood-Brain Barrier;** Award # 1R01NS117407-01; Sponsor: NIH/NINDS; Period of Performance: 9/14/20-8/31/2024; Total Awarded: ; Effort: 2.4 CM: Role PI

Other Resources: Unrestricted Gifts Available for Research Endeavors

Nan Fung Life Sciences US LLC; Received March 2020
Funds received will support work to investigate non-CNS phenotypes associated with CNS disease.

Google, Inc.
Received 2016 and June of 2018
Funds received continue to support our efforts to more precisely define neurodegenerative diseases using high throughput imaging.